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Studies on the alkaloid production of genetically transformed and non-transformed cultures of *Lobelia inflata* L.

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Key words: alkaloid, amino acid, growth regulator, hairy root, Lobelia inflata, lobeline

Abbreviations: 2,4-D: 2,4-dichloro-phenoxiacetic acid, AMS-2 medium: MS medium containing 1 mg/l kinetin and 1 mg/l 2,4-D, IAA: indole acetic acid, NAA: naphtalene acetic acid, Sz/11: 1,4-dihydroxy-phthalazine-biguanidine salt, Sz/28: N-isopropyl-benzimidazolium-chloride

Summary: The investigations of the growth and alkaloid production of cell suspension-, callus-, organized- and hairy root cultures from *Lobelia inflata* L. proved that these cultures are able to synthesize the characteristic piperidine alkaloids of the intact plant. Alkaloid precursor amino acids (Phe, Lys) and plant growth regulators affect not only the growth and differentiation of tissue cultures but also their secondary metabolism. The synthetic regulator Sz/11 combined with Phe increased the total alkaloid content considerably in callus- and organized cultures; regulator Sz/28 especially increased the lobeline content (in organized cultures in response to Lys, in callus tissues as a result of Phe application). With the aim of optimizing growth and alkaloid production of the genetically transformed hairy root cultures of *Lobelia inflata* L. we studied the effect of some growth regulators (NAA, IAA, kinetin) and precursor amino acids (Lys, Phe). The kinetin had inhibiting effect on the growth and lobeline production of the hairy roots. The IAA and NAA increased the biomass formation and lobeline production. The highest lobeline level was detected in tissues cultivated on hormone-free medium containing Phe.

Introduction

The herb of *Lobelia inflata* L. contains piperidine alkaloids. The Lobelia alkaloids are placed among the alkaloids of lysine origin, as the part that contains the nitrogen heteroatom comes from lysine. In the synthesis of these alkaloids, Phe as a precursor amino acid is also present (*Szőke* 1994).

The main alkaloid is the pharmacologically active lobeline, which has stimulative effect on the respiratory center and it has been applied in cases of asthma, collapse, gas- and narcotic poisoning. Lobeline also causes nausea. In large doses its effect resembles to that of the nicotine, so that it is used in preparations against smoking.

Wysokinska (1977) examined the influence of different regulators on growth and alkaloid production of the callus cultures of *L. inflata*. The tissues grew best on the culture medium containing 2,4-D (10^{-6} M). The culture medium containing NAA (10^{-7} M) induced rhizogenesis, while 2,4-D did not. The highest alkaloid content was attained under the influence of IAA (10^{-5} M) and NAA (10^{-7} M). 2,4-D inhibited the formation of lobeline. The cultivation of plant cells transformed by *Agrobacterium rhizogenes* is a new possibility to produce pharmacologically active secondary metabolites (*Ishimaru* et al. 1991, 1992, *Yonemitsu* et al. 1990).

We have studied the biosynthetic activity of callus-, cell suspension-, organized- and hairy root cultures of *L. inflata*

L. (*Krajewska* & *Szőke* 1989, *Krajewska* et al. 1986, 1987, *Bálványos* et al. 2000). The total alkaloid and lobeline contents were determined by photometric, densitometric and HPLC methods, respectively. ^{1}H and ^{13}C-NMR spectra of lobeline has been analysed first time by our group which will be completed with special interest to the structure- activity relationship (*Szőke* et al. 1998).

Material and methods

Cultivation

The callus tissues induced from the leaves of sterile seedlings were raised on Murashige-Skoog (MS) culture medium (*Murashige* et al 1962) in light (2500 lux). From callus tissues of leaf origin kept on a MS medium containing 1 mg/l kinetin and 1 mg/l 2,4-D (AMS-2), a vigorously growing tissue line could be produced. The regulators and the alkaloid precursor amino acids (Phe, Lys) were added to the culture medium in various concentrations.

Cell suspension cultures were raised in Braun-Melsungen Biostat-S type fermentor on basic culture medium (AMS-2), and on a culture medium containing Phe and Lys as supplement.

The changes in growth dynamics and alkaloid content of organized cultures raised on MS basic culture medium (2mg/l IAA and 0.2 mg/l kinetin) was analysed during the incubation period. The effect of alkaloid precursore amino acids (Lys and Phe) and growth regulators on the organized cultures was also studied.

