Production of podophyllotoxin from roots and plantlets of *Hyptis suaveolens* cultivated *in vitro*

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ABSTRACT

Background: *Hyptis suaveolens* was an important source of food and medicines in pre-hispanic México and is actually used popularly to treat respiratory and skin diseases, fever, pain, and cramps, between other ailments. In 2008 the presence of podophyllotoxin (PTOX) was reported in this plant. **Objective:** To establish *in vitro* cultures of *H. suaveolens* able to produce PTOX. **Materials and Methods:** Explants of *H. suaveolens* were cultivated in Murashige and Skoog (MS) medium supplemented with different concentrations of the phytohormones 6-benzylaminopurine (6-BAP), 2,4-dichlorophenoxyacetic acid (2,4-D), 1-naphthaleneacetic acid (NAA) and kinetin (Kin), in order to induce the production of podophyllotoxin. Root cultures without hormones were also established and the quantification of PTOX was performed by HPLC analysis. **Results:** The presence of growth regulators during *in vitro* cultivation of *H. suaveolens*, provoked morphological variations in explants, and induced the accumulation of different levels of PTOX. Roots grown without phytohormones accumulated PTOX at 0.013% dry weight (DW), while in three of the callus cultures cell lines growing together with roots, PTOX accumulated at concentrations of 0.003, 0.005 and 0.006% DW when NAA was combined with either Kin or BAP. In wild plant material PTOX was present in trace amounts in the aerial parts, while in the roots it was found at 0.005% DW. **Conclusion:** This study demonstrated that although it is possible to obtain PTOX in a variety of *in vitro* cultures of *H. suaveolens*, *in vitro* roots grown without the addition of growth regulators were better producers of PTOX.

Key words: *Hyptis suaveolens*, *in vitro* cultures, podophyllotoxin

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INTRODUCTION

Podophyllotoxin (PTOX) is a 2,-7'-cyclolignan that posses antiviral and cytotoxic properties,[1] and is used topically in the treatment of genital warts (*Condyloma acuminata*) caused by the human papilloma virus.[2] Synthetic derivatives of PTOX (etoposide, teniposide and etopofos), which are currently commercialized, are actually used as efficient anticancer drugs.[3] Other synthetic derivatives from PTOX have been developed for treatment of certain cancers, as well as for rheumatoid arthritis.[4] Even tough, the synthesis of 2,-7'-cyclolignans has been established; it turns out to be costly and therefore economically unviable.[5] Actually, PTOX is extracted from the plant material of the species *Podophyllum peltatum* and *Podophyllum emodi*, which are now categorized as endangered species, and whose *in vitro* propagation has as yet proven to be complicated.[6] The development of the drugs etoposide, teniposide, and etopofos, have increased the demand for PTOX, both to carry out the synthesis of such derivatives, and also for research purposes.[7] Among investigated new natural sources that accumulate PTOX are included the plant species *Linum album*,[8] *Callitris drummondii*,[9] *Jeffersonian chinensis*,[10] *Linum scabellrum*, and *Hyptis suaveolens*[6] used in this investigation.

The establishment of cell cultures (roots and cell suspensions); through biotechnological procedures have been investigated in some species expecting to favor the production of PTOX.[7] Experiments to enhance the production of PTOX have included medium optimization,[11] permeabilization,[12] addition of precursors and elicitors;[13] as well as the immobilization of cells and also the addition of phytohormones,[12] as in this study.

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However as of yet, the production of PTOX by biotechnological procedures, hasn't proven to be a good commercial alternative, due to the constant genetic spontaneous variation of the plant cells, and the high costs of production.[7]

The presence of PTOX in the plant species *H. suaveolens* has been reported in both aerial parts and in roots[6] *H. suaveolens*, ("Chan" or "Chia de Colima") was an important source of food and medicine in pre-hispanic Mexico.[14] Nowadays *H. suaveolens* is used traditionally for the treatment of respiratory diseases, fever, skin diseases, pain, and cramps.[15] It has also been reported that essential oils extracted from the leaves of this plant, exhibit antifungal,[16] antimicrobial,[17] anticonvulsant,[18] antiplasmodial,[15] anti-inflammatory[19] and antinociceptive[20] activities.

It is important to seek new alternatives for obtaining PTOX in quantities economically exploitable. This project, based on previous screening studies indicating *H. suaveolens* as a possible source of PTOX,[6] is designed to use biotechnological tools such as *in vitro* propagation, tissue culture and callus formation, in search of future sustainable and rational exploitation of this plant species. Herein we report the effect of different phytohormone combinations on the production of PTOX using *in vitro* cultivation.

MATERIALS AND METHODS

Plant material

Hyptis suaveolens (L.) Poit. (Lamiaceae) aerial parts, roots, and seeds were collected near Mérida Yucatán, and authenticated by German Carnevalli from the CICY's Herbarium. The assigned voucher number is 7086 (CICY).

Cultivation procedure

The medium used was Murashige and Skoog (MS)[21] enriched with 0.1 g/L of *myo*-inositol (Sigma Chem. Co), 30 g/L of sucrose, and 1.5 g/L of polyvinylpyrrolidone (PVP) (Sigma-Aldrich Chem. Co). The pH was adjusted to 5.7, and 1.5 g/L of phytagel (Sigma-Aldrich Chem. Co) was added; then autoclaved for 15 minutes at 121ºC for sterilization.

