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Methoxylated fatty acids in *Blumeria graminis* conidia

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Abstract

The total fatty acids (FA) composition of *Blumeria graminis* f.sp. *tritici* conidia, the causal agent of wheat powdery mildew, was analyzed as a function of their age. A total of 19 FA (C_{12}–C_{24} saturated and unsaturated) and unusual methoxylated fatty acids (mFA) were detected in young, intermediate and old conidia. Two very long chain methoxylated FA were identified by GC–MS as 3-methoxydocosanoic and 3-methoxytetracosanoic acids. Medium chain FA were predominant in young conidia (75%, including 13% of mFA) while very long chain fatty acids constituted the major compounds in old conidia (74%, including 30% of mFA). We have shown for the first time that the total FA composition is strongly correlated with the age of *B. graminis* f.sp. *tritici* (*Bgt*) conidia.

Keywords: *Blumeria* (Syn. *Erysiphe*) *graminis*; Blumeriaceae; Powdery mildew; Fatty acids; Methoxylated fatty acids

1. Introduction

Methoxylated FA are unusual and have been described only in a few organisms. For example, 10-methoxyoctadecanoic, 11-methoxy octadecanoic, 12-methoxyeicosanoic and 13-methoxyeicosanoic acid were detected in the bacterium *Thiobacillus* (Kerger et al., 1986). *Helicobacter pylori* also contains two mFA, 11-methoxyheptadecanoic and 13-methoxynonadecanoic acid (Inamoto et al., 1995). 2-Methoxy-5-hexadecenoic and 2-methoxy-6-hexadecenoic acids were identified in marine sponges (Ayanoglu et al., 1983; Carballeira et al., 1990; Carballeira and Sepulveda, 1992), whereas 7-methoxytetradec-4-enoic acid was identified from the cyanobacterium *Lyngbya majuscula* (Praud et al., 1993). In total lipids of the red alga, *Schizymenia dubyi*, four mid-chain mFA were identified as 9-methoxypentadecanoic, 9-methoxyheptadecanoic, 13-methoxy heneicosanoic and 15-methoxytricosanoic acids (Barnathan et al., 1998). The mFA seem to be unusual and, to our knowledge, have never been described from fungi. On the other hand, such compounds have been occasionally reported from different organisms as artifactual (Lough, 1964; Minnikin and Polgar, 1967; Fulk and Shorb, 1970; Orgambide et al., 1993; Navarro et al., 1997). The major fatty acids found in the present study of *Bg* were long chain FA, as reported previously (Tulloch and Ledingham, 1960; Johnson et al., 1976) and particularly, unusual *trans*-2,3 long chain monoenoic acids (Senior et al., 1993, 1995). Because of the existence of these

Abbreviations: Bg, Blumeria graminis; Bgt, Blumeria graminis f.sp. tritici; Bgh, Blumeria graminis f.sp. hordei; f.sp., formae specialis; FA, fatty acids; mFA, methoxylated fatty acids; FAMEs, fatty acids methyl esters; mFAMEs, methoxylated fatty acids methyl esters

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trans-2,3 long chain monounsaturated acids, unusual 3-methoxy FA were considered as possibly artifactual. However, using deuterated methylation reagents, we have demonstrated the presence of new and non-artifactual 3-methoxy FA in *Blumeria graminis*.

