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# *Anemia tomentosa* var. *anthriscifolia* in vitro culture: sporophyte development and volatile compound profile of an aromatic fern

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## Abstract

*Anemia tomentosa* var. *anthriscifolia* is an aromatic fern with a pleasant woody aroma and antimycobacterial activity. In this paper, we describe for the first time its spore-derived gametophyte development and the effect of indole-3-acetic acid and jasmonic acid on in vitro gametophyte/sporophyte development, as well as volatile compound production. Volatiles were obtained by simultaneous distillation and extraction (SDE) and analyzed by high resolution gas chromatography coupled with mass spectrometry. Spore-derived gametophytes were able to develop into sporophytes independently of the media culture composition, even when no plant growth regulator was added. Fifty different substances were detected in all in vitro *A. tomentosa* SDE extracts, while 20 were detected in the wild SDE plant extract. Monoterpenes were more prevalent (69.8–89.8%) than sesquiterpenes (9.4–28.7%) in in vitro plants, while sesquiterpenes represent 97.5% of the volatiles produced by the wild-grown plants. The major monoterpene components in in vitro plants were α-pinene (9.3–24.3%), *trans*-pinocarveol (20.6–27.9%), pinocarvone (15.4–25.1%) and myrtenyl acetate (6.4–12.3%). The triquinane sesquiterpenes silphiperfol-6-ene (0.6–2.9%), α-guaiene (0.5–2.5%), β-barbatene (1.1–3.9%) and 9-*epi*-presilphiperfolan-1-ol (2.5–5.6%) represent the most abundant sesquiterpenes. The changes in the monoterpene/sesquiterpene rates between micropropagated and wild plants are not related to the presence of JA or IAA in the media culture. Further studies are still needed to obtain a complete understanding of the factors leading to these results, which could be related to differences in the irradiance levels of in vitro plants versus those from a wild environment, as well as the developmental stage of the plants. This is the first report of the use of plant growth regulators on *Anemia tomentosa* in vitro culture development and their effects on volatile profiles.

**Keywords** Pteridophyta · Monoterpenes · Sesquiterpenes · Plant tissue culture · Jasmonic acid · Indole-3-acetic acid

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**Abbreviations**

| | |
|---|---|
| SDE | Simultaneous distillation and extraction |
| HRGC-MS | High resolution gas chromatography coupled with mass spectrometry |
| PGR | Plant growth regulator |
| IAA | Indole-3-acetic acid |
| JA | Jasmonic acid |
| MS | Murashige and Skoog (1962) medium |
| GC | Gas chromatography |
| FID | Flame ionization detector |
| LRI | Linear retention indices |

## Introduction

The Anemiaceae family has only one genus, *Anemia*, which comprises more than 100 species of terrestrial habit that are widely distributed throughout the New World, occurring in

the Tropical Americas, Africa and Southeast India (Juliani et al. 2004; Santos et al. 2006). *Anemia* species can be found from sea level to 1.500 m altitude (Juliani et al. 2004). In Brazil, the largest populations occur at the coastline, especially in the south and southeast regions (Schwartsburd and Labiak 2007).

*Anemia tomentosa* (Savigny) Sw. is a rupicolous species that commonly occurs on rocky shores and is frequently associated with cactus. It has four varieties of which *A. tomentosa* (Savigny) Swartz var. *anthriscifolia* (Schrader) Mickel is the most common (Pinto et al. 2009a).

The essential oil composition of this variety has been described, and it has been suggested that different chemotypes exist. Studies from Juliani et al. (2004) on the essential oil from plants collected in Argentina have described α-bisabolol as its major constituent. Santos et al. (2006) described isoafricanol as the major sesquiterpene from the oil obtained from a specimen collected in Rio de Janeiro State, Brazil; Pinto et al. (2009a, b) described triquinane sesquiterpenes as the major compounds of the oil from a specimen collected in Espirito Santo State, Brazil, in the absence of α-bisabolol or isoafricanol.

In a previous work from our group (Pinto et al. 2013), the volatiles produced by plants from an in vitro culture showed a different profile from previous studies. *A. tomentosa* var. *anthriscifolia*, which was successfully developed in Murashige and Skoog (1962) medium without growth regulators, produced an essential oil mainly composed of monoterpenes (79%), whereas those from wild-grown plants comprised mostly triquinane sesquiterpenes (97.5%) (Pinto et al. 2013). Triquinane sesquiterpenes are responsible for the pleasant woody aroma of *A. tomentosa*´s essential oil, as well as its antimycobacterial activity against *Mycobacterium tuberculosis* (Pinto et al. 2009a), giving this species huge potential to be used as medicine or in perfumery. Thus, it is of paramount importance to develop strategies to produce whole plants that can be used for essential oil extraction. Despite the previous in vitro cultures showing a decrease in the triquinane sesquiterpenes production, these techniques are still a suitable tool for large scale plants and/ or essential oil production.

Studies regarding the in vitro culture of ferns have mainly focused on spore germination and micropropagation, with an eye toward the ornamental plant market (Bharati et al. 2013). Gametophyte early development and gametophyte multiplication from a preexisting gametophyte are the micropropagation techniques described in the scientific literature, while sporophyte growth is obtained mainly through ex vitro conditions (Somer et al. 2010; Bharati et al. 2013; Kodym et al. 2016). Nevertheless, the in vitro culture could contribute to the understanding of sporophyte in vitro development and establish the appropriate conditions to improve volatile compound production, enabling plant multiplication and

eliminating any biotic or abiotic factors that could have a negative effect on the desired metabolic profile.

The main purpose of this study was to investigate the effect of two plant regulators—indole-3-acetic acid and jasmonic acid—on *Anemia tomentosa* var. *anthriscifolia* in vitro plant development and the production of volatile compounds.

## Materials and methods

### Spore collection, disinfection and inoculation

Fertile fronds of *A. tomentosa* var. *anthriscifolia* were collected from a triquinane essential oil-producing mother plant (voucher specimen RB438912) cultivated in Bom Jesus do Itabapoana, Rio de Janeiro State, Brazil (21°08′19.5″S 41°40′37.0″W). The fronds were placed into paper bags immediately after collection, transported to Rio de Janeiro and stored for 2 weeks at room temperature (25–30 °C) to allow spore release. The collected spores (12.5 g) were transferred to Eppendorf tubes (2.5 ml), soaked in distilled water for 12 h at room temperature and were centrifuged at 2000 rpm for 3 min. The supernatant was discarded and replaced with a NaOCl 1% + Tween 20 1% solution. After 10 min, the spores were spun again, and inside a laminar flow cabinet, the supernatant was discarded and the pellet was washed with sterile distilled water. During the final spin, the supernatant was removed and the pellet (spores) was suspended in 5 ml of sterile distilled water, resulting in a 2.5 g spores/ml suspension.

Spores were cultured on the surface of different media: MS (Murashige and Skoog 1962, full micro and macronutrients), MS 1/2 (MS half strength micro and macronutrients), MS 1/4 (MS a quarter of micro and macronutrients) and non-MS (zero amount of micro and macronutrients). All media were supplemented with MS vitamins, myo-inositol and 3% sucrose and were solidified with 0.8% agar, at pH 5.8.