Germination

One hundred seeds obtained from adult specimens were selected and sterilized by a process of immersion in a solution of ethanol at 70% for 10 minutes, followed by 3 immersions in sodium hypochlorite at 5, 10 and 15% for 3, 10, and 3 minutes, respectively. After each immersion, the seeds were rinsed with distilled sterilized water, and equally distributed in 10 jars using an enriched MS medium. The cultures were kept at 25 ºC in a cultivation room with a photoperiod of 16 hours.

In vitro propagation

After germination, and once the plantlets have reached a size of approximately 5 cm and developed roots, they were sub-cultured into separate vials and allowed to grow until they reached a length of 8 cm. Explants of lateral branches when extending at 2-3 cm from the stem apical meristem, were then taken for multiplication. MS-enriched medium was used as described before.

In vitro cultivation of roots

The roots produced by plantlets were separated and sub-cultured in enriched MS liquid medium without phytohormones. Erlenmeyer flasks (250 mL) with 150 mL liquid medium were inoculated with roots (10 g) each. The medium was changed every 7 days. The flasks were maintained at 25°C in a cultivation room with no light and with agitation at 60 rpm on an orbital shaker.

In vitro cultivation of *Hyptis suaveolens* explants

As above, explants of lateral branches when extended 2-3 cm long were taken. They were maintained at 25°C in a cultivation room with a photoperiod of 16 hours. Thirty different treatments resulting from the combination of 2,4-dichlorophenoxyacetic acid (2,4-D) at 0, 0.2, 1 and 2 mg/mL and kinetin (Kin) at 0, 0.06, 0.2 and 0.5 mg/mL or 6-benzylamino purine (6-BAP) at 0, 0.06, 0.2 and 0.5 mg/mL, were added to the medium. In other series of experiments, 30 treatments resulting from the combination of 1-naphthaleneacetic acid (NAA) at 0, 0.4, 1 and 2 mg/mL with Kin at 0, 0.06, 0.2 and 0.5 mg/mL or 6-BAP at 0, 0.06, 0.2 and 0.5 mg/mL were incorporated [Table 1]. All the hormones were purchased from Sigma Chem Co. The 15 explants used for each of the different combinations of phytohormones were harvested at 5 weeks. Phytohormones and the concentrations used were chosen through an exhaustive bibliographic review.

Table 1: Different combinations of phytohormones used for the *in vitro* cultivation of *Hyptis suaveolens*

Auxin/Cytokines	NAA (mg/mL)			
	0	0.4	1	2
Kin (mg/mL)				
0		1	2	3
0.06	4	5	6	7
0.2	8	9	10	11
0.5	12	13	14	15
6-BAP (mg/mL)				
0		16	17	18
0.06	19	20	21	22
0.2	23	24	25	26
0.5	27	28	29	30

Numbers 1-30 represent an hormonal combination; NAA=Naphthaleneacetic acid; Kinetin; 6-BAP=6-benzy/aminopurine

Extract preparation

Plantlets and roots obtained *in vitro* were dried by lyophilization. The wild plant was air dried. Subsequently the dried plant material was pulverized and macerated with 100% chloroform for 6 days. The extracts were filtered to remove plant material. The solvent was separated from the extracts by distillation under reduced pressure, using a rotary evaporator at a temperature of 35ºC. Once evaporated, all samples were re-suspended in methanol for HPLC analysis.

Identification and quantification of PTOX

The instrumentation used for HPLC-DAD analysis consisted of a Waters (Millipore Corp., Waters Chromatography Division, Milford, Ma.) 600E multisolvent delivery system equipped with a Waters W996 diode array detector (232 nm). Control of the equipment, data acquisition, processing, and management of chromatographic information were performed by Millenium 32 software program (Waters). The analytical HPLC separations were done on a Symmetric C_{18} column (Waters; 5 μm, 4.6 × 250 mm) with an isocratic elution of acetonitrile: water with 0.2% acetic acid (40:60), a flow rate of 1 mL/min, and a sample injection of 20 μL (1 mg/mL).

For quantification, we established a calibration curve using a standard commercial PTOX from Sigma Chem. Co. (98%) under the conditions described above. We started with an initial concentration of 1 mg/mL of PTOX in methanol, and 7 dilutions were made. For the identification of PTOX in the extracts, some dilutions were prepared at a concentration of 10 mg/mL and 8 mg/mL for extract of plantlets and roots obtained *in vitro,* respectively. In addition, UV-visible spectra were obtained, and co-elutions were made, in which a solution was prepared with 100 μL of sample solution plus 500 μL of the extract, further analyzed by HPLC. For the wild plant material, PTOX in aerial parts and roots was quantified. For roots grown *in vitro* 3 replicates were prepared. For plantlets, callus and roots subjected to hormonal treatments, 3 replicates were prepared.

Statistical analysis

A one way analysis of variance was conducted (ANOVA), and for comparison with the control, the Dunnett test was used. For the quantification of PTOX in the different extracts, 3 repetitions were done.

RESULTS

Roots grown *in vitro*

From cultures grown in solid agar media without phytohormones, five sets of 10 g of roots each, were separated and sub-cultured in liquid MS medium for five weeks. Roots were brown, flexible and thin [Figure 1], and showed a 36.7 percentage of growth at the end of the culture period (35 days), in comparison with inoculum. At 35 days in culture mean fresh weight was of 15.8 ± 1.054 g, reduced to 2.1037 ± 0.3957 g of dry plant material.

