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Artigo

LEAF AND ROOT VOLATILES PRODUCED BY TISSUE CULTURES OF *Alpinia zerumbet* (PERS.) BURTT & SMITH UNDER THE INFLUENCE OF DIFFERENT PLANT GROWTH REGULATORS

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Volatiles produced by plantlets of *Alpinia zerumbet* were obtained by means of simultaneous distillation-extraction (SDE). The effects of indole-3-acetic acid, kinetin, thidiazuron and 6-benzylaminopurine on leaf and root volatile composition obtained by tissue cultures were investigated. A higher content of β–pinene and a lower content of sabinene were observed in leaf volatile of plantlets cultured in control, IAA and IAA+ TDZ media, as compared with those of donor plants. *In vitro* conditions were favorable to increase caryophyllene content. Volatile compounds from the root were characterized mainly by camphene, fenchyl-acetate and bornyl acetate; which constitute about 60% of total volatile.

Keywords: volatile compounds; *Alpinia zerumbet*; growth regulators.

INTRODUCTION

Alpinia zerumbet (Pers.) Burtt & Smith (Zingiberaceae) is native of tropical and subtropical Asia. This species is used as a medicinal, spice and ornamental plant in several places in Asia, Africa and America.[1] In Brazil, it is used in folk medicine mainly to treat arterial hypertension.[2] Ethnopharmacological studies have showed a greater use of this species in Brazilian religious afro rituals, mainly in the Northeastern region.[3] Terpenoids represent one of the largest and most diverse classes of secondary metabolites in this species, being produced in all parts of the plant, and being responsible for the biological properties attributed to the species by the popular users, as for instance, antihypertensive, antimicrobial and antioxidative effects.[4]

Plant tissue culture techniques are a useful resource to improve the standardized and continuous production of plant raw material and bioactive metabolites.[5] This technique is an alternative to rapid large-scale production and shares the guarantee of aseptic and ethic plant materials. Besides, some studies have reported the effects of growth regulators on mevalonic acid pathway, changing so the qualitative and quantitative content of volatiles in medicinal and aromatic plants,[6] since many terpenoid compounds are found in lower proportion from their natural sources. For *A. zerumbet*, this is the first evaluation of growth regulators effects on the production of leaf and root volatiles in tissue culture; which is relevant because this species is widely used, thanks to its aromatic and medicinal properties.

In order to evaluate the effects of plant growth regulators on volatile compounds production, *A. zerumbet* was cultured *in vitro* with cytokinins and auxins supplemented media, and the respective volatile composition was analyzed by gas chromatography.

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EXPERIMENTAL

Plant material

Alpinia zerumbet was collected in October 2008, in Federal University of Rio de Janeiro (Rio de Janeiro, Brazil). A voucher specimen is kept at the Herbarium of Rio de Janeiro Botanical Garden, accession number RB 433485. Those plants were used as explants donor for tissue cultures establishment.

Tissue cultures

The *in vitro* cultures were established according to Victório.[7] Rhizome buds were excised from donor plants, sterilized and placed in glass bottles (72 x 59 mm) containing 60 mL of MS medium.[8] Plants were subcultured in different liquid media: MS0 (control) – MS0*l* (leaves) and MS0*r* (roots), MS + 2 mg L^{-1} indole-3-acetic acid (IAA 2), MS + 2 mg L^{-1} benzylaminopurine (BAP 2), MS + 2 mg L^{-1} kinetin (KIN 2), MS + 2 mg L^{-1} IAA + 2 mg L^{-1} thidiazuron (TDZ 2). Cultures were maintained at 25 ± 2 °C, photoperiod of 16/8 hours (day/night), under white light fluorescent tubes (F-20 W, General Electric®) and intensity of 30 μmoles m^{-2} s^{-1}. Proliferation rate, shoot length, number of leaves per shoot, root percentage, root length and leaf and root dry weight was recorded within 4 months when plantlets were used to analyze leaf and root volatile (Table 1). The design of the experiments was a complete randomized block, and each experiment consisted of three explants per glass and the minimum of ten replicate culture glasses per plant growth regulator treatments. Data were subjected to the analyses of variance (ANOVA) and averages were compared using Tukey's test at the $p \leq 0.05$ significance level.

