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# Modulation of anthraquinones and phloroglucinols biosynthesis in *Hypericum* spp. by cryogenic treatment

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ABSTRACT

Beside the high post-cryogenic recovery rate, a reinstated scale of secondary metabolites in recovered plant tissues represents another inevitable aspect of an effective cryopreservation protocol for medicinal plants. The current study was aimed at evaluation of the elicitation potential of cryogenic treatment on secondary metabolism of some *Hypericum* species. In agreement with our assumption, the cold stimuli applied during the pre-cryogenic phase increased the tolerance to low temperatures (−196 °C) in *H. perforatum, H. rumeliacum* and *H. tetrapterum* reaching a maximum of 46% recovery rate in St. John's wort plants. The effect of cryogenic treatment-associated stressors on the spectrum of the profiling secondary metabolites, naphthodianthrones and phloroglucinols, was ambiguous. The content of hypericins in both pre-cultured *H. tetrapterum* donor plants and *H. perforatum* shoots regenerated from cryopreserved meristems increased more than 3-times. The highest 38-fold enhancement of phloroglucinols was observed in *H. rumeliacum* shoots recovered after cryostorage. Our findings indicate that modulated biosynthesis of secondary metabolites represented by naphtodianthrones and phloroglucinols can be considered as a part of overall plant adaptations to stress conditions associated with liquid nitrogen (LN) treatment.

## 1. Introduction

Overwintering plants increase protection of their tissues against freezing (so-called freezing tolerance, FT) when they are gradually exposed to low non-freezing temperatures (Gusta et al., 2005; Thomashow, 1999). Under cold acclimation (CA), numerous protective mechanisms leading to morphological, physiological and biochemical modifications are activated (Lee et al., 2012; Wanner and Junttila, 1999). Besides, the exposition of plants or *in vitro* derived cultures to cold has been proved to enhance the cryo-tolerance (CT) expressed by the ability to survive at low temperatures (−196 °C) (Caswell et al., 1986; Chen et al., 1985; Sakai, 1960). Related to the fact that successful cryo-storage assumes both the sustained regeneration potential and biosynthetic capability of cryopreserved tissues, our understanding of cold signalling in the regulation of metabolic pathways is essential for an efficient long-term storage of plant tissues or cell cultures in cryogenic medium.

In plants, the translation of the cold stimulus into biological adaptations is mediated by hormones, especially abscisic acid (ABA) known as a central regulator of the abiotic interactions (Mariya et al., 2010; Wilkinson and Davies, 2010). In several perennial herbs and derived *in vitro* systems, the increased resistance to freezing injury has been shown after exogenous application of ABA at non-acclimating temperatures (Chen and Gusta, 1983; Ishikawa et al., 1990; Keith and McKersie, 1986; Orr et al., 1986). ABA participates in promoting the tolerance to a wide range of stressors associated with low temperature treatment (reviewed in Xue-Xuan et al., 2010). One of the mechanisms by which the application of ABA to plants or their parts induces cold-stress response is the increasing hormone level in the tissues (Chen et al., 1983; Lalk and Dörffing, 1985; Li et al., 2002). Subsequently, the activation of ABA-transduction pathway leads to the accumulation of signalling and transcription factors, metabolic enzymes and other components of stress reaction (Yamaguchi-Shinozaki and Shinozaki 2006; Zhu, 2016). Thus, the primary carbon metabolism is diverted towards the biosynthesis of cryo-protective molecules like soluble saccharides, saccharide alcohols, low-weight nitrogenous compounds, enzymatic and non-enzymatic scavengers of reactive oxygen species as well as various classes of specific proteins (reviewed by Rodziewicz et al., 2014).

On the other site, our current knowledge on the role of secondary metabolites in plant-environmental interactions is limited. However, the current metabolomics analyses indicate a connection of unique plant constituents to the complex regulatory network associated with stress adaptations and tolerance (Cramer et al., 2011). According to

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several findings, the abiotic factors involving drought and cold stressors suppress plant growth and result in modifications of plants transcriptome and metabolome leading to increased accumulation of secondary compounds (Herms and Mattson, 1992; Niinemets, 2015; Thapa et al., 2008). The cold signal has been shown to impact the biosynthesis of compounds belonging to major classes of secondary metabolites: the nitrogen-containing compounds, e.g. dimeric indole alkaloids in *Catharanthus roseus* L. (Asano et al., 2010); the terpenes, e.g. sesquiterpene artemisinin in *Artemisia* spp. (Wallaart et al., 2000) and phenolic derivatives, e.g. anthocyanin in *Nicotiana tabacum* L. (Huang et al., 2012), quercetin in *Arnica montana* L. cv. ARBO (Albert et al., 2009), and others.

Nowadays, *Hypericum perforatum* L. (St. John's wort) is the best studied taxon of the genus *Hypericum*. The aerial parts of plants (Herba Hyperici) are considered to be a unique source of broad spectrum of bioactive compounds. Among them, the anticancer, antidepressant, antimicrobial, and anti-inflammatory agents have been intensively studied (Arumugam et al., 2013; deMelo et al., 2013; Jendželovská et al., 2016; Wölfle et al., 2014). Despite a majority species of the world-wide *Hypericum* genus has not been phytochemically characterised yet, the list of taxa with known biochemical profiles is recently expanding. Along with the genus-specific metabolites, the photodynamic pigments naphthodianthrones (e.g. hypericin and pseudohypericin) and phloroglucinol derivatives (e.g. hyperforin and adhyperforin), flavonoids (hyperoside, quercitrin, isoquercitrin, rutin, quercetin), biflavones (amentoflavone, biapigenin), phenylpropanes (chlorogenic acid), and essential oil also contribute to the *Hypericum* phytocomplex (Hölzl and Petersen, 2003). In different *Hypericum* species and culture systems, several alterations in the spectrum and quantity of these compounds have been observed under various environmental conditions including dehydration, light intensity, high temperatures and factors associated with the cryopreservation, particularly the oxidative and cold stresses (Danova et al., 2012; de Abreu and Mazzafera, 2005; Petijová et al., 2014; Skyba et al., 2012; Sooriamuthu et al., 2013).

