Inhibitory effects of acetophenone or phenylethyl alcohol as fumigant to protect soybean seeds against two aflatoxin-producing fungi

Sawai Boukaew¹ · Poonsuk Prasertsan²

Revised: 5 October 2018 / Accepted: 16 October 2018 / Published online: 19 October 2018
© Association of Food Scientists & Technologists (India) 2018

Abstract The antifungal activity of acetophenone and phenylethyl alcohol prevents seed contamination by *Aspergillus flavus* TISTR 3041 and *A. parasiticus* TISTR 3276. Their effects on seed germination were investigated. In vitro results showed that 100 $\mu\text{L L}^{-1}$ acetophenone completely inhibited (by 100%) growth, conidial germination, and sporulation of the two aflatoxin-producing fungi, while phenylethyl alcohol showed only weak inhibitory activity even at 1000 $\mu\text{L L}^{-1}$. Exposure to acetophenone at 100 $\mu\text{L L}^{-1}$ for 6 h could completely kill (100% death) both fungal strains, while phenylethyl alcohol showed much lower efficacy (53.12%). In vivo results revealed that fumigation with 100 $\mu\text{L L}^{-1}$ acetophenone for 24 h completely controlled (100%) *A. flavus* TISTR 3041 on soybean seeds during a 14-day test but exhibited weak efficacy on *A. parasiticus* TISTR 3276 (31.77%). Phenylethyl alcohol (1000 $\mu\text{L L}^{-1}$) demonstrated weak inhibitory effect against both strains. The two volatile compounds had no adverse effects on seed germination. SEM confirmed that acetophenone could completely inhibit conidia germination, and abnormal growth of both fungal strains was observed. Thus, acetophenone has high

potential to protect soybean seeds against aflatoxin-producing fungi.

Keywords Aflatoxigenic fungi · Acetophenone · Fumigant activity · Fungal ultrastructure · Phenylethyl alcohol

Introduction

Soybean (*Glycine max* (L.) Merrill) is one of the most important oil seed crops and protein sources in the world. Contamination of cereal and grains by fungi is a chronic problem in the warm and humid warehouses of tropical countries. Various microorganisms, especially *Aspergillus flavus* and *A. parasiticus*, which are the most common spoilage fungi in food and stored grains, are known to produce aflatoxins (Ng'ang'a et al. 2016). Protection against these is commonly pursued by fumigation. The fumigants can be acquired from microorganisms or from natural compounds (Svircev et al. 2007).

In recent years, synthetic pesticides have been replaced by other types of volatile compounds in the control of food spoilage or food-borne pathogens (Li et al. 2010; Wang et al. 2013; You et al. 2015; Zheng et al. 2013). Volatile compounds that are active against *Penicillium italicum* include dimethyl disulfide, dimethyl trisulfide, d-limonene, aromadendrene, isolekene, caryophyllene, phenylethyl alcohol, and acetophenone (Li et al. 2010). There are reports on the efficacy of isoamyl acetate, isoamyl alcohol, and phenethyl alcohol against *A. brasiliensis* (Ando et al. 2012), phenylethyl alcohol, and α -terpineol against *A. flavus* NKDW-7 (Prakash et al. 2015), heneicosane, 2-nonanone, 2-decanone, 2-methylpyrazine, heptadecane, diethyl phthalate, β -benzene ethanamine, BHT, gentisic acid, thymol, and n-hexadecanoic acid against *Colletotrichum*

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s13197-018-3458-6>) contains supplementary material, which is available to authorized users.

✉ Sawai Boukaew
sawai.bo@skru.ac.th

¹ College of Innovation and Management, Songkhla Rajabhat University, Songkhla 90000, Thailand

² Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University, Songkhla 90112, Thailand

gloeosporioides (Zheng et al. 2013), and 2,4-di-tert-butylphenol against *Phytophthora capsici* (Sang and Kim 2012). The volatile compounds against *F. oxysporum* include acetophenone and benzyl alcohol (Raza et al. 2015), phenylethyl alcohol, 1H-indene, 2,3-dihydro-4,7-dimethyl, biphenyl, methyl *N*-(1H-benzimidazol-2-yl) carbamate (Yuelian and Qingfang 2013), and 1-phenylbut-3-ene-2-ol (Kavitha et al. 2010).

Several compounds derived from microorganisms or plant essential oils, including acetophenone and phenylethyl alcohol, have shown remarkable antimicrobial activities in previous studies (Li et al. 2010; Naznin et al. 2014; Prakash et al. 2015). They chemically belong to ketones or alcohols and are known as antimicrobial agents. They possess antifungal activity against anthracnose pathogen (*Colletotrichum* spp.) on chili (Boukaew et al. 2018).

