horticulturae

MDPI

Article

In Vitro Establishment of 'Delite' Rabbiteye Blueberry Microshoots

Carolina Smanhotto Schuchovski and Luiz Antonio Biasi *

Departamento de Fitotecnia e Fitossanitarismo, Universidade Federal do Paraná, Rua dos Funcionários, 1540. CEP 80.035-050, Curitiba, Brazil; carolina.sschu@gmail.com

* Correspondence: biasi@ufpr.br; Tel.: +55-41-3350-5682

Received: 18 January 2019; Accepted: 25 February 2019; Published: 8 March 2019

Abstract: Micropropagation is an important technique for clonal mass propagation and a tool for in vitro studies. One of the first steps to overcome in this process is the establishment of new explants in vitro. 'Delite' rabbiteye blueberry was cultured in vitro with four cytokinins (zeatin (ZEA), 6-(γ-γ-dimethylallylamino)-purine (2iP), 6-benzylaminopurine (BAP), and kinetin (KIN)) at eight concentrations (0, 2.5, 5, 10, 20, 30, 40, and 50 μM). Additionally, nine combinations of nitrogen salts were tested, using Woody Plant Medium (WPM) and a modified WPM as the basic medium. ZEA and 2iP showed better responses, but ZEA was superior at lower (2.5 μM) concentrations (89.7% survival, 81.3% shoot formation, 1.3 shoots, 13.8 mm shoot length, 10.0 leaves). BAP and KIN showed very low responses. In the combinations of salts with modified WPM, no differences were observed. However, the original WPM with treatments of 0.5 × NH_4NO_3 and 1 × $Ca(NO_3)_2$, 0.5 × NH_4NO_3 and 0.5 × $Ca(NO_3)_2$, and the modified WPM alone showed the lowest rates of survival and shoot formation and the shortest shoot lengths. The highest shoot lengths were observed in treatments with the original WPM, 1.5 × NH_4NO_3 and 0.5 × $Ca(NO_3)_2$, and 1.5 × NH_4NO_3 and 1.5 × $Ca(NO_3)_2$. This initial study with 'Delite' can be the basis for further experiments with different combinations of salts, 2iP, and ZEA.

Keywords: *Ericaceae*; *Vaccinium virgatum*; micropropagation; in vitro culture; cytokinins; zeatin; 2iP; BAP; kinetin; WPM

1. Introduction

Blueberry is a woody perennial species in the family Ericaceae and genus *Vaccinium*. The fruit is a true berry with many seeds, with color ranging from light blue to black and a waxy cuticle layer [1]. Blueberry has been gaining great importance in fruit production, especially because of its recognized taste properties and its nutraceutical qualities as an anti-inflammatory and anti-oxidant, being a health-promoting food [2]. Blueberry fruits are rich in polyphenols [3]. These blueberry polyphenols show anti-inflammation activity, related to the balances in pro-inflammatory cytokines, and they could be used as an anti-inflammatory medicine [4]. Among the phenolic compounds that appear at high levels in blueberries are anthocyanins [5], flavonols, and phenolic acids [6]. The anthocyanin found in high amounts in blueberries contributes to preventing several chronic diseases, such as neurodegenerative diseases, cardiovascular disorders, cancer, and diabetes [7].

Much research has been developed related to the propagation of blueberries. Traditionally, blueberry is propagated by softwood, semi-hardwood, and hardwood cuttings [8] or even rhizome cuttings of selected clones [6]. Some challenges in this production are a very low rooting percentage in many genotypes, the amount of time required to propagate and commercialize newly-released cultivars for mass propagation [8,9], and phytosanitary problems. In vitro culture (micropropagation) can overcome the limitations of traditional cuttings, presenting an alternative for faster growth [10]

throughout the year (with no seasonal effects) without pathogens [11]. There are some studies on the in vitro propagation of *Vaccinium* species, but only some of these have been done with rabbiteye (*V. virgatum* Ait. (syn. *V. ashei* Reade)), specifically for the 'Delite' rabbiteye cultivar that is suitable for and adapted to regions of southern Brazil. For this specific cultivar, some research concerning in vitro protocol is still required to give more information on the optimal conditions for the development of this technique.

One crucial point in tissue culture techniques is the appropriate use, type, and concentration of growth regulators and the combination of culture medium salts that allows fast, efficient development of the initial explants. Understanding the interference of factors can lead to the development of further regeneration protocols that could be useful for either micropropagation or developing regeneration techniques necessary for plant recovery after cell transformation. There is some research showing that the lack of new shoot growth can make initiation the limiting step in establishing *Vaccinium* cultures in vitro [12]. Studies also show that new growth in vitro is difficult to achieve in *Vaccinium*, especially when using plant material from the field [13].

For the initial phase of in vitro culture, a combination of cytokinins can usually be used. In the initial in vitro culture in one study using nodal segments from softwood cuttings of 'Ozarkblue' highbush blueberry (*V. corymbosum*), zeatin (ZEA) and 6-(γ-γ-dimethylallylamino)-purine (2Ip) were tested in the initial culture medium in different combinations (18 μM ZEA, 25 μM 2-iP, and 9.1 μM ZEA combined with 25 μM 2iP) using WPM as the basal medium. On medium with ZEA present, shoots developed with green and red leaves. However, on medium containing only 2iP, shoots had light red leaves and callus at the base with stunted growth [9].

In lowbush blueberry (*V. angustifolium* Ait.) cultivated in the initiation phase medium containing 5 μM ZEA or 10 μM 2iP, explants produced elongated shoots with both growth regulators. However, ZEA treatments showed a higher percentage of new shoot growth compared to 2iP for three cultivars [6].

Wild bilberry (*V. myrtillus* L.) and lingonberry (*V. vitis-idaea* L.) were tested using buds and shoot tips on a modified MS medium supplemented with 2iP variations from 9.8 to 78.4 μM. For bilberry and lingonberry, the best results were obtained with 49.2 μM and 24.6 μM, respectively. Brownish explants were observed with an increasing 2iP concentration [13]. For 'Berkeley', 'Bluecrop' and 'Earliblue', highbush blueberries, and 'O'Neal' southern highbush blueberry, a medium containing 20 μM ZEA was used in the initiation of cultures [14].

Concerning the type of basal culture medium, many studies have used WPM as the basic medium for blueberry [14]. However, some authors have tried to optimize this medium by making some modifications, such as combining MS and WPM media, creating an MW medium [14], or proposing some changes in the components [15], leading to a modified WPM. A well-balanced medium is important to prevent stunted growth and physiological disorders [16]. Some authors have discussed the importance of the balance between nitrogen forms used in tissue culture (NO_3^- and NH_4^+) as much as the total amount of nitrogen in the culture medium [17].

The objective of this work was to determine an efficient growth regulator and balance of nitrogen salts for the establishment of 'Delite' microcuttings in in vitro culture.

2. Materials and Methods

In this work, three experiments in initial in vitro culture were designed. In the first one, four different cytokinins (ZEA, 2iP, 6-benzylaminopurine (BAP), and kinetin (KIN)) were tested at eight different concentrations. The second experiment tested nine different combinations of the nitrogen salts $(NH_4)_2SO_4$, KNO_3, and $Ca(NO_3)_2 \cdot 4H_2O$, using the modified WPM [15] as the basic medium. The third experiment tested nine different combinations of two nitrogen salts, NH_4NO_3 and $Ca(NO_3)_2 \cdot 4H_2O$, using the original WPM [18] as the basic medium and compared them with treatment 10 (modified Woody Plant Medium [15]).

