

THE EFFECT OF ZINC OXIDE NANOPARTICLES ON THE GROWTH AND DEVELOPMENT OF STEVIA PLANTS CULTURED *in vitro*

Anna Krzepińko ¹, Roman Prazak ² ✉, Katarzyna Matyszczuk ¹,
Magdalena Dyduch-Siemińska ²

¹ Department of Biotechnology, Microbiology and Human Nutrition, Faculty of Food Sciences and Biotechnology, University of Life Sciences in Lublin, Skromna 8, 20-704 Lublin, Poland

² Institute of Plant Genetics, Breeding and Biotechnology, University of Life Sciences in Lublin, University of Life Sciences in Lublin, Akademicka 15, 20-950 Lublin, Poland

ABSTRACT

Stevia (Stevia rebaudiana Bertoni) is an essential herbal plant used as a sweetener. The demand for stevia is growing due to its low caloric and medicinal value, hence the need for a more thorough investigation of its nutritional and biological properties. Nanoparticles of metal oxides have been found to have broad applications in agriculture for the stimulation of plant growth and development. The study aimed to assess the effect of various zinc oxide nanoparticles (ZnONPs) concentrations on stevia plants' quantitative and qualitative traits obtained in *in vitro* cultures. Micropropagation of two stevia varieties, Candy and Morita, was carried out using explants of shoot tips placed on MS medium supplemented with 1.0 mg dm⁻³ BA and 0.1 mg dm⁻³ IBA and with ZnONPs at concentrations of 0 (control), 10, 20, 30 and 40 mg dm⁻³. The obtained results indicated that high concentrations of ZnONPs stimulated the propagation of shoots. On the other hand, they negatively influenced shoot length, root number and length, and the fresh weight of the plantlets. The presence of zinc oxide nanoparticles in the medium increased the potassium, calcium, magnesium, and zinc content while decreasing the sodium and iron content in the regenerated stevia plantlets. The total phenolic content in the Candy variety was higher in the treatments with ZnONPs than in the control plants, while it was varied in the Morita variety. In both varieties, total antioxidant content measured by the ABTS method showed significantly higher in the treatments with 20–30 mg dm⁻³ ZnONPs than in the control. The content of chlorophyll *a*, chlorophyll *b* and chlorophyll *a + b* in the Morita variety was higher in the treatments with 10 and 20 mg dm⁻³ ZnONPs than in the control. On the other hand, high concentrations of ZnONPs negatively affected the content of carotenoids in both varieties. The study showed that stevia plants obtained in *in vitro* cultures on control media and media containing ZnONPs had a high content of valuable minerals, phytochemicals with antioxidant properties, and photosynthetic pigments.

Keywords: ZnONPs, *Stevia rebaudiana Bertoni*, *in vitro* culture, antioxidants, content of metals

INTRODUCTION

Stevia (Stevia rebaudiana Bertoni) is a perennial herbal plant belonging to the family *Asteraceae*, native to north-eastern Paraguay and the Brazilian border, where local indigenous populations used it as a sweetener and as a medicine for treating wounds,

skin lesions, hypertension, and digestive disorders. It is one of 154 species of the genus *Stevia*; only two produce steviol glycosides, which are about 100–300 times as sweet as saccharose. It is used to sweeten dishes, drinks, ice cream, sweets, soy sauces, yoghurt,

and chewing gum. Although sweet, they are not glycaemic and thus are an alternative for people with diabetes [Gantait et al. 2015, Samuel et al. 2018, Galo 2019]. Apart from steviol glycosides, stevia contains various alkaloids, tannins, flavonoids, essential oils, saponins, sterols and anthraquinones. Stevia extracts strengthen the immune system and exhibit anti-inflammatory and antioxidant properties [Gantait et al. 2015, Samuel et al. 2018].

Plantlets from *in vitro* culture are usually used on professional plantations. Stevia seeds germinate poorly, and the plants obtained from them show a high degree of variability [Rosales et al. 2018, Galo 2019, Doliński and Kowalczyk 2019, Rokosa and Kulpa, 2019, Al-Taweel et al. 2021, Dyduch-Siemińska et al. 2020, Dyduch-Siemińska 2021].

The growth and development of plants in *in vitro* cultures are stimulated using various agents, including zinc oxide nanoparticles [Tymoszuik and Wojnarowicz 2020].

Many researchers have observed a positive effect of zinc oxide nanoparticles on plant growth and development. ZnONPs facilitate the uptake and transport of essential minerals and nutrients, improve photosynthesis efficiency, increase synthesis of anti-stress compounds, and modulate hormonal balance, especially of auxins and gibberellins, thereby increasing the tolerance of plants to various abiotic stressors. Nanoparticles mitigate oxidative damage induced by abiotic stressors by scavenging reactive oxygen species (ROS) and activating antioxidant defence mechanisms [Hayat et al. 2023]. ZnONPs can be used in agriculture as a zinc fertiliser to increase yield and reduce disease occurrence, owing to their broad antifungal and antibacterial effects [Tymoszuik and Wojnarowicz 2020].

Maithreyee and Gowda [2015] showed a beneficial effect of zinc oxide nanoparticles on the germination and vigour of maize seedlings. Using ZnONPs in *in vitro* culture positively influenced mung bean plants' growth and the shoots' microelements, proteins and phenols content [Sorahinobar et al. 2023]. Tymoszuik and Wojnarowicz [2020] demonstrated that ZnONPs stimulate the germination of onion seeds *in vitro*.

The study aimed to investigate the effect of zinc oxide nanoparticles (ZnONPs) on stevia's quantitative and qualitative traits (*Stevia rebaudiana* Bertoni)

in *in vitro* culture. The plants were analysed for the content of selected metals (calcium, sodium, potassium, magnesium, zinc and iron) and of chlorophyll *a*, *b*, and *a + b* and carotenoids, as well as total phenolic content (TPC) and total antioxidant capacity (TAC).

MATERIAL AND METHODS

Sigma Aldrich ZnO nanoparticles (catalogue no. 721077) are <100 nm in size as measured by dynamic light scattering (DLS) and have an average particle size of <35 nm measured using an aerodynamic particle sizer (APS). They are synthesised by hydrolysis of a zinc salt in a polyol medium heated to 160°C. Their zeta potential is +46.1 ± 1.5 mV [Wang et al. 2013].

