Isolation and Characterization of Inositol Sphingophospholipids from *Phytophthora parasitica* Dastur[1]

Maud Bruneteau[a,*], Florence Fournol[a], Corinne Gandon[a], Michel Becchi[b], and Véronique Pivot[a]

[a]Laboratoire d'Ecologie Microbienne du Sol, UMR-CNRS 5557, Université Claude Bernard Lyon 1, F-69622 Villeurbanne Cedex, France, and [b]CNRS, Service Central d'Analyses, F-69390 Vernaison, France

ABSTRACT: Several inositol sphingophospholipids (ISPL) were isolated from mycelia of *Phytophthora parasitica* Dastur, a phytopathogenic fungus of carnation. The ISPL structures were determined by fast atom bombardment. All ISPL consisted of ceramides linked to inositol phosphate. We investigated the effect of growth conditions on the ISPL produced in four different media that are commonly used for fungal cultures. We showed that *P. parasitica* Dastur synthesized four major classes of compounds with molecular weights of M_r = 751, 807, 835, and 849 containing the 16:1 base and the 16:0 or 20:0 or 22:0 or 22h:1 *N*-acyl group. The relative abundance of the different ISPL is dependent on growth conditions.
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Inositol sphingophospholipids (ISPL) have been found in plants (1–5), yeasts (6–8), fungi (9), and protozoa (10–12), and all were shown to be derived from myoinositol phosphorylceramide (7,10). The ceramides typically contain C_{16}- or C_{18}-sphingosine or C_{18}-phytosphingosine (7,10) as long-chain base (LCB), which is acylated with palmitic, stearic (10, 11), 2- or 3-hydroxyhexacosanoic or 4-hydroxy-2-docosenoic acid (7) to form the backbone of the glycosphingophospholipids (13).

In a previous work, we isolated ISPL from mycelia of *Phytophthora capsici*, a phytopathogenic fungus of pepper (*Capsicum annuum* cv. Yolo Wonder) (14). The amide-linked fatty acids were found to have chains of 16, 20, and 22 carbon atoms. The LCB were identified as trimethylsilyl derivatives and shown to be C_{16}-sphingosine and C_{16}-dihydrosphingosine. We showed that *P. capsici* synthesized one major class of compounds with molecular weights of M_r = 833 and 835 containing the 16:1 base and the 22:1 or the 22:0 *N*-acyl group, independent of the growth medium used (15).

These ISPL induce a protective response by cotyledons of young peppers against necrotic lesions caused by the pathogen *P. capsici* (14,15).

In continuation of our work on ISPL of the *Phytophthora* genus, we determined whether ISPL were present in the strain 26 of *P. parasitica* Dastur, a phytopathogenic fungus of carnation and whether their composition was dependent on growth conditions.

MATERIALS AND METHODS

Microorganism and growth. The isolate 26 of *P. parasitica* Dastur from the fungal culture collection of INRA Antibes was grown on M1, M2, M3, and M4 media.

M1 was the Huguenin medium (16) which contained (g/L): glucose, 10; K_2HPO_4, 0.2; KH_2PO_4, 0.8; K_2SO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCO_3$, 0.2; KNO_3, 1; thiamine, 0.002; $FeSO_4 \cdot 7H_2O$, 0.0005; $ZnSO_4 \cdot 7H_2O$, 0.0005; $CuSO_4 \cdot 7H_2O$, 0.00002; $MnCl_2 \cdot 7H_2O$, 0.00002; $Mo_7O_{24}(NH_4)_2$, 0.00002; after sterilization M1 was supplemented with 10 mL of alcoholic solution which contained 2 mg/mL of cholesterol. M2 was the Hall medium (17) which contained (g/L): glucose, 30; L-asparagine, 10; K_2HPO_4, 0.26; KH_2PO_4, 0.47; $MgSO_4 \cdot 7H_2O$, 0.01; $FeSO_4 \cdot 7H_2O$, 0.001; thiamine, 0.001; $CaCl_2 \cdot 2H_2O$, 0.01; $ZnSO_4 \cdot H_2O$, 0.001; $CuSO_4.5H_2O$, 0.0003; $Na_2Mo \cdot 2H_2O$, 0.0002; $MnCl_4 \cdot 4H_2O$, 0.0002; with pH adjusted to 6.5. M3 was the Plich and Rudnicki medium (18) which contained (g/L): glucose, 25; yeast extract, 5; L-asparagine, 1; KH_2PO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 1; thiamine, 0.001; with pH adjusted to 6. M4 was a chemically defined medium which contained (g/L): glucose, 20; L-asparagine, 1; KH_2PO_4, 0.6; $K_2HPO_4 \cdot 3H_2O$, 0.125; KNO_3, 0.7; $MgSO_4 \cdot 7H_2O$, 0.25; $Ca(NO_3)_2$, 0.3; $ZnSO_4 \cdot 7H_2O$, 0.004; $MnSO_4 \cdot H_2O$, 0.0015; H_3BO_3, 0.001; $Na_2MoO_4 \cdot 2H_2O$, 0.001; $CuSO_4 \cdot 5H_2O$, 0.00002; KI, 0.00002; $CaCl_2 \cdot 6H_2O$, 0.00002; $FeSO_4 \cdot 7\ H_2O$, 0.0055; sodium ethylenediaminetetraacetate, 0.0075; nicotinic acid, 0.001; pyridoxine, 0.001; calcium pantothenate, 0.001; thiamine, 0.001; with pH adjusted to 6.

