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Chemical Signals from Avocado Surface Wax Trigger Germination and Appressorium Formation in *Colletotrichum gloeosporioides*[1]

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The surface wax of the host, avocado (*Persea americana*) fruit, induced germination and appressorium formation in the spores of *Colletotrichum gloeosporioides*. Waxes from nonhost plants did not induce appressorium formation in this fungus, and avocado wax did not induce appressorium formation in most *Colletotrichum* species that infect other hosts. Bioassays of the thin-layer chromatographic fractions of the avocado wax showed that the fatty alcohol fraction was the main appressorium-inducing component. Testing of authentic *n*-C_8 to *n*-C_{32} fatty alcohols revealed that C_{24} and longer-chain alcohols induced appressorium formation. Gas-liquid chromatography/mass spectrometry analysis of free fatty alcohols revealed that avocado wax contains a high content of very long chains. Waxes from nonhost plants containing an even higher content of the very long-chain alcohols did not induce appressorium formation. Waxes from nonhost plants strongly inhibited appressorium induction by avocado wax. Thus, a favorable balance between appressorium-inducing very long-chain fatty alcohols and the absence of inhibitors allows the fungus to use the host surface wax to trigger germination and differentiation of infection structures in the pathogen.

Fungal spores use physical or chemical signals from the plant surface to trigger germination and differentiation into appressoria, which are necessary for successful infection of the host (Emmet and Parberry, 1975; Aist, 1976; Staples and Hoch, 1987). In anthracnose fungi belonging to the genus *Colletotrichum*, several species are thought to produce appressoria in response to specific physical signals and topography of leaf surface (Staples and Macko, 1980). These include *Colletotrichum capsici* (Parberry, 1963), *Colletotrichum trifolii* (Miehle and Lukezic, 1972), *Colletotrichum lindemuthianum* (Mercer et al., 1975), *Colletotrichum truncatum* (Staples et al., 1976), *Colletotrichum graminicola* (Lapp and Skoropad, 1978), and *Colletotrichum lagenarium* (Suzuki et al., 1982). In others, appressorium formation was suggested to involve chemical signals from the plant, such as Suc in *Colletotrichum piperatum* (Grover, 1971), chlorogenic acid in *Colletotrichum musae* (Swinburne, 1976; Harper and Swinburne, 1979), and phenolics in *Colletotrichum acutatum* (Parberry and Blakeman, 1978). There is also evidence to suggest that cuticular components are involved in the control of fungal differentiation on leaf surfaces (Macko, 1981; Trione, 1981).

Colletotrichum gloeosporioides Penz. is the causal agent of anthracnose disease on fruit crops (Verhoeff, 1974; Brown, 1975; Muirhead, 1981; Daykin, 1984) such as avocado (*Persea americana*) fruit (Binyamini and Schiffmann-Nadel, 1972). It was observed that on avocado fruits germination and appressorium formation of *C. gloeosporioides* spores may be triggered by chemical signals from the surface wax (Prusky and Saka, 1989; Prusky et al., 1991). In this paper, we confirm the reports that appressorium formation by the avocado pathogen *C. gloeosporioides* is induced by surface wax from avocado fruits. We also demonstrate that appressorium formation by this pathogen is induced by the host wax but not by surface wax from other plants and that avocado wax does not induce appressorium formation in the spores of *Colletotrichum* sp. that attack many other hosts. The very long-chain alcohol fraction of the wax was identified as the major chemical signal that induces appressorium formation.

MATERIALS AND METHODS

Materials

Avocados (*Persea americana* Miller var Haas) were supplied by Mission Produce (Oxnard, CA). Surface waxes were isolated by dipping intact fruit in chloroform for 30 s. The chloroform solution was extracted with acidified water to remove water-soluble materials, and then the solvent was evaporated off under reduced pressure in a rotary evaporator. Surface wax from the leaves of the other plants were extracted in a similar manner (Kolattukudy, 1980). Wax from sweet potato periderm was isolated as described before (Espelie et al., 1980). Authentic *n*-fatty alcohols and acids of even-chain lengths from C_8 to C_{32} were purchased from Analabs (Hamden, CT). Cellulose acetate/nitrate filters (HAWG, 1.3 cm diameter) were purchased from Millipore (Bedford, MA).

