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ASPECTS REGARDING ORGANOGENESIS INITATION IN *Sedum telephium ssp.maximum L.* CALLUS UNDER BLUE FLUORESCENT LIGHT

Mirela ARDELEAN[1*], Dorina CACHIȚĂ-COSMA[1], Andrei LOBIUC[2], Aurel ARDELEAN[3,4]

[1]„Vasile Goldiş" Western University of Arad, Plant Biotechnology, Institute of Life Science, Romania
* Corresponding author: Mirela Ardelean, Ph.D., „Vasile Goldis" Western University of Arad, Plant Biotechnology, Institute of Life Science Arad, no. 86, Liviu Rebreanu Street, Arad 410414, Romania Phone: +40(0)257.212.111, Fax: +40(0)257.212.111, e-mail: mirela.ardelean1@yahoo.com
[2]„ Alexandru Ioan Cuza" University of Iasi, Romania, Plant Biology Department, Carol I Bd., Romania
[3]„Vasile Goldiş" Western University of Arad, Cell Biology Department, Institute of Life Science, Romania
[4] Academy of Romanian Scientists, 54 Splaiul Independentei 050094, Bucharest, Romania

Abstract

Environmental factors may influence organogenesis on phytoinoculi metabolism. *Sedum telephium ssp. maximum L.* callus, subcultivated for 30 days on basic *Murashige-Skoog* (1962) (MS) medium proved to be a plant material with particular reactive capacities, depending on the nature of growth regulators in medium and on the light wavelength used to illuminate phytoinoculi. Our experiments showed that a certain cytokinine in the medium induced accumulation of a red pigment in the vacuole sap of meristematic cells in root apices, but not in caliptral cells or cells from the growth area. *Sedum* callus culture under blue fluorescent light cultivation synthesized the red pigment in root meristems on basic MS medium supplemented with 2,5 mg/l 2,4-dichlorphenoxyacetic acid and with 1,5 mg/l benzilaminopurine. Concerning organogenesis, rhizogenesis was stimulated by blue light. We highlight the detection of secondary metabolic processes in root meristematic and callus cells.

Keywords: Sedum, organogenesis, callus, blue fluorescent light

Introduction

In plant biotechnology, vitrocultures usually require exposure of phytoinoculi to artificial sources of light, represented, in most cases, by white fluorescent tubes. Light is required for both photosynthesis, as well as for cell

differentiation alterations, morphogenesis initiation and regulation, photoperiod and phototropism dependent organ growth etc. According to Herman (2013), the light wavelength may exert certain influences on phytoinoculi, as the light intensity and duration do. Zhu Xingui and Guo Yong (1998) (according to Herman, 1999) showed that blue light led to a 10 fold accumulation of anthocyanins in Hibiscus sabdariffa cell suspensions, compared to red or orange light.

Muleo and colab. (2001) (according to Herman, 2002), experimenting with plum shoot apices explants, observed that blue light led to a more intense bud differentiation compared to red light and consider that light quality may increase micropropagation efficiency in vitrocultures obtained from plum apices or buds.

More recently, Liu et al. (2011) and Lin et al (2011) showed that in *Oncidium* and *Dendrobium* protocorms cultivated under blue light, cell differentiation was accompanied by increases in biomass, protein and assimilating pigments synthesis and enzymes activities, therefore leading to a higher rate of shoot genesis. Combining red and blue monochromatic light further stimulated those processes, as well as a accumulation of dry biomass in these protocorms (according to Herman, 2011 a and b).

However, Park et al. (2013) (according to Herman, 2013 b), *Panax ginseng* root vitroculture exposure to blue light increased synthesis of secondary metabolism compounds, such as α-tocopherol and β-amyrine and certain phenolic compounds in cells. Meanwhile, they stated that ,,blue light could be a useful source for the production of ginseng roots-cultured in vitro-with higer nutricional value and antioxidant activities".

Alvarenga et al. (2015 –according to Herman, 2015) observed that blue light is the best for *in vitro* cultivation of *Achillea millefolium* vitrocultures. Blue LED light stimulated, in Picea and Robinia cotiledonary epidermal cells, accumulation of electron dense particles in vacuole sap or in the tonoplast (Cachiță și colab., 2015).

Considering that in the last decade the number of articles focused towards the reaction of phytoinoculi as a function of the illumination type, our study is aimed at the same issues, using *Sedum telephium* explants as a model.

