horticulturae

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Biogenic Zinc Oxide Nanoparticles Improve In Vitro Growth of Blueberries

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Abstract: Nanotechnology offers promising applications in agriculture by enhancing crop growth, yield, and antioxidant defenses. This study is the first to evaluate the effect of biogenic zinc oxide nanoparticles (ZnO-NPs) on the in vitro growth of two blueberry cultivars, Brigitta and Duke. The ZnO-NPs were synthesized biogenically using an extract of *Lemna minor* L., which is a free-floating aquatic plant, as a capping and modulating agent, and were added to the plant's growth media at different dosages (0, 2, 6, and 18 mg L^{-1}). The ZnO-NPs significantly increased the shoot number, fresh biomass, and dry weight in both cultivars without affecting shoot vitality, length, or basal callus formation. Moreover, the increases in carotenoids in both cultivars, as well as chlorophyll and and soluble proteins in the 'Brigitta' cultivar, confirm the prompted benefits and possibly evidence genotype-specific metabolic adaptations in response to ZnO-NPs. These results demonstrate that biogenic ZnO-NPs can effectively promote the in vitro growth of blueberry explants, offering improvements in micropropagation efficiency.

Keywords: *Vaccinium corymbosum* L.; in vitro propagation; plant growth stimulation; multiplication coefficient

Citation: Regni, L.; Del Buono, D.; Micheli, M.; Facchin, S.L.; Cesarini, A.; Priolo, D.; Proietti, P. Biogenic Zinc Oxide Nanoparticles Improve In Vitro Growth of Blueberries. *Horticulturae* **2024**, *10*, 1234. https://doi.org/10.3390/horticulturae10121234

Academic Editor: Sergio Ruffo Roberto

Received: 26 August 2024
Revised: 18 November 2024
Accepted: 19 November 2024
Published: 21 November 2024

1. Introduction

Nanotechnology mainly focuses on the control of materials at the nanometric scale. This results in the improvement and revolution of many sectors, as reflected in the already widespread or increasing use of nanomaterials (NMs) in fields such as computer technology, engineering, textiles, medicine, cosmetics, packaging, agriculture, and transport [1]. Nanotechnology develops organic or inorganic NMs, which include hybrids or composites. NMs are considered as such if they consist of particles less than 100 nm in size in at least one dimension (nanoparticles—NPs) [2].

Regarding agriculture, there is the idea that nanostructured materials could improve, redesign, and transform agricultural practices in the near future by strongly impacting crop productivity [2–4]. For instance, due to their effectiveness, NMs can make agrotechnologies more efficient in crop protection and lower application doses of fertilizers and other synthetic compounds compared to conventional ones still in use [5].

NMs can be obtained through physical and chemical methods. These include be top-down procedures, which start from bulk materials and progressively reduce the material size until nanometric dimensions are reached. In contrast, bottom-up strategies have the scope to progressively assemble atoms or molecules, controlling their size to obtain nanoparticles of various sizes [6]. In general, some of the most employed physical and chemical methodologies can use toxic reagents, be energy-consuming, and have a high

environmental impact [7]. These aspects represent the main drawbacks in the production of NMs [8].

As a result, there is a mounting interest in and demand for new environmentally friendly synthesis procedures for NMs. This has led to the development of green alternative strategies, such as the synthesis using biological entities and plant extracts, which has shown to be promising and is receiving increasing attention [3,9–11]. This bottom-up method, called biogenic synthesis, employs plant extracts as ligand, capping, and reducing agents to allow the biogenic synthesis of NMs [12].

Zinc oxide nanoparticles (ZnO-NPs) are particularly noteworthy in this context, as they can be efficiently obtained using plant extracts [13,14]. Some examples are the extracts obtained *Solanum surattense* Burm. f. [15], *Cnidoscolus aconitifolius* (Mill.) I.M.Johnst. [16], *Pisonia alba* Span. [11], *Brassica oleracea* L. var. botrytis [17], and coconut husk extract [18]. The NPs generated are viewed as safer and less harmful than those created from different metals and their oxides. [19].

ZnO-NPs are a subject of interest in plant science because zinc, an essential micronutrient, plays a crucial role in many different metabolic processes and controls some physiological and morphological traits of plants [8]. Furthermore, ZnO-NPs can activate antioxidant responses, as in the case of salt stress, thus serving as positive regulators to mitigate the impacts of salinity on vegetative growth and crop production [20].

The variability in the effects promoted by ZnO-NPs is influenced by factors such as the particle size and shape, the plant's developmental stage, the material application method and dosage, and the duration of exposure [3,21]. These NMs may actively stimulate plant growth at low concentrations, while at high concentrations, they can hamper or totally inhibit growth and exert phytotoxic effects [22].

For instance, high concentrations of ZnO-NPs can lead to reduced root growth in lettuce and negatively impact photosynthesis and chlorophyll fluorescence in *Cyclocarya paliurus* (Batalin) Iljinsk. [23]. Seed priming in *Triticum aestivum* L. with ZnO-NPs resulted in increased shoot and root lengths and higher fresh and dry weights of both leaf and root tissues at concentrations up to 250 ppm. In contrast, at a very high concentration of 500 ppm, these parameters were hampered by the NM's treatment [24]. In *Solanum lycopersicum* L. and *Capsicum annuum* L., ZnO-NPs positively affected growth at concentrations of 50 mg L^{-1} and 25 mg L^{-1}, in that order. However, treatments with concentrations greater than 75 mg L^{-1} have deleterious effects on their growth and metabolism [21]. In *Triticum aestivum* L., ZnO-NPs at a concentration of 80 mg L^{-1} increased plant height, seeds per inflorescence, seed mass, production, and biomass accumulation [25]. Conversely, negative effects such as increased DNA fragmentation and ROS overproduction in *Allium cepa* L. at concentrations exceeding 200 mg L^{-1} were observed [26]. In *T. aestivum* L., exposure to 100 and 200 µM of ZnO-NPs reduced photosynthetic and antioxidant activities, while elevating hydrogen peroxide levels and lipid peroxidation [27].

