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ORIGINAL PAPER

Effects of sucrose and plant growth regulators on acetylcholinesterase inhibitory activity of alkaloids accumulated in shoot cultures of Amaryllidaceae

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Abstract The influence of sucrose (30, 60, 90 and 120 g/L), activated charcoal (5 and 10 g/L), and various levels of several plant growth regulators (6-benzyladenine, naphthalene-1-acetic acid, 2,4-dichlorophenoxyacetic acid, and picloram) on organogenesis (bulb and root development) and the accumulation of alkaloid and galanthamine in shoot cultures of three Amaryllidaceae species (*Narcissus pseudonarcissus*, *Galanthus elwesii*, and *Leucojum aestivum*) was investigated in a full-factorial experiment. Alkaloid extracts were analyzed by gas chromatography–mass spectrometry, leading to the quantification of galanthamine and to the identification of other alkaloids. The different extracts were then subjected to an Ellman test to evaluate the inhibitory activity of acetylcholinesterase. The highest contents of galanthamine [0.02–0.1% dry weight (DW) depending on the plant species] were always accompanied with a high level of acetylcholinesterase inhibition (>30%). However, some samples containing low amounts of galanthamine (0.005% DW) showed high inhibitory activities (40–80%). These findings demonstrate the presence of Amaryllidaceae alkaloids that have not yet been identified as having anti-acetylcholinesterase activity.

Keywords *Narcissus pseudonarcissus* · *Galanthus elwesii* · *Leucojum aestivum* · Amaryllidaceae alkaloids · Acetylcholinesterase inhibition · Nutrient medium variation

Abbreviations

AChE	Acetylcholinesterase
BA	6-benzyladenine
2,4-D	2,4-dichlorophenoxyacetic acid
DW	Dry weight
Gal	Galanthamine
NAA	Naphthalene-1-acetic acid
PGRs	Plant growth regulators
Picloram	4-Amino-3,5,6-trichloropicolinic acid
Rt	Retention time

Introduction

Cholinesterase inhibitors are approved drugs for the palliative treatment of Alzheimer's disease, a progressive neurodegenerative disorder that is one of the most common causes of mental deterioration in the elderly population (Henchman et al. 2002). Plants of the Amaryllidaceae family produce pharmacologically active alkaloids that have interesting pharmacological properties, such as acetylcholinesterase (AChE) inhibitory activity, cytotoxicity, antitumoral activity, among others (Bastida et al. 2006).

Galanthamine (Gal) (Fig. 1a) and lycorine (Fig. 1b) are two of the best known alkaloids that have been found in plants of different Amaryllidaceae genera, including

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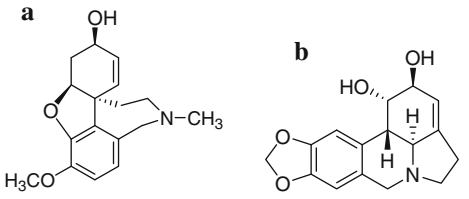

Fig. 1 **a** Galanthamine, **b** Lycorine

Galanthus, *Narcissus*, and *Leucojum*. Gal is used worldwide as an anti-AChE drug for the treatment of Alzheimer's disease. It enhances cholinergic activity by blocking AChE (Sener et al. 2003; Mroczek and Mazurek 2009). Lycorine has antimalarial properties, among others, and acts as an antiviral agent and a powerful inhibitor of cell division and growth in higher plants (Ghosal et al. 1985).

Gal has been isolated from more than 20 plant species (Novikova and Tulaganov 2002). For medical applications, it is obtained via a low-yielding and complex total synthesis (Guillou et al. 2001; Marco-Contelles et al. 2006), but it has also been isolated from *Leucojum aestivum* bulbs in particular (Berkov et al. 2005). A number of studies have reported that Gal is accumulated in Amaryllidaceae tissue cultures and that some factors can enhance its accumulation (Selles et al. 1997; Colque et al. 2004; Diop et al. 2007; Diop et al. 2006; Ptak et al. 2009; Georgiev et al. 2009; Berkov et al. 2009a, b; Ptak et al. 2010), suggesting that biotechnological methods can be considered as an alternative approach for the production of this alkaloid. Changes in the external conditions of cultures strongly affect alkaloid metabolism in plant cell and tissue cultures (Mantell and Smith 1983), but they also affect regeneration from somatic embryos (Malik 2008). Sucrose is an essential and the most common carbon source used in plant cell tissue and organ culture media. Merillon et al. (1984) demonstrated that sucrose may influence alkaloid metabolism in cell cultures. To date, the highest production of Gal was observed with the addition of 180 and 60 g/L of sucrose to liquid-shake medium of *Narcissus confusus* (Selles et al. 1997) and *Leucojum aestivum* (Georgiev et al. 2009) shoot cultures, respectively. To date, there has been no report of alkaloid production by different Amaryllidaceae species in in vitro systems showing a correlation between in vitro growth, alkaloid diversity and accumulation, and biological activity.

