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Abiotic factors influencing podophyllotoxin and yatein overproduction in *Leptohyptis macrostachys* cultivated *in vitro*

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ABSTRACT

This work describes an *in vitro* propagation protocol for the large-scale cultivation of *Leptohyptis macrostachys* (Benth.) Harley & JFB Pastore and the influence of abiotic factors on podophyllotoxin and yatein production. The plant was established from seeds collected in Chapada Diamantina, BA, Brazil and submitted to different growth mediums and physical conditions. The podophyllotoxin and yatein contents were quantified by HPLC/DAD and with pure standards and these two lignans were present in all experiments. The lignan quantities were evaluated using the Sisvar Program, compared by Tukey's test and hierarchical cluster analysis and principal component analysis. In all experiments, podophyllotoxin and yatein were detected at different concentrations. The best protocol cultivar of *L. macrostachys* was established from seeds in MS ½ medium supplemented with 1.5% sucrose and 11.55 μM of gibberellic acid A3 (GA_3) at 30 °C, which yielded the highest concentration of podophyllotoxin (5.831 mg g^{-1}). These results are important findings for the production of podophyllotoxin from the tissue culture.

1. Introduction

Podophyllotoxin (Fig. 1) is one the most active cytotoxic natural products and is used as a starting compound for the synthesis of the anticancer drugs etoposide and teniposide (Brandão et al., 2010). Currently, the commercial sources of podophyllotoxin are the rhizomes and roots of *Podophyllum emodi* Wall., Berberidaceae. The search for new and renewable sources of podophyllotoxin is important because *Podophyllum* spp is endangered due to the exhaustive extraction of this compound (Medrado et al., 2015). The presence of aryltetralin lignans, including podophyllotoxin, as minor compounds in *Eriope* and *Hyptis* species (Fig. 1) has previously been reported (Novelo et al., 1993; Santos et al., 2011). Lamiaceae is comprised of 220 genera with approximately 4000 species occurring all over the world. *Leptohyptis macrostachys* (Benth.) Harley & JFB Pastore (Harley and Pastore, 2012) is a recurrent Lamiaceae species previously classified in the genus *Hyptis*. This species occurs in the Central and South America. In Brazil, it habitats the northeastern semi-arid region and is a species found in the semi-arid region of Brazil and it is popularly known as "alfavaca-brava" and "hortelã-do-mato". In folk medicine, tea and syrup of its aerial parts are used orally to relief symptoms in asthma, cough and bronchitis used by the locals (Agra et al., 2008). Other closer species of this genus, such as *H. brevipes*, occurs all over the word and it also is used in folk medicine for the treatment of asthma, besides malaria, cancer and pest-repelling (Suárez-Ortiz et al., 2017). Previously chemical studies concerning *Hyptis* spp. describe the presence of aryltetralins lignans in different plant parts (Raffauf et al., 1987) and a set 6-heptyl-5,6-dihydro-2H-2-one derivatives (Suárez-Ortiz et al., 2013).

An understanding of the biotechnological processes of cell, tissue and organ culture or intact plant culture for the production of bioactive compounds are very important to overcome problems from cultivation, such as seasonality, environmental variation, geographical location, pest attack and political instability (Hussain et al., 2012). Tissue culture can be used to induce qualitative and quantitative changes in the production of metabolites by altering nutrients and/or regulators in the conditions of *in vitro* growth. Thus, the use of biotechnological tools, such as tissue culture, can facilitate the homogenous and well-defined production of secondary metabolites from plant sources (Affonso et al.,

