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Research Article

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Increasing the Anti-Addictive Piperidine Alkaloid Production of *In Vitro* Micropropagated Indian Tobacco by Nitrate Treatments

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Abstract

Background: *Lobelia inflata* L. (Indian tobacco) is a traditional medicinal plant native to North America. It contains several piperidine alkaloids. Interest in Lobelia alkaloids, and in particular (-)-lobeline, the most active component, has increased in recent years due to their effect on the central nervous system. Thus, lobeline is currently the subject of renewed interest for its anti-addictive activity in the treatment of drug abuse, and neurological disorders. Our studies were aimed at introducing this species into cultivation in Hungary.

Results: For direct characterization of di-substituted and mono-substituted piperidine alkaloids in extracts of *L. inflata*, a tandem mass spectrometric method was developed using electrospray ionization. The compounds (-) lobeline, norlobeline, lobelanidine, norlobelanine and other related structures were identified by HPLC-MS/MS. With the aim of increasing the alkaloid production, we have investigated the effect of changing the ammonium and potassium nitrate levels of the basic Murashige-Skoog medium. The highest dry mass, total alkaloid and lobeline content were measured in the herbs and roots cultured at 570 mg L^{-1} KNO_3 content.

Conclusions: The highest values for lobeline derivatives norlobeline and lobelidine were also recorded in the herba and roots of *Lobelia inflata* cultured on reduced KNO_3 containing MS medium. The most sensitive response to media modification was observed in the case of lobelidine. Double-concentration of NH_4NO_3 had an inhibitory effect on plant growth, total alkaloid and lobeline content.

Keywords: Micropropagated *Lobelia inflate*; Piperidine alkaloids; Anti-addictive lobeline; LC-MS/MS; Nitrate treatments

Abbreviations: MS medium: Murashige and Skoog medium; HPLC-MS/MS: High performance liquid chromatography coupled with tandem mass spectrometry; CNS: Central nervous system; AD: Alzheimer's disease; NR: Nitrate reductase; SPE: Solid phase extraction; DAD: Diode array detection; ESI: Electrospray ionization.

Introduction

Indian tobacco (*Lobelia inflata* L.) is a species native to North America (Canada and US. Eastern states) [1]. It is mainly an annual plant, but its biennial populations can also be found [2-4]. *Lobelia inflata* belongs to the order Campanulales, to the family Lobeliaceae [5]. Lobelia is named after Flemish Botanist Matthias de L'Obel (1538-1616) [6]. *L. inflata* synthesizes medicinally important compounds. The herba contains several piperidine skeleton alkaloids [7-11]. 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 [12,13]. Recently, the species has come into the limelight due to research on CNS, drug abuse and multidrug resistance [14-19]. Several studies have shown that lobeline improves memory in rodents, probably due to its involvement in cholinergic mechanisms of neurotransmission. This pharmacological profile may be of great importance in the treatment of learning disorders like Alzheimer's disease (AD), the most common cause of dementia in the elderly [20]. Another important active agent in the plant is an antidepressant known as β-amyrin-palmitate [21]. Recent studies indicated the chemo-attractant character of *Lobelia inflata* extracts, while lobeline had chemo-repellent effects [22,23]. For economic production, it is important to increase the biomass and alkaloid content of the plant by nitrogen treatments *in vitro* [24] and in the open field [25-28]. There was a favorable effect of changing the NH_4^+ and NO_3^- concentrations on the biomass formation of both *in vitro* cultures [29-31] and aquacultures [32,33]. Britto and Kronzucker [34] described the inhibitory effect of ammonia on the growth in the open field.

Nitrogen regulates the expression of specific proteins through mechanisms affecting transcription and/or mRNA stability [35,36]. 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 [37]. Nitrogen sources are important for secondary product synthesis of compounds such as alkaloids [38], anthocyanins, and shikonin from cell suspension cultures [39]. Interestingly, the NH_4^+ to NO_3^- ratio in the medium affects not only the growth of plant cell cultures [40] but also the production of secondary compounds [41]. 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 effects of the culture medium differ from one species to another and from one compound to another [42-44]. 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.

