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Interaction with and Effects on the Profile of Proteins of *Botrytis cinerea* by C_6 Aldehydes

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The natural volatile compounds *cis*-3-hexenal (*c*-3-H) and *trans*-2-hexenal (*t*-2-H) have significant antifungal activity with potential for use as postharvest fumigants of fruits and vegetables. However, the nature of their interaction with fungi and impact on fungal growth at the molecular level are largely unknown. The sites of interaction of these six carbon (C_6) aldehydes with *Botrytis cinerea*, a common pathogen of many plant species, was characterized using ^{3}H-labeled *c*-3-H and *t*-2-H. Radiolabeled C_6 aldehydes were produced with lipoxygenase and hydroperoxide lyase extracts using [9,10,12,-13,15,16-$^3H_6$]linolenic acid as a substrate. Following exposure of *B. cinerea* cultures to radiolabeled C_6 aldehydes, radiolabel was recovered in protein-enriched but not lipid-enriched fractions. Radiolabel was incorporated at higher levels (6-fold per milligram of fresh weight and 4-fold per microgram of protein) into conidia than mycelia. About 95% of the radiolabeled aldehyde recovered in the protein fraction was from the surface of the fungal tissue, while 5% was from protein in internal tissue (cell wall, membrane, and cytosol). Exposure to *t*-2-H at both 5.4 and 85.6 μmol affected the protein profile of *B. cinerea*, changing the intensity of over one-third of all proteins. Both up-regulation and down-regulation of specific proteins were observed by two-dimensional gel electrophoresis, indicating a clear effect of *t*-2-H on changes in the protein profile of *B. cinerea*. This is the first evidence that fungal proteins are targets of the volatile C_6 aldehydes and that sublethal levels of the aldehydes cause changes in the protein profile of a fungus.

KEYWORDS: *Botrytis cinerea*; radiolabeled C_6 aldehyde; *cis*-3-hexenal; *trans*-2-hexenal; site of interaction; protein profile

INTRODUCTION

Plants produce numerous volatile compounds, and many of these compounds have been studied for their biological effects on fungal growth (*1*). Since gray mold, caused by *Botrytis cinerea*, has been regarded as a major disease of fresh produce, numerous natural volatile compounds emitted by produce including aldehydes, acetate esters, alcohols, and terpenes have been tested for their antifungal effects (*2*−*6*). Among these compounds, aldehydes have been extensively studied due to their effective in vitro antimicrobial activity and potential as biologically based fumigants. Hamilton-Kemp et al. (*7*) developed a bioassay system testing the effects of volatile compounds on fungal growth and showed the effectiveness of six-carbon (C_6) and nine-carbon (C_9) aldehydes, which have antifungal activity at low concentrations. In addition, Hamilton-Kemp et al. (*7*) and Andersen et al. (*8*) demonstrated that *trans*-2-hexenal (*t*-2-H) was more effective in inhibiting hyphal growth of *B. cinerea* than the saturated aldehyde hexanal. Concentration-dependent promotion and inhibition of growth of *B. cinerea* have been observed (*9*).

The C_6 aldehydes *cis*-3-hexenal (*c*-3-H) and *t*-2-H are released from damaged leaves and fruits and produced via the lipoxygenase (LOX) pathway (*10*−*12*). It has been previously reported that wounded strawberry fruit exhibits changes in the LOX pathway within minutes, consistent with the rapid production of C_6 aldehydes (*13*). These aldehydes are thought to be involved in the defense mechanism of plants by inducing gene expression (*14*−*16*) and stress and defense responses (*17*, *18*) in plants. Linolenic acid (LNA) released from membrane lipids by lipases is converted to 13-HPOT [(13*S*)-hydroperoxy-(9*Z*,11*E*,15*Z*)-octadecatrienoic acid] by LOX, non-heme iron-containing dioxygenases (*19*). 13-HPOT formed by LOX is immediately metabolized by hydroperoxide lyase (HPL) (*20*, *21*) to form 12-oxo-(9*Z*)-dodecenoic acid and *c*-3-H (*22*). *c*-3-H isomerizes spontaneously or enzymatically into more stable *t*-2-H (*23*). The C_6 aldehydes may be further reduced to alcohols by alcohol dehydrogenase.

There have been several studies to elucidate the factors involved in the antimicrobial activity of C_6 aldehydes (*24*, *25*). The presence of an α,β-unsaturated bond adjacent to the carbonyl moiety has been shown to be significant in antifungal and antibacterial activity (*8*, *25*). The inhibitory effects of *t*-2-H

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against *B. cinerea* may be due to severe damage to fungal membranes and cell walls (*9*). Trombetta et al. (*25*) demonstrated that aldehydes including *t*-2-H caused significant changes in cellular membrane permeability, and Kubo et al. (*24*) hypothesized that the aldehydes bind nonspecifically, disrupt the hydrogen bonds in the lipid bilayer, and affect the fluidity of membrane lipids.