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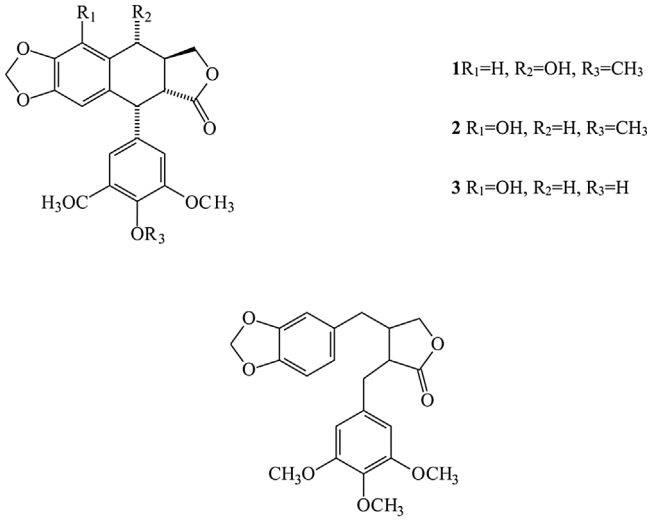

Fig. 1. Aryltetralins lignans present in Lamiaceae species.

2009). The yield of such production has been assessed by analytical methods such as HPLC/DAD, which is a broadly used technique to estimate the concentration of *in vitro* and *in vivo* bioactive compounds (Vijayan et al., 2015).

This work describes the influence of abiotic factors in the development of the *in vitro* culture of *L. macrostachys* and on the production and concentration of podophyllotoxin and yatein (**4**, Fig. 1), its direct biosynthetic precursor.

2. Materials and methods

2.1. Plant material

Leptohyptis macrostachys seeds were collected from the surroundings of Mucugê, a city above sea level over 1003 m in the region of Chapada Diamantina (13°0′22“S 11°22′34“W), Bahia, Brazil. A voucher (#127644) is deposited in the Herbarium of Universidade Federal da Bahia. The seeds were subjected to surface disinfestation by the immersion in 70% alcohol (1 min), followed by sodium hypochlorite (1.0% active chlorine) with one drop of neutral detergent (10 min), and finished with three washes with sterile distilled water to remove any trace of chlorine.

2.2. In vitro germination experiments of Leptohyptis macrostachys

Seeds were inoculated in glass tubes (20 cm × 1.5 cm) containing approximately 10 mL of MS culture medium according to Murashige and Skoog (1962), with variations in the concentration of basal salts and various supplementations. The culture media used had the following salt concentrations: 1.) MS with the total concentration of salts (complete MS); 2.) MS with the salt concentration reduced by half (½ MS); and 3.) MS medium with no added salt (agar). All media containing sucrose (3%) and solidified with agar (0.7%). Seeds were sown in MS medium, ½ MS medium with no supplementation, and ½ MS medium containing either 1.5% sucrose or 3% sucrose, all solidified with agar (0.7%). To evaluate the effect of gibberellic acid (GA3), the seeds were inoculated in ½ MS medium solidified with 0.7% agar, supplemented with 3% sucrose and either 2.88 µM, 5.77 µM or 11.55 µM gibberellic acid. The pH of the culture medium was adjusted to 5.7 ± 0.1 before autoclaving (for 15 min at 121 °C and 15 psi) all media. The cultures were maintained at 25 °C for 30 days in a growth chamber with a relative humidity of 60% and a photosynthetic radiation activity of 30 µmol m^2 s^{-1}.

2.3. In vitro multiplication experiments with L. macrostachys

The plants germinated *in vitro* by the different methods were used at 30 days of age as the source of explants. Explants (node region) of approximately 0.5 cm in length were individually inserted into test tubes with approximately 10 mL of MS or ½ MS medium, both supplemented with 3% (w/v) or 1.5% (w/v) sucrose and solidified with agar (0.7%). Cultures were maintained in a proper growth chamber at 25 °C for 30 days with 60% of relative humidity and photosynthetic active radiation of 30 µmol m^2 s^{-1}. In addition, the production of podophyllotoxin and yatein contents were carried out in all experiments.

2.4. Effect evaluation of 6-benzylaminopurine (BAP) and naphthalene acetic acid (NAA)

The explants were inserted on ½ MS medium containing 3% sucrose and BAP (0, 4.44, or 8.88 µM) combined with NAA (0, 5.37, or 10.74 µM) and solidified with agar (0.7%). The cultures were maintained at 25 ± 3 °C for 30 days in a growth chamber with a relative humidity of 60% and photosynthetic active radiation of 30 µmol $m^2 s^-$µmol m^2 s^{-1}.

