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DOSAGE OF SOME ORGANIC COMPOUNDS IN THE LEAVES OF THE *SEDUM TELEPHIUM* SSP. *MAXIMUM* L. PLANTS HARVESTED FROM NATURE COMPARED WITH PLANTS OBTAINED ON THE CULTURE OF MURASHIGE – SKOOG SUPPLEMENTED WITH GROWTH REGULATOR

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ABSTRACT. In the near future, the medicinal species of *Sedum telephium* ssp*. maximum* L. studied by us, would become general interest for the biotechnical procedures of obtaining phytopharmaceuticals sublayers, because of their secondary products of metabolism that they synthetize. The purpose of the present study was to determine the content of organic compounds of the leaves harvested from nature and the vitro-leaves taken from the vitro-plants grown on culture of Murashige – Skoog (1962) which had been treated with various growth regulators. The organic compounds determined have been: ascorbic acid, total proteins, total carbohydrates, total polyphenols and anthocyans. The absorptivity of extracts obtained has been calculated with a UV/Vis T60U spectrophotometer. The biochemical determinations carried out on the vegetal material have indicated that the quantities of organic compounds were significantly increased in the samples taken from the *in vitro*-plants of *Sedum telephium* ssp*. maximum* L., cultivated on the culture of Murashige – Skoog, especially in those with additions of kinetin and naphthaleneacetic acid, in comparison with the results gathered from the leaves harvested from plants grown in nature.

Keywords: *Sedum telephium* ssp. *maximum* L., proteins, carbohydrates, polyphenols, antocyans, growth regulators

INTRODUCTION

Sedum telephium ssp. *maximum* L. is a plant frequently found in the spontaneous flora of Romania, which belongs to the *Crassulaceae* family with specific features as the content of its vegetative organs are concerned: various valuable chemical compounds. The Romanian traditional medicine assigns to this plant some antiseptic and cicatrizing effects, the leaves or the whole plant being used to treat paralysis and paresis of peripheral nerves, hemorrhoids, wounds and burns, but also tumours.

In general, many of the medicinal plants are micro propagated *in vitro*, being used as initial material in the population of the cultures that bioreactors are filled, and from the biomass collected after a certain number of days of vitro culture- we pass on to extract and condition the compounds relevant for pharmaceutical purposes (Cachiţă *et al.*, 2004).

For populating the bioreactors with vegetal material for pharmaceutical purposes, that generate secondary products of metabolism, or for obtaining planting material, the cloning of plants is made *in vitro,* micro propagation being the unique way of extreme efficiency for enhanced multiplication of vegetal species.

As the *in vitro* cultivation of the species *Sedum* is concerned, in order to obtain some material that could be used as source of active principles, the scientific literature has a lack of such articles.

The chemical composition of the species taken in this study could be highlighted through the dosage of some important organic compounds like the proteins and the carbohydrates.

In the near future, because of its secondary products of metabolism, this species would become general interest for the biotechnical procedures that consist in obtaining the phytopharmaceutical sublayers.

MATERIALS AND METHODS

Plant material. The vegetal material was represented by the foliar limbs of the petioles of *Sedum telephium* ssp. *maximum* L. harvested in a natural environment and the foliar limbs of the petioles harvested from the vitro leaves taken from the 60-day-old vitro seedlings from the zygotic embryo of the seeds germinated on the culture of *Murashige – Skoog* (Murashige & Skoog, 1962), supplemented with various growth regulators. We have made determinations concerning their content in insoluble ash, dry substance, chemical elements, ascorbic acid, total proteins, total carbohydrates, total polyphenols and antocyans.