It is well-established that *t*-2-H can react with and modify proteins (*26*−*28*). Several lines of evidence have indicated that *t*-2-H is a reactive compound featuring two functional groups, an aldehyde group at C1 and a double bond between C2 and C3. The aldehyde and α,*β*-bond can readily attack electrophilic groups on proteins (*26*, *29*). Targets may be the sulfhydryl group of cysteine, the ϵ-amino group of lysine, and the imidazole group of histidine in protein; adducts may form via Michael addition or Schiff base reactions (*27*, *29*−*31*). Amarnath et al. (*32*) showed that the major reaction of *t*-2-H in the presence of protein in vitro was Michael addition. Thus, the chemical properties of C_6 aldehydes may result in reactions with proteins in biological systems.

The site(s) of interactions of C_6 aldehydes with fungi and their impact on molecular aspects of fungal growth are largely unknown. Protein and/or lipid modification alone via direct aldehyde interactions could influence fungal growth, though indirect effects on gene and/or protein expression patterns could also impact fungal growth. To further our understanding of the mode of action of *t*-2-H on fungal development, the goals of this study were (1) to determine whether protein and/or lipids of *B. cinerea* are radiolabeled following exposure to radiolabeled C_6 aldehydes and (2) to examine the effect of exposure of different concentrations of *t*-2-H on mycelial protein expression of *B. cinerea*.

MATERIALS AND METHODS

***B. cinerea* Isolation and Culture.** Mature strawberry fruit (*Fragaria* x *ananassa* Duch. cv. "Tribute") grown in the greenhouse in containers was harvested, placed on a wire mesh in a glass jar containing water to maintain humidity, and incubated for 5 days at 22 °C. Single spores of *B. cinerea* Pers.:Fr. were isolated from gray-colored lesions developed on the incubated fruit using an inoculation loop. The isolated single spores were point-inoculated and cultured on potato dextrose agar (PDA) medium at 22 °C for 7 days for fungal identification. The spores collected from the initial cultures were used to produce subsequent cultures on PDA. Cultures from subcultures 2−10 were used in this work. The cultures were grown for 14 days prior to use in an experiment.

In Vitro C_6 Aldehyde Production. For the production of C_6 aldehydes, a mix of *c*-3-H and *t*-2-H, the enzymatic reactions were performed in vitro by incubation of LNA (Sigma, St. Louis, MO) with LOX and HPL extracted from soybeans and watermelon leaves, respectively, based on methods described by Fukushige and Hildebrand (*33*). LOX crude extract was prepared from a −LOX3 genotype as detailed in Myung et al. (*13*). HPL crude extract was prepared from watermelon leaves (0.5 g) as described by Fukushige and Hildebrand (*34*). LOX and HPL activities were measured spectrophotometrically (Cary 50 Bio, Varian, Walnut Creek, CA) as previously described (*13*). The activities of LOX and HPL extracts were approximately 30.0 and 2.0 (μmol/min)/mg of protein, respectively. The reaction producing aldehydes was initiated in a 10 mL serum bottle which contained 4 mL of 0.012 μM LNA and 0.2 M borate buffer at pH 9.0. LOX was added to the mixture saturated with oxygen. After 2 h of incubation at 23 °C, the pH was lowered to 6−7 and HPL was added to the mixture. In preliminary experiments, various ratios of LOX, HPL, and LNA were tested, and the highest *t*-2-H production was obtained from 5 μg of LOX protein extract, 15 μg of HPL protein extract, 3.5 μg of LNA, and a 2 mL reaction volume, and these quantities were used for aldehyde generation. Following HPL addition, the bottle was immediately sealed with a poly(tetrafluoroethylene) (TFE)/silicon liner and aluminum cap and placed at 23 °C for 18 h. The content and identification of vapor-phase volatile compounds were determined by using solid-phase microextraction (SPME; Supelco, Bellefonte, PA) as described by Hamilton-Kemp et al. (*5*). The air in the bottle was sampled for 15 min periods after incubation for 2 and 18 h.

Incubation of Radiolabeled C_6 aldehydes with *B. cinerea* Cultures. The process of producing C_6 aldehydes from radiolabeled [9,-10,12,13,15,16-$^3H_6$]LNA (120 Ci/mmol at 1 mCi/mL, American Radiolabeled Chemicals Inc., St. Louis, MO) was performed according to the method described above with some modifications. After 2 h of incubation of the radiolabeled LNA with LOX and pH adjustment, the mixture was placed in 1 or 2 cm diameter glass vials, depending on the final volume of the mixture, to which HPL was added. Subsequently, a Petri dish (100 × 15 mm) containing a 14 day old culture of *B. cinerea* and a glass vial containing the reaction mixture were placed in a 15 cm diameter glass Petri dish, which was covered with the lid and immediately wrapped in Parafilm. This bioassay system was modified from that developed by Hamilton-Kemp et al. (*7*). Three different amounts (0.75, 7.5, and 15 μCi) of [^{3}H]LNA were compared in this experiment. Volumes of reaction mixture used were in proportion to LNA added (2 mL/1.5 μCi of LNA). The system was sealed for 18 h. Each experiment was repeated three times.

