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Response of the Five Highbush Blueberry Cultivars to In Vitro Induced Drought Stress by Polyethylene Glycol

Sabin Molnar [1], Doina Clapa [2,*] and Viorel Mitre [1]

[1] Faculty of Horticulture, University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca, Manastur St. 3-5, 400372 Cluj-Napoca, Romania; msabin21@gmail.com (S.M.); viorel.mitre@usamvcluj.ro (V.M.)
[2] Institute of Advanced Horticulture Research of Transylvania, University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca, Manastur St. 3-5, 400372 Cluj-Napoca, Romania
* Correspondence: doina.clapa@usamvcluj.ro

Abstract: Stress caused by drought is an important factor that affects the growth and development of highbush blueberry plants. In vitro screening for drought stress tolerance is of major importance in identifying cultivars that have optimal stress tolerance and productivity. The aim of this study was to evaluate the responses of five in vitro-grown highbush blueberry cultivars (Bluecrop, Brigitta Blue, Duke, Goldtraube and Hortblue Petite) under drought stress. Five concentrations of polyethylene glycol (PEG 6000), 0 g/L, 10 g/L, 20 g/L, 30 g/L, 40 g/L, 50 g/L, were applied to induce drought stress in the culture media. Significant differences were found in shoot length and number, proliferation rate, fresh weight, dry weight, water content, chlorophyll, and carotenoid content. Drought stress had a negative impact on shoots length, chlorophyll, and carotenoid content for all highbush blueberry varieties. The conclusion of the study highlights that Goldtraube had the highest drought tolerance efficiency, followed by Bluecrop, Hortblue Petite, Duke, and Brigitta Blue.

Keywords: abiotic stress; in vitro screening; morphological traits; water content

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1. Introduction

On a worldwide basis, water is the most limiting resource in crop production [1]. Drought is one of the most crucial factors that play important roles in decreasing plant production and probably causes the greatest loss compared to other causes [2,3], and furthermore, it weakens global food security [4]. Drought stress affects plants through their life cycle, i.e., from germination till maturity; therefore, the improvement of productivity under drought is a major goal for plant breeding. [5,6].

According to Sivritepe et al. [7], the need for water conservation and evaluation of the existing and/or newly developed germplasm of crop plants for their tolerance to drought has become a major concern.

One of the most sensitive species to water shortages in soils is blueberry (*Vaccinium corymbosum)* due to its superficial root system, most of its roots being situated in less than 40 cm depth in soil [8]. Even though blueberry plants are exposed for short periods to limited water sources (for example, a few days without rain or irrigation), the response to water stress develops quickly in blueberry, reducing photosynthesis and leading to less growth and fruit production. The growth, function, productivity, and water use of a plant are intimately related to its water status [9]. Differences between cultivars can be observed in this regard; these differences may be the result of inherent morphological and physiological features, as well as phenological factors such as the timing of fruit development. Water use appears to be especially high during fruit ripening in blueberry [10]. Strong markets for processed and fresh fruit have resulted in good returns for growers and an increase in planted surface area. [11]. Blueberry production, along with the cultivated surface area, is in continuous increase to keep up with the growing demand of consumers

for blueberries [12]. Due to the fact that blueberry fruits contain a high level of vitamins, anthocyanins and other anti-inflammatory and anti-tumor compounds [13], their production is of main interest worldwide.

In this context, the evaluation of germplasm *V. corymbosum* for drought tolerance becomes increasingly important. Currently, polyethylene glycol (PEG) -induced drought stress is one of the latest models to study drought tolerance [14]. In vitro simulation of drought stress using chemical reagents constitutes a convenient method to assess the effects of drought on plant growth and development in controlled conditions [15]. PEG is a natural water-soluble and non-ionic compound, with a molecular weight larger than 6000 (PEG 6000). They are inert and cell impermeable; therefore, the molecules are small enough to influence osmotic pressure but at the same time large enough to remain unabsorbed by plants. It was shown that PEG induces significant water stress in plants and does not have any toxic effects [16].

In vitro cultures have been used to assess drought tolerance for different species: olive (*Olea europaea*) [17], chickpeas (*Cicer arietinum* L.) [18], guava (*Psidium guajava* L.) [19],) lentil (*Lens culinaris* Medik). [20], sugarcane cultivars (Saccharum spp. Hybrids [21]. Drought stress induced by PEG negatively influenced the content of chlorophylls in cultivars on apple and cherry. For the cherry cultivars, the concentrations of chlorophyll a and chlorophyll b were generally higher compared to apple cultivars [22]. An article published in 2008 revealed that water stress caused physiological disorders in explants of cherry rootstock Gisela 5 grown in vitro, with culture media supplemented with PEG. Higher PEG concentrations resulted in more severe physiological disorders [7]. Furthermore, the study carried out on the Pyrus species [23], reveals that under water deficit conditions induced by PEG, the fresh weight of shoots decreased for all the studied cultivars. Increased concentrations of PEG resulted in a lower fresh weight value.

Even though that highbush blueberry is exceptionally suitable for micropropagation, the in vitro hydric stress simulation model has not yet been applied to this specific species [24–29]. Micropropagation of highbush blueberry was achieved more than forty years ago [30], and significant progress in plant tissue cultures have been developed since that time [31]. Further, in vitro propagation is widely used, as the micropropagated plants possess enhanced vegetative growth, an important aspect from the viewpoint of farmers, whose main concern is the rapid establishment of plants for early fruit production [32].

In addition, micropropagated plants yield higher crop production than softwood cuttings [33]. These differences were still visible after a period of 10 years observation, due to the higher number of lateral branches and greater spread of tissue cultures in micropropagated plants.

The objective of the present study was to evaluate the effects of drought on plant growth and development in five highbush blueberry cultivars (Bluecrop, Brigitta Blue, Duke, Goldtraube and Hortblue Petite) under drought stress conditions induced by different PEG 6000 concentrations which were added to the in vitro culture medium.

2. Materials and Methods

2.1. In Vitro Culture and Water Stress

Five blueberry varieties (*V. corymbosum* L.) were used for the present study: Duke, one of the earliest varieties for the European market, followed by Bluecrop, a popular variety, the Brigitta Blue originating from Australia, is a very well adapted variety with firm blueberry fruits, then Goldtraube with its extra-large fruits and finally Hortblue Petite, a variety that offers two crops/year.

Explants originating from axillary buds were used for the experiment and were cultured on Woody Plant Medium (WPM) [34] with 100 mg/L Sequestrene 138 and 1 mg/L zeatina (Z) according to the protocol [35].

The culture medium was solidified with 0.4% (*w*/*v*) plant agar. The medium was introduced in recipients of 720 mL, each with a diameter of 9 cm and a height of 13.5 cm,

with a metallic lid equipped with a ventilation filter. Each recipient was filled with 100 mL of culture medium.

The experimental design included different concentrations of PEG 6000 as follows: 0 g/L, 10 g/L, 20 g/L, 30 g/L, 40 g/L, 50 g/L. PEG 6000 was added to the culture media before adjusting the pH and before autoclavation. The pH of the media was adjusted to 5 before the addition of agar. The media was autoclaved at 120 °C for 20 min. In every vessel, 16 explants were inoculated, each explant with a length of 1.5–2 cm. After inoculation, the culture vessels were incubated in the growth room under a controlled environment (22 ± 1 °C, 32.4 mmol·m^{-2}·s^{-1}, 16-h photoperiod).