A known amount (100 µl) of sterilized spore suspension was inoculated into glass flasks (100 ml) containing 40 ml of different media. In total, 40 flasks were inoculated (10 per media culture). Sterile distilled water (1 ml) was added to the spore culture to facilitate germination at the inoculation time and subsequently once a week. All media and solutions used in the experiments were previously sterilized at 121 °C at a pressure of 1 atm for 15 min. The spores and gametophyte cultures were maintained in a growth room at $25 \pm 1$ °C with 16 h illumination provided by 50 µmol s$^{-1}$ m$^{-2}$ daylight fluorescent tubes.

Thirty days after inoculation, spore cultures were evaluated for gametophyte development and photographed using a Leica Magnifier coupled to a digital camera.

## Effects of plant growth regulators (PGR) on in vitro development

Three-month-old spore-derived gametophytes obtained on MS 1/2 were used as the initial material for the study of the effect of various plant growth regulators (PGR) on gametophyte and sporophyte development. Spore-derived gametophyte clusters (40 mg) were transferred to glass flasks (500 ml) containing 40 ml of MS 1/2 medium, prepared as described earlier, and jasmonic acid—JA (0.1; 1 and 10 µmol) and indole-3-acetic acid—IAA (2.8; 5.4 and 11.4 µmol) were added. All cultures were maintained in the growth room under the conditions described previously, with the monthly addition of 1 ml of sterile distilled water.

Fresh and dry biomass and the number of newly fully expanded leaves per responding cluster were calculated after 105 days of culture (without subculture). The experiment was performed following a randomized complete block design with six types of media and three replications, each consisting of four flasks containing five gametophyte clumps (n = 60).

## Data analysis

The data were analyzed using the software Statistica© 7.0 Stat Soft Inc. (Tulsa, OK, USA) for Analysis of Variance (ANOVA), and medium means were compared using Tukey's test at 5% level of significance.

## Extraction of volatiles by simultaneous distillation and extraction (SDE)

Volatiles from the leaves of wild (Pinto et al. 2013) and in vitro plants were extracted by simultaneous distillation and extraction (SDE) in a modified micro-SDE-type apparatus (Chaintreau 2001). Five grams of each plant material were placed in a 100-ml flask with 50 ml of distilled water; 1 ml of dichloromethane was introduced to a conical flask; 5 ml of dichloromethane was added to the cold-finger area. Both flasks were submitted to heating in a mineral oil bath. The heating temperatures for the sample and solvent flasks were controlled at $130 \pm 5$ and $55 \pm 5$ °C, respectively. After 2 h, the steam distillation was stopped, while the solvent extraction continued for 30 min. In this way, all volatile
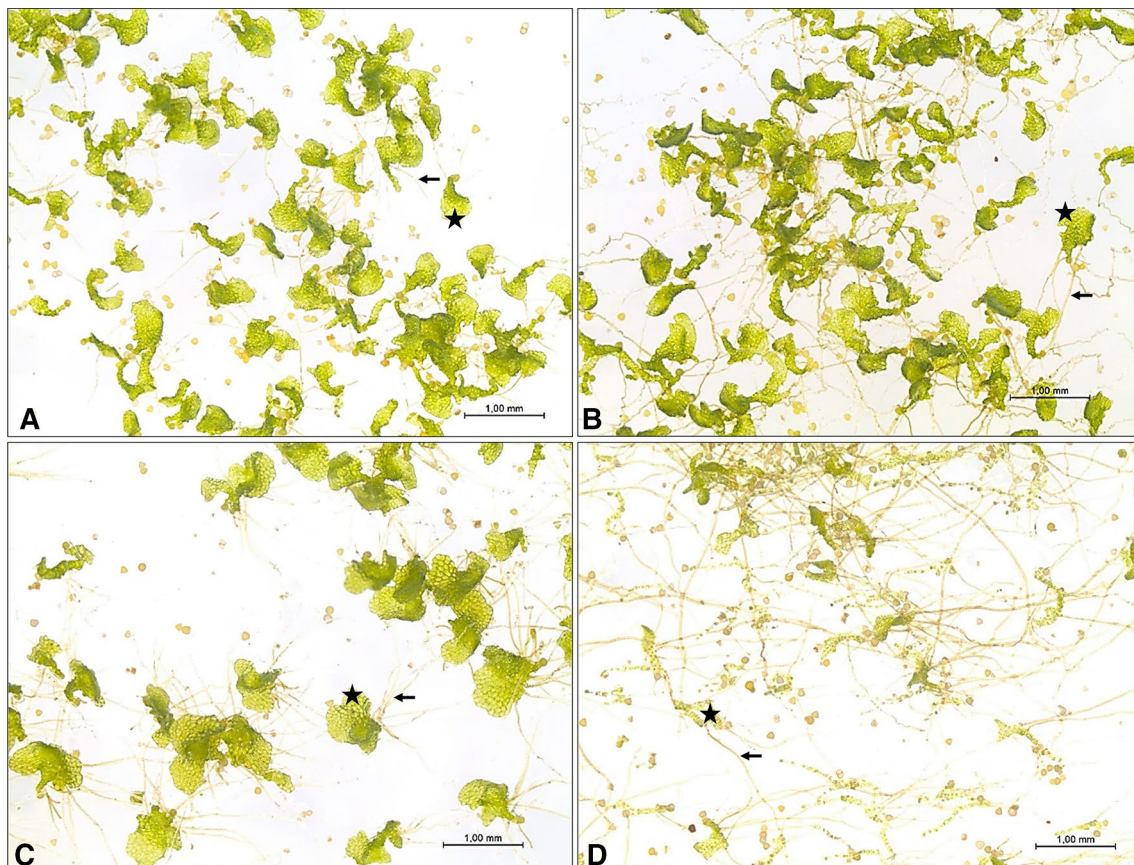


**Fig. 1** Gametophyte development after 30 days in different culture media showing gametophytes (star) and rhizoids (arrow) **a** MS; **b** MS 1/2; **c** MS 1/4; **d** non-MS

material was collected in *ca*. 1 ml of dichloromethane. Subsequently, 1 µl each of these solutions was analyzed by capillary gas chromatography (GC).

## Gas chromatographic analyses

Gas chromatographic analyses were performed within 2–3 days after extraction, using a Shimadzu GC-2010 system equipped with a flame ionization detector (FID) and a DB-5MS (5% phenyl −95% dimethylpolysiloxane) fused silica capillary column (30 m×0.25 mm×0.25 µm). Hydrogen was the carrier gas (1.0 ml min$^{-1}$ at 60 °C in a constant linear velocity). The injector temperature was kept at 250 °C, and the oven temperature programmed from 60 to 290 °C at 3 °C min$^{-1}$ (5 min in an isotherm). The detector (FID) was operated at 290 °C. One microliter of the dichloromethane solution obtained by SDE was injected in split mode (1:10). The percentages of each component were reported as raw percentages without standardization.

