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Establishment of high-productive *Isatis tinctoria* L. hairy root cultures: A promising approach for efficient production of bioactive alkaloids

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ABSTRACT

For the first time, *Isatis tinctoria* hairy root cultures (ITHRCs) induced by *Agrobacterium rhizogenes*, were established as alternative sources for the production of bioactive alkaloids (AK). The highest transformation rate (76.67%) was obtained when 3 week-old petiole explants were co-cultured with *A. rhizogenes* for 2 days by the aid of 125 μM acetosyringone and 1.5 mM arginine. Among eight *I. tinctoria* hairy root lines (ITHRLs), ITHRL III was screened as the lead line and was confirmed by the PCR amplification of *rol*B, *rol*C and *aux*1 genes. Culture conditions of ITHRCs were optimized by Box–Behnken design, and six main AK constituents were qualitatively and quantitatively determined by liquid chromatography–tandem mass spectrometry (LC–MS/MS). Under the culture temperature (24.7 °C), inoculum size (0.75%), sucrose concentration (3.14%) and initial pH (5.8), ITHRCs (23 day-old) in MS/2 medium gave the maximum biomass dry weight (DW) of 12.85 g/L and the optimal total AK content of 521.77 μg/g DW. Results showed that the proposed ITHRCs system possessed higher ability of AK production as compared to that of 2 year-old field-grown roots (464.69 μg/g DW). Overall, this work offered a promising, sustainable and high-productive biosynthesis platform that was capable of augmentation production of valuable naturally-derived AK.

1. Introduction

Isatis tinctoria L. (woad), the biennial herbaceous plant of Brassicaceae family, is an ancient indigo dye and medicinal plant in Europe and Asian countries [1]. Its dried roots (Radix isatidis) remain until now important herbal drugs in Traditional Chinese Medicine (TCM) for the treatment of influenza, pestilence, epidemic hepatitis and infections [2]. Phytochemical researches have illustrated that alkaloids, phenylpropanoids and terpenoids are recognized as principle active ingredients of Radix isatidis [3]. Among them, alkaloids including epigoitrin (EPI), isatin (ISA), indole-3-carboxaldehyde (INC), tryptanthrin (TRY), indigo (ING) and indirubin (INR) are validated to be responsible for doxorubicin resistance, leukocyte inhibition, anti-inflammation, anti-tumor, anti-endotoxin and antiviral activities [4–8]. Such versatile medical values mean that the interest and market demanded for AK from Radix isatidis is growing more and more.

The production of phytochemicals via field cultivation of intact plants is far from flawless and has various disadvantages (e.g., low yields, slow growth cycles, and fluctuations in quantities due to unfavorable environmental conditions, infestation and diseases). Plant tissue culture technology, an attractive alternatives system for the uniform production of phytochemicals, can continuously provide high-value medical, food and healthy ingredients, independent of geographical, climatic, and environmental variations [9,10]. Over the past years, hairy root cultures (HRCs) produced via *A. rhizogenes*-mediated transformation, have emerged as ideal biotechnological sources of valuable phytochemicals because of their genetic stability and sizable biomass production without the external application of phytohormones [11,12]. Additionally, HRCs always possess comparable biosynthetic capacity to the native plant roots, and often accumulate bioactive secondary molecules at higher levels as compared to undifferentiated cell cultures [13]. In this context, it is hypothesized that the transformation of *I. tinctoria* by *A. rhizogenes* could result in hairy root lines with the potential to

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biosynthesize AK for research or agricultural and pharmaceutical applications.

To date, this is the first work describing the establishment of *I. tinctoria* hairy root cultures (ITHRCs) and the clarification of AK production profile. In the present study, *A. rhizogenes*-mediated transformation of *I. tinctoria* was optimized systematically. Subsequently, the high-productive *I. tinctoria* hairy root line (ITHRL) was screened in terms of biomass production and AK accumulation. Afterwards, culture parameters of ITHRCs that ensured the efficient production of AK were optimized by Box–Behnken design (BBD), which might provide valuable data for industrial scale-up applications in bioreactors. Moreover, six main AK constituents including EPI, ISA, INC, TRY, ING and INR were qualitatively and quantitatively determined by LC–MS/MS. Furthermore, the superiority of ITHRCs was eventually evaluated as against *I. tinctoria* field grown roots (ITFGRs). This study opened up a new avenue for the utilization of ITHRCs as an efficient bioprocess system for the production of valuable naturally-derived AK.

2. Materials and methods

2.1. Seed sterilization and germination

The mature seeds of *I. tinctoria* were generously provided by Rongquan Medicine Plant Co. Ltd., Daqing, Heilongjiang Province, China. AK standards including EPI, ISA, INC, TRY, ING and INR were purchased from Weikeqi Biological Technology Co., Ltd. (Sichuan province, China). Other reagents either of analytical or optical grades were obtained from Beijing Chemical Reagents Co. (Beijing, China). To produce *I. tinctoria* aseptic plantlets, seeds were surface sterilized with 70% (v/v) ethanol for 45 s and 4% (v/v) sodium hypochlorite solution for 8 min, and then rinsed 5 times with sterilized water. After that, seeds were immediately germinated on Murashige and Skoog (MS)-based solid medium and incubated in a growth chamber under 16/8 h of light/dark photoperiod at $25 \pm 1\,^{\circ}C$.

