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Lipid composition of the extracellular matrix of *Botrytis cinerea* germlings ☆

Lol L.D. Cooper[a,b,*], James E. Oliver[c], E. David De Vilbiss[c], Robert P. Doss[a,b]

[a]*USDA-ARS, Horticulture Crops Research Unit, 3420 NW Orchard Avenue, Corvallis, OR 97330, USA*
[b]*Department of Horticulture, Oregon State University, Corvallis, OR 97331, USA*
[c]*USDA-ARS, Insect Chemical Ecology Laboratory, Building 007, BARC-W, Beltsville, MA 20705, USA*

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Abstract

Six simple lipid classes (mono-, di- and tri-acylglycerols, free fatty acids, free fatty alcohols and wax esters) were identified by TLC in the extracellular matrix of *Botrytis cinerea* germlings and the molecular components of each class were characterized using GC-MS. The relative amounts of fatty acids and fatty alcohols within each lipid class were determined by GC-FID. Over all the lipid classes, the most abundant saturated fatty acids were palmitic (ca. 30%) and stearic acid (ca. 22%). Palmitoleic and oleic acids made up ca. 21% and 24% (respectively) of the free fatty acids, while erucic (ca. 4.1%) and linoleic (ca. 3.6%) acids were the most abundant unsaturated fatty acids in the acylglycerides. The acylglycerides also contained almost 35% long chain fatty acids (C20:0 to C28:0). Six fatty acids were identified which had odd-numbered carbon chain lengths (C15:0, C17:0, C19:0, C21:0, C23:0 and C25:0). Of these, pentacosanoic acid made up almost 14% of the fatty acids in the acylglycerides. Three methyl-branched chain fatty acids, namely isopalmitic, isoheptadecanoic and anteisopalmitic, were identified in the ECM, all in small amounts. Of the fatty alcohols identified, only palmityl and stearyl alcohols were found in the free form (ca. 57% and 43%, respectively) but arachidyl alcohol (ca. 47%) and 1-octacosanol (ca. 30%) were the most abundant fatty alcohols found in the wax ester fraction. Published by Elsevier Science Ltd.

Keywords: *Botrytis cinerea*; Scelerotiniaceae; Gray mold; Extracellular matrix; Characterization; Lipids; Fatty acids; Fatty alcohols

1. Introduction

The deuteromycete (Hyphomycete) fungus, *Botrytis cinerea* Pers:Fr. is responsible for important diseases in an extremely wide range of plants, including field and glasshouse vegetables, small berry fruits including grapes, ornamentals, bulb and corm-producing monocotyledons and forest tree seedlings (Coley-Smith, 1980). It is also responsible for the post-harvest spoilage of many fruits and vegetables during storage and transport (Cole, Dewey & Hawes, 1996). Conidia of *B. cinerea*, the primary inoculum source, first attach to the host surface by a hydrophobic interaction that is easily disrupted. After germination, the germ tubes and appressoria secrete an extracellular matrix (ECM) that acts, in part, as a strong adhesive (Doss, Potter, Chastagner & Christian, 1993; Doss, Potter, Soeldner, Christian & Fukunaga, 1995; Doss, 1999).

Extracellular matrices have been observed in association with many fungal species, including, *B. cinerea* and *B. fabae* (Cole et al., 1996), *B. ellipitica* (Doss, Christian & Chastagner, 1988; Ward, 1888), *Colletotrichum graminicola* (Nicholson & Moraes, 1980), *Phyllosticta ampelicida* (Kuo & Hoch, 1995) and *Uromyces viciae-fabae* (Clement, Butt & Beckett, 1993). The ECM are believed to be involved with adhesion of the germling or mycelium to the substrate (Epstein & Nicholson, 1994; Jones, 1994; Moloshok, Leinhos, Staples & Hoch, 1993; Moloshok, Terhune, Lamboy & Hoch, 1994; Nicholson, 1996; Sugui, Leite

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* Corresponding author.

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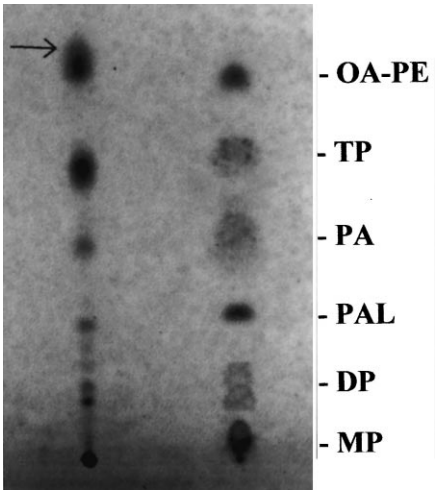

Fig. 1. TLC separation of simple lipids from the extracellular matrix of *B. cinerea* germlings. Simple lipids were extracted from ca. 1 mg ECM and separated by TLC (left) along with a mixture of simple lipid standards (right). Standards: MP — monopalmitin (100 μg), DP — dipalmitin (100 μg), PA — palmityl alcohol (1 μg), PA — palmitic acid (100 μg), TP — tripalmitin (100 μg), OA-PE — palmityl oleate (1 μg). The arrow indicates the position of the high R_f unknown material.