Transformed root cultures of *Lobelia inflata* L. 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 1000 mg/l was added to the medium for several subcultures to eliminate the bacteria. The axenic hairy roots were cultivated on solid, hormone-free culture media consisting of MS 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 concentracion of $CaCl_2$ and NaH_2PO_4 was changed 0-600 mg/l in B5 medium, but $MgSO_4$ was changed 0-2000 mg/l. The hairy roots were cultivated on B5 solid medium containing growth regulators (IAA, NAA and kinetin) at 24±2 °C, in the dark. The applied concentrations of NAA and IAA were varied between 0 and 20 mg/l. The concentration of kinetin was changed between 0 and 5 mg/l. The alkaloid precursor amino acids (Lys and Phe) were applied in 10^{-4} M concentration.

Chemical analysis of alkaloids

The total alkaloid content in the callus,- suspension- and organized cultures was determined photometrically while the lobeline content was measured by TLC-densitometry at 249 nm.The quantity of total alkaloids was given in terms of dry tissue weight %. On taking up the concentration series for the determination of the total alkaloid content we used methanolic solution of lobeline hydrochloride. The reproducibility of the determination can be represented with a CV % = 3,81 value (n = 6).

The lobeline content of the hairy root cultures was determined by HPLC. Alkaloids from the lyophilized and powdered hairy root samples were extracted with 0.1 N HCl : methanol (1:1, v/v) using a Labsonic U ultrasound device. Samples of this solution were purified by solid-phase extraction (SPE cartridge: Supelclean LC-8, 3 ml). The HPLC system used consisted of a Spectra Physics P4000 quaternary gradient pump, a FOCUS scanning UV-VIS detector in combination with a Rheodyne 7125 injector (5 µl loop volume). Separation was achieved on an Eurospher 100-C8 (5 µl) 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. (*Figure 1*).

Concerted use of two-dimensional ^{1}H- and ^{13}C-NMR spectroscopy (COSY, HETCOR) allowed assignments of the resonances of the free base dissolved in $CDCl_3$. (*Figure 2*).

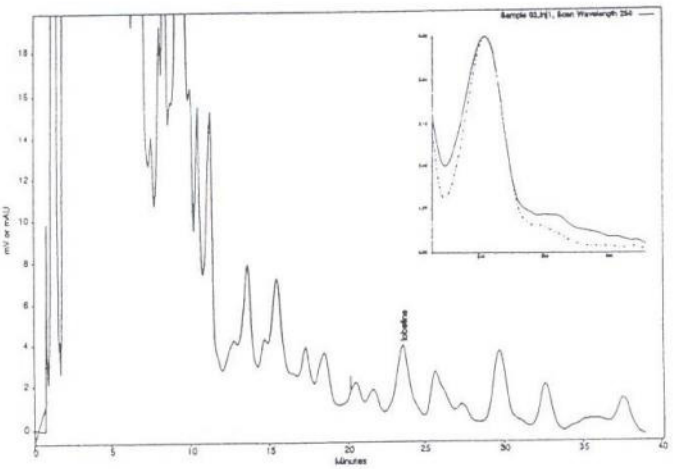

Figure 1 HPLC chromatogram from L. inflata hairy root (clone 8009/h7). Column: Eurospher 100-C8; 5mm; eluant: acetonitrile: 0.1% trifluoracetic acid (33.2: 66.8, v/v). Flow rate: 1ml/min. UV detection at 250 nm

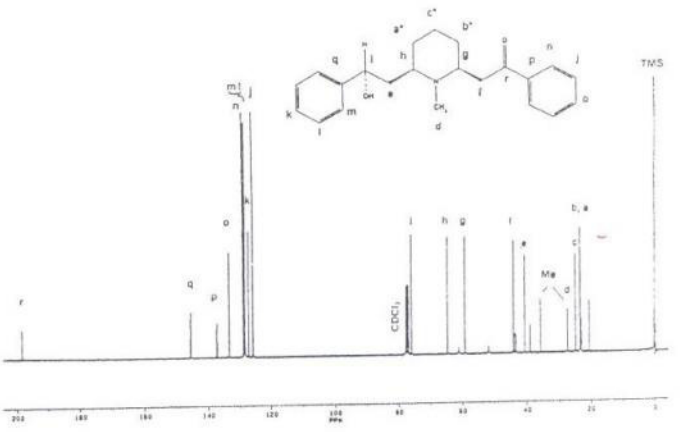

Figure 2 13C-NMR spectrum of lobeline (free base) at 75 MHz and room temperature in CDCl3 solution.

The concentration is 3% w/v. Tetramethyl-sylane is used as internal reference. Theoretical energy calculations for minimized energy geometries (Alchemy-III from Tripos Inc.) with NMR restraints resulted in candidates for the probable bioactive conformation of lobeline.

Results and discussion

Callus- and suspension cultures

Callus cultures (*Figure 3*), achiving maximum weight and alkaloid content after 8 weeks of incubation were collected for chemical analysis.

Between the 6th and the 8th week the growth of the cultures is still intensive (about 50% increase in fresh weight).

