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The fatty acid composition of *Plasmopara halstedii* and its taxonomic significance

Otmar Spring and Klaus Haas
Institute of Botany, University of Hohenheim, 70593 Stuttgart, Germany (Phone: +7114593811; Fax: +7114593355; E-mail: spring@uni-hohenheim.de)

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Abstract

The fatty acid composition of sunflower downy mildew, *Plasmopara halstedii,* was compared to other parasitic Oomycetes and to pathogenic fungi associated with sunflower by gas chromatographic analysis. *P. halstedii* revealed a characteristic profile differing from all other samples by the dominance of 5Z,8Z,11Z,14Z,17Z-eicosapentaenoic acid. The infraspecific variation between the isolates of sunflower downy mildew was low: no correlation was visible between fatty acid profiles and isolates of different pathotypes or from different geographic origins. Lipids from hyphae, zoosporangia and oospores of the pathogen contained the same fatty acids, though the content of 5Z,8Z,11Z,14Z,17Z-eicosapentaenoic acid was significantly lower in oospores. The taxonomic and diagnostic value of the fatty acid profile in *P. halstedii* is discussed.

Introduction

The hyphae and reproductive structures of the Oomycete *Plasmopara halstedii*, the causal agent of sunflower downy mildew, contain large numbers of lipid vesicles (Nishimura, 1922; Spring, 2000; Spring and Zipper, 2000). These are most obvious in the ooplast, the central structure of the oospore of many Oomycetes (Dick, 1995), where accumulated lipid accounts for more than three quarters of the total volume. For the obligate pathogen this seems to be the most important endogenous reserve to fuel oospore germination and to energize the short period between zoospore formation and infection of a new host. Despite this important role in the life cycle of *P. halstedii*, no information is available on the chemical composition of these lipids. Furthermore, fatty acid composition has long been considered to be a good phylogenetic marker (Erwin, 1973) which might play an important role in the species concept of Oomycetes (Hall, 1996). Numerous reports have shown the usefulness of lipid profiling in chemotaxonomic studies of cyanobacteria (Romano et al., 2000; Cohen et al., 1995), pathogenic bacteria (Wells et al., 1993), microalgae (Volkman et al., 1991), yeasts (Cottrell et al., 1986; Viljoen et al., 1989) and other organisms (Lösel, 1988; Wood, 1988). The current study is focused on the identification of the fatty acid composition of *P. halstedii,* its consistency in different developmental stages of the pathogen, and its potential diagnostic value in comparison to closely related Oomycetes and pathogenic fungi associated with sunflower.

Materials and methods

Strains

Isolates of *P. halstedii* were collected from commercial sunflower fields. They were propagated on sunflower seedlings and differentiated for physiological races (Rozynek and Spring, 2000). Sporangia of other Oomycetes were harvested from infected wild plants by means of a small suction device, in order to avoid contamination with host tissue. Samples are deposited in the herbarium of the author.

Table 1. Pathotype differentiation and origin of the strains analyzed of *P. halstedii*

Strain	Pathotype	Origin
Ph1-97/B8	300	Groß-Gerau, Germany; single spore strain isolated from Ph1-97
Ph1-97	330	Groß-Gerau, Germany
Ph9-98	330	Leinfelden, Germany
Ph1-00	703	INRA, France
Ph5-95	710	Unterjesingen, Germany
Ph4-93/A11	710	Fargo, USA; single spore strain isolated from Ph4-93
Ph1-94/C13	730	Eckartsweier, Germany; single spore strain isolated from Ph1-94
Ph5-96	730	Gerolzhofen, Germany

