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Release of Acetaldehyde from β-Cyclodextrins Inhibits Postharvest Decay Fungi in Vitro

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Many naturally occurring plant volatiles are known to have antifungal properties. However, they have limited use because they diffuse rapidly in air. In this in vitro study, acetaldehyde was chosen as a prototype volatile in order to study the controlled release of antifungal volatiles from cyclodextrins (CD). The major postharvest pathogens *Alternaria alternata, Botrytis cinerea*, and *Colletotrichum acutatum* were exposed to the pure volatile for 7 days at 23 °C. Acetaldehyde was most effective against *A. alternata,* followed by *C. acutatum*, and *B. cinerea*, with 0.12, 0.56, and 1.72 μL/L in air being required to inhibit fungal growth, respectively, according to the bioassay developed. Second, the effectiveness of the new β-CD−acetaldehyde release system was evaluated against *A. alternata* for 7 days at 23 °C. Sufficient volatile was released from 0.7 g of β-CD−acetaldehyde to prevent fungal growth in vitro.

KEYWORDS: ***Colletotrichum acutatum*; *Alternaria alternata*; *Botrytis cinerea*; inclusion complexes; cyclodextrins; acetaldehyde; postharvest shelf life**

INTRODUCTION

Acetaldehyde is a naturally occurring plant product and plays an important and well-known role in postharvest conservation. This volatile has been used for many years as a postharvest treatment because of its fungicidal and insecticidal properties (*1*). The antifungal activity of acetaldehyde has been tested on several postharvest pathogens, both in vitro and in vivo, with encouraging results. Prasad and Stadelbacher (1973, 1974) (*2*, *3*) controlled *Botrytis cinerea* and *Rhizopus stolonifer* rots of strawberries and raspberries with acetaldehyde. A significant reduction of *Botrytis cinerea* and *Rhizopus stolonifer* in in vitro tests was also achieved (*4*). Yuen et al. (1995) (*5*) reported that acetaldehyde was effective against *Penicillium italicum* and *Penicillium digitatum* on citrus at 20 °C. This volatile was also found to be effective in the postharvest protection of apples (*6*), sweet cherries (*7*), and stone fruit (*8*). This compound has also been shown to have inhibitory capacity against fungi when added as a liquid to culture media (*9*). In addition, acetaldehyde has been found to be metabolized by fresh produce to yield additional volatile compounds. Postharvest application of acetaldehyde vapors on blueberries, tomatoes, and pears led to the enhancement of fruit sensory qualities (*10*). Taking this into account, acetaldehyde could be very useful in preventing diseases during the postharvest period and in improving fresh product sensory qualities if it could be maintained around the product. This could be provided as a gas treatment during storage or using an active packaging format, though both are hard to achieve because of the diffusivity of acetaldehyde in air.

Cyclodextrins (CD) make up a family of cyclic oligosaccharides, composed of α-D-glucopyranoside units linked 1 → 4. These macrocyclic compounds are made from starch by means of enzymatic conversion of α-amylase by cyclodextrin glycosyltransferase. This makes them environmentally friendly because they are produced from a renewable natural material. Typical CD contain a number of glucose monomers ranging from six to eight units in a ring, namely, α-, β-, and γ-CD, respectively. Cyclodextrins are able to form host−guest inclusion complexes (ICs) with hydrophobic molecules, and as a result, these molecules have found a number of applications in a wide range of fields, mainly in the pharmaceutical industry (*11*). In this paper, we suggest a new application for CD that employs them for the stabilization and controlled release of acetaldehyde gas, which can then be used to treat fruits and vegetables during storage. This IC could be added to packaging materials, to the plastic materials used to construct greenhouses, and in conservation chambers. Such devices should be capable of slowly releasing acetaldehyde during storage when humidified by the water vapor emitted from the fresh produce. The gradual release of the volatile would significantly enhance its effectiveness by reducing its loss due to rapid diffusion. This is a novel way to extend the shelf life of fruits and vegetables, and to date, no commercial application has been made of this technique.

The major aim of this research is to develop and evaluate the effectiveness of CD−acetaldehyde ICs as a new postharvest

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fungicide system for fresh produce. However, the objective of this study was first to determine the effectiveness of acetaldehyde against the postharvest decay fungi *Alternaria alternata*, *Botrytis cinerea*, and *Colletotrichum acutatum* in vitro and, second, to use acetaldehyde as a prototype volatile to study the controlled release of antifungal volatiles from CD. This promising antifungal compound could be an economically viable alternative to currently used fungicides with little environmental impact because both CD and acetaldehyde are naturally occurring plant compounds.

MATERIALS AND METHODS

Materials. The α-, β-, and γ-cyclodextrins (purity >99%) were provided by Wacker Chemical Corporation (Adrian, MI). The volatile compound acetaldehyde (purity >99.5%) was purchased from Sigma-Aldrich Corp. (Saint Louis, MO). Single spore isolates of *Colletotricum acutatum*, *Alternaria alternata*, and *Botrytis cinerea* were originally isolated from diseased blueberry fruit.

