Enzyme and Non-Enzyme Antioxidant Activity of Micro Propagated Cape Gooseberry (*Physalis peruviana* L.)

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Summary

Several studies have represented data on in vitro clonal multiplication of *Physalis peruviana* L. using different nutrient media supplemented with diverse growth regulators, but there is still scarce information on the changes in the antioxidant potential of the plants subjected to micropropagation. The present research investigates the effect of various plant growth regulators on the antioxidant activity of micropropagated *P. peruviana* L. The seedling explants of *P. peruviana* (L.) were cultured on Murashige and Skoog (MS) medium supplemented with different concentrations of the studied citokines, 6-benzylaminopurine (BAP), zeatin and thidiazuron (TDZ).The micro-shoots produced normal roots within two weeks of cultivation on half-strength MS medium supplemented with the auxins indole-3-butyric acid (IBA) or indole-3-acetic acid (IAA) at concentrations 0.1 mg L^{-1} and 0.5 mg L^{-1}, respectively. The most efficient plant rooting was achieved with plants grown on half-strength MS medium with 0.1 mg L^{-1} IBA (number of shoots per explant 12.1 with a shoot height 5.2 cm) as well as 0.1 mg L^{-1} IAA (number of shoots per explant 10.4 with a shoot height 7.3 cm) after four weeks of cultivation. The reported micropropagation protocol here is characterized by a rapid proliferation and facilitated rooting of the microshoots. The multiple plants were successfully adapted showing 100% survival rate after two months of *ex vitro* growth. The changes of enzyme and non-enzyme antioxidant activity were found to depend on the concentration of the used cytokinins. At the lower concentration of 0.5 mg L^{-1} of studied cytikinins the antioxidant capacity was characterized by a higher concentration of metabolites with antioxidant potential. The higher cytokinin concentration of 0.1 mg L^{-1} used in the micropropagation procedure resulted in higher antioxidant enzyme activity. The influence of different growth regulators on the antioxidant potential of micropropagated *P. peruviana* L. plantlets is discussed.

Key words

antioxidants, *in vitro* propagation, *Physalis peruviana* (L.), plant growth regulators

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Introduction

Physalis peruviana L. (cape gooseberry, golden berry) from Solanaceae family is a perennial medicinal plant, native to tropical Peru and other warm temperate and subtropical regions throughout the world. The cape gooseberry plant has orange edible fruits in the shape of a smooth berry, resembling a miniature, yellow tomato. The nutraceutical values of this fruit help in their use for the synthesis of functional food (Nocetti et al., 2020) and formulation (Pereda et al., 2020) for the treatment of non-communicable diseases. They are a source of health-related valuable and bioactive compounds as phytosterols, vitanolids, carotenoids (β-carotene, α-carotene, β-cryptoxanthene) (Hassan et al., 2017), phenolic compounds, physalins, ascorbic acid, vitamin A, C, and B-complex, iron and phosphorus,with a broad spectrum of biological activities, including antitumoral, antioxidant (Bazalar et al., 2019), immunomodulatory, cytotoxic, antimycobacterial, antimicrobial and insect repellent (Nocetti et al., 2020). Withanolide present in *P. peruviana* shows anti-adipogenic activity (Kumagai et al., 2020) and it is considered as a cure for chronic obstructive pulmonary disease (Yang et al., 2020). This plant shows promising results in treatment of inflammation (Parket al., 2017), progression of colorectal adenocarcinoma (El-Beltagi et al., 2019), and cancer (Hassan et al., 2017; Yu et al., 2021). Badr and Naeem, (2019) showed that the intake of *P. peruviana* fruit was protective against cadmium-induced neurotoxicity (Moneim et al., 2014) and also it was effective against the cancer-inducing effect of aflatoxins. The fruits are also rich in iron, manganese, zinc, sodium, magnesium, potassium, calcium, phosphorus, sulphur, aluminium, boron and copper (Karasakal et al., 2021).

The high content of biologically active substances in its fruits (Karasawa and Mohan, 2018), which are also a good source of nutrients, motivated the research related to factors controlling the growth of *P. peruviana* L. (Wu et al., 2005). Sexual reproduction, through seeds utilization, is the preferred approach for *P. peruviana* natural propagation in which plants are reproduced in their native environment or under controlled conditions. Successful seed emergence depends on environmental parameters such as humidity, temperature, light and oxygen. Deviations in any of these factors negatively influence seed germination . Although Cape gooseberry plants produce a large number of seeds per fruit which usually have high germination rate, the sexual propagation of the species has certain limitations for the production of seedlings due to the plant's susceptibility to pests and diseases and to the low production of secondary metabolites (Oliveira and Fernando, 2013).