Plant material. The study was conducted using two varieties of stevia (*Stevia rebaudiana* Bertoni) – Candy and Morita – from the experimental farm of the Department of Vegetable and Medicinal Plants, University of Life Sciences in Lublin (51°14'53"N, 22°34'13"E). *In vitro*, stevia cultures were established on 20 August 2018 in the Institute of Genetics, Breeding and Biotechnology Laboratory, University of Life Sciences in Lublin [Dyduch-Siemińska 2021]. Shoot tips about 3 cm long with two or three nodes were placed in 200 mL glass jars with heat-resistant Magenta B-caps. Each jar contained 20 mL of MS medium [Murashige and Skoog 1962] with 3% saccharose, 0.8% Difco bacto-agar, 1.0 mg dm⁻³ BA and 0.1 mg dm⁻³ IBA, as well as ZnONPs at concentrations of 0 (control), 10, 20, 30 and 40 mg dm⁻³. The acidity of the medium was previously set at 5.8 pH. Cultures were conducted in a growth room at 22–24°C, 60% relative humidity, and 16 h of light per day from fluorescent lamps, with light intensity of 54 μmol m⁻² s⁻¹. After 3 months, stevia plantlets were removed from the jars, and measurements were taken: the number and length of shoots and roots and fresh weight. The plantlets were used for further analysis – determination of the content of metals (calcium, sodium, potassium, magnesium, zinc, and iron), the content of chlorophyll *a*, *b*, and *a + b* and carotenoids, total phenolic content (TPC), and total antioxidant capacity (TAC).

Determination of the content of selected minerals. Samples of plantlets were washed with distilled water and placed in a forced air furnace to dry at 70°C

for 72 h. The dried plant material was digested with a diacid mixture (HNO_3 and HClO_4). Following dilution, the digestion products were analysed for the content of calcium (Ca), potassium (K), sodium (Na), magnesium (Mg), zinc (Zn) and iron (Fe) by ASA.

Determination of the content of photosynthetic pigments. The chlorophyll content in the plantlets was determined by spectrophotometry following extraction with 80% acetone [Lichtenthaler and Wellburn 1983]. Absorbance was measured at 645 nm, 663 nm and 470 nm. Chlorophyll and carotenoid content were expressed in mg g^{-1} FW.

Determination of total phenolic content. The plant material was homogenised with a 70% v/v methanol-water solution in a weight ratio of 1:10. Then the homogenate was mixed for 24 hours on a laboratory shaker at 60 rpm and 22°C , centrifuged for 5 min at 5000 rpm. The supernatant was frozen and used to determine the total polyphenol content. The Folin-Ciocalteu spectrophotometric method, used to determine total phenolic content, exploits the capacity of polyphenols to produce coloured products of reactions with the components of Folin reagent (salts of phosphomolybdic and phosphotungstic acids). The absorbance of the solutions is proportional to the total content of phenolic compounds in the sample. Absorbance was measured after 1 h at 765 nm [Singleton et al. 1999]. The results were expressed in mg gallic acid equivalent (GAE) per g fresh weight (FW) of plantlets.

Determination of total antioxidant capacity. Antioxidant extraction was performed from plant material homogenised with distilled water in a weight ratio of 1:10. Total antioxidant capacity (TAC) was determined in the extracts by the ABTS method. This method involves spectrophotometric measurement of the colour reduction reaction between $\text{ABTS}^{+\cdot}$ cation radical and the antioxidants in the sample. Coloured $\text{ABTS}^{+\cdot}$ radicals are formed from ABTS (2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) during chemical reactions with $\text{K}_2\text{S}_2\text{O}_8$. $\text{ABTS}^{+\cdot}$ is soluble in both water and organic solvents, so it can be used to determine the antioxidant capacity of hydrophilic and lipophilic compounds. When antioxidants reduce cation radicals ($\text{ABTS}^{+\cdot}$), the colour of the solution fades, and the decrease in the intensity of the colour is proportional to the concentration of antioxidants. Spectrophotometric measurement was performed at 414 nm, 30 min after mixing the reagents [Re et al. 1999]. TAC was expres-

sed as μM Trolox equivalent (TE) per g fresh weight (FW) of plantlets.

Statistical analysis. All experiments were performed in triplicate. Statistical analysis of the results was performed by analysis of variance, determining the significance of differences using Tukey's *t*-test at $\alpha = 0.05$. Statistica v.13.1 software was used for the analysis

RESULTS

Determination of biometric features. The composition of the MS medium used in the experiment successfully induced the formation of shoots and roots from explants of shoot tips of *Stevia rebaudiana* Bertoni).

After three months of *in vitro* culture, the average number of shoots of the plantlets of both varieties was higher in the treatments with zinc oxide nanoparticles than in the control (Fig. 1). The highest average number of shoots (6.18/explant) was produced by the Candy variety in the treatment with 40 mg dm^{-3} ZnONPs. In the other ZnONPs treatments, there were also more shoots in this variety than in the control, but these results were not statistically significant. Plantlets of the Morita variety obtained in the treatments with 30 and 40 mg dm^{-3} ZnONPs had significantly more shoots than in the control. The average numbers of shoots for the Morita variety in these treatments were 4.82 and 4.81/explant, significantly higher than in control – 3.68/explant (Table 1).

The average shoot length of the plantlets of the Morita variety in the treatments with 30 and 40 mg dm^{-3} ZnONPs was 6.97 and 3.36 cm, significantly lower than in the control (10.72 cm). The average shoot length in the Candy variety for the 40 mg dm^{-3} ZnONPs treatment was 5.72 cm, significantly lower than for the control (6.75 cm). In the treatments 0, 10, 20, 30, and 40 mg dm^{-3} ZnONPs, the percentage of shoot clumps that formed roots was for the Candy variety as follows – 81.8%, 87.5%, 82.3%, 75.0%, 66.7%, and for the Morita variety – 100.0%, 98.4%, 100.0%, 94.5%, 92.0%.