Gas chromatography–mass spectrometry analyses were performed on a Shimadzu GC-2010 system with a mass selective detector coupled gas chromatograph and fitted with a DB-5MS capillary column (30 m×0.25 mm×0.25 µm) operating in electronic ionization (EI) mode at 70 eV. A transfer line was maintained at 260 °C, while the mass analyzer and ion source temperature were held at 150 and 230 °C, respectively. Helium (1.5 ml min$^{-1}$) was used as the carrier gas. The oven temperature program was from 60 to 290 °C at 3 °C min$^{-1}$, the injector temperature was kept at 260 °C and the split rate was the same as stated for GC analyses. A standard solution of *n*-alkanes (C$_7$–C$_{30}$), injected under the same column and conditions as above, was used to obtain the linear retention indices (LRI). Individual volatile components were identified by comparison of mass spectra (MS) and LRI with those reported in the literature (Adams 2007), as well as those stored in the NIST8s and NIST08. LIB mass spectral library of the GC–MS data system.

**Fig. 2** Gas chromatography/flame ionization detector (GC/FID) chromatograms of volatiles from in vitro *Anemia tomentosa* obtained by SDE. **1** MS 1/2; **2** MS 1/2+0.1 µmol JA; **3** MS 1/2+1.0 µmol JA; **4** MS 1/2+10.0 µmol JA; **5** MS 1/2+2.8 µmol IAA; **6** MS 1/2+5.4 µmol IAA; **7** MS 1/2+11.4 µmol IAA. *A* α-pinene; *B* trans-pinocarveol; *C* pinocarvone; *D* myrtenyl acetate; *E* silphiperfol-6-ene; *F* α-guaiene; *G* β-barbatene; *H* 9-epi-silphiperfolan-1-ol; *I* 2-{[(2-ethylhexyl)oxy]carbonyl} benzoic acid (contaminant)

## Results

Spore germination of *A. tomentosa* occurred in all culture media, and a different gametophyte development pattern was observed depending on nutrient content. After 30 days of culture, almost all gametophytes developing in MS 1/4 medium presented a heart-shaped developmental stage, while in non-MS medium, they were mainly in the filamentous and spatulate stages. Gametophytes from MS and MS 1/2 were in the spatulate and heart-shaped developmental stages (Fig. 1a, b). Rhizoids showed differential development within the media and was shortest in gametophytes from MS 1/4 (Fig. 1c), especially when compared to growth in non-MS medium which was clearly longer than for all others (Fig. 1d).

Gametophytes from non-MS medium were brown in color after 45 days, and their growth clearly decreased compared to other cultures. The same substandard development was observed for cultures from MS 1/4 spore-derived gametophytes (data not show). Given these results, we chose MS 1/2 to evaluate the PGR effects on sporophytes development.

Spore-derived gametophytes were able to develop into sporophytes independently of the media culture composition, even when no PGR was added. The total period needed for sporophytes to be evident in the culture was approximately 4–5 weeks, and in all media culture conditions, we observed gametophytes and sporophytes (Table 1). There were more individuals with both structures in JA treatments (62–69%) than in MS 1/2 (38%) or IAA (17–34%). For

**Table 1** *A. tomentosa* in vitro development after 105 days in various culture media

| Media | Parameters | | | |
|---|---|---|---|---|
| | Fresh biomass (mg) | Dry biomass (mg) | Individuals with gametophyte present (%) | Number of expanded leaves |
| MS 1/2 | 327[B] | 78[AB] | 38 | 11[AB] |
| MS 1/2+0.1 µmol JA | 880[A] | 107[A] | 69 | 12[A] |
| MS 1/2+1.0 µmol JA | 435[B] | 82[AB] | 62 | 10[AB] |
| MS 1/2+10.0 µmol JA | 314[BC] | 68[BC] | 62 | 8[B] |
| MS 1/2+2.8 µmol IAA | 173[C] | 52[BC] | 17 | 6[BC] |
| MS 1/2+5.4 µmol IAA | 125[C] | 41[C] | 34 | 4[C] |
| MS 1/2+11.4 µmol IAA | 177[C] | 49[BC] | 32 | 6[BC] |

