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The Role of Silver Nanoparticles in Response of *invitro* Boysenberry Plants to Drought Stress

Sevinç Şener [1,*] and Hülya Sayğı [2]

[1] Department of Horticulture, Faculty of Agriculture, Akdeniz University, Antalya, Turkey; ssener@akdeniz.edu.tr
[2] Department of Organic Agriculture, Vocational School of Yumurtalık, Çukurova University, Adana, Turkey hulutas@cu.edu.tr
* Correspondence: ssener@akdeniz.edu.tr; Tel.: +90 242 310 24 25

Abstract: Drought is one of the leading abiotic factors limiting morphological and physiological activities in blackberry cultivation. We investigated the effect of silver nanoparticles (AgNPs) on morphological and physiological processes in the boysenberry crop (*Rubus ursinus* Chamisso & Schlenhtendal) under drought stress. The experiment was performed with three drought stress levels (0% PEG, 4% PEG and 8% PEG) and three AgNPs treatments (0, 0.1, 0.2 mg L^{-1}) *invitro* conditions. The results showed that drought stress induced by PEG, reduced root and shoot development of boysenberry plants grown *invitro*. The addition of AgNPs is significantly alleviated the adverse effect of drought stress and increased the plant growth parameters. Antioxidant activity of SOD and CAT enzymes increased in boysenberry leaves when treated with AgNPs under drought conditions, while the amount of MDA decreased. Therefore, the results obtained in this study suggest that 0.1 mg L^{-1} AgNPs added to the medium improve the growth and development of *invitro* boysenberry plants under drought stress.

Keywords: blackberry; berries; boysenberry; functional food; nano silver; nano particles; water stress.

1. Introduction

The cultivation of berries is the subject of wide-ranging studies due to the fact that they have very tasty fruits and that they are recommended to be consumed at increasing rates in the daily diet [1,2]. *Rubus fructorious*, in the genus *Rubus*, is widely grown in temperate climatic regions of the world due to increasing consumer demand [3,4], and one of the most popular horticultural berry fruit species. Blackberries are used in many sectors such as food, cosmetics, health, and can be consumed fresh and processed. There for the high antioxidant properties and valuable nutritional content of fruits, it is recommended by the authorities as a healthy food [1,4,5]. Blackberries are also described as functional food in connection with their organoleptic properties and high content of polyphenol compounds [2,4,6]. Compared to many other fruit species, blackberry has advantages such as ease of cultivation and marketing, providing raw materials to the industry, being suitable for family business, high income from small areas, and low production costs [1–3,6]. This plant, which can be propagated vegetative methods by hard or softwood cuttings, one-year-old suckers, and layering or root cuttings, can also be propagated under in vitro conditions quickly and highly efficiently in a relatively short time with limited space and without seasonal variation [3]. One of the most important problems in the cultivation of this plant is the increasing drought day by day.

When usable areas in the world are classified according to stress factors, drought stress, which is a natural stress factor, contains the highest rate [7,8]. Drought stress is one of the most common environmental stresses affecting growth and yield and induces many physiological, biochemical and molecular responses in plants [9–12]. Drought stress occurs when the amount of water lost by the plant through transpiration is more than it takes. Under these conditions, competition begins between plant organs for water, the water potential gradient between different parts of the plant is disrupted, and the plant is under water stress [9,11,12]. The thornless cultivars of *Rubus* L. species, have soft and juicy fruits and also a root system that does not reach the deep soil profile [13,14]. For this reason, the plant needs frequent watering and the vegetative growth and development of this plant is significantly affected by drought [15–17]. Water stress, which has the negative effects mentioned in

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all plants, is one of the factors limiting the production in blackberry cultivation and restricting the morphological, enzymatic and physiological activities of the plant. Researchers report that drought stress can be decreased shoot, leaf and root development, leaf number and disrupts water use efficiency, and thus causes physiological and biochemical problems in blackberry plant [17–19]. In addition, under drought stress conditions, the total leaf area, leaf relative water content (RWC) and thus the photosynthesis rate decreases [20]. The loss of intracellular water causes dehydration due to the increase in concentration, and in this case, the collapse of the plasma membrane leads to ruptures and autolysis of the cytoplasm [18–20]. Protein structure and membrane stability in the plant may be adversely affected by this situation. Photosynthetic ability is determined by the total leaf area in that plant and the photosynthetic activity of each leaf. With drought stress, the total leaf area decreases and therefore photosynthesis decreases [17,19–21]. In this case, changes in the rate of photosynthesis can reduce vegetative growth and severely delay the development of plant [15,19,21]. The first response of plants to drought stress is stomatal closure, which causes oxidative stress. In order to protect against oxidative stress in plant cells, there is a highly efficient antioxidant defense system including superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) enzymes [16,22].

