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Quaternary alkaloids in *Chelidonium majus in vitro* cultures

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ABSTRACT

Chelidonium majus L. is a medicinal plant containing several alkaloids, such as chelidonine, chelerythrine, sanguinarine, protopine, and coptisine that are stored and secreted from laticifers—highly specialized elongated cells of aerial and terrestrial parts of the plant. For use as a drug, several cultivars of this specialized crop are cultivated but the content and composition of bioactive substances is variable. Based on the observation of a characteristic orange-yellow color of latex in dedifferentiated tissue of *in vitro* callus cultures we investigated the alkaloid composition and the anatomy of callus and organ cultures of *C. majus* maintained on media enriched in plant growth regulators and illuminated with white or photosynthetically active light. Alkaloid composition was analyzed using DAD-HPLC, whereas tissue and organ anatomy was studied using light and scanning electron microscopy.

The fully developed organs and rhizogenic callus compared to the undifferentiated cells, produced larger proportion of alkaloids, and complex alkaloid composition. In roots, sanguinarine was the predominant alkaloid exceeding 4 mg/g dry mass. In microshoots, coptisine was the major compound, with the maximum content exceeding 14 mg/g, depending on the medium composition. In callus cultures, either protopine or sanguinarine predominated on different media and under different illumination, with maximum content over 13 mg/g dry mass of protopine reached under PAR (Photosynthetically Active Radiation), and sanguinarine over 0.9 mg/g. The presence of laticifers was confirmed with microscopy and the scanning electron microscopy revealed an abundant extracellular matrix in the callus tissues.

These results suggest the high biosynthetic potential of *in vitro* grown organs and dedifferentiated callus tissue that are able to produce significant amounts of pharmacologically relevant alkaloids from *C. majus* in various proportions that depend on the culture conditions such as supplementation with growth substances and sugars as well as on the illumination with light of different spectrum.

1. Introduction

Chelidonium majus L. (Greater celandine) is a traditional medicinal plant, listed in several pharmacopoeias and used in form of galenic preparations or purified alkaloid fraction, marketed in many European and East Asian countries. In folk medicine of Poland, Ukarine, Russia, Balkan countries, and Great Britain, the aerial parts or latex of *C. majus* were commonly used against skin, liver and eye diseases, and also against digestive tract parasites (summerized ion our recent review by Zielińska et al., 2018). According to available literature the alkaloid composition of *C. majus* tends to be unstable and unpredictable, leading to some toxicological issues (also reviewed in Zielińska et al., 2018). The plant is common in natural and synanthropic habitats but the demand for crude drug is covered mainly by cultivation. As a crop, *C. majus* is easy to grow and has higher alkaloid content than the wild plants. A couple of registered cultivars are used as the alternative crop that is harvested twice during the growing season in Central and Eastern European countries (Mordalski et al., 2014). Given the complexity of factors, i.a. plant growth regulators, nutrients or precursor feeding influencing the content and composition of bioactive substances in this crop, using *in vitro* tissue and organ cultures may facilitate obtaining high quality plant material and desired composition of alkaloids under precisely controlled conditions. There are numerous previous studies using different species proving that biotechnological approach –

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where influence of environmental factors is minimized – it is possible to obtain plant material with a specific composition of bioactive metabolites (Espinosa-Leal et al., 2018; Younas et al., 2018; Esmaeilzadeh and Rezaiei, 2018; Szopa et al., 2018).

Isoquinoline alkaloids, contained in a mixture of latex substance, effuse from secretory structures called laticifers localized in aerial and terrestrial parts of plants belonging to *Papaveraceae* family (Hagel et al., 2008). Many of these compounds, like papaverine or morphine from *Papaver* sp., demonstrate strong phrmacological activity. From among the large variety of isoquinoline alkaloids, sanguinarine, chelerythrine and protopine, deserve no less attention. These three compounds are found in several medicinal plants such as *Fumaria*, *Coptis*, *Sanguinaria*, *Corydalis*, or *Chelidonium* and have notable pharmacological properties such as antimicrobial, antiviral, cholagogue, and antiproliferative, and are responsible for the spasmolytic and anti-inflammatory effects of these herbs (Zielińska et al., 2018; Galadari et al., 2017; Lei et al., 2018; Prescot et al., 2018; Kędzia et al., 2013; Zuo et al., 2008; Gerenčer et al., 2006; Lee et al., 2007; Bugatti et al., 1987; Malinowska et al., 2017; Noureini et al., 2017). The biosynthetic route of this group of alkaloids is more or less explored (Weiss et al., 2006). Nevertheless, it is still not decisively documented if these substances are produced or only stored in the laticifers. For the efficient extraction of the alkaloids from *C. majus*, it is important to know the exact way of their formation in the plant. Apart from isoquinoline alkaloids, several other specialized metabolites are contained in *C. majus*: flavonoids such as kaempferol, quercetin, isorhamnetin and their glycosylated derivatives, as well as hydroxycinnamic acids, other organic acids (chelidonic, malic, citric, succinic), biogenic amines, terpenoids, xanthophylls, and defence-related proteins (recently reviewed in our previous paper - Zielińska et al., 2018). However, most of these constituents (except of some resistance related proteins - nucleases, proteases, protease inhibitors and others) apparently have much lower relevance to the specific medicinal usefulness of this plant than the main alkaloids (Zielińska et al., 2018).

