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The use of natural plant volatile compounds for the control of the potato postharvest diseases, black dot, silver scurf and soft rot

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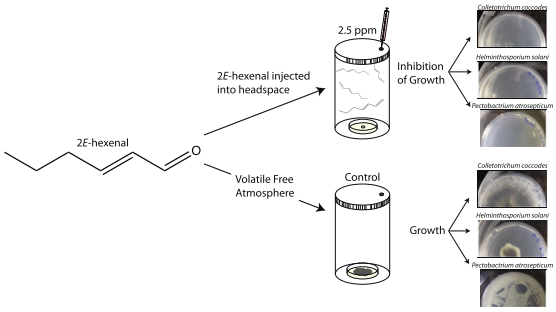
HIGHLIGHTS

- Naturally occurring plant volatiles are known for their anti-fungal properties.
- Acetaldehyde and 2*E*-hexenal tested for anti-fungal properties against potato diseases.
- Acetaldehyde did not prevent growth of potato pathogens *in vitro*.
- 2*E*-hexenal completely inhibited growth of potato pathogens *in vitro* at 10 μL/L.

GRAPHICAL ABSTRACT



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ABSTRACT

Many naturally occurring plant volatile compounds are known for their anti-fungal properties. In this study, acetaldehyde and 2*E*-hexenal were chosen as prototype volatiles in order to investigate the use of volatile compounds for control of blemish pathogens in fresh-pack potato packaging. Pure cultures of the three main potato blemish pathogens, *Pectobacterium atrosepticum* (bacterial soft rot), *Colletotrichum coccodes* (black dot), and *Helminthosporium solani* (silver scurf), were used in the study. Pathogen cultures were exposed to the pure volatiles that were injected into the atmosphere of sealed jars for 4–8 days at 23 °C. Results showed that 2*E*-hexenal was the most effective of the two volatiles with 5 μL/L providing complete inhibition of growth for all three pathogens *in vitro*. Cytological studies showed that a concentration of 2.5 μL/L of 2*E*-hexenal was capable of inhibiting germination in both fungal pathogens. These results suggest that the primary mode of action of 2*E*-hexenal was inhibiting germination for fungi and suppressing bacterial growth. The quantities required to achieve pathogen inhibition are extremely low. This study suggests that these volatiles may be used to effectively manage potato postharvest blemish diseases in storage.

1. Introduction

Pectobacterium atrosepticum (van Hall) Patel and Kulkarni, the causal agent of bacterial soft rot or pit rot of potato, is an important potato pathogen affecting postharvest storage of potatoes and causing significant economic losses. Although it is difficult to estimate exact values for loss of potato crops due specifically to bacterial soft rot, it has been estimated that soft rot bacteria may cause economic losses of $50–100 million in multiple crops world-wide every year (Perombelon and Kelman, 1980). This pathogen infects the lenticels of tubers while in the soil under wet soil conditions and can quickly spread to other potatoes in low oxygen, humid and warm storage conditions (Johnson, 2011). Currently, there are few, if any, postharvest methods of controlling this pathogen. Current control methods rely heavily on keeping harvested tubers well aerated, cool and dry to prevent ideal conditions for pathogen growth and spread (Bourne et al.,1981).

Colletotrichum coccodes (Wallr.) S. Hughes, the causal agent of black dot, and *Helminthosporium solani* (Durieu and Mont.), the

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causal agent of silver scurf, are other economically important blemish pathogens of potato. These pathogens often initially infect stems and tubers in the field and then spread during storage through distribution of air-borne conidia causing latent infection of tubers (Glais-Varlet et al., 2004, Errampalli et al., 2001). *C. coccodes* is characterized by visible black spots and lesions on the tuber skin that can lead to rejection of the produce by processors and consumers. Similarly, *H. solani* produces a well-defined silvery lesion that can also lead to rejection of the produce. Currently, the most effective methods for control of these pathogens are cultural measures. These include reducing initial field inoculum by not planting infected seed, limiting free moisture in the soil by managing irrigation and reducing the time between vinekill and digging. Studies have also shown that the length of crop duration, from 50 percent emergence to harvest, shows a close relationship to final black dot levels on progeny tubers in storage. The shorter the crop duration the less black dot occurred in storage (Wale et al., 2008). Most current control methods for these pathogens focus on reducing initial field inoculum, but there are very few effective methods of protecting tubers directly in storage.

Many plant volatile organic compounds are known for their anti-fungal properties. 2*E*-hexenal is a well studied volatile organic compound produced by bananas, tomatoes, and other fruits as an aroma volatile associated with a green or grassy smell (Hayata et al., 2002). It is also produced by plants such as common bean (*Phaseolus vulgaris*), gingko (*Gingko biloba*), and cotton (*Gossypium* sp.) in response to pathogenic infection (Croft et al., 1993). 2*E*-hexenal is biosynthesized from the breakdown of linolenic acid by lipoxygenase and hydroperoxide causing the formation of two intermediate compounds *cis*-3-hexenal and *trans*-3-hexenal (Myung, 2005). These intermediates are further altered by two isomerization factors ultimately resulting in the formation of 2*E*-hexenal (Min, 2001). In blueberries and strawberries, it was found that 2*E*-hexenal had antimicrobial properties against *Colletotrichum acutatum*, but the levels present in fruit were not correlated with host resistance (Polashock et al., 2007; Arroyo et al., 2007). Studies by Pérez et al. (1999), found that 2*E*-hexenal was the primary aldehyde in ripening strawberry fruits and its production was due to increases in lipoxygenase activity. An increase in lipoxygenase activity is not necessarily correlated with increased host resistance. In avocados, lipoxygenase breaks down antifungal dienes present in unripe fruits causing them to become susceptible to *Colletotrichum gloeosporioides* infection (Prusky and Keen, 1993). However, in several studies exogenous application of 2*E*-hexenal has been shown to slow the growth of multiple plant pathogens including *Colletotrichum truncatum, Rhizoctonia solani*, and *Sclerotium rolfsii* on soybean, *Penicillium expansum* on conference pears, *Monilinia laxa* on stone fruits, *Pseudomonas syringae* on common bean and *Aspergillus flavus in vitro* (Vaughn and Gardener, 1993; Neri et al., 2006; Neri et al., 2007; Croft et al., 1993).

