Anthocyanins in suspension cultures originating from different parts of sweet potato plant

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1. Introduction

Of all the food additives food colorants arouse a great controversy. The safety of synthetic food dyes has been questioned for many years and restrictions have been put on the use of synthetic dyes in food, which have activated more research on plant pigment. Recently developed purple-fleshed sweet potato (*Ipomoea batatas* (L.) Lam) cultivar "Ayamurasaki" accumulates high amount of anthocyanins in the storage root (1). These anthocyanins possess high thermostability (2), which makes them an attractive source of natural food colorant for the food industry. However, production of pigment from field-grown storage root has some disadvantages like seasonality, large plantation requirements, extended time required to produce roots, weather dependency, high transport and labour cost, etc. Anthocyanin production from the "Ayamurasaki" plant material by means of tissue culture techniques would overcome these disadvantages.

Plant cell culture was successfully applied to induce biosynthesis of anthocyanins *in vitro* from different plant materials (3-12). Nozue et al. (13,14) selected a storage root originated cell line from white-fleshed sweet potato cultivar "Kintoki", which accumulated relatively low level of anthocyanins (3.5 μmol/g fresh weight) under continuous illumination. We undertook this study to establish callus and suspension cultures from different parts of the "Ayamurasaki" plant, evaluate their potential for anthocyanin accumulation and the composition of the accumulated anthocyanin, and compare the quality of pigments accumulated in field-grown plant and in the suspension cultures.

2. Materials and methods

2.1. Plant material

Callus cultures were induced from storage root, leaf and stem of sweet potato (*Ipomoea batatas*, (L.) Lam.), cv. Ayamurasaki. Storage root explants were prepared from the plant material stored at 13°C for 6 months after harvest. The storage roots were scrubbed clean under running tap-water and submerged in 10% commercial bleach solution for 30 min. After rinsing 3 times with sterile distilled water the skin (0.5 cm layer) was peeled off under aseptic conditions. From the remaining tissue small cubes ($10 \times 10 \times 5$ mm) were prepared.

Leaves and stems were collected from vines grown in biotron. Leaf tissue was taken from the first three fully developed leaves. Stem internode tissue was taken from the stem apex to the fourth internode. Leaves and stem segments were surface sterilised for 15 minutes in 1.25% sodium hypochlorite solution followed by three rinses with sterile distilled water. Explants were prepared by taking 5 mm thick cross-sections of the stem and 1 cm diameter discs from the leaf blades (veins included).

2.2. Callus and suspension cultures

The explants were placed on Murashige and Skoog (1962) agar medium enriched with 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzylaminopurine (BA) in concentrations ranging from 0 to 4 mg/l. Seven explants were placed in a Petri dish (10 cm diameter). At least five plates per treatment were prepared. The experiments were repeated twice. The cultures were incubated at 27°C in the dark with unmonitored light interruptions during daily observations.

The selected calli produced by leaf and stem explants were subsequently exposed to MS medium with 2,4-D in concentrations: 0, 0.5, 1, 2 and 3 mg/l to intensify growth.

Somatic pro-embryos induced on the storage root explant surface under the influence of 2,4-D and BA were used for callusogenesis. The pro-embryos were isolated from the explants and placed on MS medium with 0.5 mg/l 2,4-D to initiate callus growth. To stimulate callus growth 2,4-D concentrations of 0, 0.5, 1, 2 and 3 mg/l in MS medium were tested. The cultures were subcultured every 3 weeks.

Suspended cell cultures were initiated by transferring about 1 g (fresh weight) of callus tissue to 25 ml of liquid medium in 100 ml flasks. As multiplication media (MM) basal MS medium supplemented with 2 mg/l 2,4-D for the callus cultures originated from storage root and stem, and 1.5 mg/l 2,4-D for the culture originated from leaf were used. The cultures were incubated on a rotary shaker (120 rpm) at 27°C in the dark. The medium was changed weekly.

2.3. Extraction of anthocyanins

The aggregates from suspension cultures maintained for 3 months on MM were used for pigment analysis. Cell aggregates were separated from the culture medium by filtration under vacuum pressure and then weighed. Fresh cells were ground and steeped in 50% acetic acid for 1 hour. The samples of intact storage root, leaf and stem were taken from chopped tissue steeped overnight in 50% acetic acid. The volume of acetic acid solution was 20 times the equivalent of the sample weight. The samples were centrifuged at 10 000 rpm for 10 min. The supernatants were used for anthocyanin identification and quality analysis.