All the components were purchased from Duchefa BiochemieBV (Haarlem, The Netherlands)

2.2. Growth Parameters

The growth response to drought stress was determined after a period of 12 weeks, using the following parameters:

1. Shoots length (cm): The shoots from 48 initial explants were measured (three vessels/treatment/variety) (SL).
2. Proliferation rate (number of shoots/explants): Three recipients/treatment/variety were measured (PR).
3. The average number of shoots/culture jar: The shoots from three recipients/treatment/variety were measured (NS).
4. Fresh weight (FW) of shoots per explant (mg): 30 explants for each treatment/variety were weighed immediately after the material was removed from the in vitro culture medium.
5. Dry weight (mg): The material was dried for three days at 45 °C and re-weighted (DW).
6. Water content (WC): Based on FW and DW, WC percentage was calculated using the formula [36]:

$$\text{WC (\%)} = ((\text{Fresh Weight} - \text{Dry Weight})/\text{Fresh Weight}) * 100$$

2.3. Photosynthetic Pigments

Chlorophyll content. Chlorophyll a (Chl a) and chlorophyll b (Chl b) levels were determined via spectrophotometry, using fresh in vitro grown shoots. The fresh samples were weighed and homogenized, and then extracted with 90% acetone in water using a magnetic stirrer until the residue was colorless. The absorbance was read at 645 and 663 nm using a Perkin Elmer Lambda 25 spectrophotometer. The following formulas were used to quantify chlorophyll a (Chl a) and chlorophyll b (Chl b):

$$\text{Chl a mg/g FW} = (11.75 \times \text{A663} - 2{:}35 \times \text{A645}) \times \text{V/g}$$

$$\text{Chl b mg/g FW} = (18.61 \times \text{A645} - 3.96 \times \text{A663}) \times \text{V/g}$$

where A645 and A663 represent the optical density at specific wavelength, V represents the volume of the extract (mL), and g represents sample s weight (mg).

Carotenoids (Caro) content. The extraction of Caro was carried out with acetone. After the separation, the organic phase was dried over anhydrous sodium sulfate until the solvent evaporated. Finally, the residue was dissolved in a known volume of hexane, and the measurements were recorded at 450 nm absorbance level using the Perkin Elmer Lambda 25 spectrophotometer. The concentration of total Caro was calculated according to the formula to follow:

$$\text{X mg carotenoids} = (\text{A} \times \text{V} \times 1000)/(\text{A 1\% 1 cm} \times 100)$$

where A represents absorbance at 450 nm, V represents volume (mL), and A1% 1 cm = 2500 and expressed as mg Caro/g fresh material [37]. All experiments were performed under subdued light.

2.4. Statistical Analysis

One-way analysis of variance (ANOVA) was performed for treatments within one cultivar and for all the cultivars within one treatment to investigate whether the differences in physiological parameters and photosynthetic pigments of the in vitro plants were affected by the presence of different concentrations of PEG 6000 added to the culture media. Post hoc testing for the ANOVAs was performed using Tukey's honestly significant difference test (Tukey test) using a $p < 0.05$ significance level to determine the statistically significant differences between the means. Values shown (in text and figures) are means ± SE (standard error). In addition, the variables determined in plants submitted to PEG 6000 treatments (SL, SN, PR, FW, WC, Chl a, Chl b and Caro) were correlated by principal component analysis (PCA) for all of the five studied *V. corymbosum* L. cultivars. The drought tolerance efficiency and parameters recorded in plants from control and PEG 6000 treatments were correlated by principal component analysis (PCA) and heat map analysis, using the XLSTAT (New York, NY, USA) and OriginPro 8.0 software (Northampton, MA, USA), respectively.

3. Results

3.1. In Vitro Growth Parameters

The obtained results show that the simulated hydric stress in the in vitro cultures of blueberries influenced the growth parameters for all five varieties studied.

The general appearance of in vitro proliferated shoots was affected by the presence of PEG 6000 in culture media; the most important changes were observed regarding the length of shoots with shorter inter-nodes and smaller leaves, as shown in Figure 1.

It has been noticed that PEG 6000 supplemented media affected the length of shoots from an average concentration of 10 g/L (Figure 2a). All the studied varieties presented shoots with the highest length when grown in a culture media without PEG 6000 with a length variating between 2.16 ± 0.29 cm (Brigitta Blue) and 1.85 ± 0.12 (Goldtraube). As expected, the length of the shoots decreased with the increase in PEG concentrations. Brigitta Blue reported the shortest shoots (0.91 ± 0.06 cm) for a concentration of PEG below 50 g/L alongside Hortblue Petite, where the shortest shoots (0.96 ± 0.07 cm) were observed for a concentration of PEG below 30 g/L (Figure 2a).

The number of shoots that were obtained in each culture vessel proved to be significantly higher on PEG supplemented media, at any given concentration, and for all varieties, as shown in Figure 2b. The analyses showed that the control variety had the lowest number of shoots/culture in the container: 32.33 ± 0.44 for Duke and 152.66 ± 5.85 for Brigitta Blue. Remarkably, the highest number of shoots/culture recipients were obtained at lower concentrations of PEG (10, 20 and 30 g/L). It was noticed, as well, that the number of shoots/culture in each vessel started to decrease at higher concentrations of PEG (40 and 50 g/L). For instance, at concentrations below 10 g/L of PEG, Brigitta Blue proved to have the highest number of shoots/culture recipients (375.33 ± 3.18), whereas levels of 50 g/L PEG resulted in the lowest number of shoots/culture container (200.66 ± 1.59).

Similarly, PEG 6000 had a positive impact on the proliferation rate/inoculum. The highest proliferation rate was obtained for PEG concentrations below 10, 20 and 30 g/L, as illustrated in Figure 2c. Higher concentrations (40 and 50 g/L) of PEG resulted in a lower proliferation rate. Withal, at higher concentrations of PEG, the proliferation rate was higher than in the control. The highest proliferation rates were recorded in Brigitta Blue: 21.75 ± 1.29 for a PEG concentration of 10 g/L and 16.85 ± 2.28 for a PEG concentration below 50 g/L compared to 9.69 ± 0.66 control. The Duke variety recorded the lowest proliferation rate.