2.1. Plant Material

One-year-old hardwood cuttings were collected during winter from field-grown rabbiteye blueberry 'Delite' mother plants at the Experimental Station of Universidade Federal do Paraná, Pinhais/PR. They were treated with an immersion in fungicide solution for 5 min (Cercobin®0.2%) and stored at a 4 °C temperature at the Micropropagation Laboratory, UFPR, Curitiba/PR for one to two months in plastic bags. Cuttings were placed in glass containers with water in the culture room at 25 °C ± 2 °C under cool day light at 40 µmol m^{-2} s^{-1} with a 16-h photoperiod. Newly formed shoots were collected and used as explants for the establishment of cultures.

Two-node segments (0.8–2 cm in length, discarding the apical portion of the donor-explant) were collected and surface sterilized with 70% (*v*/*v*) ethanol for 30 s, followed by immersion in 0.5% sodium hypochlorite solution containing 0.1% (*v*/*v*) Tween 20 for 5 min. They were washed with sterile deionized water three times inside the laminar flow chamber.

2.2. Culture Medium and Growing Conditions

Explants were isolated in culture tubes (150 × 30 mm), with each containing 6 mL of modified culture medium, differing in each of the three experiments. In all experiments, the medium was supplemented with Murashige and Skoog (MS) [19] vitamins, 30 g L^{-1} sucrose, 0.1 g L^{-1} myo-inositol, and 6 g L^{-1} agar (Vetec®). The pH of all media was adjusted to 5.2 before autoclaving at 120 °C and 1.5 atm.

2.2.1. Experiment 1: Cytokinins

Microcuttings were isolated in the modified WPM [2] (Table 1), supplemented as described above. Eight different concentrations (0, 2.5, 5, 10, 20, 30, 40, and 50 µM) of each of the four cytokinin growth regulators, ZEA, 2iP, BAP, and KIN, were tested, for a total of 32 treatments. ZEA and 2iP, when used, were sterilized through 0.22 µm filters and added to the cooled media. BAP and KIN were added to media before autoclaving.

Table 1. Modified Woody Plant Medium (modified WPM) [15] and original WPM [18] culture medium compositions.

Components	Modified WPM	Original WPM
Macronutrients	Final Concentration in the Culture Medium (mg L^{-1})	
$(NH_4)_2SO_4$	119.00	-
NH_4NO_3	-	400.00
KNO_3	893.00	-
K_2SO_4	-	990.00
KH_2PO_4	170.00	170.00
$Ca(NO_3)_2{\cdot}4H_2O$	278.00	556.00
$CaCl_2{\cdot}2H_2O$	-	96.00
$MgSO_4{\cdot}7H_2O$	370.00	370.00
Micronutrients	-	-
$FeSO_4{\cdot}7H_2O$	55.60	27.80
Na_2-EDTA	74.60	37.30
H_3BO_3	6.20	6.20
$MnSO_4{\cdot}H_2O$	22.30	22.30
$ZnSO_4{\cdot}7H_2O$	8.60	8.60
KI	0.415	-
$Na_2MoO_4{\cdot}2H_2O$	0.25	0.25
$CuSO_4{\cdot}5H_2O$	0.025	0.25

2.2.2. Experiment 2: Combinations of $(NH_4)_2SO_4$, KNO_3, and $Ca(NO_3)_2{\cdot}4H_2O$ Using the Modified WPM [15] as the Basic Medium

Explants were isolated using nine different treatments as described in Table 2, with different amounts (1×, 0.5× or 1.5×) of $(NH_4)_2SO_4$, KNO_3, and $Ca(NO_3)_2{\cdot}4H_2O$ (Table 2), using the modified

WPM [2] (Table 1) as the basic medium. Media were supplemented as described above, with the addition of cytokinin ZEA (5 µM).

Table 2. Experiment 2 with treatments 1 to 9 on the modified Woody Plant Medium (WPM) [15] with different amounts of $(NH_4)_2SO_4$ (x), KNO_3, and $Ca(NO_3)_2{\cdot}4H_2O$ (x).

Treatments	1 (Modified WPM)	2	3	4	5	6	7	8	9
NH_4: $(NH_4)_2SO_4$ (x)	1×	1×	1×	0.5×	0.5×	0.5×	1.5×	1.5×	1.5×
NO_3: KNO_3 and $Ca(NO_3)_2{\cdot}4H_2O$ (x)	1×	0.5×	1.5×	1×	0.5×	1.5×	1×	0.5×	1.5×
Components	**Final Concentration in the Culture Medium (mg L^{-1})**								
$(NH_4)_2SO_4$	119.0	119.0	119.0	59.5	59.5	59.5	178.5	178.5	178.5
KNO_3	893.0	446.5	1339.5	893.0	446.5	1339.5	893.0	446.5	1339.5
$Ca(NO_3)_2{\cdot}4H_2O$	278.0	139.0	417.0	278.0	139.0	417.0	278.0	139.0	417.0

2.2.3. Experiment 3: Combinations of NH_4NO_3 and $Ca(NO_3)_2{\cdot}4H_2O$ Using the Original WPM [1] as the Basic Medium

In this third experiment, explants were isolated in 10 different treatments described in Table 3. Nine treatments were used with different amounts (1×, 0.5× or 1.5×) of NH_4NO_3 and $Ca(NO_3)_2{\cdot}4H_2O$, using the original WPM [18] as the basic medium, and one treatment used the modified WPM [15] (Table 1). Media were supplemented as described above, with the addition of cytokinin ZEA (5 µM).

Table 3. Experiment 3 with 10 treatments. Treatments 1 to 9 with the original Woody Plant Medium (WPM) [18] with different amounts of NH_4NO_3 (x) and $Ca(NO_3)_2{\cdot}4H_2O$ (x) and treatment 10 with the modified WPM [15].

Treatments	1	2	3	4	5	6	7	8	9	10
NH_4NO_3 (x)	1×	1×	1×	1.5×	1.5×	1.5×	0.5×	0.5×	0.5×	-
$Ca(NO_3)_2{\cdot}4H_2O$ (x)	1×	0.5×	1.5×	1×	0.5×	1.5×	1×	0.5×	1.5×	-
Components	**Final Concentration in the Culture Medium (mg L^{-1})**									
NH_4NO_3	400.0	400.0	400.0	600.0	600.0	600.0	200.0	200.0	200.0	-
$Ca(NO_3)_2{\cdot}4H_2O$	556.0	278.0	834.0	556.0	278.0	834.0	556.0	278.0	834.0	278.0

2.3. Growing Conditions

After isolation, cultures were transferred to a culture room and grown at 25 °C ± 2 °C in the dark for eight initial days and then transferred to a 16-h photoperiod with a light intensity of 40 µmol m^{-2} s^{-1} provided by cool-day fluorescent lamps.

2.4. Experimental Design, Data Collection, and Statistical Analysis

The experiments were conducted in a completely randomized design. In experiment 1, a two-factor experiment (4 × 8) design was used, with four different cytokinins (ZEA, 2iP, BAP, and KIN) in eight different concentrations (0, 2.5, 5, 10, 20, 30, 40, and 50 µM). There were 32 treatments in total. Each treatment had four replicates of 10 tubes each (one plant per tube), e.g., 40 plants per treatment, resulting in a total of 1280 plants.

In experiment 2, a completely randomized design was used, with nine treatments, according to Table 2. Each treatment had three replicates of seven tubes each (one plant per tube), e.g., 21 plants per treatment, resulting in 189 plants.