Table 1. Effects of growth regulators on in vitro development

Treatments (mg L^{-1})	Proliferation rate	Number of leaves/ plants	Shoot length (cm)	Root length[a] (cm)	Shoot weight[a] (%)	Root weight[a] (%)
MS0 (control)	2.3 : 1^{a}	3.4 ± 0.4^{ab}	12.1 ± 1.5	32.4±4.1	100	100
IAA 2	1.6 : 1^{b}	3.0 ± 0.4^{ab}	13.6 ± 2.5	21.6±1.7*	95.5	331
IAA 2 + TDZ 2	3.1 : 1^{c}	4.0 ± 0.2^{a}	13.1 ± 1.4	14.7±0.8**	n.e.	n.e.
BAP 2	2.8 : 1^{ac}	2.6 ± 0.2^{b}	9.2 ± 1.5	22.6±1.2*	83.4	139
KIN 2	2.7 : 1^{ac}	3.3 ± 0.3^{ab}	9.1 ± 1.4	14.1±1.6**	91.1	270

Average ± SD, n≥30, Tukey`s test. Different letters indicate significant differences at p< 0.05. *p< 0.05, **p< 0.001 statistical differences in comparison with control. [a]Data of the dry weight compared with control. n.e. - not evaluated.

Extraction procedure

Fresh leaves (3 g) and roots (10 g) were homogenized with 80 mL of distilled water and submitted to simultaneous distillation-extraction (SDE)[9] for 1 h 30 min using 2 mL of dichloromethane as an organic collecting solvent[10] as schematized by Boix *et al*..[11]

Volatile analysis

Analytical GC (Gas Chromatographic) was carried out on a Varian Star 3400 gas chromatograph fitted with a DB-5-MS column (30 m × 0.25 mm i.d., 0.25 µm film thickness) and equipped with flame ionization detection (FID). Temperature was programmed from 60 to 290 ºC at 5 °C min^{-1}. The injection consisted of 1 µL of distilled oil diluted with dichloromethane. Hydrogen was used as carrier gas at the flow rate of 1 mL min^{-1}. The injector temperature was 270 °C. Leaf volatile samples were analyzed in splitless mode. GC-MS analyses were carried out on a Shimadzu Model GC-MS-QP 5000 fitted with a HP-5/MS fused silica capillary column (30 m x 0.25 mm i.d., 0.25 µm film thickness). GC-MS conditions were the same as above, except for helium that was used as carrier gas at flow rate of 1 mL min^{-1} and for the mass spectrometer that was operated on electron impact mode at 70 eV. Quantification was performed from GC-FID profiles using relative areas (%). A test of difference between two percentages was conducted, considering p≤ 0.05 level, *t*-test, using Statistica® 6.0 program. Identification of components in the volatiles was based on retention indices relative to *n*-alkanes (C_8 - C_{19}) and computer matching with the National Institute of Standards and Technology (NIST 98) library as well as by comparison between mass fragmentation patterns with those reported in literature.[12]

RESULTS AND DISCUSSION

Data from plantlets cultured for 4 months are shown at Table 1. The addition of 2 mg L^{-1} of IAA plus TDZ resulted in increase of both, proliferation rate (3.1:1) and number of leaves (4.0), as compared with control medium. Considering the root parameters analyzed in this study, the plantlets cultured in MS0 have shown a higher root length (32.4 cm) as compared with other tested media. The addition of IAA, which has been widely used in tissue cultures to improve rooting, increased the root weight in 331% as compared to MS0. Rooting was 100% for all tested media, but a significant reduction in root length was observed using KIN or TDZ plus IAA. These effects on rooting are probably related to additional cytokinins, as reported in tissue culture studies with others species.[13]

Volatile composition was listed in Table 2.