As such, in studies performed by our group (Ardelean şi colab., 2013), using *Sedum telephium* ssp. *maximum* explants under 16 h/day white fluorescent light, rhizo- and caulogenesis was observed (Fig 1). The apices of roots regenerated in primary cultures on Murashige-Skoog (1962) with α-naphtilacetic acid (ANA) and kinetin (KIN) supplementation 1,5 mg/l, were red colored (phenomenon described the first time in the literature by us). Researches pointed out that only in root apical, meristematic cells, and not in caliptral ones, red

pigmentation may be met, depending on the growth regulators used (Fig. 1 B și D). The pigmentation phenomenon was not observed in roots regenerated form explants on basic Murashige-Skoog (1962) (MS) (Fig. 1 A and C) without growth regulators while it was present, however less intense, in Sedum inoculi where growth regulators such as indolilbutiric acid (AIB) 1,5 mg/l was used; the phenomenon was much more intense in explants grown with α-naphtilacetic acid (ANA) or kinetine (KIN) 1,5 mg/l supplementation. Increased pigmentation was observed in plantlets grown with blue fluorescent light, 16 h/day photoperiod.

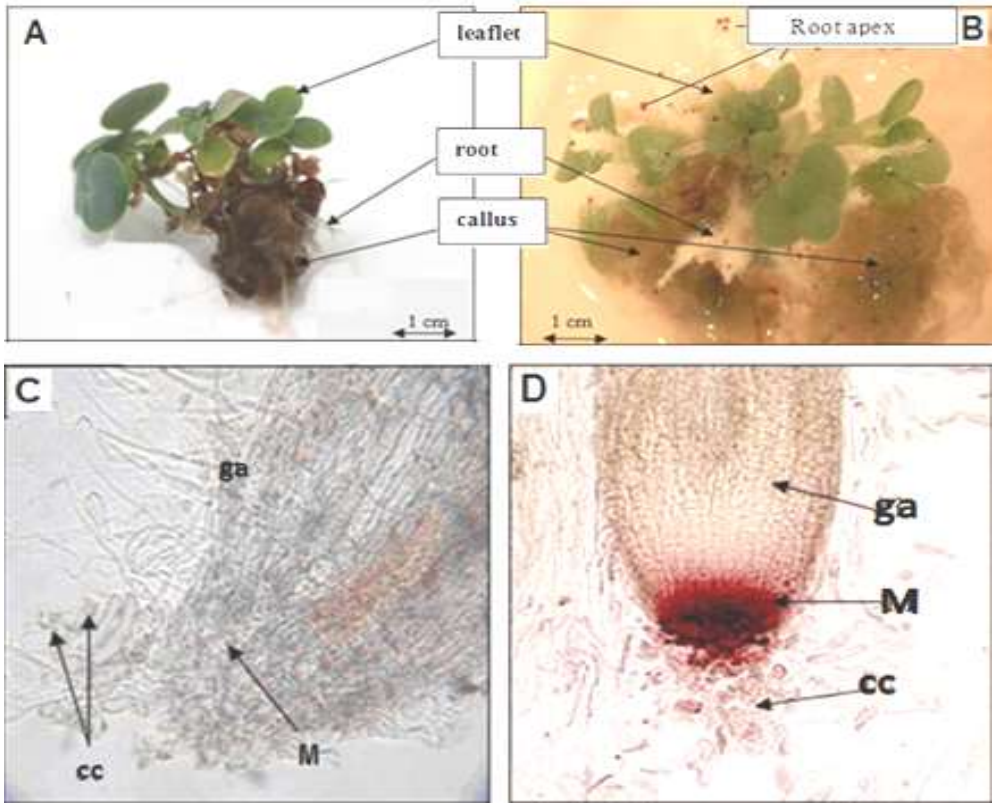

Fig. 1. Images of in vitro cultures of *Sedum telephium* ssp.maximum L. plants (after Ardelean et al., 2013) (Fig. 1 A and B), grown for 30 days in Murashige-Skoog basal medium (1962) with the addition of NAA 1.5 mg / l (A), or NAA and BAP 1.5 mg / l (B); (C, D) root apices - C representing the detail of a apex from a vitro cultures performed on MS medium, but with the addition of NAA 1 mg / l (position a), D – red colored root apex from a culture performed on MS medium with suplementation of ANA plus BAP 1 mg / l (abbreviation: cc – caliptral cells; M meristematic zone, ga- growth area stretch).