Another approach for *P. peruviana* reproduction is the asexual method, which employs micropropagation and grafting. Asexual *in vitro* propagation is an alternative option for the rapid production of healthy, genetically uniform and pathogen-free plantlets (Chaves et al., 2005;Ramar et al., 2014). The most widely used cytokinin for shoot initiation is 6-benzylaminopurine (BAP). The addition of 0.3 mg L^{-1} BAP in full and 2/3 Murashige and Skoog (MS) nutrient medium (Murashige and Skoog, 1962) has been reported to be very effective for the Cape gooseberry shoot multiplication resulting in the largest number of formed shoots (Chaveset al.,2005). Otroshy et al., (2013) have studied the effect of growth regulators kinetin (Kin) (0, 1, 2 mg L^{-1}), BAP (0, 1, 2, 3, 4 mg/L) and IBA (0.5, 1 mg L^{-1}) on *P. peruviana* rhizogenesis and organogenesis initiated from leaves and nodes. The authors have found that the highest regeneration from leaf explants with the highest number of shoots per explant has been observed on MS media supplemented with 4 mg L^{-1} BAP and 2 mg L^{-1} BAP + 2 mg L^{-1} Kin. When nodule explants were used as a starting material it was found that a higher percentage of plant regeneration was achieved on MS medium containing 3 and 4 mg L^{-1} BAP in combination with kinetin, while the best rooting was observed on medium supplemented with 1.0 mg L^{-1} IBA without cytokinins or with the addition of lower cytokinin concentrations. Ramar et al., (2014) also have used nodal explants for *P. peruviana* regeneration and observed the maximum numbers of multiple shoots of *P. peruviana* on 2.0 mg L^{-1} BAP + 1.0 mg L^{-1} GA_3 + 1.0 mg L^{-1} 2, 4-D. Using axillary meristem explants another study reported maximum shoot number per explant of *P. angulata* after two weeks of cultivation on media with 1 mg L^{-1} BAP (Kumar et al., 2015). When the MS medium containing 1 mg L^{-1} BAP was supplemented with 1.5 mg L^{-1} IAA + 0.25 mg L^{-1} GA_3 the shoot number per explant increased. The highest shootregeneration from *P. peruviana* seed calli has been observed on MS medium supplemented with 21.0 mg L^{-1} BAP (Lashin and Elhaw, 2016). Mascarenhas et al., (2019) reported results for direct shoot production from cotyledonary node and leaf explants with 12.50 µM of BAP. Several studies have been conducted on the effect of the addition of different growth regulators to the various nutrient media to achieve the optimal micropropagation of *P. peruviana*. However, there is limited information on the role of TDZ and Zeatin in the organogenesis through *in vitro* regeneration of *P. angulata* L.

During *in vitro* propagation the plantlets experience the stressful environment inside the culture tubes which is characterized by high relative humidity, reduced light intensity and poor gas exchange. Under these conditions, plant metabolism is prone to generate overproduction of reactive oxygen species (ROS), leading to oxidative stress (Cakir et al., 2014). Plants have evolved an antioxidant enzyme (comprising ascorbate peroxidase (APX), catalase(CAT), superoxide dismutase (SOD), glutathione reductase(GR)) and non-enzyme (secondary metabolites such as ascorbic acid, glutathione, and tocopherols) antioxidative defence systems for ROS scavenging (Ahmad et al., 2008). The stress provoked by micropropagation causes certain changes in the antioxidant potential of the plantlets. There is scarce information on the effect of the cytokinin additives in the MS medium on the antioxidant potential of *in vitro* propagated *P. peruviana* plantlets. Such data on the antioxidant changes during *in vitro* propagation of plantlets is essential and it will benefit future investigations of the involved molecular mechanisms.

The antioxidant activities of methanolic extracts from the leaves and roots of the *in vitro* regenerated plants of *Rehmannia glutinosa* Libosch evaluated by *in vitro* assays have demonstrated differential levels of antioxidant capacity compared to the one in seed-derived plants (Piątczak et al., 2014). The present study aimed to evaluate the effect of different growth regulators added to the MS culture medium on the antioxidant potential of *in vitro* propagated plantless of *P. peruviana* which is a medicinal species with great biotechnological potential. We tested the effect of BAP, TDZ and Zeatin on *in vitro* growth and antioxidant capacity of the micropropagated plantlets by monitoring the enzyme and non-enzyme antioxidant activities.

Material and methods

Plant material. The seeds of *P. peruviana* L. were purchased from Sortovi Semena - Sofia plc. and were used for conducting the experiments while the seed batches were still fresh (i.e. in the same year). The seeds were planted in pots containing soil:perlite mixture (2:1) to sprout. After four weeks, the plants reach approximate height of 10 cm. For the *in vitro* culture, the seedlings were surface sterilized with 0.04% mercuric chloride ($HgCl_2$) for 30 minutes, and then rinsed thoroughly three times for 15 minutes with sterile water, to remove the traces of mercuric chloride.