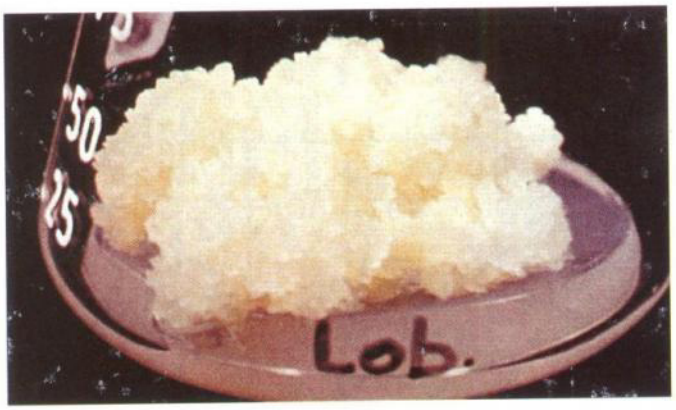

Figure 3 Callus culture of L. inflata

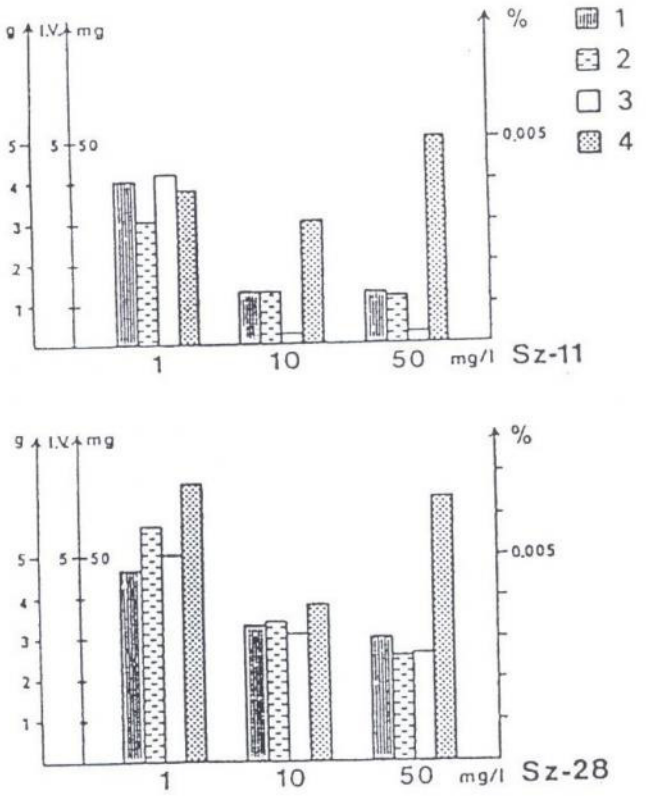

Figure 4 Effects of regulators on growth and alkaloid content of L. inflata callus tissue. (1. Fresh weight (g); 2. Increased value on dry weight basis; 3. Daily growth (mg); 4. Alkaloid content (%))

When the cultures were kept on hormone-free culture medium (S), their growth intensity decreased considerably.

The effect of synthetic growth regulators (Sz/11, Sz/28) was investigated (*Figure 4*). The Sz/11 (1,4-dihydroxy-phthalazine-biguanidine salt) regulator added at a concentration of 1 mg/l considerably increased the growth of the cultures compared to the S culture medium, while at higher concentrations (10 and 50 mg/l) it caused significant growth inhibition. On the other hand, in cultures showing the lowest rate of growth (50 mg/l Sz/11) the alkaloid content was higher than in the vigorously growing ones containing 1 mg/l Sz/11, or in those raised either on S or on AMS-2 culture medium. However, as regards the alkaloid production (per dry weight of one culture) of the callus tissues kept on a culture medium containing 1 mg/l Sz/11 are considered better.

The regulator Sz/28 (N-isopropyl-benzimidazolium-chloride) also stimulated the growth of the callus tissue, although an increase in its concentration similarly to Sz/11-resulted in significant growth inhibition.

In order to increase further the alkaloid production of the cultures, phenylalanine and lysine were added to the culture medium. Both regulators, combined with the amino acids, increased the biomass two- to threefold (compared to kinetin), and increased the alkaloid content even more. On a culture medium containing Sz/11 and 2,4-D, as well as 10^{-4} M phenylalanine, the total alkaloid content was 7.5-fold, but since the growth of the cultures is also more intensive, the alkaloid production (mg/culture) is already nearly 20-fold compared to the culture medium of similar composition but containing kinetin. On the other hand, for the biosynthesis of lobeline, the effect of Sz/28 is very favorable; the lobeline content is also highest in callus tissues raised on a culture medium containing phenylalanine.

