Revista Brasileira de Farmacognosia 27 (2017) 679–690

Brazilian Journal of Pharmacognosy
REVISTA BRASILEIRA DE FARMACOGNOSIA
www.elsevier.com/locate/bjp

Original Article

# Influence of growth regulators on distribution of trichomes and the production of volatiles in micropropagated plants of *Plectranthus ornatus*

Helna C. Passinho-Soares[a,b,1], Juceni P. David[a,*], José R.F. de Santana[b], Jorge M. David[c], Frederico de M. Rodrigues[d], Paulo R.R. Mesquita[c], Fábio S. de Oliveira[e], Moema C. Bellintani[f]

[a] *Laboratório de Pesquisa de Produtos Naturais, Faculdade de Farmácia, Universidade Federal da Bahia, Salvador, BA, Brazil*
[b] *Programa de Pós-graduação em Biotecnologia, Universidade Estadual de Feira de Santana, Feira de Santana, BA, Brazil*
[c] *Instituto de Química, Universidade Federal da Bahia, Salvador, BA, Brazil*
[d] *Empresa Baiana de Desenvolvimento Agrícola S.A., Salvador, BA, Brazil*
[e] *Centro de Ciências da Saúde, Universidade Federal de Recôncavo Baiano, Santo Antônio de Jesus, BA, Brazil*
[f] *Instituto de Biologia, Universidade Federal da Bahia, Salvador, BA, Brazil*

ARTICLE INFO

*Article history:*
Received 12 May 2017
Accepted 10 October 2017
Available online 21 November 2017

*Keywords:*
*In vitro* cultivation
Trichomes
Plant growth regulators
VOC
Monoterpenes

ABSTRACT

The profile of volatile organic compounds, the glandular and non-glandular trichomes of *Plectranthus ornatus*, obtained by *in vitro* cultivation, was evaluated in plants grown in Murashide and Skoog medium supplemented with benylaminopurine at 4.5, 9.0, and 18.0 μM + naphthaleneacetic acid at 5.37 μM, kinetin at 4.7, 9.3 and 18.5 μM + naphthaleneacetic acid (5.37 μM) or Murashide and Skoog 0 medium (as a control). Scanning Electron Microscopy was performed on samples of the third leaf node of the 90 days old plants obtained from treatment with 4.5 or 9.0 μM benylaminopurine, and 4.7 or 9.3 μM kinetin. Headspace Solid Phase Micro-Extraction of the 30, 60 and 90 days old *in vitro* plants permitted to determinate by GC/MS the composition comprised of 62 compounds. The data were analyzed using Principal Component Analysis and Hierarchical Clustering Analysis and, the major constituents of these oils after treatment and aging were monoterpenes and sesquiterpenes. Morphoanatomical analysis of trichomes, by Scanning Electron Microscopy, enabled the identification of non-glandular trichomes and four types of glandular trichomes, which comprised capitate and peltate glandular trichomes that were distributed on both sides of the leaf. We observed that the regulators influenced qualitative and quantitative profiles of the volatile organic compounds and the number and distribution of hairs on the leaf surface.



## Introduction

*Plectranthus ornatus* Codd, Lamiaceae, is a perennial, succulent herb with a pleasant smell that is commonly used as an ornamental plant in Brazilian gardens where it is known as "boldo-miudo" and "boldo-de-jardim." Moreover, in Brazil the leaves of *P. ornatus* are used by locals to treat liver and stomach problems, as a substitute for *P. barbatus* Andrews (commonly known as "falso-boldo"). Research on the chemistry of some *Plectranthus* species revealed the presence of terpenoids and phenolic compounds as typical products of these species. The terpenoids are considered to be primarily responsible for the cytotoxic, genotoxic, anti-fungal and anti-microbial activities of *Plectranthus* species (Lukhoba et al., 2006). Similarly, some *Plectranthus* species are rich sources of diterpenes. Other compounds, such as triterpenoids of the lupane and aristolane classes and sesquiterpenes and flavonoids, are also found in the *Plectranthus* genera (Abdel-Mogib et al., 2002; Gaspar-Marques et al., 2004, 2005, 2006). Forskolin-like diterpenoids, found in leaves of *P. ornatus*, have antifungal activities against *Candida* and antibacterial activities against Gram-positive and Gram-negative bacteria (Rijo et al., 2002). Furthermore, the essential oil of leaves of *Plectranthus grandis* and *P. ornatus* presented antioxidant activities and β-caryophyllene, eugenol, germacrene D and thymol were determinated as the main constituents of *P. ornatus* oils (de Albuquerque et al., 2007). Studies of micropropagation of medicinal species, using cell and tissue culture, are used to solve the variability of the production of secondary metabolites due environmental and genetic factors. On

* Corresponding author.
*E-mail:* juceni@ufba.br (J.P. David).
[1] In memoriam.

https://doi.org/10.1016/j.bjp.2017.10.001

the other hand, there appears to be a close correlation between morphological and biochemical differentiations of cells, which allows the establishment of metabolic pathways leading to the biosynthesis of secondary metabolites (Turner et al., 2000; Tisserat and Vaughn, 2008). In particular, the terpenoid biosynthesis is produced in specialized glands within or on organ surfaces. Cultures without these structures appear to be competent in the accumulation of terpenes (Tsuro et al., 2001; Kirakosyan, 2006). Previous studies dealing with the induction of callus from nodal segments of *P. ornatus* described the effects of different concentrations of the 2,4-dichlorophenoxyacetic acid (2,4-D) and 1-naphthaleneacetic acid (NAA) in the production of volatile organic compounds, mainly monoterpenes and sesquiterpenes (Passinho-Soares et al., 2013). The employment of these plant growth regulators also permitted to obtain an overproduction of the bioactive rosmarinic acid and cinnamic acid derivatives (Medrado et al., 2017).

This work describes the effect of growth regulators on the volatile organic compound profile obtained by *in vitro* cultivation of *P. ornatus* and measured using Headspace Solid Phase Micro-Extraction/gas chromatography coupled with mass spectrometry (HS-SPME/GC–MS). The effect of these regulators on the differentiation of the leaves by evaluating the morphological changes by Scanning Electron Microscopy (SEM) was also examined. Importantly, despite a large number of mainly ultrastructural and histochemical studies of glandular trichomes of many species (Serrato-Valenti et al., 1997; Anačkov et al., 2009), *in vitro* studies are rare (Avato et al., 2005) and in the case of *in vitro* cultivation of the species *P. ornatus*, have not been published previously.

## Materials and methods

### *Plant material*

The explants of *Plectranthus ornatus* Codd, Lamiaceae, were taken from mother plants cultivated in the Faculty of Pharmacy, Federal University of Bahia (UFBA). A voucher has been deposited at the Herbarium of the National Museum, Federal University of Rio de Janeiro, under number R196538.

### In vitro *cultures*

Nodal segments bearing axillary buds were selected, and the leaves were removed. The explants were cleaned by washing in running water for 40 min, followed by two washes with distilled water. Using an aseptic chamber, the explants were immersed in ethanol (70 °GL) for 1 min with agitation and then in sodium hypochlorite (commercial product, 2% active chlorine) + Tween 20 (20 drops $l^{-1}$) for 15 min. They were then rinsed three times (3 × 3 min) with autoclaved distilled water. Following disinfection, the explants (approximately 1.5 cm in length) were inoculated individually into test tubes (25 × 150 mm) containing 12 ml MS medium (Murashige and Skoog 1962), solidified with 6 g $l^{-1}$ agar and 87.64 mM sucrose and supplemented with different concentrations of BAP (4.5, 9.0 or 18.0 μM) + NAA (5.37 μM) and KIN (4.7, 9.3 or 18.5 μM) + NAA (5.37 μM). The MS medium without regulators (MS0) was used as a control. The pH was adjusted to 5.7 before autoclaving and sterilization was performed by autoclaving at 120 °C for 15 min. The incubations were performed in a growth room that was maintained at 25 ± 2 °C, with an approximately 70% average of humidity. The test tubes containing the medium and the explants were closed with film containing no plastic or PVC, kept in the dark for 8 days and then submitted to a 16 h photoperiod (cool white light of 25 μmol $m^{-2}$ $s^{-1}$ irradiance). The plants were evaluated for volatile organic compound (VOC) profiles at 30, 60 and 90 days after inoculation.

### *Extraction and VOC analyses*

For extraction and VOC analyses, headspace and solid phase microextraction (HS-SPME) (Lord et al., 2003) techniques, coupled with GC–MS analysis were employed (Eiceman et al., 2002). All extractions were performed in triplicate, on an identical mass of plant shoot (1 g) that was aged for 30, 60 or 90 days *in vitro*. Initially, the shoot was macerated using a stick and a glass container of 12 ml capacity. The bottle was sealed with the appropriate sealer and with an aluminum lid and silicone septum that was Teflon faced. The sample was then left to stand for 20 min at room temperature to equilibrate the vapor phase. A holder needle with a 100 μm fiber coated of Polydimethylsiloxane/Divinylbenzene (PDMS/DVB) mounted in a syringe-like (Supelco, Bellefonte, PA, USA) was the inserted into the sample vial where the fiber was exposed and which was heated on a heating plate at 60 °C during 20 min. After the extraction and heating, the fiber was collected on the same SPME microextrator and introduced directly into the injector of the chromatograph equipment. The volatiles were thermally desorbed in the GC injector for 3 min at 260 °C. The extraction parameters, such as sample volume (1 g), equilibrium time (20 min), extraction time (15 min), extraction temperature (60 °C), desorption time (5 min), desorption temperature (260 °C) and fiber extraction (PDMS-DVB), were established using univariate and multivariate tests. Analyses were conducted using a GC–MS system (Shimadzu CG-2010/QP-2010 high efficiency, coupled with quadrupole mass detector). Helium, used as a carrier gas, was adjusted to a linear velocity of 40 cm $s^{-1}$ (measured at 100 °C) with column flow of 1.22 ml $min^{-1}$; the injection mode was split with a rate of 1, 30; the temperature of the injector was set to 260 °C and the oven temperature gradient set to 50 °C (at 0.0 min), rising at 1.5 °C $min^{-1}$ to 80 °C (0.0 min), then 15 °C $min^{-1}$ to 160 °C (4 min), 20 °C $min^{-1}$ to 250 °C (7 min); with the transfer line set to 250 °C; the ion source to 250 °C and an impact energy of 70 eV. The total run time was 40.5 min. The essential oil constituents were identified by their retention indices (RI) that were in turn determined from calibration curves of a homologous series of *n*-alkanes ($C_8$–$C_{32}$) injected under the same chromatographic conditions as the samples, and they were also identified analyzing the fragmentation pattern in the mass spectra and comparison with data from the literature (Adams, 2007) and database NIST 147.