Shoot *in vitro* propagation and rooting stage. For shoot regeneration, the seedlings were segmented into 4–16 pieces and cultured in MS medium supplemented with 7.0 g L^{-1} agar, 3.0% sucrose and 1.0 mg L^{-1} $CaCl_2$ for three weeks. The shoots were subcultured on media containing two concentrations (0.5 and 1.0 mg L^{-1}) of BAP, Zeatin and TDZ for the evaluation of shoot multiplication. After five weeks of cultivation, the percentage of formed shoots was determined, as well as the height and the average number of shoots per explant. These *in vitro* plantlets were cultivated on half-strength MS medium, supplemented with 7.0 g L^{-1} agar, 2.0% sucrose (control variant), and auxins (IBA and IAA at a concentration of 0.1 and 0.5 mg L^{-1}) for rooting of *P. peruviana*. The percentage of root formation, number of roots per plant and root length were recorded after four weeks of cultivation. Each treatment included 20 plants.

***Ex vitro* acclimatization stage**. For acclimatization under *ex vitro* conditions, the regenerated plants with a well-established root system were carefully taken out from the vessels and washed under running tap water to remove the adhering gelling agent. They were transplanted to small plastic pots (8 cm diameter) containing soil: sand: perlite (2:1:1, v/v/v) and were kept in a growth chamber. The slightly acidic leached cinnamonic forest soil (Chromic Luvisols – FAO) used in the soil experiments is characterized with the following agrochemical characteristics: pH (H_2O) = 6.2; 8mg kg^{-1} soil total mobile nitrogen (N) (N-nitrate (NO_3^-) + N-ammonium (NH_4+)); 30 mg kg^{-1} soil phosphorus pentoxide (P_2O_5); 120 mg kg^{-1} soilpotassium oxide (K_2O); and 1.88% of organic matter. The potted plants were covered with a transparent polyethylene membrane to ensure high humidity (90%) and the cover was removed after three weeks. The survival rate of the acclimated plants was determined after five weeks.

Culture conditions. The pH of the medium was adjusted to 5.8 with 1N NaOH or 1N HCl. Sterilization of the medium was performed in an autoclave at 121 °C for 20 min at a pressure of 1.1 kg cm^{-2}. *In vitro* cultures were maintained under growth room conditions at a temperature of 22±2 °C, relative humidity of 70% and a 16/8 h photoperiod under 40 µmol $m^{-2}s^{-1}$ illumination provided by Philips 36 W cool white fluorescent tubes. *Ex vitro* plants were maintained in a growth chamber at a temperature of 24±2 °C under 16/8 h photoperiod and fluorescent light illumination of 50 µmol $m^{-2}s^{-1}$.

Antioxidant capacity assays. Dry leaves from *in vitro* regenerated five-week-old Cape gooseberry plantlets (0.3 g) 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 free radical), was determined spectrophotometrically at 517 nm, according to (Tepe et al.,2006). The percent inhibition of the DPPH• radical (I%) was calculated by the following equation: I% = ((A_{blank}–A_{sample})/A_{blank}) × 100 where A_{blank} is the absorbance of the control reaction (containing all reagents except the extract) and A_{sample} is the absorbance of the extract. The ferric reducing antioxidant potential (FRAP) was monitored by colourimetry-reduction of ferric ions (Benzie et al.,1996). Spectrophotometric quantification of water-soluble (WS-AOM) and lipid-soluble (LS-AOM) antioxidant metabolites, expressed as equivalents of ascorbate and α-tocopherol was performed through the formation of the phospho-molybdenum complex (Prieto et al., 1999). The assay was based on the reduction of Mo (VI) to Mo (V) by the sample analysis and the subsequent formation of a green phosphate/Mo (V) complex at acidic pH. Molar absorption coefficients for the quantitation of WS-AOM (3.4±0.1) x 103 M^{-1} cm^{-1} for ascorbic acid and LS-AOM (4.0±0.1) x 103 M^{-1} cm^{-1} for α-tocopherol were used.

The concentrations of total phenolic compounds were determined spectrophotometrically using the Folin–Ciocalteu reagent and calculated as caffeic acid equivalents (Pfefferetal., 1998). The flavonoids in plant tissues were measured spectrophotometrically according to (Zhishenet al., 1999) 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 according to a previously described approach (Hristozkovaet al., 2017). Total SOD (EC 1.15.1.1) activity was determined using the method described by (Giannopolitis and Ries,1977). Total CAT (EC 1.11.1.6) activity was measured according to the method of (Beers and Sizer,1952). Total APX (EC 1.11.1.1) activity was assayed according to (Nakano and Asada,1987). Total GPO (EC 1.11.1.7) activity was determined as described by (Urbanek et al.,1991). Soluble protein content was determined by the method of (Bradford, 1976)) using bovine serum albumin as a standard.

Statistical analysis. Data were subjected to one-way ANOVA analysis of variance 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). Data were presented as means ± standard error. In addition, three independent replicates of the shoot induction and rooting experiments were carried out, with 20 plantlets per variant (n = 60).

