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Cytokinins in the *in vitro* multiplication and analysis of the volatile fraction of *Hyptis marrubioides*[1]

Citocininas na multiplicação *in vitro* e análise da fração volátil de hortelã do campo

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ABSTRACT - The aim of this study was to evaluate different concentrations of two cytokinins in the *in vitro* propagation of the medicinal plant *Hyptis marrubioides* Epl. and to analyse the volatile fraction of the compounds. Nodal segments of plants were inoculated onto Murashige and Skoog (MS) solid medium with different concentrations of benzylaminopurine (BAP) or tidiazuron (TDZ) under a 16/8 h light/dark cycle at 25 ± 2 °C. After 45 days growth, the plants were evaluated for the number and length of the shoots and for shoot dry matter. The volatile constituents were analysed by headspace-GC/MS. The greatest number of shoots was obtained with 1.0 mg L^{-1} TDZ in the MS medium. The greatest values for shoot length and dry matter were obtained with BAP. Twenty-seven compounds were characterised as constituents of the essential oil of *H. marrubioides*. The major compounds of the volatile fraction were sabinene, α-thujone, β- thujone, α-copaene, β-caryophyllene, γ-gurjunene and γ-himachalene. The types and concentrations of the growth regulators influenced accumulation of the volatile fraction. Quantitative changes in the monoterpenes and sesquiterpenes in the volatile fraction of the plants were also seen in response to the type of growth regulator added to the culture medium.

Key words: Growth regulators. Proliferation. Terpenes. Medicinal plant. *Hyptis smarrubioides*.

RESUMO - objetivou-se com o presente trabalho avaliar diferentes concentrações de duas citocininas na propagação *in vitro* da planta medicinal *Hyptis marrubioides* Epl. e analisar a fração volátil dos compostos. Segmentos nodais de plantas foram inoculados em meio sólido de Murashige e Skoog (MS) com diferentes concentrações de benzilaminopurina (BAP) ou tidiazuron (TDZ) sob 16/8-h luz/escuro em 25±2 ºC. Aos 45 dias de cultivo, as plantas foram avaliadas quanto ao número e comprimento de brotações e matéria seca da parte aérea. As análises dos constituintes voláteis foram por *headspace*-CG/EM. O maior número de brotos foi obtido com 1,0 mg L^{-1} de TDZ no meio MS. Os maiores valores para comprimento e matéria seca foram obtidos com BAP. Vinte e sete compostos foram caracterizados como constituintes de *H. marrubioides* óleo. Os compostos majoritários da fração volátil foram sabineno, α-tujona, β-tujona, α-copaeno, β-cariofileno, γ-gurjuneno e γ-himachaleno. Os tipos e as concentrações dos reguladores de crescimento afetaram o acúmulo da fração volátil. Mudanças quantitativas na classe dos monoterpenos e sesquiterpenos na fração volátil das plantas, também foram observadas em resposta do efeito do tipo de regulador de crescimento suplementado ao meio de cultura.

Palavras-chave: Reguladores de crescimento. Proliferação. Terpenos. Planta medicinal. *Hypti smarrubioides*.

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INTRODUCTION

Hyptis marrubioides Epl. (Lamiaceae) occurs in the phytogeographical domains of the Cerrado and Atlantic Forest, and is found in the States of Minas Gerais and Goiás, Brazil (HARLEY *et al*., 2012). Monoterpenes, sesquiterpenes, flavonoids and phenolic compounds are reported as present in the species, and give it its medicinal properties (POVH; SANTOS; SILVA, 2012). Among the terpenes, the major compounds are α-and β-thujone, β-caryophyllene and γ-muurolene among others, whose content vary according to the culture environment (BOTREL *et al*., 2010).

H. marrubioides is propagated preferentially by seeds; however, for medicinal aromatic plants, sexual reproduction by allogamy results in plants of more-variable morphology and the production of secondary metabolites. In order to avoid this variability, micropropagation is used as an alternative form of propagation, giving homogeneous plants with the same phytochemical profile and of high phytosanitary quality (SHARAFZADEH; ZARE, 2011).

