

Cytological aspects and anthocyanin accumulation observed in *sedum telephium* ssp. *maximum* L. callus

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Abstract

Sedum telephium ssp. *maximum* L. calluses grown on Murashige-Skoog growth media supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) and benzyladenine (BAP) showed a red coloration of the vacuolar content of some of their cells. It was determined that this phenomenon is due to anthocyanins accumulation under the influence of the growth regulators. Using HPLC analysis, it was found that the highest overall anthocyanins concentration was in calluses grown on medium supplemented with 1.5mg/l 2,4-D + 2.5 mg/l BAP. Furthermore, the type of the growth regulators (cytokinines or auxines) added individually or as a mixture (in different ratios) in the growth medium, can influence the callus growth rate and what type of anthocyanins is produced preferentially.

Keywords: cyanidin 3- glucoside, growth regulators, callusogenesis

1. Introduction

Plant vitro cultures refer to procedures concerned with the inoculation and growth on aseptic culture media, in anoxic conditions of different types of explants (for example anthers, ovaries, ovules or even seeds, rootstocks, tissues, cells or protoplasts) that have been previously sterilised. Such explants can generate primary phyto-innocula cultures or if these generate a callus (as such or of organogenous or embriogenuous type), this can be replicated obtaining *subcultures*. The time interval between the moment of initiating *primary cultures* and the first *subculture* depends on the species used for phyto-innocules, on the type of explant used and vitro culture conditions such as the illumination regime, adopted photoperiod and temperature in the growth room. It also depends on the composition of the growth medium, mainly concerning the type of the growth regulators used and their concentration (CACHIȚĂ [4]). The sum of these factors induce and sustain callus generation, growth and anthocyanins accumulation in the cells vacuoles of the vitrocultivated plant material.

After the growth of the callus mass, the process of *in vitro* culture requires the operation of subcultures by dividing the callus in fragments of minimum 100 – 250 mg each

that will be subcultivated on media with adequate composition to sustain for a long time the proliferative capacity of its cells. The callus can be used as an experimental model in morphogenesis studies and also for anthocyanins production, for the phytopharmaceutical and food industry (SOCACIU [12]; STREET [13]).

Anthocyanins are water soluble vacuolar pigments responsible for the red, orange or blue coloration of fruits and vegetables (GIUSTI and WROLSTAD [9]). One of the factors that can induce anthocyanins formation in calluses are the growth regulators such as cytokinines and auxines introduced in the growth medium. Furthermore, the concentrations and ratios between cytokinins and auxines can affect anthocyanins production (CARA & al. [8]).

In the case of the experiments performed with *Sedum telephium* ssp. *maximum* L. explants, the callus was obtained from subcultures for which minicutting inoculae were used. These were obtained either from vitrostemlets fragments or from leaflets taken from vitroplantlets (ARDELEAN & al. [2]). Depending on the type of growth regulators introduced in the culture medium and their concentration, organogenesis, callusogenesis and anthocyanin formation were triggered. This observed phenomenon led to initiating studies focused on examining the morphogenesis process and anthocyanins generation in the callus obtained from *Sedum telephium* ssp. *maximum* L minicuttings. The minicuttings were generated from the embryos of seeds germinated on an aseptic Murashige-Skoog (1962) (MURASHIGE and SKOOG [11]) base culture medium, the phyto-innocules being kept for 60 days in the growth room under white fluorescent light. After 30 days of vitroculture, at the newly formed vitroplantlets level, callusogenesis was induced. The obtained callus was subcultivated on MS growth media supplemented with auxins and cytokinines in different concentrations. A few days after subculturing it was noticed that some of the callus cells had a raspberry-red coloration probably due to the anthocyanins present in their vacuolar content. To elucidate these aspects, the anthocyanins were extracted and determined using high performance liquid chromatography (HPLC).

2. Materials and method

Plant material, callus initiation and anthocyanin induction

To perform vitrocultures, leaflets from *Sedum telephium* ssp. *maximum* L vitroplantlets stemlets were taken and by cutting the petiole were inoculated *in vitro* on a agarised *Murashige-Skoog* (MURASHIGE and SKOOG [11]) base medium (MB), using as growth regulators either a mixture of benzyladenine (BAP) with indolyl butyric acid (AIB) in concentration of 1.5mg/l each (type VI) or kinetin (KIN) with α -naphthyl acetic acid (ANA) (type VII) each in concentration of 1.5 mg/l. The pH of the growth medium was adjusted to 5.5 before autoclaving at 121°C for 21 minutes. The conditions in the growth chamber were as follows: illumination of cultures for 16hrs/24hrs with white fluorescent light (intensity 1700 lux), temperature 25°C during light period and 21°C in the dark (ARDELEAN & al. [2]).

