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LCMS and GCMS for the Screening of Alkaloids in Natural and *in Vitro* Extracts of *Leucojum aestivum*

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HPLC coupled to a mass spectrometer (MS) was used for the analysis of galanthamine and lycorine in natural extracts of *Leucojum aestivum* and in their *in vitro* cultures grown with a precursor (ACC), inhibitors ($AgNO_3$, STS), or an absorber ($KMnO_4$) of ethylene. The maximum galanthamine (0.002%) and lycorine (0.02%) concentrations in tissue cultures were obtained in the presence of $KMnO_4$. GCMS was used to investigate underivatized alkaloid mixtures from *L. aestivum*. Seven alkaloids were identified in *in vivo* bulbs. $KMnO_4$ led to the highest diversity of alkaloids in tissue culture extracts.

Leucojum aestivum L., a bulbous plant belonging to the Amaryllidaceae family, contains numerous alkaloids that are known to exhibit a wide range of biological activities.[1,2] Galanthamine (Figure 1a), an isoquinoline alkaloid, is known to possess acetylcholinesterase (AChE) inhibitory activity, while N-alkylated galanthamine derivatives, present at low concentrations in plants, have shown enhanced AChE inhibitory activity.[3] Lycorine, a pyrrolophenanthridine alkaloid (Figure 1b), displayed a strong antiviral effect against poliovirus, measles, and Herpes simplex type 1 viruses, as well as high antiretroviral[4] and strong antimitotic activities.[5] *L. aestivum* is cultivated on a commercial scale[6] for the extraction of galanthamine, which is widely used as an anti-Alzheimer drug.[7] Galanthamine is also obtained via a low-yielding and complex total synthesis.[7,8] Due to the growing demand from the pharmaceutical market, the supply of this alkaloid is important. *In vitro* cultures of *Narcissus confusus* (Amaryllidaceae) have been considered as alternative sources for the production of galanthamine.[9] *In vitro* cultures of *L. aestivum* have been successfully initiated, and the influence of cell differentiation on the regulation of galanthamine accumulation has been shown.[10−12]

Compounds with AChE inhibitory activity have been detected by HPLC coupled with UV, mass spectrometry, and biochemical detection from natural extracts of *Narcissus* species.[13,14] GCMS has been used for the identification of 14 alkaloids in bulbs and *in vitro* cultures of *L. aestivum*.[6]

The present paper reports the use of LCMS for the analysis of galanthamine and lycorine in bulbs of *L. aestivum* and their *in vitro* cultures. In particular, the effects of ethylene, produced by plant tissues grown *in vitro* and accumulated in the culture vessels, on calli initiation and on galanthamine and lycorine synthesis were studied. GCMS analyses are also reported for the screening and identification of alkaloids detected by LCMS analysis.

Results and Discussion

Ethylene and *in Vitro* Culture Initiation. Ethylene produced by plant tissue grown *in vitro* can be accumulated in the culture vessels. The production of this gaseous plant hormone is stimulated by numerous factors, e.g., wounding, auxin treatment, and environmental stresses.[15] The accumulation of ethylene is often difficult to control; therefore, its role in plant tissue culture is usually studied by adding an ethylene precursor [ACC (1-aminocyclopropane-1-carboxylic acid)] and various ethylene inhibitors [$AgNO_3$, STS (silver thiosulfate), $CoCl_2$] to culture media or by using an ethylene absorbent ($KMnO_4$).[16−18] The role of ethylene in plant tissue culture is not clear, but it has an important influence on many aspects of *in vitro* morphogenesis, e.g., shoot and root induction, and somatic embryos formation.[18,19]

Somatic embryogenesis of *L. aestivum* proceeds indirectly through a callus. About 6 weeks after initiation of the culture, the growth of embryogenic nodular calli on explants cultivated on each condition was observed. The highest percentage of callus-forming explants was noted on leaves cultured on the medium added with $AgNO_3$ (1, 10 μM) and STS (10 μM) (95.2; 90.8; 91.3%, respectively) (Table 1). The highest intensity of callusing was also achieved with inhibitors of ethylene added in the culture medium ($AgNO_3$, 10 μM; STS, 10 μM) and with ethylene absorbent ($KMnO_4$) (Table 1). Addition of $AgNO_3$ into callus induction medium enhanced embryogenic callus production of male immature inflorescence culture of buffalograss.[20] $AgNO_3$ has also been successfully used in other monocotyledoneus plants, such as maize (production of callus from immature embryos) and rice (callus proliferation).[21−23]

In our experiment, the addition of ACC into induction medium of *L. aestivum* significantly decreased callus formation. The presence of $KMnO_4$ was also less effective for embryogenic callus induction.

