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Influence of Zinc Oxide Nanoparticles in In Vitro Culture and Bacteria *Bacillus thuringiensis* in Ex Vitro Conditions on the Growth and Development of Blackberry (*Rubus fruticosus* L.)

Anna Krzepiłko [1], Roman Prażak [2,*] and Katarzyna Matyszczuk [1]

[1] Department of Biotechnology, Microbiology and Human Nutrition, Faculty of Food Sciences and Biotechnology, University of Life Sciences in Lublin, St. Skromna 8, 20-704 Lublin, Poland; anna.krzepilko@up.lublin.pl (A.K.); katarzyna.matyszczuk@up.lublin.pl (K.M.)
[2] Institute of Plant Genetics, Breeding and Biotechnology, University of Life Sciences in Lublin, St. Akademicka 15, 20-950 Lublin, Poland
* Correspondence: roman.prazak@up.lublin.pl

Abstract: The blackberry, valued for its delicious fruit, has gained attention for its medicinal bioactive compounds. In vitro cultivation methods, including nanoparticle enhancement, are increasingly chosen due to their advantages over traditional propagation techniques. We tested the effect of commercial zinc oxide nanoparticles (ZnONPs) on the growth and development of blackberry (*Rubus fruticosus* L.) of the Navaho variety in an in vitro culture on MS medium supplemented with 0.6 mg dm^{-3} BA, 0.1 mg dm^{-3} IBA, 0.01 mg dm^{-3} GA_3, and various concentrations of zinc oxide nanoparticles: 0 (control), 10, 20, 30, and 40 mg dm^{-3}. The morphological features of the plantlets were assessed two and three months after the start of the culture. Selected biological characteristics of the plantlets were determined. The values of the morphological and biological parameters assessed in the plantlets from in vitro culture depended on the concentration of ZnONPs in the medium. Increasing the concentration of ZnONPs negatively affected the number and length of shoots and roots and the fresh weight of the plantlets. The total phenolic content in the plantlets from the treatments with ZnONPs was lower than in the control plants, but the total antioxidant capacity as measured by the ABTS method was higher. The content of chlorophyll *a*, chlorophyll *b*, total chlorophyll, and carotenoids in the blackberry plantlets decreased at higher concentrations of ZnONPs in the medium. The addition of zinc oxide nanoparticles increased the zinc content and reduced the iron content in the blackberry plantlets. Concentrations of 10–30 mg dm^{-3} ZnONPs increased the concentrations of potassium, calcium, magnesium, zinc, manganese, and copper, while at the highest concentration of 40 mg dm^{-3} ZnONPs, the concentrations of these minerals were similar to the control, except for a lower content of calcium and manganese. The plantlets from the in vitro culture growing in the presence of ZnONPs were acclimatized to ex vitro conditions in control soil and soil inoculated with *Bacillus thuringiensis*. Bacteria added to the ex vitro substrate favourably influenced the growth and development of the shoots and roots of the blackberry plants and their fresh weight.

Keywords: ZnONPs; blackberry; PGPB; phenols; antioxidants; photosynthetic pigments

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1. Introduction

Blackberry plants are widespread in natural habitats in the northern hemisphere. Blackberry fruits are valued for their flavour and aroma and are rich in bioactive substances and minerals. They supply large amounts of vitamins, organic acids, polyphenols, cyanidin, aromatic compounds, pectin, minerals, sugars, and dietary fibre [1]. In medicine and in pharmacy, blackberry products have anti-tumour, anti-sclerotic, anti-inflammatory, antidiabetic, and anti-allergic applications, and also help to reduce body weight [2]. Earlier research showed that blackberry and tayberry buds have valuable biological properties,

are rich in antioxidant compounds, and exhibit antibacterial activity [3]. Blackberry can easily be propagated by tip layering. This is a useful method because it does not impair the plants' ability to bear fruit. The shoot tip buried in soil quickly takes root and becomes a new shrub [4]. Species of the genus *Rubus* are highly heterozygous, and high plant variation is obtained from seed propagation [5,6]. Blackberry propagation using in vitro techniques can have a number of benefits; for example, it reduces the time needed to obtain plantlets, eliminates limitations associated with seasonality, offers greater control over growth conditions, facilitates disease prevention, and requires less plant material for explanting, which is especially important in the propagation of endangered species [7,8]. The propagation of blackberry in vitro is carried out on media supplemented with various concentrations of cytokinins and auxins [5,6,9–11]. Various growth-promoting agents are also added to the media, such as silicon, Fe-EDTA, and alternative gelling agents to agar, such as starch [12–14]. Many studies have also investigated the use of nanotechnology in plant biotechnology. Nanoparticles can have a positive impact on callus induction, embryogenesis, disinfection of plants, and metabolite production [15,16].

Zinc oxide nanoparticles are used as a fertilizer supplying this important micronutrient to plants and as an antifungal and antibacterial agent [16,17]. Zinc plays a key role in photosynthesis, carbohydrate metabolism, energy acquisition processes, biosynthesis of proteins, nitrogen metabolism, and calcium and phosphorus balance in plant cells. Key enzymes for metabolism, such as RNA polymerases, superoxide dismutase, and carbonic anhydrase, contain zinc [18]. Zinc is involved in DNA/RNA metabolism, is part of the structure of transcription factors (zinc fingers), and ensures the stability and normal functioning of chromatin [19]. It also influences the production of plant growth hormones, as it is needed for the biosynthesis of tryptophan, a precursor of indole-3-acetic acid [20]. Zn deficiency causes impairment of numerous physiological functions of plant cells and leads to restriction in plant growth. Optimal zinc content in the plant means better cell membrane permeability and increased tolerance for salinity, drought, excessive levels of heavy metals in the soil, and pathogens [21].

An important stage in the in vitro culture of plants is the rooting of plantlets, which is carried out by two basic methods: in vitro and ex vitro [22]. Plantlets with the beginnings of roots obtained in vitro and planted in soil root better in the presence of PGPRs (plant growth promoting rhizobacteria) [23,24]. Bacteria of the genus *Bacillus*, as rhizosphere bacteria, play an important ecological role by inhibiting the development of plant pathogens, supporting plant growth, and mitigating the consequences of environmental stress for plants [24]. These are Gram-positive bacteria which are widespread in natural environments, especially in soil and on plants. They are mainly saprophytes, which break down organic compounds and thereby contribute to the circulation of nutrients. Bacteria of the species *Bacillus thuringiensis* are chemo-organo-heterotrophic Gram-positive bacilli which are able to form endospores, allowing them to survive in adverse environmental conditions [24]. Previous research has demonstrated that *B. thuringiensis* is capable of growth in the presence of zinc oxide nanoparticles [25]. The MIC (minimum inhibitory concentration) value determined was higher than 1.6 mg cm^{-3} and the MBC (minimum bactericidal concentration) was higher than 1.8 mg cm^{-3} for *Bacillus thuringiensis*. The increased concentration of ZnO particles may have induced oxidative stress in *B. thuringiensis*, inhibited planktonic cell growth, stimulated endospore formation, and reduced biofilm formation. However, concentrations of ZnONPs ranging from 0.2 to 0.8 mg cm^{-3} enhance the production of IAA by *B. thuringiensis*, which is crucial for improving plant growth [25].

