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Investigation regarding the potential application of grape pomace extracts on *in vitro* plant growth and development

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Abstract. The grape pomace hydroalcoholic extracts obtained by two different extraction methods were tested for biostimulatory potential activity for *Origanum vulgare* L. cultures. The total polyphenols contents of extracts were evaluated by Folin-Ciocalteu method. Characteristics such as: pH, salinity, conductivity and total dissolved solids were determined. FTIR and UV spectra of extracts were also recorded. The effect of grape pomace extracts on growth and development of oregano plant were studied by carrying out *in vitro* propagation of oregano on Murashige and Skoog basal medium supplemented with different concentrations of grape pomace extracts. Biometric measurements, growth rate and biomass accumulation have been narrowly monitored for all samples and compared to the control sample. The results have shown that the morphogenetic response depends both on the proportion in which basal medium was supplemented and on the extraction method used. A stimulation of growth and development at a low concentration of grape pomace extracts has been noted. The best results were observed in the samples with 1 and 5 % of grape pomace extract obtained by classical extraction.

***Keywords*:** grape pomace extract; *Fetească Neagră*; valorization; *in vitro* propagation; *Origanum vulgare*; plant growth; classical extraction; ultrasound assisted extraction.

1. Introduction

Romania is an important wine producer ranked 6th in European Union countries by International Organization of Vine and Wine [1], an annual evaluation on wine consumption in Romania revealed that *Fetească Neagră* was the most consumed variety of wine [2].

Thus, the waste from wine industry represents an important environmental issue at national level as well as worldwide; therefore, the concern for the grape pomace reuse becomes a significant part of our research, in an attempt to find new solutions for its valorization in different fields. Recently, an innovative product based on honey and *Fetească Neagră* grape pomace powder was reported by our research team [3].

Grape pomace (GP) is extremely rich in dietary fiber, lipids (seeds), proteins, minerals, polyphenolic compounds (tannins, anthocyanins and flavones), one of its most important biologic activities, the antioxidant properties, is attributed to the polyphenolic composition [4, 5].

This very inexpensive by-product has permanently attracted the attention of researchers leading to an interest in the study of different uses in various domains: as ingredients in the food industry [5, 6], as additive in animal feed [7], in cosmetics, medicine, etc. [8, 9]. Other traditional applications of grape pomace have been its use as fertilizer. Grape pomace as a heterogeneous mixture of seeds, skins, pulp, and stalks usually cannot directly applied into the soil, because of the possible pathogens. It should be composted at first and then can be used as a soil conditioner. The composted marc pomace application to enhance the organic matter, nitrogen and mineral contents of vineyard soils is reported in literature [10-12].

On the other hand, one of the current trends is represented by the discovery of new ecological stimulants for plant growth and development. Plant biostimulants based on natural materials have received considerable attention. Currently, a biostimulant is theoretically defined as any substance or microorganism that is not a nutrient, pesticide or any of the soil improvers, but has the capacity to promote the health and growth of a plant during the induction of natural biological processes [13, 14].

In vitro propagation is an effective tool to multiply plant species and a suitable method for regeneration, micropropagation and long-term storage of high quality plant material. Plant cell and tissue cultures employ nutritive culture media and controlled aseptic conditions for the growth of plant cells, tissues and organs. The cell and tissue culture techniques have some important advantages in agriculture, horticulture, and other important aromatic crop plants. Aromatic and medicinal plants represent good choices for investigation

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concerning the study of growth and morphogenesis of plant tissue cultures by adding different proportion of compounds, hormones or extracts [15, 16].

Our previous research in collaboration with the Biology Department present the impact of certain products (whey [17], hormones [18], synthetic heterocyclic compounds [19]) on growth and morphogenesis of different plant tissue cultures.