The highest concentration of ZnONPs (40 mg dm^{-3}) had a significant negative impact on root number and length and the fresh weight of the plantlets of both varieties. In this treatment, the number of roots in the

Table 1. Effect of ZnONPs treatments on biometric features of *Stevia rebaudiana* Bertoni after three months of *in vitro* culture (mean value of feature/explant)

Biometrical feature	Varieties	Concentration of ZnONPs (mg dm ⁻³)					LSD _{p=0.05}
		0 (control)	10	20	30	40	
Number of shoots	Candy	3.94	4.07	4.63	4.94	6.18*	1.981
	Morita	3.68	3.77	4.58	4.82*	4.81*	0.767
Shoot length, cm	Candy	6.75	7.07	5.68	5.61	5.72*	1.705
	Morita	10.72	9.71	9.73	6.97*	3.36*	1.860
Number of roots	Candy	5.94	5.50	4.88	4.35	3.47*	1.981
	Morita	8.40	9.55	8.06	6.53	4.24*	3.519
Root length, cm	Candy	2.21	2.94	1.72	1.61	0.91*	1.302
	Morita	2.68	2.67	2.43	1.52	1.39*	0.853
Fresh weight of plant, g (increment, g)	Candy	0.54 (0.48)	0.57 (0.51)	0.42 (0.35)	0.41 (0.35)	0.30* (0.24*)	0.202 (0.195)
	Morita	0.67 (0.62)	0.61 (0.55)	0.60 (0.53)	0.50 (0.45)	0.33* (0.27*)	0.211 (0.205)

* – result significantly different concerning the control at $p = 0.05$

Morita variety (4.24/explant) was significantly lower than in the control (8.40/explant). For the Candy variety, in the treatment with 40 mg dm⁻³ ZnONPs, there were 3.47 roots per explant, compared to 5.94/explant in the control. The length of the roots in this treatment was also significantly lower than in the control; the average root length was 1.39 cm in the Morita variety – 2.68 cm in the control, and 0.91 cm in the Candy variety – 2.21 cm in the control (Table 1).

The smaller number of roots and lower shoot and root length resulted in lower weight of the plantlets in the treatments with zinc oxide nanoparticles compared to the control. Fresh weight (0.67 and 0.61 g) and the

fresh weight of the plantlets (0.62 and 0.55 g) were higher for the Morita variety in the control and the treatment with 10 mg dm⁻³ ZnONPs. In the treatment with 40 mg dm⁻³ ZnONPs, the fresh weight and increase in fresh weight of the plantlets of both varieties was just over half of the values recorded in the control plantlets. The average fresh weight and increase in fresh weight of the Morita plantlets in this treatment were 0.33 g and 0.27 g – half the control values. Similarly, in the Candy variety, the highest concentrations of the average fresh weight and increase in fresh weight of the plantlets were 0.30 g and 0.24 g – just over half of the values noted in the control (Table 1).

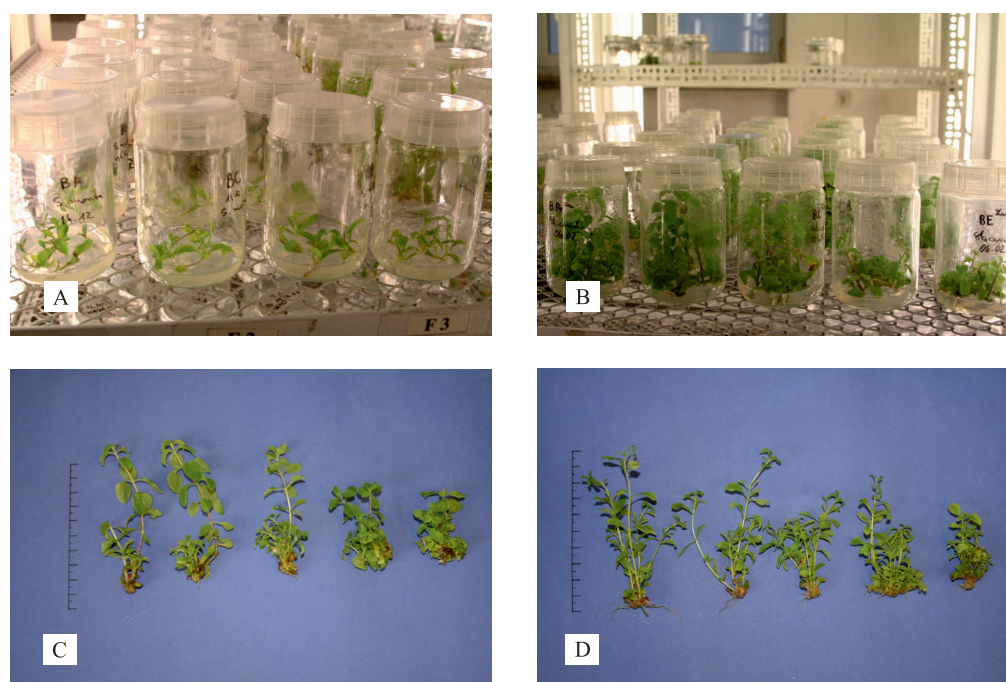


Fig. 1. Effect of different concentrations of ZnONPs (from left: 0 (control), 10, 20, 30 and 40 mg dm⁻³) in MS medium on the growth and development of *Stevia rebaudiana* Bertoni. A and B) developing explants, C) regenerated plantlets of – Candy variety, D) regenerated plantlets – Morita variety

Table 2. Effect of ZnONPs on mineral contents in plantlets of *Stevia rebaudiana* Bertoni after three months of *in vitro* culture

Variety	ZnONPs (mg dm ⁻³)	Mineral content (mg 100 g ⁻¹ DW)					
		K	Ca	Mg	Na	Zn	Fe
Candy	0	2260	278	120	395	26.9	17.8
	10	2255	372	145	360	89.0	15.6
	20	2240	340	136	348	110.0	12.4
	30	2010	278	124	290	129.0	9.8
	40	1960	260	122	266	148.0	7.9
Morita	0	1660	400	130	363	21.3	20.9
	10	2040	463	164	429	90.5	17.8
	20	2050	434	171	384	145.0	15.7
	30	1876	398	170	370	166.0	13.6
	40	1790	376	168	302	197.0	7.9
LSD _{p=0.05}		229.2	60.6	42.0	n.s.	n.s.	n.s.

n.s. – no statistically significant differences in the mineral content between Candy and Morita varieties

Determination of mineral content. In the treatments with 0–40 mg dm⁻³ ZnONPs, the stevia (*Stevia rebaudiana* Bertoni) varieties Morita and Candy differed significantly statistically in the content of the metals K, Ca, and Mg, while in the case of the other metals, i.e. Na, Zn, and Fe, there were no significant differences between varieties. In the Candy variety, the highest potassium and sodium levels were noted in the control, while calcium, magnesium and iron levels were highest in the treatment with 10 mg dm⁻³ ZnONPs (Table 2).

