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Ultraviolet radiation for flavonoid augmentation in *Isatis tinctoria* L. hairy root cultures mediated by oxidative stress and biosynthetic gene expression

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ABSTRACT

Search of cost-effective strategies that can enhance the accumulation of phytochemicals of pharmaceutical interest in plant *in vitro* cultures is an essential task. For the first time, *Isatis tinctoria* L. hairy root cultures were exposed to ultraviolet radiation (ultraviolet-A, ultraviolet-B, and ultraviolet-C) in an attempt to promote the production of pharmacologically active flavonoids. Results showed that the maximum flavonoid accumulation (7259.12 ± 198.19 μg/g DW) in *I. tinctoria* hairy root cultures treated by 108 kJ/m^2 dose of UV-B radiation increased 16.51-fold as compared with that in control (439.68 ± 8.27 μg/g DW). Additionally, antioxidant activity enhancement and cell wall reinforcement were found in the treated *I. tinctoria* hairy root cultures, indicating the positive-feedback responses to oxidative stress mediated by ultraviolet-B radiation. Moreover, the expression of *chalcone synthase* gene was tremendously up-regulated (up to 405.84-fold) in *I. tinctoria* hairy root cultures following ultraviolet-B radiation, which suggested *chalcone synthase* gene might play a crucial role in flavonoid augmentation. Overall, the present work provides a feasible approach for the enhanced production of biologically active flavonoids in *I. tinctoria* hairy root cultures *via* the simple supplementation of ultraviolet-B radiation, which is useful for the biotechnological production of these high-added value compounds to fulfil the ever-increasing demand in pharmaceutical fields.

1. Introduction

Isatis tinctoria L. (Brassicaceae family), an economically important crop, is widely distributed in Europe and Eastern Asia (Hamburger, 2002). The dried roots of *I. tinctoria* (Radix isatidis) have received great attention for the treatment of severe acute respiratory syndrome (SARS) and novel swine-origin influenza A (H1N1) (Lin et al., 2005; Wang et al., 2011). The notable pharmacological activity of *I. tinctoria* is mainly related to phenylpropanoids present in the extracts, particularly flavonoids and lignans (Nguyen et al., 2017). Nevertheless, the phytochemical profile in the field cultivation of *I. tinctoria* is highly affected by environmental, geographic, and climatic variations, which has resulted in the inconsistent treatment efficacy of this herbal medicine (Chen et al., 2015).

Currently, plant *in vitro* culture technology has been emerged an attractive alternative to the field cultivation of medicinal crops, which allows for the sustainable and standard production of valuable phytochemicals without occupation of agricultural lands (Dias et al., 2016). Among various types of plant *in vitro* cultures, hairy root cultures belonging to differentiated organs are preferred over plant cells, callus, and suspension cultures, due to genetic/biochemical stability, high growth rate independent of phytohormone, and biosynthetic capacity comparable to the parent plant (Rimando and Duke, 2013). In this context, *I. tinctoria* hairy root cultures (ITHRCs) have been established as a reliable biological platform for the efficient production of bioactive flavonoids (Gai et al., 2015).

It is well known that plant *in vitro* cultures are grown in an aseptic space with appropriate conditions of light, humidity, and temperature that are lack of environmental stresses, thus always leading to the accumulation of defense phytochemicals in low levels (Narayani and Srivastava, 2017). Generally, flavonoids are thought to be phytoanticipins that can be inducibly synthesized by biotic and abiotic stresses, which can improve plant survival under unfavorable environmental conditions (Fini et al., 2011; Giampieri et al., 2018). Based on this principle, application of external elicitors is likely to boost flavonoid biosynthesis in ITHRCs by inducing plant defense responses. In view of the bio-safety of products, atoxic elicitors should be adopted for the improvement of flavonoid yield in ITHRCs.

Notably, ultraviolet (UV) radiation is a physical elicitor that does not introduce any taints in plant *in vitro* culture system (Schreiner et al.,

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2016). Moreover, UV radiation has been acknowledged as an effective elicitor that can boost up the accumulation of various secondary metabolites such as flavonoids, phenolics, alkaloids, carotenoids, glucosinolates, and terpenoids in fruits, vegetables, and herbs (Matsuura et al., 2013; Schreiner et al., 2012), which is beneficial for the high consumption of these health-beneficial phytochemicals in nutritional and pharmaceutical industries. In view of this, it is recommendable to use UV radiation as a "clean" elicitor for promoting flavonoid production in ITHRCs.

In the present study, the elicitation effects of UV-A, UV-B, and UV-C with successive radiation doses on flavonoid accumulation in ITHRCs were initially compared. Subsequently, the profiles of eight flavonoids before and after elicitation by the selected UV-B with the optimal radiation dose were evaluated. Afterwards, antioxidant activity, cell wall modification, and biosynthetic gene expression were systematically investigated, which aimed to understanding the response mechanism of ITHRCs underlying UV-B elicitation. It is worth mentioning that there are no reports on the application of UV radiation to elevate the production of pharmacologically active flavonoids in ITHRCs.

2. Experimental

2.1. Hairy root cultures

I. tinctoria hairy roots were obtained by *Agrobacterium rhizogenes* LBA9402 mediated transformation of petiole explants in the Key Laboratory of Forest Plant Ecology, Ministry of Education, Northeast Forestry University, and all experiments in this study were conducted using an *I. tinctoria* hairy root line V (ITHRLV) due to the highest flavonoid productivity (Gai et al., 2015). The stock culture of ITHRL V was maintained on phytohormone-free MS/2-based solid medium supplemented with 30 g/L sucrose at 25 °C in the dark. According to the previous report (Gai et al., 2015), ITHRCs were initiated by culturing 1.125 g ITHRL V (fresh weight, FW) into 250 mL Erlenmeyer flask containing 150 mL of MS/2 liquid medium supplemented with 30.6 g/L sucrose, and incubated on a rotary shaker (120 rpm) at 24.71 °C in the dark.

