JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Antifungal Activity of Strawberry Fruit Volatile Compounds against *Colletotrichum acutatum*

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Eight volatile products characterizing strawberry aroma, which is generated from the oxidative degradation of linoleic and linolenic acids by a lipoxygenase (LOX) pathway, were examined because of their antifungal activity against *Colletotrichum acutatum*, one of the causal agents of strawberry anthracnose. In this study, the effects of aldehydes, alcohols, and esters on mycelial growth and conidia development were evaluated. (*E*)-Hex-2-enal was found to be the best inhibitor of mycelial growth [MID (minimum inhibitory doses) = 33.65 μL L^{-1}] and of spore germination (MID = 6.76 μL L^{-1}), while hexyl acetate was the least effective of all volatile compounds tested (MID = 6441.89 μL L^{-1} for mycelial growth and MID = 1351.35 μL L^{-1} for spore germination). Furthermore, the antifungal activity of (*E*)-hex-2-enal on susceptibility of strawberry fruits to *C. acutatum* was also confirmed. The presence of these molecules in jars containing strawberry fruits inoculated with a suspension of spores inhibited the fungus growth and prevented the appearance of symptoms. Moreover, a study of the effects of (*E*)-hex-2-enal on conidial cells of *C. acutatum* was evaluated by transmission electron microscopy. This volatile compound altered the structures of the cell wall and plasma membrane, causing disorganization and lysis of organelles and, eventually, cell death.

KEYWORDS: Antifungal activity; anthracnose strawberry; *Colletotrichum acutatum*; lipoxygenase products

INTRODUCTION

The fungal pathogen *Colletotrichum acutatum* J. H. Simmonds causes a wide range of pre- and postharvest anthracnose diseases worldwide on economically important crops such as almond, apple, avocado, blueberry, citrus, peach, strawberry, and tomato (*1*). Several species of *Colletotrichum* have been described causing strawberry anthracnose (*2*); *C. acutatum* is the most common species in Europe (*3*, *4*). Recently, it has been detected in a production field in Denmark (*5*). Lesions can occur all over the plant, including roots, leaves, blossoms, stolon, crown, and fruit, causing diseases such as crown rot, blossom blight, and fruit rot (*2*, *6*). Losses caused by fruit rot are particularly damaging economically. Although green fruits are free of visible disease symptoms, decay lesions rapidly develop during fruit ripening. In fact, when green fruits are inoculated, no symptoms are observed and the infection remains quiescent until the fruit ripens; then, lesions typically from anthracnose develop (*7*). The use of fungicides in the control of fungal diseases of strawberry fruit is limited mainly due to the restrictions imposed on their usage (*8*). Furthermore, synthetic fungicides impose selective pressure on pathogen populations and may result in fungicide-resistant strains (*9*), so viable and safe alternatives must be developed.

Naturally occurring plant-derived volatiles that are fundamental flavor and fragrance constituents seem to possess antifungal activity. Zeringue et al. (*10*) suggested that volatile compounds emitted from plants may be responsible for fungal inhibition. These volatile compounds have been studied for their potential as postharvest inhibitors of several fruits (*11*−*13*). One of the most widespread and abundant volatile compounds in plant tissues is the six-carbon aldehyde (*E*)-2-hexenal (*14*), which has proved its efficacy to control spore germination and mycelial growth of *Botrytis cinerea* (*15*, *16*), *Colletotrichum gloeosporioides* (*13*), and *Penicillium expansum* (*12*). Furthermore, Doehlert et al. (*17*) provided evidence that growth and aflatoxin production can be disrupted in vitro when *Aspergillus flavus* is exposed to volatiles released from fatty acids through soybean lipoxygenase (LOX) activity. Fatty acids, the major precursors of volatile compounds responsible for the aroma of plants, are transformed in aldehydes, alcohols, acids, and esters through the oxidative degradation by the LOX and hydroperoxide lyase (HPL) pathway. In addition to their role in aroma biosynthesis, both LOX and HPL may have physiological relevance because their products have antimicrobial and antifungal activities and are implicated in plant wounding response (*18*−*20*). Several authors have also described the relationship

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10.1021/jf0703957 CCC: $37.00 © 2007 American Chemical Society
Published on Web 06/13/2007

between LOX and defense plant response. Kesmann et al. (*21*) reported a LOX activity induced by *Pyricularia syringae* in local and systemic tissues. Moreover, several defense genes are activated by volatile compounds and induce resistance against *B. cinerea* in *Arabidopsis thaliana* (*22*). All of these findings indicate the phytogenic potential of plant volatile compounds inhibiting the development of several pathogens.

The aim of this work was to evaluate the efficacy of several volatile compounds from strawberry aroma in controlling *C. acutatum* mycelial growth and conidia germination and to understand the mode of action of these compounds, especially of (*E*)-hex-2-enal, to promote its application as a fumigant for the control of anthracnose.