Plant tissue culture allows better control of environmental factors, since suitable conditions can be ensured, such as luminosity, concentration, type of culture medium and growth regulator, in order to obtain suitable conditions for growth and multiplication. Among the growth regulators are the cytokinins, compounds which favour multiplication, promoting an increase in the number shoots per explant through the fall of apical dominance and the proliferation of meristematic zones (KYOZUKA, 2007). Among the cytokinins commonly used in tissue culture are 6-benzylaminopurine (BAP) and thidiazuron (TDZ), both efficient in the multiplication of various species. In *Passiflora foetida* L., Shekhawat *et al.* (2015) reported a greater number of shoots with 2 mg L^{-1}; in *Baco pamonnieri*, a concentration of 0.10 mg L^{-1} was the most suitable for the multiplication of the species (SHARMA *et al*., 2010).

TDZ proved to be efficient in the multiplication of *A. millefolium*. Alvarenga *et al*. (2015) found that a concentration of 0.75 mg L^{-1} promoted the best combination of shoot number, shoot length and shoot dry matter. Studies are therefore necessary to determine the best regulator and its concentration, considering that responses are specific.

Biotechnology techniques have proved to be an alternative source for the production of secondary metabolites (MURTHY *et al*., 2014; SHARAFZADEH *et al*., 2011). The success of micropropagation and the synthesis of secondary metabolites in tissue culture comprise one set of factors. Some factors have already been reported, such as the type of medium, the concentration of salts and the amount of growth regulator (MURTHY; EUN-JUNG LEE; PAEK, 2014). The growth regulator in the culture medium can alter the amount and composition of the essential oil *in vitro* (MONFORT *et al*., 2012; SIDDIQUE; ANIS, 2008). However, there has been no study on the effect of the growth regulator by an analysis of the *in vitro* volatile fraction of the genus of this species.

Considering the above, the aim of this study was to evaluate the effect of different concentrations of BAP and TDZ on the multiplication of *Hyptis marrubioides* Epl., and through analysis, on the volatile constituents of the leaves of plants grown *in vitro*.

MATERIAL AND METHODS

Multiplication

To set up the experiment, nodal segments of plants already established *in vitro* and grown in MS medium for 45 days were used. The leaves were removed, and explants with one pair of buds were inoculated horizontally into flasks (250 mL) containing 35 mL of MS medium (MURASHIGE; SKOOG, 1962) at different concentrations (0.0, 0.25, 0.5, 0.75 and 1.0 mg L^{-1}) of benzylaminopurine (BAP) and tidiazuron (TDZ) supplemented with 30 g L^{-1} sucrose and 6 g L^{-1} agar, and the pH adjusted to 5.7 ± 0.1 before autoclaving. The experiment was arranged in a completely randomised design, in a 2 x 5 factorial scheme of two growth regulators (BAP and TDZ) and five concentrations to give ten treatments, with four replications of four flasks and two nodal segments per flask. After 45 days, the number and length of the shoots (cm) and the shoot dry matter (g) (SDM) per flask were evaluated. The data were submitted to analysis of variance, and when significant by F-test the polynomial regression curves were prepared using the SISVAR 5.0 software (FERREIRA, 2007).

Analysis of the volatile constituents

Chemical analysis of the volatile fraction was carried out using the Combi PAL Autosampler System automated headspace extractor (CTC Analytic AG, Switzerland) coupled to a GC/MS system. After procedures to optimise the operating conditions, the following parameters were established: an incubation temperature of 100 °C for 30 min for the samples, with a syringe temperature of 110 °C. The injection volume was 500 µL in vapour phase, split-injected at a ratio of 50 to 1. The samples (n = 3) consisted of 100 mg of leaves, oven-dried at 40 ºC and packed in 20 mL phials sealed with a silicone/PFTE septum.