Thus, the leaves and vitro leaves on which we have carried out our physical-chemical analysis were taken from the following cultures:

- $\mathbf{V_{MN}}$ – the leaves of *Sedum telephium* ssp. *maximum* L. harvested from a natural environment;
- $\mathbf{V_0}$ – the witness variant represented by the vitro leaves harvested from the vitro seedlings generated by the MB-MS culture without growth regulators;

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-**V_1**- the experimental variant represented by the vitro leaves harvested from the vitro seedlings generated by the MB-MS culture with **BA**[1] having the concentration of 1,5 mg/l;

- **V_2**- the experimental variant represented by the vitro leaves harvested from the vitro seedlings generated by the MB-MS culture with **IBA**[2] having the concentration of 1,5 mg/l;

-**V_3** - the experimental variant represented by the vitro leaves harvested from the vitro seedlings generated by the MB-MS culture with **BA** having the concentration of 1,5 mg/l and **IBA** having the concentration of 1,5 mg/l;

-**V_4** - the experimental variant represented by the vitro leaves harvested from the vitro seedlings generated by the MB-MS culture with **KIN**[3] having the concentration of 1,5 mg/l and **NAA**[4] having the concentration of 1,5 mg/l;

Harvesting: The vegetal samples grown *in vitro*, represented by the leaves with petiole have been harvested from the recipients of the culture, while in the case of the plants grown in a natural environment, we proceeded to harvest the young leaves with a diameter almost equal to those *in vitro* (about 2 cm). For each variant, we took three specimens.

Drying and grind: After the harvesting, the samples were homogenized, removed the unusable parts and washed them with deionized water (the residual humidity evaporated at the room temperature) and they dried after 2-3 days on a white paper.

The dried vegetal products have been grinded in a mill for vegetal products until becoming a fine powder, then they were homogenized and pressed through a sieve with 30 holes/cm^2.

***Extraction*:** Extraction was performed used 50 ml ethyl ether as an extraction solvent. The specimens have been sonicated in the dark for 24 hours. The absorbance of the obtained extracts (undiluted and diluted 1:10, 1:100, 1:1000), rich in phytochemical compounds was registered on the 200 – 700 nm domain (UV – Vis) with the aid of a UV/Vis spectrophotometer T60U, PG Instruments Limited, UV WIN®version 5,05, in order to determine the total concentration of compounds.

The methods of physical-chemical analysis have been applied in accordance with the *European Pharmacopoeia* as it follows:

1. *Determination of the content of total proteins*. The total concentration of proteins was determined through two methods: Bradford, with Coomasie Brilliant Blue (Bradford, 1976) and BCA method, with bicinchoninic acid and copper sulphate (Smith *et al.*, 1985). For each determination, we have made specimen curves with bovine serum albumin (BSA).

2. *Determination of the content of total carbohydrates* was performed with the aid of a UV/Vis spectrophotometer in conformity with STAS 10 902 - 77.

3. *Determination of the content of ascorbic acid.* The ascorbic acid (Vitamin C) was determined titrimetrically, taking into consideration the fact that the ascorbic acid is a strong reducing agent, which easily loses the hydrogen-atoms, transforming the dehydroascorbic acid that also present a vitanimic action (Danny *et al.*, 2003). The vitaminic acid is lost when the lactonic cycle of the dehydroascorbic acid is hydrolyzed forming a diketogulonic acid. The method that we used was based on the titration of the ascorbic acid from the vegetal extracted with a 2,6-dichloroindophenol until a pink, persistent colour appears for at least 5 seconds. We established the titration of the solution of 2,6-dichloroindophenol by using a solution of concentration of vitamin C freshly prepared and titrated in the same conditions as the samples. The absorbance of the extract obtained on a 200 – 700 nm domain (UV – Vis) with the aid of a UV/Vis spectrophotometer in conformity with STAS 10 902 - 77.

4. *Determination of the content of total polyphenols.* The total content of polyphenols was estimated through the Folin-Ciocalteu method (Harborne, 1998). The homogenized product (m = 1±0,01g) was quantitatively treated with a 15cm^3 sultry ethylic alcohol (96%) in a conical balloon. The extraction of polyphenols was carried out through boiling for 10 minutes in a water bath (the conical balloon being equipped with an ascendant refrigerant). The specimen was filtered through a glass filter. The filtered product was passed through a 25cm^3 balloon and was brought to the quota with a 96% ethylic alcohol. In a 100 cm^3 balloon we introduced a 1 ml filtered product of Folin – Ciocalteu reagent. After 3 minutes, we added 5 ml Na_2CO_3 and we brought it to the quota with distilled water (the operation solution). We left it for 30 minutes in the dark. We prepared the witness solution: 1 ml ethylic alcohol + 1 ml Folin – Ciocalteu, after 3 minutes we added 5 ml Na_2CO_3 and we brought it to the quota with distilled water. The concentration of the polyphenols in the sample was determined through the spectrophotometer method, at the UV/Vis spectrophotometer T60U, PG Instruments Limited, UV WIN®version 5.05, at a wavelength of 750 nm. c = D/ 0,0864, (mg/ml).

