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Histochemistry of simple hairs from the foliar cavities of *Azolla filiculoides*

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Abstract

The foliar cavities of the water fern *Azolla filiculoides* have as many as 20–25 simple hairs (SH) protruding from the epidermal cells that delimit the cavity. These SH have a transfer-like ultrastructure normally associated with secretion of metabolites. The aim of this study was the chemical characterization of the compounds that accumulated in the SH of the zones F1–12 (from 1st to 12th leaves) and F13 (from 13th until the end of the sporophyte) throughout the seasons during a 1-year study. The histochemical tests show that the vacuoles of simple hairs contain a mixture of lipids, unsaturated lipids, polysaccharides, polyphenols (*o*-dihydroxyphenols, phenols with free –OH groups and tannins) and alkaloids or alkaloid-like compounds. These substances do not show seasonal variation, having been present throughout the one-year study. The histochemical analysis demonstrated that the SH always have a variety of metabolites. The function within the *Azolla-Anabaena* symbiosis is not known.

Key words: *Alkaloids, Azolla filiculoides, histochemistry, lipids, polysaccharides, tannins*

Introduction

Azolla is a small floating aquatic fern with bilobed leaves covering the rhizome. Each leaf has an aerial, thick, chlorophyllous dorsal lobe and a very thin, hyaline ventral lobe. The dorsal lobe has an ovoid cavity from which as many as 20–25 simple hairs (SH), one primary branched hair (PBH) and one secondary branched hair (SBH) protrude. These develop during the differentiation of the leaves, one primary branched hair (PBH) and one secondary branched hair (SBH). This cavity is inhabited by a community of the nitrogen-fixing cyanobacteria *Anabaena azollae*, and several genera of bacteria (Lumpkin & Plucknett, 1980). This unique association is important in rice culture when applied as a biofertilizer in some subtropical and tropical countries, as animal food (Carrapiço et al., 2000) or as a wastewater purifier (Costa et al., 1999).

Transmission electron microscopy shows that the cells of SH are transfer cells, which may be associated with the secretion of compounds, accumulated in the vacuoles and released to the cavity. This means an exchange of metabolites from *Azolla*

to the cyanobacteria (Calvert & Peters, 1981; Calvert et al., 1985).

Various histochemical methods allow the analysis of the many cellular components including proteins, carbohydrates, lipids, phenols or alkaloids. These techniques, which enable the investigation of compounds accumulated in special plant structures, such as trichomes and ducts, are widely used in angiosperms to characterize the secreted metabolites. In the *Azollaceae* such studies are very rare. Calvert et al. (1985) suggested that the SH accumulate tannins, and this was confirmed by Teixeira (1999) and Pereira et al. (2000), while Carrapiço & Tavares (1989) detected polysaccharides and lipids. No other histochemical study, either on these compounds or other compounds accumulated in the vacuoles of the SH has been conducted since then. Although the phytochemical studies performed on *Azolla* (Ishikura, 1982; Greca et al., 1989; Arai et al., 1998; Teixeira, 1999) show a great variety of metabolites, there are no histochemical studies on their *in situ* localization.

In this paper we report a histochemical study made on the vacuolar content of the SH in F1–12 and F13

leaves, during a 1-year period. These tests allowed the characterization of the substances accumulated in the SH, and an assessment of their possible seasonal changes.

Materials and methods

Plant material

A. filiculoides growing in the open was collected in Lisbon Botanical Garden (Faculty of Sciences, University of Lisbon) between October 2000 and September 2001. A voucher was deposited in the Herbarium of the Faculty of Sciences, University of Lisbon (LISU 191335).

The lateral branches of fresh *Azolla* sporophytes were removed, and the main axis separated into 3 parts: A) the apical meristem and the leaves that cannot be detached without being damaged; B) F1-12 (1st to 12th leaf); and C) F13 (from the 13th leaf to the end of the sporophyte), according to the developmental stage of the cavities (Calvert et al., 1985). F1-12 and F13 were cut longitudinally along the main axis through the foliar cavities with a razor blade.