MATERIALS AND METHODS

Fungal Inoculum/Pathogen. In all experiments, *C. acutatum* isolate CECT 20240 from Colección Española de Cultivos Tipo (CECT) was used. The fungus was grown on potato dextrose agar (PDA) from Difco Laboratories (Detroit, MI) at 25 °C under continuous fluorescent light (100 μE m^{-2} s^{-1}) for 7 days. Conidial suspensions were prepared by flooding the culture plates with 4−5 mL of sterile distilled water, scraping the colony surface with a scalpel, and filtering the suspension through sterile cheesecloth. For in vitro (mycelial growth and spore inhibition) and in vivo (fruit inoculation and ultrastructural study) tests, the concentrations were adjusted to 5×10^5 and 1×10^6 conidia mL^{-1}, respectively, using a hemocytometer.

Volatile Compounds. Eight volatile compounds, which are generated through LOX and HPL pathways for the formation of strawberry aroma (*23*), were screened for their ability to control *C. acutatum*. The compounds used in this study were the aldehydes hexanal and (*E*)-hex-2-enal; the alcohols hexan-1-ol, (*Z*)-hex-3-enol, and (*E*)-hex-2-enol; and the esters (*Z*)-hex-3-en-1-yl acetate, (*E*)-hex-2-en-1-yl acetate, and hexyl acetate. The volatile compounds, with at least 98% purity, were purchased from Sigma-Aldrich.

Inhibition of Mycelial Growth in Vitro Test. A mycelial disc (5 mm diameter) was taken from the periphery of an actively growing PDA culture and placed at the center of an 85 mm × 13 mm Petri dish (74 mL) containing 20 mL of PDA. Different amounts of volatile compounds were added on the paper filter placed on the cover inside the dish. For each compound, at least six doses were tested and varied from 33.78 to 1351.35 μL L^{-1}. For (*Z*)-hex-3-en-1-yl acetate and hexyl acetate, 2702.70, 4054.05, 6756.76, and 10135.13 μL L^{-1} were also tested. Likewise, nine doses from 1.35 to 30.40 μL L^{-1} were also tested for (*E*)-hex-2-enal. The dishes were quickly sealed with Parafilm and incubated at 25 °C. For each treatment, for each compound, and at each of the doses tested, five replicate Petri dishes were used. The control treatment consisted of a Petri dish with the mycelial disc but treated with sterile distilled water. After 8 days of incubation, the diameter of the colonies was recorded. If no mycelial growth was observed, the disc was removed from the dish and placed in a new Petri dish containing PDA to evaluate whether the activity was either fungistatic or fungicidal. A compound was considered fungistatic when the mycelia grew during the additional incubation period and was considered fungicidal if no mycelial growth was detected. The experiments were repeated twice.

Inhibition of Conidial Germination and Development of Appressoria Test. To test the activity of strawberry fruit volatile compounds on conidial germination, a droplet of 100 μL of the conidial suspension (5×10^5 conidia mL^{-1}) was placed on slides partially covered with Parafilm, which had been put in Petri dishes according to the methods previously described by Arroyo (*7*). Conidial germination as well as formation and maturation of appressoria of *C. acutatum* on slides covered with Parafilm were similar to those developed on strawberry tissues. The initial stages of infection process from conidial adhesion, germination of conidia, in which it develops a germ tube, up to the production and maturation of appressorium were observed on this artificial surface (*7*). Moreover, differentiation of this pigmented structure forming an appressorial pore, which is in the basal zone, was revealed as a light spot inside of the appressorium (*24*). Different amounts of volatile compounds were added to a paper filter, which was placed opposite to the droplet of inoculum. For each compound, at least four doses were tested and varied from 0.13 to 1351.35 μL L^{-1}. The dishes were sealed with Parafilm immediately and then incubated at 25 °C. Sterile distilled water added to the filter papers served as the control. For each compound at each of the doses tested, five Petri dishes (replications) were used. The germinated conidia and formation of appressoria were observed at 4 and 24 h after inoculation. The experiments were performed twice.

Fruit Inoculation. Because (*E*)-hex-2-enal showed the best results in vitro, this compound was the only one selected for this trial. Strawberry fruits (cv. Camarosa) harvested at commercial maturity stage were collected directly from trial fields of "El Cebollar", an agricultural experimental station located in Huelva (southwestern Spain). Fruits were superficially disinfested by immersion in a 1% sodium hypochlorite solution for 1 min, rinsed in sterile distilled water, and placed in 500 mL capacity jar, which contained filter paper moistened with 5 mL of sterile water. Fruits were inoculated by applying 100 μL of the conidial suspension (10^6 conidia mL^{-1}). Slides with pieces of filter paper, which were impregnated with different doses of the volatile compound, were placed adjacent to fruits. The doses tested were 4, 5, 10, 20, and 50 μL L^{-1}. After treatment, fruits were incubated in a growth chamber at 25 °C under continuous fluorescent light and the incidence of infected fruits was recorded and determined at 7 days. Categories based on percentage of fruit surface covered with fungi were as follows: 0, no detectable fungal growth; 1, fruit covered with mycelia. Each treatment was replicated eight times. Inoculated-nontreated fruits were selected as control.

