5813

Cellular Lipid and Fatty Acid Composition of Cyanobionts from *Azolla caroliniana*

Abstract

Cyanobacteria were separated and purified from their symbiotic host, *Azolla caroliniana*. Their major cellular lipid components were identified as monogalactosyldiacylglycerol, digalactosyldiacylglycerol, cardiolipin, phosphatidylethanolamine, phosphatidylglycerol and two unknown components. Minor lipid components (less than 1% of the total) were identified as sulfoquinovosyldiacyLglycerol, phosophatidylcholine, and bacteriohopanetetrol. Fifty-one cellular fatty acid components were detected, fully or partly-characterized and compared to those in free-living *Anabaena-Nostoc*. Major fatty acids (and mean percentage of total) included the saturated 16:0 (36.68%), the unsaturated 12:1 (2.03%), 14:1 *cis*-9 (2.90%), four unsaturated 16-carbon straight chains (total=12.25%), four unsaturated 18-carbon acids (total=31.34%); and an *iso*-branched 16:0 (1.89%). Cyanobionts also contained a 16:1 *trans*-3 (0.24%) an *iso* 30H 13:0 (0.22%) not found in free-living *Anabaena-Nostoc*, and two poly-unsaturated 20-carbon fatty acids (total=0.59%). A tentatively-characterized *iso*-branched 15-carbon fatty acid (equivalent carbon chain length: 14.35), generally present in free-living *Anabaena-Nostoc*, was absent in cyanobionts.

Keywords: symbiotic cyanobacteria, *Azolla caroliniana*, lipid, fatty acid

1. Introduction

Azolla Lamarck is an aquatic fern of world-wide distribution in tropical and subtropical regions (Lumpkin and Plucknett, 1982). *Azolla* is host to symbiotic, nitrogen-fixing cyanobacteria which enrich aquatic systems. *Azolla* is also of value as a source of green manure in rice paddy soils (Lumpkin and Plucknett, 1992). Symbiotic cyanobacteria are located in upper aerial leaves of *Azolla* within a central cavity.

The major lipid classes in cyanobacteria have been reported as being quite similar to those of photosynthetic eukaryotes (Sata and Murata, 1981; Al-Hasan et al., 1989). Cyanobacteria of the genera *Anabaena* and *Nostoc* (order Nostocales) have been reported to contain monogalactosyldiacylglycerols, digalactosyldiacylglycerols, sulphoquinosylglycerols and phosphatidylglycerols (Sata and Murata, 1981; Al-Hasan et al., 1989). The basic techniques to analyze lipid class composition have been thin-layer chromatography (TLC) followed by chemical determinations. As constituents of lipids, the fatty acid composition of *Anabaena* have been described (Kenyon et al., 1977; Starmann and Boger, 1982; Sato and Murata, 1981). They include the saturated 14:0, 16:0, and 18:0 chains, mono- and poly-unsaturated 16-, 18- and 20-carbon fatty acids and some minor isomers. The fatty acid composition in eubacteria (Lechevalier, 1977) and microalgae is generally species-specific. Recently Caudales and Wells (1992) reported that fatty acid composition, as determined by gas-liquid chromatography-mass spectrometry (GLC-MS), was an effective taxonomic tool for differentiation of free-living *Anabaena* and *Nostoc* cyanobacteria. In addition, other groups of fatty acids–(saturated odd-carbon, hydroxy-substituted, branch-chained, cyclopropane and unsaturated branched chains) were reported in cyanobacteria for the first time.

At present there are no reports of total lipid and fatty acid composition of *Azolla* cyanobionts. We describe herein an analysis of lipids of *Azolla* cyanobionts, as performed by high-performance liquid chromatography (HPLC) using an isooctane-isopropanol-water gradient system and an evaporative light-scattering detector (ELSD) developed by Moreau (1991), and an analysis of fatty acids by GLC-MS.

2. Materials and Methods

Culture of Azolla

A. caroliniana was grown in defined media, without nitrogen, modified from Van Hove et al. (1983), consisting of: 54.4 mg/l KH_2PO_4, 294 mg/l $CaCl_2 \cdot 2H_2O$, 192 mg/l $MgSO_4 \cdot 7H_2O$, 150 mg/l KCl, 12 mg/l NaCl, 12 mg/l

Fe-EDTA and 1 ml/l trace metal mix A5 (Rippka et al., 1979) + 43.87 mg/l $CoCl_2 \cdot 6H_2O$. Photon flux density was 100 $\mu E \cdot m^2 \cdot s^{-1}$ using cool-white fluorescent and incandescent light and with a 14/10 hr light photoperiod. Temperature was 26 ± 1°C in light and 16 ± 1°C during the dark period.