The aim of this study was to determine the optimal culture media for plants of three Amaryllidaceae species (*Narcissus pseudonarcissus*, *Galanthus elwesii*, and *Leucojum aestivum*) that would result in the best organogenesis (bulbs and roots) and achieve the highest alkaloid diversity, maximal yields of Gal, and the best AChE inhibitory activity. The evolution of alkaloid accumulation was followed using gas chromatography–mass spectrometry (GS–MS) analyses, and AChE inhibitory activity was evaluated by the in vitro Ellman method (Ellman et al. 1961).

Materials and methods

Plant material

Leaves isolated from *L. aestivum*, *N. pseudonarcissus*, and *G. elwesii* bulbs (from French local markets) and 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, Budapest, Hungary) and rinsed three times with sterile H_2O. Sterilized leaves were cut into thin slices (about 3–5 mm in length) and plated on culture media (Ptak et al. 2009). Voucher specimens of *L. aestivum* (911371), *N. pseudonarcissus* (820570), and *G. elwesii* (20100274) are deposited at the herbarium of the Jardin botanique, Nancy, France.

Culture media

For all experiments, MS (Murashige and Skoog 1962) nutrient medium was used, adjusted to pH 5.5 before autoclaving (120°C).

An experimental design was established using Nemrod software in order to optimize the nutrient medium for *N. pseudonarcissus* explants. A total of 21 experiments were conducted by randomly varying the proportions and concentrations of the following four factors in the medium: the auxin 2,4-dichlorophenoxyacetic acid (2,4-D; 0, 2.50, 7.50, 12.50, 17.50, 20 µM); the cytokinin 6-benzyladenine (BA; 0, 3.33, 6.67, 10, 13.33, 16.67, 20 µM); sucrose (0, 30, 60, 90, and 120 g/L); activated charcoal (0, 5, 10 g/L). Two basal media were used to investigate individual and combined effects of plant growth regulators (PGRs) and sucrose on the accumulation and types of alkaloids in shoot cultures of *G. elwesii*, *N. pseudonarcissus*, and *L. aestivum*. The first medium, designated as MSA, contained 10 µM 2,4-D and 10 µM BA along with various levels of sucrose (30, 60, 90 or 120 g/L); the second medium, designated as MSB, contained 60 g/L sucrose and 10 µM BA along with various types of auxins (10 µM 2,4-D, 10 µM NAA, or 10 µM picloram). All cultures were maintained at 25 ± 2°C, under a 16/8-h (light/dark) photoperiod (Tungsram lamp, 40 WF, 90 µmol m^{-2} s^{-1}), and subcultured once every 4 weeks under the same conditions.

Alkaloid extraction

Plant materials consisting of in vivo bulbs and shoot cultures (Fig. 2), were lyophilized and powdered; 1 g of

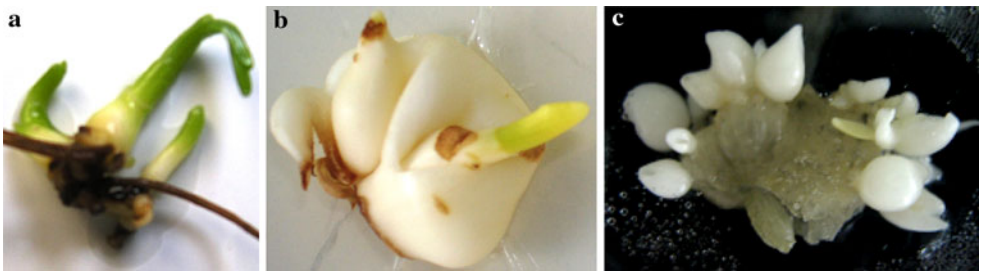

Fig. 2 Direct organogenesis from leaves of Amaryllidaceae species: shoot cultures of *Leucojum aestivum* (**a**), *Narcissus pseudonarcissus* (**b**), and *Galanthus elwesii* (**c**) cultivated on activated charcoal

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 decantation, the mixture was filtered through 0.45-µm filters, and the total MeOH extract was analyzed by chromatography (Ptak et al. 2009).

GC–MS analysis

Gas chromatography coupled to mass spectrometry analyses were performed using QP2010-Shimadzu equipment (Shimadzu, Kyoto, Japan) operating in the EI mode at 70 eV. An AT-1 column (25 × 0.32 mm × 0.30 µm) was employed with a 33-min temperature program of 80–280°C 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 alkaloids were identified by comparing the measured data with those of authentic compounds (Gal, lycorine) or with published data (Berkov et al. 2005; Ptak et al. 2009). Quantification was carried after column calibration with authentic Gal (Sigma–Aldrich, Lyon, France; 99% purity) in single ion monitoring (SIM) mode.

