Impact of Plant Growth Regulators on Greek Oregano Micropropagation and Antioxidant Activity

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This study highlights the development and achievements made for the micropropagation of Greek oregano (Origanum heracleoticum L.) using stem tip explants. The shoots were cultured on Murashige and Skoog (MS) medium followed different concentrations of plant growth regulators (PGR) -6-benzyl aminopurine, thidiazuron and zeatin at concentrations (0.5 or 1.0 mgL^{-1}). The induction of multiple shoots from stem tip segments was the highest in MS medium supplemented with 1.0mg L^{-1} zeatin. It was the most effective medium for shoot formation, which produced multiple shoots (2.7) with an average height of 3.5 cm. These shoots were transferred on half strength MS medium containing three different auxins: indole-3-butyric acid, á-naphthalene acetic acid or indole-3-acetic acid(0, 0.1 and 0.5 mg L^{-1}) for rooting, Multiple shoots were the most efficiently rooted on ½ MS medium supplemented with 0.5 mg L^{-1} indole-3-butyric acid. Rooted plants showed the best adaptation on pots containing peat: perlite (2: 1 v/v). The higher rates of shoots number and height per plant have a positive relationship with the production of metabolites with antioxidant potential as phenols and flavonoids as well as with ferric reducing antioxidant potential.

Keywords: Antioxidant activity, *ex vitro* Acclimatization, Micropropagation, *Origanum heracleoticum*.

Greek oregano (*Origanum heracleoticum* L.) is a perennial herb, naturally spread from the Mediterranean to Central Asia. Greek oregano has long been recognized as a culinary herb and medicinal plant (MP) in the food and pharmaceutical industry[1]. The essential oilsfrom this plant have strong antioxidant and antibacterial activities[2].They are insulated from 48 to 64 compounds constituting the oil as the main ingredients are carvacrol and thymol, followed by p-cement and ã-terpinene[3, 4].

In contrast to conventional methods of propagation, vegetative micropropagation, using the tip and nodal segments, allows the production of a largely homogeneous population of plants in a relatively short period of time, irrespective of the season[5]. However, attempts to increase the amount of highly valuable metabolites by applying biotechnological methods for the propagation of *O. heracleoticum* appear to be rather limited. Full strength Gamborg's B5 medium with 20 mg

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L^{-1} sucrose, 6 g L^{-1} agar supplemented with factorial combinations of 6-benzylaminopurine (BAP) (0.25 and 0.50 mg L^{-1}), and 0.01 mg L^{-1} á-naphthalene acetic acid (NAA) has been used for Greek oregano micropropagation. For *in vitro* rooting, optimal development of shoots achieved by adding 0.5 mg L^{-1} NAA[6]. Morone-Fortunato and Avato[7] reported that the best results for micropropagation in terms of percentage of growth, a number of developing shoots and shoot length of Greek oregano have been adding 0.5 mg L^{-1} BAP on a nutrient MS medium. Rooting of Green oregano was realized in a nutrient medium containing 0.15 mg L^{-1} of IAA[8]. Goleniowski et al.[9] developed a protocol for *in vitro* propagation of *Origanum vulgare × applici*, by studying the effects of the BA and NAA at various concentrations and combinations on micropropagation.

The Greek oregano could re produce by seeds germination and *in vitro* micropropagation. The small seeds have a good germination rate[10]. Unfortunately, this way of propagation has a very considerable disadvantage - as a cross-pollinating plant the off spring of seeds will represent a various population in relation to habitus, colouration, content and odour of the essential oil. Using plant micropropagation, allows the preservation of the genotypic and phenotypic characteristics of the initial plants as well plant lets are free from seasonal and somatic variations, infestations of bacteria, fungi, or insects and environmental pollution that can affect their medicinal value. Faisal et al.[11] found that the *in vitro* propagated plants *Bacopa monnieri* L. using TDZ were genetically uniform to their mother plants. On the other hand, the choice of cytokinin type and concentration exogenously supplied to the nutrient medium during plant shooting markedly influences not only shoot proliferation but also the production of metabolites with antioxidant activity[12]. There is a lack of data in the scientific papers about changes of antioxidant potential of extracts in plantlets from *in vitro* propagated *O. heracleoticum* depending on the type and concentration of PGRs added to the nutrient medium for plant micropropagation.

The main purpose of this study was the influences of three cytokinins on shoot initiation and multiplication rooting and antioxidant activity of *O. heracleoticum*. This method will allow the reproduction of an unlimited number of plants with strictly specified qualities.

