Aspects of some elicitors influence on non-morphogenic callus of *Vitis vinifera* var. Isabelle

Received for publication, December 20, 2009
Accepted, June 5, 2009

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Abstract

The aim of this investigation consists in testing the influence of different elicitors on a non-morphogenic callus of *Vitis vinifera* var. Isabelle regarding both the anthocyanin biosynthesis and the rate of cellular proliferation.

Taking into account these objectives we have studied in vitro experimental systems using different concentration of elicitors such as: manitol, abscisic acid, salicylic acid and jasmonic acid.

Based on a comparative gravimetric analysis after 30 days of culture in systems supplemented with different concentration of elicitors, we observed that 10 μmol salicylic acid has an important influence on the callus proliferation comparing to the control. Concentration of 10 μmol jasmonic acid demonstrates a positive influence on the optimization of the anthocyanin yield in this experimental system.

Keywords: non-morphogenic callus, *Vitis vinifera*, jasmonic acid, abscisic acid, salicylic acid, manitol.

Introduction

Plants synthesize thousands of metabolites that are used for their growth and development, reproduction, defense against attack by different kinds of organisms, and survival in often harsh and changing environments. The vast array of small molecular weight compounds, known as secondary metabolites are not generally essential for the basic metabolic processes of the plant, but are often critical to the proper functioning of the plant in relation to its environment [1]. These compounds are believed to play vital roles in the physiology and ecology of the plants that produce them, particularly as defense elements against pests and pathogens [2] or as attractants for beneficial organisms such as insect pollinators [3]. Because of their biological activities, some plant natural products have long been exploited by human beings as pharmaceuticals, stimulants, and poisons [4].

One example is represented by anthocyanins, described as potent anti-oxidant compounds. Such molecules neutralize the reactive oxygen species (ROS) such as hydrogen peroxide (H₂O₂), superoxide anion (O₂⁻) and hydroxyl (HO⁻), which are produced under stress or in aging process, damaging the cell membrane. Publicity of these results has led to a dramatic increase in the popularity of this secondary metabolites and consequently of the interest in using *in vitro* methods for their biosynthesis.

Plant cell cultures are successfully exploited for secondary metabolite production, including anthocyanins and provide an opportunity for extensive manipulation of the identified parameters involved in the productivity enhancement of these natural products over the levels found in intact plants. The capacity of callus culture to be a potential source of anthocyanins was reported for the first time in 1974 by Mitsuoka and Nishi, who found that
the callus of *Mellotus japonicus* could accumulate a red pigment in specific condition of cultivation. The experiments demonstrate that anthocyanins occur in a large number of callus and suspension cultures but excellent results on anthocyanin biosynthesis were obtained by us using *V. vinifera* cultivars [5].

The recent development of elicitation as a method of secondary metabolites biosynthesis improvement, particularly anthocyanins, has offered new perspectives [6]. It was suggested that elicitors generate an inducing process which increases the mRNA level for various enzymes, like phenylalanineammonia-lyase, chalconesynthase, and represent the primary answer in a chain of biochemical events [7].

The aim of the present experiment, consists in emphasizing the effect of several potential elicitors such as manitol, abscisic acid, salicylic acid and jasmonic acid (in different concentrations), on the rate of anthocyanin biosynthesis and callus proliferation starting from a long–term callus culture of *Vitis vinifera* cv. Isabelle.

**Materials and Methods**

**Biological material.**

The primary callus culture was initiated from pericarp of grape berries (*V. vinifera* L. cvs. Isabelle) at 21 days after the anthesis. The cell line that produces anthocyanins was isolated by visual selection and periodic subcultivation on fresh nutrient medium [8]. This stock culture of long-term callus was used as a source of inocula in this experiment.

**Method**

Growth medium for initiation, subcultivation and treatment with elicitors consisted in a variant of basal Gamborg-B5 (1968) medium, supplemented with 0.1 mg/l NAA (α-naphthalene acetic acid), 0.2 mg/l kinetin, 2 mg/l casein hydrolisate, 30g/l sucrose, 8g/l agar (Difco).

For testing the effect of different elicitors on the long–term *V. vinifera* callus culture, eight different experimental variants were analyzed (Table 1):

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<th>EXPERIMENTAL VARIANTS</th>
<th>ELICITORS</th>
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<td>MANITOL (mM)</td>
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In the first experiment three variants were tested comparing to the Control and to the Blank (which differ by the Control by the addition of 1 ml Methanol/l); in the second experiment lower concentrations of elicitors were used.

Methanol, added before autoclaving in a concentration of 1ml/l, was necessary to be tested, because this substance was a solvent for all the elicitors used in this experiment.

The culture conditions consist in a temperature of 24°C-26 °C, 1500 lux cold
fluorescent light and 16 hours light/day photoperiod.

