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Short communication

Influence of *in vitro* micropropagation on lycorine biosynthesis and anticholinesterase activity in *Hippeastrum goianum*

Gabriella Gusmão Pereira da Costa [a], Cristian Aldemar Gasca Silva [a], João Victor Dutra Gomes [a], Amanda Gomes Torres [a], Izulmé Rita Imaculada Santos [d], Fabrício Tavares Cunha de Almeida [c], Christopher William Fagg [b], Luiz Alberto Simeoni [a], Dâmaris Silveira [a], Kicia Karinne Pereira Gomes-Copeland [a,*]

[a] *Faculdade de Ciências da Saúde, Universidade de Brasília, Campus Universitário Darcy Ribeiro, Brasília, Distrito Federal, Brazil*
[b] *Faculdade de Ceilândia, Universidade de Brasília, Ceilândia, Distrito Federal, Brazil*
[c] *Faculdad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina*
[d] *Embrapa Recursos Genéticos e Biotecnologia, Brasília, Distrito Federal, Brazil*

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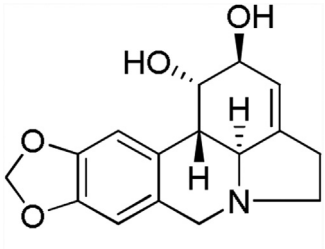
ABSTRACT

Hippeastrum goianum (Ravenna) Meerow, Amaryllidaceae, is an endemic species from the Cerrado, Brazil; there are only few studies about its chemistry or biological activity. This study aimed to investigate the occurrence of lycorine in extracts from *in vitro H. goianum* plantlets, as well as evaluate a possible inhibition of acetylcholinesterase. The ethanol extract of plantlets produced by *in vitro* seed germination and micropropagation of bulblets was obtained from seedlings from *in vitro* germination, while the ethanol extract micropropagtion of bulblets was obtained from a subculture of those seedlings. The presence of lycorine was detected in only in the micropropagation of bulblets. The micropropagation of bulblets was more active than the plantlets produced by *in vitro* seed germination, with an IC_{50} of 114.8 ± 0.95 μg/ml and IC_{50} 386.00 ± 0.97 μg/ml, respectively. These results showed that both *in vitro* germination and micropropagation of *H. goianum* can lead to the biosynthesis of lycorine. Moreover, the micropropagation led to improved anticholinesterase activity.

Introduction

The *Hippeastrum* genus, Amaryllidaceae, represented by 94 species distributed in South and Central America, stands out for its ornamental flowers and its complex and varied chemical composition. The so-called Amaryllidaceae alkaloids, compounds with a broad spectrum of bioactivity, have been found in this genus (Bastida et al., 2006). One of them, lycorine (**1**), presents several relevant pharmacological properties such as being an antiproliferative (Havelek et al., 2017), cytotoxic, antimalarial and inhibition of acetylcholinesterase (Bastida et al., 2006; Liu et al., 2015), as well as having anti-inflammatory and anti-tumor potential (Hu et al., 2015). This alkaloid can be found in bulbs of *H. goianum* (Verdana et al., 2017), an endemic species from the Brazilian midwest Cerrado, appreciated because of its flowers and is considered endangered (Ministério do Meio Ambiente, 2008). The tissue culture technique applied to plants is an interesting alternative in the study of bioprospection, as it friendly toward the conservation and non-degradation of the species. However, although scientific research involving the production of special plant metabolites using *in vitro* culture is growing every year, it remains challenging (Babashpour-Asll et al., 2016).



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* Corresponding author.
E-mail: kcopeland@unb.br (K.K. Gomes-Copeland).

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In vitro cultures of plants belonging to this family have been considered an alternative for the production of bioactive molecules (Pavlov et al., 2007). The objective of this study was to investigate the occurrence of lycorine in extracts of *in vitro* plantlets and as well as evaluate a possible inhibition of acetylcholinesterase.

Material and methods

Seeds of *Hippeastrum goianum* (Ravenna) Meerow, Amaryllidaceae, were collected from adult plants, population at 15°47′22.8S 48°02″25W in the Federal District, Brazil. The species was identified by Prof Christopher Fagg, and a voucher (UB 217068) was deposited in the herbarium of the University of Brasilia UB).

For *in vitro* germination, seeds were disinfected with ethanol (70%) for 1 min, followed by an immersion in commercial sodium hypochlorite solution (2–2.5%) for 8 min, and then washed with sterile distilled water. The seeds were inoculated in an MS medium (Murashige and Skoog, 1962) supplemented with 3% sucrose and 0.7% agar and kept in a growth room at 25 °C ± 2 °C and a 16 h photoperiod with 50 μmol m^{-2} s^{-1} intensity for 90 days.

