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Upland rice vinegar vapor inhibits spore germination, hyphal growth and aflatoxin formation in *Aspergillus flavus* on maize grains

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ABSTRACT

The efficacy of vapor-phase (VP) upland rice vinegar (URV) was investigated as a bio-fumigant for maize, to reduce consumer health risks associated with spore and toxin formation by *Aspergillus flavus*. Complete reduction of mycelial growth occurred with *in vitro* VP exposure to URV (containing 0.0017 mmol/L acetic acid) or with VP exposure to pure acetic acid (PAA) (containing 0.0023 mmol/L acetic acid). No significant differences were observed between the two materials after 90 min exposures. Using gas chromatography-mass spectrometry (GC-MS), URV vapor was shown to contain volatiles having antifungal activities. These are identified as isoamylalcohol, 1-butanol, 3-methyl-, acetate and β-phenylethyl acetate. It is suggested these volatiles increase the antifungal effectiveness of URV. Exposure to VP-URV (containing 0.0043 mmol/L AA) for 5 h completely eliminated viable spores of *A. flavus* on maize seeds (23% moisture content) previously inoculated with 4.43 ± 0.28 log spores/g). At the same time, aflatoxin production decreased, as VP-URV exposure increased. Hence, VP-URV is shown to be an effective control agent for *A. flavus* mycelial growth and aflatoxin formation on maize, so effectively reducing the potential for consumer health risks due to this widespread fungus.

1. Introduction

Aspergillus flavus is a very common mold, of yellow/green appearance. It is able to grow and multiply under a wide range of conditions including hot, cold, humid and dry (Bhatnagar-Mathur, Sunkara, Bhatnagar-Panwar, Waliyar, & Sharma, 2015). In humid tropical and subtropical areas, a wide range of food and feed products (maize, rice, peanut, cotton, ginger, chili, almond, walnut, coconut, milk etc) suffer significant fungal contamination by *A. flavus*. This often results in severe commercial loss (Klich & Pitt, 1988; Reddy et al., 2010).

A. flavus can also produce aflatoxins which are highly toxic to mammals – both human beings and farm livestock. Aflatoxins are hepatotoxic, teratogenic, mutagenic and immunosuppressive (Arrus, Blank, Abramson, Clear, & Holley, 2005). Hence, inhibition of mold growth on food and feed crops is necessary, not only to reduce commercial loss but also to mitigate risk to human and animal health. Various physical and chemical methods are used to control hyphal growth and toxin formation of *A. flavus*. Given growing consumer aversion to the introduction of synthetic chemicals to the food chain, the development of biological control methods to safe-guard food and feed quality is becoming increasingly attractive (Alberts, Gelderblom, Botha, & van Zyl, 2009).

Organic acids are well known as effective biological control agents and are also reasonably affordable. A number of organic acids are used in this way including acetic acid (AA), lactic acid and propionic acid (Higgins & Brinkhaus, 1999). It has also been reported that AA vapor (8 μl/L) completely inhibits hyphal growth and spore germination of many common fungi, including of *A. flavus* (Morsy, Abd-El-Kareem, & Abd-Alla, 2000).

Vinegar is biologically and environmentally friendly and also safe to use. The principal organic component of vinegar is AA,

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which is well known both for its safety and also its antimicrobial properties. Many reports have demonstrated the effective antimicrobial properties of AA and vinegar in liquid form, and these are already used for limiting bacterial and fungal contamination, and thus postharvest decay, in fresh produce including in apples, tomatoes, carrots, stone fruits, lettuces and strawberries (Chang & Fang, 2007; Kilonzo-Nthenge, Chen, & Godwin, 2006; Krusong, Jindaprasert, Laosinwattana, & Teerarak, 2015; Sengun & Karapinar, 2004; Sholberg, Haag, Hocking, & Bedford, 2000). Vapor-phase vinegar has also been shown to exhibit antibacterial and antifungal properties with many food products such as with eggs, tomatoes, apples, apricots, lettuces, strawberries, and coriander (Krusong, Jindaprasert, et al., 2015; Krusong, Teerarak, & Laosinwattana, 2015; Sholberg et al., 2000; Yeesibsan & Krusong, 2009). Nevertheless, many investigations have been reported that volatile - biologically active compound contains antifungal property and represents a candidates for development as biological control agents in agriculture (Masoud, Poll, & Jakobsen, 2005; Morath, Hung, & Bennett, 2012). Therefore, volatile biologically active components, especially ester compounds, occurring in acetification of upland rice vinegar (URV) were investigated in our study. They may cause to increase the effectiveness of inhibitory property of AA in URV on the mold, *A. flavus*.

The aim of this study was to determine if vapor-phase (VP) upland rice vinegar (URV) is effective as a control agent for inhibiting conidial germination, hyphal growth and aflatoxin formation in *A. flavus* inoculated onto maize seeds. An additional aim was to identify the main volatile components of URV using gas chromatography-mass spectrometry (GC-MS) analysis.

