Vitis **42** (3), 117–121 (2003)

The effect of different fertilizer application levels on anthocyanoplast development in berry skin of Pione grapevines (*V. vinifera* x *V. labrusca*)

G. Okamoto, H. Onishi and K. Hirano

Faculty of Agriculture, Okayama University, Okayama, Japan

Summary

Effects of different levels of a complete liquid fertilizer on grape berry coloration and anthocyanoplast (ACP) development in the skin were investigated. Four-year-old vines of cv. Pione (a tetraploid hybrid of *Vitis vinifera* L. and *V. labrusca* L.) were planted in root zone-restricted beds and were treated with normal (N), high (H; Nx1.5), and excessively high (EH; Nx2) levels of a commercial liquid fertilizer. Fertilizer levels were decreased to one third after veraison to accelerate berry ripening. Skin anthocyanin contents in N- and H-treated berries increased at a constant rate after veraison, while the contents in EH berries were significantly lower. ACPs were first observed in epidermal cells at veraison in N and H berries and 2 weeks later in EH berries. In each treatment formation of ACPs in hypodermal cells occurred one week later than in epidermal cells. In each treatment the number of epidermal cells containing ACPs increased during ripening of the berries but the number of hypodermal cells with ACPs increased only in N vines. The average numbers of ACPs per epidermal and hypodermal cell were largest in N vines, followed by H and EH vines (two weeks after veraison). The number of ACPs decreased thereafter because most ACPs coalesced. The average ACP diameter at full ripeness was 16.7 μm in N vines, whereas in H and EH vines it was 9.5 μm and 9.7 μm, respectively. From these results, it can be deduced that in Pione grape berries high fertilizer levels inhibit ACP formation both in epidermal and hypodermal cells at veraison and/or induce their coalescence thereafter, resulting in poor coloration.

Key words: anthocyanoplast, skin coloration, fertilizer levels, Pione grape.

Abbreviations: ACP, anthocyanoplast; GA, gibberellin; N, normal; H, high; EH, excessively high.

Introduction

Anthocyanoplasts (ACPs) (Peckets and Small 1980), are intensely pigmented organelles found in vacuoles of plant cells such as *Raphanus sativus* (Yasuda and Shinoda 1985, Yasuda and Tsujino 1988, Yasuda *et al.* 1989), *Polygonum cuspidatum* (Kubo *et al.* 1995), and many *Brassica* plants (Nozzolillo and Ishikura 1988, Hodges and Nozzolillo 1996). Nakamura (1989, 1993, 1994) has reported that ACPs are formed in vacuoles of epidermal and hypodermal cells of grape berry skin after veraison. He also noted that the enlargement of the ACP by coalescing might be related to the darker coloration of the berry skin in Kyoho grapevines (a hybrid of *Vitis vinifera* and *V. labrusca*).

It is generally known that grape berry coloration, especially in tetraploid grapes, is significantly inhibited by overfertilization (Okamoto *et al.* 1991, Hirano *et al.* 2002), although the ACP development was not observed in their studies. Hodges and Nozzolillo (1996) found that red cabbage seedlings grown under nitrogen starvation formed more ACPs than those grown under conditions of sufficient nitrogen.

In this study, using Pione grapevines (a tetraploid hybrid of Kyoho and 4x-Muscat of Alexandria), effects of different levels of a complete liquid fertilizer on ACP development, skin coloration, and berry maturation were investigated.

Material and Methods

Plant material: Twenty-four vines of 6-year-old Pione, grafted on SO 4 rootstock, were used for this study in 2002. The vines were individually planted in raised soil beds (0.9∞0.6 m wide and 0.2 m high), composed of peat, sand and loam (1:1:3 in volume), at a vine space of 1.2 x 2.0 m. The side and bottom of the beds were wrapped with water permeable but root proof polyethylene sheet to prevent the root extension out of the root zone. They were supplied with a normal level of commercial liquid fertilizer, Ohtsuka House Ekihi #1 and #2 (Ohtsuka Chemical Co.), through a drip irrigation system at a rate of 5 l per vine once a week. A 100 l of the liquid fertilizer consisted of 27.0 g KNO_3, 31.7 g $Ca(NO_3)_2 \cdot 4H_2O$, 0.2 g $MgSO_4 \cdot 5H_2O$, 5.0 g $NH_4H_2PO_4$, 1.5 g EDTA-Fe, 0.3 g H_3BO_3, 0.2 g $MnSO_4 \cdot 5\ H_2O$, 22 mg $ZnSO_4 \cdot 7H_2O$, 5 mg $CuSO_4 \cdot 5H_2O$, and $Na_2MoO_4 \cdot 2H_2O$. The concentration of total N in the solution is equivalent to 60 mg l^{-1}.