Microorganisms that are sources of volatile compounds include *Muscodor albus* (Corcuff et al. 2011), *Laetiporus sulphureus* (Petrovic et al. 2013), *Acremonium* sp. (Yuelian and Qingfang 2013), *Arabidopsis thaliana* (Naznin et al. 2014), *Aureobasidium pullulans* (Francesco et al. 2015), *Paenibacillus polymyxa* WR-2 (Raza et al. 2015), *Daldinia* cf. *concentric* (Liarzi et al. 2016), *Candida intermedia* (Huang et al. 2011), *Nocardia levis* MK-VL_113 (Kavitha et al. 2010), *Streptomyces philanthi* RM-1-138 (Boukaew et al. 2013), *S. platensis* F-1 (Wan et al. 2008), and *S. globisporus* JK-1 (Li et al. 2010).

Essential oils containing acetophenone and phenylethyl alcohol can be obtained from several plants such as *Angelica archangelica* (Prakash et al. 2015) and *Premna integrifolia* Linn (Rahman et al. 2016). Volatile acetophenone and phenylethyl alcohol at 100 $\mu\text{L L}^{-1}$ had an inhibitory effect on the disease incidence of *P. italicum* on Shatang Mandarin (Li et al. 2010). Phenylethyl alcohol at 2.0 $\mu\text{L mL}^{-1}$ added to solid media could inhibit (by 100%) *A. flavus* NKDW-7 (Prakash et al. 2015).

To date, the antifungal activities of acetophenone and phenylethyl alcohol against food spoilage fungi have not been extensively investigated. The main purposes of this study were (1) to investigate the antifungal activities of acetophenone and phenylethyl alcohol on the suppression of mycelial growth, conidial germination, and sporulation of the two aflatoxin-producing fungi through fumigation, (2) to evaluate the efficacy of the two volatile compounds in controlling soybean seed contamination by aflatoxin-producing fungi and their effects on seed germination, and (3) to study the mode of action of acetophenone on the two aflatoxin-producing fungi.

Materials and methods

Microorganisms and inoculum production

Aflatoxin producing fungi *Aspergillus parasiticus* TISTR 3276 and *A. flavus* TISTR 3041 (Sangmanee and Hongpattarakere 2014) were obtained from the Thailand Institute of Scientific and Technological Research (TISTR), Phatumthani, Thailand. They were grown on potato dextrose agar (PDA; 39 g L^{-1} ; Difco Laboratory) at 4 °C. Spore inocula of pathogenic fungi were collected in 5 mL of water from 7-day-old cultures. The spore count was performed using a hemacytometer before dilution with distilled sterilized water to the required concentration.

Technical grade pure volatile compounds

Pure commercial compounds (technical grade) of acetophenone (Fluka, for GC, $\geq 99.5\%$) and phenylethyl alcohol (Sigma–Aldrich $\geq 99\%$, FCC, Kosher, FG) were used in this study.

Evaluation of antifungal activity of acetophenone and phenylethyl alcohol on mycelial growth and conidial germination of the two aflatoxin producing fungi

The two volatile compounds were tested for antifungal activity on mycelial growth and conidial germination of two aflatoxin-producing fungi, *A. parasiticus* TISTR 3276 and *A. flavus* TISTR 3041, on PDA using an antifungal bioassay (Li et al. 2012). Four small Petri dishes (50 mm diameter $\times$ 15 mm height) were placed inside a larger Petri dish (140 mm diameter $\times$ 20 mm height with 0.5 L inner volume). Three smaller dishes contained 5 mL of PDA inoculated with a 0.5 cm diameter fungal plug from the periphery of an actively grown culture of aflatoxin-producing fungi, while the fourth dish contained a piece of autoclaved filter paper (Whatman®), to which a volatile compound was added at the concentrations 1, 10, 100, and 1000 $\mu\text{L L}^{-1}$ (droplet to filter paper in an enclosed Petri dish system). The Petri dishes were covered with a large lid, sealed with Parafilm M® and incubated at 28 ± 2 °C for 5 days. Equivalent amounts of sterile distilled water were used as a control. After the diameters of the colonies on the three plates were measured, the inhibition percentage was calculated using the following formula.

$$\text{Inhibition(\%)} = [(R1 - R2) / R1] \times 100$$

where R1 = mycelial growth of the aflatoxin-producing fungi alone on PDA (control) and R2 = mycelial growth of the aflatoxin-producing fungi on PDA fumigated with acetophenone or phenylethyl alcohol.

For the conidial germination in this assay, four small Petri dishes (50 mm diameter $\times$ 15 mm height) were placed inside a larger Petri dish (140 mm diameter $\times$ 20 mm height with a 0.5 L inner volume). Fifty μL of spore inoculum (1×10^4 spore mL^{-1}) of the aflatoxin-producing fungi were spread on three of the smaller dishes containing 5 mL of PDA and the fourth dish was filled with the gaseous components of each volatile compound that varied in concentrations of 1, 10, 100, and 1000 $\mu\text{L L}^{-1}$ (droplet to filter paper in an enclosed Petri dish system). Equivalent amounts of sterile distilled water were used as a control. After incubation at $28 \pm 2^\circ\text{C}$ for 24 h, the conidial germination on the smaller plates was observed and the inhibition of conidial germination was determined by the previously mentioned formula. There were three replicates for each treatment.

