UNIVERSIDADE FEDERAL DO PARANÁ

CAROLINA SMANHOTTO SCHUCHOVSKI

APPROACHES ON *IN VITRO* ORGANOGENESIS IN 'DELITE' RABBITEYE BLUEBERRY (*Vaccinium virgatum* Ait.) WITH MORPHOLOGICAL AND ANATOMICAL ASPECTS

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Tese apresentada ao curso de Pós-Graduação em Agronomia - Produção Vegetal, Setor de Ciências Agrárias, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Doutora em Ciências.

Orientador: Prof. Dr. Luiz Antonio Biasi

Coorientador: Prof. Dr. Bruno Francisco Sant'Anna dos Santos

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em AGRONOMIA (PRODUÇÃO VEGETAL) da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **CAROLINA SMANHOTTO SCHUCHOVSKI** intitulada: **APPROACHES ON *in vitro* ORGANOGENESIS IN 'DELITE' RABBITEYE BLUEBERRY (*Vaccinium virgatum* Ait.) WITH MORPHOLOGICAL AND ANATOMICAL ASPECTS**, sob orientação do Prof. Dr. LUIZ ANTONIO BIASI, que após terem inquirido a aluna e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de doutor está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

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Aos queridos
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Renova-te.
Renasce em ti mesmo.
Multiplica os teus olhos, para verem mais.
Multiplica os teus braços para semeares tudo.
Destrói os olhos que tiverem visto.
Cria outros, para as visões novas.
Destrói os braços que tiverem semeado.
Para se esquecerem de colher.
Sê sempre o mesmo.
Sempre outro.
Mas sempre alto.
Sempre longe.
E dentro de tudo.
(CECÍLIA MEIRELES)

RESUMO

O mirtileiro é uma espécie frutífera perene e seus frutos apresentam propriedades antioxidantes e anti-inflamatórias. É convencionalmente propagado vegetativamente, o que pode trazer muitos problemas fitossanitários. Uma alternativa seria o uso da propagação *in vitro*. Há muitos estudos em propagação e regeneração *in vitro* em espécies de mirtileiro, entretanto, a maioria está concentrada nas cultivares highbush e lowbush, e pouco nas cultivares rabbiteye, mais adaptadas ao clima subtropical. Portanto, há a necessidade de se pesquisar protocolos *in vitro* para este grupo de cultivares. O objetivo desta pesquisa foi estudar vários aspectos da micropropagação e da organogênese *de novo* de brotos *in vitro* na cultivar 'Delite' do grupo rabbiteye de mirtilo. Nossos estudos foram organizados em três capítulos (i, ii e iii). Em cada um destes capítulos nosso objetivo foi: i) definir um protocolo de estabelecimento *in vitro*; ii) otimizar um protocolo de micropropagação, definindo os estágios de estabelecimento, multiplicação e enraizamento; iii) aperfeiçoar um método de regeneração adventícia de brotos *in vitro* e estudar o processo de desenvolvimento destes brotos. De acordo com os nossos resultados: i) no estabelecimento *in vitro* os tratamentos com zeatina foram superiores nas menores concentrações testadas (2,5 µM); não houve diferenças nas combinações de sais com o meio WPM (Woody Plant Medium) modificado; e no meio WPM original, um aumento ou diminuição nas concentrações de NH_4NO_3 e $Ca(NO_3)_2$ não levou ao aumento da eficiência no estabelecimento, e, em alguns casos, houve uma menor taxa de sobrevivência, formação de brotos, ou tamanho dos brotos; ii) na micropropagação, o estabelecimento com imersão em hipoclorito de sódio por 5 min foi eficiente (96.7% explantes não contaminados) e o meio WPM com 2,5 µM zeatina levou a uma taxa de 84.5% explantes com indução de brotação; a multiplicação com 2,5 µM zeatina levou a uma taxa de 70% dos explantes proliferando novos brotos, e a posição dos explantes na vertical teve 100% de sobrevivência e 100% dos explantes proliferando novos brotos); o enraizamento *in vitro* com 500 mg L^{-1} IBA (ácido indolbutírico) levou a 100% sobrevivência e 100% de manutenção das folhas, com 37,5% de enraizamento; ou ainda, o enraizamento *ex vitro* em vermiculita com sais minerais do meio WPM, alcançando 88% de sobrevivência dos explantes, 86% dos explantes com a manutenção das folhas, 50% de indução de brotações e 68% de enraizamento. iii) uma alta taxa de regeneração (100% dos explantes, com 57 brotos/explante) no tratamento 0,5 µM TDZ (thidiazuron); o uso da superfície adaxial do explante foliar em contato com o meio, com porções basal ou apical, mostrou boa regeneração (97%) e maior número de brotos/explante (47.5). Foi observada a organogênese direta e indireta, com novos brotos formando primórdios foliares com tecidos bem desenvolvidos, observados em microscopia óptica e eletrônica de varredura. Em conclusão, este estudo define um protocolo de micropropagação *in vitro* e regeneração adventícia de brotos em mirtileiro 'Delite' do grupo rabbiteye. Além disso, estes estudos podem ser usados para futuras pesquisas de cultura de tecidos *in vitro*, biotecnologia, conservação de germoplasma e micropropagação mirtileiro.

Palavras-chave: Ericaceae. *Vaccinium virgatum.* Micropropagação. Microscopia Eletrônica de Varredura. Microscopia Óptica.

ABSTRACT

Blueberry is a perennial fruit crop with antioxidant and anti-inflammatory benefits, being recognized as a nutraceutical product. It is mainly vegetatively propagated, which can lead to phytosanitary issues. Therefore, an alternative method for traditional clonal propagation is *in vitro* culture. Although we found research on *in vitro* tissue culture in blueberry, most of this research focuses on highbush and lowbush groups. There are few works with rabbiteye cultivars, which are more suitable for subtropical climates. Therefore, there is a need to research *in vitro* protocols for this group of cultivars. The objective of this research was to study various aspects of micropropagation and *de novo* shoot organogenesis *in vitro* for 'Delite' rabbiteye blueberry. Our studies were organized into three distinct chapters (i, ii, and iii). In each of these chapters, we aimed to: i) develop an *in vitro* establishment protocol; ii) optimize a micropropagation protocol, defining the establishment, multiplication, and rooting stages; iii) improve an adventitious shoot regeneration method and study the development process of these *de novo* shoots formed. According to the results: i) at *in vitro* establishment, zeatin was superior at the lowest (2.5 µM) concentration tested. In the combinations of salts with modified WPM (Woody Plant Medium) culture medium, no differences were reported. And an increase or decrease in NH_4NO_3 and $Ca(NO_3)_2$ concentration in WPM did not improve the establishment efficiency, and, in some treatments, led to lower survival and shoot formation rates compared to the original WPM. ii) optimizing the micropropagation, the establishment with 5 min immersion in sodium hypochlorite revealed a good rate of uncontaminated explants (96.7%) and survival rate of the explants (96.7%), and WPM medium with 2.5 µM zeatin led to high rates of survival (92.3%) and explants with axillary shoot growth (84.5%). Multiplication in the presence of 2.5 µM zeatin led to a high percentage of explants proliferating new shoots (70%), and explants in the vertical orientation were efficient (100% survival, 100% explants with shoot proliferation, 1.8 new shoots formed/explant, with 5.1 cm and with 12.7 leaves/shoot). *In vitro* rooting with 500 mg L^{-1} IBA (indole-3-butyric acid) (100% survival and leaf maintenance, and 37.5% rooting), or *ex vitro* rooting in vermiculite with WPM (88% survival, 86% leaf maintenance, 50% bud induction, and 68% rooting) were the most efficient treatments. iii) A high rate of explants regenerating shoots (100%) was achieved at 0.5 µM TDZ (thidiazuron), with 57 new shoots formed/explant. The use of adaxial surface touching the medium with apical or basal portion of the leaf showed good regeneration rates (97% explants regenerating shoots) and a high number of shoots formed (47.5 shoots/explant). Direct and indirect organogenesis were observed, and new shoots forming leaf primordia with well-developed tissues were described in light and scanning electron microscopy. In conclusion, this study defines protocols of micropropagation and *in vitro* adventitious shoot regeneration in 'Delite' rabbiteye blueberry. Beyond that, these findings can be used in further *in vitro* studies, as well as to improve biotechnological applications, germplasm conservation, and micropropagation in blueberry.

Keywords: Ericaceae. *Vaccinium virgatum*. Micropropagation. Scanning Electron Microscopy (SEM). Light Microscopy.

LIST OF ABBREVIATIONS AND ACRONYMS

2iP	- 2-isopentenyladenine (6-(γ-γ-dimethylallylamino)-purine)
Ab	- abaxial
Ad	- adaxial
ANOVA	- analysis of variance
atm	- atmosphere
BAP	- 6-benzylaminopurine
Ca	- callus
CV	- coefficient of variation
CAPES	- Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
DF	- degrees of freedom
Ex	- explant
GA_3	- gibberellic acid
GC	- guard cell
IAA	- indole-3-acetic acid
IBA	- indole-3-butyric acid
KIN	- kinetin (6-furfurylaminopurine)
LP	- leaf primordium
ms	- mean squares
MS	- Murashige and Skoog medium
NA	- not available
NAA	- α-naphthaleneacetic acid
ns	- non-significant
Os	- ostiole
p	- probability
Pc	- procambium
R	- roots
®	- registered trademark
SAM	- shoot apical meristem
SE	- standard error
SEM	- scanning electron microscopy
Sh	- shoot

syn.	- synonym
TDZ	- thidiazuron
™	- trademark
Tr	- trichome
v	- volume
V. angustifolium	- *Vaccinium angustifolium*
V. ashei	- *Vaccinium ashei*
V. corymbosum	- *Vaccinium corymbosum*
V. macrocarpon	- *Vaccinium macrocarpon*
V. myrtillus	- *Vaccinium myrtillus*
V. virgatum	- *Vaccinium virgatum*
V. vitis-idaea	- *Vaccinium vitis-idaea*
VT	- vascular tissue
WPM	- woody plant medium
Zea	- zeatin (6-(4-Hydroxy-3-methylbut-2-enylamino)purine)

SUMMARY

1 GENERAL INTRODUCTION

Blueberry is a woody perennial species of the family Ericaceae and the genus *Vaccinium*. This genus is composed by shrubs or small trees, including cranberries, lingonberries and bilberries (VANDER KLOET, 1988). The fruit is a true berry, with light blue to black color and numerous seeds (RETAMALES; HANCOCK, 2012).

Blueberries have high nutraceutical benefits, with antioxidant and anti-inflammatory properties (MICHALSKA; ŁYSIAK, 2015). They are rich in polyphenol compounds that have been shown to induce neurogenesis in adults (POULOSE et al., 2017) and showed to modulate the balances of pro-inflammatory cytokinins (CHENG et al., 2014). Blueberry fruits contain anthocyanidins in high concentrations: cyanidin, delphinidin, malvidin, peonidin, and petunidin (ROUTRAY; ORSAT, 2011; AQIL et al., 2014), with health benefits and effects against a diversity of chronic diseases — cancer, diabetes, neurodegenerative and cardiovascular disorders (ROUTRAY; ORSAT, 2011). It was shown the chemo-preventive and therapeutic potential of blueberry in breast cancer (JEYABALAN et al., 2014) and potential preventing Alzheimer's disease, showing a neuroprotective activity (BUSINARO et al., 2018). Some findings show that the consumption of blueberry by older adults can improve some aspects of cognition and can be a strategy for combating the age-related neurodegenerative process (MILLER et al., 2018).