Histochemical tests

The histochemical tests were 10% ferric trichloride for *o*-dihydroxyphenols (Johanson, 1940); potassium bichromate for phenols with free —OH groups (Gabe, 1968); vanillin-hydrochloric acid for tannins (Mace & Howell, 1974); Wagner reagent (Fulton, 1932), Dittmar reagent (Fulton, 1932), Dragendorff reagent (Stahl, 1965) for alkaloids; PAS for polysaccharides (Feder & O'Brien, 1968); Sudan Black B for total lipids (Bronner, 1975); osmium tetroxide for unsaturated lipids (Ganter & Jollés, 1969); copper acetate/rubeanic acid for fatty acids (Ganter & Jollés, 1969); antimony trichloride for sterols (Hardman & Safowora, 1972); and 2,4-dinitrophenylhydrazine for terpenoids with carbonyl groups (Ganter & Jollés, 1969). All histochemical tests were compared with their controls. The sections were observed by light microscopy using a Leitz-Wetzlar Dialux and an Olympus BX60.

Results

During a 1-year period, and throughout the main axis of the sporophyte of *A. filiculoides*, histochemical and fluorescence tests were used to visualize different classes of compounds accumulated in the vacuoles of SH (Table I), either in basal or in terminal cells.

The histochemical tests with PAS, Sudan Black B, osmium tetroxide, vanillin-hydrochloric acid, potassium dichromate, and Dragendorff reagent stained

positively the vacuolar content red/pink for polysaccharides (Figure 1A), dark black for total lipids (Figure 1B), dark black for unsaturated lipids (Figure 1C), intense red for tannins (Figure 1D), slightly brown for phenols (Figure 1E), and intense brown-orange for alkaloids (Figure 1F), respectively. Figures 1B, 1D, 1F, 1H, 1J and 1M, respectively, show negative controls for the staining tests. The vacuole stained positive with 10% ferric trichloride indicating the presence of phenolic compounds, and with Dittmar reagent for alkaloids, confirming the results obtained with the Dragendorff reagent.

The tests with Wagner reagent, copper acetate/rubeanic acid, and 2,4-dinitrophenylhydrazide were negative.

During the 1-year study SH always contained lipids, unsaturated lipids, polysaccharides, *o*-dihydroxyphenols, tannins, and alkaloids with few differences in colour intensity between F1-12 and F13, and with no seasonal variations.

As for the antimony trichloride test, the 1st, 2nd, 3rd, and 10th months showed a weak positive reaction, but overall it was considered to be negative.

Discussion

Present results show that the vacuoles of SH may contain a mixture of compounds: lipids (such as the unsaturated ones), polysaccharides, *o*-dihydroxyphenols and phenols with free —OH groups, tannins and alkaloids.

Lipids and polysaccharides were always present in the vacuoles of SH throughout the 1-year study and without seasonal variation. Carrapiço & Tavares (1989) in semithin sections, and Teixeira (1999) and Pereira et al. (2000) in fresh *Azolla*, already reported the presence of polysaccharides, but not in the course of 1 year. In this sense, we can establish a possible correlation between our positive histochemical *in situ* results and the polysaccharides, and other carbohydrates, isolated by Kaplan & Peters (1998). The lipophilic nature of the secretion in SH was previously established in semithin sections (Carrapiço & Tavares, 1989), and confirmed here in fresh *A. filiculoides* with Sudan Black B and osmium tetroxide. Seasonal variation in the lipophilic component of the secretion was not observed. Sudan Black B stains triglycerides, unsaturated fatty acids, and phospholipids. The positive reaction with osmium tetroxide confirms the unsaturated nature of the lipophilic material, but it also stains phenols (Ganter & Jollés, 1969). Although osmium tetroxide may stain unsaturated fatty acids, these were not detected with the copper acetate/rubeanic acid test. Consequently, it was not possible to correlate the phytochemical (Greca et al., 1989) and the histochemical results. This probably indicates that, in

Table I. Histochemical tests for the characterization of the compounds in simple hairs of *A. filiculoides*.