Sedum telephium ssp. *maximum* L. vitroplantlets from which minicuttings, consisting of foliar laminas, were taken had 60 days of vitroculturing on the same media types VI or VII respectively (ARDELEAN & al. [2]). Also, after 60 days of vitroculture, the formed callus was subcultivated on agarised *Murashige-Skoog* (MB) base medium, using growth regulators (auxins and cytokinines) as follows:

- MB-MS with no growth regulators (V_0);
- MB-MS with: 1.5mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) (V_1);
- MB-MS with: 1.5mg/l 2,4-D + 2.5 mg/l benzyladenine (BAP) (V_2);
- MB-MS with: 1.5mg/l BAP (V_3);

- *MB-MS* with: 1.5mg/l 2,4-D + 1.5 mg/l BAP (V_4).

After 60 days we performed semithin or ultrathin section through the callus generated at their level (Fig. 1 F and Fig. 2 and 3 A – D); the preparations obtained according to the techniques described by Cachiță and Crăciun (CACHIȚĂ & al. [6]) and Hayat (HAYAT [10]) were examined either with the optical or transmission electron microscope.

The containers with the calluses were kept in the growth chamber under previously stated growth conditions. This callus was repeatedly subcultivated every 21st day for 60 days. The anthocyanin content from this callus was determined after 60 days.

Anthocyanin extraction

According to Socaciu (SOCACIU [12]), extraction of anthocyanins from callus is easier than from organized organ tissue. Briefly, the anthocyanins were extracted from *Sedum* calluses using a modified literature method (SOCACIU [12]) as follows: 500 mg callus was ground and extracted at room temperature for 10 minutes with 9 ml acidified methanol (2% hydrochloric acid in methanol) into which 10% phosphoric acid was added. Extracts [2 ml] of extract were prepared for analysis using high performance liquid chromatography hyphenated with diode array detector (HPLC – DAD) by filtering through a 0.45 μ m filter (Chromafil – Macherey – Nagel, Germany).

Chemicals and Standards

All solvents and standard used were HPLC grade. Cyanidin 3-O glucoside was purchased from Sigma-Aldrich (Germany). Formic acid, acetonitrile, methanol, hydrochloric acid and phosphoric acid were purchased from Merck (Darmstad, Germany). Water was purified using a Purelab Option Q system.

Anthocyanin Identification and Quantitation

HPLC-DAD analysis of the antocyanins extracted from calluses was performed using a Dionex Ultimate 3000 instrument equipped with a Ultimate 3000 diode array detector (DAD). The analytical column used was a Thermo Scientific Acclaim 120, C18, 5 μ m Analytic (4.6 x 250 mm) column, coupled with an Acclaim C18 guard column.

The HPLC grade solvents used as mobile phases were: 10% formic acid (solution A) and formic acid (10%), methanol (22.5%) and acetonitrile (22.5%) (solution B)[***]. Before use, the solvents were filtered through a 0.2 μ m cellulose filter and degassed.

To optimise peak separation, the gradient run was done using a modified literature method [Dionex (Thermo Scientific) – Application Note 281, 2011] as follows: isocratic 20 minutes 91% A, 5 minutes gradient to 65% A, 16 minutes gradient to 50% A, isocratic 13 minutes 50% A, step change to 91% A, 5 minutes isocratic 91% A to reequilibrate the column. The injection volume was 5 μ l, flow rate 1ml/min, column temperature 35°C. The anthocyanins were detected at 520 nm. Cyanidin 3- glucoside, being one of the most widespread anthocyanin (AZEVEDO & al. [3]), was used as an external standard to quantify the anthocyanins content. A standard curve of cyanidin 3- glucoside was established for quantification purposes.

Microscopy

Microscopic examinations were performed on the calluses that have been fixed to obtain semithin or ultrathin sections using an ultramicrotome.

For examination using the optical microscope, the sections must be around 500 nm (0.5 μ m) thick while the ultrathin sections that can be examined using transmission electron microscopy should have a thickness of about 40 – 70 nm. A typical sample preparation process for microscopy studies is presented below (CACHIȚĂ and CRĂCIUN [5]; HAYAT [10]). Briefly, the tissue samples are first pre-fixed in 2.7% glutaraldehyde prepared in 0.1M, pH 7.4, phosphate buffer solution followed by 4 successive washings with the same buffer.