2.4. Anthocyanin identification and HPLC analysis

The supernatant diluted fourfold with McLbanc's buffer solution (14.7 g/l Sodium phosphate, Dibasic and 16.7 g/l Citric acid, Anhydrous), pH adjusted to 3, was used for the measurement of the optical densities at 530 nm with spectrophotometer CS-9300PC (Shimadzu, Japan). Colour value (CV) of the pigment extract was calculated using the following formula: $CV = 0.1 \times OD_{530} \times 4 \times 20/1g$ tissue fresh weight (OD_{530} is a spectrophotometric reading at 530 nm, and 4 and 20 are the levels of dilution) according to Japan Food Additives Association (16).

HPLC analysis was performed according to the method described by Odake et al. (17) on LC-9A (Shimadzu, Japan) liquid chromatograph. Analytical HPLC was run on Inertsil ODS-2 column (250 × 4.6 mm, GL Sciences Inc.) at 35°C and monitored at 530 nm. The following solvents in water with a flow rate of 1 ml/min were used: A — 1.5% phosphoric acid and B — 1.5% phosphoric acid, 20% acetic acid and 25% acetonitrile. The elution profile was a linear gradient elution for B 25% → 85% for 40 min in solvent A. Identification of anthocyanins was carried out comparing the peaks with six standard peaks of sweetpotato YGM anthocyanins: YGM-1 [cyanidin 3-(6,6'-caffeyl*p*-hydroxybenzoylsophoroside)-5-glucoside later named YGM-1a and cyanidin 3-(6,6'-dicaffeylsophoroside)-5-glucoside later named YGM-1b], YGM-2 [cyanidin 3-(6-caffeylferulylsophoroside)-5-glucoside], YGM-3 [cyanidin 3-(6,6'-caffeylferulylsophoro-side)-5-glucoside], YGM-4 [peonidin 3-(6,6'-dicaffeylsophoroside)-5-glucoside], YGM-5 [peonidin3-(6,6'-caffeyl*p*-hydroxybenzoyl-sophoroside)-5-glucoside later named YGM-5a and cyanidin 3-(6-caffeyl-sophoroside)-5-glucoside later named YGM-5b] and YGM-6 [peonidin 3-(6,6'-caffeylferulylsophoroside)-5-glucoside] (17-19).