Acetaldehyde is another volatile compound that is highly effective in slowing the process of fruit ripening in mangos and preventing pathogenic infection of other fruits (Pesis, 2005; Utama et al., 2002). Acetaldehyde is biosynthesized from pyruvic acid by pyruvate decarboxylase, which depends on the cofactors thiamine pyrophosphate and magnesium (Dyda et al., 1993). Exogenous applications of acetaldehyde have also shown that it is fungitoxic to the blueberry pathogens, *C. acutatum*, *Botrytis cinerea*, and *Alternaria alternata* when introduced into the headspace of a jar containing the pathogens (Almenar et al., 2007). Given the effectiveness of these plant volatile compounds in controlling pathogen growth in fruits and other plants tissues, they could also be very useful in providing control of postharvest potato blemish pathogens.

The use of volatile organic compounds is a novel method of controlling postharvest potato blemish pathogens. This method of control has many potential uses such as in storage fumigation, controlled atmosphere storage, and fresh-pack packaging. These volatile organic compounds could provide a new method of protecting tubers directly, and could be an alternative to the few currently available postharvest fungicides. They have less environmental impact as both acetaldehyde and 2*E*-hexenal are naturally occurring plant compounds and registered by the US Food and Drug Administration (FDA) as food grade additives (FDA, 2011). Thus, the objectives of this study were first to determine *in vitro*, the effectiveness of acetaldehyde and 2*E*-hexenal in controlling the growth of the postharvest potato blemish pathogens, *C. coccodes*, *H. solani*, and *P. atrosepticum* and secondly to identify the minimum concentration of the volatiles required to inhibit pathogen growth.

2. Materials and methods

2.1. Fungal isolates and culture preparation

Mono-conidial cultures of the pathogens *C. coccodes, H. solani*, and *P. atrosepticum* were originally isolated from diseased potato tubers grown in Idaho. Cultures of *C. coccodes*, *P. atrosepticum*, and *H. solani* were grown on PDA (potato dextrose agar, VWR International, Randor, PA, USA) in plastic Petri dishes (1 × 10 cm diameter) for 1 week at 23 °C in the light. Conidia and mycelia from the fungal pathogens *C. coccodes* and *H. solani* were collected by flooding the surface of plates with sterile distilled water and gently scraping the surface with a sterile bent glass rod. The suspension (3 mL) was transferred to a sterile 15 mL plastic tube, which was vortexed for three 10 s intervals to dislodge the conidia from the mycelia. Conidial concentrations were determined using a hemocytometer and adjusted to 1×10^5 conidia/mL using sterile distilled water.

Cells from a 24-h-old *P. atrosepticum* culture were collected using a sterile loop (3 mm diameter) and suspended in 1 mL of sterile distilled water in a sterile 15 mL plastic tube. The suspension was vortexed for 10 s to evenly distribute the cells in each step in the dilution series used to obtain a final concentration of cells that was 1:10,000 (v/v) of the original solution.

2.2. In vitro bioassays

For the bioassay, three replicate plates for each pathogen, volatile, and volatile concentration combination were prepared. Ten microliters of each pathogen suspension was pipetted onto the center of separate 5-cm-Petri dishes that each contained exactly 10 mL of PDA. The fungal conidial suspensions were pipetted onto the top of the media in the center of each plate. Bacterial suspensions were pipetted onto the center of the media then spread evenly around the plate using a sterile bent glass stir rod to create a thin layer of the cells on top of the media. The inoculated lidless Petri dishes were carefully transferred in a sterile biosafety cabinet to sterile 1 L glass jars (VWR International). These jars were then sealed using Polytetrafluoroethylene (PTFE) tape (Mil SPEC T-27730A, Merco-Hackensack Inc, Hillburn, NY, USA) around the mouth of the jar and closed with modified screw cap lids fitted with a rubber plug septum (Shimadzu thermo-green from Sigma–Aldrich Corp., St. Louis, MO, USA) and a PTFE liner (Thomas Scientific Swedesboro, NJ, USA) to make sure that they were air-tight.

Liquid volumes of 2.5, 5, 7.5, and 10 μL of either acetaldehyde or 2*E*-hexenal, were injected into the jars using an airtight syringe (25 μL GASTIGHT Hamilton syringe, Reno, NV, USA) through the septum onto the side of the glass container. This allowed the volatile compounds to evaporate into the headspace of the jars. Liquid cyclohexanol was also injected into the jars and used as an internal standard at a constant volume of 2 μL. The jars were stored at 23 °C

for 48 h (*P. atrosepticum*) and 96 h (*C. coccodes* and *H. solani*) in the dark.

2.3. Determination of volatile compound concentrations in the jar headspace

Standards for acetaldehyde, 2*E*-hexenal, and cyclohexanol were purchased as commercial preparations with a nominal purity of at least 95% from Supelco (Bellefonte, PA, USA). The headspaces of the jars were sampled manually 24 h after injection by means of Polydimethylsiloxane/Divinylbenzene (PDMS/DVB) 16 gage 65 μm solid-phase micro-extraction (SPME) fiber (Supleco) and quantified with gas chromatography–mass spectrometry (GC–MS). The SPME fiber was exposed to the sample headspace for 10 min at 23 °C. The sample trapped on the SPME fiber was immediately desorbed for 5 min (at 280 °C) at the split-less injection port of a Shimadzu GC–MS QP5000 (Shimadzu Corporation, Kyoto, Japan) equipped with an electron ionization detector and a DB-5MS column (30 m × 0.32 mm × 0.25 μm, J&W Scientific, Agilent Technology, Santa Clara, CA, USA). The oven temperature was set at 40 °C for 5 min ramped up to 230 °C over 10 min and maintained at 230 °C for 5 min, the injection port and detector temperatures were set at 230 °C. The concentration of volatile present in the headspace of each jar was determined using GC analysis and an internal standard method of calibration.

