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Effect of heavy metal treatment on glucosinolate biosynthesis in hairy root culture of watercress (*Nasturtium officinale*)

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This study was conducted to investigate the effect of three different heavy metals, namely cobalt (Co), silver (Ag), and copper (Cu) at different concentrations on GSL biosynthesis in hairy root cultures of *Nasturtium officinale*. In this study, six different glucosinolates, namely4-hydroxyglucobrassicin, glucosiberin, glucohirsutin, glucobrassicin, 4-methoxyglucobrassicin, and gluconasturtin, were detected in the hairy root cultures of *N. officinale*. The total and individual glucosinolate levels, especially of gluconasturtiin responded greatly for the highest accumulation in response to cobalt treatment at the lowest concentration (50µMCo). Generally, for most of the cases, all treatments at low concentration selicited good responses for glucosinolate accumulations. Of the six glucosinolates, gluconasturtiin accumulation was the highest, irrespective of the heavy metal treatments. Gluconasturtiin level was 2.46, 2.02, 1.92, 1.86, and 1.82 times higher with 50-µMCotreatmentthan with the treatments with1 mMCu, 100 µMAg,0.5 mMCu, 200 µM Co, and 0.1 mMCu, respectively. Both glucosiberin and glucohirsutin accumulated at the highest levels with the10-µMAg treatment, whereas the lowest concentrations for both were observed with the1-mMCu treatment. Glucobrassicin and 4-methoxyglucobrassicinaccumulated at the highest levels with the treatments with Cu at 0.1 and 0.5 mM, respectively. Glucobrassicin concentration was 2.95, 1.95, 1.92, 1.68, 1.46, and 1.44 times higher with the 0.1-mMCu treatment than with the treatmentswith1 mMCu, 50 µMCo,100 µMCo, 50 µMAg,0.5 mMCu, and 200 µMCo, respectively. Among the glucosinolates, 4-hydroxyglucobrassicin accumulated at the highest level in the control treatment. This implies that no heavy metal treatment exerted positive effectson4-hydroxyglucobrassicinaccumulation. Therefore, treatment with heavy metal elicitors, especially cobalt at a low concentration (50µMCo), might be a valuable alternative approach to the mass production of glucosinolates in the hairy root cultures of watercress.

Keywords: Glucosinolate, hairy root induction, heavy metals, water cress.

INTRODUCTION

Nasturtium officinale L. is a hardy, perennial, aquatic plant, specifically, a herb growing in and around clear, cold water, and distributed widely in Europe, America, and Asia. It is recognized commonly as the “watercress” (Cruz et al. 2008). Generally, *N.officinale* is considered an aquatic weed in certain regions, or used in fresh salads, soups, garnishes, and other dishes in other regions (Cruz et al., 2006). According to published research findings, *N.officinale* is a valuable traditional medicinal plant. This plant is rich in vitamin C, vitamin B, pro-vitamin A, vitamin E, folic acid, carotenoids, glucosinolates, and minerals

such as Ca, Fe, I, and S (Gonçalves et al., 2009; Pourhassan-Moghaddam et al., 2014). Recently, medical studies have revealed the anti-diabetic, anti-inflammatory (Sadeghi et al., 2014), anticancer, and antioxidant activities of *N.officinale* (Gill et al., 2007).

Glucosinolates (β-thioglucoside-N-hydroxy sulfates) can be grouped into three main groups based on the modified amino acids present: aliphatic (derived from methionine, isoleucine, leucine, or valine), aromatic (derived from tyrosine or phenylalanine), and indole (derived from tryptophan) (Halkier and Gershenzon, 2006). Glucosinolate biosynthesis comprises three phases: first, side chain elongation of precursor amino acids; second, amino acid conversion; finally, secondary modification of amino acid side chains (Radojčić et al., 2014)

Elicitors have been employed to induce physiological and biochemical changes, and stimulate signal transduction for modulating the expression of defense-response genes. Secondary metabolite levels increase considerably under conditions of treatment with important biotic or abiotic elicitors through several mechanisms, and this approach has been effective in plant cell and organ cultures (Sudha and Ravi Shankar, 2002). This study was considered to evaluate the effects of abiotic elicitors, specifically heavy metals, on glucosinolate biosynthesis in hairy root cultures of *N. officinale*.