In experiment 3, a completely randomized design was used, with 10 treatments (Table 3). Each treatment had four replicates of 10 tubes each (one plant per tube), e.g., 40 plants per treatment, resulting in a total of 400 plants.

Plants were evaluated based on many aspects two months (Experiment 1) or three months (Experiments 2 and 3) after initial culture. Contaminated cultures were discarded and not included in the data analysis. Contamination rates ranged from 0 to 7.5% in experiment 1. The final number of explants evaluated is presented in Table S1. In experiment 2, contamination rates ranged from 0 to 14%; and were 0 to 35% in experiment 3. Survival rate (%) and new shoot growth (%) were recorded. The number of new shoots formed per explant was counted (nº), the length of the longest shoot (millimeters from base to shoot tip) was measured, and the number of leaves of the longest shoot was counted (nº). All the plants were evaluated and had the mean estimated from the plants in each replication, and subsequently, the mean of the three or four replications in each treatment.

In experiment 1, ANOVA, Tukey, and regression analyses did not include values for the zero concentration treatments, since it was clear that a zero concentration did not have any influence on the explant development and it is not a concentration that labs would apply in practice. In the zero concentrations, there was no shoot formation in any of the explants evaluated. Since there was no shoot formation, there was no valid evaluation of number of shoots formed, length of shoot, or number of leaves per shoot. Hence, 28 treatments were statistically analyzed using a two-factor experiment (4×7), with four different cytokinins (ZEA, 2iP, BAP, and KIN) at seven different concentrations (2.5, 5, 10, 20, 30, 40, and 50 μM). The results were first transformed to the square root scale and then two-way ANOVA was performed (Table S2) to detect any interaction between the two factors and to check for any statistically significant difference between treatments at levels 1 and 5%. In the case of interaction between factors, in the variable analyzed, two tests were performed. First, Tukey's test ($P < 0.05$) was performed for each of the cytokinins with each of the concentrations. For factor 2 (different concentrations), regression analysis was performed for each cytokinin with the original data. The best-fitting regression model was obtained and the R^2 value was recorded. In experiments 2 and 3, original data were used, and one-way ANOVA was performed to check for any statistically significant difference between treatments ($P < 0.01$). Then, Scott-Knott's test ($P < 0.05$) was performed. For these analyses, the software Assistat® was used.

3. Results

3.1. Experiment 1: Cytokinins

In all the dependent variables analyzed (survival, shoot formation, number of shoots, length of shoot, and number of leaves), there was a significant interaction (at least $P < 0.01$) between the two factors (growth regulator and concentrations) tested, indicating that their effects are not independent. In addition, there was a significant difference between the different kinds of cytokinin tested for all the variables evaluated. F values were significant (at least ($P < 0.01$)) concerning factor 1 (different cytokinins) and concerning the interaction of factor 1 (different cytokinins) with factor 2 (different concentrations). Tukey's test results are shown in Table 4. The overall development of the explants in different cytokinin concentrations can be observed in Figure 1. The use of kinetin in the culture medium did not lead to any response in new shoots formed.

Table 4. Experiment 1, treatments with four cytokinins at different concentrations, showing mean values of survival (%), shoot formation (%), number of shoots (nº), length of shoot (mm), and number of leaves (n) in initial in vitro shoot culture of 'Delite' rabbiteye blueberry.

Survival (%)							
Cytokinin	2.5 µM	5 µM	10 µM	20 µM	30 µM	40 µM	50 µM
ZEA	89.7 ± 14.2a [z]	96.4 ± 7.1a	92.2 ± 5.2a	94.7 ± 6.1a	100.0 ± 0.8a	100.0 ± 0.8a	100.0 ± 0.8a
2iP	36.9 ± 14.4b	60.0 ± 20.0b	78.1 ± 11.4a	78.6 ± 16.0a	94.7 ± 6.1a	100.0 ± 0.8a	100.0 ± 0.8a
BAP	24.2 ± 11.6b	52.5 ± 6.8b	59.3 ± 8.3a	82.2 ± 16.9a	71.9 ± 14.7a	68.9 ± 10.1a	73.6 ± 21.6a
KIN	2.8 ± 5.6c	0.0 ± 0.1c	5.0 ± 5.8b	5.3 ± 6.1b	0.0 ± 0.1b	8.3 ± 5.6b	7.8 ± 6.1b
Mean	38.4	52.2	58.7	65.2	66.7	69.3	70.4
Shoot Formation (%)							
Cytokinin	2.5 µM	5 µM	10 µM	20 µM	30 µM	40 µM	50 µM
ZEA	81.3 ± 9.2a	88.2 ± 1.8a	90.0 ± 0.1a	94.7 ± 6.1a	100.0 ± 0.0 a	100.0 ± 0.0a	100.0 ± 0.0a
2iP	0.0 ± 0.0b	0.0 ± 0.0b	30.6 ± 22.4b	42.2 ± 8.6b	53.3 ± 14.4b	70.0 ± 12.4b	95.0 ± 5.8a
BAP	0.0 ± 0.0b	0.0 ± 0.0b	0.0 ± 0.0c	0.0 ± 0.0c	5.0 ± 5.8c	7.8 ± 5.8c	7.5 ± 5.0b
KIN	0.0 ± 0.0b	0.0 ± 0.0b	0.0 ± 0.0c	0.0 ± 0.0c	0.0 ± 0.0d	0.0 ± 0.0d	0.0 ± 0.0c
Mean	20.3	22.1	30.2	34.2	39.6	44.4	50.6
Number of Shoots per Explant (nº)							
Cytokinin	2.5 µM	5 µM	10 µM	20 µM	30 µM	40 µM	50 µM
ZEA	1.3 ± 0.1a	1.4 ± 0.1a	1.3 ± 0.1a	1.5 ± 0.1a	1.3 ± 0.1a	1.5 ± 0.1a	1.6 ± 0.1a
2iP	0.0 ± 0.0b	0.0 ± 0.0b	1.0 ± 0.0a	1.1 ± 0.1a	1.0 ± 0.0a	1.5 ± 0.1a	1.2 ± 0.1ab
BAP	0.0 ± 0.0b	0.0 ± 0.0b	0.0 ± 0.0b	0.0 ± 0.0b	0.5 ± 0.6b	0.8 ± 0.5b	0.8 ± 0.5b
KIN	0.0 ± 0.0b	0.0 ± 0.0b	0.0 ± 0.0b	0.0 ± 0.0b	0.0 ± 0.0c	0.0 ± 0.0c	0.0 ± 0.0c
Mean	0.3	0.3	0.6	0.7	0.7	0.9	0.9
Shoot Length (mm)							
Cytokinin	2.5 µM	5 µM	10 µM	20 µM	30 µM	40 µM	50 µM
ZEA	13.8 ± 3.4a	8.4 ± 1.1a	5.6 ± 1.8a	6.7 ± 3.4a	4.1 ± 0.3a	3.9 ± 0.6ab	5.0 ± 0.8a
2iP	0.0 ± 0.0b	0.0 ± 0.0b	3.0 ± 0.8a	3.3 ± 0.3b	3.9 ± 0.4a	4.7 ± 0.6a	4.2 ± 0.4a
BAP	0.0 ± 0.0b	0.0 ± 0.0b	0.0 ± 0.0b	0.0 ± 0.0c	1.3 ± 1.5b	2.5 ± 2.1b	1.8 ± 1.3b
KIN	0.0 ± 0.0b	0.0 ± 0.0b	0.0 ± 0.0b	0.0 ± 0.0c	0.0 ± 0.0c	0.0 ± 0.0c	0.0 ± 0.0c
Mean	3.4	2.1	2.1	2.5	2.3	2.8	2.7
Number of Leaves (nº)							
Cytokinin	2.5 µM	5 µM	10 µM	20 µM	30 µM	40 µM	50 µM
ZEA	10.0 ± 1.3a	8.8 ± 1.3a	6.3 ± 2.3a	8.0 ± 3.2a	6.0 ± 0.9a	6.1 ± 1.5a	7.9 ± 1.1a
2iP	0.0 ± 0.0b	0.0 ± 0.0b	2.4 ± 1.1b	3.0 ± 1.3b	3.2 ± 0.3b	5.7 ± 1.0a	5.8 ± 0.7a
BAP	0.0 ± 0.0b	0.0 ± 0.0b	0.0 ± 0.0c	0.0 ± 0.0c	0.8 ± 0.0c	1.0 ± 0.0b	0.5 ± 0.0b
KIN	0.0 ± 0.0b	0.0 ± 0.0b	0.0 ± 0.0c	0.0 ± 0.0c	0.0 ± 0.0c	0.0 ± 0.0c	0.0 ± 0.0b
Mean	2.5	2.2	2.2	2.7	2.5	3.2	3.6