2.5. Effect of temperature, tube closing evaluation and photoperiod

The explants were inoculated on MS culture medium with half the total concentration of salt, containing 3% sucrose and solidified with agar (0.7%). The cultures were maintained at 25 °C for 30 days in a growth chamber with a relative humidity of 60% and photosynthetic active radiation of 30 µmol m^2 s^{-1} (control) or at 30 °C for 30 days in a growth chamber with a relative humidity of 60% and photosynthetic active radiation of 30 µmol $m^2 s^-$µmol m^2 s^{-1}. Two types of test tube closures were studied: with PVC (not allowing gas exchange) and with a plastic cover (allowing gas exchange). Cultures were maintained at 25 °C for 30 days in the same growth chamber conditions. It was also evaluated the photoperiod of 16 h of light for the control group and 8 h of light for the test group in the inoculated explants.

2.6. HPLC analysis of lignans

The chromatographic analyses were carried out using a Shimadzu® (Corp., Kyoto, Japan) HPLC equipment model 2010 with an autoinjector (SIL-10AD model), solvent pump (SCL-10AVP model) and UV–vis detector (SPD-M10A,). The chromatographic separation was performed employing a LiChroCART Purospher Star® RP18-e column (50 mm × 4 mm i.d.) (3 µm) combined with a pre-column (3 µm) (LiChroCART 4-4 LiChrospher 100 RP18, Merck Darmastad, Germany). Podophyllotoxin standard were purchased (Sigma-Aldrich, USA) and yatein was previously isolated from *Eriope blanchetii*. The yatein purity was determined by HPLC and NMR (Santos et al., 2011).

The plants grown *in vitro* were dried in a hot air circulation oven at 40 °C for 48 h. The extraction was briefly performed by adding 15 mL of ethanol to every 2 g of dried plant material, which was then mixed and maintained at 40 °C for 2 h, under agitation. The ethanolic extracts were filtered, and the solutions were completely dried in a fume hood with a heat pistol. The dried extract was then resuspended and partitioned with water and ethyl acetate (1:1). The resulting ethyl acetate phase was divided in half, and the solvent was evaporated. The semi-purified extract was then dissolved in 10 mL of aqueous solution of acetonitrile (8:2H_2O:ACN) and pre-concentrated using a Sep-Pak C18 cartridge. The compounds absorbed in the cartridge were eluted with 10 mL of acetonitrile in a measured vial and taken to dryness. The material was then redissolved in methanol, filtered through a 0.22-µm membrane (Supelco, Pennsylvania, USA) and injected onto the HPLC. All procedures were performed in triplicate. Methanol, water,

acetonitrile and acetic acid (all HPLC grade) were employed for the HPLC mobile phase, and two systems were used in the gradient elution: solvent A (MeOH:H_2O:AcOH 14:85:1) and solvent B (ACN). Initially, a mixture of A and B (8:2) was eluted for 8 min, progressively decreasing to 7.8:2.2 and 7:3 at 12 min and 5.5:4.5 by 18 min. The full analysis lasted 22 min with a flow rate of 0.8 mL min^{-1} and an injection volume of 2 µL at ambient temperature. The read range of the array detector diode was established in 210 to 400 nm, and the chromatographic acquisition was set at 290 nm. The identification of lignans was obtained by a comparison of retention times of standards and samples, as well as the obtained mass and ultraviolet spectra.

2.7. Statistical and multivariate data analyzes

To analyze the data obtained in the germination experiments and the *in vitro* multiplication of *Leptohyptis macrostachys*, the SISVAR program (Ferreira, 2011) was used and tested at 1% probability. The means were compared by Tukey's test.

The determinated content of lignans (mg g^{-1} dried material) 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 different culture conditions (Table 1). The software Origin® 2017 was used for the PCA and HCA calculations.