Regarding in vitro plant cultivation, some studies have demonstrated that NPs can positively influence seed germination, callus induction, shoot multiplication, and overall plant growth [28–31]. However, the effects of NPs on plant performance are significantly influenced by the plant genotype and the type, size, and concentration of the nanoparticles [32]. For instance, in stevia, it has been observed that elevated concentrations of ZnONPs promoted shoot proliferation. Conversely, these concentrations adversely affected shoot length, root quantity and length, and the fresh weight of the plantlets [32].

Within this framework, this research aims to evaluate the potential biostimulatory effect of biogenic ZnO-NPs on the in vitro growth of blueberry 'Brigitta' and 'Duke' cultivars.

Therefore, this study ascertained how these NPs could influence the treated explants' key physiological parameters, biomass production, and biochemical traits. This allows us to understand whether the biogenic ZnO-NPs could represent a useful tool for improving or supporting in vitro cultivation techniques.

2. Materials and Methods

2.1. Duckweed Growth Conditions and Biogenic Synthesis of ZnO-NPs

ZnO-NPs were synthesized by adopting a procedure reported in a recently published paper [4]. In particular, the synthesis used an aqueous extract derived from *Lemna minor* L. (duckweed). Full details on duckweed growth, preparation of the hydroalcoholic extract, biogenic synthesis, and characterization of ZnO-NPs are given in [3]. Briefly, the plant was grown in trays at 24 °C and 90 μmol m^{-2} s^{-1} of light intensity (photoperiod: 12/12 h light/dark), containing a nutrient solution, which was renewed every two weeks, according to [33]. For the biosynthesis of ZnO-NPs, 20 g of fresh plant tissue was harvested, washed, and dried. A total of 1 g of dried tissue was treated with 40 mL of 25% (*v*/*v*) ethanol water. Then, the suspension was maintained at 80 °C for 15 min under stirring and filtered to obtain the hydroalcoholic plant extract. Subsequently, 30 mL of this extract was added dropwise to 1.40 M $ZnSO_4$ $7H_2O$ (50 mL). The reaction mixture was maintained for 2 h at 80 °C, and the pH was slowly increased. At the end of the reaction, the precipitate was recovered by centrifugation, repeatedly washed, and then dried overnight at 80 °C. The ZnO-NPs were then obtained by calcination in a muffle at 400 °C for 4 h. The NPs showed a spherical shape and size on the order of 10–20 nm (see all characterization data in [4]).

2.2. Plant Material, ZnO-NPs Exposure, and Growth Conditions

The experiment was carried out on the blueberry cultivars 'Brigitta' and 'Duke'. 'Duke' is entirely classified as *Vaccinium corymbosum* L. and has a ripening period of 30 days with an early harvest timing, while 'Brigitta' is a hybrid made up of 96% *V. corymbosum* L. and 4% *V. angustifolium*, and has a ripening period of 60 days with a mid-season harvest timing [34].

The initial explants were represented by about 2.5 mm long shoots grown on Woody Plant Medium (WPM) [35] macro and micro-nutrients, Murashige and Skoog [36] vitamins, inositol (10 g L^{-1}), Indole-3-butyric acid (IBA) (0. 01 mg L^{-1}), zeatin (0.5 mg L^{-1}), sucrose (30 g L^{-1}), and agar (7 g L^{-1}), with a pH of 5.6. Glass vessels (500 mL capacity) were used, each containing fifteen single shoots and 100 mL of the substrate described above. The substrates and vessels were autoclaved at 115 °C for 20 min before being used under a horizontal laminar flow cabinet in aseptic conditions. In particular, four concentrations of ZnO-NPs (0, 2, 6, and 18 mg L^{-1}) were added to the above-described proliferation substrate. The solution containing ZnO-NPs was added to the substrate before autoclaving. The vessels were then placed in a growth chamber at a temperature of 22 ± 2 °C and a 16 h photoperiod with a 40 μE m^{-2} s^{-1} light intensity. For each treatment, six vessels (replicates) were used for each one of the three subsequent subcultures. At the end of each subculture (30 days), destructive measurements on the proliferated shoots of three pots were carried out and the proliferated shoots of the remaining vessels were used as the initial explants for the subsequent subcultures.

2.3. Growth Parameters

At the end of each subculture, the proliferated explants (45 for each treatment) were pulled out of the jars, taking care to remove all growth substrate residues to measure the following parameters:

viability (%): count of green and viable explants, referred to as total explants for each pot;
shoots (n): count of the number of shoots developed from each initial explant;
shoot length (mm): length of developed shoots, measured with a ruler;
callus (%): count of explants that produced basal callus, referred to as total explants for each pot;
fresh weight (mg): fresh weight per explant of leaves, shoots, and callus masses, measured with a precision balance;
total dry weight (mg): dry weight per explant of leaves, shoots, and callus masses, measured with a precision balance after drying the plant material in an oven for three days at 105 °C.

2.4. Pigment, Soluble Carbohydrate and Soluble Protein Contents

The blueberry explants (leaves and shoots) were used to determine some biochemical parameters as follows. Fresh samples were reduced to fine powder using liquid nitrogen with a mortar and pestle and stored at −80 °C. Successively, 0.25 g of samples were extracted in 2.5 mL of methanol. The methanolic extract was then centrifuged for 5 min at 20,000 rpm. The resulting supernatant was analyzed spectrophotometrically, according to Venkatachalam et al. [37], to quantify chlorophyll a (Chl a), chlorophyll b (Chl b), and carotenoid contents.