The aim of this study was to test the effect of zinc oxide nanocolloids (ZnONPs) on the quantitative and qualitative traits of blackberry in in vitro culture. Plant material collected after three months of cultivation was analysed for selected metals (calcium, potassium, magnesium, iron, zinc, copper, and manganese), chlorophyll a and b, and total chlorophyll, carotenoids, total phenolic content (TPC), and total antioxidant capacity (TAC). To improve the acclimatization of plants to soil conditions, this study also included assessment of the

effect of *B. thuringiensis* on the rooting of blackberry plantlets obtained from tissue cultures in which they were exposed to zinc oxide nanocolloids.

2. Materials and Methods

2.1. Plant Material

The subject of this study was blackberry (*Rubus fruticosus* L.) of the Navaho variety. The plants were obtained from the collection of the Institute of Plant Genetics, Breeding and Biotechnology, University of Life Sciences in Lublin.

Zinc Oxide Nanoparticles

Sigma Aldrich ZnO nanoparticles (catalogue no. 721077) were <100 nm in size as measured by dynamic light scatting (DLS) with an average particle size of <35 nm measured using an aerodynamic particle sizer (APS). They were synthesized by hydrolysis of a zinc salt in a polyol medium heated to 160 °C. Their zeta potential was +46.1 ± 1.5 mV [26].

2.2. In Vitro Culture of Blackberry Plantlets

The experiment was set up using fragments of blackberry plants obtained from in vitro culture, conducted in the laboratory of the Institute of Plant Genetics, Breeding and Biotechnology, the University of Life Sciences in Lublin. Explants of shoot tips of the Navaho variety of blackberry (*Rubus fruticosus* L.) with 3–5 leaves were placed (5 per culture jar) on MS medium (Murashige, Skoog 1962). The 200 mL jars, with heat-resistant Magenta B-caps, each contained 20 mL of medium. Media with pH 5.7 were autoclaved under a pressure of 1 atm at 121 °C for 20 min. Plant growth hormones [9], i.e., 0.6 mg dm^{-3} BA (6-benzyladenine), 0.1 mg dm^{-3} IBA (indole-3-butyric acid), and 0.01 mg dm^{-3} GA_3 (gibberellic acid), as well as various concentrations of zinc oxide nanoparticles, i.e., 0 (control), 10, 20, 30, and 40 mg dm^{-3}, were added to the media. There were 75 explants used in each treatment, and the entire experiment was carried out in triplicate. After two months, the blackberry plantlets were removed from the culture jars and subcultured on MS medium with the same composition as before and various concentrations of zinc oxide nanoparticles—0 (control), 10, 20, 30, and 40 mg dm^{-3}, but with the addition of 0.5 mg dm^{-3} of activated carbon to stimulate root formation. The in vitro blackberry cultures were carried out in a phytotron at a light intensity of 54 µmol photons·m^{-2}·s^{-1}, temperature 23 ± 1 °C, and a 16/8 photoperiod.

2.3. Assessment of the Growth Rate of Blackberry Plantlets from In Vitro Culture

Two and three months after the start of the culture, measurements were taken of the number of shoots and roots of the plantlets, shoot and root length (cm), and fresh weight (g) [7].

2.4. Determination of Selected Minerals in Blackberry Plantlets from In Vitro Culture

After two months of in vitro culture, plantlets were collected and analysed for their content of minerals by absorption spectroscopy according to PN-EN ISO 6869:2002 [27]. The 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 content of calcium (Ca), potassium (K), sodium (Na), magnesium (Mg), zinc (Zn), iron (Fe), copper (Cu), and manganese (Mn). Mineral content was expressed in mg 100 g^{-1} DW.

2.5. Preparation of Extracts from Blackberry Plantlets Grown In Vitro

After two months of in vitro culture, plantlets were collected, rinsed, dried on filter paper, and then mixed with extraction solution in a weight ratio of 1:10. The plant material was crushed using a homogenizer, and the samples were cooled in a water bath for 5 min. The homogenate was mixed for 24 h in a laboratory shaker at 60 rpm at 22 °C and then centrifuged for 5 min at 5000 rpm [3]. Methanol–water extracts (70%v/v) were

prepared for determination of total phenolic content, water extracts for determination of TAC by the ABTS method, and acetone–water extracts (80%v/v) for measurement of photosynthetic pigments.

2.6. Determination of Total Phenolic Content

The Folin–Ciocalteu method used to determine the total content of polyphenols is based on the capacity of these compounds to form coloured products of reactions with substances contained in Folin reagent (salts of phosphomolybdic and phosphotungstic acids). The absorbance measured after one hour at 765 nm is proportional to the total content of phenolic compounds in the sample [28]. The results are expressed in gallic acid equivalents (GAE) per g of fresh weight of plant material.

2.7. Determination of Total Antioxidant Capacity

Total antioxidant capacity (TAC) was assessed in the extracts using the ABTS method. The method involves spectrophotometric measurement of the colour reduction reaction between $ABTS^{\cdot+}$ cation radical and the antioxidants present in the sample—both hydrophilic and lipophilic. The spectrophotometric measurement was made at 414 nm, 30 min after the reagents were mixed [29]. Total antioxidant capacity was expressed as Trolox equivalent per g of fresh weight of plant material (μM TE g^{-1} FW).

2.8. Measurement of Photosynthetic Pigment Levels

Chlorophyll levels in the plantlets were assessed using spectrophotometry after extraction with 80% acetone [30]. Absorbance readings were taken at 645 nm, 663 nm, and 470 nm. The quantities of chlorophyll and carotenoids were reported in mg g^{-1} FW.

2.9. Preparation of a Suspension of Bacillus thuringiensis Bacteria for Inoculation of Blackberry Plantlets in Ex Vitro Cultivation

Bacillus thuringiensis strain PCM 1853 was purchased from the Polish Collection of Microorganisms (PCM), Wroclaw, Poland, and registered in the World Federation for Culture Collections (WFCC, no. 106) and the European Culture Collections' Organisation (ECCO). The PCM has the status of an international depository authority (IDA) for patent purposes. This strain is capable of growth in the presence of ZnONPs, production of IAA, biofilm formation, and biotransformation of complex compounds [25]. The bacteria were grown in nutrient broth medium (Difco) for 24 h at 37 °C. Then, the bacterial culture was diluted with distilled water to achieve a cell concentration of 1×10^7 CFU/mL. The resulting bacterial suspension was used to water the blackberry plantlets (Bacteria+) planted in soil.