& Nicholson, 1998). Previous characterization of the ECM of *B. cinerea* (Doss, 1999) had shown it to be composed of carbohydrates (ca. 20%), proteins (ca. 28%) and lipids (ca. 6%). The lipid fraction of the ECM, analyzed by TLC,was found to contain free fatty acids, mono- and tri-acylglycerols, wax esters, fatty alcohols and unidentified components (no complex lipids were found). This was the first report of lipids in fungal ECM. The present work was carried out as part of an ongoing effort to determine the composition and function of ECM of *B. cinerea*. To this end, the molecular species present in each of the simple lipid classes were identified by GC-MS. The relative amounts of the fatty acids and fatty alcohols within each lipid class were determined by GC-FID.

2. Results and discussion

Extracellular matrix produced by three-day old *B. cinerea* germlings was isolated from glass slides after the fungal mycelium had been removed by a stream of water. As noted in earlier studies (Doss et al., 1995; Doss, 1999), the ECM could not be dislodged from glass slides, even with vigorous washing, but the fungal mycelium was readily removed by this treatment. This was shown conclusively (Doss, 1999) using interference contrast optics. The absence of complex lipids, which are universally present in fungal cell membranes (Weete, 1980) in the ECM fractions also indicated that

Table 1
Relative composition (area%) of fatty acids found in each of the simple lipid classes from the extracellular matrix of *B. cinerea* germlings[a]

Fatty acid	Simple lipid classes				
	Free fatty acids (FFA)†	Mono-acylglycerides (MAG)	Di-acylglycerides (DAG)	Tri-acylglycerides (TAG)†	Wax esters (fatty acid fraction) (FAWE)
14:0	n.d.[b]	2.1 (0.1)	2.1 (0.1)	1.7 (0.1)	7.3 (0.0)
15:0	n.d.	1.6 (0.0)	1.5 (0.1)	1.4 (0.1)	1.8 (0.0)
16:1	21.0 (3.6)	2.43 (0.0)	3.0 (0.3)	3.9 (0.8)	4.4 (0.1)
16:0	40.9 (9.0)	29.6 (1.2)	26.3 (3.1)	17.0 (3.5)	33.8 (0.3)
14-CH_3 C16:0	n.d.	1.5 (0.2)	2.2 (0.4)	n.d.	2.2 (0.1)
15-CH_3 C16:0	n.d.	n.d.	n.d.	1.6 (0.3)	2.4 (0.1)
17:0	n.d.	2.4 (0.1)	1.8 (0.3)	1.3 (0.2)	2.9 (0.1)
16-CH_3 C17:0	n.d.	1.1 (0.1)	1.3 (0.2)	n.d.	n.d.
18:2	n.d.	3.4 (0.3)	4.1 (0.6)	3.4 (0.5)	n.d.
18:1	24.1 (2.7)	1.6 (0.1)	0.8 (0.4)	1.3 (0.8)	n.d.
18:0	14.0 (4.7)	31.0 (0.9)	26.1 (3.7)	16.6 (3.3)	22.9 (0.1)
19:0	n.d.	1.4 (0.2)	1.5 (0.6)	1.5 (0.4)	n.d.
20:0	n.d.	1.6 (0.2)	1.6 (0.3)	3.5 (1.9)	18.0 (0.0)
21:0	n.d.	1.2 (0.3)	0.7 (0.1)	n.d.	n.d.
22:1	n.d.	3.1 (0.2)	2.2 (0.3)	7.2 (2.3)	n.d.
22:0	n.d.	7.1 (0.1)	6.4 (0.4)	8.8 (1.8)	4.4 (0.2)
23:0	n.d.	1.1 (0.1)	7.4 (3.3)	7.0 (2.2)	n.d.
24:0	n.d.	1.8 (0.1)	2.4 (0.4)	n.d.	n.d.
25:0	n.d.	3.7 (0.0)	7.7 (2.8)	13.5 (3.0)	n.d.
26:0	n.d.	1.4 (0.3)	0.9 (0.3)	6.2 (1.9)	n.d.
28:0	n.d.	2.6 (0.3)	n.d.	4.5 (0.7)	n.d.