According to Al Murad and Muneer [38], the methanolic extracts were also used to determine the total soluble carbohydrates by the anthrone method. To this end, 50 µL of supernatant was diluted with 950 µL of distilled water and then added to 2.5 mL of sulfuric acid (H_2SO_4) containing 0.2% *w/v* anthrone. The solution was then incubated at 100 °C for 10 min and cooled before the spectrophotometric determination.

The Bradford method [39] was used to determine the total soluble protein, extracting 100 mg of blueberry explants in 1.0 mL of 50 mM phosphate buffer.

2.5. Statistical Analysis

The trials were organized according to a completely randomized design. The data collected were subjected to various tests to verify the variance hypotheses; in particular, the homogeneity of variance was assessed using Levene's test, and the normal distribution was assessed using the D'Agostino–Pearson omnibus normality test.

Collected data were subjected to a one-way analysis of variance (ANOVA), and significant differences were assayed by the Tukey HSD test ($p = 0.05$).

3. Results

3.1. Growth Parameters

The ZnO-NP treatment did not influence the explants' viability of 'Brigitta', which was equal to 100% in all treatments. On the contrary, the ZnO-NP treatment influenced the number of shoots produced by each initial explant. In particular, at 18 mg L^{-1}, an increase of 73.8% in shoots was observed (Figure 1). The shoot length was unaffected by the treatment and was equal to around 13.2 mm (Figure 1).

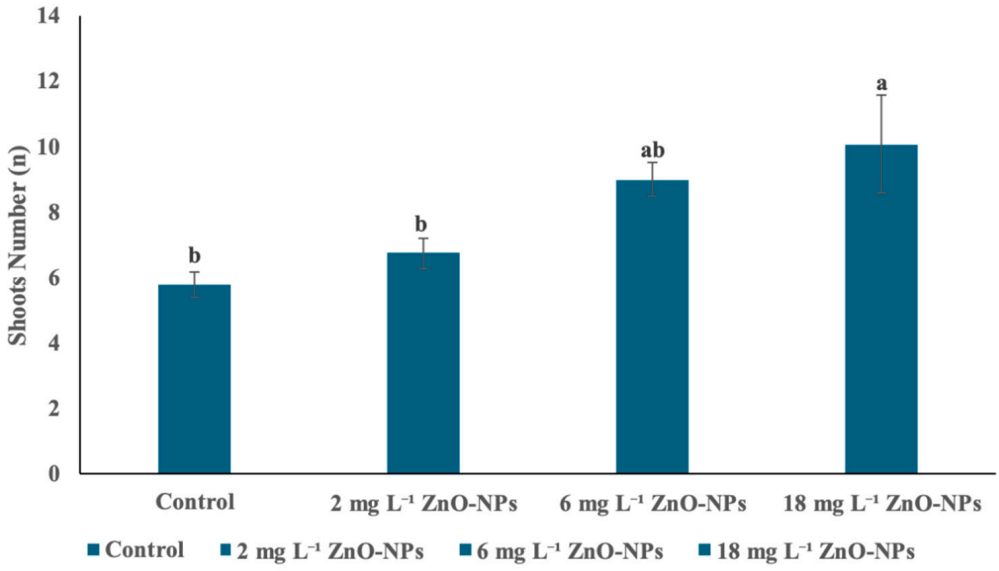

Figure 1. *Cont.*

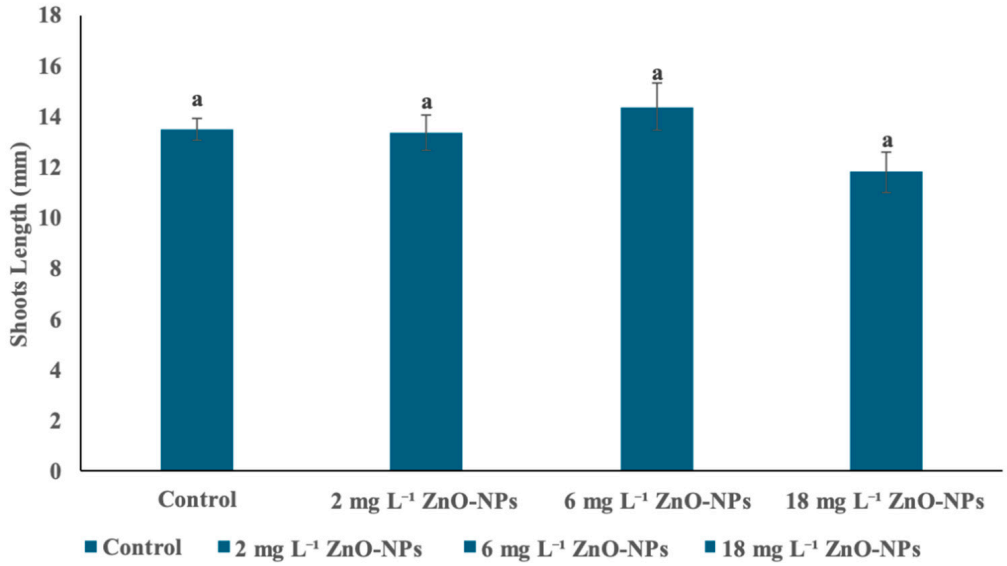

Figure 1. Shoot number and shoot length values ± SE of the Brigitta cultivar-proliferated explants. Mean values followed by different letters were significantly different ($p < 0.05$).

The treatment with ZnO-NPs did not influence the basal callus production that was present in about 35% of the explants (Figure 2).