Material and methods

For examination of morphogenesis in calluses, macro and microscopically observations were performed, in superficial and profound callus layers, depending on the type of illumination.

Microscopic examinations of the callus or the tips of the roots structure were performed either on live tissues or on tissues that have been fixed prior to their use to obtain semi-fine sections using an ultramicrotome. For examination using the optical microscope, the thick sections must be around 500 nm (0.5 pm). For callus section preparation 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 l-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 are 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 – 60^0C, 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 ultrafine 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 semi-fine 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, l99l; Hayat, 2000). The semithin sections were examined by us using a Olympus BX 5l optical microscope equipped with a CCD camera.

Results and discussions

As can be seen in figure 2, the *Sedum* callus subcultured for 30 days under white fluorescent light (Fig. 2 A), maintained the green color, while callus exposed to blue light became dark red (Fig. 2C). In addition, the callus lighting with blue fluorescent light presented caulogenesis processes and rootedness.

From a microscopic point of view, the green callus presented cells with large vacuoles and cytoplasm which contained many chloroplasts (Fig. 2 B), whereas the samples exposed to fluorescent light blue, parenchyma of callus held cells with a color almost black. Their vacuoles presented numerous particles, solitary, or conglomerates; in other areas, callus tissue presented numerous meristematic nodules (Fig. 2 D and Fig. 4 A and 5 A) with small cells and large nuclei.

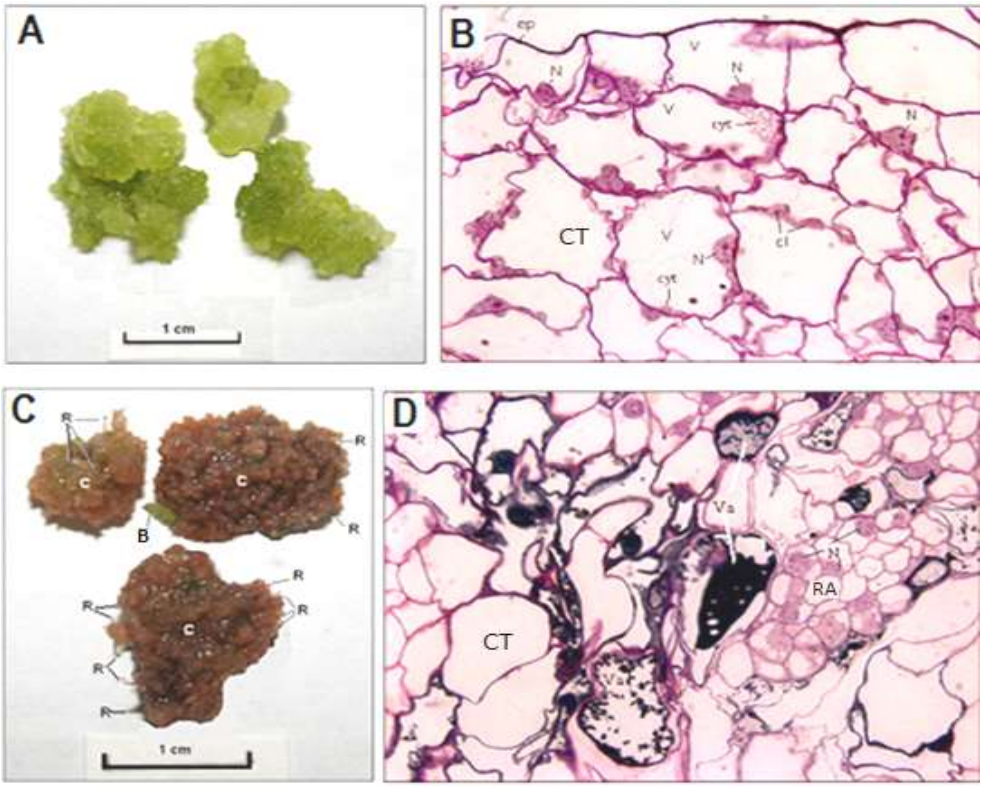

Fig. 2. The macroscopic (position A and C) and microscopic aspects (optical microscopic - 100x) of the Sedum callus grown 60 days on basic Murashige-Skoog medium (1962), with 2 4-dichlorophenoxyacetic and benzylaminopurine at concentrations of 1.5 mg / l and illuminated with white fluorescent tubes – positon A and B - or blue - positions C and D (abbreviations: cyt - cytoplasm; cl - chloroplasts ; ep - epidermis; N - nucleus; V – vacuole; Va - vacuole with anthocyanins; B – bud, R - roots; C – callus, Va - vacuole with anthocyanins, CT- callus tissue).

