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Histochemical Localization of Alkaloids in the Bulbs of In Vitro-Regenerated Snake's Head Fritillary (*Fritillaria meleagris* L.): The Effect of a Temperature Regime

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Abstract: The genus *Fritillaria* is characterized by the production of alkaloids, primarily of the isosteroidal type. The aim of this preliminary study was to perform a histolocalization screening aimed at revealing the presence of alkaloids in the bulbs of *F. meleagris*, cultured in vitro under different growth regimes and at different developmental stages, as a powerful tool to identify preferable in vitro plant material for alkaloid isolation. Histochemical localization of alkaloids was performed using Wagner's and Dragendorff's reagents in fresh sections of bulbs cultured at 24 °C or 7 °C for 4 weeks, as well as those cultured at 24 °C following the 4-week chilling treatment, which were sampled at the beginning of sprouting. A positive reaction was observed with both reagents and was particularly intense in cold-treated bulbs. Alkaloids were mainly distributed in the bulbs and partly in the sprouts of the in vitro grown *F. meleagris*. The most intense staining, indicative of high alkaloid content, was observed in the bulb scales of pre-chilled bulbs that sprouted at 24 °C, rendering them preferable as in vitro plant material for alkaloid isolation. The results suggest that alkaloid production in the bulbs of *F. meleagris* can be improved by manipulating growth in the microenvironment of in vitro cultures, in order to meet the increasing industrial demand for medicinally or commercially important metabolites used as traditional medicines and herbal remedies.

Keywords: snake's head fritillary; bulbs; alkaloids; histolocalization

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1. Introduction

Fritillaria meleagris L. (Liliaceae) is a valuable horticultural species that is mainly distributed in the temperate zones of the northern hemisphere. It is often propagated vegetatively using its underground storage organs (bulbs). Bulbous plants, such as fritillary, evade unfavorable conditions by going dormant as bulbs underground during the winter months and germinating the following year, often requiring a period of low temperatures to overcome dormancy. The absence of low-temperature exposure leads to very slow plant growth and causes various anomalies in flower formation and/or development [1]. The very low natural propagation rate of *Fritillaria* species renders their mass production and cultivation generally problematic [2]. *F. meleagris* is considered a rare and threatened species throughout its geographic range [3]. A comparison of the current status of *F. meleagris* populations in Serbia with the status 50 years ago shows that the populations have declined to such an extent that they could be considered endangered [4].

In addition to their great horticultural importance, fritillaries also have potential for medicinal use due to their phytochemical properties, with a rich pharmacological history [5]. *Fritillaria* species, which are most commonly consumed as dried bulbs [Bei Mu in Chinese], exhibit pharmacological effects due to the presence of alkaloids, terpenoids, and many other

metabolites, including antitussive, expectorant, antiasthmatic, and antitumor activity [6]. Fritillaries have been used to treat hypotension, sensory disorders, various lung infections including tuberculosis, respiratory disorders, myocardial irritation, scrofulous swellings, and chest nodules [6,7]. Alkaloids, the major compounds isolated from the crude extracts of the bulbs and identified as the active ingredients in *Fritillaria*, are considered to be useful agents that reduce coronavirus-associated lung injury [8]. To date, 120 alkaloids have been isolated from plants of the genus *Fritillaria* [9]. The alkaloids extracted from *Fritillaria* species belong to the isosteroidal and steroidal types [10]. Most alkaloids belong to the isosteroidal type (72.7%), and their contents and structure types differ among species [11,12].

Natural products from *Fritillaria* species have many advantages over conventional drugs [13]. Recently, a very thorough investigation was conducted as a strategy to produce new drugs from *Fritillaria* [14]. In view of the traditional use of the taxa belonging to *Fritillaria*, several review articles addressed the phytochemistry, chemotaxonomy, molecular phylogeny, and pharmacology of *Fritillaria* species [6,15]. Many Chinese *Fritillaria* sp. have been extensively studied, especially *Fritillariae thunbergii* bulbus [16], but the species distributed outside China have been greatly neglected in scientific research. Beyond China, the bulbs of *Fritillaria* are also approved as active ingredients in medicinal preparations in Oceania, North America, and Asia [17]. However, the disappearance of most *Fritillaria* species, including *F. meleagris*, from their natural habitats, and the decline in the number of wild-growing plants, have resulted in diminishing sources of potentially valuable medicinal compounds.