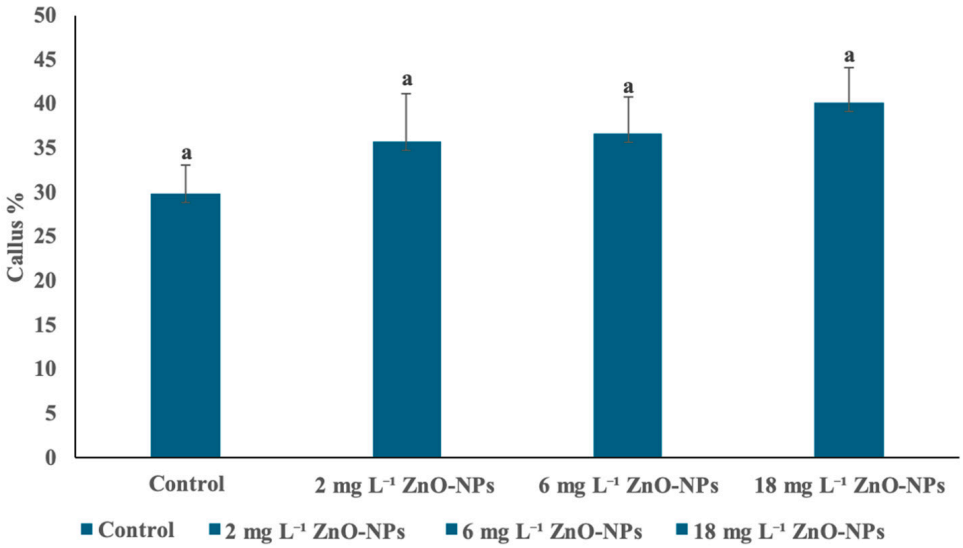

Figure 2. Incidence of explants that produced basal callus, values ± SE in the Brigitta cultivar-proliferated explants. Mean values followed by different letters were significantly different ($p < 0.05$).

At dosages of 18 mg L^{-1}, the ZnO-NPs positively influenced the fresh weight, which was higher by 57.9% than the control (Figure 3). A similar trend as that observed for the fresh weight was also found for the total dry weight, with an increase of about 56% at 18 mg L^{-1} compared to the control (Figures 3 and 4).

The ZnO-NP treatment did not influence the explants' viability, which was equal to 100% in all treatments for the Duke cultivar. The explants treated with ZnO-NPs at all concentrations exhibited a greater number of shoots compared to the control (Figure 5), while the shoot length was not influenced by the treatment with ZnO-NPs (Figure 5).

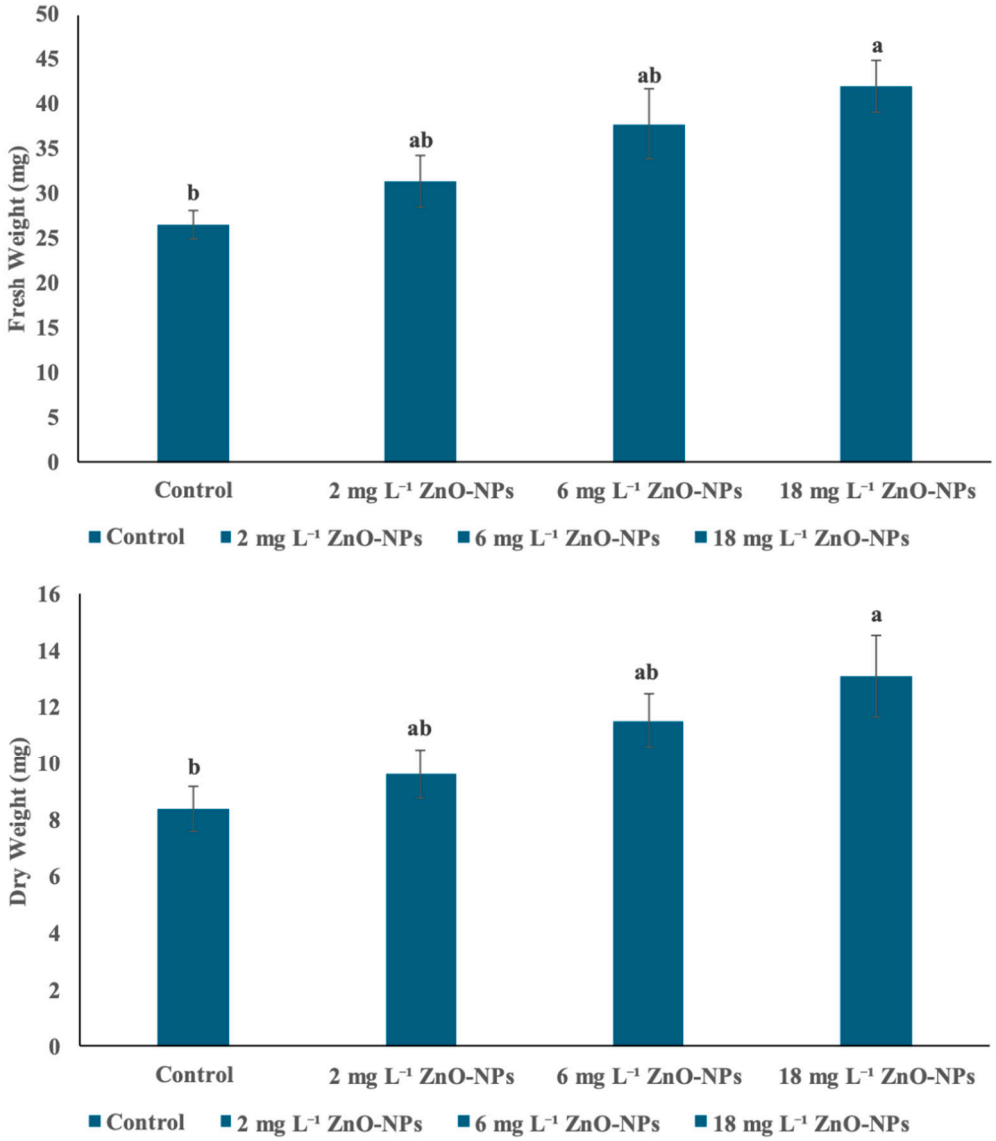

Figure 3. Fresh and dry weight values ± SE of the Brigitta cultivar-proliferated explants. Mean values followed by different letters were significantly different ($p < 0.05$).