Because of the high concentrations of many beneficial bioactive compounds and to the attractiveness of their rich flavor and texture, blueberries have been gaining attention from consumers. Then the production has been facing an increase, offering fresh fruits as much as processed products, including juices, and frozen and dried products (MICHALSKA; ŁYSIAK, 2015). The production of this crop is increasing worldwide, including South American countries (RETAMALES, 2011; RETAMALES et al., 2015).

Blueberries have become a major crop worldwide (STRIK; YARBOROUGH, 2005) with and increasing production over the last two decades, achieving 545,000 tons in 2014 (IIZUKA; GEBREEYESUS, 2017). The production and commerce have expanded widely in the last 20 years through different regions and a diversity of environments. The most cultivated species are *Vaccinium corymbosum* L. (highbush), *Vaccinium angustifolium* Ait. (lowbush), and *Vaccinium virgatum* (syn.

Vaccinium ashei Reade) (rabbiteye). Highbush cultivars are subdivided in northern, southern or intermediate types, according to their chilling requirement and winter cold hardiness (RETAMALES; HANCOCK, 2012). Rabbiteye cultivars are the least demanding in chilling hours for normal floral development and their floral buds do not tolerate temperatures much below freezing (RETAMALES; HANCOCK, 2012). These cultivars represent a possibility of expanding the production beyond the traditional producing regions and are already being produced in many countries with subtropical climates, since they need low chilling hours to develop and produce appropriately (FACHINELLO, 2008; MEDEIROS et al., 2017; SCHUCH; TOMAZ, 2019).

Blueberry production was originally concentrated in Northern Hemisphere regions, mainly the USA, Canada and European countries. But because of the great increase in demand and the high nutritional fruit quality, many countries are now producing it, such as Australia, New Zealand, China, Japan and South America countries (RETAMALES; HANCOCK, 2012). Chile, Argentina and Uruguay are the main producers in South America. Brazil first cultivated blueberries in the 1990's, with an estimated area of 400 ha in 2014. Mainly in the Southern and Southeastern regions, some cultivars with low chilling requirements (150 a 400 h) can be cultivated. Most of the cultivated blueberries in Brazil are rabbiteye and recently some southern highbush cultivars (CANTUARIAS-AVILÉS et al., 2014). The 'Delite' rabbiteye cultivar was among the first rabbiteye cultivars introduced in Brazil (BAÑADOS, 2006). In a study comparing ten cultivars (eight rabbiteye and two highbush) under humid subtropical conditions in Southern Brazil (Cerro Azul/Paraná), the authors found that the highest fruit set, with best productive performance was observed in Bluegem, Climax, Delite, and Powderblue (MEDEIROS et al., 2018). In another research in a mild winter region in Southern Brazil (Pinhais/Paraná), comparing ten cultivars, the authors described that the same four rabbiteye cultivars above mentioned showed the highest productivity (MEDEIROS et al., 2017).

Besides local market, blueberries grown in Brazil can reach international markets, since its production is concentrated in the off season of the countries of the Northern hemisphere (COUTINHO et al., 2007).

Traditionally, blueberries can be propagated by softwood, semihardwood, hardwood (MARINO et al., 2014a), and rhizome cuttings (DEBNATH, 2009b). However, these propagation methods in general have low rooting rates and a long duration of the whole process (MEINERS; SCHWAB; SZANKOWSKI, 2007; MARINO

et al., 2014a), besides many phytosanitary problems. Micropropagation can be presented as a faster growing alternative (DEBNATH, 2017), throughout the year and with higher phytosanitary quality (GOYALI; IGAMBERDIEV; DEBNATH, 2015b). *In vitro* culture techniques, furthermore, are important for their diversity of applications, such as clonal mass propagation, cryopreservation, germplasm conservation, and other biotechnological researches. The use of these techniques is dependent on the efficiency of the whole process.

In vitro blueberry research has focused more on the highbush and lowbush cultivars of *Vaccinium* (BRISSETTE; TREMBLAY; LORD, 1990; ABDELNOUR-ESQUIVEL, 1991; ISUTSA; PRITTS; MUDGE, 1994; NOÉ; BONINI, 1996; CAO; HAMMERSCHLAG; DOUGLASS, 2002; CAO et al., 2003; LITWIŃCZUK; SZCZERBA; WRONA, 2005; CAPPELLETTI; DEBNATH, 2007, 2009a, 2009b, 2011, 2017; MEINERS; SCHWAB; SZANKOWSKI, 2007; LITWIŃCZUK; WADAS, 2008; TETSUMURA et al., 2008; LIU et al., 2010; REED; RUZIĆ et al., 2012; HINE-GÓMEZ; ABDELNOUR-ESQUIVEL, 2013; IGAMBERDIEV; DEBNATH, 2013, 2015a, 2015b; MARINO et al., 2014b; PIZZOLATO et al., 2014; CAPPELLETTI; SABBADINI; MEZZETTI, 2016; HUNG et al., 2016b; MEZZETTI, 2016; FAN et al., 2017; GAO et al., 2018; GHOSH; IGAMBERDIEV; DEBNATH, 2018; QIU et al., 2018; GOYALI; GUO et al., 2019; SANTIAGO; SMAGULA, 2013; BERAUD; ULLOA, 2015; WELANDER et al., 2017; WANG et al., 2019). However, these *in vitro* protocols are specific to the genotype studied (CAPPELLETTI; SABBADINI; MEZZETTI, 2016). Only few researches in *V. virgatum* (rabbiteye cultivars, more adapted to warm winter regions, such as Brazilian Southern regions) have been reported, and not including all the steps of *in vitro* propagation and regeneration, usually referring to one of the stages only (ERIG; SCHUCH, 2005; SILVA et al., 2006, 2008; DAMIANI; SCHUCH et al., 2008; SCHUCH, 2008, 2009; SOUZA et al., 2011; PELIZZA et al., 2012; FARIAS et al., 2014; HUNG et al., 2016a; FAN et al., 2017; QIU et al., 2018; SCHUCHOVSKI; BIASI, 2019). Therefore, there is a need to increase the research on *in vitro* protocols for this specific group of cultivars.

The main objective of this research was to develop efficient micropropagation and *in vitro de novo* shoot organogenesis protocols for 'Delite' rabbiteye blueberry. The whole research was divided in three chapters, with specific objectives: i) to define a suitable establishment protocol *in vitro* with different concentrations of different growth regulators and balances of nitrogen salts in two different culture

media; ii) to optimize a micropropagation protocol, developing an efficient surface sterilization for *in vitro* establishment, testing the initial culture with different concentrations of zeatin and 2iP, the multiplication stage with different zeatin concentrations and different orientations of the explant in the medium, and finally, test *in vitro* and *ex vitro* rooting, with IBA concentrations and different substrates and mineral salts; iii) to define an adventitious shoot organogenesis protocol from leaf explants, testing TDZ concentrations, explant orientations and explant portions and developing morphoanatomical studies in *in vitro* organogenesis.

2 CHAPTER I

In vitro establishment of 'Delite' rabbiteye blueberry microshoots[1]

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ABSTRACT

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 x NH_4NO_3 and 1 x $Ca(NO_3)_2$, 0.5 x NH_4NO_3 and 0.5 x $Ca(NO_3)_2$, 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 x NH_4NO_3 and 0.5 x $Ca(NO_3)_2$,

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and 1.5 x NH_4NO_3 and 1.5 x $Ca(NO_3)_2$. 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.

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.

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 anti-inflammatory medicine [4]. Among these 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 cutting, 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 research has been done in *V. virgatum* Ait. (syn. *V. ashei* Reade), specifically for the 'Delite' rabbiteye cultivar that is suitable for and adapted to regions of southern Brazil. In 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 would allow 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 either for micropropagation or for 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 *in vitro* new growth is difficult to achieve in *Vaccinium*, especially when using plant material from the field [13].

For the initial phase of *in vitro* culture, usually a combination of cytokinins can be used. In the initial *in vitro* culture, for nodal segments from softwood cuttings in 'Ozarkblue' blueberry (*V. corymbosum*), ZEA and 2iP were tested in the initial culture medium in different combinations (18 µM of ZEA, 25 µM of 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, the explants produced elongated shoots with both growth regulators. However, ZEA treatments showed a higher percentage of new shoot growth compared to 2iP in all 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, the best results were with 49.2 µM and

for lingonberry, 24.6 µM. More brownish explants were observed with increasing 2iP concentration [13]. In 'Berkeley', 'Bluecrop' and 'Earliblue', highbush blueberry (*V. corymbosum*) and 'O'Neal' southern highbush blueberry, medium containing 20 µM ZEA was used in initiation of cultures [14].

Concerning the type of basal culture medium, many researchers have been using WPM as the basic medium for blueberry [14]. However, some authors tried to optimize this medium by doing some modifications, such as combining MS and WPM media, creating an MW medium [14], or by 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 discuss 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.

MATERIALS AND METHODS

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

Plant material

One-year-old hardwood cuttings were collected during winter from field-grown rabbiteye blueberry 'Delite' (*V. virgatum*) mother plants at the Experimental Station of Universidade Federal do Paraná, Pinhais/PR. They were treated with an immersion in fungicide solution for 5 minutes (Cercobin® 0.2%) and stored at 4°C temperature at the Micropropagation Laboratory – UFPR, Curitiba/PR for 1-2 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-hour photoperiod. Newly formed shoots were collected and used as explants for the establishment of cultures.

Two-node segments (0.8 - 2 cm in length, discarded 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 minutes. They were washed with sterile deionized water three times inside the laminar flow chamber.

Culture medium and growing conditions

Explants were isolated in culture tubes (150 x 30 mm) 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.

Experiment 1: Cytokinins

Microcuttings were isolated in the modified Woody Plant Medium (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 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 Woody Plant Medium (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
$Ca(NO_3)_2.4H_2O$	278.00	556.00
$CaCl_2.2H_2O$		96.00
$MgSO_4.7H_2O$	370.00	370.00
Micronutrients		
$FeSO_4.7H_2O$	55.60	27.80
Na_2-EDTA	74.60	37.30
H_3BO_3	6.20	6.20
$MnSO_4.H_2O$	22.30	22.30
$ZnSO_4.7H_2O$	8.60	8.60
KI	0.415	
$Na_2MoO_4.2H_2O$	0.25	0.25
$CuSO_4.5H_2O$	0.025	0.25

Experiment 2: Combinations of $(NH_4)_2SO_4$, KNO_3 and $Ca(NO_3)_2.4H_2O$ using the modified WPM [15] as the basic medium

Explants were isolated using nine different treatments as described in Table 2, with different amounts (1x, 0.5x or 1.5x) of $(NH_4)_2SO_4$, KNO_3 and $Ca(NO_3)_2.4H_2O$ (Table 2), using the modified Woody Plant Medium (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 (modified WPM) [15] with different amounts of $(NH_4)_2SO_4$ (x), KNO_3 and $Ca(NO_3)_2.4H_2O$ (x)..