MATERIALS AND METHODS

Establishment of culture and conditions of hairy root culture of *N. officinale*

Hairy root cultures of *N.officinale* were established according to the procedure established by Thwe et al.2013. Fresh established hairy roots were transferred to half-strength liquid MS (Murashige and Skoog) medium containing 30 g/l sucrose for the experiments, and then, transferred onto a gyratory shaker (100 rpm)in a growth chamber maintained at25°C,containing cool white fluorescent lamps at a flux rate of 35 $\mu mol \cdot s^{-1} \cdot m^{-2}$with a 16-h photoperiod. The hairy roots were treated with 50, 100, and 200 µMcobalt (Co) chloride ($CoCl_2$), 10, 50, and 100µMsilver nitrate ($AgNO_3$), and 0.1, 0.5, and 1mM copper (Cu) chloride ($CuCl_2$)one week after subculture. The treated hairy root cultures described above were collected after 4days, and then, frozen in liquid nitrogen, and immediately stored at −80°C for subsequent analysis.

HPLC Analysis of Glucosinolate

The collected hairy root samples were freeze-dried, and then, ground using a mortar and pestle for HPLC analysis. A previously described procedure for glucosinolate quantification by HPLC-UV analysis was followed with slight modifications in the methods (Kim et al., 2013). Then, 100mg of the freeze-dried *N. officinale* samples were treated with 4.5ml of boiling 70% (v/v) methanol, followed by incubation at a constant temperature of 70°C in a water bath for 5min. The supernatant was collected in a 15-ml tube after centrifugation at 12,000 g and 4°C for 10 min. The extracts were introduced into a DEAE anion-exchange column,preparedin advance using Sephadex A-25(GE Healthcare, Sweden) together with 0.5 M sodium acetate (Samchun, Korea), and desulfated by adding 75 µl of arylsulfatase solution (Sigma-Aldrich, St Louis, MO, USA). After desulfation, the desolated glucosinolates were eluted into a 2-ml micro centrifuge tube containing 1.5 ml of distilled water overnight (16 h) at room temperature. For analysis by HPLC, the eluates were filtered through a 0.45-µm PTFE syringe filter. For this purpose, the external standard sinigrin was procured from Sigma-Aldrich Corporation (USA).

An Agilent 1260 Infinity HPLC system (Agilent Technologies, CA, and USA) equipped with an Inertsil ODS-3 (C18)150 × 3.0 mm column I.D. and a particle size of 3 µm (GL Science, Tokyo, Japan) for glucosinolate quantification.

A flow rate of 0.4 ml/min was used in the HPLC analysis, with the column oven temperature maintained at 40°C and wavelength of 227 nm. The solvent systems used for the analysis were (A) water and (B) 100% acetonitrile. The procedure of the gradient program was as follows: 0 min with0%solvent B; 2 min with 0%solvent B; 7 min with 10% solvent B; 16 min with 31%solvent B; 19 min with 31%solvent B; 21 min with 0% solvent B (totally, 27 min). Individual glucosinolates were evaluated using the respective external standard desulfo-sinigrin and their HPLC peak area ratios and the response factor (ISO 9167-1, 1992).

RESULTS

Three different heavy metals, namely cobalt (Co), silver (Ag), and copper (Cu), were used at different concentrations to investigate the extent of glucosinolate accumulationin the hairy roots of *N. officinale*.

Table 1. Glucosinolate (μmol/g dry wt.) content in hairy root culture of *Nasturtium officinale* under Heavy Metal Treatments

Heavy Metal Treatment	Glucosinolate (μmol/g dry wt.)						
	4-Hydroxygluco brassicin	Glucosiberin	Glucohirsutin	Glucobrassicin	4-Methoxygluco brassicin	Gluconasturtin	Total
control	0.89±0.13	1.42 ± 0.16	0.62 ± 0.08	1.03 ± 0.09	1.47 ± 0.14	26.34 ± 2.07	32.14 ± 2.61
Co 50μM	0.65 ± 0.18	1.32 ± 0.07	0.52 ± 0.04	0.62 ± 0.05	1.70 ± 0.11	34.26 ± 1.77	39.29 ± 1.82
Co 100μM	0.43 ± 0.03	1.14 ± 0.04	0.40 ± 0.02	0.63 ± 0.03	1.37 ± 0.14	22.60 ± 0.62	26.90 ± 0.82
Co 200μM	0.43 ± 0.13	1.36 ± 0.06	0.51 ± 0.06	0.87 ± 0.09	1.06 ± 0.05	18.37 ± 1.11	22.87 ± 1.36
Ag 10μM	0.60 ± 0.09	1.54 ± 0.04	1.01 ± 0.61	1.00 ± 0.05	1.78 ± 0.05	28.39 ± 1.59	34.49 ± 1.03
Ag 50μM	0.21 ± 0.06	1.39 ± 0.17	0.52 ± 0.03	0.72 ± 0.02	1.62 ± 0.14	23.58 ± 1.18	28.18 ± 1.34
Ag 100μM	0.22 ± 0.07	1.41 ± 0.28	0.48 ± 0.10	0.84 ± 0.08	1.17 ± 0.22	16.98 ± 3.60	21.25 ± 4.30
Cu 0.1mM	0.11 ± 0.07	1.36 ± 0.06	0.65 ± 0.05	1.21 ± 0.13	1.93 ± 0.17	18.87 ± 2.04	24.30 ± 2.25
Cu 0.5mM	0.07 ± 0.06	0.98 ± 0.08	0.38 ± 0.01	0.83 ± 0.01	2.84 ± 0.11	17.88 ± 0.69	23.08 ± 0.77
Cu 1mM	0.01 ± 0.01	0.66 ± 0.01	0.25 ± 0.01	0.41 ± 0.00	0.81 ± 0.03	13.92 ± 0.42	16.14 ± 0.38

