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EXTRACTED FATTY ACIDS FROM *GOSSYPIUM HIRSUTUM* STIMULATORY TO THE SEED-ROTTING FUNGUS, *PYTHIUM ULTIMUM*

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Key Word Index—*Gossypium hirsutum*; Malvaceae; cotton; seed exudation; fungal germination stimulants; palmitic acid; linoleic acid.

Abstract—Specific germination of the pathogenic fungus *Pythium ultimum* in response to a viable host is a vital point in its life cycle. It must have the means to elucidate information on the proximal host, and a response to specific germination signals from the host seed was suspected. Active fractions from cotton seed extract containing unsaturated fatty acids induced high levels of germination in *Pythium*, whereas several saturated analogs did not.

INTRODUCTION

Pythium species cause seed decay, pre-emergence and post-emergence damping off, root rot of seedlings, and rot of plant storage organs [1]. They are ubiquitous in soil and aquatic environments, worldwide in distribution, and have very diverse host ranges, including many plants as well as animals, such as fish and Crustacea [1]. *Pythium* species depend upon both oospores and sporangia as survival propagules during periods without access to a substrate [2–4], and among those species with globose sporangia, sporangia may persist in soil to the same extent or longer than oospores [2].

Sporangia in soil are exogenously dormant, i.e. dormancy imposed by the soil environment, and will germinate rapidly in response to introduced nutrient stimuli or upon alleviation of fungistasis [5, 6]. Sporangia of *Pythium ultimum* Trow germinate rapidly in response to external stimuli and the hyphae produced infect seed and plant tissue within a few hours of planting [3, 4, 7–12]. The principal sources of these external stimuli are host seed and root extracts [13–16], although the nature of the compounds involved in this process has yet to be conclusively determined. The purpose of the present work was to determine the nature of *Pythium*-stimulatory compounds from extracts of cotton seeds in an attempt to explain the differential reaction of propagules to external stimuli and to provide an understanding of the nature of the *Pythium*-seed interactions.

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RESULTS

Specific activity of seed extracts

The specific activity of the seed extracts extracted with water or acetone were very similar (Fig. 1). Both extracts showed quantitative levels of activity at 10 mg ml^{-1}, reduced levels of activity at 5 mg ml^{-1}, and no activity at 1 mg ml^{-1}.

Activity of C18 *acetone/water column fractions*

The levels of activity were highest in the 10% acetone fraction for the water extract and the 75% acetone fraction of the acetone extract (Fig. 2).

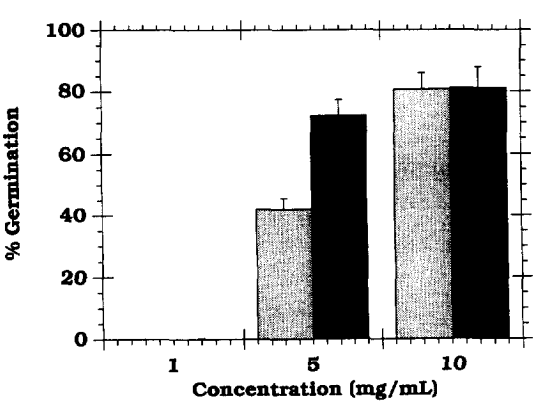

Fig. 1. Comparative specific stimulatory activity, expressed as percent germinated *Pythium* ultimum sporangia, of cotton seed extract extracted with water or acetone. ▩ = water extraction, ■ = acetone extraction (see Materials and Methods).

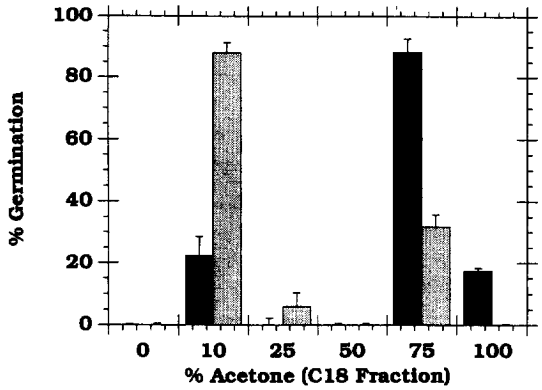

Fig. 2. Stimulatory activity of seed extract fractions eluted from a C18 column with an acetone/water step gradient (10, 25, 50, 75, and 100% acetone). Each fraction was dried and a portion reconstituted in 10 mM NH_4OAc buffer (pH 5.5) prior to sporangium germination assays. ▩ = water extraction, ■ = acetone extraction (see Materials and Methods).