AChE activity test

The AChE inhibitory activity was measured using the photometric method of Ellman (Ellman et al. 1961), with AChE obtained from *Electrophorus electricus* and acetylthiocholine as the substrate.

AChE activity was measured by following the increase in yellow coloration produced from thiocholine when it reacts with the dithiobisnitrobenzoate ion to produce the yellow anion of 5-thio-2-nitrobenzoic acid in 96-well microtiter plates. The color production rate was measured at 412 nm in a Spectra Max 384 plus plate reader (Molecular Devices, Sunnyvale, CA). At the end of the culture period, shoot cultures of the three different species and the corresponding extracts were collected into a batch of samples. The whole batch, with three repetitions per sample, was submitted to the same test (96 wells/test). AChE activity was measured during the same analysis and under the same respective conditions. The data were collected as inhibition percentages and compared to those of authentic Gal and tacrine. Each extract was evaluated at several concentrations (in the range of 10^{-9} –2×10^{-5} M) obtained with a robot Beckman Biomek 3000 and Biomek NX (Beckman-Coulter, Fullerton, CA). Percentage inhibition was calculated relative to a control sample (dimethyl sulfoxide) and reported as the the mean ± standard deviation for triplicate assays. Authentic Gal and tacrine were used as standards to which the inhibition percentages of plant extracts were compared.

Results and discussion

More than 100 structurally diverse alkaloids possessing a wide spectrum of biological activities have been isolated from various Amaryllidaceae species (Hoshino 1998). Gal, which is used worldwide as an anti-AChE drug for the palliative treatment of Alzheimer's disease, is one of the alkaloids found in *Narcissus*, *Galanthus*, and *Leucojum* species. However, tissue cultures of some Amaryllidaceae plants can be considered as an alternative source of Gal (Colque et al. 2004; Diop et al. 2006; Pavlov et al. 2007). It has been reported that cell differentiation level has a major effect on Gal accumulation in *L. aestivum* (Diop et al. 2007).

Experimental design

One of the target species in this study, *N. pseudonarcissus*, was first studied within the framework of a full-factorial experiment to determine the best conditions for organogenesis (Table 1). After 4 months of culturing *N. pseudonarcissus* shoots, the ratio of surviving explants, callusing, and organogenesis (adventitious root and bulb formation) (Fig. 2b) was measured. An average of six shoot cultures at each condition was used for analysis. However, due to the plant inherent variability, the results were variable for each explant belonging to the same species cultivated under the same conditions. Ratios (Table 1) were determined using

Table 1 Estimated ratios (using Nemrod software) for the four factors tested (sucrose, BA, 2,4-D, activated charcoal) in an *Narcissus pseudonarcissus* experimental design

Factors (*X*)	Ratios (*Y*)			
	Surviving explants	Calluses	Roots	Bulbs
	0.372***	0.468**	−0.000	0.100
Sucrose	0.186**	−0.043	0.025	0.090
BA	0.028	−0.035	−0.023	−0.085
2,4-D	−0.123*	−0.056	0.143	−0.022
Activated charcoal	−0.092	−0.161	−0.269*	−0.351**
$(\text{Sucrose})^2$	−0.133	−0.286	0.046	0.082
$(\text{BA})^2$	0.002	0.329	−0.015	0.085
$(\text{2,4-D})^2$	−0.096	−0.115	0.409**	−0.005
$(\text{Activated charcoal})^2$	0.055	0.091	0.252	0.291
Sucrose/BA	−0.115	−0.248	−0.000	0.426
Sucrose/2,4-D	−0.153	0.462	−0.612*	−0.151
BA/2,4-D	−0.007	0.355	0.568	0.178
Sucrose/Activated charcoal	0.128	−0.253	−0.095	−0.061
BA/Activated charcoal	−0.155	0.386	0.343	0.112
2,4-D/Activated charcoal	0.012	−0.555	0.332	−0.144
R^2	0.830	0.568	0.794	0.762

* Significant at 90%,
** Significant at 95%,
*** Significant at 99%

BA, 6-Benzyladenine; 2,4-D, 2,4-dichlorophenoxyacetic acid

Negative signs indicate that an increase in the factor concentration leads to a decrease in the ratio

the Doehlert matrix in order to obtain the estimated coefficients for the four factors being studied (sucrose, BA, 2,4-D, and activated charcoal). The data of survival, callusing, and organogenesis ratios were statistically analyzed using Nemrod software and the estimated coefficients for the four factors are presented in the Table 1. The addition of activated charcoal did not seem to be beneficial in any condition and always had a negative effect on survival, callusing and organogenesis; specifically, its concentration in the nutrient medium decreased the ratio of root and bulb formation. In contrast, the sucrose and PGR concentrations can be optimized. For example, the addition of sucrose increased survival of the explants, with high sucrose concentrations inducing better organogenesis and decreasing callusing. This is in agreement with previous results where the addition of sucrose to the nutrient medium also improved bulb development in *Narcissus* cultivars (Staikidou et al. 2005; Chow et al. 1992) and in *L. aestivum* (Berkov et al. 2009a, b).

