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Enhancement of betanin yield in transformed cells of sugar beet (*Beta vulgaris* L.)

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Betanin belongs to a large family of betacyanin pigments, betalains, and in the food industry it is established as a powerful antioxidant and natural colorant. The main source of betanin is a red beet (*Beta vulgaris* L.), the yield depending on abiotic and biotic factors in the field. Sugar beet cells, transformed by a wild strain B6S3 of *Agrobacterium tumefaciens*, strongly produce betanin and could be a stable production source. With the aim of enhancing the yield, cell suspensions were initiated from friable calli. Biomass accumulation, betanin content and yield were monitored over 21 days in relation to changes of sucrose concentration, modifications of minerals in nutrient medium, and the usage of elicitors. Results showed that elevating sucrose levels from 3% to 4%, 5%, or 6% (w/v) in the original medium strongly induced biomass accumulation followed by an increase in betanin yield of up to 250%. Modification of minerals in the medium additionally increased betanin yield up to 20% in a few instances: 40 mg L^{-1} was recorded at day 10 with 5% and 6% of sucrose. The highest yield at 53 mg L^{-1} was reached at day 21 on 4% Suc, again with the modified medium. High sucrose concentrations positively affected betanin accumulation only during lag phase of the cell suspension, but afterwards the trend reversed. Calcium and yeast extract were used as abiotic and biotic elicitor, respectively, in the early exponential phase of subculture (day 7). Calcium ions (at 10 fold higher concentration than in the control) failed to increase betanin yield as well as yeast extract at 0.25% (w/v). Yeast extract at 1.25% provoked excretion of betanin at day 4, and cell necrosis at day 7 after elicitation. Taken together, in our system, sucrose affected betanin yield more strongly than medium modifications or elicitors. Yeast extract could be used for reverse-sequestration of betanin where the cells can be used over an extended period.

Key words: elicitors, plant tissue culture, pigment, antioxydant, betanin, *Beta vulgaris*

Abbreviations: **DOPA** – 3,4-dihydroxyphenylalanine, **HB** – high betacyanin, **YE** – yeast extract, **Suc** – sucrose

Introduction

Betalains are a class of water soluble nitrogenous plant pigments characteristic of the order Caryophyllales (GIROD and ZRYD 1991, TANAKA et al. 2008). They are classified into red (crimson) betacyanins and yellow betaxanthins. Betalain biosynthesis starts with

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amino acid tyrosine and continues through two independent pathways from DOPA; the betalamic acid biosynthetic pathway and the cDOPA synthetic pathway. Betalamic acid is the chromophore molecule of both betacyanins and betaxanthins, and cDOPA and its derivatives are essential to betacyanin production (STRACK et al. 2003). The addition of amines or amino acids such as betalamic acid conjugates produces numerous betaxanthins. However, in comparison to flavonoids and carotenoids, biosynthetic pathways of betalains, enzymes and genes involved in the pathway are much less understood (TANAKA et al. 2008). Betalains have aroused a huge interest in the food industry as a natural food colorant as well as in biopharmaceuticals. Their advantages as food colorants are that the color does not depend on the pH, and their stability (TANAKA et al. 2008). Additionally, betalains are up to twice fold better free radical scavenger than some anthocyanins (LEE et al. 2005, GLISZCZYŃSKA-ŚWIŁGO et al. 2006). Moreover, they have desirable bioactive properties: they have an anti-inflammatory effect, they activate phase II detoxifying enzymes, or they can protect low density lipoproteins from oxidation (TESORIERE et al. 2004, LEE et al. 2005, GLISZCZYŃSKA-ŚWIŁGO et al. 2006).