The content of polyphenols was expresed in mg polyphenols/100g product and was determined with the following equation:

$$X = \frac{c \cdot V_1 \cdot V_2}{m \cdot V_3} \cdot 100$$

, where:

- x – content of total polyphenols in the product, mg/100g;
- c – concentration of polyphenols after the graph, mg/ml;

[1] BA = benzyladenine

[2] IBA = indolebutyric acid.

[3] KIN = kinetin.

[4] NAA= naphthaleneacetic acid.

- V_1 – volume of the extracted product, ml;
- V_2– volum of the specimen analyzed, ml;
- V_3 – volume of the extracted product taken for the analysis, ml;
- m – mass of the product used for analysis, g.

The precision of determination is ± 5,0%.

5. ***Determination of the total content of antocyans*****.** The total content of antocyans was assessed through the spectrophotometer method (Lee *et al.*, 2008). The homogenized product (m = 10±0,01g) was extracted with a solution of a 96% ethylic alcohol and hydrochloric acid 1,5N in a ratio 85:15 until the discoloring of the sample was achieved. The specimen was filtered through a glass filter. The filtered product was passed through a 100cm^3 balloon. The extract obtained was used to determine the total content of antocyans. We left it for 30 minutes in the dark and then, we determined the optic density. The determination of the optic density was carried out at the UV/Vis spectrophotometer T60U, PG Instruments Limited, UV WIN®version 5.05, at a wavelength of 540 nm. The total content of antocyans (Xa) was expressed in mg/100g and was determined with the following equation:

$$X_a = 100 \cdot \frac{D \cdot V \cdot R}{m \cdot K},$$

where:

Xa – total content of antocyans, mg/100g;

– D – optic density of the extract at a 540 nm wavelength;

– V – total volume filtered after the extraction, ml ;

– R – dilution coefficient of the product;

– m – mass of the specimen for research, g;

– K – coefficient equal with 98,2 for the 1 cm vat.

The precision of determination is ± 5,0%.

RESULTS AND DISCUSSION

The content of proteins and carbohydrates**:** The proteins, nitrated extracts of major importance in the growth and development of the plants were analyzed in all the samples. Comparative analysis of the content of proteins in the vitro seedlings cultivated on the MB - MS (1962) culture with growth regulator addition with the quantity of proteins existent in the leaves of the plants grown in a natural environment, we could infer great quantitative differences (Fig. 1A). This difference could have appeared due to the growth regulators added on the culture. Besides, we could notice some significant differences among the *in vitro* seedlings grown in cultures where we added auxins as compared to those grown with cytokinin.