Changing climatic conditions have increased the pressure of abiotic stress factors on agricultural production. More studies are needed to increase the drought tolerance of the plant for economical and sustainable blackberry cultivation. The development of new varieties resistant to stress conditions takes a long time and costs are high, especially in fruit growing. NPs can be used in many different ways due to their physico-chemical properties such as high surface area, high reactivity, variable pore size and particle morphology. In recent years, nanoparticles, which are environmentally friendly, the new generation of technological products have been used to alleviate the effects of abiotic stresses, including drought [23–25]. Among the remarkable properties of metal nanoparticles, which are among NPs, are their extremely large surface area/volume ratio and their ability to regulate electron exchange [26]. Silver nanoparticles, a metallic, antimicrobial/antibacterial nanomaterial, increase the chlorophyll content and photosynthesis rate, thereby promoting plant growth and development [26–31]. Various studies have reported that NP application contributes to the regulation of antioxidant enzyme activity and cellular water balance [23,34]. Also some researchers stated that AgNPs has the potential to developing resistance against drought stress in different plants such as wheat [32,33], eggplant [34], thymus [35], lettuce [36] and lentil [37]. Therefore, in this research, it was aimed to determine the effects of different concentrations of AgNPs treatments on drought tolerance of blackberries grown *invitro*.

2. Materials and Methods

2.1. Plant Material

In the study, Boysenberry (*Rubus ursinus* Chamisso & Schlenhtendal) is used as plant material which is a hybrid *Rubus* berry derived from a cross between loganberry (*Rubus loganobaccus* Bailey) and trailing blackberry (*Rubus fruticosus* L.) [38]. Stock plant materials, used for micropropagation were obtained from plants grown in Akdeniz University Research and Application Area.

2.2. AgNPs Suspension Preparation

AgNPs with an average particle size <30 nm, surface area >15 $m^2\ g^{-1}$ and purity was >99% was used as nanoparticle (Nanografi Chemical Company). Morphological study of AgNPs was done by scanning electron microscope (SEM) (ZEİSS-LEO 1430) (Figure 1). PVP-coated AgNPs were purchased as a dry powder and suspended in deionized water to prepare solutions at a ratio of 0,1 and 0,2 mg L^{-1} AgNPs (resistance > 18 MΩ cm; suspension pH = 5.8). The stock suspension was dissolved by sonicating for 30 minutes using a probe-type sonicator (Misonix, QSonica LLC, Newton, ABD) [39]. These stock solutions were used for prepared MS culture media at multiplication stage.

