PERGAMON

Phytochemistry 50 (1999) 1027–1031

PHYTOCHEMISTRY

Sterol distribution in arbuscular mycorrhizal fungi

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Received 14 June 1998; revised 14 September 1998

Abstract

The sterol composition of spores from 16 species of arbuscular mycorrhizal fungi belonging to the order Glomales were examined by GC–MS. The major compound was found to be 24-ethylcholesterol (up to 85%) followed by cholesterol (up to 15%). Several other sterols such as 24-methylcholesterol, Δ5-avenasterol and 24-ethylcholesta-5,22-dien-3β-ol were also detected. Significant amounts of α-amyrin, a common vascular plant triterpene, were present in the spores of all the fungal species analyzed. The absence of ergosterol, a classical fungal sterol, is discussed in relation to fungal evolution.

Keywords: Glomaceae; Gigasporaceae; Acaulosporacea; Endomycorrhizae; Sterols; Pentacyclic triterpenoid; Taxonomy

Nomenclature

Trivial names used: cholesterol (**1**), cholest-5-en-3β-ol; 24-methylcholesterol (**2**), (24ξ)-24-methylcholest-5-en-3β-ol; 24-ethylcholesta-5,22-dien-3β-ol (**3**), 24-ethylcholesterol (**4**), (24ξ)-24-ethylcholest-5-en-3β-ol; Δ5-avenasterol (**5**), 24-ethylcholesta-5,24 (24^1)-dien-3β-ol; 24-methylenecycloartanol (**6**), 4,4,14α-trimethyl-9β, 19-cyclo-5α-ergost-24 (24^1)-en-3β-ol; α-amyrin (**7**), ursan-12-en-3β-ol; β-amyrin (**8**), olean-12-en-3β-ol.

1. Introduction

Arbuscular mycorrhizal fungi (AMF) are soil borne organisms which establish an obligate mutualistic association with the roots of about 90% of the terrestrial plants, making them the largest widespread plant symbiosis on earth.

Already identified in fossils from the early Devonian (Taylor, Remy, Haas, & Kerp, 1995; Phipps & Taylor, 1996) these organisms are classified in the order Glomales (Morton & Benny, 1990) (Zygomycetes) and subdivided in three families: Glomaceae (*Glomus* and *Sclerocystis*) (Pirozynski & Dalpé, 1989), Gigasporaceae (*Gigaspora* and *Scutellospora*) and Acaulosporaceae (*Acaulospora* and *Entrophospora*) (Morton & Benny, 1990) based on spore morphology and the intraradical structure. However the taxonomic position of AMF is still a matter of discussion (Bruns et al., 1992; Morton & Bentivenga, 1994; Rosendahl, Dodd, & Walker, 1994; Simon, 1995).

Biochemical and physiological relationships of AMF with the Zygomycetes and other fungi still remain obscure. The lipid content of lipids from these fungi may constitute a useful tool to clarify their taxonomic position. Although the fatty acid composition of these fungi is relatively well known (Beilby, 1980; Jabaji-Hare, 1988; Sancholle & Dalpé, 1993; Graham, Hodge, & Morton, 1995), little attention has been paid to sterols (Beilby, 1980; Beilby & Kidby, 1980). The present study deals with sterol analysis of different species of Glomales in order to highlight some aspects of the systematics and phylogeny of these organisms.

2. Results and discussion

The sterol composition of 16 species of AMF analyzed in the present study, including representatives of Glomaceae, Gigasporaceae and Acaulosporaceae, is shown in Table 1. Free and esterified sterols were quantified together and identification of different sterol structures was made by GC–MS. The number of sterols detected ranged from five to fifteen depending on the species. The main Δ5-sterols identified were 24-ethylcholesterol (**4**), cholesterol (**1**), 24-methylcholesterol (**2**), 24-ethyl-

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0031-9422/99/$ - see front matter
PII: S0031-9422(98)00636-0