The tissues are then post-fixed using a 1-2% osmium tetroxide solution made in 0.1M, pH 7.4, phosphate buffer then washed 2-3 times with the same buffer. The samples are then dehydrated by placing them in baths with increased acetone concentration. After that, the samples were passed through 2-3 baths containing propylene oxide solution. The tissue samples thus prepared were placed in baths of Epon 812 (epoxy resin) mixed with anhydrous acetone with increased resin concentration, the final bath containing only the Epon. The samples impregnated with Epon were embedded in gelatin capsules. The capsules were then finish filled with resin and kept at 50 - 60°C, for 48 – 72 hours to cure the resin which will become hard and transparent.

Once the samples are cured, the hardened resin can be modeled under a stereo microscope using a new razor blade to obtain a small „pyramid trunk” at one end. For ultrathin sections, the sides of the pyramid trunk should be around 0.1 – 0.2 mm.

The sections were obtained using a Leica UC 6 ultramicrotome equipped with a Diatone diamond knife. The semithin sections were stained with *Epoxy tissue stain*, a special epoxy stain.

The sections for electron microscopy were contrasted in the first stage with uranyl acetate solution, followed by contrasting with lead citrate solution, technique which is currently used in electron microscopy laboratories, around the world (CACHIȚĂ and CRĂCIUN [5]; HAYAT [10]). The semithin sections were examined by us using a Olympus BX 51 optical microscope equipped with a CCD camera and the ultrathin sections were examined using a JEM-JEOL TEM 1010 electron microscope. Selected pictures were recorded using a MEGA WIEW III camera.

3. Results and Discussion

Morphological and cytological aspects

After 21 days of vitroculture it was observed that callusogenesis was initiated in *Sedum telephium* ssp. *maximum* L vitroculture in the presence of BAP and 2,4 - D mixture in the growth medium.

From literature (CACHIȚĂ & al. [6]), it is known that BAP, a synthetic cytokinine, induce and sustain budlet neogenesis whereas 2,4-D stimulates the callusogenesis process. It is emphasized that in the case of budlet neogenesis – on a medium with BAP from the newly formed cells a differentiation of various tissue structures occurs (generated from epidermal, mezophyll or vascular tissue). These are characteristic for a leaf structure whereas the meristem resulting at the base of the *Sedum* lamina minicutting that was inoculated and grown on a medium with a mixture of BAP and 2,4 – D has generated a callus with a different hystological structure.

Figure 1 shows *Sedum telephium* ssp. *maximum* L calluses grown on *Murashige-Skoog* (1962) growth media without and with growth regulators (2,4 – D and BAP). It was observed that through callus initiation and growth on media containing 2,4 – D and BAP, the callus started to change color from green to red.

The callus cells were of different size and randomly distributed, the intercellular spaces being sometimes very large (Fig 2 A). Generally the callus is without veins even tough in this tissue. Chloroplasts are scattered in the cytoplasm lining the interior to the cell wall. The nuclei have a normal structure, not ameboidali, but are fusiform.