Methods. *Preparation of Complexes.* A cyclodextrin/water solution (1:1 M) was prepared by adding cyclodextrins to a beaker containing hot distilled water (100 °C) and stirring at 205 rpm using a hot plate stirrer (Thermolyne Mirak hot plate/stirrer; Sigma-Aldrich Corp., Saint Louis, MO). After the solution was cooled to room temperature, it was placed into two centrifuge tubes and 70, 140, or 280 μL of acetaldehyde and was quickly added to each tube and closed. Pipet tips and the bottle of acetaldehyde were stored at −20 °C for 1 h prior to the addition of acetaldehyde to the cyclodextrin solution to avoid volatile loss. Samples were centrifuged at 1600 rpm for 40 min, and the paste obtained was dried at 60 °C for 24 h. All samples were evaluated in triplicate and stored in hermetically sealed flasks at 23 °C.

Emission of Acetaldehyde from Cyclodextrin Complexes. A simple desorption system was used to evaluate the efficacy of the ICs. Glass vials (40 mL) were filled with 1 mL of distilled water, and on the bottom of these, a 2 mL glass vial containing 0.1 g of inclusion complex was positioned (*12*). The glass vials were closed with Mininert valves (Supelco, Bellefonte, PA), and the amount of acetaldehyde released into the headspace was determined at 1, 3, 5, and 7 days. Acetaldehyde concentration was measured using a 65 μm DVB/CAR/PDMS SPME fiber (Supelco, Bellefonte, PA) and quantified with gas chromatography. The fiber was exposed to the vial headspace for 10 min. The volatile trapped in the SPME was quantified by desorbing the volatile (for 5 min) at the splitless injection port of a GC Hewlett-Packard 6890 series (Agilent Technology, Palo Alto, CA) equipped with a flame ionization detector (FID) and a HP-5 column (30 m × 0.32 mm × 0.25 μm, Hewlett-Packard, Agilent Technology, Palo Alto, CA). The oven temperature was set at 40 °C for 5 min. The injector and detector temperatures were set at 220 and 230 °C, respectively. Quantification of acetaldehyde in the headspace was determined using previously prepared calibration curves. The desorption systems were stored for 7 days at 23 °C. Three replicates were evaluated for each IC sample.

Culture Preparation and Bioassay. Cultures of *Colletotricum acutatum*, *Alternaria alternata*, and *Botrytis cinerea* were grown on PDA (potato dextrose agar, Sigma-Aldrich Corp., Saint Louis, MO) in plastic Petri dishes (9 cm diameter) for 14 days at 23 °C. Conidia were then collected by flooding the surface of the plates with sterile distilled water and gently scraping with a spatula to dislodge conidia and mycelia. A 10 mL amount of this suspension was then transferred to plastic tubes, which were shaken to dislodge the conidia from the mycelia. The conidial suspensions were then filtered through sterile cheesecloth to remove mycelial and condensed-agar fragments, and the aliquot was concentrated to 1×10^6 spores/mL. The Neubauer improved method (Bright-Line Hemacytometer, Hausser Scientific, Horshan, PA) was used to determinate spore concentration.

For the bioassay, 3.5 μL droplets of conidial suspensions of each pathogen were placed in the center of small polystyrene Petri dishes (5.5 cm diameter) containing PDA using a 100 μL Oxford autoclavable Benchmate pipet (Nichiryo, Japan). The Petri dishes were then placed inside 1 L glass jars, which were closed with screw caps and stored at 23 °C. These were used as the controls (control bioassay systems).

Other jars were modified for insertion and withdrawal of the volatile (treatment bioassay systems) by introducing a septum system in the jar cap (*12*). The desired doses of liquid acetaldehyde (from 1 to 15 μL) were applied neat to a piece of glass suspended 4 cm below the cap and allowed to evaporate. Gastight syringes were chilled inside a conventional freezer prior to injection to ensure accurate volumes, since acetaldehyde boils at 20.8 °C. Acetaldehyde was introduced by means of a 10 μL liquid-tight (Hamilton, Reno, NV) syringe through the rubber septum of the device described above. The same device was used to withdraw samples during storage. Three Petri dishes were set up for each concentration of the compound. All jars were stored at 23 °C.

Treatment bioassay systems, with 500 mL capacity, were used to test the β-CD−acetaldehyde complexes. From 0.3 to 0.7 g of complex (cyclodextrin/acetaldehyde 1:1 M) was positioned in the bottom of the jars using a piece of aluminum foil folded into a tray. Both complex and aluminum foil were previously sterilized under UV light, and jars were stored at 23 °C.

Measurement of Fungal Growth. Growth of the cultures in both control and treatment bioassay systems was evaluated daily by measuring the diameter of the colony on the agar surface. Measurement of diameters was made using a conventional ruler. Because of the optical transparency of both the glass and Petri dish, these measurements could be made without opening the jars. Each assay was tested in triplicate, and the results were analyzed statistically using analysis of variance (see below). The delay in fungal growth due to treatment exposure was expressed in centimeters or as a percentage of colony growth by comparing the treatments to the controls.

Determination of Acetaldehyde Levels in the Jar Headspace. The concentration of volatile in the vapor phase was estimated by solid-phase microextraction (SPME) sampling of the headspace combined with GC analysis. The vapor phase was generated by the evaporation of the liquid compound from a small piece of glass or in complexes with the cyclodextrin-volatile. Acetaldehyde samples in the headspace were withdrawn by inserting a needle (65 μm DVB/CAR/PDMS SPME fiber) through the septum in the screw cap (*12*). The fiber was exposed to the jar headspace for 10 min, and the trapped volatiles were immediately desorbed (for 5 min) at the splitless injection port of the GC instrument. The concentration of acetaldehyde in the headspace was determined from previously prepared calibration curves after incubation for 1, 3, 5, and 7 days at 23 °C.

