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ORIGINAL PAPER

Alkaloids biosynthesis by *Pancratium maritimum* L. shoots in liquid culture

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Abstract The process of alkaloid biosynthesis by *Pancratium maritimum* shoot culture, cultivated under submerged conditions, was investigated. Twenty-two compounds of different structural types of the Amaryllidaceae alkaloids (tyramine, narciclasine, galanthamine, haemanthamine, lycorine, pancracine, tazettine and homolycorine types) were detected in the studied samples from biomass and cultural liquid. Dominant compounds in the shoots were of tyramine, lycorine and haemanthamine types, whereas in the culture media were found mainly lycorine type compounds. Based on the multi-metabolic estimation of the alkaloid metabolism and physiological peculiarities, liquid cultures of *P. maritimum* shoots could be defined as prospective biological systems for producing bioactive molecules with acetylcholinesterase inhibitory and apoptotic activities.

Keywords Acetylcholinesterase inhibition · Amaryllidaceae alkaloids · *Pancratium maritimum* L. · Shoot culture

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
AChE	Acetylcholinesterase
ADB	Accumulated dry biomass
BAP	6-Benzylaminopurine
DTNB	Dithionitrobenzene
GC/MS	Gas chromatography/mass spectroscopy
MS	Murashige and Skoog nutrient medium
NAA	α-Naphthylacetic acid
TIC	Total ion current

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Introduction

Plant species belonging to the Amaryllidaceae family are widely distributed in the tropical and temperate countries in the world. They are extensively used in traditional medicine as their pharmacological effects are frequently associated with several typical alkaloids they synthesize (Evidente and Kornienko 2009). The genus *Pancratium* includes about 20 species distributed in the Mediterranean region, Africa and Asia (Medrano et al. 1999; Berkov et al. 2004).

Pancratium maritimum L. is a commonness species in communities occupying stable dunes on the Atlantic coast (Medrano et al. 1999). This plant species is also characteristic for sandy coastal habitats of the Mediterranean and Black sea regions where it is endangered and protected. The bulb and leaf extracts of *P. maritimum* were found to possess several biological activities—purgative (Iordanov 1964), acaridical, insecticidal (Abbassy et al. 1998), as well as antifungal (Sur-Altiner et al. 1999). Recent studies demonstrated that some alkaloids synthesized by plants from the Amaryllidaceae family are acetylcholinesterase inhibitors (Maelicke et al. 2001; Bastida et al. 2006; Berkov et al. 2008). As such, they are of pharmaceutical

importance and hence they are in the focus of research to investigate the potential of in vitro cultures as a system to produce these substances under controlled conditions. Plant in vitro systems represent a promising technology for both basic research of physiology and biochemistry of plant cells and for the production of biologically active substances (Georgiev et al. 2008; Huerta-Heredia et al. 2009). In the case with rare and threatened plants, they are prospective ecologically friendly experimental matrix for searching new bioactive metabolites. Currently, the AChE inhibitory activity is ascribed mainly to the lycorine and galanthamine type alkaloids and therefore search for new AChE inhibitors is of importance for the treatment of Alzheimer's disease (Lopez et al. 2002; Houghton et al. 2006). Till date, mainly intact plants from the genus *Pancratium* have been investigated for their alkaloid profiles (Sandberg and Michel 1968; Tato et al. 1998; Abou-Donia et al. 1991, 2002; Senar et al. 1993, 1994, 1998; Orhan and Sener 2003; Berkov et al. 2004; Sener and Orhan 2005; Kintsurashvili and Vachnadze 2007; Cedron et al. 2009; Kinghorn et al. 2009; Torras-Claveria et al. 2010). However, there is scanty data about secondary metabolites of their in vitro systems (unpublished data), and there is no information available about alkaloid profiles of in vitro systems cultivated under submerged conditions.