The analysis was carried out using an Agilent® 7890A gas chromatography system coupled to an Agilent® MSD 5975C mass selective detector (Agilent Technologies, California, USA), operated by 70 eV electron impact ionisation in sweep mode at a speed of 1.0 scan/s, with a mass acquisition range of 40-400 m/z. An HP-5MS fused-silica capillary column (30 m length x 0.25 mm internal diameter x 0.25 μm film thickness) (California, USA) was used. Helium at a flow rate of 1.0 mL/min was used as the carrier gas; the temperature of the injector and the MS transfer line was maintained at 230 °C. The initial oven temperature was 60 °C, and then ramped at a rate of 3 °C/min up to 150 °C, followed by a ramp rate of 10 °C/min to 230 °C. The concentrations of the volatile fraction constituents were expressed as the relative percentage area of each chromatographic peak.

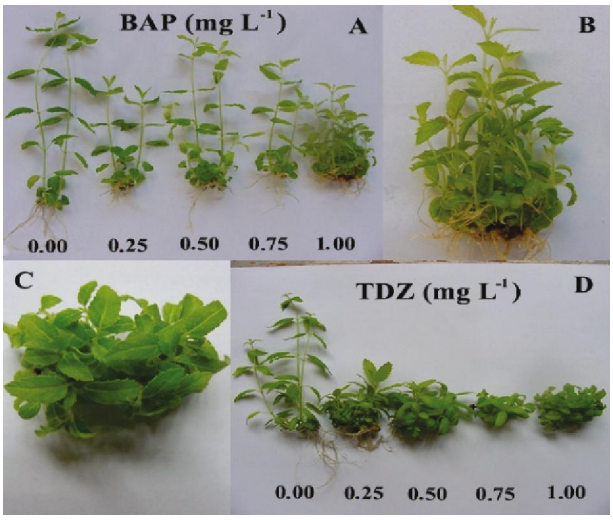
The constituents were identified by comparing their relative retention indices with an n-alkane standard solution C_8-C_{18} (Sigma-Aldrich®, St. Louis, USA), and by comparison with mass spectra from the NIST library database (NIST SPEECH GROUP WEBSITE, 2008) and from the literature (ADAMS, 2007). The retention indices were calculated using the equation proposed by Van den Dool and Kratz (1963), retention indices from the literature being consulted for the attributions (ADAMS, 2007). Identified constituents are presented as a table, including their respective calculated indices and relative areas.

RESULTS AND DISCUSSION

Multiplication

For all treatments, the cultivated plants presented normal development, showing no signs of hyperhydricity or leaf anomalies, and with a similar appearance to plants grown *ex vitro*. It was found that the formation and growth of adventitious roots occurred in the medium supplemented with BAP; however, in the medium with TDZ, root formation only occurred in the absence of TDZ and at the lowest dose (Figure 1). Similar results showed that the effects of TDZ are not significant for rooting (BASKARAN; NCUBE; VAN STADEN, 2012; MAGYAR-TA'BORI; DOBRA'NSZKI; SILVA, 2010).

Figure 1 - General appearance of *Hyptis marrubioides* grown under different concentrations of BAP and TDZ at 45 days. **A** - Explants grown under different concentrations of BAP, **B** - Detail of sprouting in BAP, **C** - Detail of sprouting in TDZ, **D** - Explants grown under different concentrations of TDZ



It was found that TDZ was more efficient than BAP for number of shoots; however, the greatest shoot lengths and dry-matter volume were seen with the presence of BAP (Table 1). Such negative effects of TDZ on some variables may be due to the stimulating effect of the regulator on the production of endogenous ethylene (SUTTLE, 1985).

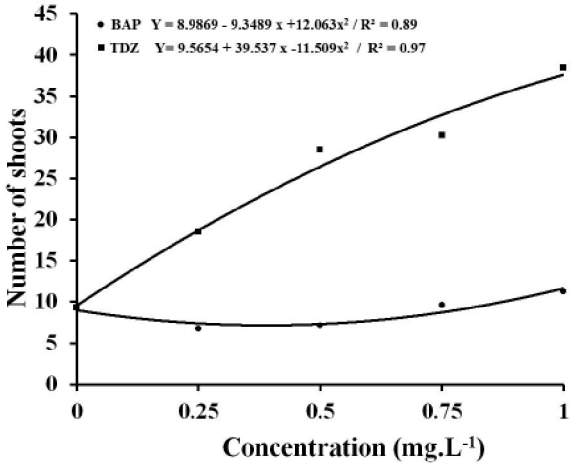
Table 1 - Comparison of the type of regulator (BAP and TDZ) on mean number of shoots (NS), mean shoot length (SL) and mean shoot dry matter (SDM) per flask of *Hyptis marrubioides* at 45 days growth