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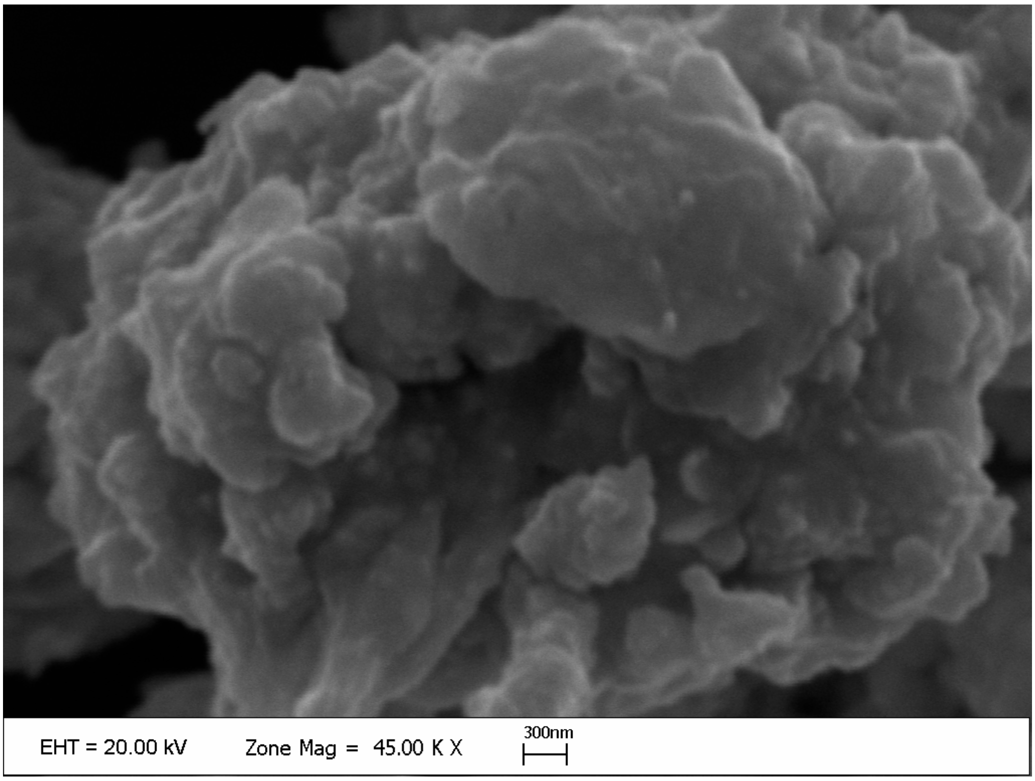

Figure 1. Scanning electron microscopy (SEM) image of AgNPs.

2.3. İnvitro Establishment of Cultures

Shoot tips and nodal cuttings (3-4 cm) of 3-year-old Boysenberry plants were washed thoroughly under running tap water for 2 hours, then surface sterilized with 70% for 30 sec and further sterilized by 30% bleach solution (5.25~ NaOCl) with 0.2 ml L^{-1} of Tween 20 for 20 min followed by explants were washed three times with sterile distilled water under a laminar airflow cabinet [40].

Murashige and Skoog (MS) [41] culture medium containing 3% sucrose and 0.7% agar and 0.44% Gelrite was prepared by dissolving in distilled water and pH was adjusted to 5.8 before autoclaving at 121°C for 20 minutes. Single bud explants were cut into 0.7-1 cm species and cultured two in each baby jar (330 ml). Explants were incubated at 24 ± 1 °C temperature, 16 h photoperiod, 2200 lx and 70–80% relative humidity in the growth room [40]. After 5 weeks young shoots were used as explant experiment.

The treatments consisted of drought stress at three levels (0, 4, 8 %) of polyethylene glycol (PEG 6000) and three levels (0, 0.1, 0.2 mg L^{-1}) AgNPs. Mixtures containing AgNPs were added to the media prepared for subculturing of plants. The addition of PEG 6000 was added to the medium before pH measurement. 135 young shoots [3 repetition x (5 explants x 3 PEG doses x 3 AgNPs doses)] were transferred to the MS media supplemented with 0.5 ml BAP + 0.5 ml IBA ve 30 g L^{-1} sucrose and cultured in the growth room same conditions as described above [40]. Shoot response was recorded 50 days after cultivation.

2.4. Growth Parameters

In order to determine the effect of applications on the vegetative development of plantlets, at the end of the experiment, shoot fresh weight (SFW) (g), shoot dry weight (SDW) (g), shoot length (SL) (mm), stem diameter (SD) (mm), root fresh weight (RFW) (g), root dry weight (RDW) (g), root length (RL) (mm), number of leaves (LN) (per plant), leaf width (LW) (mm), leaf length (LL) (mm) were determined. After measuring the fresh weights of the shoots and roots, they were placed in paper bags and kept in an oven at 70 °C for 48 hours. Dry weights were determined by weighing the dried shoots.

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2.5. Physiological Parameters

Relative water content (RWC): Measurement of the relative water contents (RWC %) was performed according to Sanchez et al. [42]. Fully expanded leaves were placed in a petridish containing wet filter papers. They were kept at 4 °C in the dark for 24 h to obtain turgid weight (TW) followed by oven dry for 4 days at 70 °C for the dry weight (DW) measurement.