After 10 weeks of culture, the formation of *L. aestivum* somatic embryos was observed. The greatest number of somatic embryos was found on the callus formed on leaves cultured on the medium containing 10 μM ACC (20 embryos/explant) (Table 2). Ethylene increased embryogenesis in citrus ovular callus.[24] Hatanaka et al. (1995) reported that the addition of ethylene antagonist, e.g., $AgNO_3$ or $CoCl_2$, inhibited the formation of somatic embryos in leaf explant of *Coffea canephora*.[25] Ethylene also inhibited this process. The addition of $AgNO_3$ had a stimulatory effect on induction of direct somatic embryogenesis in immature embryo culture of *Triticum durum*.[26]

These results suggest that the control of ethylene production can be important for somatic embryogenesis in *in vitro* culture of *L. aestivum*.

LCMS Analysis. LCHRMS was used to investigate the influence of ethylene on alkaloid accumulation, specifically galanthamine and lycorine, in *L. aestivum in vitro* cultures. It is known

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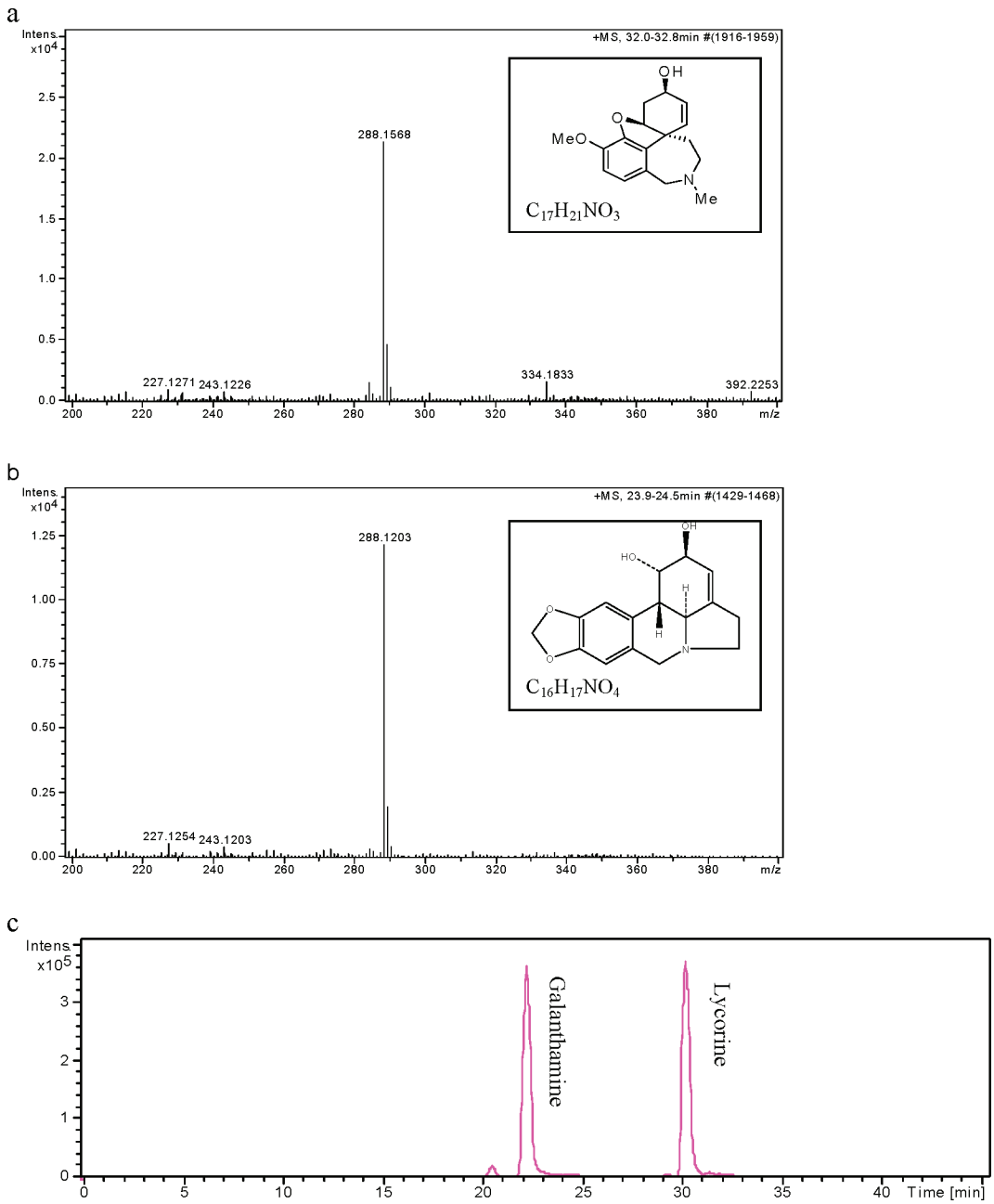