Concentration (mg L^{-1})	NS		SL (cm)		SDM	
	BAP	TDZ	BAP	TDZ	BAP	TDZ
0.00	9.34	9.34	5.77	5.77	0.36	0.36
0.25	6.75*	18.50	5.48*	1.70	0.41*	0.21
0.50	7.13*	28.50	5.97*	1.52	0.42*	0.19
0.75	9.63*	30.25	4.38*	1.34	0.37*	0.21
1.00	11.25*	38.50	2.71*	1.15	0.26^{ns}	0.23^{ns}

^{ns} Not significant; * significant by F-test at 5% probability, comparison on the rows within each parameter

For the mean number of shoots, the doses of TDZ showed values three times greater than those of BAP (Figure 2); an increasing response to the application of the TDZ regulator can be seen. The frequency and *in vitro* response of shoot regeneration is influenced by the concentration of the growth regulator (MUKHTAR *et al*., 2012).

Figure 2 - Mean number of shoots in *Hyptis marrubioides* grown under different concentrations of BAP (•) and TDZ (▪)



The high activity of TDZ is related to its stability in tissue culture. The substance is consequently not degraded by cytokinin oxidase; furthermore, diphenylurea strongly inhibits the oxidase (GALUSZKA *et al*., 2000). In addition, the difference in response between regulators can be attributed to the structures or element present in each one. BAP, a derivative of adenine, is composed of N, H and C, and acts in the proliferation and elongation of the shoots (GEORGE; HALL; DE KLERK, 2008). TDZ is a diphenylurea, containing S in its structure, and is more efficient in inducing adventitious shoots (GEORGE; HALL; DE KLERK, 2008). Regulators such as the cytokinins play several roles in plant development during meristemic formation and activity (KYOZUKA, 2007).

There was a decreasing response in shoot length to the increases in BAP (Figure 3); however, higher values were found than for the shoots under the TDZ treatment. The results of this variable are the opposite of those seen in the multiplication of other medicinal plants, such as *Varronia curassavica* (SANTOS *et al*., 2013), which resulted in greater shoot length, being more effective than the BAP. This probably indicates that each species has a specific response to each regulator.

The highest values for the accumulation of shoot dry matter were seen with 0.5 mg L^{-1} BAP (Figure 4); less SDM was accumulated in the medium supplemented with TDZ. Alvarenga *et al*. (2015) reported that an increase in TDZ concentration promoted a lower accumulation of SDM in *Achillea millefolium* L.

Figure 3 - Shoot length in *Hyptis marrubioides* grown under different concentrations of BAP (•) and TDZ (▪)

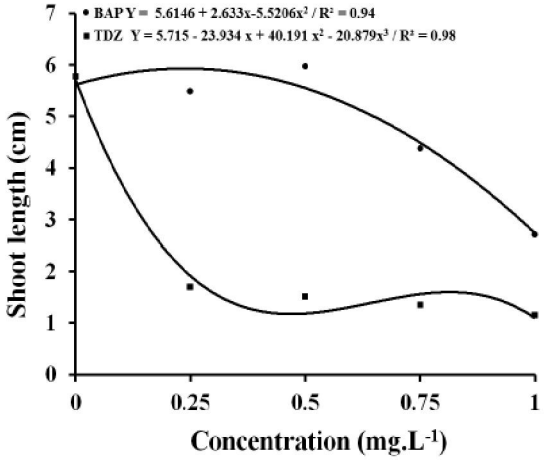
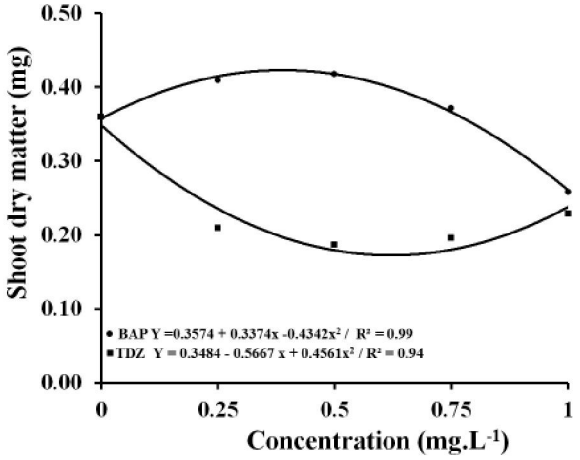