Results and discussion

***In vitro* propagation of shoots and rooting stage.** The establishment of *P. peruviana in vitro* culture depends on several factors such as the quality of the starting material, prevention of culture contamination avoiding any possible side effects on the explants originating from the treatment with mercuric chloride or plant growth regulators. The surface sterilization of seeds with $HgCl_2$ is a successful approach providing contamination-free shoots. The frequency of shoot formation depends on the type of explant and the composition of the medium. *In vitro* shoot initiation was performed on MS medium supplied with cytokinin BAP, Zeatin and TDZ in two different concentrations (0.5 and 1.0 mg L^{-1}). The maximum shoot regeneration frequency (95%)

was observed after five weeks of cultivation on MS medium supplemented with 1.0 mg L^{-1} TDZ, resulting in 6.6 average number of shoots with 1.5 cm approximate height (Table 1, Fig. 1a and 1b). MS medium with 1.0 mg L^{-1} BAP produced shoots with an average shoot number of 5.3 and height of 1.2 cm. All tested cytokinins at a concentration of 0.5 mg L^{-1} were less effective at *in vitro* multiplication stage compared to the variants in which they were applied at a higher concentration (1.0 mg L^{-1}). Our results indicated that 1.0 mg L^{-1} TDZ or BAP alone proved to be the most efficient for shoot formation, which is in line with previously published reports on successful micropropagation of *P. peruviana* (Chaves et al., 2005; Ramar et al., 2014; Mascarenhas et al., 2019). The good potential of applying BAP and IBA in combination for *in vitro* shoot induction and multiplication has been also reported (Siwach and Gill, 2011). The authors have observed the highest *Ph. peruviana* shoot numbers (6.00) on MS medium supplemented with 2 mg L^{-1} BAP + 0.4 mg L^{-1} IBA combinations and the cultivation on medium containing 2 mg L^{-1} BAP + 0.2 mg L^{-1} IBA was the most beneficial in regard of the shoot length (3.30 cm) (Guney et al., 2016). Another published study showed that *P. peruviana* shoot formation occurred on LS medium supplied with BAP, TDZ, kinetin or gibberellic acid (GA_3), alone or in combination with 0.25 mg L^{-1} IAA or IBA (Yücesan et al., 2015). Nodal segments of 1-2 cm having at least one axillary bud cultured on MS medium supplemented with BAP (1.5mg L^{-1}), Kinetin (1.0 mg L^{-1}) and IBA (0.05 mg L^{-1}) have shown the best results in shoot proliferation experiements with *P. peruviana* (Singh et al., 2016). The highest average number of shoots per explant by Cape gooseberry direct organogenesis in MS culture medium with half the normal concentration of salts with 8.88 µM BAP has also been reported (Mascarenhas et al., 2019).

The effect of the auxin concentration on the root formation of *P. peruviana* was monitored via *in vitro* cultivation of shoots on ½ MS medium containing 2.0% sucrose (controls) supplemented with 0.1 and 0.5 mg L^{-1} IBA or IAA (Table 2).

Table 1. Effect of citokinines on the micro propagation of *P. peruviana* shoots after five weeks of cultivation

PGR (mg/L)	Micropropagation rate (%)	Average number of shoots (explant^{-1})	Average shoots height (cm)
0.5 BAP	75	2.9± 0.18^{b}	2.3 ± 0.20^{e}
1.0 BAP	90	5.3± 0.36^{d}	1.2 ± 0.11^{a}
0.5 Zeatin	65	1.8 ± 0.16^{a}	1.7 ± 0.15^{c}
1.0 Zeatin	70	2.3 ± 0.19^{ab}	1.4 ± 0.12^{b}
0.5 TDZ	80	4.0 ± 0.27^{c}	2.0 ± 0.18^{d}
1.0 TDZ	95	6.6 ± 0.41^{e}	1.5 ± 0.13^{bc}

The data are presented as means of 20 plants per treatment ± standard error. Different letters indicate significant differences assessed by Fisher test (5%) after performing ANOVA multifactor analysis.

The controls showed 60% rooting, the average number of roots was 5.5 with an average root length of 2.2 cm. The most effcient root initiation was obtained on medium containing 0.1 mg L^{-1} IBA with an average number of roots 12.1 and an average root length of 5.2 cm. Half-strength MS medium containing 0.1 mg L^{-1} IAA resulted also in 95% efficiency of root formation (Table 2, Fig. 1). Our results are in line with Singh et al., (2016) who also have used IBA for root initiation of regenerated shoots by direct organogenesis. The average number of roots (10.4) and the average root length (7.3 cm per shoot) observed in their study were within the same value range.

The efficiency of various concentrations of the auxins IBA, IAA and NAA for root formation by *in vitro* cultures of *P. peruviana* has been previously studied by several authors. The MS medium

