Quantitative Determination of Lycorine and Galanthamine in Different *in Vitro* Tissues of *Narcissus tazetta* by GC-MS

Ameneh Tarakemeh[1], Majid Azizi[1*], Vahid Rowshan[2], Hassan Salehi[3], Rosella Spina[4], François Dupire[4,5], Hossein Arouei[1] and Dominique Laurain-Mattar[4*]

1. Department of Horticultural Science, Ferdowsi University of Mashhad, Mashhad, Iran.

2. Department of Natural Resources, Fars Agricultural and Natural Resources Research and Education Center, Agricultural Research, Education and Extension Organization (AREEO), Shiraz, Iran

3. Department of Horticultural Science, Shiraz University, Shiraz, Iran.

4. Université de Lorraine, CNRS, L2CM, F-54000 Nancy, France.

5. Université de Lorraine, Service Commun de Spectrométrie de Masse, F-54000 Nancy, France.

Abstract

Narcissus spp. are of great importance to pharmaceutical industries because of biosynthesis of Amaryllidaceae alkaloids. *N. tazetta* with several varieties is a widespread species, native to the Mediterranean region with considerable distribution in Iran. In the present study, calli, roots and bulblets were regenerated from *N. tazetta* var. Meskin. In order to report alkaloid profile and quantify galanthamine and lycorine, methanolic extract of different *in vitro* tissues were subjected to alkaloid purification by SPE (solid phase extraction) method, followed by GC-MS analysis. In general, galanthamine and lycorine were detected in all *in vitro* tissues, while demethylmaritidine was only identified in bulblets. Bulblet was identified as the best source of main Amaryllidaceae alkaloids regarding the highest quantities of galanthamine and lycorine with 15 and 731 µg/g dry weight (DW) respectively.

Keywords: Amaryllidaceae alkaloids, Galanthamine, Lycorine, *Narcissus,* Tissue culture.

Introduction

Narcissus spp., including widespred 65 species, 20000 cultivars and hybrids, are among the most important plants of Amaryllidaceae family (Blanchard, 1990). They are known not only for their ornamental values, but also for pharmaceutical properties due to biosynthesis of unique isoquinoline alkaloids named as Amaryllidaceae alkaloids, exhibiting wide range of biological activities (Gabrielsen et al., 1992; Weniger et al., 1995; Jin, 2013). Lycorine, a powerful inhibitor of growth, cell division and organogenesis in higher plants, algae and yeasts, is the first Amaryllidaceae alkaloid, isolated from *N. pseudonarcissus.* Heretofore more than 200 species and varieties belonging to this plant family have been investigated for their alkaloids (Bastida et al., 1998, Bastida and Viladomat, 2002). Furthermore, nearly 100 alkaloids belonging to different skeletal types of Amaryllidaceae alkaloids were also reported for *Narcissus* species.

---

* Corresponding Authors, Email: Azizi@um.ac.ir, dominique.mattar@univ-lorraine.fr

Galanthamine, galanthine, pluviine, homolycorine, lycorenine, hemanthamine, tazettine, narciclasine and montanine are the most common alkaloids existed in *Narcissus* species (Bastida et al., 2006).

Narcissus is the main natural source for galanthamine. This alkaloid registered for its reversible antiacetylcholinesterase with neurocognitive-enhancing activity, used therapeutically for the treatment of Alzheimer's disease and marketed commercially in the USA as Razadyne® and in Europe as Reminyl® (Berkov et al., 2012; Torras-Claveria et al., 2013).

Considering the fact that secondary metabolites are generally produced negligibly in plants (Ratnadewi, 2017), tissue culture could provide an alternative technique for mass production of high quality plant materials (Askari et al. 2018) and commercial *in vitro* production of desired secondary metabolites in a short time (Ratnadewi, 2017).

A literature review showed that the accumulation of Amaryllidaceae alkaloids varies in different organs during the growing season (López et al., 2003; Lubbe et al., 2013), as well as different stages of morphogenesis, i.e., callogenic, embryogenic, and organogenic stages during tissue culture practices (El Tahchy et al., 2011; Sellés et al., 1999; Ivanov et al., 2012).