Mean values with the same letter do not differ significantly in Tukey's test at a 5% level of statistical significance
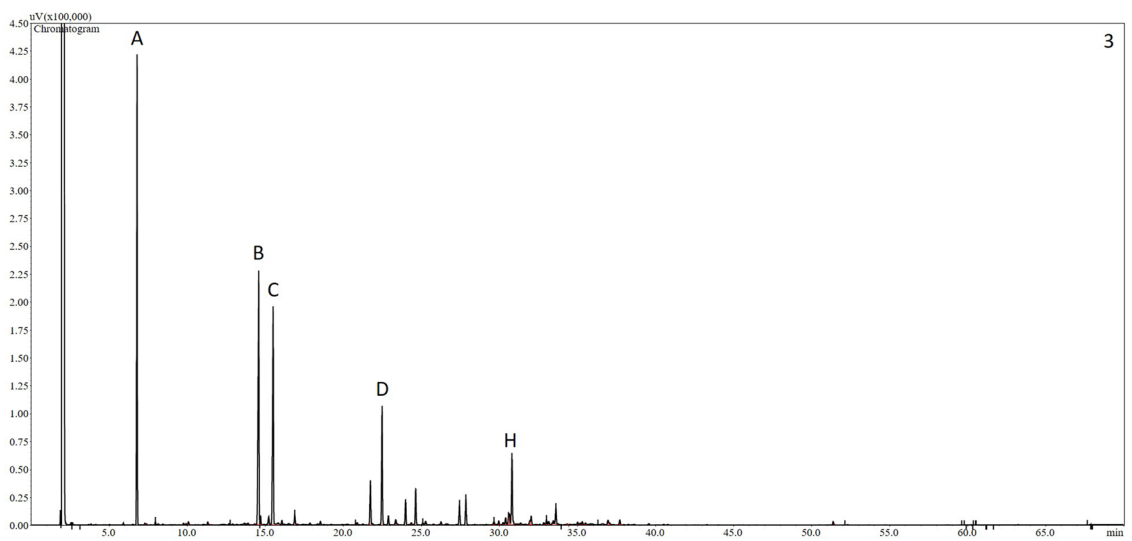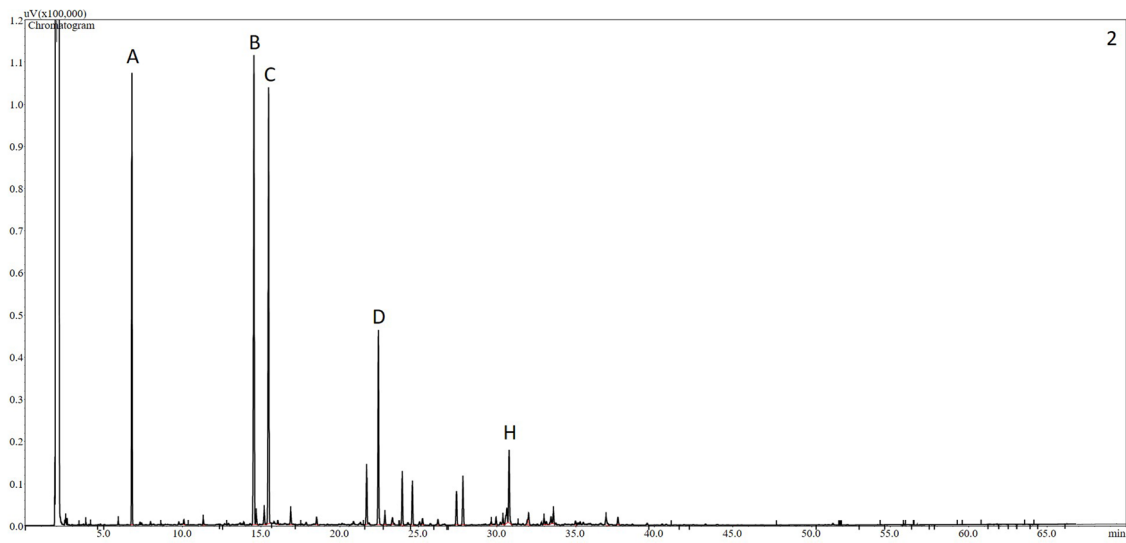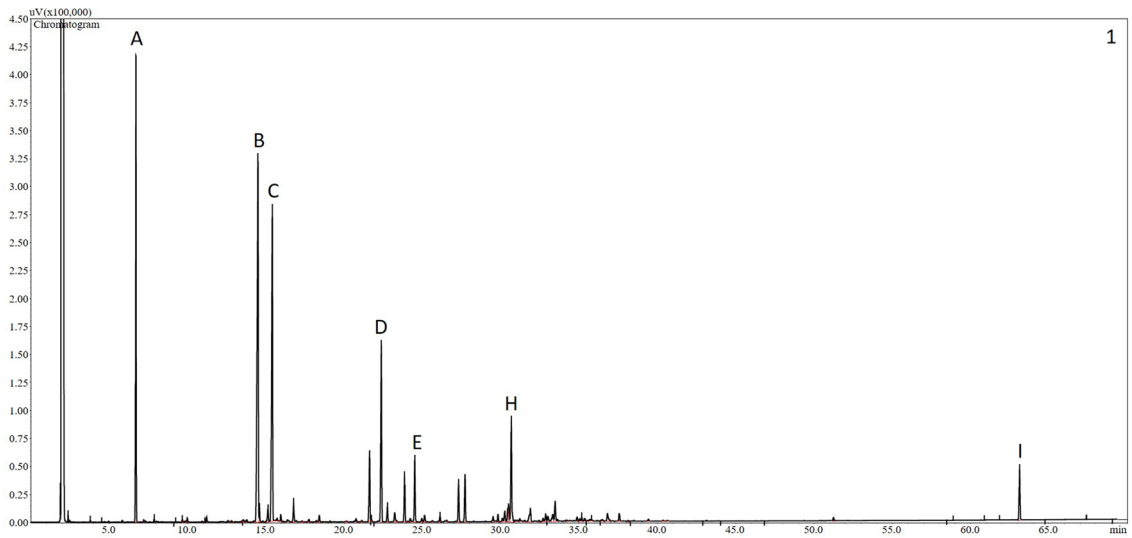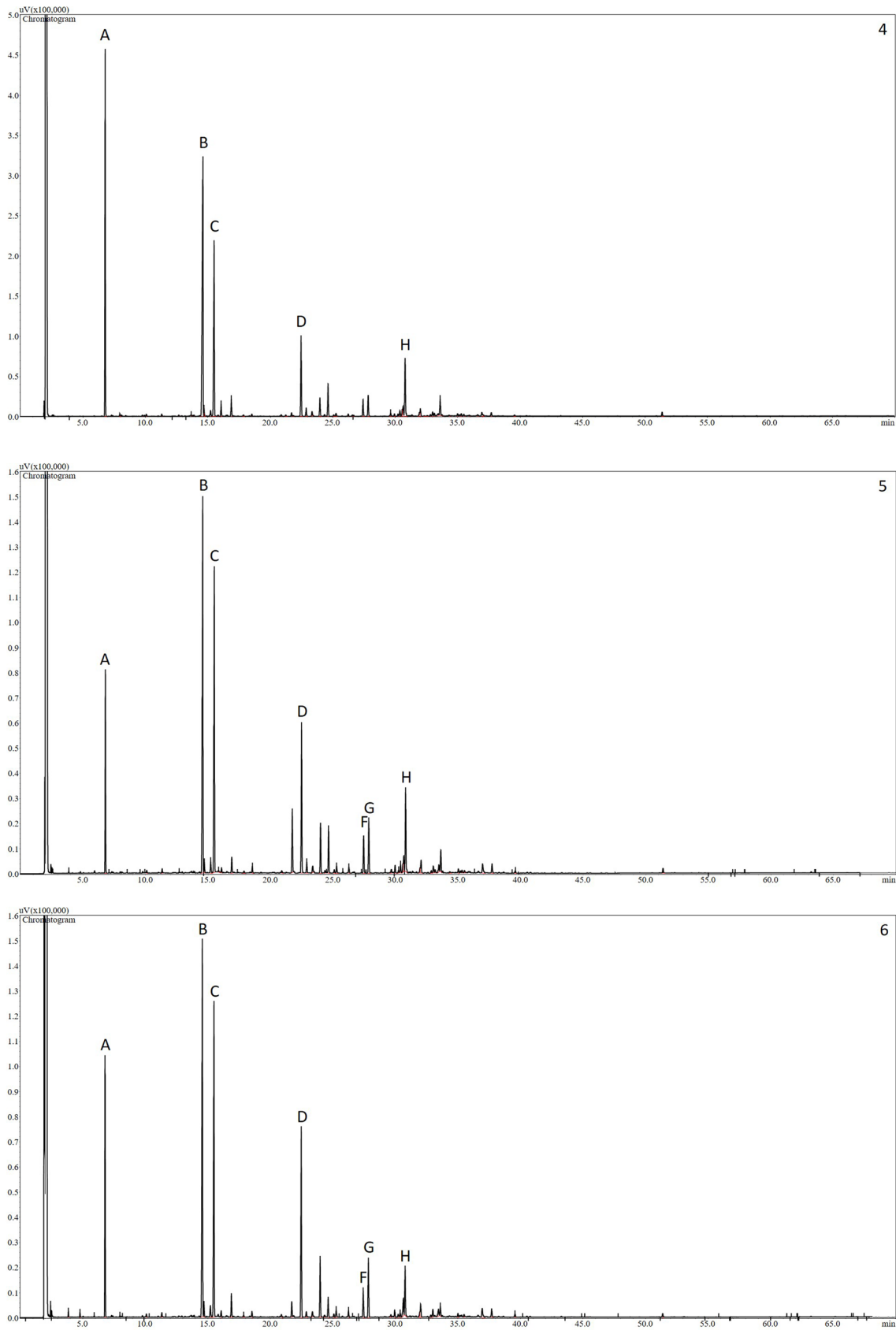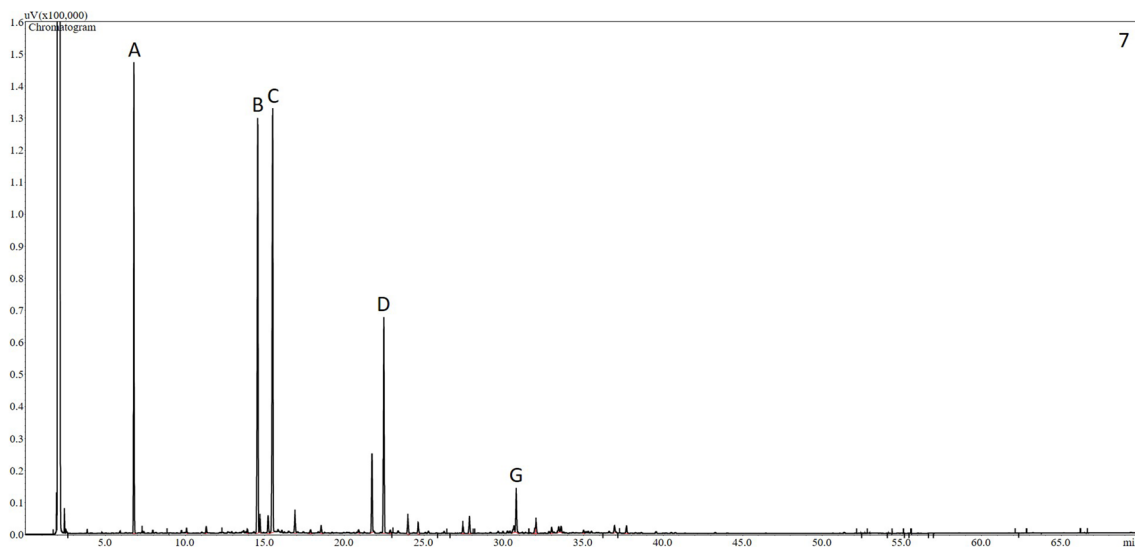
**Fig. 2** (continued)