Data from donor plants were compared with those obtained from plantlets cultured in control medium (MS0*l*). The main volatile constituents from donor plants were sabinene (9.8%), β-pinene (3.8%), 1,8-cineole (13.6%), γ-terpinene (11.8%) and terpinen-4-ol (17%). Except for γ-terpinene, which was absent in plantlets cultured in MS0, the same main compounds were produced. Plantlets of *A. zerumbet* maintained under *in vitro* conditions showed quantitative changes in terpenoids production, with significantly increased content of α–pinene, β-pinene and caryophyllene, and reduced content of sabinene, 1,8-cineole and terpinen-4-ol in leaf volatiles, as compared to donor plants. Donor plants produced less sesquiterpenes in aerial parts (4.1%), than in plantlets (39.4-52.5%). Caryophyllene and its oxide were the single sesquiterpenes detected in donor leaves. Other sesquiterpenes, as α-humulene, β-farnesene, nerolidol and carotol were also detected in aerial parts of plantlets. These terpenoids have been reported in studies about *A. zerumbet* oil composition, from different regions.[14] In addition, caryophyllene increase under *in vitro* conditions was up to 72% in relation to donor plants. Affonso *et al.*,[6] in studies about tissue cultures of *Lantana camara*, have also verified an increase in caryophyllene by addition of TDZ. β-caryophyllene has anti-inflammatory and anesthetic properties,[15] furthermore it has been used in spices, soaps, detergents, creams and lotions, and also in a variety of food products and beverages.[16] The enhancement in caryophyllene content is very interesting, considering its wide importance in pharmaceutical, cosmetic and food industries.

The chromatographic profiles of volatile oils from plantlets cultured in different media have shown similarity respecting to camphene, β-pinene, linalool, terpinene-4-ol, bornyl acetate, and caryophyllene content. The main changes were related to quantitative feature (Figures 1 and 2), only.

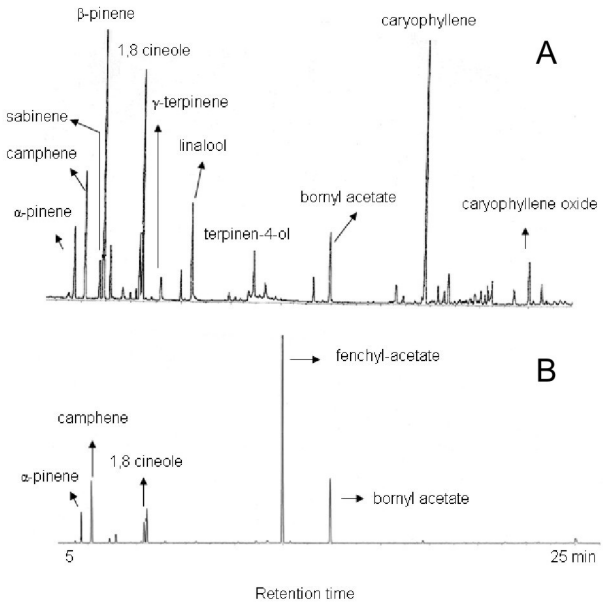

Figure 1. *Chromatographic profile (GC-MS) of Alpinia zerumbet leaf volatiles (A) and root volatiles (B) from plantlets cultured for 4 months in IAA supplemented medium, showing the main constituents*

Table 2. Composition (%) of leaf and root volatiles according to GC-FID analyses from 4-month-old plantlets of *Alpinia zerumbet* cultured under different growth regulators