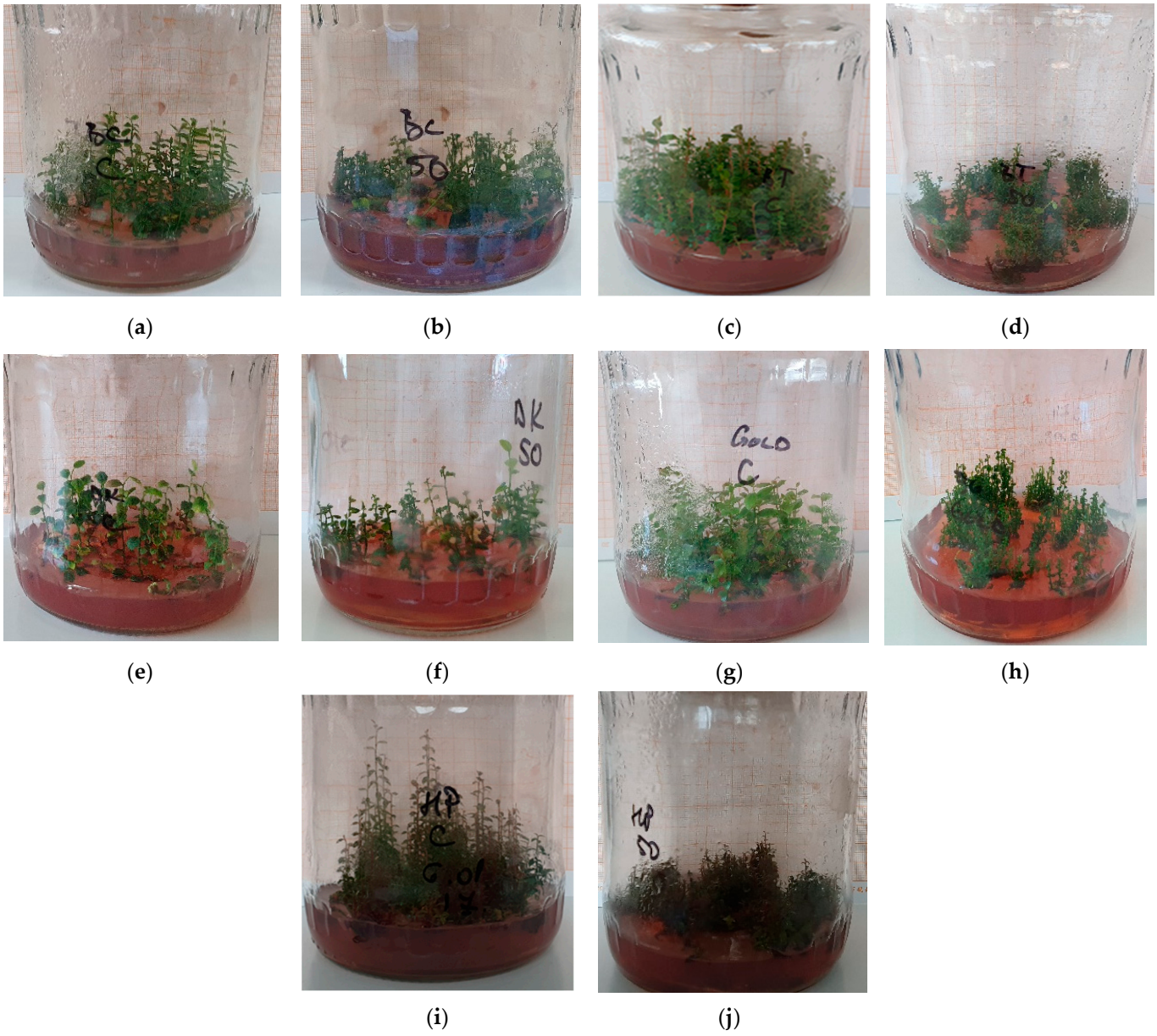
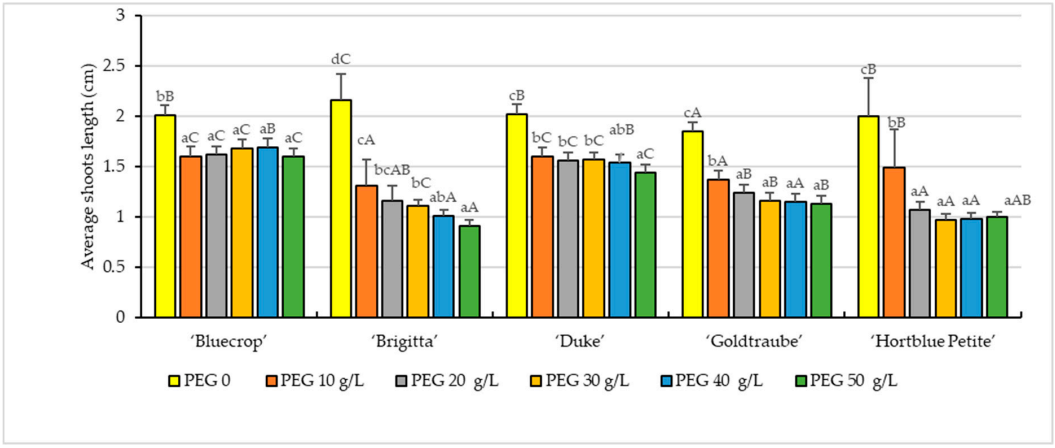


Figure 1. Effect of different concentration of polyethylene glycol (PEG-6000) on the growth and appearance of the in vitro culture of blueberry cultivars: (**a**) Bluecrop—WPM + 0 g/L PEG 6000; (**b**) Bluecrop—WPM + 50 g/L PEG 6000; (**c**) Brigitta Blue—WPM + 0 g/L PEG 6000; (**d**) Brigitta Blue—WPM + 50 g/L PEG 6000; (**e**) Duke—WPM + 0 g/L PEG 6000; (f) Duke—WPM + 50 g/L PEG 6000; (**g**) Goldtraube—WPM + 0 g/L PEG 6000; (**h**) Goldtraube—WPM + 50 g/L PEG 6000; (**i**) Hortblue Petite—WPM + 0 g/L PEG 6000; (**j**) Hortblue Petite—WPM + 50 g/L PEG 6000. Minishoots with length of 1.5–2 cm (16 inoculs/jar) of the five blueberry cultivars were used in this study, and polyethylene glycol (PEG 6000) was added to the medium [Woody Plant Medium (WPM) + 100 mg/L Sequestren 138 + 1 mg/L zeatin (Z) + 4 g/L Plant agar, pH = 5] at different concentrations (0 g/L, 10 g/L, 20 g/L, 30 g/L, 40 g/L, 50 g/L) to induce drought stress. Period of growth 12 weeks (22 ± 1 °C, 32.4 mmol·m^{-2}·s^{-1}, 16-h photoperiod).



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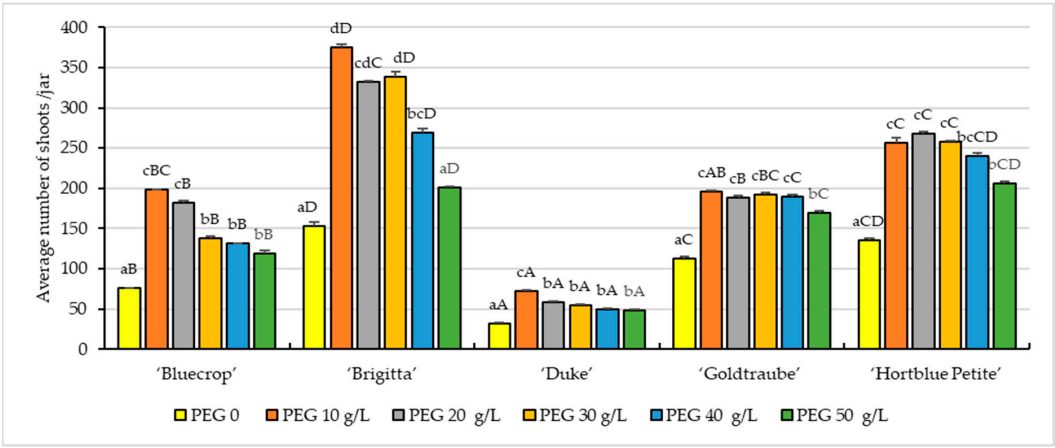