**Light microscopic analyses** were achieved using thick (semifine) sections of 1-2 μm, which were stained with 1% toluidine blue in 1% borax [9]. Squash analyses of fresh cell callus culture were also performed.

**Extraction and preparation of samples for spectrophotometric analyses**

The callus samples of 0.5 g were accurately weighted and homogenized in 5 ml of HCl/ methanol 1%. The extraction was stored overnight at 4° C in the refrigerator and after that the homogenate was centrifuged (3000 min⁻¹, 15 min).

The supernatant was collected and then used in spectrophotometric analyses.

**Spectrophotometric assay of total anthocyanins**

The total anthocyanin content was determined spectrophotometrically using a Spectro UV/VIS Thermo Scientific Helios gama and detection being made at a wavelength of 525 nm.

**Gravimetric analyses** were achieved making the difference between the weight of the callus samples grown on different medium variants and the initial weight of the sample that was uniformly chosen as 1 g.

The *long-term* callus of *Vitis vinifera* cv. Isabelle, after 30 days of cultivation in the presence of elicitors such as manitol, jasmonic acid, abscisic acid, salicylic acid, presented different cell proliferation rate, anthocyanin biosynthetic potential and morphological characteristics, depending on the type of elicitor used and its concentration.

The microscopical observations of fresh cell culture (squash probe) cultivated on the variant of Control revealed the presence of cellular aggregates of numerous cells having large sizes, predominantly oval, next to aggregates of cells with small dimensions. This morphological heterogeneity was clearly displayed in semifine sections. These offer more information about the intensity of biosynthetic process in callus mass, indicating a more intense activity on the callus surface. 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 large senescent and in small post-dividing cells. The callus cultured on this variant of Control medium has developed like a mass of cells with moderate proliferative capacity, having a hard texture, predominant superficially colored. The accumulation of anthocyanin pigments in this type of callus can be observed at the level of the entire vacuom and along tonoplast membrane like precipitates of electron – dense material, (Figure 1).

![Image](https://via.placeholder.com/150)

*a)* The macroscopic aspect of the callus after 30 days of cultivation on Control medium;  
*b)* The semifine sections of the same callus;  
*c)* The aspect of the squash probe from the callus grown on Control medium.

Under the influence of Manitol, the callus appeared as a mass of cells with significant proliferative capacity, having a lax texture, not very compact, and having large zones with cells very intense colored both at callus surface and inside the callus mass, up to the base in contact with the culture medium. The squash probes of the callus samples revealed the presence of very heterogeneous cell populations from morphologic point of view, having an
oval, oblong or reniform shape. The anthocyanin biosynthesis can be observed in all cell types which manifest different levels of color intensity. The same aspects revealed by the squash probe could be observed more intensively in the semifine sections (Figure 2).

Fig. 2. a) The macroscopic aspect of the callus after 30 days of cultivation on medium supplemented with manitol; 
b) The semifine sections of the same callus; 
c) The aspect of the squash probe from the callus grown on medium optimized with manitol.

The callus developed on the medium containing jasmonic acid as elicitor 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 this elicitor revealed the presence of spherical cells of various sizes and a heterogeneous pigment accumulation. The morphofunctional peculiarities of this culture are very well represented in the semifine sections (Figure 3).

Fig. 3. a) The macroscopic aspect of the callus after 30 days of cultivation on the medium supplemented with jasmonic acid; 
b) The aspect of semifine sections from the same callus; 
c) The appearance of the squash probe from the callus grown on medium optimized with jasmonic acid.

In the presence of salycilic acid 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 by heterogenous cell populations, having both spherical and elongated shapes. The percentage of the cells with anthocyanin accumulation was significantly increased. Among cells with different level of pigment accumulation, some cells totally deprived of anthocyanins are present. The cellular stage of development even in this case seems not to be correlated with anthocyanin accumulation, the pigments being present both in very young cells and in old ones (Figure 4).

The experimental medium variant in which abscisic acid was tested as elicitor revealed the development of a callus with a very interesting aspect, with soft texture and a very intense pigmentation, uniformly present throughout the cell mass. Groups of small cells containing anthocyanins are frequent. (Figure 5)
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Fig. 4. a) The macroscopic aspect of the callus after 30 days of cultivation on medium supplemented with salicylic acid;  
b) The aspect of semifine sections from the same callus;  
c) The appearance of the squash probe from the callus grown on medium supplemented with salicylic acid.

Fig. 5. a) The macroscopic aspect of the callus after 30 days of cultivation on medium supplemented with abscisic acid;  
b) The appearance of the squash probe from the callus grown on medium optimized with abscisic acid.