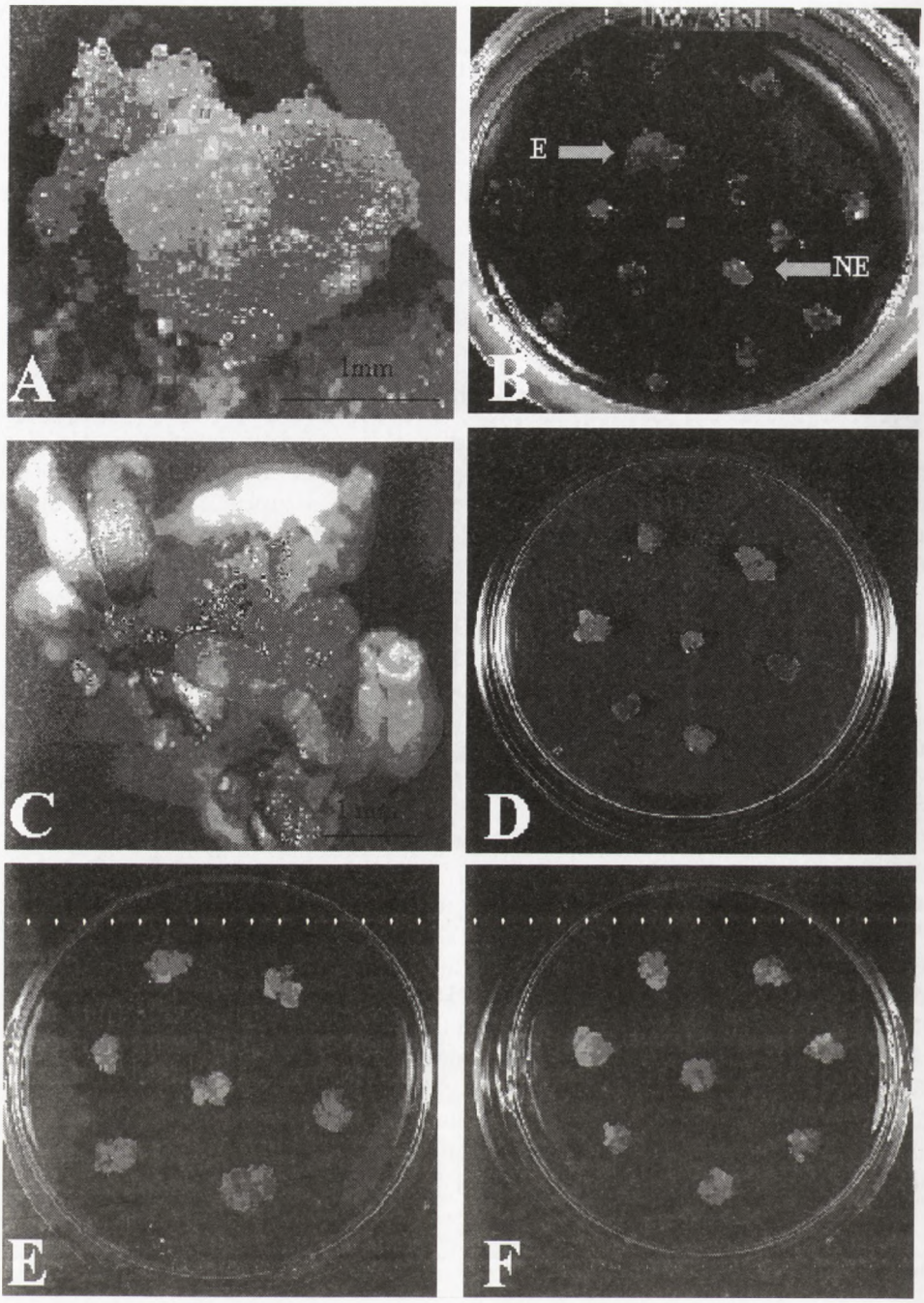

Fig. 1. A: Somatic pro-embryo on the surface of storage root explant, B: Callus induction from pro-embryo on MS medium with 0.5 mg/l 2.4-D (E: embryogenic, NE: non-embryogenic callus), C: Somatic embryo arising from embryogenic callus, D: Callus culture originating from storage root, E: Callus culture from leaf, F: Callus culture from stem.

3. Results and discussion

3.1. Establishment of anthocyanin producing cell lines

The majority of the storage root explants placed on media treatments with a range of 2,4-D and BA concentrations responded with production of a white, soft and friable callus. Beside the friable callus somatic pro-embryos appeared on the explant surface after 6-10 weeks of cultivation in the presence of 1 mg/l 2,4-D (Fig. 1A). The frequency of pro-embryos development was very low. The increase of 2,4-D concentration as well as addition of BA in the medium suppressed their development (Table 1).

TABLE 1

INFLUENCE OF 2,4-D AND BA ON THE FRIABLE CALLUS FORMATION AND SOMATIC PRO-EMBRYO DEVELOPMENT FROM THE SWEET POTATO STORAGE ROOT EXPLANTS IN THE DARK AT 27°C

BA/2.4-D (mg/l)	0		1		2		4	
	FC	SP	FC	SP	FC	SP	FC	SP
0	28	0	100	10	71	5	62	0
0.5	86	0	95	10	86	2	81	0
1	86	0	95	5	100	0	86	0

Each numerical value represents the percentage of explants formed friable callus (FC) and somatic pro-embryo (SP).

Pro-embryos subcultured on MS medium enriched with 0.5 mg/l 2,4-D developed two different types of calli: one slow growing firm yellow slightly pigmented embryogenic callus and the other fast growing white friable non-embryogenic callus (Fig. 1B). Selectively isolated pigmented embryogenic callus developed somatic embryos in 3 weeks time on MS medium without growth regulators (Fig. 1C). With an increase of 2,4-D concentration to 3 mg/l we observed an enhancement of embryogenic callus growth and reduction in the growth of non-embryogenic callus. Chee and Cantliffe (20) reported similar response of embryogenic callus developed from shoot tips of sweetpotato plants, cv. White Star, to 2,4-D, which developed somatic embryos on hormone-free medium. Low level of 2,4-D (up to 2 μM) reduced the size and developmental stage of embryos. The concentrations from 2 to 5 μM 2,4-D stimulated pro-embryonal callus growth.

