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In vitro inhibition of postharvest pathogens of fruit and control of gray mold of strawberry and green mold of citrus by aureobasidin A

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Abstract

Aureobasidin A (AbA), an antifungal cyclic depsipeptide antibiotic produced by *Aureobasidium pullulans* R106, has previously been shown to be effective against a wide range of fungi and protozoa. Here we report the inhibitory effects of AbA on spore germination, germ tuber elongation and hyphal growth of five pathogenic fungi including *Penicillium digitatum*, *P. italicum*, *P. expansum*, *Botrytis cinerea* and *Monilinia fructicola*, which are major pathogens causing postharvest diseases of a variety of fruits. AbA inhibited five pathogenic fungi by reducing conidial germination rates, delaying conidial germination initiation, restricting elongation of germ tuber and mycelium, as well as inducing abnormal alternations of morphology of germ tubes and hyphae of these fungi. The sensitivity of these fungi to AbA was pathogen species-dependent. *P. digitatum* was the most sensitive and *M. fructicola* the least. Importantly, AbA at 50 μg/ml was effective in controlling the citrus green mold and in reducing the strawberry gray mold incidence and severity, caused by *P. digitatum* and *B. cinerea,* respectively, after artificial inoculation. AbA and/or its analogs, therefore, hold promise as relatively safe and promising fungicide candidates to control postharvest decays of fruits, because AbA targets the inositol phosphorylceramide (IPC) synthase, an enzyme essential for fungi but absent from mammals.

Keywords: Aureobasidin A (AbA); Antifungal activity; Postharvest fruits; Disease controlling

1. Introduction

Storage diseases, especially those caused by fungal pathogens, are responsible for substantial postharvest loses. It is not only a problem for the producers, but also it persists throughout the distribution chain and affects the cost and availability of the product to the consumer (Arul, 1994). To prevent fruit decays, fungicides, such as sodium *o*-phenylphenate (*o*-phenylphenol), thiabendazole and imazalil, are routinely used preharvest or postharvest (Eckert and Ogawa, 1988; Holmes and Eckert, 1999). However, in consequence of the intensive use of fungicides, the emergence and development of fungicide-resistant subpopulations of postharvest pathogens have developed: decreasing the performance of fungicides in fruit production (Eckert et al., 1994; Holmes and Eckert, 1999; Zhu et al., 2006). Coupled with public concerns over chemical residues in the food chain and environmental safety, there is an urgent need to develop alternative technologies or novel fungicides with good efficacy, low residues, a lack of cross-resistance with existing fungicides, and little or no toxicity to non-target organisms for postharvest disease control (Arul, 1994; Wisniewski and Wilson, 1992).

Aureobasidin A (AbA), an antifungal cyclic depsipeptide antibiotic produced by *Aureobasidium pullulans* R106 (Takesako et al., 1991), has potent fungicidal activity against a variety of fungi, including yeast (*Saccharomyces cerevisiae*) and some important human yeast-like pathogenic fungi in the genus of *Candida* and *Cryptococcus*, and protozoa (Takesako et al., 1993; Endo et al., 1997; Zhong et al., 2000; Sonda et al., 2005; Denny et al., 2006). The mode of action of AbA is blocking the activity of inositol phosphorylceramide (IPC) synthase, an

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essential enzyme for fungal sphingolipid biosynthesis that is absent in mammals (reviewed in Obeid et al., 2002; Dickson et al., 2006; Dickson and Lester, 2002). The fact that AbA specifically targets IPC synthase of fungi makes it a particularly attractive compound for controlling opportunistic fungi infecting immuno-compromised patients, such as those with AIDs, cancer, or organ transplants (Sugimoto et al., 2004; Nagiec et al., 1997). Indeed, AbA was shown to have little toxicity to mice (Takesako et al., 1993).

In this study, we examined the antifungal activity of AbA against five postharvest fruit pathogenic fungi *Penicillium digitatum*, *P. italicum*, *P. expansum*, *Botrytis cinerea*, and *Monilinia fructicola in vitro and in vivo*. Our results showed that AbA is capable of inhibiting these fungi and providing effective control of two diseases caused by *P. digitatum* and *B. cinerea* in the laboratory setting, suggesting that AbA or its analogs are promising fungicide candidates for controlling postharvest disease of fruits and vegetables.