Histochemistry	Compound	Colour	Zone	10/2000	11/2000	12/2000	01/2001	02/2001	03/2001	05/2001	07/2001	09/2001	Avaliation
PAS	Polysaccharides	Red/bright pink	F1-12 F13	++ ++	? ?	- -	+	+	+	+	+	++ ++	+ +
10% Ferric trichloride	O-dihydroxyphenols	Black/dark brown	F1-12 F13	++ +	+? +	- -	+	+	+	+	- -	+	+ +
Potassium dichromate	Phenols with free OH group	Dark brown	F1-12 F13	+	+? +	- -	+	+	?	-	+	+	+ +
Vanillin	Tannins	Red	F1-12 F13	++ ++	+	+	++	++	++	-	++	++	++ ++
Wagner reagent	Alkaloids	Red-brown	F1-12 F13	a a	a a	a a	a	a	-	-	-	? ?	- -
Dittmar reagent	Alkaloids	Red-brown	F1-12 F13	a a	a a	a a	a	a	++	-	-	+	+ +
Dragendorff reagent	Alkaloids	Brown-orange	F1-12 F13	++ ++	++ ++?	- -	++	++	++	-	+	+	++ ++
Sudan Black B	Lipids	Dark black	F1-12 F13	+	++ ++	+	+	+	-	-	+	+	+ +
Osmium tetroxide	Unsaturated lipids	Dark black	F1-12 F13	++ ++	++ ++	+	++	++	++	+	++	++	++ ++
Copper acetate/rubeanic acid	Fatty acids	Dark green	F1-12 F13	- -	- -	- -	-	? ?	? ?	-	-	-	- -
Antimony trichloride	Sterols	Red-orange	F1-12 F13	+	-	-	+	+	+	-	-	-	- -
2,4-Dinitrophenylhydrazine	Terpenoids with carbonyl group	Red-orange	F1-12 F13	- -	- -	- -	-	-	-	-	+	-	- -

a, histochemical test not made; ?, inconclusive; -, negative; +, slightly positive; ++, strongly positive.

