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"In vitro" *Hedeoma multiflorum* Benth propagation in response to different nutritional conditions

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ABSTRACT

An efficient protocol for *in vitro* propagation of *Hedeoma multiflorum* Benth ("Tomillo de las Sierras") was established. To determine the effect of different medium and plant growth regulators on the growth rates and essential oil production, nodal segments from *in vitro* 90-days old plantlets were incubated on the following media: 1) SH (Schenk and Hildebrandt); 2) WP (Lloyd and McCown); 3) B5 (Gamborg). All the media studied were supplemented with different combinations and consentrations of growth regulators: naphtaleneacetic acid (NAA) and 6-benzyl adenine (BA).
The optimal shoot length and number of nodes of the plantlets, showing no statistical difference, were obtained in WP with the addition of 2.69:0.5 μM, 0.5:0.5 and 2.69:0.05 μM of NAA:BA and in absence of these growth regulators (control).
The best multiple shoot medium with no statistical difference, corresponded to SH with the addition of 0.5:2.25 μM and B5 with 0.05:2.25, 0.5:2.25 and 2.69:2.25 μM of NAA:BA.
The major essential oil determined by GC/MS, were common in all the plantlets grown in all the media being pulegone (62-64%) , menthone (25-28 %) and isomenthone (2-2,3%).

Keywords: *Hedeoma multiflorum; in vitro* tissue culture; plant regeneration; essential oil composition.

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Introduction

Hedeoma multiflorum Benth (Lamiaceae), known as "tomillo de las sierras", is an perennial herb, endemic of Argentina. It is grown in dry, calcareous soils and well lighting situations. (Barboza *et al.*, 2006). The volatile oils of *H. multiflorum* contains pulegone (60-70%) and menthone (23%) as main components, Other compounds are: Isomenthone, α-Pinene, β-Pinene, Mircene, Limonene, trans-β-Ocimene, cis-Isopulegone, α-Copaene, (+)-Aromadendrene, Germacrene-D, Bicyclegermacrene and Germacrene-A. (Vazquez *et al.*, 2007)

Traditional medicine documentation of this specie showed that this plant has an increasing market, with demand at national and international level. Local pharmaceutical, food and cosmetic industries, mainly use tomillo for the production of juices, sour beverages ("amargos") and in "yerbas". (Forlani, 1999). There is also an increasing demand of "alternative therapies" such as phytotherapy and aromatherapy (Goleniowski *et al.*, 2006).

Owing to its high therapeutic potential, *H. multiflorum* has been subjected to intense harvesting causing devastation of native areas and put in risk survival of remaining populations in natural habitats (Martinez *et al.*, 2006).

Plant tissue culture offers an alternative to field agriculture providing controlled laboratory enviroments. The major advantages of tissue culture techniques over traditional cultivation of wild plants are: (a) useful compounds can be produced under controlled conditions, (b) plants are free of microbes and insects, (c) plants can easily be multiplied to yield specific metabolites, (d) a stable and uniform year-round supply of selected plants is guaranteed, independent of seasonal variations. (Palacio *et al.*, 2008)

In this study, we report an efficient protocol for *in vitro* plant propagation of *H. multiflorum* using different nutritional conditions.

Experimental

Plant material and initiation of plant regeneration. Nodal segments of *H. multiflorum* were used for the study. The initial material were 3 months plantlets grown in asseptical conditions in basal Murashige and Skoog medium.

Three media were screened for shoot culture: SH (Schenk and Hildebrandt, 1972); WP (Lloyd and McCown, 1980) and B5 (Gamborg *et al.*, 1966). Treatments consisted on variations of macro and micro elements, wich are inorganic salts necessary to support growth. The media were supplemented with 30 g/L of sucrose as an energy source and different concentrations and combinations of growth regulators: naphtaleneacetic acid (NAA) and 6-benzyl adenine (BA), as shown in table 1.

The pH of the media was adjusted to 5.6-5.7 and 0.7% agar-agar, autoclaving for 15 min at 121 ºC. Cultures were incubated under controlled conditions of temperature (25 ± 2 ºC) with a 16 h photoperiod (4.35 $W.m^2$).

Essential oils analisys. In this study, a sampling method called headspace micro solid phase extraction (HS-SPME) combined to gas chromatography-mass spectrometry has been applied for the analysis of volatile fraction of essential oils. The identification of each component was made by comparing their mass spectra with the library (% match equal to ar greater than 70%).

Statistical analysis. Data on principal shoot lengh, number of nodes of the principal shoot and number of shoots were recorded after 90 days incubation. There were twelve replications for each treatment in a ramdomized statistical design with an analysis of variance (ANOVA). Differences between mean values were analysed by LSD Fisher test with a significance level of 5 %.

