Effect of Macroelements on the Growth and Lobeline Production of *Lobelia inflata* L. Hairy Root Cultures

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Abstract

***Lobelia inflata* L. contains piperidine alkaloids. The main alkaloid is the pharmacologically active lobeline. We have studied the effect of macroelements (Mg^{2+}, Na^{+} and Ca^{2+}) on the growth and alkaloid production of hairy root cultures of *L. inflata* (clone 8009/f4). Macroelements influenced characteristically the linear growth and biomass formation of hairy roots. The greatest biomass formed in B5 media containing 1000 mg/l $MgSO_4$. It was found that the highest amount of lobeline (39 μg/g, dry weight) was reached at 75 mg/l $CaCl_2$. The results suggest that the optimal nutrient mixture for the best lobeline production was the basal B5 medium (containing 150 mg/l $CaCl_2$, 150 mg/l NaH_2PO_4) supplemented with 1000 mg/l $MgSO_4$.**

INTRODUCTION

Lobelia inflata L. (Lobeliaceae) is an indigenous plant to North-America. It contains piperidine alkaloids. The main alkaloid is lobeline which has a stimulatory effect on the respiratory centre. In large doses its effect resembles that of nicotine, so that it is used in anti-smoking preparations.

The investigation of genetically transformed cultures of *L. inflata* showed that the hairy roots are able to synthesise lobeline (Ishimaru et al., 1991; 1992; Yonemitsu et al. 1990). The effect of growth regulators (NAA, IAA and kinetin) and alkaloid precursor amino acids (Lys and Phe) on the biosynthetic activity of *L. inflata* hairy roots was studied (Bálványos et al., 2000; Bálványos et al., 2001). These results show that amino acids added to the media alone or in combination with hormones (IAA and kinetin) have characteristic effects on growth and secondary metabolite production of hairy root cultures. Many experiments examined the influence of macroelements on the biomass- and alkaloid production of hairy root cultures. Earlier we studied the effect of $MgSO_4$ on the linear growth and the biomass production of hairy root clone 8009/h2 (Bálványos et al., 1998). The hairy root clone grew more intensively on the medium containing 500 mg/l $MgSO_4$ than on the basal B5 medium. We repeated the study to investigate the effect of macroelements ($MgSO_4$, NaH_2PO_4 and $CaCl_2$) on the growth and lobeline production of the hairy root clone 8009/f4. On the basis of our previous results we applied modified B5 medium containing 500 mg/l $MgSO_4$ to investigate the effect of NaH_2PO_4 and $CaCl_2$.

MATERIALS AND METHODS

Establishment and Cultivation of Hairy Roots

Hairy root cultures of *Lobelia inflata* were obtained by direct infection of the sterile, two-month-old plants with *Agrobacterium rhizogenes* strain R1601. About 20 hairy root clones were isolated after 14 days. The antibiotic combination of cefotaxime 250 mg/l and ampicillin l000 mg/1 was added to the medium for several subcultures (3-5 times) to eliminate the bacteria. The axenic hairy roots were cultivated on solid, hormone-free culture media consisting of MS (Murashige and Skoog, 1962) or Gamborg (B5) salts and vitamins (Gamborg et al., 1968) with 2% sucrose.

We studied the effect of macroelements ($MgSO_4$, NaH_2PO_4 and $CaCl_2$). The concentration of $CaCl_2$ and NaH_2PO_4 was changed between 0-600 mg/l in modified B5

medium (containg 500 mg/l $MgSO_4$), but $MgSO_4$ was changed between 0-2000 mg/l in B5 medium. The cultures were collected on the 38th day of the cultivation period (Fig. 1).

The linear growth of the hairy roots was estimated by measuring root length (mm) every 3-4 days during culturing. Five pieces of hairy root tips approx. 2 cm in length were inoculated into each Petri dishes. For the estimation of linear growth of the hairy roots, 10 parallel replications were used and 8-10 replications for the determination of biomass formation (fresh weight after the collection and dry weight of the lyophilised cultures).

HPLC Analysis of Lobeline

The alkaloids of the hairy root cultures were analysed by HPLC (Spectra Physics P4000). Alkaloids from the lyophilised and powdered hairy root samples were extracted with 0.1 N HCl : methanol (1:1, v/v) using a Labsonic U ultrasound device (Braun, Germany). After the alkalisation of extracts (pH 9, 25% NH_4OH) they were purified with 3x40 ml $CHCl_3$. Each sample was evaporated to dryness and dissolved in 0.5-1 ml methanol.