Determination of Fungicidal and/or Fungistatic Activity of Acetaldehyde. After 7 days of storage, each bioassay system was opened and the plates were assessed. Petri dishes with no fungal growth after exposure to acetaldehyde were moved from the bioassay systems and transferred to a bioassay system containing a headspace atmosphere free of volatile. Prior to their insertion into the jars, the growth-free Petri dishes were divided into two groups: (a) one group was directly placed into the new jars; (b) the other group was modified by transferring 5 mm agar plugs from the center of the plate to new Petri dishes containing fresh agar and then inserted into the new jars. Radial growth of the cultures was evaluated over a 7 day period at 23 °C as described above. Fungal growth was expressed as direct radial growth in centimeters. The fungistatic and fungicidal activities of acetaldehyde were determined by the presence or absence of fungal growth over a period of 7 days.

Determination of Acetaldehyde in the Culture Medium. The concentration of acetaldehyde absorbed by the culture medium was also estimated by SPME sampling combined with GC analysis. Petri dishes with only PDA were placed inside the bioassay system, and an amount of 3 or 5 μL of acetaldehyde was introduced as described above. After 7 days of storage at 23 °C, the Petri dishes were removed from the jars and their media transferred to 40 mL vials. The media were melted by slowly heating and stirring for 10 min on a hot plate stirrer so that the volatile was released into the vial headspace. The amount of acetaldehyde was determined by SPME sampling and GC as described above. Quantification of acetaldehyde in the medium was determined using previously prepared calibration curves and a solubility coefficient of acetaldehyde in water of 668 000 mg/L (*13*). All measurements were replicated three times by sampling from separate bioassay systems.

Absorption of Acetaldehyde by B. cinerea. Preliminary studies showed a rapid decline of acetaldehyde in bioassay systems containing

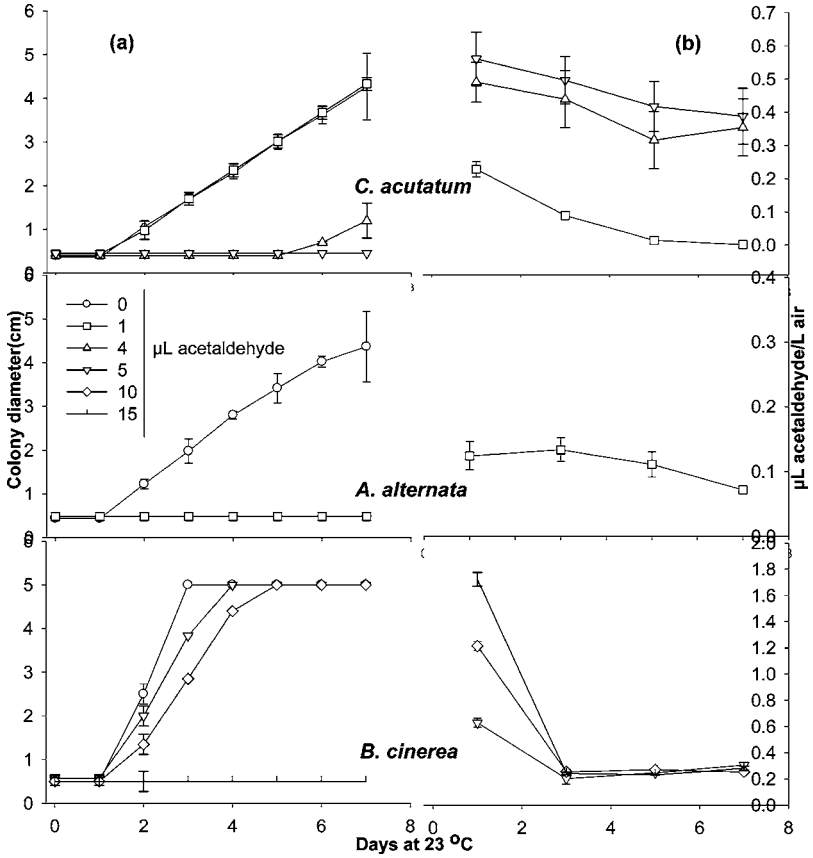

Figure 1. Comparison of the growth rates of the postharvest decay fungi *Alternaria alternata*, *Botrytis cinerea*, and *Colletotrichum acutatum* during exposure to different concentrations of acetaldehyde over a 7 day storage period at 23 °C: (**a**) colony diameter of the three fungi over the 7 day storage period; (**b**) measured concentration of acetaldehyde in the headspace of the sealed jar containing the growing fungus over the 7 day storage period.

B. cinerea but not *A. alternata* or *C. acutatum.* Thus, the absorption of acetaldehyde by *B. cinerea* was investigated as follows. Polystyrene Petri dishes (5 cm diameter) with and without spores of *B. cinerea* (3.5 μL of suspension, 1×10^6 spores/mL) were inserted into bioassay systems. After closure, an amount of 2 μL of acetaldehyde was introduced into each jar using a 10 μL liquid-tight syringe. The concentrations of volatile in the headspace and media were determined after 7 days of storage as explained previously. Before analysis, *B. cinerea* spores were carefully removed with a spatula. The amount (%) of acetaldehyde absorbed by the fungus *B. cinerea* was obtained through these expressions:

$$[\text{acet}]_{\text{total AF}} = [\text{acet}]_{\text{media AF}} + [\text{acet}]_{\text{headspace AF}} \quad (1)$$

$$[\text{acet}]_{\text{total PF}} = [\text{acet}]_{\text{media PF}} + [\text{acet}]_{\text{headspace PF}} + [\text{acet}]_{\text{fungus PF}} \quad (2)$$

$$\%\ \text{acet}_{\text{fungus}} = \frac{\{[\text{acet}]_{\text{total AF}} - ([\text{acet}]_{\text{headspace PF}} + [\text{acet}]_{\text{media PF}})\}}{[\text{acet}]_{\text{total AF}}} \times 100 \quad (3)$$

where AF and PF mean absence and presence of fungus in the bioassay system, respectively.

