

Identification of Ceramide-Phosphorylethanolamine in Oomycete Plant Pathogens: *Pythium ultimum*, *Phytophthora infestans*, and *Phytophthora capsici*

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ABSTRACT: Cellular lipids were extracted from three species of Oomycete plant pathogens (*Pythium ultimum*, *Phytophthora infestans*, and *Ph. capsici*) and analyzed via normal-phase high-performance liquid chromatography with flame-ionization detection. The most abundant polar lipids in each of the three species were the polar membrane lipids, phosphatidylethanolamine (PE), phosphatidylcholine, and a phosphosphingolipid that eluted soon after PE. Structural analysis via mass spectrometry and nuclear magnetic resonance spectrometry revealed that the phosphosphingolipid was ceramide phosphorylethanolamine (Cer-PE). The most abundant molecular species of Cer-PE in *P. ultimum* had a molecular weight of 670.5, contained an unusual 19-carbon branched triunsaturated sphingoid (C19- Δ 4, 8, 10, 9-methyl long-chain base) and palmitic acid as the amide-linked fatty acid. The most abundant molecular species of Cer-PE in *Ph. infestans* had a molecular weight of 714.5, contained a common 16-carbon 1,3 di-OH sphingoid, and erucic (*cis* 13-docosenoic, C22- Δ 13) acid as the amide-linked fatty acid. The Cer-PE in *Ph. capsici* comprised a mixture of each of the two molecular species found in *P. ultimum* and *Ph. infestans*. *Lipids* 33, 307–317 (1998).

Formerly classified as fungi, the Oomycetes have so many physiological and biochemical peculiarities that they have recently been placed in a separate kingdom, the Stramenopila (1). Unlike most fungi, the cell walls of Oomycetes contain no chitin, and instead contain structural carbohydrate polymers composed of glucans (2). Pythiaceus members of the Oomycetes (including the *Phytophthora* and *Pythium* species of this study) are unable to synthesize sterols and do not require them for growth, but must obtain sterols in their host or environment because sterols are necessary for their sexual re-

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Abbreviations: CAD, collisionally activated dissociation; CAEP, ceramide aminoethylphosphonate; Cer-PE, ceramide phosphorylethanolamine; Cer-PI, ceramide phosphorylinositol; 2D, two-dimensional; FAB, fast atom bombardment; FID, flame-ionization detector; GC/MS, gas chromatography/mass spectrometry; HPLC, high-performance liquid chromatography; LC/MS, liquid chromatography/MS; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

production (3,4). For this reason, fungicides that inhibit ergosterol biosynthesis are not effective against Oomycetes. There are several reports that certain Oomycetes contain high levels of an unusual sphingophospholipid, ceramide aminoethylphosphonate. This unusual sphingophospholipid was reported to occur in *P. prolatum* (5), *Ph. parasitica* (5), and *Ph. infestans* (6,7).

Our laboratory has recently developed sensitive high-performance liquid chromatography (HPLC) methodologies to quantitatively analyze phospholipids and other lipids in microorganisms (8,9). This study was undertaken to employ the tools of modern HPLC methodology to rigorously reinvestigate the occurrence and structure of phosphosphingolipids in three plant pathogenic species of Oomycetes, *P. ultimum*, *Ph. infestans*, and *Ph. capsici*. These species infect many agricultural commodities and cause considerable economic losses.

EXPERIMENTAL PROCEDURES

Growth of Oomycetes. *Pythium ultimum* (ATCC 26083) from the American Type Culture Collection (Rockville, MD) was maintained on potato dextrose agar at 25°C. Mycelium for lipid isolation was prepared by inoculating 20-mL aliquots of a liquid asparagine-sucrose medium (10) in petri dishes (9 cm in diameter, 2 cm in depth) with a mycelial plug, 7 mm in diameter, taken from the growing edge of a culture. The dishes were incubated for 48 h at 25°C with shaking on a gyratory shaker at 60 rpm. Mycelium was harvested by filtration on glass fiber filters, washed with water, and freeze-dried.

Phytophthora infestans (A1 strain) was obtained from Dr. W. Fry (Cornell University, Ithaca, NY) and was maintained on pea juice agar, prepared by autoclaving 283 g of frozen peas in 1 L of distilled water and filtering through cheesecloth. Agar (20 g) was added to the filtrate, which was autoclaved again before pouring culture plates. Mycelium for lipid isolation was prepared as described above for *P. ultimum* except that cultures were grown for 20 d before harvesting the mycelium.

Phytophthora capsici (ATCC 15399) from the American Type Culture Collection was maintained on V-8 juice agar,

pH 7.0, containing 200 mL V-8 juice, 4 g CaCO₃, and 20 g agar per liter. Mycelium for lipid isolation was prepared as described above for *P. ultimum* except that cultures were grown for 96 h before harvesting the mycelium.

Lipid extraction and mild alkaline hydrolysis. Lyophilized hyphae (200 mg) were homogenized in chloroform/methanol/water (8 mL/16 mL/4.8 mL) with a Polytron Homogenizer (Brinkmann, Westbury, NY), and lipids were extracted with chloroform/methanol (11). Some lipid samples were subjected to mild alkaline hydrolysis by evaporating the solvent under N₂, adding about 5 mL of 1.5 M methanolic KOH/10 mg lipid, heating the mixture to 70°C for 30 min, acidification to pH 2, and reextraction of the hydrolysate with chloroform/methanol.

Analytical normal-phase HPLC. The lipids were separated and quantified using a method similar to one we have used for hopanoid analyses in other species (9). The column was a LiChrosorb 5 Si 60 (3 × 100 mm) from Chrompack, Inc. (Raritan, NJ), with a flow rate of 0.5 mL/min. The solvents were: A, hexane; B, isopropanol; and C, 0.04% triethylamine in water (C was prepared fresh daily). The linear gradient timetable was: at 0 min, 100/0/0; at 5 min, 95/5/0; at 10 min, 85/15/0; at 15 min, 40/60/0; at 53 min, 40/51/9; at 68 min, 40/51/9; at 73 min, 40/60/0; at 78 min, 100/0/0; at 100 min, 100/0/0; (%A/%B/%C, respectively). The HPLC system consisted of an Isco (Lincoln, NE) Model 2350 pump, an Isco Model 2360 gradient programmer, and a Tremeetrics (Austin, TX) Model 945 flame-ionization detector (FID).

Semipreparative normal-phase HPLC. For purification of milligram quantities of ceramides, 10–20 mg of total lipid extract was injected in a volume of 1 mL on a column (10 × 250 mm, LiChrosorb 10 micron Silica 60A, packed by Phenomenex, Torrance, CA). The flow rate was 5.0 mL/min, and the ternary gradient was identical to that described above. The HPLC pumping system and detectors were also identical to those described above, except that the column effluent was split, using a Valco T (VICI Valco, Houston, TX), so that 10% of the flow entered the detector and 90% was collected.