2.10. Effect of B. thuringiensis on Rooting of Ex Vitro Blackberry Plantlets in Soil

Three months after the start of the in vitro culture, blackberry plantlets were planted in pots filled with sterile soil (in an autoclave, 121 °C, 20 min). The pool of plantlets from each in vitro experimental treatment was divided in two. Immediately after planting, half of the plantlets were watered a single time with 10 mL of the suspension of *B. thuringiensis* bacteria (Bacteria+), and the other half were watered with distilled water (Bacteria−). In the initial stage of ex vitro growth, plantlets were protected with plastic lids. The rooting process took place at the same time as acclimatization in a growth room at 23 ± 2 °C, with 16 h light/day and a light intensity of 80 μmol $m^{-2}s^{-1}$. In the subsequent stages of growth, all plantlets were watered with distilled water alone.

2.11. Assessment of the Growth of Blackberry Plantlets from Ex Vitro Culture

The growth parameters of blackberry plantlets were determined after one month of ex vitro culture in soil. The shoots and roots were counted, and measurements were taken of the shoot length (cm), root length (cm), and fresh weight of plants (g).

2.12. Statistical Analysis

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

3. Results

3.1. Effect of ZnONPs on In Vitro Blackberry Growth

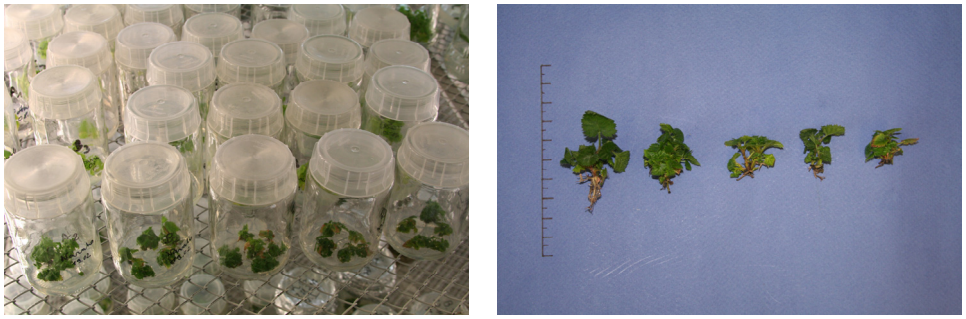
The results of the experiments show that the Navaho variety of blackberry (*Rubus fruticosus* L.) can be successfully micropropagated on MS medium supplemented with 0.6 mg dm^{-3} BA, 0.1 mg dm^{-3} IBA, and 0.01 mg dm^{-3} GA_3. The control plantlets obtained after two months had formed an average of more than four shoots of more than 2 cm in length. After three months of culture, the control plantlets had produced the longest shoots (Table 1). The addition of ZnONPs to the medium did not induce the formation of shoots from explants of Navaho blackberry. In general, the average number and length of shoots obtained during the growth of plants in a medium with zinc oxide nanoparticles were lower than in the control. This was observed in the plants grown in vitro for two months and for three months. Only the plants growing on medium supplemented with 10 mg dm^{-3} ZnONPs had more shoots than the control plants, but the values were not statistically significant. Increasing the concentration of ZnONPs in the medium caused the blackberry plantlets to produce fewer shoots on average, and in the treatment with 40 mg dm^{-3} ZnONPs, the number of shoots was statistically significantly lower than in the control (Table 1).

Table 1. Influence of increased content of ZnO-NPs in MS solid medium on biometric features of blackberry (*Rubus fruticosus* L.), Navaho variety (mean value of feature/explant).

Biometric Feature	Number of Months	Concentration of ZnO-NPs [mg dm^{-3}]					
		0 (Control)	10	20	30	40	$LSD_{p=0.05}$
Number of shoots	2	4.67 ^{a}	4.75 ^{a}	4.46 ^{a}	4.18 ^{a}	2.93 ^{b}	1.645
	3	6.08 ^{a}	6.10 ^{a}	5.54 ^{a}	5.11 ^{a}	3.92 ^{b}	2.056
Shoot length [cm]	2	2.14 ^{a}	1.91 ^{a}	1.78 ^{a}	1.38 ^{b}	0.98 ^{b}	0.679
	3	2.52 ^{a}	2.34 ^{a}	2.21 ^{a}	1.94 ^{b}	1.85 ^{b}	0.454
Number of roots	2	0.85 ^{a}	0.74 ^{a}	0.63 ^{a}	0.52 ^{a}	0.00 ^{a}	ns
	3	3.77 ^{a}	2.82 ^{a}	2.61 ^{a}	1.72 ^{b}	0.92 ^{b}	2.701
Root length [cm]	2	0.15 ^{a}	0.11 ^{a}	0.10 ^{a}	0.09 ^{a}	0.00 ^{a}	ns
	3	1.67 ^{a}	0.55 ^{b}	0.54 ^{b}	0.53 ^{b}	0.26 ^{b}	0.506
Fresh weight of plant [g]	2	0.41 ^{a}	0.34 ^{a}	0.26 ^{a}	0.24 ^{b}	0.14 ^{b}	0.172
	3	0.59 ^{a}	0.38 ^{a}	0.35 ^{a}	0.31 ^{b}	0.20 ^{b}	0.241
Increase in fresh weight of plant [g]	2	0.36 ^{a}	0.29 ^{a}	0.20 ^{a}	0.19 ^{a}	0.09 ^{b}	0.165
	3	0.54 ^{a}	0.33 ^{a}	0.30 ^{a}	0.26 ^{b}	0.15 ^{b}	0.145

Different letters indicate significant differences at $p = 0.05$, ns—no significant differences.

Zinc oxide nanoparticles reduced the length of the shoots at every concentration, and in the case of 30 and 40 mg dm^{-3} ZnONPs, this parameter was significantly lower than in the control. The rooting process of the plantlets, in terms of both the number of roots and their length, was slower than the formation and growth of shoots. The best results were noted in the control (Table 1, Figure 1). After three months of in vitro culture, the highest concentrations of ZnONPs, i.e., 30 and 40 mg dm^{-3}, were shown to have a statistically significant negative effect on the number of roots. In all media supplemented with ZnONPs, the average length of Navaho blackberry roots was significantly lower than in the control. In addition, after three months of culture with ZnONPs, blackberry plantlets had statistically significantly shorter roots (Table 1, Figure 1).



(**A**) (**B**)

Figure 1. Influence of different treatments of ZnONPs in MS medium on the growth and development of blackberry (*Rubus fruticosus* L.) of the Navaho variety. (**A**) Developing explants on micropropagation medium; (**B**) regenerated plantlets (from left): 0 (control), 10, 20, 30, and 40 mg dm^{-3} ZnONPs.

After two and three months of in vitro culture, the average fresh weight of Navaho blackberry plantlets (0.41 and 0.59 g) and the increase in fresh weight (0.36 and 0.54 g) were highest in the control. The smaller number of roots and shorter shoot length and root length in the samples growing on media with zinc oxide nanoparticles reduced the weight of the plantlets. After both two and three months of culture, the fresh weight and increase in fresh weight were statistically significantly reduced in the treatments with 30 and 40 mg dm^{-3} ZnONPs, by twofold and threefold, respectively, compared to the control (Table 1).

The biological quality of the blackberry plantlets from the in vitro culture was evaluated by determining the content of selected minerals; secondary metabolites, i.e., phenolic compounds; antioxidants; and photosynthetic pigments.

