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Physiological factors affecting polyphenolics production of *in vitro* cultivated Balkan endemic *Sideritis scardica*

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Shoot cultures of the plant, collected from the Slavyanka Mountains in Bulgaria were initiated from sterile germinated seeds with the purpose of germplasm conservation and biotechnological delivery of secondary metabolites with antioxidant properties.

Plant growth regulators were applied in order to affect *in vitro* multiplication of the plant. Total phenolic and flavonoids content and hydrogen peroxide levels, low molecular antioxidants (ascorbate and glutathione), as well as antioxidant enzymes in the shoot cultures were assayed spectrophotometrically.

Keywords: *Sideritis scardica*, shoot cultures, polyphenolics production, antioxidant enzymes, hydrogen peroxide *in vitro*

INTRODUCTION

Genus *Sideritis* (Lamiaceae) includes more than 150 species, distributed mainly in the Mediterranean region, Central Europe and West Asia [1]. The Balkan endemic *Sideritis scardica* is an aromatic medicinal plant, traditionally utilized for pulmonary treatment, as well as anti-flu and wound healing remedy [2]. The species is represented by relatively small populations [3]. The plant has been determined with a Near Threatened status, with a decreasing current population trend [4] and under the category "Endangered" in the Red List of Bulgarian vascular plants [5].

Essential oil of the species is characterized by the presence of over 100 compounds of monoterpenoid, sesquiterpenoid and diterpenoid character with variable profile, according to the origin of the plant material, while non-volatile components of the species consist of mono- and sesquiterpenoids, diterpenoids, triterpenoids, sterols, fatty acids, esters and alcohols, as well as hydrocarbons [6 and references cited within]. In addition, the phenolic compounds found in the plant are represented by phenylethanoid glycosides, flavonoid-7-*O*-diglycosides, flavonoid acetylglycosides and hydroxycinnamic acids [7, 8].

Secondary metabolite constituents of phenolic chemical structure have been shown to possess promising biological activity in *S. scardica.* Thus, different types of its extracts have been shown to possess cytotoxic effect on murine melanoma B16, human leukaemia HL-60 cells, as well as C6 rat glioma cells, these effects being attributed to reactive oxygen species induction by the phenolic constituents present in the studied preparations [9 and references cited within].

Environmental factors, such as UV radiation has been shown to play decisive role in the accumulation of compounds with polyphenolic structure in *S. scardica*, which was established for samples originating from plant individuals grown at different altitudes [10].

The endangered status of *S. scardica* in Bulgaria has motivated us to establish tissue culture of the species and to investigate its capacity as a model system for biotechnological delivery of compounds with polyphenolic structure.

EXPERIMENTAL

Plant material and in vitro culture establishment

For establishment of shoot cultures, seeds of the plant, growing in Slavyanka Mountains (Shabran peak locality) were collected. Surface sterilization was performed by 0.1 % $HgCl_2$, followed by triple rinse in sterile distilled water. Seeds were then placed in the basic Murashige and Skoog [11] culture medium containing half-strength macro-salts, Gamborg vitamins [12] and 20 $g.L^{-1}$ sucrose and 6.0 $g.L^{-1}$ agar. Germination was conducted in the dark, at 25 ± 1 ºC. The obtained axillary shoots were further maintained on plant growth regulators (PGR) - free Murashige and Skoog medium with Gamborg vitamins supplementation, 30 $g.L^{-1}$ sucrose and 6.5 $g.L^{-1}$ agar (Rm medium) with a period of 8 weeks of regular subculture.

Plant growth regulators treatments

For elucidation of the effect of plant growth regulators the following modifications were applied

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to the basic Murashige and Skoog medium, supplemented with Gamborg vitamins and 30 $g.L^{-1}$ sucrose and 6.5 $g.L^{-1}$ agar: the PGR-free control – Rm; Sm – 0.2 mg/L^{-1} benzyl adenine (BA) and 0.02 mg/L^{-1} Naphthylacetic acid (NAA); Sr_1 – 0.2 mg/L^{-1} BA and 0.5 mg/L^{-1} NAA; Sr_2 - 0.2 mg/L^{-1} BA and 1.0 mg/L^{-1} NAA; Sr_3 - 0.5 mg/L^{-1} BA and 0.5 mg/L^{-1} NAA and Sr_4 - 0.5 mg/L^{-1} BA and 1.0 mg/L^{-1} NAA.