The number of shoots per vine was limited to 6 at anthesis. All the flower clusters were treated with 25 mg l^{-1} of GA solution twice, at full bloom and 2 weeks after, to produce seedless berries. Clusters and berries were thinned 3 weeks after anthesis to 1 cluster per shoot and 40 berries per cluster. The leaf:fruit ratio was adjusted to 10 cm^2 g^{-1} by restricting the total number of lateral leaves per shoot at veraison.

Correspondence to: Dr. G. Okamoto, Okayama University, Faculty of Agriculture, Tsushima-naka Okayama 700-8530, Japan. Fax: +81-86-251-8388. E-mail: gokamoto@cc.okayama-u.ac.jp

Fertilizer application treatment: The test vines were divided into 3 groups at berry set. The vines in the first group received the normal level (N) of the liquid fertilizer continuously, while vines in the second and third group were supplied with high (1.5 times higher than N; H) and excessively high (twice as high than N; EH) levels of the liquid fertilizer, respectively. Each vine was supplied with 5 l of the liquid fertilizer twice a week until veraison, then each level was lowered to one third to enhance berry ripening (OKAMOTO *et al.* 1991).

Berry sampling and analyses: Forty to 50 berries showing average growth and maturation were sampled from each treatment at veraison (70 % of berries began coloration; July 16), at premature stage (15 °Brix; July 27), and at full ripeness (18 °Brix; August 15). The sampled berries of each treatment were randomly divided into 4 groups as replications. The skins of all berries were peeled by hand immediately and were divided into 2 groups; one was stored at -20 °C for anthocyanin analyses, the other was used for ACP observation. The flesh was homogenized, centrifuged at 10,000 rpm for 5 min, and filtrated to obtain juice samples. Total soluble solids (TSS), pH, and titratable acidity (TA, as tartaric acid equivalent) in juice were determined by a hand refractometer (Atago N-1α), a pH meter (Horiba F-22), and titration with 0.1 N NaOH, respectively. For determination of the sugar and amino acid contents, juice samples and 80 % EtOH extracts of skin samples were filtrated through a filter (Ø 0.45 µm), then analyzed by HPLC and a fully automated amino acid analyzer (JEOL JLC-300).

Anthocyanin analyses: Anthocyanins were extracted from 0.5 g of frozen skin samples with 25 ml of 50 % acetic acid 3 times for 5 min. Optical density at 520 nm was determined by a spectrophotometer (Beckmann DU530). For identification of individual anthocyanins, the extract was filtrated through a filter (Ø 0.45 µm) and applied to HPLC (JASCO). HPLC conditions: column, STR ODS (Ø 4.6 x 250 mm); column temperature, 35 °C; flow rate, 0.8 ml min^{-1}; mobile phase, solution A: 1.5 % phosphoric acid, solution B: 1.5 % phosphoric acid + 20 % AcOH + 25 % AcCN, gradient (solution A %), 0 min 75 % ➔ 0-40 min 15 %; detection at 520 nm. Identification of the peaks was based on results of MOCHIOKA *et al.* (1995) and FUJISHIMA and SHIRAISHI (1997).

ACP observation: ACP observation was carried out at 4-7 d intervals beginning at veraison. According to NAKAMURA (1989), fresh skin samples, Ø 5 mm and 0.1-0.2 mm thick, were obtained from the apical portion of sample berries using a razor blade. They were mounted on a slide glass with distilled water, and covered by a cover glass. Photographs taken under a light microscope (Olympus BH) were used for counting the number and size (maximum diameter) of ACPs both in epidermal and hypodermal cells. 20-30 cells per berry and 20-30 berries per treatment were examined.

Results

Berry growth and juice composition: The level of the liquid fertilizer did not affect significantly berry size, juice pH, and TA. TSS as well as the glucose and fructose contents, tended to be lower in EH vines than in N and H vines at veraison and in the premature stage, although these contents became similar to those in N and H vines at full ripeness (Tab. 1).