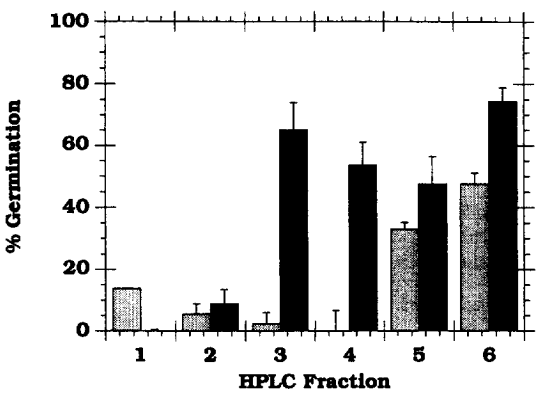

Fig. 3. Stimulatory activity of extract fractions eluted from a C18 column with 75% methanol and subjected to C18 HPLC in a CH_3CN–H_2O gradient. Fractions were collected based on time: 1 = 0–5 min., Fraction 2 = 5–10 min., Fraction 3 = 10–15 min., Fraction 4 = 15–20 min., Fraction 5 = 20–25 min., Fraction 6 = 25–30 min. ▩ = water extraction, ■ = acetone extractions (see Materials and Methods).

Activity of CH_3CN–H_2O C18 *HPLC fractions*

For both the water and acetone extracts, the majority of the activity eluted in the final 25 to 30 min fraction (Fig. 3).

Activity of MeOH–H_2O C18 *HPLC fractions*

The levels of activity in each fraction varied considerably (Fig. 4), with relatively high levels of activity detected in the very late fractions of both extracts.

UV, MS, and GC-MS analysis of HPLC fractions

The 27 to 28 min. fraction of the acetone extract from the above HPLC analysis possessed UV profiles devoid of any structurally distinct absorption maxima. The electron impact (EI) mass spectrum showed a characteristic fatty acid fragmentation with significant ions at *m/z* 43, 57, 71, 85, etc. and *m/z* 41,

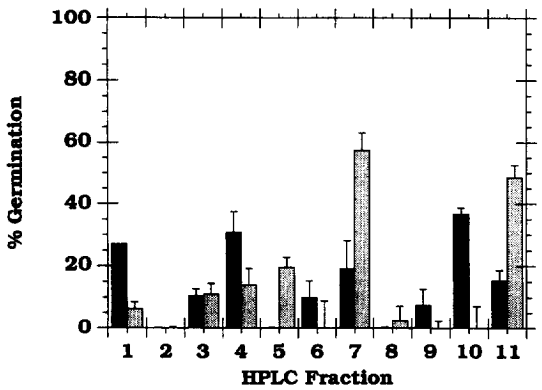

Fig. 4. Stimulatory activity of fraction 6 from the CH_3CN–H_2O HPLC (see fig. 3) and subjected to C18 HPLC in a MeOH–H_2O gradient. Eleven fractions were collected and assayed for activity: Fraction 1 = 0–10 min., Fraction 2 = 10–17 min., Fraction 3 = 17–18 min., Fraction 4 = 18–19 min., Fraction 5 = 19–22.5 min., Fraction 6 = 22.5–24 min., Fraction 7 = 24–25 min., Fraction 8 = 25–26 min., Fraction 9 = 26–27 min., Fraction 10 = 27–28 min., Fraction 11 = 28–30 min. ▩ = water extraction, ■ = acetone extractions (see Materials and Methods).

55, 69, 83, etc. [17]. Furthermore, the CI spectrum indicates that one component of this fraction possesses a high M_r of 830.

GC-mass spectrum analysis revealed the presence of at least six different fatty acid methyl esters. The predominant compounds, eluting at 10.22 and 11.05 min., were identified as palmitic acid methyl ester and linoleic acid methyl ester. The compounds eluting at 8.1 and 9.1 min. were identified as the dimethyl ester of azelaic (nonanedioic) acid and the methyl ester of myristic acid, respectively. The compounds above all displayed mass spectra with abundant ions of *m/z* 74, [McLafferty, $C_3H_6O_2^+$], an M^+-31 ion, [loss of CH_3O] and the alkyl and alkoxy fragments of *m/z* 43, 57, 71, 85, etc. and *m/z* 41, 55, 69, 83, etc. Finally, the mass spectrum of the less prominent peak at 8.57 minutes is consistent with that of 4-(2,2,4-trimethylpentyl)-phenol. Injection of a non-esterified version of the same 27 to 28 min. HPLC fraction showed the presence of palmitic acid and linoleic acid as the free acids. GC-mass spectrum analysis of a similarly purified and esterified aqueous extract revealed the presence of two major components: the methyl esters of palmitic and linoleic acid.