Six different glucosinolates, namely4-hydroxyglucobrassicin, glucosiberin, glucohirsutin, glucobrassicin, 4-methoxyglucobrassicin, and gluconasturtiin were detected in the hairy root cultures of *N. officinale* (Table 1).

There was no distinct pattern for glucosinolate accumulation in response to different heavy metal concentrations. The total as well as individual lglucosinolate levels, particularly of gluconasturtiin, acted greatly for the highest accumulation in response to the treatment with the lowest cobalt concentration (50μMCo). In general, for most of the cases, all treatments at low concentration selicited good responses for glucosinolate accumulation. The total glucosinolate concentration range was 16.14–39.29 μmol/g dry wt for the treatments. The total glucosinolate concentration was the highest with the 50-μM Co treatment, and was 2.43, 1.85, 1.72, and 1.22 times higher than that obtained with the highest concentrations of Cu (1 mM), Ag (100 μM), Co (200μM), and control, respectively.

Gluconasturtiin accumulation was much higher than that of the other glucosinolates, irrespective of the heavy metal treatments. The gluconasturtiin concentration range was 13.92–34.26 μmol/g dry wt for the heavy metal treatments. The gluconasturtin level obtained withthe 50-μMCo treatment was 2.46, 2.02, 1.92, 1.86, 1.82, 1.52, 1.45, and 1.30 times higher than that obtained with the treatments with1 mMCu, 100 μMAg,0.5 mMCu, 200 μMCo, 0.1 mMCu, 100 μMCo, 50 μMAg, and control, respectively. Both glucosiberin and glucohirsutin accumulated at the highest levels with the 10-μMAg treatment. The 10-μMAg treatment resulted in 4.04, 2.66, 2.53, 2.10, 1.94, and 1.63 times higher glucohirsutin concentration than that with the treatmentswith1 mMCu, 0.5 mMCu,100 μMCo, 100 μMAg,200 μMCo,50 μMCo, and control, respectively. Glucosiberin accumulation was similar among the treatments, excluding a few treatments. Similarly, the 10-μM Ag treatment induced glucosiber into accumulate at the highest level. With the 10-μMAg treatment, accumulation occurredat2.33, 1.57, and 1.35times higher than that with the treatmentswith1 mMCu, 0.5 mMCu, and 100 μMCo, respectively. The copper treatment was observed to elicit good responses for the accumulation of glucobrassicin and 4-methoxyglucobrassicin. Glucobrassicin accumulated at the highest level with the treatment with0.1 mMCu. Glucobrassicin concentration was 2.95, 1.95, 1.92, and 1.68 times higher with this 0.1-mMCu treatment than with the treatmentswith1 mMCu, 50 μMCo,100 μMCo, and50 μM Ag,respectively. 4-methoxyglucobrassicin accumulated at the highest level with the 0.5-mMCu treatment. The variation was considerably high among the treatments. The 4-methoxyglucobrassicin concentration range was 0.81–2.84 μmol/g dry wt for the heavy metal treatments. The treatmentswith0.5 mMCu and10 μMAg resulted in3.51, 2.68, 2.43, 2.07, and 1.93 folds higher 4-methoxyglucobrassicin concentration than that with the treatmentswith1 mMCu, 200 μMCo,100 μMAg,100 μMCo, and control, respectively. Among the glucosinolates, 4-hydroxyglucobrassicin accumulated at the highest levels in the control treatment. This implied that none of the heavy metal treatments exerted positive effects on of 4-hydroxyglucobrassicinaccumulation. The variation in 4-hydroxyglucobrassicin accumulation among the treatments was substantially high. The 4-hydroxyglucobrassicin concentration range was 0.01–0.89 μmol/g dry wt for the heavy metal treatments, of which the highest 4-hydroxyglucobrassicin level was obtained with the control treatment,which was 89.0, 12.7, 8.1, 4.2, and 4.1 times higher than that with the treatmentswith1 mMCu, 0.5 mMCu,0.1 mMCu,50 μMAg, and100 μMAg, respectively.