MATERIAL AND METHODS

The plant material used for the experiment in this study was a mature 2-year-old plant of *O. vulgare L. ssp. hirtum* (Link) Iet swaart (*O. heracleoticum* L.) collected from the green house, Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences. Stem tips about 0.8-1.0 cm were used as a source of explants which were washed in running tap water and surfaces terilized with 0.04% $HgCl_2$ for 20 min followed by rinsing them three times with sterile distilled water for 15 minutes against fungal and bacterial spores.

The explants (stem tip segment) were cut with a sterile blade and inoculated on MS[13] medium with 500 mg L^{-1} $CaCl_2$, 30 mg L^{-1} sucrose, 7 g L^{-1} agar for two passages (passage – 3 weeks). The plantlets were prepared aseptically and were implanted on MS medium prepared with different concentrations of PGRs: 6-benzylaminopurine (BAP), thidiazuron (TDZ) and zeatin (0.5 and 1.0 mg L^{-1}) for shoot proliferation and multiplication. The efficacy of the cytokinin concentration was determined by recording the percentage of formed shoots, the number of developing shoots per explant, shoot height and fresh weight and the length after three weeks of culture.

Rooting of elongated shoots (shoots obtained on MS + 1.0 mg L^{-1} zeatin) was attempted under *in vitro* conditions. Auxins (indole-3-butyric acid (IBA), á-naphthalene acetic acid (NAA) or indole-3-acetic acid (IAA) (0.1 and 0.5 mg L^{-1}) were incorporated in the agar (0.7%) solidified medium containing ½ MS salts and 2.0% sucrose. As control medium was used ½ MS basal medium. Data were recorded on percentage rooting, the mean number of roots per plant, and root length after four weeks of culture.

The well-rooted plants were carefully taken out from the vessels and washed under running tap water to remove the gelling agent for *ex vitro* adaptation. The plants (shoots obtained on MS + 1.0 mg L^{-1} zeatin and rooted on MS + 0.5 mg L^{-1} IBA) were transplanted in each potting mixture. The *in vitro* rooted plants were transferred

to 10 cm diameter pots filled with M_1 soil: sand: perlite (2: 1: 1 v/v/v) and M_2 peat: perlite (2: 1 v/v) for two months. *Ex vitro* acclimatization of plants was carried out at 24±2°C and 70-80% relative humidity under 16 h illuminations (50 imol $m^{-2}s^{-1}$). There were ten plants per treatment. The pots were covered with porous polyethylenebags for maintaining high humidity (90%). The polyethylene was removed after two weeks.The per cent of survived plants, plant height and a number of leaves were measured after eight weeks.

For the antioxidant testing dry samples (0.3 g) from three weeks *in vitro* shooted plantlets were ground and extracted with 96% (v/v) methanol. Free radical-scavenging activity by using coloured, artificial stable free radicals DPPH˙ (1,1-diphenyl-2-picrylhydrazyl), was determined spectro-photometrically[14]. The percent inhibition of the DPPH˙ radical (I %) was calculated by the following equation: I% = [(Ablank – Asample)/ Ablank] × 100, where, Ablank is the absorbance of the control reaction (containing all reagents except the extract), and Asample is the absorbance of the extract.The ferric reducing antioxidant power (FRAP) was monitored by BENZIE and STRAIN[15]. Concentrations of total phenolic compounds were determined spectrophotometrically using the Folin–Ciocalteu reagent and calculated as caffeic acid equivalents[16]. Flavonoids in plant tissues were measured spectrophotometrically according to Zhishen et al.[17], using the standard curve of catechin.

The extraction for the determination of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and guaiacol peroxidase (GPO) activities was made by Hristozkovaet al.[18]. Total SOD (EC 1.15.1.1) activity was determined by Giannopolitis and RIES[19]. Total CAT (EC 1.11.1.6) activity was measured according to Beers and Sizer[20]. Total APX (EC 1.11.1.1) activity was assayed according to Nakano and Asada[21]. Total GPO (EC 1.11.1.7) activity was determined by Urbaneket al.[22]. Soluble protein content was determined by Bradford[23] using bovine serum albumin as a standard.

Statistical analysis

The data were statistically processed by analysis ofvariance (ANOVA)for comparison of means, and significant differences were calculated according to Fisher's least significance difference (LSD) test at the 5% significance level using a statistical software package (Statgraphics Plus, version 5.1 for Windows).

