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Inhibition of *Penicillium digitatum* and Citrus Green Mold by Volatile Compounds Produced by *Enterobacter cloacae*

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Abstract

Penicillium digitatum causes green mold decay on citrus fruit, resulting in severe economic losses to citrus growers and packers worldwide. The present study is to evaluate the control of citrus green mold by volatiles produced by *Enterobacter cloacae*. An *E. cloacae* strain isolated from plant rhizospheres was able to produce three volatile organic compounds, which were identified as butyl acetate, phenylethyl alcohol, and 4,5-dimethyl-1-hexene by GC/MS chromatography. The volatile compounds produced by *E. cloacae* inhibited conidial germination and hyphal elongation of *P. digitatum* and reduced green mold severity. *E. cloacae* cultured at temperatures ranging from 16°C to 28°C, at pH values ≤6, or in a substrate carrier (sphagnum moss, vermiculite, or perlite) provided superior control against *P. digitatum*. A laboratory formulation using *E. cloacae* and perlite protected citrus fruit from green mold up to 22 days and its effectiveness outperformed fungicide application at room temperature (~25°C). The results implicate practical application of *E. cloacae* as a biofumigant for controlling citrus postharvest decay caused by *P. digitatum*. Significantly, the study provides a model for future research on how to formulate an effective biocontrol agent for disease management.

Keywords: Biocontrol; Biofumigant; Fungi; Gas-producing bacterium; Postharvest disease; Orange

Introduction

Green mold, caused by *Penicillium digitatum* (Pers.: Fr.) Sacc., is a noxious postharvest disease of citrus. *Penicillium digitatum* is an opportunistic pathogen that resides on healthy citrus fruit and attacks citrus fruit through injuries caused by rough handling during harvesting, transportation and storage. Infection of *P. digitatum* in citrus often results in tissue maceration and fruit decay. Economic losses caused by green mold decay could be enormous for citrus growers and packers worldwide. Application of fungicides, such as imazalil and thiabendazole, is a common practice to control *P. digitatum* induced fruit decay in the packinghouse [1-4]. However, repeated use of toxic chemical compounds could induce the emergence of fungicide resistant strains of the pathogen and also could increase human health risks.

To lessen the above-mentioned problems, the use of biological control is a promising alternative. Various microorganisms, including *Bacillus subtilis, B. pumilus, Burkholderia cepacia, Pseudomonas syringae, P. glathei, Pantoea agglomerans, Candida famata, C. oleophila, Trichoderma viride,* and *Myrothecium roridum* have been shown to have fungistatic or fungicidal effects to *P. digitatum* and have been explored, with some success, for reducing postharvest green mold decay in citrus [5-15].

Enterobacter cloacae is a rod-shaped, gram-negative bacterium commonly found in plants and has been shown to control a wide range of plant pathogens [16-22] and insect pests [23]. Strains of *E. cloacae* have been known to produce hydroxamate siderophore, non-volatile metabolites and inorganic volatile substances such as ammonia, all of which might contribute to biocontrol activity [19,24]. *Enterobacter cloacae* has been shown to inhibit *Pythium ultimum* sporangium germination and damping-off by competing for fatty acids [25]. Biological control using volatile compounds-producing microorganisms has been reported with increasing success on the various fruit crops and diseases [26-34]. However, little is known about volatile compounds produced by *E. cloacae*. The objectives of this study were to examine whether or not *E. cloacae* will emit volatile compounds and to assess their antimicrobial activity against *P. digitatum* and control of green mold of citrus fruit in storage.

Materials and Methods

Biological materials and culturing conditions

The P-51 and DOB-2 strains of *P. digitatum* (Pers. Fr.) Sacc. were single spore isolated from diseased orange fruit grown in Fongyuan and Dalin (Taiwan), respectively. DOB-2 strain has been characterized elsewhere [35]. The affected fruit were incubated in plastic boxes until conidia were produced. Conidia were picked and transferred onto 2% sucrose water agar. Fungal strains were cultured on potato dextrose agar (PDA; Difco) in a growth chamber with 12 h daily illumination at 25°C to 26°C. Fungal strains were identified as *P. digitatum* based on the distinct characteristics of conidia and colony morphologies formed on malt extract agar (MEA), Czapek yeast autolysate agar (CYA), and glycerol nitrate agar (GN25N) as described by Pitt [36]. The identity of *P. digitatum* was further confirmed by sequence analysis of the 5.8S ribosomal internal transcribed spacers (ITS).