Both on basic culture medium and on one containing a regulator, callus tissues synthesize extremely large amounts of a so far unknown component whose quantity is reduced to minimum if the culture medium contains alkaloid precursor amino acids. On the basis of its NMR spectrum, the compound is saturated and contains aromatic molecule parts which can be connected to lobeline, it can be regarded as a lobeline derivative. By mass spectrometry its molecular weight is 485.

It was found that in the suspension cultures, compared with the basic culture medium, the alkaloid content rose twofold in response to the alkaloid precursor amino acids (*Table 1*). The cultures also synthesize very large quantities of the 485 molecular weight compound produced in the callus tissues.

Table 1 Total alkaloid content in the callus and cell suspension cultures

Medium (%)	Culture	Total alkaloid content
AMS-2	Callus	0.003
AMS-2+Phe+Lys	Callus	0.003
AMS-2	Suspension	0.003
AMS-2+Phe+Lys	Suspension	0.006

Organized cultures

Analyzing the changes in growth dynamics and alkaloid content of organized cultures raised on MS basic culture medium (2 mg/l IAA and 0.2 mg/l kinetin) during the incubation period, it was found that up to the 8th week they increased dynamically, but hardly changed afterwards (*Figure 5*).

The total alkaloid content in the cultures is lower than in the intact plant (MS: shoot 0.232%, root 0.147%; intact: shoot 0.395%, root 0.30%), but substantially exceeds that of the callus tissue. In the shoot we identified lobeline, norlobeline, and lobelanidine.

If besides 2 mg/l IAA, Sz/11 is added to the culture medium in different concentration (0.2, 1, 10 mg/l), its increasing concentration causes a gradual decrease in the fresh weight of the herb. However, the alkaloid content of herb and root is the highest in response to 10 mg/l Sz/11. The alkaloid production substantially exceeds that of cultures raised with a similar concentration of kinetin.

With a joint application of 2 mg/l IAA and Sz/28 (in concentration of 0.2, 1 and 10 mg/l) the cultures develop well at all the three concentrations. Parallel with an increase in the concentration the alkaloid content gradually rises. In response to 10 mg/l Sz/28 the lobeline content is three-times as high (0,41 %) as when Sz/11 is applied.

All in all, it can be established that the alkaloid content of organized cultures is essentially higher if the cultures are raised on a culture medium containing both IAA and some synthetic cytokinin-like regulator (than the cultures raised on a medium containing a synthetic regulator alone).

Therefore, to exercise further influence on the alkaloid content the culture medium containing 1 mg/l IAA and 1 mg/l of a synthetic regulator (Sz/11 or Sz/28) was completed with alkaloid precursor amino acids (Phe, Lys) too (*Figure 6*).

When the compound Sz/11 is applied, the biomass- and total alkaloid production by the herb and root of the cultures will be optimum if Phe is added to the culture medium. In case of the culture medium containing 2 mg/l IAA the alkaloid precursor amino acids are added beside Sz/28, it is Lys that has an optimum effect on the biomass production of the herb and root of the cultures, as opposed to the application of Sz/11 when the effect of Phe was outstanding. It should be noted that the regulator Sz/11 with either amino acid induces the lobeline biosynthesis to a lesser extent than the combination of Sz/28 and Lys.

Finally, it can be established that the biomass- and alkaloid production of both the herb and the root are