RESULTS AND DISCUSSION

The growing conditions influenced favourably the plant development, withouttraits of deviation of their normal development, such as glassing, chlorosis, necrosisetcin cultivation

Table 1. Effect of plantgrowth regulators (PGRs) on shoot multiplication of*O. heracleoticum*shoots after 3weeks of culture

PGRs, mgL^{-1}	Shoot formation %	Number of shoots explant^{-1}	Shoot height, cm	Fresh weightg
BAP				
0.5	50	2.0±0.1^a	2.2±0.11^a	0.07±0.004^a
1.0	70	2.7±0.135^{bc}	3.5±0.175^c	0.13±0.006^b
Zeatin				
0.5	70	3.4±0.17^d	3.6±0.18^c	0.16±0.008c
1.0	80	3.8±0.19^e	3.9±0.195^d	0.19±0.01^d
TDZ				
0.5	60	2.5±0.125^b	2.7±0.135^b	0.15±0.007^c
1.0	65	2.8±0.14^c	2.4±0.12^a	0.13±0.006^b
LSD		0.2603	0.2773	0.0126

The data are presented as means of 10 plants per treatment ± standard error.Different letters indicate significant differencesassessed byFisher LSDtest (5%) afterperforming ANOVA multi-factor analysis

on MS medium(Fig. 1a). Adding on MS medium BAP, TDZ or Zeatin (0.5 and1.0 mg L^{-1}) for Greek oregano shooting the maximum frequency (80%) was observed on MS medium supplemented with 1.0 mg L^{-1} zeatin, which induced a number of shoots (3.8) with a shooth eight (3.9 cm) after three weeks of culture (Table 1, Fig. 1b).The concentration of 0.5 mg L^{-1} zeatin gave the explant responses in terms of percentage of shoot formation (70%), number of developing shoots (3.4), shoot length (3.6cm), follow 1.0 mg L^{-1} BAP gave the number of developing shoots (2.7) and shoot length (3.5 cm) (Fig. 1c). The highest fresh weight values (0.19 g) were on MS medium with 1.0 mg L^{-1} zeatin, followed by fresh weight (0.16 g) on MS medium with 0.5 mg L^{-1} zeatin. Accordingly, the most of the published protocols for *in vitro* multiplication of MPs the type and concentration of the cytokinin significantly influence the frequency of the formed buds and buds average number of explant[24, 25].The

protocols allowed the establishment of *in vitro* tip explants and also permitted *in vitro* multiplication of an adult plant with desirable agronomic features. Established conditions for maintaining growth and plant development cannot be applied with guaranteed success to all the representatives of a particular species, as the process is genotypically dependent. Protocols for *in vitro* multiplication of *O. heracleoticum* have been developed[6,7]. The investigation of multiplication protocol for *O. heracleoticum* was based on the independent addition of cytokinins (BAP, TDZ or Zeatin) to the MS medium.Greek oregano had a high *in vitro* multiplication factor in MS medium supplemented with 1.0 mg L^{-1} zeatin and gavethe best responses in terms of percentage of growth, number of developing shoots and shoot height.

The efficacy of IBA, NAA and IAA supplemented to the ½ MS mediaon rooting of *O. heracleoticum* plant lets is shown inTable 2. With

Table 2. Effect of ½ MS medium with auxins on the root induction of *O.heracleoticum* plants after 4 weeks of culture

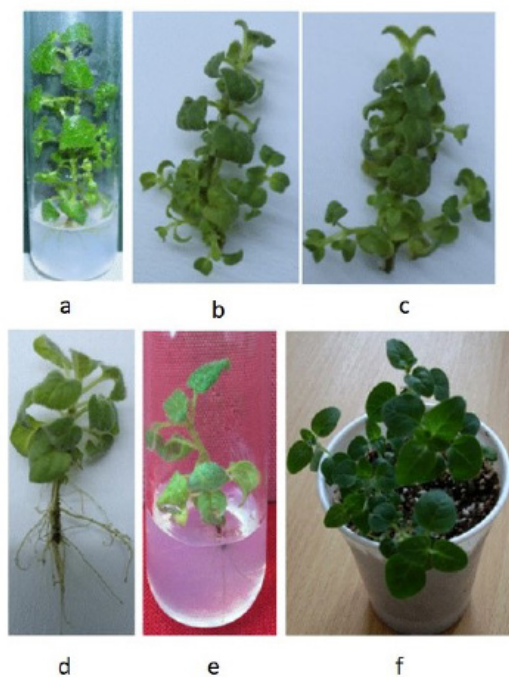| Auxin, mg L^{-1}	Rooting %	Number of roots plant^{-1}	Root length cm
Control, ½ MS	0	0	0
½ MS+ 0.1 IBA	45	1.7±0.085[c]	1.2±0.06[b]
½ MS + 0.5 IBA	80	3.3±0.165[f]	2.4±0.12[e]
½ MS+ 0.1 NAA	30	1.4±0.07[b]	0.9±0.045[a]
½ MS + 0.5 NAA	65	2.8±0.14[e]	1.5±0.075[c]
½ MS+ 0.1 IAA	40	1.2±0.06[a]	0.8±0.04[a]
½ MS + 0.5 IAALSD	50	2.2±0.11[d]	1.7±0.085[d]
		0.1984	0.1349

