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Research Article

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Influence of cytokinins and yeast extract on growth and flavone production in hairy root cultures of *Scutellaria baicalensis*

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ABSTRACT

Hairy roots produce various bioactive chemical compounds than wild-type roots which offer a promising in vitro approach for synthesizing important nutraceutical compounds. The purpose of this study is to increase the production of flavone compounds in hairy root cultures of *Scutellaria baicalensis* by the addition of elicitors such as cytokinins and yeast extract. Cytokinins such as kinetin (KIN), 6-benzylaminopurine (BAP), and Thidiazuron (TDZ) were utilized at 0.1, 0.5, and 1.0 mg/L, whereas for yeast extract treatment 50, 100, and 150 mg/L concentrations were added to the ½ SH medium. Effects of elicitors were measured in terms of dry biomass and flavone contents (baicalin, baicalein, and wogonin) using high-performance liquid chromatography (HPLC). The highest dry weight was achieved in the control hairy root than that of all cytokinins-treated hairy root cultures. In all the cytokinin-treated hairy root cultures, as the concentration increased the dry weight of the hairy root decreased. In contrast, in all the yeast extract-treated hairy root cultures as the concentration increases the dry weight of the hairy root increased, whereas the highest dry weight was achieved in 150 mg/L of yeast extract. Moving to the flavone content, baicalin was detected highest content in all the hairy root cultures supplied with cytokinin and yeast extract. The highest total flavone content was achieved in the hairy root culture treated with 1.0 mg/L of TDZ and 50 mg/L of yeast extract. This result might help the commercial agronomic sector by facilitating the in vitro mass production of nutraceuticals using *S. baicalensis* hairy root cultures.

KEYWORDS: *Scutellaria baicalensis*, Hairy root, Cytokinins, Yeast extract, Flavone compounds

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INTRODUCTION

Scutellariae baicalensis belongs to the family Lamiaceae, and it is one of the main herbs used in traditional Chinese medicine (Zhao *et al.*, 2019; Park *et al.*, 2021; Pei *et al.*, 2022). *S. baicalensis* root has been used to treat bacterial and viral infections, lower total cholesterol, and lower blood pressure, in addition, it has been used as an anti-inflammatory and anti-cancer agent (Wang *et al.*, 2022; Chmiel & Stompor-Gorący, 2023; Huang *et al.*, 2023; Yu *et al.*, 2023). Numerous flavones, phenolics, sterols, amino acids, and essential oils are present in *S. baicalensis*. More than 30 distinct types of flavonoids were rich in the dried root of *S. baicalensis*. According to phytochemical studies, the main components of flavonoids are baicalin, baicalein, and wogonin (Yeo *et al.*, 2021; Zhao *et al.*, 2022; Wang *et al.*, 2023).

Agrobacterium rhizogenes is a gram-negative soil-borne bacterium, that can infect several plant species at the wounding site and their infections lead to the development of adventitious roots with multiple hairy roots (Mauro & Bettini, 2021; Stepanova *et al.*, 2022). Hairy roots developed through *A. rhizogenes* transformation exhibit rapid growth rates, genetic stability, and possess the ability to produce useful natural compounds at comparable levels to those of wild-type roots, these hairy root cultures are attractive for the synthesis of secondary metabolites (Malarz *et al.*, 2022; Bagal *et al.*, 2023; Biswas *et al.*, 2023). The secondary metabolites production in the hairy root cultures of various plants is influenced by several variables, such as light, temperature, medium composition, pH,

elicitors, and exogenous application of plant growth regulators (Roy, 2021; Alcalde *et al.*, 2022; Morey & Peebles, 2022).

Yeast extract is one of the important sources of various essential amino acids, vitamins, and phytohormones (Taha *et al.*, 2020; Alcalde *et al.*, 2022). Active yeast extract reduces drought stress and enhances the growth and yield of pea plants (*Pisum sativum* L.) (Xi *et al.*, 2019). In addition, they found that active yeast extract enhances the growth, yield, and chlorophyll content of several plant species such as *Beta vulgaris*, *Phaseolus vulgaris*, and *Zea mays* (Kasim *et al.*, 2017). Because yeast contains amino acids and phytohormones that can improve plant growth and chlorophyll, it can therefore play a crucial role in the tolerance of environmental stress. Furthermore, it has been documented that yeast promotes the synthesis of proteins, nucleic acids, chlorophyll, and cell division and expansion (El-Desouky *et al.*, 1998; Wanas, 2002). In addition, yeast extract is widely used as a natural inducer in the in vitro culture conditions to enhance the secondary metabolites production in plants (Karalija *et al.*, 2020; Rani *et al.*, 2020; El-Beltagi *et al.*, 2022). Baque *et al.* (2010) reported that cytokinin in the media has a significant effect on cell metabolism growth and regulation of plants. In another study, it has been reported that the treatment of *Scutellaria alpina* shoot culture with auxin and cytokinin treatment enhances the baicalin, wogonoside, and verbascoside, compounds (Grzegorczyk-Karolak *et al.*, 2015).