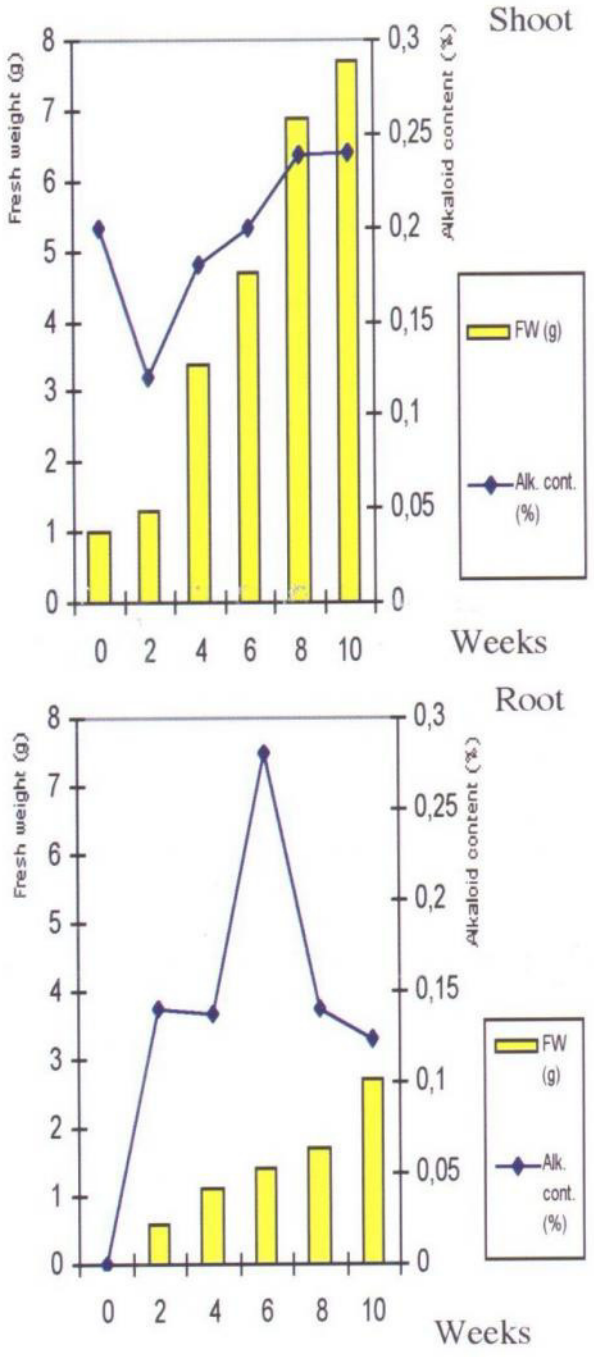

Figure 5 A, B. Changes in the growth and alkaloid content of organized culture (A: shoot; B: root) during the culturing period

stimulated by the combined application of synthetic regulators (beside IAA) and alkaloid precursor amino acids.

Vegetative Micropropagation

In order to increase the efficiency of propagation of explants in the course of in vitro micropropagation, the cultures were treated with a chloro-acetyl-hexamethylene type (TI-35) compound. Under the influence of this compound, the composition of the wax components and membrane lipids becomes similar to that of the intact plants raised in the field, that is, the wax layer thickens, the amount

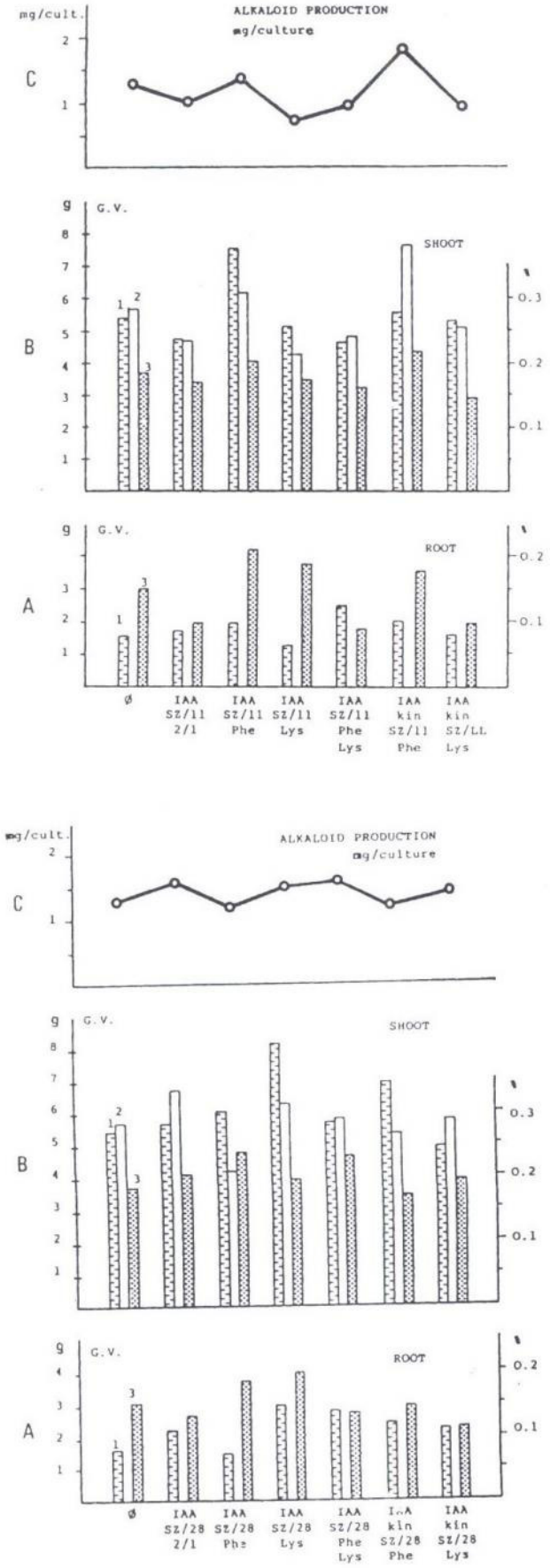

Figure 6 Effects of regulators and amino acids on the biomass- and alkaloid formation of organized L. inflata cultures (1. Fresh weight (g); 2. Growth value by dry weight; 3. Total alkaloid content (%))

of acids in it decreases, while the proportion of primary alcohols increases, which prevents the overpermeability of the leaf surface.