Ultrastructural Study of Conidia and Appressorium Treated with (*E*)-Hex-2-enal. In this trial, the volatile compound (*E*)-hex-2-enal was also selected to characterize and describe its effects on the ultrastructure of conidia and appressorium of *C. acutatum* because it had shown the best results in in vitro and in vivo trials. This experiment was carried out on detached strawberry leaves, which were superficially disinfested by immersion in a 1% sodium hypochlorite solution for 1 min, rinsed in sterile water, and then placed in Petri dishes containing filter paper moistened with 5 mL of sterile water. Also, inside Petri dishes, slides with pieces of filter paper were placed. Strawberry leaves were inoculated by applying 100 μL droplets of the conidial suspension (10^6 conidia mL^{-1}). A 2.5 μL amount of (*E*)-hex-2-enal (33.78 μL L^{-1}) was added to the paper filter as previously described. This amount was selected for causing 100% of mycelial growth inhibition in vitro of *C. acutatum* and having a fungicide effect. Three leaves were sampled at 24 and 48 h postinoculation (hpi). Strips of tissues approximately 1 mm thick and 1−2 mm long were removed from beneath inoculation droplets of leaves and were fixed in a solution 4% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 3 h at 4 °C. After they were rinsed in the same buffer, the tissues were postfixed in 1% (w/v) osmium tetroxide for 2 h at 4 °C, then dehydrated in a graded acetone series, and embedded in EMBED-812 (Polysciences; Warrington, PA) according to the manufacturer's indications. Slides with semithin sections (0.5 μm) were placed on a hotplate at 50 °C, stained for 1 min with 0.1% aqueous toluidine blue O solution, and examined using a light microscope (Leitz, Aristoplan). Ultrathin 60−80 nm sections were made with a Reichert-Jung Ultracut E ultramicrotome and a diamond knife and were collected on 300 mesh copper grids (*25*). Grids were stained with 7% aqueous uranyl acetate and lead citrate solution. Sections were observed, and images were collected using a Philips CM-10 TEM.

Statistical Analysis. Minimum inhibitory dose (MID) and 95 and 50% inhibitory doses (ID_{95} and ID_{50}, respectively) were calculated using probit analysis applied to the percentages of mycelial inhibition resulting from in vitro experiments. Regression lines between the logarithm of the compound doses and the inhibition indices transformed in probit were calculated. The data from in vivo trials (fruit inoculation) were processed using the statistical package Statistica for Windows v.5 (StatSoft, Inc.).

RESULTS

Effect of Volatile Compounds on Mycelial Growth of *C. acutatum*. The regression lines of the logarithm of the com-

Table 1. MIDs, 95% Inhibitory Doses, and 50% Inhibitory Doses (ID_{95} and ID_{50}, Respectively) ($\mu L\ L^{-1}$) of Treatments with Different Volatile Compounds from the LOX-HPL Pathway Measured by Mycelial Growth of *C. acutatum*

volatile	MID	ID_{95}	ID_{50}
(*E*)-hex-2-enal	33.65[a]	32.97	26.89
hexanal	272.16[a]	256.49	151.35
hexan-1-ol	270.27[b]	255.14	151.35
(*E*)-hex-2-enol	268.92[a]	253.78	151.76
(*Z*)-hex-3-enol	640.54[a]	595.00	230.14
(*E*)-hex-2-en-1-yl acetate	651.35[a]	584.86	221.62
(*Z*)-hex-3-en-1-yl acetate	2476.35[a]	2063.11	503.11
hexyl acetate	6441.89[a]	5100.41	718.65

[a] Mean inhibitory doses that were fungicidal. [b] Mean inhibitory doses that were fungistatic.

pound concentrations transformed in probit were highly significant (correlation coefficients between 0.98 and 0.99, $P \leq 0.05$). The most active compounds to inhibit the mycelial growth of *C. acutatum* were to be found (*E*)-hex-2-enal, hexanal, hexan-1-ol, and (*E*)-hex-2-enol. In particular, (*E*)-hex-2-enal was the best inhibitor of mycelial growth: MID = 33.65 $\mu L\ L^{-1}$, ID_{95} = 32.97 $\mu L\ L^{-1}$, and ID_{50} = 26.89 $\mu L\ L^{-1}$. For hexanal, hexan-1-ol, and (*E*)-hex-2-enol, it was necessary to increase the doses near 10 times to inhibit mycelial growth of *C. acutatum* with regard to (*E*)-hex-2-enal. At the same time, for (*Z*)-hex-3-enol and (*E*)-hex-2-en-1-yl acetate, it was necessary to increase the doses near 20 times as compared to (*E*)-hex-2-enal. The volatile compounds that showed less effectiveness against *C. acutatum* were esters (*Z*)-hex-3-en-1-yl acetate and hexyl acetate (**Table 1**). At doses tested, no mycelial growth for almost all volatile compounds was observed 8 days after the volatiles were removed. However, hexan-1-ol was fungistatic at 337.84 $\mu L\ L^{-1}$ and mycelial growth could be observed after this period (**Table 1**).