[z] Data are the means of four replicates ± standard deviation (SD). Means followed by the same lowercase letter within the column are not significantly different according to Tukey's test ($P < 0.05$).

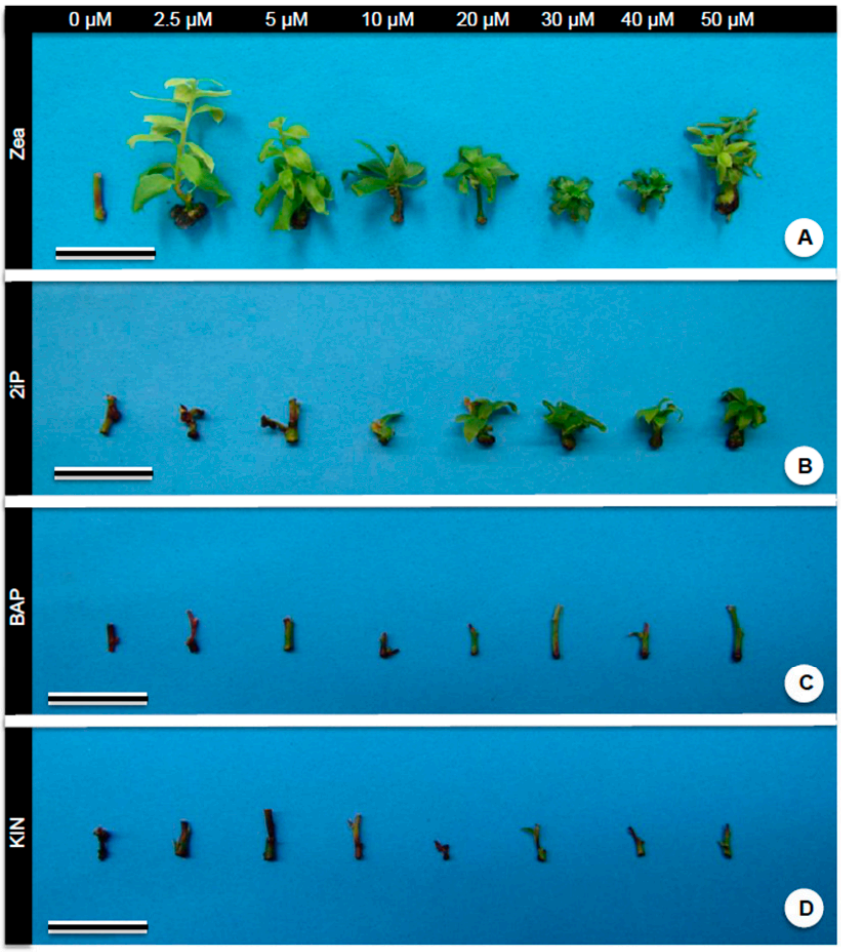

Figure 1. Initial in vitro shoot culture of 'Delite' rabbiteye blueberry in eight different concentrations (0, 2.5, 10, 20, 30, 40, and 50 µM) with four different cytokinins: (**a**) ZEA, (**b**) 2iP, (**c**) BAP, and (**d**) KIN. Bars represent 2 cm. Abbreviations: BAP, 6-benzylaminopurine; KIN, kinetin; ZEA, zeatin; 2iP, 6-(γ-γ-dimethylallylamino)-purine.

3.1.1. The Effects of Cytokinins on Survival

ZEA was superior to the other cytokinins at the concentrations of 2.5 and 5 µM. In all concentrations, KIN had the worst performance for survival rate. Finally, in the concentration range of 10–50 µM, ZEA, 2iP, and BAP all had the same effect on survival. The regression analyses can be observed in Figure 2.

