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Plant extracts containing caffeic acid and rosmarinic acid inhibit zoospore germination of *Phytophthora* spp. pathogenic to *Theobroma cacao*

Timothy L. Widmer[1,2,*] and Nathalie Laurent[1]
[1]*European Biological Control Laboratory, USDA/ARS, Campus International de Baillarguet, CS 90013 Montferrier sur Lez, 34988, St. Gely du Fesc, France;* [2]*Foreign Disease and Weed Science Research Unit, USDA-ARS, 1301 Ditto Avenue, Frederick, MD, 21702, USA *Author for Correspondence (Fax: 301-619-2880; E-mail: tim.widmer@ars.usda.gov)*

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Abstract

The three most important species of *Phytophthora* worldwide causing black pod disease of cacao are *P. palmivora*, *P. megakarya*, and *P. capsici*. Chemicals are effective in controlling this disease but more natural methods would be preferred. One alternative is to use natural plant extracts. Rosemary and lavender leaf extracts were found to be effective in reducing germination of *P. capsici*, *P. megakarya*, and *P. palmivora* zoospores when supplemented to agar plates at different dilutions. The extracts displayed the biggest impact on *P. megakarya* zoospores where it completely inhibited germination at a 25% dilution of the prepared extract. When applied to cacao leaf disks, rosemary extract reduced necrosis caused by *P. megakarya* zoospores. In a bioassay, pears first treated with lavender extract showed no symptoms of *P. megakarya* infection compared with the non-treated controls. Based upon HPLC analyses, the active compound in these extracts was determined to be caffeic acid, rosmarinic acid or some simple derivative thereof. When added to agar plates, synthetic caffeic acid and rosmarinic acid completely inhibited germination of *P. capsici*, *P. megakarya*, and *P. palmivora* zoospores at concentrations of 3 and 6 g l^{-1}, respectively. In addition, sage and rice bran extracts, which both contain caffeic acid, were also effective in reducing zoospore germination. In contrast, inhibition of *Botrytis cinerea* or *Trichoderma asperellum* conidia germination did not occur, displaying some level of specificity. These extracts could provide an economically safe method for reducing damage caused by black pod disease on cacao until resistant varieties are developed and released.

Introduction

Black pod disease caused by *Phytophthora* species is one of the most economically important diseases of cacao (*Theobroma cacao*). The three most important species of *Phytophthora* worldwide causing black pod disease are *P. palmivora*, *P. megakarya*, and *P. capsici*. In 1979, *P. megakarya* was first identified in Nigeria (Brasier and Griffin, 1979) and has now become the most predominant pathogenic species in central and west Africa. Crop losses caused by *P. palmivora* prior to the appearance of *P. megakarya* in Ghana were estimated at 4.9–13.5% (Dakwa, 1984). Losses now attributed to the more aggressive *P. megakarya* are at 60–100% (Dakwa, 1987; Opoku et al., 2000). In 2001, reduced production due to black pod disease was estimated at 450,000 tons or $423 million (Bowers et al., 2001).

Chemical control of black pod disease relies primarily upon copper and metalaxyl-based fungicides. Copper-based fungicides are reported to

be very effective for controlling black pod disease (Duguma et al., 2001). However, these chemicals are expensive for the small farmer and therefore not always applied the six or seven times annually, as recommended (Hislop and Park, 1960; Henderson et al., 1994; Opoku et al., 1997). In addition, there are environmental concerns regarding the use of chemicals and fears of exacerbating the development of resistance.

Methods other than synthetic chemical use are being investigated as alternative solutions. Developing genetic resistance against this disease is considered the most cost effective and reliable method for control (Iwaro et al., 1997, 1998). However, this method is usually slow in developing varieties for farmer use. Until resistant varieties can be developed and distributed to the farmer, the use of biological control agents and natural products are a more practical alternative for an integrated pest management strategy. This paper describes the impact of some natural plant extracts containing caffeic acid and rosmarinic acid on *P. megakarya* and other *Phytophthora* spp. in laboratory tests. It also discusses different strategies on how this knowledge can be used to reduce black pod disease.

Materials and methods

Fungal isolates

Isolates of *Phytophthora megakarya* (M309, NS269, NGR16, and NGR20), *P. palmivora* (P881, Tri1, and NS607), and *P. capsici* (MHU 76.2) were donated by Michel Ducamp, CIRAD, Montferrier sur Lez, France. These isolates were all originally isolated from diseased cacao. The isolates were maintained on clarified V8 agar (Mitchell and Kannwischer-Mitchell, 1992).

Zoospores were prepared by growing the isolates in clarified V8 broth as described by Mitchell and Kannwischer-Mitchell (1992). After 4 days of growth, the V8 broth was removed and the mycelium was rinsed three times with sterile distilled water (SDW). SDW was added to cover the mycelium and the plates were placed under a continuous light at room temperature. After 2 days, sporangia had formed and the plates were placed at 4 °C for 30 min. The plates were removed and kept at room temperature until zoospore release was observed. The zoospore suspension was carefully poured into a beaker so as not to induce encystment. Zoospore concentration was counted by adding 0.1 ml of zoospore suspension to 0.9 ml of water in a 1.5 ml Eppendorf tube. The tube was vortexed to induce encystment and counted on a hemacytometer. The zoospore suspensions were applied immediately.