RWC was calculated using the following equation:

$$RWC= [(FW-DW)/(TW-DW)]x100$$

SPAD index was measured using chlorophyll content meter (SPAD– 502, Konica Minolta Sensing, Inc., Tokyo, Japan).

2.6. Biochemical Parameters

Fresh leaves (0.5 g) from different treatments were homogenized in an ice bath containing 5 mL 50 mM potassium phosphate buffer (pH 7.6 + 0.1 mM EDTA + 2% insoluble polyvinypolypyrrolidone). The homogenate was centrifuged at 15,000 g for 15 min at 4 °C. The supernatant was stored at –20 °C and used for assays of SOD, CAT and MDA.

Leaf superoxide dismutase (SOD) activity was measured according to the method of reduction of NBT (nitro blue tetrazolium chloride) by O_2- under light [43]. The absorbance of leaf extract was spectrophotometrically measured at 560 nm wavelength. Catalase (CAT) activity is determined by following the consumption of H_2O_2 (E=39.4 mM cm $^{-1}$) at 240 nm [43]. Heath and Packer's [44] method was used to measure the content of Malondialdehyde (MDA). The absorbance of the leaf's extracts was read at 532 and 600 nm by spectrophotometer.

All photochemical quantifications were conducted on a UV-2100 spectrophotometer (UNIKO Inc, NJ, USA) at a temperature 4°C.

2.7. Statistical Analysis

All measurements in this work were replicated three times. Data of morphological and physiological indices were subjected to an analysis of variance (ANOVA) using the software SPSS 22.0. Duncan's multiple range test ($P < 0.05$) was used to determine the significance among all means between different treatments. Error bars in graphs represent ± standard error.

3. Results

3.1. Growth Parameters

The results showed that PEG-induced water stress and AgNPs had a significant effect on growth parameters. As seen in Tab. 1 addition of AgNPs is significantly alleviated the adverse effect of drought stress and increased the SFW, SDW, SL, SD, RFW, RDW and RL. SFW in the presence of 0.1 mg L^{-1} (0.72 g) and 0.2 mg L^{-1} (0.74 g) AgNPs was significantly higher compared to the control (0.39 g) (Tab. 1). In addition, the SWF value increased approximately twice with the addition of 0.1 mg and 0.2 mg L^{-1} AgNPs in plants where 4% and 8% PEG was applied to induce drought stress. The highest SDW (0.126 g) was determined in the control group in the application of 0.1 mg L^{-1} AgNPs. SDW values of *invitro* plants decreased at 4% and 8% PEG levels, where drought stress conditions were created. However, with the addition of 0.1 and 0.2 mg L^{-1} AgNPs in 4% PEG application, the negative effects of drought on SDW were eliminated. As a matter of fact, Tab. 1 shows that SDW values obtained from 4% PEG+0.1 mg L^{-1} AgNPs (0.056 g) and 4% PEG+0.2 mg L^{-1} AgNPs (0,058 g) applications are in the same statistical group as 0% PEG+0 mg L^{-1} AgNPs (0.078 g) application without drought stress. The mean SL values of the *invitro* plants obtained from different applications are also similar to the SDW results. In Tab. 1, where the effects of PEG applied at different doses are shown, it is seen that the highest mean SL values were obtained from 0.1 mg L^{-1} AgNPs applications. As with all other vegetative growth criteria, SD values were higher in the control group, where PEG was not applied. Unlike SFW, SDW and SL criteria, it is seen that the average highest SD value in 4% PEG application is obtained with the addition of 0.2 mg L^{-1} AgNPs (2.13 mm). The effect of AgNPs on root growth of plants grown *invitro* under drought stress was investigated in the study. Similar results in shoot growth of plants were also recorded for root growth (Tab. 1). It was determined that the highest RFW values were obtained with the addition of 0.1 mg L^{-1} AgNPs in all applications. The best RDW value was seen in the control group (0% PEG+0.1 mg L^{-1} AgNPS; 0.227 g). Meanwhile, the highest RL (29.43 mm) was achieved on %0 PEG+0.2 mg L^{-1} AgNPs which differed significantly from the control treatment (15.93 mm). In short, if we evaluate the effect of the applications on the growth criteria in Tab. 1, we can say the following; drought stress induced by PEG, reduced root and shoot development of boysenberry plants grown *invitro*. AgNPs application was successful in eliminating the negative effects of drought. In 0%, 4% and 8% PEG applications, better results were obtained with the addition of 0.1 mg L^{-1} AgNPs compared to the addition of 0.2 mg L^{-1} AgNPs.