Results

After 3 months of culture, different combination and concentration of BA and NAA resulted in varing plant regeneration (Figure 1, 2 and 3).

Figure 1 shows that culturing the explants on SH-6, B5-3, B5-6 and B5-9 resulted in a well adventitious buds development (12.4, 11.7, 10.2 and 9.6 respectively). The higher frequency of microshoots were obtained with a high concentration of BA (2.25 μM).

The results presented in Figure 2, show that different concentration and combination of growth regulators affected the principal shoot length. The best stem elongation, with no statistical difference, correspond to plantlets grown on WP-8, WP-5, WP-7 with the addition of NAA:BA 2.69:0.5 μM and WP-Control media. Results from this study showed that the maximum node number (24), correspond with the optimal shoot length (Fig. 3). This value was two fold than those observed to the control. Root formation occurred without any special rooting medium. B5 medium affected the plant regeneration, the plantlets grown in this condition showed a scarce growth.

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The essential oil analysis in the clonally propagated plants showed a similar composition (Table 2). The major components found were (+)-Pulegone and Menthone, in the WP-8, SH-6 and MS-0 media. Minor compounds were: α-Pinene, β-Pinene, Mircene, Limonene, trans-β-Ocimene, Isomenthone, cis Isopulegone, α-Copaene, (+)-Aromadendrene, Germacrene-D, Biciclogermacrene y Germacrene-A.

Table 1: Different combination and concentration of growth regulators

Treatments	NAA (µM)	BA (µM)
SH- Control	0	0
SH-1	0,05	0,05
SH-2	0,05	0,50
SH-3	0,05	2,25
SH-4	0,50	0,05
SH-5	0,50	0,50
SH-6	0,50	2,25
SH-7	2,69	0,05
SH-8	2,69	0,50
SH-9	2,69	2,25
WP-Control	0	0
WP-1	0,05	0,05
WP-2	0,05	0,50
WP-3	0,05	2,25
WP-4	0,50	0,05
WP-5	0,50	0,50
WP-6	0,50	2,25
WP-7	2,69	0,05
WP-8	2,69	0,50
WP-9	2,69	2,25
B5- Control	0	0
B5-1	0,05	0,05
B5-2	0,05	0,50
B5-3	0,05	2,25
B5-4	0,50	0,05
B5-5	0,50	0,50
B5-6	0,50	2,25
B5-7	2,69	0,05
B5-8	2,69	0,50
B5-9	2,69	2,25

Conclusions

The auxin and citokinin ratio are a determinant morphogenic factor. Plants that have been grown with a high concentration of BA stimulate a great number of microshoots. WP-8 and SH-6 media resulted to be a good condition for stimulate the shoot length and number of nodes.

The major components obtained in all *in vitro* clones, demonstrated to have a similar composition.

Table 2: Major volatile compounds of H. multiflorum plantlets grown in MS, SH and WP media

Compound	MS	SH	WP
	(% areas)	(% areas)	(% areas)
Pulegone	63,76	62,56	63,45
Menthone	25,83	27,8	28,12
Isomenthone	2,24	2,1	2,03
Bicyclogermacrene	0,84	0,52	0,49
Germacrene D	0,27	0,22	0,32

Note: This study was presented at the ¨II Reunión de Biotecnología Aplicada a Plantas Medicinales y Aromáticas¨. (Second Biotechnology Meeting on Medicinal and Aromatic Plants), Córdoba, Argentina, 2009.

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Figures:

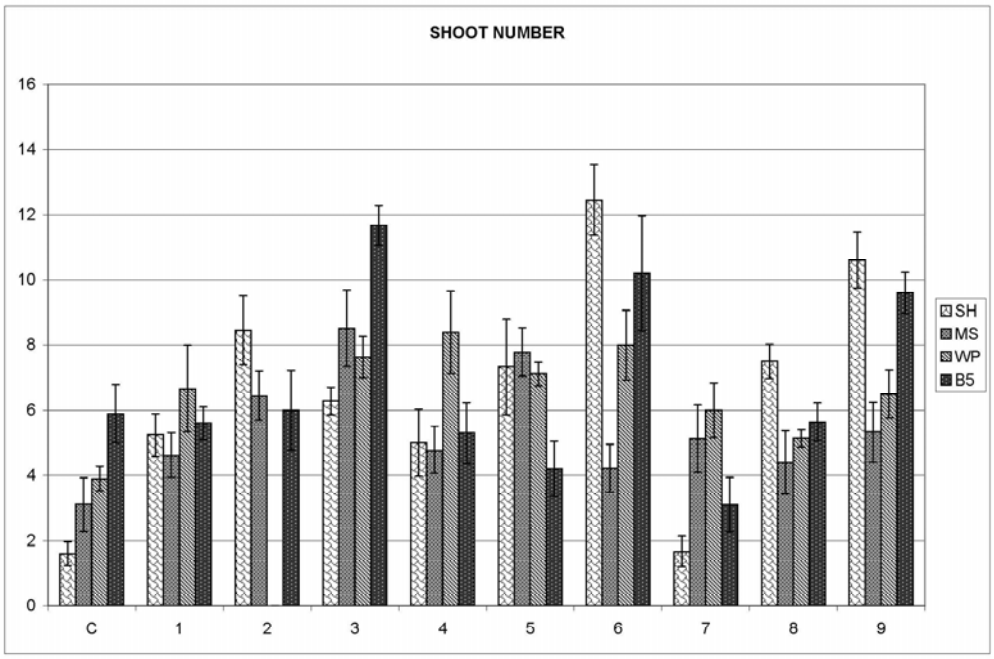

Figure 1: Shoot number of H. multiflorum plantlets grown in different media.

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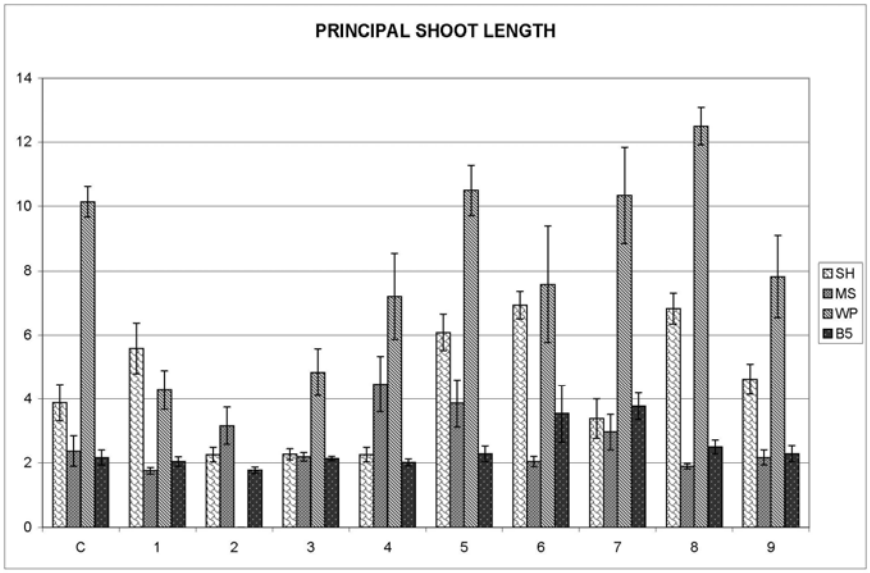

Figure 2: Principal shoot length of *H. multiflorum* plantlets grown in different media.

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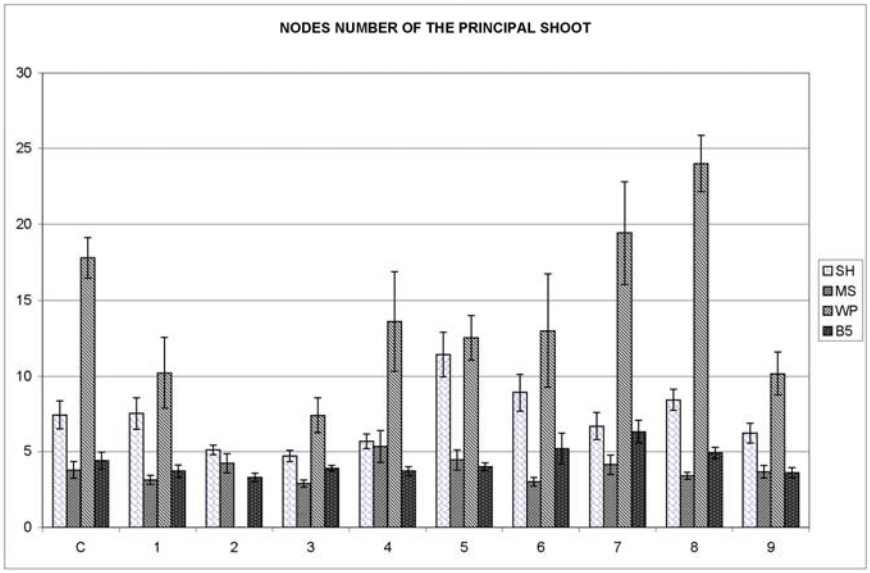

Figure 3: Number of nodes (principal shoot) of *H. multiflorum* plantlets grown in different media.