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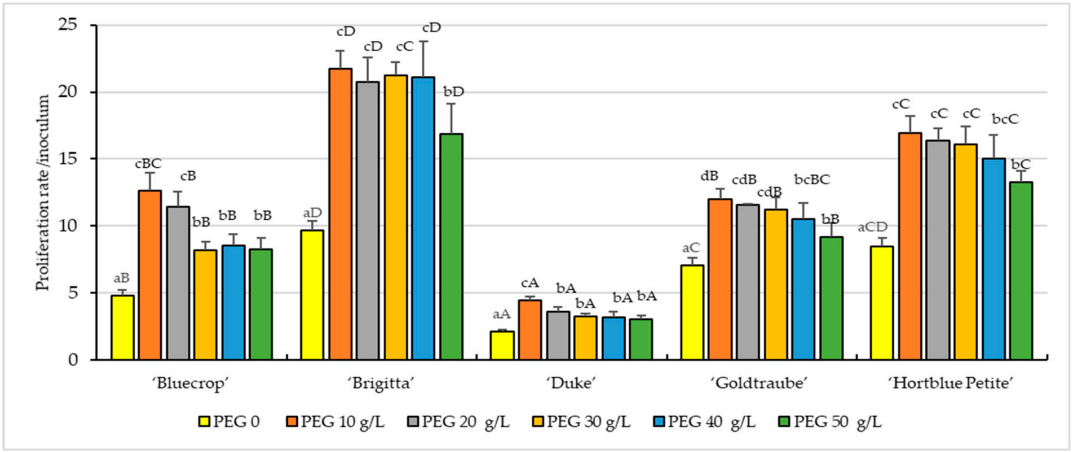
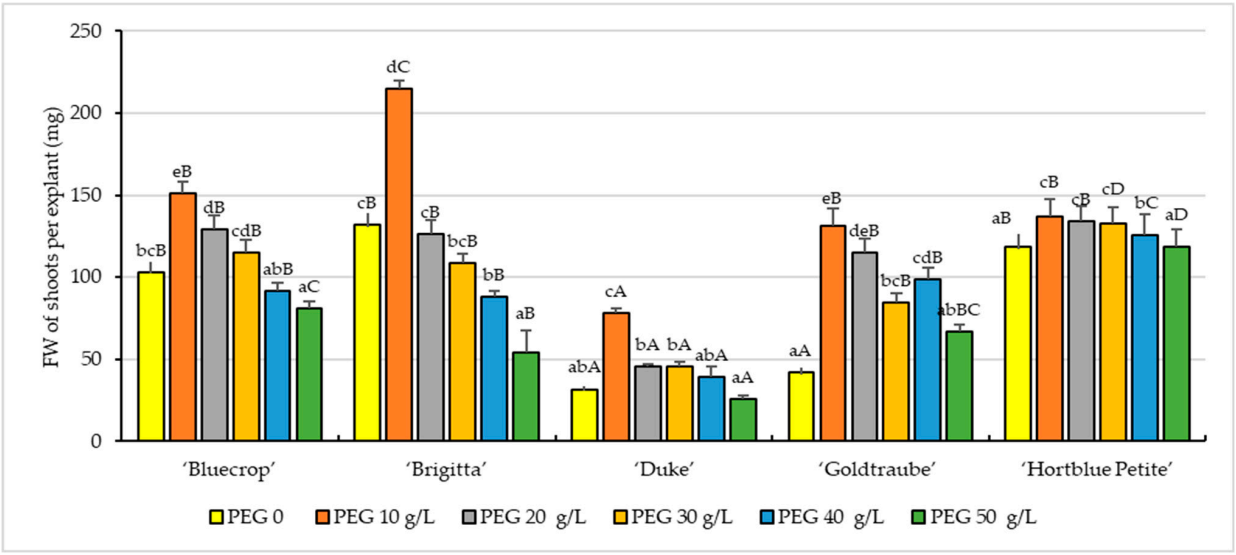


(**c**)

Figure 2. Effects of drought stress on (**a**) shoot length (cm), (**b**) average number of shoots/culture vessel, and (**c**) proliferation rate of five in vitro-grown highbush blueberry cultivars (Bluecrop, Brigitta Blue, Duke, Goldtraube and Hortblue Petite). For inducing the drought stress in culture media [Woody Plant Medium (WPM) + 100 mg/L Sequestren 138 + 1 mg/L zeatin (Z) + 4 g/L Plant agar, pH = 5] were added different concentrations polyethylene glycol (PEG 6000) (0 g/L, 10 g/L, 20 g/L, 30 g/L, 40 g/L, 50 g/L). Error bars indicate mean ± SE, and different lowercase letters within each cultivar indicate significant differences among the treatments and different capital letters indicate significant differences among the cultivars undergoing the same treatment according to Tukey's HSD test ($p < 0.05$).

Growth parameter measurements, in terms of fresh weight, were taken at the end of the 12-week culture cycle. After weighing the freshly removed inoculums from the cultured media, the results showed that concentrations of PEG below 10 g/L increased fresh weight in all tested varieties. The weight proved to be significantly higher on 10 g/L PEG cultured media than in the control (Figure 3a). However, higher concentrations of PEG (above 10 g/L) resulted in a decrease in fresh weight. At PEG concentrations below 50 g/L fresh weight had a significantly lower value than in the Bluecrop control (81.16 ± 4.35 mg compared to 103.01 ± 6.34 mg) and in Brigitta Blue (54.28 ± 3.02 mg compared to 131.61 ± 7.11 mg). For the other varieties, the differences between control and PEG concentrations of 50 g/L were not statistically significant.



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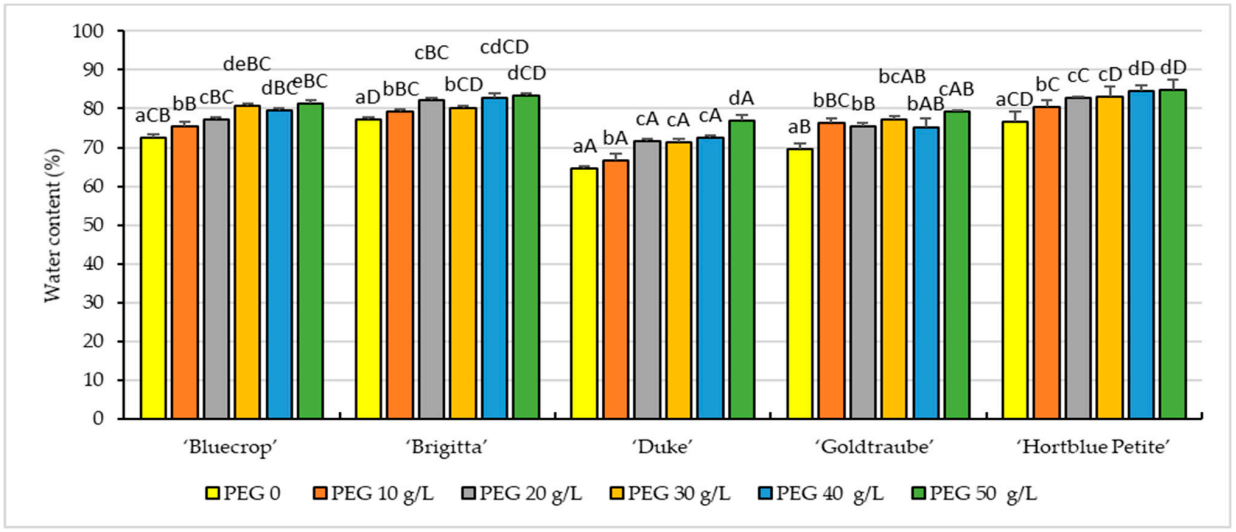


