

Anthocyanin production in adventitious root cultures of *Raphanus sativus* L. cv. Peking Koushin

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Abstract The adventitious roots of *Raphanus sativus* L. cv. Peking Koushin were established by culturing root segments of *in vitro* seedlings in 1/2 Murashige and Skoog (MS) liquid medium supplemented with 0.5 mg/l IBA. The adventitious roots cultured in 1/2 MS liquid medium supplemented with 0.5 mg/l IBA produced anthocyanin in the dark. When cultured under the 14 h/day light condition, the adventitious roots cultured with 0.5 mg/l IBA produced more anthocyanin than those cultured with 0.1 or 0.5 mg/l NAA. The acid hydrolysis of the extract prepared from the adventitious roots revealed that the main anthocyanidin was pelargonidin. In addition, the DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity of the adventitious root extract was comparable to that of roots of the intact plant grown in the field.

Key words: Anthocyanin, *Raphanus sativus*.

Anthocyanin pigment is used as a natural food color in the manufacture of foods, beverages, etc. (Shimizu et al. 1996b). Anthocyanin prepared from red radish is also used for food color (Shimizu et al. 1996a). Recently, the structures of anthocyanins from red radish were elucidated and 12 acylated anthocyanins, including six new compounds, were identified (Otsuki et al. 2002). Of these, the major anthocyanidin of the identified anthocyanins in red radish was pelargonidin. In our laboratory, productivity of secondary metabolites by adventitious root cultures of various plants has been investigated. We have been interested in Peking Koushin, of which the skin is white and the flesh is red (Figure 1). In addition, small roots that are attached to the main root are red, and there are no reports of anthocyanin production by the root culture of Peking Koushin. Therefore, the adventitious root culture of this cultivar was attempted to investigate its capability of anthocyanin production. The bioactivity of anthocyanin is well known (Ohba et al. 2000; Osawa 2001), and Osawa has indicated that much attention has been focused on polyphenols, in particular, anthocyanins, which play a significant role in the prevention of oxidative stress. In addition, it has been reported that anthocyanins produced by the sweet potato, Ayamurasaki (*Ipomoea batatas* L.), which has deep purple flesh, contribute to the antioxidative activity (Konczak-Islam et al. 2000). Therefore, the DPPH radical scavenging and antioxidative activities of field-grown roots and

adventitious roots of *R. sativus* L. cv. Peking Koushin were also investigated.

Materials and methods

Establishment and maintenance of adventitious root and field cultivation

Raphanus sativus L. cv. Peking Koushin seeds were disinfected by immersing in 75% ethanol for 30 s. Then, they were washed with sterile water once, sterilized with 2% NaOCl containing 0.1% Tween 20 for 10 min, and washed with sterile water three times. The seeds thus sterilized were placed on agar medium (0.5% agar and 0.5% sucrose), and cultured under the 14 h/day light condition at 25°C. The roots (3 cm in length including the root tip) that were excised from plantlets germinated on agar medium were inoculated into the half strength of macro elements (1/2) Murashige & Skoog (MS) (Murashige and Skoog 1962) liquid medium (50 ml/100 ml Erlenmeyer flask) supplemented with 1-naphthaleneacetic acid (NAA) or indole-3-butyric acid (IBA) and the culture was conducted at 25°C under the 14 h/day light condition or in the dark on a rotary shaker at 100 rpm. The adventitious roots were subcultured at four-week intervals. The 1/2 MS liquid medium containing various concentrations (0.1, 0.5 mg/l) of auxin (IBA, NAA) was adjusted to pH 5.7 and autoclaved at 121°C for 15 min.

The seeds were sowed in early autumn and cultivated

Abbreviations: DPPH, 1,1-diphenyl-2-picrylhydrazyl; HPLC, high performance liquid chromatography; IBA, indole-3-butyric acid; MS, Murashige and Skoog; NAA, 1-naphthaleneacetic acid; TBA, 2-thiobarbituric acid; TFA, trifluoroacetic acid.

for approximately three months in Tsukuba Medicinal Plant Research Station, NIHS, Japan.

Analysis of pigments

Extraction of anthocyanin

The adventitious roots grown under various culture conditions were harvested and their fresh weights were measured individually, and then the roots were lyophilized. The samples (50–100 mg) thus prepared were extracted with 1 ml of 80% methanol containing 1% trifluoroacetic acid (TFA) by ultrasonication for 20 min and the extract was filtered through a cotton plug. The same extraction was repeated and the volume of the pooled extract was made up to 2 ml.