Activity of fatty acids

Various commercially-available fatty acids (Sigma), both plant-derived and non-plant derived, were assayed for the ability to induce germination (Table 1). The addition of a commercial antioxidant, butylated hydroxytoluene (BHT), does not diminish the activity of the commercial fatty acids nor did periodic removal and replacement of the fatty acid (washing assay). Several diglycerides of active and inactive fatty acids

Table 1. Structure-activity relationship of commercially available fatty acids as germination stimulants for *Pythium ultimum* sporangia

	Percent germinated sporangia*			
	Concentration ($\mu g\ \mu l^{-1}$)			
Fatty acid	10.00	1.00	0.10	0.01
Saturated fatty acids				
Caprylic C8:0	0.0	0.0	20.6	0.4
Capric C10:0	0.0	1.7	0.5	0.0
Myristic C14:0	0.0	0.0	0.0	0.0
Palmitic C16:0	0.0	0.0	0.0	0.0
Margaric C17:0	0.0	0.0	0.0	0.0
Stearic C18:0	0.0	0.0	0.0	0.0
Unsaturated fatty acids				
Myristoleic C14:1	70.5	1.1	0.0	0.0
Palmitoleic C16:1	98.5	78.1	30.3	0.0
cis-10-Heptadecenoic C17:1	74.7	3.0	0.0	0.0
Oleic C18:1	0.0	55.9	55.7	2.4
cis-10-Nonadecenoic C19:1	32.8	1.2	1.1	0.0
Linoleic C18:2	100.0	73.3	0.0	0.0
Linolenic C18:3	96.5	98.2	14.2	0.0
6,9,12,15-Octadecaenoic C18:4	NT†	93.0	96.2	12.6
Arachidonic C20:4	NT†	82.3	75.5	3.3

* Sporangium germination determined after 3 hr of exposure to the fatty acid.
† NT = not tested.

were assayed but were not active, this perhaps being due to a lack of solubility.

DISCUSSION

It has long been proposed that sugar and amino acid components of seed and root extracts are the primary germination stimulants for propagules of *Pythium* spp. in nature. Agnihotri and Vaartaja [4, 18–20] suggested that sugars, amino acids, and organic acids could be responsible for stimulating sporangial germination in *P. irregulare* Buis. and *P. ultimum*. Other studies [21, 22] established correlations between carbohydrate exudation *in vitro* and decreased emergence of pea and bean seedlings in the field due to *Pythium* seed rot. It was concluded, therefore, that carbohydrates could be responsible for overcoming fungistasis, thereby stimulating the activity of *Pythium* in soil. Other studies, however, have demonstrated that propagules of *Pythium ultimum* derived from living plant tissue behave differently that those produced on synthetic media [23, 24]. In fact, the ability of various sugars and amino acids to stimulate germination of *Pythium ultimum* sporangia was lost when the sporangia were produced on plant tissue, yet these same sporangia remained fully responsive to seed extract. Additionally, propagules produced on media amended with host seeds or soybean lecithin responded similarly to those produced on plant tissue. From the results of those experiments, and the work of others (see references in [24]), Nelson and Hsu speculated that while sugars and amino acids can act as germination stimulants for *Pythium* propagules produced under starvation conditions, they may not be the primary signals for the propagules in nature.

The spectral and structure-activity data presented here support the notion that lipophilic components of cotton seed extract, such as unsaturated fatty acids (LCFAs), may be of primary importance in stimulating germination of *Pythium* propagules. Other studies indirectly support the involvement of LCFAs as stimulants of *P. ultimum* sporangium germination. Van Dijk *et al.* [25, 26] found that a transposon-induced mutant of *Enterobacter cloacae*, unable to metabolize LCFAs, was also unable to inactivate the stimulatory activity of cotton seed extract or a number of unsaturated LCFA, including linoleic acid. Complementation of the mutant strain with a 3.5 kb wild-type clone restored fatty acid metabolism as well as the ability of the bacterium to inactivate the stimulatory activity of cotton seed extract.