In the present manuscript, we present for the first time an investigation on the alkaloid fractions (composition and bioactivity) and some physiological peculiarities of liquid shoot cultures of *P. maritimum.*

Materials and methods

Shoot culture

Surface-sterilized young fruits were sliced and planted on Murashige and Skoog (1962) (MS) (Duchefa, The Netherlands) medium supplemented with 30 g/L sucrose, 5.5 g/L "Plant agar" (Duchefa), 1 or 4 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) (Sigma, USA) and 2 mg/L 6-benzylaminopurine (BAP) (Duchefa, The Netherlands) for initiation of callus induction. Cultivation was carried out at 26°C in darkness until the first calli appeared. The obtained calli were transferred on MS nutrient medium supplemented with 30 g/L sucrose, 5.5 g/L "Plant agar", 1.15 mg/L α-naphtylacetic acid (NAA) (Duchefa, The Netherlands) and 2.0 mg/L BAP for initiation of shoot cultures. Single shoot clumps, differentiated from callus tissue were propagated to give a raise of homogenous shoot lines. The cultivation of the established shoots was carried out at 26°C under illumination 16:8 h (light:dark) at a light intensity of 110 μmol/(m^2 s)—SYLVANIA Gro-Lux fluorescent lamp (F18W/GRO-LUX). The further subcultivations of obtained shoot cultures were performed every 28 days under the same conditions.

For the liquid cultivation, the shoots were transferred to 250 ml flasks with 50 ml MS medium supplemented with 30 g/L sucrose, 1.15 mg/L NAA and 2.0 mg/L BAP, and cultivated on a shaker (110 rpm) at 26°C under illumination (light:dark, 16:8 h) at a light intensity of 110 μmol/(m^2 s)—SYLVANIA Gro-Lux fluorescent lamp (F18W/GRO-LUX).

Alkaloid extraction

200–300 mg dry biomass was extracted three times with 5 ml methanol under sonification for 15 min. Combined extracts were concentrated under vacuum and dissolved in 2 ml of 2% sulfuric acid. The neutral compounds were removed by extraction (tree times) with diethyl ether. The alkaloids were fractionated after basification of the extracts with 1 ml of 25% ammonia and extraction (3 × 3 ml) with chloroform. The chloroform extracts were dried over anhydrous sodium sulphate and evaporated to dryness.

Analyses

GC/MS of alkaloids

The GC–MS analyses were performed with a Hewlett Packard 6890+/MSD 5975 (Hewlett Packard, Palo Alto, CA, USA) instrument operating in EI mode at 70 eV. A HP-5 MS column (30 m × 0.25 mm × 0.25 μm) was used. The temperature program was: 100–180°C at 15°C × min^{-1}, 180–300 at 5°C × min^{-1} and 10 min hold at 300°C. Injector temperature was 250°C. The flow rate of carrier gas (helium) was 0.8 ml × min^{-1}. Split ratio was 1:20. 1 μl of the solution was injected.

The spectra of coeluting chromatographic peaks were examined and deconvoluted using AMDIS 2.6 (NIST, Gaithersburg, MD, USA) software before area integration. The contribution of each compound in the extracts was shown in Table 1 as a percentage of the total ion current (TIC). The area of the GC–MS peaks depends not only on the concentration of the corresponding compounds but also on the intensity of their mass spectral fragmentation, so data given in the table do not express absolute values (do not represent a true quantification) but can be used for comparison of the samples, which was the objective of this work.

Alkaloid identification The compounds were identified by comparing their mass spectral fragmentations with those of standard references from NIST 08 database (NIST Mass Spectral Database, PC-Version 5.0 (2008), National Institute of Standardization and Technology, Gaithersburg, MD, USA) and/or as described literature data (Berkov et al. 2008;

Table 1 Alkaloid fraction of *P. maritimum* shoots cultivated under submerged conditions. Data are presented as % of TIC