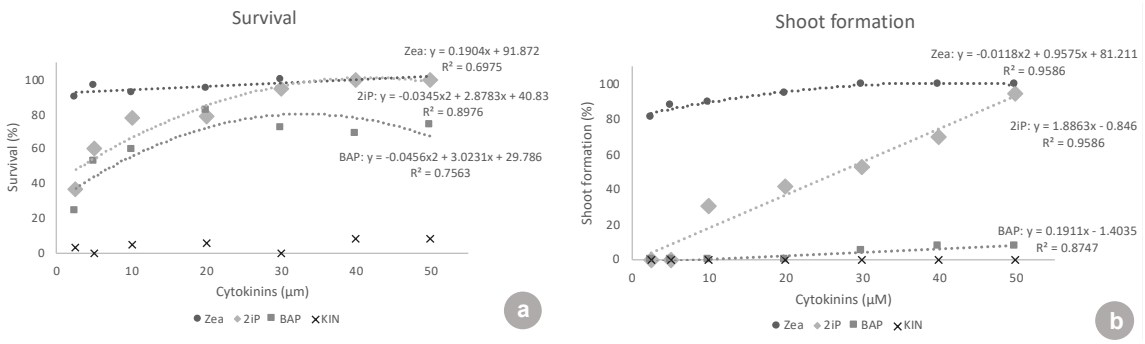

Figure 2. *Cont.*

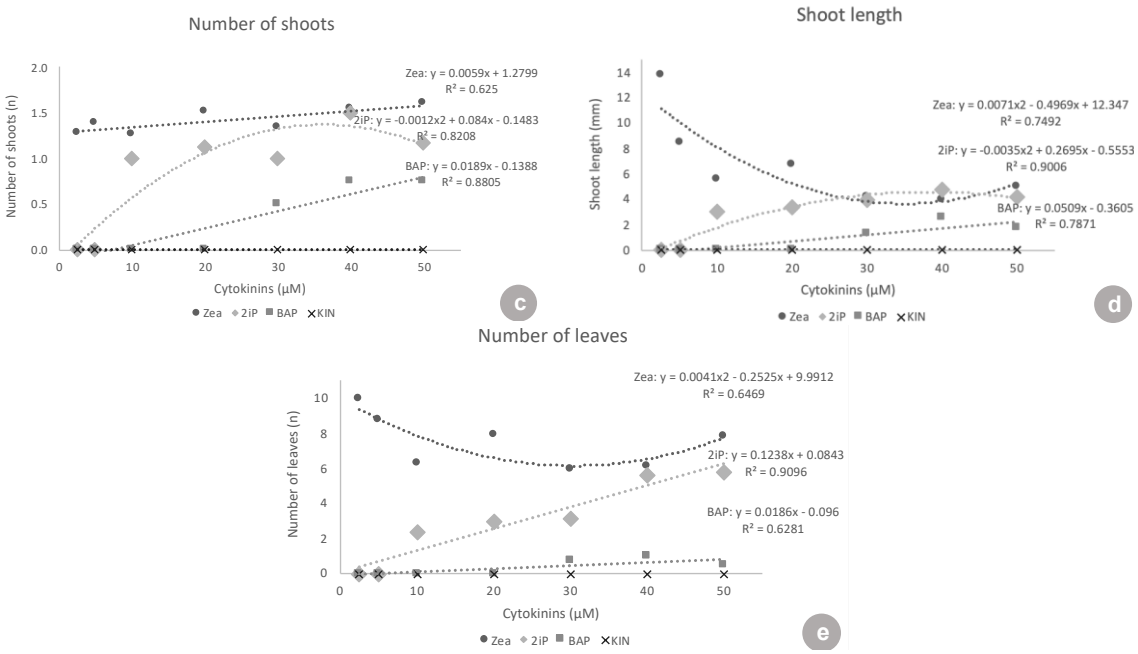

Figure 2. Regression analysis related to different cytokinin (ZEA, 2iP, BAP, and KIN) concentrations (2.5, 5, 10, 20, 30, 40, and 50 µM) on in vitro establishment of 'Delite' rabbiteye blueberry. (**a**) Survival (%); (**b**) shoot formation (%); (**c**) number of shoots (n°); (**d**) shoot length (mm); (**e**) number of leaves (n°). Abbreviations: BAP, 6-benzylaminopurine; KIN, kinetin; ZEA, zeatin; 2iP, 6-(γ-γ-dimethylallylamino)-purine.

3.1.2. The Effects of Cytokinins on Shoot Formation

Shoot formation from the initial explant was highly influenced by different cytokinins. According to the quadratic polynomial regression analysis across ZEA concentrations (Figure 2), a maximum shoot formation of 100% would be acquired at a concentration of 40.6 µM.

The evaluation of different means can be observed in Table 4, where in almost all of the concentrations tested (except 50 µM), ZEA was superior to all the other treatments, varying from 81.3 to 100% shoot formation. At concentrations of 2.5 and 5 µM, 2iP, BAP, and KIN did not show any response. 2iP showed responses from 10 to 50 µM only, presenting a rate varying from 30.6 to 95.0% in those concentrations. In concentrations of 10, 20, 30, and 40 µM, 2iP was the second cytokinin to form shoots. At a concentration of 50 µM, 2iP was equivalent to ZEA, and both were superior to BAP and KIN in this concentration. BAP did not show any response in the explants growing at the lowest concentrations of 2.5, 5, 10, and 20 µM. The first response for BAP appeared only at the concentrations of 30, 40, and 50 µM, showing a rate of shoot formation of only 5.0 to 7.8% of explants showing new shoot formation. BAP had lower shoot formation than ZEA and 2iP at all the concentrations tested.

3.1.3. The Efects of Cytokinins on the Number of Shoots Per Explant

Regarding the number of new shoots formed, we can observe that ZEA showed a linear relationship (Figure 2) and calculate that a concentration of 22.0 µM would be required to reach 1.4 shoots per explant. With 2iP, the maximum point in the curve reached 1.4 shoots per explant, which would be acquired at a concentration of 37.37 µM 2iP.

At the concentrations of 2.5 and 5 µM (Table 4), ZEA was superior to all the other cytokinins, showing 1.3 and 1.4 shoots per explant, respectively. At concentrations of 10, 20, 30, 40, and 50 µM, ZEA and 2iP had the same performance and were superior to BAP and kinetin. BAP only showed some shoot formation at concentrations of 30, 40, and 50 µM, exhibiting an average of only 0.5 to 0.8 new shoots per explant.

3.1.4. The Effects of Cytokinins on Shoot Length

Type of cytokinin had a significant influence on shoot length. Regression analysis (Figure 2) shows that ZEA followed a quadratic polynomial trend, with the concavity upward, showing an initial higher shoot length in the lowest concentrations (11.1 mm calculated at 2.5 μM), decreasing to the lowest point (3.6 mm) at 34.8 μM ZEA, and then starting to increase again. 2iP had a quadratic polynomial trend with the concavity downward. The maximum point in this curve was 4.56 mm of shoot length at 38.2 μM 2iP.

At the lowest concentrations of growth regulators, 2.5 and 5 μM ZEA was superior to all the other treatments, showing shoots with 13.8 and 8.4 mm, respectively (Table 4). In these two concentrations, 2iP, BAP, and KIN did not show any new shoots. At the other concentrations tested, 10, 20, 30, 40, and 50 μM, 2iP showed new shoots. At concentrations of 10, 30, 40, and 50 μM, 2iP treatments presented shoot lengths that did not differ from those of ZEA; ZEA and 2iP displayed an equal performance. BAP showed smaller shoots compared to 2iP and ZEA at all the concentrations except 40 μM. BAP only showed new shoots at the concentrations of 30 μM (1.3 mm), 40 μM (2.5 mm), and 50 μM (1.8 mm). Kinetin was inferior to all the others, in all the concentrations tested.

3.1.5. The Effects of Cytokinins on Number of Leaves

The number of leaves was significantly influenced by different cytokinins. The ZEA regression curve was a quadratic polynomial with concavity upward (Figure 2), similar to the curve observed for the influence of ZEA concentrations on shoot length. The minimum value in this curve was 6.2 leaves, reached at the concentration of 30.7 μM ZEA. 2iP behaved in a linear way, showing a maximum of 5.8 leaves at a concentration of 50 μM. BAP was also represented by a linear relationship, reaching a maximum of 0.8 leaves at the highest concentration of 50 μM.

At concentrations of 2.5, 5, 10, 20, and 30 μM, ZEA was superior to all the other cytokinins, showing 10.0, 8.8, 6.3, 8.0, and 6.0 leaves per shoot (Table 4). 2iP was inferior to ZEA at all concentrations, except at the highest concentrations of 40 and 50 μM, where both cytokinins were equivalent. BAP was always inferior to ZEA. At concentrations of 10, 20, 30, 40, and 50 μM, BAP was also inferior to 2iP. BAP only showed some leaves at concentrations of 30 μM (0.8 leaves), 40 μM (1.0 leaf), and 50 μM (0.5 leaves). At all of the concentrations, KIN did not show any response.

3.2. Experiment 2: Combinations of $(NH_4)_2SO_4$, KNO_3, and $Ca(NO_3)_2 \cdot 4H_2O$ Using the Modified WPM [15] as Basic Medium

There were no statistically significant differences among the nine treatments tested for any of the variables analyzed. Survival and shoot formation rates ranged from 43.7 to 76.2%, the number of shoots formed ranged from 1.1 to 1.4, shoot lengths ranged from 7.5 to 25.0 mm, and the number of leaves ranged from 9.4 to 19.7 (Table 5).

Table 5. Experiment 2 with treatments 1 to 9 on the modified Woody Plant Medium (modified WPM) [15] showing the number of explants evaluated (nº), survival rate (%), shoot formation (%), number of shoots (nº), shoot length (mm), and number of leaves (nº) in 'Delite' rabbiteye blueberry in vitro establishment. Abbreviations: CV, coefficient of variation; nº, number.