Effect of Volatile Compounds on Conidial Germination and Development of Appressoria. Results obtained in this trial are listed in **Table 2**. (*E*)-Hex-2-enal was the most effective inhibitor against conidial development of *C. acutatum* on Parafilm of all volatiles tested (**Table 2**). It inhibited the spore germination fully at 6.76 $\mu L\ L^{-1}$. Also, those spores treated with 1.35 $\mu L\ L^{-1}$ of (*E*)-hex-2-enal did not show a development similar to that of the control. Although they germinated and formed appressoria, their pigmentation and maturation were altered, whereas appressorium from control showed the typical brown color and developed a pore appressorial at 24 hpi. However, this phenomena could not be observed in those conidia treated with (*E*)-hex-2-enal. The production of secondary conidia or microcyclic conidiation, which originated from phialide hyphal or conidial, was also observed at 0.68 and 1.35 $\mu L\ L^{-1}$ but not at 6.76 $\mu L\ L^{-1}$ of (*E*)-hex-2-enal. No germinated conidia exhibited deformations and great vacuolization at 6.76 $\mu L\ L^{-1}$; what is more, they showed cell lysis at doses higher than it.

Hexanal was also a powerful inhibitor of germination of *C. acutatum* but to a lesser extent since 33.78 $\mu L\ L^{-1}$ was necessary to prevent this process fully. Microcyclic conidiation was also observed at 1.35, 6.76, and 13.51 $\mu L\ L^{-1}$ of this volatile compound but not at 33.78 $\mu L\ L^{-1}$. Pigmentation and maturation of appressoria were altered, and the appressorial pore was not viewed at 6.76 $\mu L\ L^{-1}$. At doses higher than 33.78 $\mu L\ L^{-1}$, vacuolization increased and cell lysis was observed.

Germination of *C. acutatum* on Parafilm was inhibited at 337.84 $\mu L\ L^{-1}$ for hexan-1-ol. The conidial development was similar to control at 6.76 $\mu L\ L^{-1}$, and they produced pigmented appressorium and the pore, but from 33.78 $\mu L\ L^{-1}$, the pigmentation and the formation of the pore were not observed. Microcyclic conidiation was inhibited at 337.84 $\mu L\ L^{-1}$ of this volatile.

(*E*)-Hex-2-enol altered the germination at 33.78 $\mu L\ L^{-1}$ because it was not detected at 4 hpi, but a lot of germinated spores and hyaline appressoria were observed at 24 hpi. However, neither pigmentation nor pore formation was produced when conidia were treated with 33.78 $\mu L\ L^{-1}$ of (*E*)-hex-2-enol. Moreover, the formation of appressoria was limited from 135.13 $\mu L\ L^{-1}$.

The effects of (*Z*)-hex-3-enol, (*Z*)-hex-3-en-1-yl acetate, and (*E*)-hex-2-en-1-yl acetate were similar at those doses tested (**Table 2**). Conidial development from germination to appressorial pore formation could be observed at low doses as 6.76, 33.78, and 67.57 $\mu L\ L^{-1}$. At 135.13 $\mu L\ L^{-1}$ of these volatile compounds, germination of spores was similar to the control, although they were less abundant, but the maturation of appressoria was altered. Conidia germination, appressorium formation, and secondary conidia production were fully inhibited for (*Z*)-hex-3-enol, (*Z*)-hex-3-en-1-yl acetate, and (*E*)-hex-2-en-1-yl acetate at 337.84 $\mu L\ L^{-1}$.

The least effective inhibitor of the volatile compound tested was hexyl acetate, which inhibited germination at higher doses than the rest of volatile compounds tested; 1351.35 $\mu L\ L^{-1}$ was necessary for modifying the normal development of this process. Also, maturation of appressoria was altered at 337.84 $\mu L\ L^{-1}$ because pigmented appressoria was not detected at 24 hpi, although conidia developed hyaline appressoria at this time. It affected appressorial pore formation, and this structure could not be observed at that dose. A high vacuolization level and deformation of conidia and germinative tube could be appreciated for this compound in the development of *C. acutatum* at 1351.35 $\mu L\ L^{-1}$.

Effect of (*E*)-Hex-2-enal on Susceptibility of Strawberry Fruits to *C. acutatum*. When detached mature strawberry fruits are inoculated with a suspension of conidia of *C. acutatum*, a whitish-pink mycelia develops on the fruit spreading out both superficially and inside the tissues. The addition of (*E*)-hex-2-enal to the atmosphere in the jar containing inoculated strawberry fruits limited and inhibited the development of *C. acutatum* depending on the doses used (**Table 3**). The results of the bioassays on strawberry fruits cv. Camarosa confirmed the antifungal activity of (*E*)-hex-2-enal previously reported from the conidia germination test (**Table 2**). Forty-eight hours after inoculation, visible symptoms were observed in all of the controls, which showed sunken, 1−1.5 cm size lesions in the inoculated zone and a whitish mycelia developed in this area. The mycelia increased and covered nearly all surfaces at 5 days after inoculation. However, when fruits were treated with 10 μL of (*E*)-hex-2-enal (20 $\mu L\ L^{-1}$), they did not develop symptoms after 5 days and no mycelia were observed during this period. This volatile showed a high reduction of infection (87.5%) at 10 $\mu L\ L^{-1}$, and it reached 100% at 20 $\mu L\ L^{-1}$ as compared with the control (**Table 3**). The effectiveness did not increase at higher doses than 20 $\mu L\ L^{-1}$.