Acid hydrolysis of anthocyanin

Red pigments of various samples (50–100 mg dry weight) were extracted with 2 ml of 80% methanol containing 1% TFA by ultrasonication for 20 min. The methanolic extract of each sample was filtered through filter paper (Advantec No. 2, Japan). Each extract was concentrated *in vacuo* and 3 ml of 2 M HCl was added. After heating at 100°C for 15 min, the reaction mixture was cooled in running water. Isoamyl alcohol (3 ml) was added to the acid hydrolysate and the mixture was mixed well. The isoamyl alcohol fraction was collected and concentrated by a nitrogen gas stream.

HPLC analysis

The retention times of the main anthocyanidins in the adventitious and intact root samples analyzed by HPLC (Multi pump CCPM, TOSOH) were compared with those of anthocyanidin standards (pelargonidin chloride, cyanidin chloride, peonidin chloride, and delphinidin chloride). The HPLC conditions were as follows: column, TOSOH TSKgel ODS-80Ts (4.6×150 mm); mobile phase, 0.4% TFA in H₂O–CH₃CN (9:1→7:1 in 25 min); flow rate, 0.8 ml/min; column temperature, 40°C, and detection wavelength, 520 nm (photodiode array).

Quantitative analysis of anthocyanin

Approximately 20 mg of dried sample was individually extracted with 2 ml of 80% methanol containing 1% TFA by ultrasonication for 20 min. The extract was filtered through a cotton plug and its volume was made up to 2 ml. The absorbance at 521 nm was measured (Beckman DU-500) and anthocyanin content was calculated as pelargonidin equivalent.

DPPH radical scavenging activity

Radical scavenging activity was measured by the slightly modified method using 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Tanaka et al. 1999). Ten mg extract prepared from fresh or dried root was dissolved in 1 ml of 80% methanol containing 1% TFA, and 100 μl of the prepared solution was used. Five hundred μl of 0.5 mM DPPH ethanol solution and 400 μl of 80%

methanol containing 1% TFA were added to each sample solution. After shaking gently, the reaction mixture was allowed to stand for 20 min at room temperature in the dark, and then the absorbance of the solution was measured at 517 nm, and percentage of radical scavenging activity was calculated by the following formula.

$$\text{DPPH radical scavenging activity (\%)} = 100 - (\text{absorbance of reaction sample}) / (\text{absorbance of control sample}) \times 100$$

Antioxidative activity measured by 2-thiobarbituric acid (TBA) method

Antioxidative activity was measured by the slightly modified TBA method using linoleic acid (Tamura and Takenaka 1999). A 20 mg extract prepared from fresh or dried root was dissolved in 200 μl of 80% methanol containing 1% TFA. To this solution, 1.64 ml of substrate solution [200 mg of linoleic acid and 200 mg of sodium dodecylsulfate (SDS) were dissolved in 100 ml of 30 mM tris-hydrochloric acid buffer (pH 7.4)], 2.5 mM H₂O₂ solution (80 μl) and 2.5 mM FeCl₂ solution (80 μl), were added and the reaction mixture was kept for 12 h at 37°C in the dark. A 200 μl of the reaction mixture was collected, the mixed solution (650 μl consisted of 200 μl of 5.2% SDS solution, 50 μl of 0.8% 2,6-di-*t*-butyl-4-methylphenol (BHT) glacial acetic acid solution, 1.5 ml of 0.8% TBA solution and 1.7 ml of distilled water) and 20% acetate buffer (150 μl) were added, and reacted for 1 h at 100°C in the dark, and then cooled in water. The absorbance of the solution was measured at 532 nm, and percentage of antioxidative activity was calculated by the following formula.

$$\text{Antioxidative activity (\%)} = 100 - (\text{absorbance of reaction sample}) / (\text{absorbance of control sample}) \times 100$$

Results and discussion

Establishment and growth of adventitious root culture

The roots of seedlings germinated aseptically on agar medium showed red coloration. The roots (3 cm in length including the root tip) excised from plantlets were cultured in 1/2 MS liquid medium supplemented with 0.5 mg/l NAA at 25°C under the 14 h/day light condition on a rotary shaker at 100 rpm. After four to six weeks of culture, adventitious roots grew in the liquid medium and anthocyanin formation was observed. Pigment production of adventitious roots grown in the dark was lower than that of the branched roots of intact plants cultivated in the field. In order to improve the growth of the adventitious roots in a culture system, the effects of auxin and illumination were examined. Adventitious