2.4. Measurement of fungal growth

The growth of each plate was recorded every 24 h using calipers to measure the diameter of fungal growth. The pathogens were exposed to the treated headspace for 48–96 h depending on the growth rate of each pathogen. The exposure time was determined by how long it took for cultures to completely colonize the surface of the media in control jars with a volatile free atmosphere. For *P. atrosepticum*, this was 48 h, for the fungal pathogens *C. coccodes* and *H. solani* it was 96 h. Fungal growth was expressed in millimeters of radial colony growth. Due to the transparency of the glass jars and Petri dishes, growth measurements could be collected without opening the jars. Each assay was carried out three times, and the results were analyzed statistically using a chi square test.

2.5. Measurement of bacterial growth

Bacterial growth on each plate was recorded every 24 h using photographic analysis. A 12.1-megapixel digital photograph (Cannon Powershot SX230 HS Cannon USA Inc., NY, USA) was taken of the bacterial colonies present on the media surface from the underside of the transparent jar and Petri dish against a black background. These images (3648 × 2736 pixels) were then imported into Photoshop (Adobe Systems Incorporated Creative Suite 5 version 12.0.1, San Jose, CA, USA) and cropped to eliminate any background noise surrounding each plate. The image pixels in the black background have a brightness of 0 light intensity units (LIU), while pure white pixels have a brightness of 255 (LIU). The "magic wand" selection tool, which automatically selects an area based on the brightness of the pixels in that area was set to a tolerance value no lower than 3 LIU or higher than 20 LIU and used to select each bacterial colony present on the media surface using visual differentiation between the bacterial colonies and media imperfections as well as background noise. Once all of the colonies were selected, the area of the selection was expressed in pixels and recorded using the count feature of the software. The area of each plate not containing bacterial colonies was also recorded and the percentage of colony growth determined by comparing the area covered in colonies to the area free of colonies. Each assay was carried out three times, and the results were analyzed statistically using a chi square test.

2.6. Determination of fungicidal and/or fungistatic activity of volatile compounds

After 48 or 96 h of exposure, jars were opened, the plates were carefully removed in a sterile biosafety cabinet and placed in a new container with a volatile free atmosphere in the headspace. Pathogen cultures were placed in new clean jars to test if they would resume growth. The pathogen cultures were exposed to the clean headspace for the same amount of time as they had been exposed to the treated headspace (48 or 96 h) at 23 °C in the dark. New growth of the pathogen on each plate was recorded using the methods described above. The test volatiles were considered fungistatic if the pathogen resumed growth when placed in a clean jar and fungitoxic if no growth was observed after being placed in a clean jar for up to 96 h.

2.7. Effects of 2E-hexenal on conidial germination and development in vitro

Cytological experiments were carried out in order to study the effects of 2*E*-hexenal on conidial germination and development *in vitro*. Conidial suspensions of *C. coccodes* and *H. solani* were prepared as described above. Droplets of the conidial suspension (30 μL) of each pathogen were placed on acid washed glass slides, which had been coated with silicone (Sigmacote, Supelco) to produce a neutral hydrophobic surface on the microscope slides. This was done because conidia of these fungi rarely adhere, and germinate poorly on a hydrophilic surface (Mercure et al., 1994). The slides were then placed in 1 L glass jars, where 2.5, 5, 7.5, and 10 μL of volatile compound were added as described above. For controls, slides with droplets of conidial suspension were placed in clean jars (no volatile). 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 CX41 (Olympus America Inc., Center Valley, PA, USA). Images were captured using an AmScope MA1000 digital camera (Iscope Corporation USA, Irvine, CA, USA) attached to the microscope. Adobe Photoshop's count tool was used to mark and measure germinated versus non-germinated conidia on each captured image.

2.8. Statistical analysis

Due to the fact that many of our treatments showed a total reduction in growth, we chose to analyze our data with a chi square analysis and compare the treatments with the respective untreated control.

3. Results

3.1. Effect of Acetaldehyde on the growth of P. atrosepticum, C. coccodes, and H. solani

Our results showed that acetaldehyde did not inhibit the growth of any of the pathogens tested in this study (Fig. 1). The growth of *P. atrosepticum* was not inhibited when cultures were exposed to acetaldehyde in airtight jars for up to 48 h (Fig. 1a). The results showed no significant difference (Table 1) between the growth of the untreated control and the highest treatment of 10 μL/L (Fig. 1a). Similar results were observed with both *C. coccodes* and *H. solani* (Fig. 1b and c).