The HPLC separation was performed using an Eurospher 100-C8 (5 μm) reverse-phase Vertex column (250 x 3 mm i.d.), with a pre-column (5 x 3 mm i.d.) using 33.2 : 66.8 (v/v) acetonitrile : 0.1 % trifluoracetic acid at a flow-rate of 1 ml/min. The lobeline peak was identified by the addition of authentic standard (lobeline base) and by diode-array detection (Bálványos et al., 2000).

RESULTS AND DISCUSSION

Effect of $MgSO_4$

We have studied the effect of $MgSO_4$ on the growth and lobeline content of the hairy roots (clone 8009/f4) and also on lobeline production. The concentration of $MgSO_4$ was changed between 0 and 2000 mg/l. B5 basal culture medium contains 250 mg/l $MgSO_4$ (control).

Linear growth was measured every 3-4 days during the cultivation period. The greatest linear growth was observed on B5 medium supplemented with 1000 mg/l $MgSO_4$, but higher concentration of $MgSO_4$ (2000 mg/l) stopped linear growth (Fig. 2A). The growth ceased on $MgSO_4$-free medium after 14 days.

$MgSO_4$ influenced characteristically the biomass formation of hairy roots as well. The greatest biomass formed in media containing 1000 mg/l $MgSO_4$ (Fig. 2A).

The lobeline content of the tissues was measured using an HPLC technique. The lobeline content of the control was 17 μg/g. It was found that the greatest amount of lobeline (24 μg/g, dry weight) was reached at the 1000 mg/l $MgSO_4$ concentration (Fig. 2A). Further increasing of $MgSO_4$ concentration of the B5 medium led to a decline in the lobeline content. The maximum lobeline production (1.7 μg/culture) was achieved in B5 medium containing 1000 mg/l $MgSO_4$ (Table 1).

Effect of $CaCl_2$

The concentration of $CaCl_2$ was varied between 0 and 600 mg/l in modified B5 medium containing 500 mg/l $MgSO_4$. B5 basal medium contains 150 mg/l $CaCl_2$ (control).

The greatest linear growth was measured in the control tissues. On $CaCl_2$-free medium the growth decreased after the 5th day and stopped at the end of the culturing period (Fig. 2B). The figure illustrates the dry weight and lobeline content of hairy roots on B5 media with different concentrations of $CaCl_2$ (0-600 mg/l). The greatest biomass formed in the control medium and in the medium containing 300 mg/l $CaCl_2$.

The highest lobeline level was measured at 75 mg/l $CaCl_2$ concentration (39 μg/g, dry weight). Higher concentrations of $CaCl_2$ decreased the lobeline content. Lobeline production was also calculated. The maximum lobeline production (1.7 μg/culture) was achieved in B5 medium containing 75 mg/l $CaCl_2$ (Table 2).

Effect of NaH_2PO_4

The effect of NaH_2PO_4 was investigated as well. The concentration of NaH_2PO_4 was varied in the modified B5 medium (containing 500 mg/l $MgSO_4$) between 0 and 600 mg/l. B5 basal medium contains 150 mg/l NaH_2PO_4 (control).

The linear growth of the hairy roots cultivated on media containing different concentrations of NaH_2PO_4 was similar to that of the cultures cultivated on control (150 mg/l NaH_2PO_4) media (Fig. 2C).

Maximum dry weight was measured in the control, but there was no significant difference between the biomass formation of the cultures grown on control medium and on that containing 600 mg/l NaH_2PO_4 (Fig. 2C).

As Figure 2C shows, the highest lobeline level (32 μg/g) was detected in tissues cultivated on media containing 600 mg/l NaH_2PO_4. The highest lobeline production was achieved on control medium and on medium containing 600 mg/l NaH_2PO_4 (Table 3).

CONCLUSIONS

We have studied the effect of macroelements on increasing growth and lobeline production of *L. inflata* hairy roots. It can be established that the macroelements influenced characteristically the growth and lobeline production of *L. inflata* hairy roots.

At 1000 mg/l $MgSO_4$ the linear growth of the hairy roots was very intensive. On $MgSO_4$-free and $CaCl_2$-free media, growth decreased significantly and stopped, but the linear growth of the hairy roots cultivated on NaH_2PO_4-free medium did not decrease during the culturing. The greatest biomass formation was observed in B5 medium containing 1000 mg/l $MgSO_4$. Analysing the lobeline content, it was found that the greatest amount of lobeline (39 μg/g, dry weight) was reached at the 75 mg/l $CaCl_2$ concentration.