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Genetic analysis of nitrate assimilation has been possible because mutants blocked in the pathway can be rescued by providing ammonia as a source of nitrogen [45,46]. Such mutants are obtained either by screening directly for plants defective in nitrate reduction by an *in vitro* assay or by selecting plants that are resistant to chlorate, the chlorine analogue of nitrate [47]. When chlorate is taken up and reduced by nitrate reductase (NR), toxic chlorite is produced. In higher plants, chlorate-resistant mutants are impaired in nitrate/chlorate reduction because they have either a defective NR structural gene or a defective molybdenum co-factor gene [48]. The one exception is the chl1 mutant of Arabidopsis, which is defective in nitrate/chlorate up-take [49,50]. Only in fungi and algae have regulatory mutants been described [51-53]. The biosynthetic activity of genetically transformed hairy root cultures of *L. inflata* has also been studied [54-57]. Several experiments dealt with the influence of macroelements on the growth and alkaloid production of hairy roots [58,59].

Bálványos et al. [59] ascertained that changes in the mineral element composition of the medium had a significant influence on the growth and metabolism of specific *L. inflata* hairy root cultures. Results of these studies have demonstrated the positive role of the B5 basal medium in increasing the biomass of cultures. It can be assumed that the better growth observed on the B5 medium [60] as compared with the growth on the MS medium [61] in the case of the *L. inflata* hairy root cultures, can be attributed to the lower ammonium concentration and the higher NO_3^-/ NH_4^+ ratio of the B5 medium.

The aim of this research was to study the effects of modified NH_4NO_3 and KNO_3 levels on the biomass formation and alkaloid production of *in vitro* cultivated *L. inflata*.

Materials and Methods

Plant material and culture conditions. Indian tobacco (*Lobelia inflata* L.) seeds were originated from Richters (Goodwood, Canada). *In vitro* propagation was initiated from seedlings by adventitious shoot induction on solid MS medium [61], containing 2% sucrose (pH 5.7). Culture media were solidified with 7 g L^{-1} agar (Biolab, Budapest, Hungary) and autoclaved at 121°C for 20 min. Cultivation was performed in 300 mL Erlenmeyer flasks, containing 100 mL of medium. Cultures were incubated in a climatized growth room at 22 ± 2°C, under a 12:12 hour photoperiod at 1400 lux light (cool and warm white fluorescent lamps, Figure 1).

The effects of various nitrate sources (NH_4NO_3 and KNO_3) of MS medium on the growth and alkaloid production of both roots and herbs of the plants were studied at halved or doubled concentrations. MS basal culture medium (basic NH_4NO_3 and KNO_3) was used as control.

NH_4NO_3 concentrations:

- NH_4NO_3 495 mg L^{-1}
- NH_4NO_3 990 mg L^{-1} (control)
- NH_4NO_3 1980 mg L^{-1}

KNO_3 concentrations:

- KNO_3 570 mg L^{-1}
- KNO_3 1140 mg L^{-1} (control)
- KNO_3 2280 mg L^{-1}

After 8 weeks of cultivation 30 explants were collected in every treatment, and dry mass was measured, following lyophilisation (Christ Alpha 1-4, B. Braun, Melsungen, Germany).

Figure 1: *In vitro Lobelia inflata* cultures (6 weeks) in the climatized growth room.

The statistical analysis was accomplished with SPSS v19 software [62]. The mean differences were regarded as significant at the 0.05 level.

Chemicals and reagents. (-) Lobeline hydrochloride originated from Sigma-Aldrich (St Louis, USA). Lobelanidine and norlobelanine were gifted by the Research Institute of Medicinal Plants (Poznan, Poland). HPLC grade acetonitrile and methanol were from Fisher Scientific (Loughborough, UK). Millipore Milli-Q equipment (Billerica, USA) provided the purified HPLC grade water. Further reagents were of analytical grade.

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

Determination of total alkaloid content. The total alkaloid content was determined by a spectrophotometric method [63] modified by Krajewska et al. [24].