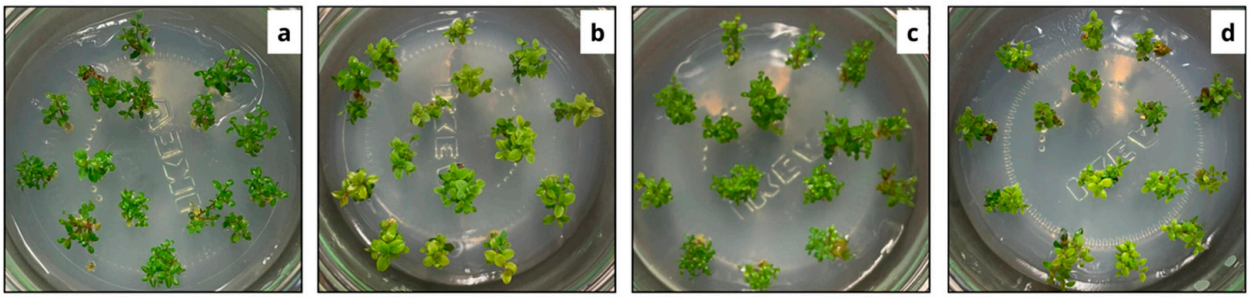

Figure 4. Explants of the Brigitta cultivar after 30 days: control (**a**), 2 ppm mg L^{-1} ZnO-NPs (**b**), 6 ppm mg L^{-1} ZnO-NPs (**c**), and 18 ppm mg L^{-1} ZnO-NPs (**d**).

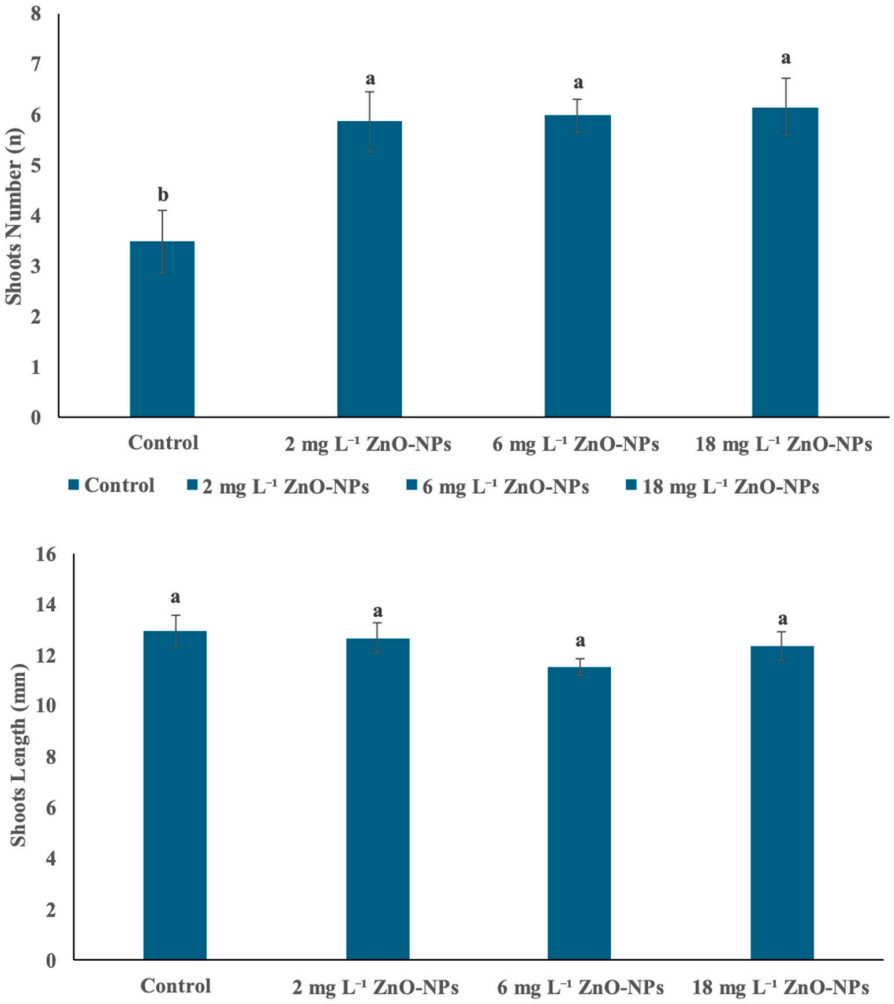

Figure 5. Shoot number and shoot length values ± SE of the Duke cultivar-proliferated explants. Mean values followed by different letters were significantly different ($p < 0.05$).

The treatment with ZnO-NPs did not influence the basal callus production that was present in about 50% of the explants (Figure 6).

