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Volatile compounds from the symbiotic system *Azolla filiculoides-Anabaena azollae* bacteria

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Abstract

Azolla filiculoides is an aquatic pteridophyte that may be used as animal food, biofertilizer and phytoremediation. Its volatile composition was never studied although several phytochemical analyses were performed. The volatile composition of *A. filiculoides* grown outdoors in a pond at the Botanical Garden of Lisbon University (BGLU) or in culture conditions as well as the effect of different harvesting times and the storage type were evaluated. The volatiles isolated by hydrodistillation and distillation-extraction were analysed by gas chromatography and gas chromatography-mass spectrometry. The oil of all the *A. filiculoides* samples studied affords a yellowish colour and an unpleasant odour in a yield of 0.01% (v/fw). Alcohols, aldehydes, alkanes and ketones dominated the culture samples, while aldehydes, alcohols, terpenoids and alkanes represented the main volatiles of the BGLU samples. Some quantitative differences were detected in seasonal and type of storage (fresh, dry or frozen at −20°C) studies of *A. filiculoides* from the BGLU. The BGLU and culture volatiles showed qualitative differences: 2-ethyl-1-hexanol was only identified in the fern culture, whereas acetophenone, pentylfuran, acetylpyridine and 2-octanone were only detected in BGLU samples. The dendrogram showed two distinct clusters (culture and BGLU samples). The possible biological origin and bioactivity of some of the volatile compounds is discussed.

Abbreviations: FID, flame ionization detector; i.d., internal diameter; v/fw, volume by fresh weight; GC, gas chromatography; GC-MS, gas chromatography-mass spectrometry; u, unified atomic mass unit

Keywords: Azolla filiculoides, *Azollaceae, gas chromatography, gas chromatography-mass spectrometry, volatiles*

Introduction

Azolla Lamarck is a small heterosporic aquatic fern (Family Azollaceae) with deeply bilobed leaves and a worldwide distribution in tropical and subtropical countries. Due to nitrogen fixation by the cyanobacteria *Anabaena azollae* Strasburger, which inhabit the foliar cavities of *Azolla*, this pteridophyte may be used as a biofertilizer in rice cultures, as feed for chickens, pigs, ducks, fish and humans. Moreover, it is used as mosquito and weed control, and in folk medicine against throat infection (Lumpkin & Plucknett 1980; Wagner 1997; Carrapiço et al. 2000).

Owing to the potential application as food for animals and humans, and in agriculture, the analysis of its chemical composition is of the utmost importance. Previous phytochemical studies performed in some *Azolla* species have revealed several compounds such as amino acids, flavones, phenylpropanoids, fatty acids and sterols (Greca et al. 1989; Marsh et al. 1998; Teixeira et al. 2001; Nakane et al. 2003), although their biological activity was unknown. Nevertheless, Greca et al. (1989) attributed to phenylpropanoid α-asarone, the growth inhibition of green algae.

To gain further knowledge on the phytochemistry of *A. filiculoides*, the aims of this study were: (1) to evaluate the composition and the seasonal variation of the volatiles produced by *A. filiculoides* grown at the Botanical Garden of Lisbon University (BGLU)

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(outdoor water pond); (2) to determine the effect of the type of storage (fresh, dry or frozen at –20°C) on the volatile composition; and (3) compare the volatiles of *A. filiculoides* from BGLU with those growing in four different culture media (H-40, IRRI1-Fe1x, IRRI1-Fe10x and IRRI2).

Materials and methods

Plant material

Azolla filiculoides (Table I) sporophytes were harvested from a pond at the Botanical Garden of Lisbon University (outdoor natural growth, BGLU) or grown in the culture media H-40 (Allen & Arnon 1955; Peters et al. 1980), IRRI1-Fe1x and IRRI1-Fe10x (Watanabe et al. 1977) or IRRI2 (T. Ventura, personal communication, 2002). A voucher from BGLU was deposited in the Herbarium of the Faculty of Sciences of Lisbon University (LISU 191335).