The callus developed on Blank, the experimental variant characterized by the addition of Methanol, was characterized by a very light pink color with a glassy aspect, colored superficially on a large area. The squash probe revealed a heterogeneous cell population from dimension point of view, with different levels of pigment accumulations, the smallest cells presenting a high degree of anthocyanin accumulation (Figure 6). Comparing to the callus tested on the Control experimental system, were not identified any significant differences, so we can conclude that the addition of Methanol into the culture medium supplemented with elicitors does not influence their effects on the cell proliferation or on the biosynthetic capacity.

Fig. 6. a) The macroscopic aspect of the callus after 30 days of cultivation on medium supplemented only with methanol;  
b) The aspect of semifine sections from the callus grown on this Blank medium;  
c) The appearance of the squash probe from the callus grown on this medium.
The randomly spreading of the cells presenting sites of anthocyanin biosynthesis throughout the callus mass has not been elucidated till now. There is the supposition that this distribution can be dependent on the intensity of light, on the nutrient and hormone quantities and on the elicitors used in culture medium that can diffuse inequitably in different areas of the callus.

Another explanation consists in the fact that the initial inoculum represents an asynchronous culture, and the reaction of different types of cells that are in diverse stages of cellular cycle, can vary under the influence of the signals received from elicitors or others environmental factors.

A direct correlation between cellular proliferation, which assures a significant cellular mass, and the intensity of biosynthetic processes could not be established. Based on similar data recorded in other experimental systems, some authors recommend the successive utilization of two different types of medium, first one for cellular proliferation and the second one for stimulation of the active principles biosynthesis [10].

In our experimental conditions this model of callus culture had no satisfactory results, so we have to find the proper condition for the stimulation both of anthocyanin biosynthesis and the cellular proliferation on the same basal nutrient medium.

After 30 days of cultivation in medium supplemented with elicitors, the comparative gravimetric analysis revealed that in case of using concentrations of 20 μM jasmonic acid, 20 μM salycilic acid, 20 μM abscisic acid and 10 mM manitol, the callus did not present any significant differentiation comparing to that grown on control medium. (Figure 7)

In the case of using smaller concentrations of elicitors: 10 μM jasmonic acid, 10 μM salycilic acid, 10 μM abscisic acid and 4 mM manito, the callus grown in the presence of salycilic acid revealed a more intense cellular proliferation comparing to that grown on control medium. The addition of 4 mM manitol to the medium seems to produce a more important cell proliferation than a concentration of 10 mM. (Figure 8)

Except for the experimental variant in which only Methanol was included in the culture medium, the entire experimental variants with Methanol and elicitors in different concentrations positively influenced the biosynthetic capacity of the callus comparing to the control.
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**Fig. 9.** The influence of the elicitors SA (20μM), MAN (10mM) on anthocyanin content. **Fig. 10.** The influence of the elicitors SA ABA (20μM), (10μM), ABA (10μM), MAN (4mM), JA (10μM) on anthocyanin content.

The values at 525 nm of the absorbance revealed some interesting aspects such as: 20 μM concentration of salicylic acid can increase the rate of anthocyanin biosynthesis (Figure 9) comparing to 10 μM concentration of this elicitor (Figure 10); a smaller concentration of manitol (4mM) and abscisic acid (10 μM) positively stimulate the biosynthetic capacity of the callus comparing to the double concentration of this two elicitors. These results suggest that would be necessary to test a larger scale of concentrations and combinations of elicitors.

The testing of the use of manitol in *Vitis vinifera* callus culture for increasing the accumulation of major anthocyanins, represents a novelty and offer new data for further studies on the influence of this compound as elicitor. The results of our research sustain the data obtained by other authors that used the elicitors salicylic acid, jasmonic acid and abscisic acid [11] on species as *Crassula multicava* [12], *Glycine max.* Merr cv. Wye [13], on cell suspensions of *Corydalis claviculata*, *Crotalaria cobalticola*, *Eschscholtzia californica*, *Rubia tinctorum*, Sarcocapnos crassifolia [14] and *Vitis vinifera* [15].

**Conclusions**

After preliminary investigations on the influences of some elicitors on the proliferative and biosynthetic capacity of *Vitis vinifera* long-term callus cultures, we can conclude that:

The concentration of 10 μM jasmonic acid stimulates the anthocyanin biosynthesis but not the cell proliferation, the same like in case of abscisic acid;

A high concentration of salicylic acid (20 μM) has a positive influence on the biosynthetic capacity of the callus;

Our data permitted us to design a new model for the modulation of callus proliferative and anthocyanin biosynthetic potential; in the first stage of culture, the use of the elicitor salicylic acid can promote a rapid cellular growth of the callus, followed by the addition in the culture medium of the elicitor jasmonic acid to stimulate the callus biosynthetic capacity.

The long-term grape callus provides a convenient experimental system for the study of anthocyanin biosynthetic pathways and of the eliciting factors that could optimize them; a more comprehensive understanding of this process will be achieved in our future studies, based on the information offered by the qualitative and quantitative anthocyanin analyses in the calli under the influence of different elicitors.
References