Alkaloids	R_t	M+	14th day of cultivation		35th day of cultivation	
			Intracellular	Extracellular	Intracellular	Extracellular
O-Methyltyramine (**1**)	6.53	151	–	–	tr.	–
Tyramine (**2**)	6.99	137	23.4 ± 2.1	–	29.0 ± 7.5	–
N-Methytyramine (**3**)	7.41	151	3.4 ± 1.1	–	1.7 ± 0.4	–
Hordenine (**4**)	7.49	165	5.8 ± 3.0	7.3 ± 1.1	3.5 ± 1.4	2.7 ± 2.0
X1 (**5**)	7.59	209	1.5 ± 1.3	–	1.0 ± 0.6	–
X2 (**6**)	11.67	–	–	–	2.6 ± 0.5	–
Ismine (**7**)	18.62	257	0.1	–	0.1	–
Trisphaeridine (**8**)	18.71	223	0.7 ± 0.1	0.3 ± 0.1	1.2 ± 0.7	0.3
Galanthamine (**9**)	20.61	287	0.1	0.5 ± 0.1	0.2	–
Buphanisine (**10**)	20.92	285	0.1	0.6 ± 0.2	0.1	4.0
N-Demethylgalanthanine (**11**)	21.21	273	4.2 ± 0.5	1.2 ± 0.3	2.8 ± 0.7	–
Vittatine (**12**)	21.63	271	4.8 ± 1.1	0.2	4.6 ± 2.0	1.9
Anhydrolycorine (**13**)	22.02	251	1.1	2.6 ± 1.1	2.6 ± 1.6	–
8-*O*-Demethylmaritidine (**14**)	22.18	273	2.6 ± 0.6	–	2.1 ± 0.9	–
Pancratinine C (**15**)	23.29	287	–	–	0.1	–
X3 (**16**)	23.61	249	1.4 ± 0.3	1.6 ± 0.1	2.2 ± 0.7	–
Haemanthamine (**17**)	24.03	301	18.9 ± 3.0	9.1 ± 2.8	14.0 ± 1.7	0.8
Tazettine (**18**)	24.23	331	0.1	6.0 ± 5.9	0.1	0.6
11-Hydroxyvittatine (**19**)	25.18	287	5.3 ± 0.1	–	6.5 ± 0.7	–
Lycorine (**20**)	25.66	287	26.1 ± 2.8	68.6 ± 9.1	23.8 ± 7.4	79.4 ± 7.1
N-Formylgalanthamine (**21**)	26.53	301	0.1	0.7 ± 0.1	0.1	0.2
8-*O*-Demethylhomolycorine (**22**)	26.62	301	0.3 ± 0.1	1.3 ± 0.7	1.7 ± 0.3	10.1

Torras-Claveria et al. 2010). Compounds **5**, **6** and **16** were left unidentified due to lack of reference data.

Inhibition of the acetylcholine esterase activity The acetylcholine esterase inhibition assays were performed after the method described by Perry et al. (2000), slightly modified as follows: The fresh enzyme solution was prepared according to Lopez et al. (2002) by dissolving 0.86 U AChE (type VI-S Sigma) in 1.0 ml phosphate buffer pH 8.0, supplied with 0.15 M NaCl (Sigma) and 0.05% Tween 80 (Duchefa, The Netherlands). For the evaluation of acetylcholine esterase inhibitory activity, 40 µl of enzyme solution and 20 µl of investigated extracts were added into 2.0 ml phosphate buffer (pH 8.0) and incubated at 4°C for 20 min in darkness. On the next step, 20 µl acetylthiocholine iodide (0.06 mM in phosphate buffer pH 7.0, Sigma) and 20 µl dithionitrobenzene (DTNB) (0.05 mM in phosphate buffer pH 7.0, Sigma) were added and the reaction mixture was incubated at 37°C for 20 min in darkness. The reaction was halted by cooling the mixture to 4°C. After that 20 µl of eserine salicylate (0.018 mM in phosphate buffer pH 7.0, Sigma) were added. The concentration of formed yellow-colored product was measured at 405 nm (Shimadzu UV/Vis mini 1240 spectrophotometer, Japan). The coloration was stable for 10 min. A blank sample (without extracts) was prepared and measured before each measurement of corresponding samples. A positive control samples were prepared for both experimental samples and blank samples, following the same procedure, with adding 20 µl of eserine salicylate (Sigma) before the initiation of the enzyme reaction with acetylcholine iodide. The results were presented as inhibition rate (%), calculated according to the following equation:

$$\text{Inhibition} = \frac{(\text{blank} - \text{blank positive control}) - (\text{sample} - \text{sample positive control})}{(\text{blank} - \text{blank positive control})} \times 100\%.$$

