

Influence of Growth Regulators in Biomass Production and Volatile Profile of in Vitro Plantlets of *Thymus vulgaris* L.

VANESSA RIBEIRO AFFONSO,[‡] HUMBERTO RIBEIRO BIZZO,[‡] CELSO LUIZ SALGUEIRO LAGE,[‡] AND ALICE SATO^{*,†}

[‡]Universidade Federal do Rio de Janeiro—UFRJ, Instituto de Biofísica Carlos Chagas Filho, CCS, Bloco G, Cidade Universitária, Ilha do Fundão, 21952-590 Rio de Janeiro, Brazil, [†]EMBRAPA Agroindústria de Alimentos, Avenida das Américas, 29501 Rio de Janeiro, Brazil, and [†]Departamento de Botânica, CCBS, Universidade Federal do Estado do Rio de Janeiro—UNIRIO, Avenida Pasteur 458, 22290-040 Rio de Janeiro, Brazil

In vitro shoots of thyme (*Thymus vulgaris* L.) were established, and the effects of the auxin indole-3-acetic (IAA) acid and the cytokinins benzyladenine (BA), zeatin (ZEA), and kinetin (KIN) at 1.0, 5.0, and 10.0 μM on rooting, biomass production, and volatile compounds production by these plants were investigated. The volatiles were extracted by solid phase microextraction (SPME) and analyzed by gas chromatography. The highest biomass shoot growth was obtained with BA at 5.0 μM , while IAA at all concentrations tested achieved 100% rooting frequency. The three major compounds were γ -terpinene (22.8–38.8%), *p*-cymene (13.8–27.9%), and thymol (6.5–29.0%). Quantitative changes of these compounds were observed in response to the effect of varying growth regulators concentrations in the culture medium. Growing *Thymus vulgaris* L. plants in media supplemented with IAA at 1.0 μM increased volatile compounds such as thymol by 315%. Nevertheless, the same major compounds were produced in all treatments and no qualitative changes were observed in the volatile profile of thyme plants.

KEYWORDS: *Thymus vulgaris* L.; volatile compounds; SPME; in vitro shoots; biomass production; growth regulators

INTRODUCTION

Thymus vulgaris L. (Lamiaceae), commonly known as thyme, is a native species to the Mediterranean region. It is cultivated and widely used as a culinary seasoning in South and Southeast Brazil (1).

Thyme aromatic leaves are used fresh or dried as flavoring in sauces, soups, meat and fish dishes, salad dressings, vegetables, confectionery products, and beverages (liquors). Traditionally, the plant has been employed in folk medicine for its stimulant, antispasmodic, and expectorant properties and also for gastrointestinal and respiratory disorders (2). The essential oil, mainly used in food, perfumery, and cosmetic industries, also presents antifungal (3, 4), antimicrobial (5–7), and antioxidant (7, 8) activities, and some of its components, such as thymol and carvacrol, are known to be biologically active (9, 10).

Studies on the chemical composition of essential oils of thyme from different geographic origins showed, besides thymol and *p*-cymene, γ -terpinene (11–13), β -caryophyllene (14), and carvacrol (15) as major compounds. Plants of *T. vulgaris* from northern California showed thymol, carvacrol, and linalol as major aroma constituents although small amounts of *p*-cymene and γ -terpinene were found (8).

Climatic conditions and genetically heterogeneous plants result in a high degree of variability in the levels of secondary metabolites. Additionally, food-processing operations may cause a loss of aroma that calls for subsequent supplementation and the growing market of flavor and bioactive compounds forces the search for alternative sources. Therefore, the use of biotechnological tools such as plant tissue culture for up-regulate metabolism pathways may create a source of homogeneous and well-defined product.

Plant tissue culture can be used to induce quantitative and qualitative modifications on the production of plant secondary metabolites (16) by changing nutrient and hormonal medium culture conditions (17). The influence of in vitro plant tissue culture and growth regulators on volatile profile and essential oil composition and yield has been studied by several authors, and a variety of effects on secondary metabolites production profiles when compared with donor plant have been reported (18–24).

Although in vitro cultures of *Thymus vulgaris* L. shoots have already been established (25, 26), to the best of our knowledge, there are no reports on the influence of phytohormones on the production of aromatic compounds by such systems.

The aim of this work was to investigate the influence of growth regulators on the production of volatile compounds by in vitro plantlets of *Thymus vulgaris* L., using solid phase microextraction (SPME) and gas chromatography/mass spectrometry as analytical tools.

*To whom correspondence should be addressed. Phone: +55 21 22445760. Fax: +55 21 28088193. E-mail: alicesato@unirio.br.