As a defense mechanism against pests, especially against attack by pathogens and insects, plants naturally produce secondary metabolites often. The concentration of secondary metabolites in any part plant, of either a mother plant or a transformed plant depends largely on their source of origin; however, it can be additionally altered by phyto hormones, elicitors, or wounding, and even by environmental factors. In this study, we used three different heavy metals, namely cobalt (Co), silver (Ag), and copper (Cu), at different concentrations to enhance GSL biosynthesis in the hairy roots of *N. officinale*. Generally, hairy root growth was observed to be inhibited by the heavy metal treatment; however, the same treatments enhanced GSL biosynthesis. It is a common practice to stimulate secondary metabolite production through the use of elicitors. The application of elicitors of either biotic or abiotic origin to the cells of higher plants significantly increased the production of different secondary compounds ,namely pigments, flavones, phyto alexins, and other defense-related compounds (Flores and Curtis, 1992; Sim et al., 1994; Bhagyalakshmi and Bopanna, 1998; Singh, 1999).

A previous study reported that cellulose stimulated sorgoleone production by 6.2 times more than that by the control (Uddin et al., 2010). Previously, low IAA concentrations were reported to result in the accumulation of glucosinolates at their highest levels in hairy root cultures of broccoli (Kim et al., 2013). In a previous study, Bong et al.,(2015) reported that auxins and wounding had positive effects on the accumulation of the biologically active glucosinolates. Five different glucosinolates were detected in hairy root cultures of the Chinese cabbage, of which the concentrations of neoglucobrassicin and 4-methoxyglucobrassicin were considerably higher than that of any other glucosinolate in response to both auxins and wounding (Bong et al., 2015). Likewise, in this study, we observed positive and negative responses for GSL accumulation in relation to the heavy metal treatments. Several previous studies have revealed that different elicitors, including heavy metals, showed either positive or negative responses for the accumulation of secondary metabolites. A few of them are discussed in this article for justifying our research in relation to previous studies. The application of elicitors at the optimum concentrations enhanced both growth status and tanshinone accumulation in hairy root cultures of *Salvia castanea* (Li et al., 2016). Xing et al., 2014 reported that the concentration of phenolic acids did not vary in response to Ag^{+} treatment, whereas the accumulation of rosmarinic acid (RA), caffeic acid, and ferulic acid increased significantly for the same Ag^{+} treatment. Salicylic acid at 15 mg l^{-1} was observed to enhance the yield of azadirachtin in the hairy roots of *Azadirachta indica* (Srivastava and Srivastava, 2014). Elicitors in combination were observed to be more effective than individual elicitors in the production of specific secondary metabolites. The concentrations of cryptotanshinone and di hydrotanshinone I increased with treatments with yeast extract (YE) + Ag^{+}, Ag^{+} + methyl jasmonate (MJ), and YE + Ag^{+} + MJ (Cheng et al., 2013) .The use of ethanol, methyl jasmonate, and $Ag(^{+})$ could increase tropane alkaloid accumulation by 1.51, 1.13, and 1.08 times after treatment for 24 h, respectively, whereas salicylic acid reduced the average tropane alkaloid concentration in *Anisodus acutangulus* (Kai et al., 2012). Ions of silver (Ag^{+};15 µM), which is an abiotic elicitor, did not stimulate RA (rosmarinic acid) accumulation; however, it dramatically increased LAB (lithospermic acid B) accumulation from approximately 5.4% to 18.8% of the dry weight in hairy root cultures of*Salvia miltiorrhiza*, (Xiao et al., 2009). Overall, the secondary metabolites directly or indirectly involved in plant defense undergo significant elicitation as a response to external physical, chemical, and biological stimuli.

CONCLUSION

Currently, hairy root culture is an innovative practice for the mass production of secondary metabolites. This practice could be a valuable alternative approach to the production of health-promoting secondary metabolites, especially glucosinolatesin*N. officinale*. Heavy metals, especially cobalt at a low concentration (50µMCo), influenced glucosinolate accumulation in the hairy roots of watercress, and furthermore, the treatments with the other heavy metals at low concentrations enhanced glucosinolate accumulation. Even now, we are undertaking efforts in our laboratory to further increase the accumulation of secondary metabolites, especially of glucosinolatesin the hairy root cultures of watercress using other treatments.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

SUP designed the experiments and also wrote the manuscript. JKK, and SJB performed hairy root culture, HPLC analysis, and data analysis. All authors read and approved the final version.

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