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Polysaccharide Composition of the Mucilage of *Azolla* Algal Packets

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Abstract

Algal packet mucilage of different species of *Azolla* was analysed for their polysaccharide content and composition. The mucilage was composed, as shown by HPLC analyses, of glucose, galactose and fucose, while rhamnose was present only in *A. pinnata* var. *imbricata*, *A. caroliniana*, *A. rubra* and *A. filiculoides*. Treatments of the fern with antibiotics to which *Anabaena* and/or bacteria are sensitive, did not change the qualitative composition of polysaccharides, but altered the amount of single monosaccharides. Fucose was the most abundant monosaccharide, both in treated and in untreated plants, suggesting its plant origin. The amount of glucose and galactose in erythromycin treated *Azolla* decreased, suggesting that these monosaccharides are produced both by *Anabaena* and by bacteria. Lectin binding was observed microscopically using fluorescein isothiocyanate-labelled lectins. Algal packet polysaccharides of untreated plants had glucose as hapten, which was also revealed on the surface of both *Anabaena* and bacteria. Galactose was a hapten both of the mucilage and of the bacteria. Hypotheses about the contribution of each component of the symbiosis to the mucilage production are put forward.

Keywords: *Azolla*, *Anabaena*, *Arthrobacter*, mucilage, polysaccharides

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1. Introduction

The symbiotic heterosporous water fern *Azolla* Lam. is utilised as green manure in rice fields. The sporophyte contains inside its leaf cavities: epidermal hair cells, the cyanobacterium *Anabaena azollae* Strasb. and a bacterial population, the main component of which is *Arthrobacter* spp. Conn and Dimmick (Forni et al., 1989; Peters and Meeks, 1989). Isolated leaf cavities, often referred to as algal packets, can be obtained by digesting *Azolla* leaves with a combination of cellulolytic enzymes (Uheda, 1986). The packet is enclosed by a thin envelope (Uheda and Kitoh, 1991) which is insensitive to digestion with cellulases, proteases, pectinases or lipases (Uheda, 1986). When algal packets are ruptured, cyanobacterial and bacterial populations are released into a mucilaginous matrix. Controversy still exists about which of the following produces mucilage: either leaf cavity hairs (Peters et al., 1978), or cyanobacteria (Robins et al., 1986) or, at least partly, the bacteria (Forni et al., 1992).

The mucilage of algal packets is rich in amino acids, ammonium compounds (Canini et al., 1990; Kitoh et al., 1992) and carbohydrates. Presence of the latter has been determined by cytochemical reactions in *Azolla filiculoides* Lam. (Forni et al., 1992).

The function of leaf-cavity mucilage has not been completely clarified. It has been hypothesized that – as well as keeping nutrients inside the leaf cavity – it can fulfil the function of retaining bacteria and *Anabaena*. It is a very viscous material and is released only upon breakage of the packet. Its polysaccharidic components, in particular, can also be important for the fern's infection by the microorganisms: the lectin-receptor interaction may play an essential role in recognition and settlement of both the cyanobacteria and the eubacteria in the fern's apical leaf cavity (Forni et al, 1992).

The present report describes the monosaccharide composition of the mucilage in algal packets, isolated both from cyanobiont-containing and cyanobiont-free *Azolla*, in order to clarify the contribution of each symbiotic component to the production of the mucilage.

2. Materials and Methods

Azolla species and culture maintenance

The *Azolla* species used are reported in Table 1. They were kindly provided by Dr. Rodulio Caudales from his collection, Rutgers University, New Brunswick (USA), presently at the University of Botswana. *Azolla filiculoides* (strain Na), originally from the Botanical Garden of the

Table 1. Species and origin of *Azolla*

Azolla species	Strain	Original designation	Original source
A. caroliniana Willd.	C1	C1	G.P.
	C6	3013	W.Z.
A. filiculoides Lam.	F14	162FI	V.H.
	Na	Na	B.G.
	Tevere	Tevere	UTV
A. microphylla Kaulfuss	M2	M2	G.P.
A. pinnata var. *pinnata* R. Brown	P1	P1	G.P.
A. pinnata var. *imbricata* R. Brown	I1	I1	G.P.