3.2. Determination of Mineral Content

In the treatments with 0–40 mg dm^{-3} ZnONPs, there were significant differences in the content of minerals in the Navaho blackberry plantlets (Table 2).

Table 2. Influence of ZnO-NPs on mineral contents in plantlets of blackberry (*Rubus fruticosus* L.) of the Navaho variety after 3 months of in vitro culture.

ZnONPs [mg dm^{-3}]	Mineral Content [mg 100 g^{-1} DW]						
	K	Ca	Mg	Fe	Zn	Mn	Cu
0	1600 ^{a}	285 ^{a}	141 ^{a}	18.5 ^{a}	53.9 ^{a}	12.8 ^{a}	0.44 ^{a}
10	2150 ^{b}	322 ^{b}	152 ^{b}	17.9 ^{a}	75.0 ^{b}	15.3 ^{b}	0.61 ^{b}
20	2010 ^{c}	310 ^{b}	149 ^{b}	16.1 ^{b}	87.4 ^{c}	14.9 ^{b}	0.58 ^{b}
30	1860 ^{d}	304 ^{b}	148 ^{b}	15.8 ^{b}	90.8 ^{c}	14.6 ^{a}	0.46 ^{a}
40	1640 ^{a}	254 ^{c}	139 ^{a}	14.5 ^{c}	99.6 ^{d}	10.9 ^{c}	0.43 ^{a}
$LSD_{p = 0.05}$	74.0	16.15	4.28	0.95	5.35	2.25	0.07

Different letters indicate significant differences at $p = 0.05$.

The content of zinc in the blackberry plantlets increased with the concentration of ZnONPs in the medium, whereas increasing the concentration of zinc oxide nanoparticles in the medium decreased the content of iron in the plantlets. The plants growing on medium with 10 mg dm^{-3} ZnONPs contained the most potassium (2150 mg 100 g^{-1} DW), calcium (322 mg 100 g^{-1} DW), magnesium (152 mg 100 g^{-1} DW), manganese (15.3 mg 100 g^{-1} DW),, and copper (0.61 mg 100 g^{-1} DW) (Table 2). At concentrations of 20 and 30 mg dm^{-3} ZnONPs, the content of potassium, calcium, magnesium, manganese, and copper was also higher than in the control. At the highest concentration (40 mg dm^{-3} ZnONPs), the content of these elements was similar to the control values, except for calcium and manganese, whose content was statistically significantly lower (Table 2).

3.3. Determination of Total Phenolic Content

In the regenerated Navaho blackberry (*Rubus fruticosus* L.) plants obtained after three months of in vitro culture, the total phenolic content (TPC) in the fresh weight ranged from 2.50 mg GAE g^{-1} FW (30 mg dm^{-3} ZnONPs) to 3.47 GAE mg g^{-1} FW (control) (Figure 2). The highest total phenolic content was noted in the control. Zinc oxide nanoparticles statistically significantly reduced TPC, by 28.5–11.5%, depending on the concentration. The lowest content, 2.50 mg GAE g^{-1} FW, was noted in the plants growing on medium with 30 mg dm^{-3} ZnONPs (Figure 2).

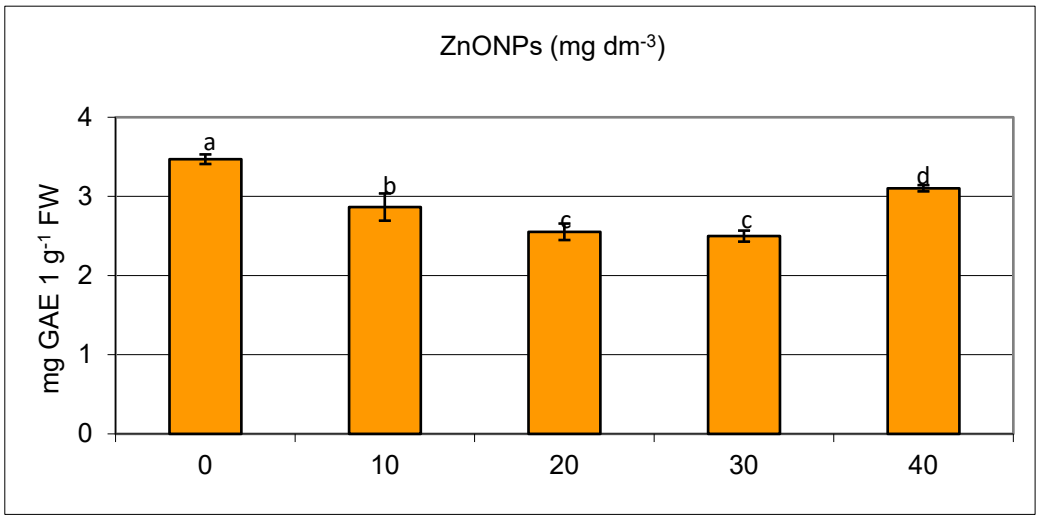


Figure 2. Effect of different concentrations of ZnONPs [mg dm^{-3}] on total phenolic content (TPC) in plantlets of blackberry (*Rubus fruticosus* L.), Navaho variety. Error bars represent ± SD. Different lowercase letters indicate significant differences at $p = 0.05$.

3.4. Determination of Total Antioxidant Capacity

The total antioxidant capacity of the blackberry plantlets after three months of culture ranged from 73.62 µM TE g^{-1} FW (control) to 82.89 µM TE g^{-1} FW (40 mg dm^{-3} ZnONPs) (Figure 3). The addition of ZnONPs increased the total antioxidant capacity of the plantlets. After three months of growth on medium with zinc oxide nanoparticles, their content of antioxidant compounds was 3.8% to 11% higher. The correlation coefficient between the concentration of ZnONPs and the TAC value was 0.978. The highest TAC value for the Navaho variety was noted in the treatment with 40 mg dm^{-3} ZnONPs, amounting to 81.89 µM TE g^{-1} FW (Figure 3).

3.5. Determination of Content of Photosynthetic Pigments

After three months of in vitro culture, the concentrations of chlorophyll *a* in the blackberry plantlets ranged from 10.26 µg g^{-1} FW (treatment with 30 mg dm^{-3} ZnONPs) to 12.83 µg g^{-1} FW (treatment with 20 mg dm^{-3} ZnONPs) (Figure 4). The content of chlorophyll *b* ranged from 17.60 µg g^{-1} FW (treatment with 40 mg dm^{-3} ZnONPs) to 20.74 µg g^{-1} FW (treatment with 10 mg dm^{-3} ZnONPs), and that of total chlorophyll from 28.25 µg g^{-1} FW (treatment with 40 mg dm^{-3} ZnONPs) to 33.05 µg g^{-1} FW (treatment with 20 mg dm^{-3} ZnONPs). The plants had the lowest content of chlorophyll *a* and total chlorophyll in the control treatment (Figure 4), and the chlorophyll *b*:*a* ratio was 1.62. Plants grown on the medium with 10 and 20 mg dm^{-3} ZnONPs had a similar content of chlorophyll *a* and *b* to the control, and the chlorophyll *b*:*a* ratio was 1.70 and 1.58, respectively (Figure 4). In these samples, there was also a statistically significant increase in the total chlorophyll concentration. Higher concentrations of zinc oxide nanoparticles, i.e., 30 and 40 mg dm^{-3}, caused a statistically significant reduction in the concentration of chlorophyll *a* and total chlorophyll, and in the treatment with 40 mg dm^{-3}, in the

content of chlorophyll *b* as well. The chlorophyll *b*:*a* ratio was highest in the treatment with 30 mg dm^{-3} ZnONPs, at 1.96, while for the highest concentration of nanoparticles it was 1.65, which was similar to the control.

