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## Screening of Amaryllidaceae alkaloids in bulbs and tissue cultures of *Narcissus papyraceus* and four varieties of *N. tazetta*.

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### Abstract

*Narcissus* spp. are an economically important crop for medicines in relation with the alkaloids production, mainly galanthamine, an acetylcholinesterase inhibitor used for the treatment of Alzheimer's disease. In this article an extensively study of the phytochemistry of both bulbs of different species and varieties of *Narcissus* grown in Iran and *in vitro* culture of these plants was investigated. In particular, the Amaryllidaceae alkaloid profile and the galanthamine and lycorine contents in wild bulbs of *Narcissus papyraceus* (G5) and four varieties of *Narcissus*

*tazetta* (*N. tazetta* var. **Shahla** (G4), *N. tazetta* var. **Shastpar** (G1), *N. tazetta* var. **Meskin** (G2), *N. tazetta* var. **Panjehgorbei** (G3)), growing in Iran are reported. The alkaloid profiles were investigated by GC-MS and LC-MS and the quantitative analysis was performed using GC-MS. In total, thirty alkaloids were identified among them nine alkaloids were observed with the both methods of analysis. The variety **Meskin** of *N. tazetta* (G2), showed the highest diversity of alkaloids and the highest content in galanthamine. On this last species (G2) and on *N. tazetta* var. **Shahla** (G4), the effects of auxins 2,4-dichlorophenoxyacetic acid (2,4-D), 4-amino-3,5,6-trichloropicolinic acid (**Picloram**) and naphthalene acetic acid (NAA) at concentrations of 25 and 50  $\mu\text{M}$  were studied on the induction of callus and its capacity to induce organogenesis and alkaloid diversity. All auxins, at the concentrations of 25 and 50  $\mu\text{M}$ , produced calli. Bulblets and roots were formed on calli grown only in the presence of 25 or 50  $\mu\text{M}$  NAA. GC-MS analyses showed the presence of galanthamine and lycorine in calli, roots and bulblets, with all auxins whatever the concentration used while demethylmaritidine and tazettine were found in differentiated tissue cultures cultivated on the medium containing NAA (25 or 50  $\mu\text{M}$ ) or in calli initiated with **Picloram** (50  $\mu\text{M}$ ). Precursor 4'-*O*-methylnorbelladine (MN) of Amaryllidaceae alkaloids feeding was found to significantly improve the accumulation of both galanthamine (82  $\mu\text{g/g}$  DW) and lycorine (1800  $\mu\text{g/g}$  DW) in bulblets of *N. tazetta* var. **Meskin** (G2).

## **Keywords**

Galanthamine ; lycorine ; *Narcissus* ; auxins ; GC-MS ; LC-MS

## **1. Introduction**

Various species of *Narcissus*, bulbous flowering plants belonging to the Amaryllidaceae family, are known to synthesize Amaryllidaceae alkaloids. There is a growing interest for these plants due to the medicinal use of one of the major alkaloids in this genus, galanthamine. The register

of the Royal Horticultural Society International lists more than 26,400 named daffodil varieties. From *N. pseudonarcissus*, Gerrard in 1877 isolated lycorine, the first Amaryllidaceae alkaloids [1]. Several studies have been published about the distribution of galanthamine in different populations of these plants as well as in their different organs and during different periods of harvesting [2]. Galanthamine is a long-acting, selective, reversible and competitive acetylcholinesterase (AChE) inhibitor, which is used in the world to treat the patients with mild to moderate Alzheimer's disease. Great interest in *Narcissus* species and varieties has risen as an industrial source for the production of galanthamine, because of its use as medicine. *Narcissus* bulbs are considered one of the most important natural sources of galanthamine other than synthetic production. Besides galanthamine, there are a number of other alkaloids found in *Narcissus* such as lycorine, lycorenine, homolycorine, haemanthamine, narciclasine and tazettine [3,4]. Some studies have shown that these alkaloids also possess some interesting biological activities such as antimalarial [5], antiviral, antitumor [6,7], antifungal [8] and insect anti-feedant activity [9].

Some species like *N. confusus*, *N. pseudonarcissus*, *N. tazetta*, *N. poeticus*, *N. broussonetii*, *N. serotinus*, *N. asturiensis*, *N. jonquilla* and different cultivars of *Narcissus* have been studied for their alkaloid content [2,3,10-18]. However to our knowledge, no investigation was reported on Amaryllidaceae alkaloids diversity and accumulation in *Narcissus papyraceus* as well as in various varieties of *N. tazetta* (*N. tazetta* var. Shastpar, *N. tazetta* var. Meskin, *N. tazetta* var. Panjehgorbei and *N. tazetta* var. Shahla), growing wild populations in Iran.

The aim of this study was to investigate both the Amaryllidaceae alkaloid profiles and galanthamine and lycorine contents of bulbs of *N. papyraceus* and four varieties of *N. tazetta* wild plants using GC-MS and LC-MS. GC-MS is a useful and reliable method for rapid separation and identification of complex mixtures of Amaryllidaceae alkaloids [19,20]. Due to the complexity of the extracts, one single analytical method is generally insufficient to identify

all alkaloids. It is for this reason that the use of another analytical instrument, LC-MS, was necessary to obtain truly comprehensive informations. Also in this work, we combined various parameters which have previously shown positive effects on alkaloid accumulation in tissue cultures of *Leucojum aestivum*, i.e. differentiated tissues such as bulblets induced by auxins and feeding with the common precursor 4'-*O*-methylnorbelladine (MN) of Amaryllidaceae alkaloids [21, 22]. The level of the alkaloid biosynthesis is strongly influenced by cellular differentiation which may be influenced by growth regulators like auxins [23, 24]. Also, we reported the metabolization of the deuterium-labeled precursor, by *L. aestivum* shoot cultures and its stimulating effect on galanthamine and lycorine biosynthesis [25]. Therefore, the biosynthetic pathway in these Iranian various varieties of *Narcissus* was estimated for the first time under the influence of various auxins and the Amaryllidaceae alkaloids precursor MN.

## 2. Materials and methods

### 2.1. Plant Materials

Bulbs of *N. tazetta* var. **Shastpar** (G1), *N. tazetta* var. **Meskin** (G2), *N. tazetta* var. **Panjehgorbei** (G3), *Narcissus tazetta* var. **Shahla** (G4), and *N. papyraceous* (G5) were harvested 2 months after full flowering stage from Behbahan, Ahvaz province in Iran in April 2016. The species have been registered and deposited in the herbarium of Fars Research Center of Agriculture and Natural Resources, Shiraz, Iran (N° 12822, N° 12823, N° 12824, N° 12825 and N° 12826 respectively).

Thin slices of leaves (about 3 – 5 mm) isolated from *N. tazetta* var. **Meskin** (G2) and *N. tazetta* var. **Shahla** (G4) bulbs chilled at 4°C for 8 weeks were used as initial explants. Plant material was surface-sterilized in 70% ethanol (30 seconds), then shaken for 10 minutes in a solution of Domestos®: water (50 mL: 250 mL, v: v) [25] and rinsed three times in sterilized water containing 0.03% of NaClO [26].