The data are presented as means of 10 plants per treatment ± standard error. Different letters indicate significant differences assessed by Fisher LSD test (5%) afterperforming ANOVA multi-factor analysis.

Table 3. Effect of mixture substrate on the survival of *O.heracleoticum* plants after 6 weeks of culture

Mixture	Plant survival %	Plant height cm	Number of leaves plant^{-1}
M$_1$ - soil: sand: perlite (2: 1: 1, v/v/v)	60	4.2±0.21[a]	4.5±0.225[a]
M$_2$ - peat: perlite (2: 1, v/v/)			
80		6.1±0.305[b]	5.3±0.265[b]
LSD		0.5936	0.5573

The data are presented as means of 10 plants per treatment ± standard error. Different letters indicate significant differencesassessed byFisher LSD test (5%) afterperforming ANOVA multi-factor analysis

increasing concentration from 0.1 mg L^{-1} to 0.5 mg L^{-1} IBA the healthy rooting percentage reached to 80% on ½ MS induced maximum average number (3.3) and length of roots (2.4 cm) after three weeks of culture (Table 2, Fig. 1d).The rooting response was observed on ½ MS medium containing 0.5 mg L^{-1} NAA, where was formeda number of roots per plant 2.8 with 1.5 cm root length (Table 2, Fig. 1e).Typical for the vegetative part of the plants was the good growing, the normal development, and the intensive green colour of the leaves. The roots were white, firmly fixed to the base of the plants, which was without callus. In most cases 0.1-0.5 mg L^{-1} IAA there were single ones, and the average percentage of rooting was lowest (40-50%). In the ½ MS medium with 0.1-0.5 mg L^{-1} IAA provokes some negative phenomena. The plants are growing but there are already pale, yellowish, and the lower leaves of most of them were fully necrotized at the end of the passage. Single roots were observed

on the medium supplemented with 0.5mg/ LIAA (Table 2). The percentage of rooting (50%) was recorded.The rooting was 0% on control ½ MS medium. However, IBA and NAA were found to be the best rooting hormone than IAA in the ½ MS medium.The rooting medium for plants cloning often is modified by the inclusion of auxin. Nanova and Slavova[8] reported that optimal rooting effect (89.6%) is reached under influence of 0.15 mg/ LIAA in MS medium. Rooting of the 15–20 mm long shoots of Greek oregano was achieved in ½ B5 medium supplemented with 1% sucrose and either 10–6 M IBA or NAA (Kintzios 2002). In our previous studies has been recommending for *in vitro Thymus vulgaris* rhizogenesis inclusion of auxins and successful adaptation under ex vitro conditions[25].

The transferring of rooted Greek oregano plantlets at *ex vitro* conditions was realized after three mounts of cultivation, in a mixture: Mix$_1$ - soil, sand and perlite (2:1:1 v/v/v) and Mix$_2$ - peat: perlite (2: 1 v/v) (Table 3).The highest percentage (80%) of plantlets survival was recorded in a mixture of peat: perlite with plant height (6.1 cm) as well as the great number of leaves (5.3) on mixture Mix 2 and acclimatized plants appeared normal (Fig. 1f). They did not show any morphological abnormalities or variations. It was found that 60% of adapted plants survived after transplanting on Mix 1 withthe plant height (4.2 cm) and a number of leaves (4.5). Usually, after one-month cultivation, their vegetative part was trimmed next to the lowest level, situated above the soil surface.After this manipulation was stimulated the development of more than one vegetative part from the grounds of the plants anduntil the end of the second month they were ready for planting in greenhouse conditions. A number of authors report successful adaptation of micropropagated plants under *ex vitro* conditions[25, 26, 27]. Micropropagated plants tend to acclimatize rapidly (three weeks) in the greenhouse at a high rate (95%) and usually are more vigorous than seed-propagated plants[27]. The soil substrate was successfully selected (1: 4 - humustim: a mixture of 1: 1 sand and pearlite) for rooting of Greek oregano plants after their one-month *ex vitro* adaptation[8].