Sample preparation by solid phase extraction (SPE) for analysis of alkaloids by HPLC. 3 mL Supelclean LC-8 columns (Supelco, Bellefonte, 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. [10] 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, 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. The HPLC-DAD chromatogram of an extract of *L. inflata* herba is presented in Figure 2.

Quantitative determination of alkaloids by HPLC. Determination of (-)-lobeline was performed by the external standard method. Standard

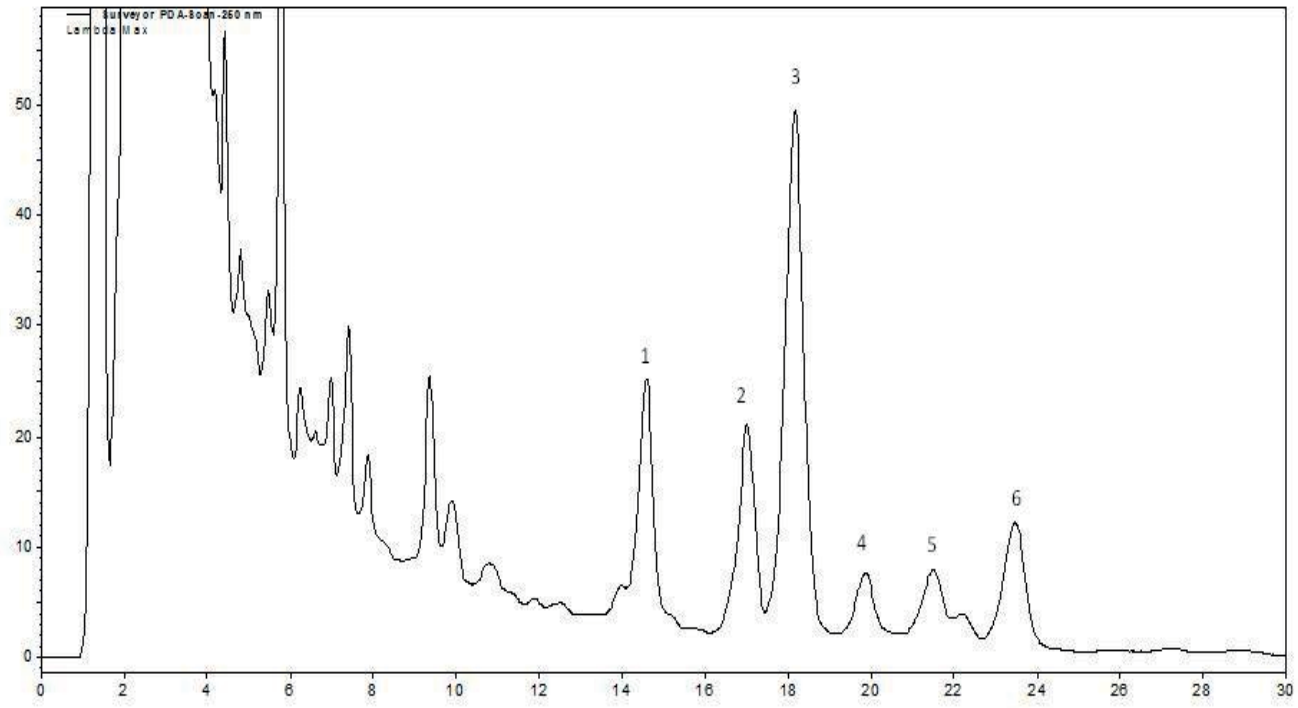

Figure 2: HPLC-DAD chromatogram of a herbs extract growing on MS medium (control). Peaks: norlobeline (1), lobelidine (2), (-)-lobeline (3), norlobelanine (4), lobelanine isomers (5-6).

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 concentrations. The amounts of lobeline in extracts was calculated from its peak area by use of the calibration plot. Validation studies proved that the repeatability of the method was good and the recovery was satisfactory (94.6%). The concentrations of lobeline derivatives: norlobeline, norlobelanine and lobelidine were expressed in lobeline.

LC-MS/MS experiments. Analysis was performed on an Agilent 6410 Triple Quad system using electrospray ionization (ESI) in positive mode. Chromatographic conditions were the same as described earlier by Kursinszki et al. [10]. The eluent was 30 mM ammonium format (pH 2.80); injection volume: 10 μL; 40% eluent could flow into the mass spectrometer (solvent splitting). Further conditions 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.