Bacterial strains were single-colony isolated from rhizospheres of Yardlong bean (*Vigna sesquipedalis* (L.) Fruwith). Bacterial cells were grown on King's medium B (KB) plate at 30°C for 2 days, harvested by low speed centrifugation (4000× rpm), suspended in 20% glycerol water, and stored at -80°C. Bacterial strains were identified as *E. cloacae* by sequence analysis of an *rpoB* gene encoding a RNA polymerase β-subunit.

Identification of and fungal bacterial strains

Microbial genomic DNA was extracted with phenol/chloroform partition and precipitated with isopropanol. Fungal 5.8S rDNA was amplified by PCR with the primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') as described by White and colleagues [37]. Bacterial DNA fragment was amplified with colony PCR as described by Fukui and Sawabe [38] using a PCR Master Mix kit (GeneMark, Taichung, Taiwan). Bacterial *rpoB* gene was amplified by PCR with the primers CM7 (5'-AACCAGTTCCGCGTTGGCCTGG-3') and CM31b (5'-CCTGAACAACACGCTCGGA-3') as described by Mollet and colleagues [39]. PCR fragments were directly sequenced. Sequences were searched against the databases at the National Center for Biotechnology Information (NCBI) using the BLAST network service to determine the similarity of the amplified fragments. Sequence data reported in this article have been deposited in the EMBL/GenBank Data Libraries under accession numbers AJ543728 (E006 *rpoB*) and AJ543682 (E010 *rpoB*).

Volatile antimicrobial assays

In-vitro volatile antimicrobial activity was assessed using a plate-to-plate method as described [27,40]. Bacterial cells grown on KB agar at 24°C for 24 h were used for antifungal activity assays. Petri dish lips were removed and the plate was turned upside down and attached onto a coverless PDA plate containing conidia or an agar plug bearing 2-day-old mycelium of *P. digitatum*. The gap between plates was tightly sealed with two layers of parafilm (American National Can, Chicago, USA) and the plates incubated at varying temperatures. Spore germination was determined microscopically 24 h after treatment. Radial growth of fungus was measured at 7 days after treatment. A KB agar plate without *E. cloacae* and a PDA plate with *P. digitatum* were used as mock controls. Fungal viability was assessed after treatments by transferring agar plugs covering with *P. digitatum* mycelium onto freshly prepared PDA. Percentage of growth inhibition of *P. digitatum* was determined by dividing the relative difference of the growth between mock control and treatment by the control growth and then multiplying by 100. Percentage of inhibition = [(A-B)/A] × 100, where A is the average diameter of *P. digitatum* without being challenged with *E. cloacae* (mock control); B is the average diameter of *P. digitatum* grown in the presence of *E. cloacae*. Effect of volatile compounds on spore germination was also assessed using *P. digitatum* conidia. Unless otherwise stated, all experiments were repeated at least twice with three replicates.

SPME/GC-MS identification of volatile compounds

Enterobacter cloacae strain was grown on KB plate or in perlite mixed with KB broth at 30°C for 2 days. Volatile compounds were extracted by a headspace solid-phase microextraction (HS-SPME) device (SUPELCO, Bellefonte, PA, USA) equipped with 50/30 μm Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS, SUPELCO) fibers. After 15 min, the HS-SPME device was removed and inserted into the injector (200°C) of a gas chromatograph (GC)-mass spectrophotometer (MS) equipped with a Model CP-3800 GC and a Saturn 2000 MS (Varian, USA) connecting to an electron capture detector (Model 902B, ECD, splitless mode). Nitrogen was used as a gas carrier and flowed at 0.2 mL/min through a VF-5MS capillary column (30.0 m × 0.25 mm ID, 0.25 μm film thickness, Agilent, Santa Clara, CA, USA). Analytical temperatures modified from Kai et al., [41] were set as following: Initial column temperature at 40°C for 2 min, followed by a ramp of 5°C/min up to 170°C with a final hold for 1 min at 170°C. KB medium without bacteria was used as controls. The identities of volatile compounds were verified by comparing them with chemical databases deposited in the GC-mass spectra (GC-MS) library (Saturn 2000, USA). Phenylethyl alcohol (PEA) and butyl acetate (BA) were purchased from Sigma-Aldrich (St. Luis, MO, USA). The toxicity of commercially available chemicals individually or in combination on the growth of *P. digitatum* also was assessed by modified plate-to-plate assays by placing a test compound onto a filter paper disc (0.5 cm in diameter).