Table 1. The effect of different AgNPs doses on *invitro* shoot growth of boysenberry under drought conditions.

PEG %	AgNP (mg L^{-1})	SFW (g)	SDW (g)	SL (mm)	SD (mm)	RFW (g)	RDW (g)	RL (mm)
	0	0.39 bc	0.074 ab	27.25 bcd	1.23 cd	0.053 b	0.051 b	15.93 bcd
0	**0.1**	0.72 ab	0.126 a	44.53 a	2.43 a	0.285 a	0.227 a	26.78 ab
	0.2	0.74 a	0.078 ab	34.13 ab	2.13 ab	0.194 ab	0.070 b	29.43 a
	0	0.30 c	0.027 b	18.50 d	1.40 bcd	0.070 b	0.029 b	13.35 cd
4	**0.1**	0.65 ab	0.056 ab	34.08 ab	1.48 bcd	0.129 ab	0.045 b	14.53 cd
	0.2	0.56 abc	0.058 ab	26.65 bcd	2.05 abc	0.076 b	0.058 b	15.90 bcd
	0	0.29 c	0.035 b	20.93 cd	1.08 b	0.050 b	0.035 b	11.93 d
8	**0.1**	0.70 ab	0.051 b	31.28 bc	1.83 abcd	0.138 ab	0.027 b	25.13 abc
	0.2	0.56 abc	0.045 b	24.63 bcd	1.78 abcd	0.027 b	0.030 b	16.58 bcd

SFW: shoot fresh weight, SDW: shoot dry weight, SL: shoot length, SD: stem diameter, RFW: root fresh weight, RDW: root dry weight, RL: root length * Different lettering in the same column shows statistically significant differences (P≤0.05).

The effect of PEG and AgNPs applied at different doses on the leaf growth of plants is given in Figure 2. It is clearly seen in Figure 1a, 1b and 1c that the addition of AgNPs under drought or normal conditions has a positive effect on the leaf growth of *invitro* boysenberry plants. In 4% and 8% PEG applications, with the addition of 0.1 or 0.2 mg L^{-1} AgNPs, similar values were obtained with the control conditions in terms of LN, LW and LL. These results show that the use of AgNPs is successful in preventing the effects of drought stress in terms of leaf growth.

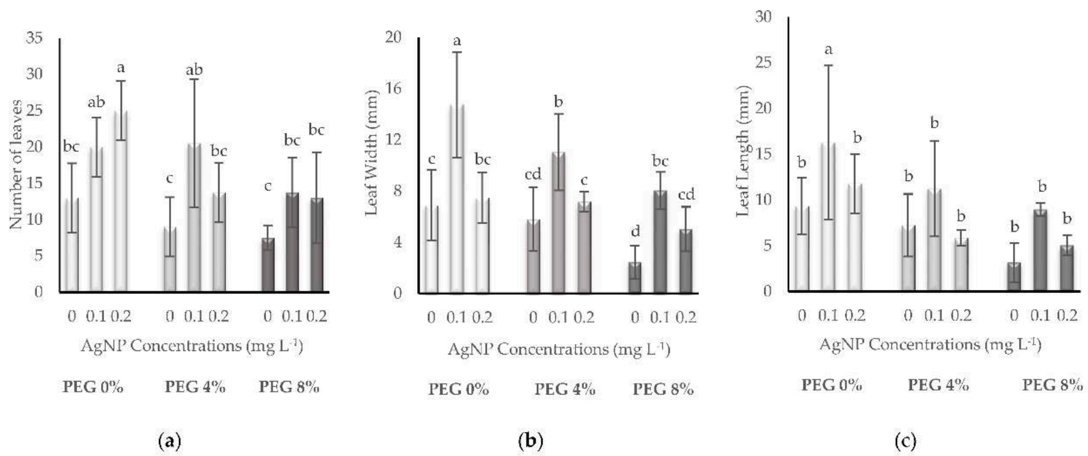

Figure 2. Influence of PEG and AgNPs treatment in *invitro* boysenberry leaves: (**a**) Number of leaves; (**b**) Leaf width (mm); (**c**) Leaf length (mm). Values are the mean ±S.D. of three replicates (n=3). Common letters are not significant (P < 0.05).