Separation of cyanobionts

Plants were harvested at 14 days and then washed in 0.2% sodium hypochlorite + 0.01% Triton-X 100 for 20 min, rinsed 10× with distilled water, and homogenized in a blender in phosphate-free BG-110 medium (Rippka et al., 1979). Cyanobacterial cells were separated from plant debris by diluting the homogenate in medium, filtering through 10 layers of cheesecloth, and by differential centrifugation. Pellets were resuspended in medium containing 2% pectinase and 2% cellulase (Sigma Chemical Co.) buffered at pH 6.0 with 10 mM MES, and incubated for 2 hr at 30°C. Pellets were then washed 2× with medium, centrifuged at 500×g (10 min) and fractionated by density gradient centrifugation in 10–90% Percoll at 2500×g (20 min). The cyanobacterial fraction was removed, examined microscopically for plant debris, and washed 2×. If plant debris was present, Percoll centrifugation and washing were repeated.

Lipid analysis

Five hundred mg of freshly-isolated cyanobionts were extracted with 4 ml of isopropanol, then with 6 ml of hexane, and partitioned against aqueous Na_2SO_4 (0.5 M) as described by Hara and Radin (1978). Extracts were dried under a stream of N_2 and redissolved in chloroform-methanol (85/15, v:v). Lipid class separation and quantification was performed by an HPLC method fully described by Moreau et al. (1990) involving separation of lipids on a silica column eluted with an isooctane-isopropanol-water gradient, and quantification by an ELSD detector (Moreau, 1991). Most of the peaks were identified by comparison of retention times with known lipid standards.

Fatty acid analysis

Approximately 400–500 mg (wet weight) of cells were saponified by mixing in 1 ml of 1.2 N NaOH in 50% aqueous methanol, and heating for 30 min in a boiling water bath. Samples were neutralized by adding 0.5 ml 6 N HCl and esterified by adding 1 ml 12% BCl_3-methanol, and heating for 5 min at 85°C, based on the method of Moss (1979). Methyl esters were then extracted with 1 ml hexane: diethylether (1:1), washed with 3 ml of 0.3 N NaOH,

and concentrated to approximately 40 μl. A two μl sample was injected into a Varian 3700 Gas Chromatograph with a flame ionization detector (Varian Associates, Sunnyvale, CA USA) and a 15 m×0.25 mm capillary glass column coated with SPB-1 (Supelco Inc., State College, PA, USA) as a non-polar stationary phase. Solvent blanks were checked periodically for impurities. Operating conditions were: helium carrier gas flow of 30 ml min^{-1}; injector temperature, 230°C; initial column temperature, 130°C; final temperature, 230°C; temperature program rate, 4°C min^{-1}. Fatty acids between 8 and 20 carbons in length were initially identified by co-chromatography with available reference standards. Major fatty acids (each constituting at least 0.90% of the total) were confirmed by mass spectrometer (Finnegan 8230 HR) and by chemical tests as previously described (Caudales and Wells, 1992). Other eluted peaks, comprising minor fatty acids (0.03 to 0.89% of the total) were confirmed chemically. Trace components (less than 0.03% of total) were provisionally identified. Equivalent carbon lengths (ECL) were calculated for each peak, and used for further confirmation of identities by reference to ECL's of published reports (Gillan and Hogg, 1984; Caudales and Wells, 1992). Unsaturation sites and isomeric positions, when indicated, were directly determined by mass spectroscopy only for major fatty acids. Eluted fractions from gas chromatography were integrated and quantified as percentages of total peak areas with a model 4270 Chromatography Data System (Varian Associates).