Evaluation of antifungal activity of acetophenone and phenylethyl alcohol on sporulation of the two aflatoxin-producing fungi on PDA plates and on soybean seeds

The effects of the two compounds on sporulation of the aflatoxin-producing fungi on PDA plates and on soybean seeds were investigated using an antifungal bioassay. In the PDA plate experiment, 50 μL of spore inoculum (1×10^4 spore mL^{-1}) of the aflatoxin-producing fungi were spread on each of the three smaller dishes containing 5 mL of PDA and the fourth dish was filled with the gaseous components of each volatile compound matching liquid concentration 1, 10, 100, and 1000 $\mu\text{L L}^{-1}$ (droplet to filter paper in an enclosed Petri dish system). Equivalent amounts of sterile distilled water were used as a control. After incubation at $28 \pm 2^\circ\text{C}$ for 5 days, the total number of conidia per plate was assessed by mixing with sterile water in a serial dilution (1×10^3) after which the number of conidia was counted under a compound microscope.

Soybean seeds were prepared by soaking in 100 mL of distilled water for 5 h and then autoclaving at 121°C for 15 min. Five soybean seeds were transferred onto each of the three smaller dishes (total of 15 seeds), and then a 50- μL spore inoculum of aflatoxin-producing fungi at 1×10^4 spore mL^{-1} was spread on each soybean seed while the fourth dish contained gaseous acetophenone or phenylethyl alcohol matching liquid concentration 1, 10, 100, and 1000 $\mu\text{L L}^{-1}$ (droplet to filter paper in an enclosed Petri dish system). Equivalent amounts of sterile distilled water were used as a control. After incubation at $28 \pm 2^\circ\text{C}$ for 5 days, the total number of conidia per soybean seed was assessed by mixing the infected soybean seeds with sterile water in a serial dilution (1×10^3) before counting the number of conidia under a compound microscope. There were three replicates for each treatment.

Fungicidal kinetics of acetophenone and phenylethyl alcohol against conidia of the two aflatoxin-producing fungi

To obtain the fungicidal kinetics of the two compounds, 50- μL spore inoculum (1×10^4 spore mL^{-1}) of each of the two aflatoxin-producing fungi was spread on PDA. The cultures were fumigated with 100 $\mu\text{L L}^{-1}$ of acetophenone or phenylethyl alcohol for 0, 1, 3, 6, 9, and 12 h. Equivalent amounts of sterile distilled water were used as a control. The cultures were removed after fumigation and incubated at $28 \pm 2^\circ\text{C}$ for 48 h. The fungicidal kinetics were determined based on the total number of conidia alive on the PDA plate (Li et al. 2013). For each treatment, there were three replicates.

Effects of acetophenone and phenylethyl alcohol in protecting soybean seeds against contamination by the two aflatoxin-producing fungi and their effects on seed germination

The protective abilities of the two volatile compounds on soybeans against the two aflatoxin-producing fungi were evaluated in an antifungal bioassay. Fifteen soybean seeds were placed on three smaller dishes (five soybean seeds per Petri dish), then 50 μL of spore inoculum (1×10^4 spore mL^{-1}) of the aflatoxin-producing fungi were spread on each soybean seed and on the fourth dish that contained the various concentrations of acetophenone or phenylethyl alcohol (1, 10, 100, and 1000 $\mu\text{L L}^{-1}$, droplet on filter paper in an enclosed Petri dish system). Equivalent amounts of sterile distilled water were used as a control. The Petri dishes were covered with a large lid, sealed with Parafilm M[®], and incubated at $28 \pm 2^\circ\text{C}$ for 5 days. Then, the soybean seeds in the dishes were individually examined for infection under a stereomicroscope (Sumalan et al. 2013) and the protection of seeds was quantified based on infected seed counts as: Percentage of protection = $\{[(\text{Control}-\text{treatment})/\text{Control}] \times 100\}$. For each treatment, there were three replicates and the experiment was conducted three times.

The influence of fumigation of acetophenone and phenylethyl alcohol on soybean seed germination was investigated. Peanut and maize seeds were also investigated for comparison. One hundred seeds were fumigated with 100 $\mu\text{L L}^{-1}$ of each volatile compound for 24 h. They were placed on moist paper and kept in a plastic box (120 mm width $\times$ 170 mm length $\times$ 68 mm height with 1 L inner volume), which was incubated at $28 \pm 2^\circ\text{C}$ for 10 days. Distilled water was used as the control treatment. The toxicity to seed germination is reported as percentage of seed germination, along with stem and root lengths after the incubation.

Effects of the fumigation period with acetophenone to control the two aflatoxin-producing fungi on soybean seeds

The efficacy by the fumigation period with acetophenone to control the two aflatoxin-producing fungi on soybean seeds was investigated using an antifungal bioassay. Five soybean seeds were placed on each of three small dishes, then a 50- μL spore inoculum (1×10^4 spore mL^{-1}) of the two aflatoxin-producing fungi was spread on each soybean seed while the fourth dish contained acetophenone at 100 $\mu\text{L L}^{-1}$ (the minimum dose that completely controlled soybean seed infection). Equivalent amounts of sterile distilled water were used as a control. The four small dishes were placed in a large Petri dish, which was covered with a lid, sealed with Parafilm M[®], and fumigated for 0, 6,

12, or 24 h. After 14 days of incubation at 28 ± 2 °C, the fungal colony growth on the soybean seeds was examined under a stereomicroscope. The seed contamination index (SCI) was determined using the formula (Doolotkeldieva 2010): % SCI = [(Number of contaminated seeds/Total number of seeds) $\times$ 100]. For each treatment there were three replicates (five soybean seeds each) and the experiment was conducted three times.