Cultures of *Botrytis cinerea* and *Trichoderma asperellum* were provided by Dr. Philippe Nicot, Institut National de la Recherche Agronomique, Avignon, France and Mr. Pierre Tondje, Institut des Recherches Agricoles pour le Développement, Yaounde, Cameroon, respectively. The cultures were maintained on half-strength potato dextrose agar (1/2PDA). *Botrytis cinerea* and *T. asperellum* spores were produced by growing the culture on 1/2PDA under continuous light at 25 °C. The conidia were removed by adding SDW to the growing culture after 10 days, gently swirling the liquid to release the conidia, and pouring the suspension through three layers of cheesecloth. The conidial concentration was counted on a hemacytometer.

Plant extract preparation

Mature leaves of rosemary (*Rosmarinus officinalis*), lavender (*Lavandula angustifolia*), a lavender hybrid (*L. angustifolia* × *L. spicata*), and sage (*Salvia officinalis*) were collected from naturally growing plants near Montpellier, France. The plant leaves listed above and rice bran, purchased from a local grocery store, were weighed (20 g) and placed into 500 ml Erlenmeyer flasks containing 200 ml of distilled water. The flasks were closed with a cotton ball, covered with aluminum foil, and autoclaved for 45 min at 121 °C at 1 atm. The liquid was filtered through three layers of sterile cheesecloth into a sterile beaker and the volume was reduced by boiling in a laminar-flow hood to an approximate volume of 20 ml. The extracts were centrifuged to remove any solids and the supernatants were poured into sterile plastic tubes and stored at 4 °C until needed.

HPLC analysis

Separation and quantification of compounds in rosemary, lavender, and sage extracts were performed using high-performance liquid chromatography, a System Gold HPLC (Beckman

Coulter, Inc., Fullerton, CA) equipped with a System Gold 168 UV detector (Beckman Coulter, Inc.) at 280 and 320 nm. Separation was performed with a Uptisphere HDO C-18, 5 μm, 4.6 mm $\times$ 25 cm column (Interchim, Montlucon, France). The data were integrated and analyzed using a 32 Karat automated software system (Beckman Coulter, Inc.). The mobile phase utilized a gradient composed of a 0.01 M sodium citrate buffer (A) pH 5.4 adjusted with 50% acetic acid and methanol (B). The following gradient was used: 0 min, 2% B; 12 min, 4% B; 20 min, 13% B; 22 min, 13% B; 26 min, 2% B. Run time was 30 min. The solvent flow rate was 1.0 ml min^{-1}, and separation was performed at room temperature.

The plant extracts prepared as described above were filtered through a 0.2 μm filter prior to analysis. Standards of caffeic acid and rosmarinic acid were prepared by dissolving the pure compounds purchased from Sigma Chemical Co. (Saint Quentin Fallavier, France) in water to concentrations of 100 mg l^{-1}. Thirty microliters of the standards and extracts were injected into the HPLC.

Solid agar bioassay

For each bioassay test, V8 agar was prepared by combining 250 ml of clarified V8 juice with 750 ml deionized water and 15 g of agar, except where noted differently. The V8 agar was supplemented with the compound or extract to be tested at various concentrations mentioned below for each test and adjusted to pH 6.7 with 0.1 N KOH, if necessary. After autoclaving for 30 min, the liquid agar was poured into plastic Petri plates (90 mm diam) and allowed to solidify. Ten thousand zoospores of various *Phytophthora* spp., prepared as described above, were pipetted onto the agar surface into 0.2 ml of SDW pipetted onto agar surface and gently swirled to evenly distribute them throughout the plate. The plates were incubated at 25 °C in the dark.

To determine the concentration of caffeic acid and rosmarinic acid necessary to inhibit *Phytophthora* spp. zoospore germination, the V8 agar preparation was supplemented with either caffeic acid at concentrations of 0, 0.1, 0.5, 1, 2, 3, 4, or 5 g l^{-1} or rosmarinic acid at concentrations of 0, 1.5, 3, or 6 g l^{-1} prior to autoclaving. *Phytophthora megakarya* isolate NGR16 and *P. palmivora* isolate NS607 were used in this bioassay. To elucidate some level of specificity of the plant extracts towards *Phytophthora* spp., a similar test was set up using 10,000 conidia of *B. cinerea* or *T. asperellum* instead of zoospores. There were three plates per concentration of caffeic acid and rosmarinic acid for each isolate and fungus tested. The plates were visually observed for any growth of mycelia resulting from zoospore or conidial germination after 48 h. The experiment was repeated once.

To quantify the effect of caffeic acid on zoospore germination, the V8 agar preparation was supplemented with 0, 0.2, 0.5, 1, 2, and 3 g l^{-1} caffeic acid. *Phytophthora megakarya* isolates M309, NS269, and NGR16 and *P. palmivora* isolate Tri1 were used in this bioassay. There were three plates per concentration of caffeic acid for each isolate tested. After 18 h incubation, the percentage of zoospores germinated was determined by observing three random fields of view on one plate through a compound microscope (10$\times$ objective lens) and counting a total of 100 zoospores per field of view. The three fields of view were averaged together for one replication. A zoospore was considered germinated if the germ tube was longer than the zoospore cyst. The test was repeated once.

