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CULTIVATION POSSIBILITIES OF IN VITRO PROPAGATED *Lobelia Inflata* IN THE AGRICULTURE

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Abstract: *Lobelia inflata* L. is a medicinally important species of the *Lobeliaceae* family. It is native to North America and contains numerous piperidine alkaloids. It is important to increase the biomass and lobeline content of *in vitro* plant by nitrogen and magnesium treatments. The aim of this research was to examine the effect of $MgSO_4$ and NH_4NO_3 fertilization on biomass and on lobeline content of *in vitro* propagated *L. inflata*.
Keywords: *Lobelia inflata*, *in vitro*, propagated, cultivation, agriculture

INTRODUCTION

Lobelia inflata L. (Indian tobacco) is a traditional medicinal plant native to North America. The plant can be introduced in Hungary. It is mainly an annual plant [19], [35], [41], but its biennial populations can also be found [13]. *Lobelia inflata* belongs to the order Campanulales, to the family Lobeliaceae [10]. The herba contains several piperidine skeleton alkaloids [16], [23], [24]. Its main alkaloid is the lobeline, which due to its stimulating effect on the respiratory centre is used in cases of gas- and narcotic poisoning [17], [40]. Recently, significant amounts of polyacetylene compounds have been isolated from above ground organs of the plant (lobetyol, lobetolin and lobetyolin) [3], [16]. Recently, the species has come into the limelight due to research on CNS, drug abuse and multidrug resistance [1], [7], [8], [14], [28], [30]. Dwoskin and Crooks [14] described a novel mechanism of action and potential use of lobeline as a treatment for psychostimulant abuse.

It is important to increase the biomass and lobeline content of the plant by nitrogen and magnesium treatments in vitro [3], [36], [37] in open field [42], [43], [44], [45], [46]. There was a favourable effect of NH_4^+ and NO_3^- on the biomass formation of in vitro cultures [11], [21], [38], and aquatic cultures [15], [31]. Britto and Kronzucker [12] described the inhibitory effect of ammonia on growth in open field conditions. Nitrogen regulates the expression of specific proteins through mechanisms affecting transcription and/or mRNA stability [27], [34]. Nitrogen is incorporated into amino acids and may also serve as a reprogramming signal for the metabolism of nitrogen and carbon, resource allocation, and root development [47]. Nitrogen sources are important for secondary product synthesis of compounds such as alkaloids [49], anthocyanins, and shikonin from cell suspension cultures [20]. Interestingly, the NH_4^+-to-NO_3^- ratio in the medium affects not only the growth of plant cell cultures [39], but also the production of secondary compounds [33]. The ammonium/nitrate ratio controls the pH of growth media, stimulates morphogenesis and embryogenesis, and thus it is important in inducing callus formation in many woody plant cultures. However, all the aforementioned effects of the culture medium differ from one species to another and from one compound to another [2], [9], [32]. Therefore, it is necessary to establish a reproducible externally applied NO_3^-/NH_4^+ ratio for the stable production of large quantities of special metabolites.

Several previous experiments examined the influence of macroelements on growth and alkaloid production of hairy roots [4], [6].

The aim of this research was to study the effects of $MgSO_4$ and NH_4NO_3 fertilisation on biomass and alkaloid/lobeline production of in vitro cultivated L. inflata in Hungary.

MATERIAL AND METHODS

– Open field description

The open field trials were carried out in 2011 in Mosonmagyaróvár, University of West-Hungary (My

PhD work was in this institute). Nitrogen and Magnesium were applied in the form of ground fertilizers. The nutrients were applied in the following methods and quantities in 2011: untreated (control), 50 kg ha^{-1} N-, 100 kg ha^{-1} Nitrogen ground fertilizer, 50 kg ha^{-1} Magnesium- and 100 kg ha^{-1} Mg ground fertilizers. Table I. summarize the specification of soil values. An extended soil analysis was carried out according to standard methods of UIS Ungarn laboratory (Hungary, Mosonmagyaróvár).