Potential applications of extracts obtained from different plants sources or industry wastes on seed germination processes and plant growth and development were reported [20]. Testing the crude extracts allows to obtain preliminary information, without previous separation and purification, with low processing costs. In the same time, it restricts the possibility to establish a concrete mechanism and a clear connection between a certain phenolic compound and the plant morphogenetic response. The good results in plant propagation using forestry wastes showed that the extracts containing polyphenols may have a similar effect to that of phytohormones [20, 21].

Taking into account all these considerations, for the present work, the investigation regarding the effect of the addition of different concentrations of grape pomace extracts on *in vitro* growth and development of oregano was initiated.

In our study, for improving the *in vitro* culture medium, GP from local *Feteasc*ă *neagr*ă variety has been selected due to its high content of different phenolic compounds with significant antioxidant effect [22, 23].

To the best of our knowledge, the use of grape pomace extracts in plant tissue culture has not been reported so far.

Knowing that medicinal plants represent suitable choices for *in vivo* or *in vitro* plant growth investigation, *Origanum vulgare* L. (oregano) has been chosen as biological material in this study. Oregano is well-known, since ancient times, for therapeutic purposes due to its chemical composition (flavonoids and phenolic acids: rosmarinic acid, caffeic acid, apigenin, luteolin, quercetin, and their derivatives) and various curative properties (digestive, expectorant, antiseptic, antispasmodic, etc.) [24, 25].

Tissue culture and micropropagation for *Origanum* species were reported in some studies by supplementing the basal medium with different compounds: 6-benzylaminopurine (BAP), α-naphthaleneacetic acid (NAA), indole-3-acetic acid (IAA), indole-3-ylbutyric acid (IBA), kinetin (KIN), 2,4-dichlorophenoxyacetic acid (2,4-D), etc. [26-29].

Therefore, the aim of current research was to evaluate the morphogenetic response for *in vitro* cultures of *Origanum vulgare* L. by supplementing the basal medium with different proportions of *Feteasc*ă *neagr*ă grape pomace extracts.

2. Experimental

2.1. Materials

All chemicals and reagents used for this study were analytical grade or purest quality purchased from Merck, Sigma, Chemical Company.

2.2. Hydroalcoholic grape pomace extracts preparation

The GP from Romanian red grape variety *Feteasc*ă *Neagr*ă (Panciu - Vrancea, Romania) was collected right away after the grape press and was stored at -18 °C. Before use, the samples were left overnight at 4 °C for slow defrost.

Classical extraction and ultrasound assisted extraction were carried out using hydroalcoholic mixture ethanol/distilled water 50/50 (v/v), raw material/ solvent rate 1/5.

Classical extraction (CE) by maceration was performed at room temperature using a magnetic laboratory stirrer (Nahita Blue, model 692) for 12 hours, leading to CE-GP extract.

Ultrasound Assisted Extraction (UAE) was performed using Digital Pro Ultrasonic PS-10A with parameters as follows: time 1 h, temperature 50 °C, ultrasonic frequency 40 kHz, ultrasonic power 80 W, heating power 50 W, leading to UAE-GP extract.

The extracts thus obtained were filtered and concentrated by evaporation under reduced pressure (using Heidolph Rotary Evaporator, Laborota 4000) and stored at 4 °C until used.

2.3. Grape pomace extracts analysis

Dry matter content in the GP extracts was established by evaporation and drying at 105 °C until a constant mass, using a Moisture Analyser (KERN MLB 50-3, Germany) and calculated as residual weight from start weight.

*p*H, salinity, conductivity and total dissolved solids (TDS) were determined using Thermo Scientific™ Orion™ Versa Star Pro™ Multiparameter Benchtop Meter (Thermo Fisher Scientific, USA).

Total polyphenols content (TPC) was determined using the Folin-Ciocalteu method [30]. The results were expressed as mg gallic acid equivalents (GAE)/mL using a calibration curve. Absorption determination was made spectrophotometrically, by using a UV/VIS Spectrophotometer (Shimadzu UV-1280, Japan), at 760 nm.