As for the lipids, the proportion of unsaturated fatty acids increases, which makes the membrane more flexible. As a result of these changes the drought tolerance of the plants increases.

Organized cultures treated with the compound TI-35 tolerated transplantation stress well compared with the control group (the rate of survival changed according to a maximum curve). At a concentration of 5 mg/l the compound had a favorable effect on the survival of the explants, in fact, nearly 100 %.

In the organized cultures treated with TI-35, the number of stomata increased significantly. This is a favorable tendency considering that this numerical increase makes it possible for the plants to react quickly to fluctuation in humidity in the very instant of transplantation.

It should be noted that the percentage of alkaloid content of the shoot in plants raised under greenhouse conditions agreed, in essentials, with that of intact plants grown in the field. It is interesting, however, that the alkaloid content of the root was in each treatment very high, about four times higher than that of in the root of the intact plant (*Table 2*).

Table 2 Changes in the alkaloid content of organized cultures of L. inflata in response to treatment (before and after setting out).

TI-35 mg/l		In vitro		In vivo	
		Total alkaloid content (%)	Alkaloid prod. mg/test tube	Tota alkaloid content (%)	Alkaloid prod. mg/plant
0*	Shoot	0.278	1.89	0.478	1.32
	Root	0.151	1.01	1.232	0.83
5	Shoot	0.206	1.58	0.425	1.66
	Root	0.163	1.11	1.630	0.43
10	Shoot	0.246	1.99	0.397	1.78
	Root	0.078	0.61	1.105	0.52
20	Shoot	0.200	1.62	0.403	1.43
	Root	0.068	0.57	1.159	1.05
1**	Shoot			0.395	
	Root			0.308	

*Control
**Intact plant

Hairy root cultures

The hairy roots (*Figure 7*) were cultivated on B5 hormonfree basal media. We have chosen clone 8009/h7 which has the greatest biomass production, in order to study the effects of plant growth regulators (NAA, IAA and kinetin) on the biosynthetic activity.

NAA applied in the concentration range 2–20 mg/l affected the growth and lobeline production of the hairy roots growing on B5 solid medium. The greatest linear growth occurred on hormone-free, B5 basal medium

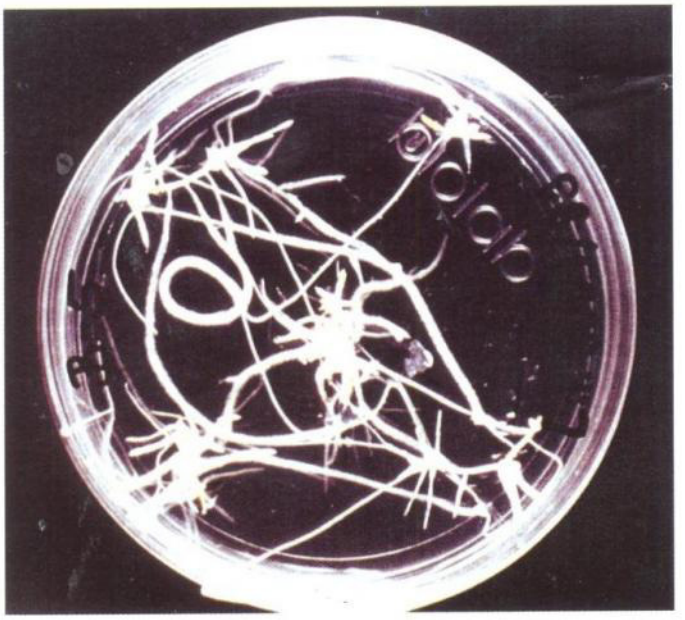

Figure 7 Hairy root culture of L. inflata

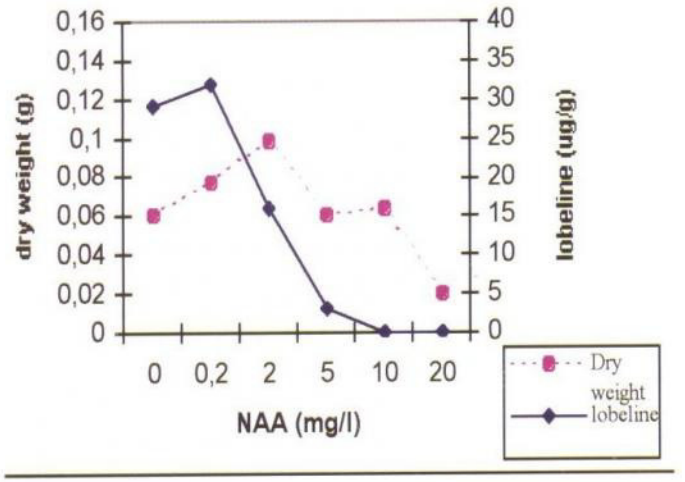

Figure 8 The effect of NAA (0.2–20 mg/l) on dry weight and lobeline content of Lobelia inflata hairy root clone 8009/h7 cultivated on B5 medium (± SD, $p < 0.05$).