Table 1. Specification of soil values (2011)

Appellation	Measure	Values
pH_{KCl}	-	7.12
salt%	m $m\%^{-1}$	0.02
humus%	m $m\%^{-1}$	3.08
$CaCO_3$%	m $m\%^{-1}$	10.7
P_2O_5	mg kg^{-1}	358
K_2O	mg kg^{-1}	518
Na	mg kg^{-1}	54.3
Mg	mg kg^{-1}	310
NO_2-NO_3-N	mg kg^{-1}	20.1
SO_4	mg kg^{-1}	8.75
Cu	mg kg^{-1}	4.21
Mn	mg kg^{-1}	20.4
Zn	mg kg^{-1}	18.5

In the open field trials, Mg (2%) - and N (34%) fertilizers were spread onto the soil surface, one day prior to transplanting. Transplanting of in vitro *Lobelia inflata* plants into open field soil was carried out on 26th May 2011. The number of plants per plot was 40. The experimental design was randomized blocks with 4 repetitions. During cultivation, mechanical weed control was applied. Plant heights (cm) were measured three times (22nd July, 29th July and 7th August) in 2011. In each treatment group 8 plants were measured (dry biomass production, g $plant^{-1}$ of *L. inflata* herb).

The first harvesting was on 9-10th August 2011. During harvesting, the plants were flowering and the biomasses were recorded. After harvesting, the plants were dried in a shaded and well-ventilated glasshouse. The dry weight determination was carried out in early September. The flowering phenophase was observed in the period of July to September [25]. The statistical analysis was preformed with SPSS v19 software [18]. The mean differences were regarded as significant at the 0.05 level.

– **Laboratory trials**

Chemicals and reagents. (-) Lobeline hydrochloride was purchased from Sigma-Aldrich (St Louis, MO, USA). Lobelanidine and norlobelanine were kindly provided by the Research Institute of Medicinal Plants, Poznan, Poland. Acetonitrile and methanol were of HPLC grade (Fisher Scientific, Loughborough, Leics, UK). Water was purified with Millipore (Billerica, MA, USA) Milli-Q equipment. All other reagents were of analytical reagent grade.

Alkaloid Extraction. The herb or roots of *L. inflata* (0.5000 g), dried and powdered, were extracted with 1 × 20 ml and 2 × 10 ml of 0.1 N HCl–methanol (1:1, v/v) by sonication (Braun Labsonic U, Melsungen, Germany) for 3 × 10 min. After centrifugation (6,000 rpm for 10 min, 2,500 g) and filtration, the methanol was evaporated and the remaining aqueous phase was diluted to a stock solution (to 25.00 ml) with 0.1 N HCl. Samples of this solution were purified with solid-phase extraction (SPE).

Determination of total alkaloid content. The total alkaloid content was determined by a spectrophotometric method [44], elaborated by Mahmoud and El-Masry [29] and modified by Krajewska [22].

Sample preparation by solid phase extraction (SPE) for analysis of alkaloids by HPLC (High Performance Liquid Chromatography). 3 ml Supelclean LC-8 columns (Supelco, Bellefonte, PA, USA), were used for SPE. 10.00 ml of the stock solution was loaded on to the SPE columns, then washed with 2.5 ml water to remove matrix. The alkaloid containing fraction was eluted with 2 × 2.5 ml methanol. According to Kursinszki et al. [23] the recovery of lobeline from the SPE step was total, determined by HPLC.

HPLC-DAD conditions. LC analysis was performed on a Surveyor LC system (Thermo Finnigan, San Jose, CA, USA) consisting of a quaternary gradient pump with an integrated degasser, a PDA detector, and an autosampler. Thermo Finnigan ChromQuest 4.0 software was used for data acquisition, processing, and reporting. Compounds were separated on a Knauer Eurospher 100-C8 (5 µm) reversed-phase column (250 × 3 mm i.d.; Berlin, Germany) integrated with a precolumn (5 × 3 mm i.d.). The column temperature was 25 °C and the injection volume 5 µL. The mobile phase was 30:70 (v/v) acetonitrile–0.1% trifluoroacetic acid. The flow-rate was 0.8 ml min^{-1}. The lobeline peak was identified by the addition of authentic standard, by diode-array and MS/MS detection.