Strains

Colletotrichum gloeosporioides, an isolate from avocado, was kindly provided by Dr. Dov Prusky (Volcani Center, Israel). *Colletotrichum lindemuthianum* was from Dr. Joseph Kuc (University of Kentucky, Lexington, KY); *Colletotrichum trifolii*,

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Colletotrichum orbiculare, and *Colletotrichum pisi* were from Dr. Sally Leong (University of Wisconsin, Madison, WI); and *Colletotrichum capsici* and *Colletotrichum coccoides* were from Dr. Ralph Nicholson (Purdue University, West Lafayette, IN). The cultures were maintained on potato dextrose agar and/or potato dextrose agar supplemented with 0.1% avocado fruit extract.

Assay for Appressorium Induction

Induction of appressorium was tested either on a glass surface or on a HAWG filter (Millipore). A chloroform solution of wax was dried within a 1-cm-diameter area on a cover glass. The dried chemical was centered in a 1-cm-diameter hole cut in a square of Parafilm, and the edges of the Parafilm around the hole were pressure sealed to the glass. Spores obtained from a 4- to 6-d-old culture were washed twice with ice-cold water, and a spore suspension (1000 spores 100 μL^{-1} of water) was applied to the glass surface coated with the wax so as to cover the entire circle. To measure the effect of addition of other plant waxes on appressorium induction by avocado wax, chloroform solutions of avocado wax and the other waxes were mixed in the desired proportions, and the resulting solution was used to coat the glass surface.

Alternatively, a chloroform solution was applied to a HAWG filter (1.3 cm diameter) on a cover glass so that the volume of solution just saturated the entire filter (usually 25 μL) as described by Prusky et al. (1991). After the chloroform had evaporated, 100 μL of spore suspension (1000 spores) were placed on the filter.

In some cases, wax was dispersed in water by sonication at twice the final desired concentration. The wax suspension (50 μL) was mixed with 50 μL of spore suspension containing 1000 spores and placed on a cover glass in a 1-cm-diameter area limited by Parafilm as described above. In all cases, the spores were incubated for 15 h at 26°C in a moist atmosphere. Spore growth was stopped by addition of lactophenol cotton blue solution. The cover glasses were placed on a grid containing 1-mm squares, and spores were counted in 1-mm squares under ×100 magnification. Typically, 10 random squares were counted for ungerminated spores, germinated spores, and appressoriated spores, and the values were averaged.

TLC

Wax samples were fractionated on 0.5-mm silica gel G layers in lined tanks using hexane:diethylether (90:10, v/v) or hexane:diethylether:formic acid (60:40:2, v/v) as the solvent system. Separated components were visualized under UV light after the plates were sprayed with a 0.1% ethanolic solution of 2,7-dichlorofluorescein, and the lipids were recovered with chloroform or chloroform:methanol (2:1, v/v); each component was identified by co-chromatography with external standards.

GLC-MS

The fatty alcohol fraction recovered from the silica gel scraped from the TLC plates was treated with bis-*N,O*-trimethylsilylacetamide (Walton and Kolattukudy, 1972), and the silyl derivatives were subjected to capillary GLC/MS analysis with a Hewlett-Packard model 5890 gas chromatograph with an HP-1 cross-linked methyl silicon gum capillary column (12 m × 0.2 mm), interfaced to a Hewlett-Packard model 5988A mass spectrometer. The temperature of the column was held at 180°C for 2 min, followed by a 10°C min^{-1} increase in temperature up to 300°C. All mass spectra were recorded at 70 eV.

RESULTS

Induction of Appressorium Formation by Avocado Wax

When *C. gloeosporioides* spores were incubated in a water layer on a cover glass, they did not germinate. Addition of Glc or Suc did not enhance germination significantly. Yeast extract caused near complete germination with very elongated germ tubes (Fig. 1). However, the germ tubes did not differentiate into appressoria. On Millipore filters, virtually all of the spores germinated without any wax, but no appressorium formation was observed. When the spores were placed on cover glasses coated with avocado wax, most spores germinated, and virtually all of the germ tubes formed appressoria within 12 h (Fig. 1). Under these conditions, <1 μg cm^{-2} wax gave complete germination and appressorium formation. When conidia were incubated on an aqueous wax suspension on a cover glass, the formation of appressoria was also observed. The amount of wax needed to stimulate appressorium formation in *C. gloeosporioides* depended on the method of application. Dose-dependence studies showed that 50% appressorium induction required 0.1 μg of wax cm^{-2} when coated on a cover glass and 0.001 μg of wax dispersed in 100 μL of water and spread on a 1-cm^2 cover glass. On the other hand, when spores were placed on moist