### *Multivariate data analyses*

The data obtained from these types of samples are multivariate and we therefore used the Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA), which are multivariate chemometric methods, to identify similarities and trends in groups of compounds induced by the different treatments. The PCA and HCA were used to evaluate the profiles of VOC from the *in vitro* explants of *P. ornatus* that were induced by the different treatments and obtained using HS-SPME/GC–MS. The areas of the chromatographic peaks of 62 VOC were measured from 21 different samples, 20 of which were run in triplicate and 1 in duplicate, and were used to construct a data matrix of size 62 × 62. The software packages Unscrambler chemometrics 8.0 (CAMO) and Statistic 7.0 (Statsoft) were used for the PCA and HCA calculations, respectively.

### *Scanning Electron Microscopy (SEM)*

In the SEM (Carl Zeiss mod LEO 1430 VP) analyses were used the third node from foliar samples of explants grown for 90 days in medium containing BAP at concentrations of 4.5 or 9.0 μM and, KIN at concentrations of 4.7 or 9.3 μM as well as with both cytokinins associated with 5.37 μM (NAA). The control was MS medium without regulators (MS0). The samples were fixed in F.A.A.

**Table 1**
HS-SPME area % of volatile organic compounds of *in vitro* and *ex vitro Plectranthus ornatus*.

| RT | RI | KI | Compound | 30 days | | | | | | | 60 days | | | | | | | 90 days | | | | | | | Matrice |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | T1[a] | T2 | T3 | T4 | T5 | T6 | T7 | T1 | T2 | T3 | T4 | T5 | T6 | T7 | T1 | T2 | T3 | T4 | T5 | T6 | T7 | *Ex vitro* |
| 5.123 | 854 | 854 | 2-Hexenal | 2.36 | 0.92 | 0.90 | 1.93 | 2.06 | 1.52 | 0.42 | 1.33 | 2.22 | 2.18 | 1.71 | 1.91 | 1.84 | 1.15 | 1.66 | 1.93 | 0.92 | 3.40 | 1.15 | 3.46 | 1.96 | 0.50 |
| 7.761 | 917 | 931 | α-Thujene | 15.76 | 32.65 | 30.16 | 28.12 | 19.09 | 31.83 | 23.54 | 17.06 | 14.11 | 12.68 | 15.95 | 19.67 | 22.14 | 31.67 | 13.46 | 24.30 | 17.66 | 14.12 | 14.01 | 25.09 | 24.15 | 13.64 |
| 8.074 | 926 | 939 | α-Pinene | 18.86 | 12.88 | 18.92 | 11.43 | 5.75 | 13.83 | 5.50 | 18.66 | 15.66 | 13.55 | 11.86 | 8.62 | 15.93 | 8.54 | 16.75 | 15.11 | 18.65 | 23.04 | 15.54 | 15.10 | 9.78 | 9.97 |
| 10.033 | 972 | 976 | Sabinene | 15.04 | 9.01 | 12.72 | 14.32 | 8.77 | 15.21 | 11.27 | 17.83 | 16.51 | 14.35 | 11.03 | 7.87 | 8.96 | 6.39 | 10.15 | 10.44 | 16.67 | 11.58 | 14.15 | 9.60 | 7.51 | 5.46 |
| 10.260 | 977 | 980 | β-Pinene | 7.32 | 4.15 | 6.37 | 5.98 | 11.59 | 4.88 | 3.59 | 7.47 | 10.56 | 6.42 | 4.83 | 4.32 | 7.43 | 3.51 | 6.52 | 5.19 | 7.15 | 6.94 | 9.43 | 4.95 | 4.50 | 3.09 |
| 10.535 | 976 | 978 | 1-Octen-3-ol | 4.38 | 2.23 | 2.68 | 4.67 | 0.56 | 3.01 | 1.75 | 5.30 | 3.34 | 5.66 | 11.11 | 8.51 | 8.88 | 4.03 | 3.41 | 9.02 | 5.59 | 7.89 | 4.30 | 4.80 | 8.10 | 9.23 |
| 11.031 | 992 | 991 | β-Myrcene | 1.28 | 1.16 | 0.98 | 1.28 | 0.79 | 0.93 | 0.45 | 0.94 | 1.56 | 1.29 | 0.47 | 0.87 | 1.44 | 0.63 | 0.88 | 1.04 | 0.88 | 0.96 | 1.22 | 0.88 | 1.10 | 0.4 |
| 11.523 | 995 | 993 | 3-Octanol | 0.75 | 1.02 | 0.63 | 1.13 | 0.91 | 0.56 | 0.37 | 0.75 | 1.88 | 1.55 | 1.84 | 2.40 | 3.10 | 1.02 | 0.59 | 3.19 | 0.36 | 1.19 | 0.94 | 0.58 | 1.38 | 0.81 |
| 12.037 | 1005 | 1004 | Hexenol acetate | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | 0.57 | – | – | – | – | – | 0.16 |
| 12.225 | 1009 | 1005 | α-Felandrene | – | – | 0.12 | – | 0.29 | – | – | – | – | – | 1.26 | – | – | – | – | – | – | – | – | – | – | |
| 12.345 | 1011 | 1011 | 3-Carene | 2.94 | 2.10 | 2.96 | 3.08 | 2.13 | 4.23 | 4.70 | 3.39 | 2.07 | 1.47 | 0.84 | 4.53 | 2.09 | 5.62 | 1.88 | – | 1.38 | 1.78 | 5.32 | 4.21 | 4.02 | |
| 13.697 | 1033 | 1031 | α-Limonene | 1.65 | 1.00 | 1.28 | 1.43 | 1.19 | 1.70 | 0.90 | 1.00 | 1.69 | 1.37 | – | 0.68 | 1.65 | 0.99 | 1.14 | 1.17 | 0.90 | 1.37 | 1.41 | 0.95 | 0.81 | 0.43 |
| 13.850 | 1035 | 1033 | 1.8-Cineole | 0.34 | 0.26 | 0.23 | 0.21 | 0.31 | 0.20 | – | – | 0.30 | 0.26 | – | – | 0.33 | – | 0.17 | 0.33 | – | 0.26 | – | – | – | 0.21 |
| 13.983 | 1038 | 1040 | (*Z*) β-ocimene | 0.17 | – | 0.06 | – | – | 0.14 | – | – | 0.30 | 0.23 | 7.04 | – | – | – | – | – | – | – | – | – | – | 2.54 |
| 14.674 | 1048 | 1050 | (*E*) β-ocimene | 4.89 | 3.20 | 2.69 | 2.80 | 1.51 | 2.69 | 1.76 | 5.35 | 8.37 | 4.73 | – | 4.88 | 5.21 | 3.51 | 5.06 | 5.25 | 5.74 | 4.62 | 4.70 | 4.85 | 4.81 | |
| 16.581 | 1074 | 1068 | 4-Thujanol | 0.28 | – | 0.22 | 0.29 | 0.09 | 0.14 | – | – | 0.37 | 0.26 | – | 0.27 | 0.36 | – | – | 0.43 | – | 0.26 | – | – | – | |
| 17.345 | 1083 | 1086 | Isoterpinolene | – | – | – | – | – | 0.12 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | |
| 17.633 | 1087 | 1088 | Terpinolene | – | – | – | – | – | 0.17 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | |
| 18.974 | 1098 | 1098 | β-Linalool | 0.51 | 0.47 | 0.33 | 0.55 | 0.44 | 0.14 | – | – | 1.00 | 0.53 | – | 1.05 | – | – | 0.56 | 0.83 | – | – | 0.94 | – | – | |
| 22.503 | 1135 | 1136 | *p*-Menth-1-ol | – | – | – | 0.28 | – | – | – | 0.97 | – | – | 0.14 | – | – | – | – | 0.13 | – | 0.22 | – | 0.21 | 0.39 | |
| 23.051 | 1141 | 1144 | *p*-Menth-8-ol | 0.15 | 0.21 | 0.15 | – | 0.13 | 0.08 | 0.09 | – | 0.49 | 0.45 | 0.14 | 0.37 | 0.38 | – | – | 0.46 | – | – | 0.19 | – | – | |
| 23.218 | 1142 | 1204 | Decanal | – | – | – | – | – | – | – | – | – | – | 0.11 | 0.14 | – | 0.28 | – | – | – | – | – | – | – | 0.1 |
| 24.751 | 1287 | 1285 | Isobornyl acetato | 0.22 | 0.21 | 0.44 | 0.48 | 1.98 | 0.73 | 0.37 | – | – | – | – | – | – | 0.78 | – | – | – | 0.17 | – | – | – | |
| 25.524 | 1356 | 1351 | α-Cubebene | 0.57 | – | – | 0.23 | – | – | – | 0.29 | 0.83 | 0.30 | 0.81 | – | 0.53 | – | 1.36 | 0.58 | 0.49 | 1.26 | 1.27 | – | – | 0.69 |
| 25.626 | 1356 | 1350 | α-Terpinyl acetate | – | 7.36 | 1.51 | 2.57 | 13.16 | 2.98 | 7.42 | – | – | – | – | 1.99 | – | 3.23 | – | – | – | – | – | 1.69 | 1.32 | |
| 25.940 | 1381 | 1376 | α-Copaene | 0.92 | 0.39 | 0.61 | 0.42 | 0.41 | 0.50 | 0.53 | 0.69 | 1.09 | 1.33 | 1.18 | 1.50 | 1.00 | 0.80 | 2.50 | 1.07 | 1.23 | 1.07 | 1.00 | 0.98 | 0.99 | 1.59 |
| 26.066 | 1389 | 1384 | β-Bourbonene | – | – | – | – | – | – | – | – | – | – | 0.13 | – | – | – | 0.95 | – | 0.15 | 0.14 | 0.05 | – | 0.82 | 0.88 |
| 26.115 | 1392 | 1390 | β-Cubebene | 1.26 | – | – | 0.60 | – | – | – | 0.99 | 1.64 | 1.16 | 1.36 | 1.41 | 1.46 | 0.78 | 1.54 | 1.23 | 1.65 | 0.82 | 0.67 | 0.69 | – | 1.35 |
| 26.209 | 1397 | 1409 | α-Gurjunene | – | – | – | – | 0.81 | 0.52 | 1.00 | – | 0.16 | 0.13 | 0.11 | – | – | – | – | – | – | 0.13 | 0.14 | 0.18 | – | 1.75 |
| 26.253 | 1399 | 1399 | Azulene | 1.24 | 1.09 | 0.67 | 0.78 | 3.96 | 1.50 | 4.47 | 0.18 | – | 0.13 | – | 0.82 | 0.17 | 3.26 | 0.17 | 0.10 | – | – | – | 0.71 | 1.34 | |
| 26.385 | 1408 | 1403 | β-Isocumene | – | 1.46 | 1.18 | 2.01 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | |
| 26.618 | 1420 | 1418 | β-Caryophyllene | 5.84 | 5.02 | 4.36 | 3.89 | 9.33 | 3.90 | 9.79 | 5.05 | 7.02 | 13.60 | 11.59 | 9.86 | 6.45 | 6.10 | 9.89 | 6.06 | 7.79 | 7.46 | 8.14 | 7.01 | 7.71 | 30.34 |
| 26.767 | 1429 | 1432 | β-Gurjunene | 0.29 | – | – | 0.10 | – | – | – | 0.21 | 0.31 | 0.26 | 0.47 | 0.44 | 0.28 | 0.36 | 0.46 | 0.28 | 0.27 | 0.26 | 0.26 | 0.28 | 0.30 | 0.41 |
| 26.902 | 1438 | 1439 | α-Guaiene | – | 0.10 | 0.10 | 0.05 | 1.09 | – | – | 0.16 | – | – | – | – | – | – | 0.15 | – | – | – | – | – | 0.11 | |
| 26.964 | 1441 | 1443 | (*Z*) β-farnesene | – | – | – | – | – | – | – | 0.16 | – | – | – | – | – | 0.28 | 0.18 | – | 0.14 | 0.09 | – | 0.26 | 0.26 | 0.15 |
| 27.014 | 1444 | 1460 | *cis*-Muurola-4(14).5-diene | 0.24 | – | 0.08 | – | – | – | – | – | 0.18 | 0.14 | 0.14 | 0.38 | 0.16 | – | 0.30 | 0.20 | 0.15 | – | – | 0.18 | – | 0.14 |
| 27.117 | 1450 | 1447 | α-Himachalene | 0.31 | – | – | – | 0.68 | 0.22 | 0.62 | 0.21 | – | 0.16 | 0.31 | 0.36 | 0.17 | 0.42 | 0.48 | 0.17 | 0.23 | 0.09 | 0.31 | 0.26 | 0.44 | |
| 27.191 | 1454 | 1458 | (*E*)β-Farnesene | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | 0.18 |
| 27.208 | 1454 | 1454 | α-Humulene | 0.68 | 0.37 | 0.52 | 0.28 | 0.79 | 0.26 | 0.74 | – | 0.30 | 0.30 | 0.87 | 0.73 | 0.43 | 0.61 | 1.00 | 0.73 | – | 0.43 | – | 0.73 | 1.00 | 1.82 |
| 27.275 | 1459 | 1459 | Aromadendrene | – | – | 0.21 | 0.28 | – | – | – | – | – | – | – | – | – | – | 0.19 | – | – | – | – | – | – | |
| 27.299 | 1460 | 1476 | γ-Himachalene | – | 0.41 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | |
| 27.433 | 1468 | 1461 | *allo*-Aromadendrene | – | – | – | – | – | – | – | – | – | – | – | – | – | 0.14 | – | – | – | – | – | – | – | 0.19 |
| 27.505 | 1471 | 1477 | γ-Muurolene | 0.14 | – | – | – | – | – | – | 0.20 | – | 0.25 | – | 0.22 | 0.21 | – | 0.43 | 0.27 | 0.38 | – | 0.34 | 0.22 | 0.27 | 0.08 |