Results

With the aim of increasing the alkaloid production, we have investigated the effect of changing the NH_4NO_3 and KNO_3 levels of the basic Murashige-Skoog medium.

The highest dry mass of herbs was measured at 570 mg L^{-1} KNO_3 concentration (200.4 mg), this value was higher than the control (167.4 mg). The highest biomass formation for roots in nitrate treatments (NH_4NO_3 and KNO_3) was measured in the control. The doubled concentrations of NH_4NO_3 and KNO_3 (1980 and 2280 mg L^{-1}) strongly inhibited the growth of the cultures (Figures 3 and 4).

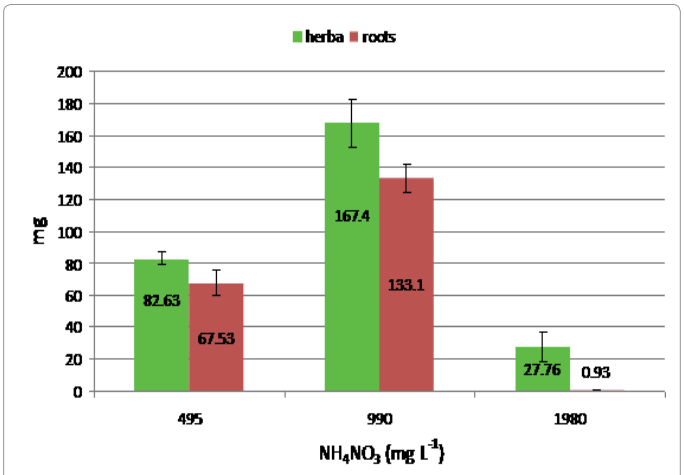

Figure 3: The effect of NH_4NO_3 on the dry mass of *in vitro L. inflata* cultures.

Studying the effect of different nitrate concentrations on the alkaloid production, we found that decreasing the levels of KNO_3 significantly increased the total alkaloid content of the cultures (Table 1). The most favourable for both herb and roots (8.3 and 9.2 mg g^{-1}) proved to be the 570 mg L^{-1} KNO_3 treatment. Decreasing the NH_4NO_3 concentration of the control MS medium did not elevate the alkaloid content.

LC-MS and LC-MS/MS analysis of alkaloids: chromatographic and mass spectrometric conditions were optimized by using lobeline and norlobelanine standards. Full scan analysis provided information about the molecular ion of standards. The MS/MS spectra obtained provided

Treatments	Roots	Herbs
	Total alkaloid content (mg g^{-1}) ± SD	
NH_4NO_3		
495 mg L^{-1}	4.3 ± 0.01	7.0 ± 0.24
990 mg L^{-1}	6.7 ± 0.17	7.7 ± 0.50
1980 mg L^{-1}	n.q.	2.3 ± 0.08
KNO_3		
570 mg L^{-1}	9.2 ± 0.26	8.3 ± 0.30
1140 mg L^{-1}	6.7 ± 0.17	7.7 ± 0.50
2280 mg L^{-1}	5.0 ± 0.07	3.7 ± 0.12

n.q.=not quantifiable

Table 1: The effect of NH_4NO_3 and KNO_3 on the total alkaloid content of *in vitro L. inflata* cultures.

Peak	R_t (min)	$[M^+H]^+$	MS-MS
1	10.7	324	82; 143; 186; 202
2	12.54	338	96; 98; 216; 218
3	13.23	338	96; 98; 105; 200; 216; 218; 320
4	14.06	322	82; 202
5	15.53	336	96; 216
6	16.87	336	96; 216

Table 2: LC-ESI-MS data of alkaloids in extract of *Lobelia inflata* herbs. 1. Peaks: norlobeline, 2. lobelidine, 3. (-)-lobeline, 4. norlobelanine, 5 and 6. lobelanine isomers.

information about the characteristic fragment ions and neutral losses which were the bases for identification of alkaloids in the extracts of *in vitro L. inflata* herb and roots [64].