The embryogenic callus cultivated on MS medium enriched with 3 mg/l 2,4-D in the dark produced mottled white, red and purple aggregates (Fig. 1D).

While cultivated on MS medium enriched with 2,4-D and BA the leaf explants responded with more intensive callusogenesis than stem explants. Callusogenesis of the leaf explants began within a week of the initial culture. Growth of callus was slow, but was most pronounced on medium containing

0.5 mg/l 2,4-D. Callusogenesis of the stem explants occurred only in the medium with 1 mg/l 2,4-D (Table 2).

TABLE 2
INFLUENCE OF 2,4-D AND BA ON THE CALLUS FORMATION FROM LEAF AND STEM EXPLANTS IN THE DARK AT 27°C

BA/2.4-D (mg/l)	0		0.1		0.5		1	
	L	S	L	S	L	S	L	S
0	0	0	SP	0	100	0	85	10
0.1	20	–	SP	–	100	–	90	–
0.5	80	–	SP	–	100	–	90	–
1	100	–	SP	–	100	–	100	–

Each value represents the percentage of explants formed friable callus. L — leaf, S — stem, SP — development of somatic pro-embryos, no callus was formed.

Leaf and stem calli selected for pigment accumulation developed intensive pigmentation with retardation of growth on MS medium at low auxin levels (0.25 and 0.5 mg/l). The highest auxin concentrations enhanced callus growth and eliminated pigmentation. The highest multiplication rate of leaf originated and stem originated calli were achieved with 2 mg/l 2,4-D and 3 mg/l 2,4-D, respectively. In two month's time heterogenous cultures developed, consisting of mottled white and slightly pigmented red aggregates (Fig. 1E and 1F).

3.2. Accumulation and composition of pigment in suspension cultures and donor tissues

The suspension cultures, maintained in MM in the dark 3 months after initiation, were assessed for the potential to accumulate pigments. The suspension cultures displayed differences in the potential for pigment accumulation. The culture originating from storage root (SRC) accumulated the highest amount of total anthocyanins expressed in colour value, followed by leaf (LC) and stem (SC) originated cultures (Fig. 2). SRC accumulated 3 times and LC accumulated 2 times higher amount of pigments than SC. Among the donor tissues, pigment content of storage root was 3 and 9 times higher than the pigment contents of leaf and stem, respectively. The amount of total anthocyanins accumulated by SRC reached 55% of that accumulated by the donor tissue of storage root. LC and SC accumulated higher amounts of pigment than their donor tissues: 138% and 170%, respectively. Similar differences in the ability to accumulate anthocyanins were found among cell lines induced from leaf, apical meristem, immature fruit and petiole of strawberry plant (7). Our suspension cultures accumulated anthocyanins on MM enriched with 2,4-D. It was reported previously that the presence of 2,4-D

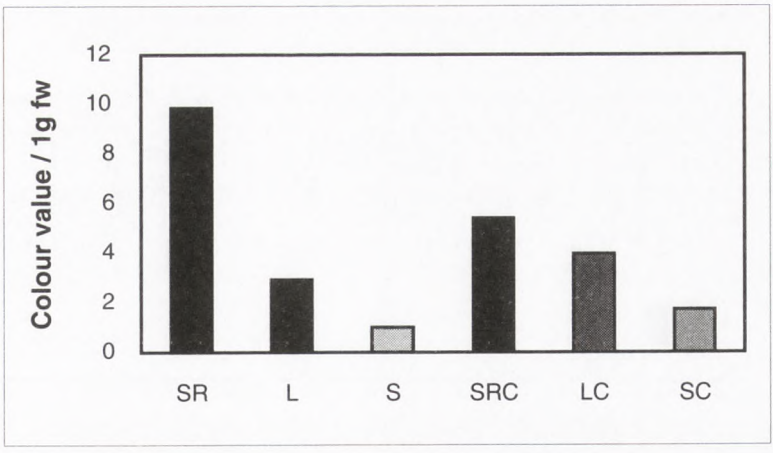

Fig. 2. Colour value of the pigment extract of the donor tissues: storage root (SR), leaf (L), stem (S) and suspension cultures originating from: storage root (SRC), leaf (LC) and stem (SC).

