Romanian Biotechnological Letters *Vol. 15, No.1, 2010*
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ORIGINAL PAPER

Two–stage system, a possible strategy for the enhancement of anthocyanin biosynthesis in a *long-term* grape callus cultures

Received for publication, September 15, 2009
Accepted, February 25, 2010

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Abstract

Polyphenols, including anthocyanins, are an essential part of human diet and constitute one of the most abundant and ubiquitous group of plant secondary metabolites. Their level is inducible by different types of stress such as fungal infections, but in the in vitro conditions the accumulation of the polyphenols can be stimulated by biotic and abiotic elicitors. In the present study an attempt has been made to maximize the long –term Vitis vinifera L. callus culture growth and anthocyanin biosynthetic capacity by optimizing the hormonal combinations in the growth medium in a two stage culture system. Different concentrations and combinations of elicitors such as: salycilic acid (SA), abscisic acid (ABA), jasmonic acid (JA) and manitol (MAN) were used. Anthocyanin accumulation was greatly enhanced by using medium variants SA-ABA (SA 10µm, ABA 10 µm), JA5-JA5 (5 µm) and JA20-JA20 (20 µm). Two of these medium variants, respectively JA5-JA5 and JA20-JA20, proved to determine the highest accumulation of browning anthocyanins in the callus cultures, while the medium containing SA-ABA determined the lowest one. In conclusion, the medium variant SA-ABA in a two stage culture system could be used as an effective strategy for enhancing the anthocyanin productivity of the long-term Vitis vinifera callus culture.

Key words: *Vitis vinifera* non-morphogenic callus, elicitors, a two-stage culture system, anthocyanins.

Introduction

Anthocyanins are a major subclass of flavonoid compounds existing widely in flowers, fruits and vegetables. Many reports have explored the relationship between flower colors and pigments composition [1]. Apart from their contribution in pigmentation, they have been receiving considerable attention due to their wide range of biological activities, including antioxidant, anti-inflammatory, antiallergic, antiulcer, antibiotic and anticarcinogenic properties [2].

Plant cell culture represents an attractive alternative method for overcoming the limitations of extracting useful metabolites from natural resources. Among recent advances in plant biotechnology, there have been a number of reports on the production of anthocyanins in various plant tissue cultures. Many factors affect cell growth and the production of these metabolites in the *in vitro* systems [3].

Recently, many studies on anthocyanin production have been reported concerned not only with producing anthocyanins on the large scale, but also with the investigation of various factors influencing anthocyanin accumulation in suspension and in callus cultures, such as UV, light, nitrogen source, osmotic stress and elicitors [4].

The elicitation process makes use of the capacity of plants and plant cell cultivated *in vitro* to react to various stress stimuli by a number of protective reactions, leading to increased accumulation of secondary metabolites. The induction of anthocyanin biosynthesis using elicitors has gained special interest in biotechnological approaches to improve the production of these secondary metabolites [5].

The endogen signal substances of plant protective reactions: jasmonic acid (JA), and salicylic acid (SA), act as elicitors in case of exogenous application [6]. The JA is generally regarded as an integral signal or elicitor signal transducer in the induction of a wide range of plant secondary metabolites, such as alkaloids, terpenoids, flavonoids and phenolic compounds [7]. This idea has been studied in several plant species by elucidating a close relationship between JA biosynthesis and secondary metabolite accumulation induced by elicitors [8]. Our previous studies revealed stimulatory effects of JA and SA elicitors on proliferative and biosynthetic capacity of *a long-term Vitis vinifera* callus culture [9]. In this paper we present another experimental model for modulation of these processes using a two stage culture system. In the first stage the possibility to stimulate the cellular mass proliferation was analyzed followed by the second one, where effects of different concentrations and combinations of elicitors under study were tested for the stimulation of anthocyanin biosynthesis.

Materials and methods

Plant Material and Growth Conditions

For this experiment the source of inocula consists in a stock culture of a *long- term* callus from the Institute of Biology collection, previously initiated from immature pericarp of grape berries (*V. vinifera L.* cvs. Isabelle) [10].