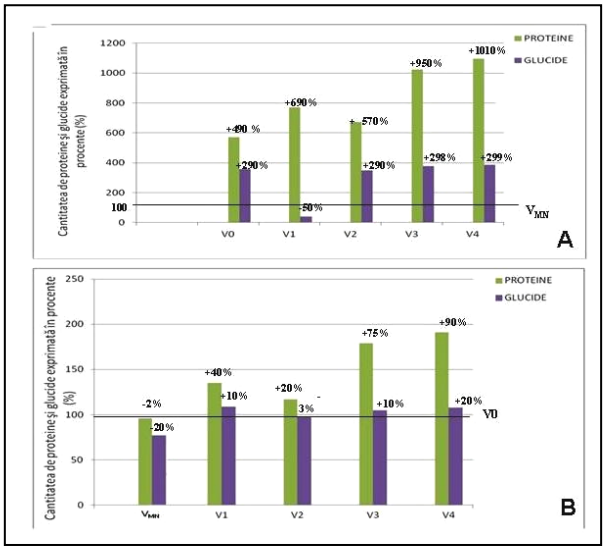

Fig. 1 A and **B**. Quantity of proteins and carbohydrates present in the extract of the vitro leaves of *Sedum telephium* ssp. *maximum* L., where: **A** – values of the samples (**V_0 - V_4**) are represented as a percentage in relation to the sample variant **V_{MN}** (extract of leaves in a natural environment) considered as a reference lot (100%) and **B** - values of the samples (**V_1- V_4**) are represented as a percentage in relation to the sample variant **V_0** (extract of vitro leaves) considered as a reference lot (100%), where: **V_{MN} –** leaves of *Sedum telephium* ssp. *maximum* L. harvested in a natural environment; **V_0** - vitro leaves of *Sedum telephium* ssp. *maximum* L. harvested in the MB – MS (1962) culture without growth regulator addition; **V_1** - vitro leaves of *Sedum telephium* ssp. *maximum* L. harvested in the MB – MS (1962) culture with **BA** addition, 1,5 mg/l; **V_2**- vitro leaves of *Sedum telephium* ssp. *maximum* L. harvested in the MB – MS (1962) culture with **IBA** addition, 1,5 mg/l; **V_3** - vitro leaves of *Sedum telephium* ssp. *maximum* L. harvested in the MB – MS (1962) culture with **BA** and **IBA** addition, 1,5 mg/l; **V_4** - vitro leaves of *Sedum telephium* ssp. *maximum* L. harvested in the MB – MS (1962) culture with **KIN** and **NAA** addition, 1,5 mg/l.

The quantities of proteins and carbohydrates synthesized *in vitro* seedlings of *Sedum telephium* ssp. *maximum* L. are by far superior to the witness sample taken from nature, significant increases being observed at the variants **V_3** and **V_4**, which indicate an acceleration of the growth processes of the vitro seedlings (Fig. 1A and B).

The content of ascorbic acid. The quantity of ascorbic acid accumulated in the leaves taken from the *in vitro* cultures of *Sedum telephium* ssp. *maximum* L. was significantly increased as compared to the variants of leaves harvested from nature. Thus, the variant **V_4** (MB - MS, with **KIN** and **NAA** each 1,5 mg *l* (Fig. 2A) registered increases of 100% as the content of vitamin C in concerned, as compared to the plants taken from nature, while with the variant **V_3** the increase was 95%. Altogether, the variant with 1,5 mg *l* **IBA** made an increase of 80%. These results could suggest that the exclusive presence of an auxin or

cytokinin in the sublayer of a *in vitro* culture cannot stimulate the accumulation of the vitamin C in tissues, while the mixture of the two, respectively **BA** with **IBA** 1,5 mg/l each and **KIN** with **NAA** 1,5 mg/l each, had a great increase of 100% as compared to the witness variants ($\mathbf{V_{MN}}$ and $\mathbf{V_0}$).

In the case in which the content of vitamin C in the leaves was related with the values of this parameter determined at the witness lot ($\mathbf{V_0}$), (Fig. 2B) leaves harvested in *vitro*, on the **MB – MS** culture without growth regulator addition, the increases were significantly and statistically raised at all variants, but moreover at the vitro cultures which contained **KIN** plus **NAA** each 1,5 mg/l, followed by the vitro cultures on the variant $\mathbf{V_3}$ (MB – MS culture supplemented with **BA** and **IBA** in a concentration of 1,5 mg/l each). At these variants, the increases compared to the $\mathbf{V_0}$ were raised, 115% for the variant $\mathbf{V_4}$ and 100% for the variant $\mathbf{V_3}$. In the case of these two variants, the result was similar with the protein content (see fig. 1 A and B), which confirms the results of the research carried out by Gershoff and his collaborators in 1993, that is that in plants, vitamin C can be found under the form of ascorbic acid and dehydroascorbic acid, associated with the proteins with which it forms a complex called ascorbigen.