Reproduction of fritillaries under natural conditions is very slow [18]. The entire growth period from seed to commercial form takes at least three years, so in vitro propagation can speed up the process of obtaining valuable plant material [19]. Propagation of *F. meleagris* bulbs in vitro can be enhanced by various tissue culture protocols. Dormant bulbs and those that begin to sprout after the cold period exhibit various physiological and morphological alterations [20]. In vitro-regenerated bulbs of *F. meleagris* also require chilling in order to overcome dormancy and continue their growth and development [21]. The physiology and biochemistry of dormancy, a developmental stage without visible morphogenesis, is not yet fully elucidated.

In recent years, great efforts have been made to investigate the in vitro culture of medicinal plants [22]. Concerning snake's head fritillary, research has focused on optimizing protocols for dormancy breaking, sprouting, and overall improvement of plant growth. A number of our previous studies [21,23–25] reported different techniques for the propagation and regeneration of *F. meleagris*. Additional physiological and biochemical studies need to be carried out to increase the production of metabolites of therapeutic interest in bulbs of in vitro-cultured *F. meleagris*. Therefore, we considered necessary a histolocalization screening aimed at revealing bioactive metabolites in the bulbs of *F. meleagris* under different growth regimes and at different developmental stages before performing in-depth phytochemical studies.

The aim of this preliminary study was to investigate the presence of alkaloids in the bulbs of *F. meleagris*, to localize the alkaloids using specific histochemical tests, and to investigate possible differences in their distribution under different growth regimes and at different developmental stages. The results may provide a new perspective for improving alkaloid production and increasing alkaloid accumulation in fritillary bulbs. Further research on this species, focusing on analytical techniques for the chemical evaluation of alkaloids, could be important from an economic point of view. This work is a preliminary study for a larger comprehensive investigation of the effects of different physical, nutritional, and hormonal factors, plant age, and bulb size, on alkaloid production in *F. meleagris*.

2. Materials and Methods

2.1. Plant Material and Culture Conditions

Bulb cultures of *Fritillaria meleagris* L. were established according to the procedure modified after Petrić et al. [25]. Briefly, seeds of *F. meleagris* were washed with running water for 1 h, surface sterilized with 0.8% (*w*/*v*) sodium hypochlorite in sterile deionized water for 20 min, and then rinsed three times with sterile deionized water. Mature zygotic embryos were isolated aseptically and used as explants. Five explants were placed in each Petri dish filled with 15 mL Murashige and Skoog (MS) medium [26] supplemented with 30 g L^{-1} sucrose, 7 g L^{-1} agar, 250 mg L^{-1} casein hydrolysate, 250 mg L^{-1} L-proline, 80 mg L^{-1} adenine sulfate, and 1 mg L^{-1} thidiazuron (TDZ). After 4 weeks of cultivation, bulblet formation was observed [25]. Obtained bulblets were cut longitudinally into four scale sections and cultured on MS medium as previously described, with the exception of 0.1 mg L^{-1} TDZ used instead of 1 mg L^{-1}, for an additional 4 weeks, where new bulblets were formed [25] and used to establish stock cultures.

Bulblet scale explants were used as primary explants for in vitro propagation of *F. meleagris*. The stock cultures were maintained on MS medium supplemented with 30 g L^{-1} sucrose, 7 g L^{-1} agar, and 0.1 mg L^{-1} TDZ for shoot and bulb multiplication. Four bulb scale explants were cultured per Petri dish. After four weeks, regenerated bulbs of about 80–100 mg were used for further experiments. The pH of all culture media was adjusted to 5.8 with 1 N NaOH before autoclaving at 121 °C for 25 min. All cultures were maintained in a growth chamber under a 16 h photoperiod, with a photon flux density of 40 µmol m^{-2} s^{-1} provided by cool white fluorescent lamps, at 24 ± 2 °C.