Our chi square analysis (χ^2) showed that *P. atrosepticum* had two statistically significant reductions in growth when treated with 2.5 and 5.0 μL/L of acetaldehyde when compared to the untreated control. However, these results were not consistent and a

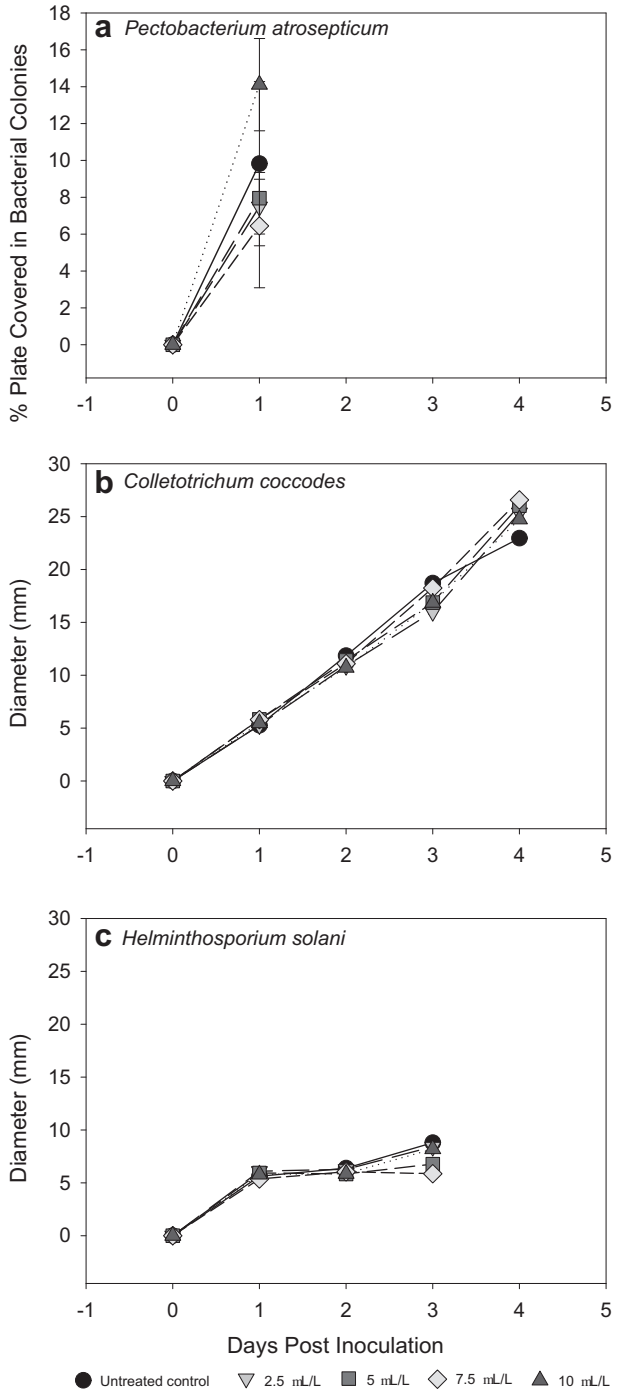

Fig. 1. The effect of acetaldehyde at different concentrations on inhibition of the pathogens (a) *Pectobacterium atrosepticum*, (b) *Colletotrichum coccodes*, and (c) *Helminthosporium solani in vitro* for up to 5 d post inoculation. Acetaldehyde did not inhibit growth of any of the pathogens at up to 10 μL/L.

statistically significant increase in growth was observed at 10 μL/L (Table 1). For the other two pathogens no significant differences between the untreated controls and the treatments were observed (Table 1).

3.2. Effect of 2E-hexenal on the growth of P. atrosepticum, C. coccodes, and H. solani in colony growth assays

As shown in Fig. 2 and Table 2, the untreated control and internal standard check showed significantly more growth than any of the 2*E*-hexenal treatments. *P. atrosepticum* showed complete inhibition at the lowest treatment concentration (2.5 μL/L) with a significant difference between the untreated controls and internal standard check (Fig. 2a, Table 2). Plates with *H. solani* also showed complete growth inhibition at 2.5 μL/L with a significant difference between the untreated controls and internal standard check (Fig. 2c, Table 2). *C. coccodes* colonies showed decreased growth at 2.5 μL/L compared to the untreated controls and internal standard check but required a higher treatment volume of 5 μL/L for total inhibition of growth (Fig. 2b, Table 2).

Table 1
Relative area under the growth progress curve (RAUGPC) and chi square values for *in vitro* growth trials with acetaldehyde for *Pectobacterium atrosepticum*, *Colletotrichum coccodes*, and *Helminthosporium solani*. Petri plates containing *P. atrosepticum* were incubated for 48 h at 23 °C and *C. coccodes* and *H. solani* plates were incubated for 96–168 h at 23 °C.

Pathogen	Acetaldehyde treatment	RAUGPC[a]	df	χ^{2b}	*P* value[c]
P. atrosepticum	2.5 ppm	76.2	1	5.65	**0.017**
	5.0 ppm	81.0	1	3.63	0.056
	7.5 ppm	65.6	1	11.86	**0.001**
	10 ppm	143.6	1	19.03	**<0.001**
C. coccodes	2.5 ppm	94.8	1	0.27	0.603
	5.0 ppm	99.6	1	<0.01	0.970
	7.5 ppm	102.5	1	0.06	0.805
	10 ppm	96.1	1	0.15	0.693
H. solani	2.5 ppm	101.0	1	0.01	0.922
	5.0 ppm	92.3	1	0.59	0.441
	7.5 ppm	91.4	1	0.74	0.389
	10 ppm	96.0	1	0.16	0.685

[a] Relative area under the growth progress curve, calculated based on the untreated control for the respective pathogen.
[b] Chi square value based on the degrees of freedom (df) and calculated by taking the treatment RAUGPC against the untreated control RAUGPC.
[c] Statistically significant values are denoted in bold ($P < 0.05$).

3.3. Antifungal or fungistatic activity of 2E-hexenal against P. atrosepticum, C. coccodes, and H. solani

After treatment for 48 or 96 h, cultures were transferred to new sterile jars with a clean headspace free of 2*E*-hexenal. Acetaldehyde was not tested because it had shown no toxicity against any of the test pathogens during the preliminary trials. Cultures were observed to see if they resumed growth in the new volatile free jar. None of the pathogens resumed growth after incubation in the clear headspace jars (Fig. 3).