## 2.2. Callus initiation and bulblets induction

Initial explants were cultivated on the Murashige and Skoog (MS) [27] medium supplemented with various auxins (2,4-D, Picloram or NAA) at different concentrations (25  $\mu$ M and 50  $\mu$ M) and the cytokinin 6-benzylaminopurine (BAP) (0.5  $\mu$ M). The MS medium was supplemented with 3% sucrose and gelling with 0.8% agar. The pH was adjusted to 5.5 before autoclaving. The cultures were maintained at  $25 \pm 2^\circ\text{C}$  under dark conditions and subcultured every 4 weeks. Treatments were arranged in a completely randomized  $2 \times 3 \times 2$  factorial design with five replications. Analysis of variance was performed using the SAS software and means were compared using LSD's test ( $p \leq 0.05$ ). Six-month-old cultures (2g) were submerged in MS liquid-shake medium (20 mL) containing BAP (4  $\mu$ M) and 2,4-D (10  $\mu$ M) and precursor 4'-O-methylnorbelladine at various concentrations (0.05 and 0.15 g/L) in 100 ml Erlenmeyer. They were incubated at  $25 \pm 2^\circ\text{C}$  and 150 rpm using the *Infors Ecotron Ht Model Shaking Incubator* for two periods of time (10 and 20 days). A stock solution for feeding experiments was prepared and was filter-sterilized directly into the flasks as reported by Saliba et al. [22].

## 2.3. Chemicals and reagents

All commercially available reagents were used without further purification. Triethylammonium acetate buffer, galanthamine, lycorine, BAP, Picloram, 2,4-D, inositol, agar-agar, magnesium sulphate heptahydrate (98%), manganese sulphate, potassium nitrate, and ethylene diamine tetra acetic acid iron (III) sodium salt were all from Sigma-Aldrich. Ammonium bicarbonate, ammonium formate, iron sulphate, zinc sulphate, copper(II)sulphate pentahydrate (99%), thiamine hydrochloride, NAA, absolute ethanol, and methanol (Hipersolv Chromanorum for HPLC – Isocratic Grade) were purchased from VWR international. Acetonitrile was acquired from Sigma-Aldrich. Pyridoxol hydrochloride, nicotinic acid, potassium dihydrogenophosphate and calcium chloride dehydrate were obtained from Merck AG Darmstadt (Germany).

## 2.4. Synthesis of the precursor

The precursor MN was synthesized according to Saliba et al. [22].

## 2.5. Alkaloid extraction

Alkaloids were extracted from plant material as previously described [25]. Methanolic crude extracts were purified by SPE procedure as reported by Saliba et al. [22].

## 2.6. GC-MS identification

Analyses were performed using QP2010-Shimadzu equipment operating in the EI mode at 70 eV. A 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 280 °C, the flow rate of the carrier gas (helium) was 1 mL/min, and the split ratio was 1:5. 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. An internal standard calibration method along with a nine-point calibration curve ( $R^2=0.99$ ) using authentic galanthamine and lycorine were used for quantitative analysis of alkaloids.

## 2.7. LC-MS analysis

The LC system consisted of an U3000-Dionex apparatus with an injector comprising a 1 μL loop and a UV detector at 280 nm. The analytical column used was an Acclaim MIXED MODE HILIC-1 ID 2.1 mm column (150 mm x 5 μm x 120 Å) and eluted at a flow rate of 200 μL/min using a gradient ranging from 2% solvent B to 25% solvent B in a time span of 26 min and 30 sec. Solvent A consisted of pure acetonitrile and solvent B consisted of 10 mM ammonium formate in H<sub>2</sub>O at pH 6.8. The ESI-HRMS was a micrOTOF<sup>TM</sup> (Bruker Daltonics) apparatus. The LC coupled with high-resolution mass spectrometry (ESI/QTOF) was used in order to confirm lycorine and galanthamine identities in sample extracts. This system was able to detect the compounds in amounts lower than 0.02 μg/mL. Authentic galanthamine and lycorine displayed  $[M + H]^+$  at an  $m/z$  of 288.1594 and 288.1234 Da, respectively. The analysis was

performed in positive mode with the compass for otofSeries 1.7 software including otofControl 3.4 for acquisition and dataAnalysis 4.2 for process. The identification of the other alkaloids was performed by comparing the measured molecular mass with the calculated values  $\pm 10$  mDa.

### 3. Results and discussion

#### 3.1. Amaryllidaceae alkaloid profile by GC-MS and LC-MS

There is no report in the literature concerning the alkaloid profiling of Iranian *Narcissus tazetta* and *N. papyraceus* bulbs. To investigate the various compounds present in the complex alkaloid fractions of four varieties of *N. tazetta* and one species of *N. papyraceus*, GC-MS and LC-MS were used. For the both methods of analysis, the identification of the alkaloids was performed by comparing the measured data with literature data [20,28]. Capillary GC-MS was used according to the method previously described by El Tahchy et al. [23]. Following the alkaloid extraction and as shown earlier by Tram et al. [29], derivatization was not required since the Amaryllidaceae alkaloids retain their characteristic EI/MS fragmentation patterns when employing GC conditions. 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 *Narcissus* varieties. Six compounds (isimine, trisphaeridine, galanthamine, lycorine, masonine, chlidanthine 1,2-dihydro) were found in all *Narcissus* varieties bulbs (Fig. 1) and showed MS fragmentation patterns characteristic of the Amaryllidaceae alkaloids (Table 1). Tazettine was identified in all varieties of *N. tazetta* and not in *N. papyraceus* (G5). This last genotype (G5), *N. tazetta* var. *Panjehgorbei* (G3) and *N. tazetta* var. *Shahla* (G4) showed the lowest distribution of the alkaloids (eight compounds) while *N. tazetta* var. *Meskin* (G2) showed the highest distribution of the Amaryllidaceae alkaloids (ten compounds) (Table 1).