Extraction of Radiolabel from *B. cinerea* Conidia and Mycelia. After 18 h of incubation, each Petri dish was opened and allowed to remain in place for 30 min prior to use. The fungal tissue was harvested from PDA medium by gentle pouring of ice-chilled water (10 mL) on the PDA medium and suspending the culture. The suspension wash was then passed though Miracloth to separate mycelia from conidia. The residue on the Miracloth was washed with water (3 × 10 mL), and the wash filtrates were combined and centrifuged at 7000*g* for 30 min. The pellet contained mostly conidia with a few pieces of mycelia, confirmed by microscopy (Stemi SV11, Zeiss, Thornwood, NY). Therefore, the residue on the Miracloth was enriched in radiolabeled mycelia, while the resulting pellet was enriched in radiolabeled conidia. Total lipid was extracted from the conidial pellet three times with methanol, chloroform, and water (2:1:0.8). For total protein extraction, conidia were boiled with sodium dodecyl sulfate (SDS) extraction buffer [950 μL of Laemmli sample buffer (Bio-Rad, Hercules, CA) mixed with 50 μL of *β*-mercaptoethanol] for 10 min and centrifuged at 14000*g* for 5 min. Total protein was retained in the supernatant. A portion of mycelial tissue was also divided into lipid and protein fractions and prepared the same as for conidia. Portions of all samples were subjected to scintillation counting (Beckman Coulter, Fullerton, CA) for measuring radiolabel.

Protein Fractionation and SDS−Polyacrylamide Gel Electrophoresis (PAGE). *Extracellular Protein.* The supernatant of the conidial pellet fraction above was labeled the extracellular protein fraction. The volume was concentrated using a 3 kDa cutoff Centricon YM-3 ultrafiltration membrane (Millipore, Bedford, MA) according to the manufacturer's protocol. The final concentration of this protein-enriched fraction was determined by the method of Bradford (*35*). All samples were stored at −80 °C until further use.

Cell Wall, Membrane, and Cytosolic Protein. The mycelial fraction was subjected to differential centrifugation by schemes previously reported (*36*, *37*). The mycelial tissue was frozen in liquid nitrogen and ground to a powder. The tissue (500 mg) was suspended in 1.5 mL of extraction buffer of 10 mM Tris−HCl, pH 8.0, 1 mM EDTA, 10 μL of chymostatin, 10 μL of aprotinin, 10 μL of leupeptin, 15 μg of phenylmethanesulfonyl fluoride (PMSF), and 2% polyvinylpolypyrrolidone (PVPP) and was then centrifuged at 3000*g* for 10 min. The resulting pellet was washed five times with each of the following ice-chilled solutions: water, 5% NaCl, 2% NaCl, and 1 mM PMSF. It was then extracted twice by boiling with 2 mL of SDS extraction buffer of 50 mM Tris−HCl, pH 8.0, 0.1 M EDTA, 2% SDS, and 10 mM DTT for 10 min. Proteins in the cell wall fraction were precipitated with 50% acetone for protein determination as described above.

The supernatant after centrifugation at 3000*g* above was further centrifuged at 10000*g* for 10 min to remove debris. The resulting supernatant was used as the membrane plus cytosol protein fraction. The sample was further centrifuged at 100000*g* for 1 h in a Ti-91 rotor

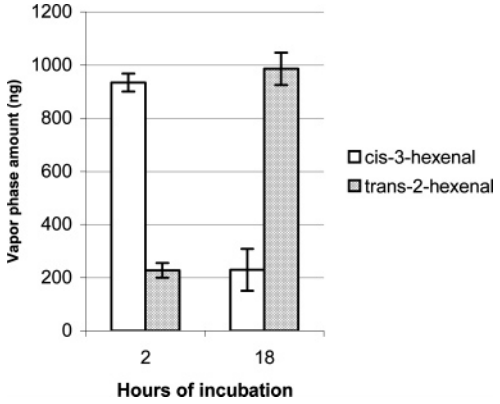

Figure 1. C_6 aldehyde production using linolenic acid as the substrate with LOX and HPL extracts. The content of aldehydes was determined using SPME. Bars indicate the SE of three independent experiments.

using an ultracentrifuge (XL-80, Beckman, Palo Alto, CA). The cytosolic supernatant was decanted, and the membrane pellet was dissolved in 500 mM NaOH.