3.4. Effect of 2E-hexenal on the germination of conidia of C. coccodes and H. solani in vitro

After treatment with 2*E*-hexenal for 24 h, slides with conidial suspensions were examined using bright field microscopy for the germination of conidia of the pathogens *C. coccodes* and *H. solani*. Acetaldehyde was not tested as it had shown no toxicity against any of the test pathogens during the preliminary trials (Fig. 1, Table 1). The germination rate of *C. coccodes* conidia dropped from 53% germination in the untreated control (no volatile) to 0% germination at the lowest concentration of 2.5 μL/L of 2*E*-hexenal (Fig. 4). Under higher magnification (40×) the conidial cytoplasm of the ungerminated *C. coccodes* conidia exposed to 2*E*-hexenal appeared disrupted and plasmolyzed (Fig. 5).

Similarly, the germination rates for *H. solani* conidia also decreased when treated with 2*E*-hexenal for 24 h. The average germination rate within the untreated control was 14% but germination decreased to 2.2% when treated with 2.5 ppm of 2*E*-hexenal. The lowest rate of germination was observed at 10 μL/L (1.9% average germination; Fig 4.). Ungerminated conidia of *H. solani* were melanized and no visible cellular dysfunctions were observed

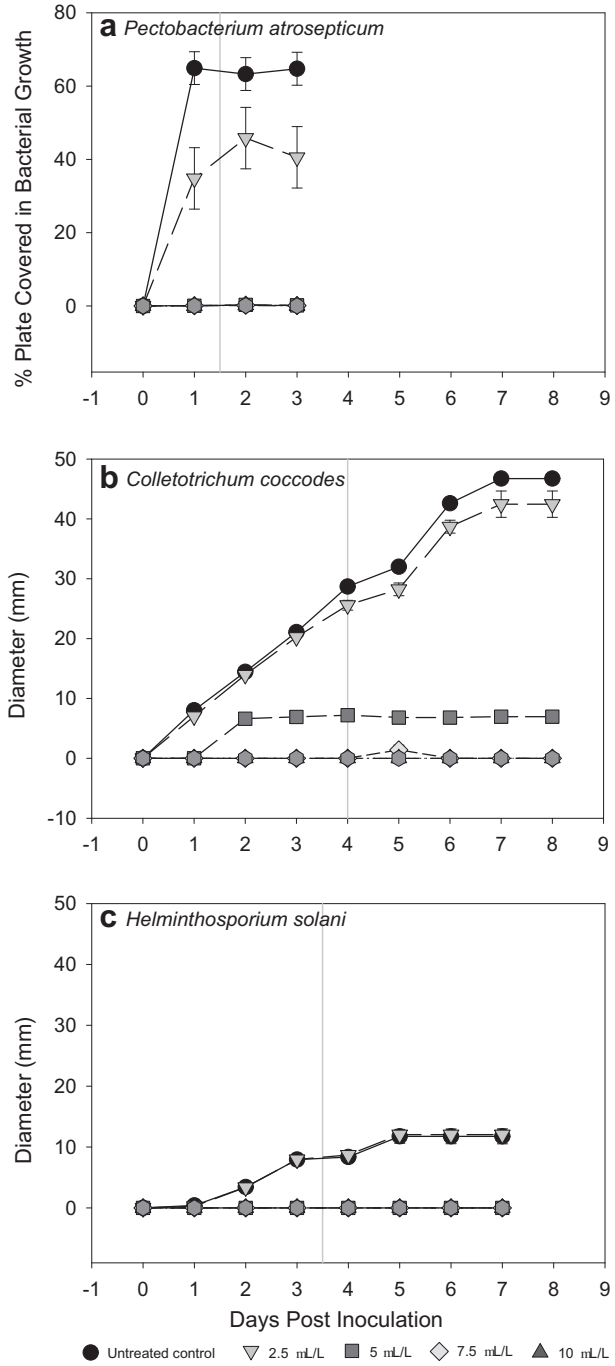

Fig. 2. The effect of 2*E*-hexenal at different concentrations on inhibition of the pathogens (a) *Pectobacterium atrosepticum*, (b) *Colletotrichum coccodes*, and (c) *Helminthosporium solani in vitro* for up to 8 d post inoculation. The volatile gave complete inhibition of *P. atrosepticum* (a) and *H. solani* (c) at 2.5 μL/L, but 5 μL/L was required for complete inhibition of *C. coccodes* (b). The internal standard (cyclohexenol) also showed some inhibitory effects on the tested pathogens, especially *P. atrosepticum* (a) but was not as effective as the 2*E*-hexenal treatments. Vertical grey line (|) indicates transfer from treatment jars to volatile free headspace.

within the conidia at 40× (Fig. 5). However, with increased dosage of 2*E*-hexenal there was slight reductions in the germination levels. All treatments were statistically different from the untreated control (Table 3).

4. Discussion

The initial objective of this study was to determine which volatile compound, either acetaldehyde or 2*E*-hexenal, was most effective at controlling potato blemish pathogen growth. Previous work by Utama et al. (2002) showed that acetaldehyde was the most effective of several aldehyde and alcohol compounds (acetaldehyde, benzaldehyde, cinnamaldehyde, ethanol, benzyl alcohol, 2-nonanone, B-ionone, ethyl formate) in inhibiting pathogen growth. Their research showed that acetaldehyde was completely effective at concentrations, 0.09 to 0.91 milimolar/dish, and totally controlled the decay pathogens *Rhizopus stolonifer, Penicillium digitatum, Cylindrocarpon musae, Erwinia carotovora* (*Pectobacterium carotovorum*), and *Pseudomonas aeruginosa in vitro*. Our results disagree with those of Utama et al. (2002), as they showed that acetaldehyde did not have any inhibitory effect on the growth of the potato pathogens *P. atrosepticum, C. coccodes* and *H. solani in vitro*. Even at the highest tested concentration of 10 μL/L, our results showed that there were no significant differences between the untreated controls and the acetaldehyde treatments.