Effects of Acetaldehyde on Spore Germination and Development in Vitro. Cytological experiments were carried out in order to study the effects of acetaldehyde on spore germination and development in vitro. Conidial suspensions of *A. alternata*, *B. cinerea*, and *C. acutatum* were prepared as described above. Droplets of the spore suspension (30 μL) of each pathogen were placed on acid washed glass slides that had been coated with polystyrene according to the method of Mercure et al. (1994) (*14*). This was done because conidia of these fungi rarely adhere and germinate poorly on a hydrophilic surface (*14*). The slides were then placed in 500 mL glass jars, to which an amount of 1, 5, or 10 μL of acetaldehyde was added as described above. For controls, slides with droplets of spore suspension were placed in jars (no acetaldehyde). After 24 h, the slides were removed from the jars and any conidia observed were assessed for their stage of development and physical appearance using bright-field microscopy using an Olympus BX 40 microscope (Olympus America Inc., NY). Images were captured using a Q-Color 3 digital camera (Olympus America Inc., NY) attached to the microscope.

Statistical Analysis. The StatGraphics Plus program, version 2.1 (Statistical Graphics Corp., 1994−1996), was used for analysis of variance (ANOVA) statistical comparison and to test significant differences between means with $p \leq 0.05$.

RESULTS AND DISCUSSION

Effect of Pure Acetaldehyde on *Colletrotichum acutatum*, *Alternaria alternata*, and *Botrytis cinerea* at 23 °C. Figure 1a shows the results obtained from the exposure of *C. acutatum*, *A. alternata*, and *B. cinerea* to different concentrations of acetaldehyde over a 7 day period at 23 °C. The different concentrations of acetaldehyde inside the bioassay system and the changes in concentration during storage were also monitored (**Figure 1b**). As shown, the addition of acetaldehyde to the bioassay system headspace significantly ($p \leq 0.05$) decreased and even prevented fungal growth for all tested fungal isolates. Concentrations of 0.12, 0.56, and 1.72 μL of acetaldehyde/L air were enough to prevent growth of *A. alternata*, *C. acutatum*, and *B. cinerea*, respectively. The toxicity of acetaldehyde was dependent on the fungal species and concentration of volatile used. For *C. acutatum*, small differences in acetaldehyde concentration had a significant impact on fungal growth. For instance, an acetaldehyde concentration in the headspace of 0.49 μL/L air reduced *C. acutatum* growth by 72% while 0.56 μL/L air reduced its growth by 100% when compared with the control. In contrast, acetaldehyde was only effective on *B. cinerea* when large amounts of volatile were present. This is in agreement

Table 1. Concentrations of Acetaldehyde (μL/L) in the Headspace and in the PDA Media of the Bioassay Systems in the Presence or Absence of *B. cinerea*

conditions inside bioassay system	acetaldehyde concentration[a] (μL/L)	
	in headspace	in PDA media
fungus present	0.0014 ± 0.0008 a	0.0016 ± 0.0005 a
fungus absence	0.2420 ± 0.0560 b	0.2570 ± 0.0830 b

[a] Numbers followed by a different letter within a column are significantly different at $p \leq 0.05$ (Fisher's test was used for comparison).

with Prasad and Stadelbacher (1974) (*3*) who reported that the antifungal action of acetaldehyde vapor against *B. cinerea* was a function of volatile concentration. In the case of *A. alternata*, treatment with volatile concentrations as low as 0.12 μL acetaldehyde/L air was enough to completely inhibit growth during a 7 day storage period.

In contrast, Utama et al. (2002) (*15*) reported that a concentration of 62 nmol acetaldehyde/mL air (3.43 μL acetaldehyde/L air) in the headspace was needed to inhibit growth of *Colletotrichum*. Avissar and Pesis (*16*) showed that as little as 0.5% or 0.25% of acetaldehyde in air during 24 and 40 h incubation reduced the growth of *B. cinerea* and *A. alternata*, respectively. All these values are significantly higher than those found in our research. Other aldehydes such as benzaldehyde and E-2-hexenal have been reported to have less effect on these three fungi, with 40 μL benzaldehyde/L air and 100 μL E-2-hexenal/L air being necessary to prevent fungal growth (*17*).

As shown in **Figure 1b**, the concentration of acetaldehyde in the headspace of the bioassay systems containing *B. cinerea* showed a rapid decrease when compared to the bioassay systems containing *A. alternata* and *C. acutatum*. To determine the reason for this unique behavior, the absorption of acetaldehyde by *B. cinerea* was investigated. After 7 days of storage at 23 °C, it was observed that the concentration of acetaldehyde in both the headspace and PDA media was lower in the presence of *B. cinerea* (**Table 1**). Acetaldehyde absorption by *B. cinerea* was calculated to be 97%. Thus, the rapid decline in the concentration of acetaldehyde in bioassay systems containing *B. cinerea* (**Figure 1b)** may be attributed to a faster growth rate for *B. cinerea* compared to *A. alternata* and *C. acutatum*, which increased its absorption area.