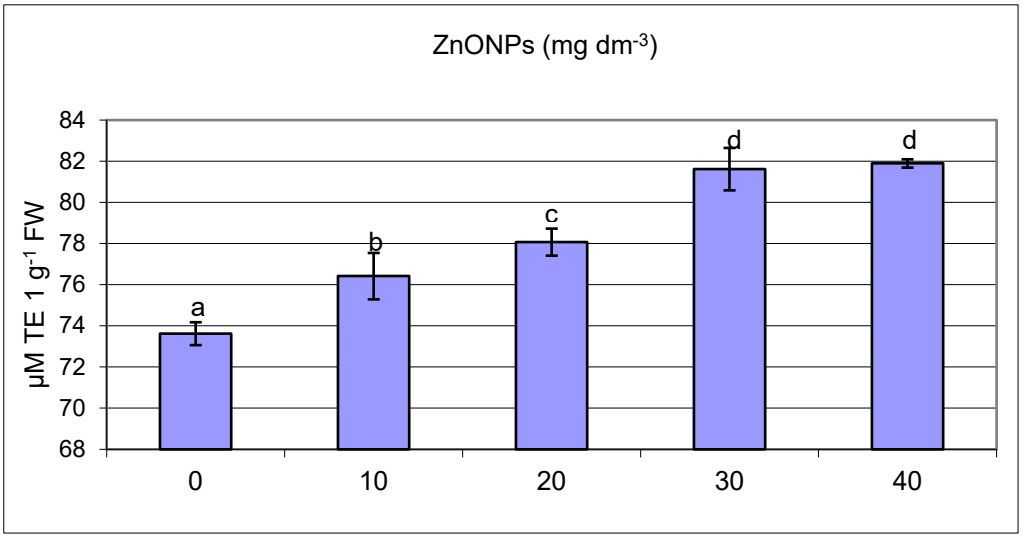


Figure 3. Effect of different concentrations of ZnONPs [mg dm^{-3}] on total antioxidant capacity (TAC) in plantlets of blackberry (*Rubus fruticosus* L.), Navaho variety. Error bars represent ± SD. Different lowercase letters indicate significant differences at $p = 0.05$.

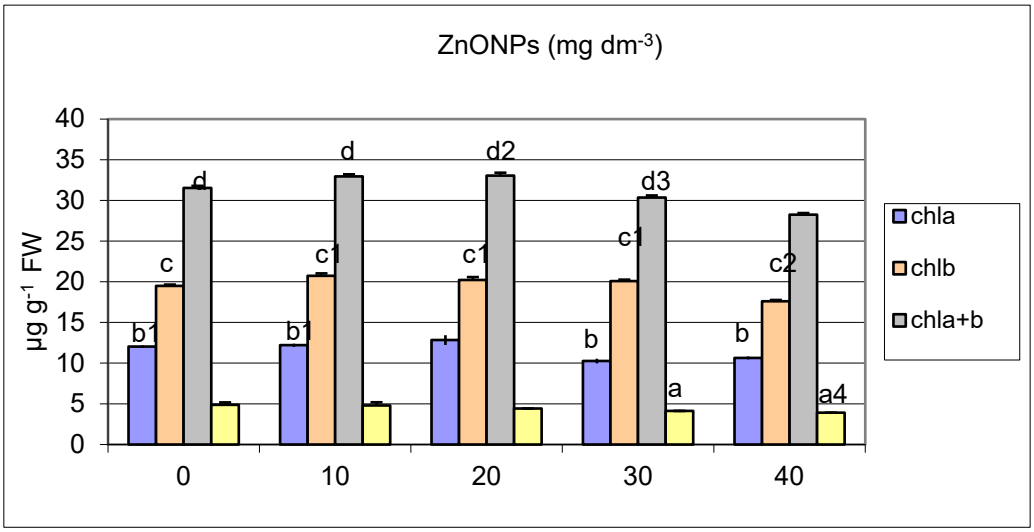


Figure 4. Effect of different concentrations of ZnONPs [mg dm^{-3}] on chlorophyll and carotenoid concentrations in plantlets of blackberry (*Rubus fruticosus* L.), Navaho variety. Error bars represent ± SD. Different lowercase letters indicate significant differences at $p = 0.05$.

Carotenoid concentrations in the blackberry plantlets ranged from 3.90 µg g^{-1} FW to 4.88 µg g^{-1} FW (Figure 4). Zinc oxide nanoparticles reduced the content of carotenoids in the blackberry plantlets; the correlation coefficient between the concentration of ZnONPs and the content of carotenoids was −0.097. Statistically significant differences in the content of carotenoids were noted for the treatments with 20–40 mg dm^{-3} ZnONPs and the control.

3.6. Effect of Bacillus thuringiensis Bacteria on the Growth and Development of Blackberry Plantlets in Ex Vitro Cultivation

After three months of in vitro culture, the blackberry plantlets were planted in soil (Figure 5). Plants from each in vitro experimental treatment were divided into two groups.

One was inoculated with bacteria (Bacteria+), while the other was not inoculated (Bacteria−). The development of the plantlets was assessed after one month of ex vitro cultivation.