Reverse-phase HPLC. Ceramide phosphorylethanolamine (Cer-PE) was purified by semipreparative normal-phase HPLC and analyzed by reverse-phase HPLC using a LiChrosorb 7 RP18 column (3 × 100 mm, Chrompack) and a mobile phase consisting of methanol/acetonitrile/water, 88:6:6, by vol, at a flow rate of 0.5 mL/min. The HPLC pumping system and detectors were as described above.

Mass spectrometry (MS). Liquid chromatography (LC)/MS measurements were performed on a Fisons Quattro SQ mass spectrometer (Beverly, MA) coupled to a Hewlett-Packard 1090 HPLC (Palo Alto, CA). The HPLC was operated with the same conditions as the reverse-phase HPLC method just described. The flow rate was 0.5 mL/min with approximately 12 µL/min of the eluant split post-column and directly introduced into the mass spectrometer. The analyte was ionized by electrospray ionization with detection of negative ions. The ion source was at 100°C, and the instrument scanned from 50–1000 *m/z* at 2.0 s/scan.

A Jeol HX-110 mass spectrometer (Peabody, MA) was employed for all other mass spectrometric measurements. The instrument was operated with 10 kV acceleration and detection of positive or negative ions. Mass resolution was 1000 (10% valley) except for high mass accuracy measurements where the resolution was 10,000 (10% valley). Analyte was ionized by fast atom bombardment (FAB) with a Xe beam and 3-nitrobenzyl alcohol matrix. For collisionally activated dissociation (CAD) measurements, helium was used as the target at a sufficient pressure to attenuate the primary beam to 30% of its original intensity. Linked scanning at constant B/E (magnetic field/electric field) provided spectra of the product ions. For high mass accuracy measurements, manual peak-matching was performed using a suitable calibrant with a mass similar to that of the unknown.

The sphingolipids were hydrolyzed by dissolution of a small amount in 3 N methanolic HCl and heating at 70°C for 4 h. FAB of the entire solution was performed for determination of the sphingoid and ceramide moieties. The fatty acid methyl esters were extracted with methylene chloride and analyzed by gas chromatography (GC)/MS to determine the fatty acid components, using a Hewlett-Packard 5970 for those of *P. ultimum* and a Hewlett-Packard 5890 GC connected to a Finnigan SSQ7000 (Sunnyvale, CA) for those of *Ph. infestans* and *Ph. capsici*. The analyses employed a J&W DB-5 column (30 m, 0.25 mm i.d., 0.025 mm film thickness; Folsom, CA), which was heated from 50 to 285°C at 8°C/min while the mass spectrometer scanned from 50 to 800 *m/z*.

Nuclear magnetic resonance (NMR) spectrometry. The NMR analyses were performed on a Bruker AMX500 spectrometer (Billerica, MA) at 25°C. The two-dimensional (2D) spectrum correlation spectroscopy experiment was acquired with 2K by 512 points which were zero-filled to make up a 2K × 2K set of data points. The projections along the two axes were from one-dimensional ¹H NMR data collected separately.

All experiments were repeated at least two times, and the data presented are the mean of duplicate samples from one experiment.

RESULTS

The yield of total lipid extract was 12–16 mg lipid per 200 mg dry weight of mycelia of *P. ultimum*, 17–20 mg lipid per 200 mg dry weight of mycelia of *Ph. infestans*, and 7–8 mg lipid per 200 mg dry weight of mycelia of *Ph. capsici*.

The total lipid extracts from each of the three organisms were analyzed with an analytical normal-phase HPLC–FID system (Fig. 1). The major phospholipids were phosphatidylethanolamine (PE) and phosphatidylcholine (PC). The chromatogram of *Ph. infestans* lipids contained an unidentified peak at a retention time of 47.3 min; because this peak was resistant to mild alkaline hydrolysis, it was postulated to be a sphingolipid (probably a ceramide) and was labeled Cer 1. The chromatogram of *P. ultimum* lipids contained PE, PC, and an unidentified peak at a retention time of 48.3 min