Figure 1. LCMS chromatogram, acquired in the positive-ion mode, from an extract of the bulb of *L. aestivum*: (a) mass spectrum of galanthamine, error of 11 ppm; (b) mass spectrum of lycorine, error of 11 ppm; (c) EIC of *m/z* 288.13 ± 0.05.

Table 1. Effects of Ethylene Biosynthesis Regulators and Ethylene Absorbent on Embryogenic Callus Induction in *L. aestivum in Vitro* Cultures

in vitro conditions	callus formation (%)	callusing[b]
control	66.0 a[a]	++
ACC (1 μM)	65.4 a	+
ACC (10 μM)	62.0 a	+
STS (1 μM)	86.9 ab	++
STS (10 μM)	91.2 b	+++
$AgNO_3$ (1 μM)	95.2 b	++
$AgNO_3$ (10 μM)	90.8 b	+++
$KMnO_4$	67.0 a	+++

[a] Means followed by different letters within columns are significantly different at $\alpha \leq 0.05$, Duncan test. [b] (+), intensity of callusing.

Table 2. Effects of Ethylene Biosynthesis Regulators and Ethylene Absorbent on *L. aestivum* Somatic Embryos Formation

in vitro conditions	no. of embryos per explant
control	1.8 a[a]
ACC (1 μM)	0.0 a
ACC (10 μM)	20.0 b
STS (1 μM)	2.0 a
STS (10 μM)	2.3 a
$AgNO_3$ (1 μM)	2.0 a
$AgNO_3$ (10 μM)	1.9 a
$KMnO_4$	0.0 a

[a] Means followed by different letters within columns are significantly different at $\alpha \leq 0.05$, Duncan test.

that ethylene can stimulate or inhibit synthesis of secondary metabolites in various plant cell and tissue cultures; for example, ginsenoside production was stimulated in *Panax ginseng* cell cultures, while the negative effect of ethylene was observed on ajmalicine production in *Catharanthus roseus* cell-suspension culture.[27,28]

The HPLC system previously used for galanthamine analysis[10] was incompatible with MS analysis. Therefore, the aqueous mobile

Table 3. Elution Gradient Employed for the LCMS Analysis of Galanthamine and Lycorine

	time (min)						
	0	1	30	31	36	37	45
solvent A	100	100	50	0	0	100	100
solvent B	0	0	50	100	100	0	0