Untargeted LCHRMS profiling was performed to generate extensive mass list corresponding to a wide variety of alkaloids present in various extracts of four varieties of *N. tazetta* and one species of *N. papyraceus*. Analysis performed in positive ion mode detected between 13 (*N. tazetta* var. **Shastpar** (G1) and 20 alkaloids (*N. papyraceus* (G5)) depending on the genotype of *Narcissus* studied (Table 2). It is important to note that the identification provided is a proposed identification. It has been reported that epimers like vittatine and crinine with the same masses show slightly different retention times [28]. In addition, the MZmine software identifies peaks via searches through an online database in which only a few Amaryllidaceae alkaloids are available. The most common alkaloids detected were lycorine (C<sub>16</sub>H<sub>17</sub>NO<sub>4</sub> [M + H]<sup>+</sup> 288.1234), trisphaeridine (C<sub>14</sub>H<sub>9</sub>NO<sub>2</sub> [M + H]<sup>+</sup> 224.0708), crinamine (C<sub>17</sub>H<sub>19</sub>NO<sub>4</sub> [M + H]<sup>+</sup> 302.1378) and albomaculine (C<sub>19</sub>H<sub>23</sub>NO<sub>5</sub> [M + H]<sup>+</sup> 346.1645) which were present in all varieties of *Narcissus* whereas galanthamine (C<sub>17</sub>H<sub>21</sub>NO<sub>3</sub> [M + H]<sup>+</sup> 288.1492) was detected only in bulbs of *N. tazetta* var. **Shastpar** (G1) and *N. tazetta* var. **Meskin** (G2). Untargeted UPLC-QTOF-MS profiling performed on various extracts of *N. pseudonarcissus* ‘King Alfred’ tissues has shown that the most abundant Amaryllidaceae alkaloid detected was crinamine followed by lycorenine in leaves [28]. Lycorenine was identified only in *N. tazetta* var. **Meskin** (G2). Surprisingly tazettine (C<sub>18</sub>H<sub>21</sub>NO<sub>4</sub> [M + H]<sup>+</sup> 316.1635), 4’ -*O*-demethylbelladine (C<sub>18</sub>H<sub>23</sub>NO<sub>3</sub> [M + H]<sup>+</sup> 302.1749), pseudolycorine (C<sub>16</sub>H<sub>19</sub>NO<sub>4</sub> [M + H]<sup>+</sup> 290.1425), haemanthidine (C<sub>17</sub>H<sub>19</sub>NO<sub>5</sub> [M + H]<sup>+</sup> 318.1318) and ismine (C<sub>15</sub>H<sub>15</sub>NO<sub>3</sub> [M + H]<sup>+</sup> 258.1111) were identified in bulbs of all varieties excepted in *N. tazetta* var. **Meskin** (G2). These results are complementary to those obtained by GC-MS analysis. Nine alkaloids were identified both with the GC-MS and LC-MS analysis in the various bulb extracts of *Narcissus* studied: trisphaeridine, assoanine, ismine, 3-epimacronine, hippeastrine, lycorenine, lycorine, galanthamine, and tazettine. However, the identification of each of these alkaloids in one genotype depended on the method of analysis considered. For example, assoanine, ismine, and

tazettine were detected in the extract of *N. tazetta* var. **Meskin** (G2) by GC-MS analysis and were not detected by LC-MS. In contrary, 3-epimacronine, hippeastrine, and tazettine were detected in the extract of *N. papyraceus* (G5) by LC-MS while they were not detected by GC-MS.

### 3.2. Lycorine and galanthamine contents

In order to investigate the contents of galanthamine and lycorine in the wild bulbs of two species of *Narcissus*, *N. tazetta* and *N. papyraceus* and also in different varieties of *N. tazetta* (G1 to G4) grown in Iran, a quantitative GC-MS analysis was employed according to the method previously described by El Tahchy et al. [23]. It is the first time that quantitative analysis of two Amaryllidaceae alkaloids was reported for *N. papyraceus* and for all the varieties of *N. tazetta*, **Shastpar** (G1), **Meskin** (G2), **Panjehgorbei** (G3) and **Shahla** (G4). All the varieties contained lycorine and galanthamine however the concentration in lycorine is much higher (from 393  $\mu\text{g/g}$  DW in *N. tazetta*: **Shastpar** (G1), to 2645  $\mu\text{g/g}$  DW in *N. papyraceus* (G5)) comparatively to the galanthamine contents (from 8  $\mu\text{g/g}$  DW in *N. papyraceus* (G5) to 105  $\mu\text{g/g}$  DW in *N. tazetta* var. **Meskin** (G2)) (Fig. 2). It is worthy to note that the bulb of *N. tazetta* var. **Meskin** (G2) showed the highest content in galanthamine (0.10 mg/g DW) and a high content in lycorine (1.8 mg/g) comparatively to the other varieties. However *N. papyraceus* (G5) has shown to have the highest content in lycorine (2.6 mg/g) and the lowest amount of galanthamine (6  $\mu\text{g/g}$  DW) comparatively to the other varieties studied. In various parts of plants of *L. aestivum*, it has been reported the concentration of galanthamine between 0.02 to 0.60 mg/g DW and lycorine between 0.11 to 0.23 mg/g DW [30].

### 3.3. In vitro culture initiation

The effects of three auxins (2,4-D, **Picloram** and NAA) in two concentrations (25 and 50  $\mu\text{M}$ ) on the induction of callogenesis and organogenesis **in the two varieties of *Narcissus*: Meskin**

(G2) and Shahla (G4), showing the highest concentrations of lycorine in bulbs, were studied (Table 3).

After 12 weeks of culture, calli were observed on the whole surface of the explants of the both varieties. All auxins and all concentrations of auxins induced calli formation. However, the callogenesis was observed from 35 % of explants for Picloram 50  $\mu$ M to 96 % of explants for NAA 25  $\mu$ M. For the both varieties, NAA with the both concentrations tested led to the best callogenesis. In addition, 2,4-D (25 and 50  $\mu$ M) induced a good callogenic response for the genotype G2 only. In contrary, Picloram (25  $\mu$ M) led to the best induction of callus in *Leucojum aestivum* [21].

In our experiment, bulblets and roots of *Narcissus* were formed after approximately 6 weeks of callus cultivation. A significant number of bulblets and roots of *N. tazetta* var. Meskin (G2) and *N. tazetta* var. Shahla (G4) were obtained in the presence of NAA (25 and 50  $\mu$ M) only. The highest numbers of bulblets and roots were obtained on the callus grown in the presence of 25  $\mu$ M NAA (92 % of explants and 77 % of explants respectively) for genotype G2 only and the lowest on the medium with 50  $\mu$ M NAA (55.20 % of explants and 53.20 % of explants respectively) for genotype G4. Picloram and 2,4-D led to no bulblet and no root development for any varieties. However Picloram is known to be the most effective auxin in initiation of embryogenic callus of bulbous plants including *Leucojum aestivum* [21].

### 3.4. Influence of auxins on the alkaloid profile

We have shown previously that Amaryllidaceae alkaloid profile in tissue cultures of *L. aestivum* is correlated with cellular differentiation controlled by various hormonal conditions [21, 23]. In the present study, the alkaloid profile of *N. tazetta* var. Meskin (G2) and *N. tazetta* var. Shahla (G4) in vitro cultures (calli, bulblets and roots) was correlated with the type of auxin (2,4-D, Picloram and NAA) and its concentration (25 and 50  $\mu$ M) used in the medium (Table 4). Quantitative GC-MS analysis was used according to the method previously described for the

analysis of *in vivo* bulbs. Following the alkaloid extraction and purification of all tissue culture samples, alkaloid fractions suitable for GC-MS analysis were obtained. The presence of alkaloids varied with auxin used for the tissue culture. It is worthy to note that galanthamine and lycorine were detected in all tissue cultures, undifferentiated cells or in roots and bulbets, with all auxins whatever the concentration used for the both varieties. These both alkaloids were also detected in all *in vivo* bulbs of the two species and four varieties of *Narcissus* studied. Two others compounds, demethylmaritidine, and tazettine were found in differentiated tissue cultures cultivated on the medium that contained NAA (25 or 50  $\mu\text{M}$ ). These two compounds were not identified in undifferentiated cells excepted in calli initiated with **Picloram** at the concentration of 50  $\mu\text{M}$ . Demethylmaritidine was not detected in the both varieties of *N. tazetta* (G2 and G4) bulbs from natural habitats while tazettine was present only in *N. tazetta* var. **Shahla** (G4) bulbs.