Other microorganisms were either strains obtained from collaborating laboratories or were isolated by the authors from surface infections of germinating sunflower seeds and were purified and cultivated on malt agar (1.5% agar, 0.2% peptone, 2% Maltzin, Diamalt GmbH, München): *Albugo candida* (Pers. ex Hook.) Kuntze, isolated from *Capsella bursa-pastoris*; *Alternaria alternata* (Fr.) Keissl, isolated from sunflower seeds; *Botrytis cinerea* Pers. ex Nocca & Balbis, isolated from sunflower seeds; *Fusarium oxysporum* f. spec. *orthoceras*, provided by J. Sauerborn, Inst. 380 University of Hohenheim; *Phomopsis helianthi* Munt.-Cvet. (strain H_0), provided by A. Heller, Inst. of Botany, University of Hohenheim; *Peronospora dipsaci* Tul. ex De By., isolated from *Dipsacus sylvestris*; *Plasmopara pusilla* (De By.) Schroet., isolated from *Geranium pratense*; *Plasmopara viticola* (Berk.&Curt. ex De By.) Berl.&De Toni, isolated from *Vitis vinifera*; *Pythium aphanidermatum*, provided by H.-U. Seitz, ZMBP, University of Tübingen, Germany; *Sclerotinia sclerotiorum* (Lib.) De By., provided by A. Heller, Inst. of Botany, University of Hohenheim. The strains of *P. halstedii* (Farl.) Berl. & De Toni used in this study are listed in Table 1. Zoosporangia are deposited in the collection of O. Spring.

Extraction of lipids, preparation of methyl esters and gas chromatography

The extraction of the lipids with $CHCl_3$/MeOH (2 : 1, v : v) and the preparation of fatty acid methyl esters by means of BF_3/MeOH (14%) was carried out according to standard procedures (Christie, 1984; Morrison and Smith, 1964). Complete extraction of lipids from ca. 10 mg sample material was achieved by 2 × 1 ml $CHCl_3$/MeOH (2 : 1, v : v) within 3 h. Extracts were taken to dryness in a stream of nitrogen. Subsequent transmethylation was carried out with BF_3/MeOH (14%, w : w) at 95 °C for 30 min. The fatty acid methyl esters were analysed by GC on a Shimadzu GC-17A gas chromatograph, equipped with a Varian-Chrompack CP-Sil 8 CB capillary column (25 m × 0.32 mm), on-column injector and FID. Operating features of the chromatograph were: detector temperature, 350 °C; flow rate of helium carrier gas, 25 ml/min. The column temperature was initially set at 160 °C for 2 min and subsequently increased by 8 °C/min to 340 °C and maintained at this temperature until separation was complete. Identification of fatty acid methyl esters was based on the comparison of retention times with known standards of C14 : 0 (myristic acid), C16 : 0 (palmitic acid), C18 : 0 (stearic acid), C18 : 1 (oleic acid), C18 : 2 (linoleic acid), 20 : 0 (arachidic acid) and C22 : 0 (behenic acid). The identification of C18 : 1, C20 : 5 (5Z,8Z,11Z,14Z,17Z-eicosapentaenoic acid), C22 : 1 (docosenoic acid) and C24 : 1 (tetracosenoic acid) was established by GC/MS analysis on a Perkin-Elmer 8420 gas chromatograph (DB 5MS column, 30 m × 0.25 mm) coupled to a Finnigan MAT ITD 800 (EI 70 eV, pos. CI MeOH).

Results

Fatty acid composition in different developmental stages of P. halstedii

Sporangiophores and zoosporangia, the only cellular structures of sunflower downy mildew that can be isolated from the surface of its host without significant contamination through plant tissue, were used for fatty acid analysis. Employing a recently described method (Spring and Zipper, 2000), it was also possible to isolate and purify several hundred mature oospores from the pith parenchyma of systemically infected sunflower seedlings. Profiles of the fatty acids from sporangiophores, zoosporangia and oospores revealed similar compositions. Besides the more common fatty acids like palmitic acid (C16 : 0), stearic acid (C18 : 0), oleic acid (C18 : 1), linoleic (C18 : 2), arachidic acid (C20 : 0) and behenic acid (C22 : 0), significant amounts of myristic acid (C14 : 0), eicosenoic

(20 : 1) and particularly of 5Z,8Z,11Z,14Z,17Z-eicosapentaenoic acid (C20 : 5) were detected (Table 2). The latter compound dominated the profiles of sporangiophores and zoosporangia with more than 25% of the total peak area. Oospores differed from the two other samples with a considerably lower amount of C20 : 5 (ca. 5% of total peak area). In contrast, the relative amount of C14 : 0, C18 : 0 and C20 : 0 was highest in oospores, while the ratio of the other compounds was more or less similar in all three samples.