The mass spectra of lobeline revealed a base peak at m/z 338, corresponding to the molecular ion $[M^+H]^+$. The product ion mass spectra were obtained by choosing the molecular ions as the precursor ions.

Fragmentation of the protonated molecular ion of lobeline in the instrument led to product ions of m/z 320, 218, 216, 200, 105, 98 and 96 (Figure 5). The subordinate product ion at m/z 320 was formed by loss of H_2O from the molecular ion at m/z 338. The ion at m/z 218 was produced by the loss of a phenyl-2-ketoethyl side chain ($C_8H_8O_1$, 120 Da). The more abundant product ion at m/z 216 was formed by loss of a phenyl-2-hydroxyethyl unit ($C_8H_{10}O_1$, 122 Da). A loss of water can be observed at m/z 200 (218-18). The most abundant product ion at m/z 96 was formed by loss of both side chains and corresponds to N-methylated 1.3-dihydropyridine ion.

The LC-ESI-MS data of alkaloids in the herb of *L. inflata* are shown in Table 2. Among them, the retention time, and the MS and MS-MS spectra of the molecular ions at m/z 338 (Peak 3), and 322 (Peak 4) were almost the same as those of lobeline, lobelanidine and norlobelanine standards respectively. Peak 3 can therefore be confirmed as lobeline, Peak 4 as norlobelanine. Above lobeline (Peak 3), another chromatographic peak of m/z 338 was also detected in its LC-MS-MS chromatogram with retention time of 12.54 min (Peak 2). The characteristic product ions at m/z 218, 216 and 96 were the same in both cases. Therefore, Peak 2 could be identified as an isomer of lobeline (lobelidine). The molecular ion at 324 (Peak 1) and its daughter ions at m/z 202, 186, 82 were all 14 Da less than the molecular ion m/z 338 of lobeline standard and its daughter ions m/z 216, 200, 96, respectively. Therefore, Peak 1 could be identified as N-dimethyl derivative of lobeline (norlobeline). Two chromatographic peaks of m/z 336 were detected in its LC-MS-MS chromatogram with retention times of 15.53 min (Peak 5) and 16.87 min (Peak 6). Fragmentation of their molecular ions showed the same pattern. Their product ions at m/z 216 and 96 and their characteristic neutral losses 120 Da and 2 × 120 Da respectively indicated that two phenyl-2-ketoethyl side chains were present in the molecule. The product ion at m/z 96 can be N-methylated 1.3-dihydropyridine ion. Therefore, Peak 5 and Peak 6 should be lobelanine isomers.

The lobeline and related piperidine alkaloid contents (μg g^{-1}) of *in vitro L. inflata* (herb and roots) in NH_4NO_3 treatments are illustrated in Table 3. The highest lobeline content was measured in the 990 mg L^{-1} NH_4NO_3 treatment for both herb and roots (280.8 and 243.6 μg g^{-1}). The norlobeline, lobelidine and norlobelanine contents were also highest in cultures cultivated on this medium. The lowest lobeline contents were measured in case of the doubled NH_4NO_3 containing medium. The results obtained on this higher NH_4NO_3 concentration were below the limit of quantification for roots.

Table 4 presents the effect of KNO_3 on lobeline and its derivatives of *in vitro L. inflata*. Out of all nitrate treatments, the lower KNO_3 concentration (570 mg L^{-1}) resulted in the highest lobeline (347.0 and 278.7 μg g^{-1}), norlobeline and lobelidine contents in both herbs and roots. The lowest lobeline, norlobeline and lobelidine contents were observed in the 2280 mg L^{-1} treatment, although the norlobelanine content of herba was lowest at the concentration of 1140 mg L^{-1} KNO_3. The most sensitive response to media modification was observed in case of lobelidine.

Conclusion

Summarizing our results, it can be stated that the decreased (570 mg L^{-1}) KNO_3 treatment exerted the most favourable effect on the growth, total alkaloid and lobeline content of *Lobelia inflata* herb and roots.