Bulbs regenerated in vitro were transferred to fresh MS medium without plant growth regulators in 100 mL Erlenmayer flasks containing 50 mL of medium. Five explants were placed in each Erlenmayer flask and cultured at different temperatures. Four replicates, each consisting of five explants, were used per treatment (n = 20). Bulbs were subjected to one of two chilling treatments (at 7 °C), performed in a cold chamber (Snaigē): (1) a chilling treatment for 4 weeks, after which the bulbs were sampled immediately; sprouting rarely occurred at this temperature, but if it did, sprouted bulbs were sampled at that time point, and (2) a chilling treatment for 4 weeks, followed by cultivation at 24 °C; after transfer to 24 °C, bulbs started to sprout and were sampled as soon as the sprouting began. Bulbs cultured at 24 °C for 4 weeks were used as a control (Figure 1).

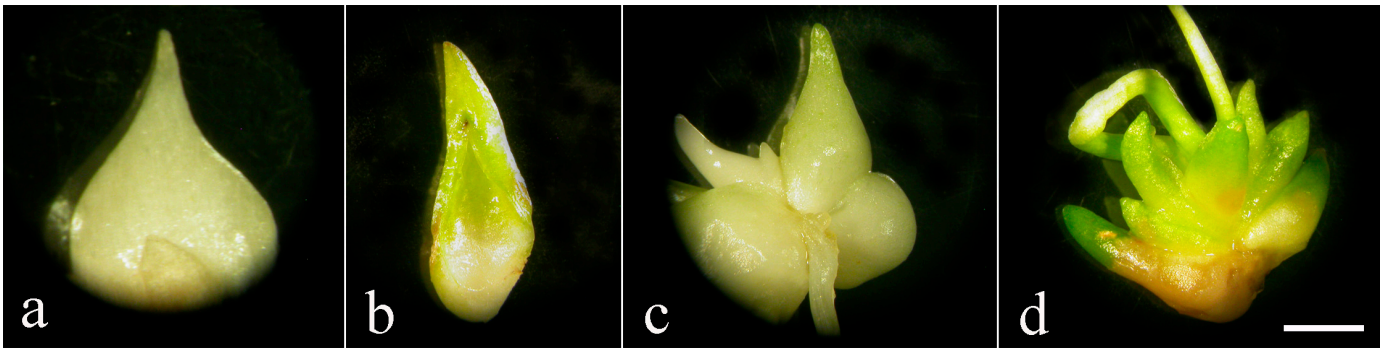

Figure 1. In vitro-cultured bulbs of *F. meleagris* collected at the beginning of sprouting, at different time points of different treatments, which were used for localization of alkaloids. (**a**) Sprouted bulb after 7 days of chilling; (**b**) bulb sprouted after 4 weeks of chilling; (**c**) control bulb sprouted after 7 days at 24 °C; (**d**) bulb sprouted after 4 weeks at 24 °C. Not all bulbs or all treatments are shown. Scale bar = 5 mm.

2.2. Determination of Alkaloids

The presence of alkaloids was detected by Wagner's or Dragendorff's reagents [27], which produce a golden-brown- or reddish-brown-colored reaction in response to alkaloids, respectively (the intensity of staining depends on the quantity of alkaloids). Wagner's reagent (iodine potassium iodide solution) was prepared by dissolving 2 g I_2 and 6 g KI in

100 mL of distilled water. The solution was left at room temperature for 24 h. Dragendorff's reagent was prepared by dissolving 0.85 g bismuth subnitrate in 10 mL glacial acetic acid and 40 mL distilled water, followed by thorough mixing with 20 mL of 40% KI aqueous solution.

Bulbs as well-as sprouted bulbs were cut with a scalpel and dipped into one of the solutions for 20 min. After staining, the longitudinal- and cross sections of the bulbs were photographed using a photomicroscope (Leica wild M3Z). The reaction using Wagner's reagent produced a golden-brown to brownish color in response to alkaloids and a blue/purple color in response to starch. Dragendorff's reagent produced an orange-brown-colored reaction with alkaloids.