Treatments	**1 (mod. WPM)**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
NH_4: $(NH_4)_2SO_4$	1x	1x	1x	0.5x	0.5x	0.5x	1.5x	1.5x	1.5x
NO_3: KNO_3 and $Ca(NO_3)_2.4H_2O$	1x	0.5x	1.5x	1x	0.5x	1.5x	1x	0.5x	1.5x
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	1,339.5	893.0	446.5	1,339.5	893.0	446.5	1,339.5
$Ca(NO_3)_2.4H_2O$	278.0	139.0	417.0	278.0	139.0	417.0	278.0	139.0	417.0

Experiment 3: Combinations of NH_4NO_3 and $Ca(NO_3)_2.4H_2O$ using the original WPM [18] 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 (1x, 0.5x or 1.5x) of NH_4NO_3 and $Ca(NO_3)_2.4H_2O$, 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 $Ca(NO_3)_2.4H_2O$ (x) and treatment 10 with the modified Woody Plant Medium [15] .

Treatments	**1 (orig. WPM)**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10 (mod. WPM)**
NH_4NO_3 (x)	1x	1x	1x	1.5x	1.5x	1.5x	0.5x	0.5x	0.5x	
$Ca(NO_3)_2.4H_2O$ (x)	1x	0.5x	1.5x	1x	0.5x	1.5x	1x	0.5x	1.5x	
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	
$Ca(NO_3)_2.4H_2O$	556.0	278.0	834.0	556.0	278.0	834.0	556.0	278.0	834.0	278.0

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-hour photoperiod with light intensity of 40 µmol m^{-2} s^{-1} provided by cool-day fluorescent lamps.

Experimental design, data collection and statistical analysis

The experiments were conducted in a completely randomized design. In experiment 1, a two-factor experiment (4x8) 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 1,280 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, 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 are presented at Table S1. In experiment 2, contamination rates ranged from 0 to 14%; and 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 zero concentration did not show any influence in 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 (4x7), with four different cytokinins (ZEA, 2iP, BAP and KIN) in 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.

RESULTS

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.

The effects of cytokinins on survival

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

The effects of cytokinins on the number of shoots per explant

Regarding the number of new shoots formed and observing the regression analysis (Figure 2), we can observe that ZEA showed a linear equation and calculate that a concentration of 22.0 µM would be required to reach 1.4 shoots per explant. In 2iP behavior, the maximum point in the curve reached 1.4 shoots per explant, which would be acquired at the 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. 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, showing an average of only 0.5 to 0.8 new shoots per explant.

The effects of cytokinins on the shoot length

Type of cytokinin had a significant influence on shoot length. Regression analysis (Figure 2) shows that ZEA follows a quadratic polynomial equation, with the concavity upward, showing 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 regression 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 showed 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.

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. Data presented 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 ($p<0.05$).

Survival (%)							
Cytokinin	2.5 µM	5 µM	10 µM	20 µM	30 µM	40 µM	50 µM
ZEA	89.7 ± 14.2a	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 ± b	0.5 ± b
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

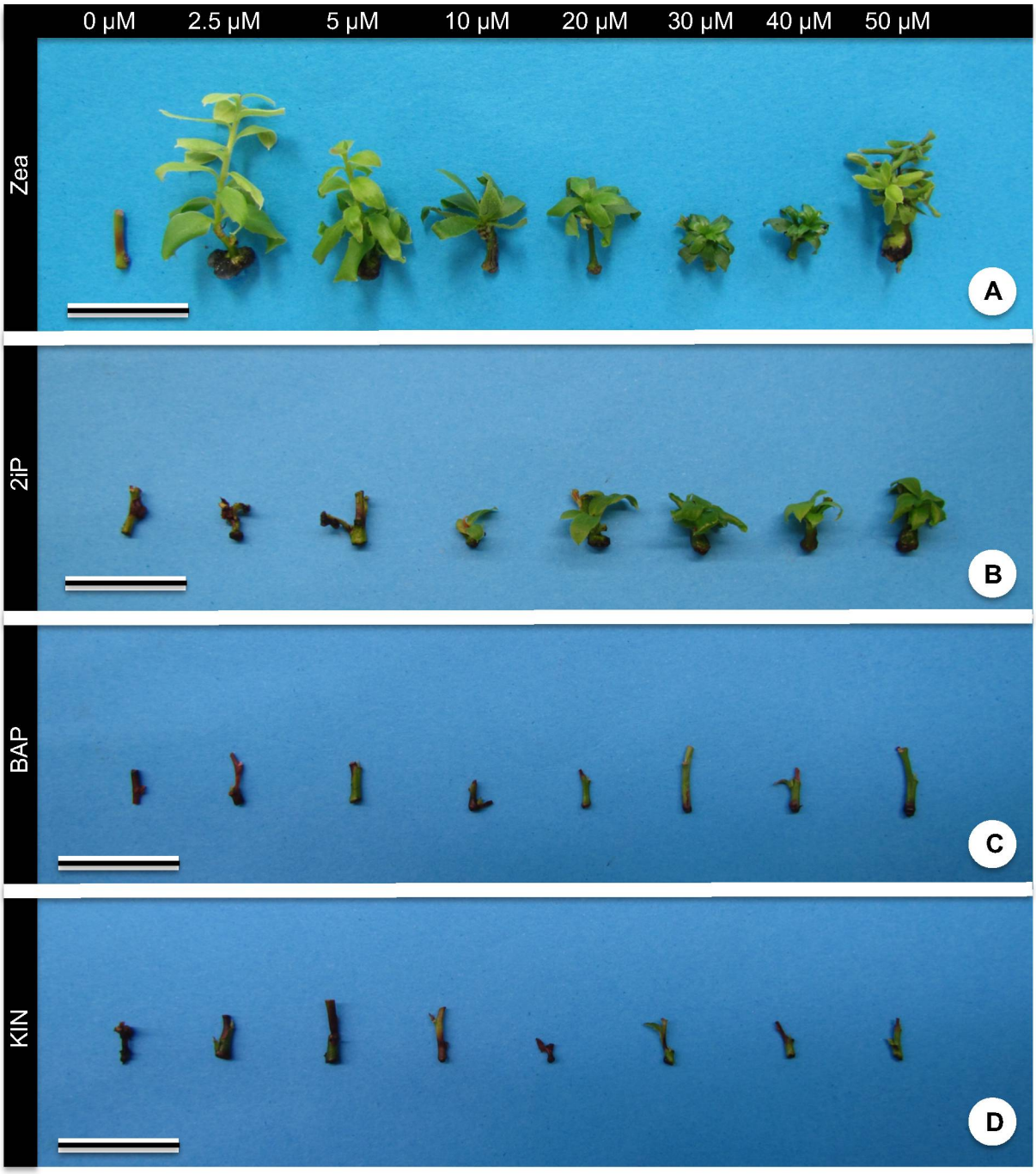

Figure 1. Initial *in vitro* shoot culture of 'Delite' rabbiteye blueberry in eight different concentrations (0, 2.5, 10, 20, 30, 40 and 50 µM) of four different cytokinins: (**a**) Zea, (**b**) 2iP, (**c**) BAP and (**d**) KIN. Bars represent 2 cm. Abbreviations: BAP, 6-Benzylaminopurine; KIN, kinetin;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

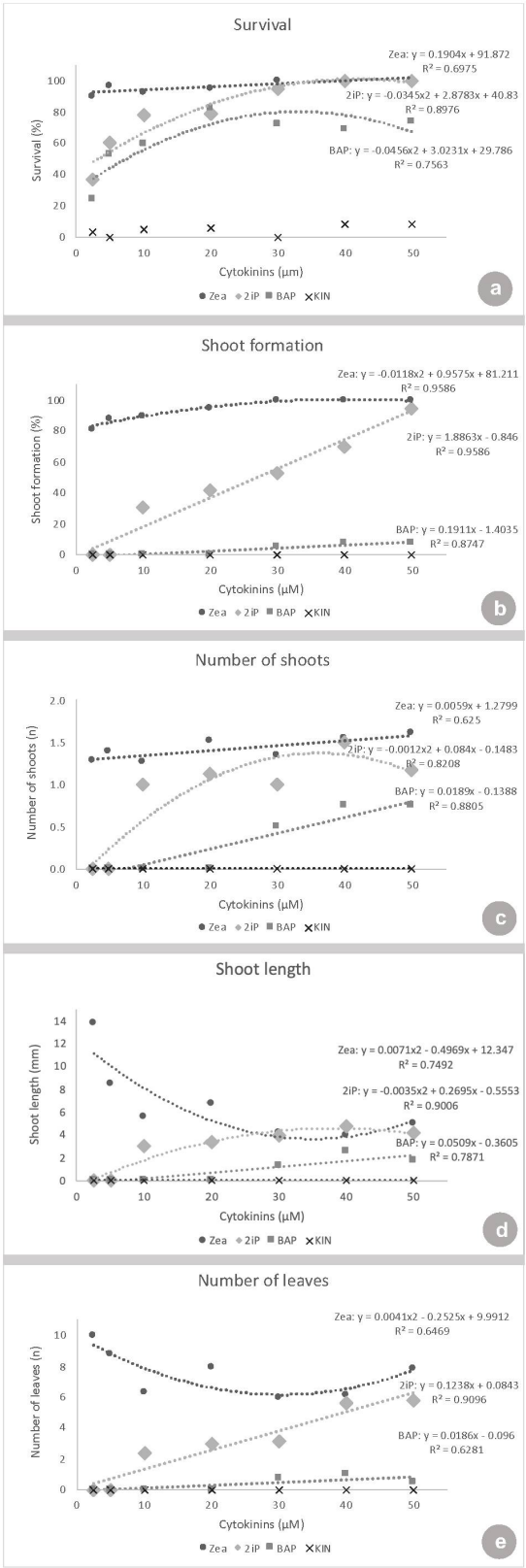

Figure 2. Regression analysis related to different cytokinin (Zea, 2iP, BAP and KIN) concentrations (2.5, 5, 10, 20, 30, 40 and 50 µM) effects on *in vitro* establishment of 'Delite' rabbiteye blueberry. (**a**) Survival (%), (**b**) Shoot formation (%); (**c**) Number of shoots (nº); **(d)** Shoot length (mm); **(e)** Number of leaves (nº). Abbreviations: BAP, 6-Benzylaminopurine; KIN, kinetin; Zea, zeatin; 2iP, 6-(γ-γ-dimethylallylamino)-purine.

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 in 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 model, showing a maximum of 5.8 leaves at a concentration of 50 µM. BAP was also represented by a linear regression, reaching a maximum of 0.8 leaves calculated 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 in all concentrations but 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 2iP. BAP showed some leaves only at concentrations of 30 µM (0.8 leaves), 40 µM (1.0 leaf) and 50 µM (0.5 leaves). At all the concentrations, KIN did not show any response.

Experiment 2: Combinations of $(NH_4)_2SO_4$, KNO_3 and $Ca(NO_3)_2.4H_2O$ 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 were from 1.1 to 1.4, with shoot lengths of 7.5 to 25.0 mm, and the number of leaves was 9.4 to 19.7 (Table 5).