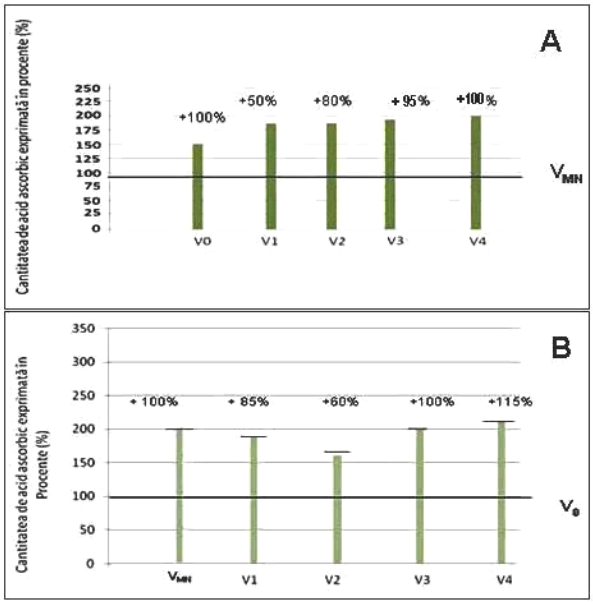

Fig. 2 A and **B**. Quantity of ascorbic acid present in the extract of the vitro leaves of *Sedum telephium* ssp. *maximum* L., where: **A** – values of the samples ($\mathbf{V_0}$ - $\mathbf{V_4}$) are represented as a percentage in relation to the sample variant $\mathbf{V_{MN}}$ (extract of leaves in a natural environment) considered as a reference lot (100%) and **B** - values of the samples ($\mathbf{V_1}$- $\mathbf{V_4}$) are represented as a percentage in relation to the sample variant $\mathbf{3V_0}$ (extract of vitro leaves) considered as a reference lot (100%), where: $\mathbf{V_{MN}}$ – leaves of *Sedum telephium* ssp. *maximum* L. harvested in a natural environment; $\mathbf{V_0}$ - vitro leaves of *Sedum telephium* ssp. *maximum* L. harvested in the MB – MS (1962) culture without growth regulator addition; $\mathbf{V_1}$ - vitro leaves of *Sedum telephium* ssp. *maximum* L. harvested in the MB – MS (1962) culture with **BA** addition, 1,5 mg/l; $\mathbf{V_2}$- vitro leaves of *Sedum telephium* ssp. *maximum* L. harvested in the MB – MS (1962) culture with **IBA** addition, 1,5 mg/l; $\mathbf{V_3}$ - vitro leaves of *Sedum telephium* ssp. *maximum* L. harvested in the MB – MS (1962) culture with **BA** and **IBA** addition, 1,5 mg/l; $\mathbf{V_4}$ - vitro leaves of *Sedum telephium* ssp. *maximum* L. harvested in the MB – MS (1962) culture with **KIN** and **NAA** addition, 1,5 mg/l.

The content of total polyphenols and antocyans. The accumulation of antocyans in the *in vitro* cultures of *Sedum telephium* ssp. *maximum* L. is sensibly increased as compared to the witness sample. In addition, we found a modification of the content of polyphenols which is significant at the variants included in the study; the differences among the various types of growth regulators that we used in our experiment were significant from a statistical point of view at the variants with mixtures of auxin and cytokinin, especially in the case of the variant $\mathbf{V_4}$ - with **KIN + NAA,** 1,5 mg/l each, (Fig.3 A), both in relation with the witness sample taken from nature and the witness sample taken from the *in vitro* culture (Fig. 3 B). We also noticed that at the specimens in the witness sample from the *in vitro* culture (Fig. 3B), the content of *antocyans* was increased with 20 – 25 % at the variants with mixtures of auxin and cytokinin as compared to the data registered when the growth regulators were administered separately (the variants $\mathbf{V_1}$ and $\mathbf{V_2}$), the greatest increase of the content of antocyans (25%) being on the variant of the **MB – MS** culture with an addition of **KIN** and **NAA,** 1,5 mg/l each.