For the micropropagation experiment, bulblets resulting from the *in vitro* germination stage were sectioned and the segments inoculated in a MS medium with 3% sucrose, 0.7% agar supplemented with 0.5 mg/l of naphthalene acetic acid (NAA), and 10 mg/l of 6-benzylaminopurine (BAP). After inoculation, the cultures were transferred to a growth room with the same conditions as described above.

Ethanol extracts were obtained from dry leaves of plantlets produced by *in vitro* seed germination (HGL1) and micropropagation of bulblets (HGL2). Before extraction, leaves were dried in a drying oven equipped with air circulation and renewal, at 37 °C for 24 h. Dried leaves were shaved and macerated in hexane for 24 h. Subsequently, a second extraction was carried out with ethanol for the same period. After this, the extract was filtrated. Filtrates were concentrated and dried using a rotary evaporator at 40 °C.

Qualitative analysis of lycorine (**1**) was performed using a Hitachi LaChrom Elite® HPLC System coupled to DAD. The sample (10 μl) was injected onto a LiChroCART Purospher RP-18 (5 μm, 4.6 × 150 mm) column coupled to a LiChroCART LiChrospher 100 RP-18 (5 μm, 4 × 4 mm) pre-column. The mobile phase consisted of 0.1% v/v trifluoracetic acid (A), methanol (B) and acetonitrile (C). The initial gradient conditions were as follows: 90%A:5%B:5%C, 0–20 min; 70%A:15%B:15%C, 25–29 min; and 55%A:22.5%B:22.5%C, 30–35 min. At 35 min, it was returned to the initial condition for 7 min. The flow rate was 1 ml/min, and detection was performed at 290 nm. Samples were solubilized in methanol at 6 mg/ml. Lycorine peak was identified by superimposing the spectrum of the peak with the corresponding standard spectrum (peak identity match >993) and by comparison of retention time (t_r). Furthermore, lycorine was confirmed by standard addition (0.075 and 0.125 μg/ml) in two samples. The parameters peak area, t_r and peak height were evaluated using HPLC software (Ezchrome Elite). The presence of lycorine was confirmed by using a single-quad LC–MS system (Model 2020, Shimadzu, Kyoto, Japan), comprising a binary pump (20AD), vacuum degasser, thermostatted autosampler (SIL 20ACXR) and column compartment (CTO 20AC), photodiode detector (SPD M 20A), and mass analyzer (MS 2020) with electrospray ionization (ESI), using the same column and pre-column used in the HPLC-DAD analysis. The mobile phase consisted of 0.1% v/v trifluoracetic acid (A) and methanol/acetonitrile 1:1 (B) with a linear gradient program: 0.0 min, 90%A:10%B; 0.1–20 min, 70%A:30%B, maintained for 5 min; 26–29 min, 55%A:45%B, maintained for 5 min. At 36 min, it returned to the initial condition and maintained for 6 min. The flow rate was 0.5 ml/min. Samples were solubilized in methanol at 6 mg/ml. Lycorine peak was identified by comparing the chromatogram of the sample with the corresponding standard of lycorine (Sigma–Aldrich), as well as by MS fragmentation profile.

The acetylcholinesterase inhibition assay was performed following the Ellman method (Ellman et al., 1961), modified by Lopez et al. (2002). The inhibitory activity was calculated as a percentage, and IC_{50} was calculated. The absorbance presented by the extracts activity was correlated to the negative control (solvent). Galantamine was used as positive control of inhibition (0.03–2.0 μg/ml). The results were analyzed using Graph Pad Prism® 7.0.

Results and discussion

After three months, all seeds of *H. goianum* had completed germination, and the seedlings had roots, bulbs, and expanded leaves (Ministério da Agricultura, 2009). In the micropropagation stage, adequate development of bulblets and leaves was observed, with up to three leaves per seedlings, some of them exceeding 13 cm in