The maximum lobeline production (1.7 μg/culture) was achieved in B5 medium containing 1000 mg/l $MgSO_4$ and it was significantly higher than the control (1.0 μg/culture). In contrast, the highest lobeline productions of cultures grown on media containing 75 mg/l $CaCl_2$ or 600 mg/l NaH_2PO_4 and control (containing 150 mg/l $CaCl_2$ or 150 mg/l NaH_2PO_4) were not significantly different.

The results suggest that the optimal nutrient mixture for the best lobeline production was the basal B5 medium (containing 150 mg/l $CaCl_2$, 150 mg/l NaH_2PO_4) supplemented with 1000 mg/l $MgSO_4$.

Literature Cited

Bálványos, I. Szőke, É. and Kursinszki, L. 1998. Effect of magnesium on the growth and alkaloid production of *Lobelia inflata* L. hairy root cultures. p.358-361. In: Magnesium and Interaction of Magnesium with Trace Elements. Ed. by Kiss, S. A. Published by the Hung. Chem. Soc., Budapest

Bálványos, I., Kursinszki, L. and Szőke, É. 2000. The effect of growth regulators on the biomass formation and lobeline production of *Lobelia inflata* L. hairy root cultures. Plant Growth Regulation, 34: 339-345.

Bálványos, I., Szőke, É. and Kursinszki, L. 2001. The influence of amino acids on the lobeline production of *Lobelia inflata* L. hairy root cultures. Plant Growth Regulation (in press)

Butenko, P. 1964. Kultura izolirovannich tkanej i physiologia morphogenesa rastenij. p.64. Moscow: Nauka.

Gamborg, O.K., Miller, R.A. and Ojima, K. 1968. Nutrient requirements of suspension cultures of soybean root cells. Exptl. Cell. Res. 50: 151-158.

Ishimaru, K, Sadoshima, S, Neera, S, Koyama, K, Takahashi, K and Shimomura, K. 1992. A polyacetylene gentiobioside from hairy roots of *Lobelia inflata*. Phytochemistry 31: 1577-1579.

Ishimaru, K, Yonemitsu, H. and Shimomura, K. 1991. Lobetyolin and lobetyol from hairy root culture of *Lobelia inflata*. Phytochemistry 30:2255-2257.

Krajewska, A. and Szőke, É. 1989. The studies on regenerated cultures of *Lobelia inflata*

L. Herba Pol. 35: 71-178.
Krajewska, A., Szőke, É., Petri, G., Botz, L. and Szarvas, T. 1987. Effect of new synthetic regulators on biomass and alkaloid production by callus tissues of *Lobelia inflata* L. Acta Bot. Hung. 33:407-411.
Murashige T. and Skoog F. 1962. A revised medium for rapid growth and bioassays with tabacco tissue cultures. Physiol Plant. 15:473-497.
Szőke, É. 1994. *In vitro* culture and the production of lobeline and other related secondary metabolites. p.289-327. In: Bajaj Y.P.S. (ed.) Biotechnology in Agriculture and Forestry 28, Medicinal and Aromatic Plants VII. Springer, Berlin, Heidelberg
Szőke, É., Bálványos, I., Kursinszki, L., Krajewska, A. Mészáros, A. and Neszmélyi, A. 2001. Studies on the alkaloid production of genetically transformed and non-transformed cultures of *Lobelia inflata* L. International Journal of Horticultural Science 2:65-71.
Yonemitsu, H., Shimomura, K., Satake, M., Mochida, S., Tanaka, M., Endo, T. and Kaji, A. 1990. Lobeline production by hairy root culture of *Lobelia inflata* L. Plant Cell Rep. 9:307-310.

Tables

Table 1. Relationship between $MgSO_4$ conc.of the medium and lobeline production (± SD, $p< 0.05$).

$MgSO_4$ (mg/l)	**0**	**250**	**500**	**1000**	**2000**
lob.prod. (µg/cult.)	nd*	1 ±0.3	1.4 ±0.2	1.7 ±0.3	1.1 ±0.2

* lobeline was not detected

Table 2. Relationship between $CaCl_2$ conc.of the medium and lobeline production (± SD, $p< 0.05$).