A previous study reported the development of *S. baicalensis* hairy root culture for the production of flavonoid compounds (Park *et al.*, 2011). Nevertheless, little research has been carried out on the effect of cytokinins and yeast extract on the synthesis of flavones in *S. baicalensis* hairy root cultures. Taken together, this study was conducted to investigate the impact of different cytokinins and yeast extract on hairy root growth of *S. baicalensis*, and their capacity to produce flavonoid (baicalin, baicalein, and wogonin) were studied.

MATERIALS AND METHODS

Establishment of Hairy Root Culture

The establishment of hairy root cultures was done according to the protocol described by Park *et al.* (2011). Every month, *Scutellaria baicalensis* hairy roots were subcultured on a new agar-solidified SH (Schenk & Hildebrandt, 1972) medium. For the further experiment, the hairy roots from the agar medium were transferred to the SH liquid culture medium. Every 10 days, the hairy root cultures were subcultured and maintained in SH liquid medium. The hairy root cultures were incubated in a growth chamber at 25 °C with shaking at 100 rpm in a standard cool white, fluorescent light, with a flux rate of 35 mol s^{-1} m^{-2} with a 16 h photoperiod.

Optimization of Cultural Conditions

For the selection of optimal concentration of cytokinins and yeast extract for enhancing the hairy root growth and flavone production, various concentrations (0.0, 0.1, 0.5, and 1.0 mg/L) of cytokinins, such as BAP (6-Benzylaminopurine), kinetin, and TDZ (Thidiazuron) and different concentrations (0, 50, 100, and 150 mg/L) of yeast extract was added to the SH liquid culture medium. After 10 days of culture, the hairy roots were harvested and freeze-dried to measure dry weight and perform further analyses. Three flasks were used for each culture condition, and the experiments were performed in duplicate.

HPLC Analysis of Flavone

Flavone concentrations (baicalein, baicalin, and wogonin) were determined in *S. baicalensis* hairy roots, as previously reported by Yeo *et al.* (2021). A hundred milligrams of dried hairy root powder were mixed in 1.5 mL of 80 % v/v aqueous methanol, and the mixture was vigorously vortexed for 30 seconds. Then the mixtures were sonicated for 1 h and were centrifuged (Hanil Science Inc., Gimpo, Korea) at 10,000 g for 15 min at 4 °C to collect the supernatant. The supernatant was filtered and sterilized into a small brown screw cap vial. Analysis of baicalein, baicalin, and wogonin was performed in HPLC (Model no. NS-6000; Futecs, Daejeon, Korea). Retention time comparison and spike tests were used to identify the metabolites of baicalein, baicalin, and wogonin. Calibration curve equations were used to quantify the metabolites.

Statistical Analysis

The results were analyzed using IBM SPPS Statistics 24 software. Tukey's Multiple Range Test with one-way ANOVA at the 5% significance level was used for the analyses. For each treatment, all experiments were done in triplicate.

RESULTS AND DISCUSSION

Effect of Cytokinin on Dry Weight of *S. baicalensis* Hairy Roots

The dry weight of hairy roots significantly changed at different cytokinin concentrations. The *S. baicalensis* hairy roots dry weight ranged from 0.36 to 0.25 g/30 mL (Figure 1). The result showed that as the concentration of cytokinin increases the dry weight of the hairy root decreases. The control showed a slightly higher dry weight content (0.36 g/30 mL) than that of the other treatments. Among the cytokinin concentrations, the highest dry weight was achieved in all 0.1 mg/L cytokinin treatments, whereas other concentrations showed a decreased pattern. At 0.1 mg/L treatment concentration, both BAP and KIN showed the highest dry weight, whereas the TDZ (0.32 g/30 mL) obtained the lowest dry weight content. In detail, in BAP-treated hairy root, the highest dry weight was achieved in 0.1 mg/L treatment (0.35 g/30 mL) followed by the 0.5 mg/L (0.32 g/30 mL) and 1 mg/L (0.29 g/30 mL) treatments. The dry weight of 0.1 mg/L BAP treatment showed the highest accumulation, which was 1.09 and 1.21 times higher than the 0.5 and 1.0 mg/L BAP treated hairy root, respectively. A similar trend was obtained in the hairy root grown at different concentrations of KIN. The TDZ-treated hairy root showed a lowered dry weight and the highest dry weight was achieved