(**b**)

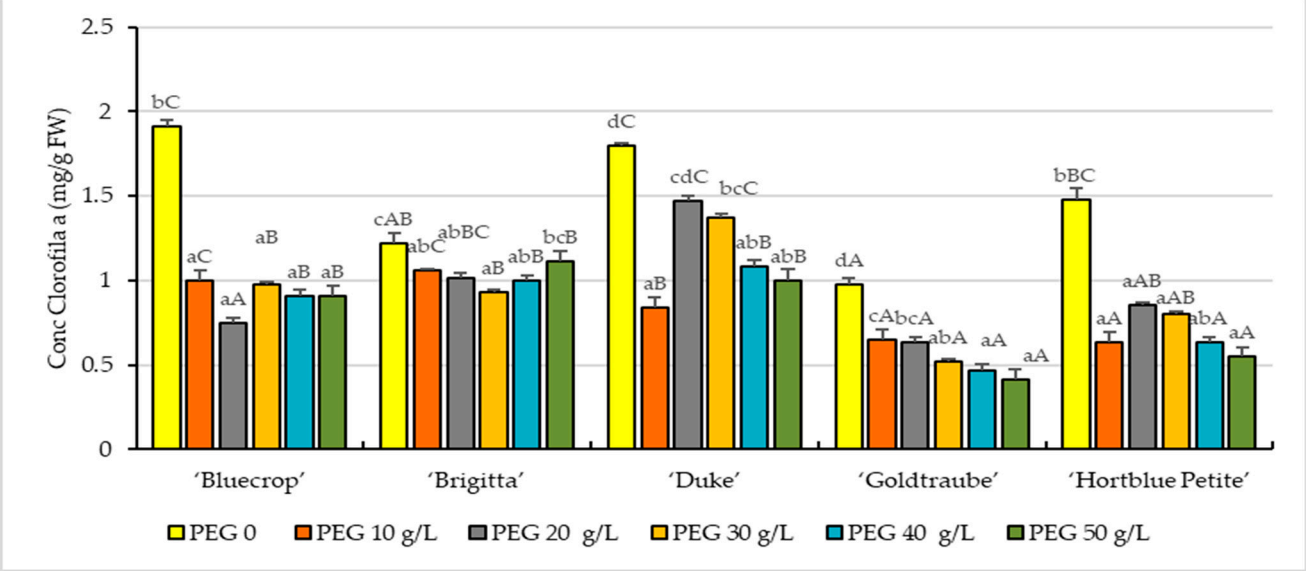
Figure 3. Effects of drought stress on (**a**) fresh weight (FW), (**b**) water content (WC) of five in vitro-grown highbush blueberry cultivars (Bluecrop, Brigitta Blue, Duke, Goldtraube and Hortblue Petite). For inducing the drought stress in culture media [Woody Plant Medium (WPM) + 100 mg/L Sequestren 138 + 1 mg/L zeatin (Z) + 4 g/L Plant agar, pH = 5] were added different concentrations polyethylene glycol (PEG 6000) (0 g/L, 10 g/L, 20 g/L, 30 g/L, 40 g/L, 50 g/L). Error bars indicate mean ± SE, and different lowercase letters within each cultivar indicate significant differences among the treatments and different capital letters indicate significant differences among the cultivars undergoing the same treatment according to Tukey's HSD test ($p < 0.05$).

The total water content was determined after drying the previously weighed leaves until a constant weight was achieved. The determinations revealed an increasing tendency in water content with the increasing of PEG 6000 concentrations for all the studied blueberry varieties (Figure 3b). Duke was the variety with the lowest parameter value for both control and PEG supplemented media (for all concentrations). In this case, the water content of the proliferated shoots in the in vitro multiplication phase showed an increase from 64.44 ± 0.59% for control to 76.88 ± 1.38% on media supplemented with 50 g/L PEG. Interestingly, Hortblue Petite had the highest water content for both PEG-free and PEG-supplemented media, showing statistically significant differences when compared to Duke.

It is noteworthy that on the culture medium without PEG 6000, all proliferated shoots had a healthy appearance in all varieties, whereas PEG concentrations caused some hyperhydricity symptoms.

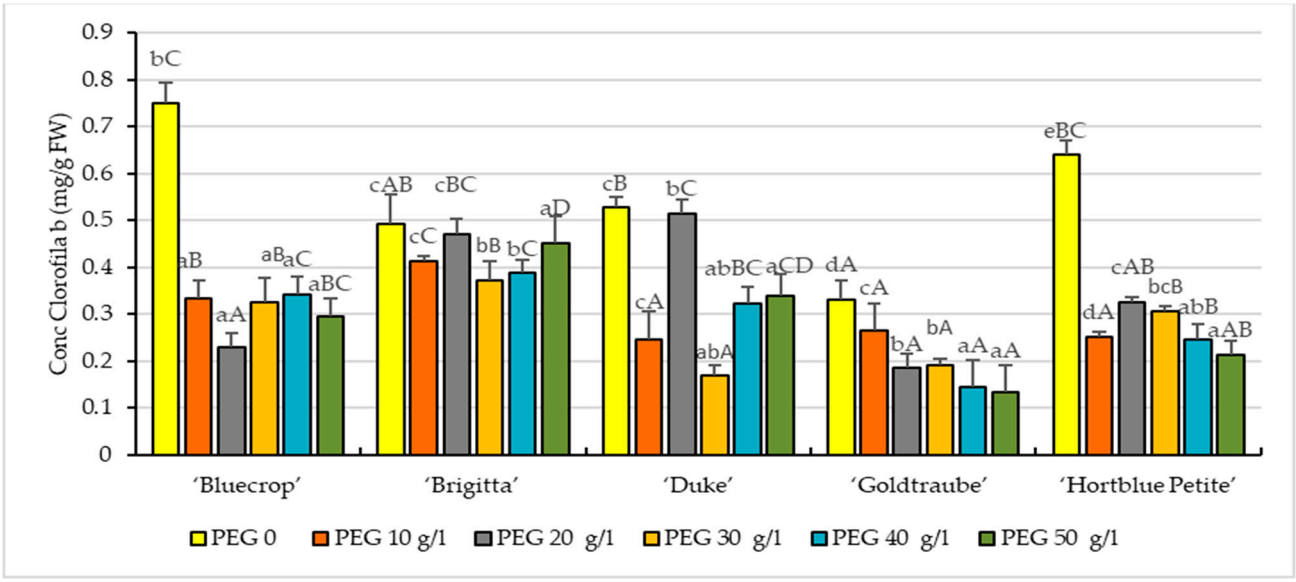
3.2. Photosynthetic Pigments

As shown in Figure 4, an increase in PEG 6000 concentrations resulted in a decrease in photosynthetic pigments. Lower levels in chlorophyll a and chlorophyll b and in carotenoids were observed in all the studied blueberry varieties. Shoots from the PEG-free culture media showed the highest level in all photosynthetic pigments for all varieties. The highest values were recorded in Bluecrop: 1.91 ± 0.04 mg/g FW chlorophyll a, 0.75 ± 0.04 mg/g FW chlorophyll b and 1.19 ± 0.02 mg/g FW for carotenoids. The lowest chlorophyll levels were identified in the shoots of Goldtraube for both PEG-free and PEG-supplemented media, as shown in Figure 4a,b. Regarding carotenoids, Duke grown on supplemented media with 30 g/L PEG recorded the lowest value: 0.31 ± 0.07 mg/g FW.