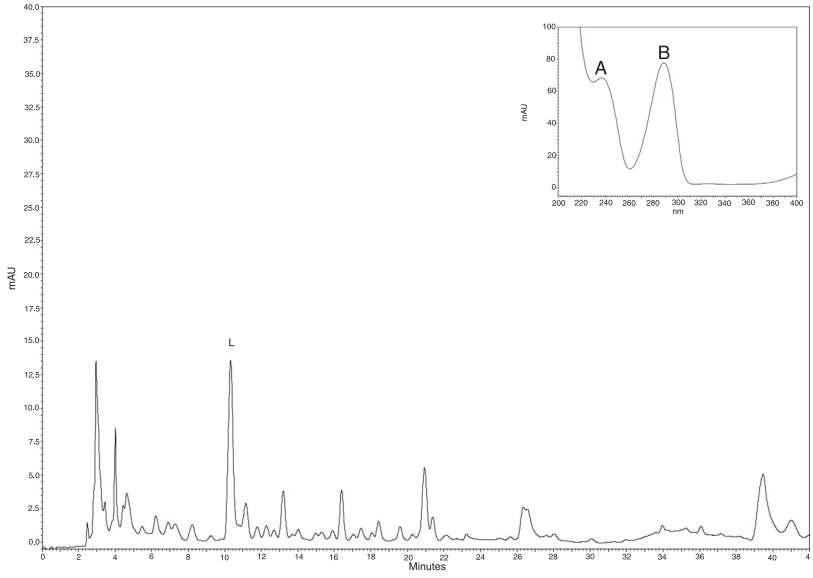

Fig. 1. HPLC-DAD Chromatographic profile of leaf ethanolic extract after bulblets micropropagation (HGL2), lycorine (L) identified with tr = 10.32 minutes. Spectrum maximum absorption at 240 and 290 nm.

Table 1
Parameters of the peak area and peak height increase when increasing amounts of the standard were added in the ethanolic extracts obtained by micropropagation of bulblets (HGL2). Samples analyzed by HPLC-DAD.

Sample	Lycorine (μg/ml)	t_r (min)	Area (mAU)	Height	Match
HGL2-a	–	10.32	2 261 344	5604	0.993
HGL2-b	0.075		5 674 424	301 616	0.994
HGL2-c	0.125		9 449 789	491 612	0.996

length. Zhang et al. (2013) obtained a high propagation coefficient of *Hippeastrum vittatum* seeds cultivated *in vitro*. Factors such as temperature, humidity, light conditions, and culture medium contribute to the progress of *in vitro* and *ex vitro* cultivation. Thus, this knowledge is essential for the development of *in vitro* seedling cultures (Zhang et al., 2013), an important tool for the preservation of plant species (Dragassaki et al., 2003).

Lycorine (**1**) presence in ethanolic extracts was evaluated using HPLC. The HGL1 chromatogram did not show this alkaloid. However, HGF2-a showed a peak at t_r 10.32 min (Fig. 1), with maximum absorption at 240 and 290 nm. Standard addition, 0.075 and 0.125 μg/ml, on two samples (HGL2-b and HGL-c, respectively) was used to confirm the presence of the alkaloid. Table 1 show that parameters as peak area and peak height increase when increasing amounts of the standard were added.

The presence of lycorine in ethanolic extracts was evaluated using LC–MS. Similar to the results obtained using HPLC, HGL1 did not present lycorine. However, HGF2-a showed a peak (L) at t_r 10.40 min (Fig. 2), comparable with lycorine standard. The comparison of the MS spectrum of L with literature data (Ptak et al., 2009) confirmed the presence of lycorine (Fig. 3).

The micropropagation of bulblets in MS medium with growth regulators NAA 0.5 mg/l and BAP 10 mg/l favored lycorine production in HGL2, as was also observed by Zayed et al. (2011), who identified this alkaloid from *H. vittatum* micropropagated bulbs. NAA and BAP may have been partly involved in the biosynthesis of lycorine.

Growth regulators are compounds that promote cell division, influence cell differentiation, and stimulate the biosynthesis of secondary metabolites in plants. Alkaloid biosynthesis in *in vitro* plant culture is directly influenced by the composition and adequate concentration of growth regulators (Verpoorte et al., 1997). In this study, the cytokinins and auxins may have synergistically interacted not only in the development of bulblets but also in the lycorine biosynthesis. However, this is a complex process and requires further research to define the relationships between bioactive compound biosynthesis and culture medium optimization (Pavlov et al., 2007).