### **3.5. Effect of 4'-O-methylnorbelladine on the alkaloid accumulation and profile**

Calli and bulblets of *N. tazetta* var. **Meskin** (G2) were subcultured in medium containing precursor 4'-O-methylnorbelladine at various concentrations (0.05 and 0.15 g/L) and incubated for two periods of time (10 and 20 days) as reported previously by El Tahchy et al. [25] and Saliba et al. [22] in *Leucojum aestivum* tissue cultures. After harvesting the calli and the bulblets, the precursor and the Amaryllidaceae alkaloids were extracted from the tissue cultures and the compounds were identified using capillary GC-MS. Regardless of the incubation duration and the concentration of precursor, the latter was detected both in calli and in bulblets indicating that it was transported from the liquid medium into the plant cells (Figure 3). The highest concentrations of the precursor were 550  $\mu\text{g/g}$  in calli and 800  $\mu\text{g/g}$  in bulblets in the presence of 0.15g/L of 4'-O-methylnorbelladine in the liquid medium after 10 days of culture. The concentrations of galanthamine and lycorine ( $\mu\text{g/g}$  DW) in calli and in bulblets are shown in Figures 4 and 5, respectively. Galanthamine was detected at the highest concentration in

bulblets (82 µg/g DW) cultivated in the presence of 0.05 g/L and 0.15g/L of precursor during 20 and 10 days respectively. In these conditions, lycorine was also detected at the highest concentration of 1883 µg/g DW. Therefore, the competition between galanthamine and lycorine biosynthetic pathways was not observed in the presence of the precursor. Indeed bulblets accumulated high quantities of both alkaloids at the same time as it has been previously reported in *Leucojum aestivum* bulblet culture [22]. However, the stimulating effect of the precursor on the alkaloid accumulation is not so clear when it is present in the culture medium of calli. The concentration of galanthamine and lycorine reached the maximum (1.17 and 58.5 µg/g respectively) in calli cultivated in the presence of 0.15 g/L and 0.05g/L of MN after 10 and 20 days of culture, respectively. In the others conditions of culture, the culture in the presence of the precursor led to the decrease of the accumulation of the galanthamine.

Capillary GC-MS was used in order to identify the various alkaloids (Table 5) present in the fractions of bulblets of *N. tazetta* var. **Meskin** (G2) cultured during 10 and 20 days with the precursor 4'-*O*-methylnorbelladine at various concentrations (0.05 and 0.15 g/L). Only galanthamine and lycorine were identified in the control bulblets after 10 days of culture and two alkaloids in addition (demethylmaritidine and tazettine) in the control bulblets after 20 days of culture. However, eight alkaloids were observed after 10 or 20 days of incubation with the precursor (0.05 or 0.15 g/L): galanthamine, lycorine, demethylmaritidine, tazettine homolycorine, narcissidine, chlidanthine, and methyl norbelladine. These results show that the precursor 4'-*O*-methylnorbelladine could be biotransformed in bulblets of *N. tazetta* var. Meskin and gives Amaryllidaceae alkaloids.

#### **4. Conclusion**

The analysis of Amaryllidaceae alkaloids in extracts of bulbs after flowering of *Narcissus papyraceus* and four varieties of *N. tazetta* grown in Iran were performed for the first time using

both GC-MS and LC-MS. It is well established that alkaloid profiles vary with the species and the varieties studied and with the analytical techniques used: 13 and 30 alkaloids were identified respectively by GC-MS and LC-MS. The obtained results of the phytochemical analysis of bulbs revealed that *N. tazetta* var. **Meskin** (G2) is a potential source of Amaryllidaceae alkaloids of interest. This study showed also that the medium containing NAA (50  $\mu$ M) is appropriate not only for callus, roots and bulblets induction, but also for leading to the highest diversity of alkaloid profile in this variety Meskin. In the same way, the 4'-*O*-methylnorbelladine feeding had a significant stimulating effect on both galanthamine and lycorine biosynthesis. The tissue culture of *N. tazetta* var. Meskin in the presence of both NAA and precursor 4'-*O*-methylnorbelladine can be an interesting approach for Amaryllidaceae alkaloids production.

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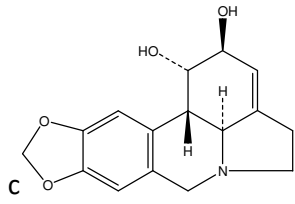
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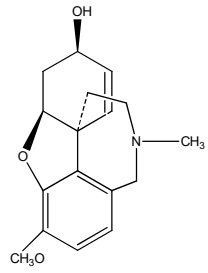
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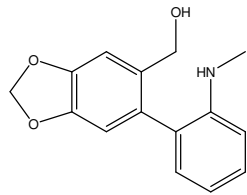
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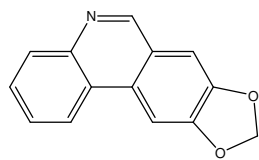
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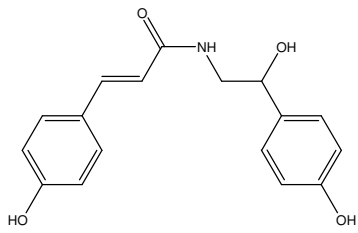
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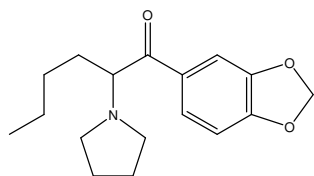
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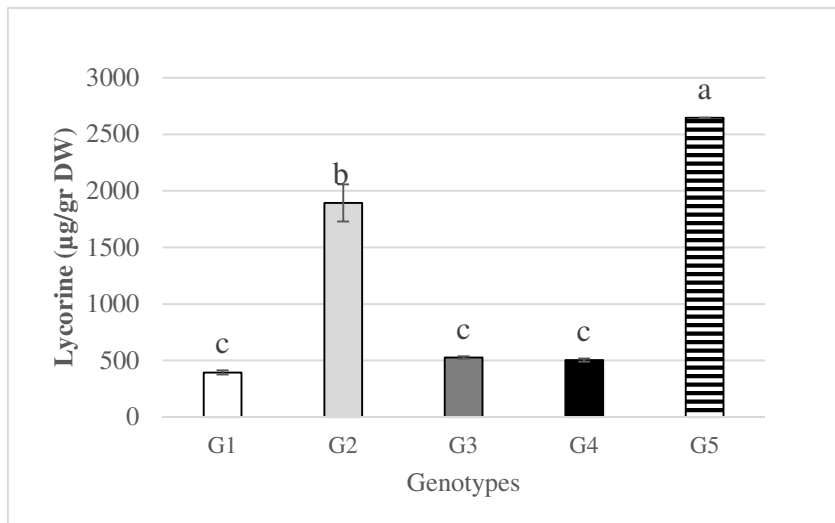
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F

Figure 1. Lycorine (A), galanthamine (B), ismine (C), trisphaeridine (D), masonine (E) and chlidanthine 1,2-dihydro (F) detected in all varieties of *Narcissus tazetta* (G1: *N. tazetta* var. *Shastpar*, G2: *N. tazetta* var. *Meskin*, G3: *N. tazetta* var. *Panjehgorbei*, G4: *N. tazetta* var. *Shahla*) and in *Narcissus papyraceus* (G5).