Also, in such cells chloroplasts were absent. Noteworthy is that on the surface of calluses colored in red white roots, a few millimeters long, were present, with red apices as a result of red pigment present in vacuolar juice of meristematic cells (Fig. 3A). This phenomenon is similar to that observed in root apices formed on the colony seedling which were obtained from in vitro cultivated apexes on MS medium suplimented with 1.5 mg /l ANA and 1.5 mg/l KIN (compare images in Fig. 1B and D in the Fig. 2 C).

Therefore, it can be said that under subculture *Sedum* callus conditions where KIN was replaced by BAP, especially when lighting was done with fluorescent tubes emitting blue light, rootedness was stimulated and more frequently caulogenesis was present (Fig. 2C). The color callus became red, a similar process to that highlighted in vacuoles of meristematic cells located in the root apices.

In native samples of *Sedum* roots (Fig. 3) meristematic cells presented a naturally pigment, evenly spread in the vacuolar juice of these cells. The application of a hydroxide solution of Na over this preparations produced a turning color of cells from red to purple, what could constitute proof that this is a anthocyanin pigment type and metabolism of these cells is not only the first type, but secondary.

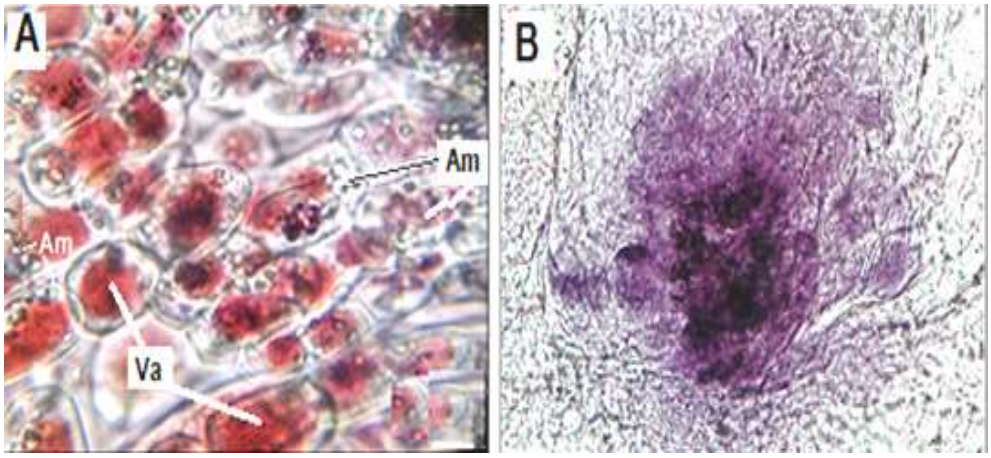

Fig. 3. Cytological aspects observed with an optical microscope (ob. 40) in the apical root regenerated from seedling or from tissue callus of *Sedum telephium spp. L. maximum* plants grown for 60 days on Murashige -Skoog (1962) culture medium with 1.5 mg / l NAA and 1.5 mg / l KIN. It can been observed the change of color of cells from red (position A) to purple (position B) by treatment with sodium hydroxide solution (abbreviations: Am- amyloplasts; Va - vacuoles containing red pigment).

Anthocyanins, a particular group of compounds, are one of the more than 6,000 members of the flavonoid family of polyphenol phytochemicals found in various plant foods. In addition to anthocyanins, the flavonoid group includes flavanols, flavones, flavanones, flavan-3-ols, and isoflavones. Anthocyanin pigments have been used in folk medicine for generations, but only recently the specific pharmacological properties of these compounds have been isolated and studied (Roy and colab., 2009).

