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Original article

Identification and quantification of hypericin and pseudohypericin in different *Hypericum perforatum* L. in vitro cultures

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Abstract

Investigations have been made to develop an efficient protocol for micropropagation allowing to improve hypericin and pseudohypericin productions in *Hypericum perforatum* L. in vitro cultures. The role of growth regulator treatments has been particularly studied. Three in vitro culture lines with different morphological characteristics were obtained during *H. perforatum* micropropagation and referred to shoots, calli and plantlets according to their appearance. Multiplication and callogenesis from apical segments from sterile germinated seedlings were obtained on solid MS/B_5 culture medium in the presence of N^6-benzyladenine (BA) (0.1–5.0 mg/l BA). Regenerative potential of shoots was assessed on medium supplemented with auxins (0.05–1.0 mg/l), indole-3-acetic acid (IAA) or indole-3-butyric acid (IBA). The main goal of the research was to summarize the influence of plant growth regulators on hypericin and pseudohypericin productions in in vitro cultures of *Hypericum*. A rapid method for naphtodianthrone quantification was developed. The use of a reversed-phase high performance liquid chromatography (HPLC) method with fluorescence detection was used. Identification of the compounds was confirmed by electrospray ionization-mass spectrometry (ESI-MS) with electrospray in negative ion mode $[M–H]^-$. Calli, shoots and plantlets of *H. perforatum* produced hypericin and pseudohypericin. The concentration range of BA from 0.1 to 2.0 mg/l improved the production of hypericin (25–50 μg/g dry mass (DM)) and pseudohypericin (170–350 μg/g DM) in shoots. In callus cultures, BA (4.0–5.0 mg/l) did not changed hypericin contents (15–20 μg/g DM) but influenced pseudohypericin productions (120–180 μg/g DM). In the presence of auxins (IAA and IBA), *Hypericum* plantlets produced hypericin (30–100 μg/g DM) and pseudohypericin (120–400 μg/g DM). The presence of IAA did not influence naphtodianthrone productions in plantlets, but IBA decreased hypericin and pseudohypericin amounts in plantlets. The specific accumulation of the naphtodianthrones in in vitro cultures was influenced by phytohormonal supplementation of the medium. Results indicated that the production of hypericin and pseudohypericin could be increased by carefully adapted in vitro cultures. *Hypericum* in vitro cultures represent promising systems for hypericin and pseudohypericin productions.

Keywords: Calli; *Hypericum perforatum* L.; Hypericin; In vitro culture; Plantlets; Pseudohypericin; Shoots

Abbreviations: BA, N^6-benzyladenine; ESI-MS, electrospray ionization-mass spectrometry; HPLC, high performance liquid chromatography; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; $[M–H]^-$, negative molecular ion, MS–MS, collision fragment ions.