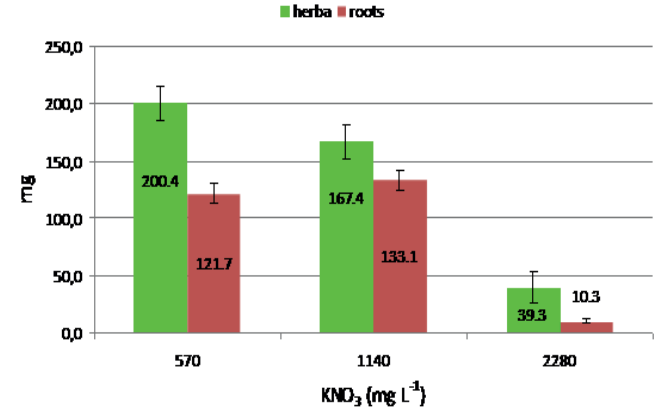

Figure 4: The effect of KNO_3 on the dry mass of *in vitro L. inflata* cultures.

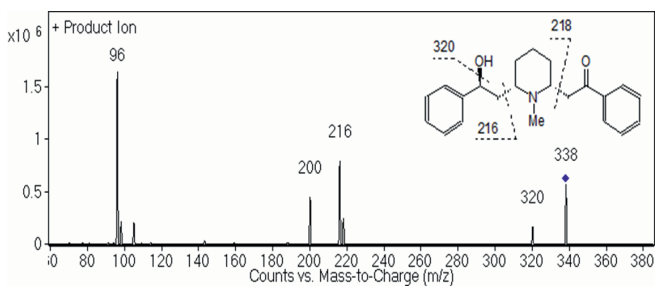

Figure 5: Predominant fragmentation pattern and MS/MS product ion spectrum of (-)-lobeline.

NH_4NO_3		lobeline	norlobeline	lobelidine	norlobelanine
495 mg L^{-1}	herbs	241.4 ± 6.63	97.4 ± 3.17	86.7 ± 1.02	24.4 ± 0.83
990 mg L^{-1}		280.8 ± 4.28	98.5 ± 2.96	95.1 ± 1.26	36.6 ± 0.18
1980 mg L^{-1}		25.9 ± 3.74	20.6 ± 0.29	3.7 ± 0.11	2.4 ± 0.05
495 mg L^{-1}	roots	75.0 ± 7.61	48.1 ± 0.73	22.7 ± 2.87	2.1 ± 0.78
990 mg L^{-1}		243.6 ± 29.20	71.7 ± 8.79	61.1 ± 4.01	29.0 ± 0.89
1980 mg L^{-1}		n.q.	n.q.	n.q.	n.q.

n.q.=not quantifiable

Table 3: The effect of NH_4NO_3 on the content of lobeline and its derivatives (µg g^{-1}) in *L. inflata* herbs and roots.

KNO_3		lobeline	norlobeline	lobelidine	norlobelanine
570 mg L^{-1}	herbs	347.0 ± 17.71	111.9 ± 5.33	118.0 ± 5.64	40.3 ± 7.78
1140 mg L^{-1}		280.8 ± 4.28	98.5 ± 2.96	95.1 ± 1.26	36.6 ± 0.18
2280 mg L^{-1}		116.4 ± 1.17	80.1 ± 2.57	33.9 ± 0.66	42.1 ± 0.45
570 mg L^{-1}	roots	278.7 ± 5.56	92.4 ± 0.57	81.8 ± 3.14	35.0 ± 3.94
1140 mg L^{-1}		243.6 ± 29.20	71.7 ± 8.79	61.1 ± 4.01	29.0 ± 0.89
2280 mg L^{-1}		45.9 ± 0.10	26.2 ± 0.34	4.8 ± 0.12	1.9 ± 0.04

Table 4: The effect of KNO_3 on the content of lobeline and its derivatives (µg g^{-1}) of *L. inflata* herbs and roots.

The highest values for lobeline derivatives norlobeline and lobelidine were also recorded on the reduced KNO_3 containing MS medium. The most sensitive response to media modification was observed in the case of lobelidine. High concentrations of NH_4NO_3 or KNO_3 strongly inhibited both biomass formation and the alkaloid biosynthesis of the cultures.

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