Skin composition: Both, glucose and fructose contents in each treatment increased after veraison, then decreased until full ripeness. These contents tended to be lower in EH vines than in N and H vines until full ripeness (Fig. 1). The anthocyanin accumulation in skins was mark-

Table 1

The effect of fertilizer levels on berry growth and ripening[1)]

Berry stage and treatment[2)]	Berry diameter (mm)	TSS (°Brix)	Juice Fructose (g 100 ml^{-1})	Juice Glucose (g 100 ml^{-1})	pH	Titratable acidity[3)] (g 100 ml^{-1})
Veraison (July 16)						
N	23.0	12.4 a	5.12 a	4.25 a	2.5	1.28
H	23.0	12.0 ab	4.94 ab	4.12 a	2.5	1.32
EH	23.1	11.1 b	4.45 b	3.79 b	2.5	1.33
Premature (July 27)						
N	23.6	15.2	6.99 ab	5.79 ab	3.0	0.73 a
H	23.9	15.3	7.19 a	5.99 a	3.0	0.64 b
EH	23.4	14.6	6.58 b	5.44 b	3.0	0.77 a
Full ripeness (Aug. 15)						
N	24.1	18.9	8.75	8.18	3.9	0.31
H	24.5	18.6	8.54	7.92	3.9	0.32
EH	24.3	18.7	8.65	7.98	3.9	0.35

[1)] Means are separated by DUNCAN's multiple range test, $p<0.05$ %; n=20 (for berry diameter) or n=4 (for others).

[2)] Applied with 5 l of liquid fertilizer containing 60 ppm (N), 90 ppm (H), amd 120 ppm (EH) of nitrogen twice a week from berry set to vraison, then the levels were lowered to one third.

[3)] As tartaric acid equivalent.

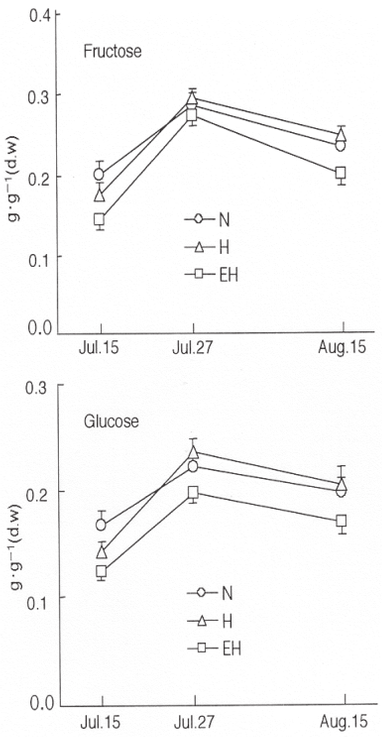

Fig. 1: The effect of fertilizer levels on the skin sugar content. Five l of complete liquid fertilizer containing 60 ppm (N), 90 ppm (H), and 120 ppm (EH) of nitrogen were applied until veraison, thereafter fertilizer levels were lowered to one third. Vertical bars represent SE, n=4.

edly inhibited in EH vines compared to N and H vines (Fig. 2). HPLC analyses of skin extracts revealed that the major anthocyanin constituents are peonidin-3-*p*-coumaryl glucoside-5-glucoside (Pn3*p*G5G) and malvidin-3-*p*-coumaryl glucoside-5-glucoside (Mv3*p*G5G), followed by malvidin-3-*p*-glucoside (Mv3*p*G), and peonidin-3-*p*-glucoside (Pn3*p*G) (Fig. 3). Anthocyanin extracts from berry skin of H vines contained Mv-3-*p*-Gl-5Gl at lower levels than those from N and H vines, although the difference was not

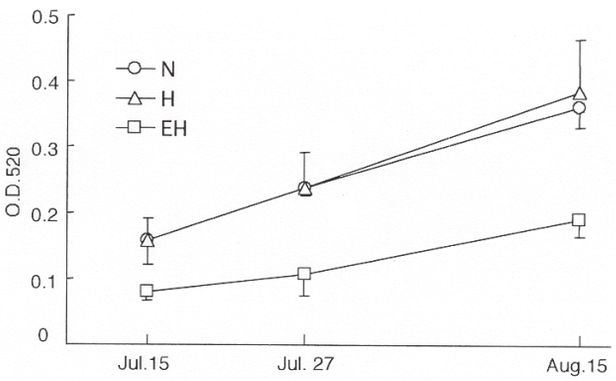

Fig. 2: Effects of fertilizer levels on the anthocyanin content of berry skins. For N, H, EH see Fig. 1. Vertical bars indicate SE, n=4.

significant. Tab. 2 shows the changes in skin amino acids between veraison and full ripeness. Major amino acids at veraison were glutamine, arginine, alanine, γ-butyric acid, serine, and glutamic acid; most of them differed insignificantly among the treatments. However, in the premature and full ripe stages, most amino acids and NH_3 contents were significantly higher in the skins of EH.