MATERIAL AND METHODS

Plant Material and Sterilization. *Thymus vulgaris* L. seeds from commercial market Isla batch no. 21094 were used as the source of plant material. Seeds were surface sterilized in 10% commercial bleach solution for 3 min, rinsed three times in distilled water, immersed in 70% aqueous ethanol, and then rinsed three times in sterile distilled water again. Seeds were inoculated on glass tubes (10 cm × 1.5 cm) containing 5 mL of Murashige and Skoog (27) basal medium (MS) supplemented with 3% sucrose and solidified with 0.8% agar, after pH adjustment at 5.8. Sterilization of culture media was performed in the autoclave at 120 °C and 1.1 kgf·cm⁻² for 15 min. Seeds were maintained in a growth room at 30 ± 2 °C under a 16/8 h light/dark photoperiod with light intensity of 23 μmol·m⁻²·s⁻¹ supplied by cool white fluorescent tubes.

Establishment of in Vitro Shoot Cultures. Thyme shoot cultures were induced from nodal segments (5–6 mm) obtained from aseptic 6-week-old seedlings on MS medium without growth regulators. Plantlets were subcultured in the MS hormone free media (MS0) until enough shoots were available to establish the experiments. After that, plantlets were subcultured to MS medium supplemented with indole-3-acetic acid (IAA) or benzyladenine (BA) or kinetin (KIN) or zeatin (ZEA) at 1.0, 5.0, and 10.0 μM. MS hormone free media served as control. Each culture flask containing 50 mL of different supplementation medium was inoculated with eight nodal segments. For each treatment four culture flasks were arranged in a completely randomized design. Cultures were maintained in a growth room under the same conditions mentioned above. Plantlet growth was determined by recording the mean of fresh and dry biomass weight. Dry weight was determined after plantlets were dried at 40 °C in an oven until constant weight was attained. Rooting percentage was obtained considering the number of rooted shoots in each treatment. After 60 days of in vitro culture, rooting and biomass data were collected and the plants were used for volatile analysis by solid phase microextraction.

SPME. The SPME apparatus was purchased from Supelco Inc. A 65 μm polydimethylsiloxane divinylbenzene (PDMS/DVB) fiber was used. For each extraction, 0.2 g of fresh leaves was placed in a 4 mL vial. In each extraction the sample was kept for 1 h at 60 °C. After this time, the SPME fiber was exposed to the headspace of the sample to adsorb volatiles for 15 min. The fiber was withdrawn into the needle and then introduced into a heated chromatographic injector for desorption for 3 min. Assays were carried out in triplicate.

GC-FID. The analyses were performed in an Agilent 6890N gas chromatograph equipped with HP-5 (5% phenylmethyl silicone) fused capillary column (30 m × 0.32 mm i.d., 0.25 μm film thickness) and a flame ionization detector (FID). The oven temperature was programmed from 40 to 180 °C at 3 °C·min⁻¹ with an initial isothermal period of 3 min. The injector was operated in splitless mode (3 min) at 250 °C and the detector at 280 °C. Hydrogen was used as carrier gas at a flow rate of 1.5 mL·min⁻¹. Quantification was performed from GC profiles using the normalized peaks area of each compound.

GC/MS. The GC/MS analyses were carried out in an Agilent 5973N mass selective detector coupled to an Agilent 6890 gas chromatograph, equipped with HP-5 MS (5% phenylmethyl silicone) fused capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness). The mass spectrometer was operated in electronic ionization mode at 70 eV. The oven and injector temperatures were the same as above. Injections were made in splitless mode, and helium was used as the carrier gas at a flow rate of 1.0 mL·min⁻¹. The components were identified by comparison of mass spectra with those in a Wiley sixth edition library, by comparison of retention indices calculated by linear interpolation to retention times of a series of *n*-alkanes (C₇–C₂₆) with those reported in the literature (28) and by injection of commercial standards of *p*-cymene, γ -terpinene, and thymol.

Statistics. Data obtained from fresh and dry weight and from volatiles compounds were subjected to one-way ANOVA, and mean values were compared by Dunnett's multiple comparison test or to the nonparametric Kruskal–Wallis test followed by Dunn's multiple comparison test as post-test using the software GraphPad InStat, version 3.01. Rooting percent data were submitted to significance test, difference between two percentages at 5% significance, using the software Statistica for Windows, version 5.0.