Fungi were grown at 24°C under 16 h of light per day on M1, M2 and M4, and in the dark on M3 medium. Mycelia were collected after 3 wk of growth on M1 and M3 media, after 10 d of growth on M4 medium, and after 3 d on M2 medium.

Extraction and fractionation of lipids. Mycelial walls were isolated from fungi as described by Fabre *et al.* (19), and were treated with chloroform/methanol (2:1, vol/vol). The extracted

[1]This work is dedicated to the memory of Professor G. Michel.

*To whom correspondence should be addressed at Laboratoire d'Ecologie Microbienne du Sol, UMR-CNRS 5557, Université Claude Bernard Lyon 1, Bâtiment 741, F-69622 Villeurbanne Cedex, France.

Abbreviations: GC, gas chromatography; FAB, fast atom bombardment; HPTLC, high-performance thin-layer chromatography; ISPL, inositol sphingophospholipid; LCB, long-chain base; MS, mass spectrometry.

lipids dissolved in a minimum volume of chloroform were separated into neutral and polar lipids by column chromatography on Bio-Sil HA silicic acid (BioRad, Ivry sur Seine, France) (20). Neutral lipids were eluted with chloroform, and polar lipids were eluted with chloroform/methanol (95:5, vol/vol; 90:10, vol/vol; 75:25, vol/vol; 50:50, vol/vol) and pure methanol. ISPL were eluted with chloroform/methanol (50:50, vol/vol).

Thin-layer chromatography of lipid fractions eluted from the silicic acid column. Lipid fractions were analyzed by high-performance thin-layer chromatography (HPTLC) on silica gel 60 plates (Merck; Darmstadt, Germany) that were developed with the solvent system described by Heape *et al.* (21). The detection of lipid fractions was carried out by spraying the plates with various reagents: 0.2% (wt/vol) vanillin in sulfuric acid/water (1:9, vol/vol), Dittmer and Lester reagent as modified by Vaskovsky and Kostetsky (22), Draggendorf reagent as modified by Wagner *et al.* (23). Identifications were made by comparison with authentic standards.

Analytical methods. Hydrolysis of phospholipids was performed with 1 M HCl for 16 h at 100°C or 6 M HCl for 48 h at 100°C. Hydrolysates were reduced and acetylated according to the method of Sawardeker *et al.* (24). Alditol acetates were analyzed by gas chromatography (GC) using Intersmat instrument (Model 120 FL; Intersmat, Lyon, France) fitted with an SP2380 glass capillary column (0.32 mm × 30 m). The column temperature was programmed from 140 to 260°C at 4°C/min.

Mass spectrometry (MS). Fast atom bombardment (FAB) mass spectra of phospholipids were recorded in the negative ion mode on a VG ZAB2 SEQ mass spectrometer (Manchester, United Kingdom). The instrument was equipped with a VG FAB standard source with a cesium ion gun delivering about 2 μA of cesium ion current at about 35 kV.

Spectra were obtained at a magnet scan rate of 20 s/decade over a mass range of *m/z* 200–2000 at 8 kV. The mass range was calibrated using cesium iodine clusters. The spectra were collected and processed using the VG II-250 J data system. Samples were dissolved in chloroform/methanol (1:1, vol/vol) and mixed with an equal volume of triethanolamine. The sample (2 μL) was deposited on the FAB probe tip mixed with matrix, i.e., glycerol.

RESULTS AND DISCUSSION

The percentages of total lipids extracted from the mycelial cell walls are given in Table 1. *Phytophthora parasitica*, strain 26, is characterized by a large total lipid content. The same percentage was observed when strain 26 was grown on M2, M3, and M4 media whereas this fungus is characterized by the lowest amount of lipids in the M1 medium.