The cell line was maintained by periodic subcultivations (once a month) on a variant of basal Gamborg-B5 (1968) medium, supplemented with 0.1 mg/l NAA (α-naphtalene acetic acid), 0.2 mg/l kinetine, 2 g/l casein hidrolisate, 30g/l sucrose, 8g/l agar (Difco), which has been referred as control.

We established an experimental system in two stages for testing the effect of different elicitors on these callus cultures. Eight variants of the experimental medium were analyzed (Table 1) for 37 days and each treatment was repeated 12 times. For the first stage of the experiment the *long- term* callus was grown 30 days on specific medium variants to ensure the callus proliferation. Further subcultivation of the callus for 7 days during the second stage of the experiment ensures the anthocyanin biosynthesis.

Table 1. Types of elicitors and their concentrations used in the two stage culture system.

EXPERIMENTAL MEDIUM VARIANTS	FIRST STAGE					SECOND STAGE				
	MANITOL (mM)	ABSCISIC ACID (μM)	SALYCILIC ACID (μM)	JASMONIC ACID (μM)	METHANOL (ml)	MANITOL (mM)	ABSCISIC ACID (μM)	SALYCILIC ACID (μM)	JASMONIC ACID (μM)	METHANOL (ml)
CONTROL	-	-	-	-	-	-	-	-	-	-
BLANK	-	-	-	-	1	-	-		-	1
SA-ABA	-	-	10	-	1	-	10	-	-	1
SA-JA	-	-	10	-	1	-	-	-	20	1
SA-SA	-	-	10	-	1	-	-	10	-	1
MAN-MAN	2	-	-	-	1	2	-	-	-	1
JA5-JA5	-	-	-	5	1	-	-	-	5	1
JA20-JA20	-	-	-	20	1	-	-	-	20	1

Morphometric parameters

Rate of growth was represented as the fresh weight of callus grown for 37 days on different medium variants starting from the idea that all treatments were initiated from callus

samples with fresh weight of 1g. The water content was calculated as difference between the fresh and the dry weight expressed as percentage from fresh weight.

Squash samples of fresh cell callus cultures were made and analyzed in light microscopy. Electron-microscopic analyses of the callus cells were performed according to the standard methods [11] using an EM-125 (Selmi-Ucraina) electron microscope.

Sample preparation and extraction of anthocyanins

Four samples of 2 g each of callus from the eight medium variants were grounded with a mortar and pestle. The disintegrated samples were extracted for 24 h with 2 ml MeOH solution containing 0.1% HCl in refrigerator, at 4^0C in a dark environment. The extract was centrifuged (4000 rpm, 15 min) and the clear supernatant was collected and used for determination of total phenolic compounds, total monomeric anthocyanin along with the indices for pigment degradation, polymeric color and browning.

Determination of total phenolic compounds

Folin Ciocalteu reagent was used to determine total phenolic compounds. A volume of 1 ml *Vitis vinifera* callus extract, diluted 5-10X with methanol (to obtain absorbance within the range of the prepared calibration curve) was mixed with 5 ml of Folin-Ciocalteu reagent (Sigma) previously diluted with distilled water. A volume of 4 ml 7.5% sodium carbonate solution was added to the mixture was let to stand for 30 min and the blue color formed was measured at 765 nm with a spectrophotometer (Hellios Gamma Thermo-Scientific). Gallic acid was used as a standard for calibration curve. The total mass fraction of phenolic compounds was calculated and expressed as gallic acid equivalent GAE/ fresh weight (mg/100 g).

Determination of total monomeric anthocyanin pigment

Monomeric anthocyanin pigments reversibly change color in a pH range from colored oxonium form which exists at pH 1.0, to the colorless hemiketal form which predominates at pH 4.5. The difference in the absorbance of the pigments at 520 nm is proportional to the pigment concentration. Results are expressed on cyaniding-3-glucoside basis as follows:

$$\text{Anthocyanin pigment (mg/l)} = \frac{A \times MW \times DF \times 10^3}{\varepsilon \times 1}$$

where $A = (A_{520nm}-A_{700nm})$ pH 1.0- $(A_{520nm}-A_{700nm})$ pH 4.5; where MW (molecular weight) = 449.2g/mol for cyanidin-3-glucoside (cyd-3-glu); DF = dilution factor established; 1 = pathlenght in cm; ε = 26900 molar extinction coefficient, in L X mol^{-1} X cm^{-1}, for cyd-3-glu; and 10^3 = factor for conversion from g to mg.