A



B

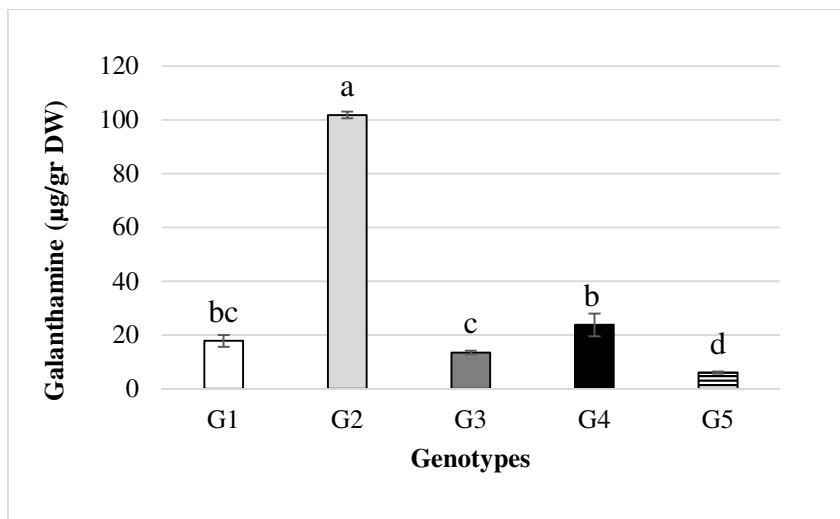


Figure 2. Lycorine (A) and galanthamine (B) contents ( $\mu\text{g/g DW}$ ) in different genotypes of *Narcissus tazetta* (G1: *N. tazetta* var. *Shastpar*, G2: *N. tazetta* var. *Meskin*, G3: *N. tazetta* var. *Panjehgorbei*, G4: *N. tazetta* var. *Shahla*) and in *Narcissus papyraceus* (G5).

The data represent the average of three replications with standard deviations. Different letters indicate the statistically significant differences (LSD's multiple range test,  $p \leq 0.05$ ) among different genotypes.

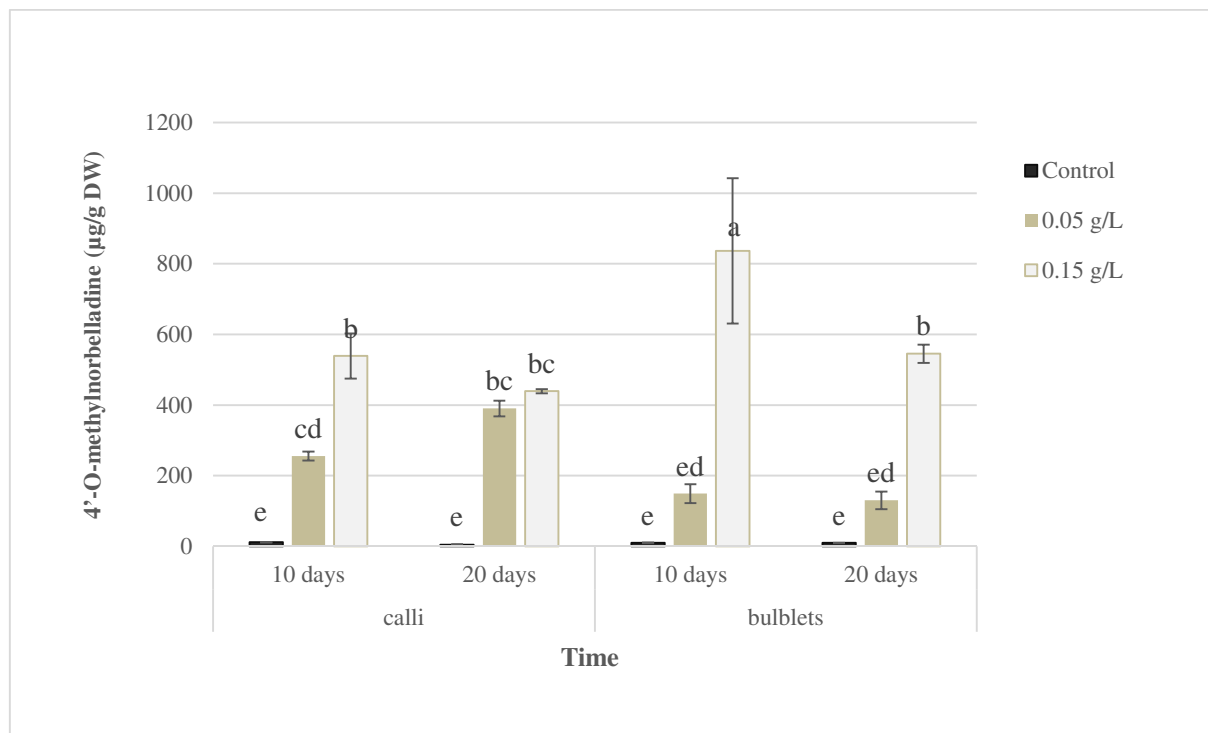


Figure 3. Accumulation of precursor 4'-O-methylnorbeldadine in calli and bulblets of *N. tazetta* var. *Meskin* (G2) during feeding with various concentrations of precursor (control, 0.05 and 0.15 g/L). The data represent the average of three replications with standard deviations. Different letters indicate the statistically significant differences (LSD's multiple range test,  $p < 0.05$ ) among different treatments at each time point.