Figure 4 - Shoot dry matter in multiple shoots of *Hyptis marrubioides* grown under different concentrations of BAP (•) and TDZ (▪)



This response may be associated with the source to drain ratios, and regulated by the meristematic activity of the shoots. As *in vitro* resources are limited, i.e. nutrients and gas exchange, the produced assimilates are distributed between multiple shoots, reducing the accumulation of dry matter through sprouting. It therefore follows that the presence of cytokinins in the culture medium promotes positive responses when the

aim is to multiply the species *in vitro*, however studies by Botrel *et al*. (2015), evaluating the micropropagation of the same species, found high rates of multiplication with no growth regulators in the medium. This ability to multiply without the need for regulators was also seen in *Plumbago indica* Linn., where the greatest number of shoots and greatest shoot lengths were seen in the treatment without the regulator (CHAROENSUB; PHANSIRI, 2004).

Constituents of the volatile fraction

The results of the chromatographic analysis showed a quantitative influence when the culture medium was supplemented with BAP or TDZ in the *in vitro* culture of *H. marrubioides*. A total of 27 constituents were identified, accounting for over 97% of the total chemical composition of the volatile fraction (Tables 2 and 3).

Table 2 - Volatile-fraction constituents of *Hyptis marrubioides* Epl. grown for 45 days under different concentrations of BAP

Constituent	RI[1]	BAP concentration (mg L^{-1})				
		0.00	0.25	0.50	0.75	1.00
α-Pinene	933	0.16	0.19	0.17	0.14	0.16
Sabinene	972	4.00	4.59	4.15	3.01	4.06
β-Pinene	976	0.63	0.73	0.66	0.54	0.63
β-Phellandrene	1028	0.21	0.20	0.20	0.14	0.19
Eucalyptol	1031	1.11	0.90	0.88	0.79	0.87
γ-Terpinene	1099	0.14	0.17	0.15	0.12	0.13
3-Carene	1106	0.20	0.22	0.15	0.20	0.22
α-Thujone	1116	15.52	15.86	15.89	14.11	15.09
β-Thujone	1140	33.28	32.51	32.96	30.01	32.72
trans-Thujanol	1149	0.65	0.68	0.65	0.62	0.65
cis-Pinocamphone	1173	2.99	2.90	2.87	2.81	2.89
α-Terpineol	1190	0.32	0.34	0.34	0.32	0.34
α-Cubebene	1349	1.05	0.98	1.04	1.16	1.06
Ylangene	1370	0.51	0.54	0.56	0.64	0.58
α-Copaene	1375	15.55	13.10	14.16	15.86	14.65
β- Cubebene	1389	0.59	0.46	0.51	0.61	0.54
β-Elemene	1391	0.27	0.26	0.26	0.35	0.27
β-Parnasisene	1400	0.30	0.33	0.33	0.38	0.35
β-Caryophyllene	1418	7.71	8.71	8.33	9.17	8.23
α-Caryophyllene	1452	0.58	0.59	0.60	0.70	0.62
γ-Muuorolene	1476	0.12	0.11	0.12	0.15	0.13
γ-Gurjunene	1480	5.61	5.88	5.80	7.45	5.92
γ-Himachalene	1482	5.92	6.71	6.77	7.83	7.02
β-Guaiene	1493	0.11	0.10	0.13	0.13	0.12
γ-Cadinene	1514	0.21	0.20	0.21	0.25	0.22
δ-Cadinene	1523	0.63	0.57	0.61	0.75	0.65
Caryophyllene oxide	1581	0.18	0.19	0.19	0.23	0.19
Monoterpenes (%)		60.32	59.64	59.05	52.81	57.97
Sesquiterpenes (%)		38.23	38.38	39.64	45.66	40.53
No of constituents		27	27	27	27	27
Total identified (%)		98.55	98.02	98.69	98.47	98.50