Fungal inoculation and assays for disease severity

Fruit of 'Gold Seal' orange (*Citrus sinensis* Osbeck), showing no visible lesions or injuries, were collected from an orchard in Gukeng (Yunlin county, Taiwan) and used throughout the experiment. Citrus fruit stored at 11°C for 5 to 7 days were washed by immersing in distilled water, surface sterilized with 70% alcohol, and dried in a sterile hood. Conidia of *P. digitatum* were harvested by flooding with sterile water containing 0.01% Tween 20 and the concentration was adjusted to 10^4 conidia per milliliter with the use of a haemocytometer. Orange fruit were wounded with 2 mm long by 1 mm wide tips (10) mounted on a steel rode as described [30] prior to inoculation. The wounded fruit were immersed in conidial suspensions or water for 10 s, dried, and placed in plastic boxes (Lock&Lock, HanaCobi, Korea) for lesion development with or without *E. cloacae* (1.2-1.8 × 10^8 cfu/mL) mixed in sterilized substrates. Fruit were soaked in a benomyl (200 ppm) or thiabendazole (2000 ppm; Mertect 41.8% SC; Sinon, Taiwan) solution for 30 min prior to inoculation as controls. Commercially available substrates: sphagnum moss (YuGuang Garden, Taichung, Taiwan), vermiculite (NanHai Co., New Taipei City, Taiwan), peat moss (BVB no. 4, Bas Van Buuren, Maasland, New Zealand) and perlite (NanHai) were sterilized and added to different concentrations of KB. A 5-fold KB stock (each liter contains 15 g peptone, 1.125 g K_2HPO_4, 1.125 g $MgSO_4 \cdot 7H_2O$, and 7.5 ml glycerol) was prepared, diluted, and mixed with substrates. Disease severity was rated in each fruit according to the following scale: 0 = No symptoms; 1 = Less than 25% of fruit surface showing symptoms; 2 = 26 to 50% of fruit surface showing symptoms; 3 = More than 50% of fruit surface showing. Percentage of disease severity was calculated from the disease rating by the formula:

$$\text{Disease Severity}(\%) = \frac{\sum(\text{Rating no.} \times \text{the no. of fruits})}{\text{Total no. of fruits} \times 3} \times 100$$

The experiment was performed three times; each replicate contained 10 fruit. The significance of treatments was determined by analysis of variance (ANOVA) and treatment means separated by nonlinear regression and Durbin-Watson Statistic normality tests using SigmaPlot 10 (Systat Software, San Jose, CA).

Result

Fungistatic effects of volatile compounds produced by *E. cloacae*

Pathogenicity assays revealed that both fungal strains were highly pathogenic to citrus (data not shown). Using plate-to-plate assays, we screened over 200 bacterial isolates and found that *E. cloacae* isolate E010 displayed inhibitory effects on vegetative growth and conidial germination of both *P. digitatum* P51 and DOB-2 strains (Table 1 and data not shown). Because *E. cloacae* and *P. digitatum* were cultured on different agar plates and never physically contacted each other, the inhibitory effects observed in *P. digitatum* were likely attributable to the production of volatile compounds that were toxic to fungus. Volatile compounds produced by the E006 strain had less effect on fungal growth and conidial germination compared to those produced by the E010 strain. Although volatile compounds produced by the E006 strain effectively suppressed the germination of conidia produced by *P. digitatum* P51 isolate, the compounds had little effect on conidial germination of DOB-2 isolate. Thus, E010 strain was chosen for further analyses. Exposure of *P. digitatum* to *E. cloacae* resulted in abnormal growth of fungal colony, producing more aerial hyphae. The effect of volatile compounds to *P. digitatum* was fungistatic as hyphae resumed normal growth after transferring onto freshly prepared PDA (data not shown).

Bacterial strain	% of inhibition[2]	
	P51	DOB-2
E006		
Conidial germination	97.6 ± 0.9a	30.2 ± 8.4b
Radial growth	15.3 ± 9.0b	27.8 ± 2.8b
E010		
Conidial germination	97.0 ± 1.0a	87.9 ± 2.3a
Radial growth	100 ± 0.0a	89.8 ± 0.3a

Table 1: Inhibition of conidial germination and radial growth of *Penicillium digitatum* by *Enterobacter cloacae* using plate-to-plate assays[1].