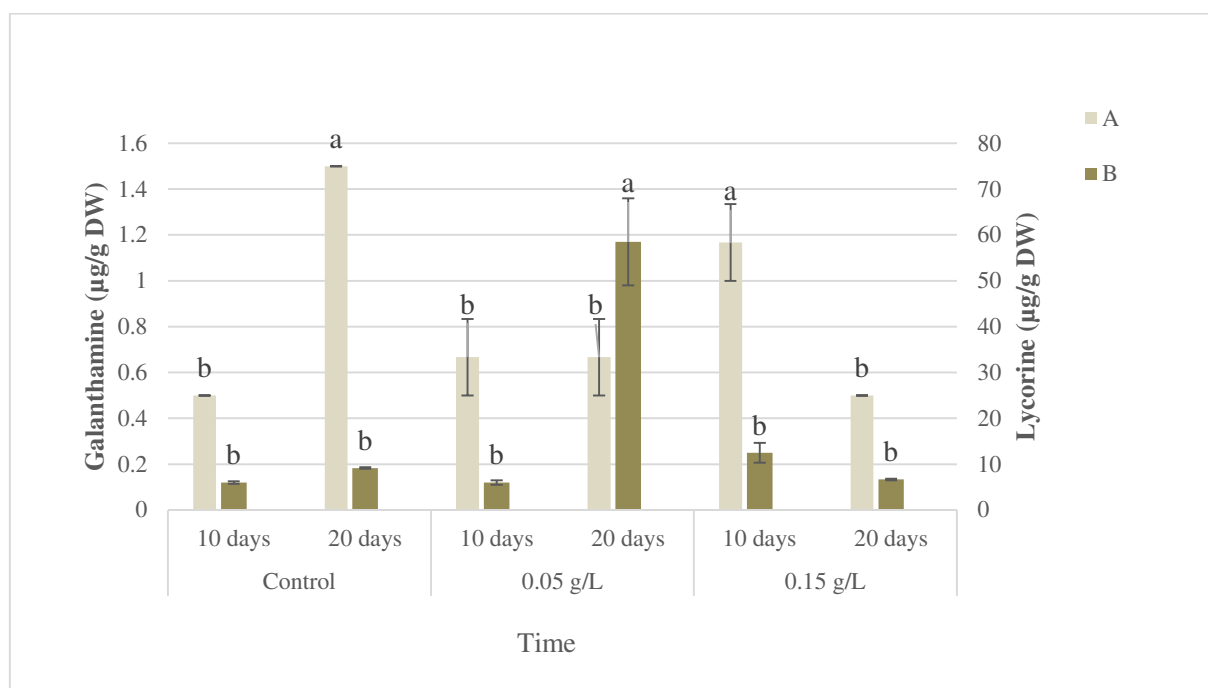


Figure 4. Galanthamine (A) and lycorine (B) concentrations in calli of *N. tazetta* var. *Meskin* (G2) during feeding with various concentrations of precursor (control, 0.05 and 0.15 g/L). The data represent the average of three replications with standard deviations. Different letters indicate the statistically significant differences (LSD's multiple range test,  $p < 0.05$ ) among different treatments at each time point.

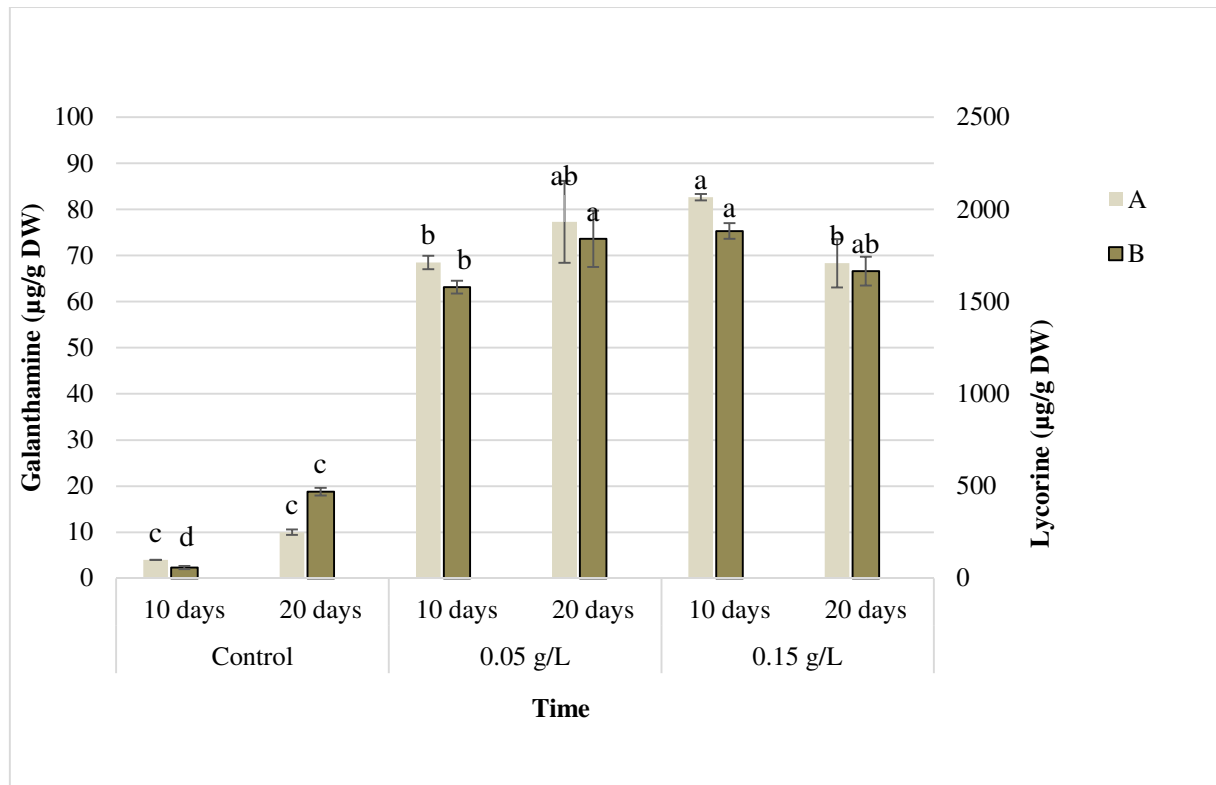


Figure 5. Galanthamine (A) and lycorine (B) concentrations in bulblets of *N. tazetta* var. *Meskin* (G2) during feeding with various concentrations of precursor (control, 0.05 and 0.15 g/L). The data represent the average of three replications with standard deviations. Different letters indicate the statistically significant differences (LSD's multiple range test,  $p < 0.05$ ) among different treatments at each time point.

Table 1. Amaryllidaceae alkaloids identified par GCMS in bulbs of four genotypes of *Narcissus tazetta* (G1-G4) and one genotype of *Narcissus papyraceus* (G5)