Table 4. Galanthamine and Lycorine Contents in *Leucojum aestivum* Bulbs and *in Vitro* Cultures (LOQ = 2×10^{-4}%)

extract	galanthamine % dry wt	lycorine % dry wt
in vivo bulbs	0.03	0.01
in vitro cultures		
control	<LOQ	0.001
ACC (1 μM)	<LOQ	<LOQ
ACC (10 μM)	<LOQ	<LOQ
STS (1 μM)	<LOQ	<LOQ
STS (10 μM)	0.001	0.002
$AgNO_3$ (1 μM)	<LOQ	<LOQ
$AgNO_3$ (10 μM)	<LOQ	<LOQ
$KMnO_4$	0.002	0.003

phase (30 mM triethylammonium acetate) was substituted by solvent A (97.5% of 10 mM NH_4HCO_3 with 2.5% of MeOH), and the organic mobile phase (100% acetonitrile) was replaced by solvent B (2.5% of 10 mM NH_4HCO_3 with 97.5% of MeOH) (pH 7.8).[14]

The optimized gradient profile (Table 3) of the HPLC mobile phase led to well-resolved peaks of lycorine and galanthamine.

The HPLC coupled with high-resolution mass spectrometry (ESi/QqTOF) was used in order to confirm galanthamine (22.2 ± 0.2 min) and lycorine (30.2 ± 0.2 min) identities in sample extracts (Figure 1c). This system was able to detect the compounds in amounts lower than 0.02 μg/mL. Galanthamine and lycorine displayed $[M + H]^+$ at a *m/z* of 288.1568 (calculated value 288.1600; error of 11 ppm) (Figure 1a) and 288.1203 (calculated value 288.1236; error of 11 ppm) (Figure 1b). The LCMS chromatogram of the different extracts showed excellent separation of the galanthamine and lycorine peaks, permitting the quantification of these alkaloids (Figure 1c).

Five types of *L. aestivum* cell cultures were analyzed: (1) calli used as control cultivated on the basal medium, (2) calli cultivated with ACC (1 or 10 μM), (3) calli cultivated with STS (1 or 10 μM), (4) calli cultivated with $AgNO_3$ (1 or 10 μM), and (5) calli cultivated with $KMnO_4$. The bulb used for the initiation of these tissue cultures was also analyzed. These tissues were cultured for 3 weeks after calli initiation prior to analysis of the alkaloids. Galanthamine and lycorine contents (% dry weight of plant material) are presented in Table 4. These compounds exhibited only the pseudo molecular ion characteristic of standard galanthamine and lycorine. *In vivo* bulb contained galanthamine (0.03% dry wt) and lycorine (0.01% dry wt). It is worth noticing that only traces of galanthamine and 0.001% dry wt of lycorine were detected in the control *in vitro* cultures. However, galanthamine and lycorine were detected at higher concentrations in calli cultivated with $KMnO_4$ (0.002% and 0.003% dry wt, respectively) or with STS at 10 μM (0.001% and 0.002% dry wt, respectively). These results suggested that the control of ethylene production can be important for alkaloid synthesis, in particular galanthamine and lycorine, in *in vitro* cultures of *L. aestivum*. Zhang and Wu (2003) and Tabata (2004) showed that taxane production in suspension cultures of *Taxus* sp. was strongly promoted by STS.[29,30] A stimulating effect of STS was also observed for tanshinone diterpenoid accumulation in *Salvia miltiorrhiza* hairy root cultures.[31] Incorporation of $CoCl_2$ or $NiCl_2$ effectively reduced ethylene accumulation and improved product accumulation, whereas exogenous application of ethrel, an ethylene-releasing compound, significantly reduced secondary metabolite production in suspension cultures of *Vaccinium pahalae*.[32]

GCMS Analysis. In order to identify the various compounds present in the complex alkaloid fractions of bulbs and *in vitro* cultures of *L. aestivum*, capillary GCMS was employed. Derivatization was not required, since the Amaryllidaceae alkaloids retain their characteristic EI/MS fragmentation patterns when employing GC conditions, as reported by Kreh et al. (1995) and Tram et al. (2002).[33,34] Following the alkaloid extraction of all samples (9), i.e., *in vivo* bulbs and tissue cultures, alkaloid fractions suitable for GCMS analysis were obtained. The GCMS chromatogram of the extract from *in vivo* bulb is presented in Figure 2a and