The GP color assays were performed by calculating the color intensity (CI) using the Glories method [31] which are widely used by wineries [32]. Color intensity corresponds to the sum of the absorbance at 420 (yellow), 520 (red) and 620 (blue) nm [33, 34]. Spectrophotometric absorbance readings were taken using a UV/VIS Spectrophotometer (Shimadzu UV-1280, Japan).

Also, the UV spectra of samples were recorded using scanning over interval 190-400 nm.

All measurements were realized in triplicate.

FTIR analyses were performed to investigate functional groups present within the samples by using FTIR Spectrophotometer (Shimadzu QATR-S, IRSpirit-L, Japan) by plotting transmittance versus wavenumber in spectral range of 4000-400 cm^{-1} with 45 consecutive scans at a resolution of 4 cm^{-1} [35].

2.4. Biological material preparation

The oregano seeds provided by Agricultural Research and Development resort from Secuieni – Neamț (Romania) were sterilized before germination by

immersion in a 5 % solution of commercial chloramine T trihydrate for 15 minutes and rinsed three times in sterile deionized water.

Vitroplants of *Origanum vulgare* obtained from seeds germinated on the Murashige-Skoog basic medium (MS) [36], without addition of any phytohormones or growth-stimulating substances were collected. Sterile explants (nodes and apex fragments) were used as biological material for *in vitro* propagation (Figure 1) [37].

The handling of the biological material was performed in sterile conditions, in a laminar flow air cabinet (model SPACE-PBI, Italy). For cultures incubation a SANYO plants growth chamber (model MLR-351, Japan) was used with the following parameters: temperature 23±1 °C, 16/8 h light/dark cycle, light intensity 2500 lux.

Figure 1. Sterile vitroplants on the hormone-free MS medium, used as explant donors.

After 2 month, 6 node or apex explants for each variant were transferred to Erlenmeyer flasks containing MS basal medium supplemented with different proportions of GP extracts (1, 5 and 10 % respectively). As well, a control sample with only MS basal medium was inoculated.

The *p*H media was adjusted to 5.7-5.8 by using NaOH 1 N.

In Table 1 are presented the sample codification for each experiment.

Table 1. Sample codification and medium composition for the experiments

Code variant	Medium composition	Proportion of GP extract [%]
MS	Murashige-Skoog basic medium	0 (control)
E_1A	MS + CE-GP extract	1
E_2A		5
E_3A		10
E_1B	MS + UAE-GP extract	1
E_2B		5
E_3B		10

The cultures were weekly monitored for a period of 8 weeks to observe the evolution of phytoinocula and their morphogenetic response. Each experiment was realized in triplicates containing 6 explants.

Biometric measurements, growth rate and biomass accumulation were assessed for all samples and compared to the control sample (MS).

3. Results and discussion

3.1. Characterization of grape pomace extracts

The GP hydroalcoholic extracts were characterized in terms of dry matter, *p*H, salinity, conductivity, total dissolved solids, total content of polyphenols and color intensity. The results were summarized in Table 2.

Table 2. Characteristics of *Feteasc*ă *neagr*ă GP extracts*

Characteristics	CE-GP extract	UAE-GP extract
Dry matter [%]	0.486±0.025	0.183±0.015
*p*H at 25 °C	4.43±0.04	4.60±0.017
TDS [ppm]	213.63±0.40	134.33±0.45
Salinity [psu]	0.211±0.013	0.132±0.006
Conductivity [µS]	428.2±2.3	271.9±1.6
TPC [mg GAE / 100 mL extract]	14.00±0.012	14.40±0.014
CI**	3.810±0.003	3.257±0.007

* Each value represents the average from three measurements. Values are expressed as mean ± SD

** Calculated as sum of the absorbance at 420 (yellow), 520 (red) and 620 (blue) nm (data not shown).

In Figure 2 is presented the UV spectrum profile of hydroalcoholic grape pomace extracts.