(**a**)

Figure 4. *Cont.*



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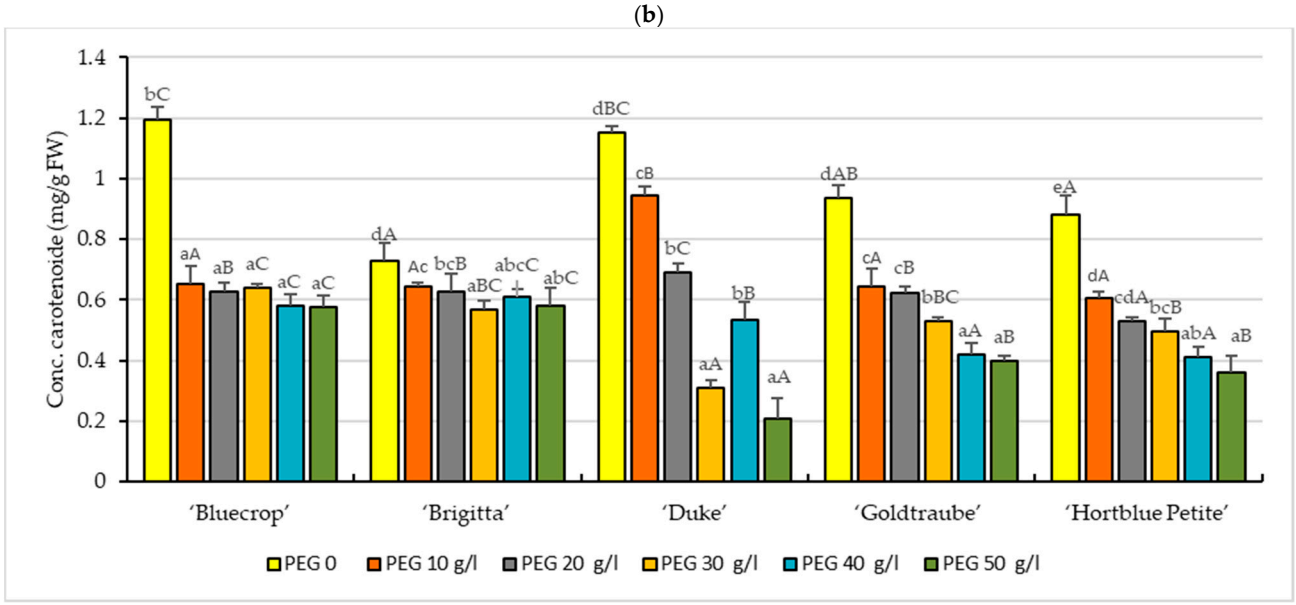


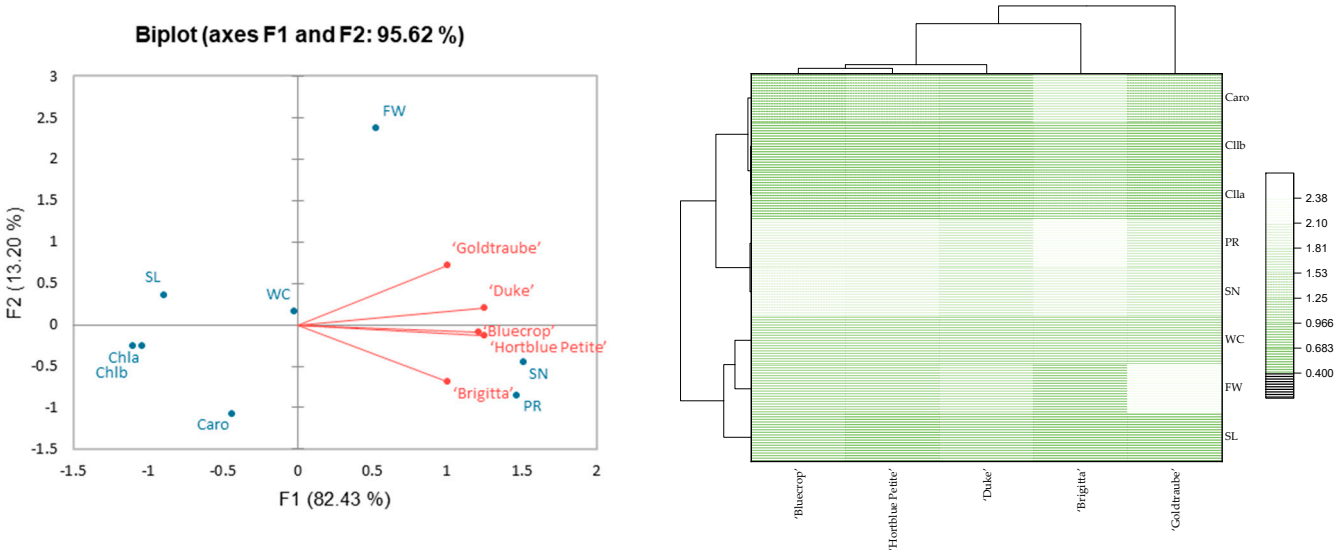
(**c**)

Figure 4. Photosynthetic pigments in the in vitro shots of the five highbush blueberry cultivars (Bluecrop, Brigitta Blue, Duke, Goldtraube and Hortblue Petite.): (**a**) chlorophyll a (Chl a), (**b**) chlorophyll b (Chl b) and (**c**) carotenoids (Caro) contents after 12 weeks of applied different concentrations polyethylene glycol (PEG 6000) (0 g/L, 10 g/L, 20 g/L, 30 g/L, 40 g/L, 50 g/L). Error bars indicate mean ± SE, and different lowercase letters within each cultivar indicate significant differences among the treatments and different capital letters indicate significant differences among the cultivars undergoing the same treatment according to Tukey's HSD test ($p < 0.05$).

3.3. Principal Component Analysis (PCA)

The evolution of the eight growth and physiological indicators (SL, SN, PR, FW, WC, Chla, Chlb and Caro) under PEG-induced water stress was analyzed by the main component PCA analysis (Figure 5a) based on drought tolerance efficiency (DTE). DTE was calculated as the quotient of the mean of five PEG 6000 induced drought treatments (10. 20, 30, 40, 50 mg/L) divided by the control (Supplementary Table S1). The effect of water stress was also assessed by PCA for each parameter (Supplementary Figure S1). Assessing DTE using PCA revealed a total variability of about 95.62% explained by two components (F1 and F2). The first component (*X*-axis) alone explains 82.43% of the variance.

All cultivars showed acute angles compared one to another, indicating a similar pattern in response to the applied treatments, as shown in Figure 5a. In addition, a heat map of Pearson correlation analysis and a dendrogram clustering were constructed on the basis of the variables in five highbush blueberry cultivars grown under different drought levels (Figure 5b).