Dry biomass

The growth of the shoots was monitored by measurement of dry biomass and the accumulated dry biomass (ADB) calculated on this base (Pavlov et al. 2007)

$$ADB = FDB - IDB$$

where ADB is accumulated dry biomass; FDB is final dry weight of shoots; IDB is initial dry weight of shoots, used for inoculation.

Sucrose, glucose and fructose

The contents of sucrose, glucose and fructose in the culture medium were determined by means of an enzyme test combination (Magazyme, Ireland).

Nitrate, ammonium and phosphate ions

Nitrate, ammonium and phosphate ions in the culture medium were determined by chemical test combinations Spectroquant® (Merck).

Statistical analysis

The data presented are averages from two independent experiments, which repeated twice, and expressed as the means with standard deviations.

Results and discussion

Shoot formation was observed when *P. maritimum* calli were planted on MS medium supplemented with 1.15 mg/L NAA and 2 mg/L BAP. The shoot clumps were developed after 48 days of cultivation, similarly as it has been reported for shoot formation from *Leucojum aestivum* L. calli (Pavlov et al. 2007). It must be underlined that the investigated shoot clumps were subcultivated for 2 years on solid medium prior experiments under submerged cultivation. During this period, they showed stable morphological characteristics and stable growth as well. The establishment of shoot clumps via callus was chosen because of absent of any bacterial and fungi contaminations. The selection of the shoot line used for further experiments under submerged cultivation was done on the basis of its stable growth, morphological characteristics, stable amounts of biosynthesized galanthamine, lycorine and norgalanthamine, as well as due to anti-AChE activity of its alkaloid mixtures. It is worth of remark that shoot clump used in the presented study are genetically homogeneous because they descend from single shoot differentiated from callus tissue.

In order to create suitable cultivation system for shoot cultures, the plant in vitro systems should be adapted to submerged conditions of cultivation. The liquid cultivation of *P. maritimum* shoots was performed after direct transfer of them from solid medium to flasks with liquid medium. No preliminary cultivations in liquid medium were necessary for adapting to the new conditions of cultivation. Shoots grew fast and in the end of growth cycle, the leaf tissue took up whole cultivation volume. The maximum in the accumulated dry biomass (0.6 g/flask) was reached on day 35th of cultivation (Fig. 1). During the growth cycle of *P. maritimum* shoots the so-called "over-flooding" effect was not observed. After the 35th day of cultivation, the shoots died fast probably due to complete exhaust of the macronutrients from the culture medium (Fig. 3).

The plant in vitro systems were mainly investigated concerning single compound production and the multi-compound metabolism was more or less neglected. Recent metabolic profiling attempts seem to be able both to provide more information on plant metabolic pathways and to give us the opportunity for better understanding and exploitation of plant cells potential for production of desired compounds, as well as compounds normally not synthesized from the intact plants (Zhong 2001; Pavlov et al. 2005). Twenty-two compounds of different structural types of the Amaryllidaceae alkaloids (tyramine, narciclasine, galanthamine, haemanthamine, lycorine, pancracine, tazettine and homolycorine types) were detected in the studied samples (Tables 1, 2). The shoots cultivated for 14 and 35 days showed very similar alkaloid profiles as compared to the alkaloid profiles of culture media, the shoots displayed relatively more compounds. The alkaloid profiles of the media obtained from cultures maintained for 14 days showed higher number of compounds as compared to those cultures maintained for 35 days. Dominant compounds in the shoot biomass were of tyramine (34 and 38% at the 14th and 35th day, respectively), lycorine (29%) and haemanthamine (32 and 27%) types, whereas in the culture media were found mainly lycorine type compounds (73 and 79%). Presence of tyramine type protoalkaloids (precursors