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1. Introduction

Over the past decade, medicinal plants have received considerable interest for their phytomedicinal chemical compounds. Among them, *Hypericum perforatum* L. has been considered according to its biochemical characteristics and secondary metabolite production. *Hypericum* species contain a number of biologically active detectable compounds naphtodianthrones, phloroglucinols, flavonoids, procyanidins, tannins, essential oils, amino acids, phenylpropanoids, xanthones and other water-soluble components [13]. Among them, naphtodianthrones such as hypericin and pseudohypericin are of interest. They are localized in the small black glandular structures located on flower petals, stamens, leaves and stems [15]. Hypericin and pseudohypericin are photodynamic pigments, produced from dimerized emodin anthrone, presumably via phenol oxidation further oxidized in hypericins [11]. Photodynamic hypericin activities displayed under the influence of light are used for therapy in various diseases. Photoactivate hypericin generates reactive oxygen species (ROS) and its photocytotoxycity properties have been proposed as photochemotherapeuticum [33]. These properties allow hypericin to acts as an antiviral agent. Attention has been focused on its use against human immunodeficiency virus type 1 (HIV-1) [20], and to enhance radiolytic sensitivity of tumor cells [14]. Hypericin has also antidepressant properties and its action alters the monoamine neurotransmission in the central nervous system [1]. Despite the strong interest of hypericin in pharmaceutical research, the principal active compound from *Hypericum* is pseudohypericin. It is two to three times more abundant than hypericin in wild *Hypericum* species from which it is isolated [2]. Commercial availability of pseudohypericin is limited because its pharmaceutical properties have not yet been studied in details. *Hypericum* sp. is considered to be an important source for therapeutic agents, according to hypericin content in extracts. Phytopharmaceutical preparations of *Hypericum* are usually produced from field-grown plants. Unfortunately, infestations by bacteria, fungi and insects can alter the content of commercial hypericin preparations. Seventeen-fold difference in hypericin- and 13-fold difference in pseudohypericin-levels can be found in different preparations [13]. The limited area of occurrence of this plant, seasonal harvesting, loss of biodiversity, variability in quality, and contamination issues, trigger to search alternative methods for hypericin production. In phytopharmaceutical industry, one solution could be the production of micropropagated plants, in sterile and standardized conditions. Many investigations have been conducted to establish and to enhance hypericin and pseudohypericin production [17,18,30,34]. Even if some biotic and abiotic factors can regulate hypericin and pseudohypericin production, the role of phytohormone supplementation in the culture medium need further investigations. Culture condition optimization could allow significant change in the level of bioactive metabolites in *Hypericum* species.

This study has been focused on two areas:

- what are the consequences of growth regulator concentrations on different plant material (calli, shoots and plantlets) in vitro;
- what are the relationships between plant growth regulator concentrations and naphtodianthrone (hypericin and pseudohypericin) production according to the developmental stages of tissue cultures.

To correctly adjust secondary metabolite production to phytohormonal changes, a rapid method for naphtodianthrone quantification was developed.

A reversed-phase high performance liquid chromatography (HPLC) method with fluorescence detection was used to quantify hypericin and pseudohypericin. To identify them with an optimal sensitivity, fluorescence emission and excitation spectra were performed like in reversed-phase HPLC conditions together with electrospray ionization-mass spectrometry (ESI-MS) with electrospray in negative molecular ion $[M–H]^-$ mode.

2. Results

2.1. In vitro culture of H. perforatum

2.1.1. Shoot formation

Surface sterilized *H. perforatum* seeds produced normal seedlings with almost 100% germination on MS/B_5 medium without hormones. After 2–3 weeks, in vitro grown seedlings produced 10–12 nodes (Fig. 1A). Shoot apices with two to four leaves were isolated as primary explants and cultivated on MS/B_5 medium supplemented with 0.1–5.0 mg/l BA. Apical segments were used as initial explants because of their high morphogenetic potential. At the beginning of culture (7–10 days), shoot apices grown in the presence of 0.1 and 0.5 mg/l BA, produced three to five axillary buds in the base of the first pair of leaves (Fig. 1B, arrow). After 20–30 days of culture, on medium with 0.1–2.0 mg/l BA, multiple shoots were obtained directly from apical or axillary buds (Fig. 1C). Red-pigmented dots, characteristic for hypericin, first were observed in developing shoots and corresponding to oil glands on leaf surfaces. Translucent spheroidal dots contain essential oils and dark oil glands on the margins of leaves described as a multicellular reservoir of hypericins [5] appeared during the first month of cultivation (Fig. 1D, arrow). Low level of 0.05 mg/l BA has not been effective on the multiplication of isolated explants. Treatments with high concentrations of BA (2.5–5.0 mg/l) reduced the number of shoots and promoted callus formation.

2.1.2. Callus induction

Calli developed on explant bases when cultivated on medium with 1.0, 1.5 and 2.0 mg/l BA were friable, small, green or brown colored (Fig. 1E). Callus formation was suppressed with shoots occurred during development. Shoot primordia could be observed on callus surface at the beginning