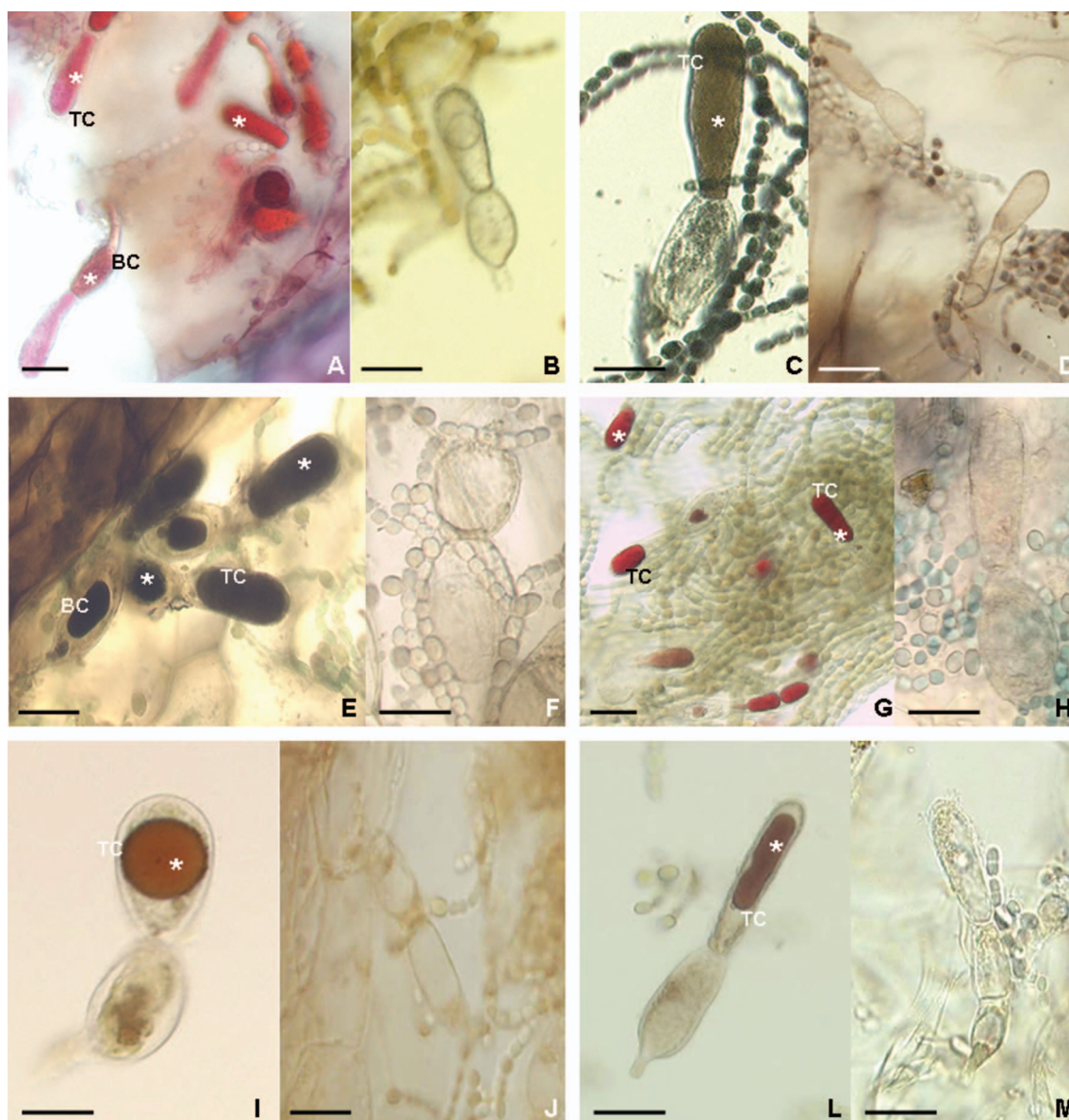


Figure 1. The histochemical tests show the staining (*) of the vacuoles of terminal (TC) and/or basal (BC) cells of the simple hairs. A: PAS. B: Negative control of PAS. C: Sudan Black B. D: Negative control of Sudan Black B. E: Osmium tetroxide. F: Negative control of osmium tetroxide. G: Vanillin-hydrochloric acid. H: Negative control of vanillin. I: Potassium dichromate. J: Negative control of potassium dichromate. L: Dragendorff reagent. M: Negative control of Dragendorff reagent. Scale bar, 5 μm . For a colour version of this figure, please visit the *Plant Biosystems* website: <http://www.informaworld.com/TPLB>

the symbiosis, fatty acids do not accumulate in SH, or may be present in amounts below the detection limit of histochemical tests.

The sterols are another group of compounds that can be detected by osmium tetroxide because of the double bond in Δ^5 (Pearse, 1995). These compounds were detected by chromatographic techniques by Greca et al. (1989) and Teixeira (1999). The histochemical tests yielded positive results in some months of the 1-year study, and hence were not conclusive.

Phenolic compounds, widely distributed in plants, include phenols, phenylpropanoids, flavonoids, tannins, and others. The positive reaction with potas-

sium bichromate in the vacuoles of SH may be due to simple phenols or phenylpropanoids. The latter seem to be present in the ferns (Berti & Bottari, 1968) and were isolated from several *Azolla* species by Greca et al. (1989), Ishikura (1982), and Teixeira (1999). Iron trichloride is used to stain the *o*-dihydroxyphenols, but as far as we know, these metabolites have never been reported in any of the *Azolla* species; however, the occurrence of simple phenols in some pteridophytes like *Polypodium vulgare* and *Asplenium lamprophyllum* has been reported (Berti & Bottari, 1968). Another type of polyphenol detected was the condensed tannins which, according to Harborne (1988), have a scattered occurrence among

the angiosperms, gymnosperms, and ferns. Their presence was reported in the cells of *Casuarina glauca* nodules infected by *Frankia* (Laplaze et al., 1999), and in *Azolla* spp. it was reported by Calvert et al. (1985), and confirmed by Teixeira (1999) and Pereira et al. (2000) in fresh *A. filiculoides*. Our observations show that tannins were always present during the 1-year study, without seasonal variations. Since it was not possible to isolate and make bioassays, we can only suggest some possible functions. The simple phenols and phenylpropanoids may have allelopathic or antimicrobial (Harborne, 1988) effects, but not on *A. azollae* and bacteria. According to Bennett & Wallsgrove (1994) and Cowan (1999), tannins act as a feeding deterrent for herbivores (as enzyme inhibitors) preventing the growth of microorganisms. In the case of *A. filiculoides*, these metabolites may bind to proteins and polysaccharides, reducing their digestibility in animals. If they accumulate in the SH, they may bind to polysaccharides and proteins of the pteridophyte itself, preventing the destruction of the fern tissue or of the symbionts. As in the case of *Casuarina glauca* (Laplaze et al., 1999), tannins may help in the establishment of the *Azolla-Anabaena* symbiosis during the germination of the megaspore, in the maintenance of the symbiosis, or in preventing secondary infection by other microorganisms.

