Chemistry of Natural Compounds, Vol. 44, No. 2, 2008

AUXIN TREATMENT IMPROVES INDIGO BIOSYNTHESIS IN HAIRY ROOT CULTURES OF *Polygonum tinctorium*

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UDC 547.913

Polygonum tinctorium Lour. is used in East Asia for large-scale production of indigo, which is both a very important natural dye and a source of indirubin, a treatment agent for chronic granulocytic leukemia [1-4]. Hairy root cultures generated by exposure of various plant species to *A. rhizogenes* have attracted considerable attention because of their genetic and biochemical stability, rapid growth rate, and ability to synthesize secondary products at levels comparable to the original plants. Thus, hairy root cultures offer a potential system for studying the biosynthesis of important secondary metabolites [5-7].

The addition of auxin, a growth-promoting plant hormone, has been shown to enhance biosynthesis of natural compounds in hairy root cultures of *Lippia dulcis* Trev and *Lobelia inflata* L. [8, 9], and Chae et al. examined the growth and indigo production in hairy root cultures of *Polygonum tinctorium* Lour. under various culture conditions [10]. However, no previous report has examined the effect of auxin on indigo biosynthesis in hairy root cultures of *Polygonum tinctorium*. In an effort to improve growth and indigo biosynthesis from hairy root cultures, we herein examined the effect of auxins on growth and indigo biosynthesis in hairy root cultures of *Polygonum tinctorium*.

Hairy root cultures of *Polygonum tinctorium* Lour. were established by infecting leaf explants with *Agrobacterium rhizogenes* A4, as previously described [10]. The hairy root cultures were grown for three weeks in Schenk and Hildebrandt (SH) liquid culture medium [11] in the presence of various concentrations (0.1, 0.5, and 1.0 mg/L) of the auxins, IAA (indole-3-acetic acid), IBA (indole-3-butyric acid), and NAA (1-naphthaleneacetic acid), and growth and indigo biosynthesis were investigated. Our results revealed that addition of the three individual auxins increased hairy root growth and indigo biosynthesis at all tested concentrations, with maximum growth and indigo yield seen in cultures treated with 0.5 mg/L IBA and 0.5 mg/L NAA (191.3 μg/g dry weight), respectively. These results show for the first time that *Polygonum tinctorium* hairy root growth and indigo biosynthesis are improved by addition of auxins to the liquid culture medium.

Establishment of Hairy Root Cultures. Excised leaves and stems of *Polygonum tinctorium* from 10 day-old seedlings were used as explants for co-cultivation with *Agrobacterium rhizogenes* A4. The excised leaves (0.7 × 0.7 cm) and stems (0.7 cm) were dipped in *Agrobacterium rhizogenes* culture in liquid inoculation medium for 15 min, blotted dry on sterile filter paper, and incubated in the dark at 25°C on Phytagar-solidified SH medium. After two days of co-cultivation, the explant tissues were transferred to hormone-free medium containing SH salts and vitamins, 30 g/L sucrose, 500 mg/L carbenicillin, and 8 g/L Phytagar. Within three to four weeks, numerous hairy roots had emerged from the wound sites. These hairy roots were separated from the explant tissue and subcultured in the dark at 25°C on Phytagar-solidified SH medium. After repeated transfer to fresh medium, rapidly growing hairy root cultures were obtained.

For experiments, isolated roots (100 mg) were transferred to 30 mL of SH liquid medium containing 30 g/L sucrose, in 100 mL flasks. Root cultures were maintained at 25°C on a gyratory shaker (100 rpm) in a growth chamber under standard cool white fluorescent tubes with a flux rate of 35 μmol/(s m^2) and a 16 h photoperiod. To identify conditions favoring hairy root growth and indigo synthesis, we tested the effects of 0.1, 0.5, and 1.0 mg/L of the auxins, indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), and 1-naphthaleneacetic acid (NAA). After 3 weeks of culture, hairy roots were harvested and the dry weight and indigo content were determined. Each experiment was carried out with three flasks per culture condition and repeated twice (Table 1).

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 0009-3130/08/4402-0272

TABLE 1. Effect of Auxins on Growth and Indigo Biosynthesis in Hairy Roots of *Polygonum tinctorium* after 3 Weeks in Culture

Auxins, mg/L	Dry weight, mg/flask	Indigo/Dry weight, μg/g
Control 0	256.2±21.7	158.8±16.3
IAA 0.1	262.5±29.1	160.3±14.5
IAA 0.5	289.8±27.3	174.5±16.6
IAA 1	309.6±28.7	179.8±15.6
IBA 0.1	292.2±28.2	169.3±16.1
IBA 0.5	361.9±37.6	183.1±17.4
IBA 1	352.4±33.1	188.7±19.3
NAA 0.1	347.8±33.9	177.9±18.7
NAA 0.5	331.7±31.7	191.3±16.2
NAA 1	293.2±27.3	183.5±22.7

Values represent the mean ± SE of nine independent measurements.

HPLC Analysis of Indigo Content. Hairy roots of *Polygonum tinctorium* were collected and fresh samples were stored frozen in sealed clear polyethylene plastic bags (168*150, Glad-Lock zipper sandwich bags, First Brands Corp., Danbury, CT) at -80°C until they were used. Collected samples were freeze dried at -80°C in brown paper bags for at least 24 h, and dried samples were ground into a fine powder (40-mesh) by mill. Samples (2 g) were extracted twice with chloroform (50 mL) for 3 hours in a water bath at 100°C. The extracts were filtered through filter paper (Whatman No. 42) and evaporated (Heidoph VV2011, 40°C). The evaporated extract was resuspended with 5 mL of 100% MeOH and prepared for HPLC analysis.

The standard chemical (95% synthetic indigo) was purchased from Sigma. Indigo was quantified by HPLC on a model LC-10A liquid chromatography (Shimadzu Co., Kyoto, Japan) equipped with a Shimadzu SPD-10A spectrophotometer operated at the wavelength of 254 nm. The separation of indigo was performed on a C18-column (Hypersil ODS, 250 × 4.6 mm) at room temperature. The initial solvent gradient consisted of 70% solvent A (water) to 30% solvent B (methanol), and the flow rate was kept constant at 1.0 mL/min. After 50 min, the solvent gradient had reached 100% B. Samples (20 μL) were detected at wavelengths of 254 nm. Identification and quantification of indigo were carried out by comparing the retention times and the peak areas respectively with those of indigo standard or by direct addition of indigo standard into the sample (spike test). Sample aliquots were filtered through a 0.45 μm poly(tetrafluoroethylene) filter prior to injection. All samples were run in triplicate.

ACKNOWLEDGMENT

This work was financially supported by the Program for Cultivating Graduate Students in Regional Strategic Industry, from the Korea Industrial Technology Foundation.

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