Quantitative determination of alkaloids by HPLC. Determination of (-)-lobeline was performed by the external standard method. Standard solutions containing lobeline at 2.25 - 80 µg ml^{-1} were prepared in 0.1 N HCl. The calibration graph for lobeline was constructed by plotting the peak areas against the corresponding concentrations. The concentration of lobeline in samples was calculated from its peak area by use of the calibration plot. Validation studies proved that both the repeatability of the method and the recovery was good [23]. The amounts of lobeline derivatives: norlobeline, norlobelanine and lobelidine were expressed in lobeline.

HPLC-MS/MS experiments. LC/MS analysis was performed on an Agilent 6410 Triple Quad system using electrospray ionization in positive ion mode. Chromatographic conditions were the same as described earlier, except that 30 mM ammonium formate (pH 2.80) was used instead of 0.1% trifluoroacetic acid. The injection volume was 10 µl. By solvent splitting, 40 % eluent was allowed to flow into the mass spectrometer. The conditions of the LC-MS/MS studies were as follows: nebulizer pressure 45.0 psi, drying gas flow rate 9 l min^{-1}, drying gas temperature 350 °C, capillary voltage 3500 V, scan range from m/z 50 to 700 at collision energy of 15 or 20 eV depending on the molecular structure.

The lobeline content was determined by HPLC method designed by Yonemitsu et al. [48] and modified by Bálványos et al. [5], Kursinszki and Szőke [24].

RESULTS

Literature references on the mineral nutrition of *L. inflata* are scarce, although it is one of the basic factors of successful cultivation of this species.

Table 2. Plant height (cm) of in vitro *Lobelia inflata* in 2011

Treatments		Height of the plants (cm) 22nd July	29th July	7th August
Control	Mean	24.75	33.75	40.25
	Number	8	8	8
	St. deviation	6.99	6.52	5.70
	Minimum	11	20	27
	Maximum	32	41	45
50 kg ha^{-1} N ground fertilizer	Mean	30.38	38.63	47.75
	Number	8	8	8
	St. deviation	12.59	11.02	7.50
	Minimum	14	23	36
	Maximum	50	56	60
100 kg ha^{-1} N ground fertilizer	Mean	29.25	37.88	45.38
	Number	8	8	8
	St. deviation	6.36	6.33	5.15
	Minimum	19	28	37
	Maximum	39	47	54
50 kg ha^{-1} Mg ground fertilizer	Mean	30.75	38.38	43.00
	Number	8	8	8
	St. deviation	6.16	6.05	6.97
	Minimum	22	29	33
	Maximum	39	47	54
100 kg ha^{-1} Mg ground fertilizer	Mean	26.25	36.50	45.13
	Number	8	8	8
	St. deviation	8.68	7.54	5.84
	Minimum	14	25	36
	Maximum	41	50	56

Our experiments were aimed at clearing the basic nutrient requirements. It could be established that in the form of ground fertilization, both nitrogen and magnesium had a favorable effect on the formation of biomass and lobeline content in open field conditions. Some information is available from in vitro hairy root experiments carried out by Bálványos [3], according to whom the various nutrients he tried (Mg, Ca, Na, N), Mg had proved to be most effective in increasing both the dry biomass and lobeline content.

Table 3. Tukey HSD test of Lobelia inflata (parameter: plant height, cm)

Date of measu-rements	Treat-ments (A)	Treatments (B)	Mean Difference (A-B)	St. Error	Significance Level
22nd July	Control	50 kg ha-1 N	-5.625	4.249	0.679 n.s.
		100 kg ha-1 N	-6.000	4.249	0.624 n.s.
		50 kg ha-1 Mg	-4.500	4.249	0.826 n.s.
		100 kg ha-1 Mg	-1.500	4.249	0.997 n.s.
29th July	Control	50 kg ha-1 N	-4.875	3.856	0.714 n.s.
		100 kg ha-1 N	-4.625	3.856	0.752 n.s.
		50 kg ha-1 Mg	-4.125	3.856	0.821 n.s.
		100 kg ha-1 Mg	-2.750	3.856	0.952 n.s.
7th August	Control	50 kg ha-1 N	-7.500	3.146	0.144 n.s.
		100 kg ha-1 N	-2.750	3.146	0.904 n.s.
		50 kg ha-1 Mg	-5.125	3.146	0.490 n.s.
		100 kg ha-1 Mg	-4.875	3.146	0.538 n.s.