Table 5. Experiment 2 with treatments 1 to 9 on the modified Woody Plant Medium (modified WPM) 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. Data presented are the means of three replicates ± standard deviation (SD). Means followed by the same lowercase letter within the column are not significantly different according to Scott-Knott's test ($p<0.05$). Abbreviations: CV, coefficient of variation; nº, number

Treatment	Sol. $(NH_4)_2SO_4$ (x)	Sol. KNO_3 and $Ca(NO_3)_2.4H_2O$ (x)	nº	Survival %	Shoot formation %	Number of shoots nº	Shoot length mm	Number of leaves nº
1-modif. WPM	1x	1x	19	48.4 ± 19.1a	48.4 ± 19.1a	1.4 ± 0.1a	13.9 ± 6.7a	11.1 ± 3.4a
2	1x	0.5x	19	62.7 ± 11.3a	62.7 ± 11.3a	1.3 ± 0.2a	24.9 ± 7.4a	19.7 ± 3.1a
3	1x	1.5x	20	43.7 ± 23.4a	43.7 ± 23.4a	1.3 ± 0.3a	17.7 ± 16.9a	13.7 ± 7.6a
4	0.5x	1x	18	73.0 ± 35.1a	73.0 ± 35.1a	1.1 ± 0.2a	9.1 ± 5.5a	10.9 ± 5.6a
5	0.5x	0.5x	19	69.5 ± 11.5a	69.5 ± 11.5a	1.1 ± 0.1a	10.7 ± 3.2a	11.8 ± 1.5a
6	0.5x	1.5x	20	45.2 ± 4.1a	45.2 ± 4.1a	1.2 ± 0.2a	25.0 ± 14.3a	18.0 ± 6.0a
7	1.5x	1x	21	76.2 ± 8.2a	76.2 ± 8.2a	1.2 ± 0.0a	7.5 ± 2.7a	9.4 ± 3.3a
8	1.5x	0.5x	20	56.3 ± 23.4a	56.3 ± 23.4a	1.1 ± 0.2a	17.2 ± 9.7a	12.7 ± 1.9a
9	1.5x	1.5x	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

Experiment 3: Combinations of NH_4NO_3 and $Ca(NO_3)_2.4H_2O$ using the original WPM [18] as the basic medium

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

The number of shoots was similar in all the treatments tested. In addition, concerning the number of leaves, the lowest number was 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).

Table 6. Experiment 3 with treatments 1 to 9 on Woody Plant Medium (WPM) with different ranges of NH_4NO_3 (x) and $Ca(NO_3)_2.4H_2O$ (x) and treatment 10 on modified Woody Plant Medium, 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. Data are presented as 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 ($p<0.05$). Abbreviations: CV, coefficient of variation; nº, number.

Treat.	Sol. NH_4NO_3	Sol. $Ca(NO_3)_2$	nº	Survival	Shoot formation	Number of shoots	Shoot length	Number of leaves
				%	%	nº	mm	nº
1-original WPM	1x	1x	38	79.4 ± 16.4a	79.4 ± 16.4a	1.2 ± 0.3a	33.3 ± 7.6a	15.3 ± 2.1a
2	1x	0.5x	40	95.0 ± 5.8a	95.0 ± 5.8a	1.1 ± 0.1a	23.5 ± 3.9b	12.2 ± 1.5a
3	1x	1.5x	38	97.5 ± 5.0a	97.5 ± 5.0a	1.2 ± 0.1a	21.0 ± 1.9b	11.4 ± 1.7a
4	1.5x	1x	27	86.8 ± 10.5a	83.7 ± 15.7a	1.1 ± 0.1a	25.4 ± 8.9b	12.1 ± 2.9a
5	1.5x	0.5x	40	92.5 ± 5.0a	90.0 ± 8.2a	1.1 ± 0.2a	32.3 ± 7.3a	14.4 ± 1.1a
6	1.5x	1.5x	40	90.0 ± 8.2a	90.0 ± 8.2a	1.1 ± 0.1a	30.7 ± 6.6a	14.1 ± 1.2a
7	0.5x	1x	39	64.4 ± 16.7b	64.4 ± 16.7b	1.1 ± 0.1a	5.0 ± 0.7d	6.6 ± 1.2c
8	0.5x	0.5x	35	55.1 ± 5.6b	55.1 ± 5.6b	1.1 ± 0.1a	18.2 ± 10.5c	10.4 ± 3.6b
9	0.5x	1.5x	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

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) with different ranges of NH_4NO_3 (x) and $Ca(NO_3)_2.4H_2O$ (x), compared to treatment 10 (modified Woody Plant Medium).

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 show a screening comparison of four different cytokinins in eight different concentrations and different balances of nitrogen salts in 'Delite' rabbiteye blueberry, presenting an efficient technique for *in vitro* plant propagation in this species.

The species and cultivars of *Vaccinium* genus show natural variation in *in vitro* responses among their species and cultivars. There is high genetic variation in growth regulator responses/needs. Our results, based on linear and quadratic polynomial regression analysis, display the effects of cytokinin concentrations and their great impact on 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 of 2.5 and 5 µM, ZEA was superior to all the other cytokinins tested, in all the variables analyzed, presenting in the following concentrations: 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. Similar results were observed in 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 µ M [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 in very low concentrations (2–4 µM) showed a good performance [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 in 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 elongation of shoots, and concentrations of 2.3 and 4.6 µM gave the best response [25].

Another aspect to be mentioned is the growth habit of '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 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), 2iP treatments did not show any response. Treatments with 2iP started to form shoots only in 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 the concentrations, except in 50 µM, where both had the same shoot formation rate. This shows that ZEA triggered a response in the explants even in 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, in the concentrations where 2iP started showing new shoots (10-50 µM), the shoots formed were equivalent in length to the shoots formed in 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 the concentrations, except 40 and 50 µM, showing again the need for higher concentrations of 2iP to show a higher number of leaves formed. In 'Brightwell' blueberry, authors found that different concentrations of 2iP (5, 10, 15, or 20 $mg \cdot L^{-1}$) and TDZ were inferior to 2 mg L^{-1} (9.12 µM) ZEA in shoot proliferation [26]. ZEA at 4 $mg \cdot L^{-1}$ (18.24 µM) was more successful than 2iP at 10 or 15 $mg \cdot L^{-1}$ (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 started showing a low response only in 30, 40 and 50 µM (5.0% - 7.8% of shoot formation). BAP was always highly inferior to ZEA at all concentrations tested, in all the variables analyzed, except 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 survival explants (maximum of 8.3% of survival), showing clearly 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 $Ca(NO_3)_2.4H_2O$. 'Delite' blueberry showed lower survival (55.7%), shoot formation (55.7%), and shoot length (15.7 mm) in the modified WPM compared with original WPM (79.4%, 79.4% and 33.3 mm, respectively).

Using the original WPM, it was possible to observe that treatments containing higher amounts of NH_4NO_3 (1x or 1.5x, instead of 0.5x) as well as the treatment with higher amount of $Ca(NO_3)_2$ (1.5x), even with lower amount of NH_4NO_3 (0.5x), 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 best 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 $Ca(NO_3)_2$ (1.5x) 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 to understand initial *in vitro* establishment. It can be useful to describe this process in other *Vaccinium* cultivars regarding the necessary adjustments to adapt the process to different genotypes.

CONCLUSIONS

The research shows a description of the effects of different cytokinins in different concentrations and different nitrogen salt ranges in 'Delite' rabbiteye blueberry *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 in '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 new

shoots of 1.3, with 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 results than the original medium.

This work is of interest for evaluating different cytokinin and salt composition in the culture medium for *in vitro* establishment, and it can contribute to developing 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 of 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 nmber 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.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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SUPPLEMENTARY MATERIALS

Table S1. 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. Abbreviation: nº (number).

Number of explants evaluated in each treatment (nº)							
	Concentration						
Cytokinin	2.5 µM	5 µM	10 µM	20 µM	30 µM	40 µM	50 µM
ZEA	37	35	39	39	39	39	40
2iP	38	38	38	38	37	36	40
BAP	37	38	37	39	39	39	39
KIN	38	38	40	39	40	37	39

Table S2. 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. Abbreviations: CV: coefficient of variation; DF, degrees of freedom; SS, sum of squares; MS, mean squares.

Evaluation	Source	DF	SS	MS	F ratio	p-value
Survival	Cytokinin type	3	1,255.80	418.60	343.45	< 0.0001
	Cytokinin concentration	6	77.44	12.91	10.59	-----
	Interaction (type X concentration)	18	57.01	3.17	2.60	0.0018
	Error	84	102.38	1.22		
CV (%): 16.2%	Total	111	1,492.63			
Shoot formation	Cytokinin type	3	1,634.83	544.94	956.68	< 0.0001
	Cytokinin concentration	6	157.89	26.32	46.20	-----
	Interaction (type X concentration)	18	230.77	12.82	22.51	< 0.0001
	Error	84	47.85	0.57		
CV (%): 18.9%	Total	111	2,071.34			
Number of shoots	Cytokinin type	3	20.13	6.71	150.13	< 0.0001
	Cytokinin concentration	6	3.54	0.59	13.19	-----
	Interaction (type X concentration)	18	4.81	0.27	5.98	< 0.0001
	Error	84	3.76	0.04		
CV (%): 35.5%	Total	111	32.24			
Shoot length	Cytokinin type	3	97.97	32.66	250.76	< 0.0001
	Cytokinin concentration	6	4.50	0.75	5.76	-----
	Interaction (type X concentration)	18	32.14	1.79	13.71	< 0.0001
	Error	84	10.94	0.13		
CV (%): 31.9%	Total	111	145.56			
Number of leaves	Cytokinin type	3	122.86	40.95	424.80	< 0.0001
	Cytokinin concentration	6	6.49	1.08	11.22	-----
	Interaction (type X concentration)	18	20.75	1.15	11.95	< 0.0001
	Error	84	8.10	0.10		
CV (%): 27.3%	Total	111	158.20			

2 CHAPTER II

Micropropagation of 'Delite' rabbiteye blueberry: a mild winter adapted cultivar[2]

Shortened title: **Micropropagation of rabbiteye blueberry**

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ABSTRACT

Blueberry is an important fruit crop, with antioxidant and anti-inflammatory health benefits. The conventional propagation method is vegetative, through cuttings, which can lead to pathogen infection. An alternative method is *in vitro* propagation. Most studies on *in vitro* culture of blueberries focus on highbush and lowbush cultivars, and only a few studies on rabbiteye cultivars — more adapted to mild winter regions. Thus, it is necessary to develop a micropropagation protocol for this group of

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cultivars. Our objective was to develop a suitable *in vitro* establishment, multiplication, and rooting using 'Delite' rabbiteye blueberry. The most promising results were: surface sterilization of nodal segments with 5 min immersion in sodium hypochlorite (96.7% of uncontaminated explants and 96.7% survival rate); *in vitro* establishment in Woody Plant Medium (WPM) with 2.5 µM zeatin (92.3% survival and 84.5% explants with axillary shoot growth); multiplication of two-node stem segments in WPM with 2.5 µM zeatin (100% survival, 70% explants with shoot proliferation, one new shoot/explant, with 3.6 cm and 11.7 leaves), and five-node stem segments in the vertical orientation (100% survival, 100% explants with shoot proliferation, 1.8 new shoots/explant, 5.1 cm long with 12.7 leaves); *in vitro* rooting with immersion in 500 mg L-1 IBA (100% survival, 100% microcuttings with maintenance of leaves and 37.5% rooting), or *ex vitro* rooting in vermiculite with WPM mineral salts (88% survival, 86% of microcuttings with leaf maintenance, 50% of microcuttings with new buds induction, and 68% rooting). In conclusion, this study provides techniques for *in vitro* propagation of 'Delite' rabbiteye blueberry using nodal segments, concerning the steps of establishment, multiplication, and rooting of the plants.