- [1]*E. cloacae* strains E006 and E010 were cultured on King's medium B and *P. digitatum* isolates P51 and DOB-2 were cultured on PDA at 24 for 24 h. Petri dish plates were attached and incubated for an additional 24 h. Experiments were repeated three times with at least 3 replicates for each treatment.
- [2]Means in the same column followed by the same letter are not significantly different according to nonlinear regression and Durbin-Watson Statistic normality tests (P = 0.05).

Effect of environmental conditions used to grow *E. cloacae* on antifungal activity

To assess if temperature would affect *E. cloacae's* ability to inhibit conidial germination, E010 strain was grown at temperatures varying between 16 and 32°C for 24 h. Conidia prepared from *P. digitatum* P51 and DOB-2 isolates were placed on PDA and the plates were attached onto the E010 plates using plate-to-plate assays and incubated at respective temperatures. In the absence of bacterial culture, the efficacy of conidial germination measured in both P51 and DOB-2 isolates increased exponentially at temperatures between 16 and 24°C and decreased sharply at temperatures greater than 28°C (Figure 1A). *Penicillium digitatum* conidia failed to germinate efficiently while grown at 32°C. The rate and magnitude of conidial germination reduced considerably when the agar plate carrying E010 strain was attached to the plates culturing fungal strains. In general, conidia of both *P. digitatum* strains germinated poorly when co-cultured with E010 bacterium grown at temperatures greater than 20°C. The pH values of medium used to culture *E. cloacae* also affected its efficacy of antifungal activity. E010 strain grown at acidic pH ≤ 6 displayed a strong inhibition on conidial germination of *P. digitatum* (Figure 1B). The inhibitory effect on conidial germination decreased markedly when E010 was cultured on medium with pH values greater than 6.

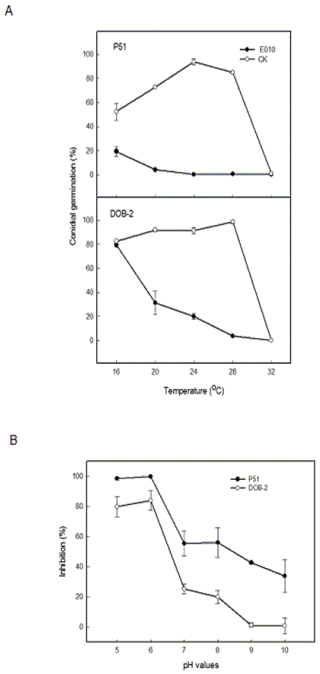
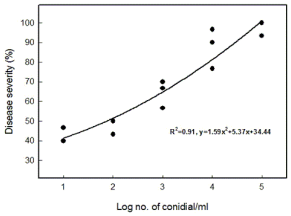


Figure 1: Effect of environmental conditions on the ability of *Enterobacter cloacae* to inhibit the germination of conidia produced by *Penicillium digitatum* strains assessed using plate-to-plate assays. (A) Effect of temperature on *E. cloacae's* ability to inhibit the germination of conidia produced by P51 and DOB-2 strains. (B) Effect of the pH valves on *E. cloacae's* ability to inhibit the germination of conidia produced by P51 and DOB-2 strains. The *E. cloacae* E010 strain was cultured on KB medium at varying temperatures for 24 h. The KB medium plates were attached to PDA plates culturing P51 or DOB-2 and incubated for an additional 24 h. The percentage of conidial germination was compared to those attached to KB medium plates only.

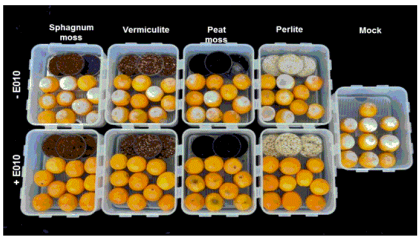
Control of citrus green mold by *E. cloacae*

Experiment was undertaken to evaluate if *E. cloacae* would be useful for controlling citrus green mold caused by *P. digitatum* and if substrates used to culture bacterium would impact *E. cloacae's* ability to inhibit test fungi. Increasing the amounts of fungal inoculum greatly increased the severity of green mold on citrus (Figure 2A).

Enterobacter cloacae and conidia suspended in KB or H_2O were mixed with sphagnum moss, vermiculite, peat moss, or perlite and incubated in a plastic box with test citrus fruit (Figure 2B). The results revealed that the substrates used to support the growth of *E. cloacae* had a profound impact on the effectiveness of reducing green mold. Fruit inoculated with *P. digitatum* developed green mold at 7 days post inoculation (dpi) in all treatments without exogenous application of *E. cloacae* (Table 2).