The most important commercial source of betalains is red beetroot (*Beta vulgaris* L.), which mostly contains betanin, a violet-red betacyanin. The yield and quality of betanin obtained from red beet root depends on field conditions and can be significantly lowered by lack of minerals, drought, salinity, pathogen attacks or even by an unfavorable political climate (RAMACHANDRA RAO and RAVISHANKAR 2002). Plant cell and tissue cultures are attractive alternative sources of bioactive plant substances, including betalain pigments. Such a type of production offers several advantages over field cultivation, notably optimal and stable growth conditions, possibility for voluntary modulation of growth parameters and constant quality control (MORENO et al. 2008). In vitro systems for producing betanin include callus cultures, cell suspensions of red beet root and hairy roots transformants of red beet root, but the yield of betanin is still lower than in field-raised red beet (GEORGIEV et al. 2008). New approaches are being tested to enhance betanin production in in vitro systems. This involves optimization of nutrient medium, elicitors – molecules of biological or non biological origin which effectively induce secondary metabolite production, or reverse sequestration using a »permeabilization, release and recovery« approach with limited oxygen supply, sonication or the use of detergents (AKITA et al. 2002, THIMMARAJU et al. 2003, UOZUMI 2004, SAVITHA et al. 2006).

We transformed sugar beet cells by infecting leaf fragments with wild strain B6S3 of *Agrobacterium tumefaciens*. Subsequently a cell line characterized by high betanin production was established (PAVOKOVIĆ et al. 2009). This tumor tissue stably maintained its phenotype during more than 10 years of subculturing. The betanin production was light-dependent. When placed in darkness, the tumor stopped producing betanin and became colorless after few subcultures. A switch from the red-violet to white phenotype was reversible (PAVOKOVIĆ et al. 2009).

The aim of present study was to optimize the betanin yield of the transformed cells by modifying the composition of nutrient media and by using elicitors. Sucrose is the best carbohydrate source as it gives the highest betalain production and yield (MONROY et al. 1994, BHAGYALAKSHMI et al. 2004). Yeast extract is frequently used as a widely available and successful biotic elicitor in a number of systems, including red beet hairy root transformants

(SÁNCHEZ-SAMPEDRO et al. 2005, SAVITHA et al. 2006, SHINDE et al. 2009) while calcium is the most successful among abiotic elicitors in elevating betalain productivity, as was recently shown (SAVITHA et al. 2006).

Material and methods

Sugar beet tumor tissue was obtained by transformation of leaf fragments by *A. tumefaciens* wild strain B6S3 (PAVOKOVIĆ et al. 2009). Tissue was cultivated on a hormone-free PG0 medium supplemented with 3% (w/v) Suc and solidified by 0.9% (w/v) agar (NEGRUTIU et al. 1975). The light (photon density of 42 mmol m^{-2} s^{-1}) / dark photoperiod was 16 / 8 hours. Cell suspensions were initiated by transferring 2 g of very friable callus to 50 mL of PG0 medium supplemented with 3% Suc. Suspensions were shaken on reciprocal shaker at 100 rpm under the same light/dark conditions as cultures on solid medium. To initiate the experiment, two-week-old cell suspensions (10 mL of packed cells volume) were added to the flask containing 35 mL of the PG0 or improved Linsmeier-Skoog medium (HB, Tab. 1) (AKITA et al. 2002). Control suspensions were supplemented with 3% Suc, while treatments were with 4%, 5% or 6% (w/v) Suc. Cells were collected at days 4, 7, 10, 14 and 21 of subculture and were weighed, lyophilized and stored until usage. Yeast extract (Sigma, Germany) as elicitor was dissolved in HB medium with 5% Suc at 0.25 and 1.25% (w/v) concentration. Calcium, as $CaCl_2$, was dissolved at 10-fold higher concentration than in HB medium. After seven days of subculture in HB with 5% Suc, old

Tab. 1. Concentrations of inorganic salts in PG0, LS and HB media, expressed in mmol L^{-1}.

Component	PG0	LS medium	HB medium
	Macroelements		
$NaH_2PO_4 \times H_2O$	1.81	–	–
KCl	8.05	–	–
NH_4NO_3	–	20.6	2.0
$(NH_4)_2SO_4$	3.03	–	–
KNO_3	19.20	18.8	18.8
$NaNO_3$	–	–	7.2
$MgSO_4 \cdot 7H_2O$	2.03	1.5	1.5
$CaCl_2$	2.04	3.0	3.0
KH_2PO_4	0.1	1.25	1.25
Na_2Fe-EDTA		0.1	0.1
	Microelements		
H_3BO_3	0.01	0.1	0.1
$MnSO_4 \cdot 4H_2O$	0.01	0.1	0.1
$ZnSO_4 \cdot 7H_2O$	0.03	0.03	0.0003
KI	0.09	0.005	0.005
$Na_2MoO_4 \cdot 2H_2O$	0.0001	0.001	0.001
$CuSO_4 \cdot 5H_2O$	0.00001	0.0001	–
$CoCl_2 \cdot 6H_2O$	0.00001	0.0001	–

medium was removed from cell suspensions and 50 mL of medium with elicitors was added to the cells (SAVITHA et al. 2006). Cell suspensions were returned to the shaker and samples were collected at days 4, 7, 10 and 14 after elicitation, and processed as mentioned above.