Figure 1: Roots of *Hyptis suaveolens* grown *in vitro* at 35 days in culture

Effect of phytohormones in plant growth and morphology

After one week of placing the seeds in agar, the percentage of germination was 40%. Two months later, one of the plantlets was selected for subcultivation, and thereby reduced the effects of biological variability on ongoing experiments. This plantlet was propagated in order to obtain 200 plantlets with roots, from which 900 explants were obtained and subjected to hormonal evaluation. For all the experiments in which 2,4-D was added as an auxin, combined either with Kin or 6-BAP, the tissue was phenolized, and died during the first week of cultivation. When NAA was added as an auxin, different results were obtained [Table 2]. Those treatments with the highest concentration of NAA (2 mg/mL) combined with any concentration of either Kin or 6-BAP (Numbers 3, 7, 11, 15, 18, 22, 26 and 30), exhibited high phenolization during the first week and finally died. Also the treatments with low NAA (0.4 and 1 mg/mL) but high 6-BAP (0.5 mg/mL) (No. 27, 28 and 29) were phenolized. In some treatments additioned with NAA at 0.4 and 1mg/mL combined either with Kin (at 0.06, 0.2 and 0.5 mg/mL) or 6-BAP (at 0.06 and 0.2 mg/mL), the explants showed protuberances within one week in culture, and after 13 days, callus appeared with three variations [Figure 2 Table 2]: 1) development of callus and roots (treatments 5, 9 and 13) [Figure 2a, Table 2], 2) development of shoots with roots and callus presence (treatments 6, 10 and 14) [Figure 2b] 3), presence of callus and shoots but defoliation during the first week (treatment 20, 21, 24 and 25) [Figure 2c].

For those hormonal combinations that contained only NAA at 0.4 (treatment 1 [Figure 3] and treatment 16), or NAA at 1.0 mg/mL (treatments 2. and 17) white and vigorous roots appeared starting in the second week and reaching big biomasses at 35 days in culture.

Table 2: Some common physiological characteristics observed for *Hyptis suaveolens* cultures supplemented with phytohormones

Treatments	Combination of phytohormones	Roots	New shoots	Callus	Phenolization
1	NAA (0.4 mg/ml)	+	+	-	-
2	NAA (1 mg/ml)	+	+	-	-
3	NAA (2 mg/ml)	-	-	-	+
4	Kin (0.06 mg/ml)	-	+	-	-
5	NAA (0.4 mg/ml);Kin (0.06 mg/ml)	+	-	+	-
6	NAA (1 mg/ml);Kin (0.06 mg/ml)	+	+	+	-
7	NAA (2mg/ml);Kin (0.06 mg/ml)	-	-	-	+
8	Kin (0.2 mg/ml)	-	+	-	-
9	NAA (0.4 mg/ml);Kin (0.2 mg/ml)	+	-	+	-
10	NAA (1mg/ml);Kin (0.2 mg/ml)	+	+	+	-
11	NAA (2 mg/ml):Kin (0.2 mg/ml)	-	-	-	+
12	Kin (0.5 mg/ml)	-	+	-	-
13	NAA (0.4 mg/ml);Kin (0.5 mg/ml)	+	-	+	-
14	NAA (1 mg/ml);Kin (0.5mg/ml)	+	+	+	-
15	NAA (2 mg/ml);Kin (0.5 mg/ml)	-	-	-	+
16	NAA (0.4mg/ml)	+	+	-	-
17	NAA (1 mg/ml)	+	+	-	-
18	NAA (2 mg/ml)	-	-	-	+
19	6-BAP (0.06 mg/ml)	-	+	-	-
20	NAA (0.4mg/ml);6-BAP (0.06 mg/ml)	+	-	+	-
21	NAA (1 mg/ml);6-BAP (0.06 mg/ml)	-	-	+	-
22	NAA (2 mg/ml);6-BAP (0.06 mg/ml)	-	-	-	+
23	6-BAP (0.2 mg/ml)	-	+	-	-
24	NAA (0.4 mg/ml);6-BAP (0.2 mg/ml)	-	-	+	-
25	NAA (1 mg/ml); 6-BAP (0.2 mg/ml)	-	-	+	-
26	NAA (2 mg/ml);6-BAP (0.2 mg/ml)	-	-	-	+
27	6-BAP (0.5 mg/ml)	-	-	-	+
28	NAA (0.4 mg/ml);6-BAP (0.5mg/ml)	-	-	-	+
29	NAA (1 mg/ml);6-BAP (0.5 mg/ml)	-	-	-	+
30	NAA (2 mg/ml);6-BAP (0.5 mg/ml)	-	-	-	+

NAA=Naphthalene acetic acid; BAP=Benzylaminopurine; Kin=Kinetin

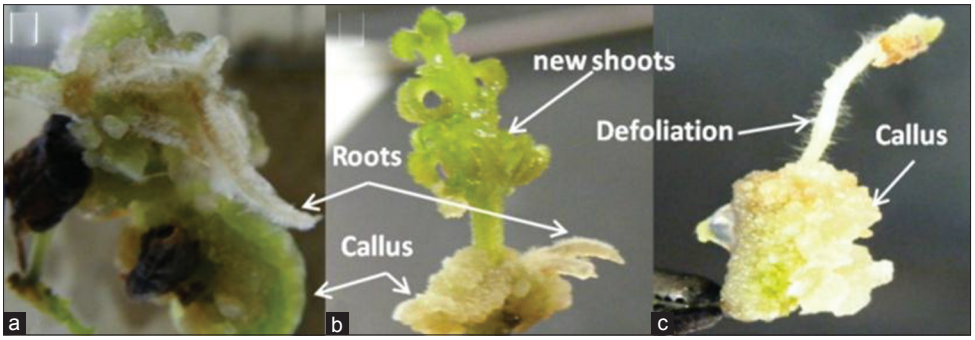

Figure 2: Variations for callus production after 35 days in culture. (a) Treatment 13 (NAA 0.4 mg/mL and Kin 0.5 mg/mL). (b) Treatment 10 (NAA 1 mg/mL and Kin 0. 2 mg/mL). (c) Treatment 24 (NAA 0.4 mg/mL and 6-BAP 0.2 mg/mlL)