**Fig. 2** (continued)

sporophyte growth, IAA addition into media culture generally decreased the overall plantlet development, while JA promoted the most vigorous sporophytes throughout the experiment. After 105 days, spore-derived gametophytes developed on MS 1/2 + 0.1 µmol JA produced the most new fully expanded leaves (12 leaves) and fresh and dry biomass (880 and 107 mg, respectively). However, *A. tomentosa* sporophytes growth on MS 1/2 + 5.4 µmol IAA showed fewer fully expanded leaves (4 leaves), as well as fresh and dry biomass (Table 1).

The gas chromatograms of the volatiles obtained from the in vitro cultures of *Anemia tomentosa* grown under the influence of different PGR are shown in Fig. 2(1–7), and the chemical identification is reported in Table 2. A total of 47 different substances were detected in all in vitro *A. tomentosa* SDE extracts, primarily consisting of mono and sesquiterpenes.

As seen in Table 2, the treatment with both PGR leads to a decrease in the number of produced volatile compounds in the in vitro plants; with IAA, many fewer components are observed. Comparison of the results obtained with JA shows that as the concentration of this hormone increases, so does the diversity of volatiles. For the sporophytes developed under MS 1/2 + 1.0 µmol JA, 41 different volatile compounds were detected; thus, at this concentration, JA favors the production of volatiles much more than other concentrations of this same hormone.

In terms of monoterpene/sesquiterpene balance, Table 2 shows that sesquiterpenes represent 97.5% of the volatiles produced by wild-grown plants, whereas the monoterpene profile is prevalent (69.8–89.8%) over sesquiterpenes (9.4–28.7%) in all in vitro plants. The major monoterpene components identified in all in vitro plants were α-pinene

(9.3–24.3%), *trans*-pinocarveol (20.6–27.9%), pinocarvone (15.4–25.1%) and myrtenyl acetate (6.4–12.3%). The triquinane sesquiterpenes silphiperfol-6-ene (0.6–2.9%), α-guaiene (0.5–2.5%), β-barbatene (1.1–3.9%) and 9-*epi*-presilphiperfolan-1-ol (2.5–5.6%), represent the most abundant identified sesquiterpenes. Despite the diminished proportion of sesquiterpenes in the volatiles obtained from in vitro plants, 9-*epi*-presilphiperfolan-1-ol is still most abundant, as well as the presence of various triquinane sesquiterpenes.

## Discussion

To contribute to understanding *Anemia tomentosa* in vitro development and volatile production, we conducted two experiments using several saline MS medium concentrations and various types of PGR and concentration. We observed that spatulate and heart-shaped gametophytes developed in rich in nutrient medium (MS and MS ½) was in contrast with those described for *Adiantum reniforme* var. *sinense*, where the development of these same gametophytes patter was greatest on MS 1/4 medium, and gametophyte development was inhibited on full and MS 1/2 medium (Wu et al. 2010). However, no significant differences were observed in *Osmunda regalis* L. spore germination and gametophyte in vitro development with media with the same saline content (Makowski et al. 2016). Differential development within the media observed for rhizoids suggests that the energetic effort for rhizoids development likely compensates for the nutritional deficit of the medium. It is well known that many factors can affect in vitro spore germination and early gametophyte development, including light, temperature, plant

**Table 2** Identification of volatile constituents from wild and in vitro *Anemia tomentosa* treated with various plant growth regulators (PGR)