| Alkaloids                                   | formula   | Molecular mass | Retention time (min) | Fragmentation m/z (Relative intensity, %)   | Genotypes of <i>Narcissus</i> |    |    |    |    |
|---|---|----------------|----------------------|---|-------------------------------|----|----|----|----|
|   |   |                |                      |   | G1                            | G2 | G3 | G4 | G5 |
| Ismine                                      | C <sub>15</sub> H <sub>15</sub> NO <sub>3</sub> | 257            | 8.1                  | 238/100; 257/39; 239/19 ; 196/8; 168/8      | +                             | +  | +  | +  | +  |
| Trisphaeridine                              | C <sub>14</sub> H <sub>9</sub> NO <sub>2</sub>  | 223            | 8.3                  | 223/100; 222/36; 138/31; 164/21; 111/18     | +                             | +  | +  | +  | +  |
| Galanthamine                                | C <sub>17</sub> H <sub>21</sub> NO <sub>3</sub> | 287            | 9.7                  | 286/100; 287/84; 174/37; 216/34; 244/ 29    | +                             | +  | +  | +  | +  |
| Alpha-dihydrocaranine                       | C <sub>16</sub> H <sub>19</sub> NO <sub>3</sub> | 273            | 10.8                 | 272/100; 273/37; 162/9; 77/8; 58/8          | -                             | -  | -  | -  | +  |
| Tazettine                                   | C <sub>18</sub> H <sub>21</sub> NO <sub>5</sub> | 331            | 13.2                 | 247/100; 70/41; 71/39; 331/21; 298/18       | +                             | +  | +  | +  | -  |
| Lycorenine                                  | C <sub>18</sub> H <sub>23</sub> NO <sub>4</sub> | 317            | 13.8                 | 243/100; 242/88; 244/18; 317/18; 268/14     | -                             | +  | -  | -  | -  |
| Lycorine                                    | C <sub>16</sub> H <sub>17</sub> NO <sub>4</sub> | 287            | 14.6                 | 226/100 ; 227/84 ; 287/38 ; 268/28 ; 286/21 | +                             | +  | +  | +  | +  |
| Masonine                                    | C <sub>17</sub> H <sub>17</sub> NO <sub>4</sub> | 299            | 15.3                 | 109/100; 108/21; 110/7; 82/6; 81/4          | +                             | +  | +  | +  | +  |
| 3-Epimacronine                              | C <sub>18</sub> H <sub>19</sub> NO <sub>5</sub> | 329            | 15.4                 | 245/100; 201;54; 109/43; 70/36; 329/26      | +                             | -  | -  | -  | -  |
| Chlidanthine, 1,2-dihydro-                  | C <sub>17</sub> H <sub>23</sub> NO <sub>3</sub> | 289            | 15.7                 | 228/100; 229/83; 289/41; 270/24; 288/21     | +                             | +  | +  | +  | +  |
| Assoanine                                   | C <sub>17</sub> H <sub>17</sub> NO <sub>2</sub> | 267            | 15.9                 | 266/100; 267/52; 104/9; 268/9; 89/9; 77/8   | -                             | +  | -  | -  | +  |
| Hippeastrine                                | C <sub>17</sub> H <sub>17</sub> NO <sub>5</sub> | 315            | 16.3                 | 125/100; 96/42; 126/11; 229/11; 124/10      | +                             | -  | +  | +  | -  |
| 4,5-Ethylene-8,9-dimethoxy-6-phenanthridone | C <sub>17</sub> H <sub>15</sub> NO <sub>3</sub> | 281            | 17.5                 | 280/100; 281/80; 264/14; 282/14; 140/13     | -                             | +  | -  | -  | -  |

G1: *N. tazetta* var. *Shastpar*, G2: *N. tazetta* var. *Meskin*, G3: *N. tazetta* var. *Panjehgorbei*, G4: *N. tazetta* var. *Shahla*, G5: *N. papyraceus*.



Table 2. Amaryllidaceae alkaloids detected in bulbs of four genotypes of *Narcissus tazetta* (G1-G4) and one genotype of *Narcissus papyraceus* (G5) by LCHRMS using positive ionization mode.

| Alkaloids                                      | Retention time (min) | Formula   | theoretical m/z | Genotypes of <i>Narcissus</i> |          |          |          |          |
|--|----------------------|---|-----------------|-------------------------------|----------|----------|----------|----------|
|  |                      |   |                 | measured m/z                  |          |          |          |          |
|  |                      |   |                 | G1                            | G2       | G3       | G4       | G5       |
| 7-Methoxyoxoassoanine (Cabezas et al. 2003)    | 2.1                  | C <sub>18</sub> H <sub>17</sub> NO <sub>4</sub> | 312.1230        | -                             | -        | -        | 312.1204 | 312.1214 |
| Bulbocapnine                                   | 2.1                  | C <sub>19</sub> H <sub>19</sub> NO <sub>4</sub> | 326.1387        | -                             | -        | -        | -        | 316.1363 |
| Oxoassoanine                                   | 2.2                  | C <sub>17</sub> H <sub>15</sub> NO <sub>3</sub> | 282.1125        | -                             | 282.1119 | -        | -        | -        |
| 5,6-dihydrobicolorine (Ortiz et al, 2012)      | 2.2                  | C <sub>15</sub> H <sub>13</sub> NO <sub>2</sub> | 240.1019        | -                             | 240.1013 | -        | 240.1003 | 240.1010 |
| Trisphaeridine                                 | 2.7                  | C <sub>14</sub> H <sub>9</sub> NO <sub>2</sub>  | 224.0706        | 240.0708                      | 240.0704 | 240.0709 | 240.0708 | 240.0702 |
| assoanine                                      | 3.1                  | C <sub>17</sub> H <sub>17</sub> NO <sub>2</sub> | 268.1332        | -                             | -        | -        | -        | 268.1312 |
| N-methylphenylalanine                          | 3.1                  | C <sub>10</sub> H <sub>13</sub> NO <sub>2</sub> | 180.1019        | -                             | -        | 180.1016 | -        | 180.1012 |
| Albomaculine                                   | 3.4                  | C <sub>19</sub> H <sub>23</sub> NO <sub>5</sub> | 346.1649        | 346.1645                      | 346.1651 | 346.1651 | 346.1638 | 346.1629 |
| Aknadicine                                     | 4.1                  | C <sub>19</sub> H <sub>23</sub> NO <sub>5</sub> | 346.1649        | -                             | 346.1630 | -        | -        | -        |
| Narcissidine                                   | 4.4                  | C <sub>18</sub> H <sub>23</sub> NO <sub>5</sub> | 334.1649        | -                             | 334.1642 | 334.1617 | -        | -        |
| Ismine   | 4.6                  | C <sub>15</sub> H <sub>15</sub> NO <sub>3</sub> | 258.1125        | 258.1111                      | -        | 258.1114 | 258.1113 | 258.1109 |
| Acetylcaranine                                 | 4.6                  | C <sub>18</sub> H <sub>19</sub> NO <sub>4</sub> | 314.1387        | 314.1355                      | 314.1358 | 314.1362 | 314.1356 | -        |
| Crinamidine                                    | 4.6                  | C <sub>17</sub> H <sub>19</sub> NO <sub>5</sub> | 318.1336        | -                             | -        | 318.1316 | 318.1300 | 318.1306 |
| 3-Epimacronine                                 | 5.1                  | C <sub>18</sub> H <sub>19</sub> NO <sub>5</sub> | 330.1336        | -                             | 330.1320 | 330.1323 | 318.1325 | 330.1321 |
| Cavinine                                       | 5.1                  | C <sub>18</sub> H <sub>21</sub> NO <sub>6</sub> | 348.1442        | -                             | 348.1456 | 348.1511 | -        | -        |
| pretazettine                                   | 6.8                  | C <sub>18</sub> H <sub>21</sub> NO <sub>5</sub> | 332.1492        | -                             | -        | -        | -        | 332.1470 |
| Haemanthamine                                  | 7.8                  | C <sub>17</sub> H <sub>19</sub> NO <sub>4</sub> | 302.1387        | -                             | 302.1378 | -        | -        | -        |
| Hippeastrine                                   | 8.5                  | C <sub>17</sub> H <sub>17</sub> NO <sub>5</sub> | 316.1179        | 316.1183                      | 316.1175 | -        | 316.1203 | 316.1176 |
| Haemanthidine                                  | 8.5                  | C <sub>17</sub> H <sub>19</sub> NO <sub>5</sub> | 318.1336        | 318.1295                      | -        | 318.1327 | 318.1300 | 318.1306 |
| Lycorenine                                     | 9.6                  | C <sub>18</sub> H <sub>23</sub> NO <sub>4</sub> | 318.1700        | -                             | 318.1606 | -        | -        | -        |
| Lycorine                                       | 10.8                 | C <sub>16</sub> H <sub>17</sub> NO <sub>4</sub> | 288.1230        | 288.1229                      | 288.1234 | 288.1234 | 288.1220 | 288.1229 |
| Apothaemanthamine                              | 11.0                 | C <sub>16</sub> H <sub>15</sub> NO <sub>3</sub> | 270.1125        | -                             | -        | -        | -        | 270.1114 |
| Erythratine                                    | 11.1                 | C <sub>18</sub> H <sub>21</sub> NO <sub>4</sub> | 316.1543        | 316.1555                      | -        | -        | 316.1540 | -        |
| Crinamine                                      | 12.4                 | C <sub>17</sub> H <sub>19</sub> NO <sub>4</sub> | 302.1387        | 302.1378                      | 302.1382 | 302.1370 | 302.1371 | 302.1378 |
| Pseudolycorine                                 | 13.4                 | C <sub>16</sub> H <sub>19</sub> NO <sub>4</sub> | 290.1387        | 290.1425                      | -        | 290.1411 | 290.1407 | 290.1397 |
| Galanthamine                                   | 15.5                 | C <sub>17</sub> H <sub>21</sub> NO <sub>3</sub> | 288.1594        | 288.1477                      | 288.1492 | -        | -        | -        |
| Anhydrolycorinone                              | 16.3                 | C <sub>16</sub> H <sub>11</sub> NO <sub>3</sub> | 266.0812        | -                             | -        | -        | -        | 266.0815 |
| Tazettine                                      | 17.1                 | C <sub>18</sub> H <sub>21</sub> NO <sub>5</sub> | 332.1492        | 332.1516                      | -        | 332.1503 | 332.1514 | 332.1496 |
| 4'-O-demethylbelladine (Vaněčková et al. 2016) | 17.4                 | C <sub>18</sub> H <sub>23</sub> NO <sub>3</sub> | 302.1751        | 302.1749                      | -        | 302.1748 | 302.1747 | 302.1738 |
| Norgalanthamine                                | 18.6                 | C <sub>16</sub> H <sub>19</sub> NO <sub>3</sub> | 274.1438        | -                             | 274.1426 | -        | -        | -        |