It is noteworthy that treatments with cytokinines (Kin or BAP) in the absence of NAA (treatments 4, 8, 12, 19 and 23), showed no root development but exhibited presence of new shoots [Figure 4].

Identification and quantification of PTOX in wild plants

To quantify PTOX, we constructed an HPLC calibration curve, where T_R (retention time) of PTOX was equal to 13 ± 0.06 min. (r^2 =1).

In the wild plant material, aerial parts showed the presence of PTOX in traces, which were not quantified because it was outside the limits of quantification. Wild roots showed a signal with a T_R = 13 ± 0.015 min. in concentration of 0.05 ± 0.0011mg_{PTOX}/g_{DPM} (mg PTOX per g of dry plant material) with a positive co-elution, in addition, UV spectrum obtained for this extract was similar to PTOX [Figure 5], which confirmed the presence of the compound.

Identification and quantification of PTOX in cultures

Roots grown in vitro

The presence of PTOX in *in vitro* roots grown without phytohormones was detected by HPLC, where a signal was obtained with a T_R = 13 ± 0.04 min, and confirmed

Figure 3: Treatment 1 (0.4 mg/mL of NAA) with roots development, after 35 days in culture

Figure 4: Treatment 8 (Kin 0.2 mg/mL), no roots were formed, and new shoots appeared