3. Results and discussion

The results were analyzed based on the parameters employed in the establishment of *L. macrostachys in vitro* cultivation. From the HPLC/DAD qualitative and quantitative informations about podophyllotoxin and yatein were possible to evaluated all results employing PCA and HCA chemometrics analysis. Thus, the best parameters employed to establish a method of *in vitro* germination for *L. macrostachys* were observed with seeds germinated on MS medium with half the concentration of salt (½ MS) supplemented with 1.5% sucrose, 11.55 µmol L^{-1} GA_3 solidified with 0.7% agar and maintained in a growth chamber with a photoperiod of 16 h at 25 °C. The complete regeneration of the plant was best achieved in two subcultures: the first favoring shoot growth and the second strengthening roots.

The identification and quantification of podophyllotoxin and yatein employing HPLC/DAD/MS allowed us to obtain chromatograms and UV and MS spectra, which indicated the presence of both lignans in all cultivation conditions. The chromatographic method quickly and adequately showed retention times recorded for podophyllotoxin between 12.2 to 12.5 min and 14.2 to 15.4 min for yatein. A comparison with external standards and the calibration curves allowed us to obtain the concentration of these two compounds in the produced matrixes. All analyses were carried out in triplicate, and the results are expressed in Table 1.

Table 1
Quantification (mg g^{-1} of dry plant) of the production of podophyllotoxin and yatein in different culture conditions.

Culture	podophyllotoxin	yatein
Control *in vitro*	0.4015 ± 0.0053	7.1839 ± 0.0548
0 µM BAP + 5.37 µM ANA	0.2926 ± 0.0120	1.8553 ± 0.0551
0 µM BAP + 10.74 µM ANA	0.4670 ± 0.0089	1.0705 ± 0.0094
4.44 µM BAP + 0 µM ANA	0.8431 ± 0.0104	6.6848 ± 0.0828
4.44 µM BAP + 5.37 µM ANA	2.243 ± 0.0112	4.2934 ± 0.0021
4.44 µM BAP + 10.74 µM ANA	1.1091 ± 0.0016	2.0343 ± 0.2745
8.88 µM BAP + 0 µM ANA	0.6678 ± 0.0081	4.1824 ± 0.0485
8.88 µM BAP + 5.37 µM ANA	4.0871 ± 0.0509	6.4908 ± 3.7483
8.88 µM BAP + 10.74 µM ANA	1.0787 ± 0.0065	3.5098 ± 0.2022
photoperiod	0.0944 ± 0.0021	15.0663 ± 0.3902
Temperature	0.8372 ± 0.1368	24.2097 ± 0.0994
Gas changes	0.1777 ± 0.0242	0.6151 ± 0.0321
½ MS, 15 g sucrose	5.8309 ± 0.1255	8.3176 ± 0.1213
½ MS, 30 g sucrose	0.6791 ± 0.4193	22.4590 ± 0.0341
MS INT, 15 g sucrose	2.8706 ± 0.0346	1.8750 ± 0.0163
MS INT, 30 g sucrose	1.5442 ± 0.3177	3.2579 ± 0.6721