KEY MESSAGE

Our study developed an *in vitro* shoot micropropagation procedure using nodal segments of 'Delite' rabbiteye blueberry using WPM culture medium supplemented with zeatin in initial and multiplication stages, and *in vitro* rooting using IBA, or *ex vitro* rooting in vermiculite and WPM mineral salts.

KEYWORDS

Ericaceae, rooting, shoot proliferation, *Vaccinium virgatum.*

INTRODUCTION

Blueberry is known for its high nutraceutical properties, with many health benefits. The true berries are an important source of antioxidants and anti-inflammatory compounds (Michalska and Łysiak 2015). Its bio compounds include polyphenols and anthocyanins, having effects against cancer, diabetes, and cardiovascular and neurodegenerative diseases (Routray and Orsat 2011). The consumption of blueberry by adults improved cognition (Miller et al. 2018), having a neuroprotective activity (Businaro et al. 2018). Besides its nutritive characteristics, blueberries are appreciated for their rich flavor and texture, with a high demand by consumers, for fresh and processed products, generating a recent increase in production in many parts of the world (Michalska and Łysiak 2015), including in South American countries (Retamales 2011; Retamales et al. 2015).

Blueberry is a fruit crop in the family Ericaceae and the genus *Vaccinium* (Vander Kloet 1988). There are many blueberry cultivars, belonging to three major species: *Vaccinium corymbosum L.*, the highbush group and its hybrids, with most of the fruit production; *Vaccinium angustifolium* Ait., the lowbush group, with about one-third of the world production; and *Vaccinium virgatum* Ait., the rabbiteye group, produced in smaller quantities (Rowland et al. 2010). Rabbiteye cultivars can grow in warmer winter climates and are high-yielding with a vigorous habit, relatively large fruits, and seeds (Ehlenfeldt et al. 2007). These cultivars represent a possibility of expanding the production beyond the traditional producing regions and are already being produced in many countries with sub-tropical climates, since they need low chilling hours to develop and produce appropriately (Fachinello 2008; Medeiros et al. 2017; Schuch and Tomaz 2019).

Commercial propagation of this crop by seeds is not common, as it presents a high heterozygosity rate (Hung et al. 2016a). The conventional propagation method is vegetative, using softwood, semi-hardwood or hardwood cuttings (Marino et al. 2014), which can represent a low level of phytosanitary standards, a low rate of rooting, and a time-consuming method, especially if the objective is to commercialize newly released cultivars (Meiners et al. 2007; Marino et al. 2014). To overcome these challenges, *in vitro* propagation is an interesting alternative for blueberry clonal propagation, with the possibility of producing a large number of plants, throughout the

year (Debnath 2017; Schuch and Tomaz 2019). Furthermore, *in vitro* techniques are the basis for further development in biotechnology (Debnath 2009a).

Blueberries have been micropropagated, with most of the research concentrated in highbush and lowbush cultivars (Brissette et al. 1990; Reed and Abdelnour-Esquivel 1991; Isutsa et al. 1994; Noé and Bonini 1996; Cao et al. 2002, 2003; Litwińczuk et al. 2005; Debnath 2007, 2009a, b, 2011, 2017; Meiners et al. 2007; Litwińczuk and Wadas 2008; Tetsumura et al. 2008; Ruzić et al. 2012; Goyali et al. 2013, 2015a, b; Santiago and Smagula 2013; Hine-Gómez and Abdelnour-Esquivel 2013; Marino et al. 2014; Beraud and Ulloa 2015; Cappelletti and Mezzetti 2016; Hung et al. 2016b; Welander et al. 2017; Fan et al. 2017; Gao et al. 2018; Guo et al. 2019; Wang et al. 2019). However, these *in vitro* protocols are specific to the genotype studied (Cappelletti et al. 2016). Only some researches in *V. virgatum* (rabbiteye cultivars) have been reported, not including all the steps of *in vitro* propagation, usually referring to one of the stages only (Erig and Schuch 2005; Silva et al. 2008, 2006; Damiani and Schuch 2008, 2009; Schuch et al. 2008; Souza et al. 2011; Pelizza et al. 2012; Farias et al. 2014; Hung et al. 2016a; Fan et al. 2017; Schuchovski and Biasi 2019). Therefore, there is a need to develop a complete and efficient micropropagation protocol for the rabbiteye group cultivars.

The main objective of this work was to develop a micropropagation procedure for 'Delite' rabbiteye blueberry using *in vitro* techniques that can be used for clonal propagation, conservation of plant biodiversity *in vitro,* and other biotechnological researches.

MATERIAL AND METHODS

Plant material and initial culture

Hardwood cuttings (one-year-old) were collected from field-grown 'Delite' rabbiteye blueberry (*V. virgatum*) mother plants during the winter. All the cuttings were treated by immersion in a thiophanate-methyl fungicide solution (0.2% Cercobin™ (Iharabras, São Paulo, Brazil)) for 5 min and stored at 4 ºC. About 30 days later, these cuttings were placed in the culture room to start bud growth. The newly grown shoots were collected and surface sterilized. They were washed with water for 10 min and immersed in 70% (v/v) ethanol solution for 30 s, then in 0.5%

sodium hypochlorite solution containing 0.1% (v/v) Tween 20 for 5 min (except in experiment 1, related to surface sterilization). All the shoots were washed three times with sterile deionized water, and they were used in the establishment of *in vitro* cultures. Shoots were placed in test tubes (160 × 23 mm), containing 6 mL of Woody Plant Medium (WPM) culture medium (Lloyd and McCown 1980) supplemented with Murashige and Skoog (MS) organic compounds (Murashige and Skoog 1962), 2.5 µM zeatin (6-(4-hydroxy-3-methylbut-2-enylamino)purine) (unless otherwise noted), and 30 $g.L^{-1}$ sucrose. The pH of the media was adjusted to 5.2 and solidified with 7 g L^{-1} agar (Vetec™, Rio de Janeiro/Brazil). After that, the media were autoclaved at 120 ºC and 1.0 atm for 20 min, and zeatin was sterilized through 0.22 µm filters and added to the cooled media. The cultures were periodically transferred to fresh medium in jars (6.5 cm x 8.0 cm) containing 30 mL of medium, with five shoots per jar and maintained at 25 ± 2 ºC under cool daylight at 40 µmol m^{-2} s^{-1} with a 16-h photoperiod.

Experiment 1: Surface sterilization of the explants

This experiment in the establishment stage tested the surface sterilization of the explants. It was conducted in a completely randomized design, with three immersion times (5, 10, and 15 minutes) in 0.5% sodium hypochlorite solution containing 0.1% (v/v) Tween 20 and a control without treatment. The explants were nodal segments containing two buds (leaves removed), 0.5-1.0 cm long, originated from the new shoots formed, as previously described. The culture medium was prepared according to the previous description, but the growth regulators used were: 10.0 µM zeatin, 1.0 µM NAA (α-naphthaleneacetic acid), and 0.5 µM GA_3 (gibberellic acid). Cultures were initially placed in the culture room in the dark. Seven days later, they were moved to the light room as previously described. Each treatment consisted of three replications (ten explants in each replication), giving a total of 30 explants per treatment, in a total of 120 leaf explants in the experiment. After 60 days of the establishment, cultures were evaluated according to the percentage of uncontaminated explants (%), the survival rate of the explants (%), and percentage of explants with axillary shoot growth (%).

Experiment 2: Different cytokinins in the establishment of the explants *in vitro*

To evaluate the initial establishment of the nodal segments *in vitro*, a two-factor (3 x 2) arrangement and a completely randomized design were used, with factor 1 being the different concentrations of zeatin (0, 2.5 and 5.0 µM) and factor 2 being the concentrations of 2iP (2-isopentenyladenine) (6-(γ-γ-dimethylallylamino)-purine) (0 and 20 µM), in a total of six treatments. The medium was prepared as previously described but supplemented with a combination of the two cytokinins. Both zeatin and 2iP were sterilized through 0.22 µm filters and added to the cooled media. The medium was placed in test tubes (160 × 23 mm), each containing 6 mL. The nodal segments were surface sterilized as previously described and placed in the tubes. Each treatment consisted of four replications (ten explants in each replication), giving a total of 40 explants per treatment, in a total of 240 explants in the experiment. Cultures were initially placed in the culture room in the dark and, seven days later, they were moved to the light room as previously described. They were evaluated seven weeks later, according to the survival rate of the explants (%), percentage of explants with axillary shoot growth (%), number of new shoots formed per explant (n), mean length of the new shoots formed (cm), and mean number of leaves per new shoot formed. The contamination rate in this experiment ranged from 23.1 to 34.9%.

Experiment 3: Multiplication with different zeatin concentrations

This experiment was conducted in a completely randomized design, with five treatments: different concentrations of zeatin (0, 2.5, 5.0, 7.5, and 10.0 µM). The initial explants (two-node stem segments) were originated from *in vitro* cultures maintained as described in "Plant material and initial culture". The explants were 0.5 to 2.0 cm long, with two leaves. The culture medium was prepared according to the previous description, but the growth regulators were used according to the five treatments described here. Cultures were placed in the culture room, as previously described. Each treatment consisted of four replications (five explants in each replication, in one jar (6.5 cm x 8.0 cm, with 30 mL of medium)), giving a total of 20 explants per treatment, and 100 explants in the experiment. After twelve weeks, cultures were evaluated according to the survival rate of the explants (%),

percentage of explants with shoot proliferation (%), number of new shoots formed per explant (n), mean length of the longest shoot (cm), and mean number of leaves of the longest shoot (n). The contamination rate varied from 0.0 to 25.0% in this experiment.

Experiment 4: Multiplication with different explant positions

This multiplication experiment was conducted in a completely randomized design, with three treatments: different explant orientations in the culture medium (vertical, 45º inclined, and horizontal). The initial explants were originated from *in vitro* cultures, as described in "Plant material and initial culture". These *in vitro* stems had the apical bud removed and the nodal segments were prepared with five buds each and 1.5 to 2.5 cm long. The two basal leaves were removed, and the three most apical leaves were kept in the explant. The culture medium was prepared as previously described, using 2.5 µM zeatin, and cultures were placed in the culture room. Each treatment consisted of seven replications (five explants in each replication, one explant per jar (6.5 cm x 8.0 cm, with 30 mL of medium)), giving a total of 35 explants per treatment, in a total of 105 explants in the experiment.

After twelve weeks, cultures were evaluated according to the survival rate of the explants (%), percentage of explants with shoot proliferation (%), number of new shoots formed per explant (n), mean length of the new shoots formed (cm), mean number of leaves per new shoot formed (n), shoot induction rate in the most basal bud (%), and shoot induction rate in the most apical bud (%). The whole experiment was repeated, with the difference that in the second experiment, the initial explants had all the five leaves removed. After twelve weeks, cultures were evaluated according to the same variables previously described. The contamination rate in this experiment varied from 0.0 to 20.0%.