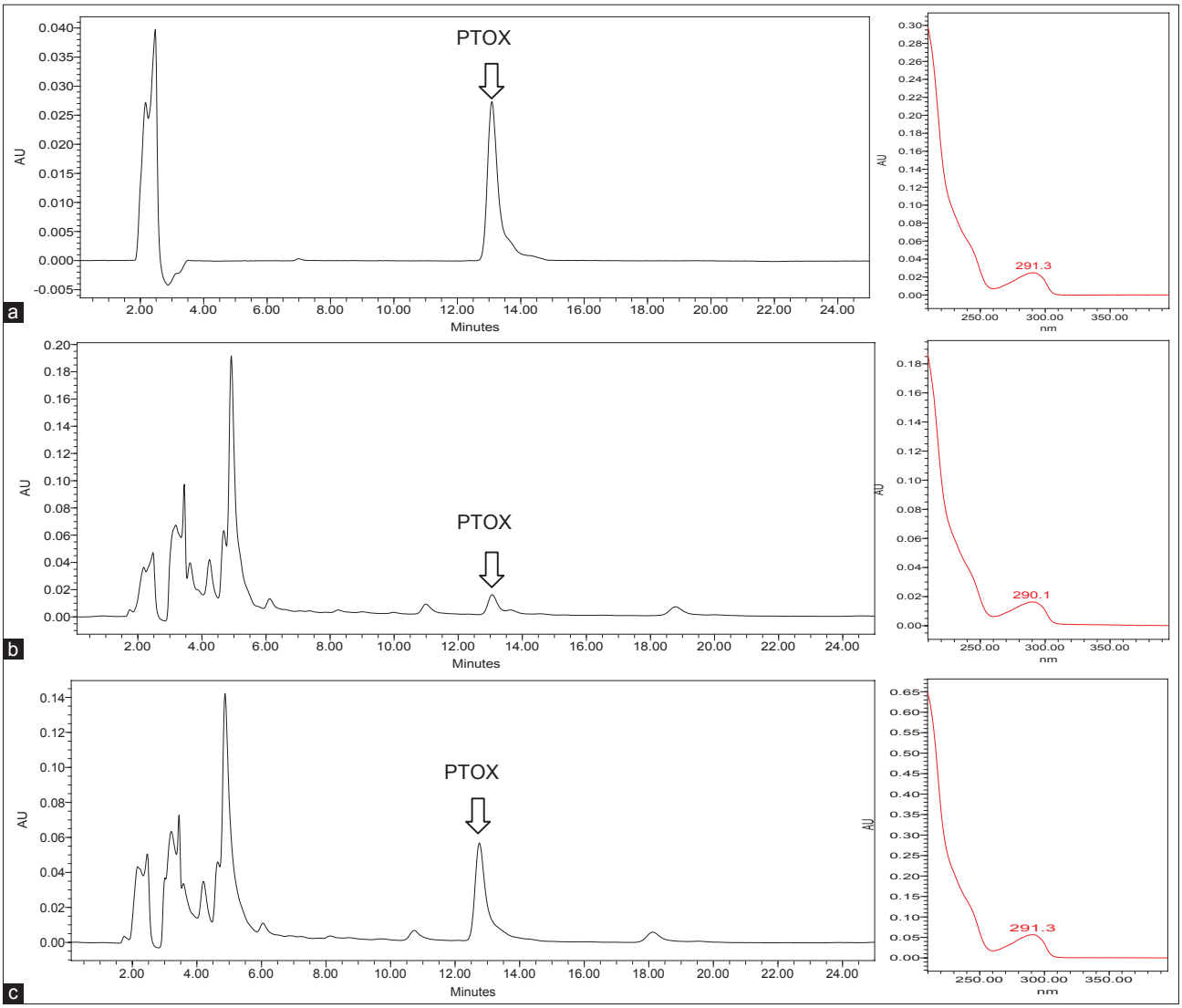

Figure 5: HPLC-DAD chromatograms and UV spectra of extracts from wild roots of *H. suaveolens*. (a) PTOX, TR = 13 ± 0.06 min. (b) wild roots, TR = 13 ± 0.015 min. equal to PTOX suggesting the presence of the compound. (c) Co-elution of wild roots extract and PTOX, TR = 13 min, where there is an increase AUC (Area under the curve) proportional to the amount of the added PTOX. UV spectra obtained from wild roots and PTOX standard were similar

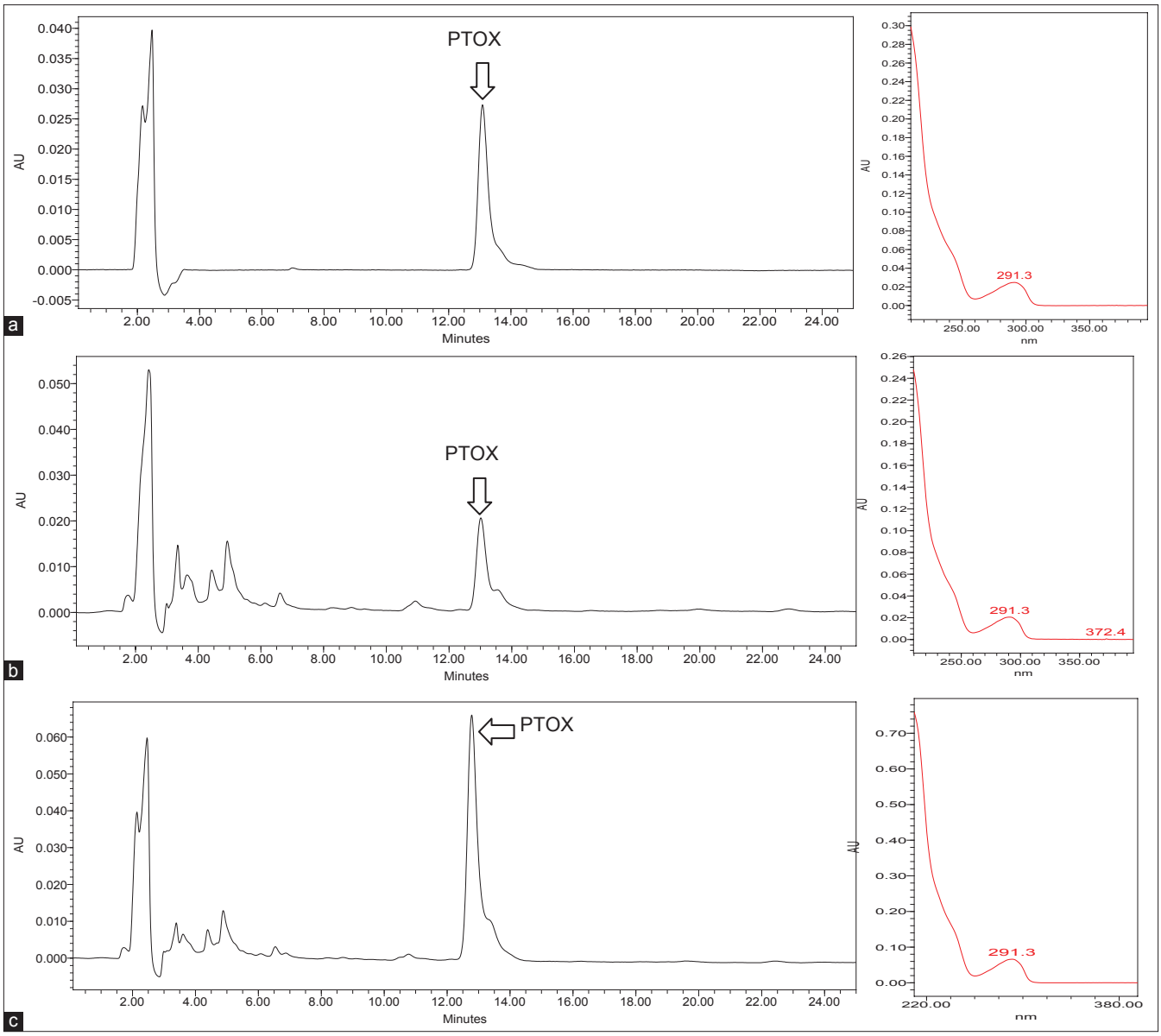

Figure 6: HPLC-DAD chromatogram and UV spectra of *in vitro* roots without hormones. (a) PTOX, TR = 13 ± 0.06 min. (b) roots grown *in vitro* showing a signal with a TR = 13 ± 0.04 min. (c) co-elution of *in vitro* root extract plus PTOX, TR = 13 min, where there is an increase in AUC proportional to the amount of added PTOX. UV spectra obtained from cultivated roots and PTOX standard were similar

for a positive co-elution, in addition, UV spectrum obtained for PTOX was similar in this extract [Figure 6]. The concentration of PTOX in these extracts was 0.12 ± 0.015 mg_{PTOX}/g_{DPM}, corresponding to 0.013% DW PTOX. Roots did not excrete PTOX into the medium.