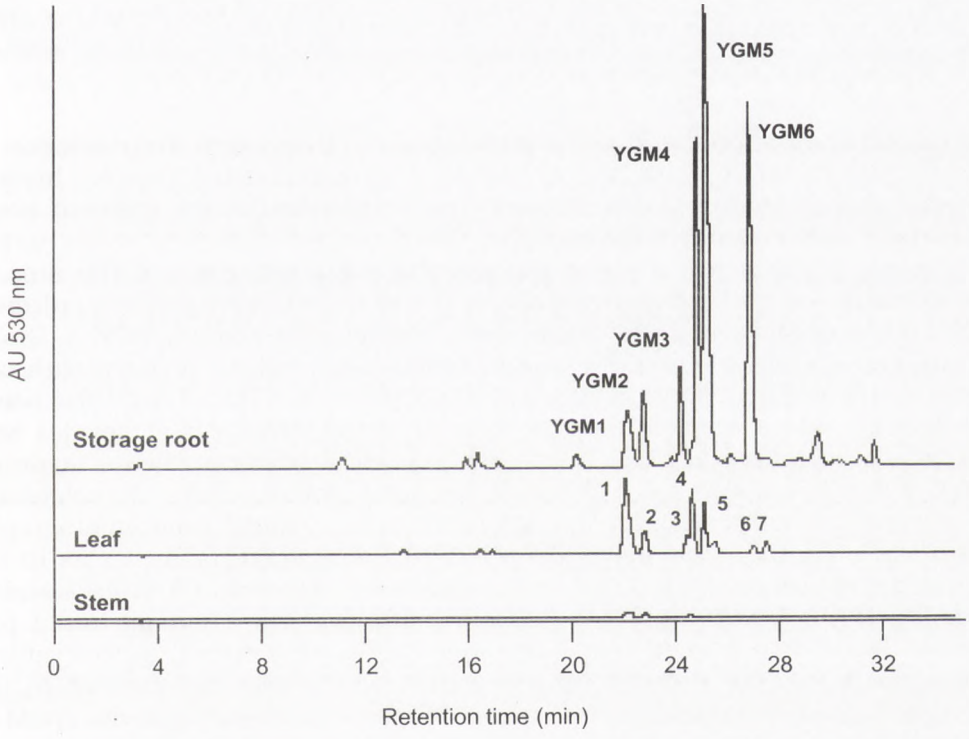

Fig. 3. HPLC chromatogram of storage root, leaf and stem extracts of sweet potato (*Ipomoea batatas* (L.) Lam), cv. Ayamurasaki.

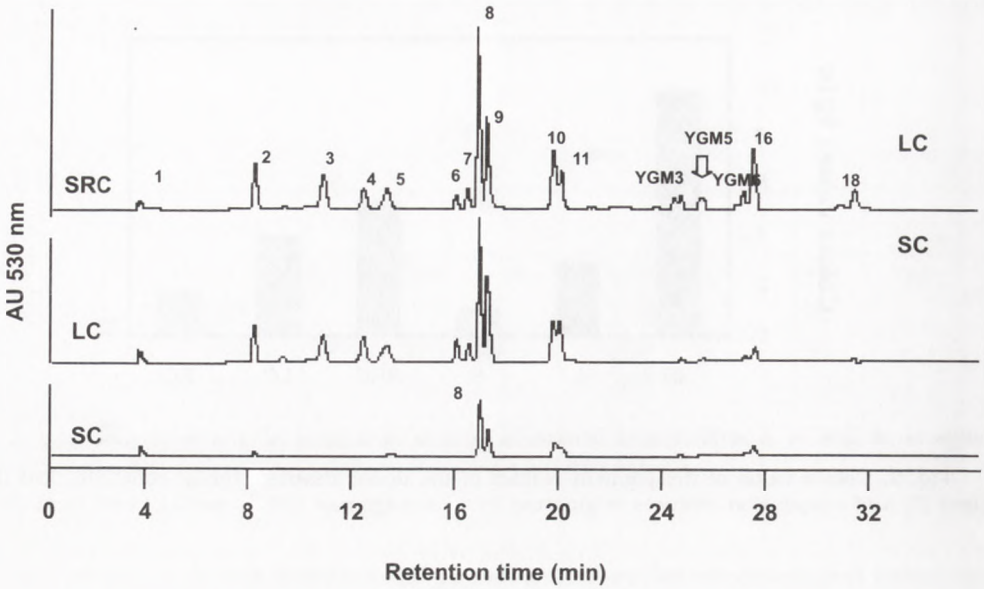

Fig. 4. HPLC chromatogram of the suspension cultures developed from storage root (SRC), leaf (LC) and stem (SC) of sweet potato plant, (*Ipomoea batatas* (L.) Lam), cv. Ayamurasaki.

in the medium suppressed or fully eliminated anthocyanin accumulation in tissue (6). Therefore, with improvements in production medium for biosynthesis of anthocyanin we can expect further increase of the pigment accumulation ability of our cultures.