2. Results and discussion

Table 1 shows that the FA composition of old *Bgt* conidia after methylation with BF_3/MeOH, BF_3/CD_4O or CH_2N_2 are similar. Nineteen FA were detected, very long chain acids (C_{22} and C_{24}) being the major ones. 3-Methoxydocosanoic and 3-methoxytetracosanoic (Fig. 1), representing about 30% of the total FA composition, were identified by comparison with data of Ryage and Stenhagen (1960). Both mass spectra showed a strong ion at *m/z* 75 ($C_3H_7O_2^+$, C_2–C_3 cleavage with rearrangement of two hydrogen ions) and *m/z* 117 ($CH^{\cdot}(OCH_3)CH_2COOCH_3$, C_3–C_4 cleavage), diagnostic of β mFA (Ryage and Stenhagen, 1960). The $C_{22:0\,3,OCH_3}$ showed a $[M]^+$ at *m/z* 384 accompanied by the related ions $[M-15]^+$ at *m/z* 369, $[M-30]^+$ at *m/z* 354 and $[M-73]^+$ at *m/z* 339, whereas the $C_{24:0\,3,OCH_3}$ showed a $[M]^+$ at *m/z* 412 accompanied by the related ions $[M-15]^+$ at *m/z* 397, $[M-30]^+$ at *m/z* 380 and $[M-73]^+$ at *m/z* 353. mFA were often reported as artifacts by (i) addition of CH_3OH on the hydroxyl group of hydroxy FA (Ryage and Stenhagen, 1960); (ii) addition of CH_3OH to the double bond of the natural monounsaturated FA catalyzed by high (Lough, 1964) or normal content of BF_3 (Navarro et al., 1997); (iii) ring opening of cyclopropane FA leading to the production of several mFA (Christie, 1970; Dawidowicz and Thompson, 1971; Minnikin, 1972; Orgambide et al., 1993; Lageot et al., 1994). The use of acid-(BF_3) or base-catalysts (CH_2N_2) for methylation showed a constant level in mFAMEs. Since BF_3/MeOH gave occasionally methoxy artifacts while CH_2N_2 does not (Kramer et al., 1997), the similarity of FA composition resulting from the use of both reagents seems to indicate that mFA are not artifactual. When using BF_3/CD_3OD, mass spectra showed a strong ion at *m/z* 78 and *m/z* 120, indicating that methylation by CD_3 was exclusively on the carboxylic end of the FA. We then can assert that mFA are naturally present in the total FA before derivatization. mFA identified from *Bgt* conidia are not artifactual. In addition to mFA, unusual very long chain monoenoic FA were detected. We identified these monounsaturated very long chain FA as methyl *trans*-2-docosenoate and methyl *trans*-2-tetracosenoate since the GC–MS data showed a *m/z* 113, characteristic of *trans*-α,β unsaturated methyl esters (Ryage et al., 1961). Such FA have been previously detected in *Bgh* conidia (Senior et al., 1993, 1995). Moreover, in agreement with Johnson et al. (1976), we found traces of medium chain FA like $C_{12:0}$, $C_{14:0}$ and $C_{15:0}$ in *Bgt* conidia.

Table 2 shows the qualitative and quantitative composition of young, intermediate and old *Bgt* conidia. C_{16} and C_{18} FA were well represented in young conidia while C_{22}–C_{24} were the major compounds in older ones. The proportions of very long chain methoxylated and very long chain monoenoic FA increases with the age of conidia. The very long chain/medium chain FA ratio increased from 0.5 in the young conidia to 4.2 in the

Table 1
FA composition of old *Bgt* conidia after methylation with BF_3/MeOH, BF_3/CD_3OD or CH_2N_2

Fatty acids	Old conidia		
	BF_3/MeOH	BF_3/CD_3OD	CH_2N_2
$C_{12:0}$	nd[a]	nd	Tr[b]
$C_{14:0}$	nd	nd	Tr
$C_{15:0}$	Tr	Tr	Tr
$C_{16:0}$	4.6	5.0	5.8
$C_{16:1}$	Tr	Tr	Tr
$C_{18:0}$	4.1	3.3	2.6
$C_{18:1}$	2.0	3.2	6.0
$C_{18:2}$	10.8	11.3	9.0
$C_{18:3}$	2.0	2.9	2.1
$C_{22:0}$	14.1	12.5	10.4
$C_{22:1}$[c]	5.3	4.8	2.5
$C_{22:1t}$[d]	6.4	9.2	6.3
$C_{22:0\,3,OCH_3}$	15.1	12.4	10.3
$C_{24:0}$	7.1	6.2	8.3
$C_{24:1}$[c]	5.9	5.1	5.1
$C_{24:1t}$[d]	6.4	8.3	11.6
$C_{24:0\,3,OCH_3}$	17.7	15.1	19.1

Values are percentages of total FA.
[a] nd, not detected.
[b] Tr, trace(<1%).
[c] $C_{22:1}$ and $C_{24:1}$, mixture of two methyldocosenoate and two methyltetracosenoate with unknown double-bond positions, which can be only detected by GC–MS.
[d] *trans*-2 unsaturated acids.