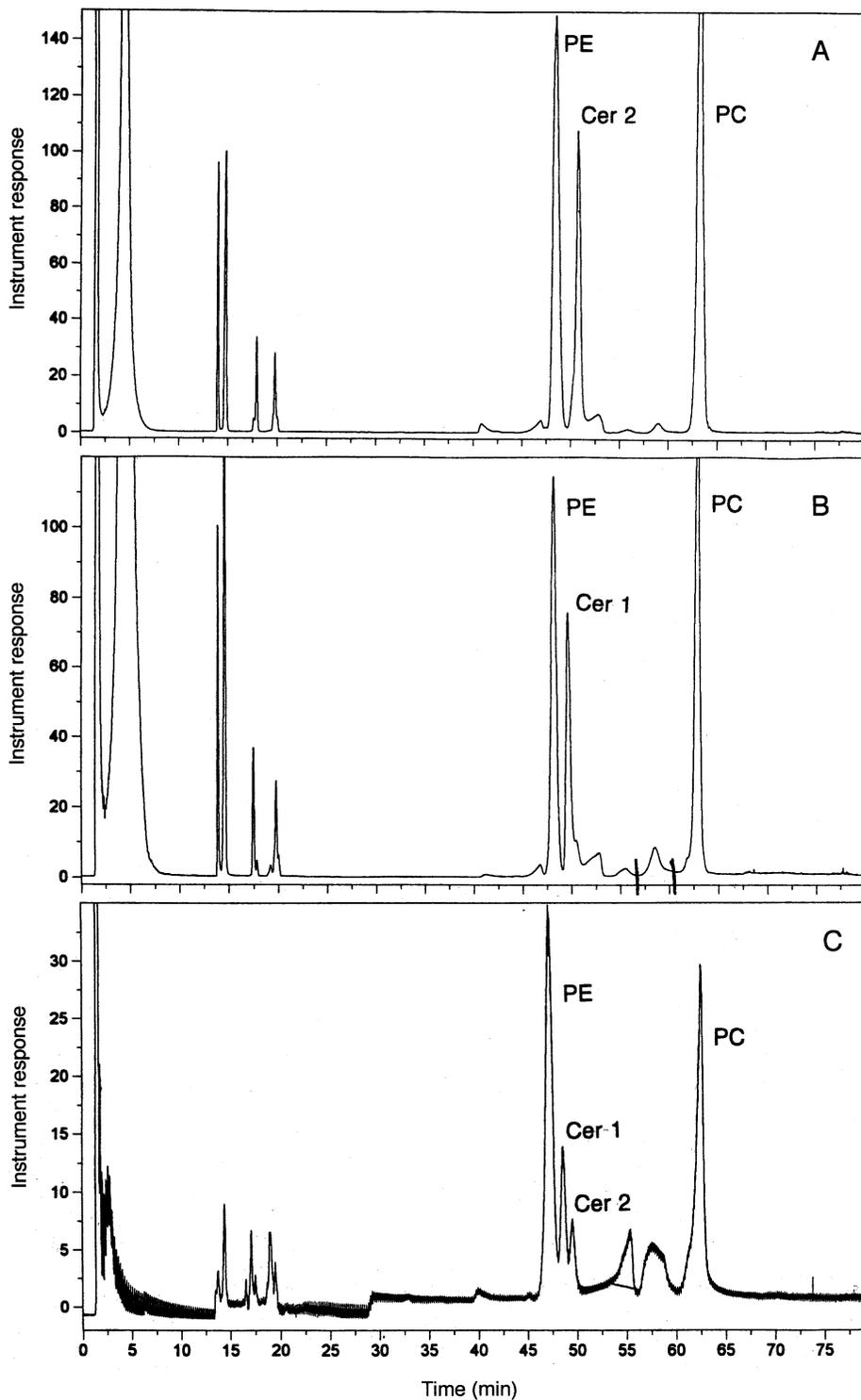


FIG. 1. Analytical normal-phase high-performance liquid chromatography-flame-ionization detector (HPLC-FID) chromatograms of the lipid classes in mycelial lipid extracts of (A) *Pythium ultimum*, (B) *Phytophthora infestans*, and (C) *Ph. capsici*. Abbreviations: PE, phosphatidylethanolamine; PC, phosphatidylcholine; Cer 1 and Cer 2, ceramide-1 and -2.

that was also resistant to mild alkaline hydrolysis. Thus it was also postulated to be a sphingolipid and was labeled Cer 2. The chromatogram of *Ph. capsici* lipids contained PE, PC, and unidentified peaks at the retention times of 47.3 and 48.3 min that were tentatively labeled Cer 1 and Cer 2.

To purify milligram quantities of Cer 1 and Cer 2 for structural analysis, we scaled up the above analytical normal-phase HPLC system to the semipreparative level, injecting larger samples (10–20 mg, in 1 mL) of total lipid extracts from each of the three species (Fig. 2). The semipreparative

chromatograms (Fig. 2) were very similar to those obtained with corresponding samples at the analytical level (Fig. 1). By using this system, several milligrams each of PE, PC, and Cer 1 from *Ph. infestans* and *Ph. capsici* and of Cer 2 from *P. ultimum* and *Ph. capsici* were purified.

Most phospholipid and sphingolipid classes that have been purified by normal-phase HPLC can be separated into their individual molecular species by reinjecting them in an appropriate reverse-phase HPLC system (12). When samples of purified PE and PC were injected in this reverse-phase system,

multiple peaks of individual molecular species were observed with each (data not shown). To attempt to separate the molecular species of Cer 1 and Cer 2, we injected the four purified samples from the semipreparative normal-phase HPLC into an analytical reverse-phase HPLC system (Fig. 3). In this chromatographic system, injection of purified Cer 1 from both *Ph. infestans* and *Ph. capsici* yielded a major peak with a retention time of 27.8 min. Injection of purified Cer 2 from *P. ultimum* and *Ph. capsici* yielded a major peak with a retention time of 13.8 min. Because the chromatograms of the two

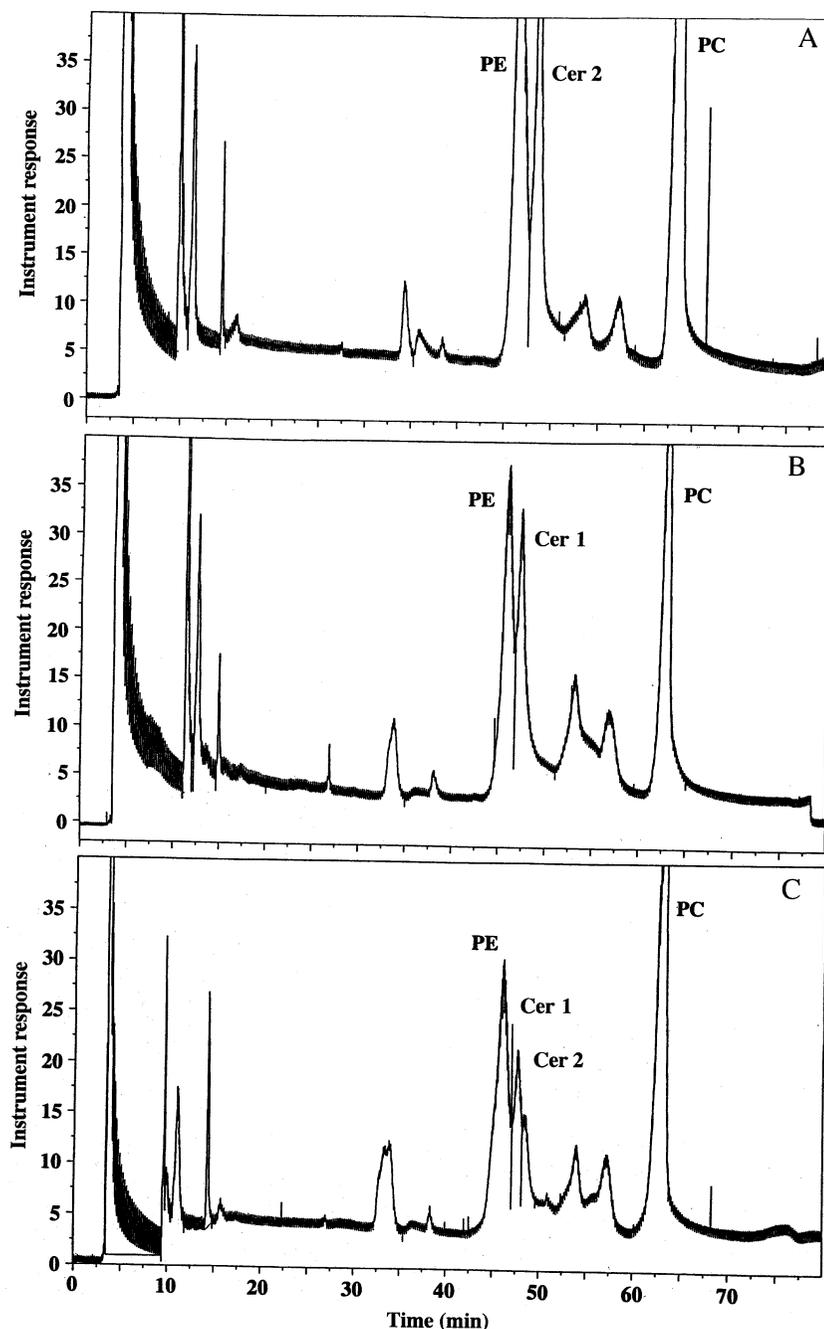


FIG. 2. Semipreparative normal-phase HPLC-FID chromatograms of the lipid classes in mycelial lipid extracts of (A) *Pythium ultimum*, (B) *Phytophthora infestans*, and (C) *Ph. capsici*. Abbreviations are defined in Figure 1.