An increase in the 2,4-D concentration decreased the survival ratio of the explants, indicating a toxic effect. Effects of BA and 2,4-D alone were not significant but the addition of 2,4-D and BA together had a positive effect on callusing and on root and bulb formation (Table 1).

The Nemrod software can provide an optimal ratio of two factors, with the other two factors being fixed. Activated charcoal was omitted from the analysis as it had only negative effects, as was sucrose at 60 g/L. Under these conditions, the combinations of BA (4 μM)/2,4-D (10 μM) were optimal for the callus formation ratio (0.9–1) and BA (5 μM)/2,4-D (12 μM) for the bulb formation ratio (0.7). Using this same type of analysis, Khanam et al. (2000) found that the highest fresh weight of *Duboisia myoporoides* calluses was obtained when the callus induction medium was supplemented with BA in combination with 2,4-D or NAA. The opposite trend in PGR concentrations was observed for the survival, with a higher concentration of BA and a lower addition of 2,4-D proving to be beneficial. The ratio of the surviving explants was optimized to 60% with the addition of 15 μM BA and 8 μM 2,4-D, while a 40% explant survival rate was reached using those combinations just mentioned which produced optimal callusing and bulb formation. Root formation was optimum (80%) with the addition of BA (6 μM) combined with 2,4-D (4 μM). Limited data have been published on the cultivation of unorganized tissue cultures of *Narcissus* species (Seabrook et al. 1976), but calluses were successfully induced in the presence of low concentrations of auxin (4.52 μM of 2,4-D). Sage et al. (2000) showed that a higher number of *Narcissus* somatic embryos was produced with 5 and 10 μM of 2,4-D than with 0.5 μM. On the other hand, reasonable yields of somatic embryos were obtained by combining 2,4-D with BA. It has also been reported that high concentrations of auxin (45.2 μM of 2,4-D) stimulates the formation of regenerative calluses of *Hyacinthus orientalis* (Takayama et al. 1991). Morini et al. (2000) showed that in vitro callus formation of quince leaves increased with increasing auxin concentrations, while roots decreased in number with increasing 2,4-D. Selles et al. (1997) reported that the transfer of the *Narcissus confusus* cultures to a solid MS medium containing low amounts of 2,4-D (4.52 μM) and high amounts of the cytokinin BA (22.2 μM) stimulated bulb formation and global growth. As a result of our experimental design, which led to the

best survival, callusing, and bulb formation ratios, the MS medium for *N. pseudonarcissus* shoot tissue subculture should be supplemented by 4 μM of BA, 10 μM of 2,4-D, enriched with 60 g/L of sucrose, and exempt from activated charcoal.

Alkaloid profile

The effects of sucrose and auxins on alkaloid profiles in the three Amaryllidaceae species were studied. Culture extracts of *L. aestivum*, *G. elwesii*, and *N. pseudonarcissus* grown on MSA or MSB medium with different sucrose concentrations (30, 60, 90, and 120 g/L) or various auxin additions (2,4-D, NAA, and picloram) were analyzed by GC–MS. This study was performed to observe the impact of various concentrations of sucrose in combination with various auxins on alkaloid accumulation and diversity.

After 4 months of culture, alkaloids were extracted from the cultured shoots (Ptak et al. 2009). For alkaloid screening, Gal [retention time (Rt) = 22.6 min] and lycorine (Rt = 26.3 min) standards were injected into the GC system to confirm their EI/MS fragmentation patterns. Derivatization was not required since Amaryllidaceae alkaloids retain their characteristic EI/MS fragmentation patterns under GC conditions, as reported by Kreh et al. (1995) and Tram et al. (2002). The extracted alkaloids were identified by comparing measured data with previous results (Ptak et al. 2009) and published data (Berkov et al. 2005). Consequently, the use of standards was not necessary. The identified alkaloids varied according to the plant species (Table 2). Surprisingly, no Gal was detected in the in vivo bulbs of the three plants.

Alkaloids isolated from *N. pseudonarcissus* in vivo bulbs were the most diverse compared to those isolated from the other in vivo species, with three alkaloids identified, namely, trispheridine, anhydrolycorine, and crinine. Overall, seven compounds showing MS fragmentation patterns characteristic of the Amaryllidaceae alkaloids were identified in vivo and in shoots of *N. pseudonarcissus* extracts, specifically, Gal, lycorine, trispheridine, anhydrolycorine, crinine, demethylmaritidine, and narwedine. The highest in vitro alkaloid diversity, namely five identified alkaloids, was obtained by culturing *Narcissus* tissues on MSA and 30 g/L sucrose.