SDS−PAGE. Samples of wash, membrane plus cytosol, cytosol, membrane, and cell wall protein were separated by 12% SDS−PAGE according to the standard protocol (Bio-Rad).

Two-Dimensional (2-D) Gel Electrophoresis and Image Analysis of Proteins. Mycelial tissue (500 mg) was harvested from fungal cultures in the absence or presence of *t*-2-H (5.4 or 85.6 μmol) after 24 h, and membrane plus cytosolic protein samples of the tissue were prepared as described above. The samples precipitated with acetone were solubilized in 100 μL of a lysis buffer (8 M urea, 4% CHAPS, 40 mM Tris). After determination of the protein concentration according to the method of Bradford (*35*), 125 μL of rehydration buffer containing 8 M urea, 2% CHAPS, 18 mM DTT, 2% pH 3−10 ampholytes (Bio-Rad), and a trace of bromophenol blue was added to the protein sample (100 μg), which was centrifuged at 12000*g* for 2 min. The resulting soluble protein was added to a focusing tray (Amersham Bioscience, Piscataway, NJ) followed by loading on a 7 cm long dry IPG strip (Bio-Rad). The strip was covered with mineral oil and left overnight for rehydration. The strip was removed from the tray, and excessive oil was rinsed with water. Isoelectric focusing was performed on a Multiphor II (Amersham Bioscience) at 20 °C by running the following program: 200 V for 1 min, 250 V for 2 min, 500 V for 10 min, increased by 250 V per 10 min up to 2750 and 3000 V for 1 h 30 min. The immobilized strip was equilibrated in equilibration buffer (1.5 M Tris−HCl, pH 8.8, 6 M urea, 2% SDS, 35% glycerol) with 1% DTT for 15 min and then in the equilibration buffer with 2% iodoacetamide for 15 min. The equilibrated strip was laid on 12% SDS−PAGE, and 1% agarose solution (1% agarose, 0.1% SDS, and 0.125 M Tris, pH 6.8) was poured to join the strip to the top of the SDS−PAGE. A second dimension run on SDS−PAGE was performed according to the standard protocol (Bio-Rad). The 2-D gels were stained with SYPRO Ruby (Bio-Rad), and images of the gels were produced by fluorescence-based scanning using Storm 860 and ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The images produced were processed and analyzed using PDQuest 7.2 (Bio-Rad). Each control and treatment were carried out three times, and the resulting triplicate gels were used for statistical analysis.

RESULTS

C_6 Aldehyde Production from Linolenic Acid. For a preliminary determination of the conversion efficiency of LNA to volatile compounds, nonradiolabeled LNA was used with LOX and HPL extracts. No volatile compounds other than C_6 aldehydes *c*-3-H and *t*-2-H were detected by GC analysis using LNA as a substrate (data not shown). At 2 h after the reaction started, *c*-3-H was the dominant volatile compound, and then it decreased to the same extent as *t*-2-H increased by 18 h (**Figure 1**). The sum of the C_6 aldehydes was the same at both 2 and 18 h. The overall yield of C_6 aldehydes was approximately 1.22 μg, indicating that 25% of the LNA was converted to C_6 aldehydes.

Table 1. Distribution of Radiolabeled Volatile Compounds Produced from 0.75 μCi (6.25 pmol) of [^{3}H]Linolenic Acid by LOX and HPL Extracts during an 18 h Exposure of *B. cinerea* to Volatile Compounds

	total radiolabel (μCi × 10^{-3})	total recovery of radiolabel (%)
[^{3}H]linolenic acid used in the reaction solution	750.0	100
nonvolatile compounds in the reaction solution after 18 h of incubation	528.0 ± 12.5	70.4
volatile compounds emitted from the reaction solution (by subtraction)	222.0 ± 5.2	29.6
total incorporation into *B. cinerea*[a]	4.4 ± 0.1	0.6

[a] Total incorporation of radiolabel into *B. cinerea* was calculated as the sum of that from all fungal extracts obtained from a 14 day old culture. Data shown are the means ± SE of three replications.

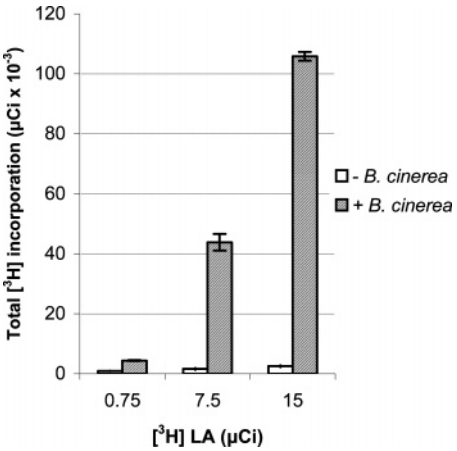

Figure 2. Total radiolabel from ^{3}H-labeled volatiles incorporated into PDA medium in the absence of *B. cinerea* (−*B. cinerea*) or in the presence of *B. cinerea* (+*B. cinerea*) Total incorporation of radiolabel in the absence of *B. cinerea* was measured from PDA medium, while the presence of *B. cinerea* was measured from all fractions (sum of the values in each fraction plus PDA medium) obtained from a 14 day old PDA culture. Three different concentrations of [^{3}H]linolenic acid, 0.75, 7.5, and 15 μCi, were incubated with LOX and HPL extracts. Bars indicate the SE of three independent experiments.