**Table 1**
(*Continued*)

| RT | RI | KI | Compound | 30 days | | | | | | | 60 days | | | | | | | 90 days | | | | | | | Matrice |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | T1[a] | T2 | T3 | T4 | T5 | T6 | T7 | T1 | T2 | T3 | T4 | T5 | T6 | T7 | T1 | T2 | T3 | T4 | T5 | T6 | T7 | *Ex vitro* |
| 27.506 | 1479 | 1485 | β-Ionone | – | – | – | – | – | – | – | – | 0.24 | – | 0.47 | – | – | – | – | – | – | – | – | – | – | |
| 27.641 | 1479 | 1480 | Germacrene d | 9.28 | 10.27 | 7.66 | 9.71 | 8.47 | 6.93 | 18.52 | 8.96 | 6.63 | 13.88 | 12.63 | 13.71 | 7.85 | 12.15 | 15.03 | 8.09 | 8.66 | 0.25 | 12.85 | 9.29 | 13.73 | 8.2 |
| 27.815 | 1489 | 1485 | β-Selinene | 0.13 | – | – | – | – | – | – | – | – | – | – | 0.19 | – | – | 0.25 | 0.13 | 0.39 | 7.29 | – | 0.16 | 0.11 | 0.25 |
| 27.908 | 1494 | 1494 | α-Selinene | 0.25 | – | – | – | – | – | – | – | – | 0.19 | 0.12 | – | – | – | 0.46 | 0.19 | 0.16 | 0.11 | – | 0.23 | 0.34 | |
| 27.920 | 1494 | 1490 | *cis*-β-Guaiene | – | – | 0.11 | – | – | – | – | – | – | – | – | – | 0.07 | – | – | – | – | 0.32 | – | – | – | 0.29 |
| 27.99 | 1498 | 1500 | *trans*-β-Guaiene | 0.34 | – | – | – | – | – | – | 0.14 | – | 0.12 | 0.12 | 0.25 | – | – | 0.12 | 0.16 | 0.16 | – | 0.35 | – | – | 3.77 |
| 28.190 | 1509 | 1499 | β-Himachalene | – | – | – | – | – | 0.14 | 0.45 | – | – | – | – | – | – | 0.52 | – | – | – | – | – | – | 0.33 | |
| 28.199 | 1510 | 1512 | β-Cucurmene | – | 0.18 | 0.13 | 0.15 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | |
| 28.218 | 1511 | 1511 | α-Himachalene | 0.14 | – | – | – | – | – | – | 0.20 | – | 0.14 | 0.12 | 0.22 | 0.20 | 0.15 | 0.44 | 0.17 | 0.18 | – | 0.33 | 0.24 | – | |
| 28.288 | 1514 | 1513 | γ-Cadinene | 1.09 | – | – | – | – | – | – | 1.96 | 0.67 | 0.73 | 1.03 | 0.82 | 0.65 | 1.08 | 1.56 | 1.01 | 2.06 | 0.24 | 0.99 | 0.69 | 1.37 | |
| 28.298 | 1515 | 1517 | α-Selinene | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | 1.09 | – | – | – | |
| 28.475 | 1525 | 1556 | Germacrene b | – | 0.08 | – | 0.55 | – | 0.40 | 0.80 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | |
| 28.489 | 1525 | 1524 | δ-Cadinene | – | 0.82 | 0.59 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | 1.54 | – | |
| 28.607 | 1532 | 1528 | Kessane | 0.27 | – | – | – | 1.12 | – | – | 0.26 | 0.31 | – | – | – | 0.41 | 1.72 | – | – | – | 0.20 | – | – | 0.84 | |
| 28.827 | 1543 | 1542 | Selina-3.7(11)-diene | – | 0.99 | 0.35 | 0.37 | 2.58 | – | 0.82 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | |
| 29.511 | 1527 | 1526 | γ-Himachalene | – | – | – | – | – | – | 0.14 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | |
| 29.636 | 1586 | 1574 | Germacrene-d-ol | 0.13 | – | 0.08 | 0.04 | – | – | – | 0.14 | 0.21 | 0.21 | 0.12 | 1.01 | 0.19 | – | 0.17 | 0.16 | – | 0.13 | – | – | – | 0.58 |
| 29.770 | 1592 | 1581 | Caryophyllen oxid | – | – | – | – | – | 0.54 | – | – | – | – | – | – | – | – | 0.15 | – | – | 0.12 | – | – | – | 0.72 |
| 30.716 | 1641 | 1641 | α-Muurolol | – | – | – | – | – | – | – | 0.14 | – | – | 0.09 | – | – | 0.29 | – | – | – | – | – | – | 0.20 | 0.08 |

[a] T1, MS0; T2, 18.5 μM KIN; T3, 9.3 μM KIN; T4, 4.7 μM KIN; T5, 4.5 μM BAP; T6, 9.0 μM BAP; T7, 18.0 μM BAP; Matrice, *ex vitro*.