Table 1. Effect of the Different Media on Rooting and Biomass Growth of Shoot Cultures Using Nodal Shoot Segments of *Thymus vulgaris* L. as Explants after 60 Days of Culture

culture media	rooting (%)	biomass fresh wt per plant (mg) ^a	biomass dry wt per plant (mg) ^a
MS0 (control)	100	21.2 ± 1.7	2.6 ± 0.2
MS + 1.0 μM IAA	100	64.2 ± 3.7**	8.1 ± 0.5**
MS + 5.0 μM IAA	100	20.2 ± 1.9	3.1 ± 0.3
MS + 10.0 μM IAA	100	22.2 ± 1.6	1.0 ± 3.7
MS + 1.0 μM BA	96.8	31.1 ± 2.5	4.9 ± 0.5
MS + 5.0 μM BA	86.0	105.4 ± 24.7**	10.0 ± 2.0**
MS + 10.0 μM BA	2.7***	44.9 ± 7.6*	5.9 ± 0.8**
MS + 1.0 μM ZEA	90.6	45.8 ± 3.7*	6.4 ± 0.5**
MS + 5.0 μM ZEA	69.4**	43.4 ± 9.9	5.8 ± 1.0*
MS + 10.0 μM ZEA	0***	31.1 ± 4.0	4.9 ± 0.5
MS + 1.0 μM KIN	93.7	47.2 ± 3.8*	6.7 ± 0.5**
MS + 5.0 μM KIN	82.8	8.0 ± 0.7	1.3 ± 0.1
MS + 10.0 μM KIN	82.3	15.8 ± 1.7	2.3 ± 0.2

^a Each value represents the mean ± standard error of the data from the respective parameter. Asterisks indicate significant differences by comparison with control: (*) $p < 0.05$; (**) $p < 0.01$; (***) $p < 0.001$. All of the results are according to a Dunnett's multiple-comparison test: IAA, auxin indole-3-acetic acid; BA, benzyladenine; ZEA, zeatin; KIN, kinetin; wt, weight. $N = 32$ plantlets per treatment.

RESULTS AND DISCUSSION

In Vitro Shoot Cultures and Micropropagation. The seed pre-treatment resulted in very low levels of contamination with less than 5% of seeds becoming contaminated. After 7 days, the rate of seed germination was 64.6%.

Development of in vitro shoots occurred in all different growth regulators supplemented media tested, and the results for rooting and fresh and dry biomass of plantlets were compared with those from plantlets cultured on control medium (Table 1).

BA at 5 μM showed the highest fresh and dry mass production in *T. vulgaris* plants, followed by IAA (5 μM) supplemented medium, with high significant differences when compared to control plants (MS0). Because the results obtained for dry weight of plantlets cultured in BA and IAA at the concentration mentioned above show the same pattern obtained for fresh weight, we can conclude that thyme biomass growth was caused not only by water uptake. The increase in IAA, ZEA, and KIN from 1.0 to 10 μM tended to decrease the biomass growth, and no significant difference was detected in plantlets grown in BA (1 μM), ZEA (10 μM), IAA or KIN (5 and 10 μM), in relation to control plants (hormone-free).

In accordance with Fraternali et al., (20) in their studies of *Thymus mastichina*, maximum biomass growth in micropropagated plants was reached in BA supplemented media. On the other hand, Santos-Gomes and Fernandes-Ferreira (21) found for *Salvia officinalis* the highest dry biomass growth in plantlets cultured on media supplemented with 2,4D and kinetin.

With respect to rooting, except for plantlets cultured on BA and ZEA 10 μM, *T. vulgaris* shoots succeed to root in all treatments. Although rooting frequency was found to be optimal in the medium supplemented with IAA at all concentrations tested, MS0 medium also resulted in 100% of rooted plants. In agreement with our findings, IAA was already reported as a potential auxin for rooting of several species (22–24, 29). The increased concentration of the cytokinins (BA, ZEA, and KIN) led to a decrease in the frequency of root regeneration. No callus formation was observed in any media tested.

Volatile Compounds Produced by in Vitro Cultures. The major volatiles components in all treatments were γ -terpinene (22.8–38.8%), *p*-cymene (13.8–27.9%), and thymol (6.5–29.0%). These same major constituents, but with higher thymol content,