Treatment	$(NH_4)_2SO_4$ (x)	KNO_3 and $Ca(NO_3)_2 \cdot 4H_2O$ (x)	nº	Survival (%)	Shoot Formation (%)	Number of Shoots (nº)	Shoot Length (mm)	Number sf Leaves (nº)
1-modified WPM	1×	1×	19	48.4 ± 19.1a [z]	48.4 ± 19.1a	1.4 ± 0.1a	13.9 ± 6.7a	11.1 ± 3.4a
2	1×	0.5×	19	62.7 ± 11.3a	62.7 ± 11.3a	1.3 ± 0.2a	24.9 ± 7.4a	19.7 ± 3.1a
3	1×	1.5×	20	43.7 ± 23.4a	43.7 ± 23.4a	1.3 ± 0.3a	17.7 ± 16.9a	13.7 ± 7.6a
4	0.5×	1×	18	73.0 ± 35.1a	73.0 ± 35.1a	1.1 ± 0.2a	9.1 ± 5.5a	10.9 ± 5.6a
5	0.5×	0.5×	19	69.5 ± 11.5a	69.5 ± 11.5a	1.1 ± 0.1a	10.7 ± 3.2a	11.8 ± 1.5a
6	0.5×	1.5×	20	45.2 ± 4.1a	45.2 ± 4.1a	1.2 ± 0.2a	25.0 ± 14.3a	18.0 ± 6.0a
7	1.5×	1×	21	76.2 ± 8.2a	76.2 ± 8.2a	1.2 ± 0.0a	7.5 ± 2.7a	9.4 ± 3.3a
8	1.5×	0.5×	20	56.3 ± 23.4a	56.3 ± 23.4a	1.1 ± 0.2a	17.2 ± 9.7a	12.7 ± 1.9a
9	1.5×	1.5×	20	54.0 ± 19.2a	54.0 ± 19.2a	1.4 ± 0.4a	21.2 ± 7.7a	16.8 ± 5.0a
Mean	-	-	-	58.8	58.8	1.2	16.4	13.8
CV%	-	-	-	33.1	33.1	16.5	57.4	33.1

[z] Data are the means of three replicates ± standard deviation (SD). Means followed by the same lowercase letter within a column are not significantly different according to Scott-Knott's test ($P < 0.05$).

3.3. Experiment 3: Combinations of NH_4NO_3 and $Ca(NO_3)_2 \cdot 4H_2O$ Using the Original WPM [18] as the Basic Medium

In this experiment, it was possible to verify that treatments 7 (0.5 × NH_4NO_3 and 1 × $Ca(NO_3)_2$), 8 (0.5 × NH_4NO_3 and 0.5 × $Ca(NO_3)_2$), and 10 (modified WPM) showed the lowest rates of survival and shoot formation and shortest shoot length (Table 6).

Table 6. Experiment 3 with treatments 1 to 9 on Woody Plant Medium (WPM) [18] with different ranges of NH_4NO_3 (x) and $Ca(NO_3)_2 \cdot 4H_2O$ (x) and treatment 10 on modified WPM [15], showing number of explants evaluated (nº), survival rate (%), shoot formation (%), number of shoots (nº), shoot length (mm), and number of leaves (nº) in 'Delite' rabbiteye blueberry in vitro establishment. Abbreviations: CV, coefficient of variation; nº, number.

Treatment	Solution NH_4NO_3	Solution $Ca(NO_3)_2$	nº	Survival (%)	Shoot Formation (%)	Number of Shoots (nº)	Shoot Length (mm)	Number of Leaves (nº)
1-original WPM	1×	1×	38	79.4 ± 16.4a [z]	79.4 ± 16.4a	1.2 ± 0.3a	33.3 ± 7.6a	15.3 ± 2.1a
2	1×	0.5×	40	95.0 ± 5.8a	95.0 ± 5.8a	1.1 ± 0.1a	23.5 ± 3.9b	12.2 ± 1.5a
3	1×	1.5×	38	97.5 ± 5.0a	97.5 ± 5.0a	1.2 ± 0.1a	21.0 ± 1.9b	11.4 ± 1.7a
4	1.5×	1×	27	86.8 ± 10.5a	83.7 ± 15.7a	1.1 ± 0.1a	25.4 ± 8.9b	12.1 ± 2.9a
5	1.5×	0.5×	40	92.5 ± 5.0a	90.0 ± 8.2a	1.1 ± 0.2a	32.3 ± 7.3a	14.4 ± 1.1a
6	1.5×	1.5×	40	90.0 ± 8.2a	90.0 ± 8.2a	1.1 ± 0.1a	30.7 ± 6.6a	14.1 ± 1.2a
7	0.5×	1×	39	64.4 ± 16.7b	64.4 ± 16.7b	1.1 ± 0.1a	5.0 ± 0.7d	6.6 ± 1.2c
8	0.5×	0.5×	35	55.1 ± 5.6b	55.1 ± 5.6b	1.1 ± 0.1a	18.2 ± 10.5c	10.4 ± 3.6b
9	0.5×	1.5×	26	85.4 ± 17.2a	85.4 ± 17.2a	1.0 ± 0.0a	24.2 ± 3.8b	14.9 ± 1.6a
10-modified WPM			32	55.7 ± 21.4b	55.7 ± 21.4b	1.2 ± 0.2a	15.7 ± 1.3c	12.9 ± 2.2a
Mean	-	-	-	80.2	79.6	1.1	22.9	12.4
CV%	-	-	-	16.4	7.4	15.0	26.9	16.6

[z] Data are the means of four replicates ± standard deviation (SD). Means followed by the same lowercase letter within the column are not significantly different according to Scott-Knott's Test ($P < 0.05$).

The number of shoots was similar in all the treatments tested. In addition, concerning the number of leaves, the lowest number was obtained with treatments 7 and 8. Observing survival, shoot formation, and shoot length, treatment 1 (original WPM) was superior to treatment 10 (modified WPM) (Figure 3).

Figure 3. Initial in vitro shoot culture of 'Delite' rabbiteye blueberry with 10 treatments. Treatments 1 to 9 with the original Woody Plant Medium (WPM) [18] with different ranges of NH_4NO_3 (x) and $Ca(NO_3)_2 \cdot 4H_2O$ (x), compared to treatment 10 (modified Woody Plant Medium [15]).

4. Discussion

In vitro establishment is an important step in tissue culture. It is a critical point where explants come from a different environment and have to adapt to in vitro conditions. One of the key steps in this process is the use of adequate growth regulators and a balance of mineral salts in a suitable concentration. Our results showed a screening comparison of four different cytokinins in eight different concentrations and varying balances of nitrogen salts in 'Delite' rabbiteye blueberry, presenting an efficient technique for in vitro plant propagation of this species.

The species and cultivars of the *Vaccinium* genus show natural variation in in vitro responses. There is high genetic variation in growth regulator responses/needs. Our results, based on linear and quadratic polynomial regression analyses, displayed the effects of cytokinin concentrations and their great impact on the survival of explants, new shoot formation, number of new shoots formed, length of shoots formed, and number of leaves in the shoots.

ZEA and 2iP resulted in better responses to in vitro establishment. At the lowest concentrations tested, 2.5 and 5 µM, ZEA was superior to all of the other cytokinins tested, in all the variables analyzed, presenting values of: 89.7 and 96.4% explant survival, 81.3 and 88.2% of explants forming new shoots, 1.3 and 1.4 new shoots formed, 13.8 and 8.4 mm of shoot length, and 10.0 and 8.8 leaves per shoot, respectively. Similar results were observed with highbush blueberry 'Polaris' and half-high blueberry 'St. Cloud', where ZEA was used at a concentration of 9.1 µM in the shoot establishment in vitro. ZEA was also efficient in inducing shoot proliferation in a liquid medium at 4.6 µM [10], instead of at higher concentrations. For *V. corymbosum* 'Oskar', *V. angustifolium* 'Emil' and 'Putte', and *V. corymbosum* × *V. angustifolium* 'Northblue' establishment, 2 mg L^{-1} (9.12 µM) ZEA was used [20]. In highbush blueberry 'Duke' propagation, ZEA at 2 mg L^{-1} (9.12 µM) was superior to 2iP or TDZ [21].