Identification and quantification of PTOX in in vitro plantlets and calluses obtained through phytohormonal combinations

From 30 hormonal combinations, only three (treatments 6, 9 and 20) showed the presence of PTOX [Figure 7] at concentrations of 0.042 ± 0.0005, 0.018 ± 0.004 and 0.049 ± 0.0005 mg_{PTOX}/g_{DPM} respectively, while in other three (treatments 5, 13 and 25) only traces of PTOX were observed.

To corroborate the identity of PTOX, UV spectra and co-elutions were performed for treatments 6, 9, 20 [Figure 8], which confirmed the presence of the compound.

For the accumulation of PTOX and according to statistical analysis, there is no significant difference between wild plants and treatments 6 and 20; however a difference is shown between wild plants and treatment 9 ($P < 0.05$). On the other hand, a significant difference was observed between *in vitro* cultivated roots without hormones, and

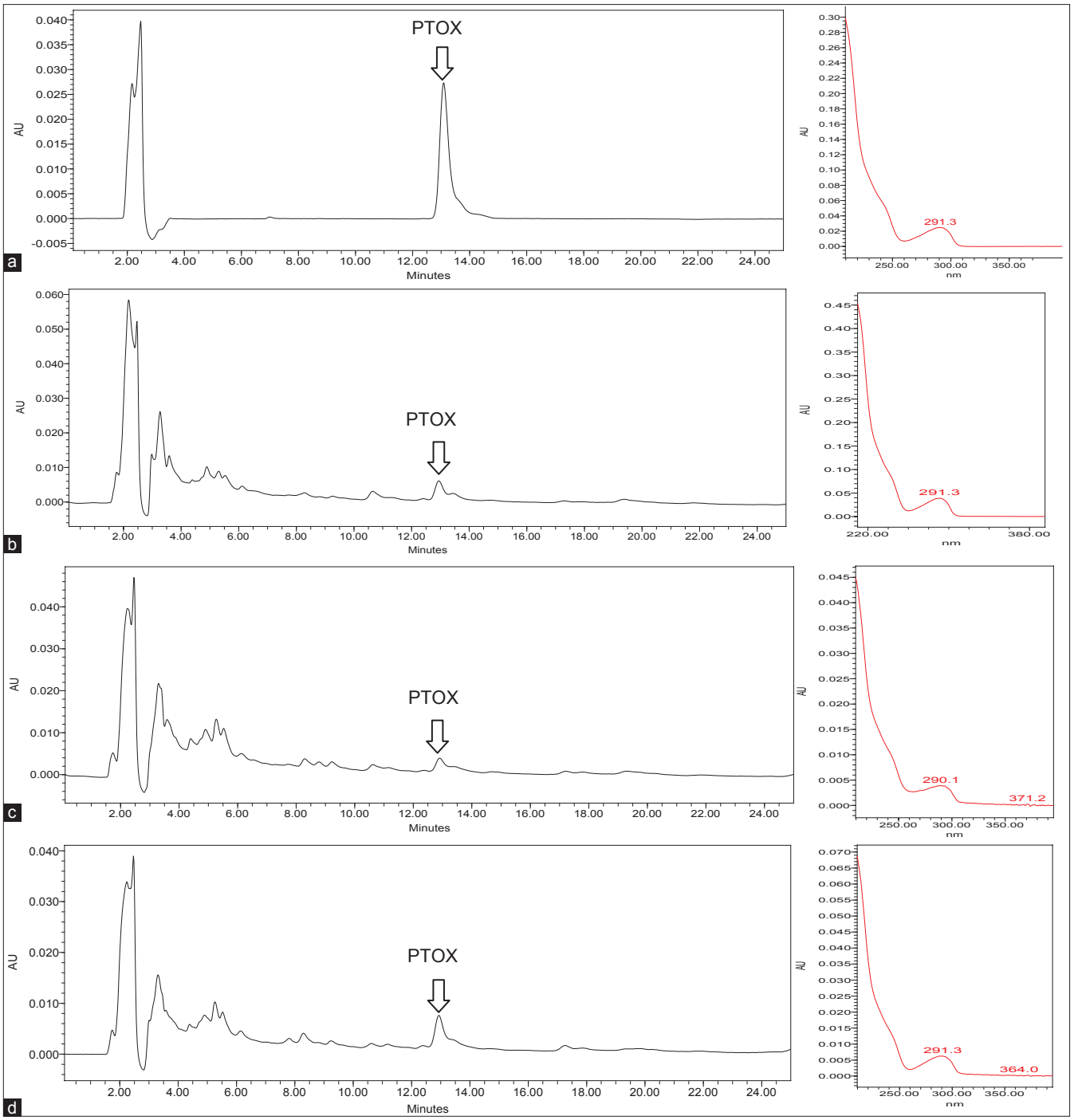

Figure 7: Chromatograms obtained by HPLC-DAD and UV spectra showing the presence of PTOX. (a) PTOX, TR = 13 ± 0.06 min. (b) treatment 20, which shows a signal with a TR = 13 ± 0.01 min. (c) treatment 9, which shows a signal with a TR = 13 ± 0.032 min. (d) treatment 6, which shows a signal with aTR = 13 ± 0.02 min. These signals are equal to the signal PTOX, suggesting the presence of the compound. UV spectra obtained from treatments 6, 9 and 20, and PTOX standard were similar

those of wild roots or those explants subjected to hormonal treatments ($P < 0.05$) [Figure 9].