G.P. = G. Peters, Virginia Commonwealth University, VA, USA; W.Z. = W. Zimmermann, University of Michigan, Dearbora, MI, USA; V.H. = C. Van Hove, Université Catholique de Louvain, Louvain-la-Neuve, Belgium; B.G. = Botanical Garden, University of Naples, Naples, Italy; UTV = University of Rome "Tor Vergata", Rome, Italy.

University of Naples (Italy), was grown for several years at the University of Rome "Tor Vergata", while *Azolla* sp. (strain Tevere) was collected from the Tiber river, 25 km North of Rome.

Plants were grown in a 2/5 strength N-free Hoagland medium (Van Hove et al., 1983) at 25°C under 16/8 hrs light/dark photoperiod, at light intensity of 5–8 klux. *Azolla filiculoides-Anabaena* free plants were obtained after treatment with erythromycin, as previously reported (Forni et al., 1991) and were grown in the above described Hoagland medium supplemented with 0. 5 g $\times$ l^{-1} $NaNO_3$ under the conditions described above.

To determine the contribution to mucilage production of each component of the association, *A. filiculoides* plants were treated with different antibiotics, either erythromycin (60 µg/ml), which affects both *Anabaena* and *Arthrobacter* (Forni et al., 1991), or, a cocktail of 60 µg/ml of each of the following antibiotics: novobiocin, cephalosporin C, gentamicin, rifampicin and vancomycin, which eliminates most of the bacterial population within leaf cavities. Surface sterilised plants were grown for 14 days in sterile 500 ml Erlenmayer flasks containing Hoagland medium with the mentioned antibiotics. All antibiotics were purchased from Sigma.

Algal packets extraction

Several attempts were done before adopting the following extraction

technique, chosen to keep algal packets intact as long as possible. Algal packets were isolated according to Uheda (1986), modified as follows: 1 g plants was washed with 0.1% (v/v) Triton X100 (Merck) and rinsed twice with distilled water. Algal packets were extracted by treatment with the following cellulolytic enzymes in 30 ml aqueous solution (w/v): 2% cellulase (Sigma), 0.1% pectolyase (Sigma) and 0.6 M mannitol, pH 6. The mixture was incubated for 24 h at 30°C, and shaken at 100 rpm (New Brunswick Rotatory Shaker). The treatment utilized did not damage the envelope structure, and the digestion did not completely release packets. Unbroken packets were collected under the stereomicroscope and resuspended by gentle pipetting in 0.1 M phosphate saline buffer (PBS) pH 7.

Extraction and analysis of polysaccharides

Algal packets were kept for 72 hrs at 4°C to solubilise the polysaccharides (PS) and then centrifuged 10 min at 15,000 g. Supernatants were collected and dialysed overnight against H_2O at 4°C with a MWCO 1000 membrane (Spectrum). A preliminary quantification of polysaccharides (PS) was performed by the Anthrone method using glucose as the standard (Dische, 1962). Samples were lyophilised, hydrolysed and analysed by high-performance liquid chromatography (HPLC) as previously described (Del Gallo and Haegi, 1990).

To determine polysaccharide haptens, algal packets of *A. filiculoides* treated or not with antibiotics were freshly isolated and stained with the following fluorescein isothiocyanate (FITC) -labelled lectins: WGA = wheat germ agglutinin, TPL = *Tetragonolobus purpureas* lectin, LCL = *Lens culinaris* lectin, CPL = chick-pea (*Cicer arietinum*) lectin, ECL = *Erythrina cristagalli* lectin, ConA = concanavalin A (*Canavalia ensiformis*) lectin. Calcofluor White (CFW) binding was performed as described by Del Gallo et al. (1989). CFW and all the lectins were purchased already labelled from Sigma, except for CPL, which was labelled according the procedure described by Nairn (1976). Packets were observed with a phase contrast and fluorescent Leitz Dialux 20 microscope equipped with an incident light illumination and filter blocks I 2/3 and A2. Analyses were done in triplicate.

3. Results

Polysaccharide composition of the algal packet mucilage extracted from different *Azolla* strains and from cyanobiont-free plants is shown in Table 2. Mucilages of the different fern species contained different amounts of glucose,

galactose, and fucose. The latter was present at the highest concentration in all the species, both in the presence and in the absence of the endophyte. Rhamnose was detected only in some *Azolla* and sometimes not in all the strains or varieties of the same species. Absence of the endophyte did not affect the PS composition, but changed the amount of the single monosaccharides, i.e. the glucose and galactose concentrations decreased, thus increasing fucose percentage (Table 2).