Mode of action of acetophenone against the two aflatoxin-producing fungi by scanning electron microscopy (SEM)

The biomass of *A. flavus* TISTR 3041 and *A. parasiticus* TISTR 3276 obtained from the 5-day-old cultures grown on PDA with and without acetophenone treatment were

Fig. 1 Effects of different concentrations of acetophenone (a, c) and phenylethyl alcohol (b, d) on the mycelial growth and conidial germination inhibition of *A. flavus* TISTR 3041 and *A. parasiticus* TISTR 3276 on PDA plates. Different letters above bars indicated significant differences (ANOVA, $P < 0.05$; Duncan's multiple range test)

subjected to scanning electron microscopy (SEM) to determine the possible mode of action of acetophenone. The fungal biomass preparation and the SEM observation followed the procedure described by Boukaew and Prasertsan (2014). The mode of action of acetophenone was assessed from the ultrastructure alterations of the two fumigated fungal strains.

Statistical analysis

Duncan's Multiple Range Test (DMRT) was used at 95% statistical significance ($P < 0.05$). The Statistical Package for the Social Sciences (SPSS) software version 15 for Windows was used to analyze the data.

Results

Evaluation of the antifungal activity of acetophenone and phenylethyl alcohol on mycelial growth and conidial germination of the two aflatoxin-producing fungi

The antifungal activities of acetophenone on mycelial growth and conidial germination of *A. flavus* TISTR 3041 and *A. parasiticus* TISTR 3276 were stronger than those of phenylethyl alcohol (Fig. 1). Significant reductions in fungal growth and conidial germination were observed in a dose-dependent manner ($P < 0.05$). At $100 \mu\text{L L}^{-1}$ fumigation, only acetophenone completely inhibited (100%) the

Fig. 2 Effects of different concentrations of acetophenone and phenylethyl alcohol on sporulation inhibition of *A. flavus* TISTR 3041 and *A. parasiticus* TISTR 3276 on PDA plates (a, b) and on soybean seeds (c, d). Different letters above bars indicate significant differences (ANOVA, $P < 0.05$; Duncan's multiple range test)

fungal growth (Fig. 1a) and conidial germination (Fig. 1c) of the two aflatoxin-producing fungi. Phenylethyl alcohol showed only weak inhibitory activities on the fungal growth (Fig. 1b) and the conidial germination (Fig. 1d) even at $1000 \mu\text{L L}^{-1}$.

Evaluation of antifungal activity of acetophenone and phenylethyl alcohol on sporulation of the two aflatoxin-producing fungi on PDA plates and on soybean seeds

Figure 2 indicates that sporulation inhibition depended on the concentration of acetophenone or phenylethyl alcohol. The two compounds only delayed sporulation at low concentrations and complete inhibition was achieved at higher concentrations. At $100 \mu\text{L L}^{-1}$ fumigation dose, only acetophenone completely inhibited (100%) sporulation of both *A. flavus* TISTR 3041 and *A. parasiticus* TISTR 3276 on PDA plates (Fig. 2a) and on soybean seeds (Fig. 2c). Phenylethyl alcohol completely inhibited (100%) sporulation only of *A. flavus* TISTR 3041 at the concentration of $1000 \mu\text{L L}^{-1}$ on the PDA plate (Fig. 2b) and at $100 \mu\text{L L}^{-1}$ on soybean seeds (Fig. 2d).

Fungicidal kinetics of acetophenone and phenylethyl alcohol against conidia of the two aflatoxin-producing fungi

Fungicidal kinetics of acetophenone and phenylethyl alcohol revealed that more than 50% death of the two aflatoxin-producing fungi occurred at 3 h and 6 h fumigation, respectively (Fig. 3). A significant reduction of conidia germination appeared as exposure time increased ($P < 0.05$). The conidia of the two aflatoxin-producing

fungi were completely killed (100% death) at 6 h of fumigation with acetophenone (Fig. 3a). However, phenylethyl alcohol showed only weak antifungal activity (51.53% and 53.12% death of *A. flavus* TISTR 3041 and *A. parasiticus* TISTR 3276, respectively) (Fig. 3b).

Effects of acetophenone and phenylethyl alcohol on protection of soybean seeds against contamination by the two aflatoxin-producing fungi and their effects on seed germination

The effects of acetophenone and phenylethyl alcohol fumigation in protecting soybean seeds from contamination by *A. flavus* TISTR 3041 and *A. parasiticus* TISTR 3276 are shown in Fig. 4. Obviously, the protection of soybean seeds against contamination depended on the concentration of either volatile compound. At $100 \mu\text{L L}^{-1}$ acetophenone fumigation dose, total protection of soybean seeds was achieved (100%) ($P < 0.05$) (Fig. 4a). Therefore, the minimum effective dose of acetophenone on soybean seeds was $100 \mu\text{L L}^{-1}$. In contrast, phenylethyl alcohol provided no inhibition of either fungal strain on soybean seeds at any concentration tested (Fig. 4b). Thus, further studies on the effects of the fumigation period on the two aflatoxin-producing fungi and the mode of action were performed with acetophenone only.