A



B

Figure 2: Citrus green mold produced by *Penicillium digitatum*. (A) Severity of citrus green mold was correlated with the amounts of inoculum prepared from conidia of *P. digitatum* strain E010. (B) Development of green mold on citrus fruit inoculated with conidial suspension of *P. digitatum* in the presence of *Enterobacter cloacae* E010 strain cultured in KB medium mixed with different substrates (3 plates) incubated at room temperature for 7 days. Experiments were repeated three times with 10 fruit for each treatment. Only representatives are shown.

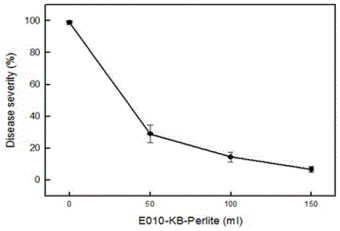
Substrate	Disease severity (%)[4]	
	+ E010	− E010
Peat moss	65.6 ± 8.7b	91.1 ± 5.6a
Perlite	0c	80.0 ± 5.1b
Sphagnum moss	0c	90.0 ± 3.3ab
Vermiculite	0c	95.6 ± 1.1a
Medium control[2]	95.6 ± 4.4a	96.7 ± 3.3a
H_2O control[3]	98.9 ± 1.1a	100.0 ± 0.0a

Table 2: Reduction of citrus green mold caused by *Penicillium digitatum* by *Enterobacter cloacae* grown in King's medium B and different substrates[1].

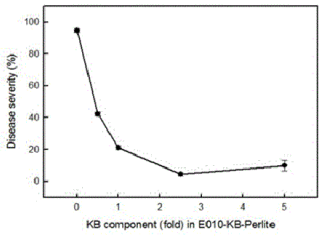
- [1]*E. cloacae* strain E010 was cultured in King's medium B (KB, 75 ml) mixed with different substrate (150 ml) in a moist chamber along with citrus fruit inoculated with *P. digitatum* for 7 days at room temperature.
- [2]Medium control contained medium ingredients without adding substrates.
- [3]H_2O control (75 ml distilled H_2O) contained no substrate and medium ingredients.
- [4]Means (n = 10) in the same column followed by the same letter are not significantly different according to nonlinear regression and Durbin-Watson Statistic normality tests (P = 0.05).

Notably, in the absence of *E. cloacae*, fruit incubated with perlite alone reduced green mold severity by 20% compared with other treatments. *Enterobacter cloacae* cultured in peat moss provided moderate protection from green mold. None of citrus fruit developed green mold symptoms when incubating with *E. cloacae* cultured in sphagnum moss, vermiculite, or perlite. *Enterobacter cloacae* cultured in KB or water (mock controls) failed to reduce green mold severity.

In order to optimize *E. cloacae*-substrate formulation for controlling citrus green mold, we further tested the effectiveness of a combined E010-KB-perlite (EKP) formula on the occurrence of the disease. Green mold severity decreased significantly as the volume of EKP increased (Figure 3A). Decreasing the ingredient concentrations in KB medium and then, mixing with perlite and E010 decreased the green mold severity substantially (Figure 3B).



A



B

Figure 3: Reduction of citrus green mold severity by E010-KB-perlite formulation. (A) *Enterobacter cloacae* E010 cultured in KB and perlite (E010-KB-perlite) was prepared in different amounts and placed in a moist chamber along with citrus fruit inoculated with *P. digitatum* P51 and incubated at room temperature for 7 days. (B) Reduction of green mold severity caused by *P. digitatum* due to the increase of KB concentrations in the E010-KB-perlite formulation. Experiments were repeated twice with 10 fruit for each treatment.

Using 5-fold KB in EKP (EK_5P) citrus fruit were protected from green mold up to 22 days at room temperature (~25°C) (Figure 4A); whereas EKP with 2.5-fold KB ingredients ($EK_{2.5}P$) provided less protection over time (Figure 4B). Further studies revealed that EK_5P reduced green mold incidence in a wide range of test temperatures (from 8 to 20°C) (Figure 4C). Uninoculated fruit never developed

green mold symptoms during the course of the experiment (data not shown). Citrus fruit soaked in benomyl (200 ppm), thiabendazole (2000 ppm), or water prior to inoculation failed to provide any protection from *P. digitatum*, whereas application of EK_5P reduced green mold development substantially (Figure 4D).