Degraded anthocyanins in the polymeric form are resistant to color change regardless of the pH and are not included in the measurements because they absorb at pH 4.5 as well as pH 1.0.

Indices for pigment degradation, polymeric color and browning

Indices for anthocyanin degradation can be derived from a few absorbance readings of a sample that has been treated with 0.68M sodium bisulfite solution. Anthocyanin pigments will combine with bisulfite to form a colorless sulfonic acid adduct. Polymerized colored anthocyanin-tannin complexes are resistant to bleching, wereas the bleaching reaction of monomeric anthocyanins will rapidly go to completion. The difference in absorbance at 420 nm, 520 nm and 700 nm of the bisulfite- treated sample serves as an index for browning. The ratio between monomeric and polymerized anthocyanin complexes can be used to determine the degradation index.

$$\text{Index degradation} = \frac{(A_{420}-A_{700})+(A_{520}-A_{700}) \times DF \text{ in water}}{(A_{420}-A_{700})+(A_{520}-A_{700}) \times DF \text{ in bisulfite}} \times 100$$

Results and discussions

Effects of medium composition on the callus growth.

The highest value of the callus growth was achieved by using medium variants SA-ABA, SA-SA and MAN-MAN, while in case of media supplemented with JA (5 and 20 µM) the rate of growth was diminished by using these elicitor concentrations. It seems that SA stimulates the callus growth like it was demonstrated by previous experiments where a media supplemented with 10 µM SA promoted an increase of the callus mass [9].

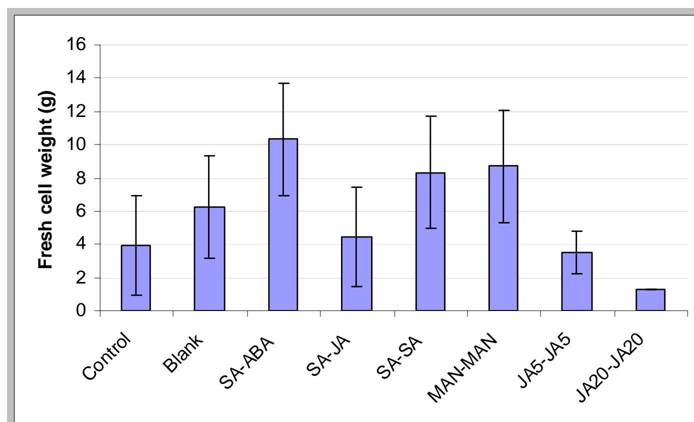

Figure 1. Fresh weight of *Vitis vinifera* callus biomass cultivated on the control and on the media supplemented with Methanol (Blank), SA, MAN, ABA, JA, at different concentrations, in a two stage experiment, after 37 days of culture. Each value represents the mean of 12 replicates; vertical lines represent standard error of replicates.

JA inhibited rate of callus growth, a fact demonstrated on the medium variants supplemented with JA, where the cell proliferation was lower than on the Control medium. The inhibitory effect of JA is emphasized also by the callus cultivation on a two stages experiment (initially on media supplemented with SA and further on JA) where the rate of growth was diminished compared with the rest of variants containing SA. Probably the initial callus proliferation was promoted by SA, but a further subcultivation on JA media induces an inhibition of the growth. Also the addition of methanol in the culture media for the elicitor dissolving had a positive effect on the callus growth.

The water content of the callus was at the highest level on the variants cultivated initially on SA, which demonstrates that SA promotes the water accumulation in the cells. A high content of the water could be also determined in case of the cultivation on medium variants supplemented with MAN, though the MAN was used as osmotic agent in this experiment. Regulation of the osmotic potential of culture medium was proved to be useful in controlling anthocyanin production. Increasing the osmotic potential of the medium in case of *V. vinifera* L. cv. Gamay Freaux cell suspension resulted in a significant increase in accumulation of anthocyanins in pigmented cells [12]. High MAN concentrations in the medium play a physical role as an osmotically active solute [13].