To determine the minimum dilution of rosemary extract that was still effective to inhibit zoospore germination, rosemary extract was prepared as described above and added to the V8 agar preparation for final dilutions of 0, 1, 5, 10, and 25% of the concentrated extract. Zoospores of *P. megakarya* isolates NGR16, NGR20, and M309 and *P. palmivora* isolate Tri1 were used in this bioassay. After 18 h incubation, the percentage of zoospores germinated was determined as described above. The experiment was repeated once.

The procedure for comparing the activity of the different plant extracts was conducted as described above. Rosemary, lavender, lavender hybrid, sage, and rice bran extracts were also prepared as described above. Liquid V8 agar was modified as described above by adding 17 ml of clarified V8 juice to 33 ml of water containing 1.335 g of agar in a 100 ml Erlenmeyer flask and cooling to 50 °C in a water bath until needed. To a sterile, 24-well microplate, 0.75 ml of the liquid agar was pipetted into individual wells containing either 0.25 ml of sterile water (control), the individual plant extracts (final concentration of 25%), 12 g l^{-1} stock

solution of caffeic acid (final concentration of 3 g l^{-1}), or 0.15 ml of sterile water and 0.10 ml of the individual plant extract (final concentration of 10%). The wells were swirled to mix well and allowed to solidify.

Zoospores of *P. megakarya* (isolates M309, NS269, and NGR20), *P. palmivora* (isolate P881), and *P. capsici* (isolate MHU 76.2) were prepared and pipetted directly onto the amended or non-amended V8 agar surface in the microwells. There were four wells per treatment per isolate. After 18 h incubation, the percentage of zoospores germinated was determined as described above. The experiment was repeated twice.

For all tests, the percentage of zoospore germination for each repetition was arcsine square root transformed to stabilize variance and then subjected to general linear model (GLM) procedure for analysis of variance (SAS Institute, Cary, NC, USA). Appropriate transformed means were separated using a Fisher's protected LSD at $P = 0.05$. Non-transformed means are presented for clarity based on transformed data.

Activity of plant extract dilution series

Dilutions of each extract were prepared so as to have equal concentrations of caffeic acid. The amount of caffeic acid in each plant extract was determined by first preparing a standard solution of 100 mg l^{-1} of caffeic acid in distilled water. Extracts of rosemary, lavender, and sage were prepared as described above. Each solution was subjected to an HPLC analysis as described above. The area of the relative peak at 26 min (corresponding to the peak for caffeic acid) was noted for each solution.

Liquid V8 agar was modified as described above by adding 25 ml clarified V8 juice to 25 ml water containing 1.5 g agar in a 100 ml Erlenmeyer flask and cooling to 50 °C in a water bath until needed. To a sterile, 24-well microplate, 0.5 ml of liquid agar was pipetted into individual wells containing either 0.5 ml sterile water (control), 0.5 ml caffeic acid solution (200 mg l^{-1}), 0.25 ml water plus 0.25 ml caffeic acid solution (200 mg l^{-1}), 0.375 ml water plus 0.125 ml caffeic acid solution (200 mg l^{-1}), 0.5 ml sage extract, 0.25 ml water plus 0.25 ml sage extract, 0.4 ml water plus 0.1 ml sage extract, 0.007 ml water plus 0.493 ml lavender extract, 0.254 ml water plus 0.246 ml lavender extract, 0.402 ml water plus 0.098 ml lavender extract, 0.27 ml water plus 0.23 ml rosemary extract, 0.385 ml water plus 0.115 ml rosemary extract, or 0.454 ml water plus 0.046 ml rosemary extract. The three different volumes of each solution (caffeic acid, sage extract, lavender extract, and rosemary extract) added to each well correlates to a caffeic acid-equivalent concentration of 100, 50, and 10 mg l^{-1}, respectively. The wells were swirled to mix well and allowed to solidify.

Zoospores of *P. megakarya* isolates M309 and NS269 were prepared and pipetted directly onto the amended or non-amended V8 agar surface in the microwells. There were four wells per treatment per isolate. After 24 h incubation at 25 °C, the percentage of zoospores germinated was determined as described above. The experiment was repeated once.

Percentage zoospore germination for each repetition was arcsine square root transformed to stabilize variance and then subjected to GLM procedure for analysis of variance. The caffeic acid-equivalent concentration in each of the plant extracts and the relationship to zoospore germination for each *P. megakarya* isolate was analyzed by regression analysis.

Effect of cinnamic acid derivatives on zoospore germination

The cinnamic acid derivatives, ferulic acid, cinnamic acid, *p*-coumaric acid, and 4-hydroxybenzoic acid, were purchased from Sigma Chemical Co. (Saint Quentin Fallavier, France). V8 agar was prepared as described above and supplemented with the cinnamic acid derivatives for a final concentration of 1 or 3 g l^{-1}. Zoospores of *P. megakarya* isolates NGR16, NGR20, and M309 and *P. palmivora* isolate Tri1 were used in this bioassay. After 18 h incubation, the percentage of zoospores germinated was determined as described above. The experiment was repeated once.