[a] Each value is the mean (±s.e.) of three or four (†) GC-FID determinations carried out on a pooled sample of three lipid extractions.
[b] n.d. — not detected.

Table 2
Relative composition (area%) of fatty alcohols found in each of the simple lipid classes from the extracellular matrix of *B. cinerea* germlings[a]

Fatty alcohol	Simple lipid classes	
	Free fatty alcohols (FFAL)	Wax esters (fatty alcohol fraction) (FALWE)†
16:0	57.1 (7.9)	3.7 (0.9)
18:0	42.9 (7.9)	4.6 (2.0)
20:0	n.d.[b]	47.2 (11.8)
21:0	n.d.	8.7 (3.1)
22:0	n.d.	5.9 (3.7)
28:0	n.d.	29.9 (7.8)

[a] Each value is the mean (±s.e.) of three or four (†) GC-FID determinations carried out on a pooled sample of three lipid extractions.
[b] n.d. — not detected.

washing resulted in the isolation of the ECM from the fungal mycelium (Doss, 1999).

Based upon weight lost occurring as a result of the extraction of the ECM with $CHCl_3$:MeOH (2:1), lipids were estimated to comprise $6.7\% \pm 0.4\%$ (mean ± s.e. for three determinations) of the ECM. This is very similar to The value of $(6.2\% \pm 1.1)$ reported previously (Doss, 1999). Total lipids were extracted from preparations of ECM and separated into simple lipid classes by one-dimensional TLC (Christie, 1982). Lipid extract from ca. 1 mg ECM was used for each of three prep. TLC analyses. Fig. 1 illustrates the chromatographic separation of a representative sample of ECM lipids in comparison to a mixture of simple lipid standards. Six lipid classes were identified in the ECM based upon their comigration with standard compounds. The lipid classes in the ECM were designated free fatty acids (FFA), mono- (MAG), di- (DAG) and tri-acylglycerols (TAG), free fatty alcohols (FFAL) and wax esters (WE). The WE class was made up of two fractions, fatty acids (FAWE) and fatty alcohols (FALWE). In addition there was one spot observed on TLC plates (indicated by the arrow) that did not comigrate with any of the simple lipid standards, or with any other standards available. This material was designated the high R_f unknown.

In the preliminary characterization of the ECM components by Doss (1999), diacylglycerides were not detected in the ECM, whereas in this study, they were easily identified by TLC. This may have been a function of the amount of ECM lipid extract applied to the plate, as in this study lipid extract from ca. 1 mg ECM was applied to the plate, whereas in the previous work (Doss, 1999) only ca. 200 μg ECM was used.

Most of the simple lipid classes identified in the ECM have been reported to occur in various structures of other fungi (Weete, 1980). Triacylglycerols are commonly produced as storage reserves of energy and carbon skeletons for growth and development since they are twice as efficient for metabolic energy than either proteins or carbohydrates. Diacylglycerols are present in trace amounts in fresh animal and plant tissues, and may be intermediates in the synthesis of triacylglycerols (Christie, 1989). Fatty alcohols have been identified as components of wax esters in fungi (Clark & Watkins, 1978) and in the free form in the extracellular environment of certain bacteria (Boulton, 1989), but free fatty alcohols in fungi were only recently reported for the first time by Doss (1999).

The fatty acid (FA) composition of the simple lipid classes was more complex (Table 1) than expected, considering what is known about the FA composition of fungal lipids (Weete, 1980; Harwood & Russell, 1984). The most abundant saturated FAs in all classes were palmitic (C16:0, ca. 30%), and stearic acid (C18:0, ca. 22%). There was a relatively a high proportion of free palmitoleic (C16:1, ca. 21%) and oleic (C18:1, ca. 24%) acids; whereas in the MAG, DAG, TAG classes and the FAWE fraction, the palmitoleic (ca. 3%) and oleic acids (ca. 2%) comprised a much smaller proportion of the total FAs. Unlike the FFA or the FAWE, the acylglycerides contained ca. 4% of each of the unsaturated FAs linoleic acid (C18:2) and erucic acid (C22:1). Also somewhat unusual is the proportion of long chain fatty acids (C20:0 to C28:0) found in the MAG (ca. 24%), DAG (ca. 29%) and TAG (ca. 51%) classes. Generally, very long chain fatty acids (>C20:0) are a minor component of the fungal fatty acids (Harwood & Russell, 1984), although conidia of *Erysiphe graminis* were found to contain a high proportion (90%) of long chain FAs (Senior, Hollomon & Holloway, 1993), and an albino mutant of *Monascus purpureus* has been reported to contain 36% long chain FAs (Juzlova, Rezanka, Martinkova & Kren, 1996). Six fatty acids were identified which had odd-numbered carbon chain lengths (C15:0, C17:0, C19:0, C21:0, C23:0 and C25:0). Of these, the pentacosanoic (C25:0) acid made up almost 14% of the fatty acids in the acylglycerides.