Figure 1. Whole view and cross section of field-grown root. Radish plants were cultivated for three months in Tsukuba Medicinal Plant Research Station, NIHS, Japan. Bars: 5 cm



Figure 2. Adventitious roots cultured in a 100 ml Erlenmeyer flask (1/2 MS liquid medium with 0.5 mg/l IBA) for four weeks at 25°C under the 14 h/day light condition.

roots were inoculated into 1/2 MS liquid medium supplemented with NAA (0.1, 0.5 mg/l) or IBA (0.1, 0.5 mg/l) at 25°C under the 14 h/day light condition or in the dark on a rotary shaker at 100 rpm, and subcultured at four-week intervals. The growth of the adventitious roots was not affected by both the light/dark condition (data not shown). In contrast, when the adventitious roots (*ca.* 45 mg of inoculum fresh weight/100 ml flask) were cultured in 1/2 MS liquid medium supplemented with 0.5 mg/l IBA under the light condition for four weeks, pigment formation was markedly improved (Table 1, Figure 2). It has been reported that in some plant species such as *Amsonia elliptica*, *Lippia dulcis* and *Digitalis lanata*, the hairy roots of these plants turned green and grew faster when cultured under the light condition (Toda et al. 2002). In our radish culture, the growth of adventitious roots did not change and the roots did not turn green under the light condition, whereas the anthocyanin formation was stimulated. It is thought that the pigment formation was regulated by the light. Taking this phenomenon into account, the adventitious root culture may be a good alternative material for investigating the anthocyanin biosynthesis induced and regulated by the light.

Analysis of anthocyanidin by HPLC

The red pigment extracts prepared from the adventitious (five weeks of culture) and field-grown roots (*ca.* three months of cultivation) were hydrolyzed with 2 M HCl, and the hydrolysates were analyzed by HPLC and compared with four anthocyanidin standards: pelargonidin chloride, cyanidin chloride, peonidin chloride and delphinidin chloride. Otsuki et al. reported the structures of acylated anthocyanins isolated from red

Table 1. Effect of auxin on anthocyanin productivity of adventitious roots.

| | Red pigment (Anthocyanin) | |
|--------------|---------------------------|------------|
| | Dark | 14 h/Light |
| 0.1 mg/l NAA | ± | + |
| 0.5 mg/l NAA | ± | ++ |
| 0.1 mg/l IBA | ± | ++ |
| 0.5 mg/l IBA | ± | +++ |

± : very low anthocyanin productivity

+ : low anthocyanin productivity

++ : intermediate anthocyanin productivity

+++ : high anthocyanin productivity

Adventitious roots were cultured in 1/2 MS liquid medium supplemented with NAA (0.1 or 0.5 mg/l) or IBA (0.1 or 0.5 mg/l) for four weeks at 25°C under the 14 h/day light condition or in the dark.

radish were determined by spectroscopic analyses, and the major anthocyanins of red radish were acylated (caffeoyl, *p*-coumaroyl and feruloyl) pelargonidin with sugar moieties (Otsuki et al. 2002). HPLC analysis demonstrated that the main anthocyanidin of the adventitious roots and the field-grown roots of Peking Koushin was pelargonidin (Figure 3). The anthocyanin content of the lyophilized adventitious roots (0.15% dry weight) was slightly higher than that of the field-grown roots (0.11% dry weight), although the difference was not significant at 5% level of F test (Figure 5).

Time course of growth and anthocyanin content in adventitious root culture

The time courses of growth and the anthocyanin contents in adventitious root cultures were investigated. The adventitious roots (*ca.* 45 mg fresh weight) were cultured in 1/2 MS liquid medium supplemented with