Table 2. Monosaccharide composition, determined by HPLC, and total amount of polysaccharides isolated from mucilages of *Azolla* spp. algal packets

Azolla species	Glucose %	Galactose %	Rhamnose %	Fucose %	Total PS $\mu g\ mg\ dw^{-1}$
A. pinnata var. *imbricata* I1	9.2	4.7	0.8	85.2	63.3
A. pinnata var. *pinnata* P1	11.5	0.2	–	88.4	51.7
A. caroliniana C6	5.9	0.3	2.4	91.3	58.6
A. caroliniana C1	9.5	0.5	1.6	88.4	96.0
A. mexicana X1	12.0	5.1	tr	82.8	55.0
A. mexicana X10	21.8	1.6	–	76.4	16.9
A. microphylla M2	12.9	4.9	–	82.2	36.7
A. rubra R4	6.4	5.8	2.3	85.5	20.3
A. filiculoides F14	17.5	0.3	1.5	80.6	45.4
A. filiculoides Na	42.1	5.2	tr	52.6	76.8
A. filiculoides Na-*Anabaena* free	8.7	1.3	–	90.0	95.5
Azolla Tevere	16.6	tr	tr	83.3	54.5

– = Absent; tr = traces.

Table 3. Effect of erythromycin – an antibiotic against *Anabaena* and *Arthrobacter* – and of an antibiotic cocktail, without erythromycin – against the associated bacterial population – on PS composition of algal packets mucilage isolated from *Azolla filiculoides* Na

Culture conditions	Glucose %	Galactose %	Fucose %	Total PS $\mu g\ mg\ dw^{-1}$
Control	44.2	0.5	55.2	50.0
Erythromycin	8.8	tr	91.1	15.0
Antibiotic cocktail	46.5	7.0	46.5	4.3

tr = Traces.

Table 3 shows the data concerning experiments with plants treated with antibiotics. The total number of bacteria, $10^6/10^7$ per algal packet, was not affected by treatment, but the antibiotic cocktail reduced the various *Arthrobacter* species to only one (see also Fig. 1a and b). In erythromycin treated plants, glucose and galactose decreased drastically, while the relative concentration of fucose increased. Treatment with an antibiotic cocktail strongly decreased, in respect to the control, the total amount of polysaccharides present in the leaf cavity (Table 3 and Fig. 1). However, the treatment did not change the monosaccharides composition, but it changed the relative amount of glucose and galactose.

In Table 4 observations on lectin binding in plants treated with antibiotics are reported. The presence of antibiotics in the medium did not change PS qualitative composition but it affected the type of hapten present in PS of both bacteria and mucilage. In fact, bacterial mucilage and *Anabaena* cells of control plants contained PS which bound to lectins specific for glucose (Con A, Fig. 1 a and c). Mucilage PS, instead, were composed of fucose (TPL), galactose (ECL) and reacted with WGA; whereas in erythromycin treated plants bacterial PS did not bind to ECL lectin and reacted weakly with WGA. Major changes were detected in PS extracted from plants treated with the antibiotic cocktail; there was no reaction with most of the lectins, with the exception of Con A.

Figure 1. Lectin and CFW binding of algal packets of *A. filiculoides* plants, treated and untreated with an antibiotic cocktail, observed at the fluorescence microscope (× 1250). (a) Con A – green fluorescence – and CFW – blue fluorescence – binding of bacteria of a control plant. It is possible to observe a mucilaginous material surrounding the bacteria and, to a lesser extent (fading fluorescence) filling the cavity. All bacteria are stained with ConA, while a few also with CFW. (b) Con A binding of bacteria in the leaf cavity of plants treated with a cocktail of antibiotics. There are fewer bacteria with respect to Figure (a), and the halo around them is smaller. No stained mucilages are present. (c) Con A binding of *Anabaena azollae* cells in leaf cavity of a control plant. It can be noticed that ConA stains the material surrounding the cell, not the cell itself. (d) CFW binding of *Anabaena azollae*, bacteria (black arrows) inside of the leaf cavity, and debris of fern epidermal cells (white arrows) of an antibiotic cocktail treated plant. Note that there are very few bacteria, except for the cluster indicated by the upper black arrow and no mucilaginous material containing β-linked PS is present within the cavity, only close to the cell wall. *Anabaena* cells of the treated plants show fluorescence on cell walls, particularly on the heterocysts. Bar = 3 μm. ConA = concanavalin A lectin, CFW = Calcofluor white.