Three different methyl-branched chain fatty acids were identified in the ECM: isopalmitic (15-CH_3 C16:0), isoheptadecanoic (16-CH_3 C17:0) and anteiso-

palmitic (14-CH_3 C16:0). Isopalmitic acid was found in small amounts (ca. 2%) in the TAGs and FAWEs. There was ca. 1% isoheptadecanoic acid in the MAG and DAG classes. Anteisopalmitic acid was found in the MAGs, DAGs and FAWEs (ca. 2%). Methyl-branched chain fatty acids are common in bacteria (Weete, 1980), and have also been known to occur in fungi, for example *Pithomyces chartarum*, *Cylindrocarpon radicicola* and *Stemphylin dendriticum* (Hartman, Hawke, Morice & Shorland, 1960; Hartman, Morice & Shorland, 1962).

Fatty alcohols were identified in the ECM in both the free form (FFAL) and esterified to fatty acids in the WE class (FALWE) (Table 2). Of the fatty alcohols identified, only palmityl (C16:0) and stearyl (C18:0) alcohols were found in the free form (ca. 57% and 43%, respectively). The FFAL class also contained appreciable amounts of FAs, primarily palmitic and stearic acids. This may have been a result of FAs comigrating with the FFALs during TLC separation. A similar situation was observed when the palmityl alcohol standard was eluted from a mixture of standards separated by TLC. After derivatization and GC-MS analysis, we observed a considerable amount of palmitic acid in the standard as well.

The FALWE fraction contained approximately 47% arachidyl alcohol (C20:0) and 30% octacosanol (C28:0). Wax esters composed of long chain fatty acids and fatty alcohols (C28:0 to C36:0) constitute a major non-glyceride portion of fungal lipids (Weete, 1980). We also observed a considerable amount of FAs in the FALWE fraction. The FAs may not have been completely separated from fatty alcohols during partitioning of the hydrolyzed wax ester. In the FALWE, most of the FA found in the sample was behenic (C22:0). In a separate GC-MS analysis, we detected that the OA-PE standard was contaminated with a number of fatty acids. Carryover of fatty acids, primarily palmitic and oleic acids into the palmityl alcohol fraction (as a result of incomplete partitioning) was observed when the wax ester standard (palmityl oleate) was subjected to chromatographic separation, elution, derivatization and GC-MS analysis.

One spot on the TLC plate did not correspond to any of the simple lipid standards tested (including wax ester or cholesterol oleate). The R_f of the unidentified material was slightly higher than those of the wax ester unknowns and standards. Although the spots on the TLC plate shown in Fig. 1 were not well resolved, they, in fact, represent two different fractions. The separation is more apparent when there is less lipid extract loaded on the TLC plates, as in Doss (1999). The high R_f unknown was hydrolyzed and the saponifiable fraction was partitioned from the diethyl ether-soluble nonsaponifiable components and methylated. It was found to contain a variety of FAs, the most abundant one being the branched chain FA, anteiso-palmitic and appreciable amounts of palmitic and stearic acids. Almost half of the FAs were 20 carbons in length or longer. TMSi derivatives for GC-MS analysis were made from the nonsaponifiable fraction and it was found to contain a mixture of FAs and FALs. The most abundant FA was pentacosanoic acid, with palmitic, stearic and behenic acids as well. The following FALs were found in the mixture; behenyl, heneicosanyl (C21:0), palmityl, stearyl and arachidyl. Based upon the migration of this material on TLC and the analysis of its chemical composition, we have concluded that it is probably neither a sterol ester nor a wax ester.

3. Experimental

3.1. Fungal culture

An isolate of *B. cinerea* (Bc-1) used in earlier studies was cultured on potato dextrose agar as described previously (Doss et al., 1993; Doss, 1999). This isolate has been provided to the American Type Culture Collection (accession number 204446).

3.2. Collection of ECM

Extracellular matrix preparations were obtained as described in Doss (1999), with the following modifications. Glass microscope slides inoculated with 1.5 ml conidial suspension (in 0.1 × potato dextrose broth) were incubated at 18–20°C and 100% relative humidity under a near UV light (Doss et al., 1993). Isolation of the ECM was accomplished as described previously (Doss, 1999). Samples for lipid analysis were dried in vacuo and stored at −20°C. Dried ECM preparations were weighed using a Cahn-31 microbalance.