Total lipids were fractionated on a column of Bio-Sil HA silicic acid to measure the relative amounts of neutral (eluted with chloroform) and polar (eluted with chloroform containing increasing concentrations of methanol) lipids as detailed in the Materials and Methods section (Table 1). The differences indicate that the relative abundance of neutral and polar lipids is dependent on growth conditions. The presence of L-asparagine in the medium or the time of growth of the fungus affected the percentage of polar lipids. The amount of polar lipids found in strain 26 grown on medium M3 was lower than in M1 medium without L-asparagine for the same time of growth. In the M4 medium the percentage of polar lipids was higher than in the M3 medium. M3 and M4 media contained the same amount of L-asparagine, but the time of growth was longer in M3 medium. The polar lipids consisted of phospholipids as revealed by HPTLC and spraying with reagent for phospholipids. HPTLC analysis of phospholipids eluted with chloroform/methanol (50:50, vol/vol) showed the presence of a major compound that comigrated with and had the molecular ion of, ISPL, as previously described (14,15). No other additional peaks were observed. This indicates that the ISPL was essentially pure, and that any other phospholipids eluting with it are present in very small amounts. No compound was revealed by specific reagent for tertiary amines. *Phytophthora parasitica* does not produce phosphatidylcholine. Previously, Pivot *et al.* (25) have shown that

TABLE 1
Lipid Accumulation in *Phytophthora parasitica* Dastur, Strain 26[a]

Culture medium	Total lipid content[b] (%, w/w)	Percentage of each lipid fraction[c] (%, w/w)	
		Neutral lipids	Polar lipids
M1	16.5	52.8	47.2
M2	32.0	55.0	45.0
M3	35.0	81.8	18.2
M4	35.0	28.5	71.5

[a]Strain 26 of *P. parasitica* Dastur was grown on M1, M2, M3, and M4 media. M1 was the Huguenin medium (Ref. 16); M2 was the Hall medium (Ref. 17); M3 was the Plich and Rudnicki medium (Ref. 18); M4 was a chemically defined medium. The compositions of M1, M2, M3, and M4 media and the conditions of cultures were described in the Materials and Methods section.
[b]These cultures were used to prepare the lipid extracts from fungal cell walls. The wt% of total lipids content was expressed as a percentage of the dry weight of fungal cell walls.
[c]The weight percentage of neutral and polar fractions eluted with chloroform and chloroform containing increasing concentrations of methanol and pure methanol, respectively, from Bio-Sil HA silicic acid chromatography (BioRad, Ivry sur Seine, France) was expressed as a percentage of total lipids extracted.

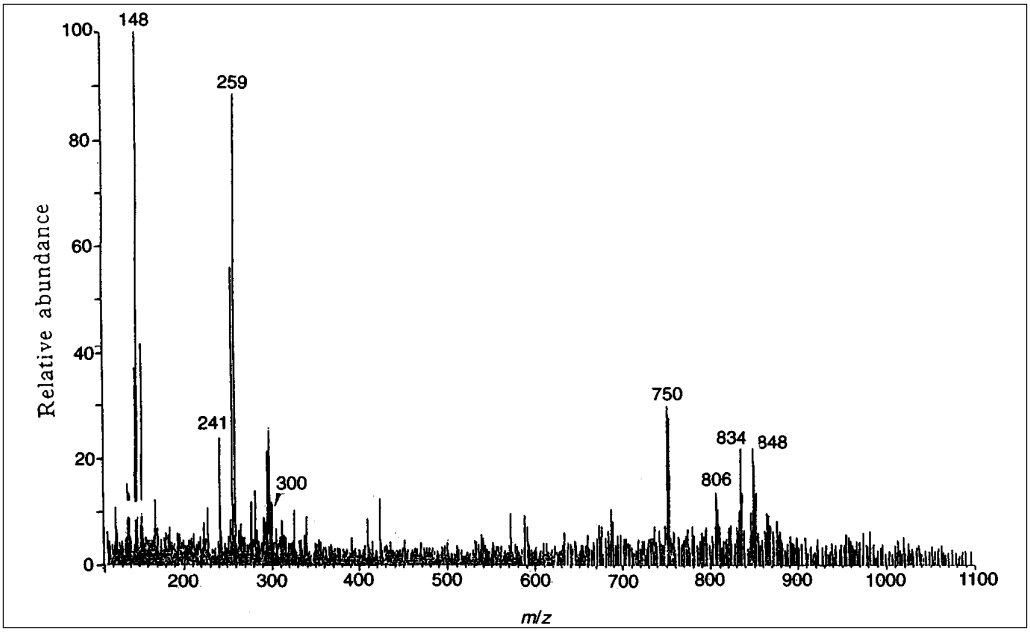

FIG. 1 . The negative fast atom bombardment mass spectrum of inositol sphingophospholipids from *Phytophthora parasitica* Dastur strain 26, grown on M2 medium. The ions at *m/z* 750, 806, 834, 848 correspond to individual molecular species present. Fragments in the spectrum at *m/z* 241, 259, and 300 are characteristic of an inositol sphingophospholipid as reported by Dommon and Costello (Ref. 27) and Ohashi (Ref. 29).