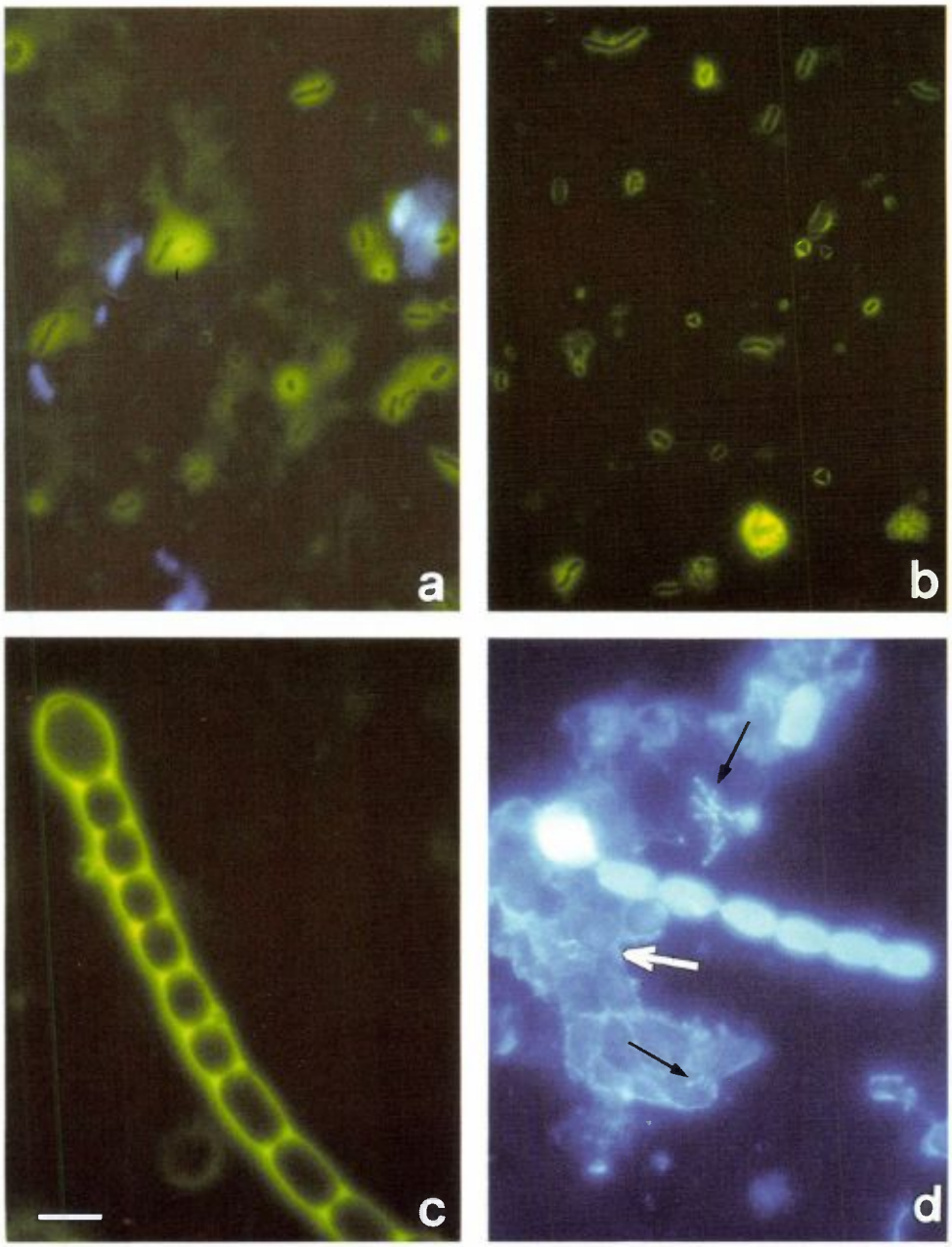

Figure 1. See legend on previous page.