The effects of acetophenone and phenylethyl alcohol on seed germination were tested on soybean, peanut, and maize seeds. The results revealed that the two volatile compounds had no significant adverse effects on seed germination or on stem and root length of the seeds compared to the control ($P < 0.05$) (Table S1; Supplementary data). This indicates that acetophenone and phenylethyl

Fig. 3 Growth inhibition kinetics of the conidia germination of the tested *A. flavus* TISTR 3041 and *A. parasiticus* TISTR 3276 by acetophenone (a) and phenylethyl alcohol (b) fumigation and incubated at $28 \pm 2^\circ\text{C}$. Different letters above bars indicate significant differences (ANOVA, $P < 0.05$; Duncan's multiple range test)

Fig. 4 Effects of different concentrations of acetophenone (A) and phenylethyl alcohol (B) fumigation on the protection of soybean seeds inoculated with *A. flavus* TISTR 3041 and *A. parasiticus* TISTR 3276 after incubation for 5 days at 28 ± 2 °C. Different letters above bars indicated significant differences (ANOVA, $P < 0.05$; Duncan's multiple range test)

alcohol fumigation can be applied to control spoilage microorganisms during seed storage.

Effects of the fumigation period with acetophenone on control of the two aflatoxin-producing fungi on soybean seeds

The efficacy of acetophenone was tested during a 24-h fumigation period for control of *A. flavus* TISTR 3041 and *A. parasiticus* TISTR 3276 on soybean seeds (Fig. 5). Fumigation using acetophenone for 24 h significantly reduced the soybean seed contamination by the two aflatoxin-producing fungi ($P < 0.05$), whereas the fumigation periods of 6 h and 12 h had no preventive effect (100%

Fig. 5 Duration of fumigation with acetophenone of soybean inoculated with *A. flavus* TISTR 3041 and *A. parasiticus* TISTR 3276 after incubation for 14 days at 28 ± 2 °C. Different letters above bars indicate significant differences (ANOVA, $P < 0.05$; Duncan's multiple range test). Seed contamination index (SCI) was calculated as $\text{SCI} \% = [(\text{Number of contaminated seeds} / \text{Total number of seeds}) \times 100]$

SCI) compared to the control. Interestingly, the 24-h fumigation treatment exhibited significant reduction and completely controlled *A. flavus* TISTR 3041 infection on soybean seeds during the 14-day test ($P < 0.05$). *A. parasiticus* TISTR 3276 appeared to be more resistant to acetophenone fumigation than *A. flavus* TISTR 3041.

Mode of action of acetophenone on the two fumigated aflatoxin-producing fungi assessed by scanning electron microscopy (SEM)

Ultrastructure alterations of the two aflatoxin-producing fungi 5 days after treatment with acetophenone are seen in the SEM images of Fig. 6. In the control, there were no morphological changes in either *A. flavus* TISTR 3041 (Fig. 6a) or *A. parasiticus* TISTR 3276 (Fig. 6d, e). The conidiophores appeared globular and formed spherical heads containing swollen conidia with normal appearing smooth cell walls. The morphological changes were evident after exposure to acetophenone in both fungal strains. Exposure to acetophenone at $100 \mu\text{L L}^{-1}$ completely inhibited conidia germination of *A. flavus* TISTR 3041 (Fig. 6b, c), whereas damage and morphological alterations of conidia were exhibited along with abnormal growth of *A. parasiticus* TISTR 3276 (Fig. 6f). Markedly shriveled and crinkled cell walls and flattened hyphae of the two aflatoxin-producing fungi were also evident in the images (Fig. 6b, c, and f).

Discussion

The efficiency of acetophenone and phenylethyl alcohol as volatile antimicrobial agents has been reported in various studies (Huang et al. 2011; Li et al. 2010; Naznin et al.

Fig. 6 Scanning electron micrographs (SEM) of normal hypha, conidiophore, and spores of *A. flavus* TISTR 3041 (a) and *A. parasiticus* TISTR 3276 (d, e) without exposure to acetophenone (the control) after incubation at $28 \pm 2^\circ\text{C}$ for 5 days. Mycelia treated with acetophenone at $100 \mu\text{L mL}^{-1}$ of *A. flavus* TISTR 3041 (b,

c) and *A. parasiticus* TISTR 3276 (f) after incubation at $28 \pm 2^\circ\text{C}$ for 5 days. Red circle indicates the size of the vesicle of *A. parasiticus* TISTR 3276 exposed to acetophenone (e) and the damage can be compared to control without exposure to acetophenone (f)