$CaCl_2$ (mg/l)	**0**	**75**	**150**	**300**	**600**
lob.prod. (µg/cult.)	0.7 ±0.1	1.7 ±0.4	1.5 ±0.3	1.3 ±0.3	0.8 ±0.2

Table 3. Relationship between NaH_2PO_4 conc.of the medium and lobeline production (± SD, $p< 0.05$).

NaH_2PO_4 (mg/l)	**0**	**75**	**150**	**300**	**600**
lob.prod. (µg/cult.)	0.8 ±0.3	0.8 ±0.1	1.4 ±0.2	0.9 ±0.2	1.6 ±0.8

Figures

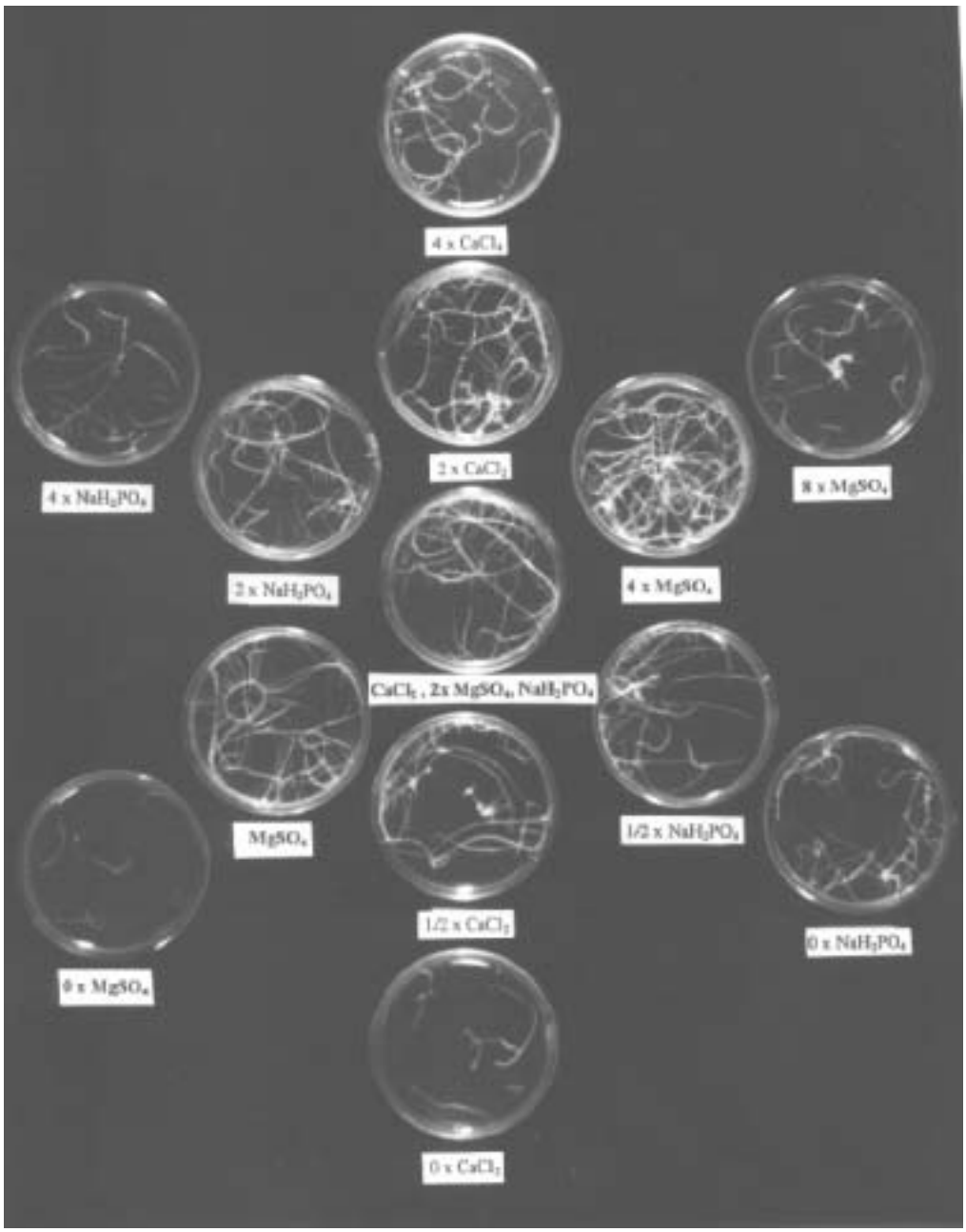

Fig. 1. *Lobelia inflata* hairy roots cultivated on B5 media containg $MgSO_4$ (0-2000 mg/l) and on modified B5 media (500 mg/l $MgSO_4$) containg $CaCl_2$ and NaH_2PO_4 (0-600 mg/l).