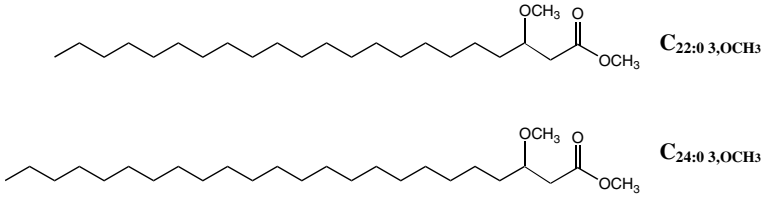

Fig. 1. Structure of 3-methoxydocosanoic and 3-methoxytetracosanoic acids.

Table 2
FA composition of young, intermediate and old *Bgt* conidia (methylation with only BF_3/MeOH)

Fatty acids	Conidia		
	Young	Intermediate	Old
$C_{12:0}$	Tr[a]	nd[b]	nd
$C_{14:0}$	Tr	Tr	nd
$C_{15:0}$	Tr	Tr	Tr
$C_{16:0}$	15.0	5.3	3.5
$C_{16:1}$	Tr	Tr	Tr
$C_{18:0}$	12.8	2.9	1.0
$C_{18:1}$	12.0	4.4	Tr
$C_{18:2}$	19.5	14	9.0
$C_{18:3}$	3.5	6.7	5.0
$C_{22:0}$	4.6	9.0	10.8
$C_{22:1}$[c]	3.5	4.3	5.0
$C_{22:1t}$[d]	2.8	7.2	9.8
$C_{22:0\,3,OCH_3}$	5.1	8.6	10.0
$C_{24:0}$	2.0	6.2	7.0
$C_{24:1}$[c]	3.4	5.7	6.0
$C_{24:1t}$[d]	3.5	11.0	15.0
$C_{24:0\,3,OCH_3}$	7.8	15.6	17.4
% monoenoic	25.2	32.6	36.3
% methoxy FA	12.9	24.2	27.4
Ratio vlc/mc FA[e]	0.5	2.0	4.2
Total content μg mg^{-1} dw	44.2	19.4	23.9

Values are percentages of total FA.
[a] Tr, trace (<1%).
[b] nd, not detected.
[c] $C_{22:1}$ and $C_{24:1}$, mixture of two methyldocosenoate and two methyltetracosenoate with unknown double-bond positions, which can be only detected by GC–MS.
[d] *trans*-2 unsaturated acids.
[e] vlc/mc, very long chain (C_{22}–C_{24})/medium chain (C_{12}–C_{18}).

older ones, indicating that the FA composition evolves with age. A majority of medium chains are present in young conidia whereas the very long chain FA are predominant in old conidia. Concomitantly the amount of total FA which is high (44.2 μg mg^{-1} dw) in young conidia decreases almost by half in intermediate and old conidia. Similar changes in the sterol composition of *Bgt* conidia were reported by Muchembled et al. (2000). The presence of such original mFA leads to two questions. Do they have specific physiological functions in relation to the age of conidia? Could they be used as biochemical markers for the identification of species of powdery mildews?

3. Experimental

3.1. Fungal material

Wheat powdery mildew was maintained on wheat seedlings of the cultivar 'Sideral', carrying no genes for resistance to powdery mildew. Plants were grown in a fitotron™ (Sanyo, P660) (photoperiod: 14–10 h; temperature d/n: 18–10 °C; RH: 90%; light: 250 μmol m^2 s^{-1}) and inoculated by spraying Bgt conidia suspension (5×10^5 conidia ml^{-1}) in Fluorinert (FC43, 3 M). Eight days after inoculation, symptoms arose and detached leaves (10 cm) were placed in square clear plastic boxes (12 × 12 cm) containing water–agar (7.5 g l^{-1}) supplemented with benzimidazole (10 mg l^{-1}) in order to prevent leaf senescence. A sheet of aluminum foil was deposited on surface of water–agar. Conidia of *Bgt* were harvested 12, 15 and 18 days after inoculation by shaking gently the leaves above the aluminum foil. The corresponding young, intermediate and old conidia were stored at −80 °C prior to freeze-drying and analysis.