Table 4. Lectin binding of the different components (B = bacteria; M = mucilage; A = *Anabaena*) of *A. filiculoides* Na algal packets treated with selective antibiotics. WGA = wheat germ agglutinin, TPL = *Tetragonolobus purpureas* lectin, LCL = *Lens culinaris* lectin, CPL = chick pea lectin, ECL = *Erythrina cristagalli* lectin, ConA = concanavalin A lectin.

Treatments	WGA	TPL	LCL	CPL	ECL	ConA
	B M A	B M A	B M A	B M A	B M A	B M A
Control	+ + −	− w −	− − −	− − −	+ + −	+ + +
Erythromycin	w + −	− w −	− − −	− − −	− w −	+ w w
Cocktail	− − −	w − −	− − −	− − −	− w −	− w +

+ = Binding; − = no binding; w = weak binding.

The presence of β-linked polysaccharides was determined by CFW binding (Fig. 1a and d); CFW stained the PS of *Anabaena* cells, the epidermal cells, and, to a lower extent and only in some cases, the mucilage surrounding the bacteria.

4. Discussion

In the literature there is still controversy about the origin of mucilage polysaccharides of fern-algal packets, i.e. about their possible plant origin (Peters et al., 1978), endophyte origin (Robins et al., 1986) or bacterial origin (Forni et al., 1989; Forni et al., 1992). In a previous study (Forni et al., 1992) we found that pure cultures of *Arthrobacter* strains, isolated from *Azolla* leaf cavities, produce mucilage, containing as main components, exopolysaccharides (EPS), i.e. PS loosely bound to the bacterial cells, which are excreted into the surrounding environment. Their monosaccharide composition was glucose and galactose, while rhamnose was present only in one strain.

The data presented in this paper suggest that algal packet mucilage is produced by all three partners of the association. Comparing polysaccharide composition of the algal packets with those produced in pure culture by *Arthrobacter* (Forni et al., 1992), we confirm a bacterial contribution to mucilage production *in vivo*, since both polysaccharides contain glucose and galactose. In Fig. 1 b, it is possible to observe a decrease of PS production by this bacterium, particularly glucose containing PS, inside plants treated with an

antibiotic cocktail. On the other hand, we detected a decrease in the amount of this monosaccharide in endophyte-free plants, therefore we cannot exclude a sharing of the polysaccharide production between cyanobiont and bactobiont. In this case we cannot exclude also a lack of stimulation by the cyanobacterium on PS production by *Arthrobacter*. The constant presence of fucose also in endophyte-free plants along with the evidence that *Arthrobacter* did not produce fucose in pure culture (Forni et al., 1992), strongly supports the assumption that fucose is of plant origin. Since rhamnose was not fern-species specific, its presence may depend on bacterial population of the leaf cavity, in fact some *Arthrobacter* strains produced rhamnose in pure culture (Forni et al., 1992).

Antibiotic treatments of the association did not change the polysaccharide composition of algal packets, only the relative amounts of single monosaccharides. Decrease of glucose and galactose in erythromycin-treated plants confirm what is suggested above, i.e. both *Anabaena* and *Arthrobacter* are involved in the production of these two monosaccharides.

Lectin-binding studies showed that treatments with antibiotics can affect the type of hapten available for lectins and can decrease binding. Monosaccharides present in PS and involved in binding were glucose, galactose and fucose. In particular, lectins specific for glucose bound to bacteria and mucilage; Con A – specific for glucose and mannose – bound also to *Anabaena* as already reported by Berliner and Fisher (1987). Since *Anabaena* did not bind to LCL – lectin specific for mannose – it is likely that the hapten of *Anabaena* is glucose. Another hapten for both bacteria and mucilage is galactose: in fact both bind to ECL. On the other hand, the lack of endophyte binding to ECL indicated the absence of a galactose hapten on its cell surface. These data are in agreement with findings of Ladha and Watanabe (1984) who reported the absence of binding *in situ* between *Azolla* lectin, specific for galactose, and *Anabaena*. In the control plants, TPL lectin, specific for fucose, did not bind to both bacteria and *Anabaena*, but only very weakly to the mucilage, indicating that this hapten is scarcely present. CFW and Con A binding microscopical observations suggested the presence of more than one polysaccharidic chain, some of them linked in position β.

