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Elicitation of galanthamine biosynthesis by *Leucojum aestivum* liquid shoot cultures

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ABSTRACT

The effects of methyl jasmonate and jasmonic acid on galanthamine production, phenolic acid content and growth of *Leucojum aestivum* L. shoot culture, cultivated in submerged conditions were investigated. The best time-point for addition of elicitors was during the exponential phase of the culture growth. The maximal contents of galanthamine and lycorine (226.9 μg/flask and 491.4 μg/flask, 1.36 and 1.67-fold higher compared to the control, respectively) were achieved after elicitation with jasmonic acid, whereas the elicitation with methyl jasmonte resulted in maximal accumulation of phenolic acids. It was demonstrated that the boosting effect of jasmonic acid on Amaryllidacea alkaloid biosynthesis was due to induction of the activity of tyrosine decarboxylase, whereas methyl jasmonate stimulates the biosynthesis of phenolic acids by inducing mainly the activity of phenylalanine ammonia-lyase.

Introduction

One of the most powerful long acting, reversible and selective inhibitors of acethylcholine esterase is galanthamine (Berkov et al., 2009a; Ptak et al., 2012). This alkaloid is synthesized in acceptable amounts by many plants of the Amaryllidaceae family (Heinrich and Lee Teoh, 2004). Galanthamine, obtained by extraction of fresh *Leucojum aestivum* L. and *Narcissus* spp. Biomasses, is used as an active ingredient in commercial medicines Razadine® and Nivalin®, respectively (Berkov et al., 2009a).

During the last decade, plant *in vitro* cultures have been the focus of scientific and commercial attention as a prospective alternative for galanthamine production, due to depletion of wild populations of *L. aestivum* L. (Georgiev et al., 2012). Both undifferentiated and differentiated plant *in vitro* systems have been investigated for their potential to biosynthesize galanthamine. The alkaloid was found to present in calli at extremely low concentrations. Further dedifferentiation to cell suspensions additionally decreased galanthamin contents, and only traces were detected (Pavlov et al., 2007). Based on this fact, extensive research with differentiated plant *in vitro* systems has been performed. By definition, secondary metabolite biosynthesis is a function of cell differentiation in plant tissue (Steingroewer et al., 2013). It was established that hairy roots did not possess potential to synthesized galanthamine (Diop et al., 2007). However, shoot type plant *in vitro* systems had the highest ability to produce this alkaloid among other type plant *in vitro* systems (Berkov et al., 2009b). It has been found that a number of factors strongly influenced galanthamine production, including illumination (Pavlov et al., 2007), concentrations of the macronutrients (Georgiev et al., 2009; Tahchy et al., 2011) and growth regulators (Tahchy et al., 2011). Reports on the optimization of production process are also available for nutrient medium optimization (Georgiev et al., 2009) and cultivation in different bioreactor systems (Georgiev et al., 2012; Ivanov et al., 2012, 2011; Schumann et al., 2012a). However, the elicitation of the process of galanthamine biosynthesis has not been studied in detail. Primary investigations on this powerful tool for stimulating plant secondary metabolism have been performed (Colque et al., 2004; Mu et al., 2009; Schumann et al., 2012b). However, there are still many questions to be answered about the most appropriate elicitor, about the time of treatment as well as how the elicitors influence other biochemical pathways.

Abbreviations: MS, Murashige and Skoog; ADB, accumulated dry biomass; C4H, cinnamic acid 4-hydroxylase; C3H, p-coumarate-3-hydroxylase; COMT, cafeoil *O*-methyltransferase; PAL, phenylalanine ammonia-liase; TD, tyrosine decarboxylase; PAL/TAL, phenylalanine/tyrosine ammonia-lyase.

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The aim of this study was to evaluate the effects of treatment of *L. aestivum* liquid shoot culture with jasmonic acid and methyl jasmonate on galanthamine biosynthesis. We also investigated the plant cells' response by monitoring changes in the activities of phenylalanine ammonium lease and tyrosine decarboxylase (TD).

Materials and methods

Leucojum aestivum L. shoot culture

The shoot cultures were established by planting the previously obtained calli of *Leucojum aestivum* L. on Murashige and Skoog (MS) nutrient medium using the protocol described previously (Pavlov et al., 2007). The conditions of maintenance and subcultivation have been described in detail elsewhere (Georgiev et al., 2012).

Conditions of elicitation

The experiments on the elicitation were performed in 500 ml Erlenmeyer flasks containing 200 ml optimized MS medium (Georgiev et al., 2009). Cultivation was performed at 26 °C, on a shaker (11.6 rad/s) under illumination with a photoperiod of 16 h light/8 h dark. For inoculation, 11.5–12.0 g fresh weight 28-day-old shoots grown on a solid medium were used. For elicitation, preliminary sterilized thought filtration (0.22 μm, Sartorius sterile filters) solutions of jasmonic acid and methyl jasmonate were used. The elicitors were added to the flasks on day 28 and 35 from the beginning of the cultivation at a final concentration of 25 μM. The samples were taken as follows: (1) in the case of elicitation, on the 28th day of cultivation and on the 29th, 32nd, 35th and 42nd days of cultivation; (2) in the case of elicitation, on the 35th day of cultivation and on the 36th, 39th, and 42nd days. All samples were monitored for alkaloid content (intracellular and extracellular), phenolic acids, dry biomass, and enzyme activities of phenylalanine ammonium lease and tyrosine decarboxylase. Control samples without added elicitor were analyzed as well.