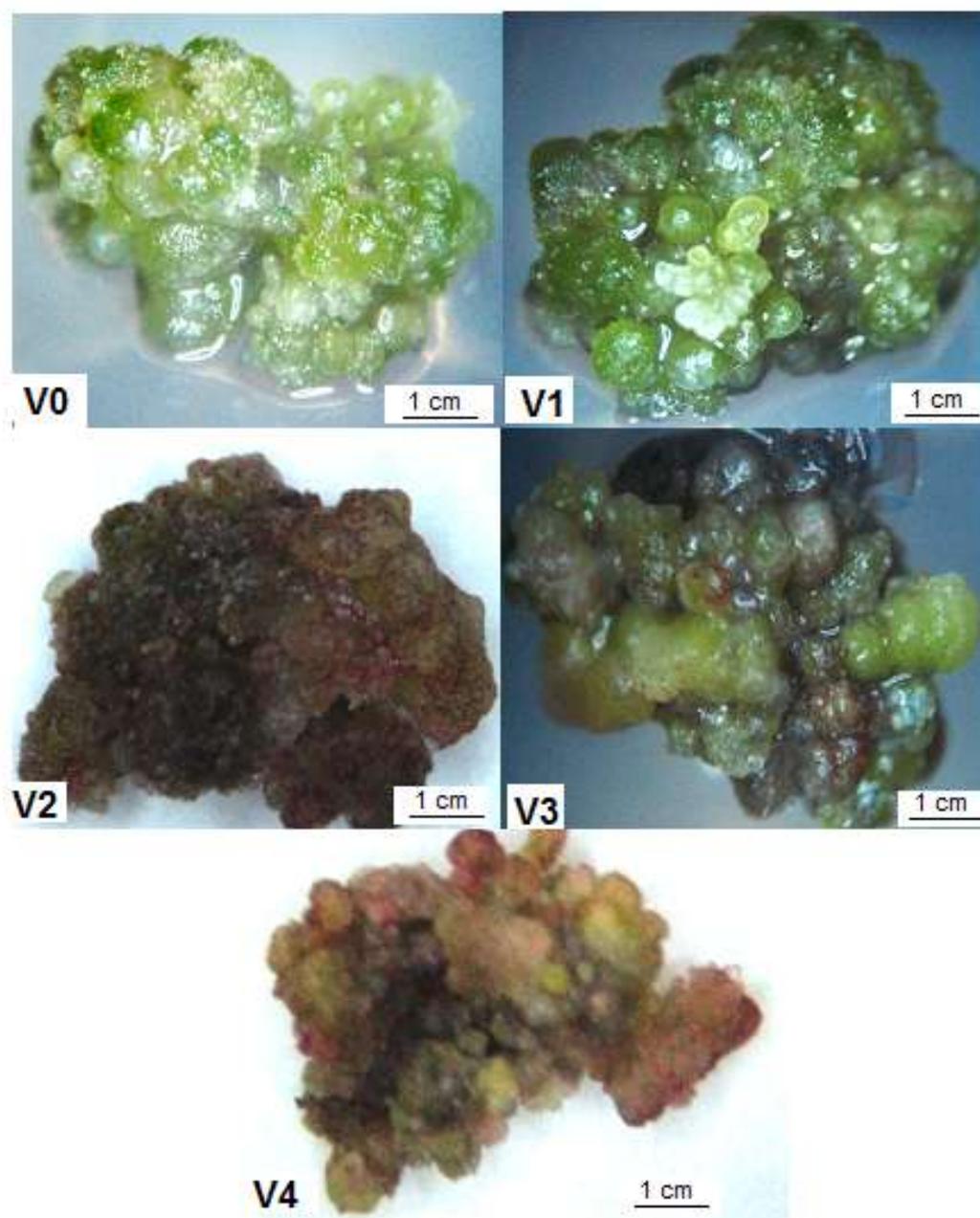


Fig. 1. *Sedum telephium* ssp. *maximum* L calluses grown on Murashige-Skoog (1962) growth media without (V_0) and with 2,4-dichlorophenoxyacetic acid (2,4-D) and benzyladenine (BAP): V_1 - 1.5mg/l 2,4-D; V_2 - 1.5mg/l 2,4-D + 2.5 mg/l BAP; V_3 - 1.5mg/l BAP; V_4 - 1.5mg/l 2,4-D + 1.5 mg/l BAP.

After 21 days of subculture it was noticed that the highest anthocyanin accumulation occurred in calluses grown on media with 1.5mg/l 2,4-D + 2.5 mg/l BAP (V_2). This was further confirmed by HPLC analysis. Furthermore, an inverse correlation between callus growth and anthocyanin accumulation was observed. The highest anthocyanin accumulation was associated with a low growth rate. This was previously reported by other authors,

studying the correlation between callus growth and anthocyanin accumulation and their relationship with the hormonal regime (CALLEBAUT & al. [7]; TAHA & al. [14].

The histological structure of the callus is lacking stomata (Fig. 2 A).