Alkaloids are frequent in the angiosperms but, in ferns, they have been found only in few species, such as *Pteridium aquilinum* and *Lycopodium* sp. (Berti & Bottari, 1968; Evans, 1996), *Arachniodes standishii* (Watson et al., 2001), and *Huperzia* (Sengbush, 2003). The histochemical tests for alkaloids (Dragendorff and Dittmar reagents) gave positive reactions in SH. The most consistent result was obtained with the Dragendorff reagent, widely used to detect alkaloids in thin layer chromatography (TLC) plates (Harborne, 1991). To our knowledge, it is the first time that the existence of alkaloids or alkaloid-like compounds is reported in the *Azollaceae*. The Dragendorff reagent is also used in TLC plates to detect tertiary amines (Harborne, 1991) and can also precipitate proteins (Evans, 1996), so that results must be interpreted with caution. The genus *Azolla* has diamines (secondary amines) and polyamines (Marsh et al., 1998), none of which are tertiary amines; however, if secondary amines are present at high concentrations, they can be detected by the Dragendorff reagent. While inside the vacuoles alkaloids can bind with phenols, making both compounds biologically inactive (Ferreira et al., 1998). These metabolites act as deterrents (Harborne, 1988; Bennett & Wallsgrove, 1994), and have antibiotic properties (Cowan, 1999). Their function in the *Azolla-Anabaena* symbiosis is not

known, but we propose that they may function as a feeding deterrent or act against microorganisms. If the presence of alkaloids is confirmed by chromatographic methods, then the *Azollaceae* can be regarded as evolved pteridophytes. This will also indicate that *Azolla* species have the enzymes for the biosynthesis of alkaloids and their precursor compounds. A chemical characterization of the vacuolar compounds by chromatographic methods is currently being performed, especially for tannins and alkaloids.

SH accumulate a complex mixture of lipids and unsaturated lipids, polysaccharides, *o*-dihydroxyphenols and phenols with free —OH groups (such as phenylpropanoids), tannins, and alkaloids or alkaloid-like compounds. This study confirms, by *in situ* methods, previous reports on the presence of tannins and phenylpropanoids in several *Azolla* species, and adds the information that these compounds are present in the fern throughout the year with no seasonal variation. Furthermore, the existence of alkaloids has never been mentioned before in *Azolla*. The function of these bioactive metabolites in the *Azolla* symbiosis is still not well understood, and needs further research.

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References

- Arai Y, Nakagawa T, Hitosugi M, Shiojima K, Ageta H, Abdel-Halim OB. 1998. Chemical constituents of the aquatic fern *Azolla nilotica*. *Phytochemistry* 48:471–474.
- Bennett RN, Wallsgrove RM. 1994. Secondary metabolites in plant defence mechanisms. *New Phytol* 127:617–633.
- Berti G, Bottari F. 1968. Constituents of ferns. *Prog Phytochem* 1:589–685.
- Bronner R. 1975. Simultaneous demonstration of lipids and starch in plant tissue. *Stain Technol* 50:1–4.
- Calvert HE, Pence MK, Peters GA. 1985. Ultrastructural ontogeny of leaf cavity trichomes in *Azolla* implies a functional role in metabolite exchange. *Protoplasma* 129:10–27.
- Calvert HE, Peters GA. 1981. The *Azolla-Anabaena azollae* relationship. IX. Morphological analysis of leaf cavity hair populations. *New Phytol* 89:327–335.
- Carrapiço F, Tavares R. 1989. New data on the *Azolla-Anabaena* symbiosis. I. Morphological and histochemical aspects. In: Skinner FA, Boddey RM, Frederik I, editors. Nitrogen fixation with non-legumes. Dordrecht: Kluwer Academic. pp 89–94.
- Carrapiço F, Teixeira G, Diniz MA. 2000. *Azolla* as a biofertiliser in Africa. A challenge for the future. *Revista de Ciências Agrárias* 23:120–138.
- Costa ML, Santos MC, Carrapiço F. 1999. Biomass characterization of *Azolla filiculoides* grown in natural ecosystems and wastewater. *Hydrobiologia* 415:323–327.