DISCUSSION

The presence of PTOX in the wild roots of *H. suaveolens* was confirmed and these results are consistent with those previously obtained by Lautié,[6] who reported accumulation of PTOX in the roots of this plant using the β-glucosidase extraction procedure.[22,23] In the present study PTOX content in the wild roots was 0.05 ± 0.0011 mg_{PTOX}/g_{DPM} (0.005%) and for *in vitro* root extracts, PTOX was equal to 0.12 ± 0.015 mg_{PTOX}/g_{DPM} (0.13%). In other roots PTOX has been reported to occur in *Juniperus sabina* and

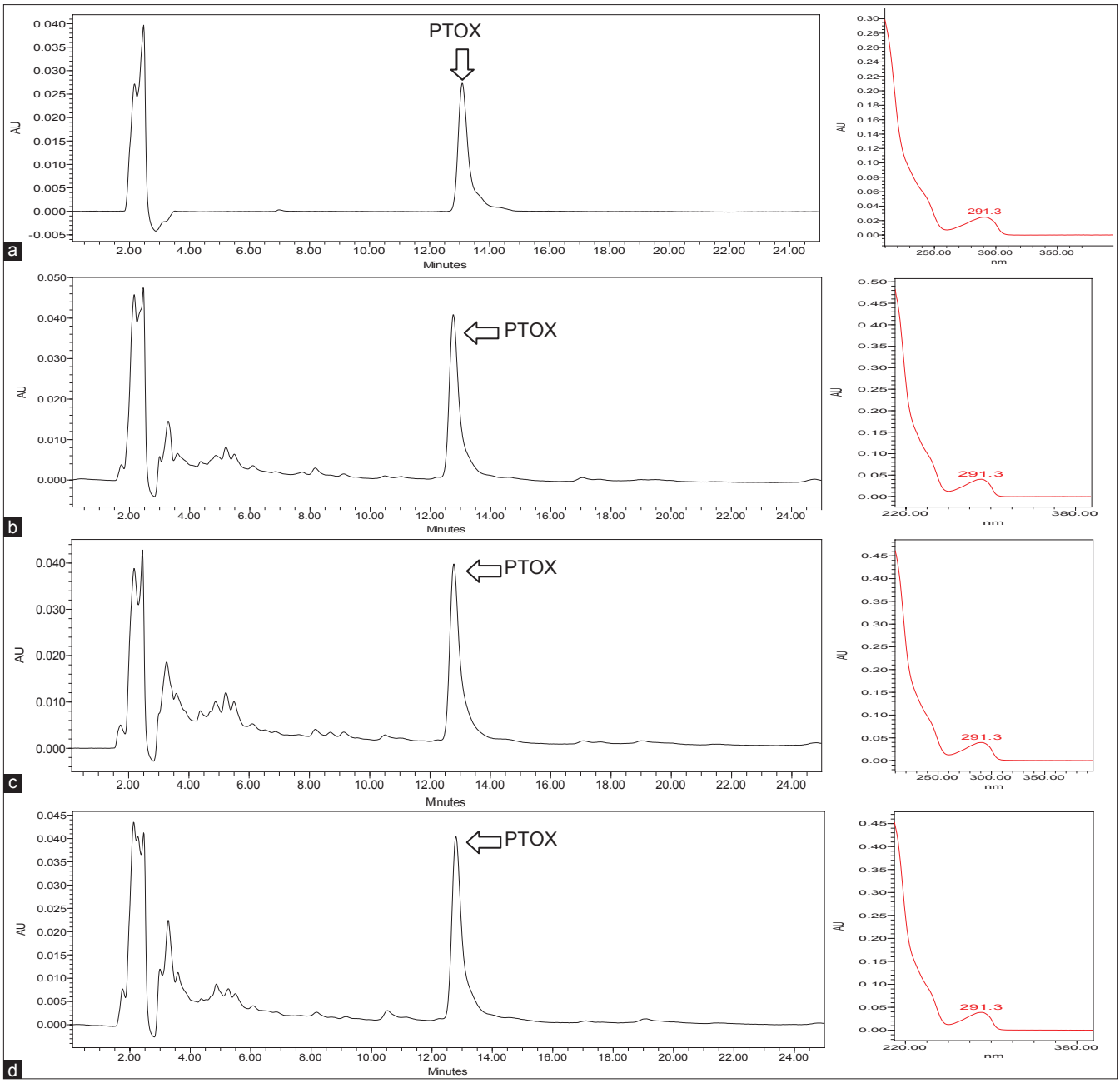

Figure 8: Chromatograms obtained by HPLC-DAD and UV spectrums showing the presence of PTOX. (a) chromatogram of PTOX, TR = 13 ± 0.06 min. (b) chromatogram obtained by co-elution of treatment 6 and PTOX TR = 13. (c) chromatogram obtained by co-elution of treatment 9 and PTOX, TR = 12.9 min. (d) chromatogram obtained by co-elution of 20 and PTOX, TR = 12.9 min. UV spectra from co-elutions were similar to the one from PTOX

J. squanata at a concentration of 0.06 and 0.09 mg_{PTOX}/g_{DPM} respectively,[24,25] values similar to the one obtained for wild roots, but lower to that for the *in vitro* roots in our study. Other studies on the accumulation of PTOX in wild plant material include *L. album* (0.0005% FW of PTOX);[26] *P. peltanum* and *H. verticillata* (0.25% DW in both cases); and *P. emondi* wild plant material (4.3 to 5.2% DW).[4] Some reports indicate that roots in culture are good producers of PTOX, for example in *L. album in vitro* roots, PTOX is accumulated at 1.5% of DW,[27] while in reports of callus cultures, PTOX accumulated at a concentration that varies from 0.018 to 0.78% DW.[28-30] In our study, treatments 6, 9 and 20 that accumulated PTOX exhibited the presence of calluses growing together with roots.