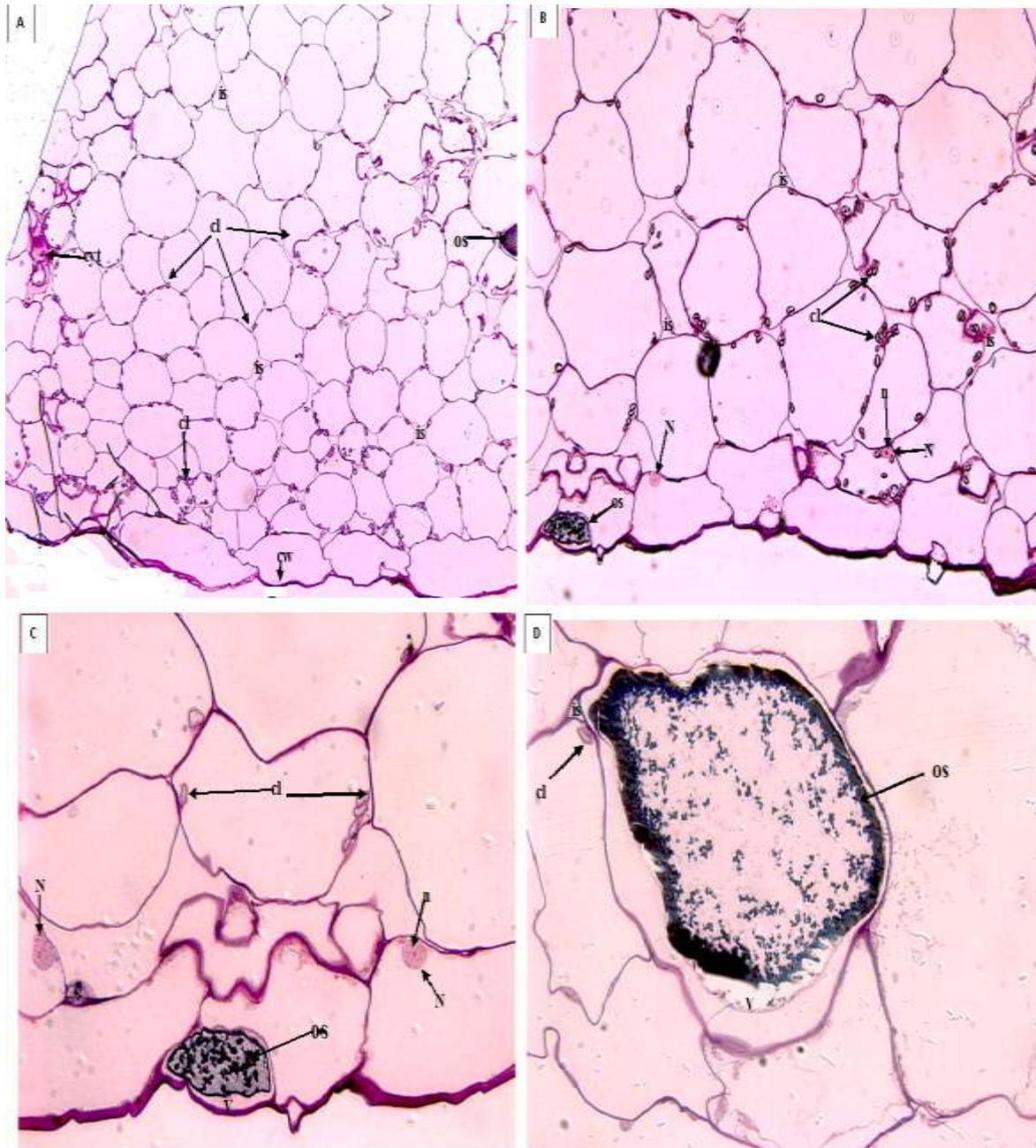


Fig. 2. Structural aspects of the callus generated at the minicutting level consisting of foliar laminas taken from *Sedum telephium* ssp. *maximum* L. plantlets vitrostemlets that were vitrocultivated on agarised *Murashige - Skoog* (1962) medium supplemented with 1.5mg/l 2,4-D and 2.5 mg/l BAP; Fig. 2A (ob. 20x) – callus marginal zone; Fig. 2B – D – deeper samples from the callus (B -ob. 40x; C and D – ob. 100x) (abbreviations: cyt – cytoplasm; os – osmophile deposits identified in the vacuolar content; is – intercellular space; cl – chloroplasts; N – nucleus; n – nucleolus; cw – cellular wall; V – vacuole).