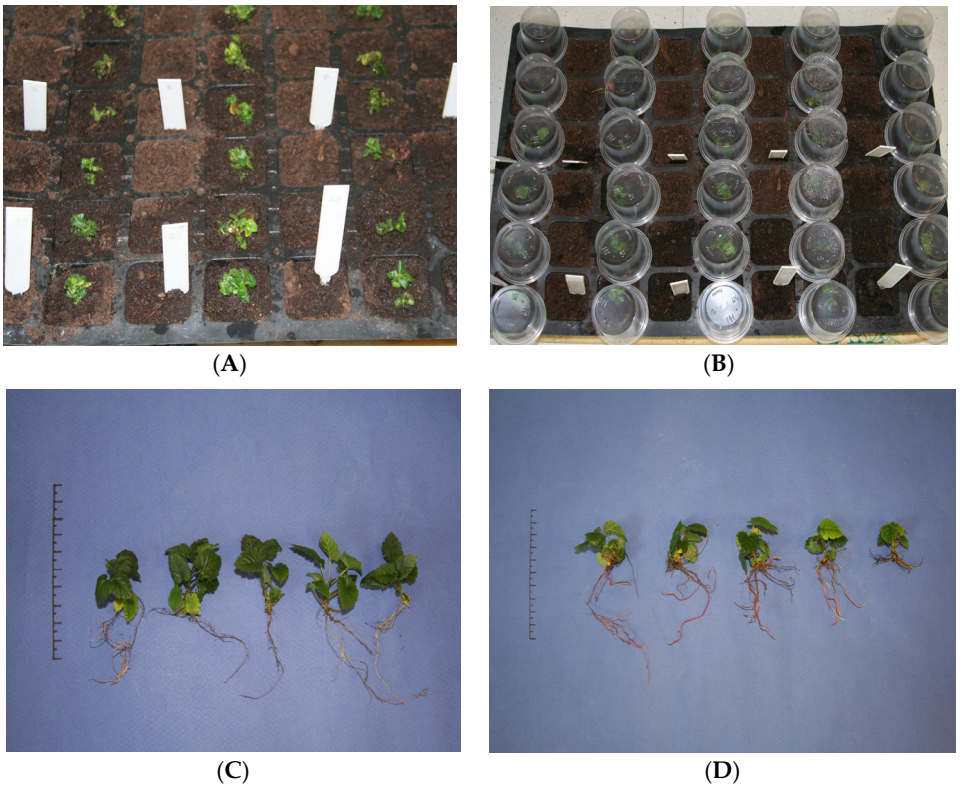


Figure 5. Influence of *Bacillus thuringiensis* on Navaho blackberry plantlets obtained in vitro on media with different concentrations of ZnONPs and growing ex vitro in subsoil. (**A**) Regenerated plantlets in micro-pots filled with sterile soil; (**B**) regenerated plantlets covered with plastic lids; (**C**) regenerated plantlets on Bacteria+ subsoil (with added *Bacillus thuringiensis* inoculum with (from left): 0, 10, 20, 30, and 40 mg dm^{-3} ZnONPs; (**D**) regenerated plantlets on Bacteria− subsoil with (from left): 0, 10, 20, 30, and 40 mg dm^{-3} ZnONPs.

Plantlets from the in vitro control were generally larger than plantlets from the treatments with ZnONPs. In comparison with the control, the shoot number and length were statistically significantly lower for the plantlets from the treatments with 30 and 40 mg dm^{-3} ZnONPs, and the root number and length were significantly lower for 10–40 mg dm^{-3} ZnONPs (for both Bacteria+ and Bacteria–). Inoculation of the substrate with *B. thuringiensis* promoted the development of all plantlets obtained in vitro. Shoot number and length, root number and length, and fresh weight were higher in the Bacteria+ samples than in the Bacteria– treatment (Table 3). Plantlets from the Bacteria– treatment with 40 mg dm^{-3} ZnONPs had the lowest values for these parameters. However, the Bacteria+ 40 mg dm^{-3} ZnONPs plantlets had about 46% more shoots, 42% longer roots, and about 32% higher fresh weight than the plants from the 40 mg dm^{-3} ZnONPs Bacteria–treatment.

The highest number of shoots (8.18/explant) were formed by the plantlets obtained from in vitro culture with 10 mg dm^{-3} ZnONPs on a substrate treated with *Bacillus thuringiensis* bacteria. Plantlets from this treatment also had similar shoot length and root length to the controls (Table 3).

Table 3. Influence of *Bacillus thuringiensis* on biometric features of blackberry (*Rubus fruticosus* L.), Navaho variety plantlets obtained in vitro on media with ZnONPs after 1 month of ex vitro culture in subsoil (mean value of feature/explant).

Biometrical Feature	Subsoil without or with Added Bacteria (−/+)	Plantlets from In Vitro Culture in Nutrient Medium Supplemented with ZnONPs [mg dm^{-3}]					
		0 (Control)	10	20	30	40	LSD p = 0.05
Number of shoots	−	6.67 ^{a}	7.23 ^{a}	5.46 ^{b}	5.18 ^{b}	2.93 ^{c}	1.342
	+	7.25 ^{a}	8.18 ^{a}	7.10 ^{a}	6.40 ^{b}	5.40 ^{b}	1.580
Shoot length [cm]	−	3.14 ^{a}	2.91 ^{a}	2.48 ^{b}	1.95 ^{c}	1.80 ^{c}	0.472
	+	3.25 ^{a}	3.22 ^{a}	2.96 ^{a}	2.44 ^{b}	2.03 ^{c}	0.332
Number of roots	−	9.85 ^{a}	6.24 ^{c}	5.63 ^{c}	4.52 ^{d}	2.89 ^{e}	1.982
	+	10.33 ^{a}	8.51 ^{b}	7.34 ^{b}	5.75 ^{c}	3.50 ^{d}	2.328
Root length [cm]	−	8.65 ^{a}	7.11 ^{b}	7.10 ^{b}	4.19 ^{c}	3.30 ^{d}	2.319
	+	9.78 ^{a}	9.34 ^{a}	8.38 ^{a}	6.21 ^{b}	5.63 ^{c}	3.214
Fresh weight of plant [g]	−	0.61 ^{a}	0.51 ^{b}	0.49 ^{b}	0.36 ^{c}	0.34 ^{c}	0.214
	+	0.74 ^{a}	0.58 ^{b}	0.54 ^{b}	0.52 ^{b}	0.50 ^{b}	0.343

Different lowercase letters indicate significant differences at p = 0.05.

4. Discussion

The topic of the micropropagation of blackberry has been taken up by a number of researchers [5,6,9–11]. In the present study, the direct organogenesis of the Navaho cultivar of blackberry (*Rubus fruticosus* L.) from shoot tips and the use of MS medium supplemented with 0.5 mg dm^{-3} IBA and 0.01 mg dm^{-3} GA_3 enabled the efficient micropropagation of this plant, and the addition of activated carbon in the amount of 0.5 g dm^{-3} stimulated root formation. Other authors have also generally conducted in vitro cultures of blackberry from shoots on MS media supplemented with cytokinins and auxins. BA concentrations proposed by other authors as optimal for the micropropagation of blackberry are 1.0 mg dm^{-3} BA [8,11], 0.6 mg dm^{-3} BA [7], 0.5 mg dm^{-3} BA [6], and 0.3 mg dm^{-3} BA [13].

Navaho blackberry plantlets obtained in the control had on average 4.67 shoots per explant with a length of about 2 cm after two months of in vitro culture, and 7.25 shoots per explant with a length of over 3 cm after three months (Table 1). The growth parameters of *Rubus fruticosus* plantlets growing on the same medium, such as shoot number and length or root number and length, may be cultivar traits, as shown for the 'Čačanska bestrna', 'Chester Thornless', 'Loch Ness', and 'Navaho' blackberry varieties [13].

