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Production of Baicalin, Baicalein and Wogonin in Hairy Root Culture of American Skullcap (*Scutellaria lateriflora*)by Auxin Treatment

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The hairy root culture of American Skullcap (*Scutellaria lateriflora*) was studied to investigate the biomass and flavonoids content (baicalin, baicalein and wogonin) in response of various auxin concentrations.The growth rates of the hairy roots varied significantly only at IBA 0.1 mg/L and for all other auxin treatments did not vary significantly. The biomass of hairy roots was 8% higher when treated with IBA 0.1 mg/L and biomass was almost similar and slightly lower levels when treated with various IAA concentration and NAA, respectively. However, the auxins treatments responsed positively to increase flavone production in American Skullcaphairy root culture. The auxin indole-3-butyric acid (IBA) at 1 mg/L performed the best for the accumulation of baicalin and wogonin. The auxin IBA at 1 mg/L accumulated 1.64 and 2.92 times higher baicalin and wogonin, respectively compared to control treatment. Meanwhile, the highest levels of baicalein were observed for hair root cultures in the presence of 1-naphthaleneacetic acid (NAA) at 0.1 mg/L achieving 2.38 times higher than that of accumulated in the control. These findings indicate that hairy root cultures of *S. lateriflora*using liquid 1/2MS medium supplemented with auxin could be a valuable alternative approach for flavonoid production.

Keywords: Auxins, Flavonoids, Hairy root cultures, *Scutellaria lateriflora*.

Auxins are plant hormones that are synthesized in young leaves and then transported to the basal regions of the plant, such as roots[1]. These naturally occurring hormones regulate plant growth and the morphology of roots[2,3]. The effects of auxins are manifested both at the whole-plant level, as observed in tropisms, apical dominance, and root initiation, and at the cellular level in terms of cell division, extension, and differentiation[4].

The genus *Scutellaria* L. in the family Lamiaceae comprises over 350 species, some of which are used in traditional medicine to

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treat anxiety, nervous disorders, liver disease, and cancers[5]. The main *Scutellaria* species are American skullcap (*S. lateriflora* Labiatae) and Chinese skullcap (*S. baicalensis* Georgi),which are typically grown in North America and Europe and in Asia (e.g., China, Korea and Japan), respectively[6,7]. American skullcap is a perennial herb and grows in glasslands. It is important in traditional medicine culture and is used to treat anxiety[8,9]. It has been reported that the anxiety levels of rats were affected by aqueous extracts of *S. lateriflora*[10]. In Canada, skullcap is used as a tea, with health food, and as a medicine in combination with other herbs[11]. Although several flavonoids have been studied in *S. baicalensis*, less is known about the compounds in *S. lateriflora*[12]. Mono- and diterpenes; flavonoids, including flavones such as the flavonoid glycosides baicalin, dihydrobaicalin, ikonnikoside I, and scutellarin, and the aglycones baicalein, oroxylin A, and wogonin; amino acids; and chalcones are contained in American skullcap. These compounds are the main phenolic components. Moreover, baicalin, baicalein, and wogonin are the main bioactive compounds in Radix Scutellariae[13].

Baicalin(5,6-dihydroxy-flavone 7-*O*-glucuronide), which is contained in various vegetables, fruits, and drinks derived from plants, has biological activities such as anti-inflammatory, antitumor, antioxidant, and antiviral activities[14,15]. Baicalein is the aglycone of baicalin, with which it has similar activities, and can induce apoptosis and inhibit cell proliferation in prostate cancer[16-20]. Wogonin (5,7-dihydroxy-8-methoxy flavone) is used for its bioactivities, such as that against the anti-respiratory syncytial virus, andhas properties similar to those of baicalin and baicalein[21,22].

Hairy root cultures (HRCs) are in vitro plant tissue culture systemsthat provide good models for biochemical and molecular studies[23,24]. HRCs have been used to produce beneficial proteins and secondary metabolites,and these systems have many advantages, including prevalent genetic/biochemical stability, a high level of secondary metabolite production, fast growth, the opportunity to identify new chemical compounds, and the ability to produce the same compounds as the mother plants[25-27]. HRCs can be established by infecting the host plant with *Agrobacterium rhizogenes* containing the hairy root-inducing(Ri) T-DNA plasmid, which transfers genes from the Ri plasmid into host plants[28-30].