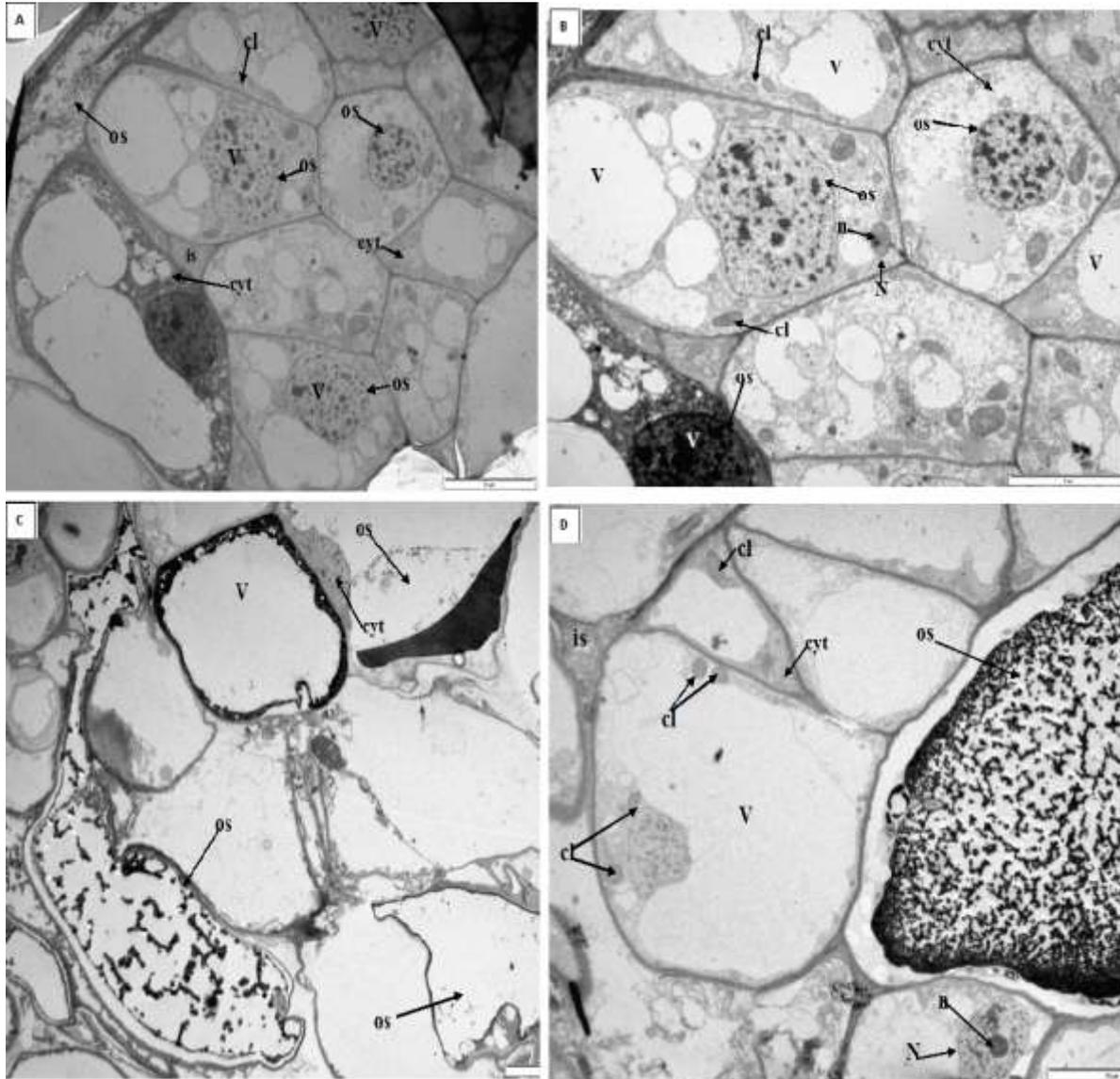


Fig. 3. Transmission electron micrographs of the cells detached from the callus tissue of *Sedum telephium* ssp. *maximum* L. cultivated on agarised *Murashige - Skoog* (1962) medium supplemented with 1.5 mg/l 2,4-D and 2.5 mg/l BAP. In the cells, corpuscular, electron dense formations of different sizes can be observed or similar deposits seen on the tonoplast (abbreviations: cyt – cytoplasm; os – osmophile deposits identified in the vacuolar content; is – intercellular space; cl – chloroplasts; N – nucleus; n – nucleolus; cw – cellular wall; V – vacuole).

Regarding the endocellular structure of the callus tissue regenerated at the minicuttings level consisting of *Sedum* foliar laminas (6-7 mm in length and 4-5 mm in width), even from the first 30 days of vitroculture, callus regeneration was noticed in the part implanted in the growth medium. The callus cells had a highest number of chloroplasts (Fig. 2 and 3, A – D). The vacuoles of many callus cells contained different electron dense, osmophile granular formations of different sizes (Fig. 3 B – D). Such deposits were also noticed on the tonoplast and is believed they are of anthocyanin nature (Fig. 3 A – D).

Anthocyanins characterisation

The anthocyanins extracted from *Sedum* calluses were separated using HPLC (Fig. 4).