2.3. Morpho-Anatomical Study

Bulbs cultured for 4 weeks at 7 °C and 24 °C, as well as sprouted bulbs, were fixed in FAA fixative (5 mL of 40% formaldehyde, 5 mL of glacial acetic acid, and 90 mL of 70% ethanol) at 4 °C for 24 h, according to the procedure of Jensen [28]. The fixed material was washed, dehydrated in a graded ethanol series, and embedded in paraffin. Cross sections (5–7 µm thick) were stained with Alcian blue and photographed using a Leitz DMRB photomicroscope (Leica, Wetzlar, Germany).

3. Results

F. meleagris bulbs grown for 4 weeks in a cold chamber (which did not sprout) showed alkaloids scarcely distributed in the whole bulb (Figure 2a). When the bulbs started to sprout, alkaloids were mostly observed in the bulb scales, as revealed by the more intense golden-brown staining, and also occasionally displaced between the reserve parenchyma cells, which contained an abundance of amyloplasts (Figure 2b). These amyloplasts could conceal a positive reaction for alkaloids, because they stained dark blue in response to the Wagner's reagent and dark brown in response to the Dragendorff's reagent. Alkaloids were also detected in the newly formed sprouts. The strongest positive reaction for alkaloids was observed in the bulb scales of the sprouted bulb at the end of cold treatment (after 4 weeks), indicative of a high alkaloid content (Figure 2c).

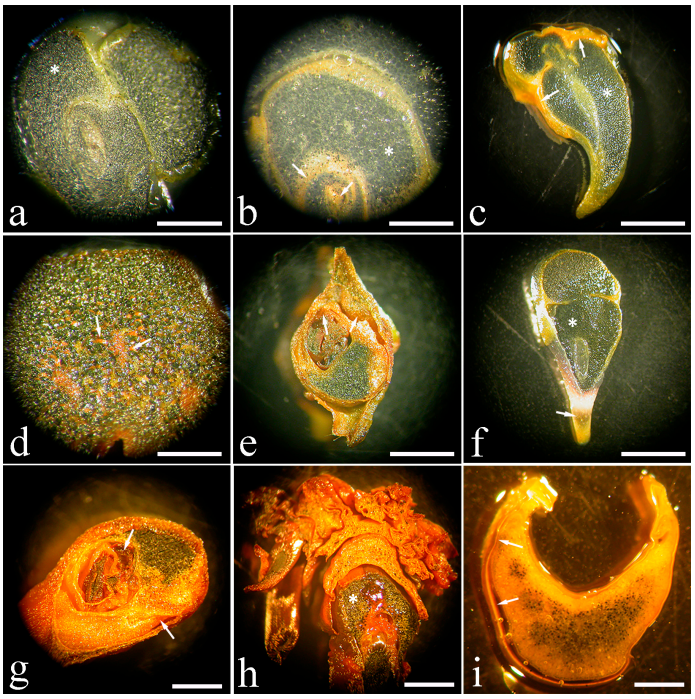

Figure 2. In situ histochemical localization of alkaloids in the bulbs of in vitro-regenerated *Fritillaria meleagris* cultured at different temperature regimes. (**a**–**c**) Bulbs cultured at 7 °C after (**a**) 4 weeks; bulb did not sprout (scale bar = 1 mm); (**b**) 7 days; beginning of sprouting (scale bar = 1.7 mm); (**c**) 4 weeks;

bulb sprouted (scale bar = 5 mm); (**d**–**f**) Control bulbs, cultured at 24 °C after: (**d**) 4 weeks; bulb did not sprout (scale bar = 3.5 mm); golden-brown colored drops (*arrows*) indicate alkaloids; (**e**) 10 days; beginning of sprouting (scale bar = 5 mm); (**f**) 14 days; bulb sprouted (scale bar = 5 mm); (**g**–**i**) *F. meleagris* bulbs pre-chilled for 4 weeks in cold chamber then cultured at 24 °C where they sprouted after (**g**) 7 days (scale bar = 5 mm); (**h**) 3 weeks (scale bar = 1.7 mm); (**i**) bulb scale of the bulb sprouted after 2 weeks at 24 °C (scale bar = 1 mm). Alkaloids are mostly detected as orange- or golden-brown staining (*arrows*) in bulb scales, whereas the dark-blue staining indicates starch granules (*asterisks*).