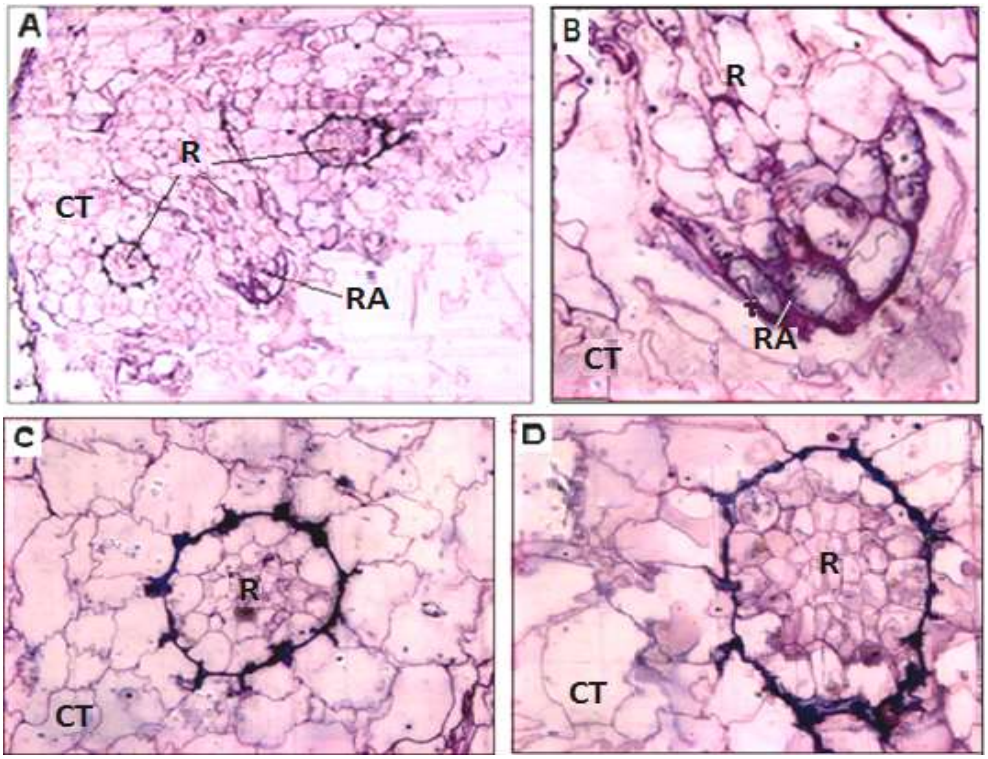

Fig. 4 A - D. Optical microscopy aspects observed in callus mass of Sedum telephium spp. L. maximum seedlings, on the 60th day of subculture's basic medium MS (1962) with the addition of 2.5 mg / l 2,4-D plus 1.5 mg / l BAP, increased by blue fluorescent light 16 hours / day, where: A - image seen by ob. 10; B - visualized image ob. 90 conducted a longitudinal section through the apex of a root mass that penetrates callus on its way to its exterior; C and D - images illustrating the appearance of cross sections made by roots that pierce parenchyma observed ob 40 (abbreviations: R - roots that pierce the mass of callus formation; CT- callus tissue; RA – root apex, longitudinal section through the meristematic).

Another aspect not mentioned in the literature is that of how initiation of callus formation in the mass organogenesis is produced. As can be seen in figures 4 A and 5 A both rootedness and caulogenesis begins in the profound layers of the callus tissue.