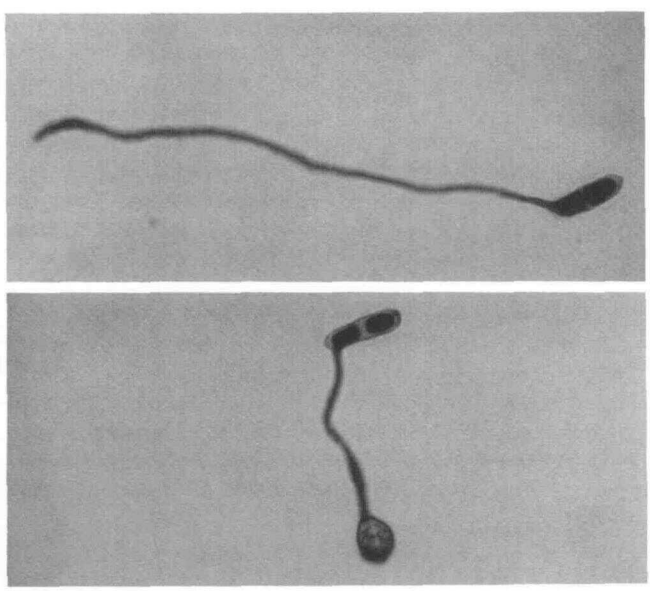

Figure 1. *C. gloeosporioides* spores germinated in the absence (top; yeast extract) and presence (bottom) of avocado wax (4 μg of avocado wax cm^{-2} on cover glass).

Table I. *Effect of various plant waxes on appressorium formation by C. gloeosporioides*

Assays were done by the coated cover glass method, using 4 μg cm^{-2} of wax; assays done with 20 μg cm^{-2} of wax gave similar percentages.

Source of Wax	Percentage of Appressoria
Avocado fruit	78
Broccoli leaves	1
Cabbage leaves	4
Pea leaves	4
Senecio odoris leaves	5
Jade leaves	4
Sweet potato tuber	6
No wax	3

Millipore filter, germination occurred without appressorium formation, and presoaking of the filter with avocado wax induced appressorium formation as previously noted (Prusky et al., 1991). However, the amount of wax that gave 50% appressorium formation under these conditions was much higher (300 μg cm^{-2}) than required by the other assay methods. For subsequent experiments to test the ability of waxes and their components to induce appressorium formation, the cover glass-coating method was used.

Effect of Various Plant Waxes on Appressorium Formation by *C. gloeosporioides*

To test whether the stimulation of appressorium formation by *C. gloeosporioides* is specific to the wax from avocado, surface waxes from different plants were tested for their ability to induce appressorium formation. The results showed that appressorium formation was induced only by avocado wax and not by wax from the other sources tested (Table I).

Effect of Avocado Wax on Appressorium Formation by Different Species of *Colletotrichum*

To test whether avocado surface wax induces appressorium formation selectively in the *Colletotrichum* sp. that attack avocado, conidia from various *Colletotrichum* spp. were tested on cover glasses coated with avocado wax for their ability to be induced by the avocado wax to form appressoria (Table II). With a level of avocado wax that gave 70% appressorium formation for *C. gloeosporioides* spores, the cucurbit pathogen *C. orbiculare* showed 25% germination but only 2% appressorium formation, and the other pathogens did not show any significant germination or appressorium formation (Table II). The only exception was *C. lindemuthianum*, which also showed stimulation of appressorium formation by avocado wax.

Effect of Concentration of Total Wax on Appressorium Formation by *C. gloeosporioides*

As the amount of wax coated on the cover glass increased, germination and appressorium formation began at a concentration of approximately 0.002 μg cm^{-2} at which 5 to 10% of the spores germinated and virtually all (approximately 90%)

Table II. *Effect of avocado wax on appressorium formation by various Colletotrichum spp. of fungal pathogens*

Assays were done using the coated cover glass method with 20 μg of avocado wax cm^{-2}. For *C. gloeosporioides*, *C. orbiculare*, and *C. lindemuthianum*, the values (1–3%) obtained with no wax (control) have been subtracted. The other organisms did not germinate under these conditions.

