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Glutathione Enhanced Anthraquinone Production in Adventitious Root Cultures of *Rubia tinctorum* L.

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Abstract

The adventitious root cultures were induced from leaf segments of *Rubia tinctorum* L. (madder) in MS medium supplemented with 15 μM indoleacetic acid and 0.5 μM kinetin. The effects of $CuCl_2$ and glutathione on phytochelatins (class III methallothioneins) induction and the production of anthraquinone pigments were studied. Addition of Cu ions to MS medium induced phytochelatins and affected the production of anthraquinone pigments. The addition of glutathione which did not induce phytochelatins by itself, augmented the content of phytochelatins induced by Cu ions at an early stage of culture. The addition of glutathione alone resulted in a marked increase in production of anthraquinone pigments, particularly lucidin-3-*O*-primeveroside.

1. Introduction

Many attempts have been made to produce useful secondary metabolites by plant tissue culture techniques. The roots of *Rubia tinctorum* L. (madder) produce anthraquinone (AQ) pigments [1], and are a source of natural dyes and a natural food colorant in Japan.

We established adventitious roots of *R. tinctorum* to study the relative levels of the uptake of metals between intact plant roots and *in vitro* cultures [2]. The comparative metal uptake levels of the adventitious roots and the roots of an intact plant were studied to estimate the transference of metal ions (Ca, Mg, Fe, Mn, Zn, Mo, and Cu ions) in culture medium into food additives produced by means of tissue-culture techniques [2]. Results indicated that the uptake ratio of Cu ion from the medium was the highest, though the concentration of Cu ion in the adventitious roots was the same as that in the roots of the intact plant.

Fujita *et al.* reported that Cu ions had marked effects on the production of shikonin derivatives by suspension cultures of *Lithospermum erytrorhizon* [3]. Christen *et al.* reported that Cu^{2+} remarkably enhanced both the growth and the alkaloid yield in hairy root cultures of *Hyoscyamus albus* [4]. Since a high uptake ratio of Cu ions was observed in *R. tinctorum* [2], Cu ions may also have an effect on AQ production.

We have previously reported that the adventitious roots induced thiol (SH)-containing peptides called phytochelatins which are classified as class III methallothioneins, in response to various heavy metal ions including Cu^{2+} [5-7]. The general structures of phytochelatins are $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ and those with $n=2\sim11$ have so far been described. Moreover, the desglycyl derivatives $(\gamma\text{-Glu-Cys})_n$ (hereafter the abbreviation PC is used to include normal phytochelatin and the desglycyl derivative) have also been found [5, 8]. PCs are thought to sequester heavy metals and protect plants against metal toxicity [9]. Therefore, the addition of Cu ion might induce PCs preferentially. We have studied the influence of the addition of Cu ions and glutathione (GSH), which is a precursor of PCs, on the induction of PCs and production of AQ in adventitious root cultures of *R. tinctorum*.

2. Materials and Methods

2. 1 *Authentic AQ pigments*

Munjistin and pseudopurpurin were gifts from Dr. Y. Hirose (University of Kumamoto). Lucidin, lucidin-3-*O*-primeveroside [10], and nordamnacanthal [11] were isolated from the dried roots of *R. tinctorum*. Alizarin and purpurin were purchased from Wako Pure Chemical Industries, Ltd., and ruberythric acid was purchased from Funakoshi Co., Ltd.

2. 2 *Culture of adventitious roots*

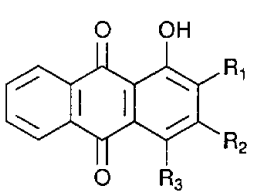
Established adventitious roots [5] were cultured in Murashige-Skoog (MS) liquid medium [12] supplemented with 5 μM indoleacetic acid, 0.5 μM kinetin, and 3% sucrose, on a rotary shaker at 100 rpm in the dark at 25°C. They were subcultured at 3 week intervals.

2. 3 Treatment of adventitious roots with $CuCl_2$ and GSH

For all experiments, the adventitious roots (0.25 g) were cultured in 10 m*l* of medium in a 50 m*l* Erlenmeyer flask for 7 days. The roots were then treated with 100 μM $CuCl_2$ and/or 2 mM GSH [13] and were cultured for 8 h and 1, 3, 5, and 7 days (n=2). The roots cultured in the flask were individually harvested and washed with distilled water. The fresh weight was determined and then the cultures were stored at −80 °C until HPLC analysis.