Percentage zoospore germination for each repetition was arcsine square root transformed to stabilize variance and then subjected to GLM procedure for analysis of variance. Appropriate transformed means were separated using a Fisher's protected LSD at $P = 0.05$. Non-transformed means are presented for clarity based on transformed data.

Leaf disk bioassay

Young cacao (*Theobroma cacao*) leaves were collected just above the point of lignification on the cacao stem from a *Phytophthora*-susceptible cacao variety grown in a greenhouse in Montpellier, France. The leaves were surface-sterilized by dipping in 70% ethanol for 1 min and rinsing two times in SDW. Squares (approximately 1 cm^2) were cut from the leaves and placed on a moist paper towel in a plastic box. Non-diluted rosemary extract prepared as described above was pipetted onto the leaf surface (20 μl per leaf disk) either 1 day or immediately before zoospore application. A caffeic acid suspension (3 g l^{-1} water), rosmarinic acid suspension (6 g l^{-1} water), and a combination of caffeic acid and rosmarinic acid suspension (1.5 and 3 g l^{-1}, respectively) were also applied to leaf disks in the same manner. Controls consisted of leaf disks treated only with distilled water and inoculated with zoospores and leaf disks treated with distilled water alone, the plant extracts, and caffeic acid and rosmarinic acid suspensions without zoospore inoculation. There were 10 leaf disks per treatment.

Phytophthora megakarya (isolate NS269 and NGR16) zoospores were prepared as described above. Zoospores were pipetted onto the leaf disk surface (10,000 zoospores per leaf disk) and the plastic box was covered and placed in a 25 °C incubator. After 8 days, three different people who were not familiar with the treatments estimated the necrosis of each leaf disk as a percentage of the total disk area. These percentages were averaged together for each leaf disk representing one value for analysis. The experiment was repeated once.

The % necrosis for each repetition was arcsine square root transformed to stabilize variance and then subjected to general linear model (GLM) procedure for analysis of variance (SAS Institute, Cary, NC, USA). Appropriate transformed means were separated using a Fisher's protected LSD at $P = 0.05$. Non-transformed means are presented for clarity based on transformed data.

Detached fruit bioassay

The lavender extract prepared as described above was diluted by measuring 5 ml in a graduate cylinder, adding 50 μl of the adjuvent Silken (Agriliance LLC, St. Paul, MN, USA) or Sticman (Agridyne, Agen France), and bringing the volume up to 20 ml with distilled water. The final dilution of the extract was 25% with an adjuvent concentration of 0.25%. Pears (*Pyrus communis* variety William) were purchased at a local supermarket, carefully washed in soapy water, and rinsed in tap water. After the pears had air-dried, three pears were placed in a plastic tub and sprayed equally over the whole pear with half of the 15 ml of water plus 0.25% Silken, water plus 0.25% Sticman, the lavender extract dilution as described above with either 0.25% Silken or 0.25% Sticman adjuvent, the rosemary extract dilution as described above with either 0.25% Silken or 0.25% Sticman adjuvent, or 3 g l^{-1} caffeic acid plus 0.25% Silken. These solutions were allowed to air-dry and then the remaining half of the treatment solutions was sprayed in the same manner. After the final application of the solutions air-dried, the pears were placed on a plastic grill in a plastic box that had a small layer of water in the bottom. The box was placed in a 25 °C incubator.

Zoospores of *P. megakarya* isolate NS269 were prepared and counted as described above. Five thousand zoospores were pipetted onto the surface of each of the pears. The plastic boxes were enclosed with a lid. The lids were removed after 16 h. The pears were observed daily for any symptoms. The experiment was repeated twice.

Results

HPLC analysis

The comparison between the controls, caffeic acid and rosmarinic acid, and the extracts are shown in Figure 1. All chromatograms show a major peak after 26 min.

Solid agar bioassay

Visual observations showed no mycelial growth of the *P. palmivora* or *P. megakarya* isolates tested on V8 plates containing 3 g l^{-1} or more of caffeic acid or 6 l^{-1} rosmarinic acid (data not shown). In the same test, mycelial growth was observed on the plates supplemented with the lower concentrations tested. Zoospore germination on caffeic acid-supplemented V8 agar at different concentrations is summarized in Figure 2. For all isolates tested,

there was significant reduction in zoospore germination with concentrations at 0.5 g l^{-1} and above ($P < 0.001$). *Botrytis cinerea* and *T. asperellum* conidia germinated at all concentrations tested (data not shown).

Complete inhibition of zoospore germination occurred on the rosemary extract-amended plates at a concentration of 25% of the prepared extract (data not shown). At a concentration of 10%, some zoospores germinated (< 2%) but it was noted later that the growth did not continue. No differences in zoospore germination from the non-amended control were noted for concentrations of 1 and 5%.

There was some difference in activity between the different plant extracts (Table 1). At a concentration of 25% of the original prepared extract, all plant extracts tested, except sage, completely inhibited zoospore germination of *P. megakarya* isolate M309. Against the same isolate, germination occurred in the 10% concentrations except with the rosemary extract, which completely inhibited germination. There was variability in the activity of the different extracts to inhibit germination against the other *P. megakarya* isolates. The lavender and sage extracts had very little impact on *P. capsici* zoospore germination.