The differences between our results and those of Utama et al. (2002), could be due to differences in actual volatile concentration, pathogens tested, or overall experimental setup. Further testing at concentrations above 10 μL/L may show inhibition of the potato blemish pathogens used in our experiments. However, in the interest of developing storage fumigation, active packaging for fresh pack potatoes, and other modified atmosphere storage conditions with volatile compounds in the airspace of a storage container, lower treatment volumes are preferred to reduce costs. Our preliminary trials indicate that 2*E*-hexenal was much more effective than acetaldehyde in controlling potato blemish pathogens at very low concentrations *in vitro*.

Our acetaldehyde results are also in contrast with those of Almenar et al. (2007), which showed that a concentration of 1.72 μL/L is capable of inhibiting the blueberry pathogens *A. alternata, C. acutatum*, and *B. cinerea in vitro*. These differences may be due to the unique characteristics of the individual pathogens tested and perhaps host related pathogen selection pressures. For example, *C. coccodes* and *H. solani* are primarily soil borne surface saprophytes of potato tubers colonizing the periderm of potato tubers causing blemishes, but do not invade the cortical tissues causing tissue necrosis. In contrast, *C. acutatum* acts as a hemibiotroph infecting blueberry fruits and remaining quiescent on the fruit surface until it ripens. Upon ripening the pathogen invades the underlying fruit cortext tissues causing tissue necrosis (Wharton and Schilder, 2008).

Table 2
Relative area under the growth progress curve (RAUGPC) and chi square values for *in vitro* growth trials with 2*E*-hexenal for *Pectobacterium atrosepticum*, *Colletotrichum coccodes*, and *Helminthosporium solani*. Petri plates containing *P. atrosepticum* were incubated for 48 h at 23 °C and *C. coccodes* and *H. solani* plates were incubated for 96–168 h at 23 °C.

Pathogen	2*E*-hexenal treatment	RAUGPC[a]	df	χ^{2b}	*P* value[c]
P. atrosepticum	2.5 ppm	0.3	1	99.39	**<0.001**
	5.0 ppm	0.2	1	99.58	**<0.001**
	7.5 ppm	0.1	1	99.70	**<0.001**
	10 ppm	0.1	1	99.77	**<0.001**
C. coccodes	2.5 ppm	15.2	1	71.86	**<0.001**
	5.0 ppm	0.6	1	98.76	**<0.001**
	7.5 ppm	0.0	1	100	**<0.001**
	10 ppm	0.0	1	100	**<0.001**
H. solani	2.5 ppm	0.0	1	100	**<0.001**
	5.0 ppm	0.0	1	100	**<0.001**
	7.5 ppm	0.0	1	100	**<0.001**
	10 ppm	0.0	1	100	**<0.001**

[a] Relative area under the growth progress curve, calculated based on the untreated control for the respective pathogen.
[b] Chi square value (χ^2) based on the degrees of freedom (df) and calculated by taking the treatment RAUGPC against the untreated control RAUGPC.
[c] Statistically significant values are denoted in bold ($P < 0.05$).

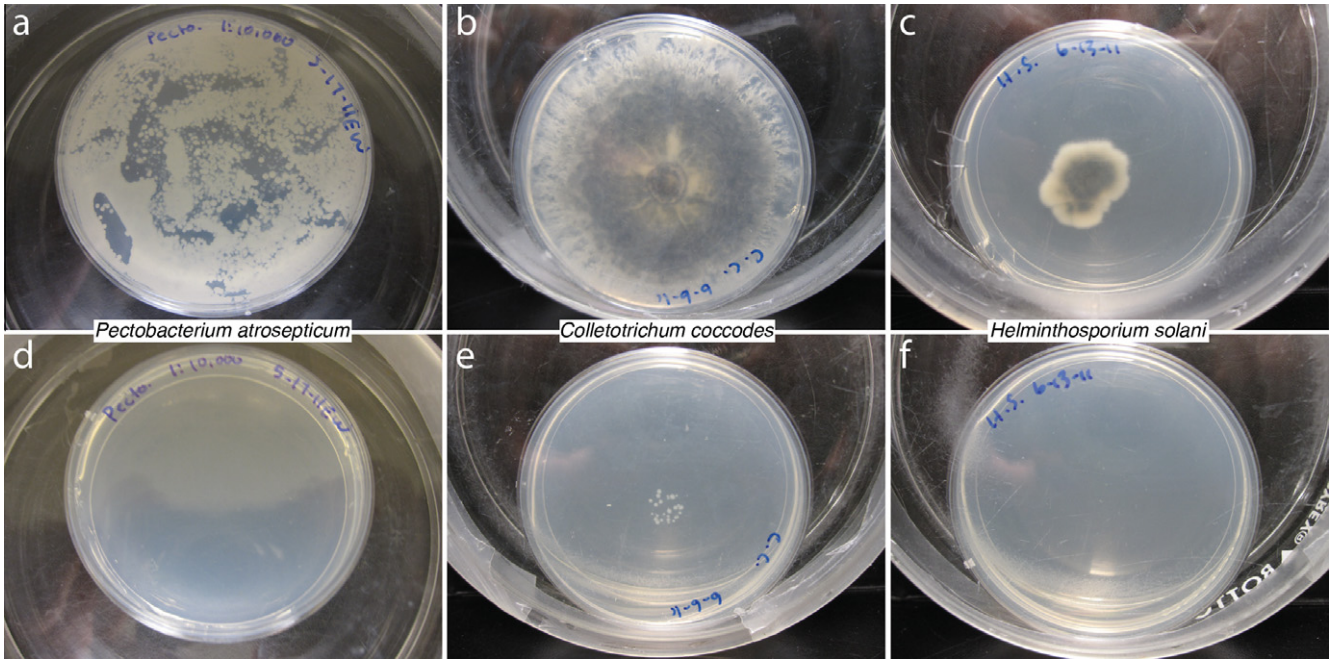

Fig. 3. The effects of 2*E*-hexenal on growth of the blemish pathogens *Pectobacterium atrosepticum* (a, d), *Colletotrichum coccodes* (b, e) and *Helminthosporium solani* (c, f). The volatile was toxic to all three pathogens with none of the cultures resuming growth after treatment, even when they were placed in new jars free of the volatile. (a–c) Untreated controls; pathogens grown in air and then transferred to clean jars with air. (d–f) Pathogens grown in 1 L jars treated with 2.5 μL of 2*E*-hexenal and then transferred to clean jars with air and incubated for 48 h (*P. atrosepticum*) or 96 h (*C. coccodes* and *H. solani*).