For betanin quantification, 20 mg of the lyophilized sugar beet tumor cells were pulverized in a bead mill homogenizer (Retsch MM 200, Retsch GmbH, Germany) and resuspended in 1 mL of 80% methanol (v/v) supplemented with 50 mM ascorbic acid. Homogenates were clarified by centrifugation for 20 minutes at 15000 × *g*. Pigments were reextracted twice with additional 2 mL of the same buffer. Extracts were joined and the absorbance was measured spectrophotometrically at 534 nm (ATI/Unicam UV4-100, Cambridge, UK). Betanin was quantified using molar absorption coefficient of betanin $\varepsilon = 60\ 000\ L\ cm^{-1}\ mol^{-1}$.

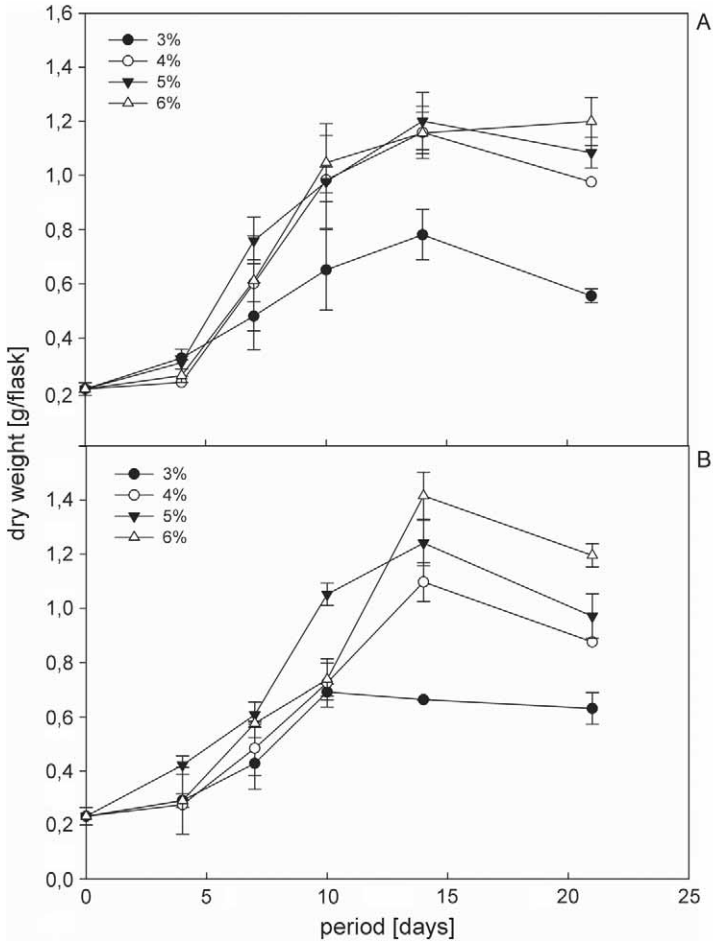

Fig. 1. Biomass accumulation of sugar beet tumor line grown using 0.8 g inoculum in 50 mL of A) PG0 or B) HB liquid medium with sucrose concentrations ranging from 3% to 6% (w/v). Each data point is a result from two independent experiments with 4 replicates ±SD.

Results

A sugar beet tumor line producing betanin was subcultured in liquid PG0 medium with 3% (w/v) Suc. In this control medium, exponential growth of the cells began after day 4 and continued until day 14 when the growth ceased (Fig. 1A). Elevation of Suc concentration in the PG0 from 3% to 4%, 5% or 6% resulted in the increased dry biomass (Fig. 1A). Maximum biomass at these concentrations was produced at day 14, except on 6% Suc where the highest biomass was accumulated at day 21. Changing nutrient composition from PG0 to HB induced slightly higher accumulation of dry biomass (Tab. 1, Fig. 1B). The positive influence of Suc on dry biomass was apparent after 10 days of subculture in both media, but only between 3% and the higher concentrations of Suc.