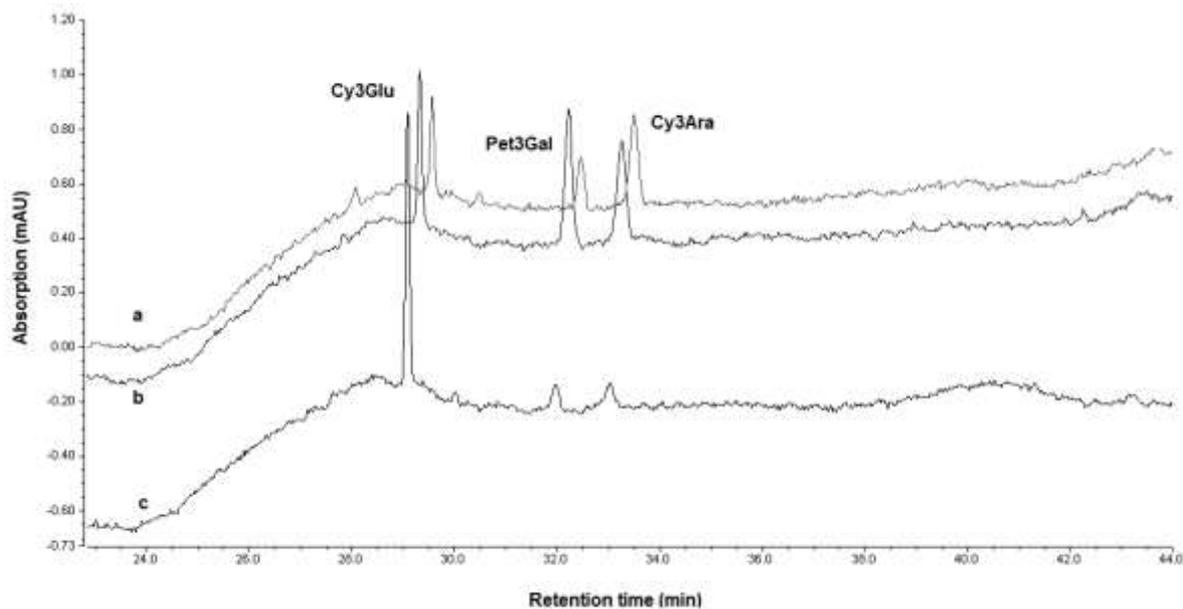


Fig. 4. HPLC chromatograms of anthocyanins extracted from *Sedum* calluses grown on media with different growth factors.

Three anthocyanins peaks were observed in the HPLC chromatograms (Fig.4). Cyanidin 3- glucoside was identified by comparing the retention time with that of the standard. The other two anthocyanins were tentatively identified as petuidin 3- galactoside and cyanidin 3 -arabinoside based on literature data (MURASHIGE and SKOOG [11]). Figure 5 shows the overall and different anthocyanins concentration extracted from *Sedum* calluses grown on media supplemented with growth regulators: 2,4-D and BAP added separately or in combination.

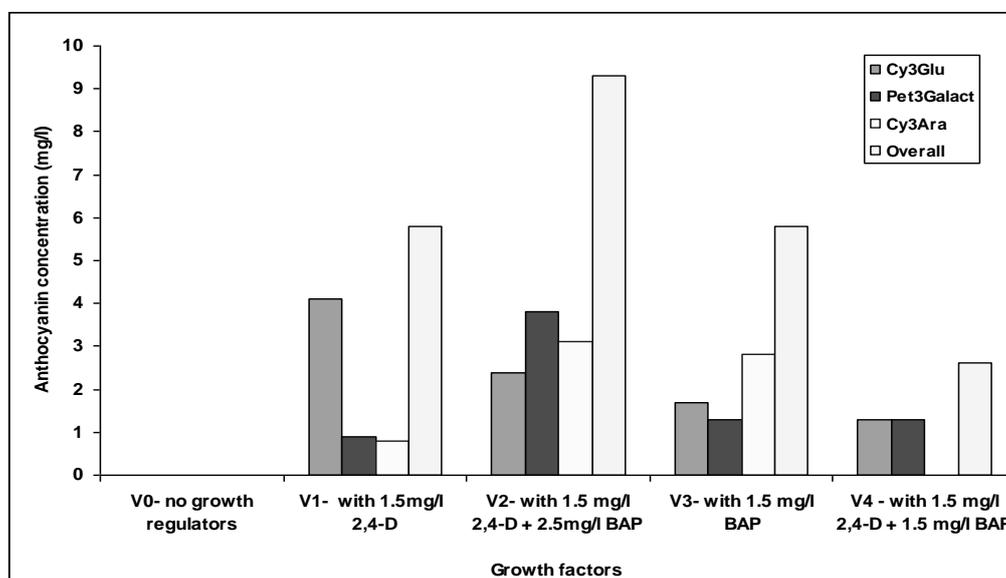


Fig.5. Anthocyanin concentration vs. growth factors types and concentrations, extracted from *Sedum* calluses.

The highest overall anthocyanin content was observed in the calluses grown on MS medium with 1.5mg/l 2,4-D + 2.5 mg/l BAP. At equal amounts of 2,4-D and BAP (Fig. 4) the callus exhibits a good growth rate whereas the overall anthocyanin content is the lowest. These results are in agreement with those of other authors (ABEDA & al.[1]). Furthermore, the type and concentration of the growth factors can also affect the type of anthocyanin that is accumulating during callus growth (Fig. 1) (ABEDA & al.[1]). The auxin, in our case 2,4-D induces predominantly the accumulation of cyanidin 3-glucoside compared with the same concentration of cytokinine (BAP), however the overall anthocyanin concentration is the same (Fig. 5).