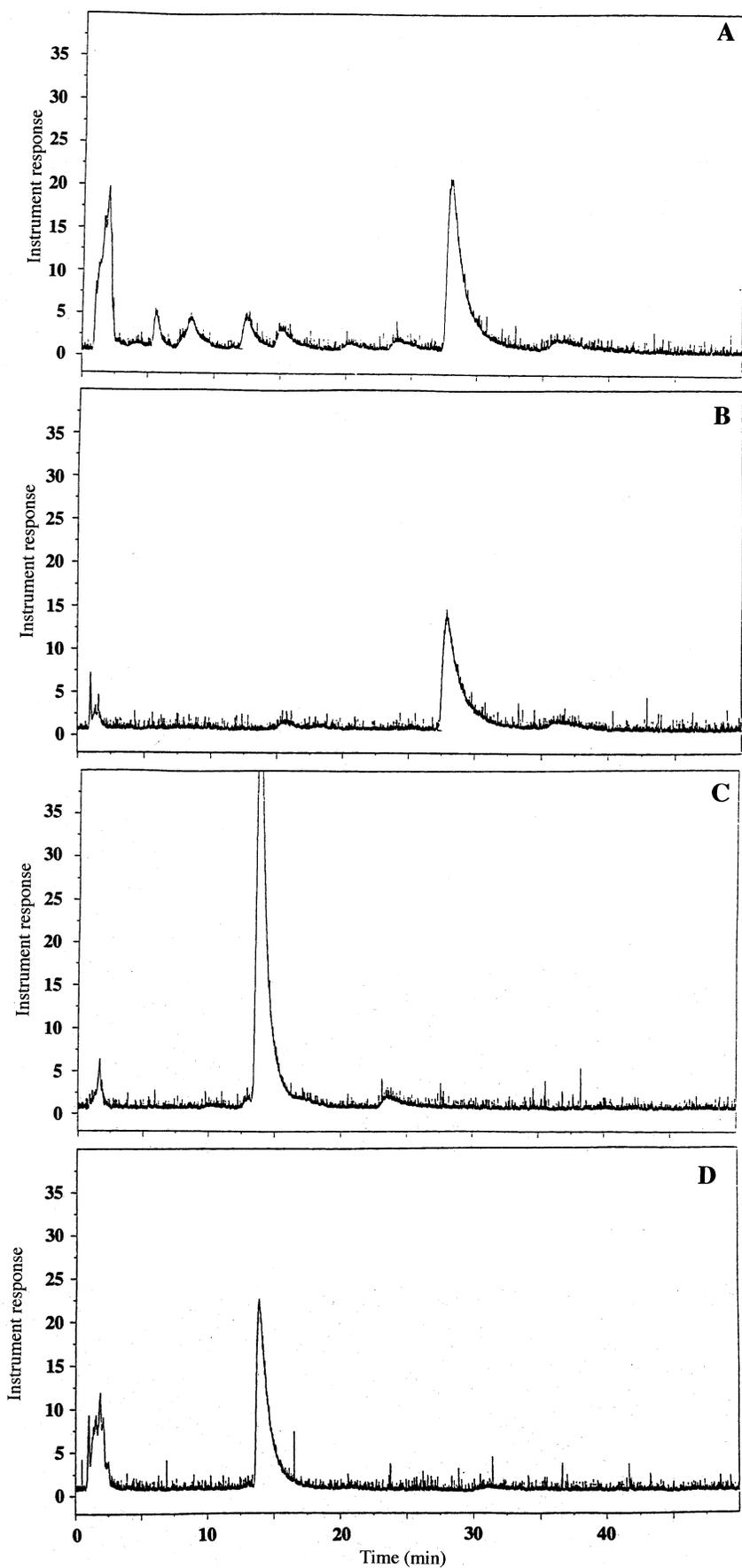


FIG. 3. Analytical reverse-phase HPLC-FID chromatograms of the molecular species of ceramides in the three species: (A) Cer 1 peak from *Phytophthora infestans*, (B) Cer 1 peak from *Ph. capsici*, (C) Cer 2 peak from *Pythium ultimum*, and (D) Cer 2 peak from *Ph. capsici*.

Cer 1 samples each show only one major peak and because the retention times are identical, it is likely that the samples contain the same major component. Similarly, because the two Cer 2 samples each only have one major peak, they apparently only contain one major molecular species; and because the retention times for these are identical, it is likely that their chemical structures are identical.

The Cer 1 and Cer 2 samples isolated by semipreparative normal-phase HPLC were then analyzed by electrospray LC/MS, using the above reverse-phase HPLC system, to determine the molecular weight of the major sphingolipid molecular species in the lipid fractions. The LC/MS chromatograms were similar to those from the reverse-phase HPLC-FID analyses shown in Figure 3. The mass spectra of the major components which corresponded to the intense peaks in Figure 3 are shown in Figure 4. The molecular weights of these components are readily determined as the negative ion electrospray produces the deprotonated molecular ions. In summary, the molecular weight of the major component in *P. ultimum* is 670.4 Da; in *Ph. infestans*, it is 714.5 Da; and in *Ph. capsici*, the major components are 670.4 and

714.5 Da. This supports the proposition that the sphingolipids in *Ph. capsici* are identical to those found in *P. ultimum* and *Ph. infestans*.

The FAB mass spectra, Figure 5, of the sphingolipid fractions, isolated by semipreparative normal-phase HPLC, confirmed the molecular weights of the major components. The mass spectrum for the *P. ultimum* sphingolipid shows the protonated molecular ion at m/z 671.4 while the *Ph. infestans* sphingolipid has m/z 715.5 as the protonated molecular ion. Additionally, the peaks at m/z 141 lower are seen in each of these spectra at m/z 530.4 and 574.5, respectively. This is indicative of loss of phosphorylethanolamine from the molecular ion (13). In the case of *Ph. capsici*, both the m/z 671.4 and 715.5 peaks and the m/z 530.4 and 574.5 peaks are observed.