(control). Increasing the concentration of NAA decreased and postponed linear growth, but the number of lateral roots increased considerably.

The dry weight of the cultures increased, reaching a maximum on medium containing 2 mg/l NAA. At 0.2 mg/l NAA lobeline content increased to the greatest lobeline level (*Figure* 8).

Higher concentrations of NAA decreased the lobeline content, and in the cultures on medium supplemented with 10 and 20 mg/l NAA lobeline was not detected at all. In the hairy root clone (8009/h7), one and half times greater lobeline production was detected than in control tissues cultivated on 0.2 mg/l NAA containing media (*Table 3*).

The concentration of IAA was varied between 0.2 and 20 mg/l. At higher concentrations of IAA the linear growth was reduced and delayed, but the number of lateral roots increased in a way similar to that found in NAA treated cultures. The greatest biomass formed in media containing 5 mg/l IAA. This is more than twice the dry weight of cultures grown on basal B5 medium, and more than the dry weight of the cultures on media containing NAA at any concentration.

It was found that the greatest amount of lobeline was reached at the 0.2 mg/l IAA concentration (*Figure 9*).

Increasing further the IAA concentration of the medium led to a decline in the lobeline content. It could not be

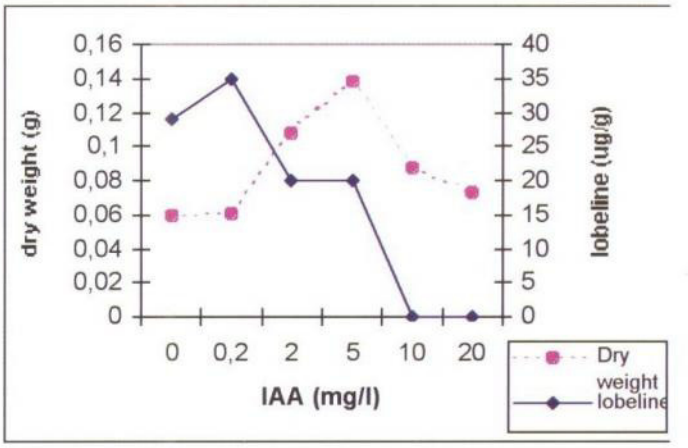

Figure 9 The effect of IAA (0.2–20 mg/l) on dry weight and lobeline content of Lobelia inflata hairy root clone 8009/h7 cultivated on B5 medium (± SD, $p < 0.05$).

detected in the cultures at the 10- and 20 mg/l IAA concentration, which was similar to the inhibition effect of NAA.

The maximum lobeline production (2.8 µg/culture, dry weight) was achieved in B5 medium containing 5 mg/l IAA (*Table 3*).

We also studied the simultaneous effect of IAA and kinetin in combination over the concentration range 0.2–5 mg/l. We established that IAA increased the biomass formation in the hormone combinations, but kinetin had a negative effect on the growth of the hairy roots. Administration of kinetin in culture medium decreased not only the biomass but also the lobeline content.

Table 3 Relationship between NAA and IAA concentration (0–20 mg/l) of the medium and lobeline production (± SD, $p < 0.05$).

NAA (mg/l)	0	0.2	2	5	10	20
lobeline prod. (µg/culture)	1.7	2.5	1.5	0.2	n.d.*	n.d.*
IAA (mg/l)	0	0.2	2	5	10	20
lobeline prod. (µg/culture)	1.7	2.1	2.2	2.8	n.d.*	n.d.*

*lobeline was not detected

The effect of amino acids and growth regulators on the biomass formation of the 6 weeks-old cultures was also studied. Between the dry weight of cultures grown on hormone-free media containing only Phe or Lys and the control were not significant differences. But the dry weight was decreased by the addition of Phe+Lys to the basal media. On the other hand, the growth wasn't inhibited if the Phe+Lys containing medium was supplemented with growth regulators.