In the case of *N. tazetta*, Shawky et al. (2015) recorded roots and bulbs as the main target organs for alkaloid accumulation. Our previous investigation indicated *N. tazetta* var. Meskin, as a potential source of Amaryllidaceae alkaloids due to higher diversity of alkaloids, galanthamine and lycorine contents, compared with other varieties of *N. tazetta* including Shahla, Shastpar and Panjehgorbei (Tarakemeh et al., 2019).

In this paper, alkaloids profile, lycorine and galanthamine quantities were investigated in different *in vitro* tissues (calli, roots and bulblets) of an Iranian native variety of *N. tazetta* var. Meskin.

Material and Methods

Plant material

This experiment initiated by collecting wild bulbs of *Narcissus tazetta* var. Meskin after full flowering stage from Behbahan, khuzestan province, Iran in April 2016. Registration and deposition of the species were done in the herbarium of Fars Research Center of Agriculture and Natural Resources, Shiraz, Iran (No: 12823). All the *in vitro* cultures and phytochemical experiments were done in the laboratory L2CM in the Faculty of Science and Technology, Lorraine university of Nancy in France. *In vitro* cultures were established by separating immature leaves from the bulbs, having been chilled at 4°C for 12 weeks. The surface sterilization was performed by immersing leaves in 70% ethanol for 30 seconds and subsequently dipping in Domestos®: water (50 mL: 250 mL, v:v) for 10 min. To minimize the contamination, final rinsing was done three times by sterilized water containing 0.03% of NaClO (Sodium hypochlorite) (Askari et al., 2014). The decontaminated leaves were cut to obtain the primary explants in 3 to 5 mm long. Murashige and Skoog (MS) medium was used as a culture medium. For regeneration of calli from leaf explants, MS medium was supplemented with 3% sucrose, 0.8% agar, 4 µM BAP (6-Benzylaminopurine) and 10 µM 2,4-D (2,4-dichlorophenoxy acetic acid). The pH was also adjusted to 5.5. All the cultures were kept in the dark in a climate room at 25 ± 2°C. Every four weeks the explants were sub-cultured. Calli were observed after 12 weeks. To induce the organogenesis, the calli were transferred to MS medium contained 3% sucrose, 0.8% agar, 5 µM BA and 12 mM 2,4-D. Roots and bulblets were regenerated approximately 8 weeks following culturing of calli on the medium. Different *in vitro* tissues including calli, roots and bulblets (Fig. 1) were used for evaluation of Amaryllidaceae alkaloids profile, galanthamine and lycorine contents.
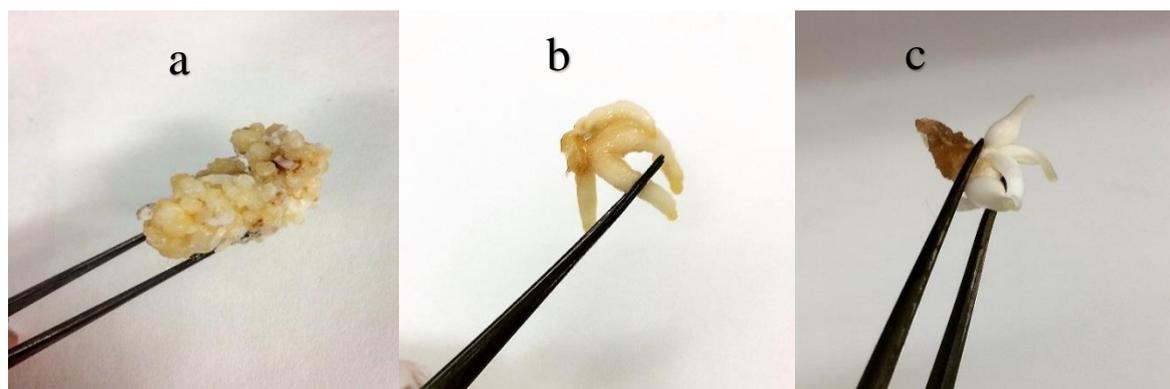
Fig. 1. Different regenerated tissues a) callus b) root c) bulblet of *N. tazetta var. Meskin*

