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Original Article

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

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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

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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.