In all treatments with zinc oxide nanoparticles, the plantlets of the Morita variety had substantially lower potassium, magnesium, zinc and iron content than the control plants. In the Candy variety, in the treatments with 10–30 mg dm⁻³ ZnONPs, the calcium and magnesium content was higher than or similar to the control. Increasing the concentration of zinc oxide nanoparticles caused a decrease in the content of potassium, sodium and iron in the plantlets of this variety. In the plantlets of the Morita variety, the highest calcium and sodium contents were noted in the treatment with 10 mg dm⁻³ ZnONPs, while potassium and magnesium contents were highest in the treatment with 20 mg dm⁻³ ZnONPs. The highest iron content was noted in the control plants of the Morita variety. As the concentration of zinc oxide nanoparticles in the medium increased from 0 to 40 mg dm⁻³ ZnONPs,

the zinc content in the plants increased as well – from 26.9 to 148.0 mg 100 g⁻¹ DW in the Candy variety and from 21.3 to 197.0 mg 100 g⁻¹ DW in the Morita variety. Overall, the Morita plantlets contained more calcium, magnesium, sodium, zinc and iron, while the Candy plantlets had a higher potassium content (Table 2).

Determination of the content of photosynthetic pigments. The content of chlorophyll *a*, chlorophyll *b* and chlorophyll *a + b* in the Candy variety did not differ significantly statistically from the control in any of the treatments with ZnONPs. Chlorophyll *a* content in the Candy variety ranged from 11.36 µg g⁻¹ FW (treatment with 30 mg dm⁻³ ZnONPs) to 12.50 µg g⁻¹ FW (40 mg dm⁻³ ZnONPs), content of chlorophyll *b* ranged from 17.90 µg g⁻¹ FW (40 mg dm⁻³ ZnONPs) to 20.72 µg g⁻¹ FW (10 mg dm⁻³ ZnONPs), and that of chlorophyll *a + b* ranged from 30.37 µg g⁻¹ FW (40 mg dm⁻³ ZnONPs) to 32.92 µg g⁻¹ FW (10 mg dm⁻³ ZnONPs). Higher concentrations of zinc oxide nanoparticles were shown to affect the content of carotenoids negatively. Carotenoid content in the treatments with 30 mg dm⁻³ ZnONPs (3.87 µg g⁻¹ FW) and 40 mg dm⁻³ ZnONPs (3.82 µg g⁻¹ FW) differed statistically significantly from the control with 0 mg dm⁻³ ZnONPs (4.66 µg g⁻¹ FW) – Fig. 2.

The contents of chlorophyll *a*, chlorophyll *b* and chlorophyll *a + b* in the Morita variety were statistical-

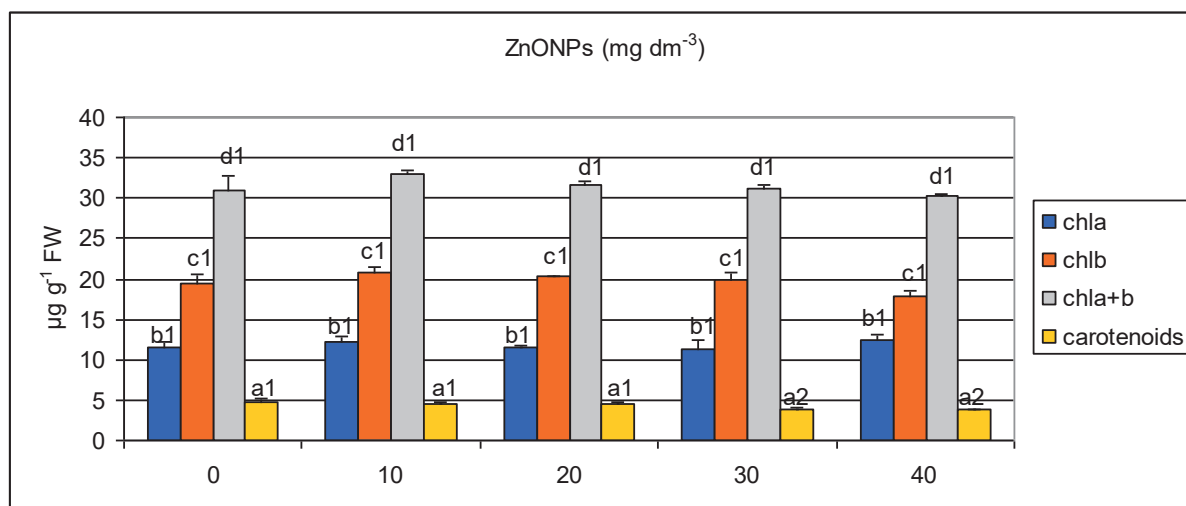


Fig. 2. Content of chlorophyll *a*, *b*, and *a + b* and carotenoids in the fresh weight of *Stevia rebaudiana* Bertoni plantlets of Candy variety. Error bars represent ±SD. Different lowercase letters indicate significant differences at $\alpha = 0.05$