3.2. Physiological Parameters

According to the results of the study, the effect of PEG and AgNPs application on SPAD index and RWC values of boysenberry leaves grown *invitro* was found to be statistically significant (P <

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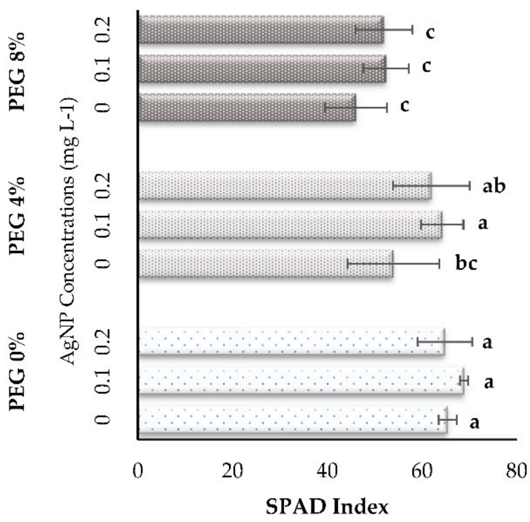
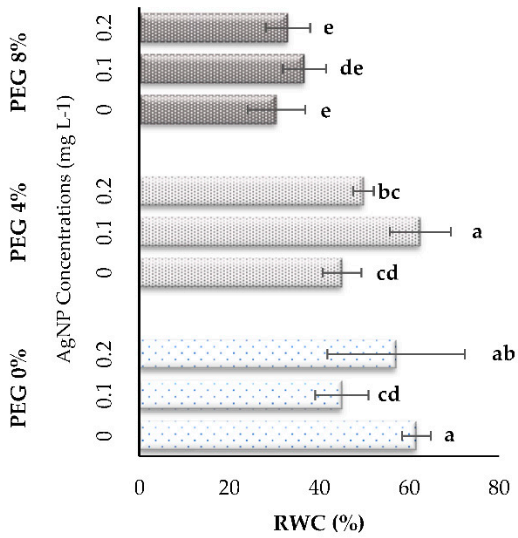
0.05) (Figure 3). The highest SPAD index values were recorded in the control group plants. The mean value obtained from the application of 4% PEG + 0.1 mg L^{-1} AgNPs (64.28) was found to be statistically similar to the control group. The lowest SPAD index averages were observed in the 8% PEG group in which the highest dose was applied. Among the different treatments, the highest RWC values were reached in plants without drought stress and without AgNPs application (61.59%), and also in plants where 4% PEG+0.1 mg L^{-1} AgNPs (62.51%) was applied. In this case, it can be said that the addition of AgNPs under 4% PEG-induced drought stress is effective in maintaining the SPAD index and RWC content of *invitro* plants. However, in 8% PEG application, the RWC content and SPAD index values of the plants were lower than the control group plants, despite the addition of AgNPs (Figure 3).



(a)



(b)

Figure 3. Effect of PEG and AgNPs treatment on SPAD index (a) and RWC (b) *invitro* boysenberry leaves. Values are the mean ±S.D. of three replicates (n=3). Common letters are not significant ($p < 0.05$).

3.3. Biochemical Parameters

As seen in Figure 4 (a,b,c), *invitro* plants showed an increasing trend in SOD and CAT activities due to drought stress caused by PEG and AgNPs application. The highest SOD (281 U g^{-1} fw) and CAT (14.74 U mg^{-1} fw) activity values were determined in the group in which 8% PEG+0.2 mg L^{-1} AgNPs was administered. MDA content decreased with the addition of AgNPs in all treatments. While the maximum MDA contents were found in the presence of 8% PEG (4.36 nmol g^{-1} FW), the lowest value was found in the application of 0% PEG+0.2 mg L^{-1} AgNPs (1.87 nmol g^{-1} FW).