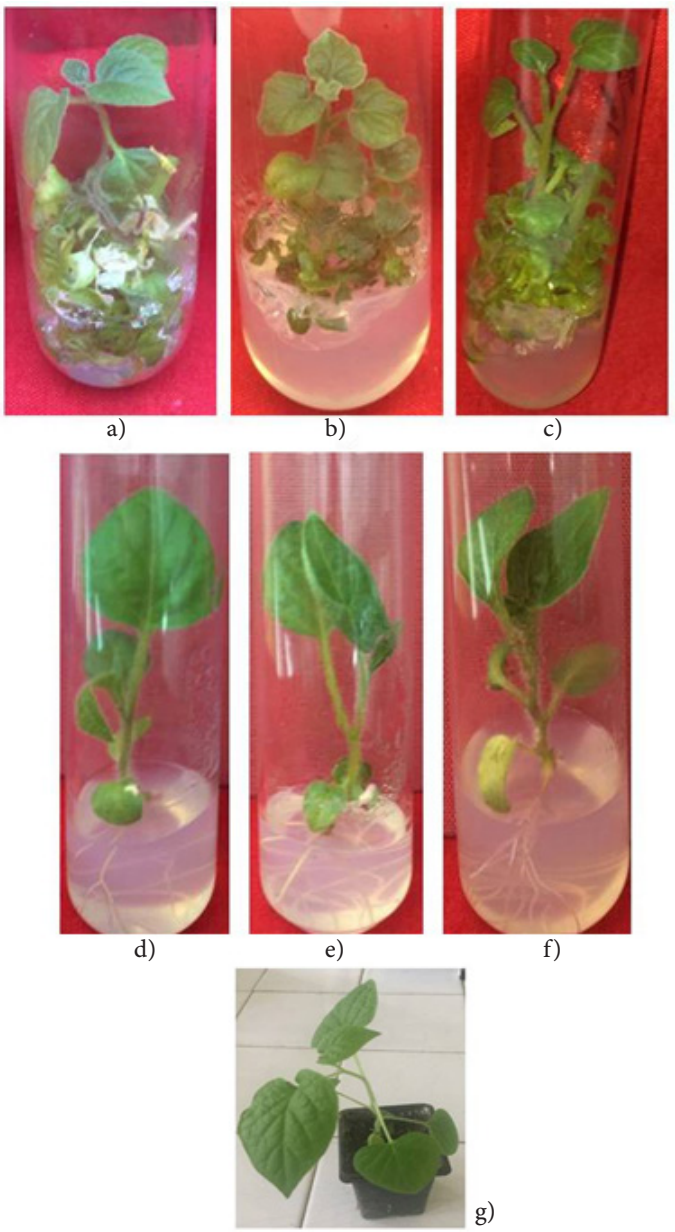

Figure 1. Micro propagation of *P. peruviana*: Shoot formation on MS medium supplemented with: a) 1.0 mg L^{-1} BAP after five weeks of cultivation; b) 1.0 mg L^{-1} Zeatin after five weeks of culti-vation; c) 1.0 mg L^{-1} TDZ after five weeks of cultivation. In vitro rooting of *P. Peruviana* on half-strength MS medium with: d) 0 mg L^{-1} auxins – control; e) 0.1 mg L^{-1} IBA after four weeks of cultivation; f) 0.1 mg L^{-1} IAA after four weeks of cultivation. g) *Ex vitro* acclima-tized plants grown on soil: sand: perlite (2: 1: 1 v/v/v) for two months.

Table 2. Effect of auxins on the plant root induction of *P. peruvianaafter* four weeks of cultivation

Auxin (mg L^{-1})	Rooting (%)	Average number of roots (plant^{-1})	Average root length (cm)	Acclimated individuals (%)
Control, ½MS	60	5.5 ± 0.48^{a}	2.2 ± 0.19^{a}	80
½ MS + 0.1 IBA	95	12.1 ± 0.64^{d}	5.2 ± 0.45^{b}	100
½ MS + 0.5 IBA	90	8.5 ± 0.52^{bc}	3.9 ± 0.3^{ab}	100
½ MS + 0.1 IAA	95	10.4 ± 0.69^{c}	7.3 ± 0.63^{c}	100
½ MS + 0.5 IAA	85	6.8 ± 0.5^{b}	5.5 ± 0.52^{b}	100

The data are presented as means of 20 plants per treatment ± standard error. Different letters indicate significant differences assessed by Fisher test (5%) after performing ANOVA multifactor analysis.

supplemented with IBA, has shown efficient root induction and further development of healthy root system (Mahmoud et al., 2013; Kumar et al., 2015; Singh et al., 2016). Singh et al., (2016) have used IBA for root initiation of regenerated shoots by direct organogenesis. The authors of this study recorded the highest root initiation, rooting percentage, root length and number of roots at half-strength MS medium fortified with 0.05 mg L^{-1} IBA . In a comparative experiment the application of IAA and NAA at various concentrations for root initiation in Cape gooseberry cultures 2.0 mg L^{-1} and 1.0 mg L^{-1} NAA application has been found to be the most efficient (Guney et al., 2016). In another study the addition of 1.0 mg L^{-1} IBA alone or in combination with low concentrations of cytokinins has exhibited the best *in vitro* rooting (Mahmoudet al., 2013). In our previous studies, the best effect of 0.1 mg L^{-1} IBA in half-strength MS medium on rooting was reported for some other medicinal plants, such as *Stevia rebaudiana* Bertoni and *Artemisia annua* (Zayova et al., 2017, 2018).