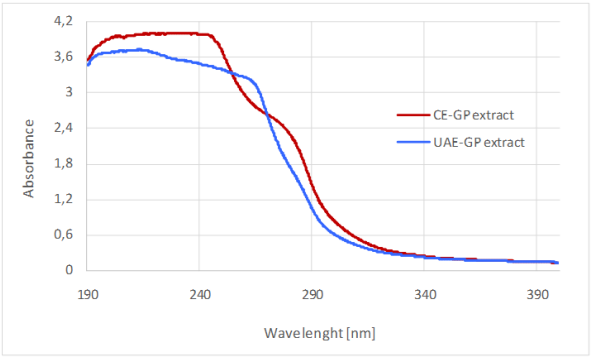

Figure 2. UV profile of hydroalcoholic *Feteasc*ă *neagr*ă grape pomace extracts.

The IR spectrum profile of extracts is shown in Figure 3.

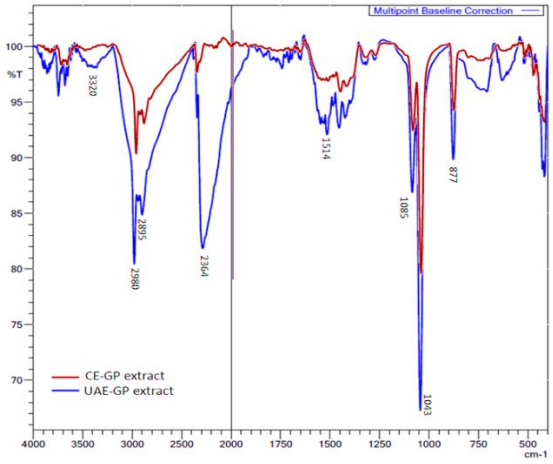

Figure 3. IR profile of hydroalcoholic *Feteasc*ă *neagr*ă grape pomace extracts.

From Table 2 it can be perceived that the hydroalcoholic extracts obtained by classical extraction and ultrasound assisted extraction, respectively, did not

show very important differences in any characteristics that have been measured.

A slightly noticeable difference can be observed at the level of total dissolved solids and the dry matter percentage which are higher for the samples obtained by classical extraction.

The *p*H of the both extracts were similar with values of 4.43 and 4.60, respectively, which is close to those reported in the literature [35].

It can be noticed that the color intensity is slightly influenced by the *p*H values, so for a higher *p*H value, chromaticity decreases which is in accordance with the literature [38]. The color intensity for CE-GP extract is 15 % higher than the UAE-GP extract. As for wine, the color of grape pomace extracts represents a summation of three components: tannins (yellow), free anthocyanins (red and blue) and combinations of tannins and anthocyanins (blue) [33, 34]. The data registered for the three absorbances proved that the yellow-orange component represents slightly over 50 % of CI value and the red pigments less than 35 %.

The total polyphenols content determined by Folin-Ciocalteu method did not differ significantly, although it can be observed that the UV profiles of hydroalcoholic grape pomace extracts (Figure 2) are relatively different with a maximum of absorption at λ_{max} = 239 nm for CE-GP extract and λ_{max} = 211 nm for UAE-GP extract, respectively.

Data registered from IR profile of hydroalcoholic grape pomace extracts (Figure 3) are in accordance with the literature [39, 40]. It can be observed the peaks around 2900-3000 cm^{-1}, which relates to the C-H groups vibrations, usually observed for phenols. The band at 3320 cm^{-1} indicates the involvement of the OH groups [40]. The peaks around 1514 cm^{-1} represent aromatic squeal vibration and around 1100 cm^{-1} aromatic CH in plane bending vibration.

3.2. Morphogenetic response of in vitro inoculated explants on media supplemented with various proportions of GP extracts

The morphogenetic response of the apex and nodule explants, inoculated on the tested nutrient media, varied depending on the type and concentration of the GP extract introduced into the nutrient medium.

Two types of morphogenetic reaction have been identified: shoot generation (caulogenesis) and root formation (rhizogenesis).

The caulogenesis was highlighted on all tested experimental variants, including the control one (Figure 4).