Alkaloid extraction of in vitro regenerated tissues

Alkaloid extraction and purification were performed for *in vitro* regenerated tissues (calli, roots and bulblets) according to Saliba et al. (2016) method. Total extracts were obtained by 24-hours macerating of lyophilized crushed plant materials in methanol followed by 60 min of sonication. To purify the extracts, crude extracts were diluted in CH_3CN (1:3 $MeOH:CH_3CN$ v/v) after adding 300 µL of the hyoscyamine stock solution and then transferred onto a silica cartridge (UPTI-CLEAN SI 1300MG SPE CARTRIDGES) preconditioned with 3 mL of $MeOH:CH_3CN$ (1:3 v/v). After removing the salts from the crude extracts by acetonitrile eluting, the cartridge was washed with 15 mL of the mobile phase of $MeOH:H_2O:HCOOH$ (85:10:5 v/v). The extract obtained from previous operation was transferred to a SCX cation exchanger column (preconditioned with 2 mL of 2% HCOOH in MeOH). To continue 2 mL of 2% HCOOH in MeOH, 3 mL of 2% HCOOH in CH_3CN and 3 mL CH_3CN were used to rinse the cartridge. The alkaloids deterged as free bases with 15 mL of 5% NH_4OH in CH_3CN. Finally alkaloid extracted from different *in vitro* regenerated tissues was injected to the GC-MS apparatus.

GC-MS identification of alkaloid extracts

QP2010-Shimadzu equipment operating in the EI mode at 70 Ev was used for GC-MS analyses. An agilent DB5MS column (30 m × 0.25 mm × 0.25 µm) was employed with a 29 min temperature program of 190-320 °C at 5 °C/min. The injector temperature was adjusted on 280 °C, the flow rate of the helium as carrier gas was 1 mL/min, and the split ratio was adjusted to 1:5. Alkaloids identified by comparing the measured data with those of authentic compounds (galanthamine, lycorine) and NIST08 from LIB database.

Quantitative GC-MS analysis

A quantitative GC-MS analysis by using external standards of galanthamine and lycorine following the method previously reported by El Tahchy et al, (2011), was used to compare the galantamine and lycorine contents in different *in vitro* regenerated tissues of *N. tazetta* var. Meskin. An internal standard calibration method along with a nine-point calibration curve (R2=0.99) was performed.

Statistical analysis

This experiment was analyzed in a completely randomized design (CRD). SAS software was used for the analysis of variance and LSD's test ($p \leq 0.05$) was used for comparing the means.

Results

Amaryllidaceae alkaloids profile of different in vitro regenerated tissues

Alkaloids were identified by comparing the measured data with those of authentic compounds (galanthamine and lycorine) and NIST08 from LIB database. Alkaloid

profile of *N. tazetta* var. Meskin was slightly influenced by the type of tissues. Galanthamine and lycorine were detected in all tissues (Fig. 2), whereas demethylmaritidine was only detected in bulblets as shown in Table 1.
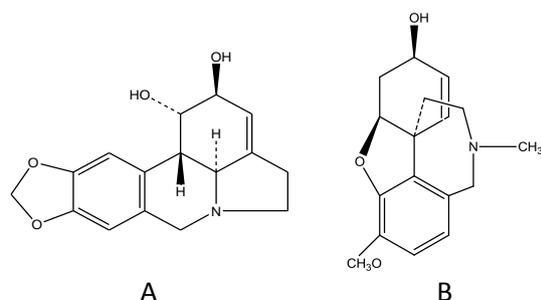

Fig. 2. Lycorine (A) and galanthamine (B) identified in callus, root and bulblet of *N. tazetta* var. Meskin

Galanthamine content in different in vitro tissues

Using an authentic galanthamine, the quantitative GC-MS analysis of regenerated calli, roots and bulblets indicated that the galanthamine concentrations were significantly influenced by the degree of differentiation (Fig. 3). The higher concentration of galanthamine was detected in bulblets (15 µg/g DW), representing a three-fold increase when compared to the roots (5.2 µg/g DW) and eight-fold increase when compared to the calli (1.9 µg/g DW) tissues.

Lycorine content in different in vitro tissues

According to the quantitative GC-MS analysis, using the standard of lycorine, significant differences were found among lycorine contents of the tissue *in vitro*. As depicted in Fig 4, it is clear that Lycorine quantities were strongly increased with the level of differentiation. The highest amount of lycorine was observed in bulblets (731 µg/g DW) which exhibited 34-fold increase in comparison with the lowest amount of lycorine that was detected in calli (21.56 µg/g DW). Lycorine content was 131.6 µg/g DW in roots, which is six-fold smaller than the lycorine content recorded for the bulblets.