2014; Prakash et al. 2015; Yuelian and Qingfang 2013). However, the antifungal activities of the two volatile compounds against aflatoxin producing *A. flavus* and *A. parasiticus* have not been extensively investigated. In the present investigation, only fumigation with acetophenone ($100 \mu\text{L L}^{-1}$) completely inhibited (100%) mycelial growth and sporulation as well as conidial germination of the two aflatoxin-producing fungi on PDA and on soybean seeds. This effective concentration of acetophenone ($100 \mu\text{L L}^{-1}$) is identical to that inhibiting *P. italicum* in an in vitro test (Li et al. 2010). In addition, acetophenone-derived Mannich bases have the potential for developing novel antifungal agents against dermatophytes (Gul et al. 2001, 2002). Phenylethyl alcohol showed only weak antifungal activity in the in vitro test, and no efficacy against aflatoxin-producing fungi on soybean seeds even at $1000 \mu\text{L L}^{-1}$ in this study, or at $100,000 \mu\text{L L}^{-1}$ in the

prior study by Li et al. (2010). However, the mycelial growth of *A. flavus* NKDW-7 was completely inhibited (100%) using phenylethyl alcohol at $2.0 \mu\text{L mL}^{-1}$ ($2000 \mu\text{L L}^{-1}$) (Prakash et al. 2015). The effective dose of phenylethyl alcohol varies by microorganism, being $50 \mu\text{g mL}^{-1}$ ($50,000 \mu\text{L L}^{-1}$) for *F. oxysporum* f. sp. *cubense* (Yuelian and Qingfang 2013).

The spores are important to survival and spread of aflatoxigenic fungi (Rabea et al. 2003). Both acetophenone and phenylethyl alcohol had strong antifungal activity against *A. flavus* TISTR 3041 and *A. parasiticus* TISTR 3276. More than 50% of conidia were killed in all groups at an exposure time of 3 h for acetophenone and at 6 h for phenylethyl alcohol. In addition, the antifungal activity gradually strengthened with exposure time. Exposure for 6 h of the conidia to acetophenone could completely kill 100% of the conidia of the two aflatoxigenic fungi, while

phenylethyl alcohol failed to kill the conidia completely under the same conditions.

Although the *in vitro* tests of acetophenone and phenylethyl alcohol are important first steps to check their antifungal potential, *in vivo* tests are still needed to confirm the results. Acetophenone fumigation on soybean seeds could decrease the growth of *A. flavus* TISTR 3041 and *A. parasiticus* TISTR 3276 considerably with 100% inhibition at 100 $\mu\text{L L}^{-1}$. Therefore, at this effective concentration of acetophenone, soybean seeds could be significantly protected from infection by the two aflatoxin-producing fungi ($P < 0.05$), while the effective dose of the phenylethyl alcohol was 1000 $\mu\text{L L}^{-1}$. Therefore, the minimum effective dose of acetophenone at 100 $\mu\text{L L}^{-1}$ was 10 times lower than the dose of 1000 $\mu\text{L L}^{-1}$ used to control *P. italicum* in the *in vivo* test reported by Li et al. (2010). This may be due to the different treatment methods and different types of organism used.

In general, some compounds produced by microorganisms (Boukaew et al. 2011) or plant essential oils (Kordali et al. 2009; Qiming et al. 2006; Young and Bush 2009) have been reported to inhibit seed germination. In this study, the fungicides acetophenone and phenylethyl alcohol were found to be effective for controlling the two aflatoxin-producing fungi in colonizing soybean seeds during 14 days of storage. In addition, the two volatile compounds at a concentration of 100 $\mu\text{L L}^{-1}$ had no adverse effects on seed germination, or early stem length or root length of the soybean, compared to the control.

According to the *in vivo* assay, increasing the fumigation period of acetophenone to 24 h resulted in a significant reduction in soybean contamination, by potent fungicidal effects ($P < 0.05$). Therefore, fumigation with 100 $\mu\text{L L}^{-1}$ of acetophenone for 24 h provided complete (100%) protection of the soybean seeds from infection by *A. flavus* TISTR 3041 during the 14 days of incubation. *A. parasiticus* TISTR 3276 appeared to have more resistance to acetophenone fumigation than *A. flavus* TISTR 3041 at the concentrations tested. The minimum fumigation period of acetophenone for protecting soybean seeds was 24 h. Exposure of the seeds to acetophenone and phenylethyl alcohol exhibited powerful antifungal activity against the two aflatoxin-producing fungi. Similar results have been obtained by several researchers with soybeans and other crops (Chavan and Padule 2005; Nasir 2003).

Results from *in vitro* and *in vivo* testing showed that acetophenone completely inhibited (100%) growth, conidial germination, and sporulation of *A. flavus* TISTR 3041 and *A. parasiticus* TISTR 3276 at a concentration of 100 $\mu\text{L L}^{-1}$. The data and SEM results confirmed that acetophenone could damage the hyphae, conidiophores, and spore germination of the two aflatoxin-producing fungi. Some chemical compounds including natural

cinnamaldehyde, citral, and eugenol have been reported to damage the hyphae, conidiophores, and spores of *Aspergillus* spp. (Hua et al. 2014).