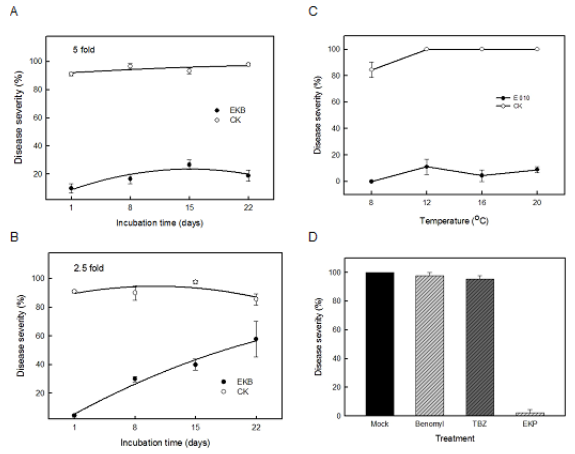


Figure 4: Control of citrus green mold by *Enterobacter cloacae* E010 cultured in KB and perlite. (A) Using 5-fold KB medium in the E010-KB-perlite (EK_5P) formulation, citrus fruit were protected from green mold caused by *P. digitatum* up to 22 days at room temperature. (B) Using 2.5-fold KB medium in the E010-KB-perlite formulation provided moderate protection against *P. digitatum*. (C) Using E010-KB-perlite (EK_5P) formulation citrus fruit were protected from green mold at temperatures ranging from 8 to 20°C. (D) E010-KB-perlite (EK_5P) had superior effect on the reduction of citrus green mold compared to fungicides benomyl (at 200 ppm) and thiabendazole (TBZ at 2000 ppm). Mock control (CK) was treated with water only. Each treatment contained 10 fruit.

GC/MS identification of volatile compounds produced by *E. cloacae*

Volatile compounds produced by *E. cloacae* grown on KB medium were identified after gas trapping and GC/MS separation (Figure 5). Compounds commonly identified from the gas phase of both the control and the *E. cloacae*-growing culture were eliminated. The volatile substances 4,5-dimethyl-1-hexene and phenylethyl alcohol uniquely found in the bacterium-grown medium were identified by comparing them in the GC-mass spectra (GC-MS) database library. Apart from these two compounds, butyl acetate was identified from gas phase of *E. cloacae* grown on KB medium mixed with perlite (Figure 5). The identity of phenylethyl alcohol and butyl acetate was further verified by comparison with authentic standards that were commercially available. Although 4,5-dimethyl-1-hexene is commercially available, we were unable to obtain this compound due to a shipping restriction of the product.

Suppression of *P. digitatum* and citrus green mold by volatile compounds

Co-incubation of *P. digitatum* P51 or DOB-2 isolate with commercially available butyl acetate or phenylethyl alcohol alone at test concentrations had little or no effect on conidial germination (data not shown) and radial growth (Figure 6).

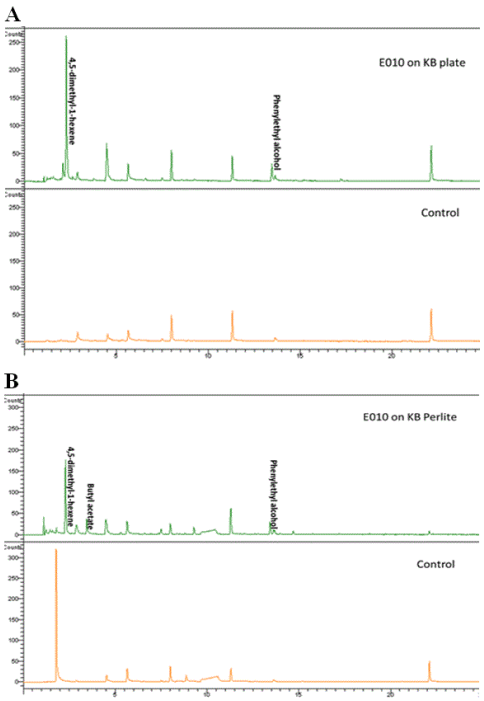


Figure 5: SPME/GC-MS chromatogram. GC/MS chromatogram of volatile compounds identified from gas phase of *Enterobacter cloacae* E010 cultured in (A) KB or (B) KB mixed with perlite (KB-perlite). Mock control (CK) contained KB medium only. E010 strain was grown in KB broth at 30°C for 2 days. Volatile compounds were collected using a solid-phase microextraction (SPME) device and analyzed by GC/MS chromatography.

