BIOSYNTHESIS OF LYCORINE BY *IN VITRO* CULTURES OF *PANCRATIUM MARITIMUM* L. (AMARYLLIDACEAE)

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ABSTRACT

Seeds of Pancratium maritimum collected from the natural populations along Bulgarian Black Sea coast were in vitro germinated in 2005 and cultures were maintained under equal conditions for three years (MS based medium with BAP and NAA; 23±2°C and 16/8 light/dark photoperiod). Directly regenerated bulblets with diameter of about 6-8 mm were cultured in parallel for two months on agar solidified and in liquid media containing 3 mg/L BAP and 0.1 mg/L NAA. Alkaloid determination by HPLC proved biosynthesis of lycorine which antiviral and antitumor properties are well known. Lycorine accumulation was up to 2.90 and 1.53 mg/g DW in the cultures grown on agar and in liquid medium, respectively, which was highly promising as first trials to produce this alkaloid in vitro. Additionally, bulblets were regenerated from callus cultures and comparison was done concerning the morphology of directly and indirectly obtained plantlets. Bulblet growth varied in accordance with the sucrose quantity in the medium as well.

Keywords: alkaloids, long-term cultures, Sea Daffodil

Introduction

The alkaloid lycorine has been isolated from many Amaryllidaceae plant species belonging to various genera: *Lycoris, Pancratium, Leucojum, Urginea, Narcissus, Galanthus, Amaryllis, Crinum, Hymenocallis, Nerine, Sternbergia, Zephyranthes, Eustephia, Haemanthus and Vallota* (1). It is usually accompanying galanthamine and some other isoquinoline alkaloids. In *P. maritimum* plants originated form the Black Sea coast of Bulgaria there were detected 16 alkaloids some of them with pharmacological activities such as lycorine and galanthamine (2). Lycorine and galanthamine were also determined in the flowers of this species (3).

Lycorine has been studied for its inhibitory activities against human immunodeficiency virus (HIV-1), severe acute respiratory syndrome-associated corona virus (SARS-CoV), poliovirus, coxsackie virus, measles virus and herpes simplex virus type 1 (HSV-1) (4, 5, 6). Extracts of *P. maritimum* plants growing in Turkey were proved to inhibit *Plasmodium falciparum* growth due to the presence of the alkaloids lycorine, hydroxyhaemanthamine and haemanthamine (7). *In vivo* and *in vitro* activities of lycorine have been assessed by many authors, whereas its *in vitro* biosynthesis was reported mostly as related alkaloid in cultures aiming at production of galanthamine (8, 9, 10).

Previously, we studied the opportunity to apply biotechnological techniques for *ex situ* conservation of *P. maritimum* (11, 12). In consequence of the lost of its natural habitats and populations the species is endangered in Bulgaria (13) and under protection by the Biodiversity Act (2002). We succeeded in the enhancement of its *in vitro* multiplication by growing of directly regenerated bulblets in liquid culture followed by subcultivation on solid medium. Our present study was aimed at testing the ability of the liquid cultures of *P. maritimum* to produce alkaloids. We also tried to additionally enhance bulbing by indirect organogenesis.

Materials and methods

In vitro cultivation of P. maritimum

Initiation of *in vitro* cultures from mature seeds of *P. maritimum* collected from natural populations along Bulgarian South Black Sea coast, subcultivation of seedlings, and shoot multiplication were described previously (11, 12). Regenerated bulblets of equal size (about 6-8 mm in

diameter) were cultured in parallel on solid and in liquid MS based media (14) supplemented with 3 mg/L BAP and 0.1 mg/L NAA for 2 months in Magenta containers with 125 mL medium (3 bulblets per vessel, 2 repetitions). Fresh weight (FW) was measured after the first and the second month of cultivation. At the end of the second month plantlets were dried at 65˚C and dry matter was calculated as percentage of dry weight (DW) towards fresh weight of the biomass: DW/FW.100. To assess the rate of the biomass augmentation taking into account both the increase of the fresh weight as well as the percentage of the dry matter we inserted growth coefficient (GC) which allowed comparison of the cultures' growth efficiency: $GC = DW/FW_{initial}$.