Pathogen	Host	Percentage of Appressoria Formation
C. gloeosporioides	Avocado	70
C. trifolii	Alfalfa	0
C. orbiculare	Cucumber, watermelon	2
C. pisi	Peas	0
C. capsici	Cotton, peppers	0
C. lindemuthianum	Bean	70
C. coccoides	Tomato	0

of the germ tubes differentiated into appressoria (Fig. 2). With increasing amounts of wax, a higher fraction of the spores germinated and formed appressoria until about 1 μg cm^{-2}, at which level about 90% or more of the spores formed appressoria. Further increases in wax showed smaller increases in appressorium formation until near complete appressorium formation was obtained with 2 μg of wax cm^{-2}.

Effect of TLC Fractions from Avocado Wax on Appressorium Formation by *C. gloeosporioides*

The total surface wax from avocado was fractionated by TLC, and various fractions were isolated and purified by repeated TLC. These fractions were tested for their ability to stimulate appressorium formation by conidia of *C. gloeosporioides*. Of all the fractions tested, the primary alcohol fraction showed the maximal stimulation of appressorium formation (Table III). The adjoining fractions from the thin-layer chromatogram, including those that contained ω-hydroxy fatty acids, showed some appressorium-inducing activity (Table III). Hydrocarbon, which constituted the major component

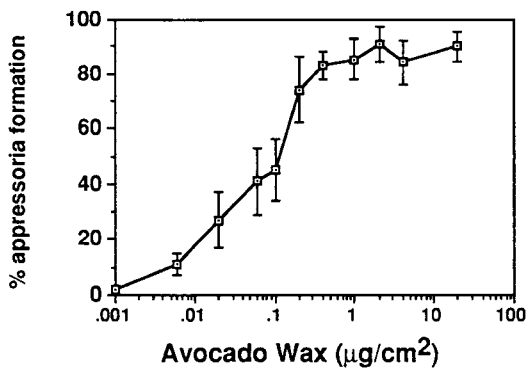

Figure 2. Effect of increasing amounts of avocado wax on appressorium formation in *C. gloeosporioides*. Assays were done using the coated cover glass method, as described in "Materials and Methods." The percentage of appressoria formed in the presence of water only (3–6%) was subtracted.

Table III. *Appressorium-inducing activity of various TLC fractions from avocado wax on C. gloeosporioides*

Assays were done using the coated cover glass method; the amount of each fraction used was the equivalent of the amount contained in 4 μg of total wax. Appressoria formation is expressed as a percentage of the response to 4 μg of total wax cm^{-2}. Both silica gel and 2,7-dichlorofluorescein were tested and were shown to have no effect on germination or appressoria formation.

Fraction	Appressorium Formation	Wax Composition
	%	% of wax by weight
Hydrocarbon	1	62
Wax ester + ketone	7	6
Aldehyde	11	7
Secondary alcohol	23	4
Fatty acid	70	4
Primary alcohol	95	5
Origin	74	11

of surface wax, and the other nonpolar components of the avocado surface wax did not stimulate germination or appressorium formation.

Effect of Chain Length of Fatty Alcohols and Fatty Acids on Their Ability to Induce Appressorium Formation in *C. gloeosporioides*

If fatty alcohols, rather than some co-migrating component in the avocado wax, induce appressorium formation, authentic synthetic alcohols should induce appressorium formation. Synthetic alcohols of various chain lengths were tested for induction of appressorium formation, and corresponding fatty acids were tested for comparison (Table IV). Fatty alcohols up to C_{16} showed very little appressorium-inducing activity. As the chain length increased from C_{18} to C_{22}, increasing levels of appressorium-inducing activity were observed. C_{24} and longer fatty alcohols showed high levels of appressorium-inducing activity that remained high up to C_{32}, which was the longest alcohol tested. The fatty acids of the corresponding chain lengths failed to induce either germination or appressorium formation.

Analysis of Fatty Alcohol Fraction of Avocado and Other Waxes

To determine whether the fatty alcohols in the surface wax of avocado fruits contain the very long-chain alcohols that appear to be highly effective appressorium inducers, the free fatty alcohol fraction was analyzed by capillary GLC/MS (Table V). Alcohols of C_{26} and longer-chain lengths constituted more than 80% of the alcohols, with C_{30} and C_{32} accounting for more than half of the total. Because the surface wax from the other sources tested did not induce appressorium formation, we analyzed these waxes for the presence of free fatty alcohols by TLC. Because all of them showed the presence of free fatty alcohols, the chain length composition of the fatty alcohol fraction from each wax was determined by combined GLC/MS (Table V). All of the waxes contained very long-chain alcohols including C_{30} and C_{32}; waxes from the leaves of *S. odoris* and jade showed a much higher content of very long-chain fatty alcohols than avocado wax, whereas the other plant waxes tested contained fewer very long-chain alcohols.