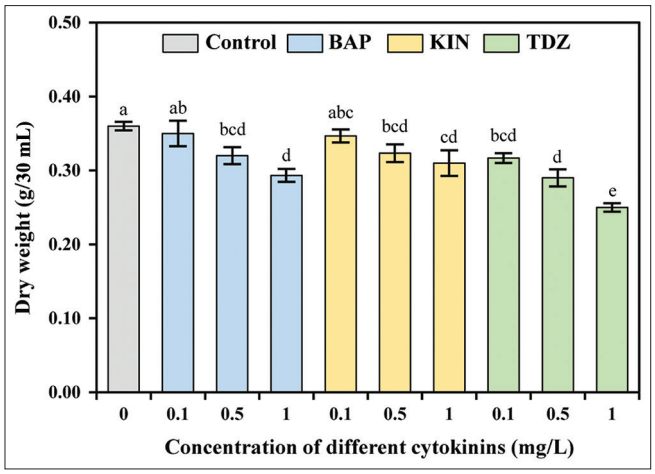

Figure 1: Effect of different cytokinins and their concentrations (mg/L) on the dry weight of *Scutellariae baicalensis* hairy root grown for 10 days on ½ SH medium supplemented with BAP, KIN, and TDZ

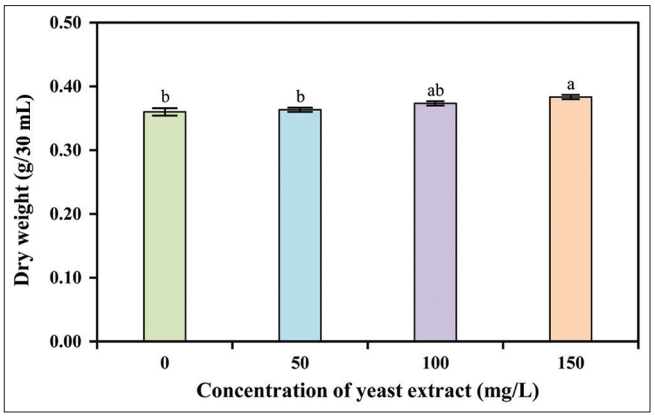

Figure 2: Effect of different concentrations of yeast extract (mg/L) on the dry weight of *Scutellariae baicalensis* hairy root grown for 10 days on ½ SH medium

at the concentration at 0.1 mg/L concentration, which was 1.10- and 1.28- times higher than the 0.5 and 1.0 mg/L TDZ-treated hairy root. From this result, it is shown that 0.1 mg/L BAP and KIN are the most important cytokinin concentrations for enhancing hairy root growth in *S. baicalensis*. Our results were in line with those of (Jeong *et al.*, 2007), who examined the impact of plant growth regulators on the development of hairy roots in *Panax ginseng*. From this result, it is shown that the addition of BAP and KIN enhanced the biomass of hairy roots.

Effect of Yeast Extract on Dry Weight of *S. baicalensis* Hairy Roots

The dry weight of hairy roots significantly changed at different yeast extract concentrations. The *S. baicalensis* hairy root's dry weight ranged from 0.38 to 0.36 g/30 mL (Figure 2). The result showed that as the concentration of yeast extract increased the dry weight of the hairy root was also significantly increased, whereas the lowest dry weight was achieved in control and 50 mg/L yeast extract treatment. In detail, the highest hairy root was found in the 150 mg/L of yeast extract treatment, which was 1.05- and 1.03- times higher than the 50 mg/L and 100 mg/L yeast extract treated hairy root. From this result, it is shown that 150 mg/L of yeast extract treatment will significantly increase the hairy root growth in *S. baicalensis*.

The dry weight of hairy roots significantly increased with increasing the yeast extract concentrations. This supports the previous study result that yeast extract increased the shoot fresh and dry weight of the *Lupinus termis* plant. This might be due to that yeast extract is one of the natural sources of cytokines that can stimulate cell division and proliferation or their impact on nutrient-rich signal transduction, generating growth factors and mitigating the harmful effects of stress conditions (Medani & Taha, 2015; Taha *et al.*, 2020). In other studies, it has been reported that yeast extract is one of the biotic elicitors that promoted the root development and biomass production in *Gentiana dinarica* and *Stevia rebaudiana* (Bayraktar *et al.*, 2016; Krstić-Milošević *et al.*, 2017). Thus in this study, the improvement in dry weight might be due to the yeast extract since it is rich in proteins, nutrients, and vitamins that have an optimistic influence on improving hairy root growth (Medani & Taha, 2015; Taha *et al.*, 2020).