Kadkade in 1982[31] observed that callus cultures of *P. peltatum* increased the accumulation of PTOX from 0.0094% to 0.065%, when they were cultivated in media enriched with NAA and 2,4-D; and when they were grown in a combination of 2,4-D and Kin, an increase

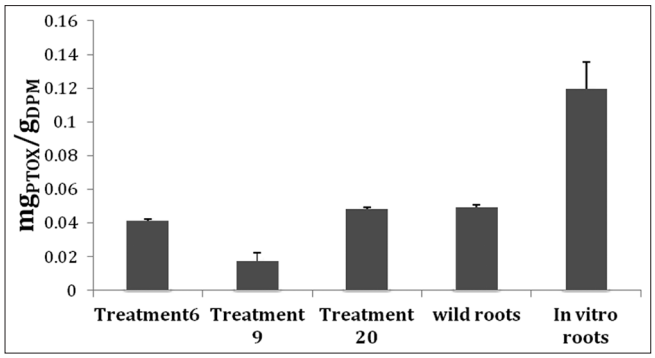

Figure 9: PTOX concentration in the analyzed different extracts. Experiments with (*) indicate significant differences compared to extracts from wild roots according to the analysis of variance that was performed via Dunnett sample ($P < 0.01$). n = 3

of 0.57% production of PTOX was obtained. Farkya and Bisaria in 2008[27] reported that in *L. album* root cultures without phytohormones PTOX was obtained at 6.20 mg_{PTOX}/g_{DW}, when Kin, NAA or 2,4-D were added separately the production of PTOX increased to 6.6, 8.9, and 12.5 mg_{PTOX}/g_{DW} respectively; but when NAA was added together with Kin or 2,4-D together with Kin, the production of PTOX decreased to 5.89 and 2.26 mg_{PTOX} g_{DW}, respectively. Furthermore Smollny *et al.*,[32] reported that in cells suspension cultures of *L. album* grown in MS medium supplemented with NAA, the PTOX production increased by 0.5% compared to control cultivation. In the study done by Heyenga *et al.*[28] with cultures of *P. hexadrum*, the presence of 2,4-D did not promote the production of PTOX.

Results obtained in the present study indicate that in those treatments in which 2,4-D was added, the tissue was phenolized and died during the first week of cultivation. However when NAA was the only phytohormone added, no presence of PTOX was detected, while in some treatments where NAA was combined with Kin or 6-BAP (treatments 6, 9 and 20) PTOX was detected at a similar level as in wild roots.

According to the results obtained in this project, the extracts of roots, treatments **6** (NAA 1 mg/mL and Kin 0.06 mg/mL) and 20 (NAA 0.4 g/mL and 6-BAP 0.06 mg/mL), had a PTOX content by dry weight of 0.004 and 0.005% respectively, accumulating PTOX at a level similar to that obtained for *J. chinensis* 0.005%.[10] However, treatment 9 (NAA 0.4 mg/mL and Kin 0.2 mg/mL) produced just 0.002% of PTOX dry weight, or half of the amount produced with treatments 6 and 20. Notably the accumulation of PTOX in roots grown *in vitro* without hormones was 0.12 ± 0.015 mg_{PTOX}/g_{DPM}, corresponding to 0.013% DW PTOX, a concentration 2.6 times higher than that accumulated for treatments 6 and 20.

Plant material in tissue culture that was supplemented with phytohormones, showed no statistically significant increase in the production of PTOX, but nevertheless did show an increase in production of PTOX compared to the control (*in vitro* plants without phytohormones). On the contrary, in our study it was notable that those *in vitro* roots rather than wild roots were the better producers of PTOX, and that the presence of exogenous hormones is not critical for its accumulation. Though as yet not studied, there is the possibility that endogenous hormones in the *in vitro* roots of this plant that were grown in the dark, are activated to become adequate inducers for increased accumulation of PTOX.

CONCLUSION

Hyptis suaveolens is a Mexican plant species that accumulates podophyllotoxin in aerial parts as well as in roots of wild specimens. This is the first report on the use of *in vitro* tissue culture techniques applied to *H. suaveolens* in order to induce plant genotypes able to express the accumulation of PTOX. Our investigation established that by the use of growth regulators, it was possible to obtain *in vitro* cultures, provoke morphological variations in explants, and establish callus and root lines of *Hyptis suaveolens*. It was also proved that three of the cultures of calluses growing together with roots cultivated in the presence of phytohormones, were able to accumulate PTOX at similar concentrations as wild roots, indicating that the metabolic pathway for this type of compounds is actively expressed in the *in vitro* obtained material. A new aspect in this study is the observation that root cultures without the addition of phytohormones produced higher levels of PTOX compared to wild plants that were collected in a specific location of México.

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