- Cowan MM. 1999. Plant products as antimicrobial agents. *Clin Microbiol Rev* 12:564–582.
- Evans C. 1996. Trease and Evans' pharmacognosy. London: WB Saunders.
- Feder N, O'Brien TP. 1968. Plant microtechnique: Some principles and new methods. *Am J Bot* 55:123–142.
- Ferreira JFS, Duke SO, Vaughn KC. 1998. Histochemical and immunocytochemical localization of tropane alkaloids in *Erythroxylum coca* var. *coca* and *E. novogranatense* var. *novogranatense*. *Int J Plant Sci* 159:492–503.
- Fulton CC. 1932. The precipitation agents for alkaloids. *Am J Pharm* 104:244–271.
- Gabe M. 1968. Techniques histologiques. Paris: Masson et Cie.
- Ganter P, Jollés G. 1969. Histochimie normale et pathologique. Paris: Gauthier-Villars.
- Greca MD, Monaco P, Onorato M, Previtera L. 1989. Lipid composition of *Azolla filiculoides* Lamarck: non-polar lipids. *Gazz Chim Ita* 119:553–556.
- Harborne JB. 1988. Introduction to ecological biochemistry. London: Academic Press.
- Harborne JB. 1991. Phytochemical methods. A guide to modern techniques of plant analysis. London: Chapman & Hall.
- Hardman R, Safowora EA. 1972. Antimony trichloride as a test for steroids especially diosgenin and yamogenin in plant tissues. *Stain Technol* 47:205–208.
- Ishikura N. 1982. 3-Desoxyanthocyanin and other phenolics in the water fern *Azolla*. *Bot Mag Tokyo* 95:303–308.
- Johanson DA. 1940. Plant microtechnique. New York: McGraw-Hill.
- Kaplan D, Peters GA. 1998. The *Azolla-Anabaena azollae* relationship. XIV. Chemical composition of the association and soluble carbohydrates of the association, endophyte-free *Azolla*, and the freshly isolated endophyte. *Symbiosis* 24:35–50.
- Laplaze L, Gerbi H, Frutz T, Pawlowski K, Franche C, Macheix J-J, et al. 1999. Flavan-containing cells delimit *Frankia*-infected compartments in *Casuarina glauca* nodules. *Plant Physiol* 121:113–122.
- Lumpkin TA, Plucknett DL. 1980. *Azolla*: botany, physiology, and use as a green manure. *Econ Bot* 33:111–153.
- Mace MZ, Howell CR. 1974. Histochemistry and identification of condensed tannin precursors in roots of cotton seedlings. *Can J Bot* 52:2423–2426.
- Marsh BH, Corbin JL, Peters GA. 1998. Changes in soluble amino acid and polyamine composition associated with increasing plant density and the onset of sporulation in *Azolla*. *Symbiosis* 24:315–326.
- Pearse AGE. 1995. Histochemistry. Theoretical and applied. Edinburgh: Churchill Livingstone.
- Pereira AL, Sevinate-Pinto I, Antunes T, Teixeira G, Carrapiço F. 2000. Morphology, histochemistry and ultrastructure of the trichomes in the foliar cavities of *Azolla filiculoides* Lam. In: Weber HC, Imhof S, Zeuske D, editors. Programs, abstracts and papers of the Third International Congress on Symbiosis. Marburg: Philipps University of Marburg. p 170.
- Sengbush PV. 2003. Vascular cryptogams. Available from: http://www.botany.wisc.edu/courses/botany_401/Lecture/02Lecture.html, accessed: 21 January 2002.
- Stahl E. 1965. Thin layer chromatography. A laboratory handbook. London: Academic Press.
- Teixeira GMM. 1999. Estudo biológico e fitoquímico de *Azolla filiculoides* Lam. e de *Azolla pinnata* subsp. *africana* (Desv.) R. M. K. Saunders & K. Fowler [dissertation]. Lisbon: University of Lisbon. p 222.
- Watson AA, Fleet GWJ, Asano N, Molyneux RJ, Nash RJ. 2001. Polyhydroxylated alkaloids—natural occurrence and therapeutic applications. *Phytochemistry* 56:265–295.