| # | Compounds | LRI-calc. | LRIlit. | Percentage [(%), SD] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Wild plant[a] | MS1/2 | MS1/2+0.1 µM JA | MS1/2+1.0 µM JA | MS1/2+10.0 µM JA | MS1/2+2.8 µM IAA | MS1/2+5.4 µM IAA | MS1/2+11.4 µM IAA |
| 1 | **α-Pinene** | **934** | **932** | **0.5** | **15.6±0.0** | **16.7±0.0** | **24.3±0.2** | **21.9±0.1** | **9.3±0.2** | **12.0±0.1** | **19.3±0.1** |
| 2 | Camphene | 950 | 946 | – | 0.1±0.0 | – | 0.2±0.0 | 0.2±0.0 | – | – | – |
| 3 | β-Pinene | 972 | 974 | – | 0.1±0.0 | – | 0.1±0.0 | 0.1±0.0 | – | – | – |
| 4 | p-Cymene | 1025 | 1020 | – | 0.1±0.0 | – | 0.1±0.0 | 0.1±0.0 | 0.1±0.0 | t | – |
| 5 | 1,8-Cineole | 1033 | 1026 | – | 0.2±0.0 | 0.2±0.0 | 0.2±0.0 | 0.1±0.0 | 0.1±0.0 | – | 0.2±0.0 |
| 6 | n.i. (MW:137) | 1065 | n.i. | – | 0.2±0.0 | 0.3±0.0 | 0.2±0.0 | 0.1±0.0 | 0.2±0.0 | 0.2±0.0 | 0.3±0.0 |
| 7 | trans-p-Mentha-2,8-dien-1-ol | 1123 | 1119 | – | 0.1±0.0 | – | – | – | – | – | – |
| 8 | α-Campholenal | 1128 | 1122 | – | 0.1±0.0 | – | 0.1±0.0 | 0.1±0.0 | – | – | 0.2±0.0 |
| 9 | **trans-Pinocarveol** | **1144** | **1135** | **0.7** | **22.6±0.1** | **23.9±0.1** | **20.6±0.1** | **27.9±0.1** | **24.4±0.1** | **25.2±0.1** | **24.3±0.1** |
| 10 | trans-Verbenol | 1146 | 1140 | – | 0.6±0.0 | 0.5±0.0 | 0.5±0.0 | 0.5±0.0 | 0.6±0.0 | 0.7±0.0 | 0.7±0.0 |
| 11 | n.i. (MW:134) | 1159 | n.i. | – | 0.7±0.0 | 0.8±0.0 | 0.6±0.0 | 0.5±0.0 | 0.8±0.0 | 0.8±0.0 | 1.0±0.0 |
| 12 | **Pinocarvone** | **1165** | **1160** | **1.3** | **17.1±0.1** | **22.2±0.1** | **16.4±0.1** | **15.4±0.1** | **19.3±0.0** | **20.5±0.1** | **25.1±0.1** |
| 13 | p-Mentha-1,5-dien-8-ol | 1172 | 1166 | – | 0.1±0.0 | – | 0.1±0.0 | 0.1±0.0 | – | – | – |
| 14 | cis-Pinocamphone | 1178 | 1172 | – | 0.3±0.0 | 0.2±0.0 | 0.3±0.0 | 0.9±0.0 | 0.3±0.0 | 0.4±0.0 | – |
| 15 | p-Cymen-8-ol | 1188 | 1179 | – | 0.1±0.0 | – | – | – | – | – | – |
| 16 | Myrtenol | 1197 | 1194 | 1.6 | 0.9±0.0 | 0.7±0.0 | 0.8±0.0 | 1.4±0.0 | 1.0±0.0 | 1.5±0.0 | 1.1±0.0 |
| 17 | n.i. (MW:151) | 1234 | n.i. | – | 0.3±0.0 | 0.4±0.0 | 0.2±0.0 | 0.2±0.0 | 0.4±0.0 | 0.4±0.0 | 0.4±0.0 |
| 18 | Bornyl acetate | 1287 | 1284 | – | 0.1±0.0 | – | 0.1±0.0 | 0.1±0.0 | 0.1±0.0 | 0.2±0.0 | 0.2±0.0 |
| 19 | **n.i. (MW:134)** | **1307** | n.i. | – | **3.4±0.1** | **3.1±0.2** | **3.2±0.1** | **0.3±0.0** | **3.8±0.1** | **1.0±0.0** | **4.8±0.1** |
| 20 | **Myrtenyl acetate** | **1324** | **1324** | **0.6** | **8.7±0.0** | **9.8±0.0** | **8.3±0.1** | **6.4±0.1** | **9.5±0.0** | **12.2±0.1** | **12.3±0.1** |
| 21 | **Presilphiperfol-7-ene** | **1333** | **1334** | **3.0** | **0.7±0.0** | **0.6±0.0** | **0.7±0.0** | – | **0.7±0.0** | **0.4±0.0** | **0.2±0.0** |
| 22 | 7-epi-Silphiperfol-5-ene | 1344 | 1345 | 1.6 | 0.5±0.0 | 0.4±0.0 | 0.4±0.0 | 0.4±0.0 | 0.5±0.0 | 0.4±0.0 | – |
| 23 | **n.i. (MW:204)** | **1358** | n.i. | – | **2.1±0.0** | **2.6±0.0** | **1.8±0.0** | **1.6±0.0** | **3.3±0.0** | **3.8±0.0** | **0.9±0.0** |
| 24 | n.i. (MW:204) | 1367 | n.i. | 0.8 | 0.2±0.0 | – | 0.1±0.0 | 0.1±0.0 | 0.2±0.0 | 0.5±0.0 | – |
| 25 | **Silphiperfol-6-ene** | **1373** | **1377** | **11.7** | **2.9±0.0** | **2.1±0.0** | **2.6±0.0** | **2.7±0.0** | **2.8±0.0** | **1.3±0.0** | **0.6±0.0** |
| 26 | n.i. (MW:204) | 1384 | n.i. | – | 0.2±0.0 | – | 0.1±0.0 | 0.1±0.0 | 0.2±0.0 | 0.2±0.0 | – |
| 27 | α-Isocomene | 1388 | 1387 | – | 0.3±0.0 | 0.4±0.0 | 0.3±0.0 | 0.3±0.0 | 0.5±0.0 | 0.5±0.0 | 0.1±0.0 |
| 28 | β-Isocomene | 1411 | 1407 | – | 0.3±0.0 | 0.3±0.0 | 0.2±0.0 | 0.2±0.0 | 0.4±0.0 | 0.4±0.0 | – |
| 29 | **α-Guaiene** | **1439** | **1437** | **7.0** | **1.8±0.0** | **1.9±0.0** | **1.5±0.0** | **1.4±0.0** | **2.5±0.0** | **1.8±0.0** | **0.5±0.0** |
| 30 | **β-Barbatene** | **1449** | **1440** | – | **2.3±0.0** | **2.6±0.0** | **2.0±0.0** | **1.8±0.0** | **3.6±0.0** | **3.9±0.0** | **1.1±0.0** |
| 31 | n.i. (MW:220) | 1492 | n.i. | 1.7 | 0.3±0.0 | 0.3±0.1 | 0.3±0.0 | 0.3±0.0 | 0.3±0.0 | 0.3±0.0 | – |