Anthocyanoplast (ACP) development: ACP was first found in epidermal cells on July 16 in N and H berries and on July 30 in EH berries (Fig. 4), in hypodermal cells on July 23 in N and H berries and on July 27 in EH berries. The number of epidermal cells containing ACPs in N berries was almost twice that of H berries at an immature stage on July 27. The average number of ACPs per epidermal cell and the total number of ACPs in epidermal cells were much greater in N berries than in H berries (Tab. 3). The number of epidermal cells containing ACPs increased remarkably thereafter in N and H berries. Cell numbers with ACPs were much lower in EH berries at full ripeness (Tab. 3). The ACP had coalesced during the ripening stage (Fig. 4 B). This resulted in an increased ACP size and decreased ACP number per cell in N and H berries. In the EH berries, however, ACPs did not coalesce. The total number of ACP per

Table 2

The effect of fertilizer levels on the amino acid and NH_3 content in berry skin at various stages of berry development[1)]

Berry stage and treatment[2)]	ALA	GABA	ARG	PRO	GLU	GLN	SER	THR	ASP	Others	Total	NH_3
						(µmol g^{-1} d.w.)						
Veraison (July 16)												
N	4.54	3.89 a	4.47	0.00 b	0.90 ab	5.21	1.57	0.84	0.83	0.54 b	23.10	16.49
H	3.27	3.22 b	3.93	0.00 b	0.69 b	4.50	1.29	0.66	0.77	0.54 b	19.14	12.41
EH	3.56	2.91 b	5.63	0.24 a	1.03 a	6.41	1.43	0.76	0.99	1.27 a	24.26	10.85
Premature (July 27)												
N	5.80 b	0.67	7.19 ab	0.98 b	5.21	2.65 b	1.32 b	0.88 b	1.17 b	1.59	27.46 b	4.00 b
H	7.65 ab	1.16	6.18 a	1.52 ab	5.37	2.99 b	1.43 b	0.97 b	1.12 b	2.55	30.92 b	4.46 b
EH	10.57 a	0.88	9.25 a	1.69 a	5.63	6.37 a	2.15 a	1.30 a	1.85 a	2.56	42.26 a	6.59 a
Full ripeness (Aug. 15)												
N	8.86 b	4.61	5.66 b	3.29	2.91	2.16 b	1.66 c	1.15 b	0.74	2.78 b	33.81 b	4.91 b
H	10.05 b	4.25	5.68 b	3.47	2.68	2.12 b	1.93 b	1.22 b	0.73	2.65 b	34.78 b	5.32 b
EH	13.66 a	4.95	10.54 a	3.80	2.01	3.98 a	2.67 a	1.75 a	1.11	4.14 a	48.59 b	5.86 a

[1)] Means in each column and each stage are separated by Duncan's multiple range test ($p<0.05$, n=4).
[2)] Refer to Tab. 1.