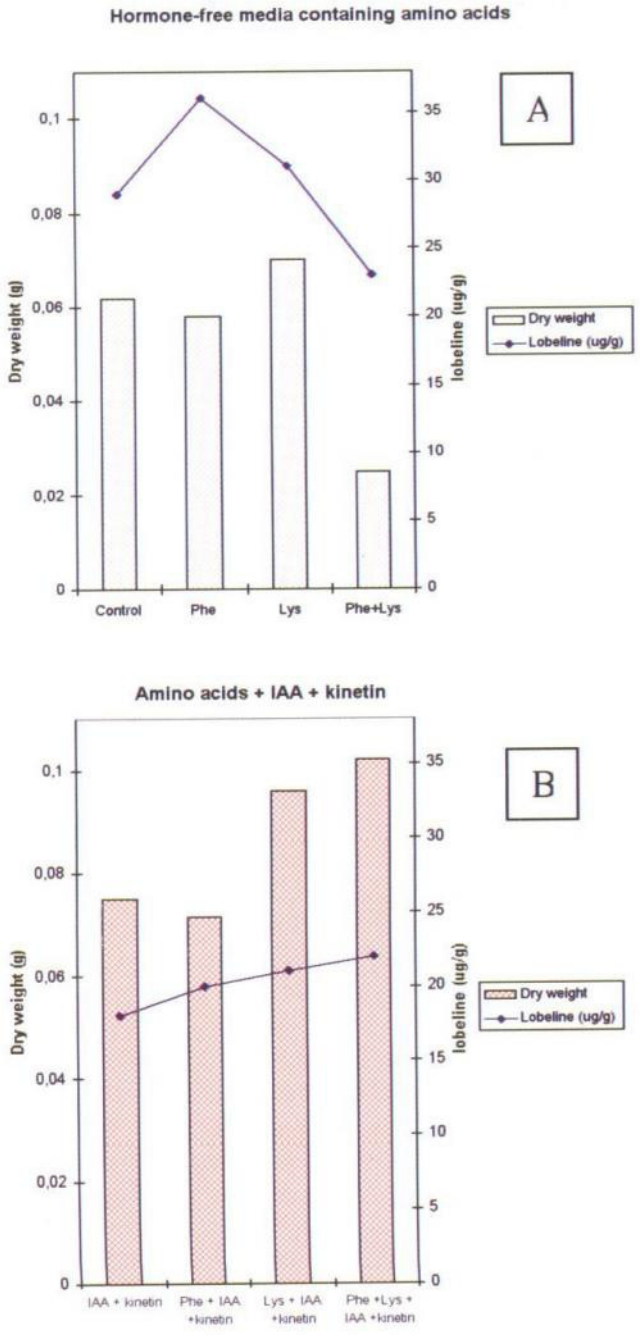

Figure 10 Dry weight (g) and lobeline content (mg/g) of Lobelia inflata hairy roots (± SD, $p< 0.05$). A/ Cultivation on hormone-free B5 media containing amino acids (10-4 M Phe, 10-4 M Lys and 10-4 M Phe+Lys). B/ Cultivation on B5 media containing amino acids (as in the foregoing) and growth regulators 2 mg/l IAA+ 0.2 mg/l kinetin. Control: B5 basal medium.

Administration of growth regulators (2 mg/l IAA and 0.2 mg/l kinetin) to the medium affected the biomass formation of the tissues resulting a maximal dry weight on the medium containing both amino acids and hormones (*Figure 10*).

The highest lobeline level (36 µg/g) was detected in tissues cultivated on hormone-free media supplemented with Phe. The lobeline content increased also on hormone-free media for the effect of Lys, but the Phe+Lys in combination had negative effect on the lobeline synthesis. Conversely the lobeline content decreased in the tissues grown on hormone containing media (with the exception of the cultures grew on media containing Phe+Lys).

By the addition of alkaloid precursor amino acids and hormones it succeeded to increase the lobeline production. The highest levels of lobeline production was achieved on hormone-free medium containing only Phe or Lys. If both amino acids were added to the basal B5 medium the lobeline production decreased greatly, but for the effect of growth regulators the lobeline production was maximal. In the last case the lobeline production was high because of the greater biomass chiefly (*Table 4*).

Table 4 Relationship between the content of amino acids, hormones in the medium and lobeline production (µg/culture) of the hairy root cultures

	Amino acid-free medium	Phe	Lys	Phe + Lys
Hormone-free medium	1.7 *	2.1	2.2	0.6
IAA+kinetin	1.3	1.4	2.0	2.2

*Control

It can be established that the application of alkaloid precursor Phe or Lys in hormone-free medium resulted an increase of lobeline production without that the growth of the cultures delayed. The lobeline production increased as the hormones were combined with Lys or Phe+Lys but it was not higher than the maximum levels on hormone-free medium.

These results show that amino acids added to the media alone or in combination with hormones have characteristic effects on growth and secondary metabolite production of *L. inflata* genetically transformed and non-transformed cultures.

Acknowledgement

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