For in vitro shoot proliferation in cranberry (*V. macrocarpon* Ait.) cultivars, ZEA at very low concentrations (2–4 µM) showed good results [22]. In *V. ashei* at the multiplication stage, ZEA increased shoot formation compared to 2iP. However, 2iP showed longer shoots with a higher number of nodes [23]. For initial culture of highbush blueberry, 1 mg L^{-1} (2.85 µM) zeatin riboside was used [24]. In lowbush blueberry, the authors tested 0, 2.3, 4.6, or 9.10 µM ZEA on the elongation of shoots, and concentrations of 2.3 and 4.6 µM gave the best response [25].

Another important aspect is the growth habit of the 'Delite' rabbiteye blueberry cultivar in this study. In particular, in the presence of ZEA and 2iP, it showed a low number of new shoots per explant, but longer shoots, which means that a new subculture could be performed using the nodal segments of the long shoot instead of using new axillary or adventitious shoots formed.

At the lowest concentrations (2.5 and 5 µM), there were no responses to 2iP. Treatments with 2iP started to form shoots only at the concentrations of 10, 20, 30, 40, and 50 µM. Concerning the percentage of explants forming new shoots, 2iP was inferior to ZEA in all of the concentrations, except 50 µM, where both had the same shoot formation rate. This showed that ZEA triggered a

response in the explants, even at inferior concentrations (2.5 and 5 μM), and that 2iP was able to lead to some shoot formation only at higher concentrations (10 μM and above). Concerning shoot length, at the concentrations where 2iP started showing new shoots (10–50 μM), the shoots formed were equivalent in length to the shoots formed with ZEA. At concentrations of 10, 20, 30, and 50 μM, both were superior to BAP and KIN. However, when analyzing the number of leaves, ZEA was superior to 2iP at almost all concentrations, except 40 and 50 μM, again demonstrating the need for higher concentrations of 2iP to produce a higher number of leaves. In 'Brightwell' blueberry, the authors found that different concentrations of 2iP (5, 10, 15, or 20 mg·L^{-1}) and TDZ were inferior to 2 mg L^{-1} (9.12 μM) ZEA in shoot proliferation [26]. ZEA at 4 mg·L^{-1} (18.24 μM) was more successful than 2iP at 10 or 15 mg·L^{-1} (49.2 or 73.8 μM) in establishing *V. corymbosum* blueberry cultivars [12].

BAP did not show any response at the lowest concentrations of 2.5, 5, 10, and 20 μM. BAP only started showing a low response to 30, 40, and 50 μM (5.0–7.8% shoot formation). BAP was always highly inferior to ZEA at all concentrations tested, in all of the variables analyzed, except for the shoot length at 40 μM. Additionally, BAP was inferior to 2iP from 10–50 μM concerning shoot formation, number of shoots, shoot length, and number of leaves. In the same way, in 'Bluejay' and 'Pink Lemonade' blueberry, the authors found that BAP induced fewer axillary shoots than ZEA, as well as smaller shoots [27].

Kinetin showed no response concerning shoot formation and had almost no surviving explants (maximum of 8.3% survival), clearly showing that it was not suitable for 'Delite' rabbiteye blueberry initiation culture.

In this study, different balances of nitrogen salts were tested. Using the modified WPM medium, no differences were observed among all combinations of nitrogen salts: $(NH_4)_2SO_4$, KNO_3, and $Ca(NO_3)_2 \cdot 4H_2O$. 'Delite' blueberry showed lower survival (55.7%), shoot formation (55.7%), and shoot length (15.7 mm) in the modified WPM compared with the original WPM (79.4%, 79.4%, and 33.3 mm, respectively).

Using the original WPM, it was observed that treatments containing higher amounts of NH_4NO_3 (1× or 1.5×, instead of 0.5×), as well as the treatment with a higher amount of $Ca(NO_3)_2$ (1.5×), even with a lower amount of NH_4NO_3 (0.5×), showed the same performance as in WPM without modification. Similarly, in red raspberries, it was found that combinations of intermediate to high NO_3^- and intermediate to high NH_4^+ developed the most growth in most cultivars [28].

However, changing the ranges of $Ca(NO_3)_2$, in addition to increasing or decreasing the total amount of nitrogen and its nitrate form, would also change the Ca^{+2} ion. Therefore, the result seen in the treatment $Ca(NO_3)_2$ (1.5×) could be related to either nitrogen or calcium in higher amounts, or even both.

This study in a rabbiteye blueberry cultivar represents a basic framework that can be used to understand initial in vitro establishment. It can be useful to describe this process in other *Vaccinium* cultivars regarding the adjustments necessary to adapt the process to different genotypes.

5. Conclusions

This research showed the effects of different cytokinins at different concentrations and different nitrogen salt ranges on 'Delite' rabbiteye blueberry during in vitro establishment, and it provides basic knowledge for further experiments in rabbiteye blueberry tissue culture.

In conclusion, focusing on an efficient strategy for in vitro establishment of 'Delite' rabbiteye blueberry, we recommend the lowest concentration tested, 2.5 μM ZEA, which promoted a high survival rate (89.7%), as well as a good response on explants forming new shoots (81.3%). This concentration yielded a number of 1.3 new shoots, a high shoot length (13.8 mm), and 10.0 leaves per shoot. Concerning salt composition, we recommend the original WPM. An increase or decrease in the NH_4NO_3 and $Ca(NO_3)_2$ concentration did not promote better growth than the original medium.

This work is of interest for evaluating different cytokinin and salt compositions in the culture medium for in vitro establishment of rabbiteye blueberry, and it can contribute to developing a

deeper knowledge of large-scale propagation, germplasm conservation, and development of other biotechnology techniques in other research fields, such as morphology, plant breeding, and physiology.

Future studies could be developed beyond the research presented here, focusing on fine-tuning the salts composition and concentrations of the growth regulator needed for an efficient response, as well as combining the two most successful cytokinins tested, ZEA and 2iP.

Supplementary Materials: The following are available online at http://www.mdpi.com/2311-7524/5/1/24/s1, Table S1: Results of the two-way ANOVA of experiment 1 studying the influence of cytokinin type and concentration on survival (%), shoot formation (%), number of shoots (nº), length of shoot (mm), and number of leaves (nº) on initial in vitro shoot culture of 'Delite' rabbiteye blueberry, Table S2: Number of explants evaluated after contamination in experiment 1 in each of the treatments (cytokinin type by concentration) on initial in vitro shoot culture of 'Delite' rabbiteye blueberry.

Author Contributions: C.S.S. contributed the design of the study, ran the laboratory work, processed the data, and drafted the paper. L.A.B. supervised the research and provided critical reading for the final version of the manuscript.

Funding: This research was funded in part by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior—Brasil (CAPES)—Finance Code 001 under a PhD scholarship grant.

Acknowledgments: We are very grateful to Marino Schiehl and Kassius Augusto da Rosa for their support and collaboration in the laboratory work.