Enzymatic activities determination

Enzyme extraction was performed after Yuan et al. [13] as follows: 0.2 g fresh weight (FW) of the whole shoots were ground with 0.05 g polyvinylpyrrolidone into fine powder with liquid nitrogen in 4 ml of 100 mM potassium phosphate buffer pH 7.2 containing 2 mM EDTA and 8 mM mercaptoethanol. After centrifugation at 15 000 rpm for 25 min at 4 ºC aliquots of the supernatant were immediately used for the below described assays. All enzymatic activities were expressed per milligram protein. The total protein content was measured by the method of Lowry et al. [14] using a calibration curve performed with bovine serum albumin.

Ascorbate peroxidase, APx, EC 1.11.1.11;

Determination of AP activity was performed at room temperature in 2 ml reaction mixture consisting of 50 mM potassium phosphate buffer pH 7.0, 0.5 mM AsA (ascorbic acid), 0.1 mM H_2O_2 and 0.04 ml supernatant. Reaction was initiated with the addition of AsA. Change in absorbance was monitored at 290 nm at a 15 sec interval within 3 min, extinction coefficient 2.8 mM cm^{-1} [15].

Glutathione reductase, GR, EC 1.8.1.7;

Determination of GR was performed at room temperature in 2 ml of reaction mixture, containing 300 mM potassium phosphate buffer pH 7.5, 3 mM $MgCl_2$, 0.1 mM EDTA, 10 mM GSSG, 0.15 mM NADPH and 0.04 ml supernatant. The reaction was initiated with the addition of NADPH. Extinction change was monitored at 340 nm at a 15 sec interval within 3 min, extinction coefficient 6.2 mM 1 cm^{-1} [16].

Guaiacol peroxidase; GPOX, EC 1.11.1.7;

Determination of GPOX was performed at room temperature in 2 ml reaction mixture, containing 100 mM potassium phosphate buffer (pH 7.0), 20 mM guaiacol, 200 µl extract, 1 mM H_2O_2. The oxidation of guaiacol was measured by following the increase in absorbance at 470 nm for 2 min after initiation with the addition of H_2O_2, extinction coefficient 25.5 mM cm^{-1} [17].

Catalase, CAT, EC 1.11.1.6

Estimation of CAT activity was performed at room temperature in 2 ml of reaction mixture, containing 100 mM potassium phosphate buffer pH 7.0, 15 mM H_2O_2 and 0.01 m1 supernatant. The reaction was initiated by the addition of H_2O_2 and its decomposition was recorded by the decline of absorbance at 240 nm for 3 min, extinction coefficient 39.4 mM^{-1} cm^{-1} [18].

Phenylalanine amonialyase, PAL, EC 4.3.1.24

Estimation of PAL was performed as follows: 0.1 ml enzyme extract was mixed with 0.25 ml 20 mM phenylalanine (dissolved in 100 mM borate buffer, pH 8.8), 2ml of 100 mM borate buffer and 1 ml of distilled water were added. Control samples contained the supernatant and buffer instead of phenylalanine. After 30, 60 and 90 min of incubation at 30 ºC, absorption was measured at 290 nm. Activity unit was calculated as $\Delta A = 0.01$, equivalent to the production of 3.09 nmol cinnamic acid [13].

Non-enzymatic antioxidants determination

AsA was estimated as the decrease in absorbance for 1 min at 265 nm, in a reaction mixture, consisting of 100 mM potassium phosphate buffer, pH 5.6, 5µl ascorbate oxidase and 0.02 ml supernatant [19]. The reaction was initiated with the addition of the supernatant. The concentrations of glutathione were determined with an enzyme recycling assay. The assay was based on sequential oxidation of glutathione by 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and reduction by NADPH in the presence of GR [20].