Control bulbs (non-chilled) that did not sprout showed a more intense golden-orange staining in cross sections (Figure 2d) compared to the bulbs cultured in the cold chamber. At the beginning of sprouting (after 10 days of sprouting), alkaloids were mainly detected in the bulb scales and the newly formed sprout, while the parenchyma cells were greenish blue in color, indicating the absence of alkaloids (Figure 2e). Non-chilled, sprouted bulbs (after 14 days of sprouting) showed almost no alkaloids in the center of the bulb, while the thickened, outer parts of the bulb and sprout remained faintly colored, suggesting lower levels of alkaloids (Figure 2f).

Bulbs cultivated in the cold chamber for 4 weeks and then transferred to 24 °C, where they sprouted, showed the highest alkaloid content (Figure 2g–i), already at the beginning of sprouting after 7 days (Figure 2g). After 3 weeks of sprouting, most of the starch was found in the sprout, while the bulb scales mostly showed no staining for starch but a positive reaction for alkaloids (Figure 2h). Compared to the non-sprouted bulbs, the scales of the sprouted pre-chilled bulbs showed a very intense brown staining after 2 weeks of sprouting at 24 °C (Figure 2i).

Histoanatomical analysis did not reveal a distinctive difference between chilled and non-chilled bulbs (Figure 3). Microscopic observations showed that the scales of both non-chilled (Figure 3a) and chilled bulbs (Figure 3b) contained densely packed parenchyma cells. Amyloplasts with numerous starch granules were clearly visible in the scales, but they were more abundant in the chilled bulbs (Figure 3b). The scales of bulbs sprouted after cold treatment showed ruptured cells with a lower number of amyloplasts (Figure 3c), as revealed by the absence of blue-colored starch in the cross sections (Figure 2i).

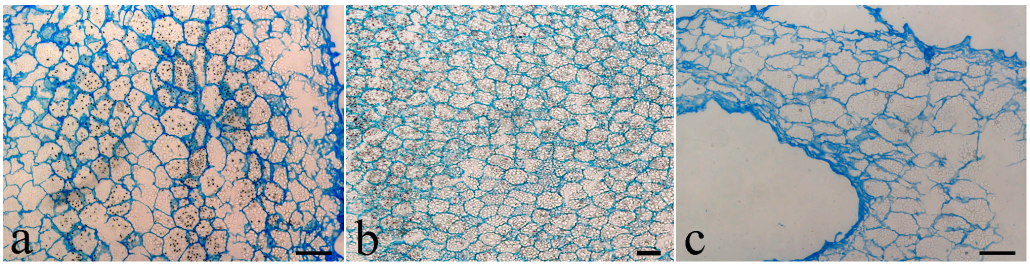

Figure 3. Histoanatomical analysis of chilled and non-chilled bulbs of *F. meleagris* cultured on medium without plant growth regulators. (**a**) Non-chilled bulb (control); (**b**) bulb cultured for 4 weeks at 7 °C; (**c**) bulb scale of the bulb sprouted after 4 weeks at 7 °C. Scale bars = 100 µm.

4. Discussion

All studied *Fritillaria* species contain alkaloids, which are essential constituents of the bulbs [29] and are responsible for the alleviation and treatment of pulmonary diseases. Nevertheless, *Fritillaria* species differ in their phytochemical composition and thus in their pharmacological activity [6]. We therefore hypothesized that *F. meleagris* might contain some specific valuable alkaloids that remain to be determined. Since *F. meleagris* is very rare and endangered and is already extinct in some places in Serbia [4], in vitro propagation techniques provide a very effective method for its large-scale propagule generation, thus expanding the source of the sought alkaloids. In the present study, we relied on our previous studies on in vitro propagation of *F. meleagris* and the possibilities of overcoming dormancy by cold treatment [23,25]. Our results showed that the highest alkaloid accumulation was