Ultrastructural Study of Cells of Conidia Treated with (*E*)-Hex-2-enal. Under electron microscopic examination of the untreated conidium, a typical structure with a cell wall and a plasma membrane, which had an intact appearance (**Figures 1−3**), was shown. The conidium wall consisted of an approximately 0.1 μm thick electron transparent layer whose outer surface was covered by thin coating fibers. The conidium cytoplasm contained many free ribosomes, an extensive system

Table 2. MIDs (μL L^{-1}) of Treatments with Different Volatile Compounds from the LOX-HPL Pathway Measured by Different Stages of Conidial Development: Conidia Germination, Appressorium Formation, Appressorial Pigmentation, Appressorial Pore Formation, and Secondary Conidia Production or Microcyclic Conidiation

volatile compound	germination	appressorium formation	appressorial pigmentation	pore appressorial formation	microcyclic conidiation
(*E*)-hex-2-enal	6.76[a]	6.76	1.35	1.35	6.76
hexanal	33.78[a]	33.78	6.76	6.76	33.78
hexan-1-ol	337.84[a]	337.84	33.78	33.78	337.84
(*E*)-hex-2-enol	135.13[a]	33.78	33.78	33.78	135.13
(*Z*)-hex-3-enol	337.84[a]	337.84	135.13	135.13	337.84
(*Z*)-hex-3-en-1-yl acetate	337.84[a]	337.84	135.13	135.13	337.84
(*E*)-hex-2-en-1-yl acetate	337.84[a]	337.84	135.13	135.13	337.84
hexyl acetate	1351.35[a]	1351.35	337.84	337.84	1351.35

[a] Cell lysis at higher doses from it.

Table 3. Antifungal Activity of (*E*)-Hex-2-enal on Strawberry Fruits cv. Camarosa Inoculated with a Suspension of 10^6 Conidia mL^{-1} of *C. acutatum*[a]

treatment	doses (μL L^{-1})	fruit damage rating
control		1 ± 0.00
(*E*)-hex-2-enal	4	1 ± 0.00
	5	1 ± 0.00
	10	0.125 ± 0.125
	≥ 20	0 ± 0.00

[a] Fruit damage rating after 7 days (0 = intact, and 1 = covered with mycelia). Data represent means and standard errors of eight replicates.

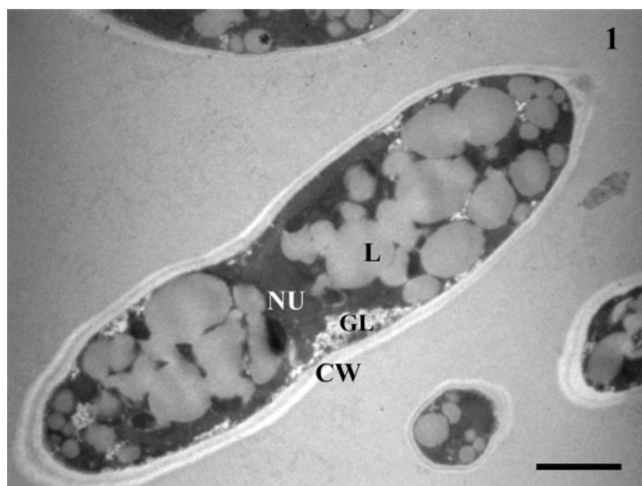

Figure 1. Transmission electron micrographs of conidia of *C. acutatum* unexposed to (*E*)-hex-2-enal. Longitudinal section of a conidium, which exhibits a well-defined organized structure and cell wall (CW); visible in the cytoplasm are the nucleus (NU), lipid droplets (L), and glycogen accumulations (GL). Scale bar = 1 μm.

tubular endoplasmic reticulum, numerous mitochondria, lipid droplets, vacuoles, and microtubules. Also, accumulations of glycogen surrounded by mitochondria were observed and a simple prominent nucleus was evident positioned near the center of the longitudinal axis of the conidium. Four hours after inoculation, the nucleus had undergone mitosis and the resulting daughter nuclei moved to opposite ends of the conidium. A germ tube then developed from the conidium, which aroused typically close to either end of this fungal cell. The germ tube cytoplasm was very dense. Just prior to or during germ tube emergence, a septum developed near the middle of the conidium, dividing it in two uninucleate cells. Twenty-four hours after inoculation, appressoria were observed, which differentiated them from the tip of the long germ tubes or from very short germ tubes (sessile