2. Materials and methods

2.1. Strains and chemicals

P. digitatum and *P. italicum* were isolated from diseased citrus fruits with typical green and blue mold symptoms, respectively. *B. cinerea* was collected from infected strawberry showing typical gray mold symptom. To obtain single-spore cultures of these pathogens, appropriately diluted conidial suspensions were spread on PDA medium and individual single-spore colonies were picked and the identities of these isolates were later confirmed based on their respective morphological characteristics of conidiophores and conidia, as well as their growth features (Lu, 2001). *P. expansum*, isolated from an infected apple, was a generous gift from Dr. Yu (Yu et al., 2006), while *M. fructicola* isolated from an infected peach, was kindly provided by Dr. Ma (Ma et al., 2003). The strains were cultured at 25 °C on the potato-dextrose agar (PDA) and maintained at 4 °C. Conidial suspensions were prepared by flooding 7-day-old sporulating cultures with sterile distilled water, filtering the conidial solution through three-layers of cheese-clothes, centrifuging and then re-suspending it. Spore concentrations were determined by a hemacytometer and diluted with sterile distilled water as required. Aureobasidin A (AbA) (analytical pure), purchased from Takara (Japan), was dissolved in methanol solution (2 mg/ml) and stored at 4 °C until used.

2.2. Effect of AbA on spore germination

The effect of AbA on conidial germination of *Penicillium* spp., *B. cinerea* and *M. fructicola* were assessed on PDA amended with AbA at concentrations of 2, 4, 8, and 16 μl/mg. Ten-microliter of spore suspension (2×10^5 spores per ml) was transferred to the medium loaded on the sterilized glass previously and spread evenly using a sterilized bent glass rod. For the control, instead of AbA an amount of same volume of methanol was added to the medium. After 12 h incubation at 25 °C, about 200 conidia per replicate were examined microscopically (Nikon YS100). The number of germinated conidia (with germ tube longer than two times of their diameters of corresponding conidia) for each treatment was counted and the respective percentage of inhibition of germination was calculated. Each treatment was replicated three times and the experiment was conducted twice. The time course of germination inhibition on 8 μg/ml AbA-containing-PDA for each fungus was conducted by microscopically determining the germination percentage at 2 h intervals that started and ended at 8 and 24 h after incubation, respectively.

2.3. Effect of AbA on germ tube elongation and mycelial growth

The spore suspensions were prepared as described previously and spread on PDA amended with 8 μg/ml AbA. The germ tubes were measured using Carl Zeiss MicroImaging GmbH system (Axioskop 2 plus, Germany) after 12 h incubation for *B. cinerea* and *M. fructicola,* and 20 h for *Penicillium* spp. at 25 °C. Inhibitory effects of AbA on mycelial growth of the five fungi were investigated by spot-inoculating 2 μl of each conidial suspension (2×10^5 conidia per ml) on PDA containing AbA at graded concentrations. The lowest AbA concentrations in the PDA plates that resulted in the failure to form colony after 96 h incubation were regarded as the MIC (minimum concentration that completely inhibits growth).

2.4. Effect of AbA on the morphologies of the five phytopathogenic fungi

To investigate how AbA affects spore germ tube formation, we compared the morphological aspects of the germ tubes formed on AbA-free with those on AbA-containing medium (4 μg/ml for *Penicillium* spp., 8 μg/ml for *B. cinerea* and *M. fructicola*) after incubation (12 h for AbA-free and 24 h for AbA-added medium) under a microscope. To further test the effect of AbA on mycelial morphology, a two-microliter spore suspension (2×10^4) was spot-inoculated on the AbA-free PDA plate. After 48 h of inoculation, two and half microliter AbA with respective concentrations of 2, 4, 8, 16, 32, and 64 μg/ml was added to the edge of the vigorous growth colony (tip of hyphae). The morphological change was determined by examining the mycelial tips grown in AbA-free and AbA-added location after 48 h incubation. The photographs were taken using Carl Zeiss MicroImaging GmbH system (Axioskop 2 plus, Germany).