Hydrogen peroxide and malondialdehyde levels determination

An amount of 120 mg FW of the shoots were homogenized in a mortar at 4 °C with 0.1 % trichloroacetic acid and centrifuged for 20 min at 15 000 rpm. For malondialdehyde (MDA) estimation, 0.5 ml of the supernatant were mixed with 0.5 ml phosphate buffer pH 7.4 and after the addition of 1 ml 0.5 % thiobarbituric acid dissolved in 20 % trichloroacetic acid, the samples were boiled for 30 min [21]. After rapid cooling of the samples in an ice-bath, absorption was measured at λ = 532 and 600 nm using the extinction coeficient 155 mM^{-1} cm^{-1} [22].

For the hydrogen peroxide (H_2O_2) assay, 0.5 ml of the supernatant were mixed with 0.5 ml phosphate buffer pH 7.4 and after the addition of 1 ml of 1 M KI, samples were incubated in the dark for 60 min and absorption was measured at λ = 390 nm. The content was calculated using a standard curve of H_2O_2 in the range of 1–100 nmol/ml of hydrogen peroxide [23].

Total phenolics and flavonoids determination

100 mg dry weight (DW) of the plant material was extracted with hot 80 % (v/v) ethanol and then centrifuged at 15 000 rpm for 15 min. Total phenolics were determined by the Folin & Ciocalteu's colorimetric method of Singleton et al. [24], modified by us as follows: an aliquot of the extract was placed in test-tube and distilled water, 1:1 Folin & Ciocalteu's reagent and 20 % Na_2CO_3 were added. The absorbtion was measured at $\lambda = 730$ nm and the total phenolics were calculated by means of a calibration curve of chlorogenic acid (in the range of 30µg ml^{-1} to 100 µg ml^{-1}) and expressed as mg of chlorogenic acid equivalent per 1 g DW of the sample. Total flavonoids content of the whole shoot samples of the plant was measured using a colorimetric assay in accordance with the method of Zhishen [25] modified as follows: 100 mg DW of the samples were extracted with hot 80 % (v/v) ethanol and then centrifuged at 15 000 rpm for 15 min. Aliquots of the extract were placed in test-tube and distilled water, 5 % $NaNO_2$ and 10 % $AlCl_3$ were added. After the addition of 1N NaOH and distilled water, the absorption at $\lambda = 510$ nm was measured and the concentration was calculated by means of a calibration curve of (+)catechin (in the range of 2 µg ml^{-1} to 80 µg ml^{-1}). The total flavonoids of the samples were expressed in mg of (+)catechin equivalent per 1 g DW of the sample. All measurements were performed in triplicate with three repetitions.

Statistical analysis

Material was collected of at least 15 individual plantlets, cultivated in 5 separate culture vessels. Comparison of means was conducted by the Student t test for unequal variances. The differences were compared at $P \leq 0.05$.

RESULTS AND DISCUSSION

Seed germination within two weeks was 1%. Within the 8 weeks of regular subculture of the stock plants in the PGR-free Rm medium, clear evidence of leakage of phenolics into the medium and browning and necrosis of the plantlets was evident.

Further observation on the effect of PGR on the productivity of non-enzymatic antioxidants showed that PGR stimulated the production of ascorbate and total phenolic and flavonoid compounds as compare with the PGR-free control (Fig.2).

As far as glutathione levels were concerned, they were not significantly affected by PGR with the exception of Sr_1 (0.2 $mg.L^{-1}$ BA and 0.5 $mg.L^{-1}$ NAA) where glutathione levels dropped significantly.

Supplementation of plant growth regulators (PGR) to the medium increased significantly the explants' survival rate and multiplication index (Fig.1).

In addition, our observations showed that PGR increased the subculture period of plants (not shown).

In addition to increasing of number of axillary shoots formed, the study showed that with the exception of the Sr_3 treatment (Fig. 1E, 0.5 $mg.L^{-1}$ BA and 0.5 $mg.L^{-1}$ NAA), PGR significantly increased the length of obtained plantlets. The leaves of the PGR modifications Sr_2 (0.2 $mg.L^{-1}$ BA and $mg.L^{-1}$ NAA) and Sr_4 (0.5 $mg.L^{-1}$ BA and 1.0 $mg.L^{-1}$ NAA) were visually characterized by larger leaf area, as compared with the other treatments (Figs. 1D and 1F).