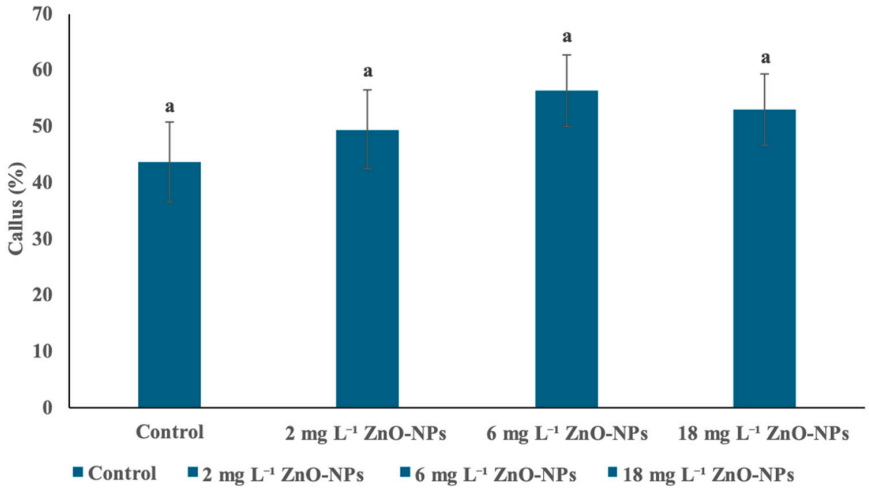

Figure 6. Incidence of explants that produced basal callus, values ± SE in the Duke cultivar-proliferated explants. Mean values followed by different letters were significantly different ($p < 0.05$).

The ZnO-NPs at all the applied dosages positively influenced the fresh and dry weights, which showed higher values than those found for the control (Figures 7 and 8).

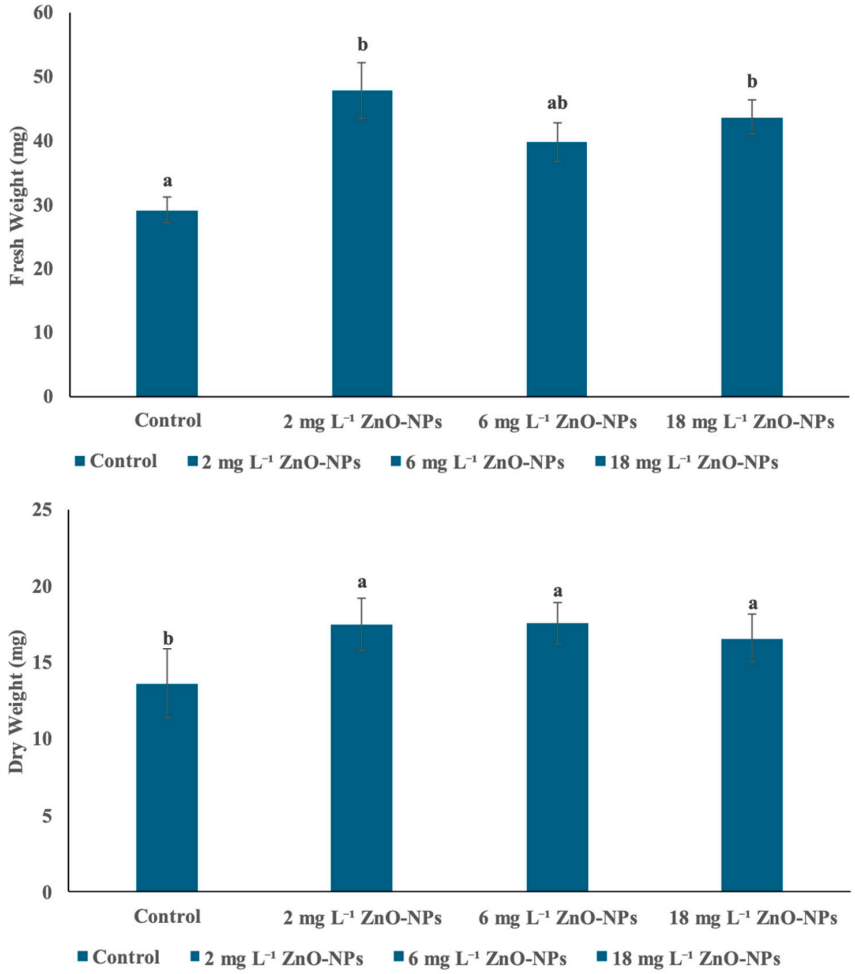

Figure 7. Fresh and dry weight values ± SE of the Duke cultivar-proliferated explants. Mean values followed by different letters were significantly different ($p < 0.05$).

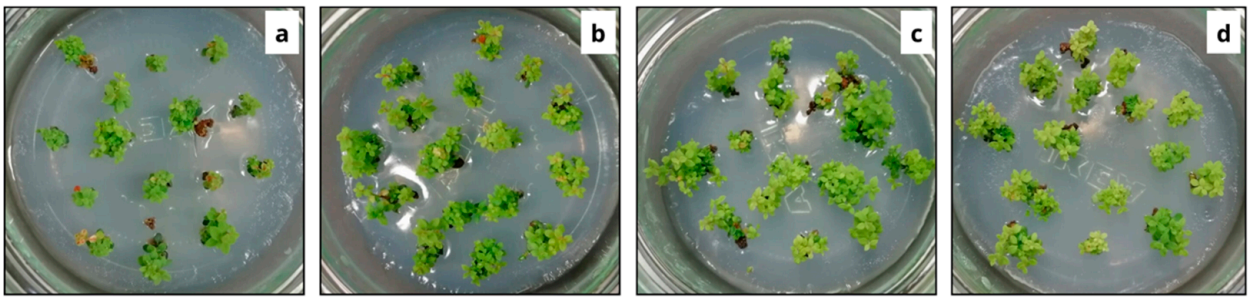

Figure 8. Explants of the Duke cultivar after 30 days: control (**a**), 2 ppm mg L^{-1} ZnO-NPs (**b**), 6 ppm mg L^{-1} ZnO-NPs (**c**), and 18 ppm mg L^{-1} ZnO-NPs (**d**).