***Ex vitro* acclimatization stage**. The survival of *P. peruviana* plants after two months under *ex vitro* conditions was evaluated (Table 2, Fig. 1g). The plants' *ex vitro* adaptability on a potting mixture consisting of soil, perlite and sand (2:1:1 v/v/v) was found to be appropriate. Initially, the rooted plants were transferred to plastic pots with a mixture substrate and kept covered with polyethylene membranes for three weeks. The high levels of relative humidity enhanced the initial survival of the potted plants. At the acclimatization stage, the survival percentage of the micro plants was 100% in all studied variants. The well-developed root system of the micropropagated plants and the appropriate substrate provides rapid acclimatization at *ex vitro* conditions. Several researchers also reported that the use of soil, perlite and sand mixture (2: 1: 1 v/v/v) has been suitable for rooted plant acclimatization (Zayova et al., 2013; Guney et al., 2016).

Antioxidant activity. The formation and removal of ROS are in a delicate balance under normal plant growth conditions. This balance can often be disturbed during abiotic or biotic stress conditions (Gill and Tuteja, 2010). Also, plants tolerance to stress is related to their enzyme and non-enzyme antioxidant capacity. Some of the most important antioxidant enzymes responsible for restoring ROS balance in plants exposed to stress are SOD, CAT, APX and GPO (Bowler et al., 1992). SOD is the enzyme that plays a crucial role in the antioxidant systems as the main molecular player for the dismutation of O_2^-into H_2O_2 and O_2. In our experiments, the highest SOD activity was detected in *P. peruviana* micro plantlets grown on media containing low BAP concentration (0.5 mg L^{-1}). It was found to be three times higher than that measured in the samples derived from plantlets grown on media containing 1 mg L^{-1} BAP (Fig. 2). A similar trend was obseved for *in vitro* propagation variants treated with Zeatin and TDZ. CAT activity, which operates in the peroxisomes and converts H_2O_2 to H_2O, was higher at low concentrations of the auxins with 38-45% increased compared to the variants grown in the presence of higher auxin concentrations. We observed a similar trend in APX activity (15 - 71%) and GPO changes (18 - 35%) (Fig. 2).

The obtained results indicated significant differences in non-enzyme antioxidant capacity among the different experimental variants as evident from the measurements of the total phenols and total flavonoids contents in the leaves of *P. peruviana* micro plantlets (Fig. 3). Cakir et al., (2014) have also reported high phenolic contents in the leaf extracts of the micro propagated plantlets. In our experiments, the maximum amount of phenols was produced in the micropropagated plantlets grown on medum with 1 mg L^{-1} BAP, and the maximum flavonoid content was registered in the samples derived from micro plantlets propagated in the presence of 1 mg L^{-1} Zeatin. The different concentrations of zeatin and thidiazuron in the MS culture medium did not affect the content of phenols in the leaves of the plantlets. However,, the leaves tend to accumulate higher amount of flavonoids with the increase of the cytokinin concentration in the growth mediaing (24-25%). The metabolites with antioxidant properties have diverse chemical structures and can be classified into two major groups, depending on their ability to dissolve in water - hydrophilic water soluble metabolites with antioxidant potential or hydrophobic lipid soluble metabolites with antioxidant potential. In general, water-soluble antioxidants react with the oxidants in the cell cytoplasm, while lipid-soluble antioxidants protect cell membranes from lipid peroxidation (Manganaris et al., 2018)). The highest WS-AOM level was reported in the leaves of plants grown on BAP-supplemented MS medium, and LS-AOM in medium containing zeatin at both concentrations. Again, at higher cytokinin concentrations, higher contents of WS-AOM and LS-AOM were recorded (12% - 23%) (Fig. 3). Phenolic compounds are important plant constituents with free radicals scavenging ability which is due to the presence of hydroxyl groups in their molecular structure (Sarangarajan et al., 2017).