3. Results

The major lipid components of *A. caroliniana* cyanobionts (Table 1 and Fig. 1) were monogalactosyldiacyglycerol (MGDG), comprising 66% of the total; digalactosyldiacylglycerol (DGDG), 9.41%; cardiolipin (DPG), 7.10%; phosphatidylethanolamine (PE), 3.38% and phosphatidylglycerol (PG), 1.18%; and two unknown components lipid, 4 (6.46%) and lipid 5 (3.82%) that eluted between MGDG and DGDG. Minor lipid components, which also co-chromatographed with standards, were sulfoquinovasyldiacylglycerol (SQDG), 0.61%; phosphatidylglycerolcholine (PC), 0.51%; bacteriohopanetetrol (BH), 0.88% and three minor unknown lipids (lipids 1, 2, and 3). BH was identified by comparison with standards purified from *Frankia* cells (Berry et al., 1990; Moreau and Gerard, 1992, in press).

The cellular fatty acid composition of *A. caroliniana* cyanobionts included 13 major components accounting for 90.89% of total fatty acids: the saturated 14:0, 16:0 and 18:0 chains; the unsaturated straight-chain 12:1, 14:1 *cis*-9, 16:3 *cis*-6, 16:1 *cis*-9 and *cis*-11, 18:1 *cis* and *trans*-9, 18:2 *cis*-9, 18:3 *cis*-9; and the

Table 1. Cellular lipids of cyanobionts of the water fern *Azolla caroliniana* in order of elution by high-performance liquid chromatography (HPLC) with an evaporative light-scattering detector (ELSD) (Moreau, 1990).

Component No.	Lipid class	Abbreviation	Retention time (min)	Area %
1	Pigments	P		
2	Lipid 1	L1	20.3	0.41
3	Lipid 2	L2	20.6	0.10
4	Lipid 3	L3	21.3	0.14
5	Bacteriohopanetetrol	BH	22.6	0.88
6	Monogalactosyldiacylglycerol	MGDG	23.0	66.00
7	Lipid 4	L4	25	6.46
8	Lipid 5	L5	29	3.82
9	Digalactosyldiacylglycerol	DGDG	30	9.41
10	Cardiolipin	DPG	33	7.10
11	Phophatidylethanolamine	PE	35	3.38
12	Phosphatidylglycerol	PG	36	1.18
13	Sulphoquinovosyldiacylglycerol	SQDG	40	0.61
14	Phosphatidylglycerolcholine	PC	44	0.51

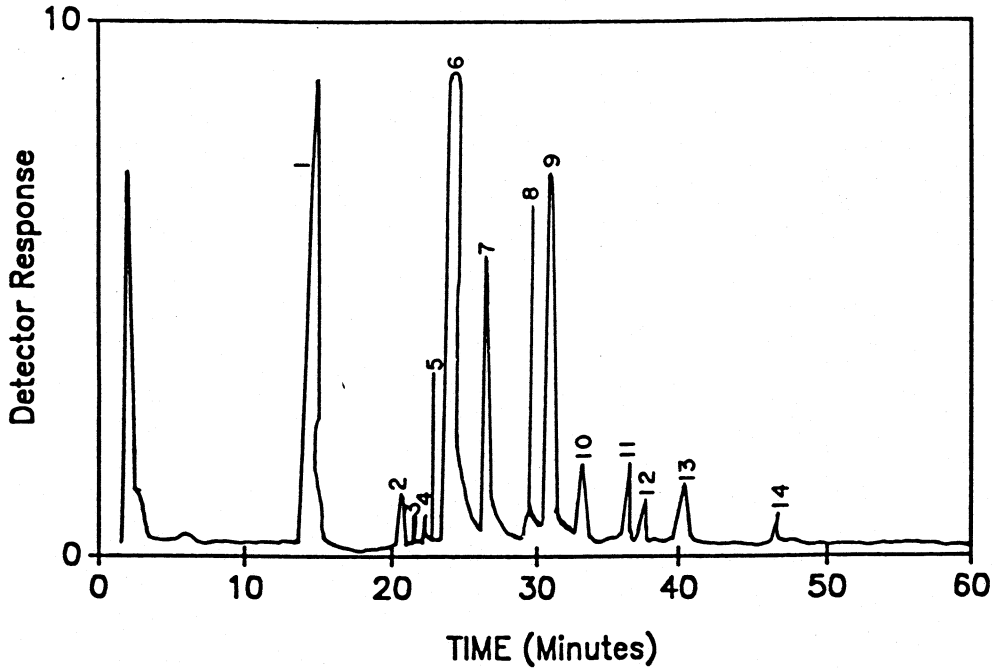

Figure 1. Separation of lipids from cyanobionts of *Azolla caroliniana* by HPLC with an evaporative light-scattering detector. Numerical symbols for each lipid are defined in Table 1. Units for the Y axis are relative response, 10 = 100%.