G1: *N. tazetta* var. *Shastpar*, G2: *N. tazetta* var. *Meskin*, G3: *N. tazetta* var. *Panjehgorbei*, G4: *N. tazetta* var. *Shahla*, G5: *N. papyraceus*.

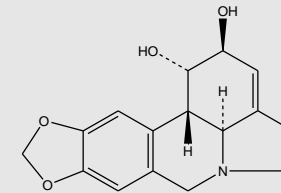
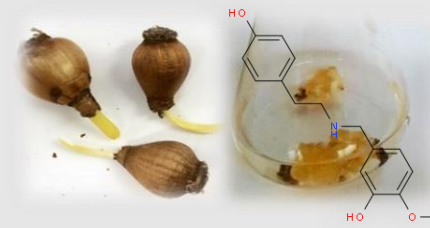
Table 3. Effects of various auxins on the percentage of explants showing callus induction and bulblets and roots development in *N. tazetta* var. *Meskin* (G2) and *N. tazetta* var. *Shahla* (G4).

| Auxins        | Genotype | Callus (%)              | Bulblets (%)             | Root (%)                      |
|---------------|----------|-------------------------|--------------------------|-------------------------------|
| NAA           | G2       | 96.00±4.00 <sup>a</sup> | 92.00±4.89 <sup>a</sup>  | <b>77.00±1.22<sup>a</sup></b> |
| 25 [ $\mu$ M] | G4       | 87.00±5.39 <sup>a</sup> | 63.20±5.63 <sup>b</sup>  | <b>63.20±5.63<sup>b</sup></b> |
| NAA           | G2       | 90.00±6.12 <sup>a</sup> | 58.20±5.21 <sup>bc</sup> | <b>56.40±3.91<sup>c</sup></b> |
| 50 [ $\mu$ M] | G4       | 88.00±4.90 <sup>a</sup> | 55.20±3.32 <sup>c</sup>  | <b>53.20±3.19<sup>c</sup></b> |
| 2-4-D         | G2       | 88.20±7.36 <sup>a</sup> | 0.0 <sup>d</sup>         | <b>0.0<sup>d</sup></b>        |
| 25 [ $\mu$ M] | G4       | 53.20±3.20 <sup>c</sup> | 0.0 <sup>d</sup>         | <b>0.0<sup>d</sup></b>        |
| 2-4-D         | G2       | 91.00±5.57 <sup>a</sup> | 0.0 <sup>d</sup>         | <b>0.0<sup>d</sup></b>        |
| 50 [ $\mu$ M] | G4       | 70.20±3.09 <sup>b</sup> | 0.0 <sup>d</sup>         | <b>0.0<sup>d</sup></b>        |
| Picloram      | G2       | 63.2±5.63 <sup>bc</sup> | 0.0 <sup>d</sup>         | <b>0.0<sup>d</sup></b>        |
| 25 [ $\mu$ M] | G4       | 70.00±5.00 <sup>b</sup> | 0.0 <sup>d</sup>         | <b>0.0<sup>d</sup></b>        |
| Picloram      | G2       | 69.60±2.20 <sup>b</sup> | 0.0 <sup>d</sup>         | <b>0.0<sup>d</sup></b>        |
| 50 [ $\mu$ M] | G4       | 35.80±1.71 <sup>d</sup> | 0.0 <sup>d</sup>         | <b>0.0<sup>d</sup></b>        |

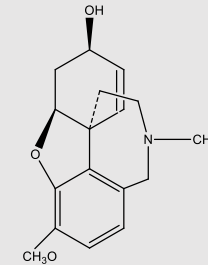
Numbers followed by different letters are significantly different at  $P \leq 0.05$ ,  $P \leq 0.05$ , Duncan's test  $\pm$  S.D.



*Narcissus papyraceus*  
*N. tazetta* var. *Meskin*  
*N. tazetta* var. *Shastpar*  
*N. tazetta* var. *Panjegorbei*  
*N. tazetta* var. *Shahla*



**Lycorine**



**Galanthamine**