Acetaldehyde has been shown to cause phytotoxicity in fresh produce depending on both volatile concentration and product tolerance. Fruits and vegetables such as strawberries, grapes, and cherries showed symptoms of phytotoxicity when acetaldehyde was used in high concentrations (*3*, *7*, *18*). However, in in vivo studies acetaldehyde was reported to be effective and did not cause phytotoxic damage when used in low concentrations. For instance, Stewart et al. (1980) (*19*) used concentrations of 38−50 μL/L of acetaldehyde to fumigate head lettuce without causing phytotoxicity. Thus, the acetaldehyde concentrations used in our research could be presumed to

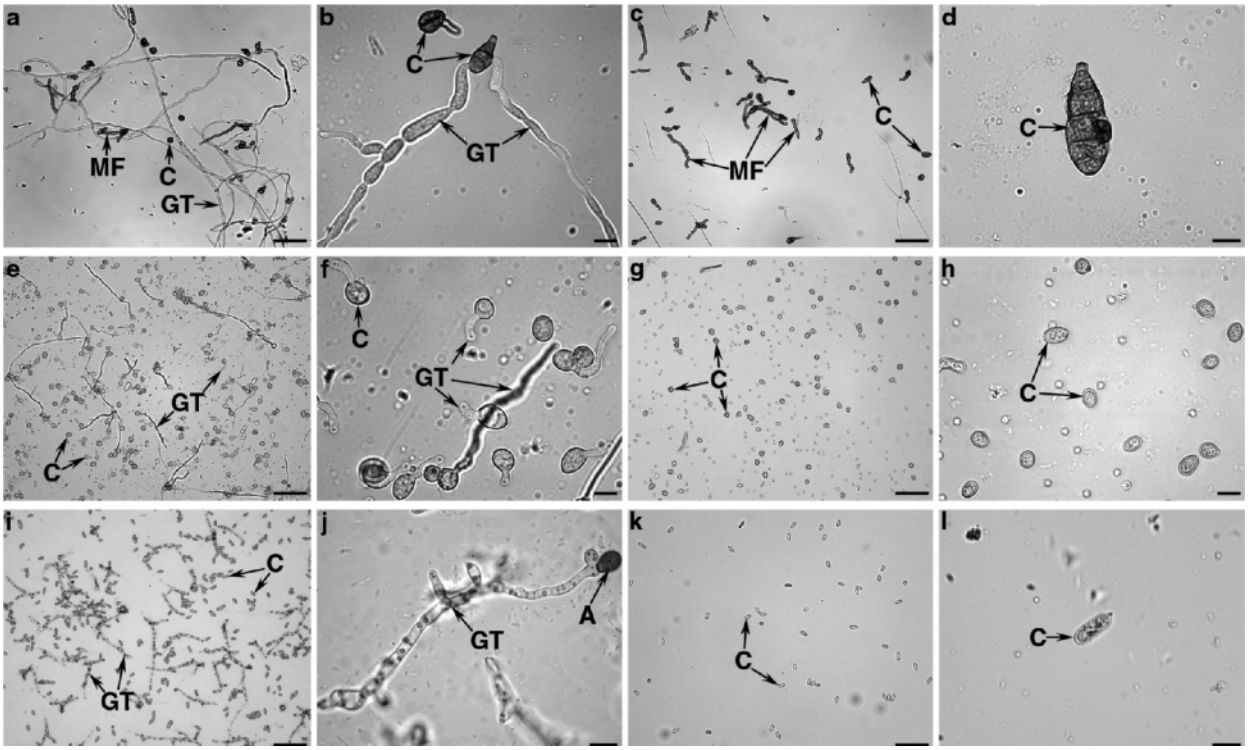

Figure 2. Effects of 1 μL of acetaldehyde (inside 0.5 L glass jars) on spore germination and development of *A. alternata* (**a**–**d**), *B. cinerea* (e–h), and *C. acutatum* (**i**–**l**) in vitro. (**a**, **b**) Conidial (C) germination and germ-tube (GT) formation of *A. alternata* 24 h after incubation in air at 23 °C. Germ tubes have proliferated over the slide surface (**a**). and their cytoplasm appears healthy (**b**). (**c**, **d**) Melanized conidia (C) and mycelial fragments (MF) of *A. alternata* have failed to germinate 24 h after being placed in an acetaldehyde atmosphere. (**e**, **f**) Conidial germination and germ tube formation in *B. cinerea* 24 h after incubation in air at 23 °C. Most of the conidia have germinated, producing germ tubes that appear healthy. (**g**, **h**) Conidia of *B. cinerea* have failed to germinate 24 h after being placed in an acetaldehyde atmosphere, and their cytoplasm appears plasmolyzed and disrupted (h). (**i**, **j**) Conidial (C) germination and germ-tube (GT) formation of *C. acutatum* 24 h after incubation in air at 23 °C. Most conidia have germinated, producing long germ tubes with melanized appressoria (A). (**k**, **l**) Conidia of *C. acutatum* placed in an acetaldehyde atmosphere failed to germinate after 24 h, and their cytoplasm appeared plasmolyzed and disrupted (l). (**a**, **c**, **e**, **g**, **i**, **k**) bars = 50 μm; (**b**, **d**, **f**, **h**, **j**, **l**) bars = 10 μm.

prevent fungal growth in vivo without causing phytotoxicity because the concentrations were lower than 38−50 μL acetaldehyde/L air. In addition, Paz et al. (1981) and Pesis et al. (1998) (*10*, *20*) have reported an enhancement in quality of certain produce such as avocados, pears, blueberries, and tomatoes when exposed to low concentrations of acetaldehyde vapors during postharvest storage.

