Effect of Cytokinin 4PU30 on Organ Differentiation and Dihydroisocoumarin Production in Amacha Callus Cultures

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Effect of a suitable combination of 1-naphthaleneacetic acid (NAA) and cytokinin *N*-(2-chloro-4-pyridyl)-*N'*-phenylurea (4PU30) concentrations, regarding organ differentiation and dihydroisocoumarin production, was examined in *Hydrangea macrophylla* var. *thunbergii* callus cultures. The callus cultured in a Linsmaier-Skoog medium containing 10^{-5} M NAA and 10^{-5} M 4PU30 under a light condition gave the highest level for callus growth, shoot formation, and phyllostulcin 8-*O*-glucoside content.

Key words *Hydrangea macrophylla* var. *thunbergii*; callus culture; 4PU30; NAA; dihydroisocoumarin

Hydrangeae Dulcis Folium, the fermented and dried leaves of *Hydrangea macrophylla* SERINGE and *thunbergii* MAKINO (amacha), has been used as a taste-adding modifier and mouth refresher in the Japanese Pharmacopoeia XIII (ed. by Nippon-koteisho Kyokai, 1996). Its sweetening and antifungal component, dihydroisocoumarin phyllostulcin (Phy),¹⁾ is mainly formed by the enzymatic hydrolysis of its 8-*O*-glucoside (Phy-G) at the time of fermentation.²⁾ We here examined the effects of 2,4-dichlorophenoxyacetic acid (2,4-D), 1-naphthaleneacetic acid (NAA), kinetin, and the cytokinin *N*-(2-chloro-4-pyridyl)-*N'*-phenylurea (4PU30)³⁾ on organ differentiation and dihydroisocoumarin production in the amacha callus, and found that a combination of NAA and 4PU30 was most suitable for organ differentiation and Phy-G content.

MATERIALS AND METHODS

Callus Induction and Subculture Fresh leaves of amacha picked in August were sterilized with an antiformin solution and washed in sterile water. The leaves were placed in a Linsmaier-Skoog (LS) agar medium⁴⁾ containing 10^{-6} M 2,4-D, 10^{-6} M 2,4-D and 10^{-6} M kinetin, 10^{-6} M 2,4-D and 15% coconut water, 10^{-6} M 2,4-D and 10^{-6} M NAA, or 10^{-6} M NAA and 10^{-6} M 4PU30, and then incubated at $25 \pm 2^\circ\text{C}$ under a dark condition for 50 d. The induced callus was transplanted into fresh medium at 50 d intervals and subcultured in the dark for 1 year.

Effects of Combinations of NAA and 4PU30 Concentrations The callus subcultured in medium containing 10^{-6} M NAA and 10^{-6} M 4PU30 was transplanted into 50 ml of agar media containing various combinations of 10^{-7} – 2×10^{-5} M NAA and 10^{-7} – 2×10^{-5} M 4PU30 in 100 ml flasks. The cultures were grown at $25 \pm 2^\circ\text{C}$ under a light condition (white light, 4000 lux, 12 h/12 h) for 50 d, with 5 replicates for each treatment.

HPLC Analysis To prepare samples for HPLC determination, MeOH was added to the dried culture and filtered following extraction for 30 min under ultrasonication. After repeating this procedure 3 times, the filtrate was concentrated, and the residue was dissolved in MeOH. The sample was quantitatively analyzed using the standard dihydroiso-

coumarins, Phy, Phy-G, hydrangenol (Hyd), and Hyd-G together with the coumarin umbelliferone (Umb), described in the literature.²⁾ The HPLC conditions were as follows: column, YMC 120A ODS (4.6 mm $\times$ 25 cm); mobile phase, tetrahydrofuran: 2% AcOH (10:90–40:90); flow rate, 1.0 ml/min; column temp., 40°C ; detection, UV 307 nm.

RESULTS AND DISCUSSION

Among the calluses subcultured in media containing 2,4-D, NAA, kinetin, or 4PU30, the callus in medium containing a combination of NAA and 4PU30 showed the best growth and organ differentiation (data not shown). Only in this callus, were the glucoside Phy-G and the free Phy and Hyd also detected. Therefore, the effects of various combinations of NAA and 4PU30 concentrations on organ differentiation and dihydroisocoumarin production were examined using the callus subcultured in medium containing NAA and 4PU30.

As shown in Table 1, the cytokinin 4PU30 of concentrations of 10^{-7} – 10^{-5} M in combinations with 10^{-5} M and 2×10^{-5} M NAA produced a shoot-forming activity proportional to callus growth. The better combinations were found to be 10^{-5} M NAA and 10^{-6} M or 10^{-5} M 4PU30, and 2×10^{-5} M NAA and 10^{-5} M 4PU30 for both callus growth and shoot formation. A small plantlet, moreover, was seen at the combination of 10^{-5} M NAA and 10^{-5} M 4PU30, as well as at those of both regulators of lower concentrations. A combination of NAA and 4PU30 also showed a strong shoot-forming activity in *Gentiana scabra* var. *buergerii* and *Scutellaria baicalensis* GEORGI calluses (unpublished results). It was reported that 4PU30 produced a marked shoot formation and callus growth for tobacco callus.⁵⁾ Shudo's studies also showed that 4PU30 promoted characteristic shoot differentiation along with the cell division in various callus cultures.³⁾ All the amacha calluses cultured under a light condition, except for those in media containing 2×10^{-5} M NAA and/or 2×10^{-5} M 4PU30, were green in color during the long incubation period, which is probably due to the chlorophyll-maintaining activity of 4PU30.³⁾