2. 4 Analysis of AQ pigments

The root cultures (*ca.* 0.25 g) were extracted 3 times with 1 m*l* of MeOH-HCl (10: 1) by ultrasonication for 5 min. The extract was diluted with H_2O to 5 m*l* and filtered (0.22 μm, Millipore, Bedford, MA, USA). A 20-μl portion of each sample was applied to an HPLC (Shimadzu LC-6A, Kyoto, Japan) connected to a UV-VIS detector (Shimadzu SPD-6AV) and a photodiode array detector (Waters 991J, Milford, MA, USA). The AQ pigments were analyzed as described previously [14]. The structures of authentic samples are shown in **Fig. 1**. Under our HPLC conditions, munjistin and pseudopurpurin were not well separated. The photodiode array detection spectrum of the peak at 28.9 min. in the sample suggested that most of the peak originated from pseudopurpurin. Therefore, the peak at 28.9 min. was assigned to pseudopurpurin. The pigments identified were ruberythric acid (retention time (Rt)=16.2 min.), lucidin-3-*O*-primeveroside (Rt=17.3 min.), pseudopurpurin (Rt=28.9 min.), lucidin (Rt=33.2 min.), alizarin (Rt=33.7 min.), and nordamnacanthal (Rt=61.2 min.). A peak at 46.0 min. was a degradation product of pseudopurpurin.



	R_1	R_2	R_3
Alizarin	OH	H	H
Ruberythric acid	O-Glc^6→^{1}Xyl	H	H
Lucidin	CH_2OH	OH	H
Lucidin-3-*O*-primeveroside	CH_2OH	O-Glc^6→^{1}Xyl	H
Nordamnacanthal	CHO	OH	H
Munjistin	COOH	OH	H
Pseudopurpurin	COOH	OH	OH
Purpurin	H	OH	OH

Fig. 1 Structures of the AQ pigments in *R. tinctorum*.

2. 5 Analysis of PCs

PCs were analyzed according to the method of Grill *et al.* [15] with some modifications as reported previously [5]. The induced PCs were analyzed as SH-containing peptides by postcolumn derivatization HPLC.

3. Results and Discussion

In a preliminary experiment, the effect of various concentrations (10 μM, 100 μM, and 1 mM) of $CuCl_2$ with 2 mM GSH on the growth of adventitious roots were examined after 5 days of treatment. After addition of 1 mM $CuCl_2$, the color of the roots changed to brown and growth of the roots stopped. Pigment production of adventitious roots cultured with 100 μM $CuCl_2$ was higher than that with 10 μM $CuCl_2$. Therefore, 100 μM $CuCl_2$ was used for further experiments.

Adventitious roots were cultured in MS liquid medium supplemented with or without 100 μM $CuCl_2$ and with or without 2 mM GSH for 8 h and 1, 3, 5, and 7 days. From an initial inoculum (0.25 g/10 m*l* of medium), the fresh weight increased more than 10 times in controls (without $CuCl_2$ and GSH) after 7 days treatment (**Fig. 2**). The adventitious roots cultured with 100 μM $CuCl_2$ for 7 days changed to brown and seemed dead. Both Cu ions and GSH added to MS medium suppressed the growth of the adventitious root cultures. The lowest level of growth was observed when both were added. This seems to be a simple additive effect of Cu ions and GSH.

The influence of Cu ions on PC induction was investigated in the presence and absence of GSH during a 7-day treatment (**Fig. 3**). PC_n (n=2~4) means PC with n units of γ-Glu-Cys. PC_2, PC_3, and PC_4, which were absent in the control (without Cu ion and GSH) were induced by the addition of Cu ions (**Fig. 3** left

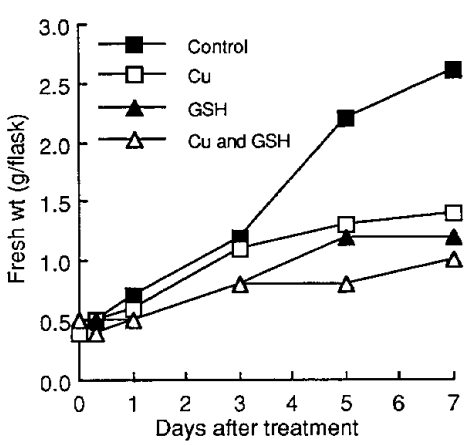


Fig. 2 Time course of growth of *R. tinctorum* adventitious roots.
Adventitious roots were treated with or without $CuCl_2$ (100 μM) and with or without GSH (2 mM). Values are the means of two observations.

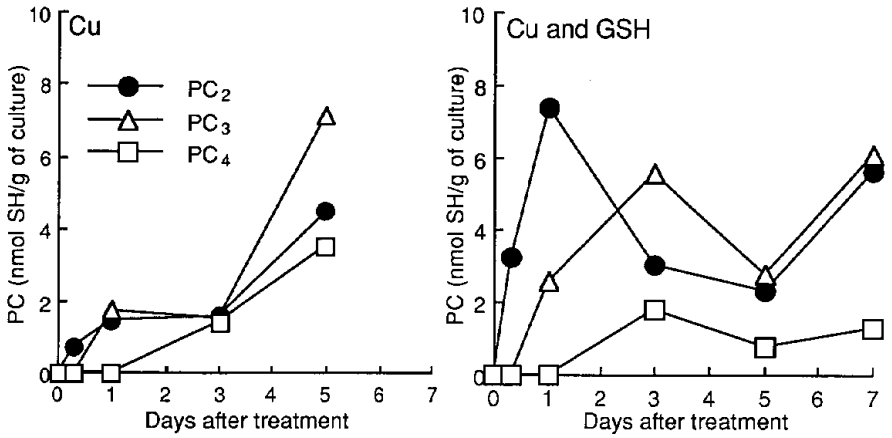


Fig. 3 Time course of the induction of PCs in root cultures.
Adventitious roots were treated with $CuCl_2$ (100 μM) and with or without GSH (2 mM). PC_n (n=2~4) indicates PC with n units of γ-Glu-Cys. Data after 7 days is not shown due to death of roots.