Table 1. Identified alkaloids by GCMS in different *in vitro* regenerated tissues of *N. tazetta* var. *Meskin*

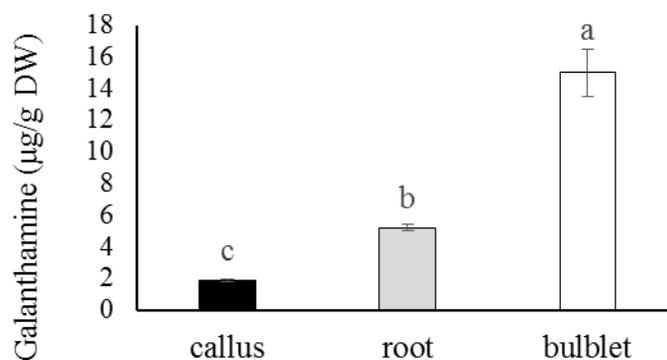|	Lycorine	Galanthamine	Demethylmaritidine
Formula	$C_{16}H_{17}NO$	$C_{16}H_{19}NO_3$	$C_{16}H_{19}NO_3$
Base peak	226	286	272.90
Molecular mass	287	287	273
in vitro tissues			
callus	+	+	-
root	+	+	-
bulblet	+	+	+

Fig. 3. Galanthamine (µg/g DW) contents of different in vitro tissues of N. tazetta var. Meskin. The data represent the average of three replications with standard error. Different letters indicate the statistically significant differences (LSD's multiple range test, p≤0.05).
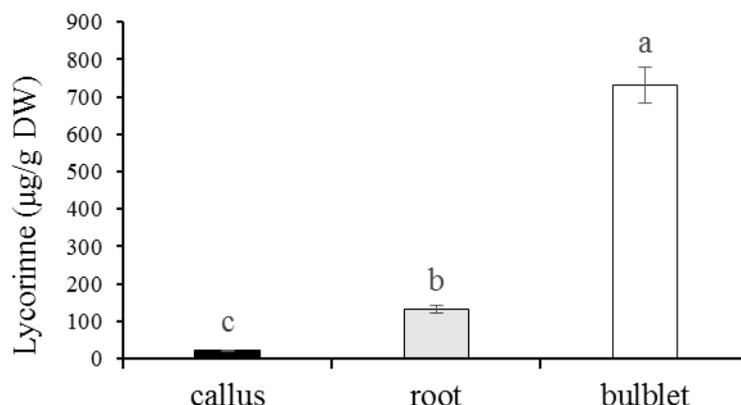
Fig. 4. Lycorine (µg/g DW) contents of different tissues of *N. tazetta* var. Meskin. The data represent the average of three replications with standard error. Different letters indicate the statistically significant differences (LSD's multiple range test, p≤0.05).

Discussion

Galanthamine accumulation in Amaryllidaceae is greatly varied among species, from trace amounts to 0.5% of DW (López et al., 2003). This alkaloid was previously detected in roots and bulbs, whereas lycorine was found not only in roots and bulbs, but also in aerial parts of *in vivo* produced *N. tazetta* (Shawky et al., 2015).

In the present study, galanthamine quantity was ranged from 1.9 to 15 µg/g DW, and lycorine contents from 21.56 to 731 µg/g DW in different *in vitro* tissues of *N. tazetta* var. Meskin. Both of these quantified alkaloids were previously reported in much higher quantity for wild bulbs of this variety (Tarakemeh et al., 2019). A possible explanation for the mentioned differences is due to this fact that plants grown in natural habitats contain higher secondary metabolites than the ones produced by *in vitro* culture practices (Karuppusamy, 2009; Dias et al., 2016).

As prior studies demonstrated, *in vitro* Amaryllidaceae alkaloids biosynthesis capacity was associated with factors including, the type of the cells and organs during ontogenic stages, as well as cellular differentiation in tissue culture samples, which is actually controlled by hormonal treatments (El Tahchy et al, 2011; Diop et al., 2006; Ivanov et al., 2012; Berkov et al., 2014; López et al., 2003; Lubbe et al., 2013).