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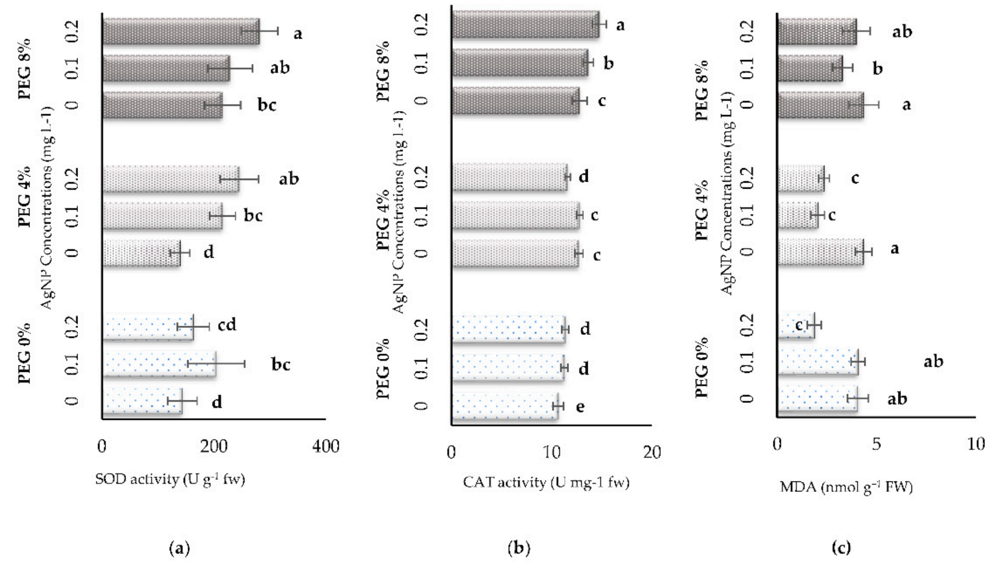

Figure 4. Effect of PEG and AgNPs treatment on SOD activities (a), CAT activities (b) and MDA content (c) of boysenberry leaves under *invitro* conditions. Values are the mean ±S.D. of three replicates (n=3). Common letters are not significant ($p < 0.05$).

4. Discussion

The negative effects of drought stress on the growth of plants are known *invitro* and *invivo* [45–49]. When the data obtained as a result of our study were evaluated, we determined that shoot and root development of *invitro* boysenberry plants decreased under drought stress conditions. Many researchers have reported similar results in berries under drought conditions *invitro* [24,49–51]. Sharma et al. [52] similarly reported that drought stress caused by PEG application at different doses (5, 10 and 15 concentrations) significantly decreased the germination percentage and shoot-root lengths at the seedling stage of fifteen wheat genotypes. Due to the physical and chemical properties of different nanoparticles, their positive effects on plant growth and their ability to eliminate abiotic stress factors are among the current research topics [53–57]. According to the results obtained, it can be said that AgNPs, whose effectiveness against drought stress was evaluated within the scope of this study, were effective in the production of boysenberry under *invitro* conditions. Silver nanoparticles are one of the materials that have become widespread in agricultural production in recent years and attract attention due to their positive effects on plant growth and development [57–60]. There is also report on the effect of AgNPs with shoot and root growth promotion in strawberry by Tung et al. [61]. It is thought that this effect of AgNPs, especially on root growth, may be related to their ability to block Ethylene signal [59,62]. Ethylene production, which disrupts auxin translocation, causes hyperhydricity and causes tissue death, can be inhibited by low concentrations of $AgNO_3$ [63,64]. Ag^+ interferes with the binding of ethylene receptor site and helps reduce ethylene production with promotion of polyamine biosynthesis [65]. Salama et al., in their study, reported that small concentrations of silver nanoparticles had a stimulating effect on the growth of common bean and corn plantlets, while increased concentrations had an inhibitory effect [28]. In addition, it is known that silver particles, one of the most used materials after carbon nanotubes among different nanoparticles, are a wonderful plant-growth stimulator [66,67]. Besides lots of researchers reported that silver nitrate is effective in increasing plant growth and development under the *invitro* conditions [61,63,68–70]. Although it is mentioned in a large number of studies that AgNPs have a positive effect on plant growth and development [27,28,61,62,66,71–73], it is also stated that these particles can cause toxicity in some studies [74–76]. It has been reported that nano silver preparations can affect the metabolism, respiration and reproduction of microorganisms and therefore may cause toxicity [77]. In our study, no inhibitory effect of this material was found. The effect of nano silver particles, which have more surface area in contact with outer space due to their small size, and thus increase the amount and efficiency of adhesion to the cell surface [78], may vary according to the dose of use and the growing medium. Some researchers have stated that when metallic ions such as silver or heavy metals accumulate in high concentrations in plant tissue, they cause the polymerization of phenol with the peroxidase enzyme that chelates heavy metals, and this leads to toxicity [72,79,80].