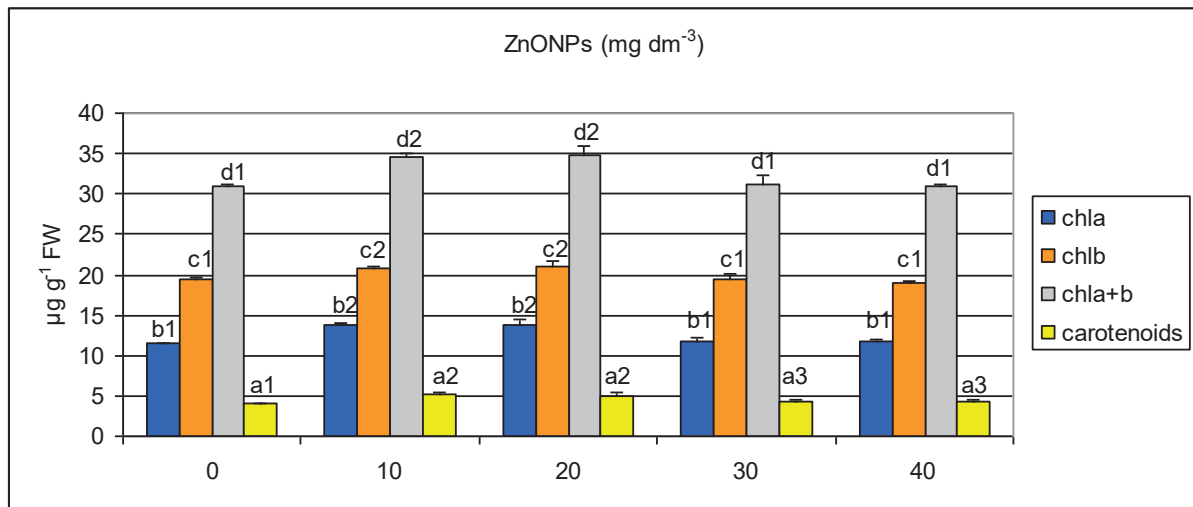


Fig. 3. Content of chlorophyll *a*, *b*, and *a + b* and carotenoids in the fresh weight of *Stevia rebaudiana* Bertoni plantlets of Morita variety. Error bars represent \pm SD. Different lowercase letters indicate significant differences at $\alpha = 0.05$

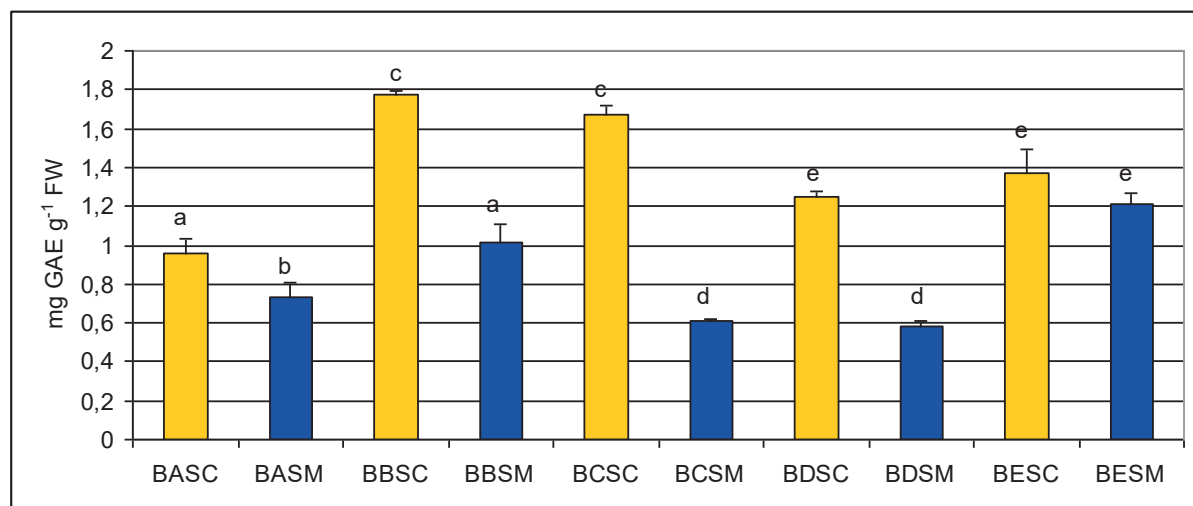


Fig. 4. Total phenolic content (TPC) in methanol extracts from the fresh matter of *Stevia rebaudiana* Bertoni plantlets. Varieties: SC – Candy, SM – Morita. ZnONPs treatments: BA – 0 (control), BB – 10 mg dm⁻³, BC – 20 mg dm⁻³, BD – 30 mg dm⁻³, BE – 40 mg dm⁻³. Error bars represent \pm SD. Different lowercase letters indicate significant differences at $\alpha = 0.05$

ly significantly higher in the treatments with 10 and 20 mg dm⁻³ ZnONPs than in the control. The chlorophyll *a* content in the Morita variety ranged from 11.53 µg g⁻¹ FW (control) to 13.89 µg g⁻¹ FW (at 20 mg dm⁻³ ZnONPs); chlorophyll *b* content ranged from 19.05 µg g⁻¹ FW (at 40 mg dm⁻³ ZnONPs) to 20.93 µg g⁻¹ FW (at 10 mg dm⁻³ ZnONPs); and content of chlorophyll *a + b* ranged from 30.90 µg g⁻¹ FW (40 mg dm⁻³ ZnONPs) to 34.82 µg g⁻¹ FW (20 mg dm⁻³ ZnONPs) –

Fig. 3. A statistically significant stimulatory effect of zinc oxide nanoparticles on the content of chlorophyll *a* and *a + b* in stevia of the Morita variety was noted in the treatments with 10 and 20 mg dm⁻³ ZnONPs, and the case of chlorophyll *b*, in the treatment with 10 mg dm⁻³ ZnONPs – Fig. 3. Carotenoid content in the Morita variety in all treatments with ZnONPs was significantly statistically higher than the control. The highest carotenoid content in the Morita variety