Additional structural information was obtained from the FAB mass spectra of the total hydrolysate solution and is summarized in Schemes 1 and 2 (spectra not presented). The *P. ultimum* sphingolipid hydrolysate showed a major peak at m/z 433.3 which would correspond to the hydrolytic loss of a 16:0 fatty acid chain from the sphingolipid. An additional peak at m/z 310.3 shows further loss of phosphorylethanol-

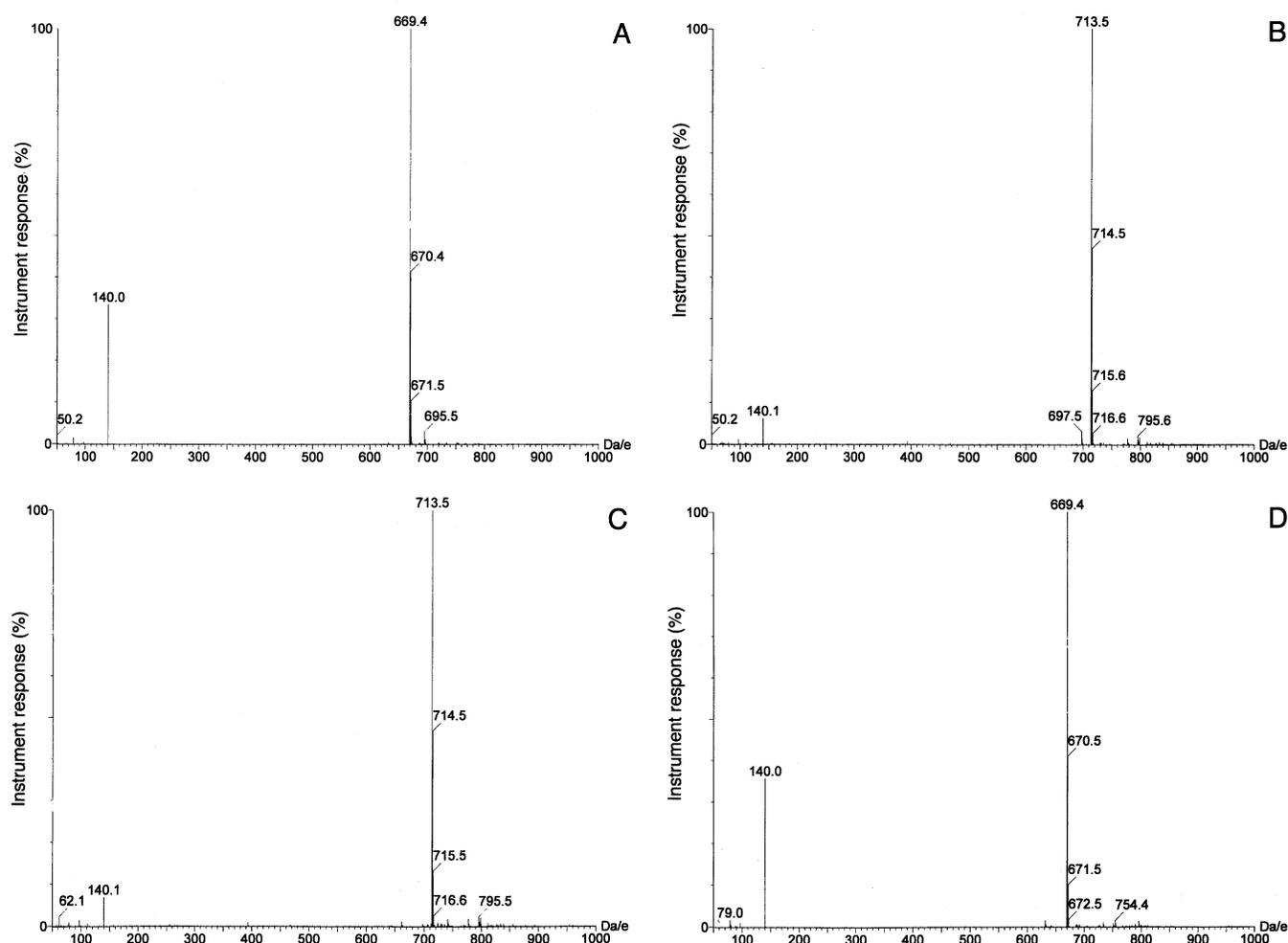


FIG. 4. Liquid chromatography-mass spectrometry electrospray analysis of (A) *Pythium ultimum*, (B) *Phytophthora infestans*, (C) *Ph. capsici* Cer 1, and (D) *Ph. capsici* Cer 2. The mass spectra of the major components were obtained using a micromass quadrupole mass spectrometer operating in the negative ion mode. For abbreviations see Figure 1.