Experiment 5: *In vitro* and *ex vitro* rooting with different IBA (indole-3-butyric acid) concentrations

To evaluate rooting of *in vitro* micropropagated microcuttings, a two-factor (2 x 3) arrangement and a completely randomized design were used, with factor 1 being the two different environments: *in vitro* (at the culture room), and *ex vitro* (at the

greenhouse) and factor 2 being the IBA concentrations (0, 250, and 500 mg L^{-1}), in a total of six treatments. For the three treatments *in vitro*, the medium used was as previously described, with no growth regulators. For the three treatments *ex vitro*, vermiculite was used as substrate. The IBA was diluted according to the proper concentration, using a solution of deionized water and 99% ethanol (9:1, v/v). The IBA solutions were autoclaved and microcuttings (2.5 to 4.0 cm long) had their bases immersed in the IBA solution for 5 s and then placed either in the jars (5 microcuttings per jar) containing the culture medium with 30 mL per jar, or in the 128 cells-tray in the moisture vermiculite substrate. Each treatment consisted of four replications (ten microcuttings in each replication), giving a total of 40 microcuttings per treatment, and 240 microcuttings in the experiment.

Cultures were maintained in the culture room, as previously described; or in the greenhouse, with intermittent mist (for 15 s at every 30 min). Manual sprinkling irrigation was done every week in the greenhouse. All the microcuttings were evaluated seven weeks later, according to the survival rate of the microcuttings (%), leaf maintenance rate (%), callus formation rate (%), rooting rate (%), number of roots per microcutting (n), and length of the longest root (mm). The contamination rate in this experiment ranged from 0.0 to 25.0%.

Experiment 6: *Ex vitro* rooting with different substrates and mineral salts

In this experiment, the *ex vitro* rooting of micropropagated microcuttings was tested, in a two-factor (2 x 2) arrangement and a completely randomized design, with factor 1 being two different substrates: Plantmax™ (a commercial substrate, Brazil) and vermiculite; and factor 2 being the mineral salt formulation: mineral salts of WPM culture medium and Basacote™ Plus 3M NPK 15-08-12 (a commercial fertilizer, Compo, Germany), in a total of four treatments. All the substrates were placed in plastic boxes, with semi-opened lids. The substrates treated with liquid WPM culture medium received 200 mL/box. Those treated with Basacote™ received 3 kg m^3 of substrate and received 200 mL per box of sterilized deionized water. All microcuttings were originated from *in vitro* culture, as previously described, 3.0-3.5 cm long. The base of the microcuttings was immersed in 1000 mg L^{-1} IBA in a solution of deionized water and 99% ethanol (9:1, v/v) for 10 s.

Each treatment consisted of five replications (ten microcuttings in each replication, in one box), giving a total of 50 microcuttings per treatment, and 200 microcuttings in the experiment. Cultures were maintained in the culture room, as previously described and watered every three days. All the explants were evaluated ten weeks later, according to the survival rate of the microcuttings (%), leaf maintenance rate (%), bud induction rate (%), callus formation rate (%), rooting rate (%), number of roots per microcutting (n), and length of the longest root (mm). The contamination rate in this experiment ranged from 0.0 to 4.0%.

Experimental Design and Statistical Analysis

All experiments were designed completely randomized. The contaminated cultures were excluded (except in the experiments in the establishment of nodal segments, where contamination was a variable measured). Thereafter, all the explants were individually evaluated, and the mean was estimated for each replication. After that, the mean of the three, four, five, or seven replications in each treatment was calculated. Levene's test was performed to confirm the homogeneity of the variance among treatments. Then, analysis of variance (ANOVA) (Sup. Table S1, S2, S2, S3, S4, S5, and S6) was performed to detect statistical differences between treatments, followed by Tukey's multiple range test ($p<0.05$) to identify the superior treatments. Results are presented as mean ± standard error in the tables.

In the experiment 3, in multiplication with different zeatin concentrations, linear regression analyses were performed with the variable that confirmed to have a statistical significance in the analysis of variance of the regression. This variable was "shoot length". For the other variables analyzed, it was not possible to adjust a statistically significant equation with a biological explanation.

All the statistical analyses were performed using R software (R Core Team 2020).

RESULTS AND DISCUSSION

In this study, a complete protocol for micropropagation in 'Delite' rabbiteye blueberry is described.

Experiment 1: Surface sterilization of the explants

The results of this experiment can be seen in Table 1. The percentage of uncontaminated explants varied from 93.3 to 96.7%, and there was no significant difference among the treatments (Table S1). Related to the survival rate of the explants and percentage of explants with axillary shoot growth, the treatments did not show any significant difference, with general means of 91.7 and 77.5, respectively.

In Fig. 1a–1d, it is possible to observe the four treatments (0, 5, 10, and 15 min). The contaminated explants showed only bacterial contamination and no fungus at all. All the treatments showed a good development of shoots, and most of the explants had an intense callus formation on the base of the explants.

The surface sterilization process before *in vitro* establishment can sometimes be demanding, especially in woody species (HUH et al., 2015). Some authors used more toxic products, such as mercuric chloride ($HgCl_2$) for this process in highbush blueberry cultivars (SEDLAK; PAPRSTEIN, 2009), or biocide agents in the culture medium (HUH et al., 2015). In this study, only ethanol or ethanol combined with sodium hypochlorite proved to be efficient to surface sterilize nodal segments for *in vitro* culture. Part of the low rates of contamination found here could be explained by the process used of collecting stems from mother plants and letting them sprout in the culture rooms, so these new shoots formed were less prone to bring microorganisms for the *in vitro* environment, compared to shoots collected directly from field-conditions.

Experiment 2: Different cytokinins in the establishment of the explants *in vitro*

In this experiment (Table 2) combining zeatin and 2iP, when evaluating the survival rate of the explants, there was no interaction between the two factors (zeatin concentrations and 2iP concentrations) (Table S2). However, there was a difference between control and zeatin treatments, showing that the concentrations of 2.5 and 5.0 µM gave results superior to those obtained on the medium without zeatin, with 89.9%, 80.4%, and 39.2% survival rate, respectively. And 20 µM of 2iP showed the same survival rate than the control (72.5 and 67.2% respectively).

Observing the percentage of explants with axillary shoot growth (Table 2), there was an interaction among the two factors ($p<0.05$). In the absence of 2iP, shoot

formation was induced only in the presence of zeatin, where the treatments with 5 and 2.5 μM zeatin did not differ significantly among each other, but both were superior to the medium without zeatin (57.6, 84.5, and 0.0% explants with axillary shoot growth, respectively). In the treatments with 20 μM 2iP, the concentrations of 5.0 and 2.5 μM zeatin had similar results to each other, and both were superior to 0 μM (81.7, 60.5, and 8.1%, respectively). In the treatments with 2.5 μM zeatin, the treatment with no 2iP showed a higher percentage of explants with axillary shoot growth than the treatment with 20 μM 2iP. In Fig. 2a–2f it is possible to observe explants in the six treatments.

For the variable number of new shoots formed, there was no interaction among the two factors ($p<0.05$). None of the means differ, and for all the six treatments one new shoot was formed per explant (Table 2). This number could seem a low rate compared to other species, however, the behavior found in rabbiteye blueberries in this study was the habit of sprouting the pre-existent bud in the nodal segment. All the shoots were formed from the sprout and growth of the axillary bud existent in the original nodal segment explant. Since we departed from a nodal segment with only 2 buds, and in most cases one of the buds got immersed in the culture medium, we observed the sprouting of only one bud, forming one long shoot. This shoot can be further cut into smaller nodal segments to continue the process of subculturing and multiplication *in vitro*.

Observing the variable mean length of shoots, there was an interaction ($p<0.05$) between the two factors. But since one of the treatments resulted not available, the factors were evaluated as not interacting. Treatments without 2iP resulted superior to 20 μM 2iP (1.0 and 0.4 cm long, respectively). And 2.5 μM zeatin was superior to 0 and 5 μM zeatin (1.0, 0.4 and 0.4 cm long, respectively).

Considering the variable number of leaves per shoot, there was no interaction between the two factors. Treatments 0 μM and 20 μM 2iP showed no difference, with 8.5 and 7.6 leaves/shoot. Observing the treatments with zeatin, the concentration 2.5 μM zeatin did not differ statistically from 5 μM zeatin (10.2 and 6.9 leaves/shoot, respectively). But 2.5 μM zeatin was superior to 0 μM zeatin.

From the observations in this experiment, it is possible to consider that for many variables observed (survival rate, number of new shoots formed, and number of leaves per shoot), the addition of 2iP in the medium (20 μM) did not give better

results than the medium without 2iP. And in some cases, it interfered negatively (decreasing the mean length of shoots, for example). Some authors observe that zeatin is less phytotoxic to the blueberry explant than 2iP (REED; ABDELNOUR-ESQUIVEL, 1991). In a research in cranberry (*Vaccinium macrocarpon*) propagation (DEBNATH, 2008), the author argues that zeatin in the culture medium could induce the juvenile branching characteristics in tissue culture shoots, leading to more vegetative growth, compared to conventionally propagated plants.

Zeatin treatments (2.5 and/or 5.0 μM), on the other hand, showed to be superior to the treatment with no zeatin in many variables (survival, shoot induction, mean length of shoots, and number of leaves per shoot). Comparing the two treatments containing zeatin (2.5 and 5.0 μM) among each other, the treatment 2.5 μM proved to be similar or even better than 5.0 μM. Concerning the overall performance and the cost, the treatment 2.5 μM zeatin with no 2iP proved to be the best option for this establishment *in vitro*, showing 92.3% survival rate, 84.5% shoot induction rate, 1.0 new shoot formed per explant, and shoots 1.5 cm long with 10.8 leaves per shoot, after seven weeks in culture.

Some authors discuss the use of 2iP on *in vitro* establishment of blueberries, such as the work with 'Avonblue' cultivar (*V. corymbosum*), where 2iP in the culture medium induced the greatest length of shoots (HINE-GÓMEZ; ABDELNOUR-ESQUIVEL, 2013). Another research in lowbush blueberry (*V. angustifolium*) used 59 μM 2iP for the establishment (BRISSETTE; TREMBLAY; LORD, 1990).

Evaluating 'Florida' rabbiteye cultivar (ERIG; SCHUCH, 2005), the authors compared 2iP alone or in combination with NAA and GA_3 in the medium and found that the concentration 24,6 μM 2iP alone showed the highest establishment rate.

In the establishment of microcuttings of 'Berkeley', 'Blue Heaven', 'Collins', 'Darrow', 'Early Blue', 'Jersey' and 'Late Blue' blueberry cultivars (*V. corymbosum*) (ECCHER et al., 1986), the authors found that in BAP (6-benzylaminopurine) treatments there was no growth of the shoots, and only on 2iP treatments the axillary shoots developed. The percentage of sprouted explants increased with 2iP concentrations, up to 15 mg L^{-1} (73.8 μM).

Evaluating the initial growth in other *Vaccinium* species (JAAKOLA et al., 2001), the research described an optimum concentration of 49.2 μM 2iP for bilberry (*Vaccinium myrtillus* L.) and for 24.6 μM for lingonberry (*Vaccinium vitis-idaea* L.). In

a study with 'Delite' rabbiteye blueberry (*V. virgatum*) (SCHUCHOVSKI; BIASI, 2019) testing different cytokinins, zeatin, 2iP, BAP, and kinetin, in eight concentrations, it was found that zeatin and 2iP were superior to BAP and kinetin for the initial establishment of nodal segments. However, the authors observed that zeatin was superior to all the other cytokinins tested using the lowest concentrations (2.5 μM and 5 μM). In another study in bilberry (NIN et al., 2019), the nodal segments were established *in vitro* in a medium containing either zeatin (9.1 and 18.2 μM) or 2iP (24.6 and 49.2 μM), however, media with zeatin allowed higher initiation rates and more shoots per explant. These results are in accordance to our findings.