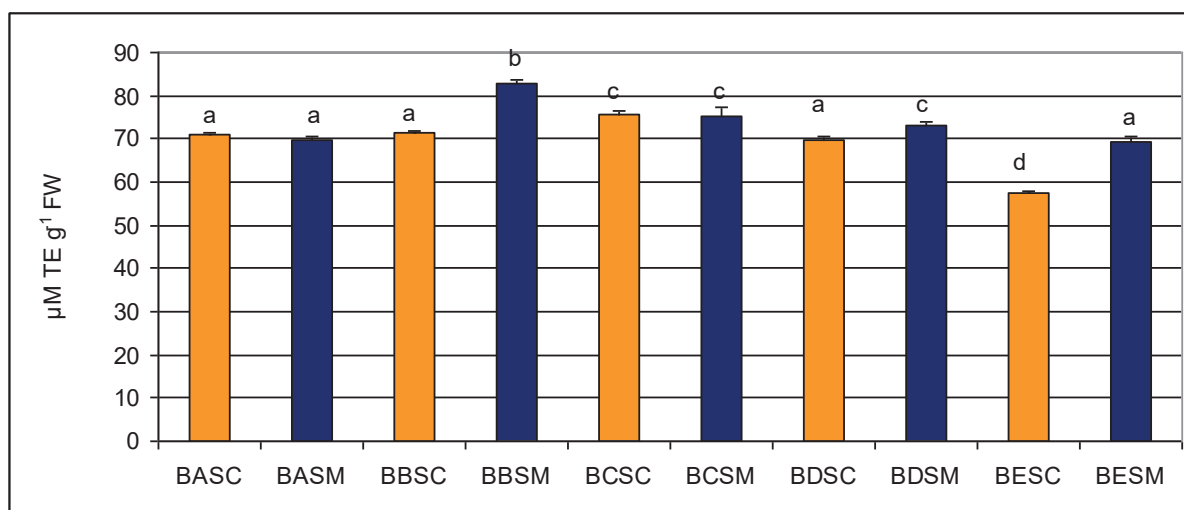


Fig. 5. Total antioxidant capacity (TAC) in the fresh matter of *Stevia rebaudiana* Bertoni plantlets determined by the ABTS method. Varieties: SC – Candy, SM – Morita. ZnONPs treatments: BA – 0 (control), BB – 10 mg dm⁻³, BC – 20 mg dm⁻³, BD – 30 mg dm⁻³, BE – 40 mg dm⁻³. Error bars represent ±SD. Different lowercase letters indicate significant differences at $\alpha = 0.05$

was noted in the treatments with 10 mg dm⁻³ ZnONPs (5.28 μg g⁻¹ FW) and 20 mg dm⁻³ ZnONPs (5.06 μg g⁻¹ FW), which was significantly higher than in the control (3.98 μg g⁻¹ FW) – Fig. 3.

Determination of total phenolic content (mg GAE g⁻¹ FW). The content of phenolic compounds in the fresh matter of regenerated plants of the two varieties of *Stevia rebaudiana* Bertoni – Candy and Morita – was compared. Methanol extracts were prepared from the fresh plantlets. Total phenolic content (TPC) in the fresh weight extracts of the *Stevia rebaudiana* Bertoni varieties fell within a fairly wide range, from 0.58 mg GAE g⁻¹ FW (Morita) to 1.77 mg GAE g⁻¹ FW (Candy) – Fig. 4.

The TPC of the Candy variety was higher than that of Morita and ranged from 0.96 to 1.77 mg GAE g⁻¹ FW, depending on the treatment (Fig. 4). In the Candy variety, TPC in all treatments with ZnONPs was significantly statistically higher than in the control. In the case of Morita, TPC was significantly higher in the treatments with 10 and 40 mg dm⁻³ ZnONPs (1.01 and 1.21 mg GAE g⁻¹ FW), while in the treatments with 20 and 30 mg dm⁻³ ZnONPs (0.61 and 0.58 mg GAE g⁻¹ FW), it was significantly lower than in the control (0.73 mg GAE g⁻¹ FW) – Fig. 3.

Determination of total antioxidant capacity (μM TE g⁻¹ FW). For the ABTS method, TAC was quan-

tified as Trolox equivalent (TE) per g fresh weight of plants, following the approach described by Re et al. [1999]. In the treatments with ZnONPs, the total antioxidant capacity (TAC) in the fresh matter of stevia plantlets determined by the ABTS method was higher in the Morita variety than in the Candy variety. The Morita variety had the highest TAC in the treatment with 10 mg dm⁻³ ZnONPs – 82.88 μM TE g⁻¹ FW (Fig. 5).

The TAC of the Candy variety ranged from 57.43 μM TE g⁻¹ FW (40 mg dm⁻³ ZnONPs) to 75.75 μM TE g⁻¹ FW (20 mg dm⁻³ ZnONPs) (Fig. 5). The TAC in plantlets of the Candy variety was significantly statistically higher than in the control only in the treatment with 20 mg dm⁻³ ZnONPs. In the remaining treatments, it did not differ statistically from the control or was significantly lower (20 mg dm⁻³ ZnONPs). In the case of the Morita variety, TAC in most treatments with ZnONPs was significantly higher than in the control (Fig. 5).

DISCUSSION

Stevia is a valuable medicinal plant in high demand. Improvement of methods of *in vitro* culture of stevia enables the selection and breeding of plants with identical genetic traits, high resistance and rapid growth, and high content of beneficial bioactive compounds [Shahnawaz et al. 2021]. *Stevia rebaudiana* is

propagated *in vitro* from various parts of the plants, such as the leaves, stems with nodes, or cell suspensions, but stem fragments with nodes are considered suitable for direct micropropagation [Thiyagarajan and Venkatachalam 2012, Rokosa and Kulpa 2019, Dyduch-Siemińska 2021]. In our control experiment, the medium (MS without ZnONPs) promoted the development of plantlets (Table 1). The Morita variety had slightly better growth parameters and shoot-forming capacity. Dyduch-Siemińska [2021] obtained an average of 4.67 shoots/explant, with an average length of 4.26 cm. Doliński and Jabłońska [2015] obtained, on average, from 4.71 to 11.36 shoots/explant, ranging from 1.44 to 1.99 cm in length. However, in the stevia micropropagation culture, the highest number of shoots and leaves was observed in the MS medium enriched with 0.5 mg·dm⁻³ cytokinin 6-benzyl aminopurine. At the same time, the most significant number of the longest roots were developed by stevia on the MS medium enriched with auxin 1 mg·dm⁻³ indole-3-acetic acid [Rokosa and Kulpa 2019].

The addition of zinc oxide nanoparticles to tissue culture media can positively influence callus development, rooting, and somatic embryogenesis in various plant species, including *Panicum virgatum* L., *Phoenix dactylifera* L., *Coffea arabica* L., and *Solanum lycopersicum* MILL [Shafique et al. 2020, Al-Mayahi 2021, Devasia et al. 2020, Alharby et al. 2016]. The biological properties of nanoparticles are primarily influenced by their structure, size, and synthesis method. Our experiment, in which we used commercially manufactured ZnONPs, showed that they exert a concentration-dependent effect on the growth and development of stevia plants in *in vitro* culture. The study showed that concentrations of 30 and 40 mg dm⁻³ ZnONPs in MS medium in the case of the Morita variety and 40 mg dm⁻³ ZnONPs in the Candy variety had a beneficial effect on shoot regeneration *in vitro*. Zinc oxide nanoparticles synthesised for scientific experiments often have properties different from those produced industrially. Javed et al. [2018] used 34 nm nanoparticles, which were synthesised by the co-precipitation method from a solution of zinc acetate dihydrate using sodium hydroxide. ZnO nanoparticles obtained in this manner had a pronounced beneficial effect on the growth of stevia plants only at concentrations of 0.1 and 1 mg dm⁻³,