An increase in Suc concentration increased betanin content in both media at days 4 (Fig. 2). At day 7 differences in betanin content were observed between 4% and 3% Suc on PG0 and between 6% and 3% on HB, and at day 10 only between 6% and 3% Suc on HB. Afterwards, the trend reversed, and a higher Suc concentration negatively affected betanin content. Compared to PG0, HB medium increased the pigment content in only a few instances: at day 4 with 4% Suc and at day 21 with 4 and 6% Suc.

The resulting effect of increase of Suc concentration was visible as a higher total betanin yield at days 7 and 10 of the subculture on PG0, and at days 4, 7, and 10 of the sub culture on HB (Fig. 3). When compared to PG0, HB medium produced higher pigment

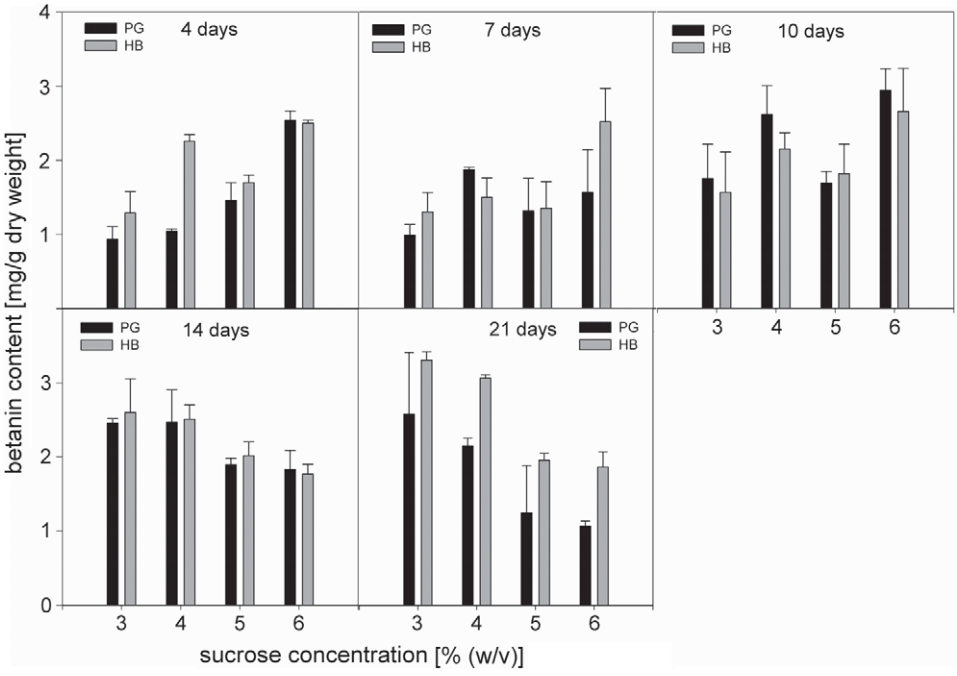

Fig. 2. Betanin content of sugar beet tumor line subcultured during 21 days in either PG0 or HB medium in relation to 3 – 6% (w/v) sucrose. Each data point is a result from two independent experiments with 4 replicates ±SD.