3.2. Pigment, Soluble Carbohydrate, and Soluble Protein Contents

Regarding the 'Brigitta' cultivar, an increase of 56% in chlorophyll a was observed in samples treated with the highest ZnO-NP concentration (18 mg L^{-1}), whereas the other concentrations did not determine differences which were statistically different, despite an increasing trend following the application dosage (Table 1). Chlorophyll b was not affected by any of the treatments. Differently, all ZnO-NP dosages significantly increased the

carotenoid content, which rose by 506%, 489%, and 578%, for 2, 6, and 18 ppm, compared to the untreated controls. ZnO-NPs did not influence the content of soluble carbohydrates but increased the content of the soluble protein by 159% at the 18 mg L^{-1} dosage.

Table 1. Chlorophyll a, chlorophyll b, carotenoid, soluble carbohydrate, and soluble protein contents of 'Brigitta' cultivar-proliferated explants.

Treatment	Chl a (mg g^{-1} FW)	Chl b (mg g^{-1} FW)	Carotenoids (mg g^{-1} FW)	Soluble Carbohydrates (mg g^{-1} FW)	Soluble Protein (mg g^{-1} FW)
Control	4.94 ± 1.12 b	2.58 ± 1.21 a	0.18 ± 0.01 b	7.27 ± 1.39 a	0.119 ± 0.034 b
2 mg L^{-1} ZnO-NPs	4.80 ± 1.46 b	2.30 ± 0.90 a	1.09 ± 0.32 a	6.63 ± 0.87 a	0.170 ± 0.043 b
6 mg L^{-1} ZnO-NPs	6.21 ± 1.41 ab	1.58 ± 0.64 a	1.06 ± 0.27 a	6.63 ± 0.91 a	0.185 ± 0.041 b
18 mg L^{-1} ZnO-NPs	7.72 ± 0.44 a	2.51 ± 2.03 a	1.22 ± 0.36 a	6.70 ± 0.68 a	0.308 ± 0.066 a

Mean values (± SE) followed by different letters were significantly different ($p < 0.05$).

Concerning the 'Duke' cultivar, the ZnO-NP application did not produce any measurable effect on chlorophyll a and chlorophyll b contents, as the amount recorded for these pigments remained almost unchanged regardless of the dosage applied (Table 2). In contrast, a significant impact was observed in carotenoids, where 18 mg L^{-1} of ZnO-NPs resulted in a pronounced increase, rising by 116% in the number of carotenoids compared to the control samples. On the contrary, soluble carbohydrates and protein were unaffected by ZnO-NPs, regardless of the NP concentration applied.

Table 2. Chlorophyll a, chlorophyll b, carotenoid, soluble carbohydrate, and soluble protein contents of 'Duke' cultivar-proliferated explants.

Treatment	Chl a (mg g^{-1} FW)	Chl b (mg g^{-1} FW)	Carotenoids (mg g^{-1} FW)	Soluble Carbohydrates (mg g^{-1} FW)	Soluble Protein (mg g^{-1} FW)
Control	5.57 ± 1.15 a	1.69 ± 0.38 a	0.19 ± 0.06 b	6.22 ± 1.12 ab	0.169 ± 0.035 ab
2 mg L^{-1} ZnO-NPs	4.76 ± 1.51 a	2.70 ± 0.26 a	0.17 ± 0.05 b	5.23 ± 0.86 b	0.190 ± 0.047 a
6 mg L^{-1} ZnO-NPs	5.26 ± 0.13 a	1.52 ± 0.12 a	0.16 ± 0.01 b	6.06 ± 0.36 ab	0.131 ± 0.027 ab
18 mg L^{-1} ZnO-NPs	5.16 ± 0.60 a	2.40 ± 0.41 a	0.41 ± 0.05 a	7.96 ± 1.29 a	0.102 ± 0.034 b

Means values (± SE) followed by different letters were significantly different ($p < 0.05$).

4. Discussion

The influence of biogenic ZnO-NPs obtained from duckweed in the proliferation phase of in vitro-grown 'Brigitta' and 'Duke' cultivars was examined. The results showed that the addition of ZnO-NPs to the proliferation medium at an 18 mg L^{-1} concentration for the cv. 'Brigitta' and at concentrations of 2, 6, and 18 mg L^{-1} for the cv. 'Duke' enhanced the number of shoots and both fresh and dry weight. Zinc plays a crucial role in protein synthesis, the functioning and development of chloroplasts, and the metabolism of carbohydrates, lipids, and nucleic acids. [40]. In addition, Zinc is a micronutrient involved in the stability of photosystem proteins, particularly those of photosystem II (PSII), thus guaranteeing their optimal functioning for photosynthetic purposes [41]. The NMs can stimulate photosynthetic efficiency, leading to better light energy use and biomass production [42], as observed in our experiments. As a result, the ZnO-NP integration into the substrate for in vitro cultivation during the proliferation phase facilitated and stimulated the multiplication and growth of the explants.

The improvement in plant growth parameters noted in vitro due to the addition of ZnO-NPs is consistent with findings from previous research [43,44] conducted with other species. ZnO-NPs added to the growth substrate stimulated the proliferation rate, the shoot length, and biomass production in pomegranate (*Punica granatum* L.) [28]. In *Brassica nigra*, Koch ZnO-NPs promoted the differentiation of calli to shoots at 10 mg L^{-1}, while at higher concentrations, a decrease in shoot biomass was observed [44]. Green-synthesized zinc and copper nanoparticles improved the in vitro germination traits of *Citrus reticulata* Blanco [43]. Biogenic ZnO-NPs improved various growth parameters in olive tree explants cultured in vitro, including the number of shoots and both fresh and total dry weight. [8]. ZnO-NPs and CuO-NPs were used as nano-elicitors in callus cultures of *Vigna radiata* L., significantly enhancing the glycoside and phenolic content of the cultures, respectively [45]. The ZnO-NPs significantly influenced the growth of tobacco calli and their physiological indices compared to other forms of ZnO. In particular, the accumulation of zinc ions in the calli treated with nanoparticles led to increased growth and protein content [46].