Table 1
Relative sterol content (%) of arbuscular mycorrhizal fungi

	Sterols[a]								
Strains	1	2	3	4	5	6	7	8	9
Glomaceae									
Glomus aggregatum									
Spores and hypha	7	Tr	Tr	44	–	–	22	Tr	27
Glomus borealis									
Spores	–	Tr	–	70	–	–	10	Tr	20
Glomus caledonium									
Matures spores	Tr	Tr	–	72	–	–	15	Tr	13
Glomus clarum									
Mature spores	Tr	Tr	Tr	79	Tr	–	21	Tr	Tr
Glomus etunicatum									
Mature spores	9	Tr	Tr	74	Tr	–	17	Tr	–
Glomus fasciculatum									
Mature spores	3	Tr	Tr	72	Tr	10	10	Tr	5
Glomus intraradices									
Young spores	7	Tr	–	76	–	–	17	–	–
Mature spores	7	–	–	77	–	–	14	–	2
Hypha	9	–	–	52	–	–	19	–	20
Glomus lamellosum									
Mature spores	4	Tr	–	48	Tr	–	22	–	26
Glomus macrocarpum									
Mature spores	Tr	Tr	Tr	85	–	–	11	Tr	4
Glomus mosseae									
Young spores	3	Tr	Tr	65	–	–	16	–	16
Spores and peridium	13	15	–	56	Tr	–	16	Tr	–
Glomus pustulatum									
Mature spores	15	–	Tr	80	–	–	5	–	–
Glomus tortuosum									
Young spores	8	–	–	60	–	–	17	–	15
Mature spores	Tr	–	–	66	–	–	12	–	22
Glomus versiforme									
Mature spores	Tr	Tr	Tr	67	Tr	–	33	Tr	Tr
Glomus vesiculiferum									
Mature spores	–	Tr	Tr	77	Tr	9	9	Tr	5
Gigasporaceae									
Gigaspora magargarita									
Mature spores	Tr	Tr	Tr	77	Tr	–	22	Tr	Tr
Acaulosporaceae									
Acaulospora scrobiculata									
Mature spores	Tr	Tr	Tr	84	Tr	–	10	Tr	5

[a]Sterols, 1: cholesterol, 2: 24-methylcholesterol, 3: 24-ethylcholesta-5,22-dien-3β-ol, 4: 24-ethylcholesterol, 5: Δ5-avenasterol, 6: 24-methylenecycloartanol, 7: α-amyrin, 8: β-amyrin, 9: nonidentified sterols.

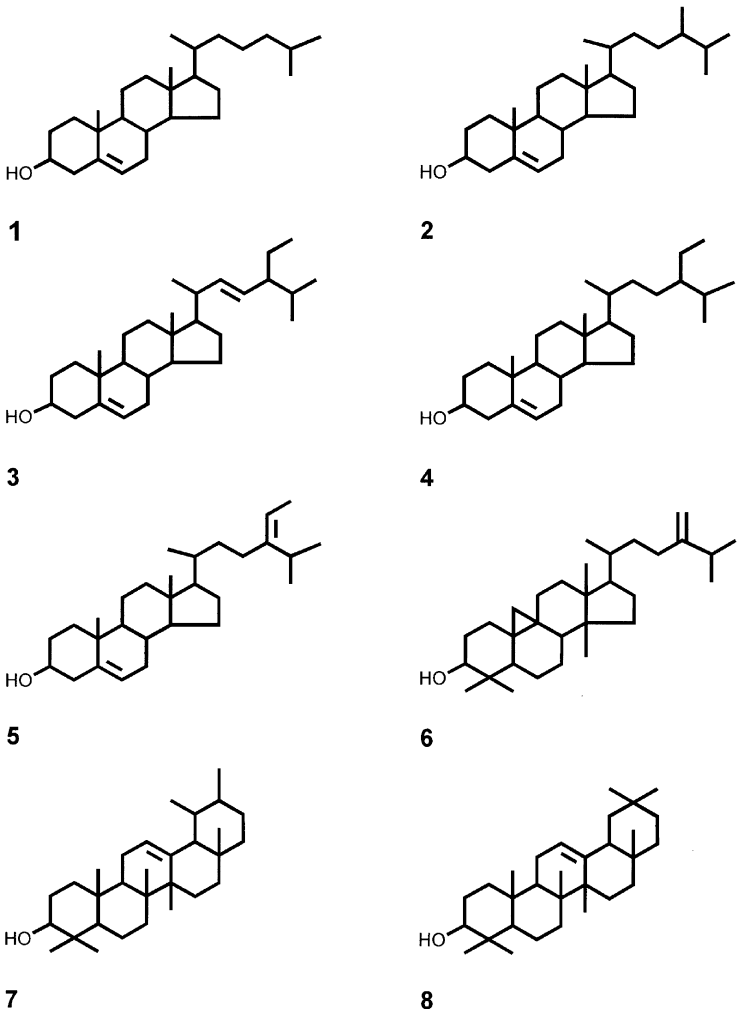
Tr means trace, not quantified, detected only by GC–MS analysis.