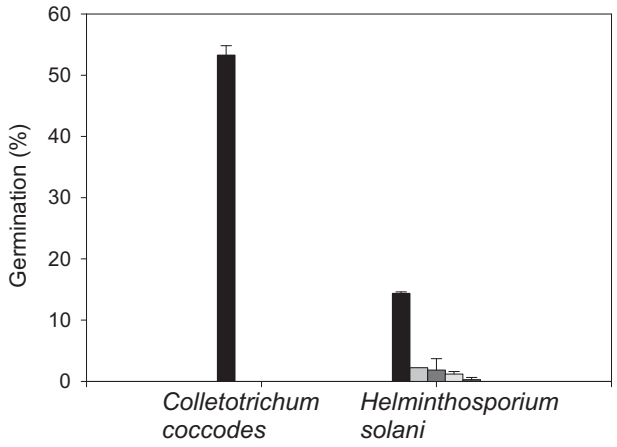

Fig. 4. The effect of 2*E*-hexenal on the *in vitro* conidial germination of *Colletotrichum coccodes* and *Helminthosporium solani* 24 h after inoculation on glass slides coated with SigmaCote which was used to create a hydrophobic surface. Germination of *C. coccodes* conidia was highest in the untreated control and completely inhibited by all concentrations of 2*E*-hexenal. Germination of *H. solani* conidia was highest in the untreated control but there was also some germination after treatment with 2*E*-hexenal.

In contrast to acetaldehyde, the volatile 2*E*-hexenal was shown to have strong inhibitory properties. At the lowest tested headspace concentration of 2.5 μL/L, 2*E*-hexenal decreased growth for all three pathogens when compared to the untreated controls and internal standard check *in vitro*. A concentration of 2.5 μL/L was able to completely inhibit *P. atrosepticum* and *H. solani in vitro*. However, 5 μL/L was required to completely inhibit the growth of *C. coccodes in vitro*. These data indicate that a concentration of 5 μL/L of 2*E*-hexenal is capable of inhibiting growth of all three major potato blemish pathogens *in vitro*. These results are in agreement with those of Arroyo et al. (2007). Their study showed that a concentration of 6.76 μL/L of 2*E*-hexenal was capable of completely inhibiting conidial germination and 33.65 μL/L was the minimum dose required to inhibit mycelial growth of *C. acutatum* on strawberry fruit.

None of the pathogens resumed growth after incubation in the clean headspace jars, indicating that 2*E*-hexenal is microcidal and antimicrobial to the three potato blemish pathogens tested in this *in vitro* study. These results have important implications for the use of this volatile on a commercial scale for control of blemish diseases in storage. Since the volatile is microcidal, it means that tubers may only need to be treated once going into storage in order to provide season long control of the diseases. These results are again in agreement with Arroyo et al., who also found 2*E*-hexenal to have fungicidal effects on *C. acutatum in vitro*.

The results of the cytological experiments agree with our colony growth experiments and showed that a concentration of 2.5 μL/L was capable of significantly decreasing the rate of conidial germination for the two fungal pathogens *C. coccodes* and *H. solani*. The colony growth experiments showed that 5 μL/L is necessary to completely inhibit the mycelial growth of *C. coccodes*. However, 2.5 μL/L could completely prevent conidial germination. *H. solani* conidial germination was also significantly decreased when exposed to 2*E*-hexenal for 24 h. A minimum concentration of 2.5 μL/L was capable of reducing the germination rates from 14% in the untreated controls to 2.2%. The *H. solani* conidia had overall lower rates of germination than that of *C. coccodes* but the germination was never completely inhibited in our experiments. The lowest germination rate was found at 10 μL/L with 1.9% germination in *H. solani* conidia. Taken together, these cytological studies provide a basic insight into the mode of action of the volatile in inhibiting pathogen growth, although more research is necessary to fully understand the functionality of this highly effective volatile.

Studies of *C. acutatum* from blueberry (Almenar et al., 2007) and strawberry (Arroyo et al., 2007) have shown that exogenous application of 2*E*-hexenal causes plasmolysis of the conidia as well as the disruption of the organelles within the conidium. Other studies