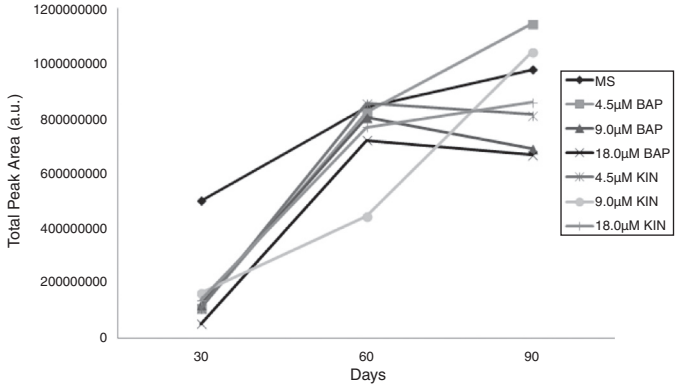


**Fig. 1.** Average of absolute areas of VOC resulting from different treatments.

[37% formaldehyde, glacial acetic acid and 70% ethanol (in a ratio of 2, 1, 1)] and subsequently dehydrated in increasing concentrations of acetone. Subsequently, the specimens were brought to critical point drying using liquid $CO_2$ (BAL-TEC CPD 030), then metalized (BAL-TEC SCD 050) and examined using SEM.

## Results and discussion

The HS-SPME/CG–MS-derived constituents of essential oils of *P. ornatus* obtained from plants grown *ex vitro* and *in vitro* with different concentrations of the cytokinins BAP and KIN and following 30 (30 D), 60 (60 D) or 90 (90 D) days of inoculation, are shown in Table 1. A number of 62 volatile compounds were found, which mainly comprised monoterpenes and sesquiterpenes in the *in vitro* plants. The major constituents of essential oils following treatment and aging were α-thujene (12.7–32.7%), α-pinene (5.5–23%), sabinene (7.51–17.8%), β-pinene (3.5–11.6%), 1-octen-3-ol (0.6–11.1%), 3-carene (0.84–5.6%), (*E*) β-ocimene (1.5–8.4%), α-terpinyl acetate (1.3–13.2%), β-caryophyllene (3.9–13.6%) and germacrene D (0.3–18.5%).

With regard to the quantitative measurements with GC–MS, we observed higher average absolute areas of VOC in plants cultivated in MS0 medium, independent of the period of inoculation (Fig. 1). In contrast, for explants grown with regulators, the highest values for VOC were obtained with 4.5 μM and 18.0 μM BAP and 18.5 μM KIN at 60 D incubation and with 4.5 μM and 9.0 μM BAP and 18.5 μM KIN at 90 D incubation. When we analyzed the different treatments within each age, we observed that for the 30 D incubation period, the major VOC, α-pinene (18.9%), 1-octen-3-ol (4.4%) and (*E*)-β-ocimene (4.9%), reached their highest concentrations when cultivated in MS0 (Table 1). In explants from the same incubation period (30 D), the production of sabinene (15.0%) and α-limonene (15.2%) appeared to be independent of the presence of regulators, given that their concentrations did not differ significantly with the addition of 9.0 μM BAP. Furthermore, VOC from explants grown in MS0 only also had the highest concentrations of 2-hexenal (2.4%), β-myrcene (1.28%), 1.8-cineol (0.3%), (*Z*)-β-ocimene (0.2%), 4-thujanol (0.3%), β-linalool (0.51%), *p*-menth-8-ol (0.2%) and α-copaene (0.9%). The presence of β-selinene, α-selinene, β-guaiene, α-himachalene, γ-muurolene and γ-cadinene was observed exclusively in explants incubated for 30 D in MS0 (Table 1). Additionally, major relative quantities of α-cubebene, β-gurjunene and cis-muurola-4(14),5-diene were observed with this same treatment and incubation period. Moreover, isoterpinolene and terpinolene were detected only in explants grown in BAP for 30 D days, alloaromadendrene only in plants incubated in BAP for 60 D and β-himachalene only in explants grown in BAP at all three periods. Hexanol acetate, β-isocumene, γ-himachalene, β-cucurmene and α-selinene were detected only in the explants cultured with KIN.

When we compared the concentrations of VOC in each treatment group with respect to age (Table 1), the 60 D and 90 D explants reached the highest percent total compound concentration, especially in those undergoing treatment with MS0, 4.5 μM BAP or 18.5 μM KIN. In Table 1, we show the 33 VOC present in the matrix from *ex vitro* plants, where the main compounds (>1%) are α-thujene (13.6%), α-pinene (~10%), sabinene (5.5%), β-pinene (3.1%), 1-octen-3-ol (9.2%), (*E*)-β-ocimene (2.54%), α-copaene (1.6%), β-cubebene (1.4%), α-gurjunene (1.8%), β-caryophyllene (30.3%), α-humulene (1.8%), germacrene D (8.20%) and *trans*-β-guaiene (3.8%).

There was a qualitative and quantitative change in the profile of volatile compounds of *ex vitro* plants when compared with VOC from *in vitro* plants. When the relative percent of major VOC of *ex vitro* plants were compared with the percent VOC from *in vitro* plants grown in culture medium without any growth regulators (MS0), we observed a yield of 14% and 21% in α-thujeno in plants aged for 30 D and 60 D, respectively.

The percent yield for α-pinene at the periods analyzed (30 D, 60 D and 90 D) was 89.6% 87.2% and 68.5%, respectively, and for sabinene, the yields were 172.7%, 223.6% and 85.5%, respectively. Further, the β-pinene yield was 135.5%, 141.9% and 109.7%, the (E)-β-ocimene yield was 96%, 116% and 104% and the germacrene D yield was 13.4%, 9.8% and 82.95% for 30 D, 60 D and 90 D, respectively. However, there was a significant loss in percent content of β-caryophyllene in plants incubated with medium MS0 *in vitro*, with a decrease of approximately 522% yield compared to the content of this compound in *ex vitro* plants. The considerable production of caryophyllene in *ex vitro* plants may be related to the biotic and abiotic stress factors that they are subject to. The synthesis of β-caryophyllene in response to attack by herbivores has been documented (Köllner et al., 2008).

When we compared the VOC results from *ex vitro* plants (using the highest results from each treatment group at the three different ages) with those of the *in vitro* plants, we noted that the percent yield of VOC obtained *in vitro* is much larger than those obtained in the *ex vitro* plants. Some VOC showed a gain of over 370%, such as in the case of β-pinene.

The chromatographic profile of VOC of *in vitro* and *ex vitro* plants (Table 1, Fig. 1) reveals a qualitative and quantitative change that is independent of the use of regulators that aid *in vitro* cultivation (using results from MS0 incubations). An exception to this pattern is β-caryophyllene whose percentage is significantly higher in *ex vitro* plants. However, the BAP and KIN regulators, for the 60 D to 90 D ages and at concentrations of 4.5 μM and 18.5 μM, respectively, positively influenced the qualitative and quantitative VOC profile.

Previous studies with *Lavandula viridis* L'Hér. (Gonçalves et al., 2008), *Lantana camara* L. (Affonso et al., 2007) and *Melissa officinalis* L. (Silva et al., 2005) showed that the content of essential oils found in *in vitro* plants are higher than those from plants grown *ex vitro*. A cytokine-induced increase in the production of VOC was observed for *Thymus vulgaris* L. (Affonso et al., 2009) and *Lavandula dentata* L. (Sudriá et al., 1999). It is noteworthy that although the defense systems of higher plants are correlated with adaptation and the demands of their natural environment, they can also be activated by *in vitro* cultivation conditions (Tregear et al., 2002).

The graph of the regulator scores (PC1 × PC2) is shown in Fig. 2. Each point represents a particular treatment. The score evaluations reveal a cluster with a significantly similar chromatographic profile (G1). In contrast, the other identified groups (G2, G3 and G4) are composed of treatments that have higher dispersions in their chromatographic profiles.

Fig. 3 illustrates the loadings PC1 × PC2, where each point represents a single VOC. It appears that the vast majority of VOC present values of loadings that are close to zero. These data indicate that VOC contribute little to the variability of the data set and the

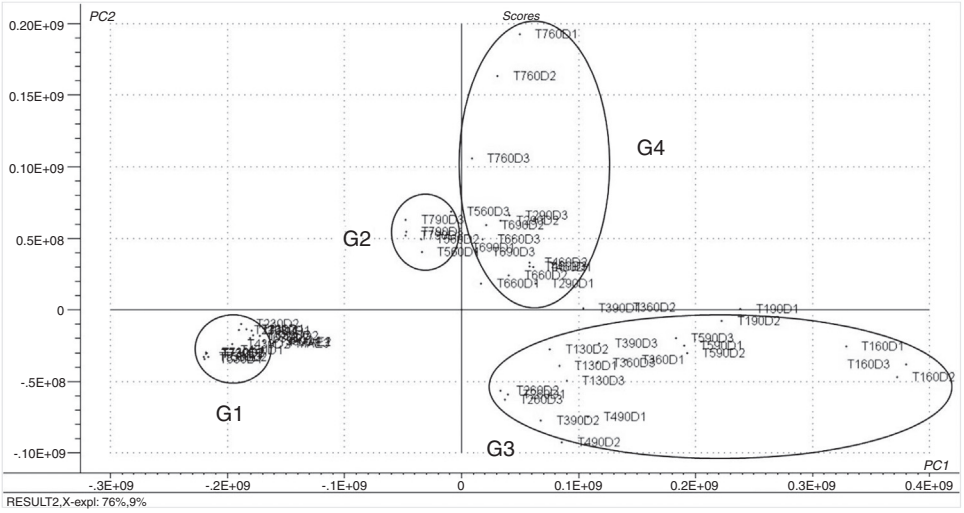


**Fig. 2.** Graph of scores (PC1 × PC2) obtained by PCA from data obtained in the chromatographic analysis of oils showing the evaluation of different regulators. The two first principal components together explain 85.4% of the total variance of the data set. T1 = MS0; T2 = 18.5 μM KIN; T3 = 9.3 μM KIN; T4 = 4.7 μM KIN; T5 = 4.5 μM BAP; T6 = 9.0 μM BAP; T7 = 18 μM BAP.