[1]Retention index for the n-alkane C_8-C_{18} series in an HP-5MS column by order of elution

Table 3 - Volatile constituents of *Hyptis marrubioides Epl*. grown for 45 days under different concentrations of TDZ

Constituent	RI[1]	TDZ concentration (mg L^{-1})				
		0.00	0.25	0.50	0.75	1.00
α-Pinene	933	0.16	0.17	0.20	0.20	0.12
Sabinene	972	4.00	4.41	4.93	5.92	2.95
β-Pinene	976	0.63	0.67	0.78	0.81	0.48
β-Phellandrene	1028	0.21	0.19	0.20	0.26	0.15
Eucalyptol	1031	1.11	1.35	1.36	1.42	1.00
γ-Terpinene	1099	0.14	0.13	0.15	0.15	0.12
3-Carene	1106	0.20	0.15	0.17	0.17	0.12
α-Thujone	1116	15.52	13.01	13.50	13.07	11.49
β-Thujone	1140	33.28	28.73	28.53	29.51	26.14
trans-Thujanol	1149	0.65	0.60	0.66	0.54	0.50
cis-Pinocamphone	1173	2.99	2.67	2.77	2.71	2.42
α-Terpineol	1190	0.32	0.29	0.31	0.29	0.27
α-Cubebene	1349	1.05	1.07	1.07	0.90	1.14
Ylangene	1370	0.51	0.45	0.48	0.37	0.53
α-Copaene	1375	15.55	17.81	17.00	14.79	18.38
β- Cubebene	1389	0.59	0.63	0.61	0.56	0.69
β-Elemene	1391	0.27	0.35	0.34	0.69	0.46
β-Parnasisene	1400	0.30	0.27	0.29	0.23	0.33
β-Caryophyllene	1418	7.71	8.87	8.38	8.85	9.38
α-Caryophyllene	1452	0.58	0.73	0.74	0.74	0.89
γ-Muuorolene	1476	0.12	0.15	0.16	0.16	0.20
γ-Gurjunene	1480	5.61	8.29	7.83	9.69	11.19
γ-Himachalene	1482	5.92	5.87	6.31	4.97	7.48
β-Guaiene	1493	0.11	0.15	0.16	0.15	0.19
γ-Cadinene	1514	0.21	0.28	0.29	0.24	0.34
δ-Cadinene	1523	0.63	0.87	0.87	0.83	1.13
Caryophyllene oxide	1581	0.18	0.19	0.28	0.17	0.21
Monoterpenes (%)		60.32	52.43	53.57	55.04	45.12
Sesquiterpenes (%)		38.23	45.92	44.80	43.35	52.18
No of constituents		27	27	27	27	27
Total identified (%)		98.55	98.35	98.37	98.39	97.30