The enzyme antioxidant potential, estimated as SOD, CAT, GPO and APX activity was greatly influenced by PGRsBAP, Zeatin and

Fig. 1. Micropropagation of *O. heracleoticum*: a) *In vitro* shoots with 500 mg L^{-1} CaCl$_2$; b) *in vitro* propagated shoots with 1.0 mg L^{-1} zeatin after three weeks of culture; c) *in vitro* propagated shoots with 1.0 mg L^{-1} BAP after three weeks of culture; d) rooted plants with 0.5 mg L^{-1} IBA after 4 weeks of culture; e) rooted plants with 0.5 mg L^{-1} NAA after 4 weeks of culture, f) ex vitro adapted plants after eight weeks of culture

TDZ used for *O. heracleoticum* micropropagation. Figure 2

When plants were *in vitro* propagated with higher concentration BAP (1.0 mg L^{-1}),the enzymes activities decreased, while when have been used zeatin and TDZ the activity increased (Fig. 2). The same models of changes of the content of total phenols and flavonoidsas well as the Figure 3 antioxidant activities of the Greek oregano plantlets extract assayed by free radical-scavenging activity (DPPH) and ferric reducing antioxidant power (FRAP) were recorded (Fig. 3).

Addition of PGRs as auxins and cytokinins on MS is necessary to induce shooting and rooting because they are key factors and strongly participate in cell cycle regulation and cell division[28, 29, 30].PGRs influencing essential oil production[31], but little is known about their effects on antioxidant enzymes and metabolites of *in vitro* cultures. The environment inside the tubes used in *O. heracleoticum* micropropagation is characterized by high relative humidity, the poor