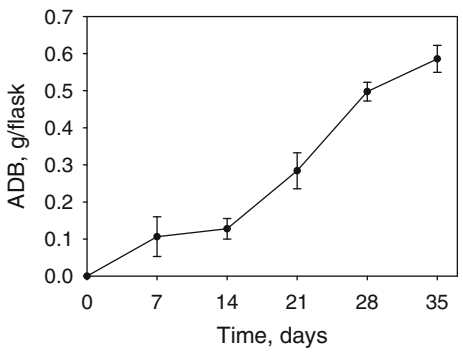

Fig. 1 Time course of growth of *P. maritimum* shoots under submerged cultivation. Data represent average of four replications with the standard deviations

Table 2 Contribution of the alkaloid types in the alkaloid fractions of *P. maritimum* shoot cultures and culture media, expressed as a % of TIC

Alkaloid group	14th day of cultivation		35th day of cultivation	
	Intracellular	Extracellular	Intracellular	Extracellular
Tyramine	34.1	7.3	37.8	2.7
Narciclasine	0.8	0.3	1.3	0.3
Galanthamine	4.4	2.4	3.1	0.2
Haemanthamine	31.7	9.9	27.3	6.7
Lycorine	28.6	72.8	28.6	79.4
Pancracine	–	–	0.1	–
Tazettine	0.1	6.0	0.1	0.6
Homolycorine	0.3	1.3	1.7	10.1

of the Amaryllidaceae alkaloid biosynthesis) in relatively high abundance in the shoot cultures of *P. maritimum* is related with the degree of tissue differentiation (unpublished data). It is noteworthy that the excretion of lycorine type compounds and lycorine (**20**), in particular, in the cultural media. This is probably due to cell defence mechanisms of the shoots because this alkaloid is well-known for its cytotoxic, antibacterial and antifungal activities (Bastida et al. 2006). 8-*O*-Demethylhomolycorine (**22**) was found in relatively higher amounts in the culture media from shoots cultivated for 35 days. In contrast to lycorine, however, its biological properties are not so clear. It is assumed that compound **22** also participates in the plant defence mechanisms due to its hypotensive and cytotoxic effects (Bastida et al. 2006). From the detected alkaloids, galanthamine (**9**) and *N*-demethylgalanthamine (**11**) are known to be AChE inhibitors, galanthamine being about 10 times more active (Lopez et al. 2002). Less galanthamine type compounds were found in the medium obtained from cultures cultivated for 35 days (0.2%), which correlates with their lower AChE inhibitory activity when compared with the media obtained from cultures cultivated for 14 days. The AChE inhibitory activity of extracellular alkaloids fraction of 14 days old liquid culture was found to be 4.01-fold higher than the corresponding activities at the day 35 of cultivation (Table 3). More interesting is the fact, that the intracellular alkaloids fraction showed different tendency—the AChE inhibitory activity in day 35 of cultivation is 3.12-fold higher than those, recorded in 14 days old culture. This higher AChE inhibitory activity correlates with higher galanthamine contribution (0.2% of all alkaloids in the fraction from shoot cultures cultivated for 35 days). Despite that the production of galanthamine is not satisfactory, the in vitro cultures of *P. maritimun* are good system for production of an other interesting alkaloid lycorine, which has been proved to be a potent inducer of apoptosis in human tumoral cells (Liua et al. 2004; McNulty et al. 2009).