Infraspecific variation in fatty acid profiles

Eight strains belonging to 5 different physiological races (pathotypes) of *P. halstedii* were tested for the consistency of fatty acid composition. Zoosporangia of all samples showed similar profiles except for some minor variations in the proportion of the compounds (Table 3). There was no correlation of the fatty acid profiles with the virulence behaviour of the strains (which is usually used to differentiate between physiological races; Tourvieille et al., 2000) or with their geographical origin. Thus isolates from sunflower fields in South Germany could not be distinguished from those of France (strain Ph1-00) or the US (strain Ph3-94/A11).

Characteristic features of the fatty acid composition of sunflower downy mildew

The fatty acid profile of *P. halstedii* differed significantly from that of its host plant, *H. annuus* (Table 4). Besides the high proportion of myristic acid (C14 : 0) and docosenoic acid (C22 : 1), the occurrence of 5Z,8Z,11Z,14Z,17Z-eicosapentaenoic acid (C20 : 5) distinguished the pathogen from sunflower.

Eicosapentaenoic acid was detected in the other Oomycetes as well, although in much smaller amounts. Similarity of the fatty acid profile of *P. halstedii* with that of the other Oomycete samples was also seen in the occurrence of myristic acid, arachidic acid, behenic acid and the smaller amounts of linoleic acid.

In contrast, other pathogenic organisms frequently associated with sunflower differed significantly in their fatty acid profile. Myristic acid and fatty acids with 20 or more carbons were generally rare in fungi and none of these samples contained eicosapentaenoic acid.

Table 2. The fatty acid composition (ratio of total peak area) of *P. halstedii* (strain Ph1-97, pathotype 330)

	Fatty acid									
	C14 : 0	C16 : 0	C18 : 0	C18 : 1	C18 : 2	C20 : 0	C20 : 1	C20 : 5	C22 : 0	C22 : 1
Sporangiophores	2.3	19.2	1.0	12.9	20.2	0.2	4.0	26.6	1.0	4.8
Zoosporangia	2.3	20.3	0.7	14.6	21.0	0.2	2.3	26.2	0.9	4.6
Mature oospores	5.5	23.3	5.4	12.6	28.0	1.5	7.4	4.9	1.9	6.2

Fatty acids are designated as number of carbon atoms : number of double bonds.

Table 3. The fatty acid composition (ratio of total peak area) of zoosporangia from field isolates of *P. halstedii* differing in pathotype (indicated in brackets) and geographic origin (as listed in Table 1)

	Fatty acid									
	C14 : 0	C16 : 0	C18 : 0	C18 : 1	C18 : 2	C20 : 0	C20 : 1	C20 : 5	C22 : 0	C22 : 1
Ph1-97/B8 (300)	2.9	17.9	0.3	10.4	22.0	tr	1.6	34.6	0.7	4.8
Ph1-97 (330)	2.3	20.3	0.7	14.6	21.0	0.2	2.3	26.2	0.9	4.6
Ph9-98 (330)	2.3	19.2	0.9	11.5	22.7	tr	1.7	31.6	0.7	3.1
Ph1-00 (703)	1.9	15.5	0.6	5.5	25.6	0.2	1.6	35.6	0.6	3.0
Ph5-95 (710)	1.8	19.6	2.1	11.0	24.1	0.4	2.6	26.4	0.8	3.3
Ph3-94/A11 (710)	2.0	16.4	0.8	15.8	19.5	tr	1.8	27.5	0.6	3.0
Ph1-94/C13 (730)	1.0	17.2	1.0	9.3	22.3	0.3	1.6	35.0	0.9	3.5
Ph5-96 (730)	1.0	13.4	1.1	9.9	18.5	0.2	1.7	30.3	0.8	2.8
Mean value	1.9	17.4	0.9	11.0	22.0	tr	1.9	30.9	0.8	3.9
Standard deviation	0.6	2.3	0.5	3.2	2.3		0.4	3.9	0.1	3.9

tr = trace amounts (<0.1%).