Instead, in our study the MAN in a concentration of 2 mM did not positively influence the increase of the anthocyanin biosynthetic grape callus capacity (Figure 6). An explanation

for this effect could be that the concentration 2mM is insufficient to promote a high osmotic stress that is capable to positively influence the anthocyanin accumulation. Instead this concentration induced an increase of the callus growth and water content, presumably due to its nutritional role as a carbon source.

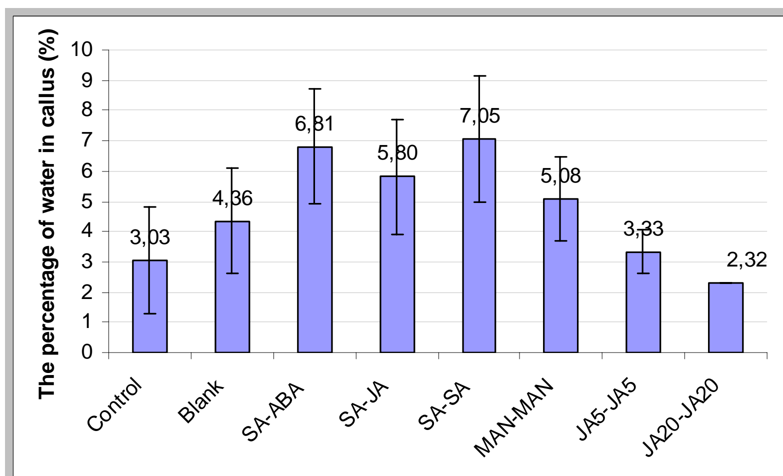

Figure 2. The percentages of water content in the calli grown on all the medium variants. Each value represents the mean of 4 replicates; vertical lines represent standard error of replicates.

Morphological characteristics of callus cells on different media composition

The squash analyses on callus grown on media supplemented with elicitors showed their influence on proliferative capacity and also on the accumulation of colored secondary metabolites as anthocyanins at the cell level.

The callus cultured on Control variant has developed like a mass of cells with moderate proliferative capacity, predominantly superficially colored. The microscopical observations of fresh cell culture (squash probe) cultivated on Control variant revealed the presence of cellular aggregates with different shapes and a heterogeneous pigment accumulation.

In the presence of SA the callus appeared as a mass of predominantly pale red cells with a high proliferative capacity, heaving a hard texture. Only in some areas could be identified small islands with a very intense red color. The squash sample revealed that the callus is composed of heterogenous cell populations, having both spherical and elongated shapes. There are cells with a small level of pigment accumulation. The biosynthesis of phenolic compounds is not restricted only at one type of cells, this being evident by the presence of anthocyanin pigments both in senescent and in post-dividing cells.

Under the influence of MAN, the callus appeared as a mass of cells with significant proliferative capacity having large zones with cells very intense colored both at callus surface and on the deepness of it till the base. The squash probe revealed in callus samples the presence of very heterogeneous cells from morphologic point of view, having an oval, oblong or reniforme shape with a high accumulation of anthocyanins (Figure 3).

The callus developed on the medium containing JA was characterized by a moderate proliferative capacity, a soft texture with a very intense red color spread throughout, both on the surface and in the deepness of the callus mass. The squash samples of the callus cultured in the presence of JA in a concentration of 5 μM revealed the presence of very elongated and spherical cells of various sizes having an important pigment accumulation (Figure 3).

Regarding the pigment accumulation the same observation could be made in case of the callus grown on medium variant SA-JA (Figure 3).

The high percentage of the cells with the anthocyanin accumulation and a very intense and uniform pigmentation throughout the cell mass was observed in the callus grown on the medium variant JA20-JA20 (Figure 3).