Table 5. Alkaloids Identified in Bulbs and in Calli of *Leucojum aestivum*

alkaloid	retention time (min)	base peak	fragmentation *m/z* (relative intensity, %)	*in vivo* bulbs	*in vitro* cultures							
					1[a]	2	3	4	5	6	7	8
trisphaeridine	21.9	223	222(39), 167(10), 166(10), 164(5)	+	−	−	−	−	+	−	+	+
galanthamine	22.75	286	287(83), 244(23), 230(12), 216(31), 174(28)	+	+	−	−	−	+	−	+	+
narwedine	23.4	284	285(84), 242(18), 216(24), 119(22), 174(40)	+	−	−	−	−	−	−	−	−
crinine	23.5	271	270(20), 199(10), 187(5), 173(10), 115(22)	+	−	+	−	−	−	−	−	−
demethylmaritidine	23.7	273	230(25), 201(86), 189(54), 175(22), 157(16), 128(19), 115(20)	+	−	−	−	−	−	−	−	−
anhydrolycorine	23.8	250	251(45), 220(4), 204(2), 192(14), 191(12), 165(6), 152(2), 139(4), 96(7), 95(9)	+	−	−	−	−	−	+	−	+
nor-galanthamine	24	272	273(98), 230(33), 202(27), 174(12)	−	−	−	−	−	−	−	−	−
pluviine	25.8	242	287(75), 286(44), 268(45), 254(4), 228(23), 198(4), 182(3), 151(4)	−	−	−	−	−	−	−	−	−
lycorine	26.3	226	287(27), 286(19), 268(20), 250(20), 227(80), 147(8), 119(12)	+	+	−	−	−	+	−	−	+
N-formylnorgalanthamine	26.9	301	272(2), 243(6), 230(7), 225(15), 211(16), 128(11), 115(10)	−	−	−	−	−	−	−	−	−

[a] 1: control; 2: ACC 1 μM; 3: ACC 10 μM; 4: STS 1 μM; 5: STS 10 μM; 6: $AgNO_3$ 1 μM; 7: $AgNO_3$ 10 μM; 8: $KMnO_4$.