Contrary to what was so far hypothesized, we here report evidence that all three partners of the symbiosis are involved in production of mucilage polysaccharides inside *Azolla* leaf cavity. Further studies are necessary, however, to verify the biochemical characteristics and chemical structure of polysaccharide chains produced by the three partners and their specific role in the symbiosis.

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REFERENCES

Berliner, M. and Fisher, R.W. 1987. Surface lectin binding to *Anabaena variabilis* and to cultured and freshly isolated *Anabaena azollae*. *Current Microbiology* **16**: 149–152.

Canini, A., Grilli Caiola, M., and Mascini, M. 1990. Ammonium content, nitrogenase activity and heterocyst frequency within the leaf cavities of *Azolla filiculoides* Lam. *FEMS Microbiology Letters* **71**: 205–210.

Del Gallo, M., Negi, M., and Neyra, C. 1989. Calcofluor- and lectin-binding exocellular polysaccharides of *Azospirillum brasilense* and *Azospirillum lipoferum*. *Journal of Bacteriology* **171**: 3504–3510.

Del Gallo, M. and Haegi, A. 1990. Characterization and quantification of exocellular polysaccharides in *Azospirillum brasilense* and *Azospirillum lipoferum*. *Symbiosis* **9**: 155–161.

Dische, Z. 1962. General color reactions. *Methods Carbohydrate Chemistry* **1**: 477–479.

Forni, C., Grilli Caiola, M., and Gentili, S. 1989. Bacteria in the *Azolla-Anabaena* symbiosis. In: *Nitrogen Fixation with Non-Legumes*. Skinner, F.A., Boddey, R.M., and Fendrik K., eds. Kluwer Academic Publisher, Dordrecht, pp. 83–88.

Forni, C., Tel-Or, E., Bar, E., and Grilli Caiola, M. 1991. Effects of antibiotic treatments on *Azolla-Anabaena* and *Arthrobacter*. *Plant and Soil* **137**: 151–155.

Forni, C., Haegi, A., Del Gallo, M., and Grilli Caiola, M. 1992. Production of polysaccharides by *Arthrobacter globiformis* associated with *Anabaena azollae* in *Azolla* leaf cavity. *FEMS Microbiology Letters* **93**: 269–274.

Kitoh, S., Nobuyuki, S., and Uheda, E. 1992. Free amino acid and ammonia in the *Azolla-Anabaena* association growth with N_2, NH_4^+ and NO_3. *Soil Science Plant Nutrition* **38**: 289–296.

Ladha, J.K. and Watanabe, I. 1984. Antigenic analysis of *Anabaena azollae* and the role of lectin in the *Azolla-Anabaena* symbiosis. *New Phytologist* **98**: 295–300.

Nairn, R.C. 1986. *Fluorescent Protein Tracing*. Fourth edition. Churchill and Livingstone, Edinburgh.

Peters, G.A., Toia, R.E. Jr., Raveed, D., and Levine, N.J. 1978. The *Azolla-Anabaena azollae* relationship. VI. Morphological aspects of the association. *New Phytologist* **80**: 583–593.

Peters, G.A. and Meeks, J.C. 1989. The *Azolla-Anabaena* symbiosis: basic biology. *Annual Review Plant Physiology Plant Molecular Biology* **40**: 193–210.

Robins, R.J., Hall, D.O., Shi, D.-J., Turner, R.J., and Rhodes, M.J.C. 1986. Mucilage acts to adhere cyanobacteria and cultured plant cells to biological and inert surfaces. *FEMS Microbiology Letters* **34**: 155–160.

Uheda, E. 1986. Isolation of empty packets from *Anabaena*-free *Azolla*. *Plant and Cell Physiology* **27**: 1255–1262.

Uheda, E. and Kitoh, S. 1991. Electron microscopic observations of the envelopes of isolated algal packets of *Azolla*. *Canadian Journal of Botany* **69**: 1418–1419.

Van Hove, C., Diara, H.F., and Godard, P. 1983. *Azolla in West Africa*. Oleffe Ed. Court-St-Etienne.