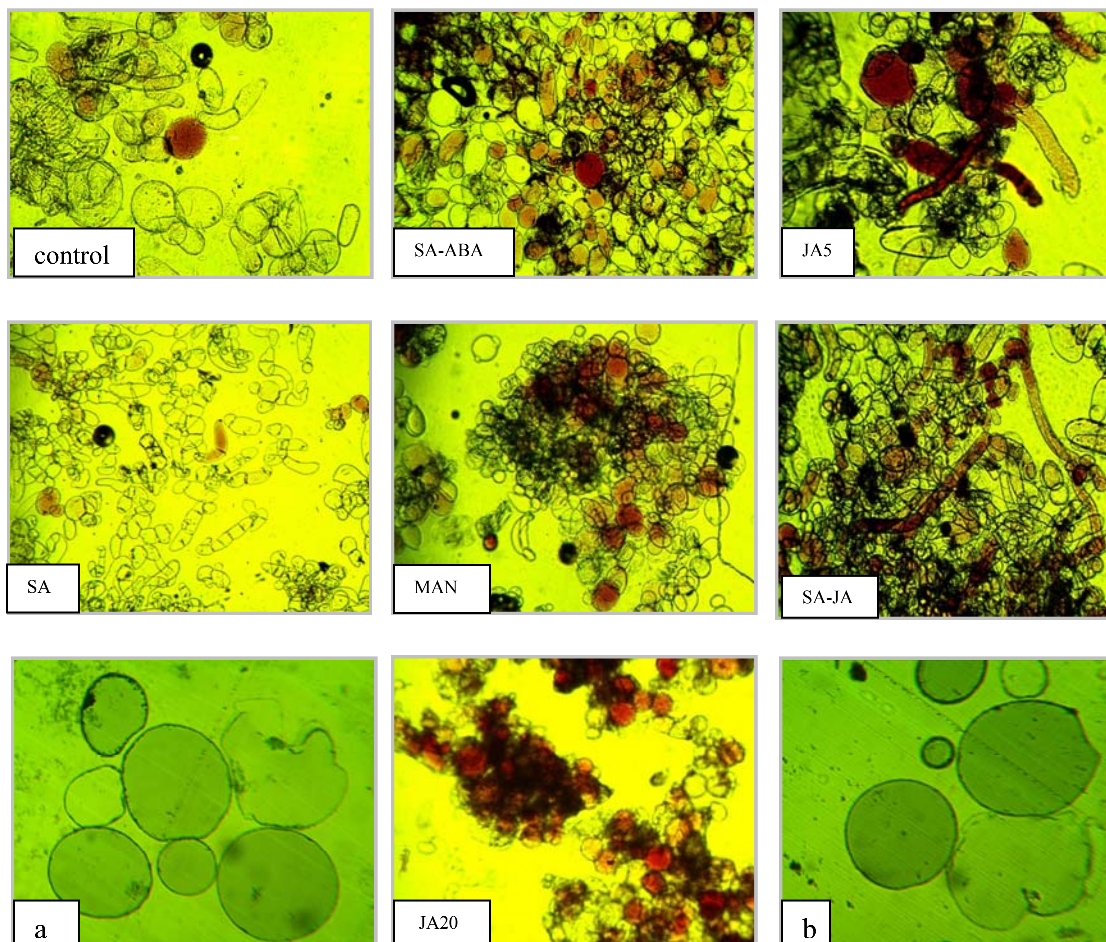

Figure 3. The most representative appearance of callus cells in squash probes and in semifine sections, function of different variants of medium.