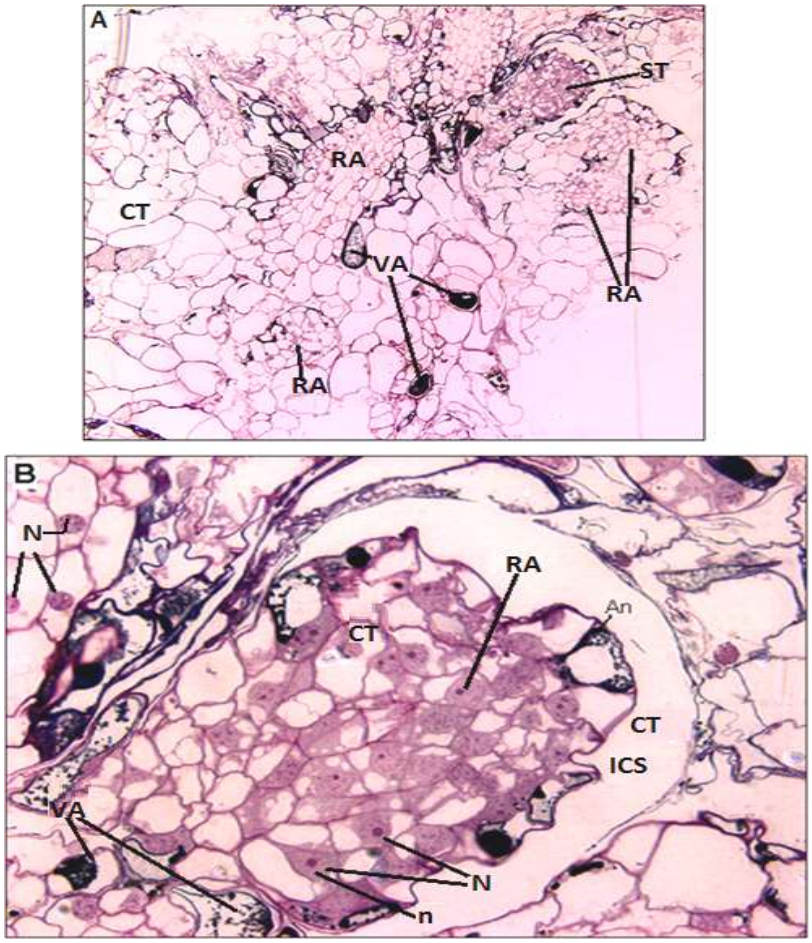

Fig. 5. Optical microscopy aspects observed to *Sedum telephium* spp. L. *maximum* callus , on the 60th day of subculture's basic medium MS (1962) with the addition of 2.5 mg / l 2 4-D plus 1.5 mg / l BAP, increased by blue fluorescent light 16 hours / day, (abbreviations: A - image seen by ob. 20, it can identify a bud neogenesis and caulinar apex seen with ob. 40, in the image of Fig 5 B (abbreviations: R - roots that pierce the mass of callus formation; CT- callus tissue; RA – root apex; ST-stem formation; Va - vacuole with anthocyanins; N – nucleus; n - nucleolus; CT - callus tissue; ICS - intercellular space).

Thus, in figure 4 A, in an area where organogenesis is triggered, roots developing in parenchyma callus can be seen. These show in their apical area (Fig. 4B), a reduced number of cells (probably initial meristematic cells) presenting cell walls stained in shades of purple after cytochemical reactions.

This phenomenon is noticed during young roots advancement throughout callus tissue (Fig. 4 C and D); at interfering points of future rhizodermal cells with parenchyma callus mass, in intercellular spaces - in the contact area - the presence of small conglomerates, black cherry, dark (almost black) can be observed, which we consider mucilaginous type as *Sedum* tissues have - in general - much mucilage; mucilage could facilitate slipping of root apices through the callus mass.

In an adjacent area where roots are produced, structures which will generate stems may arise, structures which present a well defined meristem in their apex (Fig. 5B); some of the future epidermal cells present a red pigment in their vacuolar sap, probably of an anthocyanin type (Fig. 4 A – D).

Conclusions

The experiments were aimed at cultivation "in vitro" explants *Sedum telephium* spp. L. *maximum* explants on *Murashige-Skoog* basal medium (1962) (MS) with the addition of ANA plus KIN in concentrations of 1.5 mg / l, or AIB with BAP, each 1.5 mg / l of each - being exposed to white fluorescent light - it was found that at the level of leaflets or stems randomly some epidermal cells were stained red raspberry, pigment considered by us as being due to accumulation of anthocyanins in their vacuolar juice. Apices of roots regenerated from in vitro cultures were also colored red raspberry about 1-2 mm from the tip (Ardelean et al, 2013), which is quite surprising, with the more so since this pigment is synthesized by the plant cells through a process of secondary metabolism, which was considered absent in meristematic cells.

In this paper, making a green *Sedum* subculturing of callus on a medium MS, but with the addition of 2,4-D 2.5 mg / l BAP plus 1.5 mg / l, exposed to fluorescent light blue (light is known - literature - that the plants stimulates secondary processes of metabolism), we noticed with surprise that not only apices of roots regenerated from the callus were stained red, but the whole callus acquired claret-red coloration. Microscopic examinations revealed that both in some cells from the surface of callus reddened, and the parenchyma thereof, presented their juice vacuolar a conglomeration of

glomerular particles or black plates, interpreted by us as anthocyanins. So, Sedum callus - depending on the nature of the growth regulators present in the culture substratum - under the exposure of light fluorescent blue, has acquired the ability to synthesize the pigment anthocyanin, unreported in the literature, in both apices of roots regenerated from callus and in vitroplantlets.

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