In the current study, the *Hypericum* representatives were selected as follows: *H. perforatum* L. (section *Hypericum*) – the cosmopolitan species and the best-studied member of the genus *Hypericum* that serves as a model plant within the genus; *H. rumeliacum* Boiss. (section *Drosocarpium*) – the endemic representative of the Balkan flora which has been known as one of the best producers of hypericins; and *H. tetrapterum* Fries. (St. Peter's Wort, section *Hypericum*) that has previously been shown to increase its biosynthetic capacity after a short period of cold treatment at low but non-freezing temperatures (Petijová et al., 2014). All of the selected *Hypericum* species have been confirmed to tolerate freezing when acclimated at 4 °C (Petijová et al., 2014) and have been recognised as the producers of the main bioactive molecules of the *Hypericum* genus − the naphthodianthrones and phloroglucinols (Suppl. Fig. S1).

The main objectives of the current study were: i) to improve the post-cryogenic survival by application of a cold acclimation period alone or in combination with high, non-physiological level of exogenously applied ABA prior to cryopreservation via direct immersion into LN; ii) to find out whether the unique biochemical profiles would be retained in the post-cryogenic regenerants; and iii) to consider the prospective elicitation effects of a cold stress on the secondary metabolic pathways leading to polyketide derivatives under *in vitro* cultivation. According to our hypothesis, the sudden decrease of temperature (a cold shock) could enhance the accumulation of specific phytochemicals in *Hypericum* species which are naturally adaptable to cold environment. Notwithstanding the elevated level of secondary metabolites is predominantly included in the biotic plant-pathogen interactions, these molecules could act as a part of the complex defence reaction in plants which are weakened by unfavourable abiotic conditions, e.g. the low-temperature.

## 2. Material and methods

### 2.1. Plant material and culture conditions

The experimental plant material includes *H. perforatum* L., *H. rumeliacum* Boiss., and *H. tetrapterum* Fries seed-derived plants multiplied on the basal solid MS medium containing Murashige-Skoog's salt mixture (Murashige and Skoog, 1962), Gamborg's B5 vitamins (Gamborg et al., 1968), 30 g/L sucrose, 100 mg/L myoinositol, 2 mg/L glycine. The media were solidified with 0.6% (w/v) agar with pH adjusted to 5.6 prior to autoclaving. Each of the plant growth vessels contained 12 plantlets growing in the 30 mL of medium. The standard culture conditions represented 22 ± 2 °C temperature, 40% relative humidity, 16/8 (day/night) photoperiod and 35 μM/$m^2$/s of PAR (photosynthetically active radiation).

### 2.2. Pre-cryogenic plant preparation

For cold and exogenous abscisic acid (ABA; Fluka Chemie, AG) treatment, the *in vitro*-derived plants on the 21st day of a subcultivation interval, were used (Suppl. Fig. S2A). The cold pre-adaptation was performed by exposure of the growth containers to: i) 0 °C for 7 days, ii) 4 °C for 7 days, or iii) gradually-decreasing temperature from 22 °C up to 4 °C at a rate of 1 °C/day. For the exogenous application of ABA, the whole plants were cautiously pulled out of the solid basal MS medium, transferred into liquid basal MS media containing 76 μM ABA and cultured at a room temperature on an orbital shaker at 90 rpm for 7 days. For a combined ABA-cold treatment, the ABA-treated plants were transferred to a solid basal MS medium and exposed to a cold treatment.

### 2.3. Cryopreservation procedure, cooling and post-cryogenic conditions

For cryopreservation, the shoot tips consisting of one apical and one pair of axillary meristematic domes with surrounding leaf primordia were isolated from cold-acclimated and/or ABA-treated *Hypericum* donor plants. Shoot apices were treated with 10 mL loading solution (LS) containing 2.0 M glycerol and 0.4 M sucrose (Nishizawa et al., 1993) at 120 rpm for 20 min at room temperature. Subsequently, shoot tips were transferred into 1.8 mL cryovials filled with 1 mL PVS3 (plant vitrification solution) cryoprotectant mixture consisting of 50% (w/v) sucrose and 50% (w/v) glycerol (Nishizawa et al., 1993). The samples were equilibrated on ice for 180 min, plunged directly into LN and stored for at least 24 h. The number of explants used per cryovial was 12. The rapid thawing of the samples in a water bath at 42 °C for 2 min was followed by 20 min washing the shoot tips in 10 mL liquid MS medium containing 1.2 M sucrose on the orbital shaker at 90 rpm at room temperature. Next, the shoot tips were gently rinsed with MS liquid medium and moderately dried on a filter paper moistened with the MS medium.

### 2.4. Post-cryogenic recovery

For regeneration, the explants were put onto a semi-solid MS medium containing 2.2 μM benzylaminopurine (BA; Lambda Life) and cultured for 14 days in the darkness at room temperature. Subsequently, the cultivation containers were exposed to a 10 μM/$m^2$/s of PAR for 7 days followed by the culturing under standard light conditions. The recovery rate (%) was defined as a ratio of the number of explants regenerating shoots during a 12-week interval after the rewarming (Suppl. Fig. S2B) to the total number of cryopreserved shoots. The clusters of regenerated shoots were put on the basal MS media and cultured for 28 days under standard culture conditions before sampling (Suppl. Fig. S2C).