In the present study, the concentrations of galanthamine and lycorine in calli (1.9 µg/g DW and 21.56 µg/g DW respectively) were confirmed the low ability of undifferentiated cells to produce these metabolites. Our finding regarding a trace amount of galanthamine in undifferentiated tissues of *N. tazetta* var. Meskin are in line with previous reports on *Leucojum aestivum* callus and undifferentiated tissues of *N. confusus* (Pavlov et al., 2007, Sellés et al., 1999). Galanthamine content of 0.03 µg/g DW for dedifferentiated calli and 0.1 µg/g DW for an embryogenic callus of *N. confusus* has been previously reported (Sellés et al., 1999), which demonstrate a strong correlation between galanthamine accumulation and the level of differentiation.

Callus induction is an important step of micro-propagation. Because of its simple structure, callus could be a useful experimental system for monitoring alkaloid biosynthesis, though it is not the most effective tissue for alkaloid production (Sellés et al., 1999).

Galanthamine quantity for root was 5.2 µg/g DW. In contrast, Diop et al (2006) did not detect any galanthamine in the *in vitro* roots of *L. aestivum*. Based on our results, bulblet was the best source for both galanthamine and lycorine production, based on their highest contents (15 µg/g DW and 731 µg/g DW respectively) in this

tissue. It should be mentioned that both galanthamine and lycorine had higher concentrations in the differentiated tissues (roots and bulblets) compared to their concentrations in the calli. This is indicative of higher ability of differentiated cells to produce and compart secondary metabolites. Saliba et al. (2015) reported that lycorine content in *in vitro* bulblets of *L. aestivum* is ranged between 0.04 and 0.2 mg/g DW.

In the current study, demethylmarithidine was only identified in bulblets of *N. tazetta* var. Meskin, while it was not detected in the wild bulbs of this variety (Tarakemeh et al., 2019).

Despite the fact that the tissue of synthesis is not being always same as the tissue of storage in the plants, identification of the synthesis tissue and accumulation tissue is an important step to stabilize and upscale desired secondary metabolites (Ratnadewi, 2017). This issue should be considered for further investigations on Amaryllidaceae alkaloid biosynthesis.

Acknowledgements

References

1. Askari N, Wang Y.G, Klerk G.J.D. 2014. In tissue culture of *Lilium* explants may become heavily contaminated by the standard initiation procedure. Propagation of ornamental plants 14, 49-56.

2. Askari N, Visser R.G.F, Klerk G.J.D,. 2018. Growth of Lily Bulblets *In Vitro*, a Review. International Journal of Horticultural Science and Technology 5, 133-134.

3. Bastida J, Viladomat F, Codina C. 1998. *Narcissus* alkaloid, In: A.U. Rahman (Ed.), Studies in Natural Products Chemistry, Elsevier Science, 323-406.

4. Bastida J, Viladomat F. 2002. Alkaloids of *Narcissus*. In*: Narcissus* and Daffodil: The Genus *Narcissus*, Taylor and Francis, 141-214.

5. Bastida J, Lavilla R, Viladomat F. 2006. Chemical and Biological Aspects of *Narcissus* Alkaloids. In: The Alkaloids: Chemistry and Biology, Elsevier 87-179.

6. Berkov S, Codina C, Bastida J. 2012. The Genus *Galanthus*: A Source of Bioactive Compounds, In: Rao V, Phytochemicals - A Global Perspective of Their Role in Nutrition and Health, IntechOpen, 235-254.

7. Berkov S, Ivanov I, Georgiev V, Codina C, Pavlov A. 2014. Galanthamine biosynthesis in plant *in vitro* systems. Engineering in Life Sciences 14, 643-650.

8. Blanchard J.W. 1990. *Narcissus*: A Guide to Wild Daffodils. Alpine Garden Society, Woking, Lye End Link, Surrey, UK.

9. Dias M.I, Sousa M.J, Alves R.C, Ferreira I.C. 2016. Exploring plant tissue culture to improve the production of phenolic compounds: A review. Industrial Crops and Products 82, 9-22.

10. Diop M.F, Ptak A, Chre´tien F, Henry M, Chapleur Y, LaurainMattar D. 2006. Galanthamine content of bulbs and *in vitro* cultures of *Leucojum aestivum* L. Natural Product Communications 1, 475-479.