2.5. Efficacy of AbA in controlling citrus green mold and strawberry gray mold in vivo

The disease- and wound-free citrus and strawberry fruits were purchased from a public market and washed with tap-water and sterilized with 70% ethanol. Once they had dried, the fruits were dipped in 50 μg/ml AbA solution for 1 min, and dried again at room temperature. For the control, the fruits were dipped in sterilized water or 250 μg/ml imazalil solution

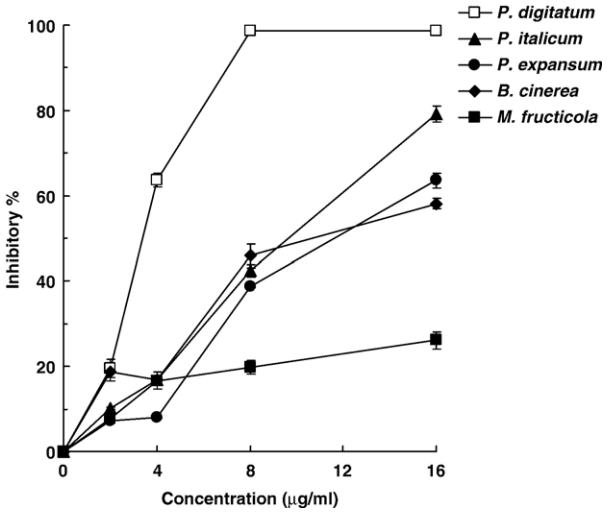

Fig. 1. Inhibitory activity of AbA on conidial germination of five fungal pathogens *in vitro*. Bars represent the standard deviations of three replicates.

(Deccozil, 250 EC, Elf Atochem North America, Inc.). Five microliter conidial suspension (2×10^5) of *P. digitatum* and *B. cinerea* was spot-inoculated on the equator of the citrus and strawberry fruits, respectively. For citrus, the fruits were wounded approximately 1-mm-deep using a sterilized needle before the inoculation. After they had dried, the inoculated fruits were kept in enclosed plastic trays to maintain a high relative humidity at room temperature (20±2 °C). The incidences of disease were determined daily after treatment. The fruits with water-soaked, white mold that gradually turned into green were regarded as infected. There were three replicates with 10 to 15 fruits per replicate and the experiment was conducted twice.

2.6. Statistical analyses

The data were analyzed by an analysis of the variance (ANOVA) for the number of treatments was more than three and followed by LSD if the treatment was significant at $P=0.05$, or by a student *t*-test for two treatments, in a statistical program (DPS, Tang and Feng, 2002).

3. Results

3.1. Inhibitory effect of AbA on spore germination

The effect of AbA on spore germination of *P. digitatum*, *P. italicum*, *P. expansun*, *M. fructicola* and *B. cinerea* was examined on PDA containing various concentrations of AbA after 12 h incubation. As shown in Fig. 1, AbA inhibited conidial germination of all pathogens tested, and the degree of inhibition was pathogen-specific. *P. digitatum* was the most sensitive to AbA treatment, followed by *P. italicum, P. expansum* and *M. fructicola*. For *P. digitatum*, more than 50% of the conidia were inhibited by 4 μg/ml AbA, and almost complete inhibition of spore germination was observed when AbA concentration was increased to 8 μg/ml. In contrast, germination of less than 30% of conidia of *M. fructicola* was inhibited when the AbA concentration was increased to 16 μg/ml (Fig. 1).

To test whether AbA could kill conidia or just delay the germination initiation, the time courses of inhibitory effect of AbA at concentration of 8 μg/ml on conidial germination of theses pathogens were further investigated at 2 h interval, started at 8 h and ended at 24 h after treatment. As shown in Fig. 2, inhibition of conidial germination decreased as the incubation period extended. After 24 h of incubation, the inhibition percentages for *P. digitatum*, *P. italicum*, *P. expansun* were 74.6, 57.4 and 46.8%, respectively. The inhibition rate decreased further with the extension of the incubation time (data not shown), but the germination initiation was markedly delayed and the morphologies of the germ tubes of these fungi were abnormal. For *B. cinerea* and *M. fructicola*, the inhibition rates, after 24 h of incubation, decreased to 2.0% and 6.0%, respectively. These results clearly indicate that AbA at 8 μg/ml operated by delaying the conidial germination initiation rather than killed these spores, especially in the cases of the fungi of *B. cinerea* and *M. fructicola*. This experiment corroborated the prior experiment that AbA was more effective against conidial germination of *Penicillium* spp. than against that of *B. cinerea* and *M. fructicola*.