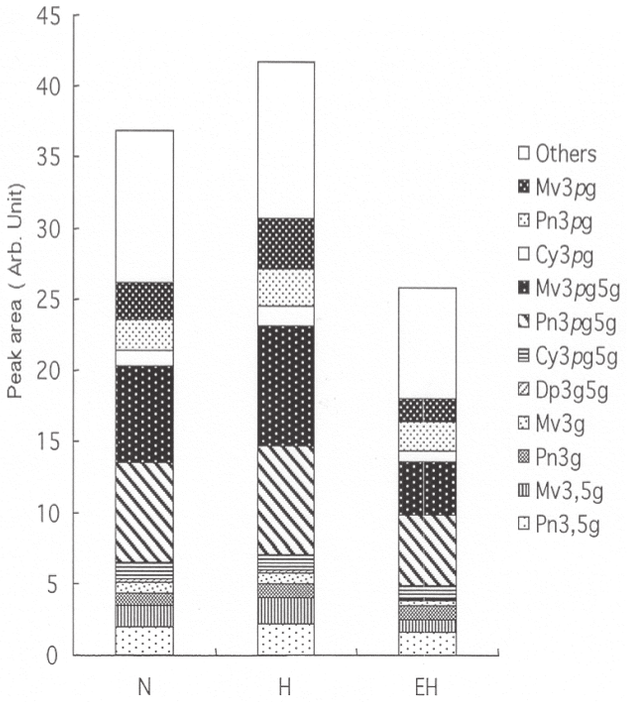

Fig. 3: Anthocyanin composition of berry skins after treatment with three different levels of fertilizer. For N, H, and EH see Fig. 1.

100 epidermal cells, calculated by multiplying the cell number containing ACP with the average number of ACP per cell, was highest in the N-treated vines at both stages (Tab. 3). In the hypodermal tissue, the ACP was localized mainly at the outermost cell layer of the tissue, and thus the data were taken for this layer only. The number of hypodermal cells containing ACPs and the average number of ACPs were much higher in the N berries in the premature stage. There was no significant difference in their number between H and EH (Tab. 3). The decrease in the ACP number per cell, caused by coalescence of the ACP, was also detected at full ripeness in each treatment. Here again, the total number of ACPs in epidermal and hypodermal tissues was considerably higher in N than in H and EH berries.

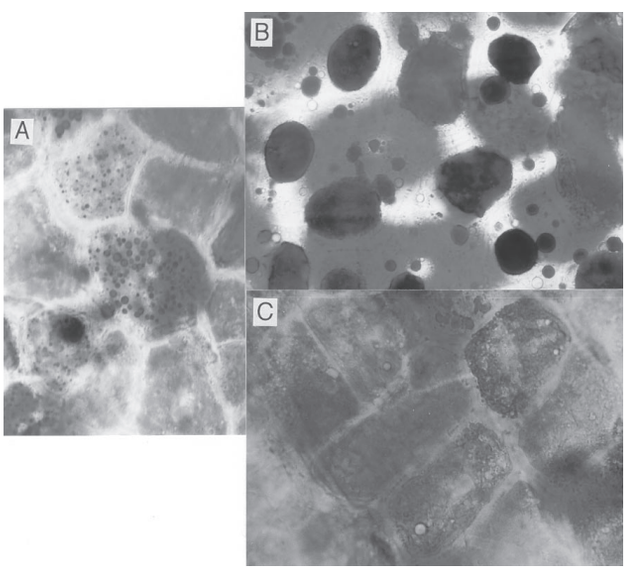

Fig. 4: Anthocyanoplasts (ACPs) in epidermal cells of berry skins. **A**: Newly formed small ACPs in normally fertilized vines at veraison (X 100); **B**: Coalesced and enlarged ACPs in normally fertilized vines at full ripeness (X 200); **C**: Epidermal cells without ACPs in excessively fertilized vines (X 200).

Table 3

The effect of fertilizer levels on ACP development in epidermal and hypodermal cells[1)]

Berry stage and treatment[2)]	No. of cells with ACPs per 100 cells	No. of ACPs per cell[3)]	Total no. of ACPs per 100 cells
Epidermal cells			
Premature (July 27)			
N	1.9	40.5	76.2 a
H	0.9	15.8	14.2 b
EH	0.0	--	--
Full ripeness (Aug. 15)			
N	16.9 a	3.0	50.8 a
H	9.0 b	3.8	34.4 a b
EH	3.0 b	4.6	13.8 b
Hypodermal cells			
Premature (July 27)			
N	6.2	59.6	368.6 a
H	2.2	43,7	96.1 b
EH	0.0	--	--
Full ripeness (Aug. 15)			
N	9.5 a	32.3	307.7 a
H	1.9 b	4.0	7.4 b
EH	0.6 b	4.0	2.6 b

1) Means in each column at each stage are separated by Duncan's multiple range test ($p<0.05$, n=17-31).
2) Refer to Tab. 1.
3) Counted only for cells containing ACPs.

The distribution and means of ACP sizes in epidermal cells, calculated from maximum diameters of 50 ACPs at full ripeness, are presented in a box plot graph (Fig. 5). In N berries, the range of ACP diameter in 90/10 percentiles of the total was between 3 and 30 µm, the range in 75/25 percentiles was between 6 and 27 µm, and the mean diameter was 16.7 µm. However, in both H and EH berries the ranges in 75/25 percentiles of 50 ACPs were between 4 and 20 µm, and the mean diameters were about 10 µm, *i.e.* significantly smaller than in N berries.