In the structure-activity studies, the low concentration required for activity of the most active fatty acids makes it more likely that these lipophilic compounds are present in the water-soluble spermosphere of the host seed in sufficient quantities to induce sporangium germination. Furthermore, the GC-mass spectrum analyses reveal that palmitic and linoleic acid are the largest lipophilic component of the aqueous extract. Additionally, if the previously water-extracted seeds are then subjected to the later acetone extraction, high levels of stimulatory activity are observed, thus indicating superiority of acetone in

solubilizing this activity. It is noteworthy that while the free fatty acids possess activity, the esters (including the triglycerides) show no activity. Although the free fatty acids are extractable from the seed, lipases present during germination would release fatty acids from the storage triglyceride and this release could signal the presence of a proximal host. This fact may enhance the importance of these LCFAs as signal molecules giving both spatial and temporal information.

The data presented here also show that a degree of unsaturation enhances the stimulatory activity of LCFA. Papavizas and Adams [27], working with endoconidia and chlamydospores of *Thielaviopsis basicola*, and Harman *et al.* [10], working with conidiospores of *Alternaria alternata*, noted similar requirements in order to induce germination. However, Harman, *et al.* [28] found that palmitic acid stimulated germination of conidiospores of *Fusarium solani f.* sp. pisi, in addition to oleic and linoleic acids, while stearic and linolenic acids were ineffective. It therefore appears that while a degree of unsaturation in the fatty acid generally enhances its germination activity in fungi, there are notable exceptions.

Harman *et al.* [28, 29] have proposed that the volatile peroxidation products of the fatty acids, i.e. 2,4-hexadienal, may be the actual stimulants of germination for *Alternaria alternata*. Whether the volatile peroxidation products of unsaturated fatty acids stimulate germination of *Pythium* spp. propagules is not known, but the assays conducted with the addition of BHT and the washing assays discount the role of peroxidation products in this system. However, we cannot rule out totally the role of volatile peroxidation products in stimulation of germination of *Pythium*. Unsaturated fatty acids are oxidized readily by molecular oxygen, and reactions catalysed by lipoxygenase enzymes are widespread in the plant and fungal kingdoms [3]. It could be argued, however, that the importance of this degree of unsaturation is related instead to a mechanism for the pathogen to obtain distance information about the host. As these unsaturated fatty acids enter the soil from the seed, various reactions result in the oxidation of the fatty acid and this could result in lower levels of the fatty acid at increasing distance from the seed.

Fatty acids have also been shown to be attractants for zoospores of *Phytophthora palmivora* [31], to be involved in host specificity of *Rhizobium* [32], to be growth stimulants and carbon sources for *Phytophthora parasitica* var. *nicotianae* [33], and to be involved in the regulation of dormancy and growth in some plant species [34]. This study clearly demonstrates that fatty acids constitutive to cotton seed elicit high levels of germination of sporangia of *Pythium ultimum* that are raised under conditions thought to closely mimic natural sporangia produced on plant tissues. This underscores the potential importance of these fatty acids as germination signals in the soil environment of cotton seeds.

EXPERIMENTAL

Culture of P. ultimum *and preparation of sporangia.* *Pythium ultimum* isolate P4 was used with the culture and induction of sporangium formation was accomplished as previously described [23, 35].

Collection and preparation of seed extracts. Seeds of cotton (cultivar Acala SJ-2) collected in 1988–89 and of good quality were sorted to remove damaged and deformed seeds, then surface sterilized in a 0.5% NaOCl solution for 5 min., rinsed thoroughly with sterile distilled H_2O, and dried overnight (room temp.). About 50 g (dry wt) of seeds were added to flasks containing H_2O (300 ml) or Me_2CO (300 ml), placed on a rotary shaker at 27° for 4 hr (H_2O) or 10 min. (Me_2CO). In the case of the Me_2CO extract, Me_2CO was then decanted after the initial 10 min. period and fresh Me_2CO (300 ml) was added for an overnight extraction. The resulting solns were filtered (Whatman no. 1 filter paper) and then passed sequentially through 0.8-, 0.45- and 0.2-μm filters. All filtrates were evaporated *in vacuo* at 45° and the resulting residue weighed. These were individually reconstituted in 10 mM NH_4OAc buffer (pH 5.5) to a concentration of 10 $\mu g\ \mu l^{-1}$ for germination studies.