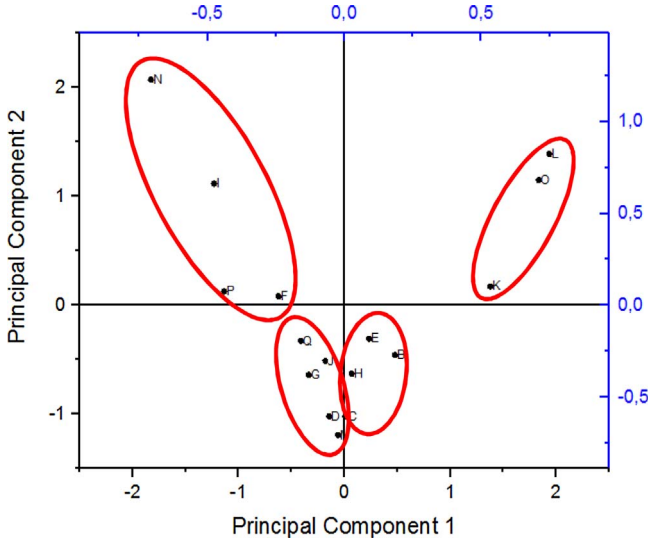

Fig. 2. PC1 versus PC2 das diferentes condições de cultivo de *Leptohyptis macrostachys*, baseadas na quantificação de podofilotoxina e yateína. Onde: B = Control *in vitro*, C = 0 µM BAP + 5.37 µM ANA, D = 0 µM BAP + 10.74 µM ANA, E = 4.44 µM BAP + 0 µM ANA, F = 4.44 µM BAP + 5.37 µM ANA, G = 4.44 µM BAP + 10.74 µM ANA, H = 8.88 µM BAP + 0 µM ANA, I = 8.88 µM BAP + 5.37 µM ANA, *J* = 8.88 µM BAP + 10.74 µM ANA, K = photoperiod, L = Temperature, M = Gas changes, N = ½ MS, 15 g sucrose, O = ½ MS, 30 g sucrose, P = MS INT, 15 g sucrose e Q = MS INT, 30 g sucrose.

The obtained data were evaluated by chemometric methods of exploratory analysis (PCA and HCA). The multivariate analyzes of PCA applied to the contents of podophyllotoxin and yatein from the different culture conditions of *L. macrostachys* are summarized in Fig. 2. Two principal components (PC) are responsible for 100% data variance (53.93% for PC1 and 46.07% for PC2). From these two components was possible to separate *L. macrostachys* plantets in four similar groups accordance with the culture conditions. PC1 showed a good separation between K, L and O (positive scores) from D, F, G, I, J, M, N, P and Q culture conditions (PC1 negative scores). However, in PCA the separation of cultures K, L and O from the cultures B, C, E and H were just confirmed by the positive and negative scores of PC2. In the same plot the PCA permitted to identify the culture conditions (C, H and E) which presented similarity with the *in vitro* control experiment. These conditions showed positive and negative scores in PC1 and PC2, respectively.

The results obtained by HCA are in agreement with the PCA results with regard to the resemblance in treatments. The HCA (Fig. 3) permitted to group the results in two distinct regions in the Dendogram and to classify the sixteen cultures of *L. macrostachys*. The culture conditions B, E, I, N, C, G, D, M, P, F, H, J and Q (black) were considerated class 1 and the conditions K, L and O (red) were named Class 2. This two class showed no similarities. Thus, the experiments K, L and O are characterized culture conditions that are differentiated in terms of podophyllotoxin and yatein productions. Comparing the culture conditions of class 2 (K, L and O), the experiment K clearly is different from the other two conditions, especially in yatein production.

The production of podophyllotoxin was influenced both by the concentration of sucrose and salt in the culture medium. The yield of this compound was greater when ½ MS supplemented with 1.5% sucrose was employed. However, the production of yatein was influenced by only the concentration of basal salt in the cultivation medium. The greatest yield of yatein was observed in the medium containing half the concentration of salt (Table 2).

The podophyllotoxin content was on average higher for the ratio of plant growth regulators composed of 8.88 µM of BAP and 5.37 µM of