Discussion

Application of complete liquid fertilizer containing 60 ppm of nitrogen has been shown to induce the most appropriate shoot growth in tetraploid grapevines, such as cv. Kyoho (OKAMOTO *et al.* 1991, HIRANO *et al.* 2002). We also reported that lowering the fertilizer level to half or one third at veraison usually promotes berry ripening and results in the production of berries with higher sugar content and darker skin coloration. Pione, used for this study, was bred by cross-pollination between Kyoho and 4x-Muscat of

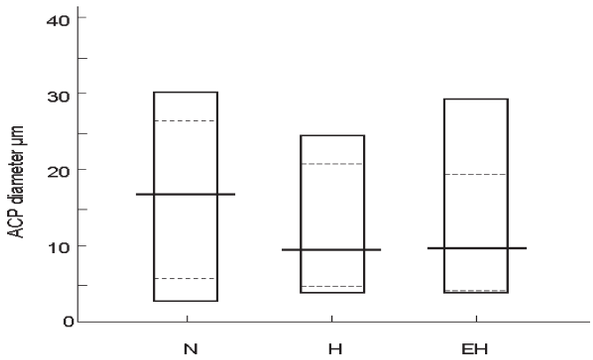

Fig. 5: The effect of different fertilizer levels on the distribution and mean of ACP diameter in skins. Top and bottom lines of each box indicate the range of 90/10 percentiles of 50 ACPs, dotted lines 75/25 percentiles, and thick line the mean value.

Alexandria by Hideo Ikawa, a Japanese grape breeder, in the 1960s and is now widely cultivated in Japan. Pione vines are known to produce larger berries than cv. Kyoho and the berries show poor coloration if vines are excessively fertilized or over-bearing. In this study, shoot growth and leaf color were estimated to be optimal at the recommended fertilizer level (N), although berry growth, juice composition, and skin coloration were similar to those in H treated vines. EH vines that received twice as much of the liquid fertilizer recommended, exhibited apparent over-fertilized features such as delayed sugar accumulation (Tab. 1) and poor skin coloration (Fig. 2).

Glucose and fructose contents in both skin and juice tended to be lower in EH than in N and H berries at veraison and in the premature stage, which was observed in the skin of EH berries as well when fully matured (Fig. 1, Tab. 1). The lower sugar content, especially glucose, in the skin of EH berries at veraison and in the premature stage may be one cause of poor coloration because a glucose moiety is an important part of most grape anthocyanins (FUJISHIMA and Shiraishi 1997). Furthermore, the amino acids and NH_3 contents in skins were often remarkably higher in EH vines than in N and H vines. It is generally accepted that abundant nitrogen usually inhibits the pigment accumulation in fruit such as grapes (HIRANO *et al*. 2002), apples (FAUST 1965 a, 1965 b, KVALE 1971), and peaches (JIA *et al*. 1999, 2000).

ACPs are found in vacuoles where anthocyanins are abundant; the mechanisms of anthocyanin accumulation into ACPs are not yet clear. SMALL and PECKET (1982) reported that ACPs in the hypocotyl cells of red cabbage seedlings had a membrane of 10 nm thickness. NAKAMURA (1993) observed the ACP membrane under light microscope in Kyoho berry skins; he also found that ACPs in the excised hypodermis of Kyoho berries coalesced within a few minutes of microscopic observation.

Our current study suggests that ACP development in grape berry skin is significantly affected by the nutritional status. The low sugar content and high levels of nitrogenous compounds, detected in the berry skin of EH berries, reduces the formation and thereafter coalescence of ACPs that are indispensable for marketable skin coloration. Furthermore, their smaller number and size in H berry skins indicates that even moderately high fertilizer levels may inhibit ACP development, although the concentrations in skin and juice constituents did not differ significantly. Levels of plant hormones and minerals in skins and flesh of berries should be investigated.

References

FAUST, M.; 1965 a: Physiology of anthocyanin development in McIntosh apple. I. Participation of pentose phosphate pathway in anthocyanin development. Proc. Am. Soc. Hort. Sci. **87**, 2-9.

FAUST, M.; 1965 b: Physiology of anthocyanin development in McIntosh apple. II. Relationship between protein synthesis and anthocyanin development. Proc. Am. Soc. Hort. Sci. **87**, 10-19.