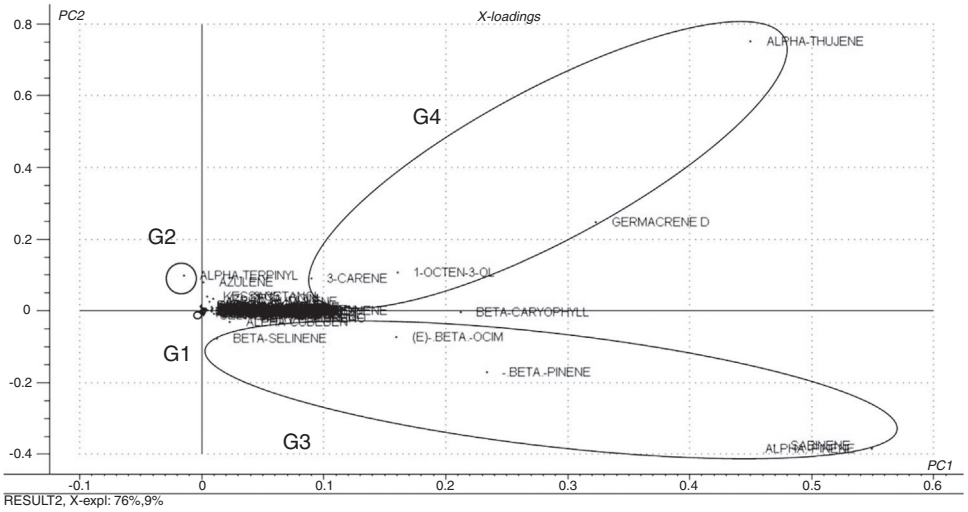


**Fig. 3.** Graph of the loadings (PC1 × PC2) obtained by PCA from data obtained in the chromatographic analysis of oils showing the evaluation of different regulators. The first two principal components together explain 85.4% of the total variance of the data set.

values of peak areas of these VOC are approximately constant in the experiments.

As shown in Fig. 3, the replicates contained within the G2, G3 or G4 groups do not have similarity in their chromatographic profiles that is comparable to that of the G1 group, as demonstrated by the dendrogram obtained from the analysis using the Hierarchical Cluster Analysis (Fig. 3).

The G1 group includes 30 D explants treated with 18.5 μM KIN (T2 30D), 9.3 μM KIN (T3 30D), 4.7 μM KIN (T4 30D), 4.5 μM BAP (T5 30D), 9.0 μM BAP (T6 30D), 18 μM BAP (T7 30D) and the *ex vitro* plant.

The G2 group comprises the 60 D explant treated with 4.5 μM BAP and the 90 D explant treated with 18 μM BAP, codified as T5 60D and T7 90D, respectively. The G3 group is composed of explants cultivated in MS0 for 30, 60 or 90 days, encoded as T1 30D, T1 60D and T1 90D, respectively, in addition to the explants cultivated for 60 D with 18.5 μM KIN (T2 60D) or 9.3 μM KIN (T3 60D) and 90 D explants cultivated with 4.7 μM KIN (T4 90D) or 4.5 μM BAP (T5 90D).

The G4 group includes the explants cultivated for 60 or 90 days with 9.0 μM BAP, codified as T6 60D and T6 90D, respectively, and the explants cultivated for 60 or 90 D with 18 μM BAP (T7 60D), in addition to explants cultivated for 60 or 90 D and treated with 4.7 μM KIN (T4 60D), 18.5 μM KIN (T2 90D) or 9.3 μM KIN (T3 90D).

Evaluation of the results presented in Figs. 1 and 2 shows that the G2 group of samples is in the same quadrant as α-terpinyl acetate, indicating that treatments grouped in G2 have, on average, levels of α-terpinyl acetate that are slightly lower when compared with other treatments.

Similarly, the group is in the same quadrant of the graph as the VOC α-pinene, sabinene, β-pinene and (*E*)-β-ocimene. The high values of loadings indicate that, on average, the levels of these VOC are much higher in the G3 group than in other groups.

The high values of loadings of the VOC α-thujene, germacrene D, 1-octen-3-ol and 3-carene, which are in the same quadrant as the treatments contained in G4, indicate a correlation between this group and these VOC. Further, the levels of these compounds, on average, are higher in G4 than in other groups.

Of the VOC that are in the same quadrant as treatments of the G1 group, β-isocumene, selin-3.7(11)-diene and α-gurjunene are present at lower loadings values, indicating that there is little variation in their levels throughout the evaluated data set.

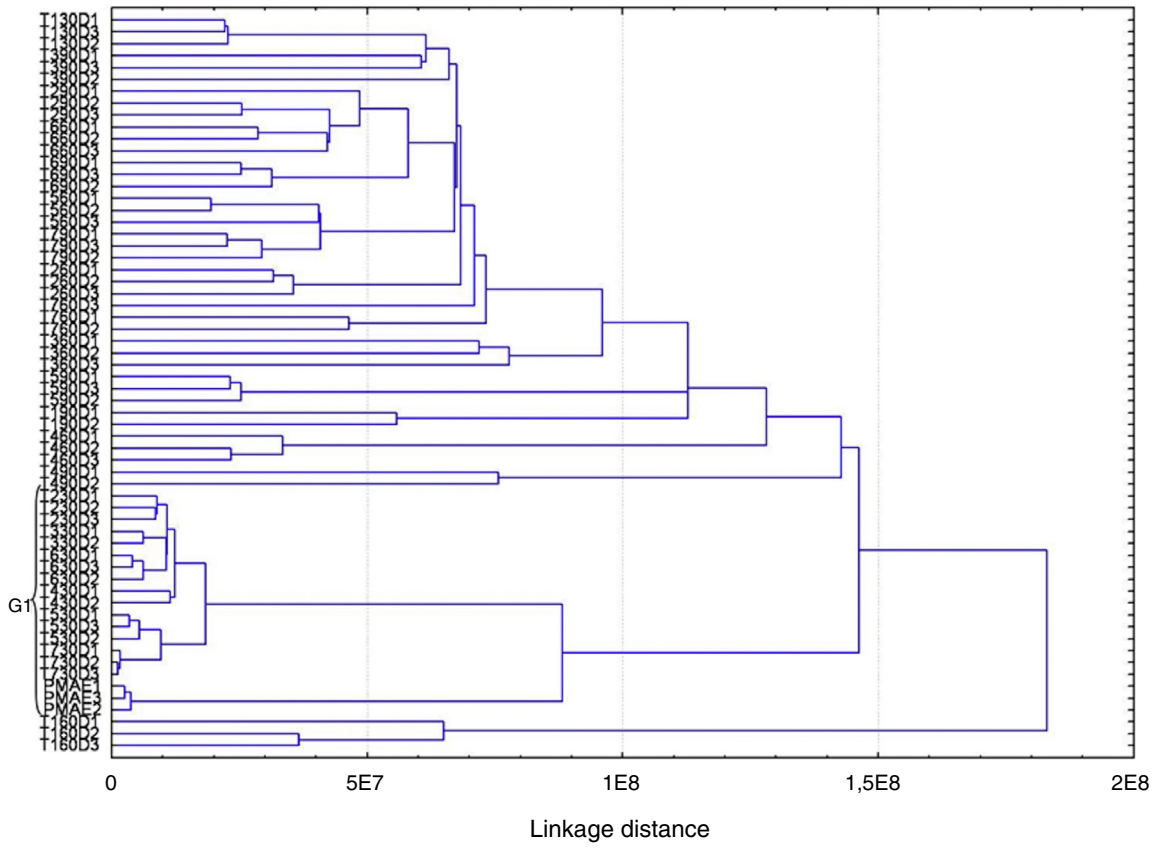


**Fig. 4.** Dendrogram obtained from Hierarchical Cluster Analysis (HCA). T1 = MS0; T2 = 18.5 μM KIN; T3 = 9.3 μM KIN; T4 = 4.7 μM KIN; T5 = 4.5 μM BAP; T6 = 9.0 μM BAP; T7 = 18 μM BAP; PMAE = *ex vitro* plant.

In Fig. 4, we show the dendrogram obtained from the HCA. The results obtained by HCA are in agreement with the PCA results with regard to the resemblance in treatments of the G1 group (this group shows great similarity in levels of different VOC). Moreover, the wide dispersion of scores in the levels of VOC of other treatments, including the disparate replicates, reflects the difficulty in defining the other groups by HCA.

The data obtained by HCA indicate that treatments that are not part of the G1 group can be framed into a large group. However, these results do not disagree with those obtained by PCA because in this latter technique, the groups are made by visual inspection and will depend upon human inspection. Thus, even with PCA, the data can be considered as two groups only, those belonging to G1, which are much more similar to each other, and other treatments gathered into another large group. Thus, it can be concluded that the best methods for the *in vitro* production of VOC in *P. ornatus* are to incubate in MS0 (control), followed by treatment with 4.5 μM BAP or by treatment with 18.5 μM KIN.

Using SEM, we observed that *in vitro P. ornatus* has non-glandular trichomes and capitate and peltate glandular trichomes that are distributed on both adaxial and abaxial leaf surfaces. Three types of non-glandular trichomes were observed. Type I (Fig. 5A–D) are simple, short, bicellular, uniseriate trichomes with an acute apex and granular surface that are arranged on a broad base that is unicellular or bicellular with a smooth surface. Similar trichomes were observed for the species *Micromeria marginata* (Sm.) Chater (Moon et al., 2009). The non-glandular trichome type II (Fig. 5B and C), measuring 75–500 μm in length, were uniseriates, multicellular, consisted of three to eight cells with a granular surface and were arranged on a basal cell with a smooth surface of 40 μm in width. We observed (Fig. 5A–C) that the union of each cell was clearly marked. The presence of these types of trichomes is in agreement with reports in the literature (Ascensão et al., 1999) of *in vivo* trichomes of *P. ornatus*. Type III were non-glandular trichomes and were simple and unicellular, with a smooth surface and rounded apex, and lay on a unicellular base.