while further increasing their concentration caused various toxic effects. In our experiment, zinc oxide nanoparticles at a concentration of 30 or 40 mg dm⁻³ significantly increased the number of shoots in both stevia varieties (Table 1), but they were shorter than in the control. Javed et al. [2018] obtained an average shoot length of 4.2 cm in the control plants, and the addition of zinc oxide nanoparticles at concentrations of 0.1, 1 and 10 mg dm⁻³ only slightly increased their length. In an *in vitro* culture of *Phoenix dactylifera* L., the best regeneration effects and the most shoots were obtained using MS medium supplemented with ZnONPs at a concentration of 150 mg dm⁻³ [Awad et al. 2020]. *Chrysanthemum × morifolium* (Ramat.) Hemsl. regenerated the most shoots when ZnONPs were applied at 500 mg dm⁻³ [Tymoszuć et al. 2022]. Analysis of the effect of zinc oxide nanoparticles (0.0, 2.43, 4.86, and 7.29 mg dm⁻³) on the regeneration of olive explants showed that the nanoparticles induced callus growth in response to all concentrations but prevented root growth [Taheri et al. 2024]. These examples of the effectiveness of zinc oxide nanoparticles for the regeneration of plants in *in vitro* cultures indicate the specific properties of various forms of zinc oxide nanoparticles as an elicitor of plant growth and the individual response of a given plant species. One of the advantages of zinc oxide nanoparticles is their antimicrobial activity [Gharpure and Ankamwar 2020]. In our experiment, no microbiological contamination was noted during the *in vitro* culture, but other authors emphasised the advantages of using zinc oxide nanoparticles as an antimicrobial agent in *in vitro* culture [Al-Mayahi 2021].

Zinc is crucial for the regulation of plant growth. It is essential for maintaining the hormonal balance of plants, especially auxin activity [Hassan et al. 2020], which explains the more beneficial effect of zinc on shoot regeneration than on root growth and development. This was confirmed in our experiment, as an increase in the concentration of zinc oxide nanoparticles inhibited shoot growth in stevia as well as root number and length, leading to a lower increase in the fresh weight of the plants (Table 1). However, the differences were statistically significant only at the highest concentration of ZnONPs (40 mg dm⁻³). In the treatments with 10–30 mg dm⁻³ ZnONPs, the average values of these biometric features were lower

than in the control, but these values were not statistically significant.

According to Lin and Xing [2007], depending on the plant's susceptibility, ZnO nanoparticles at 2000 mg dm⁻³ inhibited root elongation in radish, rapeseed, ryegrass, lettuce, maize and cucumber. Much lower concentrations of 5 and 50 µg dm⁻³ ZnONPs inhibited root growth in *Allium cepa*. With the increasing concentrations of ZnO NPs, the mitotic index decreased with the increase of pyknotic cells of *Allium cepa*. On the other hand, micronuclei and chromosomal aberration indexes increased [Kumari et al. 2011]. They were shown to adhere to the root's surface and accumulate in it, causing a loss of cell membrane integrity, chromosomal aberrations, and DNA damage [Sun et al. 2019]. However, how nanoparticles affect roots may depend on their physicochemical properties and concentrations. Tomato plants responded to 75 mg dm⁻³ ZnONPs with increased shoot and root length and increased content of chlorophyll and antioxidants [Rehman et al. 2023].

Roots in the form of Zn²⁺ cation and organic chelates can take up zinc. It has been demonstrated that zinc can easily be transported by phloem and stimulate leaf development [Haslett et al. 2001, Erenoglu et al. 2002, Schaff et al. 2004, Riesen and Feller 2005].

Zinc is essential in photosynthesis processes. Hajiboland and Amirazad [2010] observed that Zn deficiency in red cabbage (*Brassica oleracea* L. var. *capitata* f. *rubra*) plants grown in hydroponic cultures reduced chlorophyll *a* content, the chlorophyll *a/b* ratio, and soluble carbohydrates and starches, while increasing in the content of anthocyanins and free phenols.

Chlorophyll content in leaves is a commonly used indicator of a plant's capacity for photosynthesis. In the present study (Figs 1 and 2), we showed minor changes in the content of photosynthetic pigments in the Candy variety and significant changes in the Morita variety in the treatments with 10 and 20 mg dm⁻³ ZnONPs. In the traditional cultivation of tomatoes, the chlorophyll content of the leaves increased substantially following the application of ZnONPs [Rehman et al. 2023]. However, there are also conflicting reports in which no changes in chlorophyll were observed. These discrepant results may be due to differences in dosage, exposure concentration, and delivery method. The use of ZnONPs has been shown to have a beneficial effect

on the growth of cotton plants, significantly increasing both shoot length (130.6%) and biomass (131%). ZnONPs increased the soluble protein level (179.5%), decreased levels of malondialdehyde (MDA), and effectively increased levels of chlorophyll *a* and *b* and carotenoids (141.5%, 134.6% and 138.6%, respectively) in the leaves [Venkatachalam et al. 2017]. In addition, there was an increase in the enzymatic activity of superoxide dismutase (264.3%) and peroxidase (182.2%), which further improved the growth of cotton plants [Venkatachalam et al. 2017].

Excessive amounts of zinc or zinc oxide nanoparticles can negatively affect plants. Adhikari et al. [2020] emphasise the destructive effect of zinc nanoparticles on homeostasis, hormonal signalling, transport of heavy metals, and photosynthesis, with a reduction in the content of chlorophyll and carotenoids in maize *Zea mays* L. Lee et al. [2010] showed a negative effect of high concentrations of zinc oxide nanoparticles on germination, root elongation, and leaf number in thale cress. According to the authors, sediments and waste containing these nanoparticles should be disposed of ecologically. Lin and Xing [2007] studied the effect of Zn-NPs on seed germination and root growth in rapeseed, radish, soybean, cabbage, maize, carrot and cucumber. The study showed that they did not affect seed germination, while root growth inhibition varied significantly depending on the plant species. Nanoparticles at a concentration of 2000 mg dm⁻³ completely inhibited root elongation in the plant species. Concentrations of 50 mg dm⁻³ in radish and 20 mg dm⁻³ in rapeseed resulted in 50% inhibition.