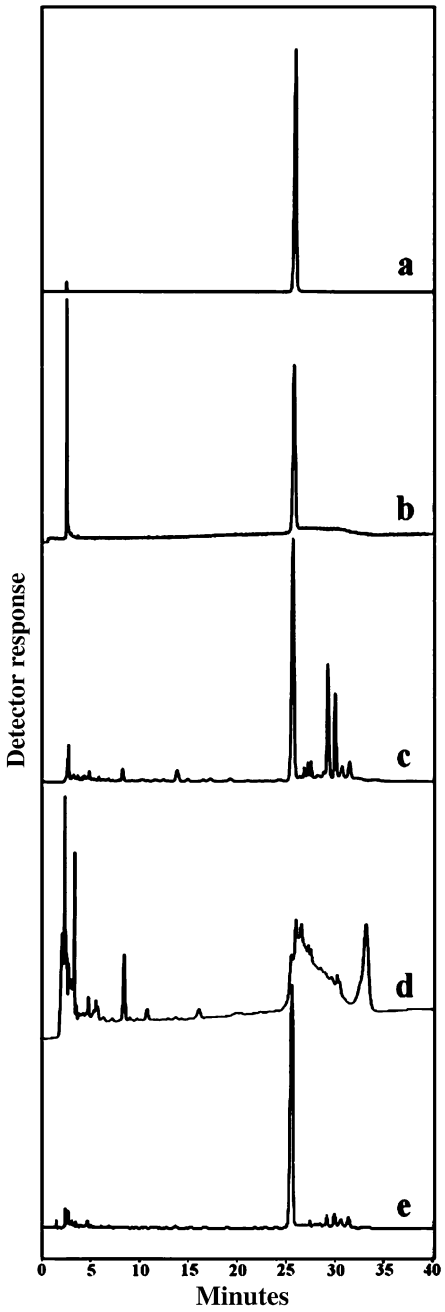

Figure 1. HPLC chromatograms of (a) caffeic acid standard (100 mg l^{-1}); (b) rosmarinic acid standard (100 mg l^{-1}); (c) prepared lavender (*Lavandula angustifolia*) extract; (d) prepared rosemary extract (*Rosmarinus officinalis*); and (e) prepared sage (*Salvia officinalis*) extract.

Activity of plant extract dilution series

The HPLC peak after 26 min of the sage and lavender extracts was equivalent in area to each

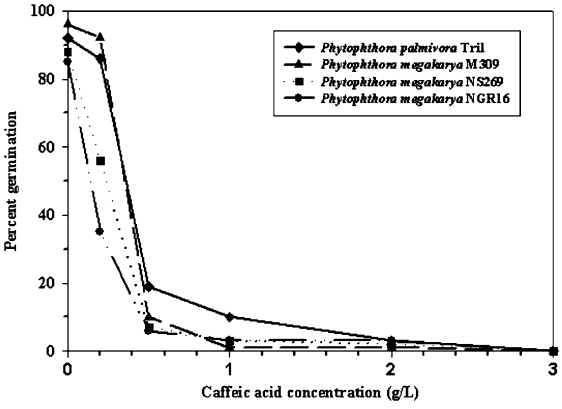

Figure 2. Percentage of germinated *Phytophthora megakarya* and *P. palmivora* zoospores on V8 agar amended with diffferent concentrations of caffeic acid after 18 h incubation. Equations of regression analysis are for *P. palmivora* Tri1: $y = 0.88–0.97x + 0.23x^2$ ($r^2 = 0.86$); *P. megakarya* M309: $y = 0.92–1.12x + 0.28x^2$ ($r^2 = 0.81$); *P. megakarya* NS269: $y = 0.72–0.89x + 0.22x^2$ ($r^2 = 0.81$); and *P. megakarya* NGR16: $y = 0.61–0.78x + 0.2x^2$ ($r^2 = 0.73$), where x is the concentration of caffeic acid and y is the percentage of zoospore germination.

Table 1. Percentage of germinated *Phytophthora* spp. zoospores on V8 agar amended with different concentrations of plant extracts after 18 h incubation

Treatment[2]	*Phytophthora* spp. isolate[1]				
	M309	NGR20	NS269	P881	MHU 76.2
Water – control	78 a[3]	89 a	88 a	92 a	93 a
Lavender hybrid – 10%	9 c	–	–	95 a	–
Lavender hybrid – 25%	0 d	0 b	–	< 1 c	32 c
Lavender – 10%	16 c	–	–	95 a	–
Lavender – 25%	0 d	< 1 b	5 bc	1 c	28 c
Rosemary – 10%	0 d	–	< 1 c	0 c	3 d
Rosemary – 25%	0 d	0 b	0 c	0 c	0 d
Sage – 10%	39 b	–	9 b	13 b	51 b
Sage – 25%	18 bc	–	< 1 c	3 c	31 c
Rice bran – 50%	0 d	–	0 c	–	–
Caffeic acid – 3 g l^{-1}	0 d	0 b	–	0 c	0 d

[1]*Phytophthora megakarya* isolates M309, NS269, and NGR20; *P. palmivora* isolate P881; *P. capsici* isolate MHU 76.2.
[2]Lavender hybrid – *L. angustifolia* × *L. spicata*; lavender – *Lavandula angustifolia*; rosemary – *Rosmarinus officinalis*; and sage – *Salvia officinalis*.
[3]Means within the same column followed by the same letter are not significantly different ($P \leq 0.05$) according to least significant differences.

other and a standard 100 mg l^{-1} solution of caffeic acid (data not shown). All subsequent dilutions of the extracts were based upon these peak values. The rosemary extract showed the highest peak at 26 min with an area 2.4 times higher than a standard solution of 100 mg l^{-1} caffeic acid.