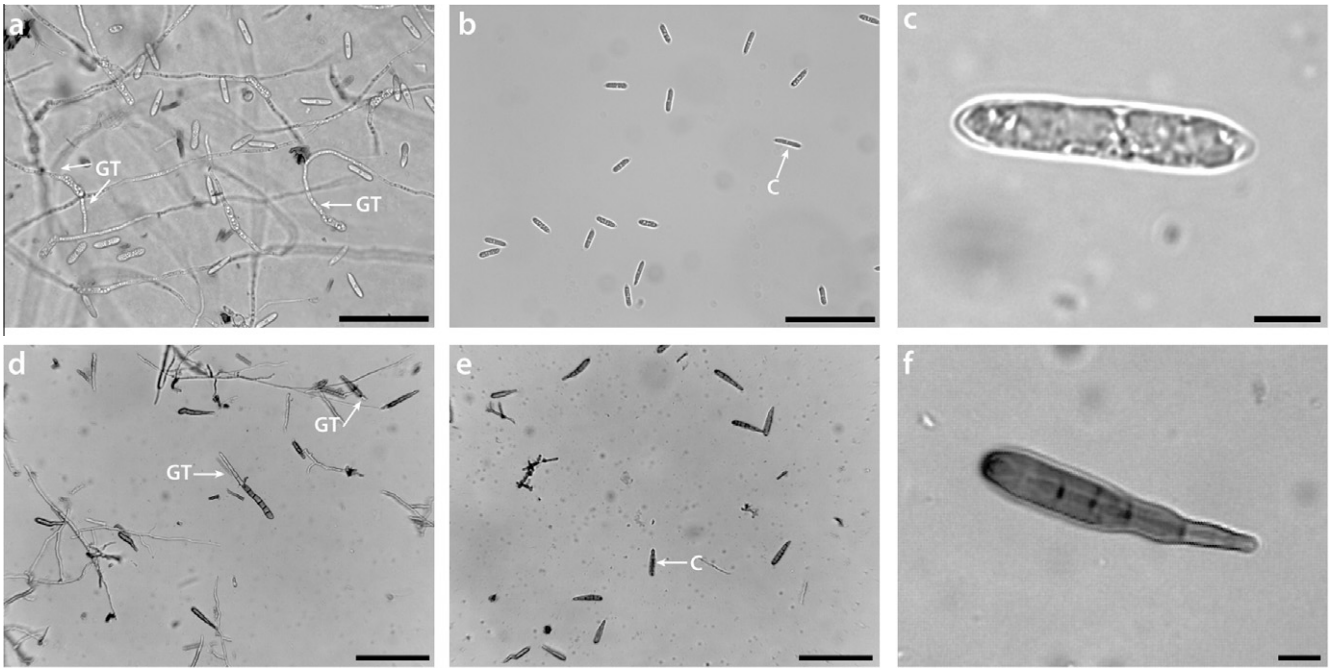

Fig. 5. Effects of 2.5 μL of 2*E*-hexenal (inside 1 L glass jars) on spore germination and development of *Colletotrichum coccodes* (a–c) and *Helminthosporium solani* (d–f). (a) Conidial germination and germ-tube (GT) formation of *C. coccodes* 24 h after incubation in air at 23 °C. Germ tubes have proliferated over the slide surface and their cytoplasm appears healthy. (b, c) Conidia (C) of *C. coccodes* have failed to germinate 24 h after being placed in a 2*E*-hexenal atmosphere and their cytoplasm appears plasmolyzed and disrupted (c). (d) Melanized conidia (C) of *H. solani* germinating to produce branched germ tubes 24 h after incubation in at 23 °C in air. (e, f) Melanized conidia of *H. solani* have failed to germinate 24 h after being placed in a 2*E*-hexenal atmosphere and their cytoplasm appears plasmolyzed (f). (a, b, d, e) Bars = 100 μm; (c, f) bars = 5 μm.

Table 3
In vitro germination trials with 2*E*-hexenal and chi square values for *C. coccodes* and *H. solani* after 24 h of incubation at 23 °C.

Pathogen	2*E*-hexenal treatment	Relative germination (%)[a]	df	χ^{2b}	*P* value[c]
C. coccodes	2.5 ppm	0.0	1	100	**<0.001**
	5.0 ppm	0.0	1	100	**<0.001**
	7.5 ppm	0.0	1	100	**<0.001**
	10 ppm	0.0	1	100	**<0.001**
H. solani	2.5 ppm	15.5	1	10.28	**0.001**
	5.0 ppm	12.9	1	70.61	**<0.001**
	7.5 ppm	12.6	1	85.36	**<0.001**
	10 ppm	8.3	1	96.32	**<0.001**

[a] Relative germination (%) calculated based on the untreated control for the respective pathogen.
[b] Chi square value (χ^2) based on the degrees of freedom (df) and calculated by taking the treatment relative germination against the untreated control.
[c] Statistically significant values are denoted in bold ($P < 0.05$).

have shown that hexenals can act as nonionic surfactants at the lipid–protein interface and can disrupt the H^+ gradient compromising ATP production, which ultimately leads to cell death (Kubo et al., 2003). *In vivo* exogenous application of 2*E*-hexenal might be even more effective when applied to plant surfaces because it is a natural antibiotic that can act as a fatty acid signaling compound capable of triggering a hypersensitive response (Farmer et al., 1998).

Similar studies to ours have been performed using volatile compounds to control plant pathogens. However, most of the research has been focused on fruits, many of which naturally emit volatile compounds as defense mechanisms (Arroyo et al., 2007), as a response to wounding (Vaughn and Gardener, 1993), during fruit ripening (Pesis, 2005) and as flavor related compounds (Song et al., 1996). Potatoes are dissimilar to fruits as they do not emit these volatile compounds. The potato periderm provides a very effective barrier against pathogen ingress when intact. However, the periderm may get damaged during harvest by bruising and wounding. These natural openings allow for the infection of tubers by storage pathogens. Furthermore, potato tubers may be stored for up to 9 months which gives pathogens plenty of time to cause disease. Because of the value, and consequently the potential for economic loss, the control of storage pathogens has become increasingly important. The use of naturally occurring volatile compounds to control potato blemish diseases is a potentially new method of controlling diseases without introducing fungicides onto the edible product. Naturally occurring plant volatiles such 2*E*-hexenal are ideal compounds for developing controlled atmosphere packaging, storage fumigation, or modified atmosphere fresh pack potato packaging. This is due to the fact that they are highly effective in controlling postharvest potato blemish pathogens at low concentrations and have already been approved as food additives by the FDA (FDA, 2011). The results of these studies indicate that further research in the form of *in vivo* studies as well as large-scale trials under realistic potato storage conditions is the next step in the development of these volatiles for the commercial use in the control of potato blemish pathogens.

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