FUJISHIMA, H; SHIRAISHI, M.; 1997: Studies on the evaluation of genetic resources in *Vitis*. A rapid screening test of skin anthocyanin. J. Japan. Soc. Hort. Sci. **66**, 208-209.

HIRANO, K.; KATO, J; OKAMOTO, G.; 2002: Effects of fertilizer concentration and crop level on berry maturation of Kyoho grape. (Japanese with English summary). J. ASEV Jpn. **13**, 15-21.

HODGES, D. M.; NOZZOLILLO, C.; 1996: Anthocyanin and anthocyanoplast content of cruciferous seedlings subjected to mineral nutrient deficiencies. J. Plant Physiol. **47**, 749-754.

JIA, H.; HIRANO, K.; OKAMOTO, G.; 1999: Effects of fertilizer levels on tree growth and fruit quality of 'Hakuho' peaches (*Prunus persica*). J. Japan. Sci. Hort. Sci. **68**, 487-493.

JIA, H.; OKAMOTO, G.; HIRANO, K.; 2000: Effect of amino acid composition on the taste of 'Hakuho' peaches (*Prunus persica* Batsch) grown under different fertilizer levels. J. Japan. Sci. Hort. Sci. **69**, 135-140.

KUBO, H.; NOZUE, M.; KAWASAKI, K.; YASUDA, H.; 1995: Intravacuolar spherical bodies in *Polygonum cuspidateum*. Plant Cell Physiol. **36**, 1453-1458.

KVALE, A.; 1971: The effect of different nitrogen levels of the trees on pigment content, ground color and soluble solids of the apple cultivars Prins, Gravenstein, James Grieve and Ingrid Marie. Acta Agric. Scandinavica **21**, 207-213.

MOCHIOKA, R.; YAMAGUCHI, M; HORIUCHI, S; MATSUI, H; KUROOKA, H.; 1995: Chemotaxonomic classification of native wild Japanese grapes by anthocyanins in berry skin. (Japanese with English summary). J. Japan. Sci. Hort. Sci. **64**, 463-470.

NAKAMURA, M.; 1989: Development of anthocyanoplasts in relation to coloration of 'Kyoho' grapes. J. Japan. Soc. Hort. Sci. **58**, 537-543.

NAKAMURA, M.; 1993: Anthocyanoplasts in 'Kyoho' grapes. J. Japan. Soc. Hort. Sci. **62**, 353-358.

NAKAMURA, M.; 1994: Distribution and formation of anthocyanoplasts in the skin of 'Kyoho' grapes. J. Japan. Soc. Hort. Sci. **62**, 717-723.

NOZZOLILLO, C.; ISHIKURA, N.; 1988: An investigation of the intracellular site of anthocyanoplasts using isolated protoplasts and vacuoles. Plant Cell Rep. **7**, 389-392.

OKAMOTO, G; NODA, M; IMAI, S; FUJIWARA, T.; 1991: Effect of levels of fertilizer application on vine growth and fruit development of 'Kyoho' grape planted on a restricted rooting volume. (Japanese with English summary). Sci. Rep. Fac. Agric. Okayama Univ. **78**, 27-33.

PECKET, R. C.; SMALL, C. J.; 1980: Occurrence, location and development of anthocyanoplasts. Phytochemistry **19**, 2571-2576.

SMALL, C. J.; PECKET, R. C.; 1982: The ultrastructure of anthocyanoplasts in red-cabbage. Planta **154**, 97-99.

YASUDA, H.; MITSUI, T.; ONISHI, M.; 1989: The studies on the spherical bodies containing anthocyanins in plant cells. III. Observations on the developments of anthocyanoplasts in the radish hypocotyls. Cytologia **54**, 673-678.

YASUDA, H.; SHINODA, H.; 1985: The studies on the spherical bodies containing anthocyanins in plant cells. I. Cytological and cytochemical observations on the bodies appearing in the seedling hypocotyls of radish plants. Cytologia **50**, 397-403.

YASUDA, H.; TSUJINO, Y.; 1988: The studies on the spherical bodies containing anthocyanins in plant cells. II. The effect of light on the pigmentation of spherical bodies in the seedling hypocotyls of the radish plant. J. Fac. Sci. Shinshu Univ. **23**, 1-6.

Received January 28, 2003