G. elwesii in vivo bulbs contained a low diversity of alkaloids, with only crinine and demethylmaritidine being detected. Shoot cultures were more efficient in terms of an increased alkaloid diversity, with a total of five different alkaloids identified. For example, Gal, trispheridine, crinine, and demethylmaritidine were observed in extracts of *G. elwesii* tissues grown on MSA and 60 g/L sucrose.

The lowest diversity of alkaloids were found in *L. aestivum* in vivo bulbs, with only crinine being detected. The highest in vitro diversity, with four identified alkaloids, was obtained in tissues grown on MSA and 30 g/L sucrose.

Similarly, the tissue cultures of the three plants grown on MSB supplemented with different auxins (2,4-D, NAA, and picloram) were established. Extracts were analyzed by GCMS screening and the results are shown in Table 3.

Compared to in vivo alkaloid patterns, an increase of alkaloid diversity was observed in all shoot cultures which was species-independent. A similar finding in *Catharanthus roseus* was also reported by Pati et al. (2010). Georgiev et al. (2009) established an optimized MS medium containing 8 μM NAA combined with 9 μM BA, which led to the detection of 12 Amaryllidaceae alkaloids of the narciclasine, Gal, hemanthamine, lycorine, and homolycorine types in *L. aestivum* shoot culture. When the medium contained 0.8 μM NAA combined with 8.8 μM of BA, the alkaloid diversity decreased to six (Berkov et al. 2005).

The highest diversity in alkaloids was reached, namely, six identified alkaloids (Gal, lycorine, trispheridine, crinine, demethylmaritidine, and narwedine), by culturing *G. elwesii* shoots on MSB and 10 μM of NAA or picloram (Table 3). The best alkaloid variability was obtained from *N. pseudonarcissus* shoots cultured on MSB and 10 μM of NAA, with five alkaloids detected: Gal. lycorine, tripheridine, crinine, and demethylmaritidine. Among all species, the lowest diversity of alkaloids was obtained from *L. aestivum* shoots cultured on MSB and 10 μM of 2,4-D, with four identified alkaloids: Gal, lycorine, crinine, and demethylmaritidine. The addition of NAA or picloram to the culture medium was less efficient in terms of promoting alkaloid diversity in *L. aestivum*, with only two alkaloids being detected (Table 3).

It should be mentioned that the effects of sucrose and auxins on the Amaryllidaceae alkaloid profiles varied dramatically depending on the species. In terms of species, the best diversity of alkaloids was obtained with *G. elwesii* cultured on medium supplemented with sucrose (60 g/L) and NAA or picloram, with *N. pseudonarcissus* cultured with sucrose (30 g/L) and NAA, and with *L. aestivum* cultured on sucrose (30 g/L) and with 2,4-D.

Gal accumulation and AChE activity

The influence of sucrose and PGRs on Gal accumulation, alkaloid pattern and AChE activity were investigated. Gal was quantified by GC–MS in SIM mode. The reference was authentic Gal hydrobromide (Rt = 22.6 min). AChE inhibitory activity was measured using the Ellman test (Ellman et al. 1961). Authentic Gal (83.8 ± 2.1% of inhibition) and tacrine (98.8 ± 0.3% of inhibition) were used as standards to which the inhibition percentages of the plant extracts were compared. The accumulation of Gal and AChE inhibitory activity differed between species cultured under the same conditions.

Table 2 Gas chromatography–mass spectrometry (GC–MS) screening of extracts of Amaryllidaceae shoot cultures grown on MS basal media (MSA) supplemented with various concentrations of sucrose (30, 60, 90, and 120 g/L)

Amaryllidaceae species	Sucrose (g/L)	Alkaloids: Molecular formula:	Galanthamine $C_{17}H_{21}NO_3$	Lycorine $C_{16}H_{17}NO_4$	Trispheridine $C_{14}H_9NO_2$	Anhydrolycorine $C_{16}H_{13}NO_2$	Crinine $C_{16}H_{17}NO_3$	Demethylmaritidine $C_{16}H_{19}NO_3$	Narwedine $C_{17}H_{19}NO_3$
		Base peak:	286	226	223	250	271	273	284
Galanthus elwesii in vivo			−	−	−	−	+	+	−
Galanthus elwesii in vitro	30		−	−	−	−	−	−	−
	60		+	−	+	−	+	+	−
	90		+	+	−	−	+	−	−
	120		−	−	+	−	−	−	−
Narcissus pseudonarcissus in vivo			−	−	+	+	+	−	−
Narcissus pseudonarcissus in vitro	30		+	−	−	+	+	+	+
	60		−	−	−	−	+	−	−
	90		+	+	−	−	+	+	−
	120		+	−	−	+	+	+	−
Leucojum aestivum in vivo			−	−	−	−	+	−	−
Leucojum aestivum in vitro	30		+	+	−	−	+	+	−
	60		+	+	−	−	−	−	−
	90		−	−	−	−	−	−	−
	120		−	−	+	−	−	−	−