3.2. Inhibitory effect of AbA on elongation of germination tube and mycelial growth

After 24 h incubation in AbA at a concentration of 8 μg/ml failed to completely inhibit spore germination of *P. digitatum*, the most sensitive strain, and did not stop the germination of either *B. cinerea* or *M. fructicola* conidia. Indeed, the complete inhibition of spore germination of *B. cinerea* and *M. fructicola* did not occur when the AbA concentration was increased to 64 μg/ml (data not shown). However, AbA at 8 μg/ml did markedly retard the elongation of germ tubes for all pathogens tested (Fig. 3). The inhibitory effect of AbA on the elongation of germ tube increased with the increase of AbA concentration in the medium (data not shown).

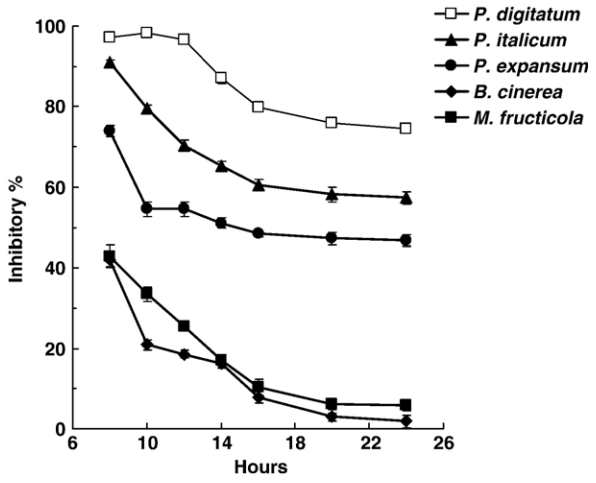

Fig. 2. Time courses of inhibitory effect of AbA on conidial germination of five pathogens. Bars represent the standard deviations of three replicates. AbA was at concentration of 8 μg/ml.

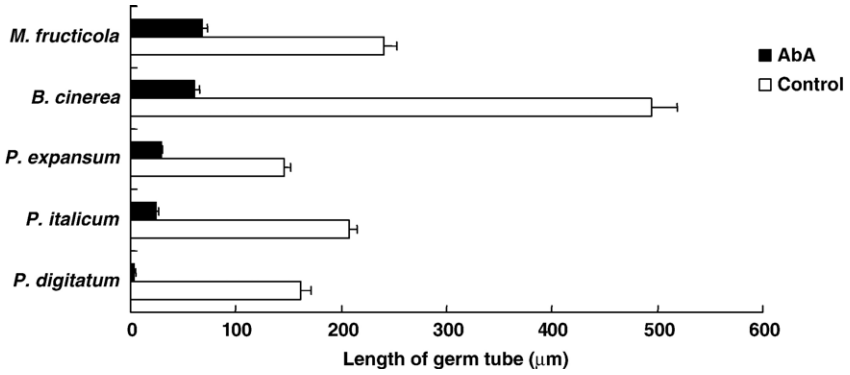

Fig. 3. Inhibitory effect of AbA on germ tuber elongation of five pathogens. Bars represent the standard deviations of three replicates. AbA was at concentration of 8 μg/ml.

About 48 h after starting the culture, *P. digitatum* formed colony with thick mycelia on the PDA plate free of AbA (Fig. 4 A). In contrast, *P. digitatum* on 2 μg/ml AbA-amended PDA produced colonies with sparse mycelia even after 96 h incubation (Fig. 4 B), and sign of mycelial growth was not observed when the AbA in the medium was increased to 6 μg/ml (MIC, the minimum concentration that resulted in the failure of colony formation after 96 h of incubation). The mycelial growth of the other pathogens was also greatly affected when the level of AbA in the medium was above 4 μg/ml. The colonies of *B. cinerea* produced on 8 μg/ml AbA-amended and AbA-free media were compared and presented in Fig. 4 C and D. The MIC (no formation of colonies after 96 h of incubation) for *P. italicum*, *P. expansum*, *B. cinerea* and *M. fructicola* was determined to be 24, 32, 32 and 48 μg/ml, respectively.