* The mean difference is significant at the 0.05 level.
n.s. = not significant

Table 2 and Table 3 summarize the effect of fertilizers on plant growth in 2011. As expected and shown by the analysis of variance, as well as Tukey HSD test, the growth parameters show significantly different values for plant height.

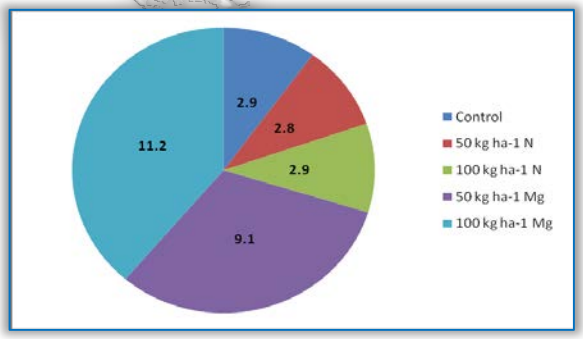

Figure 1. Dry biomass (g plant-1) production of in vitro L. inflata herb

Table 4. Tukey test of in vitro L. inflata herb (parameter: plant biomass, g plant-1)

Treat-ments (A)	Treatments (B)	Mean difference (A-B)	St. Error	Signifi-cance level
Contr ol	50 kg ha-1 N	0.095	1.232	1.000 n.s.
	100 kg ha-1 N	-0.040	1.232	1.000 n.s.
	50 kg ha-1 Mg	-6.180	1.232	0.021 *
	100 kg ha-1 Mg	-8.225	1.232	0.006 *

* The mean difference is significant at the 0.05 level.
n.s. = not significant

Figure 1 and Table 4 illustrates the dry biomass values recorded for herbs during growing seasons. The highest value was 11.2 g plant^{-1} in the 100 kg ha^{-1} Magnesium treatment groups. The control values were 2.9 g plant^{-1}. Figure 2 and Table 5 shows lobeline content of herbs. The herb was also favourably influenced by the Magnesium fertilization. The lowest values were recorded in the 100 kg ha^{-1} N ground fertilizer (336 µg g^{-1}) group. The highest value was in the 100 kg ha^{-1} Mg fertilizer treatment group (635 µg g^{-1}).

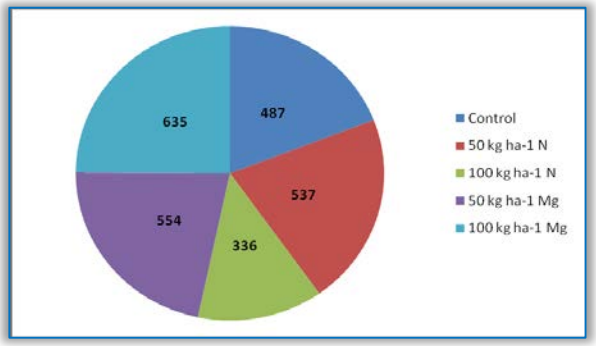

Figure 2. Lobeline content (µg g-1) of Lobelia inflata herb

Table 5. Tukey test of in vitro L. inflata herb (parameter: plant lobeline content, µg g-1)

Treat-ments (A)	Treatments (B)	Mean difference (A-B)	St. Error	Signifi-cance level
Control	50 kg ha-1 N	-50.250	58.422	0.900 n.s.
	100 kg ha-1 N	151.500	58.422	0.207 n.s.
	50 kg ha-1 Mg	-66.750	58.422	0.781 n.s.
	100 kg ha-1 Mg	-147.800	58.422	0.221 n.s.

* The mean difference is significant at the 0.05 level.
n.s. = not significant

Figure 3 and Table 6 presentation the total alkaloid content of herbs. The lowest values were recorded in the 100 kg ha^{-1} N ground fertilizer (355 mg 100 g^{-1}) group. The highest value was in the 50 kg ha^{-1} Mg fertilizer treatment group (514 mg 100 g^{-1}). The control values were 388 mg 100 g^{-1}.