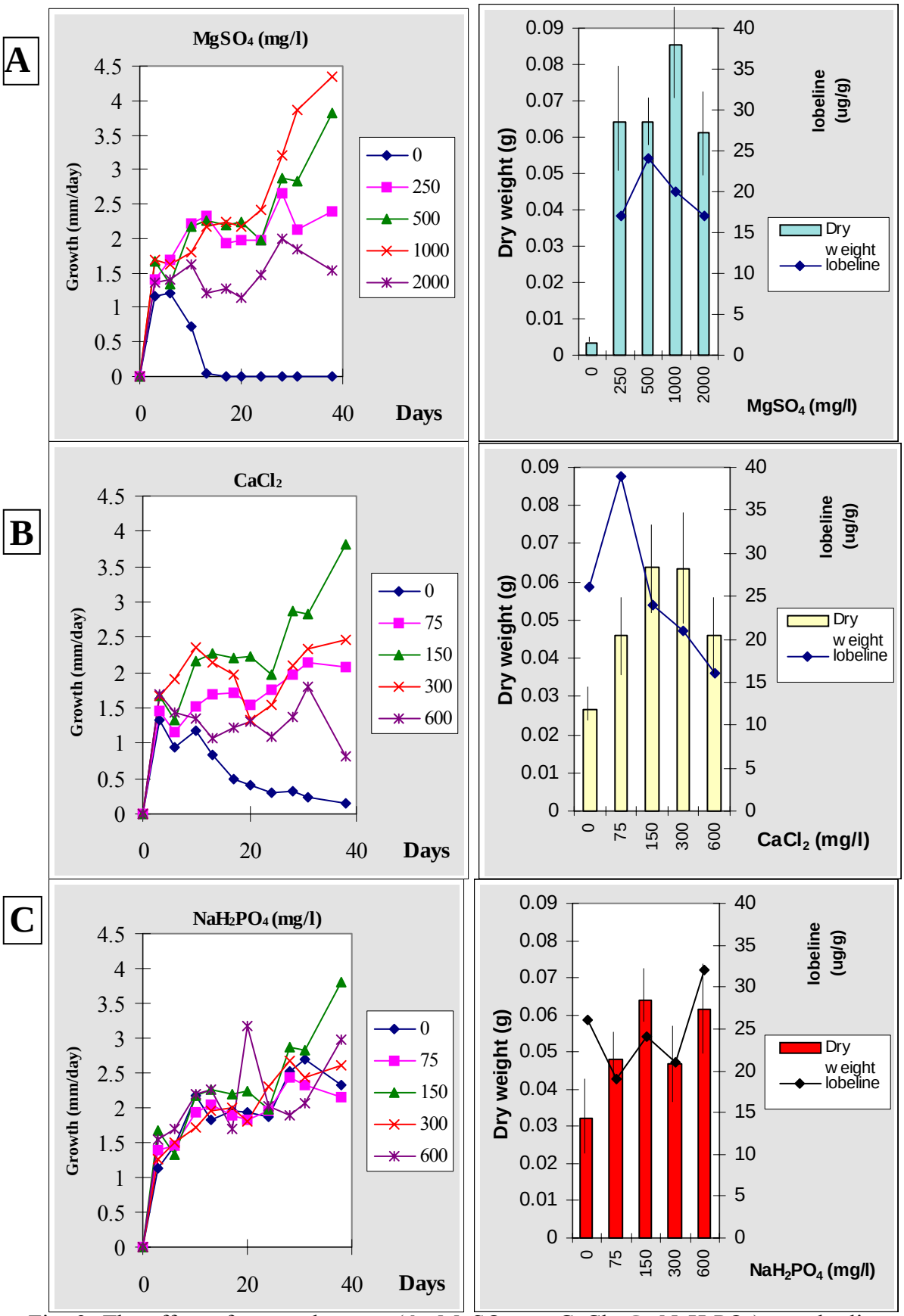

Fig. 2. The effect of macroelements (**A:** $MgSO_4$, **B:** $CaCl_2$ **C:** NaH_2PO_4) on the linear growth, dry weight and lobeline content of *L. inflata* hairy roots (clone 8009/f4).