– means not detected.

cholesta-5,22-dien-3β-ol (**3**) and Δ5-avenasterol (**5**), in agreement with previous reports concerning the sterol composition of spores from *Acaulospora laevis* (Beilby, 1980) and *Glomus caledonium* (Beilby & Kidby, 1980). The major sterol in all species analyzed is by far 24-ethylcholesterol, which amounts up to 85% of total

sterol. Cholesterol is the second most abundant Δ5-sterol. Significant amounts of 24-ethylcholesterol were found only in *Glomus mosseae*. In two species of *Glomus*, *G. fasciculatum* and *G. vesiculiferum*, 24-methylenecycloartanol (**6**), a biosynthetic precursor of plant sterols was detected at a level of about 10%. Surprisingly, quite high concentrations of α-amyrin (**7**) were found in all the AMF species analyzed. This compound is known to be present in higher plants (Boar & Allen, 1973) and has not been reported thus far to occur in fungi. Traces of β-amyrin (**8**) were identified in a great number of AMF species (Scheme 1). Two reports mention the presence of this compound in the spores of *Anthurus muellerranus* (Basidiomycotina) (Herber, Villoutreix, Granger, & Chapelle, 1983) and in cultures (mycelium and spores) of *Aspergillus nidulans* (Deuteromycotina) (Gealt, 1983). Interestingly, β-amyrine was recently detected in symbiosomes from pea root nodules i.e. in the plant derived membrane that encysted the bacteria *Rhizobium* and accounts for 16% of total lipids (Hernandez & Cooke, 1996). This pentacyclic triterpene was not present in either the free living bacteria or in the pea root plasma membrane.

Representatives of the three genera studied presented rather similar sterol profiles. Some qualitative variations of fungal sterols were observed, particularly in the case of *G. mosseae*, *Glomus intraradices*, *Glomus borealis* and *Glomus tortuosum*; they seem to be related to the biological stage of the fungus (mature spores, young spores or hyphae). The similarity of sterol profiles of the various Glomales species studied here revealed metabolic relationships between these organisms, which are currently related taxonomically on the basis of their obligately symbiotic way of life. Such a convergence in sterol composition between species may be considered as a potential tool for chemotaxonomic regrouping of these species, supporting general morphological classification and strengthening the hypothesis that AMF belong to a monophyletic group. Indeed, the analysis of gene sequence data for the small subunit rRNA of 37 fungal



Scheme 1. Structures of sterols and triterpenes found in arbuscular mycorrhizal fungi. **1**, cholesterol; **2**, 24-methylcholesterol; **3**, 24-ethylcholesta-5,22-dien-3β-ol; **4**, 24-ethylcholesterol; **5**, Δ5-avenasterol; **6**, 24-methylenecycloartanol, **7**, α-amyrin, **8**, β-amyrin.

species reported by Simon, Bousquet, Lévesque and Lalonde (1993) and Berbee & Taylor (1993) supports their grouping in the specific order Glomales. Our analysis of sterols matches perfectly with these results.

Ergosterol ($\Delta^{5,7,22}$ sterol) was not detected in any of the analyzed species, even though this sterol was recognized to be the most common one found in fungi (Weete, 1989), including the Zygomycotina (Weete & Gandhi, 1996). Therefore, ergosterol cannot be definitively used to estimate the AMF biomass inside colonized roots as suggested by Frey, Vilarino, Schüepp, & Arines (1994).