In the current study we demonstrate quaternary alkaloid production *in vitro* both in organ and dedifferentiated tissue cultures of *C. majus*. The most abundant specialized metabolites produced in aerial and underground parts of this species are derivatives of benzophenantridine (chelidonine, chelerythrine, sanguinarine) protoberberine (berberine, coptisine, stylopine), and protopine (Sowa et al., 2018; Sarkozi et al., 2006). Previous research on *C. majus in vitro* cultures showed the presence of isoquinoline alkaloids in different plant organs, as well as high organogenic potential of the plant. A total alkaloid content expressed as chelidonine was measured by Ciric et al. (2008). However, their observations did not show the actual content of the individual compounds. Our earlier studies (Zielinska et al., 2017) showed that supplementation of the culture medium with kinetin influenced the induction of rhizogenesis processes in callus culture and stimulated the production of alkaloids in dedifferentiated cells. For this reason, in the present work we focused on the production of isoquinoline alkaloids in *C. majus* cell and organ cultures separately. The results showed large qualitative and quantitative differences in the alkaloids productions between shoots and roots grown *in vitro*. It was also shown that callus cells cultured on growth media retained their highly organogenic potential, which corresponded directly to higher alkaloid production and more complex composition.

2. Material and methods

2.1. In vitro plant material establishment

Seeds of *C. majus* collected from Botanical Garden of Medicinal Plants at the Department of Pharmaceutical Biology in 2016 were used to obtain shoot and callus cultures. The seeds were surface sterilized with 5% sodium hypochlorite, rinsed with sterile distilled water, and sawn on basal Murashige and Skoog (1962) (MS) medium with reduced of macro-, and microelements concentration (1/2 MS) and 6 g/l plant tissue culture grade agar (Biocorp, Poland).

4-week-old aseptic seedlings were cut into explants. Shoot tips containing apical meristem were excised and transferred to a shoot proliferation medium in the 500 ml volume culture jars (WECK Rundrand-glas 60, J. Weck & Co Germany). The proliferation media consisted of agar-solidified (6 g/L) B5 (Gamborg et al., 1968), and MS, and 1/2 MS containing 3% or 1.5% of sucrose, and optionally supplemented with double amount of $NH4^+$ ions and simultaneous depletion of an equivalent amount of nitrate ions. MS proliferation media for callus tissue induction and organogenic response were supplemented with plant growth regulators (PGRs), three cytokinines: 6-benzylaminopurine – BA (Duchefa Biochemie B. V., The Netherlands), kinetin – KIN (Sigma Aldrich, USA), and thidiazuron – TDZ (Sigma Aldrich, USA), and two auxins: 1-naphthaleneacetic acid – NAA (Sigma Aldrich, USA), and 2,4-dichlorophenoxyacetic acid – 2.4-D (Sigma Aldrich, USA).

Fragments of cotyledons (3 × 5 mm), leaves (3 × 5 mm), hypocotyls (5 mm), and roots (5 mm) were used to initiate callus cultures. A total of 720 explants (180 explants of each type) were used for the callus tissue initiation. After 42 days of culture, the percentage of explants producing callus tissue was recorded.

Cultures were maintained in growth chamber at 25 ± 2 °C under 16/8 h (light/dark) photoperiod with LED lamps 8000–10,000 K (white light), and LED lamps emitting light of the Photosyntetically Active Radiation range (PAR) at a photon flux density of 180 μM $m^{-2} s^{-1}$, and 40% of humidity. The intensity of white (8000–10,000 K), and PAR (blue: 430 nm 5%, 460 nm 10%, red: 610 nm 10%, 630 nm 35%, 660 nm 35%, 730 nm 5%) light was measured with a portable LightMeter HD 2302.0 equipped with LP 471 PAR and LP471 UVA detectors.

After 28 days of culture data were recorded on percentage of axillary bud break, length of main shoots, number of nodes per explant, number of leaves per explant, length of leaf lamina, number of roots per explant, mean length of roots, and morphogenetic response of callus cells.

2.2. Light microscopy

Pieces of the callus and roots were e fixed in a mixture of 2.5% glutaraldehyde with 2.5% formaldehyde in a 0.05 M cacodylate buffer (Sigma; pH 7.2) overnight or for 5 days, washed three times in a 0.1 M sodium cacodylate buffer and post-fixed in a 1% osmium tetroxide solution at room temperature for 1.5 h. Dehydration using a graded ethanol series, infiltration and embedding using an epoxy embedding medium kit (Fluka) followed. Following polymerisation at 60 °C, sections were cut using a Leica ultracut UCT ultramicrotome. Semi-thin sections (0.9–1.0 μm thick) were stained for general histology using aqueous methylene blue/azure II (MB/AII) for 1–2 min (Humphrey and Pittman, 1974) and examined with an Olympus BX60 light microscope (Olympus Corp., Japan).

2.3. Scanning electron microscopy

For SEM, the callus fragments with roots were fixed (as above) and later dehydrated and subjected to critical-point drying. They were then sputter-coated with gold and examined at an accelerating voltage of 20 kV or 10 KV using a Hitachi S-4700 scanning electron microscope (Hitachi, Tokyo, Japan), which is housed in the Institute of Geological Sciences, Jagiellonian University in Kraków).

2.4. Phytochemistry

2.4.1. Reagents and standards

Alkaloid standards such as protopine, allocryptopine, berberine, chelidonine, chelerythrine, and sanguinarine were purchased from Sigma (St. Louis, MO) and coptisine from ChromaDex (USA).