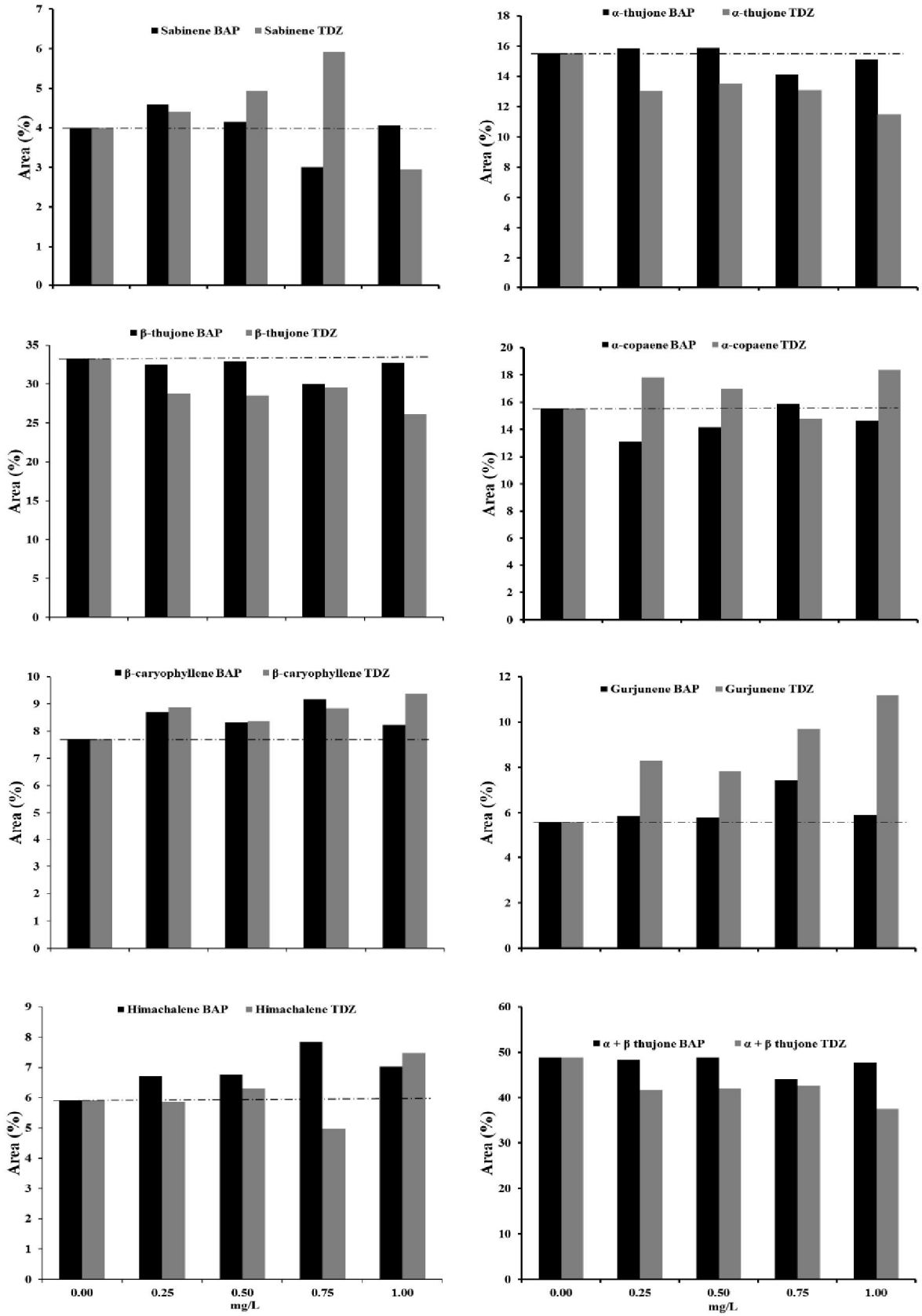
[1]Retention index for the n-alkane C_8-C_{18} series in an HP-5MS column by order of elution

Seven major constituents were identified in plants of *H. marrubioides* grown in a medium supplemented with BAP and TDZ (sabinene, α-thujone, β- thujone, α-copaene, β-caryophyllene, γ-gurjunene and γ-himachalene), accounting for more than 86% of the volatile fraction. Botrel *et al*. (2015) reported for the *in vitro* culture, five major compounds totalling 80.21% (thuja-2,4-(10)-diene, terpinolene, α-thujone, β-bourbonene and β-caryophyllene); small amounts of the constituents β-thujone, α-copaene and γ-himachalene were also detected.

The major compounds (α-thujone, β-thujone, α-copaene and β-caryophyllene) and their respective levels were very close to those found by Botrel *et al*. (2010) when working with the same species grown in the field.

In this study, the production of α-copaene was approximately 2.5 times greater than levels found by

Figure 5 - Major constituents in leaves of *Hyptis marrubioides* grown under different concentrations of BAP and TDZ



Botrel *et al*. (2010) in plants grown in the field (5.27%) and greenhouse (5.83%), showing how this compound favours production in *in vitro* culture. The difference in major compounds may be due to the growing conditions of each plant: light intensity, culture medium, type of analysis (headspace volatile fraction or essential oil) and other factors.