All plant extracts inhibited zoospore germination of *P. megakarya* isolates NS269 and M309 effectively up to a caffeic acid-equivalent concentration of 25 mg l^{-1} (Table 2). A caffeic acid-equivalent concentration of 10 mg l^{-1} in the rosemary and sage extract was still effective in inhibiting zoospore germination of isolate NS269, but not against isolate M309. Increased dilutions of caffeic acid and rosmarinic acid were not effective in inhibiting zoospore germination although a dilution effect was noted. Regression analysis showed a significance in concentration for all of the plant extracts tested ($P < 0.01$). There was no difference in the regression analysis between the two isolates within each extract ($P = 0.741$) so the data were combined to produce equations that fit the curves for caffeic acid: $y = 0.88 - 4.9x$ ($r^2 = 0.88$); for rosemary extract: $y = 0.78 - 0.06x + 8.36x^2$ ($r^2 = 0.86$); for lavender extract: $y = 0.82 - 0.06x + 7.9x^2$ ($r^2 = 0.94$); and for sage extract: $y = 0.79 - 0.06x + 8.23x^2$ ($r^2 = 0.87$), where x is

Table 2. Percentage of zoospore germination of *Phytophthora megakarya* isolates after 18 h exposure on V8 agar amended with dilutions of extracts equivalent to various concentrations of caffeic acid

Caffeic acid concentration[2]	NS269[1]					M309				
	CA[3]	RA	R	S	L	CA	RA	R	S	L
100	32	79	–	–	–	47	66	–	–	–
50	56	–	0	0	0	73	–	0	0	0
25	71	–	0	0	0	84	–	2	2	0
10	–	–	2	1	14	–	–	17	24	32
0	85	85	85	85	85	91	91	91	91	91

[1]*Phytophthora megakarya* isolate tested.
[2]Caffeic acid-equivalent concentration (in mg l^{-1}), as determined by HPLC, supplemented in V8 agar.
[3]Caffeic acid (CA), rosmarinic acid (RA), rosemary (*Rosmarinus officinalis*) extract (R), sage (*Salvia officinalis*) extract (S), and lavender (*Lavandula angustifolia*) extract (L).

the caffeic acid-equivalent concentration (mg) and y is the % zoospore germination. There was no significant difference between the different plant extracts ($P = 0.489$).

Effect of cinnamic acid derivatives on zoospore germination

Results of zoospore inhibition by all cinnamic acid derivatives tested were similar, regardless of the *P. megakarya* isolate. All of the cinnamic acid derivatives at either 1 or 3 g l^{-1}, except 1 g l^{-1} caffeic acid, completely inhibited zoospore germination (Table 3).

Leaf disk bioassay

The average percentage of necrosis is summarized in Table 4. The control leaf disks without any zoospores did not show any necrosis. The majority of the damage on the extract-treated leaf disks occurred along the mid-vein. Rosmarinic acid alone did not give very good control. There did not appear to be an advantage to applying

Table 3. Percentage of germinated *Phytophthora megakarya* zoospores on V8 agar amended with different concentrations of cinnamic acid derivatives after 18 h incubation

Treatment[1]	*P. megakarya* isolate	
	M309	NS269
Control	84 a[2]	81 a
Caffeic acid (3 g l^{-1})	0 b	0 b
Caffeic acid (1 g l^{-1})	1 b	3 b
Ferulic acid (3 g l^{-1})	0 b	0 b
Ferulic acid (1 g l^{-1})	0 b	0 b
Cinnamic acid (3 g l^{-1})	0 b	0 b
Cinnamic acid (1 g l^{-1})	0 b	0 b
p-Coumaric acid (3 g l^{-1})	0 b	0 b
p-Coumaric acid (1 g l^{-1})	0 b	0 b
4-Hydroxybenzoic acid (3 g l^{-1})	0 b	0 b
4-Hydroxybenzoic acid (1 g l^{-1})	0 b	0 b

[1]V8 agar amended with various cinnamic acid derivatives.
[2]Means within the same column followed by the same letter are not significantly different ($P \leq 0.05$) according to least significant differences.