Effect of Other Plant Waxes on Appressorium Induction by Avocado Wax

Because the fatty alcohol content alone could not explain the observed selective induction of appressoria by avocado wax, we tested whether other plant waxes contained antagonists that prevented appressorium induction. Addition of other waxes with avocado wax severely inhibited appressorium induction by the avocado wax (Table VI). For example, under conditions that gave appressorium induction in 74% of the spores by avocado wax, addition of 2 μg of broccoli leaf wax or jade leaf wax depressed appressorium formation by 80%, and 2 μg of *S. odoris* wax depressed appressorium formation by 70%.

DISCUSSION

Appressorium formation in some pathogenic fungi has been shown to depend on physical stimuli (Staples and Macko, 1980). There are very few examples of chemical stimulation of germination and differentiation of fungal spores (Grover, 1971; Swinburne, 1976; Parberry and Blakeman, 1978; Harper and Swinburne, 1979). In these cases, it was usually the germination that was stimulated, and the differentiation of germ tube into appressorium depended on the physical nature of the substratum. Involvement of a chemical signal from the epicuticular wax was implicated for the avocado anthracnose fungus, *C. gloeosporioides* (Prusky and Saka, 1989; Prusky et al., 1991). We have attempted to investigate this possibility and to determine the nature of the chemical signal present in the epicuticular wax from avocado fruit that is responsible for the induction of appressorium formation in *C. gloeosporioides*.

The epicuticular wax from avocado fruit indeed stimulated

Table IV. *Effect of chain length of fatty alcohols and fatty acids on their ability to induce appressorium formation in C. gloeosporioides*

Assays were done with the coated cover glass method, using 4 μg of fatty alcohol or fatty acid cm^{-2}. nd, Not determined.

Chain Length	Appressorium Formation	
	Alcohol	Fatty acid
	%	
C_8	8	nd
C_{10}	7	nd
C_{12}	5	3
C_{14}	9	3
C_{16}	9	2
C_{18}	16	2
C_{20}	23	2
C_{22}	30	2
C_{24}	82	2
C_{26}	82	4
C_{28}	75	2
C_{30}	88	2
C_{32}	91	2

germination of conidia of *C. gloeosporioides* and appressorium formation under various conditions. It seems likely that appressorium formation was dependent on chemical signals from the wax rather than on the physical nature of the surface. On Millipore membranes containing no wax, the conidia germinated but failed to form appressoria, whereas few germinated in the controls when the conidia were incubated on cover glasses without the wax. The higher concentrations of wax needed for the stimulation of appressorium formation on Millipore membranes probably reflects the fact that wax is embedded into the filter with only a small portion available to the spore, and the present results are consistent with the higher levels found to be necessary by Prusky et al. (Prusky and Saka, 1989; Prusky et al., 1991). When wax was coated on a glass surface, only minute quantities were needed to stimulate appressorium formation, and wax dispersions were even more potent. For example, if we assume a mol wt of 400 (the mol wt of a C_{30} alcohol is 438) for appressorium inducer, 10^{-8} M showed 40% induction of appressorium formation with dispersion. Even though the effect of wax coating of glass surfaces could possibly be viewed as being due to alterations of the surface property, our observation that extremely low concentrations of wax dispersed in water by sonication can induce appressorium formation makes it unlikely that the wax induction is by a physical modification of the surface. This observation strongly suggests that avocado wax acts as a chemical signal.

If the induction of appressorium formation in *C. gloeosporioides* by avocado wax is of biological significance, waxes from nonhost plants might not be effective in stimulating appressorium formation in *C. gloeosporioides*. Experimental results presented here clearly show such a specificity. Thus, it seems that nonhost waxes may either lack the chemical signals needed to stimulate appressorium formation or contain chemicals that inhibit the appressorium formation of *C. gloeosporioides*. To examine further for biological specificity, we tested whether avocado wax can induce appressorium formation by other anthracnose fungi that are not pathogens on avocado. Results from these experiments indicated that induction of appressorium formation by avocado wax is quite specific for *C. gloeosporioides*; the exception was that appressorium formation was induced also in *C. lindemuthianum*, indicating that there may be qualitative differences that exist between various anthracnose fungal conidia in their ability to perceive the right host.