The subsequent experiments focused on investigating some biochemical parameters linked to the effect of the ZnO-NPs in plants. It is well known that Zn can carry out and control some pivotal processes in plants, particularly those regulating chloroplast structures and function [47,48]. This element can also play a crucial role in photosynthesis and is an essential cofactor for several enzymes and proteins, particularly those involved in chlorophyll biosynthesis [49,50]. ZnO-NPs significantly increased the Chl a content in 'Brigitta' (Table 1), and such an effect may reflect the benefits mentioned above, which are promoted by Zn on chlorophyll metabolism and chloroplast and protochlorophyllide development. On the other hand, the 'Duke' cultivar did not respond in the same way to ZnO-NP treatment (Table 2); this can be ascribed to the cultivar-specific responses to treatments. Therefore, it can be assumed that 'Duke' could have distinct metabolic adaptive mechanisms activated in response to ZnO-NP treatments that did not affect the chlorophyll content.

Furthermore, zinc, as a multifaceted element which is part of antioxidant enzymes such as superoxide dismutase (SOD), may decisively contribute to reactive oxygen species (ROS) scavenging [51]. Therefore, the increase in Zn can represent a stimulus that, among other things, can sharply increase the content of the carotenoids. These biomolecules are, in fact, light-harvesting pigments, but at the same time, they show a vital signature in carrying out antioxidant functions involved in ROS removal [52]. Tables 1 and 2 show that ZnO-NPs generally elevated the carotenoid content in treated plants, but the increases were particularly marked for 'Brigitta'. This can be explained by the fact that some genotypes can be more responsive than others to ZnO-NPs, as already observed for chlorophylls. Finally, it is worth mentioning that the increases in carotenoid content are of nutraceutical interest for the human diet, considering that these biomolecules are bioactive with health-beneficial effects [53]. In fact, carotenoids can serve as antioxidants to contain or deactivate oxidants and very reactive radical species [54]. Such a protective action has been correlated with the prevention of cancer and chronic, age-related, and cardiovascular-related diseases, just to name a few examples [52].

Soluble sugars were investigated in the samples of this experimentation for the Zn involvement in their biosynthesis and regulation. Zn acts as a cofactor for some specific Calvin cycle enzymes, including, among others, the aldolase and carbon anhydrase [55]. Soluble sugar homeostasis remained unaffected in 'Brigitta', while in 'Duke', an increase in soluble sugars was recorded in response to NMs at the highest dosage (Tables 1 and 2). Soluble carbohydrates can serve as energy reserves and represent signaling molecules that influence plant development and growth [56]. In addition, plants can increase soluble carbohydrates under stress conditions to optimize energy management and promote adaptive strategies that avoid energetic and osmotic imbalances [57]. 'Brigitta' did not show increases in soluble carbohydrates, suggesting that metabolic resources were directed toward protein, pigment, and antioxidant synthesis rather than carbohydrate accumulation, as generally observed in our experiments. Differently, Duke increased the content of soluble

carbohydrates at the highest dosage in response to ZnO-NPs; in parallel, the protein content was decreased. Clearly, this cultivar managed its metabolism in a completely different way, diverting energies towards carbohydrates and reducing protein synthesis. This could have been functional to a homeostatic adjustment finalized to deal with ZnO-NPs. Combined with what was observed for the carotenoid content, this result can be of interest for using bioactivating NMs to prepare plants for environmental stressors. In fact, the Zn-NPs effects in 'Duke' included increasing the antioxidants and the osmoprotective potential of carbohydrates.

The impact of nanomaterials on the environment must be considered carefully. Despite this, the use of NPs for in vitro culture can be considered safer than applying nanomaterials directly in agricultural fields. In fact, this cultivation technique minimizes or eliminates the risk of environmental contamination, as it is a controlled setup that prevents the release of NPs into the environment. Finally, low dosages, such as those chosen for this study (2, 6, and 18 mg L^{-1}), significantly reduce ecological risks and potential phytotoxicity.

5. Conclusions

This research demonstrated the beneficial effects of biogenic ZnO-NPs on the in vitro growth of 'Brigitta' and 'Duke' blueberry cultivars for the first time. Adding the nanostructured material to the growth media significantly increased the number of shoots and fresh and dry weights in both cultivars without affecting vitality, shoot length, or basal callus production. Moreover, ZnO-NPs stimulated the carotenoid content, especially for 'Brigitta'. This effect is worth mentioning, given their role in assisting the plant light-harvesting capacity and as antioxidant protective biomolecules. Also, different metabolic responses were stimulated by ZnO-NPs. In particular, 'Duke' reprogrammed its metabolism, diverting it towards carbohydrate formation, while 'Brigitta' increased the biosynthesis of proteins, pigments, and antioxidants, confirming a cultivar-dependent response.

In light of these findings, the data of this study suggest that green biogenic ZnO-NPs could represent a promising nanostructured material to enhance micropropagation techniques and promote positive traits in "in vitro"-cultured plant material.

As a final point, it is to be underlined that a holistic approach is essential to assess the potential use of nanostructured materials in agriculture. In particular, a clear understanding of their effect on plant metabolism makes it possible to exclude toxicity effects or eventually ascertain their causes.

Author Contributions: Conceptualization, L.R. and D.D.B.; methodology, L.R., D.D.B. and M.M.; investigation, L.R., D.D.B., M.M., S.L.F. and D.P.; data curation, L.R. and D.D.B.; writing—original draft preparation, L.R., A.C., D.D.B., D.P. and P.P.; supervision, P.P. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest: The authors declare no conflicts of interest.

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