Among the phylogenetically advanced taxa (Ascomycotina and Basidiomycotina, as well as imperfect or anamorphic forms) most of the fungi examined for sterol content contain ergosterol as a major or unique sterol (Weete, 1989; Weete & Gandhi, 1996). The most primitive fungi (Mastigomycotina) generally produce no ergosterol, but mainly cholesterol and/or 24-alkyl/alkylidene derivatives (Weete & Gandhi, 1996). The Zygomycotina differ from other classes of lower fungi in including both ergosterol-producing and nonproducing organisms. For example, Mucorales, which comprise both saprophytic and parasitic fungi as Zoopagales and Dimargaritales (mycoparasitic Zygomycetes) contain high concentrations of ergosterol (Lösel, 1988; Weete & Gandhi, 1996; Weete & Gandhi, 1997). On the other hand, insect parasites from the order Entomophtorales totally lack ergosterol, and in that way, resemble lower fungi with 24-alkylcholesterol (Δ^5 sterols) as the major sterol. The Kickxellales, fungal soil saprophytes, could be considered as an intermediate case with their abundant production of 22-dihydroergosterol ($\Delta^{5,22}$ sterol) (Weete & Gandhi, 1997).

Zygomycetes appear to be a transitional taxon, in which some groups resemble more closely the less advanced taxa with respect to sterol content, and others the more advanced fungi. By comparing the sequences of genes encoding the small ribosomal subunit ARN, Berbee & Taylor (1993), tentatively estimated the point where the major groups of fungi diverged. Their results revealed that the Glomales probably evolved as an ancestral group, prior to Ascomycetes and Basidiomycetes. The analysis of sterols from Glomales reported in the present paper agrees with this hypothesis, lending support to the statement that Glomales are a primitive order of Zygomycetes.

AMF are known to be obligate symbionts. The present sterol analysis indicates that these organisms exhibit a very similar sterol profile, with 24-ethylcholesterol as the major compound. The stereochemistry at C-24 of this sterol remains to be determined. The presence of α-amyrin in all the species analyzed as well as the occasional occurrence of 24-methylenecycloartanol raises fundamental questions: are AMF able to synthesize their own sterols and precursors or do they depend on the plant partner for sterol needs? What is the role played by amyrins? It is well established that enzymes involved into the cyclization of squalene oxide into cycloartanol as well as into α- or β-amyrins only occur in higher plant cells (Abe, Rohmer, & Prestwich, 1993). Thus, it is likely that these compounds are taken up from the plant partner by the fungus. The potential to grow some AMF species on T-DNA transformed roots (Bécard & Fortin, 1988, 1992), in a two-compartments culture system where the mycelium and spores are physically separated from transformed roots (St-Arnaud, Hamel, Vimard, Caron, & Fortin, 1996), could provide additional data on the sterol metabolism by AMF. In vitro studies of the ability of these fungal organisms to synthesize their own lipids are in progress in the laboratory.

3. Experimental

3.1. Fungal material

Arbuscular mycorhizal fungi were propagated in vermiculite/perlite potculture over leek plants (*Allium porrum* L.). Spores were isolated by wet-sieving and sucrose gradient techniques, surface sterilized and preserved by lyophilization (Dalpé, 1993). The 16 studied species are listed filed in the National Mycological Herbarium, Ottawa, Canada access number (DAOM): *Glomus aggregatum* Schenck & Smith emend Koske (DAOM 211639), *G. borealis* (Thaxter) Trappe & Gerd. (DAOM 220896), *G. caledonium*, (Nicol, & Gerd.) Trappe and Gerd. (DAOM 193528), *G. clarum* Nicol. and Schenck (DAOM 198446), *G. etunicatum* Becker & Gerd. (DAOM 212150), *G. fasciculatum* (Thaxter sensu Gerd.) (DAOM 220895), *G. intraradices*, Schenck and Smith (DAOM 197198), *G. lamellosum* Dalpé (DAOM 212349), Koske and Tews, *Glomus macrocarpum* Tul. and Tul. (DAOM 220899), *G. mosseae*, (Nicol. and Gerd.) Gerd. and Trappe (DAOM 198394), *G. pustulatum* Koske, Friese, Walker and Dalpé (DAOM 220900), *G. tortuosum* Schenck and Smith (DAOM 211736), *G. versiforme*, (Karsten) Berch (DAOM 212939), *G. vesiculiferum* (Thaxter) Gerd. and Trappe (DAOM 196674), *Gigaspora margarita* Becker and Hall (DAOM 211552), *Acaulospora scrobiculata* Trappe (DAOM 220898).