+, Present; –, absent

Table 3 GC–MS screening of extracts of Amaryllidaceae in vitro shoot cultures grown on MS basal media (MSB) supplemented with various phytoregulators (2,4-D, NAA, and picloram)

Amaryllidaceae species		Alkaloids:	Galanthamine	Lycorine	Trispheridine	Anhydrolycorine	Crinine	Demethylmaritidine	Narwedine
		Molecular formula:	$C_{17}H_{21}NO_3$	$C_{16}H_{17}NO_4$	$C_{14}H_9NO_2$	$C_{16}H_{13}NO_2$	$C_{16}H_{17}NO_3$	$C_{16}H_{19}NO_3$	$C_{17}H_{19}NO_3$
		Base peak:	286	226	223	250	271	273	284
Galanthus elwesii in vivo			−	−	−	−	+	+	−
Galanthus elwesii in vitro	2,4-D		+	−	+	−	+	+	−
	NAA		+	+	+	−	+	+	+
	Picloram		+	+	+	+	+	+	−
Narcissus pseudonarcissus in vivo			−	−	+	+	+	−	−
Narcissus pseudonarcissus in vitro	2,4-D		+	+	−	−	+	+	−
	NAA		+	+	+	−	+	+	−
	Picloram		+	+	−	−	+	+	−
Leucojum aestivum in vivo			−	−	−	−	+	−	−
Leucojum aestivum in vitro	2,4-D		+	+	+	−	+	+	−
	NAA		+	+	+	−	−	−	−
	Picloram		−	+	−	−	+	−	−

The highest contents in Gal (0.1% DW in *N. pseudonarcissus*, 0.07% DW in *L. aestivum*, and 0.02% DW in *G. elwesii*) (Fig. 3) were often accompanied with high AChE inhibition (>30%). However, some samples were shown to have low amounts of Gal also showed significant inhibitory activity (20–80%), predominantly in in vivo plant extracts of *G. elwesii* and *N. pseudonarcissus* [Fig. 3a, b; samples contained low amounts of Gal (0.005% DW) and showed high AChE activity (35–80%)]. These interesting results could be explained by the presence of other alkaloids, such as crinine and demethylmaritidine in *Galanthus*, and trispheridine, anhydrolycorine, and crinine in *Narcissus*. Nevertheless, other unknown alkaloids have been also detected in the in vivo bulbs of both plants and be found to have the same biological activity or the presence of synergic alkaloids. The search for more effective AChE inhibitors from natural sources has revealed that some Amaryllidaceae alkaloids, such as sanguinine, habranthine, and 1-*O*-acetyllycorine, possess an inhibitory activity similar to or stronger than Gal (Lopez et al. 2002; Orhan and Sener 2003).

In *G. elwesii*, an in vitro improvement in Gal accumulation (0.02% DW) of 20-fold was reached by culturing explants on culture media enriched with 60–90 g/L of sucrose and 10 µM of 2,4-D associated with 10 µM of BA [Fig. 3a(a)]. This improvement was associated with a parallel increase in the inhibitory activity (38.5%) with a factor of 1.19 compared to in vivo plants showing 0.001% DW of Gal and exerting 34.4% of inhibition. In addition, the substitution of NAA or picloram for 2,4-D did not show any beneficial effect on both Gal accumulation and antiAChE activity [Fig. 3a(b)].

Among the three species, shoot cultures of *N. pseudonarcissus* had the highest Gal content (0.1% DW) when cultured in the presence of 30 g/L of sucrose, 10 µM 2,4-D, and 10 µM BA [Fig. 3b(a)]. Variations in PGRs did not have any significant effect on Gal accumulation in *Narcissus* shoot cultures [Fig. 3b(b)]. The lowest production of Gal (0.015% DW) was obtained with 60 and 90 g/L of sucrose. However, Gal contents increased to 0.08% DW with 120 g/L of sucrose. The highest production of Gal-type alkaloids was reached using high concentrations of sucrose. These results were in accordance with Selles et al. (1997).