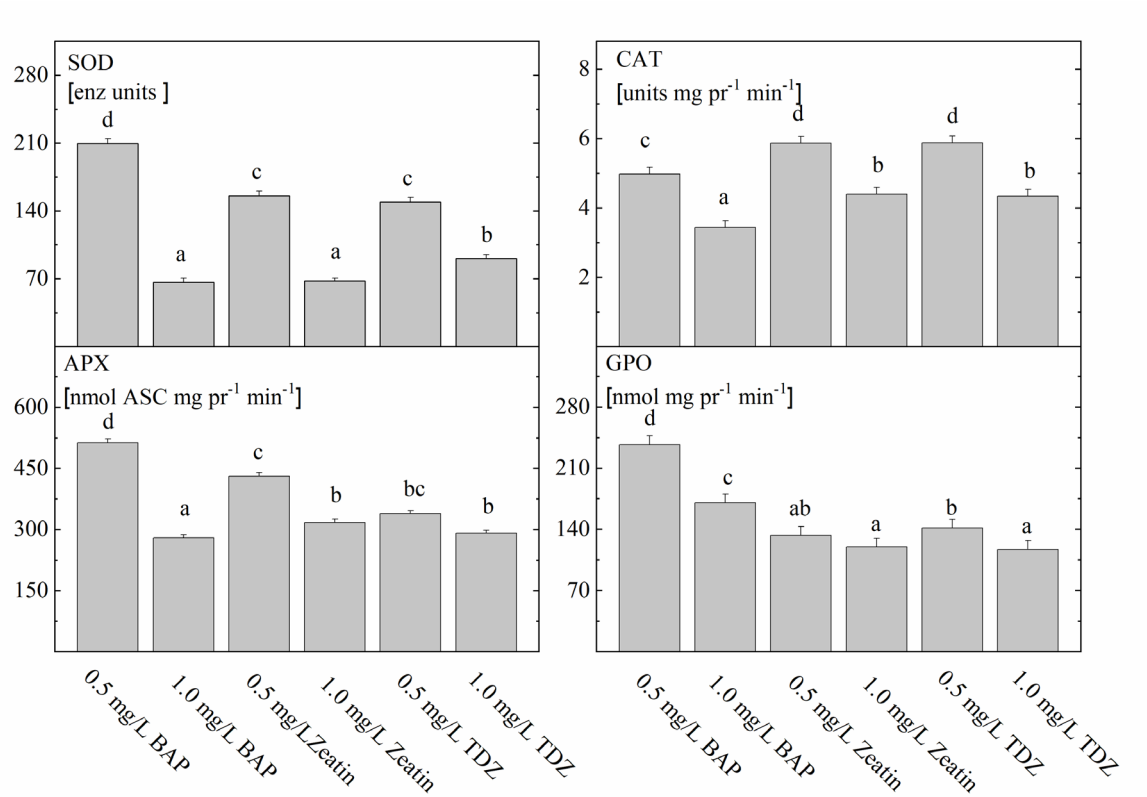

Figure 2. The activity of antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPO), and ascorbate peroxidase (APX) in the *in vitro* propagated *P. peruviana*. Values are means ± SE, n=3; different letters indicate significant differences assessed by Fisher LSD test (P≤0.05) after performing ANOVA multi-factor analysis.

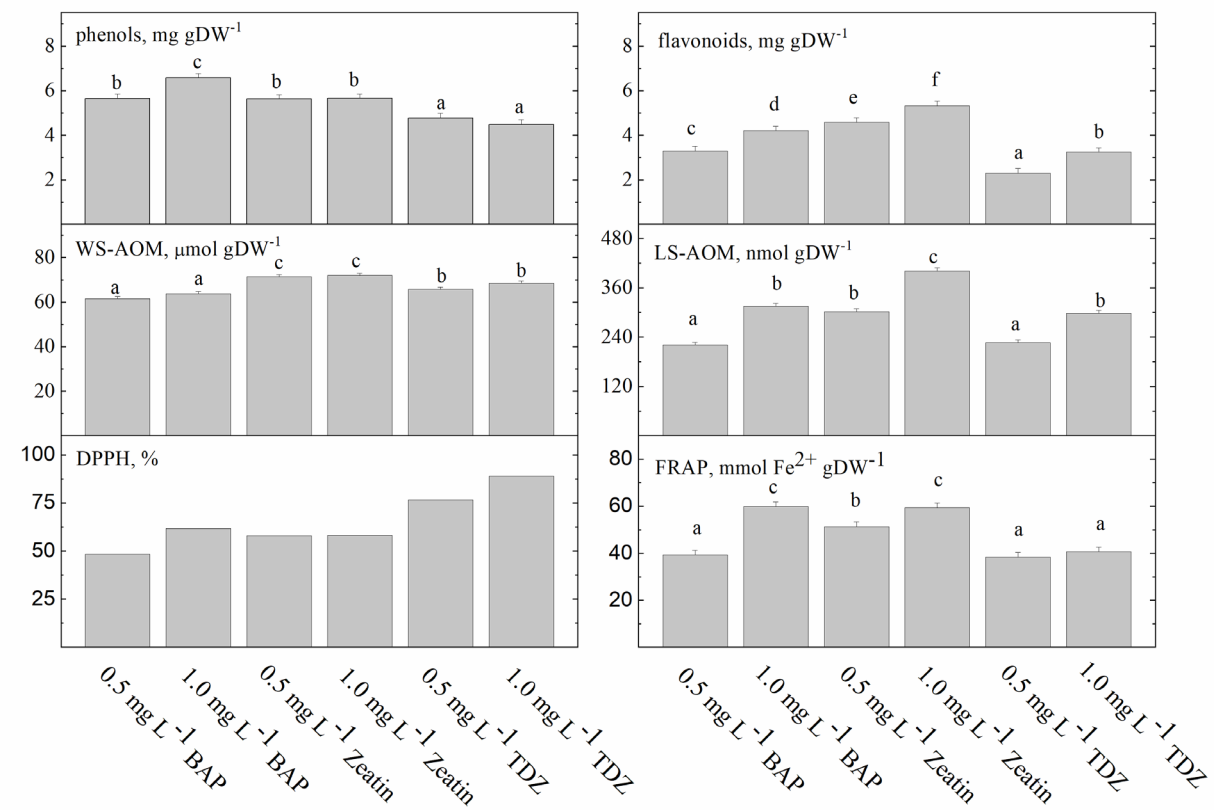

Figure 3. Content of metabolites with antioxidant properties and antioxidant potential in the *in vitro* propagated *P. peruviana*. Values are means ± SE, n=3; different letters indicate significant differences assessed by Fisher LSD test (P≤0.05) after performing ANOVA multi-factor analysis.