Isolation procedure

The volatiles were isolated by distillation-extraction for 3 h using a Likens-Nickerson-type apparatus (Likens & Nickerson 1964) with distilled *n*-pentane as organic solvent and by hydrodistillation for 3 h using a Clevenger-type apparatus (Anonymous 1996). The samples isolated by hydrodistillation were used to estimate the yield, and those isolated by distillation-extraction to determine the percentage composition of the volatile compounds, since the chance of artefact formation must be considered smaller when the latter method is used.

Gas chromatography (GC)

GC analyses were performed using a Perkin-Elmer 8700 gas chromatograph equipped with two flame ionization detectors (FIDs), a data handling system and a vaporizing injector port into which two columns of different polarities were installed: a DB-1 fused-silica column (30 m × 0.25 mm i.d., film thickness 0.25 μm; J & W Scientific) and a DB-17HT fused-silica column (30 m × 0.25 mm i.d., film thickness 0.15 μm; J & W Scientific). Oven temperature was set at 45°C –175°C at 3°C/min, subsequently at 15°C/min up to 300°C, and then held isothermal for 10 min; injector and detector temperatures were 280 and 290°C, respectively; the carrier gas, hydrogen, was adjusted to a linear velocity of 30 cm/s. Samples were injected using the split sampling technique, at a ratio of 1:50. The percentage composition of the volatiles were computed by the normalization method from the GC peak areas, which were calculated as mean values of two injections of each sample, without using response factors.

Gas chromatography-mass spectrometry (GC-MS)

The GC-MS unit consisted of a Perkin-Elmer Autosystem XL gas chromatograph, equipped with a DB-1 fused-silica column (30 m × 0.25 mm i.d., film thickness 0.25 μm; J & W Scientific), and interfaced with a Perkin-Elmer Turbomass mass spectrometer (software version 4.1). Injector and oven temperatures were as above; transfer line temperature at 280°C; ion trap temperature at 220°C; the carrier gas, helium, was adjusted to a linear velocity of 30 cm/s; the split ratio was 1:40; ionization energy was 70 eV; ionization current was 60 μA; the scan range was 40–300 u; scan time was 1 s. The identity of the components was assigned by comparison of their retention indices, relative to C_7–C_{21} *n*-alkanes, and GC-MS spectra from a home-made library based on the analysis of reference oils, laboratory-synthesized components and commercially available standards.

Statistical analysis

The normalized percentage composition of the volatiles was used to establish the relationship between the eight samples of *A. filiculoides* using Pearson's correlation coefficient to measure the similarity of the samples (Rohlf 2000). The correlation was evaluated as very high ($S_{corr} > 0.9$), high ($0.6 < S_{corr} < 0.9$), moderate ($0.3 < S_{corr} < 0.6$) and low ($0 < S_{corr} < 0.3$) (Callegari-Jacques 2003).

The dendrogram was obtained with sequential, agglomerative, hierarchical and nested clustering

Table I. Origin, harvesting and storage method of *A. filiculoides* samples used for the isolation of the volatiles.

Origin	Harvesting	Type of storage	Abbreviation
BGLU (water pond, outdoor)	From 1998 until 2001	Dry at 30°C –35°C	BGLUDry
BGLU (water pond, outdoor)	December 2001	Fresh	BGLUFreshDec
BGLU (water pond, outdoor)	April 2002	Fresh	BGLUFreshApril
BGLU (water pond, outdoor)	April 2002	Frozen at –20°C	BGLUFrozApril
Culture in IRRI2	28 days	Frozen at –20°C	CultIRRI2
Culture in IRRI 1-Fe10x	28 days	Frozen at –20°C	CultIRRI1-Fe10x
Culture in H-40	28 days	Frozen at –20°C	CultH-40
Culture in IRRI 1-Fe1x	28 days	Frozen at –20°C	CultIRRI1-Fe1x

(SAHN) based on the unweighted pair group method with arithmetic mean (UPGMA) cluster analysis with the NTSYS-pc software (version 2.1, Exeter Software). The cophenetic correlation (Mantel test) was determined to test the goodness-of-fit between the dendrogram and the similarity matrix (Rohlf 2000).