Germination assays. The seed extract, chromatographic fr. (see next section), or commercial fatty acid (Sigma) was dissolved in 10 mM NH_4OAc buffer (pH 5.5) to a concentration of 10 $\mu g\ \mu l^{-1}$ (or with later HPLC fr., where no weight could accurately be determined, to a standardized vol. (100 μl) of 10% MeOH–NH_4OAc buffer) and then serially diluted to the appropriate concentration range (1.0, 0.1, and 0.01 $\mu g\ \mu l^{-1}$). Butylated hydroxytoluene (BHT) was added to a final concn of 0.1%. Leached discs containing sporangia were placed on sterile glass slides (three replicate discs per slide), 10 μl of the appropriate soln was added and cultures were incubated at 24° for 4 hr. Washing assays (see discussion) were conducted as follows: each hour after original application of the fatty acid, the soln was washed off the discs by rinsing $\times 3$ thoroughly with H_2O, allowing the discs to remain immersed in H_2O for 2 min. between each rinse. The fresh fatty acid soln was then reapplied. In all assays, germination of sporangia was assessed as previously described [23].

Fractionation of seed extracts. Seed extract was dissolved in Me_2CO–H_2O and applied to a 20 m $\times$ 200 mm C18 (Waters) column. The extract was washed from the column with an Me_2CO–H_2O step gradient, consisting of 10, 25, 50, 75, and 100% acetone. Each fr. was dried and a portion was reconstituted in 10 mM NH_4OAc buffer (pH 5.5) and assayed for sporangial germination activity. The 75% Me_2CO fr. was then dissolved in a small amount of CH_3CN and passed through a C18 Sep-Pak (Waters). The extract, dissolved in CH_3CN, was then injected onto a Waters HPLC system with a 30-minute step/linear gradient from 5:95 CH_3CN–H_2O to 100:0 CH_3CN–H_2O (0–3 min, 5%; 3–5 min, 10%, 5 min, 20%; 5–6 min, 20–

30% linear; 6–8 min, 30–40% linear; 8–11 min, 40–75% linear; 11–13 min, 75–85% linear; 13–18 min, 85–95% linear; 18–20 min, 95–100% CH_3CN linear; 2–30 min, 100% CH_3CN). Absorption was monitored at 262 nm at 0.001 AUFS and HPLC trace profiles were recorded on a Hewlitt–Packard 3393A integrator. Six crude time dependent frs, consisting of 5 min. each, were collected and dried *in vacuo* at 40°. A portion of each fr. was reconstituted as above and assayed for activity. The 25–30 min. fr. from this run were then redissolved in 100% MeOH and injected onto the Waters HPLC system with a 30 minute step/linear gradient from 5:95 MeOH–H_2O to 100:0 MeOH–H_2O (same gradient profile as with CH_3CN). Absorption was monitored at 262 nm at 0.001 AUFS. 11 frs were collected, based on time: 0–10, 10–17, 17–18, 18–19, 19–22.5, 22.5–24, 24–25, 25–26, 26–27, 27–28, and 28–30 min. All of these frs were dried *in vacuo* at 40° and a portion was reconstituted and assayed as described above.

Ultraviolet and mass spectral analysis of active fractions. UV absorbance spectra were obtained by scanning samples from 200 nm to 500 nm in a Perkin-Elmer Lambda 6 spectrophotometer at a scan rate of 500 nm min^{-1}. Purified samples were submitted to the University of Illinois Mass Spectrometry Laboratory for analysis. Samples were subjected to electron impact ionization (EI), chemical ionization (CI), and fast atom bombardment (FAB) mass spectral analysis in positive ion modes.

GC-MS analysis of extract fractions. The cotton extract was fractionated as described above and esterified (except where noted) in excess MeOH–H_2SO_4. The dried residue from this reaction was diluted with ether (0.1%) and 1 μl injection were made onto a HP 5890 gas chromatograph with a HP 5972 mass selective detector, scanning a mass range of 50–550 a.m.u. A solvent delay of 2 min., inlet temp. of 250°, and a temp. program that consisted of a 70° initial temp. for 2 min., followed by a 20° min^{-1} ramp to 300°. Run times ranged from 12–20 min. and a 30 m HP-5 GC column, with a 0.25 mm diameter and 0.25 m film thickness, was used. Mass spectra of the samples were compared to an NBS75K standard library for identification and identity was confirmed by retention time comparison to a commercially-obtained standard (Sigma), except where noted.

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