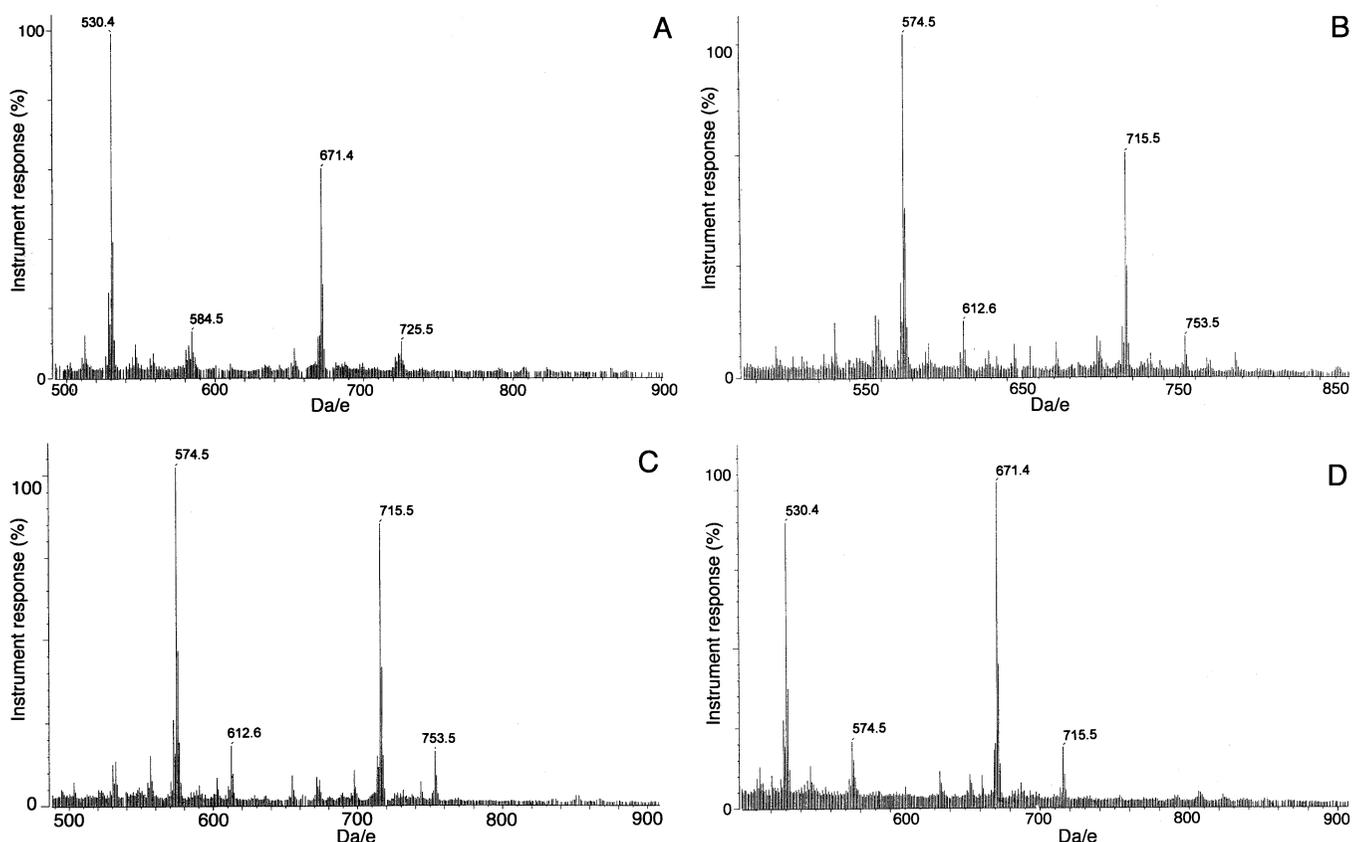


FIG. 5. The fast atom bombardment mass spectra of the sphingolipid fractions of (A) *Pythium ultimum*, (B) *Phytophthora infestans*, (C) *Ph. capsici* Cer 1, and (D) *Ph. capsici* Cer 2. The mass spectra were obtained using a Jeol HX-110 double focusing mass spectrometer (Peabody, MA) operating in the positive ion mode. Fractions were prepared in a 3-nitrobenzyl alcohol matrix. For abbreviations see Figure 1.

amine leaving m/z 310.3 as the protonated molecular ion of the sphingoid. The FAB mass spectrum of the *Ph. infestans* hydrolysate solution showed a major peak at m/z 395.3. This corresponds to the hydrolytic loss of a 22:1 fatty acid

from the sphingolipid. The other significant hydrolysis product m/z 272 reflects further loss of phosphorylethanolamine. The *Ph. capsici* hydrolysate showed both pairs of hydrolysis products, further reinforcing the supposition

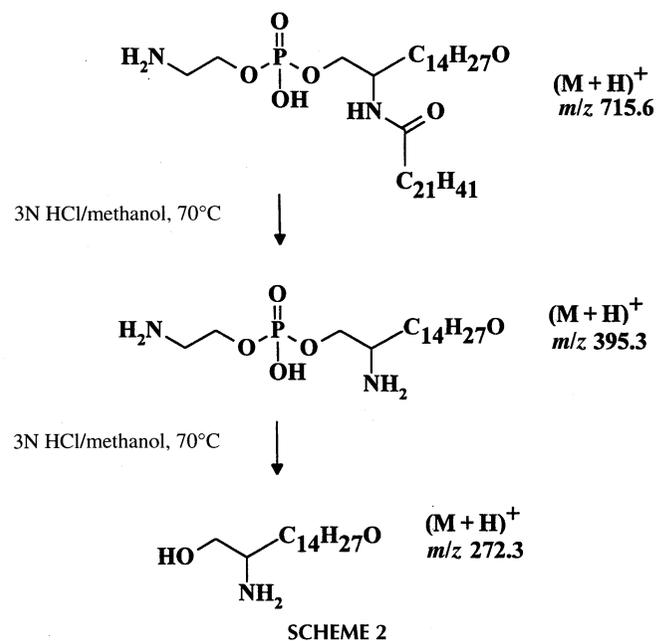
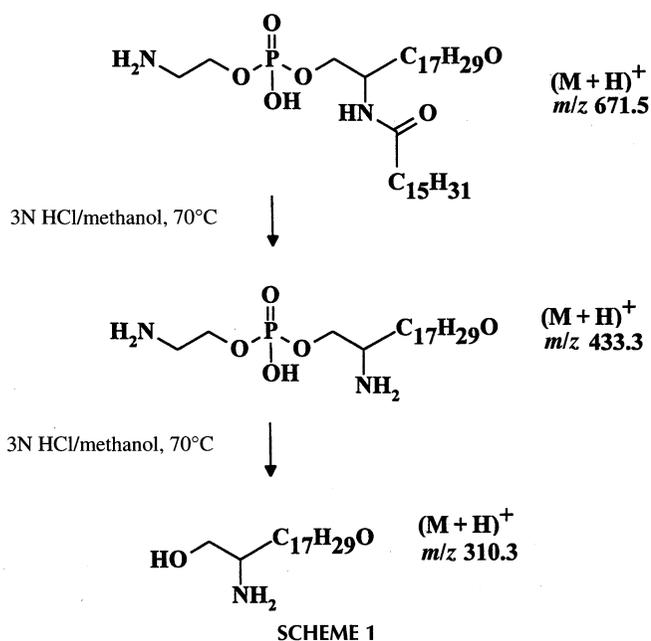


TABLE 1
Summary of High-Resolution Accurate Mass Measurements

Measured mass	Proposed formula	$\frac{\text{Measured mass} - \text{calculated mass}}{\text{Calculated mass}} \times 100$ (%)
<i>Pythium ultimum</i> ^a		
671.5136 (M + H) ⁺	C ₃₇ H ₇₂ O ₆ N ₂ P	0.00012
669.4983 (M - H) ⁻	C ₃₇ H ₇₀ O ₆ N ₂ P	0.00017
530.4919 (M - PE) ⁺	C ₃₅ H ₆₄ O ₂ N	-0.00034
140.0111 (PE) ⁻	C ₂ H ₇ O ₄ NP	-0.00012
<i>P. ultimum</i> hydrolysate		
433.2819 (M + H) ⁺	C ₂₁ H ₄₂ O ₅ N ₂ P	-0.00029
310.2729 (M + H) ⁺	C ₁₉ H ₃₆ O ₂ N	-0.00055
<i>Phytophthora infestans</i>		
715.5748 (M + H) ⁺	C ₄₀ H ₈₀ O ₆ N ₂ P	-0.00008
574.5532 (M - PE) ⁺	C ₃₈ H ₇₂ O ₂ N	-0.00054
<i>Ph. infestans</i> hydrolysate		
395.2658 (M + H) ⁺	C ₁₈ H ₄₀ O ₅ N ₂ P	-0.00043
272.2601 (M + H) ⁺	C ₁₆ H ₃₄ O ₂ N	0.00042

^aPE, phosphatidylethanolamine.

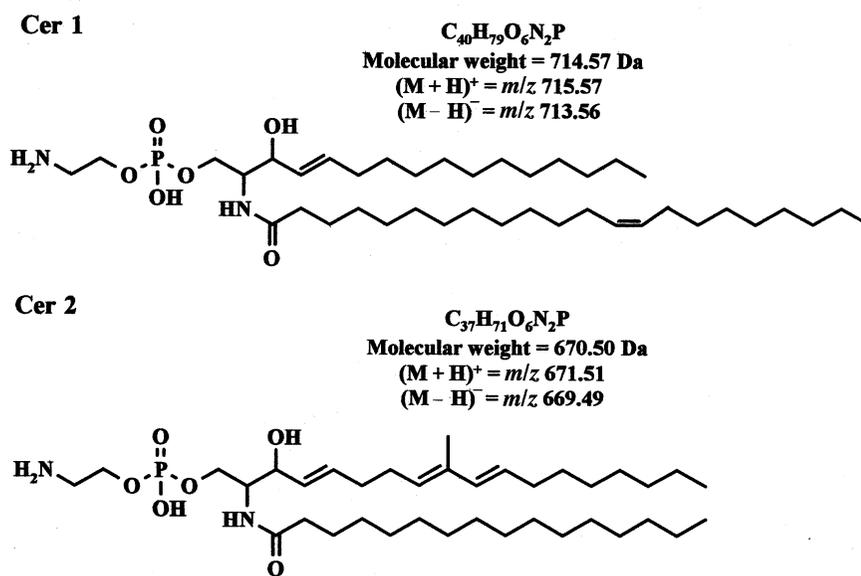
that the sphingolipids in *P. ultimum* and *Ph. infestans* are both present in *Ph. capsici*.