3.3. Dramatic effect of AbA on morphology of germling and hyphae of five phytopathogenic fungi

The effect of AbA on the morphology of these pathogens was examined by comparison the microscopic morphologies of germlings and mycelia on AbA-free PDA with those on AbA-contained PDA. We found that the germling grown in AbA-free PDA exhibited highly polarized growth through apical extension. The branching never occurred in tip cell of germling. The tip cell of the germling was long with constant width

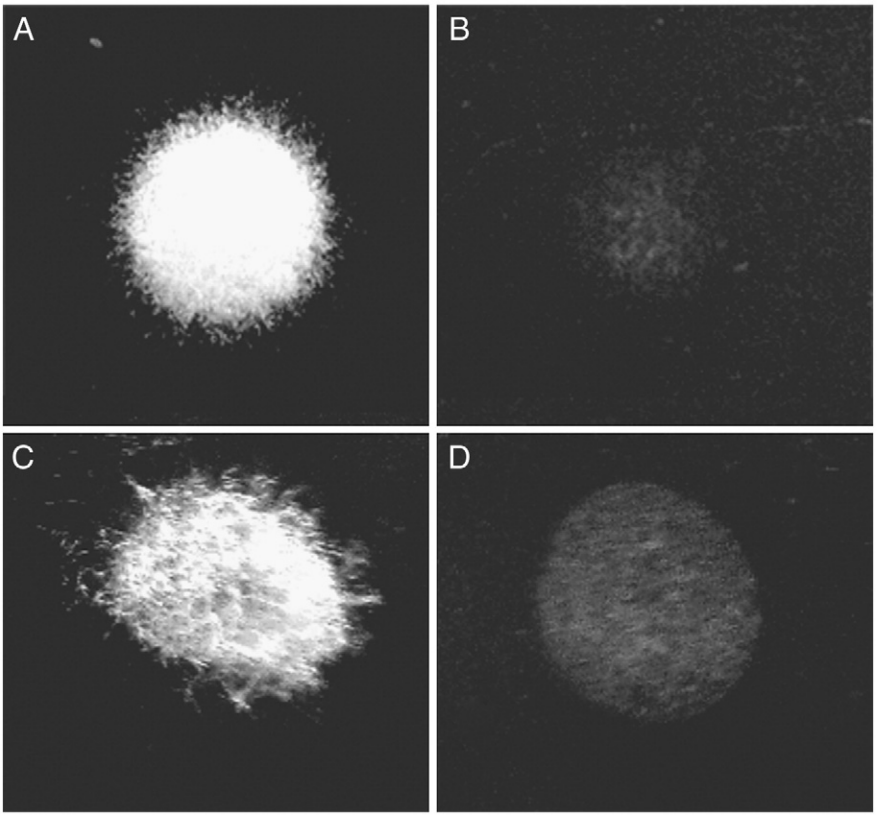

Fig. 4. Effect of AbA on mycelial growth of *P. digitatum* (A and B) and *B. cinerea* (C and D). Conidial suspensions of both pathogens were spot-inoculated onto the AbA-free (A and C) or AbA-amended PDA (B, 2 μg/ml; and D, 8 μg/ml). The photos of colonies were taken after 48 h of culture.