Studying *V. corybosum* cultivars (REED; ABDELNOUR-ESQUIVEL, 1991) and comparing the effects of zeatin at 4 $mg \cdot L^{-1}$ (18.24 μM) and 2iP at 10 $mg \cdot L^{-1}$ (49.2 μM) or 15 $mg \cdot L^{-1}$ (73.8 μM) on initiation growth of single-node pieces, the authors found that the medium with 4 mg L^{-1} (18.24 μM) zeatin showed the highest rates of new shoot growth. Although our research focused on lower concentrations of 2iP, we also found it to be less efficient than zeatin.

Our findings are in accordance to a study with lowbush blueberry (*V. angustifolium*) (DEBNATH, 2009b), where the authors compared zeatin (5 μM) and 2iP (10 μM) in the establishment of nodal segments and found that, although both treatments produced elongated shoots, the better initiation rates occurred on zeatin medium. The same behavior was reported in a study in 'Delite' rabbiteye cultivar (SILVA et al., 2006), demonstrating the differences among 2iP (25 μM) and zeatin (18 μM) in the establishment of nodal segments, showing that zeatin improved the survival, establishment rate, and decreased oxidation.

We could observe in our study that a low concentration of zeatin (2.5 μM) in the medium was effective to induce shoot growth and initiate the culture. This is an important observation, considering the high cost of zeatin, that in low concentrations could be more viable for use in research and commercial laboratories.

Experiment 3: Multiplication with different zeatin concentrations

Observing the experiment of multiplication with different zeatin concentrations, all the treatments showed 100% survival rate of the explants. New

shoot formation did not occur in the control without zeatin. And it varied from 70.0 to 85.0% among the media containing zeatin, not differing among each other (Table 3).

The number of new shoots formed per explant did not differ among the treatments with zeatin, and so did not the number of leaves per shoot (9.0 to 12.2 leaves per explant) (Table 3).

In Fig. 3a it is possible to observe the five treatments (0, 2.5, 5.0, 7.5, and 10 μM) compared. The regression graphic (Fig. 3b) shows a quadratic simple linear regression explaining how shoot length starts higher in the concentration of 2.5 μM and there is a decrease in the shoot length along the concentrations, until the concentration of 8.1 μM zeatin.

According to these results, it is possible to choose 2.5 μM as the most indicated concentration, considering it was as efficient as the other treatments concerning survival rate of the explants, the percentage of explants with shoot proliferation, number of shoots per explant and number of leaves of the longest shoot, and beyond that, it showed a higher mean length of the longest shoot than the other treatments, yet having a more reduced-cost compared to the higher concentrations.

Our findings are similar to a work testing shoot proliferation in cranberry (*V. macrocarpon*) cultivars, where the authors studied four zeatin concentrations (1.0, 2.0, 4.0, and 6.0 μM) in the medium and found that the best proliferation occurred in the media with 2–4 μM concentration of zeatin (DEBNATH, 2008).

Zeatin is one of the most used cytokinins in blueberry micropropagation (CAPPELLETTI; MEZZETTI, 2016). These authors tested shoots of 'Duke' highbush blueberry (*V. corymbosum*) with different cytokinins, zeatin (alone) and TDZ (alone or combined with 2iP), and found that the highest axillary bud proliferation occurred on media with zeatin.

In a study with stationary and temporary immersion bioreactor (DEBNATH, 2017), testing the multiplication of nodal explants of half-high blueberry cultivar 'St. Cloud' in liquid medium with 0, 2.3, 4.6, or 9.1 μM zeatin, the authors found that the best shoot proliferation occurred in the medium containing 4.6 μM zeatin in both bioreactors tested. This system was not tested in our work, but it represents an efficient system for mass clonal propagation.

In a study with 'Bluejay' (*V. corymbosum*) and 'Pink Lemonade' blueberries (*Vaccinium ashei*) (FAN et al., 2017), the authors tested combinations of 4.56, 9.12, and 13.67 µM zeatin and 0.05, 0.27, and 1.34 µM NAA. The most appropriate medium was 13.68 µM zeatin and 0.27 µM NAA for 'Bluejay' multiplication, whereas in 'Pink Lemonade' 9.12 µM zeatin with 0.05 µM NAA showed the highest proliferation. For both cultivars tested by the authors, the efficient concentrations of zeatin were higher than the optimum concentration found in our work.

The concentrations of zeatin tested in our study during the multiplication step could be expanded to other cytokinins or even combinations with auxins and GA_3, trying to reduce the amount of zeatin and the whole cost of the process.

Experiment 4: Multiplication with different explant positions

This experiment was performed two times. In the first experiment, the survival rate of the explants was not statistically different among treatments, varying from 90.7 to 100% (Table 4 and Sup. Table S4). The percentage of explants with shoot proliferation rate was higher in the vertical treatment (100.0%), compared to the horizontal treatment (83.6%). And the treatment 45º (94.3%) was similar to both vertical and horizontal treatments regarding survival rate. The number of new shoots formed per explant in the horizontal orientation (2.2 new shoots) was higher than at 45º (1.7 new shoots); and the number obtained in the vertical position (1.8 new shoots) was similar to both 45º and horizontal positions. The mean length of the new shoots formed, was not statistically different among the three treatments, varying from 4.8 to 5.7 cm. The mean number of leaves per shoot was 14.2 in the treatment horizontal and was superior to the treatment 45º (10.9 leaves). The shoot induction rate in the most basal bud was not statistically different among the treatments. It varied from 46.4 to 59.3%. On the other hand, the shoot induction rate in the most apical bud was 69.1% in the treatment horizontal, being superior to the treatment vertical (17.9%). In the treatment 45º, it was 37.9%, and it did not differ statistically from the other treatments.

In the second experiment, in six of the variables evaluated, the treatments were not statistically different. However, in the last variable, shoot induction rate in the most apical bud, the behavior of the treatments was similar to the previous experiment. The horizontal treatment showed more shoot induction (76.7%) than the

vertical treatment (21.7%). And the treatment 45º was 37.9, not differing statistically from the other treatments.

The experiment is seen in Fig. 4a-4o. The beginning of the experiments is shown in Fig. 4a-4f, with the three orientations: vertical (Fig. 4a and Fig. 4d), inclined at 45º (Fig. 4b and Fig. 4e), and horizontal (Fig. 4c and Fig. 4f). The results of the vertical orientation are shown in Fig. 4g, Fig. 4j, and Fig. 4m. The 45º treatments are in Fig. 4h, Fig. 4k, and Fig. 4n. And horizontal treatments are demonstrated in Fig. 4i, 4l, and 4o.

In Fig. 5, there is a diagrammatic representation of the differences between the explant orientations on shoot induction rate in the most apical bud (%) and shoot induction rate in the most basal bud (%). It is possible to evaluate that there is an increase in the shoot induction rate of the most apical bud when the explant orientation goes from the vertical to the horizontal treatment. This situation occurred in both experiments (the first and the second).

Considering only the horizontal and the vertical orientations, we did not observe differences among these two treatments, related to the survival of explants, number of shoots per explant, shoot length, and number of leaves per shoot, in both first and second experiment. We can notice, however, a significant difference occurring in the percentage of explants with shoot proliferation, where the vertical orientation was superior (100% explants forming shoots) compared to the horizontal orientation (83.6% explants forming shoots) in the first experiment. Based on our results, we could recommend the use of the explants in the vertical position.

However, studying five woody species *Amelanchier*, *Acer*, *Forsythia*, *Malus*, and *Betula*, using vertical or horizontally placed explants in the culture medium (MCCLELLAND; SMITH, 1990), the authors found that explants in the horizontal orientation produced more shoots per explant, and favored the shoot initiation rates, compared to the vertical.

Also, in a study with lingonberry (*V. vitis-idaea*) nodal explants placed vertically or horizontally in the medium (DEBNATH, 2005), the cuttings placed horizontally had an increased shoot number per explant, with smaller shoots and lower number of leaves per shoot.

A study in 'Troyer' citrange (*Citrus sinensis* [L.] Osbeck x *Poncirus trifoliata* L. Raf.), observed the adventitious bud formation in epicotyl segments with different orientations in the culture medium. The explants placed horizontally had higher

adventitious bud formation in the apical end of the explant, compared to the vertically upright position. And observing the adventitious bud formation of the basal end of the explant, the vertical position showed the highest number of new shoots formed, compared to the horizontal treatment (GARCÍA-LUIS et al., 1999). This study in citrange develops a possibility, not fully supported by their study, that the highest formation of shoots in the apical end of the explant positioned horizontally in the medium, compared to the vertical position, could be explained by the readily availability of cytokinins from the culture medium to this most apical bud, touching the medium when placed horizontally.

The blueberry is a shrub, composed of shoots that start emerging from buds located in the crown, and grow in two or more flushes during the growing season (RETAMALES; HANCOCK, 2012). These growth flushes are due to apical abortion, called 'black tip'. And then, growth restarts after an axillary bud is released from dormancy (BARKER; COLLINS, 1963; RETAMALES; HANCOCK, 2012). So, the fact that shoots emerge from the base of the plant is a natural habit in the species. Besides, when we prepared the explants, and the apical bud was removed, part of the apical dominance was lost, and the following more basal axillary buds were more prone to sprout. The shoot emergence in the most apical bud was the lowest in the vertical position of the explant and increased when the explant was inclined. Finally, the sprouting of the most apical bud in the explants positioned horizontally was higher than the other explant positions. The changes in the sprouting of the most apical buds in the horizontal position could be explained by a difference in the balance between auxins and cytokinins already available in the stems. Or else, we could explain these results considering that the horizontally positioned explants had a better contact (and especially, the most apical bud) with the medium containing cytokinin, favoring the sprouting of the most apical bud.

Experiment 5: Rooting in different environments (*in vitro* and *ex vitro*) combined with different IBA (indole-3-butyric acid) concentrations (0, 250, and 500 mg L^{-1}).

In this rooting experiment, there was an interaction between the two factors (environment x IBA concentrations) only in the variable analyzed callus formation rate (Sup. Table S5). Observing the survival rate of the microcuttings, *in vitro*

treatments were superior (99.2%) to *ex vitro* treatments (88.3%). And treatments with (250 and 500 mg L-1) or without IBA immersion did not interfere in the survival rate of the explants.

Observing leaf maintenance rate, the results obtained with *in vitro* treatments (99.2%) were also superior to those of *ex vitro* treatments (86.7%). But they were not affected by IBA concentrations.

Evaluating the variable callus formation rate, looking at the level *in vitro* treatments, the IBA concentrations did affect callus formation, where 500 mg L^{-1} was superior to 0 mg L^{-1}. Whereas looking at level *ex vitro* treatments, the IBA concentrations did not show differences.

The rooting rate was higher in the *in vitro* treatments (22.1%) than *ex vitro* treatments (9.2%). And 500 mg L^{-1} showed higher rooting rate (25.0%) than 0 mg L^{-1} (4.4%). But did not differ from 250 mg L^{-1} (17.5%). Fig.6 shows the differences between the six treatments (Fig. 6a – 6f). More detailed rooting is observed in Fig. 6g and Fig. 6h.

The number of roots formed per microcutting did not differ among treatments and vary from 1.0 to 1.9 roots. The length of the longest root was higher in the *in vitro* treatments (16.5 mm) compared to the *ex vitro* treatments (5.6 mm). And there was no difference among the IBA treatments.