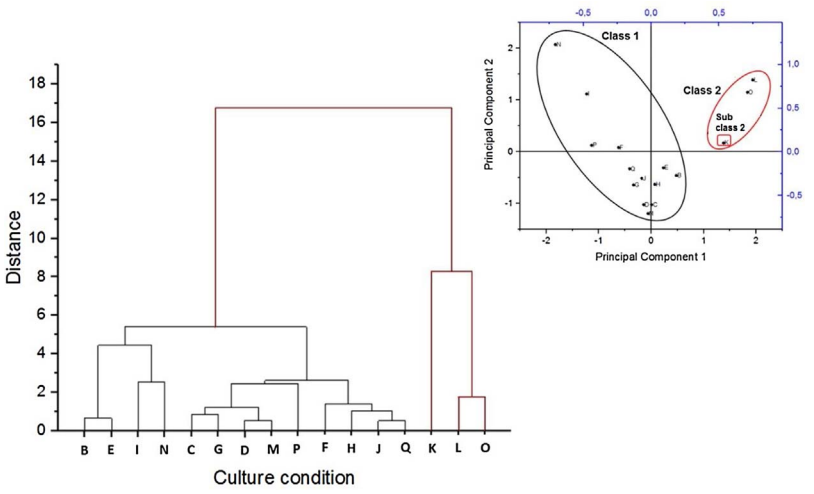

Fig. 3. Dendograma correspondente à classificação de diferentes condições de cultura de *Leptohyptis macrostachys*, baseadas na quantificação de podofilotoxina e yateína. Onde: B = Control *in vitro*, C = 0 μM BAP + 5.37 μM ANA, D = 0 μM BAP + 10.74 μM ANA, E = 4.44 μM BAP + 0 μM ANA, F = 4.44 μM BAP + 5.37 μM ANA, G = 4.44 μM BAP + 10.74 μM ANA, H = 8.88 μM BAP + 0 μM ANA, I = 8.88 μM BAP + 5.37 μM ANA, J = 8.88 μM BAP + 10.74 μM ANA, K = photoperiod, L = Temperature, M = Gas changes, N = ½ MS, 15 g sucrose, O = ½ MS, 30 g sucrose, P = MS INT, 15 g sucrose e Q = MS INT, 30 g sucrose.

Table 2
Average values of podophyllotoxin and yatein in *Leptohyptis macrostachys* plants as a function of the salt and sucrose concentrations in the culture medium.

Concentration of salts	Yatein	Podophyllotoxin	
		1.5% sucrose	3.0% sucrose
MS ½	3.5449	5.8309	0.6791
MS complete	1.9026	2.8705	1.5442

Values showed significant differ at 5% probability by Tukey's test.

Table 4
Average values of podophyllotoxin and yatein in *Leptohyptis macrostachys* plants as a function of temperature, type of cover and photoperiod.

	Temperature (°C)		Types of Closing		Photoperiod (h)	
	25	30	PVC film	plastic cover	16	8
podophyllotoxin	0.4015	0.8372	0.4015	0.1777	0.4015	0.0944
yatein	7.1839	24.2097	7.1839	0.6151	7.1839	15.0663

Analyzing each factor separately (Temperature, Types of closing and Photoperiod), values differ by 5% of probability by the Tukey test.

NAA. The yatein appears to have been inhibited by the presence of NAA, and its average was higher in the absence of this auxin (Table 3).

In a summary of the analysis of variance on the effect of temperature, it was evident that both podophyllotoxin and yatein showed statistically significant differences (Table 1). The comparison of the means shows that yatein and podophyllotoxin, on average, were produced in greater numbers when cultured at 30 °C (Table 4).

A comparison of means shows that the production of podophyllotoxin and yatein was favored under a PVC film sealant, but also in a greater volume of medium when a plastic cover was used (Table 5).

The comparison of averages shows that podophyllotoxin on average was better produced in a photoperiod of 16 h, whereas yatein was better produced in a photoperiod of 8 h (Table 4). Thus, podophyllotoxin production can be improved *in vitro* if maintained on MS medium with half the concentration of salt, supplemented with 1.5% sucrose, sealed with PVC film, and kept in a growth room at 30 °C with photoperiod of 16 h. With the exception of the concentration of salt in the culture medium and the concentration of sucrose, these same conditions favor the number of leaves and/or buds.

Wild *Leptohyptis macrostachys* specimens collected in the region of Diamantina Chapada (Bahia, Brazil), when subjected to the same methods of extraction and quantification, showed podophyllotoxin on leaves with a yield of 0.089 mg g^{-1} dried plant material (Brandão et al., 2017). Cultivated *in vitro* on MS medium contained 1.5% sucrose, *Leptohyptis macrostachys* produced 5.83 mg g^{-1} (Fig. 4).