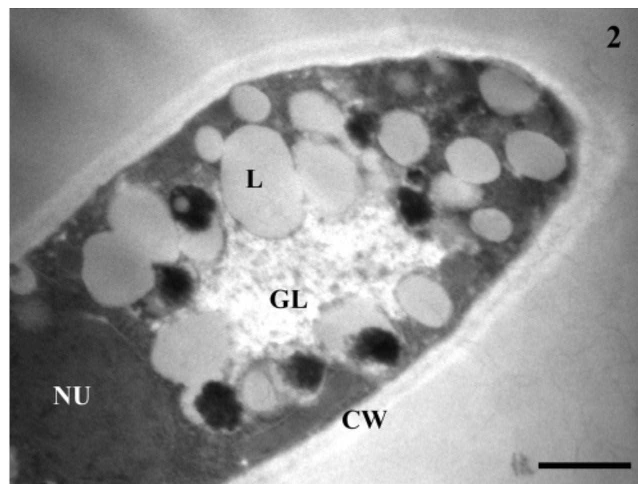

Figure 2. Transmission electron micrographs of conidia of *C. acutatum* unexposed to (*E*)-hex-2-enal. Section of a conidium at higher magnification. Visible in the cytoplasm are the nucleus, lipid droplets, and glycogen accumulations. Scale bar = 0.5 μm.

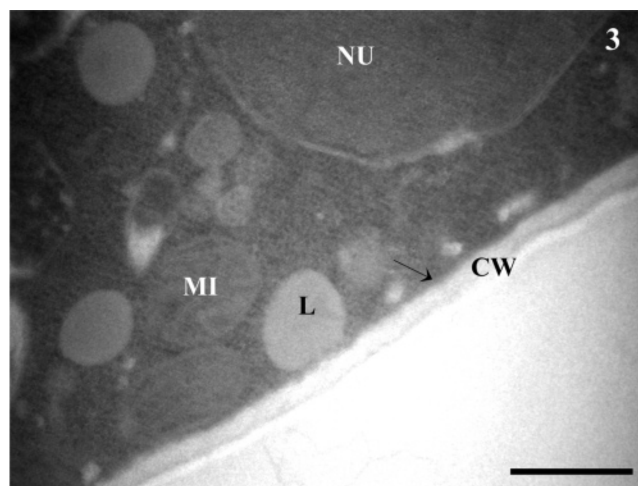

Figure 3. Transmission electron micrographs of conidia of *C. acutatum* unexposed to (*E*)-hex-2-enal. Section of a conidium at higher magnification exhibiting a well-organized structure of the nucleus and organelles as mitochondria (MI). Also, the plasma membrane (arrow) and cell wall show a well-defined intact appearance. Scale bar = 0.5 μm.

appressoria). On the other hand, conidia treated with (*E*)-hex-2-enal showed considerable disruption of the plasma membrane and cell wall and a high disorganization of cell components. In fact, the morphology of the organelles was lost and was impossible to distinguish them (**Figures 4** and **5**).

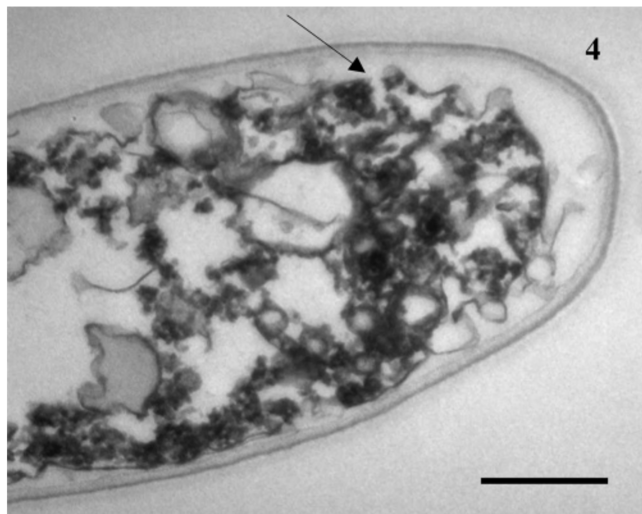

Figure 4. Transmission electron micrographs of conidia of *C. acutatum* exposed to 33.78 μL L^{-1} of (*E*)-hex-2-enal. Longitudinal section of a conidium, which exhibits a disorganized structure, and disruption of the plasma membrane (arrow). Organelles cannot be distinguished.