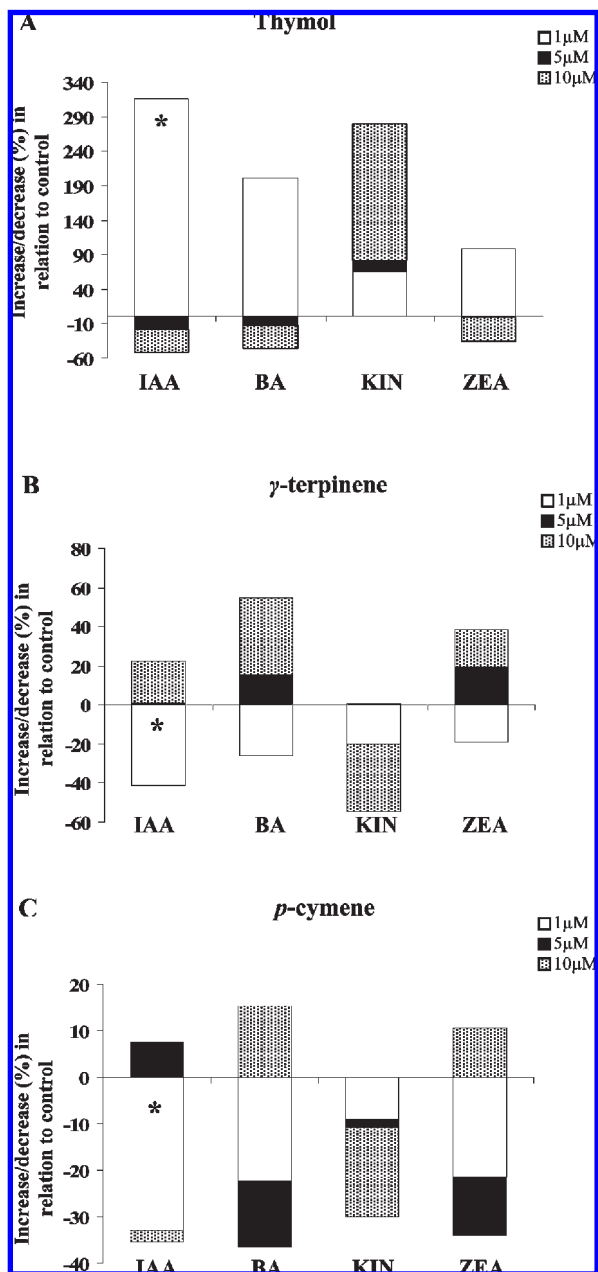


Figure 1. Percentage production in major volatile compounds of 60 days old in vitro plantlets of thyme when comparing plantlets in media supplemented with growth regulators to control plants (MS0): (A) thymol; (B) γ -terpinene; (C) *p*-cymene. Asterisks indicate significant differences by comparison with control: (*) $p < 0.05$; IAA, auxin indole-3-acetic acid; BA, benzyladenine; ZEA, zeatin; KIN, kinetin.

were also found in *T. vulgaris* oils obtained by hydrodistillation from two other Brazilian areas, namely, South and Southeast Brazil (12, 13).

By comparison of volatile compounds data from in vitro plantlets cultivated in MS0 medium (control) with those cultivated in media supplemented with different types of growth regulators, it can be observed that all growth regulators tested at 1.0 μM resulted in increased thymol levels, up to 315% (IAA) and 200% (BA) (Figure 1A).

It is interesting to note that the production of thymol by plantlets is negatively affected by higher concentrations of IAA, BA, and ZEA (5.0 and 10 μM). On the other hand, in these experiments γ -terpinene production was increased (Figure 1B).

It was also observed that the auxin IAA and all tested cytokinins (BA, KIN, and ZEA) at 1.0 μM caused a decrease in *p*-cymene production, while the highest concentration of BA and ZEA increased its proportion (Figure 1C).

Among the cytokinins tested, BAP and ZEA showed the same pattern of production of the three major compounds considered in this work. The cytokinin KIN demonstrated the ability to decrease production of *p*-cymene and γ -terpinene and positively influenced the content of thymol when compared to control (hormone-free medium).

Despite few significant differences among treatments and control, our study provided evidence that the type and concentration of growth regulators apparently influenced the accumulation of the volatile compounds. Also, a correlation between the production of γ -terpinene, *p*-cymene, and thymol when IAA, BAP, or ZEA is added into the culture medium was observed, since increased levels of thymol result in a decreased production of γ -terpinene and *p*-cymene and vice versa.

The data presented here are in agreement with results from a study of Poulou and Croteau (30), whose experimental evidence pointed out that thymol is biosynthesized through a pathway in which γ -terpinene is converted into *p*-cymene, which in turn yields thymol.

Plant tissue culture with phytohormones addition into the medium improved biomass production and induced quantitative modifications on the production of the major volatile compounds in *Thymus vulgaris* L. plants, although the same major compounds were produced in all treatments. In vitro plant should be more exploited as a means to obtain volatile compounds in vitro. Plantlets readily produce desirable volatile compounds, which may not be produced in cell suspension or callus cultures, since undifferentiated cultures of volatile-producing plants are unable to produce these metabolites and a partial differentiation in specialized cells is required often with production of lower amounts of these compounds (31, 32).

Supporting Information Available: Comparative gas chromatograms of volatile profile from 60 days old in vitro *Thymus vulgaris* L. using SPME. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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