Table 4. The fatty acid composition (ratio of total peak area) of Oomycetes and of pathogenic fungi associated with sunflower

	Fatty acid									
	C14 : 0	C16 : 0	C18 : 0	C18 : 1	C18 : 2	C20 : 0	C20 : 1	C20 : 5	C22 : 0	C22 : 1
Oomycetes										
Albugo candida	4.7	6.5	30.1	16.7	18.0	3.0	tr	0.7	3.7	tr
Peronospora dipsaci	4.1	14.9	10.4	8.0	8.5	0.5	2.0	6.4	1.0	4.5
Plasmopara halstedii (mean)	1.9	17.4	0.9	11.0	22.0	0.6	1.9	30.9	0.8	3.9
Plasmopara pusilla	1.7	16.6	9.9	13.4	19.7	0.4	0.5	4.8	0.4	1.6
Plasmopara viticola	tr	9.8	5.3	10.7	24.9	4.6	1.0	0.9	9.9	—
Pythium aphanidermatum	9.5	37.6	3.1	6.3	6.0	6.1	3.0	0.2	3.1	5.8
Pathogenic fungi associated with sunflower										
Alternaria alternata	—	16.5	3.5	26.3	50.5	—	—	—	—	1.0
Botrytis cinerea	—	13.5	2.5	46.6	36.5	—	—	—	—	—
Fusarium oxysporum	tr	22.3	7.1	22.5	37.5	—	—	—	2.7	—
Phomopsis helianthi H_0	0.7	28.6	7.8	25.2	33.1	0.4	tr	—	0.5	—
Sclerotinia sclerotiorum	0.5	25.6	4.3	18.0	44.2	—	—	—	—	—
Host plant										
H. annuus HA89 pericarp	tr	9.6	8.7	22.6	44.1	0.5	—	—	1.0	—

tr = trace amounts (<0.1%); — = not detected.

Discussion

In contrast to the wealth of lipid data compiled from taxa of almost all groups of microorganisms, relatively few investigations have been published on lipids of Oomycetes. Lösel (1988) reviewed the data for the Saprolegniales and Leptomitales. With the exception of *Phytophthora* and *Pythium*, the lack of data from the Peronosporales is explained by the inability to grow taxa of this order on synthetic media, thus causing difficulties to access sufficient amounts of material for fatty acid analysis. However, the present study has shown that certain structures like zoosporangia, sporangiophores and oospores of obligate parasitic Peronosporales are accessible from infected plants in amounts sufficient for such analysis.

Comparison of the fatty acid profiles indicated a clear distinction of the Oomycetes from the investigated taxa of the Eumycota. This was most obvious in the occurrence of 5Z,8Z,11Z,14Z,17Z-eicosapentaenoic acid. Similar results were reported for Oomycetes of the Saprolegniales (Lösel, 1988). Whether the failure to find this compound in previously investigated Peronosporales of the genera *Phytophthora* and *Pythium* results from methodological differences or from the fact that these strains were cultivated on synthetic media remains unclear. In this context it should be noted that the only species of the current study not isolated from host tissue was *Pythium aphanidermatum*, and it showed the lowest concentration of this fatty acid. To what extent the host nutrition could influence the fatty acid profile of the parasitic Oomycetes has not yet been investigated.

Except for some Chytridiomycetes, no group of the fungi has been reported to contain 5Z,8Z,11Z,14Z,17Z-eicosapentaenoic acid. However, this fatty acid was obtained from heterocontophytic algae like the Bacillariophyceae, Chrysophyceae, Xanthophyceae or Phaeophyceae (Wood, 1988). Together with the characteristic sterol composition (Lösel, 1988) and numerous other taxonomically relevant characters, including 18S-rRNA data (Van de Peer and de Wachter, 1997), this underlines the close phylogenetic relationships of the Oomycetes with stramenopilate algae.

Among the Oomycetes investigated, there was considerable variation with respect to the relative proportion of fatty acids, thus prohibiting further taxonomic interpretation within this group until many more taxa have been studied. However, in *P. halstedii* the fatty acid composition appears to be species specific with a relatively low degree of variation in the different developmental stages. The differences observed in the profile of oospores may be the result of intensive metabolic rearrangements during the maturation process (Beakes, 1980). For zoosporangia, neither the geographic origin nor the virulence of the tested strains affected the profiles significantly. Thus, together with the host specificity, the fatty acid composition could be a good diagnostic marker for the classification of species in

obligate biotrophic downy mildews which often lack good morphological characters for differentiation.

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