However, podophyllotoxin production is very low compared to yatein production (Figs. 5 and 6). Therefore, more studies are needed to move the turnover of the biosynthesis reaction to enhance the formation of podophyllotoxin.

4. Conclusions

The concentration of podophyllotoxin can be improved in the *in vitro* culture of *Leptohyptis macrostachys* if maintained on MS medium with half the concentration of salt, contained 1.5% sucrose, sealed with PVC film and kept in a growth chamber at 30 °C with a photoperiod 16 h. Also, in this case, subculture may further increase yields, as the concentration of yatein, the precursor of podophyllotoxin, is favored under conditions (photoperiod and temperature, for example) that are averse to podophyllotoxin production. Comparing with previous studies dealing with new sources of podophyllotoxin from collected plant and/or *in vitro* induced plant the present good results showing the production of this compound, especially with cultures employing 8.88 μM BAP + 5.37 μM ANA. From *Juniperus scopulorum* the highest level of podophyllotoxin characterized was 486.7 mg/100 g DW (Och et al.,

Table 3
Average values of podophyllotoxin and yatein in *Leptohyptis macrostachys* plants for different concentrations of BAP and NAA.

NAA (μM)	Podophyllotoxin BAP (μM)			Yatein BAP (μM)		
	0.00	4.44	8.88	0.00	4.44	8.88
0.00	0.4015 Ab	0.8431 Ca	0.6678 Ba	7.1838 Ab	6.6848 Ad	4.1824 Ba
5.37	0.2926 Aa	2.2430 Bc	4.0871 Cc	1.8553 Aa	4.2934 Ae	4.3272 Aa
10.74	0.4670 Ac	1.1091 Bb	1.0787 Bb	1.0705 Aa	2.0343 A	3.5098 Aa

For Podophyllotoxin: Values followed by the same lowercase letter do not differ in each column, followed by the same capital letter do not differ in each row. For Yatein: means followed by the same lower case letter in each column do not differ at 5% probability by Tukey's test. Followed by the same capital letter do not differ in each row.

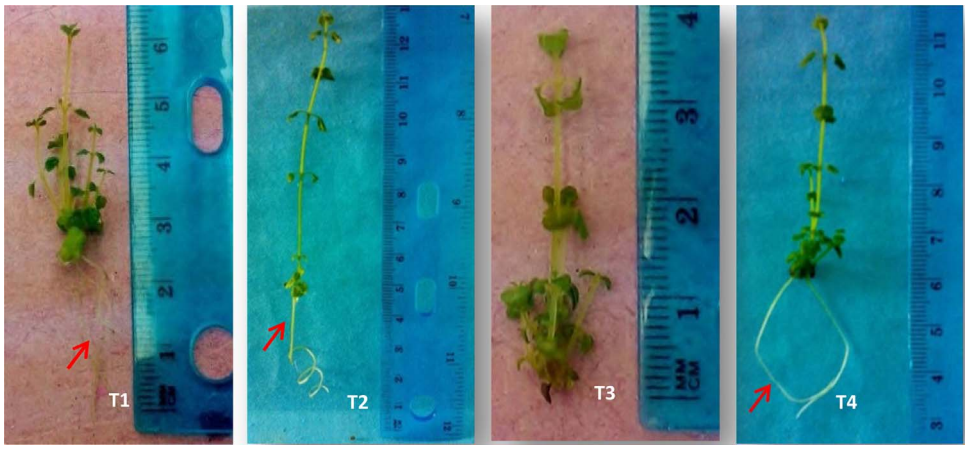

Fig. 4. *In vitro* establishment of *Leptohyptis macrostachys* (T1 = ½ MS and 1.5% sucrose; T2 = ½ MS 3.0% sucrose; T3 = 1 MS 1.5% sucrose; T4 = 1 MS 1.5% sucrose). Roots are indicated by the red arrows. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