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

Abbreviations

BAP	6-Benzylaminopurine
CV	coefficient of variation
DF	degrees of freedom
KIN	kinetin: 6-furfurylaminopurine
MS	mean squares
SS	sum of squares
WPM	Woody Plant Medium
ZEA	zeatin: 6-(4-Hydroxy-3-methylbut-2-enylamino)purine
2iP	6-(γ-γ-dimethylallylamino)-purine

References

1. Retamales, J.B.; Hancock, J.F. *Blueberries*; CABI: Oxfordshire, UK, 2012.
2. Michalska, A.; Łysiak, G. Bioactive compounds of blueberries: Post-harvest factors influencing the nutritional value of products. *Int. J. Mol. Sci.* **2015**, *16*, 18642–18663. [CrossRef] [PubMed]
3. Poulose, S.M.; Miller, M.G.; Scott, T.; Shukitt-Hale, B. Nutritional factors affecting adult neurogenesis and cognitive function. *Adv. Nutr. Int. Rev. J.* **2017**, *8*, 804–811. [CrossRef] [PubMed]
4. Cheng, A.; Yan, H.; Han, C.; Wang, W.; Tian, Y.; Chen, X. Polyphenols from blueberries modulate inflammation cytokines in LPS-induced RAW264.7 macrophages. *Int. J. Biol. Macromol.* **2014**, *69*, 382–387. [CrossRef] [PubMed]
5. Balducci, F.; Capocasa, F.; Mazzoni, L.; Mezzetti, B.; Scalzo, J. Study on adaptability of blueberry cultivars in center-south Europe. *Acta Hortic.* **2016**, 53–58. [CrossRef]
6. Debnath, S.C. A scale-up system for lowbush blueberry micropropagation using a bioreactor. *HortScience* **2009**, *44*, 1962–1966. [CrossRef]
7. Routray, W.; Orsat, V. Blueberries and their anthocyanins: Factors affecting biosynthesis and properties. *Compr. Rev. Food Sci. Food Saf.* **2011**, *10*, 303–320. [CrossRef]
8. Marino, S.R.; Williamson, J.G.; Olmstead, J.W.; Harmon, P.F. Vegetative growth of three southern highbush blueberry cultivars obtained from micropropagation and softwood cuttings in two Florida locations. *HortScience* **2014**, *49*, 556–561. [CrossRef]
9. Meiners, J.; Schwab, M.; Szankowski, I. Efficient in vitro regeneration systems for *Vaccinium* species. *Plant Cell Tissue Organ Cult.* **2007**, *89*, 169–176. [CrossRef]

10. Debnath, S.C. Temporary immersion and stationary bioreactors for mass propagation of true-to-type highbush, half-high, and hybrid blueberries (*Vaccinium* spp.). *J. Hortic. Sci. Biotechnol.* **2017**, *92*, 72–80. [CrossRef]
11. Goyali, J.C.; Igamberdiev, A.U.; Debnath, S.C. Propagation methods affect fruit morphology and antioxidant properties but maintain clonal fidelity in lowbush blueberry. *HortScience* **2015**, *50*, 888–896. [CrossRef]
12. Reed, B.M.; Abdelnour-Esquivel, A. The use of zeatin to initiate in vitro cultures of *Vaccinium* species and cultivars. *HortScience* **1991**, *26*, 1320–1322. [CrossRef]
13. Jaakola, L.; Tolvanen, A.; Laine, K.; Hohtola, A. Effect of N6-isopentenyladenine concentration on growth initiation in vitro and rooting of bilberry and lingonberry microshoots. *Plant Cell Tissue Organ Cult.* **2001**, *66*, 73–77. [CrossRef]
14. Tetsumura, T.; Matsumoto, Y.; Sato, M.; Honsho, C.; Yamashita, K.; Komatsu, H.; Sugimoto, Y.; Kunitake, H. Evaluation of basal media for micropropagation of four highbush blueberry cultivars. *Sci. Hortic.* **2008**, *119*, 72–74. [CrossRef]
15. Wang, Y.; Wang, S.; Xu, J.; Yan, Z.; Bao, H. Culture Medium for Proliferating Blueberry Tissue, Comprises Improved Woody Plant Medium Culture Medium, Naphthaleneacetic Acid, Gibberellic Acid, Zeatin, Sucrose and Agar. Patent Number CN104082142-A; CN104082142-B, 8 October 2014.
16. Reed, B.M.; Wada, S.; DeNoma, J.; Niedz, R.P. Mineral nutrition influences physiological responses of pear in vitro. *In Vitro Cell. Dev. Biol. Plant* **2013**, *49*, 699–709. [CrossRef]
17. Ramage, C.M.; Williams, R.R. Inorganic nitrogen requirements during shoot organogenesis in tobacco leaf discs. *J. Exp. Bot.* **2002**, *53*, 1437–1443. [CrossRef] [PubMed]
18. Lloyd, G.; McCown, B. Commercially-feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot-tip culture. *Int. Plant Propagators Soc. Comb. Proc.* **1980**, *30*, 421–427.
19. Murashige, T.; Skoog, F. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. Plant.* **1962**, *15*, 473–497. [CrossRef]
20. Welander, M.; Sayegh, A.; Hagwall, F.; Kuznetsova, T.; Holefors, A. Technical improvement of a new bioreactor for large scale micropropagation of several *Vaccinium* cultivars. *Acta Hortic.* **2017**, 387–392. [CrossRef]
21. Cappelletti, R.; Sabbadini, S.; Mezzetti, B. The use of TDZ for the efficient in vitro regeneration and organogenesis of strawberry and blueberry cultivars. *Sci. Hortic.* **2016**, *207*, 117–124. [CrossRef]
22. Debnath, S.C. Zeatin-induced one-step in vitro cloning affects the vegetative growth of cranberry (*Vaccinium macrocarpon* Ait.) micropropagules over stem cuttings. *Plant Cell Tissue Organ Cult.* **2008**, *93*, 231–240. [CrossRef]
23. Schuch, M.W.; Damiani, C.R.; Silva, L.C.; Erig, A.C. Micropropagação como técnica de rejuvenescimento em mirtilo (*Vaccinium ashei* Reade) cultivar climax. *Ciênc. Agrotecnol.* **2008**, *32*, 814–820. [CrossRef]
24. Hung, C.D.; Hong, C.-H.; Kim, S.-K.; Lee, K.-H.; Park, J.-Y.; Nam, M.-W.; Choi, D.-H.; Lee, H.-I. LED light for in vitro and ex vitro efficient growth of economically important highbush blueberry (*Vaccinium corymbosum* L.). *Acta Physiol. Plant.* **2016**, *38*, 152. [CrossRef]
25. Debnath, S.C. A two-step procedure for adventitious shoot regeneration on excised leaves of lowbush blueberry. *In Vitro Cell. Dev. Biol. Plant* **2009**, *45*, 122–128. [CrossRef]
26. Jiang, Y.; Zhang, D.; He, S.; Wang, C. Influences of media and cytokinins on shoot proliferation of "Brightwell" and "Choice" blueberries in vitro. *Acta Hortic.* **2009**, *810*, 581–586. [CrossRef]
27. Fan, S.; Jian, D.; Wei, X.; Chen, J.; Beeson, R.C.; Zhou, Z.; Wang, X. Micropropagation of blueberry 'Bluejay' and 'Pink Lemonade' through in vitro shoot culture. *Sci. Hortic.* **2017**, *226*, 277–284. [CrossRef]
28. Poothong, S.; Reed, B.M. Optimizing shoot culture media for *Rubus* germplasm: The effects of NH_4^+, NO_3^-, and total nitrogen. *In Vitro Cell. Dev. Biol. Plant* **2016**, *52*, 265–275. [CrossRef]