(**a**) (**b**)

Figure 5. (**a**) Principal component analysis biplot obtained for five highbush blueberry cultivars (Bluecrop, Brigitta Blue, Duke, Goldtraube Hortblue Petite) and drought tolerance efficiency calculated on the basis of the eight growth and physiological indicators: SL, SN, PR, FW, WC, Chla, Chlb and Caro. The first two components explained 95.62% of the data variance. (**b**) Hierarchical clustering and heatmap visualization of the highbush blueberry cultivars based on the drought tolerance efficiency. Columns indicate the highbush blueberry cultivars and rows indicate the drought tolerance efficiency which was calculated for the eight growth and physiological indicators: SL, SN, PR, FW, WC, Chla, Chlb and Caro. The white and green colored cells represent the positive correlation between samples. The dendrogram of the rows resulted from the correlation between the drought tolerance efficiency for the eight growth and physiological indicators; the column dendrogram showed the correlation between the five highbush blueberry cultivars. Different concentrations of polyethylene glycol (PEG 6000): 0 g/L, 10 g/L, 20 g/L, 30 g/L, 40 g/L, 50 g/L. were added to induce drought stress in the culture medium [Woody Plant Medium (WPM) + 100 mg/L Sequestren 138 + 1 mg/L zeatin (Z) + 4 g/L Plant agar, pH = 5]. After 12 weeks treatment (22 ± 1 °C, 32.4 mmol·m^{-2}·s^{-1}, 16-h photoperiod), 8 indices were measured and drought tolerance efficiency of each cvs. of each index were calculated: SL-shoot length (cm); SN-average number of shoots/culture vessel), (PR-proliferation rate; FW-fresh weight (mg); WC-Water content (%); Chla-chlorophyll a (mg/g FW); Chlb-chlorophyll b (mg)/g FW); Caro-Carotenoid (mg/g FW).

4. Discussion

In recent years, Europe has been subjected to severe droughts during the summertime in many of its regions. Because of this undesirable situation, it is essential to understand how several commercial varieties respond to drought conditions before they reach the field, especially because expended areas confront water deficits, mostly during critical stages in plant development.

Blueberry is a highly sensitive species to water deficit, and because of this fact, the plants are mainly grown under irrigation [38,39]. Furthermore, dry years are more frequent due to climate change, and therefore, the probability of water deficit in blueberry plants is increasing [36]. As a consequence, the cultivation of drought-resistant blueberry varieties and the development of new cultivars that are hydric-stress resistant is of major importance.

Drought stress causes a number of morphological, physiological and biochemical changes in plants. Drought stress reduces leaf size, stem extension and root proliferation, disturbs plant water relations and reduces water-use efficiency. It disrupts photosynthetic pigments and reduces the gas exchange leading to a reduction in plant growth and productivity [40].

In the present study, the possible drought tolerance of five blueberry varieties was tested by examining their morphological, physiological and biochemical parameters subjected to drought-like stress conditions obtained by adding five concentrations of PEG 6000 to in vitro culture media. Currently, there is a large variety of experimental drought models based on osmotic stress, and established by supplementation of growth medium with polyethylene glycol (PEG), which can be classified as soil-based, aqueous-culture or agar-based; however, research shows that the agar-based PEG infusion model has several advantages [14].

Previous reports indicate that in the in vitro culture simulation of drought stress using chemical reagents, such as PEG, constitutes a convenient way to assess the effects of drought on plant growth and development [15,41]. For example, drought tolerance of five apples and five cherry cultivars was determined via induced osmotic stress. PEG-6000 was added at a concentration of 0, 5, 10, 25, and 50 g/L to the medium. Physiological and biochemical parameters under progressive drought-like stress conditions showed that plant wilting, leaf twisting, and reduction in water content, are among the primary manifestations of drought stress [22].

In this study, four growth indicators, shoot length (SL), the average number of shoots/culture vessel (SN), proliferation rate (PR) and fresh weight (FW), were influenced by in vitro simulated drought stress. A single growth indicator (SL) decreased with increased PEG concentrations (Figure 2a) in all the five studied varieties indicating the damaging effects of PEG-induced drought stress. Regarding SL, the most affected variety was Brigitta Blue which presented shoots under PEG 50 were 2.38 times shorter than the control without PEG, and the least affected was Bluecrop, in which under PEG 50, the shoots were 1.26 times shorter than in control without PEG. Shoot growth was inhibited by the concentration of PEG in culture media in other species also under drought-like stress conditions induced by PEG. As shown in *Arachis hypogaea* L cultivated in vitro under conditions of drought stress, maximum shoot height (5.4 cm) was observed in the control treatment (medium devoid of PEG), and among the PEG treatments, medium supplemented with 60 g/L PEG recorded the least shoot height (1.6 cm) [42]. Similarly, PEG-induced water stress negatively affected shoot length as seen at the cherry rootstock Gisela 5 (significant decrease being observed at 2% and 4% PEG) [7] as well as in cactus [43], stevia [44], vanilla [45], myrtle [46], thyme [47], and strawberry [48].

Water stress greatly suppresses cell expansion and cell growth due to the low turgor pressure [49]. Thus, lack of water availability and associated decrease in nutrient availability led to a reduction in cell multiplication that resulted in an overall reduction in offshoot length.

Growth indicators such as SN, PR, FW had their maximum values under different treatments with PEG (Figure 2). Analyzing these indicators, there was a general tendency to increase the values of SN, PR, FW when treatments below concentrations of 10 and 20 PEG were applied. Further, a decrease in the growth indicators was observed below 30, 40 and 50 PEG. For example, in Bluecrop below 10 PEG, PR increased 2.6 times compared to control while under 50 PEG PR increased 1.71 times. At the other end of the spectrum was Goldtraube, where PR heightened 1.7 times below 10 PEG and 1.3 times below 50 PEG. This tendency to increase the number of shoots has also been reported in *Stevia rebaudiana* Bertoni [50], which at 4% of PEG concentration has been proven very effective in increasing the organogenesis of the shoot. As well, PEG treatments significantly increased shoot regeneration, and shoot number was significantly higher in *Prunus dulcis* (Mill.) [51]. In other species subjected to drought stress simulated with different concentrations of PEG, the total number of shoots was negatively affected by PEG content in the medium, as

well as the number of shoots was reduced for explants that were cultured in the media containing PEG compared to the PEG-free control explants. Supplementation of cultures with a high PEG concentration (50 g/L) in *Dendrobium officinale* induced a significant decrease in multiplication rate [52].

Aazami et al. [53] showed that the shoot number from cotyledonary nodal explants of four tomato cultivars was decreased with increasing PEG concentrations; in all cultivars, the number of shoots in each explant was significantly decreased as the PEG level increased. Stimulation of lateral shoot regeneration of the drought-sensitive genotypes under drought conditions was probably due to growth inhibition of shoot apical meristem, or necrosis and decay of shoot apical meristem [51].