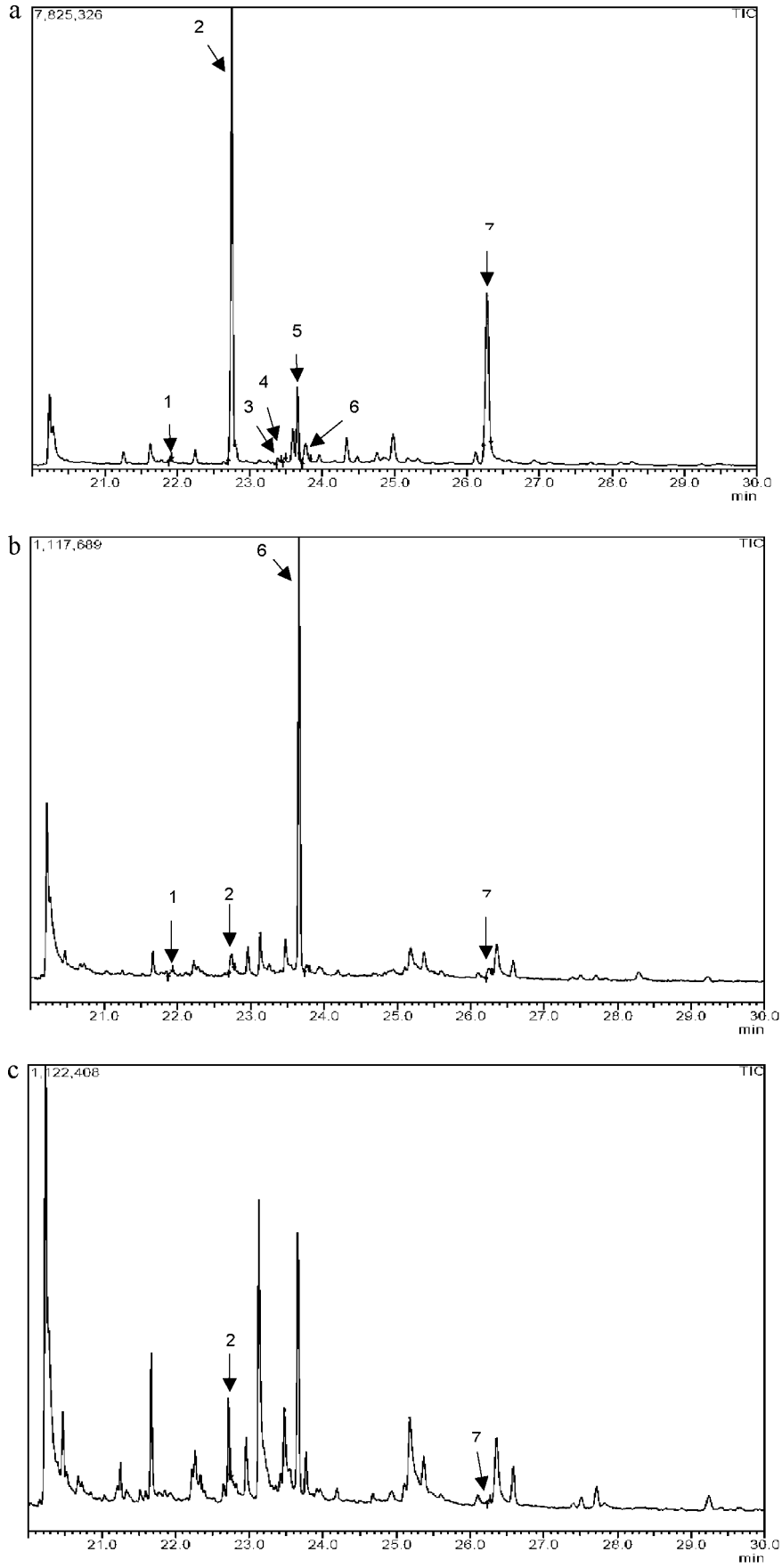

Figure 2. GCMS chromatogram (total ion current) of the alkaloid fractions obtained (a) from *in vivo* bulb of *Leucojum aestivum*, (b) from calli cultivated with $KMnO_4$, and (c) from calli control. The numbers of the peaks correspond to the following compounds: 1: trisphaeridine, 2: galanthamine, 3: narwedine, 4: crinine, 5: demethylmaritidine, 6: anhydrolycorine, 7: lycorine.

demonstrates the excellent separation of many compounds in the alkaloid mixture.

Seven compounds from *in vivo* bulbs and calli cultures showed MS fragmentation patterns characteristic of the Amaryllidaceae alkaloids (Table 5). The identification of these alkaloids was performed by comparing the measured data with literature data.[6] The use of standards was not necessary; however, galanthamine and lycorine standards were injected in order to compare and confirm their EI/MS fragmentation patterns with literature results. The distribution of the alkaloids varied according to the initial material and the factors influencing ethylene synthesis or accumulation in tissue cultures. Two compounds, narwedine and demethylmaritidine, were detected only in *in vivo* extract. The five other compounds, trisphaeridine, galanthamine, crinine, anhydrolycorine, and lycorine, were observed in bulbs and in some *in vitro* cultures. Calli cultured with the absorber of ethylene, $KMnO_4$ (*in vitro* culture no. 8), showed the highest distribution of the alkaloids. Galanthamine and lycorine were identified only in calli obtained with ethylene inhibitors (STS, 10 μM; $AgNO_3$, 10 μM), with an ethylene absorber ($KMnO_4$), and in controls. These results are in accordance with the results obtained with the LCMS analysis. The accumulation of these alkaloids in calli seemed to decrease with the presence of ethylene. In the same way, the culture of calli with a precursor of ethylene (ACC, 10 μM) or with an inhibitor of ethylene at low concentration (STS, 1 μM) led to the absence of any alkaloids in the extracts.

Several other compounds were also detected in *in vivo* and *in vitro* extracts, but they could not be identified. Quantitative and qualitative differences were observed between *in vitro* and *in vivo* synthesized alkaloids (Figure 2).