Table 2. Percentage (of the total) of cellular fatty acids of *Azolla caroliniana* cyanobionts

Fatty acids		
Chemical class	ECL*	Mean percentage**
A. Saturated, even-carbone straight chains:		
8:0	8.0	0.01
10:0	10.0	0.02
12:0	12.0	0.24
14:0	14.0	1.02
16:0	16.0	36.68
18:0	18.0	2.78
20:0	20.0	0.21
Class A total		40.96
B. Saturated, odd-carbon straight chains:		
9.0	9.0	0.02
15:0	15.0	0.80
17:0	17.0	0.36
19:0	19.0	0.20
class B total		1.38
C. Unsaturated, straight chain acids:		
12:1	11.8	2.03
13:1	12.8	0.05
14:1 *cis*-7	13.8	0.01
14:1 *cis*-9	13.9	2.90
16:4	15.2	0.20
16:?	15.5	1.89
16:4 *cis*-4	15.55	0.39
16:3 *cis*-6	15.6	3.29
16:1 *cis*-9	15.8	4.83
16:1 *trans*-9	15.85	0.01
16:1 *cis*-11	15.9	2.24
16:1 *trans*-3	15.95	0.24
18:3 *cis*-6	17.2	0.58
18:4 *cis*-6	17.5	0.23
18:2 *cis*-9	17.7	7.61
18:3 *cis*-9	17.75	16.65
18:1 *cis*-9	17.8	5.18
18:1 *trans*-9	17.85	1.90
20:4 *cis*-5	19.2	0.47
20:2 *cis*-11	19.6	0.12
Class C total		50.82
D. Hydroxy-substituted:		
2OH-10:0	11.15	0.19
3OH-10:0	11.4	0.26
2OH-12:0	13.2	0.14
3OH-12:0	13.5	0.12
iso 3OH-13:0	14.1	0.22
iso 3OH-17:0	18.1	0.01
3OH-17:0	18.4	0.31
Class D total		1.27

E. Branched chain acids:		
iso-13:0	12.6	0.16
iso-15:0	14.6	0.01
anteiso-15:0	14.7	0.01
iso-16:0	15.6	1.86
iso-17:0	16.6	0.34
anteiso-17:0	16.7	0.23
iso-19:0	18.6	0.17
Class E Total		2.78
F. Cyclopropane acids:		
cyclo-17:0	16.9	0.55
cyclo-19:0	18.9	0.53
Class F total		1.08
G. Unsaturated branch-chained fatty acids:		
iso-15:1	14.35	0.10
iso-17:1	16.4	0.24
iso-18:1	18.4	0.24
Class G total		0.58
Unidentified components		1.13

*. ECL = equivalent (carbon) chain length
**. Mean percentage of triplicated tests

branch-chained *iso* 16:0 (Table 2). The most abundant component was 16:0, with a mean percentage of 36.68 of the total.

There were 36 minor fatty acid components detected and identified, comprising 9.01% of the total, and 8 trace-level components. These included hydroxy-substituted acids (1.25%), branched chains in addition to *iso*-16:0 (1.89% of a total of 2.76%), and cyclopropane acids (1.08%) (Table 2). Also of interest were two fatty acids, 16:1 *trans*-3 and *iso* 3-OH 13:0, present in cyanobionts but not previously reported in *Anabaena* or *Nostoc*. In *Azolla* cyanobionts, poly-unsaturated acids averaged 29.34%, the more abundant being 18:3 *cis*-9 and 18:2 *cis*-9. Total mono-unsaturated acids averaged 17.38%, and the 16-carbon and 18-carbon chains averaged 51.95 and 32.15% of the total, respectively.

4. Discussion

Cyanobacteria, as oxygenic photosynthesizing organisms, have the same gross membrane organization as Gram-negative prokaryotic eubacteria except that they have thylakoids, analogous to chloroplasts in higher plants (Drews and Weckesser, 1982; Golecki and Drews, 1982). The major lipid components in *Azolla* cyanobionts were MGDG, DGDG, DPG, PG, the unknown lipids 4 and 5, and PE. Minor lipids were BH, SQDG, PC and three unknown lipids (lipids 1, 2, and 3). Lipid composition was similar to that found

in plant thylakoid membranes, reported to consist of MGDG, 53%; DGDG, 27%; SQDG, 7%; PG, 7%; and phosphatidylinositol (PI), 2% (Selstam et al., 1990). No PI was found in cyanobionts. Some of these differences may result from our analyses being for total lipids and not restricted to thylakoid membranes. Furthermore, unknown lipid 4 was not present in unicellular cyanobacteria (Caudales, unpublished results), which may indicate that it is part of heterocyst membranes. Heterocysts account up to 30% of total cells of this cyanobiont (Hill, 1975).