2. Materials and methods

2.1. Materials

Maize seeds were purchased from a local market in the Ladkrabang area of Bangkok, Thailand. Upland rice wine vinegar (URV) containing 8 ± 0.1% (v/v) of acetic acid was produced at the Laboratory of Fermentation Technology, Faculty of Agro-Industry, King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand. Pure acetic acid (PAA) was obtained from Merck KGaA (Darmstadt, Germany). A vapor exposure box (see Fig. 1) for exposing maize samples to vapor-phase (VP)-URV or to VP-PAA consisted of a 37.5 L plastic box (0.25 × 0.30 × 0.25 m) with a slide cover to prevent pressure build up during vapor treatment. To generate the VP, ambient clean air was bubbled at 0.5 L/min through 500 mL of URV or PAA in a 1000 mL closed bottle. Both the URV and the PAA solutions were diluted to contain 8% (v/v) of AA. The VP-URV or VP-PAA from the headspace of the bottle was delivered at the same rate (0.5 L/min) to the vapor exposure box via a spreader manifold. The AA content (mmol/L) of the introduced air was calculated based on the weight loss rate of the liquid URV or PAA and the volume flow rate (0.5 L/min) of the air.

2.2. Sample preparation and microbiological method

After removal of soil and foreign matter, the random maize-seed samples were checked for moisture content (MC). Selected seeds of MC 10–13% were packed in aluminum-foil bags, sealed and stored at −20 °C. Before use, they were thawed to room temperature and the seeds surface-sterilized by dipping in 3% sodium hypochlorite for 3 min. They were then washed several time with sterile distilled water (Abd-Alla, 2005) and dried aseptically in laminar airflow for 30 min (modified method of Krusong, Teerarak, et al., 2015).

To prepare the maize seeds for studying the effects of VP-URV on maize inoculated with *A. flavus* conidia, the seeds were then heated at 121 °C for periods of 0 (control), 15, 20, 25 or 30 min to ensure no contamination by *A. flavus* or other molds.

2.3. Survey of Aspergillus flavus on maize and preparation of conidial suspension as inoculum

Surface-sterilized seeds without grinding or shredding were transferred to Petri dishes containing potato dextrose agar (PDA; Merck KGaA, Darmstadt, Germany) and incubated for 7 d at 30 ± 2 °C. The growth of fungal colonies was observed after incubation. The isolates of the fungal species were single spore to obtain pure cultures. Pure cultures of *Aspergillus* species were then subcultured and transferred onto three differential media consisting of malt extract agar, Czapek yeast extract agar and Czapek dox agar and incubated for 7 d at 30 ± 2 °C for species identification (Oyeleke, Egwim, & Auta, 2010). Identification was based on macro and micro morphological characteristics to species level together with taxonomic keys according to Pitt and Hocking (1985) and Raper and Fennell (1965). The characteristics consisted of colonial characteristics such as size, surface appearance, texture and color of the colonies. Additionally, microscopy exposed vegetative mycelium including the presence or absence of cross walls, mycelial diameter and the structural types of sexual and asexual reproductive. The isolated namely *A. flavus* exhibited a yellowish grey coloration. Then, the pure culture was maintained on PDA slants at 4 ± 1 °C and later formally confirmed to be *A. flavus* by the Department of Medical Sciences, Ministry of Health, Thailand.

To prepare a suspension of *A. flavus* conidia for use as inoculum, the fungus was cultured on PDA slants for 7 d at 30 ± 2 °C, by which time good levels of sporulation were observed. The conidia were harvested, suspended homogeneously in sterile distilled water

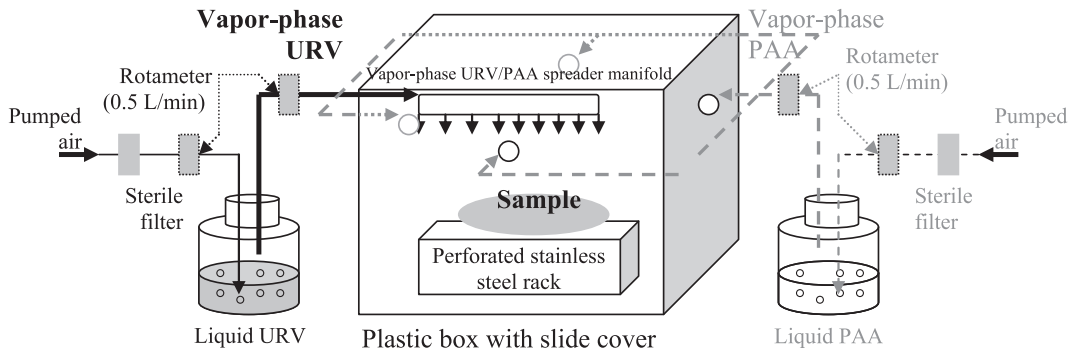

Fig. 1. Schematic of setup used to expose maize seed samples to vapor-phase upland rice vinegar (URV) or pure acetic acid (PAA). The sealed plastic box (0.25 × 0.30 × 0.25 m) containing the sample was injected with a stream of air that had been bubbled through either liquid URV (containing 8% AA) or liquid PAA (containing 8% AA).