By analysing the seven major compounds, the effect of the BAP and TDZ growth regulators on the content can be seen (Figure 5). The compounds that showed a decrease in the presence of both regulators were α and β-thujone, where there was a linear drop as the doses increased; when the medium was supplemented with TDZ, the drop was more pronounced (Figure 5). In the culture medium supplemented with TDZ there was an increase in the compounds sabinene, α-copaene, β-caryophyllene, γ-gurjunene and γ-himachalene. However, in the medium supplemented with BAP, there was a greater increase in β-caryophyllene and γ-himachalene, and a decrease in the α-copaene constituent. This increase or decrease in compounds can be explained by the fact that the growth regulators are related to plant development and growth *in vitro*. These compounds are extremely active at low concentrations in the culture medium. The class of cytokinins (BAP and TDZ) is very important for regulating growth and morphogenesis in tissue culture.

Variations in constituent levels in the culture medium supplemented with growth regulator may therefore have occurred due to the activation or inhibition of various enzymes during the different steps for synthesising the essential oils and secondary metabolites. This difference in the levels of the compounds and metabolites is demonstrated in several studies, (GONÇALVES; ROMANO, 2013; SHARAFZADEH; ZARE, 2011).

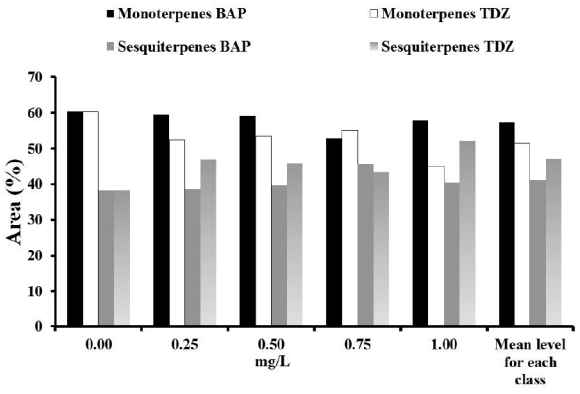
The results of this study corroborate those reported by Affonso *et al*. (2007), who found a significant increase in the trans-caryophyllene and γ-gurjunene content when TDZ was added to the MS medium in the *in vitro* culture of *Lantana ca*mara. Alvarenga *et al.* (2015) also saw significant changes in the volatile constituents of *A. millefolium*, with an increase in the levels of borneol and β-caryophyllene. Prins *et al*. (2013), comparing different *in vivo* culture environments and the application of the BAP regulator in *Cymbopogon citratus*, found a negative correlation between the concentration and the citral content of the essential oil, with a reduction of approximately 50% in the levels of the constituent.

The results indicate that the application of growth regulators can affect the production and composition of the essential oil, which corroborates research reported by other authors (HAZZOUMI *et al*., 2014; NOURAFCAN *et al*., 2014).

When comparing the mean levels of the monoterpene and sesquiterpene volatile-constituent classes, it was found that with BAP there was a predominance of monoterpenes in relation to the medium supplemented with TDZ. However, when the medium was supplemented with TDZ, there was a predominance of sesquiterpenes (Figure 6).

The application of cytokinins promotes changes in the activity, expression and action of terpene synthase enzymes, which alters the volatile chemical composition, mainly with an increase in sesquiterpene constituents (EL KELTAWI; CROTEAU, 1987). It therefore follows that both BAP and TDZ promote important alterations in the volatile constituents of *H. marrubioides*. For that reason, studies that evaluate the influence of growth regulators on the production of constituents are important, since the changes that occur can alter the biological activities of the species.

Figure 6 - Cumulative total monoterpene and sesquiterpene content in leaves of *Hyptis marrubioides* grown under different concentrations of BAP and TDZ



CONCLUSIONS

1. The species responds positively to the presence of cytokinins in the number of shoots; TDZ was more efficient than BAP, however the greatest shoot lengths and volume of dry matter were found in the presence of BAP;
2. The seven major compounds suffered an increase or decrease depending on the medium being supplemented with BAP or TDZ;

3. The medium supplemented with BAP maintained a predominance of monoterpenes, the medium with TDZ had a higher sesquiterpene content.

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