Table 4. Average percentage of necrosis of treated or non-treated *Theobroma cacao* leaf disks (10 leaf disks/treatment) inoculated with *Phytophthora megakarya* zoospores

Treatment[1]	Time applied[2]	*P. megakarya* isolate	
		NGR16	NS269
Control	Day 0	62 a[3]	67 a
Rosemary extract	Day 0	4 c	17 b
Rosemary extract	Day −1	11 bc	12 b
CA (3 g l^{-1})	Day 0	7 c	5 b
CA (3 g l^{-1})	Day −1	5 c	5 b
RA (6 g l^{-1})	Day 0	50 a	11 b
RA (6 g l^{-1})	Day −1	27 b	13 b
CA (1.5 g l^{-1}) + RA (3 g l^{-1})	Day 0	18 bc	3 b
CA (1.5 g l^{-1}) + RA (3 g l^{-1})	Day −1	6 c	3 b

[1]Control – water alone; Rosemary extract – undiluted preparation; CA – caffeic acid; RA – rosmarinic acid.
[2]Time difference between application of *T. cacao* leaf disks with treatment and application of zoospores.
[3]Means within the same column followed by the same letter are not significantly different ($P \leq 0.05$) according to least significant differences.

the treatment 1 day before zoospore application. Only when the rosmarinic acid suspension alone was applied 1 day before inoculation with the NGR16 isolate was there a difference in necrosis. No difference was observed with the NS269 isolate.

Detached fruit bioassay

After 4 days, necrosis appeared on the inoculated pears treated with water plus adjuvant only, regardless of the adjuvant used (Figure 3). No necrotic symptoms were observed on the pears that were first treated with the lavender extract and inoculated with zoospores. No necrotic symptoms were observed on any of the non-inoculated controls (data not shown). Similar results were observed in all experiments.

Discussion

Cinnamic acid derivatives including caffeic acid and rosmarinic acid are secondary plant metabolites. These compounds have been studied intensively for their antimicrobial and antioxidant activities (Ravn et al., 1989; Shetty, 1997; Nascimento et al., 2000; Debersac et al., 2001; Bais et al., 2002). Agar plate assays and bioassays on leaf disks and pears demonstrated that 3 g l^{-1} of purified caffeic acid or 6 g l^{-1} of purified rosmarinic acid was sufficient to inhibit zoospore germination. Other cinnamic acid derivatives had an activity at 1 g l^{-1}. Although these concentrations are relatively high, activity against zoospore germination was consistent and was not the main focus of this study. The emphasis of this study was the activity of a natural compound extracted from a plant source. All plants from the same genera used in this study are reported to contain caffeic acid and rosmarinic acid in their tissue (Kovatcheva et al., 1996; Nascimento et al., 2000; Ibanez et al., 2003). To validate that caffeic acid or rosmarinic acid is the active compound in the extracts, the extracts were diluted to equivalent concentrations of caffeic acid and tested for their activity to inhibit zoospore germination. Zoospore inhibition from the plant extracts should have been equal to those observed with known concentrations of caffeic acid and decreased as the dilutions increased. Although inhibition did decrease with dilution for each of the extracts, the natural plant extracts demonstrated a greater inhibition than the synthetic caffeic acid at equivalent concentrations. This does not eliminate the likelihood that the active compound is caffeic acid. The most likely explanation for this difference in the inhibitory concentration between the synthetic caffeic acid and that found in the plant extracts is a slight difference in chemical structure in the naturally extracted compound. Secondary plant products are known to produce different isomers or have slight modifications such as glycosylations, methylations, hydroxylations, or intercalations with metal ions that may lead to differences in function or activity (Len et al., 1996; Kurihara et al., 1997; Vinson et al., 1998; Alam, 2004; Asahina et al., 2005). These slight differences may not be distinguished easily by HPLC analysis alone. More in-depth analyses such as infrared spectrophotometry or mass spectrometry would have to be conducted to confirm that any differences between the natural extracted compound and the synthetic compound occur. Of course, there is also the possibility that

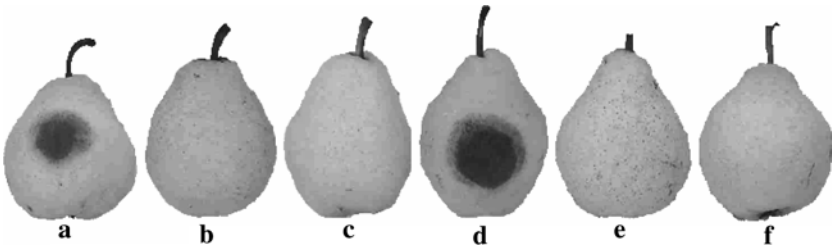

Figure 3. Detached fruit bioassay 4 days after inoculation with 5000 zoospores of *Phytophthora megakarya* isolate NS269 using pears (*Pyrus communis* variety William) treated with (a) water plus 0.25% Silken adjuvent; (b) 3 g l^{-1} caffeic acid plus 0.25% Silken adjuvent; (c) 25% lavender extract plus 0.25% Silken adjuvent; (d) water plus 0.25% Sticman adjuvent; (e) 3 g l^{-1} caffeic acid plus 0.25% Sticman adjuvent; or (f) 25% lavender extract plus 0.25% Sticman adjuvent.

another compound unrelated to caffeic acid that displays a peak at the same time under the same HPLC conditions is responsible for inhibiting zoospore germination. However, since all plant extracts and cinnamic acid controls tested showed the same peak, we find this to be very unlikely.