Concentration Dependence of Incorporation of Radiolabeled C_6 Aldehydes into Fungal Tissue. Using [9,10,12,13,-15,16-$^3H_6$]LNA for studying the incorporation of radiolabeled C_6 aldehydes into *B. cinerea*, it was found that 70% of the total radiolabel remained in the reaction solution (**Table 1**). Thus, 30% of the radiolabel was incorporated into volatile compounds, most likely C_6 aldehydes, though a direct measurement of radiolabeled C_6 aldehyde production was not attempted. Only 0.6% of the radiolabel was recovered from the mycelia. Radiolabel recovered on PDA medium without *B. cinerea* was $(0.9 \pm 0.1) \times 10^{-3}$ μCi (<0.1% of the radiolabel).

To determine whether the incorporation of radiolabeled C_6 aldehyde into *B. cinerea* was dependent on the total [^{3}H]LNA, the radiolabeled C_6 aldehydes generated from 0.75, 7.5, and 15.0 μCi of [9,10,12,13,15,16-$^3H_6$]LNA (specific activity of 120 Ci/mmol) were allowed to interact with the fungus (**Figure 2**). The incorporation of radiolabel was concentration-dependent, as an increase in [^{3}H]LNA from 0.75 to 7.5 μCi resulted in about an approximately 10-fold increase in incorporation of radiolabel, and with a 2-fold increase from 7.5 to 15 μCi, the radiolabel incorporated into *B. cinerea* also increased 2-fold.

Table 2. Radiolabel in Protein from Conidia or Mycelia of *B. cinerea* after Exposure to C_6 [^{3}H]Aldehydes for 18 h[a]

	total FW (mg)	total protein (μg)	radiolabel amt (μCi $\times 10^{-6}$)	radiolabel amt (μCi $\times 10^{-6}$/mg of FW)	radiolabel amt (μCi $\times 10^{-6}$/μg of protein)
conidia	42 ± 6	55 ± 9	2228 ± 300	53 ± 2	41 ± 7
mycelia	501 ± 22	434 ± 31	4151 ± 242	8 ± 1	10 ± 1

[a] [^{3}H]Linolenic acid at 7.5 μCi (62.5 pmol) was used to generate C_6 [^{3}H]aldehydes. The radiolabel was recovered from tissue after extensive gentle washing of conidia and mycelia for removal of external radiolabel. Total proteins were extracted by boiling fungal tissues with SDS sample buffer. Data shown are the means ± SE of three replications. FW stands for fresh weight.

This demonstrated that radiolabeled C_6 aldehydes can be consistently produced and incorporated into *B. cinerea* and that incorporation was dependent on the initial concentration of [^{3}H]LNA.

Distribution of Radiolabeled C_6 Aldehydes between Conidia and Mycelia. A comparison of radiolabel incorporation into conidia versus mycelia was determined after gentle washing to remove external radiolabel. Radiolabel was not found in the lipid fraction of conidia or mycelia (data not shown), but it was recovered in the protein fraction. Of the total radiolabel, approximately 2-fold more was incorporated into mycelia than into conidia (**Table 2**). However, as the total mass and protein contents of mycelia on PDA medium were 12- and 8-fold greater, respectively, than those of conidia, radiolabel per unit fresh weight (FW) and protein of conidia was greater than that of mycelia.

Distribution of Radiolabeled C_6 Aldehydes in Protein Fractions. Extracellular protein as well as protein from the cell wall and membrane + cytosol was recovered, and the radiolabel of each fraction was measured. The majority of the radiolabel on the fungal tissue, $(39.7 \pm 3.8) \times 10^{-3}$ μCi (95 ± 9%), was recovered in the wash fraction, while $(0.5 \pm 0.1) \times 10^{-3}$ μCi (1 ± 2%) was recovered in the cell wall fraction, and $(1.5 \pm 0.4) \times 10^{-3}$ μCi (4 ± 1%) was recovered in the membrane + cytosol fraction.