A combination of butyl acetate and phenylethyl alcohol had a moderate effect on the suppression of conidial germination prepared from *P. digitatum* P51 strain, but inhibited the germination by nearly 40% of conidia prepared from *P. digitatum* DOB-2 strain. Mixture of butyl acetate and phenylethyl alcohol suppressed radial growth of both P51 and DOB-2 strains (Figure 6). When tested for green mold control, application of phenylethyl alcohol alone did not provide apparent protection against citrus green mold; however, application of butyl acetate alone or in combination with phenylethyl alcohol provided low levels of protection of citrus fruit from green mold (Figure 7). Notably, butyl acetate and phenylethyl alcohol had no synergistic effects in the context of disease reduction. Lack of commercially available 4,5-dimethyl-1-hexene prevented us from testing its toxicity toward *P. digitatum*.

Discussion

In Taiwan, benzimidazole is the primary fungicide recommended to control postharvest citrus diseases and more than 97% of *P. digitatum* isolates were found to be resistant to this fungicide [35]. In the present study, we identified an E010 bacterium collected from rhizospheres of Yardlong bean as *E. cloacae* based on sequence analysis of an *rpoB* gene and have demonstrated its ability of producing antimicrobial

volatile compounds, as well as its potential use as a biocontrol agent for controlling citrus green mold.

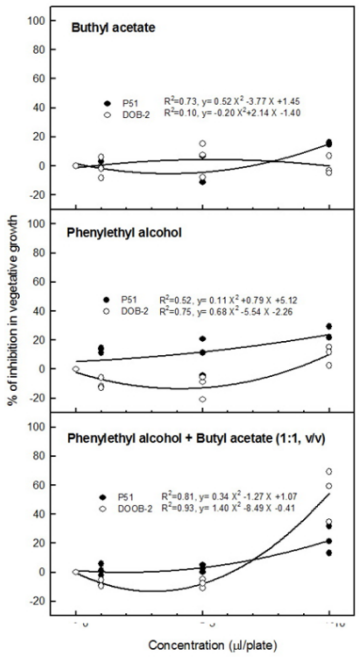


Figure 6: Effects of commercially available compounds: butyl acetate (BA) and phenylethyl alcohol (PEA) individually or in combination on vegetative growth of *P. digitatum* strains (P51 and DOB-2). Fungal strains were placed on PDA and the plates were attached to petri dish plates containing a filter paper disc with a test compound. Experiments were repeated three times with 3 replicates for each treatment. Only representatives are shown.

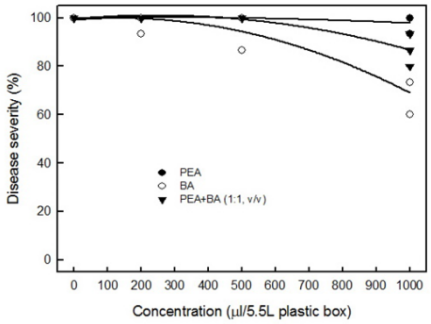


Figure 7: Effects of commercially available compounds: butyl acetate (BA) and phenylethyl alcohol (PEA) individually or in combination, on the reduction of citrus green mold caused by *P. digitatum*. Filter paper discs spotted with various with chemicals were placed in a petri dish along with citrus fruit inoculated with conidial suspension of the *P. digitatum* P51 strain and incubated in a moist box for 7 days at room temperature.

The chemical properties and the biological activities of volatile compounds produced by *E. cloacae* were characterized to be inhibitory to fungal pathogen *P. digitatum* causing citrus green mold. The environmental conditions that might influence the production of volatile compounds by *E. cloacae* and its effectiveness of controlling green mold were also investigated. Temperatures and substrates used to culture *E. cloacae* and pH values of medium all affected *P. digitatum*-induced disease severity on citrus fruit. The efficacy of a well-known volatile antimicrobial producer *Muscodor albus* as a biofumigant also is affected by temperatures [42,43]. Nevertheless, our results provide valuable information in determining the importance and practicability of utilizing *E. cloacae* as a biocontrol means in controlling citrus green mold.