Constituent	Relative area (%) [a]							
	Donor plant (leaves)	*In vitro* leaves [c]					*In vitro* roots [c]	
		MS0*l*	IAA 2	IAA 2 + TDZ 2	BAP 2	KIN 2	MS0*r*	IAA 2
tricyclene	3.6	-	-	-	-	-	-	0.4
α-pinene	1.7	4.3*	3.9	2.6	0.3	-	5.4	6.5
camphene	0.2	7.6*	6.6*	5.2*	0.4	-	10.3	13.5
sabinene	9.8	1.1	1.9	0.4	0.2	-	-	-
ß-pinene	3.8	14.3*	13.7*	7.1*	0.9	-	-	0.9
myrcene	0.9	1.9	1.7	1.2	-	-	0.6	1.4
α-terpinene	2.5	-	-	-	-	-	-	-
ρ-cymene	3.6	-	-	-	-	-	-	-
limonene	1.6	3.1	2.9	2.1	-	-	2.6	4.4
1,8 cineole	13.6	4.7*	6.8*	2.4*	0.3	6.3*	3.4	4.9
n.i. [b]	-	-	-	-	-	-	3.4	2.6
n.i. [b]	-	-	-	-	-	-	0.5	0.6
n.i. [b]	-	-	-	-	-	-	0.7	0.8
γ-terpinene	11.8	-	0.9	0.2	-	-	-	-
ρ-2-mentha-4(8)-diene	0.8	1.7	1.5	1.1	-	-	-	-
linalool	1.4	3.7	3.6	2.6	0.6	7.7*	-	-
terpinen-4-ol	17	6.5	5.0	6.0	1.0	-	-	-
endo-fenchyl-acetate	-	-	-	-	-	-	40	38
decenal	-	1.1	1.0	1.3	2.5	-	-	-
bornyl acetate	-	2.5	2.7	2.1	4.0	3.7	16.7	13.7
daucene	-	1.2	1.2	1.9	1.6	-	-	-
trans-β-caryophyllene	2.4	21.1*	17.1*	27.2*	20*	22.8*	1.9	0.5
clovene	-	1.2	1.2	1.8	1.4	-	-	-
α-humulene	-	0.7	2.3	0.8	-	3.4	-	-
β-farnesene	-	2.7	0.6	3.4	4.3	-	-	-
β-dihydro agrofuran	-	1.5	0.7	2.3	2.6	-	-	-
γ-cadinene	-	1.5	1.6	2.1	3.0	-	-	-
cubebol	-	-	1.6	-	-	-	-	-
cis-calamenene	-	1.6	1.8	2.6	3.4	-	-	-
(E)-nerolidol	-	0.7	0.9	1.1	2.0	2.2	-	-
caryophyllene oxide	1.7	2.3	2.5	3.8	3.6	3.7	-	-
carotol	-	1.3	1.5	2.1	2.6	3.8	-	-
Monoterpenes (%)	72.2	48.9	48.5	30.9	3.7	17.7	83.6	87.7
Sesquiterpenes (%)	4.1	39.4	37.3	52.5	51.2	35.9	1.9	0.5
Total identified	90.4	88.1	86.5	83.4	54.9	53.6	89.6	88.2

[a]Data show the averages. [b]not identified. [c]Plant material obtained from *in vitro* cultures of *A. zerumbet*. *p<0.05 (*t*-Test) - Statistical differences in comparison with donor plants.

Under *in vitro* conditions (MS0*l*, IAA 2 and IAA 2 + TDZ 2), leaf volatiles showed remarkable increase in β–pinene and caryophyllene content, as compared with oil from donor plants. On the other hand, sabinene, one of the most expressive monoterpene of this species, appeared in very low percentage. Leaf volatiles of *A. zerumbet* plantlets cultured under KIN effects showed significantly high amounts of linalool (7.7%), a remarkable compound in the fragrance industry.[17] Addition of BAP to MS medium resulted in increase of sesquiterpenes (51.2%) in leaf volatiles, as compared with monoterpenes (3.7%). β–pinene, 1,8-cineole, linalool and terpinene-4-ol were the most influenced monoterpenes in this experiment. These results are not in agreement with studies using hydrodistillation technique for oil extraction, where monoterpene production was increased under BAP supplementation.[6] Sudriá *et al.*[6] verified a positive influence of BAP in induce oils production and accumulation in tissue cultures of *Lavandula dentate*. Low concentration of γ–terpinene was found only in leaf volatiles from plantlets treated with IAA 2 (0.9%) or IAA 2 plus TDZ 2 (0.2%). Using MS0*l* medium, γ–terpinene production was suppressed, suggesting an inhibition of limonene conversion in γ-terpinene.[18]