observed in the bulbs after cold treatment, particularly in those that sprouted. The positive reactions to Dragendorff's and Wagner's reagents were localized in the bulb scales as well as in the newly formed sprouts. The strongest positive reaction for alkaloids, indicative of high alkaloid content, was observed in the bulb scales of pre-chilled bulbs that sprouted at 24 °C. Histological analysis showed that chilled and non-chilled bulbs differed only in the number of amyloplasts and that the number of amyloplasts with starch granules was greatly reduced after the onset of sprouting. Li et al. [30] also showed that alkaloid accumulation in the bulbs of *F. thunbergii* depends on the season. Therefore, for potential alkaloid production, it is preferable to cultivate the bulbs at low temperatures and use them for alkaloid isolation.

In vitro-derived bulblets of *F. cirrhosa* showed the presence of peimissine, the main isosteroidal alkaloid detected in commercial crude drug samples [31]. Lower but significant amounts of this alkaloid were also found in the callus, the concentration depending on light conditions during cultivation [31]. Considering that bulbs are the main repository for the alkaloids, the higher number of bulbs obtained in vitro is a significant result, since under natural conditions about one bulb per plant develops in about one year [32]. Moreover, the bulbs take several years to reach the desired size as they are geophytes and have a long life cycle of 270–280 days below ground and 80–90 days above ground [33]. The levels of isosteroidal alkaloids in the bulbs of *F. cirrhosa* are strongly influenced by environmental conditions and fluctuate with the age and reproductive stage of the plant [34]. The above results are very similar to our observations for the bulbs of *F. meleagris*. The environmental conditions led to a strong accumulation of alkaloids in the bulbs, which is in agreement with the findings that the occurrence of alkaloid-containing plants and the amount of alkaloids contained in their vegetative tissues depend on their growth habit and ecogeographical distribution [35]. Genetic studies also showed that in vitro-regenerated bulbs of *F. cirrhosa* had a higher alkaloid content than bulbs under natural conditions [36]. The differences in alkaloid content could be the result of evolutionary progress and the defensive role of alkaloids as barriers against fungal, microbial, and insect attack. The need to allocate defensive compounds to a particular plant part primarily depends on its susceptibility to predators but also varies with the developmental stage of the plant and environmental conditions [37]. The biosynthesis of alkaloids is often affected by temperature, which also influences respiration and photosynthesis [38]. Enhanced respiration and photosynthesis may lead to elevated protein synthesis and alkaloid production [38]. Despite the seasonal variation, Konchar et al. [34] pointed out that the reproductive stage of the plant is more consistent with the observed differences in alkaloid accumulation than any variation in the environment. However, in vitro-cultured plants are by definition considered as juvenile-stage plants [39]. Therefore, it is also possible that alkaloid production in *F. meleagris* bulbs grown at low temperatures is less influenced by environmental conditions than by genetic factors, as the developmental stage is triggered by temperature conditions.

Further improvement of in vitro techniques for bulblet propagation, such as liquid medium in bioreactors, could help to increase the number of new bulblets for the commercial production of alkaloids. The logical next step for further experimentation is to investigate the effects of additional factors that could enhance alkaloid production in the bulbs of *F. meleagris*, such as plant developmental stage, bulb size, in vitro culture conditions, plant growth regulators, and combinations thereof. The next stage of research must be the determination of alkaloids and their pharmaceutical activity. An interesting follow-up study would be to investigate the alkaloid shift in *F. meleagris* at different developmental stages and to sample more frequently at different time points. Histolocalization remains a powerful tool for solving the problem of cost-effective isolation of natural products, whose content varies according to the growth phase, and for optimizing research in the field of phytochemistry and phytotherapy.

5. Conclusions

This preliminary investigation revealed that alkaloids were mainly distributed in the bulbs and partly in the sprouts of in vitro grown *F. meleagris*. The most intense staining, indicative of high alkaloid content, was observed in the bulb scales of pre-chilled sprouted bulbs, rendering them preferable as in vitro plant material for alkaloid isolation.

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Data Availability Statement: Data are contained within the article

Conflicts of Interest: The authors declare no conflict of interest.

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