Comparison and Analysis of Protein Profiles in the Absence or Presence of *trans*-2-Hexenal. To characterize the effect of *t*-2-H on the protein profile of *B. cinerea*, the protein present following exposure to 0, 5.4, and 85.6 μmol of *t*-2-H was analyzed using 2-D gel electrophoresis (**Figure 3**). Proteins were both up- and down-regulated in the presence of *t*-2-H. Of 184 distinct proteins found, the intensity of 15 and 18 proteins increased more than 4-fold, but the intensity of 10 and 14 proteins decreased, in the presence of 5.4 and 85.6 μmol of *t*-2-H, respectively (**Table 3**). Significant intensity changes in proteins caused by 5.4 μmol of *t*-2-H were not necessarily observed in proteins affected by 85.6 μmol of *t*-2-H, nor were they always observed to change in the same direction if they did change in response to both amounts (data not shown). However, approximately 60% of the proteins were not affected (less than 2-fold change) by *t*-2-H at either concentration.

Of five proteins identified exhibiting significant changes in intensity (**Figure 3**), both proteins 1 and 2 were reduced greater than 8-fold when exposed to both amounts of *t*-2-H, proteins 3 and 4 were increased 10.1-fold and 5.2-fold, respectively, only when exposed to *t*-2-H at 5.4 μmol, and protein 5 increased 5.5-fold only when exposed to 85.6 μmol. The five proteins of interest (**Figure 3**) were subjected to MALDI-TOF peptide analysis, but a database search did not yield any significant matches of spectra except for protein 1. There is no database of proteins of *B. cinerea*, though other fungal species are represented. The spectrum of protein 1 matched that of a glucokinase from *Salmonella typhimurium* where four out of the nine peptides matched (data not shown).

DISCUSSION

C_6 Aldehyde Production from Linolenic Acid. In the in vitro C_6 aldehyde generating system used in this study, the C_6 aldehydes *c*-3-H and *t*-2-H were the major volatiles derived from LNA, agreeing with previous studies (*21*, *33*, *38*, *39*). High *c*-3-H production and low *t*-2-H production were detected at 2 h of incubation (**Figure 1**), similar to that reported by Fukushige and Hildebrand (*33*) after 2 min. The total yield of C_6 aldehydes did not change between 2 and 18 h, suggesting the reaction was complete and had reached a maximum production by that time and that *c*-3-H isomerized to *t*-2-H, a possibly spontaneous rearrangement of *c*-3-H (*22*). C_6 aldehyde production from LNA by various sources of LOX and HPL in different incubation systems has been reported to be complete within 1−4 h of incubation (*21*, *38*, *39*). The 25% conversion of LNA to C_6 aldehyde in this study was similar to the 26% conversion of Noordermeer et al. (*21*), but lower than the 50.3% conversion possible under optimum conditions using a LOX3−LOX1 mutant (*33*).

Incorporation of Radiolabeled C_6 Aldehydes into *B. cinerea*. Though 30% of the LNA was converted to C_6 aldehydes, less than 1% was recovered from fungal tissues (**Table 1**). This suggests that an appreciable amount was lost through evaporation when the closed incubation system was opened and/or was bound to glass surfaces and media. There was a concentration dependence of radiolabeled C_6 aldehyde incorporation into the fungal tissue, as about 10-fold more radiolabel was recovered when 10-fold more LNA was used as the substrate (**Figure 2**). There was no relationship between radiolabel concentration and that recovered on PDA medium without *B. cinerea* (data not shown), so the concentration dependence with fungal tissue presence suggested an abundance of sites of interaction were present in the tissue that were not saturated at the lower amounts of LNA and C_6 aldehyde production.

As shown in **Table 2**, greater incorporation of C_6 aldehydes into conidia than mycelia per milligram of FW and microgram of protein was observed. Conidia of *B. cinerea* had more potential sites of interaction as they contained more protein per milligram of FW than mycelia, 1.3 μg/mg vs 0.9 μg/mg, respectively. In addition, it appeared that the conidial protein was more likely to interact with the radiolabeled aldehyde. This may be because a greater proportion of the conidial protein was extracellular and more accessible to the volatile aldehydes, or it presented more sites for potential interaction. The protein thiol content was 25% higher in conidia than in mycelia of *Neurospora crassa* (*40*), and thiols can react with aldehydes, suggesting the possibility of greater binding potential to conidial rather than mycelial protein. Previously, it was reported that spore germination was apparently more sensitive to the *t*-2-H concentration than was mycelial growth (*9*). The greater relative interaction of C_6 aldehydes with conidia may be correlated to their greater sensitivity.