The root volatiles were characterized especially by camphene, fenchyl acetate and bornyl acetate, all of them constituting more than 60% of total volatiles. Root volatiles showed a predominance of fenchyl acetate (38-40%). *A. zerumbet* root volatiles were for the first time reported. Comparing the composition of root volatiles from plantlets cultured in MS0*r* to that of IAA treatment, differences were observed in the quality of monoterpene production. β-pinene (0.9%),

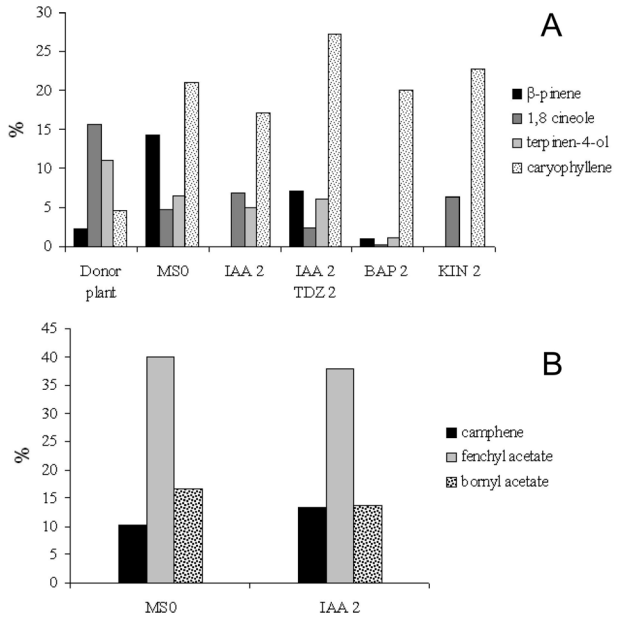

Figure 2. *Composition (%) of the major contents of leaf (A) and root (B) volatiles, according to GC-FID analyses, from plantlets of Alpinia zerumbet cultured for 4 months under different growth regulators*

for example, was detected only under IAA treatment. Regarding the sesquiterpene fraction of root volatiles, both caryophyllene concentrations (1.9%, MS0*r* and 0.5%, IAA) were very scarce as compared with monoterpenes, which have accounted for more than 80%. The higher root length of plantlets developed in MS0 did not result in positive changes in volatiles amount, as compared with shorter roots. No correlations were verified between the plant development and improvement of terpenoids content.

The effects of growth regulators on plantlets, probably, constitute a stress factor inducing defense responses that modify the biosynthesis pathway of terpenoids. Silva *et al.*[6] have observed a high amount of nerol and geraniol, after treating *Melissa officinalis* with IAA supplementation.[6] Plants in environment are exposed to larger uncontrolled environmental factors. The advantage of tissue culture is the ability to reproduce standardized conditions, as previously evaluated.

By comparing leaf (MS0*l*) and root (MS0*r*) volatiles produced by plantlets, it was possible to find α–pinene, camphene and 1,8 cineole in common, as the main compounds. Bornyl acetate was produced by roots and leaves of plantlets in all treatments, but not by donor plants. In root volatiles, its production was higher, between 13 and 17%. Probably, this monoterpene represents a plant defense aspect, as the one resulting from *in vitro* conditions. Some plants produce bornyl acetate as an antifeeding effect.[19]

In vitro conditions, such as humidity, temperature, light intensity and medium composition, particularly growth regulator types and concentrations, may be manipulated to provide a suitable microenvironment to secondary metabolites production.

This study revealed the main *A. zerumbet* volatiles produced under *in vitro* conditions, indicating their changes in composition according to used medium. The effects of growth regulators IAA auxin and BAP, TDZ and KIN cytokinins on *A. zerumbet* volatile composition were evidenced. Quantitative and qualitative changes in terpenoids produced by aerial parts of plantlets, as compared with donor plants were also observed; with sabinene, β-pinene (MS0*l* and IAA 2), γ-terpinene and caryophyllene presenting, among others, an inverse concentration in leaf volatiles. A remarkable positive effect on caryophyllene production by leaves was achieved in tissue cultures. Furthermore, additional sesquiterpenes were found in leaf volatiles from plantlets, and were not detected in donor plants. Root volatiles exhibited slight changes in their composition by addition of IAA, as compared to MS0*r*.

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