Effect of Cytokinin on Flavone Contents in *S. baicalensis* Hairy Roots

Three different types of flavone compounds were identified in *S. baicalensis* hairy roots treated with different types of cytokinin (Table 1). Among the three individual flavone compounds, baicalin, baicalein, and wogonin were significantly higher in the 1.0 mg/L TDZ, control, and 0.1 mg/L KIN, respectively. Among the individual flavone content, the baicalin content was significantly high in both control and all cytokinin-treated *S. baicalensis* hairy root cultures. The highest baicalin content was achieved in *S. baicalensis* hairy root culture treated with the highest concentration of cytokinins such as 1.0 mg/L of KIN, BAP, and TDZ, which was 1.15-, 1.17-, and 1.38- times higher than that in control, respectively. In contrast, the baicalein content was lower in all the cytokinin-treated hairy roots than in the control hairy root. The wogonin content was highest in the KIN treatment, whereas the 0.1 mg/L KIN treatment showed the highest wogonin content. In contrast, in the TDZ treatment, the highest wogonin content was achieved in the 1.0 mg/L treatment. The total flavone content ranged from 100.62–129.49 mg/g dry weight in *S. baicalensis* hairy roots treated with different types of cytokinin. The 1.0 mg/L TDZ treated hairy root culture showed the highest total flavone content (129.49 mg/g dry weight), which was 1.23-, 1.06-. and 1.29- times higher than that in 0.1 mg/L TDZ, 0.5 mg/L TDZ, and control-treated hairy root culture, respectively.

The current study findings showed that variations in the types and dosage of cytokinin administered can have a significant impact on accumulation. For instance, it has been observed that cytokinins enhance the formation of anthocyanins in *Oxalis linearis* callus (Meyer & Van Staden, 1995), lignans in *Phyllanthus amarus* shoots (Nitnaware *et al.*, 2011), and polyphenolics compound (verbascoside, baicalin, wongonoside contents) in *Scutellaria alpina*, alkaloids in *Catharanthus roseus*

Table 1: Effect of different cytokinins and their concentrations (mg/L) on flavone contents (mg/g dry weight) in hairy root cultures of *Scutellariae baicalensis* grown for 10 days on ½ SH medium supplemented with BAP, KIN, and TDZ

Treatments	Baicalin	Baicalein	Wogonin	Total
Control	86.7±1.71^{d}	8.45±0.01^{a}	5.47±0.09^{ab}	100.62±1.81^{c}
KIN 0.1	99.77±3.36^{c}	8.29±0.24^{ab}	5.81±0.01^{a}	113.87±3.61^{b}
KIN 0.5	99.04±1.09^{c}	7.5±0.35^{b}	5.06±0.01^{bc}	111.6±1.45^{b}
KIN 1.0	100.43±6.85^{c}	7.5±0.14^{b}	4.93±0.04^{bcd}	112.86±7.03^{b}
BAP 0.1	100.42±4.27^{c}	7.88±0.21^{ab}	5.4±0.03^{ab}	113.7±4.51^{b}
BAP 0.5	99.86±5.62^{c}	6.2±0.71^{c}	4.37±0.4^{d}	110.43±6.73^{b}
BAP 1.0	101.11±2.44^{c}	5.91±0.68^{cd}	4.99±0.46^{bc}	112.01±3.58^{b}
TDZ 0.1	94.7±5.41^{c}	5.79±0.84^{cd}	4.71±0.64^{cd}	105.19±6.89b^{c}
TDZ 0.5	111.84±0.21^{b}	5.4±0.53^{cd}	4.87±0.44^{bcd}	122.1±1.18^{a}
TDZ 1.0	119.32±3.96^{a}	5.26±0.18^{d}	4.92±0.1^{bcd}	129.49±4.24^{a}

cell cultures (Decendit *et al.*, 1992). On the other hand, they have also been shown to suppress the development of rutin in the adventitious root and callus culture of *Morus alba* (Lee *et al.*, 2011) and secoiridoid in *Blackstonia perfoliata* shoots (Sabovljevic *et al.*, 2006). From this result, it is shown that different types of cytokinins and concentration showed different accumulation of individual flavone compounds.