Experimental Section

Plant Material. Leaves isolated from *Leucojum aestivum* L. bulbs (from Polish and French local markets) chilled for 12 weeks at 5 °C were surface-sterilized in 70% EtOH (1 min), then shaken for 15 min in 15% Domestos (with NaOCl and NaOH content below 5%; Unilever, Hungary) and rinsed three times with sterile H_2O. Sterilized leaves were cut into thin slices (about 2−3 mm in length) and plated on culture.

Callus and Somatic Embryo Induction. Initial explants were cultivated on Murashige and Skoog (MS, 1962)[35] medium supplemented with auxin [4-amino-3,5,6-trichloropicolinic acid (Picloram) (25 μM)] and cytokinin [benzyladenine (BA) (0.5 μM) (control)]. The medium was also enriched with (1 or 10 μM) ACC, $AgNO_3$, or STS. $KMnO_4$ was introduced as a 4.5 g solid, placed in an open tube within the culture atmosphere. The media were supplemented with 3% sucrose. The media were adjusted to pH 5.5 before autoclaving and gelled with 0.8% agar (purified agar from Difco). ACC, $AgNO_3$, and STS were filter sterilized and added to the autoclaved medium. The cultures were maintained at 25 ± 2 °C in the darkness and subcultured every 4 weeks.

Statistical Analysis. The results of observations were evaluated by analysis of variance and by using Duncan's multiple test.

Chemicals and Reagents. Triethylammonium acetate buffer, galanthamine, lycorine, picloram, 6-benzylaminopurin, inositol, agar-agar, $MgSO_4 \cdot 7H_2O$ (98%), $MnSO_4$, KNO_3, and EDTA iron(III) sodium salt were all from Sigma-Aldrich Chemie GmbH (Stennheim, Germany). NH_4HCO_3, iron(II) sulfate, $ZnSO_4$, $CuSO_4 \cdot 5H_2O$ (99%), thiamine hydrochloride, 2,4-dichlorophenoxyacetic acid, absolute EtOH, and MeOH (Hipersolv Chromanorum for HPLC-Isocratic grade) were purchased from Prolabo VWR International bvba/sprl. Pyridoxol hydrochloride, nicotinic acid, NaH_2PO_4, and anhydrous $CaCl_2$ were obtained from Merck AG Darmstadt (Germany). Acetonitrile was acquired from Carlo Erba Reagenti (SDS).

Alkaloid Extraction. Plant materials, *in vivo* bulbs and *in vitro* cultures, were lyophilized and powdered, and 150 mg of powder was macerated in MeOH (10 mL) for 24 h, with sonication for 90 min in an ultrasonic bath (Transsonic 460/H Elma) at room temperature. After centrifugation at 4000 rpm for 20 min, the mixture was filtered through 0.2 μm filters, and the total MeOH extract was analyzed using LCMS and GCMS systems.

LCMS Analysis. The LC system consisted of a U3000-Dionex and an injector with a 1 μL loop. The column used was a reversed-phase Acclaim PepMap C_{18} analytical column (150 × 1 mm i.d., 3 μm particle size) (Dionex-France) and was eluted at a flow rate of 40 μL/min using a gradient ranging from 0% solvent B to 100% solvent B in a time span of 36 min. Solvent A consisted of 97.5% 10 mM NH_4HCO_3 pH 7.8 with 2.5% MeOH, and solvent B consisted of 97.5% MeOH and 2.5% 10 mM NH_4HCO_3 pH 7.8. The ESI-HRMS was a microTOF$_Q$ (Bruker Daltonics) apparatus. An external standard method was utilized for calibration (five-point calibration), with reference to solutions of authentic galanthamine and lycorine (Sigma Aldrich).

GCMS Identification. Analyses were performed using QP2010-Shimadzu equipment operating in the EI mode at 70 eV. An SLB5 column (30 m × 0.25 mm × 0.25 μm) was employed with a 30 min temperature program of 80−280 at 10 °C/min followed by a 10 min hold at 280 °C. The injector temperature was 280 °C, the flow rate of the carrier gas (helium) was 0.8 mL/min, and the split ratio was 1:50. The identification of the alkaloids was performed by comparing the measured data with those of authentic compounds (galanthamine, lycorine) or with literature data as specified in the text.

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