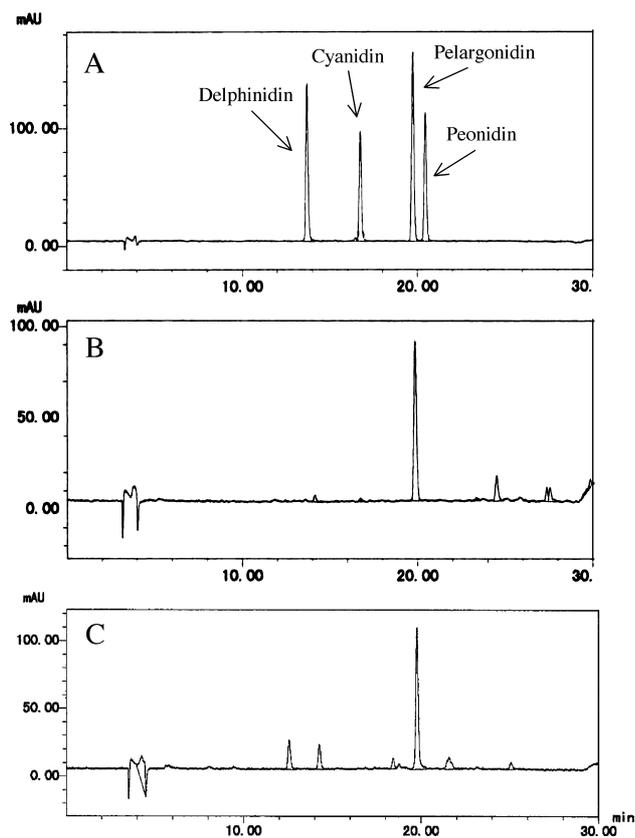


Figure 3. HPLC profiles of standard compounds (A), and hydrolysates of dried roots of field-grown plants (B) and adventitious root culture (C).

0.5 mg/l IBA at 25°C under the 14 h/day light condition on a rotary shaker at 100 rpm. The fresh and dry weights of the adventitious roots increased rapidly in the first two weeks, and the increase became gradual up to the 7th week (Fig. 4A). Anthocyanin was produced at the early stages of culture, the largest amount being observed at week 5 (*ca.* 250 $\mu\text{g}/100\text{ ml}$ flask), after which its production decreased gradually (Figure 4B).

DPPH radical scavenging activity

The DPPH radical scavenging activity of the 0.2 and 1 mg/ml reaction solutions prepared from the various samples was investigated. The 0.2 mg/ml reaction solution showed much lower activity than the 1 mg/ml reaction solution (Figure 6). The activity of the lyophilized field-grown roots of the 1 mg/ml reaction solution was 25%, and those of the fresh field-grown roots and the fresh adventitious roots were approximately 18% and 17%, respectively (Figure 6). However, the radical scavenging activity of the lyophilized adventitious roots was low at approximately 8%. Although Aokubi daikon does not form red pigments, very weak activity was observed (*ca.* 5%). In the lyophilized adventitious roots, low DPPH radical scavenging activity was detected compared to the fresh

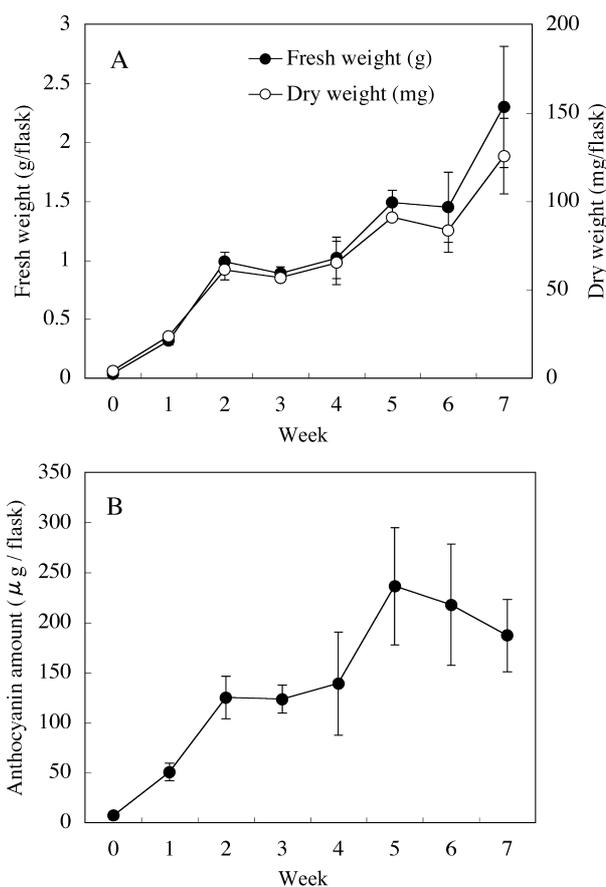


Figure 4. Time courses of growth (A) and anthocyanin contents (B) of adventitious root cultures of Peking Koushin. Adventitious roots were cultured in 1/2 MS liquid medium supplemented with 0.5 mg/l IBA for four weeks at 25°C under the 14 h/day light condition. Data are averages of triplicate experiments. Bars represent standard error.