In the case of nutrient media supplemented with GP extract obtained by the CE (EA variants), the best caulogenetic reaction was identified on the variants E_1A and E_2A, the number of shoots on these media varying between 3 - 4 / explant.

The morphogenetic reaction is analogous to that describes in several studies on oregano *in vitro* propagation using phytohormones in different concentrations as a supplement in nutrient media (BAP + IAA/IBA/NAA) [27-29].

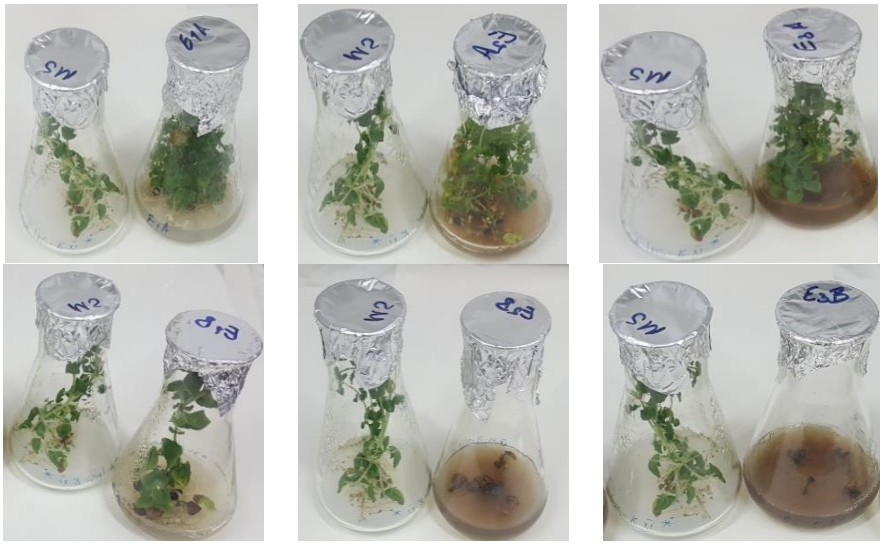

Figure 4. Shoots obtained on tested experimental variants, comparing with the control one (MS).

The shoots developed well, but the size of the leaves was different. On the E_1A medium compared to the MS control medium, the leaves of the shoots were small, but numerous on the stem. On E_2A variant at the level of the shoots, larger leaves developed (similar in size to those of the shoots obtained on the MS medium), but less in number / shoot (Figure 5, a-b). In the case of shoots from E_2A media, some leaves showed chlorophyll deficiencies.

a. b.

Figure 5. Aspect of shoots on E_1A and E_2A variants: a) numerous shoots with small leaves; b) shoots with large leaves.

Inoculation of the explants on the variant E_3A with the highest proportion of GP extract, induced a weak caulogenetic reaction (Figure 6, a-b), the number of regenerated shoots from phytoinocula was reduced at 1-2 / explant. Also, the process of growing in height was much slower compared to the shoots of the previously discussed variants. In few samples, some shoots degenerated rapidly after a short growth period (Figure 6, b).

A slowly degeneration of shoots on the E_3A variant was highlighted in most regenerants after about 3 weeks from their growth and development.

At increased concentration, the morphogenetic response decreases which is in accordance with the literature compared to other extracts containing polyphenols [20, 21].

The presence of a high concentration of GP in the medium (10 %) favored a good development of the leaves, which had much larger dimensions compared to E_1A, E_2A and MS variant.

Rhizogenesis was highlighted in all experimental samples, the most intense being observed in the case of E_1A, E_2A variants. The roots, measuring over 4.5 cm in size, were well developed, some with secondary branches and absorbent hairs. In the case of regenerants in E_1A sample, it can be noticed the appearance of adventitious roots at the level of stem nodes (Figure 5, a-b). It is well known the role of adventitious roots related to the absorption and fixation for plants.

The weakest rhizogenesis was observed on the E_3A variant (Figure 6, a), the roots being reduced in number and size, as well as very thin, without secondary branches.

a. b.