Using plate-to-plate assays, it was noticed that *E. cloacae* grown at temperatures ranging from 16°C to 28°C was able to suppress the germination of conidia produced by *P. digitatum*. Green mold incidence on citrus stored at temperatures ranging from 8°C to 20°C also was suppressed using E010-KB-Perlite (EKP) formulation. *Enterobacter cloacae* grown in acidic pH tended to exhibit stronger toxicity toward *P. digitatum* than that grown in alkaline conditions. This could be related to bacterial growth as *E. cloacae* grew much better in acidic environments [44]. We found that the composition of the medium can impact the bacterial growth and the type of volatile compounds produced by *E. cloacae*. In the end, a combination by growing *E. cloacae* E010 strain in 5-fold KB medium and perlite (EK_5P) was found to be the most effective formula for controlling green mold. EK_5P seems to provide much protection against green mold, keeping citrus fruit from the disease up to 3 weeks. Perlite is a volcanic stone comprising silicon dioxide, ammonium oxide, and many other minerals, and has been widely used in the industry and agriculture sectors. Owing to its high permeability and low water retention, perlite is often used as a soil amendment and as a carrier for fertilizers, herbicides and pesticides [45].

Enterobacter cloacae is not a prolific producer of volatile compounds. Only an isolate of *E. cloacae* has been reported to emit ammonium [19]. Although only three volatile compounds were identified from analysis of gas phase of *E. cloacae* in this study, the bacterium demonstrates its impressive biological activity against *P. digitatum* and citrus green mold. It is very likely that changing the composition of growth medium may increase the number and the type of volatile compounds produced by *E. cloacae* as suggested in gas-producing fungus *Muscodor albus* [28]. Moreover, a combination of three volatile compounds (4,5-dimethyl-1-hexene, butyl acetate and phenylethyl alcohol) is likely required to exert a stronger inhibitory effect against the target test *P. digitatum*. Phenylethyl alcohol is a common gas released by bacteria and fungi and exerts antimicrobial activities against various plant pathogenic fungi at very high levels or in cooperation with other volatile compounds [33,40,41,46-48]. Phenylethyl alcohol has rose-like flavors and has been demonstrated to induce germination of ascospores, but suppress conidial germination in *Neurospora crassa* [49,50]. Phenylethyl alcohol affects membrane permeability, causes the alternation of amino acid and sugar transport systems, and inhibits the synthesis of macromoleculars and thus, is inhibitory to microorganisms [51,52]. Like trytophol, phenylethyl alcohol is involved in fungal quorum sensing [53]. Butyl acetate, also known butyl ethanoate, has sweet smell of banana or apple and can be inhibitory to bacteria [54].

Although artificial application of volatile compounds did not fully suppress fungal growth and conidial germination, they mimicked the inhibitory effects to some extent. The differences in activity between the *E. cloacae*-involved production of volatiles and commercially

available products may relate to the quantitative production of the volatile compounds produced by E010, which cannot be determined by our GC/MS methodology. Another explanation for less inhibitory effect of commercially available products on *P. digitatum* is that volatile compounds exert their maximal toxicity in a cooperative manner. Exogenous application of butyl acetate or phenylethyl alcohol individually had little effect on conidial germination and radial growth of *P. digitatum*, whereas a combination of both compounds inhibits conidial germination and vegetative growth. The results confirm the synergistic effect of volatile compounds in terms of their toxicity toward *P. digitatum*. Similar synergism among volatile organic compounds in increasing antimicrobial activity has also been observed [28,55]. The toxicity of 4,5-dimethyl-1-hexene alone to *P. digitatum* remains uncertain. However, of three volatile compounds identified from gas phase of *E. cloacae*, 4,5-dimethyl-1-hexene is the predominant compound. Judging from differences in activity between the *E. cloacae*-producing volatile compounds and commercially available butyl acetate and phenylethyl alcohol, it appears that 4,5-dimethyl-1-hexene plays an important role in fungistatic effects against *P. digitatum*, as well as in suppressing green mold incidence. Evaluation of the toxicity of 4,5-dimethyl-1-hexene will be the next logical step toward better understanding of how *E. cloacae* exerts its antimicrobial ability.

Delayed application of EKP could be ineffective in controlling green mold, since volatile compounds emitted by *E. cloacae* only inhibit but do not kill *P. digitatum*. Further experiments to determine the optimal growth condition for *E. cloacae* that provides the most effective measure in controlling citrus green mold in a closed chamber during storage and shipment are warranted. It will also be of interest to determine if volatile compounds produced by *E. cloacae* can be inhibitory to other plant pathogenic fungi and if EKP formula is useful in controlling other plant diseases. The study also paves a way for future research on how to formulate an effective biocontrol agent for disease management.

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