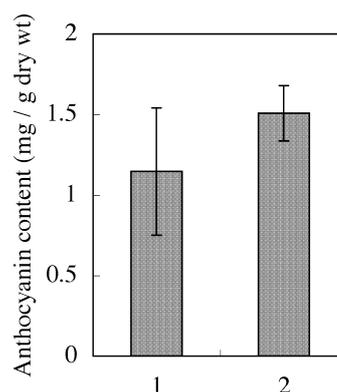


Figure 5. Anthocyanin contents of field-grown plants and adventitious root culture. Anthocyanin contents represent pelargonidin equivalent. Plants cultivated for three months in the field and four-week-old adventitious roots cultured in the light condition were analysed. Data are averages of triplicate experiments. Bars represent standard error. 1, Dried roots; 2, Dried adventitious root culture.

adventitious roots. Taken together, we conclude that the bioactive compounds responsible for the DPPH radical scavenging activity in the adventitious roots were

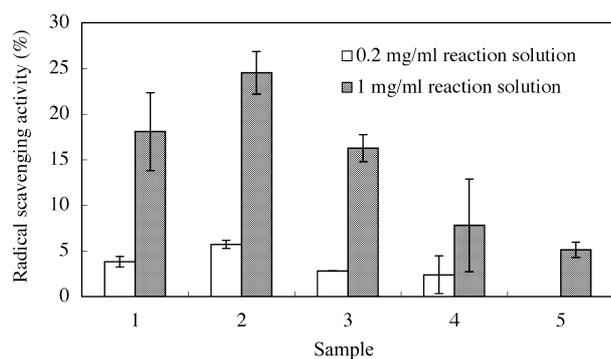


Figure 6. DPPH radical scavenging activity of anthocyanin in various samples. Data are averages of triplicate experiments. Bars represent standard error. 1, Fresh roots of Peking Koushin; 2, Dried roots of Peking Koushin; 3, Fresh adventitious root culture of Peking Koushin; 4, Dried adventitious root culture of Peking Koushin; 5, Fresh roots of Aokubi.

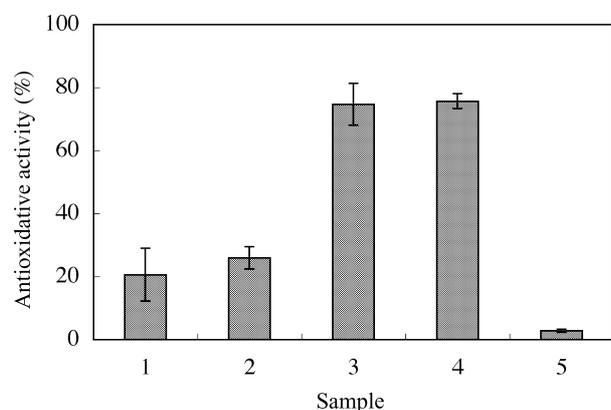


Figure 7. Antioxidative activity (TBA method) of anthocyanin in various samples. Data are averages of triplicate experiments. Bars represent standard error. 1, Fresh roots of Peking Koushin; 2, Dried roots of Peking Koushin; 3, Fresh adventitious root culture of Peking Koushin; 4, Dried adventitious root culture of Peking Koushin; 5, Fresh roots of Aokubi.

decomposed more easily than those in field-grown roots. Further investigation is necessary to reveal the decrease of the DPPH radical scavenging activity in adventitious roots.

Antioxidative activity measured by TBA method

The antioxidative activity of field-grown and adventitious roots was determined by the TBA method. The antioxidative activity of dried and fresh roots of field-grown plants was approximately 25%, respectively (Figure 7). The extract of Aokubi daikon, which does not form red pigments, exhibited both low antioxidative activity and low DPPH radical scavenging activity. Interestingly, the dried and fresh adventitious roots

established from the *in vitro* seedlings had high antioxidative activity, more than three times that of the intact roots of plants grown in the field. The dried adventitious roots showed a relatively high antioxidative activity although the DPPH radical scavenging activity was low. It is not clear whether the antioxidative activity of the adventitious roots was caused by anthocyanin or other compounds having antioxidative activity. Further investigation is needed to clarify this matter.

From these results, the adventitious root culture of *R. sativus* L. cv. Peking Koushin, which produces anthocyanins and exhibits the DPPH radical scavenging and the antioxidative (TBA method) activities, may be used as an alternative source of food color with bioactivity.

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