Furthermore, cultures were maintained on medium with 30 or 60 g/L sucrose and 2/0.15 mg/L BAP/NAA, solidified with 6 g/L Plant agar (Duchefa, NL) in Vitro Vent containers (Duchefa, NL). Medium with auxin predominance was used for callus initiation: 0.9/1.8 mg/L BAP/NAA. To compare direct and indirect bulblet regeneration 18 *in vitro* bulblets were used as initial source. They were subcultured by longitudinal cut to 4 sectors and explants were put on media with 30 g/L sucrose, supplemented with 2/0.15 and 0.9/1.8 mg/L BAP/NAA, respectively. Once obtained calli were further grown on medium with 2/0.15 mg/L BAP/NAA to produce shoots. Propagation coefficient (PC) was calculated as number of bulblets obtained per initial *in vitro* bulblet. Medium with 0.2/1.0 mg/L BAP/NAA was used for rooting of the bulblets. All cultures were grown under 16/8 h light/dark period, 40.5 $\mu mol m^{-2} s^{-1}$, at 23±2°C and transferred to fresh medium every two months.

Alkaloid determination

Each sample consisted of three *in vitro* plantlets grown in one container. Each analysis was carried out in two repetitions. Alkaloids were extracted from dried (65˚C) and powdered plant material. Samples (50 mg DW) were macerated with methanol (3 ml) in ultrasonic bath, 3 times for 30 min every 8 h at 25˚C. After filtration (FILTRAK 390∅), centrifugation at 9500g for 10 min and evaporation with liquid nitrogen, the total methanol extract was diluted with a mixture of 2.5% methanol and 1.7% acetonitril in water, and filtrated through a 0.45 µm filter (Waters). Chromatographic analysis was carried out on Waters HPLC system supplied with quaternary pump 600E and PDA 996 detector; Alltech Ultrashere-Octyl RP-C8 column (150 × 4.6 mm i.d., 5 µm) protected by a Symmetry guard column C8 (20 × 3.9 mm i.d., 5 µm). The mobile phase consisted of acetonitril/methanol/water (containing 7.5 mM triethanolamine, pH up to 6.9 with phosphoric acid) (20/15/65); column temperature: 35°C; flow rate: 1.0 ml/min; injection volume: 20 µl). Data acquisition and analysis were made by Empower chromatographic software. Determination was done for lycorine, homolycorine, galanthamine, norgalanthamine, ungiminorine and galanthaminone.

The effect of liquid medium on lycorine biosynthesis was assessed by ANOVA single factor analysis.

Results and Discussion

Chromatographic analysis confirmed biosynthetic activity of all tested 3-year old *in vitro* cultures of *Pancratium maritimum*, grown on agar solidified and in liquid medium. Lycorine was detected as the only alkaloid produced under *in vitro* conditions. Its concentration was significantly higher in plantlets grown on solid medium ($P < 0.001$) (**Fig. 1**). However, the growth coefficient of the liquid cultures was three times higher due to the fast augmentation of the fresh weight related to slight decrease of the dry matter (**Table 1**). Finally, the amount of lycorine per container was higher in the case of cultivation of plantlets in liquid medium (**Fig. 1**).

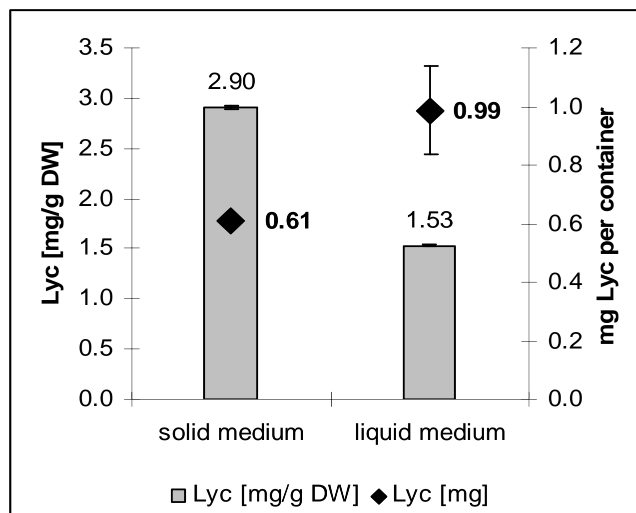

Fig. 1. Lycorine concentration (mg/g DW) and amount per container (mg) of *P. maritimum* plantlets grown on agar solidified and in liquid medium with equal composition.