Radiolabel from C_6 aldehydes was not found in the lipid fraction of *B. cinerea* (data not shown). This suggests that hydrophobicity may not be a critical factor affecting the reactive property of C_6 aldehydes. This is in agreement with Niknahad et al. (*41*), who indicated that cytotoxicity of alkenals such as the C_6 aldehydes in our work was related to their electrophilicity,

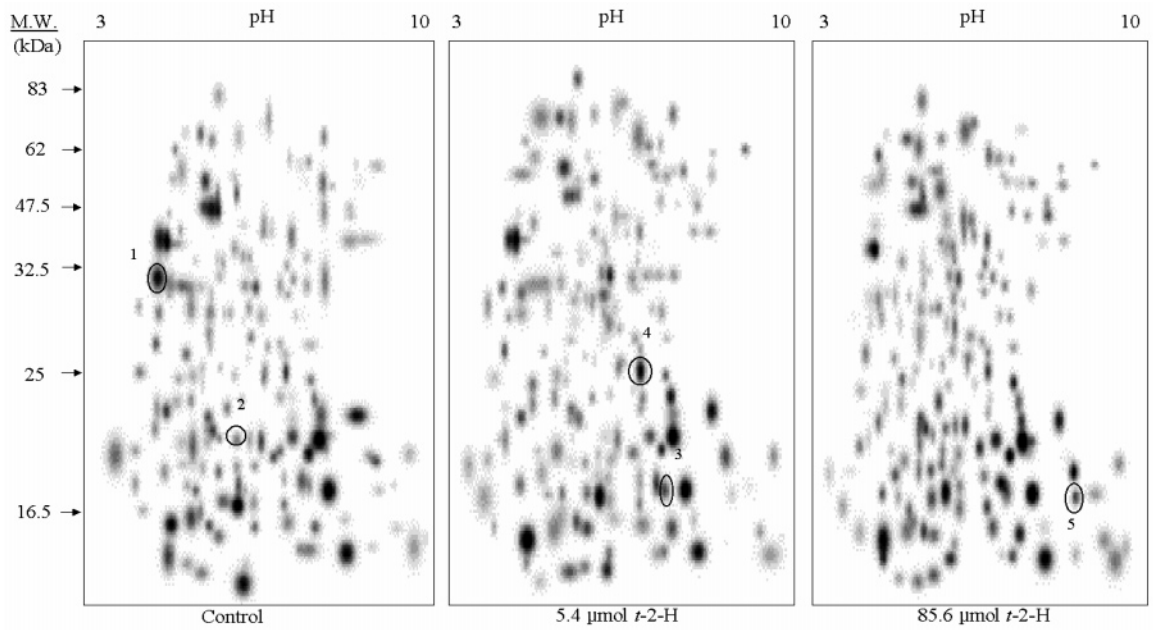

Figure 3. Comparisons of protein profiles of *B. cinerea*, using two-dimensional gel electrophoresis, in the absence or presence of *t*-2-H. The three images were obtained from two-dimensional gel electrophoresis using image analysis software. Five protein spots in circles showed significant changes in intensity and were further analyzed by MALDI-TOF MS.

Table 3. Changes in the Protein Intensity of *B. cinerea* in the Absence or Presence of *t*-2-H[a]

		no. of proteins affected by the amount of *trans*-2-hexenal	
degree of intensity changes of proteins	change in protein intensity	5.4 μmol	85.6 μmol
high (>4-fold)	up	15	18
	down	10	14
moderate (2–4-fold)	up	20	28
	down	21	17
little (<2-fold)		118	107
total		184	184

[a] A total of 184 protein spots were visible and analyzed by image analysis software. The intensity levels of each protein spot obtained from triplicate samples of the control and 5.4 and 85.6 μmol of *t*-2-H treatments were averaged and compared. Gels were reproducible three times and subjected to image analysis.

not their hydrophobicity. Previously, Haynes et al. (*42*) also suggested that hydrophobicity is not closely related to the toxicity of aldehydes. However, Kubo et al. (*24*) suggested that alkenal interaction with the membrane, mediated by the balance between hydrophobic and hydrophilic traits, was key to their antifungal activity. The similar antifungal activity of alkanals, lacking an α,β-unsaturated bond, versus alkenals supported this conclusion. However, in that work, the lowest effective level of both *trans*-2-alkenals and alkanals in solution cultures with *Saccharomyces cerevisae* was in the 6 μg/mL range. On the basis of the efficiency of conversion of LNA to C_6 aldehydes noted above, headspace levels of C_6 aldehydes in the Petri dishes containing fungal cultures were estimated to be about 0.3 ng/mL, over 1000-fold less than the levels used by Kubo et al. (*24*). In our previous work with *t*-2-H (*9*), headspace levels of 20−500 ng/mL, as used in the present work to study the impact of the aldehydes on the fungal protein profile (**Table 3**), were employed. These levels are 10−300-fold lower than those used by Kubo et al. (*24*). Though the present data do not preclude interaction of C_6 aldehydes with membranes, it is clear that interactions of C_6 aldehydes with protein occur at very low headspace levels with effects observed at the molecular level (i.e., protein profile) as well. The observation that alkanals and alkenals had comparable effects on *S. cerevisae* (*24*) may have been due to the concentrations used and obscured effects that may exist with lower concentrations. The differing approaches used in that work versus the present study, fungi and alkenals in solution culture as compared to the vapor phase, may have also contributed to the differing results and interpretations. The presence of an α,β-unsaturated bond adjacent to the carbonyl moiety of *trans*-2-alkenals such as *t*-2-H has been shown to be important to antifungal and antibacterial activity (*8*, *25*). In our work, only significant interaction with protein was noted. The electrophilicity of C_6 aldehydes is essential for interaction with proteins via Michael addition or Schiff base formation. It should be noted that *t*-2-H can react with proteins via both reactions, while *c*-3-H can react with proteins only via Schiff base formation because it lacks the α,β-unsaturated bond. Therefore, the significant interaction with protein in this work suggests that this may mediate an important effect on the fungus.