According to our results, the effectiveness of acetaldehyde varies depending on the fungal species. The response of each fungus to this volatile could be due to several factors, such as the isolate of the pathogen and/or culture conditions used. A difference in the affinity of the volatile for the cell membrane of each fungus may play a role in its susceptibility to the volatile, since toxicity of volatile molecules has been reported to be linked to their affinity for the cell wall (*21*). Inouye et al. (2001) (*22*) showed that the effectiveness of gaseous contact on bacteria would depend on its permeability and/or interaction with the cell membrane. Knobloch et al. (1988) (*23*) reported that variations in the fungicidal activity of naturally occurring compounds such as essential oils seem to depend on its solubility as well as its capacity to interact with the plasma membrane. Thus, acetaldehyde may have the potential to be a wide-spectrum antifungal compound, as it completely inhibited growth of these three common rot pathogens. In order to determine the response/interaction of each fungus to this volatile, a cytological study was carried out.

Results from these cytological experiments (**Figure 2**) showed that acetaldehyde was highly toxic to all three species of fungi, inhibiting germination completely when 1 μL of volatile was introduced into the bioassay systems. In the control bioassay systems, 24 h after being placed on slides, conidia of all three fungi had germinated producing germ tubes. *A. alternata* was the fastest germinating of the three fungi and had produced long branched hyphae that spread out across the slide surface 24 h after the start of incubation (**Figure 2a**). Conidia of *C. acutatum* and *B. cinerea* also produced numerous germ tubes; however, these were much shorter than those of *A. alternata* (**Figure 2e**,**i**). Under higher magnification, the cytoplasm of the hyphae of all three species appeared healthy, with no signs of plasmolysis or degradation of the plasma membrane (**Figure 2b**,**f**,**j**). On slides that had been incubated in an acetaldehyde-rich atmosphere, there was no growth of the three fungi 24 h after exposure. There were no differences when 1, 5, and 10 μL of acetaldehyde were used (results not shown). An amount of 1 μL of acetaldehyde completely inhibited conidia germination of all three species (**Figure 2c**,**g**,**k**). Under higher magnification it was observed that the conidial cytoplasm of all three species appeared disrupted and plasmolyzed (**Figure 2d**,**h**,**l**). These observations are consistent with the theory that acetaldehyde causes leakage of electrolytes from fungal mycelia (*4*).

Fungicidal and Fungistatic Capacity of Acetaldehyde. The fungicidal and/or fungistatic properties of acetaldehyde were investigated by studying the inhibition of fungal reproduction and growth. Acetaldehyde was considered fungistatic if growth of the fungus occurred after it was transferred from the bioassay system containing the volatile to media in a jar free of the volatile. If no growth was observed after transfer to an empty jar, then it was considered fungicidal. **Figure 3** shows the effects of acetaldehyde on the growth of *C. acutatum*, *A. alternata*, and *B. cinerea* during storage at 23 °C. The fungicidal/fungistatic effects of acetaldehyde varied depending on the fungal species and concentration of volatile. A concentration of 0.56 μL/L acetaldehyde in air had a fungistatic effect on *C. acutatum* because mycelial plugs started to grow again 3 days after being placed on fresh media in an acetaldehyde-free atmosphere. At the end of storage, fungal growth was reduced by 10% compared to the control. However, when cultures were maintained on the same media, no growth was observed. At a concentration of 0.83 μL/L acetaldehyde in air, fungal growth of *C. acutatum* on new media was completely inhibited. Thus, at this concentration acetaldehyde was considered fungicidal to *C. acutatum*.

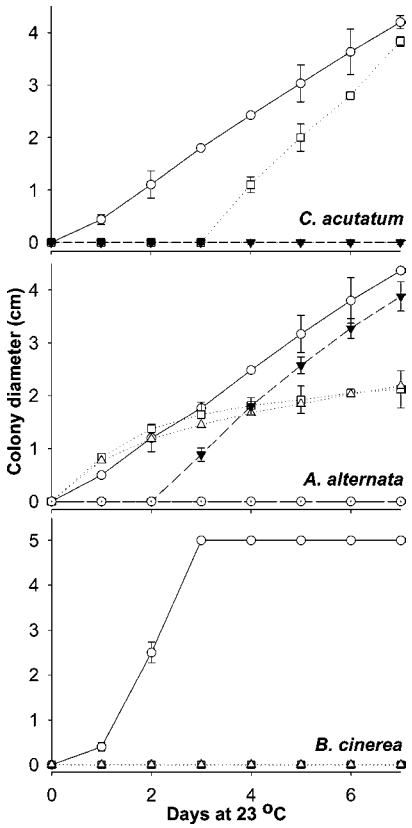

Figure 3. Growth of *C. acutatum*, *A. alternata*, and *B. cinerea* after being transferred to an acetaldehyde-free atmosphere bioassay systems in new or old media. *C. acutatum*: (○) control, (□) 0.56 μL acetaldehyde/L air (new medium), (△) 0.56 μL acetaldehyde/L air (old medium), and (▼) 0.83 μL acetaldehyde/L air (new medium). *A. alternata*: (○) control, (□) 0.12 μL acetaldehyde/L air (old medium), (△) 0.12 μL acetaldehyde/L air (new medium), (▼) 0.88 μL acetaldehyde/L air (new medium), (◇) 0.88 μL acetaldehyde/L air (old medium), and (Θ) 1.21 μL acetaldehyde/L air (new medium). *B. cinerea*: (○) control, (□) 1.72 μL acetaldehyde/L air (old medium), and (△) 1.72 μL acetaldehyde/L air (new medium).

