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PRODUCTION OF ACYLATED ANTHOCYANINS BY BLUE FLOWERS OF *HYACINTHUS ORIENTALIS* REGENERATED *IN VITRO*

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Key Word Index—*Hyacinthus orientalis*; Liliaceae; acylated anthocyanin; gibberellic acid; organogenesis; pigmentation; regenerated flowers; sucrose; temperature.

Abstract—Acylated anthocyanins were produced by flowers of *Hyacinthus orientalis* that had been regenerated *in vitro*. The anthocyanin production was affected by the concentration of gibberellic acid (GA_3) and sucrose in Murashige and Skoog's (MS) medium and by temperature. The highest concentration of anthocyanin was obtained when the regenerated flowers were cultured for three weeks at 15° on MS medium containing 1 $mg l^{-1}$ GA_3 and 30 $g l^{-1}$ sucrose. The concentration of anthocyanin in the regenerated flowers was *ca* 1.2 times higher than that in field-grown flowers. The anthocyanins in the regenerated flowers were compared with those in field-grown flowers by chromatographic and spectroscopic methods, showing that these flowers produce almost the same anthocyanins with a little difference in composition.

INTRODUCTION

Many anthocyanins have been produced by cell cultures [1]. However, production of anthocyanin in flowers cultured *in vitro* has been limited to *Impatiens balsamina* [2], "Baccara" rose [3] and *Petunia hybrida* [4]. In the case of *I. balsamina*, anthocyanidins produced in *in vitro* culture were determined and shown to be different from those of intact flowers. It therefore seemed necessary to examine the anthocyanin profile of the flowers cultured *in vitro* in order to see whether they are the same as in the original plant. We now report the first establishment of a stable system for production of acylated anthocyanins in the regenerated flowers of hyacinth and demonstrate that the regenerated flowers produce essentially the same anthocyanins as field-grown flowers.

RESULTS AND DISCUSSION

Effects of the culture condition on anthocyanin production

In the regenerated flowers, anthocyanins were synthesized only at the distal ends on medium containing 2 $mg l^{-1}$ benzylaminopurine (BA). GA_3, sucrose and temperature, which were important for anthocyanin production in flowers [2–4], were investigated for the regenerated flowers. On a medium containing the optimal concentration of GA_3 (1 $mg l^{-1}$) and sucrose (30 $g l^{-1}$), the maximum concentration of anthocyanin (410 μg/fr. wt flowers) was obtained at 15° after 3 weeks of culture. GA_3 and sucrose had the stimulatory effect on the growth as well as the anthocyanin production. Higher temperature (20–35°) was stimulatory for growth. On the other hand, a low temperature (15°) had a stimulatory effect on the anthocyanin production.

The importance of GA_3, sucrose and a low temperature for anthocyanin production in flowers has also been reported in other systems using early-stage flowers, *I. balsamina* [2], "Baccara" rose [3] and *P. hybrida* [4]. The factors (GA_3, sucrose and temperature) were also important for the anthocyanin production in the regenerated flowers of hyacinth. The physiological characteristics of the regenerated flowers of hyacinth correspond to those of flowers at a relatively early stage of development *in vivo* with regard to pigmentation.

Comparison of anthocyanins in regenerated flowers and field-grown flowers

One major and six minor anthocyanins of the regenerated flowers of hyacinth were detected by HPLC analysis. The anthocyanins were identical with those of field-grown flowers in regard to retention times in HPLC (Table 1). The anthocyanins (**1**–**7**, except for **2** that could not be isolated) produced in the regenerated flowers were isolated and compared with those of field-grown flowers.

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Table 1. Analytical HPLC data for the anthocyanins in the flowers regenerated *in vitro* and field-grown flowers of *H. orientalis* cv. Delft Blue

	Regenerated flowers		Field-grown flowers	
Anthocyanin	R_t (min)	Composition (Area % at 533 nm)	R_t (min)	Composition (Area % at 533 nm)
1	5.54	3.3	5.71	5.4
2	7.07	0.2	6.81	0.2
3	12.08	3.6	12.06	2.1
4	14.56	78.9	14.61	82.9
5	19.93	4.5	19.81	5.6
6	23.77	8.2	23.96	2.6
7	33.61	1.3	33.26	1.2
Total*		410		340

*Total anthocyanin concn is in μg per g fr. wt flowers.