In previous studies, treatments with different concentrations of auxin were used to enhance hairy root biomass production and the accumulation of secondary metabolites in different plant[31,32]. To date, however, there has been no documented report regarding hairy root establishment and secondary metabolite accumulation in American skullcap. Here, we report the effects of various auxins at different concentrations on hairy root production and accumulation of flavonoid compounds inAmerican skullcap.

MATERIALS AND METHODS

Plant materials

Seeds of *S. lateriflora* Labiatae were washed for 1min in 70% ethanol and then sterilized for 10min in 4% sodium hypochlorite containingTween 20. Following sterilization, the seeds are washed four to five times in distilled water. Seven seeds were placed on 20mL of growth medium in Petri dishes (100 × 15mm). The growth medium consisted of 1/2MS (Murashige & Skoog, 1962) containing 30g/L of sugar solidified with 8g/L agar. The seeds were germinated in a growth chamber at 25°C. The resulting seedlings were subsequently transferred to 50mL of 1/2MS solid medium until use.

Induction of hairy roots

A. rhizogenes strain R1000 was cultured overnight at 28°C for 16 h with shaking (200 rpm) in liquid Luria-Bertani medium (1% tryptone, 0.5% yeast extract and 1% NaCl, pH 7.0) until the mid-log phase culture had anOD_{600} of 0.5. The *A. rhizogenes* cells were collected by centrifugingfor 10min at 2500 rpm and then resuspended in liquid inoculation medium (1/2MS containing 30g/L sucrose). Finally, the density of *A. rhizogenes* cells was adjusted toA_{600}= 1.0. Excised cotyledons of *S. lateriflora* were dipped in the *A. rhizogenes*culture for 15 min, dried using sterile filter paper, and then incubated in the dark at 25°C on agar-solidified 1/2MS medium for2 days for co-cultivation with *A. rhizogenes*. After co-cultivation, the explant tissues were washed using sterile water and transferred to a hormone-free 1/2MS medium containing 250 mg/L cefortaxime. Hairy roots were observed from the

excised tissues within 4 weeks. Only isolated hairy roots were transferred in fresh 1/2MS solidified medium containing 30g/L sucrose and 8g/L agar and grown for 1month.

Treatment with different concentrations of auxin (IBA, IAA, NAA)

Hairy roots (100mg) were transferred to 30mL of 1/2MS liquid medium containing 30g/L sucrose and different concentrations (0.1, 0.5, 1.0 mg/L) of IBA (indole-3-butyric acid), IAA (indole-3-acetic acid), or NAA (1-naphthaleneacetic acid). The hairy roots were cultured at 25°C on a gyratory shaker (100 rpm) for 3 weeks, after which they were harvested and freeze-dried for measurements of dry weight and flavonoids.

HPLC analysis

Harvested hairy roots were ground using liquid nitrogen and 0.05g of freeze-dried powder was extracted with 10ml of 70% ethanol for 1h at 60°C. After centrifugation at 12,000rpm, the supernatant was passed through a0.45-μm poly filter and analyzed by HPLC. The analysis was monitored at 275nm and performed using a C_{18} column (250mm ×4.6mm, 5μm; RStech, Daejeon, Korea).

Table 1. The effects of auxins on the growth of hairy root cultures of *S. lateriflora* for 3 weeks

Auxins (mg/L)	Dry Weight (g)
Control	0.284±0.040
IAA 0.1	0.284±0.043
IAA 0.5	0.286±0.023
IAA 1.0	0.283±0.029
NAA 0.1	0.264±0.035
NAA 0.5	0.259±0.050
NAA 1.0	0.274±0.056
IBA 0.1	0.307±0.059
IBA 0.5	0.290±0.024
IBA 1.0	0.255±0.039

RESULTS AND DISCUSSION

Secondary metabolite biosynthesis in transformed roots is largely controlled genetically but can be affected by nutritional and environmental factors. We initially transformed explants of *S. lateriflora* with*A. rhizogenes* strainR1000 in order to induce hairy roots. Following induction,the hairy roots of *S. lateriflora*were transferred to liquid 1/2MS medium and allowed to grow in the presence of various concentrations (0.1, 0.5, and 1 mg/L) of different auxins (IAA, IBA, and NAA) for 3 weeks to study the effects on growth and flavone production. Our results revealed that the growth rates of the hairy roots did not vary significantly between the various auxin treatments, with the exception of IBA at 0.1 mg/L (Table 1). IBA at 0.1mg/L produced the highest biomass followed by IBA at 0.5mg/L. Compared to the control treatment, the biomass of hairy roots was 8% higher when treated with 0.1 mg/L IBA.The biomass of hairy rootstreated with different concentrations of IAA was similar to that of the control, whereas NAA treatment resulted in a biomass that was slightly lower than that of the control.