Analyses

Dry biomass

The growth of *L. aestivum* 80 shoot culture was monitored by accumulated dry biomass (ADB) according to Georgiev et al. (2009).

Quantification of alkaloids

Extraction of intracellular alkaloids. Freeze-dry biomass (0.2–0.3 g) was extracted three times with 5.0 ml of methanol in an ultrasonic bath for 15 min. The combined extracts were concentrated under a vacuum and dissolved in 2× 2.0 ml of 3% sulfuric acid. The neutral compounds were removed by extraction (three times) with diethyl ether. The alkaloids were fractionated after basification of the extracts with 1.0 ml of 25% ammonia and extraction with chloroform (3× 3.0 ml). The chloroform extracts were then dried over anhydrous sodium sulfate and evaporated to dryness.

Extraction of extracellular alkaloids. Fifty milliliters of culture liquids were evaporated to dryness and dissolved in 10.0 ml methanol. After centrifugation and separation of pellets, 8.0 ml of supernatants were evaporated to dryness and residuals were dissolved in 2 × 2.0 ml of 3% sulfuric acid. The obtained solution was processed as described above.

For alkaloid determination, a Waters HPLC system, equipped with a Dual λ absorbance detector (Waters 2487, Milford, USA) and binary pump (Waters 1525, Milford, USA) was used. The chromatographic conditions have been reported previously (Ivanov et al., 2011).

Phenylalanine ammonium lease (E.C. 4.3.1.5)

Samples of fresh biomass (1.5–2.0 g) were frozen at −20 °C and immediately homogenized in 4.0 ml borax buffer (100 mM, pH 8.8) cooled to 4 °C. Homogenates were centrifuged (4200 rcf for 15 min) and supernatants were collected. The volumes of supernatants were adjusted to 4.0 ml with 100 mM borax buffer (pH 8.8) and used for the determination of phenylalanine ammonium lease activity. For evaluation of PAL activity, the method of Hino et al. (1982) was used with slight modifications as follows: 0.4 ml of the investigated extracts were added to 2.0 ml 3 mM L-phenylalanine dissolved in 100 mM borax buffer. The samples were temperate at 37 °C for 30 min. The reaction was stopped by adding 0.2 ml 25% HCl. After that, reaction mixtures were centrifuged at 10,000 rcf for 15 min to remove precipitated proteins. Formed cinnamic acid was detected spectrophotometrically at 290 nm against control samples, which were developed in the same way except that HCl was added prior to adding the enzyme solution to the reaction mixtures. Calculations of the formed cinnamic acid were performed with a molar coefficient of absorption of 10,700 mol/cm (Gomez-Vasquez et al., 2004).

Tyrosine decarboxylase (E.C. 4.1.1.25)

Frozen shoots (2.0 g fresh weight) were homogenized in a mortar for 2 min with 3.0 ml extraction buffer cooled to 4 °C and containing 200 mM-Tris–HCl buffer (pH 8.4), 50 mM KCl, 1 mM EDTA, 12 mM 2-mercaptoethanol, 10 mM $MgSO_4 \times 7H_2O$ and 0.5% (w/v) polyvenylpyrrolidone. The homogenate was centrifuged at 4200 rcf for 15 min. Supernatants were used for the determination of tyrosine decarboxylase following the method of Phan et al. (1983), modified as follows: 0.5 ml of the investigated extracts were added to 0.5 ml reaction buffer consisting of 100 mM Tris–HCl buffer (pH 8.4), 3 mM L-tyrosine and 0.3 mM pyridoxal-5-phosphate as a co-enzyme. The enzyme reaction was performed at 37 °C for 45 min. The reaction was stopped by adding 1.0 ml of 1 M Na_2CO_3. After the end of reaction, 1.0 ml of 0.01 M picrylsulfonic acid and 3.0 ml toluene were added to the mixtures. The samples were then gently homogenized and centrifuged at 4200 rcf for 15 min to separate water and toluene phases. The formed product (condense compound of picrylsulfonic acid and tyramine) was detected spectrophotometrically at 340 nm against control samples, which were developed the same way except that Na_2CO_3 was added before enzyme solution. Calculations of the formed color product were performed with a molar coefficient of absorption of 9300 mol/cm (Phan et al., 1983).

Quantification of phenolic acids

Extraction of phenolic acids. Frozen shoots (1.0 g fresh weight) were homogenized in a mortar with 3.0 ml 70% ethanol and extracted three times at 70 °C in a water bath for 15 min. The biomass was removed by filtration through filter paper, and the combined ethanol extracts ware evaporated to dryness under reducing pressure. The dry residues were dissolved in 1.0 ml 70% ethanol and were used for the next analyses.

HPLC analyses of phenolic acids. Qualitative and quantitative determinations of phenolic acids were performed using Waters 1525 Binary Pump HPLC systems (Waters, Milford, MA, USA) equipped with a Waters 2484 dual λ Absorbance Detector (Waters, Milford, MA, USA) and Supelco Discovery HS C18 column (5 μm, 25 cm × 4.6 mm), operated under the control of Breeze 3.30 software. For separation of phenolic acids, a mobile phase of 2% (v/v)