Effect of Yeast Extract on Flavone Contents in *S. baicalensis* Hairy Roots

Three different types of flavone compounds were identified in *S. baicalensis* hairy roots treated with different concentrations of yeast extract (Table 2). The accumulation of baicalin, baicalein, and wogonin was identified in both control and different concentrations of yeast extract. Among the identified compounds, the accumulation of baicalin was much more than that of the other identified compounds. The highest baicalin content was found in the 50 mg/L of yeast extract, which was 1.05-, 1.08, and 1.07- times higher than that of the 100 mg/L of yeast extract, 150 mg/L of yeast extract, and control, respectively. A higher level of baicalein was found in 100 mg/L of yeast extract, followed by 150 mg/L of yeast extract, 50 mg/L of yeast extract, and control. Similar to the baicalein content the highest wogonin content was achieved in 100 mg/L of yeast extract (9.71 mg/g dry weight), which was 1.12-, 1.04-, 1.78- times higher than that in 50 mg/L of yeast extract, 150 mg/L of yeast extract, and control, respectively. The total flavone content ranged from 100.62-110.25 mg/g dry weight in *S. baicalensis* hairy roots treated with different concentrations of yeast extract. The 50 mg/L of yeast extract showed the highest content of flavone content (110.25 mg/g dry weight), which was 1.03-, 1.06-, and 1.09 times higher than that of the 100 mg/L of yeast extract, 150 mg/L of yeast extract, and control, respectively. From this result, it is shown that different concentrations of yeast extract showed different accumulations of individual flavone compounds.

From the above result, we found that the yeast extract treatment might enhance or inhibit the production of specific secondary metabolites. Chen and Chen (2000) reported that the cell culture of *Salvia miltohirzia* treated with high concentrations of yeast extract inhibits rosmarinic acid production, whereas the

Table 2: Effect of different concentrations of yeast extract (mg/L) on flavone contents (mg/g dry weight) in hairy root cultures of *Scutellariae baicalensis* grown for 10 days on ½ SH medium.

Treatments	Baicalin	Baicalein	Wogonin	Total
C ontrol	86.7±1.71^{a}	8.45±0.01^{a}	5.47±0.09^{b}	100.62±1.81^{a}
YE 50	92.65±5.59^{a}	8.96±0.76^{a}	8.64±0.65^{a}	110.25±7.0^{a}
YE 100	87.89±6.38^{a}	9.21±0.85^{a}	9.71±0.83^{a}	106.81±8.06^{a}
YE 150	85.42±4.85^{a}	9.07±0.68^{a}	9.28±0.47^{a}	103.77±6.0^{a}

lower concentrations of yeast extract increased cryptotanshinone production. In addition, some of the volatile compounds were elevated or absent after yeast extract treatment (Hassan *et al.*, 2018). In another study, it was reported that *Lupinus termis* plants treated with activated yeast extract markedly enhanced the plant growth, leaf photosynthetic pigments, total protein, total soluble sugars, and seed yields of two lupine cultivars grown under salinity conditions (Taha *et al.*, 2020). In another study, the *Polygonum minus* root culture treated with yeast extract showed that the volatile compounds were increased at the lowest concentration of yeast extract, whereas at the decreased concentrations of yeast extract the volatile compounds were increased. A similar result was obtained in this study treatment of *S. baicalensis* hairy roots with higher concentrations of yeast extract inhibit the flavone production while lower yeast concentration of yeast extract increased the production of flavone compounds. From this result, it is shown that the yield of the secondary metabolites depended on the concentration of the yeast contract and elicitation time.

CONCLUSIONS

The current study describes the impact of different elicitors affecting the biomass and secondary metabolite content in hairy root cultures of *S. baicalensis*. All concentrations of cytokinins showed a decrease pattern in the dry weight content, whereas in the yeast extract the dry weight significantly increased with increasing the concentrations than that of the control. The increase in flavone content was achieved in all concentrations of cytokinins and yeast extract in the treated *S. baicalensis* hairy root. In conclusion, cytokinin and yeast extract are some of the most suitable and effective elicitors to enhance the flavone content in *S. baicalensis* hairy root. This result might help to produce important bioactive nutraceutical substances that can be scaled up without needing a lot of time or money.

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AUTHOR'S CONTRIBUTIONS

J.K.K. and S.U.P. designed the experiments. H.K., H.-H.K., M.C., B.V.N., K.K., performed the experiments and analyzed the data. J.K.K. and S.U.P. revised the manuscript. All authors read and approved the final manuscript.

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