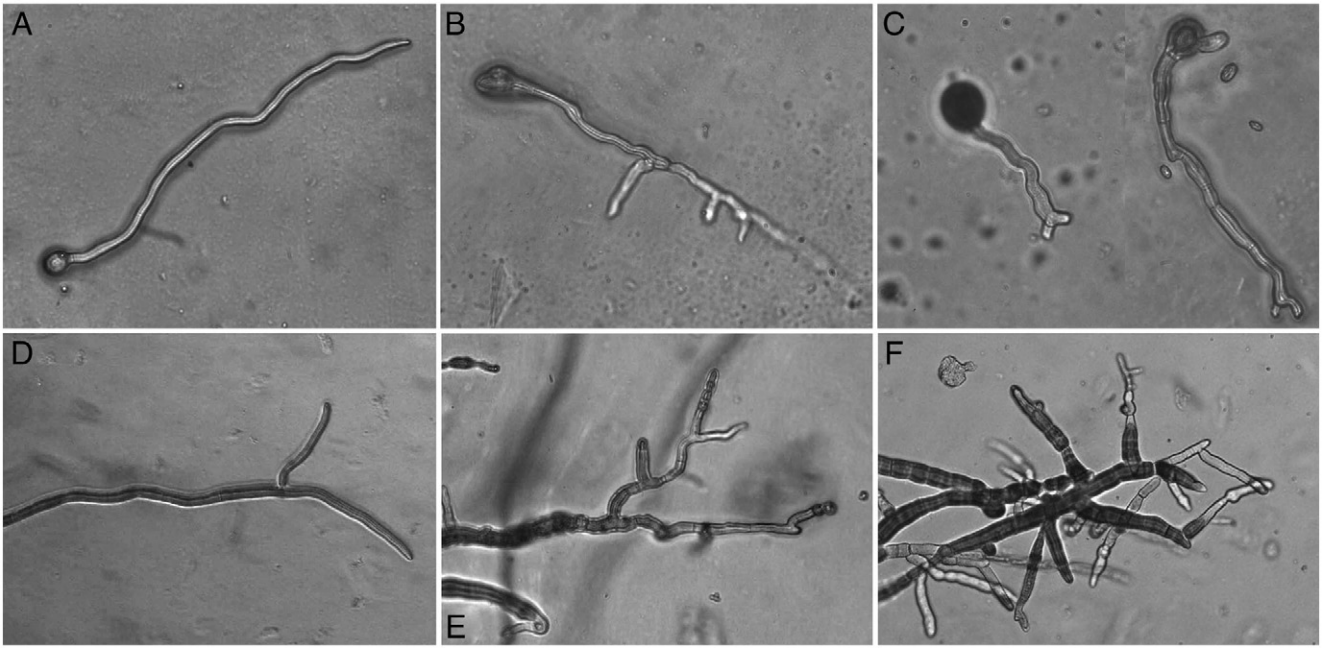

Fig. 5. Morphologic effect of AbA on germling (A–C) and mycelium (D–F) of *B. cinerea.* A. Germlings developed in AbA-free PDA; B and C, Germlings developed in 8 μg/ml AbA-amended PDA. D, Hyphae grew in AbA-free PDA ; E and F, Hyphae grew in 16 μg/ml AbA-amended PDA.

(Fig. 5 A, *B. cinerea* was only presented). In contrast, the germling grown in PDA with 4 μg/ml AbA for *Penicillium* spp. and 8 μg/ml for *B. cinerea* and *M. fructicola* exhibited severe morphological abnormality as indicated by the occurrence of branching near the tip. The cells of germling were swollen with inconsistent width and shortened due to the abnormal increase of septum. The cytoplasm was condensed to different degree (Fig. 5, B to C, only *B. cinerea* is shown).

Microscopic examination on mycelia treated with AbA after it had grown for 48 h revealed that AbA below 4 μg/ml did not cause significant morphological changes to mycelia of all five fungi. Significant alternations were observed when the AbA concentrations were above 16 μg/ml for *P. digitatum* and *B. cinerea*, and 32 μg/ml for the other fungi, indicating that the mycelia, for which the polarized growth had been established, were less sensitive to AbA than the germlings. The morphological alternations, induced by AbA, of hyphae of these five fungi were similar to those observed in their corresponding germlings (Fig. 5 D to F, *B. cinerea* was only presented).

Table 1
Effect of AbA on disease incidence of citrus green mold and strawberry gray mold

Pathogens	*P. digitatum*		*B. cinerea*	
Incidence of disease	Mean [a]	Se	Mean [a]	Se
Water	100.0±0.0	A	100.0±0.0	A
AbA (50 μg/ml)	3.0±3.0	C	57.3±4.0	B
Imazalil (250 μg/ml)	24.4±3.4	B		

[a] Means in a column followed by different letter are significantly different (LSD, $P<0.01$ for both *P. digitatum* and *B. cinera*, *t*-test). The observation was made 7 days after treatment for citrus and 3 days for strawberry.

3.4. Efficacy of AbA in controlling citrus green mold and strawberry gray mold

As shown in Table 1, AbA (50 μg/ml) provided excellent (better than that of imazalil) control of green mold of citrus fruits and also was effective in reducing infection rate of strawberry gray mold under artificial inoculation conditions. Seven days after inoculation, 100% control citrus fruits developed water-soaked lesions with initially white and gradually turned into green mold, whereas the 97.0% AbA-treated fruit formed a fine and dried dot on the wounded pericarp. For the strawberry, 57.3% AbA-treated fruits were infected compared with the 100% incidence of disease in the control. Moreover, the disease produced on the infected strawberries of the AbA-treated fruits was much less severe than that of the control in terms of both the extent of spoilage and the mold density (data not shown).