The GC/MS analysis of the hydrolysate extract gave qualitative information on the fatty acid components of the sphingolipids. For *P. ultimum* the methyl esters of 14:0, 16:0, 18:0, 18:1, 20:0, and 20:1 were detected. For *Ph. infestans* the methyl esters of 16:0, 18:0, 22:0, and 22:1 fatty acids were found.

Strong structural evidence is provided by the accurate mass measurements of the sphingolipids and their hydrolysates. Table 1 summarizes the data showing the measured mass, the proposed formula, and the difference between the expected and measured mass. Mass errors of less than 0.001% are generally considered a good match. The difference in composition between the protonated molecular ion of the *P. ultimum* sphingolipid and the peak corresponding to the

loss of *m/z* 141 almost exactly accounts for loss of phosphoylethanolamine. This strong indication of loss of phosphoylethanolamine is also shown for the *Ph. infestans* sphingolipid fragments. Similarly, the assignments of the hydrolysate products are very near the measured values. All of the assignments in Table 1 are within 0.00055% of the proposed masses, giving strong support for the proposed structures (Scheme 3).

The connectivity in these sphingolipids is further elucidated by the CAD spectra of the various molecular ions. These CAD spectra provide a fingerprint of the molecule and are a sensitive indicator of molecular structure. The major ions resulting from the fragmentation of the molecular ions are shown in Table 2. The notation is similar to that used in Pivot *et al.* (14). These data provide clear evidence of the connectivity of the various moieties mentioned in the above re-



SCHEME 3

TABLE 2
The Collisionally Activated Dissociation Spectra of Cer 1 and Cer 2

	Ion mode	(M + H) ⁺	(M - H) ⁻	Y	U	Y - B	Y - F - H ₂ O	XPO ₄ H ₃	XPO ₄
Cer 1	Positive	715.5		573		362	236	142	
	Negative		713.5		501				140
Cer 2	Positive	671.5		530		280	274	142	
	Negative		669.4		419				140

sults. Based on these CAD spectra and the above data, the structures of the Cer 1 and Cer 2 can be assigned with certainty and are shown in Scheme 3. Additionally the CAD spectra of Cer 1 and Cer 2 from *Ph. capsici* are essentially the same as these shown here, proving that the sphingolipids in *Ph. capsici* have the same structure as those in *Ph. infestans* and *P. ultimum* (Scheme 3).

The only ambiguities which are unresolved by the above MS analyses are in the position of the three double bonds and branching in the *P. ultimum* sphingoid moiety. Proton NMR was used to address this point and provide further evidence for the proposed structures.

The positions of the olefinic groups along the sphingoid (or sphingatriene) chain were determined in a 2D NMR spectrum correlation spectroscopy experiment (15) that was carried out on a 500 MHz NMR spectrometer. In this experiment, signals arising from protons three bonds apart are correlated in the 2D map as shown in Figure 6. There are two sets of signals in the 2D plot. The diagonal peaks running from the top right corner of the figure to the bottom left end are essentially the same as the normal one-dimensional proton NMR signals that are also shown on the top as well as left side of the 2D plot. The off-diagonal peaks indicate the connectivities among neighboring proton species. Horizontal and vertical dashed lines are drawn on the 2D spectrum to assist with these correlations. For example, the long vertical line at 5.34 ppm at the olefinic methine proton (8 on the structure) shows two correlations to 1.69 and 2.16 ppm as highlighted by the two horizontal dashed lines. As indicated on the left side of the figure, these correspond to the methylene 7 and methyl group at 19. The rest of the correlations are assigned in a similar fashion, as indicated with several dashed lines on the spectrum, and identify the *P. ultimum* sphingoid as a C19-Δ 4,8,10, nine-methyl-long chain base. A summary of the proton chemical shift assignment for the sphingoid (or sphingatriene) side chain is given in Table 3. The *J* coupling between the protons across the double bond for pairs 4-5 and 10-11 is 15.3 Hz—indicative of *trans* configuration of the two olefins. We could not accurately measure the *J* coupling between 8-9 (CH₃) owing to severe overlap and weak coupling. Thus, we could not determine its configuration.

Based on the above structural information, Cer 1 and Cer 2 appear to be molecular species of Cer-PE, with proposed structures shown in Scheme 3. The analytical HPLC data presented in Figure 1 were used for the quantitative analysis of each of the lipid classes in the lipid extracts of the three species (Table 4). To accomplish this quantification, several samples of each of the major peaks purified by semipreparative HPLC (Fig. 2) were injected, and the FID was calibrated to convert peak area to mass in the range of 1-50 μg for each of the major lipid classes. The levels of total lipids and non-polar lipids varied significantly among the three species. Although no attempt was made to quantify the individual non-polar lipid classes, they appeared to be mostly triacylglycerol, with some free fatty acids, and several other minor unidentified peaks. For *P. ultimum* and *Ph. infestans* the most abundant polar lipid class was PC, followed by PE, and then Cer-PE. For *Ph. capsici*, the most abundant polar lipid class was PE, followed by PC, and then Cer-PE.

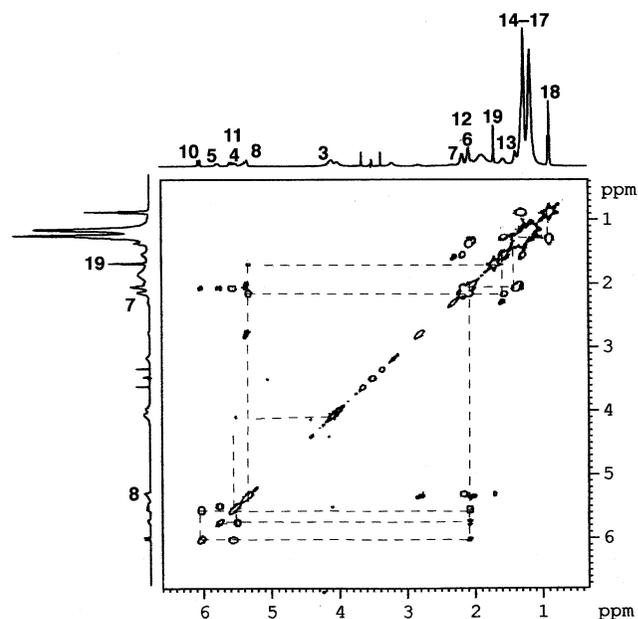


FIG. 6. The two-dimensional nuclear magnetic resonance spectrum correlation spectroscopy of *Pythium ultimum* ceramide phosphorylethanolamine.