Biochemically, the main parameters we followed do not significantly modify in relation with the witness variants. The protein synthesis and the biosynthesis of the ascorbic acid is intense at the *in vitro* seedlings of *Sedum telephium* ssp. *maximum* as compared to the leaves harvested from the plants of nature (witness sample $\mathbf{V_{MN}}$). These data indicate favourable conditions for the *in vitro* seedlings and the increased capacity of adaptation and synthesis in the case of the plants *Sedum telephium* ssp. *maximum* L.

We consider that the *in vitro* seedlings grown on the culture variants supplemented with a mixture of auxin and cytokinin: the variant $\mathbf{V_4}$ (vitro leaves of *Sedum telephium* ssp. *maximum* L. grown on the MB - MS (1962) culture with addition of **KIN** and **NAA** with a concentration of 1,5 mg/l each) and the variant $\mathbf{V_3}$ (vitro leaves of *Sedum telephium* ssp. *maximum* L. grown on the MB - MS (1962) culture with addition of **BA** and **IBA** with a concentration of 1,5 mg/l each) had the best results. The growth regulators used in this case can positively influence the growth ratio and the multiplication with indirect effects on the synthesis of the secondary metabolites.

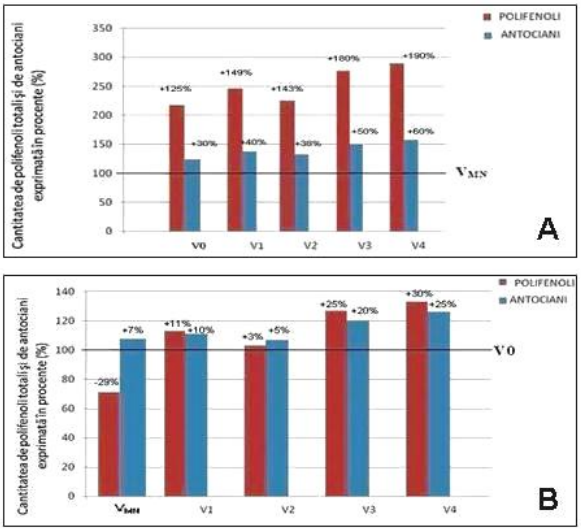

Fig. 3 A and **B**. Quantity of total polyphenols and antocyans present in the extract of the vitro leaves of *Sedum telephium* ssp. *maximum* L., where: **A** – values of the samples (**V_0 - V_4**) are represented as a percentage in relation to the sample variant **V_{MN}** (extract of leaves in a natural environment) considered as a reference lot (100%) and **B** - values of the samples (**V_1- V_4**) are represented as a percentage in relation to the sample variant **V_0** (extract of vitro leaves) considered as a reference lot (100%), where: **V_{MN} –** leaves of *Sedum telephium* ssp. *maximum* L. harvested in a natural environment; **V_0** - vitro leaves of *Sedum telephium* ssp. *maximum* L. harvested in the MB – MS (1962) culture without growth regulator addition; **V_1** - vitro leaves of *Sedum telephium* ssp. *maximum* L. harvested in the MB – MS (1962) culture with **BA** addition, 1,5 mg/l; **V_2**- vitro leaves of *Sedum telephium* ssp. *maximum* L. harvested in the MB – MS (1962) culture with **IBA** addition, 1,5 mg/l; **V_3** - vitro leaves of *Sedum telephium* ssp. *maximum* L. harvested in the MB – MS (1962) culture with **BA** and **IBA** addition, 1,5 mg/l; **V_4** - vitro leaves of *Sedum telephium* ssp. *maximum* L. harvested in the MB – MS (1962) culture with **KIN** and **NAA** addition, 1,5 mg/l.

CONCLUSION

The biochemical analyses performed at the level of the vegetal material made of leaves and *in vitro* leaves indicated the fact that the main parameters followed, that is total proteins, total polyphenols and ascorbic acid have been significantly increased at the samples taken from the *in vitro* seedlings of *Sedum telephium ssp. maximum* L., compared to the foliary samples harvested from the plants taken from nature. However, in the case of antocyans, with the specimens taken from the Murashige-Skoog culture, with a 1,5 mg/l addition of **KIN** and **NAA**, their content was increased with 60% as compared to the values obtained at the witness variant and in case of the leaves harvested from plants in nature.

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