The results from this study demonstrated that all plant extracts displayed inhibition towards the various *Phytophthora* spp. pathogenic to cacao to some degree. Under the conditions prepared, an extract concentration of 25% was effective in inhibiting zoospore germination although 10% also reduced germination. The pH of the solutions and amended agar used in the solid agar bioassay was checked but was not different from the non-amended controls (data not shown).

In this study, the rosemary extract appears to be the most effective in inhibiting zoospore germination. However, this is probably an effect of concentration of the responsible compound within the extract. The HPLC chromatogram of the plant extracts show a higher peak at 26 min, corresponding to the peak of caffeic acid, for the rosemary extract than the other plant extracts. When equivalent dilutions were made to have the same level of caffeic acid within each plant extract, the zoospore inhibition was similar for each extract (Table 2).

The impact of this research could be significant in terms of two future research areas in an integrated control system of *Phytophthora* diseases of cacao. First, the concentrations of caffeic acid and rosmarinic acid within cacao tissue or pods could be used as an indicator for breeding for resistance. Although to the authors' knowledge no studies have been conducted to analyze cacao leaves or pods for caffeic acid, it is found in the beans (Sanbongi et al., 1998). A study conducted to determine if there is a correlation between cinnamic acid derivative concentration *in planta* and black pod resistance could be interesting.

The second area of research would be to investigate the possibility for transgenics. If a correlation between caffeic acid and black pod resistance was determined then the cacao plants could be genetically modified to have cinnamic acid derivatives *in planta* at high enough concentrations to inhibit *Phytophthora* spp. zoospore germination. Another approach would be to insert the gene(s) responsible for caffeic acid production into a microorganism so that it will exogenously produce the chemical and the organism could be used as a biological control agent. The first step in this approach would be to understand the pathway for caffeic acid production. Nair et al. (2002) have already done part of this work where they have identified CYP98A3 to be responsible for production of caffeic acid in recombinant yeast cells. This strategy would have several advantages over applying a plant extract if it became ethically acceptable. The costs of application would be reduced significantly since, theoretically, the organisms would only need to be applied one time per year if established on the cacao plant. Once established, wash-off due to rain would be less likely. The plant extract would most likely need to be applied as often as chemical fungicides.

The immediate impact of this research, however, is also promising in that it provides a safer alternative than synthetic fungicides. Rosemary and lavender extracts are often used in food preparations for human consumption; thus, there should not be a major concern over toxicity. Leaf disk and detached fruit bioassays demonstrated the effectiveness of the extracts on plant tissue when inoculated with *P. megakarya* zoospores. Although application on live trees and cacao pods would have been preferable, access to this material for this type of study was not possible. Previous studies have shown that leaf disk bioassays are a reliable indicator of what would occur on a live plant (Tahi et al., 2000; Nyassé et al., 2002). Future studies will need to be conducted on live trees under natural environmental conditions to demonstrate efficacy under these conditions. Of course, caffeic acid and rosmarinic acid are chemicals and over time, some degree of resistance could be expected as is observed with synthetic fungicides. However, these natural plant extracts could give the local farmer a safe alternative for some immediate control until resistant varieties are developed and become commercially available.

Inhibition of *T. asperellum* conidia was examined specifically because *Trichoderma* spp. are being examined as a biocontrol agents against cacao diseases (Holmes et al., 2004; Bateman et al., 2005). This potential biocontrol agent mycoparasitizes hyphae of *Phytophthora* spp. Field trials involving the application of *T. asperellum* conidial are being conducted at this time. Since the plant extracts were not inhibitory towards

T. asperellum conidial germination, the plant extracts could be used in a joint formulation with the biocontrol fungus. This could provide a synergistic control potential by limiting the spread of the disease by inhibiting the primary inoculum source with the plant extract and reducing the established disease agent with the biocontrol fungus.

More work still needs to be investigated in different areas to make this a practical option for farmers. Probably the most important factor in determining whether the farmer will accept any new method is cost. A study will need to be conducted to determine the cost effectiveness of producing and applying these plant extracts. Extracts of other plants that may be more readily available to the local farmer and contain caffeic acid and rosmarinic acid, such as lemon-balm (*Melissa officinalis*) and basil (*Ocimum basilicum*), should be tested for their activity against *Phytophthora* spp. Demonstration in this study of the effectiveness of a rice bran extract could lead to a very cost-effective solution since this substrate is often discarded after rice processing in the same regions where cacao is grown. In order to reduce costs, efficient extraction methods will need to be developed to optimize yields of the essential compound. This study used a very simple hot water extraction method, but techniques that are more efficient in extracting the efficient compounds have been specifically designed for rosemary (Basile et al., 1998; Ibanez et al., 2003). Another important area that will need to be addressed is the formulation of a rain-fast product. Caffeic acid and rosmarinic acid are both water soluble and so a formulation will need to be developed so that the extracts do not immediately wash off the plant after the first rain. This is a problem shared with synthetic fungicides. Different adjuvants have been tested and proven to increase rain fastness (Roggenbuck et al., 1990; Reddy and Singh, 1992; Sun et al., 1996). Despite all of the work that still needs to be done, this study provides the basis for an alternative method to synthetic chemical application that can be developed quickly for farmer use.

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