3.3. Extraction of lipids

Total lipids were extracted using $CHCl_3$:MeOH (2:1) (Christie, 1989). Extracted lipids were taken to dryness at 40°C with a stream of N_2 and reconstituted in 25 μl $CHCl_3$. Lipid extracts, if not analyzed immediately, were stored at −20°C under N_2 until required. All solvents used in the extraction, separation, elution and chromatographic steps contained 50 ppm butylated hydroxytoluene (BHT) as an antioxidant (Christie, 1989).

3.4. Lipid separations

Prep. TLC was carried out in one dimension using silica gel plates (150 Å, 250 μm layer thickness). Lipids extracted from ca. 1 mg ECM were spotted onto TLC

plates that had been prewashed with $CHCl_3$:MeOH (1:1). Simple lipid standards were spotted alongside the extract. Separation of the simple lipid classes was achieved using a hexanes:Et_2O:HCO_2H (80:20:2) solvent system. After development, the lanes containing the standards were removed (by breaking the plate) from the TLC plate and visualized by spraying with H_2SO_4:MeOH (1:1) and charring. The locations of simple lipid classes were identified by comparison with the migration of the charred standards. After scraping the separated ECM lipid fractions from the TLC plates, they were eluted from the silica gel with EtOAc. Eluted lipids were taken to dryness at 40°C with a stream of N_2 and reconstituted in 25 μl CH_2Cl_2. Extracts not analyzed immediately were stored at −20°C under N_2 until needed. Standard compounds (monopalmitin, dipalmitin, tripalmitin, palmityl oleate and palmityl alcohol) were eluted from prep. TLC plates after separation and derivatized for GC-MS analyses.

3.5. Preparation of derivatives

Fatty acid methyl esters were prepared from free fatty acids (FFA) and from fatty acid fraction (FAWE) of the wax esters by reacting with ethereal CH_2N_2. Mono-, di- and tri-acylglycerols (MAG, DAG, TAG) were transesterified using 5% methanolic HCl (Christie, 1989). Before GC-MS analysis, the transesterified samples were treated with ethereal CH_2N_2 to ensure all FAs were in the methyl ester form. Wax esters were hydrolyzed by refluxing with 1 M KOH in 95% EtOH. The fatty alcohol fraction (FALWE) was partitoned from the hydrolyzate by diethyl ether extraction and derivatized. The hydrolyzate was then acidified and the fatty acids were partitioned out by diethyl ether extraction and methylated (Christie, 1989). TMSi derivatives of FFAL and the FALWE fraction were prepared using TMSCl:HMDS:pyridine (1:3:9). A second derivatization step was carried out using 1% TMSCl in BSTFA before GC-MS analysis.

3.6. Gas chromatography

The derivatized lipid samples were analyzed on an SPB-1 WCOT, capillary column (30 m, 0.25 mm i.d., 0.25 μm film thickness) with an FID (300°C), connected to an HP Chemstation (Version 3.02) integrator. The operating conditions were as follows; 1 μl injections in CH_2Cl_2 (splitless, inlet temperature 280°C) using He carrier gas at 3.7 ml min^{-1} (temp. program: 150°C for 4 min, increased 4°C min^{-1} to 300°C and held for up to 20 min). Individual fatty acid methyl esters and TMSi ethers were identified by comparison with authentic standards. Initially, three independent sets of simple lipid derivatives (one set from each of three prep. TLC analyses) were analyzed independently using GC-FID. After determining that all three preparations yielded similar fatty acid and fatty alcohol profiles, the three sets were pooled in order to have sufficient sample for mass spectral analysis. Relative mass% of the components of each simple lipid class was determined. Means and s.e. are based on three (MAG, DAG, FAWE, FFAL) or four (FFA, TAG, FALWE) replicate runs on the GC-FID. Unknown compounds were identified by comparing their retention times to those of authentic standards.

3.7. Mass spectrometry

Mass spectral analysis was performed on a GC-MS with a DB-5 WCOT, capillary column (60 m, 0.25 mm i.d., 0.25 μm film thickness), using a temperature program as follows: 150°C for 4 min, then increasing 7°C per minute to 280°C, and held for 40 min. Samples were introduced in hexanes using 1 μl injections (splitless, inlet temp. 250°C) using He carrier gas at 1.3 ml min^{-1} (ionization energy; 70 eV). The mass spectra were analyzed using a Wiley 275 library using HP Chemstation software (version 3.01). The mass spectra of the unknown compounds were compared to those of authentic standards for identification.

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