11. El Tahchy A, Bordage S, Ptak A, Dupire F, Barre E, Guillou C, Henry M, Chapleur Y, Laurain-Mattar D. 2011. Effects of sucrose and plant growth regulators on acetylcholinesterase inhibitory activity of alkaloids accumulated in shoot cultures of Amaryllidaceae. Plant Cell Tissue Organ Culture 106, 291-390.

12. Gabrielsen B, Monath T.P, Huggins J.W, Kefauver D.F, Pettit G.R, Groszek G, Hollingshead M, Kirsi J.J, Shannon W.M, Schubert E.M, Dare J, Ugarkar B, Ussery M.A, Phelan M.J. 1992. Antiviral (RNA) activity of selected Amaryllidaceae isoquinoline constituentsand synthesis of related substances. Journal of Natural Products 55, 1569-1581.

13. Ivanov I, Georgiev V, Berkov S, Pavlov A. 2012. Alkaloid patterns in *Leucojum aestivum* shoot culture cultivated at temporary immersion conditions. Journal of Plant Physiology 169, 206-211.

14. Jin Z. 2013. Amaryllidaceae and sceletium alkaloids. Natural Product Reports 2013, 30, 849-868.

15. Karuppusamy S. 2009. A review on trends in production of secondary metabolites from higher

16. plants by *in vitro* tissue, organ and cell cultures. Journal of Medicinal Plants Research 3, 1222-1229.

17. López S, Bastida J, Viladomat F, Codina C. 2003. Galanthamine pattern in *Narcissus confusus* plants. Planta Medica. 69, 1166-1168.

18. Lubbe A, Gude H, Verpoorte R, Choi Y.H. 2013. Seasonal accumulation of major alkaloids in organs of pharmaceutical crop *Narcissus* Carlton. Phytochemistrybastida 88, 43-53.

19. Pavlov A, Berkov S, Courot E, Gocheva T, Tuneva D, Pandova, B, Georgiev M, Georgiev V, Yanev S, Burrus, M. 2007. Galanthamine production by *Leucojum aestivum in vitro* systems. Process Biochemistry 42, 734-739.

20. Ratnadewi D. 2017. Alkaloids in Plant Cell Cultures. In: Alkaloids-Alternatives in Synthesis, Modification and Application, IntechOpen 1-23.

21. Saliba S, Ptak A, Laurain-Mattar D. 2015. 4'-O-methylnorbelladine feeding enhances galanthamine and lycorine production by *Leucojum aestivum* L. shoot cultures. Engineering in Life Sciences 15, 640-645.

22. Saliba S, Ptak A, Boisbrun M, Spina R, Dupire F, Laurain-Mattar D. 2016. Stimulating effect of both 4'-O-methylnorbelladine feeding and temporary immersion conditions on galanthamine and lycorine production by *Leucojum aestivum* L. bulblets. Engineering in Life Sciences 16, 731-739.

23. Sellés M, Viladomat F, Bastida J, Codina C. 1999. Callus induction, somatic embryogenesis and organogenesis in *Narcissus confusus*: Correlation between the state of differentiation and the content of galanthamine and related alkaloids. Plant Cell Reports 18, 646-651.

24. Shawky E, Abou-Donia A.H, Darwish F.A, Toaima S.M, Takla S.S, Pigni, N.B, Bastida J. 2015. HPTLC and GC/MS study of Amaryllidaceae alkaloids of two *amary* species. Chemistry and Biodiversity 12, 1184-1199.

25. Tarakemeh A, Azizi M, Rowshan V, Salehi H, Spina R, Dupire F, Arouie H , Laurain-Mattar D. 2019. Screening of Amaryllidaceae alkaloids in bulbs and tissue cultures of *Narcissus papyraceus* and four varieties of *N. tazetta*. Journal of Pharmaceutical and Biomedical Analysis 172, 230-237.

26. Torras-Claveria L, Berkov S, Codina C, Viladomat F, Bastida J. 2013. Daffodils as potential crops of galanthamine. Assessment of more than 100 ornamental varieties for their alkaloid content and acetylcholinesterase inhibitory activity. Industrial Crops and Products 43, 237-244.

27. Weniger B, Italiano L, Beck J.P, Bastida J, Bergoñón S, Codina C, Lobstein A, Anton R. 1995. Cytotoxic activity of Amaryllidaceae alkaloids. Planta Medica 61, 77-79.