Figure 6. Aspect of shoots on E_3A variant: a) some very elongated shoots, low caulogenesis, roots presence; b) some degenerated shoots.

The presence in the nutrient media of GP extract obtained by UAE (EB variants), induced the formation of new shoots (caulogenesis) in the all tested environmental variants, but the intensity of the process was different.

The best caulogenetic reaction was observed on the medium supplemented with 1 % GP extract (variant E_1B), the regenerated shoots (about 3-4 / explant) showed slow growth, most having smaller dimensions compared to the control sample shoots.

On the other hand, the leaves size was clearly superior both to the control variant and to the shoots leaves obtained on the media supplemented with the GP-CE variants EA (Figure 7).

Even if the number of shoots is small, the large size of the leaves can be an advantage in their use in the food, pharmaceutical and cosmetic industries; harvesting can be done after 2-3 weeks of *in vitro* development.

a. b.

Figure 7. Aspect of shoots on E_1B variant: a) medium intensity of caulogenesis; b) large leaves.

Although the shoots regenerated on the E_1B medium developing very large leaves, it was noticed that it grow to a certain size (2-2.5 cm), then, after about 3 weeks, its start to degenerate quickly and die. The degeneration of biological material also occurred if the regenerated shoots were transferred to fresh media, from the same nutritional variant.

On E_2B and E_3B media, not all inoculated explants reacted; few explants generated new shoots, they grew about 2.5 cm, but in a very short time they degenerated. In Figure 8 (a-b), it can be observed sporadic appearance of shoots (in about 1 week) and degeneration of explants almost immediately after the formation of shoots in about 2 weeks – Figure 8 (c-d).

a. b. c. d.

Figure 8. Aspect of shoots on E_2B and E_3B variants: (a-b) sporadic appearance of shoots after one week after inoculation); (c-d) degeneration of explants after 2 weeks after inoculation.

Grape pomace ultrasound assisted extraction seems to lead to a chemical content of extract which it does not inhibit caulogenesis but not allow the survival of the plant materials; some of its compounds probably have an inhibitory effect on shoots development, if proportion of UAE-GP extracts added overpass 5 %. Inhibition of shoot growth may be due to some compounds from the extract or their interaction with the compounds from nutrient medium. In order to elucidate this result, further investigations should be performed for exhaustive analysis of the chemical composition of the extracts.

Rhizogenesis was absent in all variants supplemented with UAE-GP extracts, regardless of concentration.

3.3. Biometric measurements of regenerants obtained in vitro

The biometric measurements are essential to quantify the effect of the grape pomace extracts introduced in the nutrient media. All samples regenerants harvesting was performed 8 weeks after the start of the experiment. The regenerated shoots from the explants were counted, measured and weighed to determine the fresh biomass for each variant.

Regarding the number of regenerated shoots from explants for each variant, it was observed that the highest value of shoots was obtained in the case of variant E_1A, followed closely by variant E_2A, with a number of shoots more than two times higher than the control variant (Figure 9).

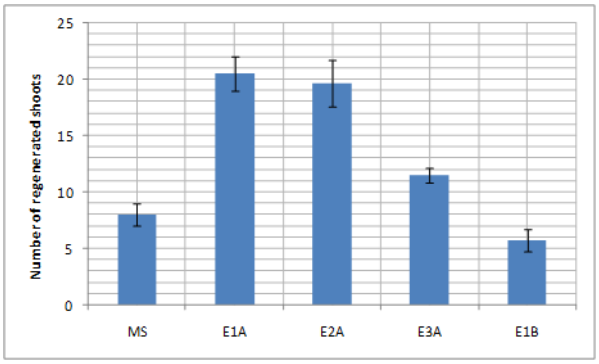

Figure 9. Influence of GP extracts on the regenerated shoots number.

These results are comparable to those of other *in vitro* studies on oregano in which hormonal combinations were used (BAP in combination with NAA/IAA/IBA) [27-29].