Figure 3 shows the reverse phase HPLC chromatogram of the storage root, leaf and stem, and Figure 4 shows the same of the suspension cultures: SRC, LC and SC. The main peaks of the storage root extract: YGM-1, 2 and 3 were identified as cyanindin 3-sophoroside-5-glucosides acylated with caffeic and/or ferulic acid and/or p-hydroxybenzoic acid (18,17) and the peaks YGM-4, 5 and 6 were identified as peonidin 3-sophoroside-5-glucosides with the same acylation pattern (17-19). The cyanidin-based pigments made up 15.2% and peonidin-based 72.4% of the total anthocyanins, as calculated according to the peak area. In the leaf extract, using co-chromatography with YGM standards, we have identified the same major pigments as in the storage root extract (Fig. 3), but cyanidin-based pigments [YGM-1, 2 and 3] dominated and made up 62.1% of total anthocyanins. Peonidin-based pigments [YGM-4, 5 and 6] made up only 22.7% of the total anthocyanins. Additionally, a new peak [7] was detected, which was not present in the storage root chromatogram. Only traces of cyanidin-based pigments [YGM-1, 2 and 3] were detected in the stem tissue. There were clear differences in the proportions between cyanidin- and peonidin-based pigments in the leaf and stem and storage root tissues. Quantitative and qualitative differences

in anthocyanin accumulation between the different parts of sweet potato plant *in vivo* might be due to the different physiological status of the leaf, stem and storage root and environmental modification of the secondary metabolism in those plant organs.

The suspension cultures established from leaf, stem and storage root displayed similar anthocyanin profiles, which were simpler than the anthocyanin profile of donor tissues (Fig. 4). The main peaks appeared with earlier retention time than the YGM peaks. The peaks, which appeared with earlier retention time on ODS-column HPLC are more hydrophilic. It suggests that they have a simpler structure and might be precursors to the biosynthesis of highly acylated YGM1-6. Among the suspension cultures, SRC displayed the richest composition of anthocyanins. Only here the peaks YGM-3, 5 and 6 were detected by co-chromatography, although their concentrations were very low: 2, 2.2 and 3%, respectively.

Our spectral data indicate that the main peaks in the suspension culture chromatograms [8 and 10] are not present in the chromatograms of the donor tissues. These two pigments made up 29.5% in SRC, 31.3% in the LC and 47.3% in SC of the total anthocyanins as calculated by the peak area. The presence of new peaks in the chromatogram of suspension cultures suggests differences in the biosynthetic pathways of anthocyanin in cell cultures and respective donor tissues. Such differences in anthocyanin composition and profile of cultured cells and intact organs were also reported for *Vitis* hybrid (5), strawberry (7) and cranberry (12).

4. Conclusions

The suspension cultures developed from different parts of sweet potato plant, cv. Ayamurasaki, accumulated pigment in the dark when maintained in MS media enriched with 2,4-D. The cultures displayed a similar anthocyanin profile, regardless of the source of explants, with the domination of less metabolically evolved components. In this aspect, they differed significantly from the respective donor tissues, which accumulated highly acylated YGM cyanidins and peonidins. The storage root originated culture (SRC) displayed the highest potential for anthocyanin accumulation *in vitro* and was selected for further research.

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Summary

Anthocyanin accumulating cell lines were established from storage root, leaf and stem explants of sweet potato *(Ipomoea batatas* (L.) Lam.*)*, cv. Ayamurasaki. The calli developed on MS basal medium enriched with 2,4-D at 27°C accumulated pigments in the dark. The storage root originated suspension culture generated the highest amount of total anthocyanins expressed as colour value (5.9) followed by the cultures originating from leaf (4.3) and stem (1.7). The cultures displayed similar anthocyanin profile regardless of source of explants. The major pigments derived from suspension cultures appeared with earlier retention time on ODS-column HPLC than the YGM pigments accumulated *in vivo*, which suggests that they are highly hydrophilic and have simpler chemical structure.

Key words:
Ipomoea batatas, embryogenic callus, suspension culture, anthocyanin.

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