It is worthy to note that Gal synthesis was more favored in shoots (0.1% DW) compared to in vivo contents where only traces of Gal were detected. In contrast, in vivo extracts of *Narcissus* showed better AChE inhibition (80%), i.e., inhibition approaching that of authentic Gal and tacrine. However, the highest activity of the shoot extracts did not exceed 50% of the inhibition exhibited by extracts of tissues grown in presence of 90 g/L of sucrose, conditions which induced the maximal Gal concentration (0.1% DW). The GC–MS screening of plant and shoot samples of

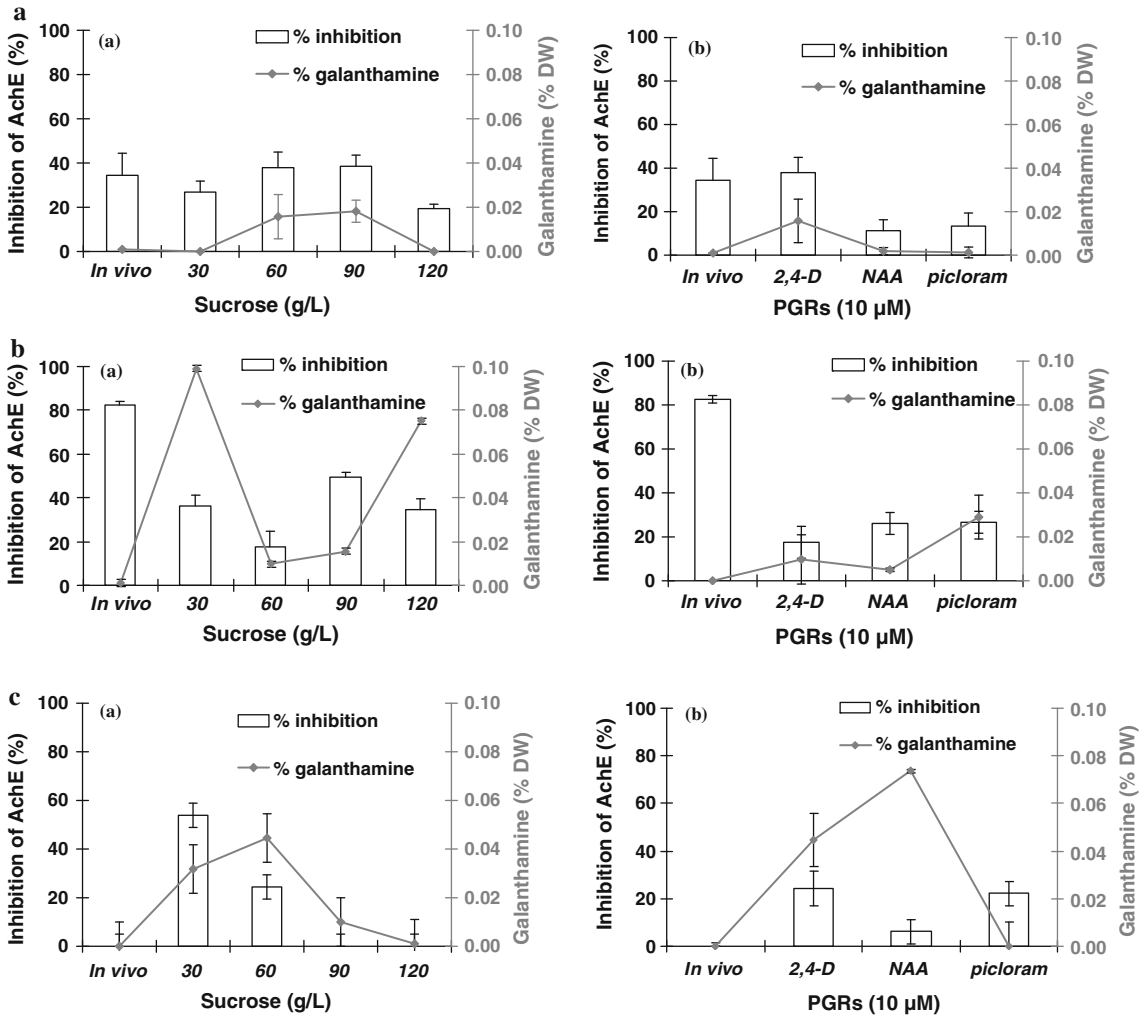

Fig. 3 Evolution of the percentage of galanthamine and acetylcholinesterase (*AChE*) inhibition of *G. elwesii* (**a**), *N. pseudonarcissus* (**b**), and *L. aestivum* (**c**) shoot culture as a function of different sucrose additions (*a*) and different plant growth regulator (*PGR*) additions (*b*)

the three plant species also revealed compounds of unknown MS spectra (Fig. 4). The Gal-type compounds, namely, Gal, norgalanthamine, narwedine, habranthine, and *N*-formylnorgalanthamine, are known to be potent AChE inhibitors (Torras-Claveria et al. 2010), with habranthine being even tenfold more potent than the approved drug Gal (Lopez et al. 2002). Therefore, the presence of these compounds could explain the important AChE inhibitory activity of some extracts possessing low amounts of Gal. Thus, the presence of Gal in high amounts and the associated weak AChE inhibition might be due to the absence of synergic alkaloids or the presence of antagonist compounds.