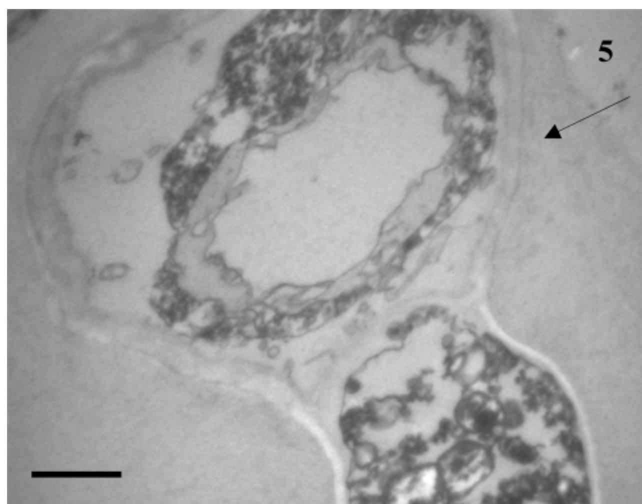

Figure 5. Transmission electron micrographs of conidia of *C. acutatum* exposed to 33.78 μL L^{-1} of (*E*)-hex-2-enal. Another longitudinal section of a conidium, which exhibits a highly degraded cell wall (arrow) and a highly disorganized cytoplasm. Scale bar = 1 μm.

DISCUSSION

In this study, eight volatile compounds responsible for the aroma of strawberry fruits (cv. Camarosa) (*23*), which are generated from the oxidative degradation of linoleic and linolenic acids through LOX and HPL pathways, showed antifungal activity against *C. acutatum* when they were tested in vitro. The aldehydes hexanal and (*E*)-hex-2-enal were the most effective to inhibit the mycelial growth of pathogen, but other volatiles such as the alcohols hexan-1-ol, (*Z*)-hex-3-enol, and (*E*)-hex-2-enol might also contribute to the antifungal activity. Similar findings are in agreement with those reported by Boué et al. (*26*) against *A. flavus*. Volatiles generated from LOX soybean including the aldehydes hexanal and (*E*)-hex-2-enal and other volatiles such as alcohols inhibited *A. flavus* mycelial growth and aflatoxin production. However, the antifungal activity of alcohols is weakly active when compared to aldehydes. As well, Zeringue et al. (*27*) reported that the compounds showing the highest fungal inhibition were alkenals. Hexanal and (*E*)-hex-2-enal, which are more reduced compounds than alcohols and esters, were the most effective in the fungal inhibition. These compounds are at the beginning of LOX and HPL pathways and are produced from linoleic and linolenic acids, respectively. Likewise, Hamilton-Kemp et al. (*16*) demonstrated that the unsaturated aldehyde (*E*)-hex-2-enal was considerably more active in their inhibition of hyphal growth of *Alternaria alternata* and *B. cinerea* than its corresponding saturated aldehyde, hexanal. As the unsaturation and the oxidation of volatile compounds decreased, their antifungal activities also decreased, which is in agreement with data reported by Andersen et al. (*28*) on the germination of *A. alternata*. However, this rule was not observed for the alcohol tested because hexan-1-ol, which is less unsaturated than (*Z*)-hex-3-enol and (*E*)-hex-2-enol, showed a higher antifungal activity on mycelial growth of *C. acutatum*. From (*E*)-hex-2-enal to esters, the (*Z*)-hex-3-en-1-yl acetate, and hexyl acetate, it was necessary to increase the doses to near 100 and 200 times, respectively, to inhibit the mycelial growth of *C. acutatum*. Croft et al. (*29*) reported that (*Z*)-hex-3-enol is 20 times less effective than (*E*)-hex-2-enal at inhibiting bacterial growth. Besides being the best mycelial growth inhibitor, the volatile (*E*)-hex-2-enal proved to be the best conidial germination inhibitor of *C. acutatum*, being more active than its corresponding saturated aldehyde hexanal. Neri et al. (*12*) also observed this behavior against *P. expansum*. The efficacy of (*E*)-hex-2-enal has also been reported against bacterial pathogens (*30*, *31*) and is the principal antimicrobial agent from olive oil (*32*). This unsaturated aldehyde known as "leaf aldehyde" (*14*) is widely distributed in many plants. In fact, in response to wounding, strawberry fruit emits (*E*)-hex-2-enal and its precursor (*Z*)-hex-3-enal (*33*). Moreover, Hamilton-Kemp et al. (*34*) have reported the increase of (*E*)-hex-2-enal production in fruit tissues from the activation of LOX−HPL in response to wounding or injury.

LOX and HPL activities from strawberry fruits have been located in the subcellular fraction, mainly in the microsomal fraction; the linolenic acid is the preferred substrate for LOX (*35*). Pérez et al. (*35*) found that the (*E*)-hex-2-enal is the major endogenous aldehyde found in strawberry fruits. During strawberry development and ripening, from green fruits to dark red overripe fruits, these authors observed a light decrease, near 5 nmol/g FW (25% of reduction), of (*E*)-hex-2-enal contents. A decrease of contents of this volatile fairly coincides with the beginning of bright red maturation stage. At the same time, the appearance of visible symptoms starts when strawberry fruit is red (*7*). It could be related to quiescent infection of *Colletotrichum* spp. Latent infections become active during or shortly after fruit ripening and lead to extensive fruit damage (*36*, *37*). Furthermore, Prusky et al. (*38*) reported that latent infection of *C. gloeosporioides* occurring in unripe avocado fruits is related to the activity of LOX.