Results

A yellowish oil with an unpleasant odour was isolated from *A. filiculoides* with a yield of 0.01% (v/fw). The identified volatiles, 40 and 43 for *A. filiculoides* grown in culture and BGLU, respectively, are listed in Table II. Among these, 12 components were ketones (3.4%–8.0%), 10 compounds were aldehydes (2.5%–32.9%) and eight components were alcohols (10.2%–26.6%).

Aldehydes and alcohols were the main fraction of the volatiles of *A. filiculoides* grown in culture and BGLU, but they differed quantitatively in the terpenoid, alkane, ketone and fatty acid fractions. Two samples of BGLU showed the highest amounts of terpenoids (18.2%).

1-Octen-3-ol (9.0%–13.5%) was the main component of the volatiles isolated from three of the four culture samples; the other sample (CultIRRI1-Fe1x) was dominated by *n*-octane (20.3%). *cis*-3-Hexenol (3.7%–21.9%), *n*-heptanal (6.5%–13.7%) and phytol acetate (0.6%–18.2%) were the major compounds in the volatiles from BGLU.

The chemical variability was confirmed by the dendrogram with UPGMA cluster analysis (Figure 1). The goodness-of-fit between the dendrogram and the similarity matrix was very high with a cophenetic correlation value of 0.92. The dendrogram revealed two clusters with a low correlation degree (S_{corr} = 0.17), one with the samples of *A. filiculoides* in culture (S_{corr} = 0.28) and another with the BGLU samples (S_{corr} = 0.48). This is due not only to the differences in the major and minor components, but also to the absence of some compounds in both culture and BGLU samples. 2-Ethyl-1-hexanol (up to 4.6%) was only detected in the volatiles from *A. filiculoides* grown in artificial media but not in BGLU samples (Table II). On the other hand, in the volatiles isolated from *A. filiculoides* grown at BGLU, acetophenone (1.8%–4.4%), 2-octanone (up to 1.0%), pentylfuran (up to 1.2%) and acetylpyridine (up to 0.9%) were identified but not in the pteridophyte grown in the four culture media (Table II).

Only quantitative and no qualitative differences were observed in the seasonal study of *A. filiculoides* grown at BGLU (BGLUFreshDec and BGLUFreshApril), with a high degree of correlation (S_{corr} = 0.82, Figure 1).

When evaluating differences between fresh (BGLUFreshApril), frozen at –20°C (BGLUFrozApril) and dried material (BGLUDry), again only quantitative variations were observed, with *cis*-3-hexenol (21.9%), *trans*-2-hexenal (13.6%) and *n*-heptanal (8.1%) as the main compounds (Table II), respectively. Probably, these differences determined a low to moderate degree of correlation between the samples (Figure 1), with the fresh samples being a more correlated cluster (S_{corr} = 0.82) than the group of frozen and dry samples (S_{corr} = 0.56).

Discussion

To the best of our knowledge, this is the first study on the volatile composition of the symbiotic system *A. filiculoides-A. azollae* bacteria. However, knowledge of its phytochemical composition is important since this pteridophyte can be used as food or biofertilizer (Lumpkin & Plucknett 1980). The study conducted using histochemical methods (Pereira & Carrapiço 2007) could be related with some of the volatile compounds detected by GC-MS, if these accumulate inside the vacuoles of the simple hairs of the foliar cavities, but it was not possible to verify this.

When performing a phytochemical analysis, it is better to use fresh material but, often, this is not possible and the plants are either frozen or dried (Harborne 1991) which may modify the volatile composition. The three types of storage of *A. filiculoides* revealed quantitative but not qualitative differences, with a moderate/high degree of correlation between the samples. The growth, development, and biosynthesis of secondary metabolites are also influenced by temperature, precipitation and photoperiod (Evans 1996). In the case of *A. filiculoides*, seasonality modifies the relative amounts of the volatiles. Moreover, the outdoor and culture growth conditions appear to affect the volatile composition due probably to a shift in the biosynthetic pathway. The culture conditions seem to induce the synthesis of 2-ethyl-1-hexanol, whereas the samples from BGLU had acetophenone, 2-octanone, pentylfuran and acetylpyridine.