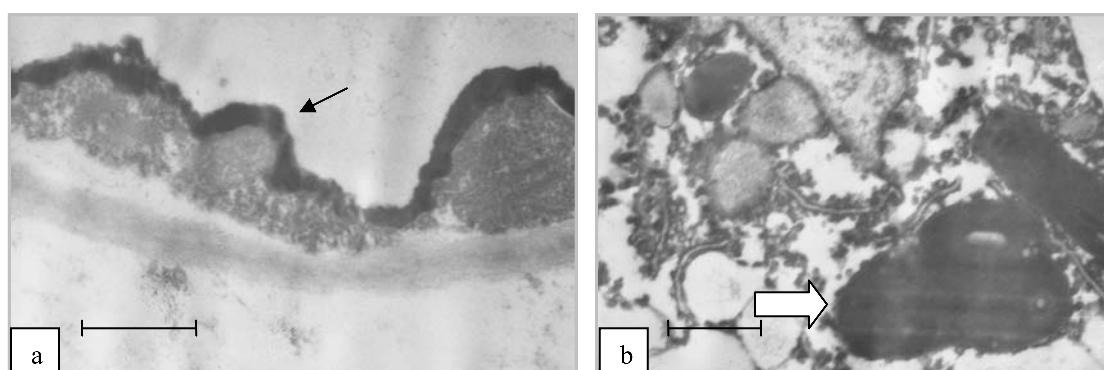

Figure 4. Ultrathin sections through callus mass revealing anthocyanins biosynthesis. Deposition of electron dense formations along tonoplast membrane (see arrow) and inside plastids displaying high electron dense stroma (see double arrow). The scale bar is 1µm.

At subcellular level, cells involved in anthocyanin biosynthesis present a specific peculiarity according to their specialization (Figure 4). Polymorphic plastids with high electron dense stroma and intra thylacoidal space which present phenolic- like accumulations can be observed. Numerous profiles of endoplasmic reticulum and Golgi bodies as well as electron-dense particles in cytoplasm are present (Fig.4b). Frequently the tonoplast is boarded by electron - dense material of phenolic nature (Fig. 4a).

The total phenolic content of callus cultivated on media supplemented with elicitors.

Total phenolic content in the *long-term* callus was determined as gallic acid equivalents reported to the callus mass and showed an increase of phenolic compounds production for all experimental variants, compared to the control. An important production of phenolic concentration was determined by the addition of JA to the culture media while low concentration of phenolic products was observed at the initial cultivation stage on SA media. Initial addition of methanol lead to the increase of phenolic compounds synthesis, while the cultivation on SA and after the transfer on SA and ABA determined a decrease in the phenol accumulation. A similar behavior could be observed in case of cultivation on MAN.

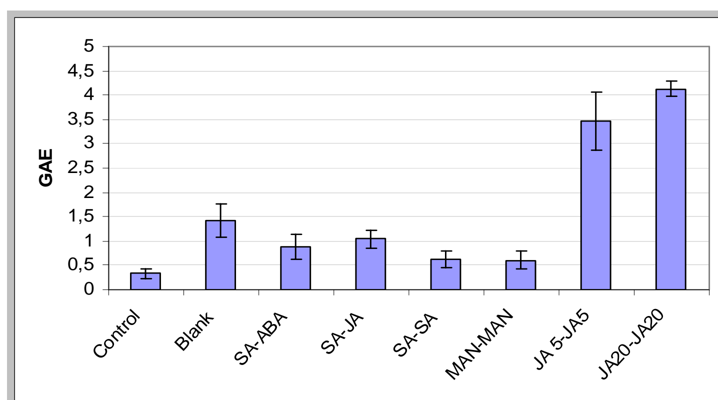

Figure 5. The total mass fraction of phenolic compounds present in the calli grown on different variants of culture media, expressed as gallic acid equivalent GAE/ (mg/100 g). Each value represents the mean of 4 replicates; vertical lines represent standard error of replicates.

Total phenolic compounds production was significantly greater when the growth regulator JA at 5 and 20 μM concentrations was used, compared to the variants containing SA, SA-ABA, SA-JA and MAN. (Figure 5).

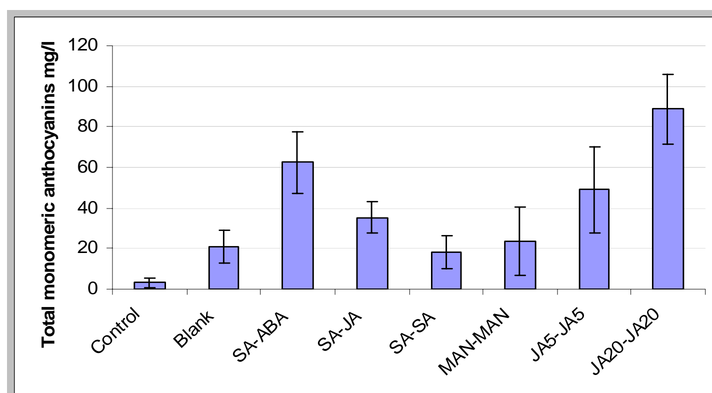

Figure 6. Values of the total monomeric anthocyanins in *long- term* calli grown on all the medium variants. Each value represents the mean of 4 replicates; vertical lines represent standard error of replicates.