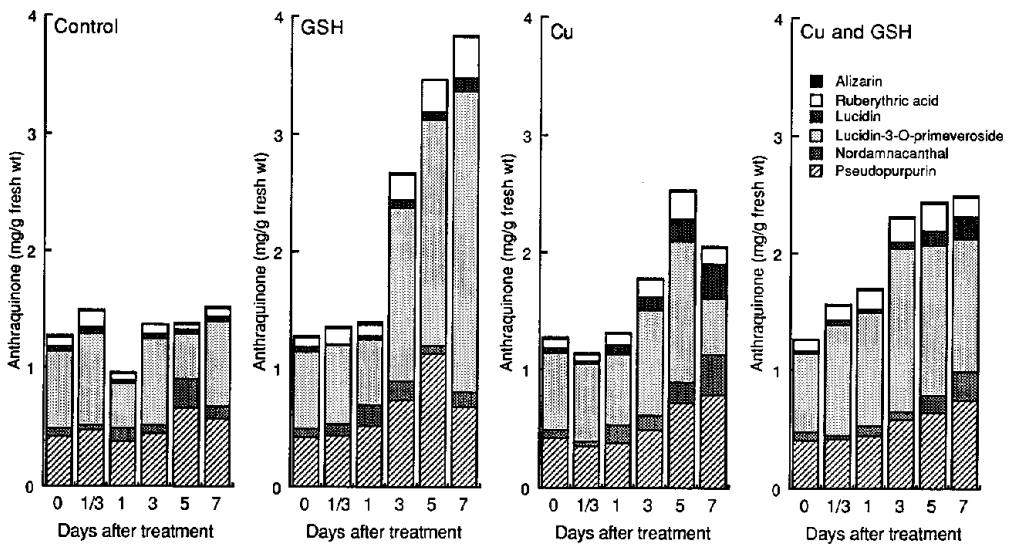


Fig. 4 Time course of the contents of AQ pigments in root cultures.
Adventitious roots were treated with or without $CuCl_2$ (100 μM) and with or without GSH (2 mM). Values are means of two samples on a fresh weight basis.

side). GSH alone did not induce PCs (data not shown), but GSH augmented the amounts of PCs induced by Cu ions at an early stage of culture (**Fig. 3** right side).

The maximal amount of PCs which were induced by the addition of Cu ions was about 20 nmol SH per whole culture (15.2 nmol SH/g of root cultures × 1.34 g at day 5 without GSH), whereas 1 μmol of Cu ion was added to the culture medium. In the Cu-PC complex, the Cu ion is bound to the SH groups of the PC molecule [16]. Since the molar amounts of SH group in the induced PCs was much less than that of the added Cu ions, it was considered that most of the additional Cu ions were not sequestered into PCs. The Cu ions may be present as Cu-GSH complexes. Therefore, it was considered that Cu ions that were not sequestered into PCs (free Cu ions or Cu-GSH complexes) could accelerate AQ-pigment production.

The production of the AQ pigments under the four conditions is shown in **Fig. 4**. The quantitative composition of the AQ spectrum was not markedly affected by the addition of Cu ions and GSH. Lucidin-3-*O*-primeveroside was detected as a major AQ pigment in most cases throughout the culture period. The production of the AQ pigments in the control was almost constant during the experimental period (the yield of AQ pigments increased with growth).

The production of AQ pigments increased in the presence of Cu ions with and without GSH (**Fig. 4**). However, the yield of AQ pigments in those cases was lower than in the control, because of the lower growth rate (**Fig. 2**). Without GSH, the content of AQ pigments decreased at day 7 and the proportion of AQ pigments was different from others, namely the relative content of lucidin-3-*O*-primeveroside was markedly decreased and those of nordamnacanthal and lucidin were increased at day 7. This may have been because the roots died. The decreased content of AQ pigments may be ascribed to the leakage of the pig-

ments from the cells.

The addition of only GSH resulted in a marked increase in AQ pigment production, particularly of lucidin-3-*O*-primeveroside (**Fig. 4**). Moreover, the addition of GSH without $CuCl_2$ yielded higher amounts of AQ pigments than the control (about 40% higher at 3 and 5 days). GSH might participate in the biosynthesis of the AQ pigments, namely in the shikimic acid-succinyl benzoic acid route [17]. However, further studies are required to clarify why the AQ pigments are increased by GSH.

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