The alcohols, ketones and aldehydes, such as *trans*-2-hexenal, 3-octanone, 1-octen-3-ol and other compounds, may contribute to the unpleasant odour of the *A. filiculoides* volatiles, which will be a disadvantage when considering its use as animal feed. The fish *Cichlasoma fenestratum* select preferably *A. microphylla* instead of *A. pinnata* as food (Antoine et al. 1986). The difference in volatile composition of *A. filiculoides* grown outdoors and in artificial culture combined with the unpleasant odour may account for their repellent and/or attractive properties.

Table II. Percentage composition of the volatiles produced by *A. filiculoides* grown in four different culture media or in BGLU. The values represent the arithmetic mean of two GC injections for each sample.

		A. filiculoides from culture[1]				*A. filiculoides* from BGLU[1]			
Components	RI	CultIRRI2	CultIRRI1-Fe10x	CultH-40	CultIRRI1-Fe1x	BGLUDry	BGLUFreshDec	BGLUFrozApril	BGLUFreshApril
n-Hexanal	756	4.4	4.2	2.1	0.5	5.0	*t*	9.7	1.0
n-Octane	800	0.8	0.8	*t*	20.3	4.5	4.1	2.3	9.9
trans-2-Hexenal	866	4.1	3.0	4.1	*t*	2.1	*t*	13.6	1.5
cis-3-Hexenol	868	*t*	*t*	*t*	3.2	3.7	17.0	11.6	21.9
n-Hexanol	882	6.2	5.7	6.9	1.5	4.0	0.7	3.2	1.3
2-Heptanone[2]	886	1.4	2.2	1.8	*t*	1.3	0.7	*t*	2.0
n-Heptanal	897	0.9	0.9	1.1	*t*	8.1	13.7	6.5	12.8
Benzaldehyde	927	*t*	*t*	*t*	*t*	1.0	0.5	0.8	*t*
n-Heptanol	952	1.0	2.8	1.5	*t*	0.9	*t*	0.8	*t*
6-Methyl-5-hepten-2-one	960	0.8	*t*	0.8	*t*	*t*	*t*	*t*	*t*
3-Octanone	961	*t*	0.9	1.4	6.0	0.4	1.3	1.6	0.8
1-Octen-3-ol	961	11.0	13.5	10.9	9.0	0.7	0.5	5.7	1.3
2-Octanone	966	—	—	—	—	1.0	*t*	*t*	*t*
Pentylfuran[2]	972	—	—	—	—	1.2	*t*	*t*	*t*
n-Octanal	973	0.7	1.0	1.0	*t*	0.8	*t*	*t*	*t*
Isolimonene	978	*t*	1.3	1.3	*t*	t	*t*	*t*	*t*
Acetylpyridine	988	—	—	—	—	t	0.9	*t*	*t*
2,6,6-Trimethylcyclohexanone	1003	1.7	2.2	2.2	*t*	t	t	*t*	*t*
2-Ethyl-1-hexanol[2]	1004	3.3	4.0	4.6	*t*	—	—	—	—
Acetophenone	1017	—	—	—	—	3.6	4.4	1.8	2.4
n-Octanol	1045	*t*	0.6	0.7	*t*	0.9	0.7	0.9	*t*
2-Nonanone	1058	*t*	1.0	*t*	*t*	1.1	*t*	*t*	*t*
n-Nonanal	1073	0.8	1.1	0.8	*t*	3.3	5.1	1.3	2.2
4-*keto*-Isophorone	1090	*t*	*t*	*t*	*t*	*t*	*t*	*t*	*t*
n-Nonanol	1151	*t*	*t*	*t*	*t*	*t*	*t*	*t*	*t*
Safranal[2]	1160	*t*	*t*	*t*	*t*	*t*	*t*	*t*	*t*
2-Decanone	1166	*t*	*t*	*t*	*t*	*t*	*t*	*t*	*t*
n-Decanal	1180	0.5	0.6	0.9	*t*	*t*	*t*	*t*	*t*
Geraniol	1236	*t*	0.7	*t*	*t*	*t*	*t*	*t*	*t*
n-Decanol	1259	*t*	*t*	*t*	*t*	*t*	*t*	*t*	*t*
2-Undecanone	1271	0.7	0.9	0.9	*t*	*t*	*t*	*t*	*t*
2-Pentadecanone	1391	1.1	*t*	*t*	*t*	*t*	*t*	*t*	*t*
n-Tetradecane	1400	*t*	*t*	*t*	*t*	*t*	*t*	*t*	*t*
trans-β-Ionone-epoxide[2]	1452	1.3	0.9	0.7	*t*	3.1	*t*	*t*	*t*
β-Ionone	1455	1.6	1.6	1.3	*t*	1.9	3.3	1.5	1.8
n-Tridecanal	1499	2.4	1.1	0.9	*t*	0.8	1.1	*t*	*t*