We also observed peltate glandular trichomes located on the abaxial leaf surface that were composed of a basal cell and a bulky rounded head measuring 60–100 μm in height and 45–70 μm in diameter (Fig. 5E and F) and composed of eight secretory cells arranged in a circle (Fig. 5F). Based on the morphology of the glandular head and the dimensions of the stalk, four types of capitate glandular trichomes were identified, type I (Fig. 6A–C), type II (Fig. 6D–G), type III (Fig. 6H–I) and type IV (Fig. 6I). Type I capitate trichomes have a round, unicellular, glandular head (Fig. 6A) or bicellular head (Fig. 6B) and a short unicellular or multicellular stalk (Fig. 6C).

Type II capitate trichomes are unicellular, with a conical stalk of variable length (Fig. 6D–G) and a glandular head that is rounded and unicellular. Below the glandular head, we observed a clearly differentiated neck-cell (Fig. 6E and F).

Type III capitate trichomes are multicellular and uniseriate. They consist of 4–7 smooth surface cells, a differentiated neck-cell and an oval, unicellular and glandular head. As in non-glandular trichomes, there are clear marks to show the union of the cells that form its stalk (Fig. 6H).

The type IV capitate trichome is multicellular, uniseriate, is composed of four visibly marked cells, has a granular cuticle and is arranged on a bicellular broad base with a smooth surface. The glandular head has a rounded shape. Capitate and peltate glandular secretory structures are characteristics of the Lamiaceae family, but the number of secretory cells varies, as does the length of the stalk cell, the density and its arrangement in the leaf epidermis.

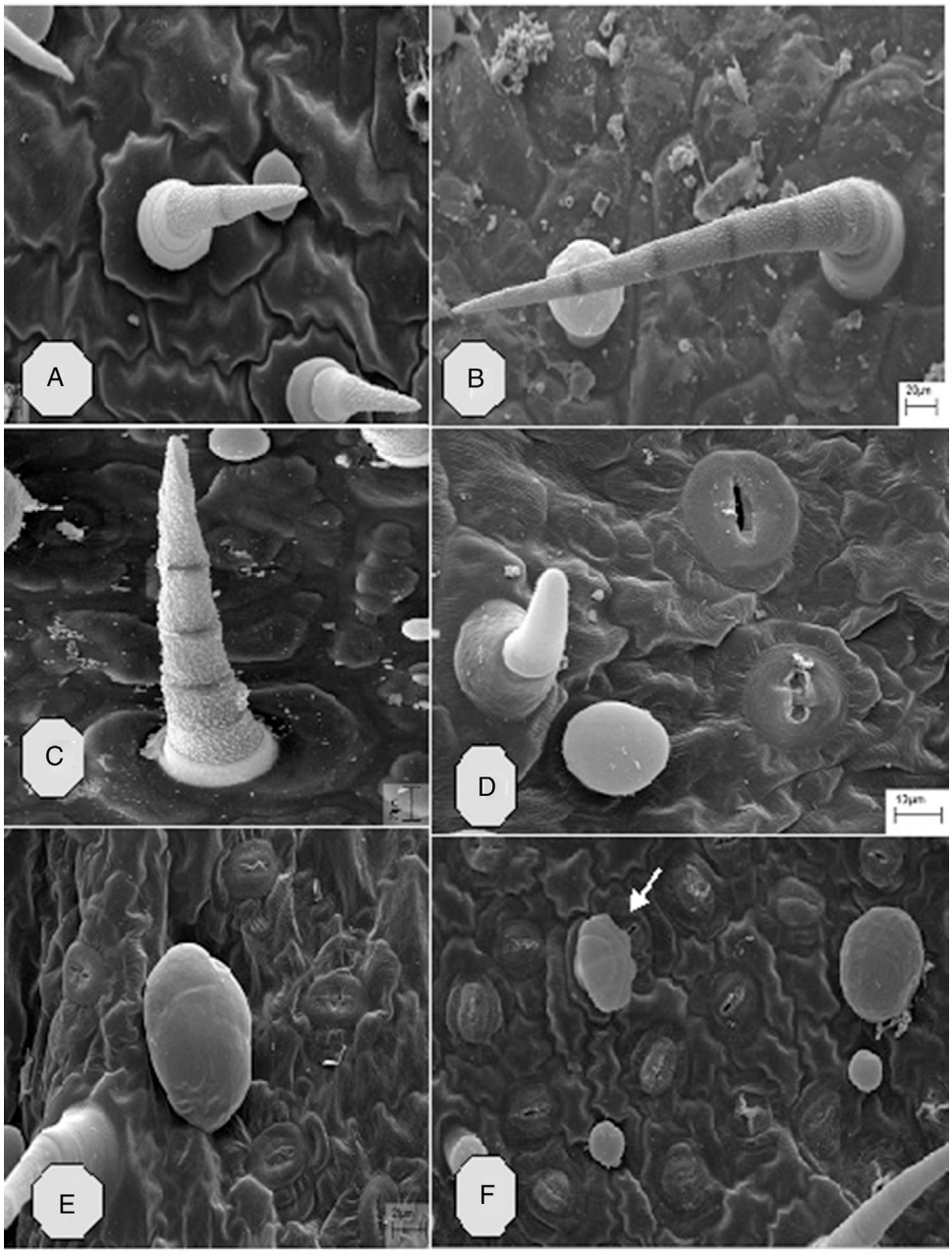


**Fig. 5.** Scanning Electron Micrograph of non-glandular (A–D) and glandular peltate trichomes (E–F) in leaves of *in vitro Plectranthus ornatus*.

Our results contrasted with reports from the literature using *in vivo* cultivated *P. ornatus* (Ascensão et al., 1999). We found that neither *in vitro* nor *in vivo* cultivated *P. ornatus* showed the presence of glandular digitiform trichomes. Moreover, unlike the observations made with cultivated *in vivo P. ornatus*, where the presence of two types of capitate trichomes only were reported, here, using cultivated *in vitro P. ornatus*, we identified four morphological types of capitate glandular trichomes (Fig. 6A–J). The effect of growth regulators on the trichome distribution on the adaxial and abaxial sides of foliar lamina is shown (Fig. 7A–J). In addition, this figure illustrates the distribution of trichomes on adaxial (Fig. 7A) and abaxial sides (Fig. 7B) of plants grown in MS0. The leaves treated with 4.5 μM BAP + NAA (5.4 μM) are illustrated in Fig. 7C and D. Treatment with MS0 and with 4.5 μM BAP + NAA 4.5 μM induced a larger number of peltate glandular trichomes when compared with other treatments and the *ex vitro* plant (Figs. 7E–J and 8A and B). The effect of treatment with 9.0 μM BAP + 4.5 μM NAA is illustrated in Fig. 7(E and F) and shows a reduction in the number of non-glandular trichomes, especially on the abaxial side (Fig. 7F) when compared with *ex vitro* plants (Fig. 8B). The effect of treatment with 4.7 μM and 9.3 μM KIN + 5.4 NAA is illustrated in Fig. 7(G–I). When this treatment is compared with plants cultivated in MS0 medium and with the *ex vitro* plant, we observed a significant decrease in the total number of glandular and non-glandular trichomes.

These results indicate that the growth regulators added to the culture medium of *in vitro P. ornatus* affected not only the qualitative and quantitative profile of VOC, but also the density of trichomes. Similar results were observed using *L. dentata* (Sudriá et al., 1999).

Trichomes are important for the passive resistance of plants to pathogens, parasites and others stressors (Moon et al., 2009) and can play an important role in adapting to environments with high