Various factors are used to stimulate the micropropagation of blackberry. Apart from changes in the type and concentration of plant hormones, silicon and Fe-EDTA are added to the medium. Media other than classic MS are also used, such as starch medium [12–14]. To the best of our knowledge, the effect of ZnONPs on the micropropagation of blackberry in in vitro culture has not yet been investigated. Other authors have used ZnONPs in in vitro culture to improve the effectiveness of propagation of other plant species, including *Phoenix dactylifera* L., *Coffea arabica* L., *Solanum lycopersicum* MILL, and *Panicum virgatum* L. [31–34]. In the present study, four concentrations of zinc oxide nanoparticles were used to assess their effect on the growth and selected biological quality characteristics of blackberry plantlets in in vitro culture. Many studies have confirmed that ZnONPs can stimulate plant growth at various stages of development, supplying plants with zinc, an important nutrient. Zinc regulates the growth and development of plants by taking part in carbohydrate metabolism, biosynthesis of proteins, respiration, DNA/RNA metabolism, and photosynthesis [19,35]. Zinc contributes to the production of growth substances—auxins, and a lack of zinc disturbs the balance between plant hormones—auxins and cytokinins, and cytokinins and abscisic acid [20].

Zinc oxide nanoparticles stimulate the germination of seeds of various plants and improve seedling vigour and growth in plants such as maize, onion, and mung bean [36–38]. They also increase the content of microelements, protein, and phenols in mung bean shoots [38]. The addition of zinc oxide nanoparticles to the medium in in vitro culture can positively influence callus development, rooting, and somatic embryogenesis in various plant species [31,33,34]. In the present study, the addition of zinc oxide nanoparticles reduced the number of shoots formed, except for the concentration of 10 mg dm^{-3} ZnONPs. The shoot and root growth of the blackberry plantlets on media supplemented with nanoparticles was also poorer, resulting in a smaller increase in the fresh weight of the plants (Table 1). Statistically significant differences were shown most often at the highest concentrations of ZnONPs (30–40 mg dm^{-3}). The reduction in the growth parameters of regenerated plants at higher concentrations of ZnONPs (Table 1, Figure 1) may be associated with the mechanism of action of metallic nanoparticles on plant cells. At the cellular level, they induce the formation of free radicals which interact with virtually all cellular mechanisms, leading to protein modifications, lipid peroxidation, and DNA damage, ultimately resulting in necrosis or cell death [32,33]. ZnONPs can affect the functions of the cell wall and membrane of plant cells and restrict the electron transport chain in mitochondria and chloroplasts, leading to oxidative bursts, increased ROS concentrations, and cell death due to elevated levels of reactive oxygen species [33].

Zinc oxide nanoparticles had a smaller effect on shoot regeneration than on root growth and development in blackberry plantlets (Table 1). Zinc is essential for hormonal balance in plants, especially auxin (IAA) activity [39]. After three months of culture, plantlets growing on the medium with the highest concentration of ZnONPs had about 1.5 fewer shoots and were about 1.4 times shorter, while they produced about one fourth as many roots, which were 6.5 times shorter than in the control plantlets. The effect of ZnONPs on the regeneration of plants in in vitro culture depends on multiple factors, mainly the species of plant, the type of explant used, and the concentration and type of nanoparticles. In a culture of *Musa paradisiacal* using shoot tips as explants, ZnONPs also stimulated callus formation, shoot and root regeneration, and the amount of biomass produced [40]. In an in vitro culture of *Solanum lycopersicum*, in which cotyledons of plants were used as explants, regeneration of plants in media with ZnONPs (15 mg dm^3) was rapid, and the plantlets also reacted better to salt stress [31]. In an in vitro culture of *Brassica nigra*, ZnONPs (1 mg dm^3) reduced the fresh weight of the callus [41]. On the other hand, regeneration of *Brassica napus* cv. Hayola from the hypocotyl in the presence of ZnONPs (10 mg/L) increased the amount of callus produced [42]. Javed et al. [15] found that the regeneration of stevia plants was positively influenced only by concentrations of 0.1 and 1 mg dm^{-3} ZnONPs, while higher concentrations caused various toxic effects.

The addition of ZnONPs to the in vitro medium affected the mineral composition of regenerated blackberry plants (Table 1). Plantlets from the treatments with 10–30 mg dm^{-3} ZnONPs contained significantly more potassium, calcium, zinc, manganese, and copper than the control, while the iron concentration was lower in all cultures with ZnONPs.

The increase in the concentration of these elements (Table 1) can be explained by the fact that ZnO nanoparticles promote the production of organic acids secreted by the roots, mainly oxalic, citric, malic, and fumaric acids, which facilitate the absorption of nutrients by plants [43]. ZnONPs can also create new pores in cell walls, increasing access to water and nutrients and promoting uptake of minerals [43].

Plants from the treatments with ZnONPs had lower iron content in comparison to the control (Table 1). Zinc disrupts iron uptake by roots and its translocation to other parts of plants. An antagonistic relationship between Fe and Zn has been noted in many plants, while complex relationships in the uptake and distribution of these mineral nutrients have been demonstrated in others [44]. The Zn concentration affects Fe uptake, and excessive Zn has been shown to result in physiological Fe deficiency. Due to their chemical similarity, bivalent cations of Fe and Zn can interact with transport channel proteins, including iron-regulated transporter 1 (IRT1) [44]. IRT1 has a wide range of substrates among divalent

metals, including zinc. In *Arabidopsis* grown in excess Zn, expression of IRT1 in the root is reduced, leading to decreased iron accumulation [44].

Comparison of the mineral composition of the plants obtained in the present study (Table 2) with the literature data revealed that the plantlets obtained from the in vitro culture in the control treatment had about 27 times as much zinc as mature leaves from blackberry shrubs grown in the ground (2.01 mg Zn 100 g^{-1} DW, according to [45]), while plantlets from the treatment with the highest concentration of nanoparticles contained about 50 times as much zinc as mature blackberry fruits from that study. The control plantlets also contained 29 times as much zinc as Navaho blackberry buds grown in the ground (1.85 mg 100 g^{-1} DW zinc, according to [3]). The control plantlets from in vitro culture also contained more iron and manganese, but less potassium, calcium, magnesium, and copper than blackberry leaves in the study cited [45]. The addition of ZnONPs to the medium reduced the content of iron (Table 2), but its lowest concentration, obtained in the treatment with 40 mg dm^{-3} ZnONPs, was higher (40.5 mg 100 g^{-1} DW) than in the buds (11.77 mg 100 g^{-1} DW [3]) and leaves of blackberry grown in the ground (6.16 mg 100 g^{-1} DW [45]). Given that zinc and iron are among the main deficient minerals in the human diet, plants grown in vitro could be used to produce dietary supplements, especially for vegetarians [46]. The blackberry plantlets obtained from a medium with 10 mg dm^{-3} ZnONPs had very high content of zinc and iron, and at the same time had good growth parameters (Tables 1 and 2).

Zinc oxide nanoparticles can affect plants not only as a nutrient, but also as elicitors modifying the metabolic reactions of plants. Owing to their nano-size, they can be absorbed into cell walls, and their physicochemical effects can influence the functions of the cell wall and cell membrane, the ultrastructure of the organelles, and the cell metabolism of plants [47]. Nanoparticles can induce the formation of pores in the cell wall structure, which allow them to enter plant cells [48], where they can generate reactive oxygen species and cause oxidative stress in cells [47]. In stress conditions, ROS play a key role in regulating the secondary metabolism of plants, which leads to increased production and storage of secondary metabolites [49].