2.2. Induction and establishment of hairy roots

Different types of explants from aseptic seedlings were pre-incubated on half-strength MS (MS/2)-based solid medium for 2 days prior to the following infection. Thereafter, the explants were immersed into the overnight grown bacterial suspension of *A. rhizogenes* strain LBA9402 ($OD_{600\,nm}$ = 0.6–0.8) for 6 min, dry-blotted on sterile filter paper, and incubated in the dark at $25 \pm 1\,^{\circ}C$ on MS/2-based solid medium. After a period time of co-cultivation, the explants were transferred to hormone-free MS/2-based solid medium containing sodium cefotaxim (300 mg/L) to eliminate the residual bacteria. Putative transgenic hairy roots were observed emerging from the wound sites of explants within a certain time of incubation. Subsequently, the root initials were isolated from the explants and sub-cultured on fresh hormone free MS/2-based solid medium at $25 \pm 1\,^{\circ}C$ in the dark every 2 weeks. During the subcultivation, the concentration of antibiotic was gradually decreased and finally omitted until the bacteria were completely eliminated.

2.3. Molecular characterization of hairy roots

Integration of T-DNA responsible for hairy root formation was confirmed by PCR analysis using *rol*B, *rol*C, *aux*1 and *vir*D specific primers according to previous reports [14,15]. Genomic DNA was isolated from the selected ITHRL using a DNeasy Plant Mini Kit (Tiangen China) following the manufacturer's instructions. PCR amplification of *rol*B, *rol*C, *aux*1 and *vir*D genes was performed by a S1000 thermal cycler (Bio-Rad Hercules, CA) according to the program supplemented in Table S1. Genomic DNA isolated from *I. tinctoria* aseptic plantlets and Ri-plasmid (pRi) DNA from *A. rhizogenes* LBA9402 were used as negative and positive controls, respectively. PCR products were analyzed by electrophoresis on a 2.5% (w/v) agarose–ethidium bromide gel along with 1000 bp DNA marker.

2.4. Optimization of culture conditions

After the high-productive ITHRL identified, certain amount of hairy roots was transferred into 250 mL Erlenmeyer flasks containing 100 mL of MS/2 liquid medium and incubated on a rotary shaker (120 rpm) at an appropriate temperature in the dark. ITHRCs were harvested by filtration after a certain period of cultivation and dried in a vacuum drier at 60 °C till constant weight. Thereafter, the biomass DW and total AK (TAK) content (sum amount of EPI, ISA, INC, TRY, ING and INR) were measured and determined, respectively.

In order to obtain the optimal biomass DW and TAK content during the culture process, BBD was applied to survey the effects of four independent key variables (culture temperature 20–30 °C, sucrose concentration 2–4%, inoculum size 0.4–1% and harvest time 18–30 days) on dependent variables (biomass DW and TAK content). The inoculum size was calculated based on the fresh weight of hairy roots. The regression analysis was carried out to evaluate the response function as a quadratic polynomial:

$$Y = \beta_0 + \sum_{j=1}^{k} \beta_j X_j + \sum_{j=1}^{k} \beta_{jj} X_j^2 + \sum \sum_{i<j} \beta_{ij} X_i X_j (k = 4)$$

where, Y is the predicted response; β_0, β_j, β_{jj} and β_{ij} are regression coefficients for intercept, linearity, square and interaction, respectively; X_i and X_j are independent coded variables; and k represents the number of variables. The actual and coded levels of independent variables used in the experimental design are summarized in Table 1. The experiment data were analyzed statistically with Design-Expert 7.0 (State-Ease, Inc., Minneapolis MN). Analyses of variance (ANOVA) were performed to calculate and simulate the optimal values of the tested parameters.

2.5. Extraction of AK

Powders (0.5 g DW) of hairy roots and 2 year-old field grown roots were extracted with 80% ethanol solution (20 mL) in an ultrasonic bath for 120 min. For the complete extraction, the above procedure was repeated for 3 cycles. Subsequently, the supernatant extracts were combined and condensed to dryness using a rotary evaporator under vacuum with oil pump at 45 °C. Thereafter, the resulting extracts were re-dissolved in 20 mL of acetonitrile (HPLC grade) and then filtered through a 0.45 μm membrane for LC–MS/MS analysis.

2.6. LC–MS/MS analysis of AK

An Agilent 1100 series HPLC (Agilent, San Jose, California, USA) coupled to an API 3000 triple tandem quadrupole MS (Applied Biosystems, Concord, Ontario, Canada) equipped with a Phenomenex Gemini C18 110A reversed-phase column (250 mm × 4.6 mm I.D., 5 μm) was applied for the analysis of AK. The binary mobile phase consisted of acetonitrile (A) and water (B) using the gradient program as follows: 0–8 min, 45–55% (A); 8 and 9 min, 55–85% (A); 9–14 min, 85% (A); 14 and 15 min, 85–45% (A); and 15–17 min, 45% (A). The column temperature was maintained at 30 °C, the flow rate 1.0 mL/min and the injection volume 10 μL. Mass spectra of analyses were performed in the selected reaction monitoring (SRM) transitions with an electrospray ionization source in the negative ion mode. Analytical conditions were