Site of Interaction of C_6 Aldehydes with *B. cinerea*. The distribution of radiolabel recovered from *B. cinerea* indicates that approximately 95% of C_6 aldehydes did not penetrate the fungal tissue and were removed with gentle washing. Proteins recovered in the wash fraction may be those secreted from mycelia of *B. cinerea*, as the fungus is known to secrete cuticle- and cell-wall-degrading enzymes (*43*, *44*). Interaction of C_6 aldehydes with the secreted proteins could modify them and thus affect the growth of *B. cinerea*. Protein modification at sufficient levels may cause loss of pathogenicity. Alberti-Segui et al. (*45*) showed that the absence of glycosidase activity, a secreted protein from yeast, reduced adherence to target cells and delayed colonization. Also, Benderdour et al. (*46*) found that inactivation of $NADP^+$−isocitrate dehydrogenase occurred upon incubation with 4-hydroxynonenal via post-translational modifications, in which the aldehyde bound to a cysteine residue near the substrate's binding site. However, it is not yet known whether C_6 aldehydes can modify and affect the activity of secreted proteins.

Comparison and Analysis of Protein Profiles of *B. cinerea* in the Absence or Presence of *trans*-2-Hexenal. Treatment with *t*-2-H increased or decreased the intensity of about 40% of the proteins of *B. cinerea* (**Figure 3**, **Table 3**). It is likely that the changes resulted from an effect on de novo biosynthesis of proteins and/or post-translational modification of proteins because *t*-2-H exposure of *B. cinerea* was for 24 h. The period of incubation was chosen because it was previously shown that the headspace *t*-2-H concentration in the presence of *B. cinerea* markedly declined over 24 h, indicating that *B. cinerea* absorbed a significant amount of the volatile compound and fungal growth was affected (*9*). Alméras et al. (*47*) indicated that *t*-2-H not only damages cells, but it can also selectively affect gene expression of *Arabidopsis*. In addition and of greater pertinence to *Botrytis*, Chitarra et al. (*48*) demonstrated that the volatile germination self-inhibitor 1-octen-3-ol produced significant effects on the protein profile of *Penicillium paneum* conidia. Sixteen proteins specific to 1-octen-3-ol treatment were observed, though not identified. Though effects on protein expression likely occurred in the present work, since *t*-2-H can interact or modify proteins, the possibility that the locations of some of the fungal proteins may have been shifted due to such modification cannot be excluded. Because approximately 60% of the proteins remain unaffected (a less than 2-fold change) by both amounts of *t*-2-H (**Table 3**), the response to C_6 aldehyde exposure would appear to be somewhat specific.

In conclusion, using a procedure designed to produce *t*-2-H from [9,10,12,13,15,16-$^3H_6$]LNA with LOX and HPL extracts, radiolabeled C_6 aldehydes were synthesized in vitro and used to characterize C_6 aldehyde interaction with *B. cinerea*. The results demonstrated that the majority of C_6 aldehydes recovered from fungal tissue had interacted with proteins on the surface of the fungal tissue, though some C_6 aldehyde did penetrate the tissue. Radiolabeled C_6 aldehydes were preferentially incorporated into conidia rather than mycelia, a result correlated to the greater sensitivity of spore germination than mycelial growth to C_6 aldehyde (*9*). The effect of the C_6 aldehyde *t*-2-H on the protein profile of *B. cinerea* was evaluated for the first time in this study. The results showed that *t*-2-H treatment can appreciably alter the fungal protein profile. Further analysis of both the affected extracellular proteins and the expressed proteins may provide a more specific understanding of how C_6 aldehydes influence *B. cinerea* spore germination and fungal growth. Furthermore, the results provide a basis for further studies on the nature of C_6 aldehyde and fungal interactions and could lead to more effective strategies and focused targets for their use as biologically based postharvest fumigants for fungal control on fresh fruits and vegetables.

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