The Navaho blackberry plantlets from the in vitro culture with the addition of zinc oxide nanoparticles had a reduced content of phenolic compounds, which are among the most important plant secondary metabolites (Figure 2). On the other hand, they were shown to have a positive effect on the content of antioxidants measured by the ABTS method (Figure 3). The literature data confirm that antioxidant capacity is closely correlated with the content of phenolic compounds [3,15,31]. A high positive correlation has been obtained between the TP content and TAC of blackberry bud extracts [3]. However, the antioxidant activity of plant extracts is believed not to be determined by the presence of polyphenols alone. Other compounds with antioxidant properties include antioxidant enzymes, ascorbic acid, glutathione, carotenoids, and salicylic acid. These antioxidant compounds work together to form a comprehensive defence system in plants, helping them survive various environmental stressors [41,50]. The role of ZnONPs as an elicitor of plant metabolites in in vitro culture is not yet well understood. Stevia plants from in vitro culture treated with 100 mg dm^{-3} ZnONPs had the highest total phenolic content (TPC), total flavonoid content (TFC), and total antioxidant capacity (TAC) [15]. The callus of *Linum usitatissimum* cv. Barbara growing on a medium with ZnONPs was shown to have increased concentrations of phenolic compounds and flavonoids, and a concentration of 10 mg dm^{-3} increased the content of lignans and neolignans in the callus [48]. In a cell suspension of *Nicotiana tabacum*, ZnONPs reduced the content of phenolic compounds [50], whereas they increased nicotine content in the callus of the related species, *Nicotiana benthamiana* [51]. A higher flavonoid concentration than in the control was obtained in extracts from the callus of *Echinacea purpurea* from a culture with various concentrations of ZnONPs [52].

The substantial changes in the content of phenolic compounds and other antioxidants measured as TAC (Figures 2 and 3) suggest that oxidative stress takes place in cells in the presence of ZnONPs. Other authors also confirm that ZnONPs can cause changes in

parameters such as antioxidant enzyme activity and antioxidant concentrations, as well as damage to cell components. In the callus of *Solanum lycopersicum*, ZnONPs caused an increase in the activity of the main antioxidant enzymes, superoxide dismutase and catalase [31]. In the callus of *Linum usitatissimum* cv. Barbara, these nanoparticles were shown to increase free radical production, lipid peroxidation, the concentration of 8-oxoguanin—a marker of oxidative damage in DNA, and the activity of antioxidant enzymes [53]. On the other hand, a reduction in the activity of superoxide dismutase and peroxidase and high activity of protease and caspase were noted in a culture of *Nicotiana tabacum* cells treated with ZnONPs [51]. Cucumber plantlets at lower concentrations of zinc oxide nanoparticles had oxidative stress parameters similar to the control, but at higher concentrations they had increased levels of superoxide dismutase and catalase activity in the leaves [54].

The response of plants to ZnONPs depends largely on the concentration used. Blackberry plantlets on a medium with 10 or 20 mg dm^{-3} ZnONPs had similar content of chlorophyll *a* and *b* and total chlorophyll to the controls (Figure 4). A negative effect of ZnONPs on blackberry plantlets was manifested at 30 and 40 mg dm^{-3} as a reduction in the concentration of chlorophyll *a* and total chlorophyll, and at 40 mg dm^{-3}, in chlorophyll *b* as well. Zinc oxide nanoparticles also reduced the content of carotenoids in the blackberry plantlets (Figure 4). ZnO nanoparticles—directly or through released zinc ions—enter into interactions with cells and can damage organelles, such as chloroplasts. ZnONPs have also been shown to decrease chlorophyll in mature plants: green pea [55], wheat leaves [56], and the seedlings of mung bean and chickpea [57]. In mesophyll cells of barley [47], zinc oxide nanoparticles caused a reduction in the size of chloroplasts, which had an abnormal structure and reduced photosynthetic activity. The phytotoxic effect of high concentrations of ZnONPs on chloroplast function in tomato plants was manifested as a decreased content of chlorophyll *a* and chlorophyll *b*, reduced photosynthetic efficiency, and reduced chlorophyll fluorescence parameters [58].

Micropropagated strawberry plantlets adapt well to ex vitro conditions (Table 3, Figure 5), as also confirmed by other authors [12]. In the Bacteria− treatment, only the lowest concentration of ZnONPs had no negative effect on the number and length of shoots (Table 3). However, even this concentration of zinc oxide nanoparticles inhibited root growth and the formation of fresh biomass compared to the control. The results from the Bacteria− trial clearly indicate that prolonged cultivation enhances the toxicity of nanoparticles for the plantlets, and the effect depends on the concentration of ZnONPs. The reduction in growth parameters and signs of oxidative stress induced by ZnONPs in the present study prompted us to inoculate plantlets with *B. thuringiensis*. *Bacillus thuringiensis* is mainly known for its ability to produce toxins with insecticidal properties [22–24]. It belongs to the group of PGPBs (plant growth-promoting bacteria), because it has the capacity for endophytic colonization, production of vitamins, phytohormones, and other substances supporting plant growth, and reduction in plant pathogens [19,23]. *B. thuringiensis* positively affects plant growth and development, which has been described for many crops, including soybean, field pea, lentil, and maize [59,60]. In the present study as well, *Bacillus thuringiensis* bacteria (Bacteria+) added to the substrate in which the blackberry plantlets took root favourably influenced their growth and development (Table 3, Figure 5). They formed more shoots and roots, which were of greater length, and their fresh weight was higher than that of the plantlets rooted in the substrate without bacteria (Table 3). Other studies indicate accelerated growth and development of seeds and plants inoculated with PGPRs [19,23,61,62]. PGPRs have the ability to support plant growth by carrying out processes such as nitrogen fixation, production of growth hormones, e.g., auxins, cytokinins, and gibberellins, and reduction in the concentration of ethylene. PGPRs facilitate uptake of minerals and water by the plant [23,24,59,62].

5. Conclusions

The use of nanoparticles in the in vitro culture of plants makes it possible to learn about many aspects of plant growth and development in controlled environmental con-

ditions. The present study showed that the concentration of nanoparticles should be optimized to suit the requirements of a given plant. In the experiment on micropropagation of blackberry in vitro in a medium with zinc oxide nanoparticles, the best growth and development parameters of the plantlets and good biochemical parameters, such as the content of polyphenols, antioxidants, and photosynthetic pigments, were obtained for the concentration of 10 mg dm^3 ZnONPs. Higher concentrations of ZnO nanoparticles inhibited morphogenesis and reduced biological quality parameters, except for TAC. Zinc oxide nanoparticles also acted as elicitors of metabolic processes, as they increased the concentrations of minerals in the tissues of the plantlets and the total content of antioxidants. Plantlets in which the toxic effects of ZnONPs were observed were better acclimatized to ex vitro conditions in the treatments inoculated with *B. thuringiensis*, which confirms the positive effects of bacteria on the growth and development of blackberry plants.

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