Table VI. *Effect of addition of other plant waxes on appressorium formation induction in C. gloeosporioides by avocado wax*

Assays were done using the coated cover glass method with 4 μg of avocado wax. The percentage of appressoria formed with avocado wax alone (74%) was used as the control value. Assays of avocado wax alone up to 8 μg did not result in a decrease in percentage of appressoria formation.

Addition	Amount	Appressorium Induction
	μg	% of control
Broccoli wax	0.5	14
	2.0	19
Jade wax	0.5	38
	2.0	18
S. odoris wax	2.0	27

To determine the nature of the chemical signal(s) from the avocado wax that stimulate appressorium formation by *C. gloeosporioides*, the total epicuticular wax was chromatographically fractionated. Even though the hydrocarbon fraction constituted the major component of the wax, making up almost two-thirds of the total wax, it showed virtually no effect on appressorium formation. The maximal stimulation of appressorium formation was obtained with the primary alcohol fraction of the wax. The fractions adjoining the alcohol fraction in the thin-layer chromatogram also showed some appressorium-inducing activity. Thus, the fatty acid fraction and the fraction that represents the more polar components of the wax also resulted in some stimulation of appressorium formation. It is possible that other components in the wax that migrate with fatty acids and with the more polar components have some appressorium-inducing activity. However, the finding that synthetic fatty alcohols, but not the corresponding fatty acids, induced appressorium formation strongly supports the conclusion that fatty alcohols in the wax constitute the major appressorium-inducing component of the avocado wax.

The effect of the chain length of the fatty alcohols on the appressorium-inducing activity showed that the very long-chain fatty alcohols normally found in the surface wax of

Table V. *Chain length distribution of free fatty alcohols in some plant waxes*

Free fatty alcohols were isolated by TLC of the total waxes and, as their trimethylsilyl derivatives, were analyzed by GLC/MS as described in the text.

Plant	Alcohol Content						
	Chain length						
	24	26	28	30	32	34	36
	μg mg^{-1} of wax						
Avocado fruit	0.2	0.5	1.7	6.7	8.1	0.3	0
Broccoli leaves	0.9	6.6	4.9	1.9	0.1	0	0
Cabbage leaves	5.9	16.0	8.0	1.6	0	0	0
Jade leaves	0.1	1.0	11.0	51.0	60.0	19.0	4.0
Pea leaves	0.2	5.6	8.4	0.6	0.2	0	0
S. odoris leaves	0.3	5.9	89.0	46.0	39.0	14.0	15.0
Sweet potato tuber	0.3	0.3	1.1	8.5	0.2	0	0

plants (Kolattukudy, 1980) are the best inducers. Because free fatty alcohols of the host wax (rather than esterified alcohols) would be expected to be the most available for the spores, free fatty alcohol fractions of the various waxes were examined. The surface wax of avocado fruits was found to have a higher proportion of the very long-chain fatty alcohols than found in the surface wax of many other plants (Kolattukudy, 1980). However, it appears clear that the content of very long-chain fatty alcohols cannot explain the observed selectivity of appressorium induction by avocado wax. The plant waxes that showed no appressorium-inducing activity also contained very long-chain fatty alcohols. Although surface wax of *S. odoris* and jade leaves contained much higher (5- to 8-fold) amounts of very long-chain fatty alcohol (C_{30} to C_{32}) than avocado wax, these waxes did not induce appressorium formation.

Obviously, the quantity of the very long-chain fatty alcohol was not the sole factor that determined whether these plant waxes had appressorium-inducing capability. Therefore, we tested for the existence of inhibitors of induction of appressorium formation in waxes of nonhost plants. In fact, appressorium induction caused by avocado wax was severely inhibited by other plant waxes. Both *S. odoris* wax and jade wax, which showed a much higher content of fatty alcohols that possess the capability to induce appressorium formation, inhibited appressorium induction by avocado wax. The component responsible for this inhibition is not known. It has been previously reported that avocado peel in unripe fruits contains inhibitors of appressorium formation that disappear as the fruits ripen (Prusky et al., 1991). It appears likely that the selectivity observed here for the host wax in inducing appressorium formation is the result of a favorable balance between appressorium inducer(s) and antagonists.

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