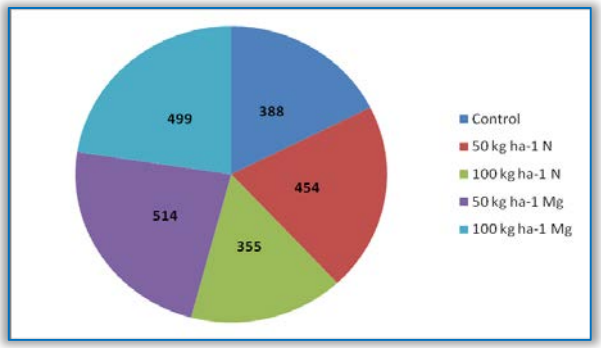

Figure 3. Total alkaloid content (mg 100 g^{-1}) of *Lobelia inflata* herb

Table 6. Tukey test of in vitro L. inflata herb (parameter: plant total alkaloid content, mg 100 g-1)

Treat-ments (A)	Treatments (B)	Mean difference (A-B)	St. Error	Signifi-cance level
Control	50 kg ha-1 N	-65.950	39.446	0.519 n.s.
	100 kg ha-1 N	32.400	39.446	0.913 n.s.
	50 kg ha-1 Mg	-126.650	39.446	0.109 n.s.
	100 kg ha-1 Mg	-110.850	39.446	0.165 n.s.

* The mean difference is significant at the 0.05 level.
n.s. = not significant

Table 7 illustrates calculated price for the lobeline content derived from open field cultivated *Lobelia inflata* plants of different treatment groups, based on yield and price in 2012. The Lobelia herb selling price was $5.6 per 100 capsules (42.5 g) in 2012 [50]. 100 kg ha^{-1} Mg treatment means a yield of 1,638 kg of dried plant material per hectare. 1% of total plant material was the lobeline content. The 16.38 kg lobeline content price was $ 2,158 per hectare.

Table 7. Calculated price for lobeline in 2012 (parameter: lobeline price ha-1, $)

Treat-ments	Herb dry mass (g)	Lobeline content (µg g-1)	Plant m2-1	Lobeline content ha-1 (µg)	Lobeline price ha-1 ($)
Control	2.9	487	18	254.21	33.49
50 kg ha-1 N	2.8	537	18	270.65	35.66
100 kg ha-1 N	2.9	336	18	175.39	23.11
50 kg ha-1 Mg	9.1	554	18	907.45	119.57
100 kg ha-1 Mg	11.2	635	18	1,280	168.66

CONCLUSIONS

The results in 2011 indicate that N and Mg fertilization increased growth (height in cm) to 47.75 cm (in the 50 kg ha-1 N fertilization group) compared to 40.25 cm of control. The value measured in the 50 kg ha-1 Mg treatment group was 43.0 cm. The highest dry biomass production (g plant-1) of herb was 11.2 g plant-1 (in the 100 kg ha-1 Mg treatment group). The 50 kg ha-1 Mg ground fertilizer group's value was 9.1 g plant-1. The biomass values were significant in the 50 - and the 100 kg ha-1 Mg treatments. The control value was equal to the value measured in the 100 kg ha-1 N treatment group (2.9 g plant-1). The highest lobeline content (µg g-1) of herb was 635 µg g-1 (in the 100 kg ha-1 Mg treatment group). The value of the control was 487 µg g-1. The 50 kg ha-1 Mg treatment resulted in 554 µg g-1,

and the 50 kg ha-1 N fertilizer resulted 537 µg g-1 lobeline.

There were several economy experiments on lobeline content in the 1970s in the United States. 1% of the dry matter content was lobeline. In the 1970s, selling prices ranged from $0.25 to $0.80 per pound (1 pound = 453 g), which means that a yield of 1,700 pounds (770 kg) of dried plant material would gross $425.00 to $1,360.00 per acre (1 acre = 4,047 m2) [26].

The conclusions of the experiments is, that open field conditions the MgSO4+ and the NH4NO3 treatments were successful for the lobeline production of Lobelia inflata.

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