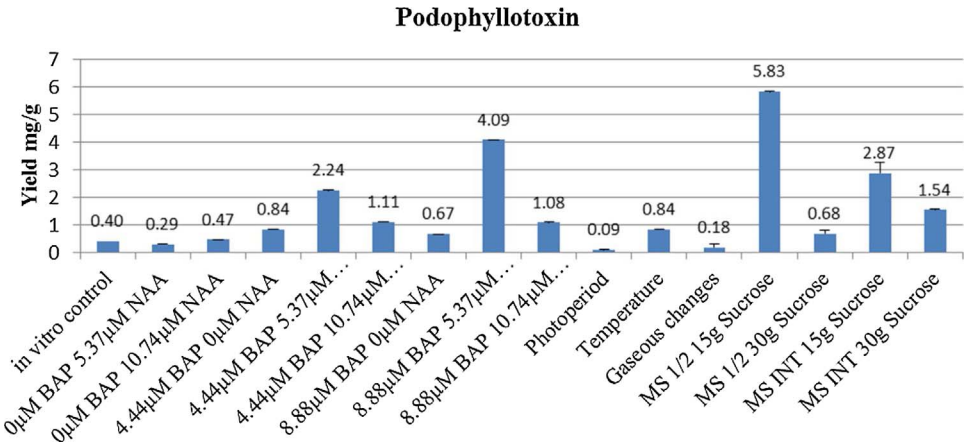

Fig. 5. Comparison of yield (mg g^{-1} dried plant material) podophyllotoxin in *Leptohyptis macrostachys* plants cultured *in vitro*.

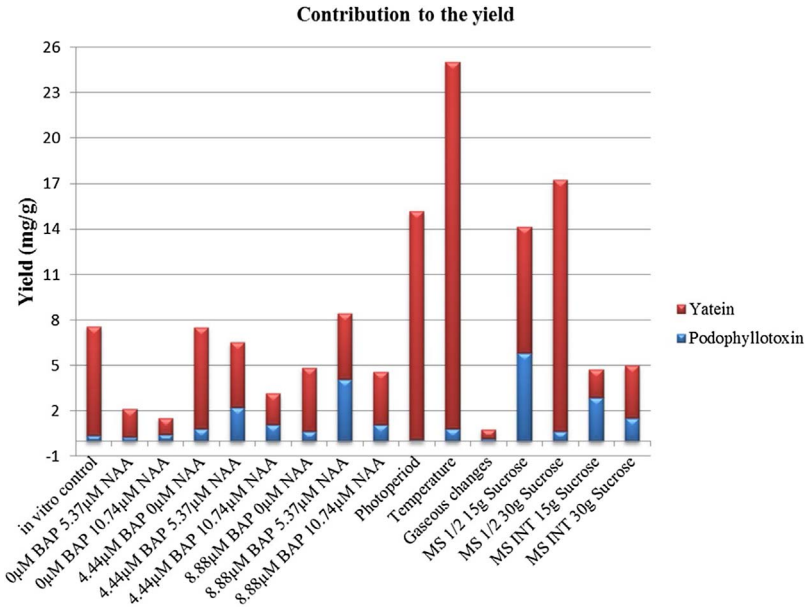

Fig. 6. Comparison of mean considering the yield (mg g^{-1} of dry plant) of podophyllotoxin × yatein in *Leptohyptis macrostachys* plants cultivated *in vitro*.

2015). And, previous studies with *in vitro*–induced roots and roots regenerated from calli of *Podophyllum hexandrum* Royle produced 11.625% and 10.5% enhanced quantities of podophyllotoxin of dry weight of the cultures while in plant drug it was 9.3% of the dry weight of plant root rhizome (Sagar and Zafar, 2005). Therefore, this work demonstrated that species of Lamiaceae could potentially serve as an alternative source of podophyllotoxin or its biossinthetic precursors by *in vitro* cultures.

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