(*continued*)

Table II. (*Continued*).

Components	RI	*A. filiculoides* from culture[1]				*A. filiculoides* from BGLU[1]			
		CultIRRI2	CultIRRI1-Fe10x	CultH-40	CultIRRI1-Fe1x	BGLUDry	BGLUFreshDec	BGLUFrozApril	BGLUFreshApril
n-Pentadecane	1500	*t*	1.0	0.5	*t*	*t*	*t*	*t*	*t*
n-Tetradecanal	1596	3.1	t	*t*	*t*	*t*	1.1	*t*	*t*
n-Hexadecane	1600	*t*	0.8	*t*	*t*	*t*	*t*	*t*	*t*
n-Pentadecanal	1688	2.3	4.1	5.2	2.0	*t*	1.3	1.0	*t*
n-Heptadecane	1700	3.3	3.0	3.0	5.0	3.0	3.7	0.8	1.7
2-Heptadecanone[2]	1787	*t*	*t*	*t*	*t*	0.6	t	t	t
Palmitic acid	1908	3.1	4.2	5.5	1.5	2.4	t	t	17.3
Phytol acetate	2047	2.1	2.3	1.9	4.2	1.8	18.2	0.6	18.2
Grouped components									
Aldehydes		19.2	16.0	16.1	2.5	21.1	22.8	32.9	17.5
Ketones		5.7	7.2	7.1	6.0	8.0	6.4	3.4	5.2
Alcohols		21.5	26.6	24.6	13.7	10.2	18.9	22.2	24.5
Terpenoids		2.1	4.3	3.2	4.2	1.8	18.2	0.6	18.2
Terpenoids of degradation		2.9	2.5	2.0	*t*	5.0	3.3	1.5	1.8
Alkanes		4.1	5.6	3.5	25.3	7.5	7.8	3.1	11.6
Nitrogenous compounds		—	—	—	—	t	0.9	*t*	*t*
Fatty acids		3.1	4.2	5.5	1.5	2.4	*t*	*t*	17.3
Furanes		—	—	—	—	1.2	*t*	*t*	*t*

RI – retention index relative to C_7–C_{21} on DB-1 column; *t* – trace < 0.05%.

[1]For abbreviations, see Table I.

[2]Identified only on mass spectra.