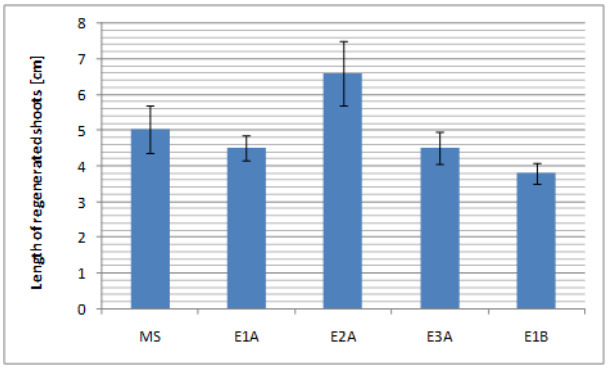

Figure 10. Influence of GP extracts on the regenerated shoots length.

The lowest number of regenerated shoots was identified on variant E_1B (Figure 9). The highest value of regenerated shoots length average was recorded in the samples supplemented with 5 % CE-GP extract (E_2A) and the shortest length average on the E_1B medium (Figure 10).

The results obtained in the evaluation of biomass accumulation are graphically presented in Figure 11. After weighing the shoots, the highest amount of biomass was found for the E_1A and E_2A variants. This result is correlated with those obtained for the number and length of the regenerated shoots. In these samples supplemented with 1 and 5 % CE-GP extracts, the biomass accumulation increased by more than 30 % compared to the control variant MS. The lowest amount of biomass was recorded on the E_1B medium.

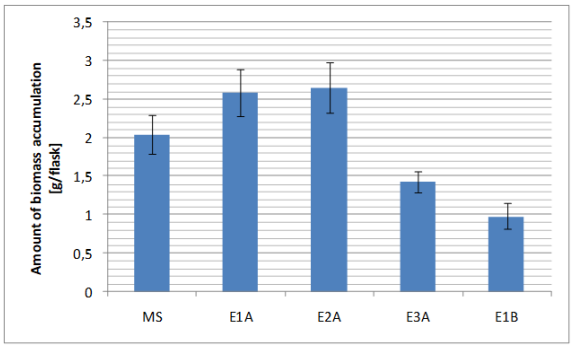

Figure 11. Influence of GP extracts on biomass accumulation.

4. Conclusions

In the present study, the effect of *Fetească neagră* grape pomace extracts introduced into the nutrient medium was investigated. It was found that GP hydroalcoholic extracts influenced the regeneration processes of *Origanum vulgare* explants inoculated *in vitro*. The stimulatory effect on the morphogenetic response depends both on the proportion in which basal medium was supplemented and on the extraction method used.

In the case of samples with the UAE-GP extracts addition, 1 % supplementation induced a low intensity caulogenesis process. Compared to the control samples, addition to UAE-GP extracts lead to a larger leaves development but their survival rate is very low. The shoots degenerated immediately after the formation stage, a phenomenon observed for all explants inoculated with UAE-GP extracts. Inhibition of shoot growth may be due to some compounds from the extract or their interaction with the compounds from nutrient medium.

The best results concerning stimulation of *in vitro* growth and development has been observed at a low concentration for CE-GP extracts in the medium. At increased concentration (over 5 %), the morphogenetic response decreases, but without degeneration phenomena.

Based on caulogenesis response and on biometric measurements, supplementing the culture medium with 1 and 5 % GP extracts obtained by classical extraction seems to have a significant role in caulogenesis. In these samples, the best results were highlighted by the

regeneration of a larger number of shoots compared to the control variant. Differences were also observed regarding the size of the shoots; thus, the longest shoots have been developed on the medium with 5 % CE-GP extract.

The present research has allowed obtaining significant information concerning the influence of the extraction method on the quality content of extracts and consequently on the potential application of these grape pomace extracts on *in vitro* plant growth and development.

Conflict of interest

Authors declare no conflict of interest.

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