gaseous exchange between the internal atmosphere of the tube and its surrounding environment. Those conditions may induce physiological disorders. Plants protect themselves against the effect of the oxidative damage by antioxidant enzymes and low molecular secondary metabolites with antioxidant potential. The activities of enzymes SOD, CAT, GPO, and APX were increased with increased concentration of zeatin and TDZ, while it decreased when was used BAP. PGRs modify the plant growth and development pattern exerting a profound influence on many physiological processes[32]. Accordingly to SANTOS-GOMES et al.[33] the total phenol content in sage (*Salvia officinalis* L.) leaf extract is changed depending onthe type of cytokines adding to the nutrient medium during micropropagation. The authors reported that the lowest value of specific production of total antioxidant phenolichave occurred when plants are micropropagated with the supplementation of 1.5 mg L^{-1} benzyladenine. However, under these conditions, the shoot proliferation and linear
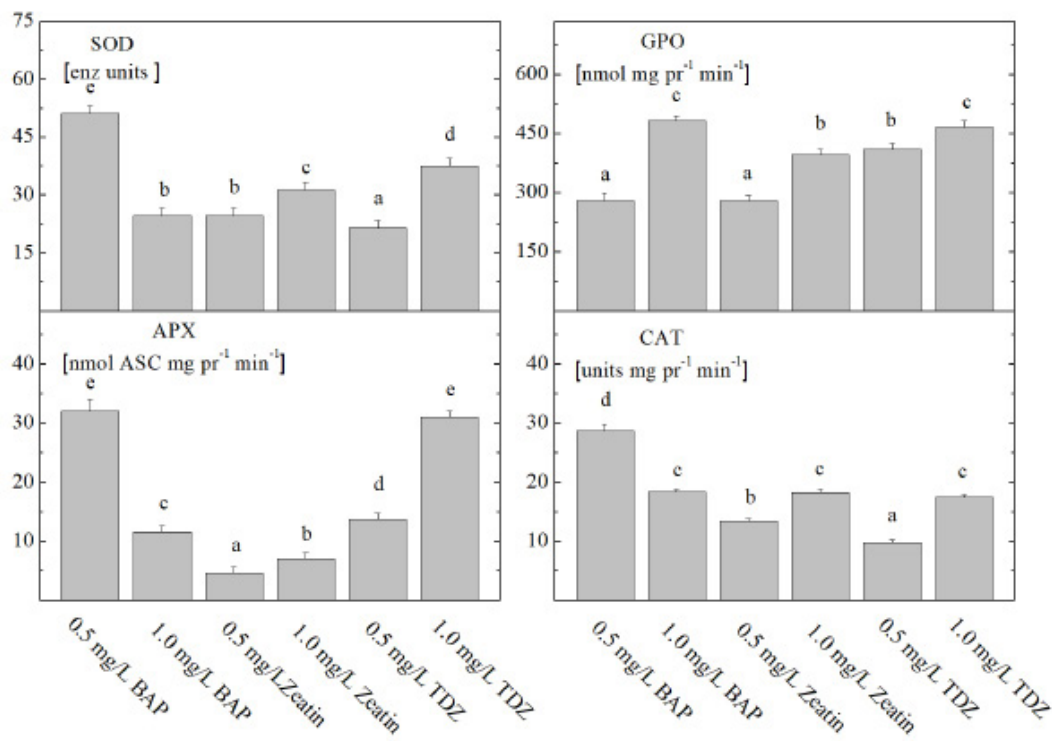
Fig. 2. The activity of antioxidant enzymes CAT, SOD, GPO, and APX in the *in vitro* propagated *O. heracleoticum* on the MS supplied with BAP, TDZ or Zeatin (0.5 and 1.0 mgL^{-1}). Values are means ± SE, n=3; different letters indicate significant differences assessed by Fisher LSD test (Pd"0.05) after performing ANOVA multi-factor analysis
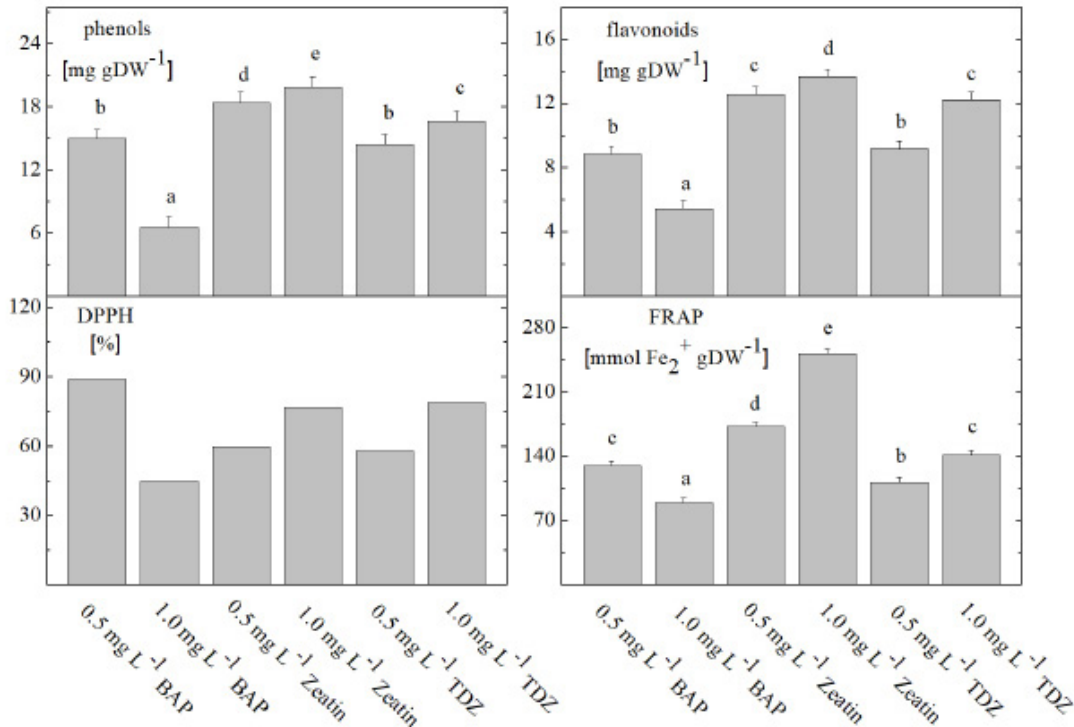
Fig. 3. The content of total phenols and flavonoids and antioxidant potential in the *in vitro* propagated *O. heracleoticum* on the MS supplied with BAP, TDZ or Zeatin (0.5 and 1.0 mg L^{-1}). Values are means ± SE, n=3; letters in common within a graph indicate no significant differences assessed by Fisher LSD test (Pd"0.05) after performing ANOVA

shoot growth have been higher. In contrast, our data showed that the highest height and number of shoot per explant in variants with MS medium supplemented with 0.5 and 1.0 mg L^{-1} zeatinare in correlation with the highest content of total phenols and flavonoids. In general, high rates of biomass accumulation have a positive relationship with the production of secondary metabolites as phenols and flavonoids as well as with ferric reducing antioxidant potential.