Table 2 shows the effects of various combinations of NAA and 4PU30 concentrations on the contents of Phy-G and Umb in the amacha callus. Although Umb content was supe-

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Table 1. Effects of NAA and 4PU30 on the Callus Growth and Organ Differentiation in *Hydrangea macrophylla* var. *thunbergii* Callus Cultures

NAA (M)	0	10 ⁻⁷				10 ⁻⁶				10 ⁻⁵				2×10 ⁻⁵			
4PU30 (M)	0	10 ⁻⁷	10 ⁻⁶	10 ⁻⁵	2×10 ⁻⁵	10 ⁻⁷	10 ⁻⁶	10 ⁻⁵	2×10 ⁻⁵	10 ⁻⁷	10 ⁻⁶	10 ⁻⁵	2×10 ⁻⁵	10 ⁻⁷	10 ⁻⁶	10 ⁻⁵	2×10 ⁻⁵
Growth (dry wt., g/flask) ^{a)}	0.16	0.38	0.56	0.35	0.51	0.45	0.55	0.48	0.53	0.56	0.82	1.03	0.51	0.32	0.50	0.80	0.51
Form ^{b)} s	+	+	+++	++	+++	+	+++	++++	++++	+++	++++	+++++	+++	++	+++	++++	++
sp	—	+	+	—	—	+	+	—	—	—	+	+	—	—	—	—	—
c	+	++	+++	++	++	++	+++	++	+++	+++	++++	+++++	++	++	++	++++	+++
Color ^{c)}	G	G	G	G	IG	G	G	G	IG	G	G	G	YG	IG	dG	IYG	YG

Static culture in an LS basal medium was performed under a light condition at 25±2 °C for 50 d. a) Mean of 5 replicates. b) The symbol+ corresponds to the number and size of s (shoot), sp (small plantlet), and c (callus) formed. c) G, green; IG, light green; YG, yellowish green; dG, dark green; IYG, light yellowish green.

Table 2. Effects of NAA and 4PU30 on the Contents of Phy-G and Umb in *Hydrangea macrophylla* var. *thunbergii* Callus Cultures

Concentrations (M) of		Contents (% of dry wt.) of	
NAA	4PU30	Phy-G	Umb
0	0	0.28	2.60
10 ⁻⁷	10 ⁻⁷	1.15	2.95
10 ⁻⁷	10 ⁻⁶	1.87	3.91
10 ⁻⁷	10 ⁻⁵	2.23	4.47
10 ⁻⁷	2×10 ⁻⁵	3.37	3.78
10 ⁻⁶	10 ⁻⁷	2.06	4.33
10 ⁻⁶	10 ⁻⁶	2.31	3.94
10 ⁻⁶	10 ⁻⁵	2.48	3.59
10 ⁻⁶	2×10 ⁻⁵	2.84	3.52
10 ⁻⁵	10 ⁻⁷	1.42	3.33
10 ⁻⁵	10 ⁻⁶	2.35	3.64
10 ⁻⁵	10 ⁻⁵	5.62	3.64
10 ⁻⁵	2×10 ⁻⁵	5.32	3.19
2×10 ⁻⁵	10 ⁻⁷	1.02	1.78
2×10 ⁻⁵	10 ⁻⁶	4.00	4.56
2×10 ⁻⁵	10 ⁻⁵	5.17	2.28
2×10 ⁻⁵	2×10 ⁻⁵	0.21	1.85

Static culture in an LS basal medium was performed under a light condition at 25±2 °C for 50 d. The contents of Phy-G and Umb represent the mean of 5 replicates.

rior to Phy-G content at the combinations of both regulators of lower concentrations, the latter content was superior to the former content at the combination of 10⁻⁵ M NAA and 10⁻⁵ M or 2×10⁻⁵ M 4PU30, or 2×10⁻⁵ M NAA and 10⁻⁵ M 4PU30. At the combination with 10⁻⁵ M or 2×10⁻⁵ M NAA, the Phy-

G content increased along with the increase in 4PU30 concentration from 10⁻⁷ M to 10⁻⁵ M. As a result, the optimum combination for Phy-G production was 10⁻⁵ M NAA and 10⁻⁵ M 4PU30, and was consistent with that for callus growth and shoot formation, as previously described. It is also interesting that the combination of 2×10⁻⁵ M NAA and 10⁻⁵ M 4PU30 resulted in an increase in Phy-G content along with a decrease in the Umb content. The contents of Phy and Hyd were 0.015—0.020% and 0.027—0.26%, respectively, for all the calluses incubated. The other glucoside Hyd-G was detected only at the combination of 10⁻⁷ M NAA and 10⁻⁶ M 4PU30, and 2×10⁻⁵ M NAA and 10⁻⁵ M 4PU30. Its content was 0.34% in the former combination, and 0.44% in the latter.

In conclusion, the amacha callus cultured in an LS medium containing 10⁻⁵ M NAA and 10⁻⁵ M 4PU30 under a light condition produced the highest callus growth, shoot formation, and Phy-G content.

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