2.2. ITHRCs treated by UV radiation

In order to enhance flavonoid production without affecting biomass yield of hairy roots, ITHRCs cultured under the aforementioned conditions for 24 days (the optimal duration for harvest) were adopted for UV radiation treatments. Prior to elicitation experiments, a series of flasks containing ITHRCs (24 day-old) with 150 mL of fresh culture media were placed in a laminar flow cabinet, and the sterile membranes of these flasks were taken off for exposing hairy root cultures to UV radiation. The UV light lamps (UV-A: 40 W, λ_{max} = 365 nm; UV-B: 40 W, λ_{max} = 313 nm; UV-C: 40 W, λ_{max} = 254 nm; Beijing Institute of Electric Light Source, China) were mounted on fixtures at the top of hairy root cultures, and the distance of lamps from cultures was adjusted to obtain a constant radiation intensity (3 W/m^2) measured with a DRC-100X photometer (Spectronics, USA). The UV radiation dose (kJ/m^2) in this work was calculated by multiplying the fixed output value (3 W/m^2) with the exposure duration. During elicitation experiments, three groups of ITHRCs (24 day-old) were individually subjected to UV-A, UV-B, and UV-C with successive time points of 0, 1, 2, 4, 6, 7, 8, 9, 10, 11, 13, and 15 h, which were equivalent to UV radiation doses of 0, 10.8, 21.6, 43.2, 64.8, 86.4, 97.2, 108, 118.8, 140.4, and 162 kJ/m^2, respectively. For control, a group of ITHRCs (24 day-old) underwent the same conditions in the dark. After elicitation experiments, hairy roots were collected, rinsed by distilled water, and divided into three parts: one being dried in a vacuum oven for the liquid-solid extraction of flavonoids, one being handled quickly in fresh state for the evaluation of antioxidant enzyme activity, and one being frozen immediately with liquid nitrogen and stored at −80 °C for the extraction of total RNA. Also, the culture media were collected for the liquid-liquid extraction of flavonoids.

2.3. Flavonoid extraction and liquid chromatographic tandem mass spectrometry (LC–MS/MS) analysis

The dried hairy root samples were ground into fine powders using a mortar and pestle. The complete extraction of flavonoids from the resulting powders was carried out according to the method previously described (Gai et al., 2015). The flavonoids in culture media were extracted twice by phase partitioning with ethyl acetate, and the organic phase was collected and condensed to dryness using a rotary evaporator under vacuum. All extracts from roots and media were re-dissolved in acetonitrile (20 mL) and filtered through a nylon filter (0.45 μm) prior to LC–MS/MS analysis.

According to the previous report (Gai et al., 2015), the identification and quantification of eight target flavonoid derivatives was conducted by a LC–MS/MS method with the precursor–product ion combinations of m/z 609.1 → 300.0 (rutin), m/z 609.5 → 301.4 (neohesperidin), m/z 591.4 → 283.1 (buddleoside), m/z 255.9 → 119.0 (liquiritigenin), m/z 301.0 → 151.0 (quercetin), m/z 315.0 → 300.1 (isorhamnetin), m/z 285.3 → 183.1 (kaempferol), and m/z 255.4 → 118.9 (isoliquiritigenin). The content of each analyte was calculated by the corresponding calibration curve, and expressed as microgram per gram of the dry weight (DW) of root samples.

2.4. Antioxidant activity determination

Activities of four typical antioxidant enzymes *i.e.* superoxide dismutases (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR) in fresh hairy root samples were measured according to the method described by Arbona et al. (2003). Enzymatic activity was expressed as unit per mg of protein that was detected in enzyme extracts. Non-enzymatic antioxidant activities of extracts from ITHRCs were determined using DPPH radical-scavenging assay and β-carotene/linoleic acid bleaching test reported by Yao et al. (2013). Antioxidant activity of non-enzymatic sample was expressed as the IC_{50} value that was the concentration of extracts required to scavenge 50% of DPPH radicals or inhibit 50% of β-carotene bleaching ratio.

2.5. Fourier transform infrared (FTIR) analysis

Prior to FTIR analysis, the dry hairy root samples (1%) together with KBr (spectroscopic grade) were ground into fine powders using a mortar and pestle, and pressed into transparent discs. The measurement of FTIR spectra in the wavelength range of 4000–400 cm^{-1} was conducted on an Affinity-1 spectrophotometer (Shimadzu, Japan). The data were corrected *via* the elimination of KBr background, and recorded as plots of transmittance (%) versus wavelength (cm^{-1}).

2.6. Scanning electron microscopy (SEM) observation

The hairy root samples were initially processed to dryness tissues using the method described by Marsh et al. (2014). Prior to SEM observation, the dry root samples were mounted on an aluminium specimen holder with double stick tape, and evenly sputtered with a thin layer of gold. The micrograph examination of samples was conducted on a Quanta-200 environmental SEM system (FEI Company, USA), and photographed under high vacuum at an accelerating voltage of 12.5 kV.

2.7. Quantitative real-time PCR (qRT-PCR) analysis

Total RNA was extracted from frozen hairy root samples using a MiniBEST Plant RNA Extraction Kit (TaKaRa, Dalian, China), and RNA was reverse-transcribed to cDNA using a PrimeScript™ RT reagent Kit (TaKaRa, Dalian, China). Specific primers of the associated genes

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