The benefits of stevia arise not only from the sweet taste of the products derived from it, owing to the presence of steviol glycosides, but also from the content of essential minerals for humans. Deficiencies of these minerals, usually resulting from a diet limited to a few essential products, frequently cause numerous diseases. Geşiński et al. [2013] demonstrated a higher content of microelements in young stevia leaves than in older ones. Young stevia leaves contained 2.5 mg 100 g⁻¹ DW zinc and 30.8 mg 100 g⁻¹ DW iron, while old ones contained 2.1 mg 100 g⁻¹ DW zinc and 25.7 mg 100 g⁻¹ DW iron. According to the authors, stevia leaves had a higher content of minerals than cereal grains, potato tubers, cornsalad, quinoa, beet, and the green parts of maize. In the present study, in the plan-

lets of the two stevia varieties, the zinc content in the control was much lower – 26.9 mg 100 g⁻¹ DW in the Candy variety and 21.3 mg 100 g⁻¹ DW in the Morita variety, while iron content was similar, amounting to 17.7 mg 100 g⁻¹ DW in the Candy variety and 20.9 mg 100 g⁻¹ DW in Morita. In a study by Befa et al. [2020], the Ca content (359.6 mg 100 g⁻¹ DW) in the leaves of stevia grown in Ethiopia was similar to the values obtained in the present study. Levels of potassium (347.4 mg 100 g⁻¹ DW), sodium (102.9 mg 100 g⁻¹ DW), and zinc (3.7 mg 100 g⁻¹ DW), however, were much lower, while levels of magnesium (324.1 mg 100 g⁻¹ DW) and iron (297.9 mg 100 g⁻¹ DW) were higher than in the present study. According to Gerdzhiova et al. [2018], the content of minerals in the leaves and stems of stevia from field cultivation in Bulgaria was as follows: potassium 1546.0 and 1811.0 mg 100 g⁻¹ DW, calcium 1103.0 and 498.0 mg 100 g⁻¹ DW, magnesium 252.0 and 120.0 mg 100 g⁻¹ DW, sodium 120.0 and 130.0 mg 100 g⁻¹ DW, zinc 45.3 and 25.2 mg 100 g⁻¹ DW, and iron 45.3 and 25.2 mg 100 g⁻¹ DW.

In the present study, in the treatments with ZnONPs, plantlets of the Morita variety of stevia had markedly higher potassium, magnesium, and zinc content and markedly lower iron content than the control plants. Increasing the concentration of zinc oxide nanoparticles resulted in a decrease in the potassium, sodium and iron content in the dry weight of the plantlets.

In addition to steviol glycosides, stevia contains other bioactive compounds and secondary metabolites with health-promoting properties. Plant tissue culture can be a source of various substances for use in the cosmetic or pharmaceutical industry, and zinc oxide can act as a stimulator of biosynthesis of secondary metabolites [Yeşil-Çeliktaş et al. 2007, El-Tohamy et al. 2009, Bagheri et al. 2023, Singh et al. 2023]. All parts of mature stevia plants contain large amounts of phenols, flavonoids, tannins, and antioxidants, but their content is highest in the roots – a material that has rarely been used [Singh et al. 2012]. Stevia plants from *in vitro* culture can also be a source of health-promoting substances. The results of our experiment demonstrate that a well-chosen concentration of ZnONPs added to the *in vitro* culture medium can increase phenolic content and total antioxidant capacity in stevia plants (Figs 3 and 4). The total phenolic content in the *Stevia rebaudiana* Bertoni varieties ranged

from 0.58 mg GAE g⁻¹ FW to 1.77 mg GAE g⁻¹ FW, and the total antioxidant capacity – from 57.43 µM TE g⁻¹ FW to 82.88 µM TE g⁻¹ FW. In the research by Topdemir and Buran [2023], the total phenolic content of methanol extracts of *Ocimum basilicum* L. calluses was between 0.701 and 2.547 mg of GAE g⁻¹, and the antioxidant content was between 1.050 and 4.186 mM TE 1 g⁻¹. In extracts of *Echinacea purpurea* obtained from *in vitro* cultures of calluses treated with various concentrations of ZnONPs, the flavonoid concentration was higher than in the control [Karimi et al. 2018]. Javed et al. [2018] obtained the highest total phenolic content (TPC), total flavonoid content (TFC), total antioxidant capacity (TAC), and free-radical scavenging activity of 1,1-diphenyl-2-picrylhydrazyl (DPPH) using 100 mg dm⁻³ ZnONPs. The inherent antioxidants in medicinal plants are crucial for shielding against various environmental pressures. Metal nanoparticles interact with plants at different levels, leading to several molecular, biochemical, and physiological changes. Zinc oxide nanoparticles can protect plants against stress caused by various factors [Alharby et al. 2016, Bagheri et al. 2023, Hayat et al. 2023]. In a study conducted by Sheikhalipour et al. [2021], the TiO₂NPs foliar treatment of stevia plants exposed to different levels of salinity improved plant height and weight, photosynthesis index, chlorophylls and carotenoids contents [Sheikhalipour et al. 2021]. Singh et al. [2023] provide examples of various nanoparticles, including silver, copper, copper oxide, and titanium dioxide, which are nutrient sources and elicit secondary metabolites in the growth medium. Javed et al. [2018] have found that ZnO and CuO nanoparticles can serve as abiotic elicitors for inducing the production of secondary metabolites from *Stevia rebaudiana* callus. In our opinion, ZnONPs can enrich the mineral composition of stevia cultured *in vitro* and increase the content of bioactive compounds.

CONCLUSIONS

Concentrations of 30 and 40 mg dm⁻³ ZnONPs increased the regenerated stevia shoots. In the case of the other morphological features, higher concentrations of zinc oxide nanoparticles had a negative effect. Differences between stevia varieties were observed in

the content of certain metals, chlorophyll, carotenoids, phenols, and antioxidants. When the concentration of zinc (in the form of ZnONPs) in the medium was increased, synergism was observed in magnesium uptake and antagonism in iron uptake in both varieties. Overall, stevia plants regenerated on media with ZnONPs had a higher content of valuable minerals (except for iron), phytochemicals with antioxidant properties, and photosynthetic pigments (except for carotenoids at higher concentrations) than the control plants.

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