In conclusion, the present study demonstrated that acetophenone exhibits higher efficacy than phenylethyl alcohol against *A. flavus* TISTR 3041 and *A. parasiticus* TISTR 3276 in both *in vitro* tests and on soybean seeds. There were no adverse effects on soybean seed germination, so acetophenone in particular could potentially be an effective fumigant to prevent fungal spoilage of seeds used as food.

Acknowledgements This research work was financially supported by the Agricultural Research Development Agency (Public Organization) (PRP5905021490) and Thailand Research Fund (RTA6080010).

References

- Ando H, Hatanaka K, Ohata I, Yamashita-Kitaguchi Y, Kurata A, Kishimoto N (2012) Antifungal activities of volatile substances generated by yeast isolated from Iranian commercial cheese. *Food Control* 26:472–478
- Boukaew S, Prasertsan P (2014) Suppression of rice sheath blight disease using heat stable culture filtrate of *Streptomyces philanthi* RM-1-138. *Crop Prot* 61:1–10
- Boukaew S, Chuenchit S, Petcharat V (2011) Evaluation of *Streptomyces* spp. for biological control of *Sclerotium* root and stem rot and *Ralstonia* wilt of chili. *Biocontrol* 56:347–365
- Boukaew S, Plubrukam A, Prasertsan P (2013) Effect of volatile substances from *Streptomyces philanthi* RM-1-138 on growth of *Rhizoctonia solani* on rice leaf. *Biocontrol* 58:471–482
- Boukaew S, Petlamul W, Bunkrongcheap R, Chookaew T, Kabbua T, Thippated A, Prasertsan P (2018) Fumigant activity of volatile compounds of *Streptomyces philanthi* RM-1-138 and pure chemicals (acetophenone and phenylethyl alcohol) against anthracnose pathogen in postharvest chili fruit. *Crop Prot* 103:1–8
- Chavan VA, Padule DN (2005) Effect of seed dressers on seed germination, seedling vigour index and control of purple stain disease of soybean. In: Khetarpal R, Gupta K, Chalam VC, Gour HN, Thakore BBL, Bhargava S (eds) Proceedings of second global conference on plant healthgava,n: wealth, 25 proceedings of second global confere ncMaharana Pratap University of Agriculture and Technology, India, pp 359–360
- Corcuff R, Mercier J, Tweddell R, Arul J (2011) Effect of water activity on the production of volatile organic compounds by *Muscodor albus* and their effect on three pathogens in stored potato. *Fungal Biol* 115:220–227
- Doolotkeldieva TD (2010) Microbiological control of flour-manufacture: dissemination of mycotoxins producing fungi in cereal products. *Microbiol Insights* 3:1–15
- Francesco AD, Ugolini L, Lazzeri L, Mari M (2015) Production of volatile organic compounds by *Aureobasidium pullulans* as a potential mechanism of action against postharvest fruit pathogens. *Biol Control* 81:8–14
- Gul M, Gul HI, Vepsalainen J, Erciyase E, Hanninen O (2001) Effect of acetophenone derived Mannich bases on cellular glutathione level in Jurkat cells. *Arzneimittelforschung* 51:679–682
- Gul HI, Ojanen T, Hanninen O (2002) Antifungal evaluation of bis mannich bases derived from acetophenones and their corresponding piperidinols and stability studies. *Biol Pharm Bull* 25:1307–1310