As a result of the study, it was determined that AgNPs had a significant effect on the RWC and SPAD index of boysenberry plants under drought stress *invitro* growing conditions. Similar effects of AgNPs on RWC and SPAD index are similar to various studies [71,72] reported that Fenugreek seeds had higher shoot and root growth in *invitro* conditions with the addition of AgNPs, which may be related to increased water and nutrient uptake of seeds treated with AgNPs. Mubashir et al., [81] in their study investigating the efficacy of nano-nutrient solutions (NNS) on growth and biochemical attributes of tomato under drought stress, reported that NNS administration resulted in more osmolyte production such as sugars and free amino acids, which may have contributed significantly to the maintenance of tissue water content. Osmolites, which have the ability to maintain the water potential gradient, have a significant effect on plant growth and productivity and have a protective effect in drought conditions [82,83]. Some researchers state that AgNPs play an important role on photosynthetic activity and improve plant growth and development criteria by promoting the production of Indole acetic acid (IAA) [84,85]. And also, the increase of the SPAD index is thought to be related to the inhibition of ethylene. As a matter of fact, it is reported that ethylene causes tissue death and reduces chlorophyll content [64].

Antioxidant activity of SOD and CAT enzymes increased in boysenberry leaves when treated with AgNPs under drought conditions. Some researchers similarly report that AgNPs application increases SOD and CAT enzymes [86,87]. Drought stress causes an increase in ROS accumulation and causes significant oxidative damage through lipid peroxidation [88]. Reactive oxygen species (ROS) accumulated in leaves due to stress cause oxidative damage to cell organelles and membrane elements [89]. ROS causes lipid peroxidation by interacting with phospholipids and fatty acids, thereby increasing the amount of MDA. For this reason, lipid peroxidation is usually measured by MDA content [90,91]. The results obtained from the study revealed that AgNPs application decreased the amount of MDA and therefore had a protective effect against oxidative stress. It is stated that there may be increases in the secondary metabolite content of plants exposed to nanoparticles [92–94]. It has been reported that AgNPs increase oxidative stress and increase phenolic and flavonoid levels in potato [86,95]. Phenols and flavonoids are known to have the ability to protrude into the lipid bilayer, preventing ROS-mediated lipid peroxidation and maintaining membrane fluidity and function [96,97]. Increased phenol and flavonoid synthesis in plant tissues can improve the antioxidant system under control and drought conditions [98–100].

5. Conclusions

The shoot and root development of plants decreased gradually with increasing PEG dose applications, however, the addition of AgNPs managed to prevent this decrease. Especially, the 0.1 mg L^{-1} AgNPs dose added to the medium, even in 4% and 8% PEG applications, was instrumental in obtaining results close to the shoot and root growth values obtained under normal (non-stress) conditions. When the results obtained at the end of the study are evaluated; it was determined that the antioxidant system of boysenberry plants in which AgNPs applied *invitro* improved further under control and drought conditions. These results show that AgNPs addition at appropriate doses is an active ingredient to be applied to *invitro* boysenberry plants under drought stress.

Author Contributions: “Conceptualization, S.S. and H.S.; methodology, S.S and H.S..; data curation, S.S. and H.S.; writing—original draft preparation, S.S. and H.S.; writing—review and editing, S.S. ; supervision, S.S.; project administration, S.S.. All authors have read and agreed to the published version of the manuscript.”

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

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