The total content of monomeric anthocyanins showed a high accumulation of these compounds in calli grown on media supplemented with JA and ABA. In this context we can draw the idea that SA inhibited accumulation of anthocyanins, while the JA promoted the anthocyanin biosynthesis. Likewise seems that the elicitor ABA has a positive effect on anthocyanin biosynthesis while MAN does not induce the pigment accumulation. The value determined is almost equal with that determined for the callus grown on medium with methanol.

Figure 6 summarizes values obtained by using the pH-differential method. The values representing the monomeric anthocyanins content of the callus grown on medium SA-ABA and JA20-JA20 were elevated in comparison with the values of the callus grown on the other medium variants, especially on the control. Instead, the callus grown on JA20-JA20 has also one of the highest content of browning anthocyanins revealed by the determination of the indices for anthocyanin degradation (Fig.7). This method reveled also a very low concentration of degraded anthocyanins in the calli grown on medium variant SA-ABA, which makes this variant a potent growth medium for the enhancement of the anthocyanin production.

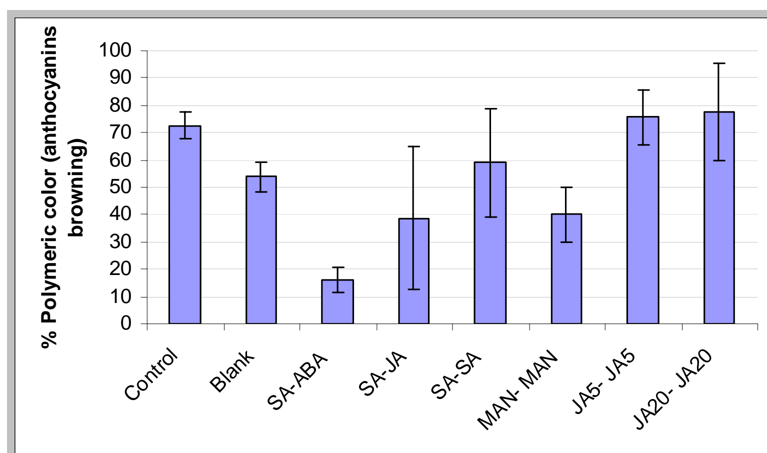

Figure 7. The browning anthocyanin content in the callus grown on the medium variants tested. Each value represents the mean of 4 replicates; vertical lines represent standard error of replicates.

As for *the browning/polymerized anthocyanins production* from the total anthocyanin content, high values were recorded on the medium variants supplemented with JA, compared to the Control medium. For all the other experimental variants the percent of polymerized anthocyanins decreased. Probably ABA, MAN and SA determine the inhibition of anthocyanin oxidation. The lower values were expressed on the variant SA-ABA which presents in parallel high monomeric anhocyanin content and high proliferative rate. This suggests that the two stage culture system with the variant SA-ABA represents the optimum alternative among all the tested ones, by promoting both the cell proliferation and the enhancement of the antocyanin accumulation in our callus culture system.

Conclusions

In this experiment a two-stage culture system was used, including a growth stage for cell proliferation and a production stage for the biosynthesis of anthocyanin pigments. In the first growth stage the callus was maintained on a variant of basal Gamborg-B5 (1968)

medium supplemented with different elicitors and growth regulators.

The enhancement of callus growth was especially achieved by using the medium variants containing the elicitors SA (10µM) or MAN (2mM). The results of the monomeric anthocyanin and the total phenolic content determinations, after the production stage, revealed that the treatment with the elicitor JA in two concentrations, respectively 5 and 20 µM, triggers out an important enhancement of these two values. Medium containing SA (10µM)-ABA (10µM) also induces an increase of the pigment content in the callus mass along with the smallest quantity of browning/polymerized anthocyanins.

Based on our experimental data we may conclude that the two stage culture system having SA (10µM) in the growth stage and ABA (10 µM) in the production stage, could become an experimental model for the stimulation of the *in vitro* anthocyanin biosynthetic processes in long-term callus and cell cultures of *Vitis vinifera L.*

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