BAP and NAA have been reported to play an important role in the biosynthesis of secondary metabolites in plants grown as *in vitro* cultures (Shilpashree and Rai, 2009; Sayd et al., 2010). Our data are in agreement with the results reported by Sayd et al., (2010), who found that antioxidant capacity, characterised by antioxidant activity, total phenolics and flavonoids content in leaves of regenerated plantlets *Gardenia jasminoides* increased upon the addition of plant growth regulators (Kin, BAP and 6-(γ, γ- dimethylallyamino) purine) to the MS medium. The study reported an increased antioxidant capacity of the plants grown in the presence of higher BAP concentration (3 mg L^{-1}) (Sayd et al., 2010). It is suggested that the accumulation of total phenolics and flavonoids in shoots and calli could be due to the presence of growth regulators in the culture medium. Taviera et al., (2009) reported that shoot cultures had strong antioxidant potential and were able to produce high amount of phenolic compound on MS nutrient medium supplemented with 2 mg L^{-1} BAP and 0.1 mg L^{-1} NAA.

In our study we also observed higher total antioxidant activity at increasing concentrations of cytokinins in the MS culture medium. We used two different methods to measure the antioxidant activity in the samples: DPPH (employing free radical compound DPPH• to test the free radical scavenging ability of antioxidant compounds) and FRAP (based on the reduction of Fe_3^+ to Fe_2^+ in the presence of 2,4,6-trypyridyl-s-triazine, accompanied by the formation of a coloured complex with Fe_2^+) (Fig. 3). It can be assumed that higher cytokinin levels in the medium could accelerate the formation of ROS, which subsequently are neutralized through the generation of higher amounts of metabolites with antioxidant potential. Among the three tested cytokines, the highest free radical scavenging activity was recorded in *P. peruviana* plants grown on MS medium supplemented with 0.5 and 1.0 mg L^{-1} TDZ followed by the groups which were cultivated in the presence of 0.5 and 1.0 mg L^{-1} zeatin, and 0.5 and 1.0 mg L^{-1} BAP. The highest ferric reducing antioxidant potential (FRAP) was determined in the leaves of Cape gooseberry plantlets grown on MS medium with 1.0 mg L^{-1} BAP and 1.0 mg L^{-1} TDZ. Similar studies evaluating the effect of different plant growth regulators on the antioxidant potential of *in vitro* propagated palnts were performed in *Stevia rebaudiana* (Radić et al.,2016). It was found that the changes in DPPH radical scavenging activity in the leaves of the micropropagated plants were dependent on the concentrations of the various tested growth regulators applied alone or in combination (GA_3, GA_3+BA, IAA and IBA). A similar trend for positive effect of the auxins IAA and IBA on the content of total phenols and flavonoids in samples derived from *Stevia rebaudiana* leaves and callus has been reported (Radić et al.,2016).

Conclusions

In conclusion, our results confirmed the practical aspects of tissue cultures as an alternative method for large scale propagation of *P. peruviana*. This technique represents an advantageous alternative to the classic methods of propagation, which allows the rapid production of high-quality seedlings. *In vitro* germination capacity of the *P. peruviana* seeds is good, showing 100% germination rate. For the most efficient multiplication of the stem tips, we recommend the use of MS medium supplemented with 1.0 mg L^{-1} TDZ since it results in multiplication rate of 6.6 shoots per an explant and the average length of the shoots is 1.5 cm. The highest rooting level followed by a succssesful *ex vitro* acclimation (100%) was achieved on half-strength MS containning 0.1 mg L^{-1} IBAor 0.1 mg L^{-1} IAA. Generally, the *in vitro* technique described here provides a promising method for the rapid propagation of *P. peruviana* as a biomass source containing valuable bioactive compounds for medicinal purposes.

The present study demonstrates that the application of certain growth regulators at various concentrations markedly influences *in vitro* production and antioxidant capacity of *P. peruviana*. The analyses revealed that the plants grown in the presence of 0.1 $^{-1}$cytokines (BAP, TDZ or Zeatin) had lower antioxidant enzyme activities as compared to the ones measured in plants grown on media containing higher concentration (0.5 mg L^{-1}) of the hormone. In contrast, a higher content of the metabolites with antioxidant properties was observed in the samples derived from plants cultivated on media containing 0.1 mg L^{-1} cytokines (BAP, TDZ or Zeatin) . It was established that at a lower cytokinin concentration, 0.5 mg L^{-1} the antioxidant capacity is predominantly determined by a higher metabolites concentration, while at a higher cytokinin concentration, 1.0 mg L^{-1} it is determined predominantely by the antioxidant enzyme activities.

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