TABLE 3
The Proton Chemical Nuclear Magnetic Resonance Shift Assignments for the *Pythium ultimum* Sphingatriene Sidechain

Position on structure	¹ H chemical shift (ppm)	Position on structure	¹ H chemical shift (ppm)
3	4.09	10	6.02
4	5.51	11	5.57
5	5.76	12	2.08
6	2.08	13	1.38
7	2.17	14–17	1.3
8	5.32	18	0.88
9			

DISCUSSION

This study is the first report of the occurrence of Cer-PE in a eukaryotic microorganism. It is also the first report of a 19-carbon-9-methyl-triunsaturated ($\Delta 4, 8, 10$) long chain base in a microbial sphingolipid. Several reports of the lipid composition of *P. ultimum* (16–18) failed to identify any sphingolipids. One of these reports noted the presence of a double peak of PE and several other unidentified phospholipid peaks, any of which could have been Cer-PE (16).

The current study now brings into question earlier reports of the occurrence of ceramide aminoethylphosphonate (CAEP) in *P. prolatum* (5), *Ph. palmitovora* (5), and *Ph. infestans* (6,7). In the cases of *P. prolatum* and *Ph. palmitovora*, it is not possible to comment on whether these species contain CAEP or Cer-PE, since these species were not examined in our current study. However, for *Ph. infestans*, all of the rig-

TABLE 4
Quantitative Analysis (mg/g dry wt) of Ceramides and Other Lipid Classes in *Pythium ultimum*, *Phytophthora infestans*, and *Ph. capsici*^a

Lipid class ^b	<i>Pythium ultimum</i> ^b	<i>Phytophthora infestans</i>	<i>Ph. capsici</i>
Nonpolar lipids	38.3	86.9	28.7
Polar lipids			
CL	trace	trace	trace
PE	9.44	5.29	3.66
Cer-PE 1	n.d.	3.03	1.39
Cer-PE 2	4.72	trace	0.84
PI	trace	trace	trace
PC	12.6	6.71	2.93
Total lipids	65.1	102.0	37.5

^aQuantification is based on the normal-phase high-performance liquid chromatograms reported in Figure 1. Standard curves of each lipid class, injected as several concentrations, were used to calculate the mass of each lipid class.

^bAbbreviations: n.d., not detected; CL, cardiolipin; Cer-PE 1 and 2, ceramide phosphorylethanolamine 1 and 2, with structures shown in Scheme 3; PI, phosphatidylinositol; PC, phosphatidylcholine. For other abbreviation see Table 1.

orous battery of mass spectral and NMR data that was employed in this study point to the occurrence of only Cer-PE (and no CAEP was detected). The two previous reports of CAEP in *Ph. infestans* (6,7) did not employ MS or NMR, and instead, the structural identification was based mainly on cochromatography of the hydrolyzed polar head group with commercial standards of aminoethylphosphonate. In one of the previous reports of CAEP in *Ph. infestans* (6) the major fatty acid moiety of CAEP was reported to be arachidonic acid. However, in the current study we found that erucic acid (*cis*-13-docosenoic, C22- Δ 13) was the most abundant fatty acid in the Cer-PE of *Ph. infestans*. The identification of arachidonic acid in CAEP in the previous study (6) was performed by gas-liquid chromatography with packed columns. It is not known whether the arachidonic acid identified in the previous study could be adequately separated from erucic acid with the technique used. However, because the identification of erucic acid in Cer-PE in the current study is based on capillary gas-liquid chromatography with mass spectral analysis, we feel quite confident that this is the major fatty acid in the Cer-PE of *Ph. infestans*.

Ceramide phosphorylinositol (Cer-PI) was recently reported to be the major sphingolipid in *Ph. capsici* (14,19,20) and *Ph. parasitica* (21). Inositol sphingolipids such as these have been reported to occur in yeast and fungi (22,23). In our current study we did not see any evidence for the occurrence of Cer-PI or other inositol sphingolipids as major membrane lipids in *P. ultimum*, *Ph. infestans*, or *Ph. capsici*. While it is possible that Cer-PI is present in *Ph. parasitica* but absent from *Ph. infestans* and *P. ultimum*, we have no explanation for the failure to detect Cer-PI in *Ph. capsici* in our study except for the possibility of (i) differences in lipid composition between individual isolates, (ii) differences in the growth conditions, or (iii) differences in extraction procedures used in our study as compared to others (14,19,20).

Although this study is the first report of the occurrence of Cer-PE in a eukaryotic microorganism, Cer-PE has been reported to occur in *Sinotia histrica*, a freshwater snail (24) and three genera of anaerobic Bacteroides (25).

The unusual 19-carbon-9-methyl-triunsaturated ($\Delta 4, 8, 10$) long chain base (sphingoid) that was found in the Cer-PE in *P. ultimum* (Scheme 3) has also been reported to occur in the glucosylceramides of *Asterias amurensis*, a starfish (26), and *Ophidiaster ophidiamus*, a sea star (27). Two fungal species, *Lentinus edodes* and *Schizophyllum commune*, have been reported to contain a somewhat similar 19-carbon-9-methyl, diunsaturated ($\Delta 4, 8$) long chain base in their glucosylceramides (28,29).

The subcellular localization and physiological significance of Cer-PE in Oomycetes are not known. However, because most of the sphingolipids in other species [including inositol sphingolipids in yeast (30), glucocerebrosides in plants (31), and sphingomyelin in animal cells (32)] are all localized in the plasma membrane, it is reasonable to expect that most of the Cer-PE in Oomycetes is localized in the plasma membrane. Cer-PE would be expected to be resistant to phospho-

lipases A1, A2, and B, since these enzymes hydrolyze the fatty acid-oxygen ester bonds and there are no such bonds in Cer-PE. Since many plants (including potatoes and tomatoes, major hosts of *Ph. infestans*) contain high levels of phospholipase B [also called lipolytic acyl hydrolase (7)], the localization of Cer-PE in the plasma membrane could help protect the membrane from hydrolysis by plant phospholipase B. However, certain hydrolytic enzymes that are also common in certain plant tissues, phospholipase C and D, would presumably be capable of hydrolyzing Cer-PE.

The pathway leading to Cer-PE remains to be elucidated. Nevertheless, the discovery of Cer-PE as a novel class of sphingolipid found in *Phytophthora* and *Pythium* species but not in plants or mammals prompts the speculation that enzymes involved in biosynthesis of these lipids may represent potential biochemical targets for the discovery of new agricultural fungicides (Stramenopilicides).

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