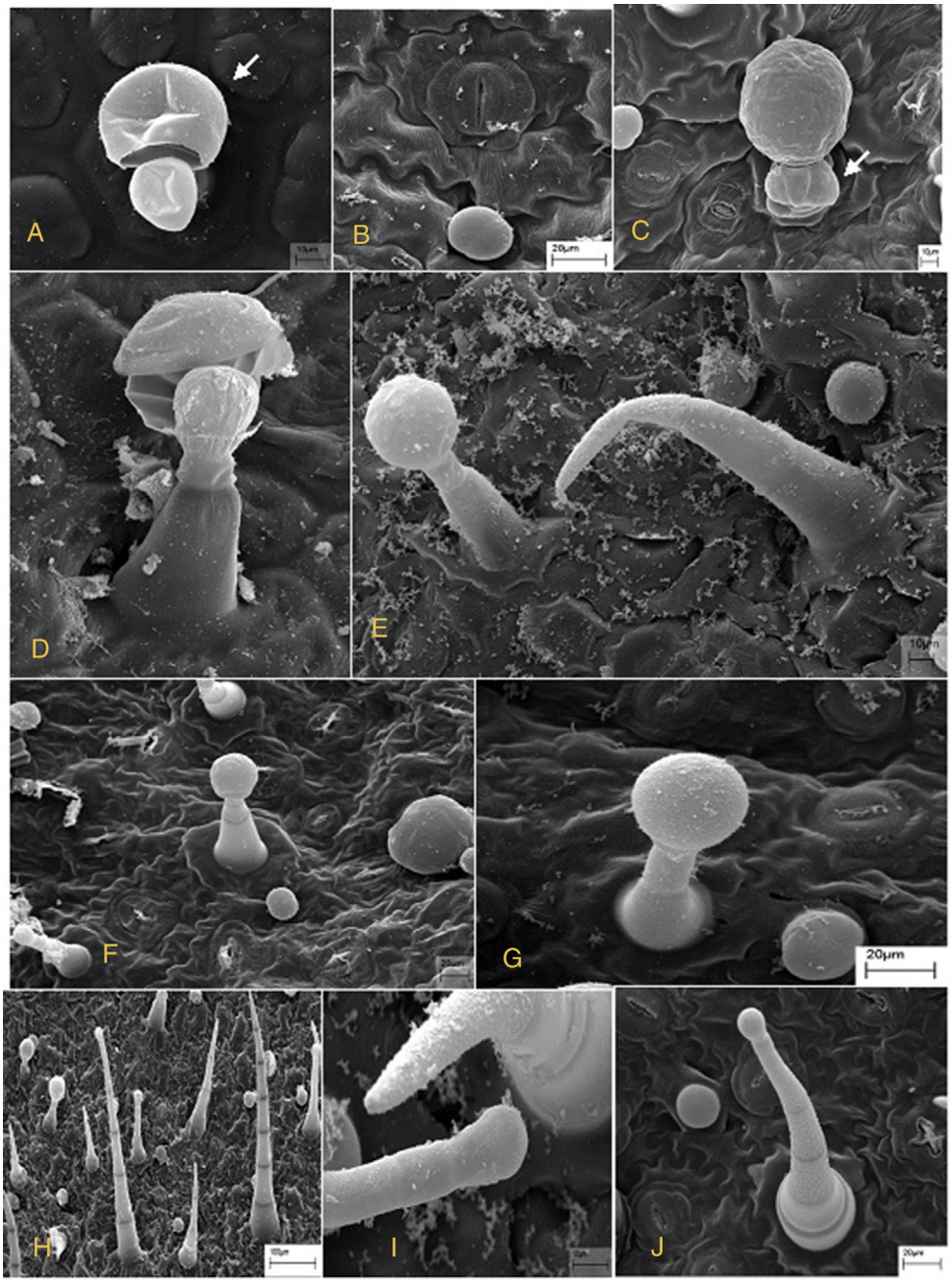


**Fig. 6.** Scanning Electron Micrograph of glandular capitate trichomes in leaves of *Plectranthus ornatus* cultivated *in vitro*. A–C, type I capitate trichomes. A, Cuticle cover recovering the head of the glandular trichome. B, Peltate trichome showing the glandular bicellular head. C, Unicellular glandular head and base of the multicellular trichome. D–G, Type II capitate trichomes. D, Cuticle cover recovering the head of multicellular glandular trichome. E, The neck cell of the trichome. F, Type I and type II capitate trichomes and peltate trichome. H-I, Type III trichomes. H, Panoramic view of the adaxial epidermis with non-glandular trichomes and type III capitate trichomes. I, A glandular oval head. J, Type IV trichomes, showing a bicellular base and stalk cells with clearly visible cells.

levels of irradiance (Stenglein et al., 2005). The glandular trichomes are responsible for the storage and secretion of several phytochemical compounds that can alter the leaf taste or that can be toxic, thus protecting plants against herbivore attack. Several trichome chemicals have proven pharmacological effects (Siebert, 2004; Sousa et al., 2008; Martins et al., 2009).

The density of trichomes on plants can vary due to genetic and environmental factors (Forkner and Hare, 2000). In this study, we observed that the leaves of plants cultivated *in vitro* in MS0 or in medium supplemented with 4.5 μM BAP showed higher numbers of abaxial side glandular trichomes. This finding may be correlated with the increased production of VOC that was observed with these conditions because trichomes are directly involved with the storage and secretion of phytochemical compounds (Alaimo et al., 2005; Bussotti et al., 1977).

Therefore, even though physiological, anatomical and biochemical characteristics of plants are genotype-derived, factors such as the use of phytoregulators and the composition of culture medium exert a robust influence on the expression of regulators involved in the defense process and organogenesis *in vitro*.

## Conclusions

In this work was observed that the regulators influenced qualitative and quantitative profiles of the VOC and the number and distribution of hairs on the leaf surface. Understanding the

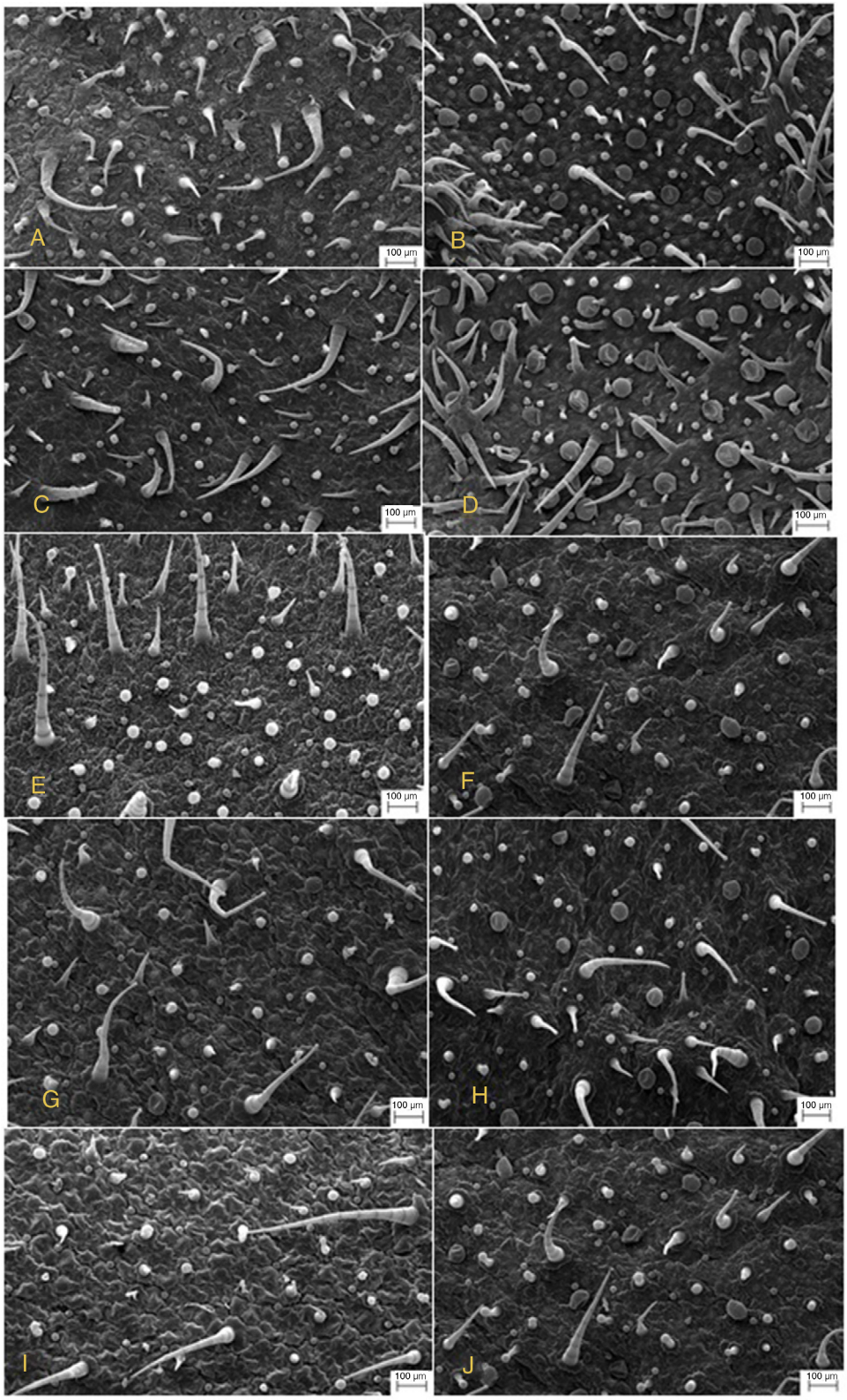


**Fig. 7.** A Scanning Electron Micrograph demonstrates the effect of growth regulators in the distribution of trichomes on the adaxial and abaxial faces, respectively, from the leaves of *Plectranthus ornatus in vitro*. A and B, MS0. C and D, 4.5 μM BAP + 5.4 μM NAA. E and F, 9.0 μM BAP + 5.4 μM NAA. G and H, 4.7 μM KIN + 5.4 μM NAA. I and J, effect of 9.3 μM KIN + 5.4 μM NAA.

mechanisms and factors that influence the biosynthesis of metabolites and the differentiation of plants grown *in vitro* contributes to the development of new approaches for producing plants competent in acclimatization, thus producing new compounds of interest.

## Author contributions

HCP-S was encharged of the *in vitro* cultivation and SEM analysis. JPD was the supervisor and deal with data analysis and

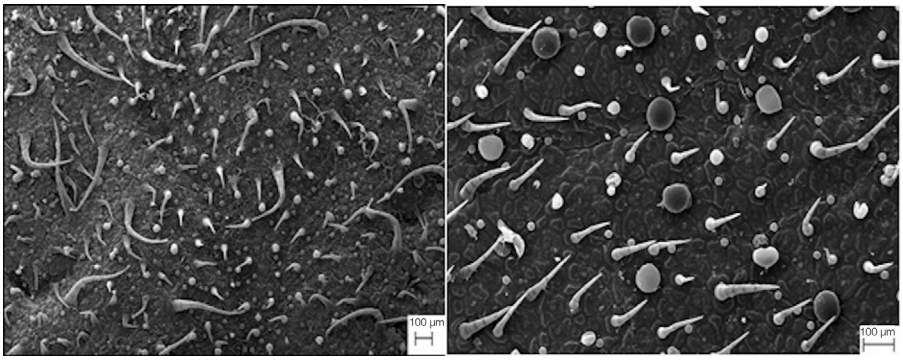


**Fig. 8.** A Scanning Electron Micrograph of *ex vitro* plants. A, adaxial face. B, abaxial sides.

discussion and writing. JRFS and MCB were the responsibles for all the *in vitro* cultivation and micropropagation supervising. JMD contributed with chemical analysis and critical reading. FMR and PRRM did the HSPM extractions and GC analysis. FSO worked with PCA and HCA analysis.

## Conflicts of interest

The authors declare no conflicts of interest.