Figure 1. Development of *Sideritis scardica* Griseb. shoot cultures in Rm (A), Sm (B), Sr_1 (C), Sr_2 (D), Sr_3 (E) and Sr_4 (F) culture media.

The two compounds having specific functions cannot be considerate as two interchangeable molecular antioxidants [27]. For example, while glutathione has been shown to possess critical functions in embryo and meristem development, ascorbate deficiency causes lethality at the seedling

stage [26 and references cited within]. Having a higher redox potential as compared with glutathione, the ascorbate is a key cofactor in a number of biosynthetic pathways. On the other hand, glutathione functions mainly as a sulfur source (reductant) in metabolism, being necessary for the formation of reduced glutathione conjugates involved in biosynthesis, transport, and detoxification [26]. Noteworthy is the observation that while in the PGR-free control glutathione significantly exceeds ascorbate levels, the PGR treatments strongly affect this ratio by bringing the values of the two parameters in comparable levels.

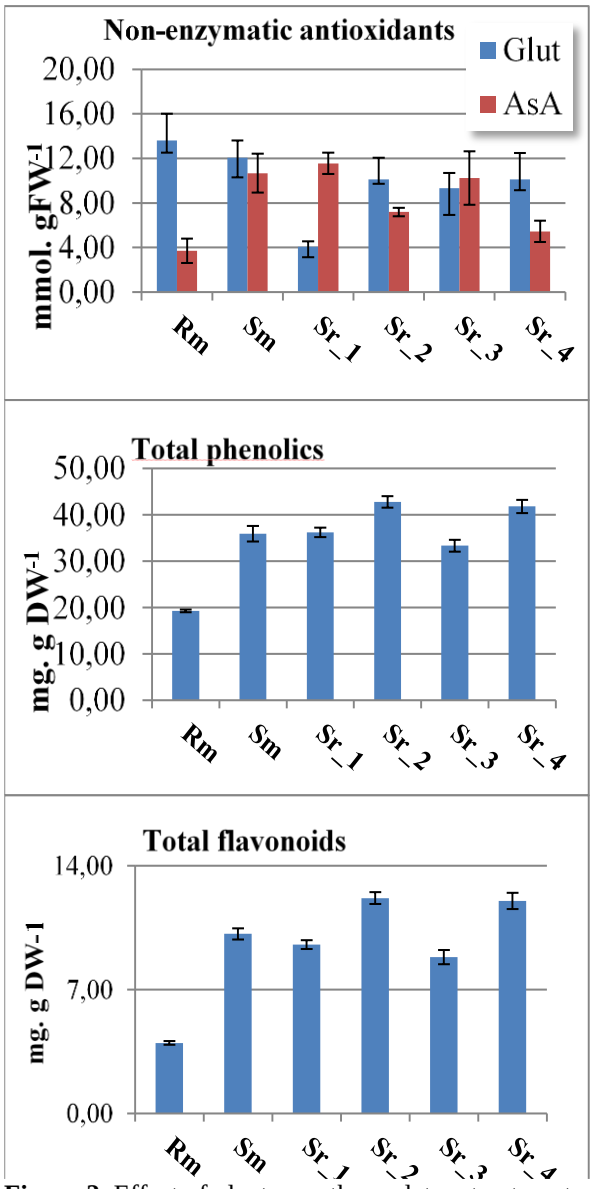

Figure 2. Effect of plant growth regulators treatments on non-enzymatic antioxidants in *Sideritis scardica* Griseb. *in vitro*. *Glut – glutathione; AsA – ascorbate.*

The Ascorbate and glutathione function in close interrelation within the ascorbate-glutathione cycle in the plant cell, catalyzing the detoxification of hydrogen peroxide, generated as a result of redox processes [26]. The two compounds having specific functions cannot be considerate as two interchangeable molecular antioxidants [27]. For example, while glutathione has been shown to possess critical functions in embryo and meristem development, ascorbate deficiency causes lethality at the seedling stage [26 and references cited within]. Having a higher redox potential as compared with glutathione, the ascorbate is a key cofactor in a number of biosynthetic pathways. On the other hand, glutathione functions mainly as a sulfur source (reductant) in metabolism, being necessary for the formation of reduced glutathione conjugates involved in biosynthesis, transport, and detoxification [26]. Noteworthy is the observation that while in the PGR-free control glutathione significantly exceeds ascorbate levels, the PGR treatments strongly affect this ratio by bringing the values of the two parameters in comparable levels. The only exception to this tendency are the Sr_2 and Sr_4 treatments, where glutathione still exceeds ascorbate levels.