The creation of biotechnologies based on the plant in vitro systems aims receiving either maximal yields of the target metabolite or maximal bioactivity of a group of metabolites. The first step of this complicated process is the definition of the relationships between biosynthesis of the target metabolites and the utilization of the main components of the medium, as well as the next statistical optimization of the nutrient medium (Pavlov et al. 2007; Georgiev et al. 2008). It is well-known that sucrose is the most suitable carbon source for the cultivation of plant in vitro systems (Georgiev et al. 2008) and therefore its hydrolysis and the next catabolism of glucose and fructose were investigated during the cultivation of *P. maritimum* shoots under submerged conditions (Fig. 2). The sucrose was completely hydrolyzed to glucose and fructose until day 28th of cultivation (Fig. 2). The most intensive of this process was before the exponential phase of growth, until the day 14th of cultivation. At the same time glucose and fructose utilization followed the time course of growth of *P. maritimum* shoots. Thus, the relationship between sucrose hydrolysis and the start of exponential phase of growth of the liquid shoot culture clearly indicated that the concentration of the sucrose will significantly influence on the alkaloid production. Moreover, the Amarillidaceae alkaloid biosynthesis is growth dependent process in liquid shoot cultures (Pavlov et al. 2007). Beside the sucrose concentration, the most frequent for the intensification of secondary metabolism, the medium content for cultivation of plant in vitro systems is modified with the respect to the ratio between the main inorganic ions—nitrate, ammonium and phosphates. The results from the investigation of their utilization (Fig. 3) clearly demonstrate that decrease in the concentrations of the nitrate, ammonium and phosphate ions followed the growth of the *P. maritimum* shoots under submerged conditions of cultivation as in the end of cultivation (35th day) they were completely exhausted. It should be underlined that the shoots utilized all three ions in parallel (Fig. 3), which is specificity of the culture, because usually the plant in vitro systems metabolized with

Table 3 AChE activity of intra- and extracellular alkaloid fractions of *P. maritimum* shoots cultivated under submerged conditions (means ± SD)

Alkaloids	Anti-AChE activity, IC_{50}	
	14th day of cultivation	35th day of cultivation
Intracellular fraction, mg Dry Biomass	19.07 ± 1.76	6.12 ± 1.32
Extracellular fraction, ml Culture liquid	72.18 ± 21.62	289.5 ± 42.5

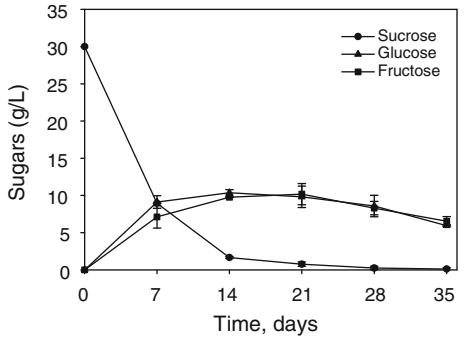

Fig. 2 Time courses of utilization of sucrose, glucose and fructose from *P. maritimum* shoots during their cultivation under submerged conditions. Data represent average of four replications with the standard deviations

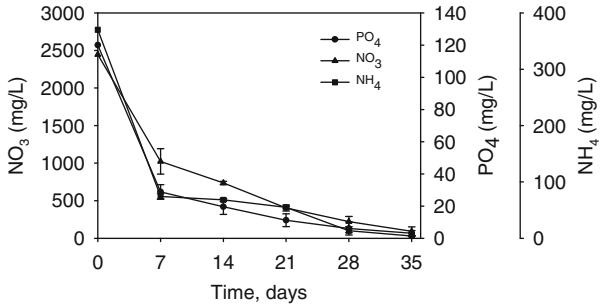

Fig. 3 Time courses of utilization of ammonium, phosphate and nitrate ions from *P. maritimum* shoots during their cultivation under submerged conditions. Data represent average of four replications with the standard deviations

preference phosphate and ammonium ions prior the nitrates (Pavlov et al. 2007; Georgiev et al. 2008).

In conclusion, presented data showed that a multi-component analysis of the bioactive secondary metabolites is a power tool for both the investigation of the physiology and biochemistry of the culture and for creation of the algorithms for discovering of the new metabolites with valuable biological activities. In the case it should be noted that liquid cultures of *P. maritimum* shoots are prospective biological systems for producing bioactive molecules with AChE inhibitory and apoptotic activities from the culture medium which is a good base to create a two-phase cultivation system for their obtaining.

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