In many species of eubacteria PE is a major component, comprising up to 95% the total lipid in *E. coli* and 40% in *Rhizobium* sp. (Moreau, unpublished data). However, in cyanobionts PE is a minor component (3.38%) and probably localized in the cell membrane.

In some cyanobacteria, as in other prokaryotes, several types of hopanoids have been found (Rohmer et al, 1984). In *Azolla* cyanobionts only one was detected. Hopanoid derivatives or hopanoid-like compounds have also been found in some plants and in *Tetrahymena* (Rohmer, 1988).

Fatty acid composition of *Azolla* cyanobionts included the same classes of fatty acids as previously reported for the free-living *Anabaena-Nostoc* (Caudales and Wells, 1992). Two fatty acids, however, the 16:1 *trans*-3 and *iso* 3OH-13:0, were present in cyanobionts but not in *Anabaena-Nostoc.*

Fatty acid composition was also different in cyanobionts compared to eubacteria, possibly due in part to the high proportion of thylakoids in cyanobacteria. In eubacteria, branch-chained fatty acids are major constituents, and straight chains constitute less than 10% of total fatty acids (Kaneda, 1991). In the cyanobacteria the 16- and 18-carbon straight chains were the major fatty acids, similar to the pattern found in plants (Christie, 1987), and the branched chains were minor components. Another difference was in the high percentage of poly-unsaturated acids in the cyanobacteria compared to their absence in most eubacteria (Johns and Perry, 1977; DeLong and Yayanos, 1989). These fundamental differences in composition may have a direct bearing on fluidity of membrane lipids. Cyanobionts, because of an obligate life cycle regulated by the host fern (Peters and Meeks, 1989), could be a close evolutionary link between eubacteria and plant chloroplasts.

REFERENCES

Al-Hasan, R.H., Ali, A.M., and Radwan, S.S. 1989. Effect of light and dark incubation on the lipid and fatty acid composition of marine cyanobacteria. *J. Gen. Microbiol.* **135:** 865–872.

Berry, A.M., Moreau, R.A., and Jones, A.D. 1991. Bacteriohopanetetrol: Abundant lipid in *Frankia* cells and in nitrogen-fixing nodule tissue. *Plant Physiol.* **95:** 111–115.

Caudales, R. and Wells, J.M. 1992. Differentiation of the free-living *Anabaena* and *Nostoc* cyanobacteria on the basis of fatty acid composition. *Int. J. System. Bacteriol.* **42:** 246–251.

Christie, W.W. 1987. *High-Performance Liquid Chromatography and Lipids, a Practical Guide.* Pergamon Press, New York, pp. 48–49.

DeLong, E.F. and Yayanos, A.A. 1986. Biochemical function and ecological significance of novel bacterial lipids in deep-sea procaryotes. *Appl. Environ. Microbiol.* **51:** 730–737.

Doolittle, W.F. 1982. Molecular evolution. In: *The Biology of Cyanobacteria. Botanical Monographs, vol. 19.* N.G. Carr and B.A. Witton, eds. University of California Press. Berkeley, Los Angeles, CA, pp. 307–332.

Drews, G. and Weckesser, J. 1982. Function, structure and composition of cell walls and external layers. In: *The Biology of Cyanobacteria. Botanical Monographs, Vol. 19.* N.G. Carr and B.A. Witton, eds. University of California Press. Berkeley, Los Angeles, CA, pp. 333–357.

Gillan, F.T. and Hogg, R.W. 1984. A method for the estimation of bacterial biomass and community structure in mangrove-associated sediments. *J. Microbiol. Methods* **2:** 275–293.

Golecki, J.R. and Drews, G. 1982. Supramolecular organization and composition of membranes. In: *The Biology of Cyanobacteria. Botanical Monographs, Vol. 19.* N.G. Carr and B.A. Witton, eds. University of California Press. Berkeley, Los Angeles, CA, pp. 125–141.