All PGR treatments significantly enhanced polyphenolics productivity *in vitro*. This observation is logic given the well-known role of polyphenolics as reactive oxygen species (ROS) scavengers in the plant cell.

Environmental stress is being alleviated by the plant organism through a rise of radical scavenging processes and by elevation of the levels of pre-existing or *de novo* synthesized compounds functioning in detoxification of ROS. In this manner, the plant not only manages with oxidative stress but also utilizes oxygen activation as a defence system ("respiratory burst") in certain external and internal "battle fields" such is the apoplast of the plant [27].

Antioxidant flavonoids have been shown to inhibit the activities of a wide array of kinases which suppress key steps of growth and differentiation in eukaryotic cells. For example quercetin inhibits auxin efflux specific proteins which may control developmental processes at the organismal level, being potentially involved in "stress-induced morphogenic responses" and "flight" strategy of sessile organisms. Stress has been shown to lead to stimulation of the activities of class III peroxidases, possibly leading to stress-induced morphogenic responses through stimulation of the ROS scavenging system and regulation of tissue-specific auxin levels with the help of "antioxidant" flavonoids, such as quercetin. Thus, antioxidant flavonoids have been hypothesized to act as developmental regulators of the whole plant and individual organs by controlling key steps of cell growth and differentiation [28] and references cited within.