## Acknowledgements

This work was in part funded with grants and fellowships by CNPq and Fundação de Amparo a Pesquisa do Estado da Bahia. We are in debt with Dr. Jailson B. de Andrade (UFBA) by the facilities for all the GC/MS data analysis and quantification of VOC.

## References

Abdel-Mogib, M., Albar, H.A., Batterjee, S.M., 2002. Chemistry of the genus *Plectranthus*. Molecules 7, 271–301.

Adams, R.P., 2007. Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry, 4th ed. Allured Publishing Corporation, Carol Stream, IL, pp. 804.

Affonso, V.R., Bizzo, H.R., Lage, C.L.S., Sato, A., 2009. Influence of growth regulators in biomass production and volatile profile of *in vitro* plantlets of *Thymus vulgaris* L. J. Agric. Food Chem. 57, 6392–6395.

Affonso, V.R., Bizzo, H.R., Lima, S.S., Esquibel, M.A., Sato, A., 2007. Solid phase microextraction (SPME) analysis of volatile compounds produced by *in vitro* shoots of *Lantana camara* L. under the influence of auxins and cytokinins. J. Braz. Chem. Soc. 18, 1504–1508.

Alaimo, M.G., Vizzı, D., Melati, M.R., 2005. Histochemical properties and trace element concentrations in *Parietaria* L. from urban sites (Palermo, Italy). Aerobiologia 21, 21–31.

Anačkov, G., Božin, B., Zorić, L., Vukov, D., Mimica-Dukić, N., Merkulov, L., Ružica, I., Jovanović, M., Boža, P., 2009. Chemical composition of essential oil and leaf anatomy of *Salvia bertolonii* Vis. and *Salvia pratensis* L. (Sect. *Plethiosphace*, Lamiaceae). Molecules 14, 1–9.

Ascensão, L., Mota, L., Castro, M.M., 1999. Glandular trichomes on the leaves and flowers of *Plectranthus ornatus*. Morphology, distribution and histochemistry. Ann. Bot. 84, 437–447.

Avato, P., Fortunato, I.M., Ruta, C., D'Elia, R., 2005. Glandular hairs and essential oils in micropropagated plants of *Salvia officinalis* L. Plant Sci. 169, 29–36.

Bussotti, F., Grossoni, P., Bottacci, A., 1977. Sclerophylly in beech (*Fagus sylvatica* L.) trees, its relationship with crown transparency, nutritional status and summer drought. Forestry 70, 267–271.

de Albuquerque, R.L., da Silva, M.G.V., Machado, M.I.L., Matos, F.J.A., de Morais, S.M., Stone-Neto, J., 2007. Chemical composition and antioxidant activity of *Plectranthus grandis* and *P. ornatus* essential oils from north-eastern Brazil. Flavour Fragr. J. 22, 24–26.

Eiceman, G.A., Nazarov, E.G., Miller, R.A., Krylov, E.V., Zapata, A.M., 2002. Micromachined planar field asymmetric ion mobility spectrometer as a gas chromatographic detector. Analyst 127, 466–471.

Forkner, R.E., Hare, J.D., 2000. Genetic and environmental variation in acyl glucose ester production and glandular and nonglandular trichome densities in *Datura wrightii*. J. Chem. Ecol. 26, 2801–2823.

Gaspar-Marques, C., Rijo, P., Simões, M.F., Duarte, M.A., Rodriguez, B., 2006. Abietanes from *Plectranthus grandidentatus* and *P. hereroensis* against methicillin- and vancomycin-resistant bacteria. Phytomedicine 13, 267–271.

Gaspar-Marques, C., Simões, M.F., Rodríguez, B., 2005. A trihomoabietane diterpenoid from *Plectranthus grandidentatus* and an unusual addition of acetone to the *orto*-quinone system of cryptotanshinone. J. Nat. Prod. 68, 1408–1411.

Gaspar-Marques, C., Simões, M.F., Rodríguez, B., 2004. Further labdane and kaurane diterpenoids and other constituents from *Plectranthus fruticosus*. J. Nat. Prod. 67, 614–621.

Gonçalves, S., Serra, H., Nogueira, J.M.F., Almeida, C.R.L., Romano, A., 2008. Headspace-SPME of *in vitro* shoot-cultures and micropropagated plants of *Lavandula viridis*. Biol. Plant. 52, 133–136.

Kirakosyan, A., 2006. Plant biotechnology for the production of natural products. In: Cseke, L.J., Kirakosyan, A., Kaufman, P.B., Warber, S., Duke, J.A., Brielmann, H.L. (Eds.), Natural Products from Plants. , 2nd ed. CRC Press, Taylor & Francis, Boca Raton, FL, pp. 222–256.

Köllner, T.G., Held, M., Lenk, C., Hiltpold, I., Turlings, T.C.J., Gershenzon, J., Degenhardt, J., 2008. A maize (*E*)-β-caryophyllene synthase implicated in indirect defense responses against herbivores is not expressed in most American maize varieties. Plant Cell 20, 482–494.

Lord, H.L., Grant, R.P., Walles, M., Incledon, B., Fahie, J.B., Pawliszyn, B., 2003. Development and evaluation of a solid-phase microextraction probe for *in vivo* pharmacokinetic studies. Anal. Chem. 75, 5103–5115.

Lukhoba, C.W., Simmonds, M.S.J., Paton, A.J., 2006. *Plectranthus*, a review of ethnobotanical uses. J. Ethnopharmacol. 103, 1–24.

Martins, M.B.G., Graf, R.R., Cavalheiro, A.J., Rodrigues, S.D., 2009. Pharmacognostic characterization of leaves and seeds of *Albizia lebbeck* (L.) Benth, (Fabaceae). Rev. Bras. Farmacogn. 19, 106–114.

Medrado, H.H., dos Santos, E.O., Ribeiro, E.M.O., David, J.M., David, J.P., Araújo, J.F., do Vale, A.E., Bellintani, M.C., Brandão, H.N., Meira, P.R., 2017. Rosmarinic and cinnamic acid derivatives of *in vitro* tissue culture of *Plectranthus ornatus*. Overproduction and correlation with antioxidant activities. J. Braz. Chem. Soc. 28, 505–511.

Moon, H.K., Hong, S.P., Smets, E., Huysmans, S., 2009. Phylogenetic significance of leaf micromorphology and anatomy in the tribe Mentheae (Nepetoideae, Lamiaceae). Bot. J. Linn. Soc. 160, 211–231.

Passinho-Soares, H., Meira, P.R., David, J.P., Mesquita, P.R.R., do Vale, A.E., Rodrigues, F.M., Pereira, P.A.P., Santana, J.R.F., Oliveira, F.S., de Andrade, J.B., David, J.M., 2013. Volatile organic compounds obtained by *in vitro* callus cultivation of *Plectranthus ornatus* Codd. (Lamiaceae). Molecules 18, 10320–10333.

Rijo, P., Gaspar-Marques, C., Simões, M.F., Duarte, A., Apreda-Ojas, M.D., Cano, F.H., Rodriguez, B., 2002. Neoclerodane and labdane diterpenoids from *Plectranthus ornatus*. J. Nat. Prod. 65, 1387–1390.

Serrato-Valenti, G., Bisio, A., Cornara, L., Ciarallo, G., 1997. Structural and histochemical investigation of the glandular trichomes of *Salvia aurea* L. leaves, and chemical analysis of the essential oil. Ann. Bot. 79, 329–336.

Siebert, D., 2004. Localization of salvinorin A and related compounds in glandular trichomes of the psychoactive sage, *Salvia divinorum*. Ann. Bot. 93, 763–771.

Silva, S., Sato, A., Lage, C.L.S., Gil, R.A.S.S., Azevêdo, D.A., Esquibel, M.A., 2005. Essential oil composition of *Melissa officinalis* L. *in vitro* produced under the influence of growth regulators. J. Braz. Chem. Soc. 16, 1387–1390.

Sousa, F.C.F., Melo, C.T.V., Citó, M.C.O., Félix, F.H.C., Vasconcelos, S.M.M., Fonteles, M.M.F., Barbosa-Filho, J.M., Viana, G.S.B., 2008. Medicinal plants and their bioactive constituents, a scientific review of bioactivity and potential benefits in the anxiety disorders in animal models. Rev. Bras. Farmacogn. 18, 642–654.

Stenglein, S.A., Arambarri, A.M., Menendez-Sevillano, M.C., Balatti, P.A., 2005. Leaf epidermal characters related with plant's passive resistance to pathogens vary among accessions of wild beans *Phaseolus vulgaris* var. *aborigineus* (Leguminosae-Phaseoleae). Flora 200, 285–295.

Sudriá, C., Piñol, M.T., Palazón, J., Cusidó, R.M., Vila, R., Morales, C., Bonfill, M., Cañigueral, S., 1999. Influence of plant growth regulators on the growth and essential oil content of cultured *Lavandula dentata* plantlets. Plant Cell Tissue Org. 58, 177–184.

Tisserat, B., Vaughn, S.F., 2008. Growth, morphogenesis, and essential oil production in *Mentha spicata* L. plantlets *in vitro*. In Vitro Cell. Dev. Biol.-Plant 44, 40–50.

Tregear, J.W., Morcillo, F., Richaud, F., Berger, A., Singh, R., Cheah, S.C., Hartmann, C., Rival, A., Duval, Y., 2002. Characterization of a defensing gene expressed in

oil palm inflorescences, induction during tissue culture and possible association with epigenetic somaclonal variation events. J. Exp. Bot. 53, 1387–1396.

Tsuro, M., Inoue, M., Kameoka, H., 2001. Variation in essential oil components in regenerated lavender (*Lavandula vera* DC) plants. Sci. Hortic. 88, 309–317.

Turner, G.W., Gershenzon, J., Croteau, R.B., 2000. Development of peltate glandular trichomes of peppermint. Plant Physiol. 124, 665–679.