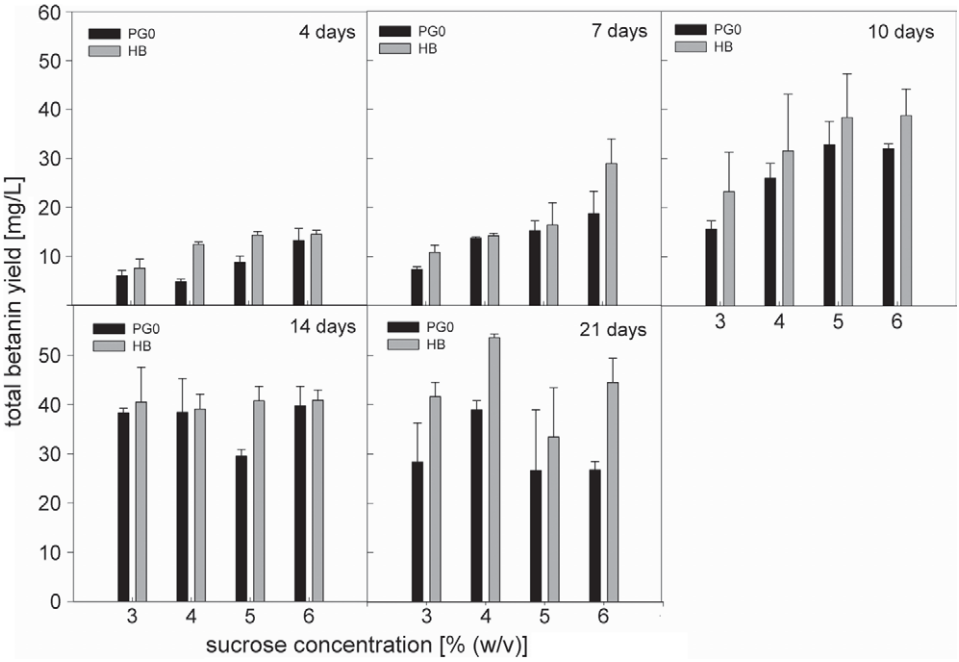

Fig. 3. Total betanin yield of sugar beet tumor line subcultured during 21 days in either PG0 or HB medium in relation to 3 – 6% (w/v) sucrose. Each data point is a result from two independent experiments with 4 replicates ±SD.

yield at day 4 with 4% and 5% Suc, at day 7 and 14 with 6% of Suc. The highest total betanin yield was obtained in HB medium and was 40 mg L^{-1} at day 10 and 14 at maximum Suc concentration and 50 mg L^{-1} at day 21 with 4% Suc.

As elicitors of betanin biosynthesis, calcium at 10-fold of the normal concentration, and YE at 0.25 and 1.25% (w/v) were used for cells grown in the HB medium supplemented with 5% Suc (Fig. 4). YE at 0.25% and Ca did not significantly improve betanin yield. YE at 1.25% provoked expulsion of betanin in liquid medium which decreased betanin yield. Cell necrosis followed 7 days after elicitation with higher concentration of YE.

Discussion

Previously, it was shown that a sugar beet cell line transformed with *A. tumefaciens* wild type B6S3 produced betanin (PAVOKOVIĆ et al. 2009). No phenotype changes were observed during more than ten years of subculturing of the tumor tissue. Suc was a better source of carbohydrates for high betanin yield than monosaccharides glucose, fructose or their combination (PAVOKOVIĆ et al. 2009). In order to optimize betanin content and yield, cell suspensions from the transformed line were initiated and cells were incubated in increasing concentrations of Suc, in different composition of mineral nutrients and using biotic and abiotic elicitors.

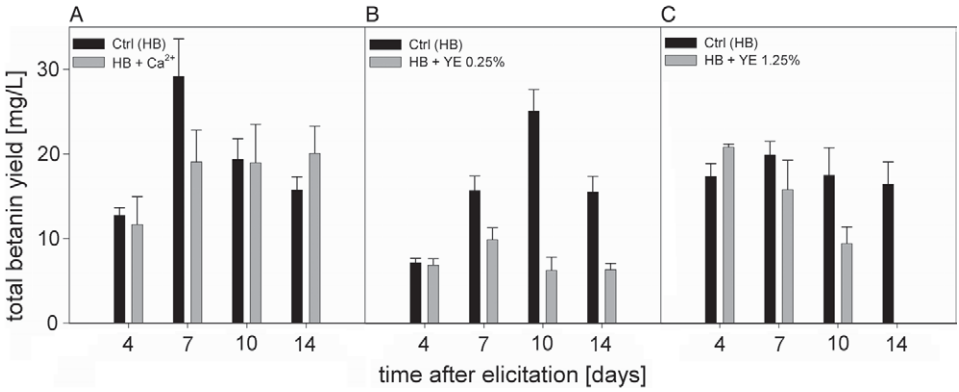

Fig. 4. Total betanin yield of sugar beet tumor line grown in HB medium with 5% sucrose for 7 days and treated with either: A) Calcium at 10 fold concentration than in HB medium. B) 0.25 % (w/v) yeast extract. C) 1.25 % yeast extract. Each data point is a result from two independent