**Table 2** (continued)

| # | Compounds | LRI-calc. | LRIlit. | Percentage [(%), SD] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Wild plant[a] | MS1/2 | MS1/2 + 0.1 µM JA | MS1/2 + 1.0 µM JA | MS1/2 + 10.0 µM JA | MS1/2 + 2.8 µM IAA | MS1/2 + 5.4 µM IAA | MS1/2 + 11.4 µM IAA |
| 32 | **β-Dihydro agaro-furan** | **1502** | **1503** | **4.4** | – | – | – | – | – | – | – |
| 33 | **Silphiperfolan-6-α-ol** | **1507** | **1507** | **3.2** | – | – | – | – | – | – | – |
| 34 | n.i. (MW:212) | 1507 | n.i. | – | 0.1±0.0 | – | 0.2±0.0 | 0.2±0.0 | 0.5±0.0 | 0.4±0.0 | – |
| 35 | Cameroonan-7-α-ol | 1511 | 1510 | – | 0.5±0.0 | 0.4±0.0 | 0.5±0.0 | 0.5±0.0 | 0.6±0.0 | 0.3±0.0 | 0.1±0.0 |
| 36 | 7-epi-Silphiperfolan-6-β-ol | 1517 | 1518 | – | 0.9±0.0 | 1.0±0.2 | 0.8±0.3 | 0.8±0.3 | 1.2±0.2 | 1.4±0.2 | 0.5±0.0 |
| 37 | **9-epi-Silphiperfolan-1-ol** | **1521** | **1518** | **31.3** | **5.4±0.0** | **4.1±0.0** | **5.6±0.0** | **5.5±0.0** | **5.5±0.1** | **3.2±0.1** | **2.5±0.0** |
| 38 | Silphiperfolan-7-β-ol | 1521 | 1520 | 0.6 | – | – | – | – | – | – | – |
| 39 | Nopsan-4-ol | 1526 | 1529 | 0.9 | – | – | – | – | – | – | – |
| 40 | Silphiperfolan-6-β-ol | 1544 | 1546 | 0.7 | – | – | – | – | – | – | – |
| 41 | n.i. | 1546 | n.i. | 0.7 | – | – | – | – | – | – | – |
| 42 | Silphiperfol-5-en-3-one B | 1550 | 1550 | 0.7 | – | – | – | – | – | – | – |
| 43 | Silphiperfol-5-en-3-ol A | 1553 | 1557 | 0.6 | – | – | – | – | – | – | – |
| 44 | n.i. (MW:222) | 1552 | n.i. | – | 0.9±0.2 | 0.3±0.0 | 0.5±0.3 | 0.7±0.0 | 0.9±0.0 | 1.0±0.0 | 0.7±0.0 |
| 45 | n.i. (MW:204) | 1572 | n.i. | – | 0.1±0.0 | 0.7±0.0 | 0.2±0.0 | 0.1±0.0 | 0.2±0.0 | – | – |
| 46 | n.i. (MW:204) | 1576 | n.i. | – | 0.3±0.0 | 0.4±0.0 | 0.4±0.0 | 0.3±0.0 | 0.5±0.0 | 0.5±0.0 | 0.4±0.0 |
| 47 | Prenopsan-8-ol | 1580 | 1577 | 1.3 | 0.2±0.0 | 0.2±0.0 | 0.2±0.0 | 0.2±0.0 | 0.2±0.0 | – | – |
| 48 | n.i. | 1576 | n.i. | 1.1 | – | – | – | – | – | – | – |
| 49 | n.i. (MW:218) | 1588 | n.i. | – | 0.4±0.0 | 0.4±0.0 | 0.3±0.0 | 0.2±0.0 | 0.5±0.0 | 0.6±0.0 | 0.4±0.0 |
| 50 | **Presilphiper-folan-8-ol** | **1591** | **1585** | **21.2** | **1.0±0.0** | **0.7±0.0** | **1.2±0.0** | **1.5±0.0** | **1.5±0.0** | **0.7±0.0** | **0.4±0.0** |
| 51 | Silphiperfol-6-en-5-one | 1623 | 1625 | 0.9 | – | – | – | – | – | – | – |
| 52 | trans-Isolongi-folanone | 1628 | 1625 | – | 0.2±0.0 | – | 0.2±0.0 | 0.2±0.0 | 0.3±0.0 | – | – |
| 53 | Caryophylla-4(12),8(13)-dien-5-α-ol | 1636 | 1639 | 0.9 | 0.2±0.0 | – | 0.2±0.0 | 0.2±0.0 | 0.2±0.0 | – | – |

**Table 2** (continued)

| # | Compounds | LRI-calc. | LRIlit. | Percentage [(%), SD] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Wild plant[a] | MS1/2 | MS1/2 + 0.1 μM JA | MS1/2 + 1.0 μM JA | MS1/2 + 10.0 μM JA | MS1/2 + 2.8 μM IAA | MS1/2 + 5.4 μM IAA | MS1/2 + 11.4 μM IAA |
| 54 | n.i. (MW:222) | 1641 | n.i. | 0.8 | 0.1±0.0 | – | – | – | 0.2±0.0 | 0.2±0.0 | – |
| 55 | n.i. | 1651 | n.i. | 0.9 | – | – | – | – | – | – | – |
| 56 | n.i. (MW:218) | 1679 | n.i. | – | 0.5±0.0 | 0.6±0.0 | 0.5±0.0 | 0.4±0.1 | 0.9±0.0 | 0.7±0.0 | 0.5±0.0 |
| 57 | Ishwarone | 1680 | 1680 | 0.9 | – | – | – | – | – | – | – |
| 58 | n.i. (MW:220) | 1699 | n.i. | – | 0.4±0.0 | 0.4±0.0 | – | – | 0.7±0.0 | 0.6±0.0 | 0.5±0.0 |
| 59 | 2-{[(2-Ethylhexyl)oxy]carbonyl}benzoic acid* | 2526 | n.i. | – | 2.6±0.0 | – | – | – | – | – | – |
| | Total of detected substances | | | 27 | 47 | 32 | 41 | 40 | 39 | 33 | 28 |
| | Total of identified substances | | | 21 | 31 | 20 | 27 | 26 | 23 | 20 | 18 |
| | Monoterpenes (%) | | | 2.5 | 71.3 | 78.7 | 76.1 | 76.2 | 69.8 | 75.0 | 89.8 |
| | Sesquiterpenes (%) | | | 97.5 | 23.0 | 20.5 | 20.8 | 19.7 | 28.7 | 22.4 | 9.4 |

Bold indicates the major compounds

*LRI* linear retention index, *n.i.* not identified, (*–*) not detected, *t* traces (<0,1%)

*Identified by NIST8s and NIST08.LIB (contaminant)

[a] Adapted from Pinto et al. (2013)

growth regulators, nutrient and sucrose content (Fernández and Revilla 2003; Wu et al. 2010). Medium with reduced saline concentration can reduce the stress on plant mineral nutrition (Menéndez et al. 2010), but for *A. tomentosa* var. *anthriscifolia*, this led to poor subsequent growth, although that reduction led to profitable germination and initial gametophyte development. The total period required for sporophytes to be evident in the culture was approximately 4–5 weeks, fewer than the previously reported time for other fern species (Somer et al. 2010) Although it is not the main objective of the present paper, we briefly described, for the first time, the gametophyte development pattern and the moment when sporophytes emerged; this result was not possible in our previous work with this same fern species (Pinto et al. 2013). Characterization of gametophyte morphogenesis and early gametophyte development is of paramount importance for characterizing fern taxa, fern taxonomy and *in situ* studies on the ecology of fern gametophytes (Praptosuwiryo 2017).

In the second experiment, we transferred a cluster of spore-derived gametophytes to media culture containing IAA or JA over a concentration range. Our results for *A. tomentosa* var *anthriscifolia* sporophytes indicate that IAA is not useful to promote fresh biomass and leaf increase in *A. tomentosa*. The addition of various auxins to *Cyathea spinulosa* cultured in vitro from gametophytes was not able to promote a higher number of leaf development (Parajuli and Joshi 2014), similar to our results. A decrease in the gametophyte fresh biomass of *Cyathea gigantea* was observed when IAA concentration exceeds 4.5 μmol, and the length of sporophytes substantially increased when IAA was combined with kinetin (Das et al. 2013). IAA directly influences development and plant cell elongation, functioning as an inhibitor or enhancer (Hou et al. 2004). However, most findings are related to the large Spermatophyta group, as almost nothing is known about the Pteridophytes. For ferns, the specific literature has shown that addition of different PGR results in growth inhibition (Fernández and Revilla 2003; Sommer et al. 2010; Bharati et al. 2013; Parajuli and Joshi 2014). In our experiments, JA appears to promote sporophyte development from spore-derived gametophytes once the highest number of new leaves was observed in plantlets developed under this PGR. At the same time, we observed that JA was able to remain in the gametophytic stage even when sporophytes were already developed. JA was previously shown to be involved in the early development of gametophyte and sporophyte protoplast culture in *Platycerium bifurcatum* (Cav.) C. Chr. (Camloh et al. 1996); it promoted rhizoids and adventitious shoot development, and after 20 days of culture, the highest percentage of new leaves was observed at 10 μmol JA (Camloh et al. 1999). In contrast to our results and those of other authors, JA inhibited the sporophyte development of *Equisetum arvense* (Kuriyama