Hara, A. and Radin, N.S. 1978. Lipid extraction of tissues with low toxicity solvents. *Anal. Biochem.* **90:** 420–426.

Hill, D.J. 1975. The pattern of development of *Anabaena* in *Azolla-Anabaena* symbiosis. *Planta* **122:** 179–184.

Johns, R.B. and Perry, G.J. 1977. Lipids of the marine bacterium *Flexibacter polymorphus. Arch. Microbiol.* **114:** 267–671.

Kaneda, T. 1991. *Iso* and *anteiso* fatty acids in bacteria: Biosynthesis, function, and taxonomic significance. *Microbiol. Revs.* **55:** 288–302.

Kenyon, C.N., Rippka, R., and Stanier, R.Y. 1972. Fatty acid composition and physiological properties of some filamentous blue-green algae. *Arch. Mikrobiol.* **83:** 216–236.

Lechevalier, M.P. 1977. Lipids in bacterial taxonomy – a taxonomist's view. *CRC Critical Reviews in Microbiol.* **5:** 109–210.

Lumpkin, T.A. and Plucknett, D.L. 1982. Botany and Ecology. In: *Azolla as a Green Manure: Use and Management in Crop Production.* Westview Press, Coulder, CO, pp. 15–38.

Moreau, R.A. 1991. Plant lipid class analysis by HPLC. In: *Plant Lipid Biochemistry, Structure and Utilization.* P.J. Quinn and J.L. Harwood, eds. Portland Press Ltd., London, pp. 20–22.

Moreau, R.A., Asmann, P.T., Norman, H.A. 1990. Quantitative analysis of the major classes of plant lipids by high performance liquid chromatography and flame ionization detection (HPLC-FID). *Phytochemistry* **29:** 2461–2466.

Moreau, R.A. and Gerard, H.C. High performance liquid chromatography as a tool for lipid chemistry and biochemist. *CRC Handbook of Chromatography: Lipids.* Vol. 3. H.K. Manfold, K.D. Murkherjee and N. Weber, eds. Chemical Rubber Co., Boca Raton, FL, 1992 (in press).

Moss, C.W. 1979. Analysis of cellular fatty acids of bacteria by gas-liquid chromatography. In: *Legionnaires: the Disease, Bacteria and Methodology.* G.L. Jones and G.A. Herbert, eds. U.S. Dept. Health, Education and Welfare, Centers for Disease Control, Atlanta, GA, pp. 117–122.

Peters, G.A. and Meeks, J.C. 1989. The *Azolla-Anabaena* symbiosis: basic biology. *Ann. Rev. Plant Physiol.* **40:** 193–210.

Rippka, R., Deruelles, J., Watebury, J.B., Herdman, M., and Stanier, R.Y. 1979. Genetic assignments, strain histories and properties of pure cultures of cyanobacteria. *J. Gen. Microbiol.* **111:** 1–61.

Rohmer, M. 1988. The hopanoids, prokaryotic triterpenoids and sterol surrogates. In: *Surface Structure of Microorganisms and Their Interactions with the Mammalian Host.* E. Schrinner, M.H. Richmond, G. Seibert, and U.C. Schwarz, eds. VCH Publications, New York, pp. 227–242.

Rohmer, M., Bouvier-Nave, P., and Ourisson, G. 1984. Distribution of hopanoid triperpenes in prokaryotes. *J. Gen. Microbiol.* **130:** 1137–1150.

Sato, N. and Murata, N. 1981. Studies on the temperature shift-induced desaturation of fatty acids in monogalactosyl diacylglycerol in blue-green algae (cyanobacterium), *Anabaena variabilis.* *Plant Cell Physiol.* **22:** 1043–1050.

Selstam, E., Brentel, I., and Lindblom, G. 1990. Phase structure of lipids of the photosynthetic membrane. In: *Plant Lipid Biochemistry, Structure and Utilization.* P.J. Quinn and J.L. Harwood, eds. Portland Press Ltd., London. pp. 39–46.

Starmann, G. and Boger, P. 1982. Volatile hydrocaarbons from photosynthetic membranes containing different fatty acis. *Lipids* **17:** 35–41.

Van Hove, C., Diara, H.F., and Godard, P. 1983. *Azolla* in West Africa. Published by: *Azolla* project of the West Africa Rice Development Association and Belgian Administration for Development and Cooperation, Brussels, Belgium.