The conidial germination of *C. acutatum* was more sensitive than mycelial growth to inhibition by exposition of volatile compounds. These findings are in agreement with those reported by Fallik et al. (*15*) and Neri et al. (*12*) especially for (*E*)-hex-2-enal. In this study, the smallest dose used to completely inhibit germination of conidia of *C. acutatum* was 6.76 μL L^{-1}, corresponding to (*E*)-hex-2-enal. Also, the development and maturation of appressoria of this pathogen were altered and the appressorial pore was not formed with 1.35 μL L^{-1} of this volatile. Production of the pore and the penetration peg formation in pigmented appressorium are related with a light spot inside this fungal structure, which have been revealed through a digital image analysis using light microscopy (*24*). Ultrastructures of the early stages of the infection process of

C. acutatum in strawberry tissues have been described by Arroyo et al. (*39*) being the pore appressorial and penetration peg structures, which are essential for a successful penetration through the plant cuticle. Appressorial pigmentation is an essential stage for morphogenesis and maturation of this structure, and it plays a determinant role in the infection process (*40*). Furthermore, Prusky et al. (*41*) reported that latent infection of *C. gloeosporioides* in unripe avocado fruits is characterized by inhibition of hyphal growth after appressorium production. Therefore, if maturation of the appressorium is altered, the infection process it could be stopped, for not lethal amounts of volatile compounds, and it could be restarted when the amounts of volatile decreased still more. The antifungal activity of volatile compounds affected various stages of developmental of *C. acutatum* differently. Germ tube elongation and formation of appressorium are stages even more sensitive than conidial germination, which require higher doses to prevent it.

(*E*)-Hex-2-enal avoided the development of symptoms in strawberry red fruit inoculated with *C. acutatum.* The efficacy of this compound for retarding or reducing the development of the disease symptoms has been reported in *A. thaliana* (*22*), strawberry, blackberry, and grapes against *B. cinerea* (*11*, *15*) strawberry against *B. cinerea*, *A. alternate*, and *C. acutatum* (*13*), and on pears against *P. expansum* (*12*). The use of volatile compounds as (*E*)-hex-2-enal and hexanal to reduce the growth potential of naturally occurring bacteria in the packaging atmospheres of fresh sliced apples has also been reported by Corbo et al. (*42*). In contrast, an extended presence of volatile had phytotoxic effects on strawberry fruits and can affect its quality. The phytotoxic response by (*E*)-hex-2-enal has been described on other fruits and plants (*12*, *29*, *42*). Fallik et al. (*15*) reported loss of fruit fresh mass and others deleterious effects, but they could be minimized. In this way, the inclusion of low levels of hexanal in the packaging atmospheres increased the color stability of sliced apples (*42*). Results seem to justify further works, which must be made to avoid adverse effects on fruit quality.

The ultrastructures of the conidia and different maturation stages of the appressorium of *C. acutatum* including pore formation in the basal zone to formation of penetration peg are in agreement with those results described by Arroyo et al. (*39*). Furthermore, observations by transmission electrom microscopy (TEM) of conidia treated with (*E*)-hex-2-enal revealed a disruption and disorganization of plasma membrane and cell components and, eventually, cell lysis. The severe damage caused by (*E*)-hex-2-enal in the fungal membranes and cell walls, which led to the morphological deformations, collapse, and deterioration of the conidia, has been reported in relation with others pathogens (*15*, *29*, *30*). Dehydrated fungal hyphae and disrupted cell wall and membranes of *B. cinerea* have been observed by Fallik et al. (*15*) with light microscopy. In this work, we have corroborated the cell damage from conidia of *C. acutatum* at the ultrastructural level by using TEM. The high electrophilic properties of (*E*)-hex-2-enal make this compound particularly reactive with protein sulfydryl and amino groups of the pathogen (*12*). Alkenals act as nonionic surfactants at the lipid−protein interface; therefore, the conformation of the membrane may be changed and the H^+-ATPase may be inhibited, which would lead to cell death (*30*).

Our results support the idea that (*E*)-hex-2-enal could provide an additional alternative to chemical products to control *C. acutatum* infections on strawberry fruits, as a postharvest "green chemical" fumigant. The beneficial advantages to using volatiles for postharvest decay control must be exploited; however, further investigations are required to test (*E*)-hex-2-enal safety and mammalian toxicity to elucidate its practical application.

ACKNOWLEDGMENT

We gratefully acknowledge the collaboration of Dr. Carlos Sanz from the Department of Physiology and the Vegetal Products Technology Department, Instituto de la Grasa (CSIC, Sevilla), for their assistance and supply of the chemicals used in this study. We thank Remedios García Navarro (Department of Cell Biology, University of Sevilla) and José María Sanabria (Electron Microscopy General Service, University of Sevilla) for their technical assistance with electron microscopy.

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Received for review February 12, 2007. Revised manuscript received April 30, 2007. Accepted May 5, 2007.

JF0703957