4. Conclusions

The foliar lamina type minicutting with the petiole removed and inoculated on *Murashige-Skoog* (1962) base medium with added growth regulators (KIN and ANA) showed both a regenerative capacity and callus formation in their basal zone cells. The resulting callus was subcultivated on the same base medium but with added 2,4 – D and BAP. During growth, some of the callus cells had their vacuolar content colored in red.

This phenomenon, in microscopy observation was translated by the accumulation in the vacuolar content of electron dense, osmophile granular formations of different sizes. These formations were proved to be anthocyanins following extraction and HPLC analysis.

It was found that adding 1.5mg/l 2,4-D + 2.5 mg/l BAP in the growth medium induces the highest anthocyanin accumulation and lowest growth rate. Also, to the best of our knowledge, this is the first time that the presence of cyanidin 3-glucoside in *Sedum telephium* ssp. *maximum* L. calluses, was reported.

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References

1. ABEDA, H. Z., KOUASSI, M. K., YAPO, K. D., KOFFI, E., SIE, R. S., KONE, M., KOUAKOU H. T. 2014. Production and Enhancement of Anthocyanin in Callus line of Roselle (*Hibiscus sabdariffa* L.) Int. J. Rec. Biotech. 2 (1): 45-56.
2. ARDELEAN, M., CACHIȚĂ, C.D., CRĂCIUN, C. 2012. Morphogenesis in the caulinary, apical and foliar minicuttings of *Sedum telephium* ssp. *maximum* in vitro on Murashige - Skoog (1962) medium culture. Studia Universitatis „Vasile Goldiș”, Seria Științele Vieții, Vol. 22, issue 2, April, p.185-191.
3. AZEVEDO, J., FERNANDES, I., FARIA. A., OLIVEIRA, J., FERNANDES, A., FREITAS, V., MATEUS, N. 2010. Antioxidant properties of anthocyanidins, anthocyanidin-3-glucosides and respective portisins. Food Chem., 119:518-523.
4. CACHIȚĂ, C.D. 1987. Metode in vitro la plantele de cultură – baze teoretice și practice. Edit. Ceres, Bucuresti.
5. CACHIȚĂ, C.D., CRĂCIUN, C. 1990. Ultrastructural studies on some ornamentals, p. 57 – 94. Handbook of Plant Cell Culture, Vol. 5, Ammirato, P.V., Evans, D.A., Sharp, W.R. (Eds) Y.P.S. Bajaj. McGraw-Hill & Comp., New York.

6. CACHIȚĂ, C.D., DELIU, C., RÁKOSY-TICAN, L., ARDELEAN, A. 2004. Tratat de biotehnologie vegetală, Ed. "Dacia", Cluj Napoca, Vol. 1, 433 p.
 7. CALLEBAUT, A., TERAHARA, N., DE HAAN, M., DECLEIRE, M. 1997. Stability of anthocyanin composition in *Ajuga reptans* callus and cell suspension cultures. *Plant Cell Tiss. Org. Cult.*, 50:195-201.
 8. CARA, R., WELCH QINGLI, W.U., JAMES SIMON, E. 2008. Recent Advances in Anthocyanin Analysis and Characterization. *Curr. Anal. Chem.*, 4(2): 75–101.
 9. GIUSTI, M.M., WROLSTAD, R.E. 2003. Acylated anthocyanins from edible sources and their applications in food systems. *Biochemical Engineering Journal*, 14: 217-225.
 10. HAYAT, M.A. 2000. Principles and techniques electron microscopy, p. 45–61. In *Biological Application*. Cambridge University Press.
 11. MURASHIGE, T., SKOOG, F. 1962. A revised medium for rapid growth and bioassays with tabaco tissue culture. *Physiol. Plant*, 15: 473-497.
 12. SOCACIU, C. 2007. Food colorants: chemical and functional properties. Boca Raton: Taylor & Francis. 616-617.
 13. STREET, H.E. 1977. Cell (suspension) cultures-techniques, p. 61-102. In: *Plant Tissue and Cell Culture*. Univ California Press, Berkeley, Los Angeles.
 14. TAHA, H.S., ABD EL-RAHMAN, R.A., FATHALLA, M.A., ALY, U.E. 2008. Successful application for enhancement and production of anthocyanin pigment from calli cultures of some ornamental plants. *Austr. J. Basic Appl. Sci.*, 2(4):1148-1156.
- ***, 2011, Thermoscientific Application Note 281. Rapid and Sensitive Determination of Anthocyanins in Bilberries Using UHPLC.