et al. 1993). BA is the most investigated phytohormone with respect to the role of PGR on sporophyte development and fern multiplication. Most studies with JA are related to angiosperms, but in ferns, jasmonates were detected using the radio-immunoassay techniques with jasmonate-specific antiserums (Parthier 1996). It is well known that JA plays an important role as a signaling agent between the biosynthetic routes of anti-herbivory and attractiveness compounds for pollination; these are primarily volatile compounds, specifically terpenes. In addition, this compound modulates many plant responses to environmental processes such as stress (by ozone, osmotic, cold or light) and adaptation to seasonal and circadian rhythms (Wasternack 2014).

For volatile composition, the total number (47) of different substances detected in all in vitro *A. tomentosa* SDE extracts was more than twice the number of substances (20) detected in the wild-grown SDE plant extract (Pinto et al. 2013). Our results reveal that neither IAA nor JA can reproduce in vitro the monoterpene/sesquiterpene balance found in wild-grown plants, as previously described by our group (Pinto et al. 2009a, b). Thus, the results presented here are surprising because JA is an important PGR that is well-known for its relationship to the production of volatile compounds in plants (Pangesti et al. 2013); it can induce Monoterpene Synthase 1 gene *SlMTS1* activity (van Schie et al. 2007). However, a few transcriptional factors regulated by the jasmonate hormone signaling cascade have been reported to activate the transcription of sesquiterpenoid biosynthetic genes (De Geyter et al. 2012). Jasmonic acid is essential for the induction of defenses in the glandular trichomes of many plant species, and a monoterpene synthase from tomato (Tomato Terpene Synthase 1, *LeMTS1*) was identified that is specifically induced by JA in trichomes (van Schie et al. 2007). In ferns, as in other higher plants, terpenoid VOCs are formed via similar JA-sensitive pathways (Kosakivska et al. 2016).

In addition to noting the IAA or JA changes in the volatile profile in in vitro plants, we noted that the hormone-free medium (MS 1/2) used in this new set of experiments confirmed our previous results (Pinto et al. 2013) about the monoterpene profile prevalence over sesquiterpenes in all in vitro plants. Assuming that *A. tomentosa* in vitro sporophytes present an inverted volatile profile compared to wild-growth plants, we wondered which factor could affect terpenes biosynthesis. Plant volatile compositions vary within each species depending on the genotype/chemotype and leaf development, as well as on seasonal and environmental conditions such as temperature, day length and light intensity (Bassolino et al. 2014; Grausgruber-Gröger et al. 2012; Chang et al. 2008). As the chemical profile of micropropagated plants of *Salvia dolomitica* was investigated, similar to our findings, the monoterpenes were found to accumulate predominantly in in vitro plants, while sesquiterpenes were

the major compounds in the essential oil of the cultivated in vivo (greenhouse) material (Bassolino et al. 2014). These findings may suggest that changes in the monoterpene/sesquiterpene rates were found between the in vitro and wild-grown plants of *A. tomentosa* could be related to different irradiance levels in in vitro and wild environments, which is much higher than any in vitro light intensity. It is well known that many MEP pathway enzymes in higher plants are in some way affected by light (Hemmerlin et al. 2012). Although the compartmentalization of two C10 and C15 pathways allows them to operate independently, metabolic cross-talk between cytosol and plastids pathways has been reported (Hemmerlin et al. 2003; Dudareva et al. 2005; Nagegowda 2010). Despite these facts, terpenes are subject to light-mediated regulation. Some MVA-derived products include down-regulation by light. This suggests a function in plant tissue synthesis, when plants have a need for high levels of substances such as steroids that are used in cell elongation and multiplication (Hemmerlin et al. 2012).

Another possibility is that volatile terpenoids are often biosynthesized and emitted from specific plant tissues at a particular time (Nagegowda 2010). The change observed in the chemical profile of in vitro *A. tomentosa* plants is related to the leaf stage, as it is well-known that higher production of monoterpenes is restricted to young leaf tissues due to its capacity for biosynthesis (Grausgruber-Gröger et al. 2012; Azam et al. 2013). The change in monoterpene/sesquiterpene rates found between the micropropagated and wild plants of *Anemia tomentosa* could also be related to the developmental stage of the plants, since it is well known that in vitro plants are maintained in a continuous juvenile state. In this case, the gene expression of monoterpene synthases and actual monoterpene formation is highest (Grausgruber-Gröger et al. 2012; Azam et al. 2013). Similarly, Azam et al. (2013) found that the development phase had an impact on leaf volatile production in *Citrus* cultivars, and some volatiles increased during the developmental transition from young to mature leaves, while quantitative and qualitative cultivar-specific changes also occurred. This showed that young leaves produced higher amounts of volatiles than mature leaves in most cultivars, with a predominance of monoterpenes.

Our experiments were not designed to determine how light or the juvenility of tissues can impact the volatiles profile of in vitro plants, but our findings show that JA and IAA could not recover the volatile profile found in wild-grown plants; in addition, there was a decrease in the amount of sesquiterpenes with a woody-like aroma and antimycobacterial activity, while monoterpenes increased to high levels. Apart from the many studies reporting the mono and sesquiterpene in vitro production in angiosperm and gymnosperm species, pteridophyte species remain underexploited. The in vitro *A. tomentosa* plants presented

a more diverse composition compared to wild plants. Further studies are needed for a complete understanding of the factors that led to these results. For spore germination, gametophyte and sporophyte development, in this paper we describe in detail all of the in vitro development stages of *Anemia tomentosa* var. *anthriscifolia* characteristics that were not previously available. These findings are useful in characterizing fern taxa and will contribute to more accurate fern taxonomy. Although in vitro cultures of many ferns have been reported, almost all describe *ex vitro* sporophyte development, in contrast with our cultures, which were able to turn spore-derived gametophytes into sporophytes *in vitro*. The saline content of the culture media significantly affected gametophyte development, since a reduction below one-half of the original Murashige and Skoog basal salt concentration led to unsatisfactory growth followed by plantlets death. In general, adding IAA to culture media led to worse sporophyte growth than in JA cultures.

The potential of the aromatic fern *Anemia tomentosa* var. *anthriscifolia* (Schrad.) Mickel is undeniable and has been described in recent years, primarily through analyses of wild-grown plant extracts by our group. The new findings reported here will be useful in future plant multiplication, with an eye toward future ornamental/pharmaceutical plant markets or volatile compound production.

**Author contributions** JFFN, CVVC and CSB conducted the experiments; SCP and CVVC analyzed all chromatographic data, substance identification and assembled the supplementary data; SGL and NCBS conceived this work, analyzed the data and wrote the paper. All authors contributed in the writing of this paper.

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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