L. aestivum in vivo extracts exerted no significant inhibitory activity (Fig. 3c), while their shoot cultures exhibited significant inhibitory activity. Shoots grown in the presence of 30–60 g/L of sucrose and 10 µM of 2,4-D and BA contained the highest Gal content (0.032–0.045% DW) associated with the highest AChE inhibition (24–54%). The substitution of NAA for 2,4-D was beneficial in terms of Gal accumulation (0.074% DW), with a factor of 1.7 more Gal compared to the amounts obtained with 2,4-D (0.045% DW); surprisingly, change led to a simultaneous decrease in inhibitory activity (6.1%).

Picloram was found to have similar effects to 2,4-D. Extracts of shoot cultures grown in the presence of picloram exhibited 22% AChE inhibition. However, the addition of picloram was not beneficial for Gal accumulation in shoot cultures where few traces of Gal were detected in corresponding extracts. Therefore, the best results were obtained with shoots grown with 60 g/L of sucrose and 10 µM of NAA in terms of optimal Gal accumulation (0.074% DW), and with 30 g/L of sucrose and 10 µM of 2,4-D for better AChE inhibition (54%). There have been a number of recent reports on experiments aimed at optimizing Gal accumulation in *N. confusus* and *L. aestivum* shoot cultures (Selles et al. 1997; Berkov et al. 2009a, b;

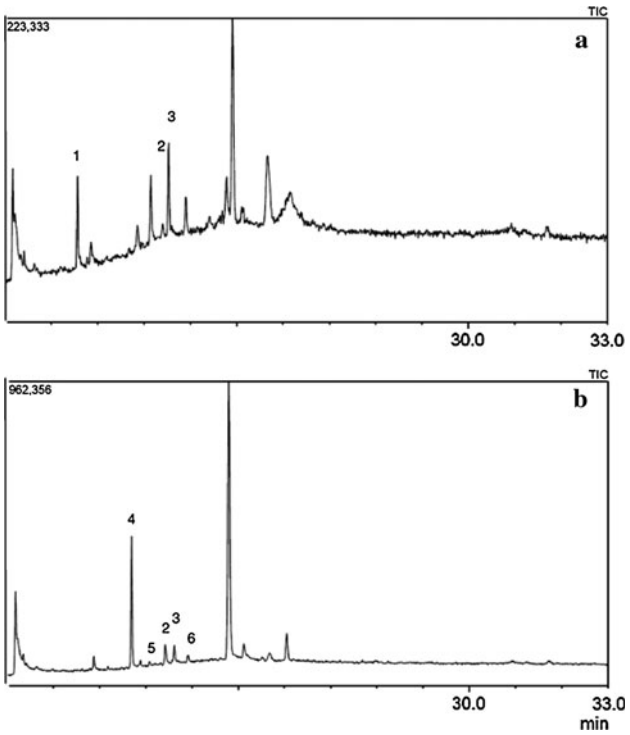

Fig. 4 Gas chromatography–mass spectrometry chromatogram of extracts of *N. pseudonarcissus* in the in vivo bulb (**a**) and in vitro tissues (**b**) grown on MSA and 30 g/L. *1* Trispheridine, *2* crinine, *3* anhydrolycorine, *4* galanthamine, *5* narwedine, *6* demethylmaritidine

Colque et al. 2002; Georgiev et al. 2009). Gal content in *L. aestivum* shoot-clumps ranged from traces to 0.05% DW and up to 0.25% DW per culture of *N. confusus*, of which 0.197% DW was released into the medium (Berkov et al. 2009a, b). In total, the optimal concentrations of Gal were obtained in the established shoot cultures of the three Amaryllidaceae species. To the best of our knowledge and compared to published data, our experiments led to a higher Gal accumulation in shoot cultures (0.02, 0.1, and 0.075% DW of Gal, respectively, in *G. elwesii*, *N. pseudonarcissus*, and *L. aestivum*) considering that alkaloid quantification in the culture media was not evaluated.

In conclusion, our results on the effect of sucrose on Gal accumulation are in accordance with published values. However, the addition of various combinations and concentrations of PGRs to the culture medium led to a better improvement in Gal content and alkaloids variability in all three species. Some extracts with low Gal content showed an important AChE inhibitory activity and may lead to research on other novel alkaloids possessing more potent AchE inhibitory activity. This is the first report of a complete protocol for the in vitro propagation of three major Amaryllidaceae species, i.e., *N. pseudonarcissus*, *G. elwesii*, and *L. aestivum*, with the aim of optimizing Gal extraction. It also highlights the culture conditions for enhanced Gal accumulation and AChE inhibition.

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