The response of *A. alternata* to acetaldehyde was similar to that of *C. acutatum* but different from that of *B. cinerea*. At a concentration of 0.12 μL/L air, acetaldehyde had fungistatic activity against *A. alternata*. However, unlike *C. acutatum* a higher concentration of volatile (0.88 μL/L air) was not fungicidal because development was observed on fresh media 3 days after a mycelial plug was placed on the fresh media in a volatile-free atmosphere. As with *C. acutatum*, no growth was observed when *A. alternata* was kept on old media in a volatile-free atmosphere. Fungicidal activity was observed when the concentration of acetaldehyde was increased to 1.21 μL/L air. Acetaldehyde only had a fungicidal effect on *B. cinerea*. At a concentration of 1.5 μL/L air it was completely fungicidal regardless of whether mycelial plugs were transferred to fresh media or kept on old media in a volatile-free atmosphere. These results correlate well with those reported by Utama et al. (2002)

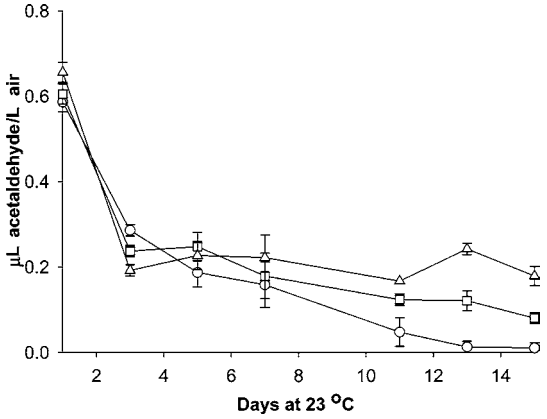

Figure 4. Concentration of acetaldehyde released from ICs obtained for different molar relationships between β-cyclodextrin and acetaldehyde: (○) 0.5 acetaldehyde and 1 β-CD; (□) 1 acetaldehyde and 1 β-CD; (△) 2 acetaldehyde and 1 β-CD.

(*15*) who showed that acetaldehyde had a fungicidal action against fungi such as *Rizhopus stolonifer*, *Penicillium digitatum*, and *C. musae*. Other authors have also reported the fungicidal effect of acetaldehyde (*24*, *25*).

According to Utama et al. (2002) (*15*), the fungicidal effect of acetaldehyde against fungi such as *Rizhopus stolonifer*, *Penicillium digitatum*, and *C. musae* was through the destruction of spores and shrinkage of mycelia. Avissar et al. (1990) (*4*) reported a leakage of electrolytes, sugars, and amino acids from fungal mycelia when *R. stolonifer* and *B. cinerea* were exposed to acetaldehyde. In contrast, other authors link the fungicidal effects of acetaldehyde to the augmentation of fruit resistance via synthesis of antifungal compounds. For example, acetaldehyde is the precursor of mevalonic acid, which is the precursor of all the monoterpenes, including the phytoalexin limonene (*26*) which protects orange fruit from fungal decay (*27*).

The fungi tested in this study showed different fungal growth patterns when transferred from an acetaldehyde-rich atmosphere to an acetaldehyde-free atmosphere. These growth patterns depended on whether or not mycelial plugs were transferred to fresh media or the cultures were left on the same media. This suggests that acetaldehyde was being absorbed by the growth media when the cultures were in an acetaldehyde-rich atmosphere. To determinate whether acetaldehyde absorption did occur, culture media (without fungus) was analyzed after 7 days of exposure to 3 and 5 μL of acetaldehyde in the bioassay systems. The results obtained demonstrated that PDA was able to absorb the volatile and that absorption was proportional to the amount of volatile used. Thus, 0.26 and 1.24 μg acetaldehyde/mg PDA, equivalent to 0.08 and 0.38 μL acetaldehyde/L air, were absorbed when 3 or 5 μL of acetaldehyde was introduced into the bioassay system. These concentrations of volatile were close to those necessary to inhibit the growth of *A. alternata* and *C. acutatum* (0.12 and 0.56 μL acetaldehyde/L air, respectively; **Figure 1**). Therefore, it is quite feasible that the fungi did not grow initially or their growth rates changed because of the acetaldehyde present in the media. This is in agreement with Utama et al. (2002) (*15*) who reported that acetaldehyde was absorbed by the growth media. However, these authors observed that the concentration of acetaldehyde absorbed was much greater than that remaining in the headspace, something that was not observed in this study.

Because fruits and vegetables are mainly composed of water like PDA, it can be postulated that they are capable of absorbing small amounts of volatile agents. Therefore, acetaldehyde may be absorbed by fresh produce and thus exerts its fungicidal and fungistatic activity during storage, thus extending product shelf life. The adsorption/absorption of acetaldehyde in fruits and vegetables needs to be more thoughly addressed.