From the spectral data, **1** and **3**–**7** were identified as delphinidin 3-*O*-(6-*O*-*cis*-*p*-coumaroyl-β-D-glucoside)-5-*O*-(6-*O*-malonyl-β-D-glucoside) (**1**), delphinidin 3-*O*-(6-*O*-*trans*-*p*-coumaroyl-β-D-glucoside)-5-*O*-β-D-glucoside (**3**), delphinidin 3-*O*-(6-*O*-*trans*-*p*-coumaroyl-β-D-glucoside)-5-*O*-(6-*O*-malonyl-β-D-glucoside) (**4**), petunidin 3-*O*-(6-*O*-*trans*-*p*-coumaroyl-β-D-glucoside)-5-*O*-(6-*O*-malonyl-β-D-glucoside) (**5**), cyanidin 3-*O*-(6-*O*-*trans*-*p*-coumaroyl-β-D-glucoside)-5-*O*-(6-*O*-malonyl-β-D-glucoside) (**6**) and pelargonidin 3-*O*-(6-*O*-*trans*-*p*-coumaroyl-β-D-glucoside)-5-*O*-(6-*O*-malonyl-β-D-glucoside) (**7**), respectively. The six anthocyanins (**1** and **3**–**7**) isolated were the same as those found in field-grown flowers which had been reported previously [5]. Compound **2** was tentatively identified as delphinidin 3-*O*-(6-*O*-caffeoyl-β-D-glucoside)-5-*O*-(6-*O*-malonyl-β-D-glucoside) which had also been reported previously [5] to be present in field-grown flowers (Table 1). The concentration of anthocyanin that were produced in the regenerated flowers was *ca.* 1.2 times higher than that obtained from field-grown flowers. There were minor differences in the relative amounts (Table 1).

EXPERIMENTAL

Tissue culture. The regenerated flowers used in this work were initiated from tepal explants of *H. orientalis* L. cv Delft blue. The culture medium for regeneration of flowers was MS [6] agar medium containing 30 $g l^{-1}$ sucrose, 0.5 $g l^{-1}$ casamino acid, 2 $mg l^{-1}$ BA and 0.1 $mg l^{-1}$ 2,4-D. For development, the regenerated flowers were subcultured on the above medium without 2,4-D. Detailed culture conditions have been described previously [7].

Production of anthocyanin. Effects of the concn of GA_3 (0–2 $mg l^{-1}$) and sucrose (0–40 $g l^{-1}$), and of the temperature (10–35°) on the production of anthocyanin were examined using the regenerated flowers.

Measurement of growth and determination of concentration of anthocyanin. Growth was measured by determining fr. wt at inoculum and harvest. Anthocyanins were extracted from 1 g of fr. flowers collected, respectively, from field-grown and regenerated flowers with 1% HCl in methanol (5 ml, ×3) at 4°. Since the absorption of chlorophyll in acidic methanol at λ_{max} (533 nm) is about 25% of that at 657 nm [8], the concentration of anthocyanin [μ g/fr. wt of flowers (g)] in flowers was calculated as follows: $[A_{533} - 0.25 \times A_{657}]$, based on delphinidin 3-*O*-(6-*O*-*trans*-*p*-coumaroyl-β-D-glucoside)-5-*O*-(6-*O*-malonyl-β-D-glucoside) as standard.

HPLC separation of anthocyanins. Fr. flowers (1 g) was collected from field-grown flowers and from the regenerated flowers that had been cultured at 15° for 3 weeks on medium containing 3% sucrose and 1 $mg l^{-1}$ GA_3, and they were extracted with EtOH–H_2O–HOAc (10:9:1, 5 ml, ×3) at 4°. Extracts were concentrated under reduced pressure, diluted with 5% HOAc in H_2O and passed through an activated Sep-Pak tC18 cartridge to adsorb the anthocyanins. After washing of the Sep-Pak cartridge with 5% HOAc in H_2O, the anthocyanins were eluted with 1 ml of methanol containing 5% HOAc and analysed by HPLC. Anthocyanins were sepd on a Chromatorex-ODS (5μm) column by elution with a mixture of CH_3CN–HOAc–H_2O–TFA (9:11.25:79.25:0.5) at a flow rate of 1.0 ml min^{-1}. The eluate was monitored at 533 nm, which was the λ_{max} of the main anthocyanin in the eluent.

Isolation and identification of anthocyanins. From freeze-dried blue regenerated flowers (30 g), anthocyanins were extracted and isolated according to the methods reported previously [5]. Six anthocyanins (**1** and **3**–**7**) were obtained as TFA salt (**1**; 0.8 mg, **3**; 1.0 mg, **4**; 78.2 mg, **5**; 6.7 mg, **6**; 1.1 mg, **7**; 2.1 mg). UV-visible spectra were recorded in MeOH containing 0.1% HCl. FAB-MS were obtained in a positive mode with glycerol as a matrix. ^1H NMR(500 MHz) spectra were obtained using 10% TFA-*d*-methanol-d_4 as a solvent. It was confirmed that spectral data of the six isolated anthocyanins

(**1** and **3–7**) were identical with those reported previously [5].

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