CONCLUSION

The stem tip explants were frequently used for micropropagation of *O. heracleoticum*. The presence of 1.0 mg L^{-1} zeatin alone on MS medium was proved best for the multiple shoot formation of Greek oregano (average number of shoots 3.8, average shoot height 3.9 cm). The best rooting was observed on ½ MS medium containing 0.5 mg L^{-1} IBA, where a number of roots plant

(3.3) measuring an average 2.4 cm were formed. The culture of *O. heracleoticum* has great potential for promotion and industrial production*,* one of the important MPs.Well-developed techniques are currently available to help growers meet the demand of the pharmaceutical and food industry. The higher antioxidant potential (determined by the content of total phenols and flavonoids and antioxidant enzymes activity) was recorded when MS medium was supplied with higher zeatin or TDZ concentration for *O. heracleoticum* shooting. On the contrary, using BAP as cytokine for shooting the antioxidant potential decreased.

REFERENCES

1. Baricevic D. and Bartol T. The biological/pharmacological activity of the Origanum genus. *In*: Medicinal and aromatic plants-Industrial profiles, Oregano. The genera Origanum and Lippia, S. E. Kintzios ed., vol. 25, CRC Press, London,2002; 177-213.

2. KarakayaS., ElS., Karagözlü N. andª ahin,S. Antioxidant and antimicrobial activities of essential oils obtained from oregano (*Origanumvulgare* ssp. *hirtum*) by using different extraction methods. *J. Med. Food,* 2011; 14(6):645-652.

3. Goliaris A.H., ChatzopoulouP.S. and Katsiotis S.T. Production of new Greek oregano clones and analysis of their essential oils. *J Herbs Spec. Med. Plants,* 2002; 10(1): 29-35.

4. Hussain A., Anwar F., Rasheed S., Nigam P., Janneh O. and Sarker S.Composition, antioxidant and chemotherapeutic properties of the essential oils from two *Origanum* species growing in Pakistan. *Braz. J. Pharmac.,* 2011; 21(6):943-952.

5. Kintzios S. The biotechnology of oregano (Origanum sp. and Lippia sp.). *In*: Medicinal and aromatic plants-Industrial profiles, Oregano. The genera Origanum and Lippia, S. Kintzios,ed.., Taylor and Francis, London, 2002; 25: 237-242.

6. Iconomou-PetrovichG. andNianiou-ObeidatI. Micropropagation of *Origanum vulgare* subsp. *hirtum* (Mt. Taygetos). In: Progress in Botanical Research, TsekosI., MoustakasM., eds., Springer, Berlin, 1998; 509-512.

7. Morone-Fortunato I. and Avato P., Plant development and synthesis of essential oilsin micropropagated and mycorrhiza inoculated plants of *Origanum vulgare L. ssp. hirtum* (Link) Ietswaart. *Plant Cell Tiss. Organ Cult.,* 2008; 93: 139-149.

8. Nanova Z. and Slavova Y. Mass vegetative propagation of winter marjoram (*Origanum vulgare* ssp. *Hirtum* (Link) Jetswaart). *Bul. J. Agr. Sci.,* 2006; 12: 531-536.

9. Goleniowski M. E., Flamarique C.,Bima P. Micropropagation of oregano (*Origanum vulgare×applii*) from meristem tips. In Vitro *Cell. Dev. Biol. Plant,* 2003; 39(2): 125-128.

10. YankulovY. Winter marjoram, *In*: Basic Aromatic Plants. Zemizdat, *Sofia,* 2000; 122-141 (Bg).

11. FaisalM., AlatarA. A., El-SheikhaM. A., Abdel-Salama E. M. and Qahtan A. A. Thidiazuron induced *in vitro* morphogenesis for sustainable supply of genetically true quality plantlets of Brahmi, *Ind. Crops Prod.,* 2018; 118: 173-179.

12. Amoo S., Aremu A. and StadenV. *In vitro* plant regeneration, secondary metabolite production and antioxidant activity of micropropagated *Aloe arborescens* Mill. *Plant Cell Tiss. Org. Cult.,* 2012; 111(3): 345-358.