Determination of the Effective Concentrations of β-Cyclodextrin−Acetaldehyde. To get ICs that slowly release the volatile in order to maintain a headspace composition that has adequate fungicidal effectiveness, different types of cyclodextrin (α, β, and γ) were tested in combination with different concentrations of acetaldehyde (from 70 to 280 μL). Inclusion complexes of β-cyclodextrin−acetaldehyde had better effectiveness compared to α- and γ- CDs, which had a 25-fold lower capacity to form ICs. This correlates with results reported by Shoesmith et al. (2003) (*28*), who found different solubility characteristics for cyclodextrins, with β-cyclodextrin being the most soluble of the three. **Figure 4** shows the volatile concentration of acetaldehyde released from the different ICs during a 15 day storage period at 23 °C and 100% RH. As can be seen, the microencapsulated content of acetaldehyde was not affected by the amount of volatile inserted into the cyclodextrin-distilled water paste except for the last 4−5 days of storage. In all cases, the highest acetaldehyde concentration in the headspace was reached after 1 day of storage with concentrations then decreasing during the rest of the storage period. Andersen et al. (1994) (*29*) also indicated that vapor-phase levels rise to a maximum within a few hours of encapsulation and then decline. Since the vapor-phase concentrations of encapsulated acetaldehyde were not constant during storage, the volatile concentration measured initially was reported as the effective concentration. In contrast with these results, release of volatiles such as hexanal from ICs has been shown to be dependent on the molar relationship between the volatile and CD (*12*). Further studies are being carried out using an electrobalance to

Table 2. Colony Diameter (cm) of *Alternaria alternata* Cultures after Exposure to Acetaldehyde Vapor Released from Different Amounts of β-Cyclodextrin–Acetaldehyde Inclusion Complexes during a 7 day Storage Period at 23 °C

	amount of β-CD–acetaldehyde IC[b] (g)							
	0.3		0.4		0.6		0.7	
time (days)[a]	colony ø[d]	volatile detected[c]	colony ø[d]	volatile detected[c]	colony ø[d]	volatile detected[d]	colony ø[d]	volatile detected[c]
1	0.4	0.05	0.4	0.09	0.4	0.11	0.4	0.15
3	0.4	0.05	0.4	0.09	0.4	0.09	0.4	0.15
5	1.5	0.06	0.7	0.08	0.4	0.08	0.4	0.12
7	2.7	0.01	2.5	0.04	1.4	0.03	0.4	0.08

[a] Cultures of *A. alternata* were placed inside sealed bioassay jars, and jars were stored at 23 °C for 7 days. [b] Inclusion complexes were obtained by means of incorporation of acetaldehyde into β-cyclodextrins during the centrifugation process. [c] The concentration of acetaldehyde (μL/L air) in the headspace of the bioassay jar was quantified by SPME sampling and GC analysis. [d] Colony diameter was measured in centimeters by means of calipers and a conventional ruler.

understand and model the kinetics of the release of different aldehydes from cyclodextrins.

Effectiveness of β-Cyclodextrin−Acetaldehyde Inclusion Complexes at Inhibiting Fungal Growth. Since *A. alternata* was the most sensitive of the three fungi to acetaldehyde, the effectiveness of the β-cyclodextrin−acetaldehyde ICs was determined for this fungus during a 7 day storage period at 23 °C. The antifungal effects of these ICs and their durability are shown in **Table 2**. Amounts ranging from 0.3 to 0.7 g of ICs were chosen because 0.7 g was necessary to reach the effective concentration of 0.12 μL acetaldehyde/L air inside the bioassay system. Lower amounts of ICs had less antifungal activity because of less acetaldehyde released. Thus, 0.6, 0.4, and 0.3 g of ICs were able to reduce *A. alternata* growth by over 92, 75, and 70%, respectively, after 7 days of storage at 23 °C.

Table 2 also shows how acetaldehyde concentration remained constant in the headspace of the bioassay systems in storage at 23 °C. In contrast to the pure volatile, a reduction in acetaldehyde levels was observed only near the end of storage when ICs were used. The decrease was less noticeable in jars with ICs than those with pure volatile because of the continuous release of acetaldehyde from the IC during the storage period. In both cases, the drop in concentration could be related to volatile absorption by the culture medium as described previously. These results demonstrate the ability of ICs to release and maintain a constant concentration of volatile in the headspace. Thus, with ICs the concentration of acetaldehyde can be maintained at a near constant level in a closed system during a fixed storage period. Nakamura and Hatanaka (2002) (*30*) also reported that the effectiveness of complexes containing aldehydes such as (3*E*)-hexenal, (3*Z*)-hexenal, (2*E*)-hexenal, and *n*-hexanal decreased with incubation time.

The results presented in this paper demonstrate the potential for the use of β-CD−acetaldehyde as an environmentally friendly fungicidal system because it has been shown to be effective against major postharvest pathogens such as *A. alternata*. Since acetaldehyde is generally recognized as a safe food additive (U.S. FDA/CFSAN: EAFUS list), β-CD−acetaldehyde complexes could be used to reduce or prevent postharvest diseases of fresh produce during the marketable period. Its commercial potential would depend upon several factors, including application amount, consumer acceptance of such systems, and the development of a system that could be used in fresh product packaging during storage.

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