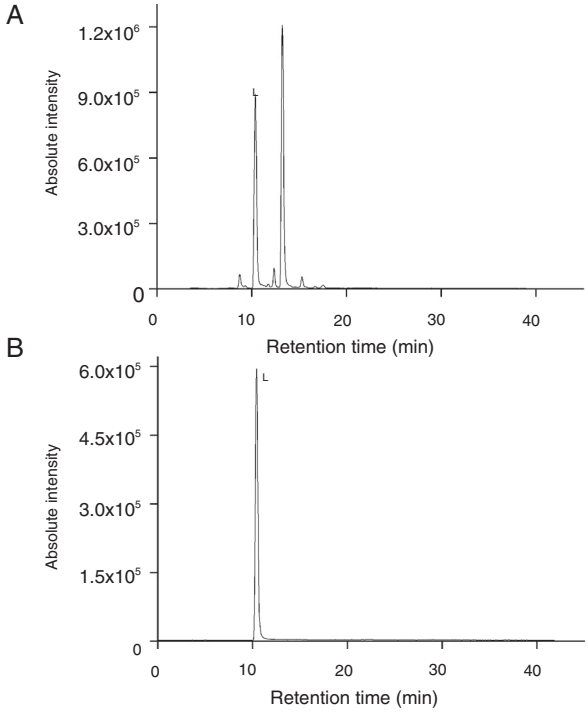

Fig. 2. LC-MS Chromatographic profile of leaf ethanolic extract after bulblets micropropagation (HGF2), lycorine (L) identified with tr = 10.40 minutes (A). LC-MS Chromatographic lycorine Standard (B).

The micropropagation method allowed this molecule biosynthesis as well as other alkaloids, thus contributing to the bioprospection of species with pharmacological interest without degradation, a relevant fact for species that are at risk of extinction.

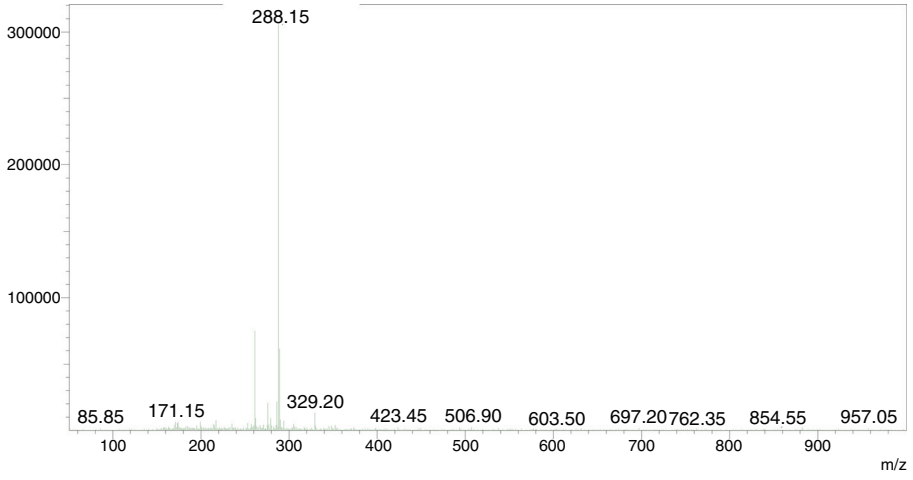

Fig. 3. Mass spectrum of lycorine.

Ethanolic extracts were evaluated for their anticholinesterase activity. The HGL1 showed an IC_{50} value of 386.00 ± 0.97 μg/ml, while HGL2 showed an IC_{50} of 114.80 ± 0.95 μg/ml. This result showed that the supplemented medium may also be able to increase the production of bioactive compounds in *H. goianum*. Galantamine activity was 0.17 ± 0.01 μg/ml. Thus, HGL2 activity is not considered high for this assay, but an improved inhibition was noticed. Lycorine is an alkaloid commonly found in Amaryllidaceae species. However, it is not possible to exclude other possible bioactive compounds (alkaloids or not) responsible for the activity (Tahchy et al., 2011). Cortes et al. (2015) obtained IC_{50} values of 28.13 ± 1.68 μg/ml and 25.73 ± 1.75 μg/ml, respectively, from extract fractions of *Hippeastrum barbatum* and *Hippeastrum puniceum* and correlated this result to the presence of lycorine, which was identified and quantified in all extracts, but showed high levels in *H. puniceum*. The inhibitory activity of acetylcholinesterase is mainly attributed to lycorine and galantamine, and species of the genus *Hippeastrum* have predominantly lycorine in their structural chemical composition.

Conclusion

Lycorine production was detected in plantlets obtained by micropropagation of bulblets, and it was possible to evaluate the inhibition of acetylcholinesterase of *in vitro* plantlets. Our results suggest that *in vitro* micropropagation is a promising technique for lycorine biosynthesis. To our knowledge, this is the first study of lycorine biosynthesis and inhibition of acetylcholinesterase in *in vitro* grown plantlets of *H. goianum*. Further studies are now in progress to determine the effect of auxins and cytokinins on the metabolism of alkaloids and bioactive compounds.

Authors contributions

GGPC and AGT contributed to *in vitro* culture, enzymatic assay and chemical extraction studies. CAGS and JVDG performed the chromatographic analysis. CWF contributed in collecting plant sample and identification. FTCA, IRIS and LAS contributed to critical reading of the manuscript. KKPGC and DS designed the study, supervised the laboratory work and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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