Kim *et al*.[33] reported a similar or slightly different trend in the biomass of *Scutellaria baicalensis*hairy roots,showing thatthe growth rates of hairy roots did not vary significantly between

Table 2. The effect of auxins on baicalin, baicalein and wogonin production in hairy roots of *S. lateriflora* for 3 weeks

Auxins (mg/L)	Baicalin (mg/g)	Baicalein (mg/g)	Wogonin (mg/g)
Control	8.611±0.051	0.895±0.066	2.337±0.015
IAA 0.1	11.226±0.077	1.217±0.167	2.402±0.010
IAA 0.5	11.372±0.083	1.336±0.346	2.634±0.012
IAA 1.0	10.818±0.428	1.080±0.152	2.776±0.069
NAA 0.1	8.810±0.048	2.131±0.060	3.429±0.004
NAA 0.5	9.013±0.040	1.755±0.177	3.536±0.016
NAA 1.0	9.135±0.131	1.473±0.268	3.022±0.012
IBA 0.1	7.135±0.028	1.126±0.148	3.688±0.010
IBA 0.5	9.336±0.303	1.122±0.114	2.871±0.091
IBA 1.0	14.124±0.482	1.995±0.422	6.834±0.256

auxin treatments. In a further study examining the adventitious roots of *Podophyllum hexandrum,* the authors found that, when concentrations of exogenous auxin were increased, there was a corresponding increase in biomass (fresh weight and dry weight),with highest dry weight being obtained following treatment with 3.0 mg/L IBA[34].

In the present study, the different auxin treatments had a positive effect in terms of increasing flavone production in American skullcaphairy root culture. In this respect, IBA at a concentration of 1 mg/L was most effective in enhancing baicalin and wogonin accumulation (14.124 mg/g and 6.834 mg/g, respectively) (Table 2). The second highest baicalin levels was observed in the presence of 0.5 mg/L IAA. With the exception of IBA 0.1 mg/L, no other auxin treatment showed lower baicalin levels than the control. Baicalin accumulation in hairy roots treated with 1 mg/L IBA was 1.64 times higher compared with the control treatment. The lowest amount of baicalin was accumulated in the presence of 0.5 mg/L IBA. Baicalein accumulation increased with all the auxin treatments (Table 2). The highest amount of Baicalein was accumulated in the presence of 0.1 mg/L NAA followed by 1.0 mg/L IBA. Treatment with 0.1 mg/L NAA and 1.0 mg/L IBA yielded 2.38 and 2.23 times higher baicalein levels, respectively, compared with the control treatment. The lowest amount of baicalein was obtained with an IAA concentration of 1.0 mg/L. Wogonin levels varied considerably within different auxin treatments. The levels of this flavone increased with all auxin treatments (Table 2). The highest level of wogonin was produced in the presence of 1 mg/L IBA and was 2.92 times higher relative to that of the control. Treatments with 0.1 mg/L IBA and 0.5 mg/L NAA increased the accumulation of wogonin by 1.58 and 1.51 fold, respectively, compared with the control. The lowest amount of wogonin was obtained at an IAA concentration of 0.1 mg/L.

In terms of secondary metabolite production in hairy root cultures, optimization of the medium can play an important role in the growth of roots and secondary metabolite yields. These findings are consistent with previous studies that have investigated growth and secondary metabolite biosynthesis in hairy root cultures of *Lobelia inflata*[35], *Centranthus rubber*[36], *Fagopyrum esculentum*[37], and *Withania somnifera*[38]. Auxins play important roles in plant growth and root development. The enhancement of hairy root growth and secondary metabolite production observed in the present study is similar to the results of previous studies showing that exogenous auxin treatments enhanced growth and natural compound production in hairy root cultures of *Lippia dulcis*[39], *Lobelia inflata*[40], and a *Panax* hybrid[41].

CONCLUSION

Our findings indicate that *S. lateriflora* hairy culture can be a valuable alternative approach for the production of flavones. By using a selective culture and exogenous auxin treatments, a relatively high flavone production and improved root growth can be achieved. Further investigations for the improvement of flavone production in hairy root cultures of *S. lateriflora*are in progress in our laboratory.

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