13. Murashige T. and Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.,* 1962; 15: 473-497.

14. Tepe B., Sokmen M., Akpulat H. and Sokmen A. Screening of the antioxidant potentials of six Salvia species from Turkey. *Food Chem.,* 2006; 95: 200–204.

15. Benzie I. and Strain J. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. *Anal. Biochem.,* 1996; 239: 70-76.

16. Pfeffer H., Dannel F. and Römheld V. Are there connection between phenol metabolism, ascorbate metabolism and membrane integrity in leaves of boron-deficient sunflower plants? *Physiol. Plant.,* 1998; 104: 479-485.

17. Zhishen J., Mengcheng T. and Jianming W. The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. *Food Chem.,* 1999; 64: 555-559.

18. Hristozkova M., Geneva M., StanchevaI., IlievI. andAzcon-AguilarC. Symbiotic association between golden berry (*Physalis peruviana*) and arbuscular mycorrhizal fungi in heavy metal-contaminated soil. *J. Plant Prot. Res.,* 2017; 57: 173-184.

19. Giannopolitis C. N. and RiesS. K., Superoxide dismutases I. Occurrence in higher plants. *Plant Physiol.,* 1977; 59: 309-314.

20. Beers F. and Sizer I. F., A spectrophotometric method for measuring breakdown of hydrogen peroxide by catalase. *J. Biol. Chem.,* 1952; 195: 133-140.

21. Nakano Y. and Asada K. Purification of ascorbate peroxidase in spinach chloroplasts: its inactivation in ascorbate-depleted medium and reactivation by monodehydroascorbate radical. *Plant Cell Physiol.,* 1987; 28: 131-140.

22. Urbanek H., Kuzniak-Gebarowska E. and Herka K., Elicitation of defence responses in bean leaves by *Botrytis cinerea* polygalacturonase. *Acta Physiol. Plant.,* 1991; 13: 43-50.

23. Bradford M. M. A rapid and sensitive method for the estimation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.,* 1976; 72: 248-254.

24. Sidhu Y. *In vitro* micropropagation of medicinal plants by tissue culture, *The Plym. Stud. Sci.,* 2010; 4(1): 432-449.

25. Zayova E., Stancheva I., Geneva M., Hristozkova M., Dimitrova L., Petrova M., Sichanova M., Salamon I. and Mudroncekova S. Arbuscular

mycorrhizal fungi enhance antioxidant capacity of *in vitro* propagated garden thyme (*Thymus vulgaris* L.), *Symbiosis,* 2017; **74**(3): 177-187.

26. Zayova E.,Nikolova M., Dimitrova L. and Petrova M. Comparative study of *in vitro, ex vitro* and *in vivo* propagated *Salvia hispanica* (Chia) plants: morphometric analysis and antioxidant activity, *Agro Life Sci. J.*, 2016; **5**(2):166-173.

27. Baricevic D. Experiences with oregano (Origanum spp.) in Slovenia. In: Promoting the conservation and use of underutilized and neglected crops – Oregano, S. PADULOSI,ed., 1997; **14**: 111–121.

28. Francis D. and Sorrell D. A. The interface between the cell cycle and plant growth regulators: a mini review. *Plant Grow. Reg.,* 2001; **33**: 1-12.

29. Fehér A., Pasternak T. P. and Dudits D. Transition of somatic plant cells to an embryogenic state.

Plant Cell, *Tiss. Org. Cult.,* 2003; **74**: 201-228.

30. Gaj M. D. Factors influencing somatic embryogenesis induction and plant regeneration with particular reference to *Arabidopsis thaliana* (L.) Heynh. *Plant Grow. Reg.,* 2004; **43**: 27-47.

31. Prins C. L., VieiraI. J. C. and Freitas S. P. Growth regulators and essential oil production review, *Braz. J. Plant Physiol.* 2010; **22**(2): 91-102.

32. Çavuþoðlu K., and Kabar K. The effects of pre-treatments of some plant growth regulators on germination and seedling growth of radish seeds under saline conditions. Dumlupinar *Uni. J. Soc. Sci.* 2007; **14**: 27-35.

33. Santos-Gomes P., Seabra R., Andrade P. and Fernandes-FerreiraM. Phenolic antioxidant compounds produced by *in vitro* shoots of sage(*Salvia officinalis* L.), *Plant Sci.,* 2002; **162**: 981-987.