4. Discussion

Effects of the antifungal agent Aureobasidin A (AbA) on five postharvest fruit pathogenic fungi *in vitro*, and green mold and gray mold of postharvest citrus and strawberry respectively *in vivo* were investigated in the present study. AbA was capable of inhibiting these fungal pathogens and providing effective control of citrus green mold and some control strawberry gray mold caused by *P. digitatum* and *B. cinerea* respectively in the artificial inoculation setting. We show that AbA exerts its antifungal effect by affecting various aspects of fungal development, including: 1) reducing spore germination rate, 2) retarding germination initiation, 3) disrupting polarized growth of germ tube and slowing its elongation, 4) abolishing established polarity of mycelia. These are consistent with the previous findings that AbA

altered the normal bud growth in yeast, and disrupted polarized growth of germ tuber and abolished the established polarity of *A. nidulans* hyphae *via* aberrant actin assembly (Endo et al., 1997; Cheng et al., 2001). The disruption of normal polarized growth, induced by AbA, of all the five fungi ultimately resulted in dramatic alternations in their morphology, characterized by excessive branching near the tip of germ tuber and hyphae. The fact that, in the artificial inoculation setting, AbA provided effective control of citrus green mold and achieved significant reduction of strawberry gray mold infection indicates that AbA or its analogs could be used as a novel type of fungicides for fighting plant disease and postharvest decay.

Among the five pathogens examined, *P. digitatum* was the most sensitive to AbA, whereas *M. fructicola* exhibited the least sensitivity. Moreover, for the same fungal pathogen, the stage of germling was more sensitive than the stages of spore germination initiation and hyphal extension. However, in comparison with yeast and *Candida* spp., the sensitivities of these presently investigated fungi to AbA are much lower and species-dependent (Takesako et al., 1993; Zhong et al., 2000). It was reported that the low sensitivity of some *Aspergillus* spp. to AbA was associated with the increased efflux, and that co-treatment of AbA and mammalian multidrug resistance modulator sverapmil, chlorpromazine, and trifluoperazine dramatically increased the sensitivity of *A. fumigatus* to AbA (Zhong et al., 2000; Ogawa et al., 1998). It would be interesting to determine whether the low sensitivity of *M. fructicola* is due to increased efflux of AbA.

It has been demonstrated that AbA targets the inositol phosphorylceramide synthase (IPC1p), a key enzyme catalyzing sphingolipid synthesis in fungi. Homologues of *AUR1*/*IPC1* gene were identified in a number of human pathogenic fungi in *Candida, C. neoformans*, *A. fumigatus* and other fungi, including *Schizosaccharomyces pombe*, *A. nidulans*, *A. niger*, *A. oryzae*, thus IPC1p is thought to be evolutionally conserved across various fungi (Kuroda et al., 1999; Do et al., 2005; Heidler and Radding, 1995, 2000). Indeed, the homologues of *AUR1* were found in some plant pathogenic fungi such as *B. cinerea*, *Fusarium graminearum, Sclerotinia sclerotiorum* and *Ustilago maydis* (unpublished data), for which their partial genomic sequences are available, suggesting it is likely that most in phytopathogenic fungi contains the AbA target. The results presented in this study clearly validate this hypothesis. The demonstration that AbA is effective in controlling green mold and gray mold of citrus and strawberry respectively in this study further indicates that AbA or its homologous compounds could be promising fungicide candidates for control of plant, especially postharvest fruit and vegetable disease. Rustmicin is an antifungal antibiotic possessing such an inhibitory mechanism (Mandala et al., 1998).

Unlike in fungi, the predominant sphingolipids in plant tissues are glucosylceramides although complex glycophosphosphingolipids including inositolphosphorylceramides (IPCs), which are the predominant sphingolipids in fungi (Lester and Dickson, 1993), are found (Dunn et al., 2004; Lynch, 1993; Bromley et al., 2003). Homologues of the fungal IPC synthase (*AUR1*/*IPC1*) gene can not be readily identified in the complete genome database of *Arabidopsis thaliana* (Dunn et al., 2004). Surprisingly, search of the complete genomic database of *Magnaporthe grisea* failed to reveal a candidate homologue of *AUR1* (this study, unpublished data), suggesting that considerable divergence of IPC synthase could occur during evolution between plants and fungi or within the plants or fungi. Whether application of AbA is safe to plants remains to be explored. However, our limited inoculation study revealed no injury on AbA-treated strawberry and citrus. It is possible that AbA might be incapable of targeting plant IPC synthases because their structure is so different from that of the fungal IPC synthases.

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