Reduction in fresh and dry biomass is another typical physiological response of plants to drought stress that represents the unfavorable impact of water stress [22]. A decrease in fresh weight was observed in several plant species, with increasing concentration of PEG, such as in Actinidia species [54], *Physalis angulata* L. [55], *Carrizo citrange* [56], eight rice lines [57] and apple and cherry cultivars [22]. Contrary to these results, in the present study, FW had the highest value under PEG 10 for all blueberry varieties. Fresh weight decreased with increasing PEG concentration. Thus, under PEG 20 and 30, four of the varieties (Bluecrop, Duke, Goldtraube, Hortblue Petite) had higher FW than control, and under PEG 40, only three varieties (Duke, Goldtraube, Hortblue Petite) had FW higher than control. Regarding Goldtraube variety, FW increased in comparison with the control by 3.13 times below the concentration of PEG 10 and by 1.59 times below PEG 50. Hortblue Petite variety presented a relatively constant FW value under all treatments (1.16 times higher below 10 PEG).

In the present study, the minimum fresh weight (FW) of shoots (0.16 g) were shown by the MS medium lacking PEG 6000. In a previous study, the fresh weight (FW) of *Stevia rebaudiana* shoots (0.52 g) were found under treatment supplemented with PEG stress of 4% concentration, followed by 2%, 1%, and 0.5% of PEG 6000 concentration [50].

In the same manner, the water content is a parameter affected by water deficit conditions. Sivritepe et al. [7] reported that water stress induced by the incorporation of 1, 2 and 4 % polyethylene glycol (PEG-8000) into the MS medium was applied for 6 weeks on sweet cherry (*Prunus cerasus* × *P. canescens*) rootstock Gisela 5 lead to a progressive decrease in WC. Similarly, the WC in the tissues in both fruit species was negatively affected with an increase in PEG concentration at all apple and cherry cultivars [22]. Contrary to these results, in this experiment, WC increased in all varieties with increasing PEG concentration (Figure 3a). This aspect has also been reported in *Dendrobium officinale* cultured in vitro under PEG-6000 (10, 30, or 50 g/L) [52] and *Beta vulgaris* L. cv. Felicita under different PEG 6000 treatments [58]. These studies have indicated that PEG-6000 at various concentrations increase the hyperhydric rate, which explains the increase in water content in plants under water stress induced by PEG.

Drought stress also affects the composition of the photosynthetic pigment, namely chlorophyll a and chlorophyll b and carotenoids. Therefore, this indicator is considered an important marker for the functional condition of plants in drought conditions [14]. Furthermore, when experiencing water stress, plant tissue is dehydrated, which can result in an increase in oxidative stress and oxidative stress is associated with a loss of chlorophyll [59]. A significant reduction in photosynthetic pigments under water stress conditions induced by PEG has been reported for many plant species [44,45,60–62]. Similarly, in our study, increasing PEG concentration resulted in a decrease in photosynthetic pigments for all the studied varieties. It is noteworthy that, in terms of these parameters, Goldtraube was the most sensitive to water stress induced by PEG (Figure 4).

An important aspect was the relevance between tissue culture evaluation and field performance. Similar results were reported [36] for four groups of highbush blueberry plants Brigitta Blue, propagated through different vegetative methods (stem cutting and tissue culture). To induce slight and moderate drought stress, two PEG 8.000 concentrations (3% and 7%) were tested and deionized water (0%) was used as a control. Each pot

was watered with 20 mL PEG solution or 20 mL water (control) at one-day intervals for three weeks. As in the case of in vitro cultures, in culture media supplemented with PEG, and in the present study as well, the most affected parameter was the growth in the height of the shoots. This aspect has also been highlighted for other species subjected to simulated water stress in vitro, and in the field with PEG [54].

The heat map and PCA verified the results obtained by a means comparison of stressed and non-stressed plants. The results of the heat map and PCA clearly and briefly summarize the pattern of the five studied cultivars, which responded very similarly to the increasing PEG 6000 concentrations in the culture media (Figure 5 and Supplementary Figure S1).

In conclusion, we can say that all five blueberry varieties were affected by the presence of different concentrations of PEG 6000 added to the culture media. Parameters adversely affected by drought-like stress conditions induced by PEG were chlorophyll a, chlorophyll b, carotenoids content followed by shoot length. However, the heat map analysis (Figure 5b) grouped the five varieties into two groups, namely Goldtraube, which had the highest DTE, followed by the second group: Bluecrop, Hortblue Petite, Duke and Brigitta Blue. This grouping is supported by biplot PCA (Figure 5a). To the best of our knowledge, this is the first report that brings to the spotlight how five in vitro blueberry cultivars (Bluecrop, Brigitta Blue, Duke, Goldtraube and Hortblue Petite) react to PEG 6000 induced water-stress. Our findings suggest that even though at lower concentrations of PEG plants are able to adapt and try to overcome the water-stress, higher PEG concentrations are not well tolerated.

Drought-stress resistant blueberry varieties should be further studied in order to obtain enhanced and sustainable large-scale production. More studies are needed to select the most promising plants that are water-stress resistant and can withstand drought, as drought is becoming more frequent globally. It appears to be worthwhile to continue further studies using these methods at various varieties in order to make a more in-depth analysis.

This study showed that PEG 6000 is an effective way to discover which blueberry varieties are more severely affected by drought, and it could prove of high importance in time and cost reduction for further studies.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy12030732/s1, Table S1: Drought tolerance efficiency of five *V. Corymbosum* cultivars under drought stress. Different concentrations of polyethyleneglycol (PEG 6000): 0 g/L, 10 g/L, 20 g/L, 30 g/L, 40 g/L, 50 g/L. were added to induce drought stress in the culture medium [Woody Plant Medium (WPM) + 100 mg/L Sequestren 138 + 1 mg/L zeatin (Z) + 4 g/L Plant agar, pH = 5]. After 12 weeks treatment (22 ± 1 °C, 32.4 mmol·m^{-2}·s^{-1}, 16-h photoperiod), 8 indices were measured and drought tolerance efficiency of each species of each index were calculated. [SL—shoot length (cm); SN—average number of shoots/culture vessel), (PR—proliferation rate; FW—fresh weight (mg); WC—Water content (%); Chla—chlorophyll a (mg/g FW); Chlb—chlorophyll b (mg)/g FW); Caro—Carotenoid (mg/g FW)]. Figure S1: Principal component analysis. Changes in all the measured parameters with respect to the control, PEG-free medium in five cultivars of *V. corymbosum* L. in correlation to the PEG 6000 concentration of the culture medium. The parameters examined were: (a) shoot length (cm); (b) average number of shoots/culture vessel; (c) proliferation rate; (d) fresh weight (mg); (e) Water content (%); (f) chlorophyll a (mg/g FW); (g) chlorophyll b (mg/g FW); (h) Carotenoid (mg/g FW)]. Different concentrations of polyethyleneglycol (PEG 6000): 0 g/L, 10 g/L, 20 g/L, 30 g/L, 40 g/L, 50 g/L. were added to induce drought stress in the culture medium [Woody Plant Medium (WPM) + 100 mg/L Sequestren 138 + 1 mg/L zeatin (Z) + 4 g/L Plant agar, pH = 5] and mini shoots of 1.5–2 cm from five highbush blueberry cultivars (Bluecrop, Brigitta Blue, Duke, Goldtraube and Hortblue Petite were inoculated. After 12 weeks of this conditions (22 ± 1 °C, 32.4 mmol·m^{-2}·s^{-1}, 16-h photoperiod) 8 indices were measured.

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