3.2. Fatty acid extraction, analysis and identification

The freeze dried conidia was extracted (3×) using 3 ml of CH_2Cl_2/MeOH (2:1, v/v) at 70 °C according to Grandmougin-Ferjani et al. (1999). The total lipid extract was saponified with 1 ml of 6% (w/v) methanolic KOH for 1 h at 90 °C. After addition of 1 ml water, the non-saponifiable fraction was extracted (3×) with 3 ml of hexane prior to extraction of total FA. The aq. phase was adjusted to pH 1 with concentrated HCl. FA were extracted (3×) in 5 ml of hexane and dried over dry Na_2SO_4. After evaporation under N_2, FA were methylated using 1 ml of BF_3/MeOH (14%) according to Morrison and Smith (1964). The deuterated reagent was prepared by mixing CD_3OD with BF_3 etherate (48%) in order to get a 14% BF_3 concentration after evaporation of Et_2O under N_2. Methylation with CH_2N_2 was done according to Schlenk and Gellerman (1960). FAMEs and mFAMEs were isolated by TLC on silica gel using Et_2O/hexane (10:90, v/v) as a solvent system. Spots were visualized under UV light (350 nm) after spraying with primulin (0.1%, w/v) in aq. aceton (80%). FAMEs (R*f* 0.75) and mFAMEs (R*f* 0.45) were scraped off and eluted in CH_2Cl_2, then analyzed by a GC-FID equipped with a glass capillary column (FFAP, 30 m × 0.25 mm id, H_2 1.2 ml min^{-1}). The temperature program was: 60–160 °C (20 °C min^{-1}), 160–195 °C (1 °C min^{-1}), 195–230 °C (2.5 °C min^{-1}) and finally 230 °C for 10 min. FAMEs were quantified using heinecosanoic acid methyl ester ($C_{21:0}$) as an internal standard. GC–MS identification was performed on a Shimadzu GC17A chromatograph linked to a HS-QP5000 mass spectrometer (70 eV), using a 30 m × 0.22 mm id column coated with bpx-70. The carrier gas was helium (8 ml min^{-1}). Column temperature was programmed from 180 to 300 °C (3 °C min^{-1}). Analyses of young and intermediate conidia were duplicate, those of old conidia were triplicate.

Relative retention times on FFAP: $C_{12:0}$ 0.32; $C_{14:0}$ 0.37; $C_{16:0}$ 0.44; $C_{16:1}$ 0.46; $C_{18:0}$ 0.61; $C_{18:1}$ 0.63; $C_{18:2}$ 0.69; $C_{18:3}$ 0.77; $C_{22:0}$ 1.10; $C_{22:1}$ (mixture of two methyl docosenoates with unknown double-bond positions)

1.14; $C_{22:1t}$ 1.21; $C_{22:0\,3,OCH_3}$ 1.26, $C_{24:0}$ 1.27, $C_{24:1}$ (mixture of two methyl tetracosenoates with unknown double-bond positions) 1.32; $C_{24:1t}$ 1.42, $C_{24:0\,3,OCH_3}$ 1.50.

Relative retention times on bpx-70: $C_{12:0}$ 0.65; $C_{14:0}$ 0.73; $C_{16:0}$ 0.80; $C_{16:1}$ 0.81; $C_{18:0}$ 0.88; $C_{18:1}$ 0.89; $C_{18:2}$ 0.91; $C_{18:3}$ 0.945; $C_{22:0}$ 1.04; $C_{22:1}$ 1.055; $C_{22:1}$ 1.06; $C_{22:1t}$ 1.10; $C_{24:0}$ 1.12, $C_{22:0\,3,OCH_3}$ 1.125, $C_{24:1}$ 1.135; $C_{24:1}$ 1.14; $C_{24:1t}$ 1.18, $C_{24:0\,3,OCH_3}$ 1.20.

3.3. MS data

trans-2-Docosenoic: 352 $[M]^+$ (3), 320 (4), 278 (2), 269 (2), 255 (4), 236 (2), 222 (2), 141 (5), 127 (5), 113 (15), 97 (13), 87 (28), 74 (20), 69 (18), 57 (32), 55 (48), 43 (100), 41 (50).

trans-2-Tetracosenoic: 380 $[M]^+$ (2), 348 (3), 306 (2), 283 (3), 264 (2), 141 (5), 127 (5), 113 (14), 97 (13), 87 (35), 74 (19), 69 (18), 57 (32), 55 (75), 43 (100), 41 (80).

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