- Hua H, Xing F, Selvaraj JN, Wang Y, Zhao Y, Zhou L, Liu X, Liu Y (2014) Inhibitory effect of essential oils on *Aspergillus ochraceus* growth and ochratoxin A production. *PLoS ONE* 9(9):e108285
- Huang R, Li GQ, Zhang J, Yang L, Che HJ, Jiang DH, Huang HC (2011) Control of postharvest botrytis fruit rot of strawberry by volatile organic compounds of *Candida intermedia*. *Dis Control Pest Manag* 101:859–869
- Kavitha A, Prabhakar P, Narasimhulu M, Vijayalakshmi M, Venkateswarlu Y, Rao KV, Raju VBS (2010) Isolation, characterization and biological evaluation of bioactive metabolites from *Nocardia levis* MK-VL_113. *Microbiol Res* 165:199–210
- Kordali S, Kakir A, Akcin TA, Mete E, Akcin A, Aydin T, Kilic H (2009) Antifungal and herbicidal properties of essential oils and n-hexane extracts of *Achillea gypsicola* Hub-Mor and *Achillea biebersteinii* Afan. *Ind Crops Prod* 29:562–570
- Li Q, Ning P, Zheng L, Huang J, Li G, Hsiang T (2010) Fumigant activity of volatiles of *Streptomyces globisporus* JK-1 against *Penicillium italicum* on *Citrus microcarpa*. *Postharvest Biol Technol* 58:157–165
- Li Q, Ning P, Zheng L, Huang J, Li G, Hsiang T (2012) Effects of volatile substances of *Streptomyces globisporus* JK-1 on control of *Botrytis cinerea* on tomato fruit. *Biol Control* 61:113–120
- Li WR, Shi QS, Ouyang YS, Chen YB, Duan SS (2013) Antifungal effects of citronella oil against *Aspergillus niger* ATCC 16404. *Appl Microbiol Biotechnol* 97:7483–7492
- Liarzi O, Bar E, Lewinsohn E, Ezra D (2016) Use of the endophytic fungus *Daldinia cf. concentrica* and its volatiles as bio-control agents. *PLoS ONE* 11(12):e0168242
- Nasir N (2003) Effect of fungicides in limiting the growth of seed borne fungi of soybean. *Pak J Plant Pathol* 2:119–122
- Naznin HA, Kiyohara D, Kimura M, Miyazawa M, Shimizu M, Hyakumachi M (2014) Systemic resistance induced by volatile organic compounds emitted by plant growth promoting fungi in *Arabidopsis thaliana*. *PLoS ONE* 9(1):e86882
- Ng'ang'a J, Mutungi C, Imathiu S, Affognon H (2016) Effect of triple-layer hermetic bagging on mould infection and aflatoxin contamination of maize during multi-month on-farm storage in Kenya. *J Stored Prod Res* 69:119–128
- Petrovic J, Glamoclija J, Stojkovic DS, Ciric A, Nikolic M, Bukvicki D, Guersoni ME, Sokovic MD (2013) *Laetiporus sulphureus*, edible mushroom from Serbia: investigation on volatile compounds, in vitro antimicrobial activity and in situ control of *A. flavus* in tomato paste. *Food Chem Toxicol* 59:297–302
- Prakash B, Singh P, Goni R, Raina AKP, Dubey NK (2015) Efficacy of *Angelica archangelica* essential oil, phenyl ethyl alcohol and α -terpineol against isolated molds from walnut and their antiaflatoxic and antioxidant activity. *J Food Sci Technol* 52:2220–2228
- Qiming X, Haidong C, Huixian Z, Daqiang Y (2006) Allelopathic activity of volatile substance from submerged macrophytes on *Microcystin aeruginosa*. *Acta Ecol Sin* 26:3549–3554
- Rabea EI, Badawy MET, Stevens CV, Smagghe G, Steubaut W (2003) Chitosan as antimicrobial agent: applications and mode of action. *Biomacromol* 4:1457–1465
- Rahman A, Shanta ZS, Rashid MA, Parvin T, Afrin S, Khatun MK, Sattar MA (2016) In vitro antibacterial properties of essential oil and organic extracts of *Premna integrifolia* Linn. *Arab J Chem* 9:S475–S479
- Raza W, Yuan J, Ling N, Huang Q, Shen Q (2015) Production of volatile organic compounds by an antagonistic strain *Paenibacillus polymyxa* WR-2 in the presence of root exudates and organic fertilizer and their antifungal activity against *Fusarium oxysporum* f. sp. *niveum*. *Biol Control* 80:89–95
- Sang MK, Kim KD (2012) The volatile-producing *Flavobacterium johnsoniae* strain GSE09 shows biocontrol activity against *Phytophthora capsici* in pepper. *J Appl Microbiol* 113:383–398
- Sangmanee P, Hongpattarakere T (2014) Inhibitory of multiple antifungal components produced by *Lactobacillus plantarum* K35 on growth, aflatoxin production and ultrastructure alterations of *Aspergillus flavus* and *Aspergillus parasiticus*. *Food Control* 40:224–233
- Sumalan RM, Alexa E, Poiana AM (2013) Assessment of inhibitory potential of essential oils on natural mycoflora and *Fusarium mycotoxins* production in wheat. *Chem Cent J* 7:1–12
- Svircev AM, Smith RJ, Zhouc T, Hernandez M, Liu W, Chud CL (2007) Effects of thymol fumigation on survival and ultrastructure of *Monilinia fructicola*. *Postharvest Biol Technol* 45:228–233
- Wan M, Li G, Zhang J, Jiang D, Huang HC (2008) Effect of volatile substances of *Streptomyces platensis* F-1 on control of plant fungal diseases. *Biol Control* 46:552–559
- Wang C, Wang Z, Qiao X, Li Z, Li F, Chen M, Wang Y, Huang Y, Cui H (2013) Antifungal activity of volatile organic compounds from *Streptomyces albobiflavus* TD-1. *FEMS Microbiol Lett* 341:45–51
- You C, Zhang C, Feng C, Wang J, Kong F (2015) *Myroides odoratimimus*, a biocontrol agent from the rhizosphere of tobacco with potential to control *Alternaria alternata*. *Biocontrol* 60:555–564
- Young GP, Bush JK (2009) Assessment of the allelopathic potential of *Juniperus ashei* on germination and growth of *Bouteloua curtipendula*. *J Chem Ecol* 35:74–80
- Yuelian L, Qingfang L (2013) Inhibitory effects of *Acremonium* sp. on *Fusarium* wilt in bananas. *Afr J Agric Res* 8:6241–6249
- Zheng M, Shi J, Shi J, Wang Q, Yanhua L (2013) Antimicrobial effects of volatiles produced by two antagonistic *Bacillus* strains on the anthracnose pathogen in postharvest mangos. *Biol Control* 65:200–206