3.2. Sterol extraction and analysis

The freeze dried spores were extracted and analyzed according to a technique adapted from Costet-Corio and Benveniste (1988) (under dark conditions). A mean of 100 freeze-dried spores were extracted ($\times 3$) with 3 ml of CH_2Cl_2–MeOH(2:1) at 70°C. The total lipid extract was dried in a rotary evaporator and saponified with 1 ml of 6% (w/v) methanolic KOH for 1 h at 90°C. After addition of 1 volume water, the unsaponifiable fraction was extracted ($\times 3$) with 3 volumes of hexane and submitted to acetylation in a toluene:Ac_2O:pyridine mixture (1:2:1,

v/v/v) for 16 h at room temperature. After evaporation of the reagents, the crude acetates were purified by TLC using CH_2Cl_2 (one run). Under these conditions, steryl acetates migrated as a single band that was scraped off and eluted in CH_2Cl_2 to form the final extract, which was then analyzed by GC–FID equipped with a glass capillary column (BP1 SGE, 25m × 0.25 mm i.d., H_2 2 ml min^{-1}). The temperature program used includes a fast rise from 60 to 270°C (30°C · min^{-1}) then a slow rise from 270 to 310°C (2°C · min^{-1}). Steryl acetates were quantified using cholesterol as an internal standard. Sterol structures were identified by GC–MS at an ionizing potential of 70 eV.

3.3. Identification of sterols and triterpenoids

For the analysis of sterol and triterpenoid acetates, cholesterol was used as internal standard, by introducing a defined amount of free cholesterol into every sample just before running it on GC. Thus, endogenous cholesterol could be quantified as cholesteryl acetate. Each compound was identified by its relative retention time with regard to free cholesterol and its specific fragmentation pattern as well. Data were compared with those obtained with reference compounds and those reported in the paper of Rahier & Benveniste (1989). Relative retention time; cholesterol: 1.00, **1** 1.11, **2** 1.21, **3** 1.255, **4** 1.31, **5** 1.33, **6** 1.475, **7** 1.36, **8** 1.285.

3.4. MS data

Cholesterol (**1**): 368 $[M\text{-}60]^+$ (100), 353 (32), 255 (30), 213 (29); 24-methylcholesterol (**2**): 382 $[M\text{-}60]^+$ (100), 367 (18), 274 (14), 255 (18); 24-ethylcholesta-5,22-dienol (**3**): 394 $[M\text{-}60]^+$ (100), 379 (8.5), 255 (39), 228 (7), 213 (10); 24-ethylcholesterol (**4**): 396 $[M\text{-}60]^+$ (100), 381 (13), 288 (18), 255 (12), 228 (2), 213 (9) Δ^5-avenasterol (**5**): 394 $[M\text{-}60]^+$ (20), 296 (100), 281 (22), 253 (9), 228 (6), 213 (10); 24-methylenecycloartanol (**6**): 482 $[M]^+$ (10), 467 (4), 422 (100), 407 (68), 357 (5), 339 (6.5), 300 (57), 297 (26), 255 (10); α-amyrin (**7**): 468 $[M]^+$ (4), 218 (100), 203 (28), 189 (33), 175 (10); β-amyrin (**8**): 468 $[M]^+$ (2), 218 (100), 203 (64), 189 (27), 175 (13).

Acknowledgements

We wish to thank the Région Nord-Pas de Calais for financial support and Dr S. Redhead for critical reading of manuscript.

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