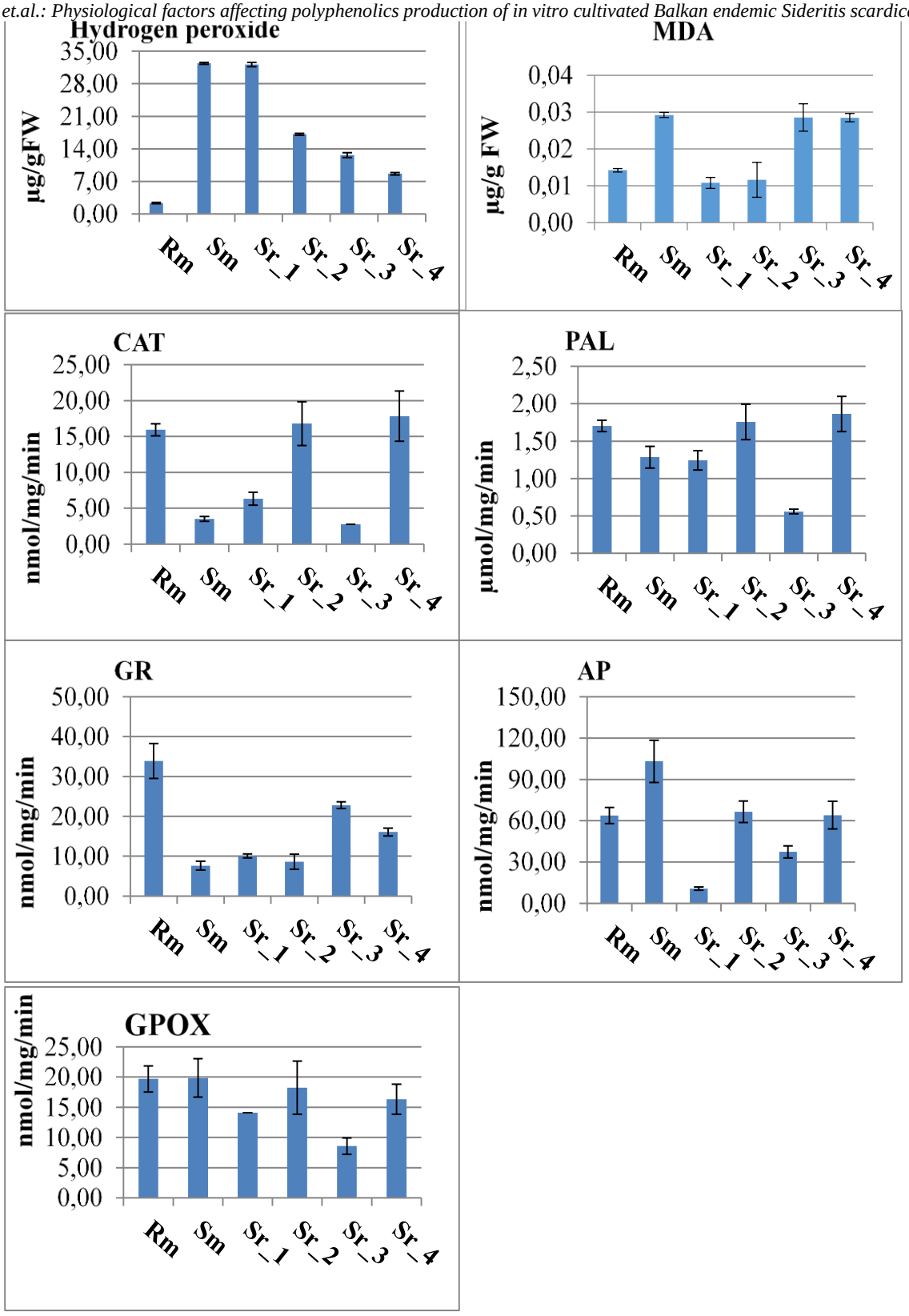

Figure 3. Effect of plant growth regulators treatments on oxidative stress (expressed as hydrogen peroxide content), lipid peroxidation (expressed as malondialdehyde content) and enzymatic activity in *Sideritis scardica* Griseb. *in vitro*.

Further observations of *S, scardica* shoot cultures showed that in all the PGR-treated plants oxidative stress (expressed as hydrogen peroxide levels) and lipid peroxidation (expressed as malondialdehyde levels, with the exception of Sr_1 and Sr_2 treatments) were significantly higher than the non-treated control (Fig. 3). Noteworthy was the peak in hydrogen peroxide levels in Sm and Sr_1 treatments as compared not only with PGR-free Rm plants, but also with the rest of the PGR-treatments. This effect

was accompanied with the suppression of also catalase and glutathione levels and only slight suppression of the activity of phenylalanine ammonia lyase.

Interestingly, PAL activity was not significantly affected by any of the treatments, except for Sr_3 (0.5 mg.L^{-1} BA and 0.5 mg.L^{-1} NAA), in which the lowest activity of activity of the enzyme correlated with also suppressed CAT and GPOX activities. As discussed above, this treatment was also related to levels of glutathione and ascorbate, which did not differ significantly and by considerable disturbance in plant development expressed as significant length suppression, compared to the rest of the PGR-treatments.

Hydrogen peroxide has a varied role in maintenance of the physiological status of the plant organism. On one hand its elevated levels are a consequence of the damaging impact of reactive oxygen species, but on the other it is also a messenger for induction of the plant's antioxidant defense and a mediator for establishment of resistance of the plant to environmental stress stimuli [29].

CONCLUSIONS

The results of the conducted experiment showed that the combination of 1.0 mg/l naphthaleneacetic acid with both 0.2 mg/l and 0.5 mg/l benzyl adenine (Sr_2 and Sr_4, respectively) were found to be most favorable for morphological development, polyphenolic production and optimal antioxidant enzymes functioning. In these treatments glutathione to ascorbate ratio was also closest to the one in the PGR-free control.

Plant tissue culture development of rare, endemic or endangered species is a method for the conservation of valuable medicinal plant germplasm. On one hand it has the potential of rapid production of large quantities of planting material of a species of interest for a short period and introduction of the produced plantlets either into their natural environment or in controlled plantations for large scale field cultivation. On the other hand, the method provides an experimental model system for obtaining the fundamental knowledge of factors affecting the production of secondary metabolites of interest which could further be applied for large scale biotechnological production of target secondary metabolites.

Further investigation of the qualitative characteristics of the produced polyphenolics is in process in order to assess the optimal conditions for the biotechnological delivery of potential phytopharmaceuticals of the Balkan endemic *S. scardica*.

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