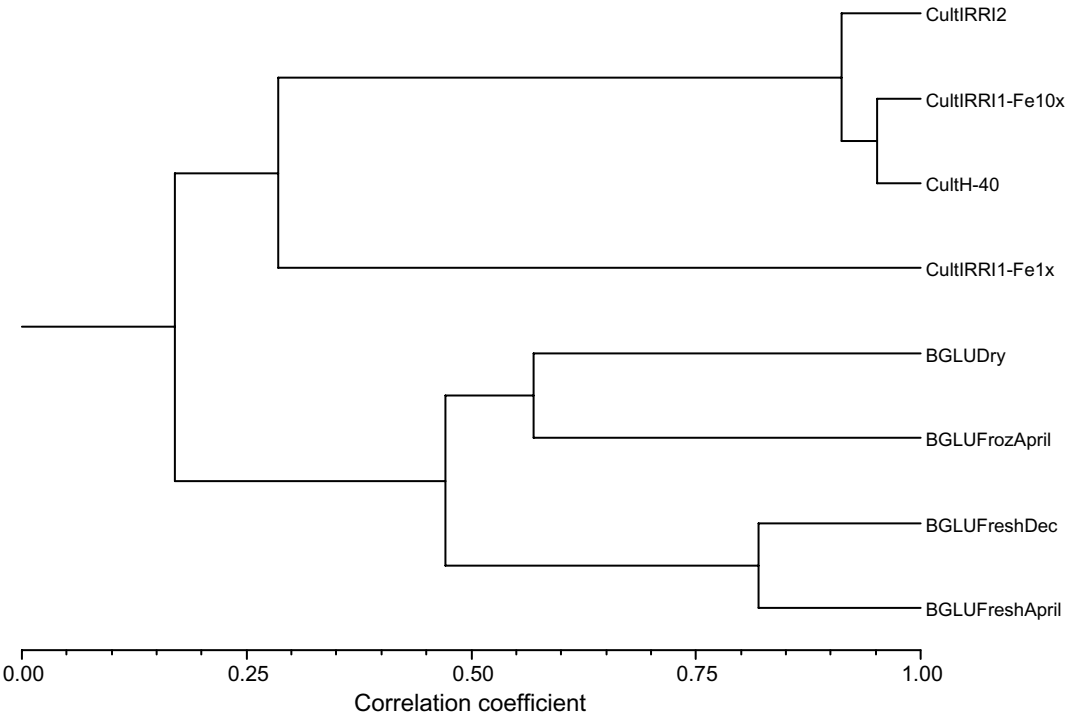

Figure 1. Dendrogram obtained with the correlation coefficient and UPGMA cluster analysis based on the percentage composition of volatiles from eight *A. filiculoides* samples. BGLU: Botanical Garden of the Lisbon University water pond, outdoor; Cult: grown in the culture media (see Materials and methods section and Table I for abbreviations).

2-Ethyl-1-hexanol, only identified in the samples of *A. filiculoides* grown in culture, was also detected in the tubercle of *Solanum tuberosum* when infected with *Phytophthora infestans* and *Fusarium coeruleum* (de Lacy Costello et al. 2001). The analysis of the volatile component of plants infected by pathogenic microorganisms allows early detection when the symptoms are not visible. In the case of the symbiotic system under investigation, it is not possible to exclude that *A. filiculoides* from culture released 2-ethyl-1-hexanol due to some type of stress.

trans-β-Ionone in *A. filiculoides* probably derives from the epoxydation of β-ionone in the 5,6-position or oxidation of β-carotene (Giuliano et al. 2003). In addition, 4-*ceto*-isophorone and 2,6,6-trimethylcyclohexanone may derive from the oxidation of β-carotene or other xanthophylls with post-structural rearrangements during distillation. This points to *A. filiculoides* as a source of carotenoids.

Aldehydes, alcohols and ketones can be obtained from the oxidation of fatty acids by the lipoxygenases (LOX) and later converted into other components. LOX gene expression is regulated by the physiological state of the plant, necrosis, water stress and pathogen attack (Siedow 1991), and the C_6-volatiles formed by LOX can act as signalling molecules (Porta & Rocha-Sosa 2002). The only reference to LOX in *Azolla* were made by Greca et al. (1989) and although the purpose of the present study was not the detection of LOX, the major compounds detected in all eight samples could derive from the degradation of fatty acids by LOX.

In *Arabidopsis*, *trans*-2-hexenal presents fungicidal activity against *Aspergillus flavus* (Gardini et al. 2001) and can also function as a signalling molecule involved in mechanisms of plant defence, such as the expression of genes of the phenylpropanoid pathway (Bate & Rothstein 1998). Since *A. filiculoides* never harbours hyphae or spores of fungi in the foliar cavities, *trans*-2-hexenal may have fungicidal activity, but this subject requires further studies.

In conclusion, the present data show that growth conditions of *A. filiculoides* influence volatile composition. Only quantitative differences were detected in response to the season and to the type of storage. Since some of the identified compounds may act as repellent or attractant, or have some other biological activity, additional studies on the biological role of these volatiles would be important.

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