strain 107 of *P. capsici* grown on a chemically defined medium (26) produced dihexadecanoyl phosphatidylcholine.

The ISPL structure was determined by FAB MS. The negative ion FAB mass spectrum of the ISPL fraction from strain 26 grown on M1, M2, M3, and M4 media showed molecular species with $[M - H]^-$ ions at *m/z* 750, 752, 806, 808, 832, 834, 848, and 850. As an example, Figure 1 shows the mass spectrum of the ISPL fraction from strain 26 grown on M2 medium. This mass spectrum is similar to that of the ISPL previously described (15). It shows the characteristic ion corresponding to the cleavage of the inositol–phosphate bond [M − H − 162], and ions at *m/z* 241 and 259, the formation of which has been previously reported (11,27–29). The results indicate that in strain 26 of *P. parasitica* Dastur grown on M1, M2, M3, and M4 media the major ISPL correspond to the compounds of molecular weights 751, 807, 835, and 849. Although the same ISPL are present in the strain 26 grown on all media, there are significant differences in their relative abundance depending on growth conditions. This is especially true in regard to the ISPL corresponding to the compounds of molecular weights 751, 835, and 849. The ISPL, $M_r = 751$ is a major compound when strain 26 is grown on Huguenin medium (M1) and on Hall medium (M2). The ISPL with $M_r = 835$ and $M_r = 849$ were observed to be the most abundant compounds found in strain 26 grown on Plich and Rudnicki medium. Strain 26 grown on synthetic medium (M4) produced the four molecular species in similar percentage. Earlier studies have shown that *P. capsici*, strains 197, 107, and 375 synthesize only one major class of compounds with molecular weights of $M_r = 833$ and 835. It seems that *P. parasitica* is different and is characterized by the presence of four major molecular species, the amounts of which are affected by growth conditions.

The data from tandem MS, reported earlier (15,27), suggested that these different ISPL were compounds mainly of a C_{16}-dihydrosphingosine LCB linked *via* a hexacosanoyl (16:0) or eicosanoyl (20:0) or docosanoyl (22:0) or hydroxydocosenoyl (22:1) amide bond (Fig. 2).

ISPL produced by *P. parasitica* Dastur are not glycosylated. Hydrolysis of ISPL fraction with 1 M or 6 M HCl at 100°C for 16 h or 48 h gave only inositol, identified by GC of the acetyl derivative. No sugar was found in the hydrolysate. They are similar to ISPL isolated from *P. capsici* (15). This structure seems characteristic of the genus *Phytophthora*. It is similar to ISPL from *Saccharomyces cerevisiae* (7) and *Leishmania donovani* (10).

The biological activity of the sphingophospholipids had already been previously demonstrated (14,15). They induce a

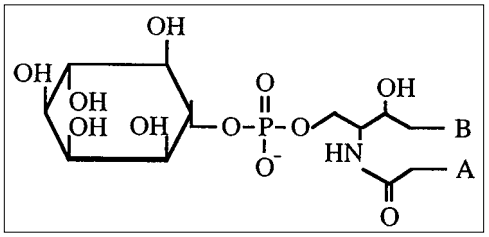

FIG. 2 . Structures of major molecular species of inositol sphingophospholipids of molecular weights 751 (B = -CH=CH-$(CH_2)_{10}$-CH_3; A = -$(CH_2)_{14}$-CH_3), 807 (B= -CH=CH-$(CH_2)_{10}$-CH_3; A = -$(CH_2)_{18}$-CH_3), 835 (B= -CH=CH-$(CH_2)_{10}$-CH_3; A = -$(CH_2)_{20}$-CH_3) and 849 (B= -CH=CH-$(CH_2)_{10}$-CH_3; A = -CH=CH-CH(OH)-$(CH_2)_{17}$-CH_3).

protective response by cotyledons of young peppers against necrotic lesions caused by the pathogen *P. capsici*. They are good models for studies in host–parasite interactions.

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