Multiplication of *P. maritimum* was enhanced by indirect bulblet regeneration (**Table 2**). Numerous plantlets were obtained when calli were transferred on medium containing 2/0.15 mg/L BAP/NAA, however they were slow growing and needed more time to reach size suitable for subcultivation. Bulblets were easily rooted on medium with 0.2/1 mg/L BAP/NAA but this combination of growth

regulators also stimulated callus induction. Roots appeared spontaneously on the medium used for shoot formation and cultures maintenance as well, usually 2 to 4 roots per bulblet. In order to faster accumulate mass, small bulblets with average weight of 0.07 g were transferred in liquid medium after removal of their roots. Large bulblets were subcultured aiming further culture multiplication.

TABLE 1

Growth of *P. maritimum* plantlets on solid and in liquid media with equal composition (data per container)

Medium	FW in (g)	FW 1m (g)	FW 2m (g)	DW/FW%	DW (g)	GC
agar solidified	0.70 ± 0.02	1.29 ± 0.04	2.10 ± 0.15	10.1 ± 0.7	0.21 ± 0.00	0.30 ± 0.01
liquid	0.65 ± 0.05	3.06 ± 0.09	8.00 ± 0.94	8.0 ± 0.4	0.64 ± 0.11	0.99 ± 0.09

TABLE 2

Comparison of direct and indirect organogenesis of *P. maritimum*: 8 months cultivation.

Bulblets	Total number	PC	% Rooted	Large	Middle size	Small
Directly regenerated	54	6.0	88.9	24	30	0
Indirectly regenerated	286	31.8	100	27	83	176
Average weight (g)				1.05	0.32	0.07

Sucrose concentration in media influenced plantlet growth. Elongation of the leaves was typical when the medium contained 30 g/L sucrose. Increase of sucrose to 60 g/L led to faster enlargement of the bulblets which shortened the period between two subcultivations. *In vitro* rooting was observed regardless the concentration of sucrose.

Conclusions

In vitro regenerated plantlets of *Pancratium maritimum* kept their viability and regeneration potential in 3-year old cultures. Moreover, they expressed biosynthetic activity producing lycorine in considerable amounts: 1.53 and 2.90 mg/g DW in cultures grown in liquid and on agar solidified medium, respectively. We determined similar lycorine content (2.85 mg/g DW on agar solidified and 1.57 mg/g DW in liquid medium) in the shoot-clumps of one clone of *Leucojum aestivum* grown under conditions of long-term cultivation (8, 15). *In vitro* biosynthesis of lycorine was also reported in cell cultures of *Ungernia victoris*, with related alkaloids galanthamine and norgalanthamine, however in much lower concentration: 0.42 mg/g DW for the total amount of the three alkaloids (10). This was most probably due to the relationship between the biosynthetic activity and the degree of tissue differentiation that has been confirmed by several authors for different Amaryllidaceae species (16, 17, 18). It should be interesting to determine the content of lycorine in the indirectly regenerated plantlets of *P. maritimum* in our future studies.

The alkaloid concentration in *P. maritimum* plantlets obviously decreased in liquid culture. Similar effect was noticed for *in vitro* organ cultures of *L. aestivum* grown in parallel on agar and in liquid medium (15) as well as for shoot-clump cultures of *Narcissus confusus* (19). It should be related with some release of the alkaloids in the liquid medium.

Lycorine was the only alkaloid we detected in *P. maritimum* cultures grown in liquid as well as on agar solidified medium. This alkaloid was found in the bulbs, leaves and roots of native plants collected from the Black sea cost of Bulgaria but as a minor component in the plant tissues (2). Authors considered that crinane type alkaloids haemanthamine and crinine were the main alkaloids in the Bulgarian *P. maritimum*. However, such conclusion seems premature taking into account the possible population heterogeneity, as well as the influence of the mother plant hemotype on the biosynthetic capacity of the corresponding *in vitro* cultures. Such determination of the alkaloid production was recently reported about 16 *in vitro L. aestivum* clones keeping the alkaloid profile of their plants of origin during several years of cultivation (8, 9).

On the other hand, for the present we cannot conclude that lycorine is the only alkaloid typical of our *P. maritimum in vitro* cultures, although there were no other chromatographic picks. We observed clearly expressed dynamics of the content of lycorine and galanthamine, the main alkaloids of 12 *L. aestivum* clones, in the course of 30

months *in vitro* cultivation under continuous conditions (9). The dynamics of the galanthamine biosynthetic activity differed from that established for the plants of this bulbous species grown *in situ* and under field conditions. It this relation it should be of interest to study the *in vitro* biosynthesis of *P. maritimum* during longer period.

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