Increase of Suc concentration from 3% to 4%, 5% or 6% had a positive effect on biomass accumulation and total betanin yield during subculture (Figs. 1, 3). Suc concentrations higher than 6% did not additionally increase betanin yield and were not used (AKITA et al. 2002). When Linsmeier-Skoog medium optimized for betanin production was used instead of PG0, a slight increase in biomass and betanin yield was observed on few instances (Tab. 1, Figs. 1, 3). These improvements were most likely due to the modification of ratio of ammonium to nitrate ions (1:14), reduction of zinc ions and complete removal of cobalt and copper ions from the medium (AKITA et al. 2002).

The effect of increasing Suc concentrations on betanin content was complex. During the first days of subculture, a higher Suc concentration increased betanin content, but this trend quickly changed and after 10 days high Suc negatively affected betanin content (Fig. 2). The initial and positive effect of high Suc on betanin yield could be a result of the high osmolarity of the medium which caused an osmotic stress on the cells. It is known that this type of stress can stimulate production of secondary metabolites such as anthocyanins in *Vitis vinifera* L. cv (grape) cells, saponins in *Panax notoginseng* cells or alkaloids in *Catharanthus roseus* cells (DO and CORMIER 1990, ZHANG et al. 1995, GODOY-HERNÁNDEZ et al. 2000). On the other hand, the inhibitory effect of Suc on betanin accumulation after 10 days was also reported in cell suspensions of *Beta vulgaris*, *Phytolacca americana* and *Chenopodium rubrum* (BERLIN et al. 1986, SAKUTA et al. 1987, AKITA et al. 2002). A possible explanation lies in the positive correlation of betacyanin accumulation and cell division (HIROSE et al. 1990, SAKUTA et al. 1994), where betacyanins are produced during intensive cell division. When a number of cell divisions was reduced, as in the stationary phase, betanin accumulation was also decreased (Fig. 3). This suggests that the main factor contributing to increased betanin yield with high Suc concentrations could be the increase in cell number, rather than increased accumulation of betanin per cell.

Production of secondary metabolites can be enhanced even further using abiotic or biotic elicitors. Calcium ions, at 10 fold higher concentration than found in HB medium, were used as they displayed the highest potential as abiotic elicitors of betanin production in hairy roots of red beet (SAVITHA et al. 2006). In our system, however, calcium failed to

provoke similar cellular response (Fig. 4). Calcium acts as the secondary messenger in signaling the responses following elicitation (NISHI 1994) but is also a messenger in a number of other signaling processes involving cellular growth, development, redox homeostasis, or light signal transduction (DEMIDCHIK and MAATHUIS 2007, TUTEJA and MAHAJAN 2007, YANG 2008), which might impede yield of betanin. Yeast extract at 0.25 and 1.25% failed to increase and decreased yield of betanin, respectively (Fig. 3). The same concentrations of YE were only marginally successful in increasing betanin yield in red beet hairy roots (SAVITHA et al. 2006). However, the higher concentration of YE used was toxic. It caused extraction of the pigment from vacuole to extracellular space and after 7 days of elicitation it induced cell necrosis. Although toxic, this effect of YE could be used for repeated sequestration of betanin. In such a process, cells are briefly introduced to a tightly controlled stressor, such as abrupt changes of pH, sonication, temperature shock or hypoxia (THIMMARAJU et al. 2003). This causes non-lethal expulsion of pigments from the cells to medium which can be harvested. Then the conditions of cultivation are returned to the original state and pigments can be harvested again.

Taken together, betanin yield was successfully induced in sugar beet tumor cells mostly by increasing carbohydrate concentration and to a lesser extent by modifying composition of nutrient medium. While our results are comparable with in vitro red beet production systems (AKITA et al. 2002, PAVLOV et al. 2005), they are lower than red beet hairy root transformants (SAVITHA et al. 2006) suggesting further testing, involving a broader number of elicitors or betanin precursor feeding should be performed to additionally increase the betanin content and yield of the tumor cells.

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