Plants in the greenhouse, after acclimatization, can be observed in Fig. 6i.

Observing *in vitro* and *ex vitro* rooting methods in wild bilberry (NIN et al., 2019), the authors found that both methods led to successful rooting. Based on the results of our work, it is possible to consider that *in vitro* treatments were more favorable to rooting explants, being superior to *ex vitro* treatments in four variables evaluated (survival, leaf maintenance, rooting rate, and length of the longest shoot). Considering the number of roots per explant, both treatments were equivalent. However, considering that *ex vitro* treatments already accomplish the rooting combined with part of the acclimatization stage, and they showed slightly lower efficiency in the survival rate of the microcuttings and the leaf maintenance rate, compared to the *in vitro* treatments, we suggest that *ex vitro* rooting could be further analyzed, trying different substrates, or longer immersion time in IBA, in an attempt to achieve higher rooting rates.

The IBA treatments were very similar in many variables evaluated; however, the rooting rate obtained with 500 mg L^{-1} IBA was higher than the result obtained

without IBA treatment. Despite the rooting rate obtained with 250 mg L^{-1} did not differ statistically from that obtained with 500 mg L^{-1}, we could choose the treatment 500 mg L^{-1} as the best option, since the rooting rate in treatment 250 mg L^{-1} did not differ itself from the rooting rate in the treatment without IBA.

Evaluating *in vitro* and *ex vitro* rooting of 'Bluetta' blueberry cultivar (*V. corymbosum*) (ECCHER et al., 1986), the authors tested two IBA concentrations in a quick basal dip (500 and 1000 mg L^{-1}), and a control using agar medium or peat-perlite mixtures. Similar to our work, they found that 1000 mg L^{-1} IBA resulted in the highest percentage of rooting.

Experiment 6: *Ex vitro* rooting with different substrates and mineral salts

In this *ex vitro* rooting experiment, it was possible to identify interaction among the two factors studied (substrate and mineral salts) only in two observed variables (bud induction rate and callus formation rate) (Sup. Table S6). Observing survival rate, there was no difference among the treatments Plantmax™ (54.3%) and vermiculite (60.0%). However, observing the mineral salts, WPM was superior (84.0%) to Basacote™ (30.3%) (Table 6). This low survival rate in this treatment could be explained by a phytotoxic effect over the explants due to the high concentration of Basacote™ in this study (3 kg m^{-3}). This concentration is commonly used in plant propagation substrates in open systems, with frequent irrigation, where part of the nutrients ends up lixiviated. However, in our closed system, nutrients could have been gradually released, but would have probably accumulated in the boxes.

The leaf maintenance rate was similar to the observations in survival rate, Plantmax and vermiculite did not differ statistically (54.3 and 59.0 respectively). WPM was superior (83.0%) than Basacote™ (30.3%).

Observing bud induction rate, looking at the treatments with Plantmax™, WPM treatment was similar to Basacote™. However, observing the treatments with vermiculite, WPM treatment (50.0%) was superior than Basacote™ (12.0%). The callus formation rate was similar in the treatments WPM and Basacote™, observing the treatments with Plantmax™. Although, there is a difference when the two treatments are compared inside the treatments with vermiculite (76.0% callus induction in the treatment WPM and 8.0% in the treatment Basacote™).

The rooting rate was superior in vermiculite (44.0%) compared to Plantmax™ (13.0%) and was also superior in WPM (47.0%) than Basacote™ (10.0%). In the treatment combining Plantmax™ and Basacote™, there was no rooting at all.

The other three treatments, where roots were produced, did not differ statistically from each other, varying from 2.8 to 4.9 roots/explant. Vermiculite showed a longer length of the root (35.0 mm) than Plantmax™ (9.0 mm). WPM (25.0 mm) and Basacote™ (35.7 mm) did not differ statistically.

The differences among the four treatments can be seen in Fig. 7a – Fig. 7h. Detail in rooting after 28 days in vermiculite is seen in Fig. 7i. Plants acclimatized in the greenhouse can be observed in Fig. 7j and Fig. 7k.

It was possible to conclude that the best option was vermiculite combined with WPM mineral salts, both promoting higher rooting rates than Plantmax™ and Basacote™.

CONCLUSIONS

This study contributed to establish an *in vitro* shoot culture protocol for 'Delite' rabbiteye blueberry (*V. virgatum*). It proposes the establishment of cultures using nodal segments, with surface sterilization as the first step, with ethanol (30 min at 70% solution) and sodium hypochlorite (immersion for 5 min in 0.5% solution). In sequence, initial *in vitro* establishment of nodal segments using WPM culture medium supplemented with zeatin, followed by the multiplication stages using zeatin, and explants in the vertical orientation. The rooting step can be done *in vitro* using IBA, or *ex vitro* in vermiculite and WPM mineral salts. The results can help better understand *the in vitro* techniques in 'Delite' rabbiteye blueberry contributing to further development of *in vitro* plant propagation, biotechnology, and *in vitro* germplasm conservation.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

SUPPORTING INFORMATION

Supplemental material for this article is available online.

SUPPLEMENTAL TABLE S1. Analysis of variance (ANOVA) of experiment 1, of surface sterilization evaluating the effect of different immersion times in 0.5% sodium hypochlorite solution containing 0.1% (v/v) Tween 20, using nodal segments on *in vitro* shoot culture in 'Delite' rabbiteye blueberry, with WPM culture medium. Abbreviations: DF, degrees of freedom; MS, mean squares, ns, non-significant; WPM, Woody Plant Medium.

SUPPLEMENTAL TABLE S2. Analysis of variance (two-way ANOVA) of the experiment 2, evaluating the effect of different combinations of two cytokinins, zeatin (0, 2.5, and 5.0 µM) and 2iP (0, 2.5, and 5.0 µM), on *in vitro* nodal segments establishment in 'Delite' rabbiteye blueberry. Abbreviations: 2iP, 2-isopentenyladenine; DF, degrees of freedom; MS, mean squares.

SUPPLEMENTAL TABLE S3. Analysis of variance (ANOVA) of the experiment 3, evaluating the effect of different concentrations of zeatin (0, 2.5, 5.0, 7.5, and 10 µM) in the multiplication *in vitro* of shoots in 'Delite' rabbiteye blueberry, using WPM culture medium. Abbreviations: DF, degrees of freedom; MS, mean square; WPM, Woody Plant Medium.

SUPPLEMENTAL TABLE S4. Analysis of variance (ANOVA) of the experiment 4, evaluating the effect of different explant orientations (vertical, 45º, and horizontal) in the multiplication of shoots *in vitro* of 'Delite' rabbiteye blueberry, using WPM culture

medium. Abbreviations: DF, degrees of freedom; MS, mean square; WPM, Woody Plant Medium.

SUPPLEMENTAL TABLE S5. Analysis of variance (two-way ANOVA) of the experiment 5, evaluating the effect of different rooting environments (*In vitro* and *ex vitro*) combined with different IBA (indole-3-butyric acid) concentrations (0, 250, and 500 mg L^{-1}) on the rooting of micropropagated shoots of 'Delite' rabbiteye blueberry. Abbreviations: DF, degrees of freedom; IBA, indole-3-butyric acid; MS, mean square.

SUPPLEMENTAL TABLE S6. Analysis of variance (two-way ANOVA) of the experiment 6, evaluating the effect of different *ex vitro* rooting substrates (Plantmax™ and vermiculite) combined with different mineral salts (WPM culture medium salts and Basacote™) on the rooting of micropropagated shoots of 'Delite' rabbiteye blueberry. Abbreviations: DF, degrees of freedom; MS, mean square; WPM, Woody Plant Medium.

AUTHORS CONTRIBUTIONS

CSS contributed to the design of the study, ran the laboratory work, processed the data and drafted the paper. LAB supervised the research, collaborated in critically reading and the final version of the manuscript.

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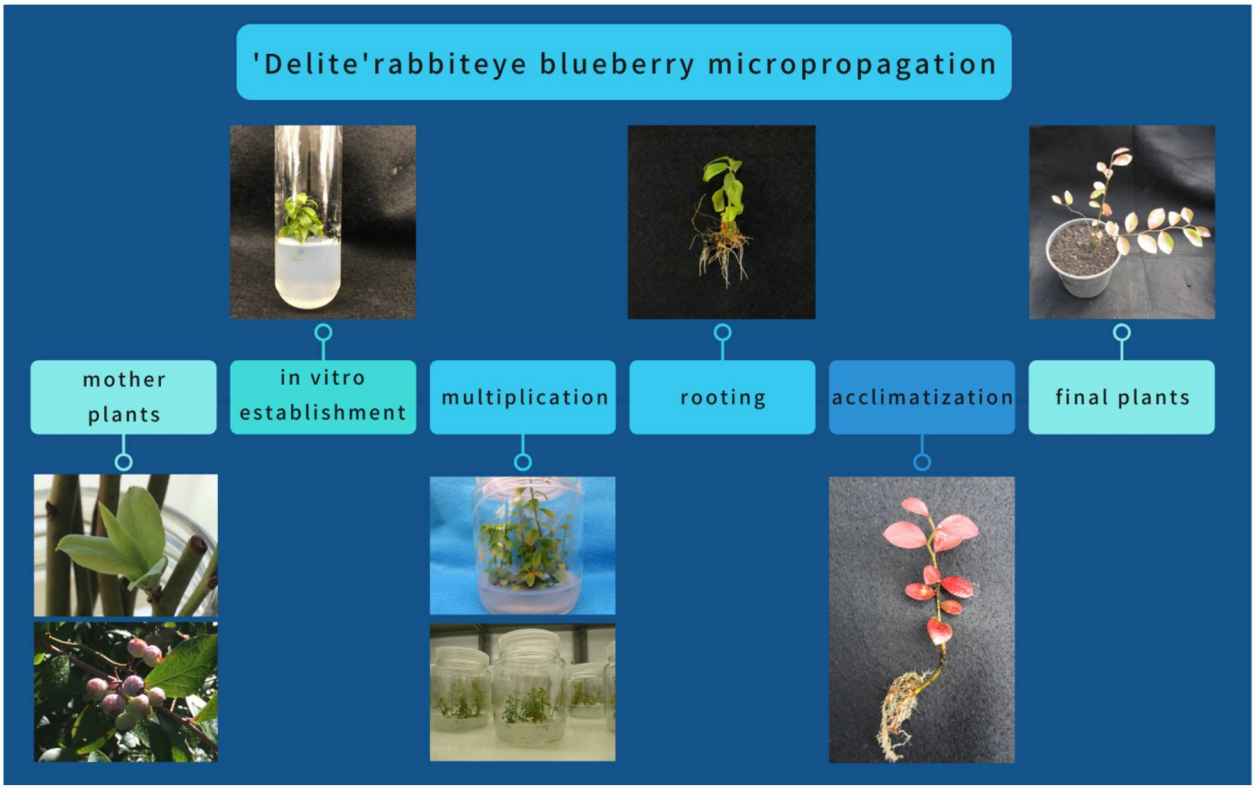
COVER SUBMISSION

COVER SUBMISSION 1. Micropropagation of ‘Delite’ rabbiteye blueberry (*Vaccinium virgatum*).

GRAPHICAL ABSTRACT



GRAPHICAL ABSTRACT. Micropropagation of ‘Delite’ rabbiteye blueberry (*Vaccinium virgatum*).

TABLES

Table 1. Effect of different immersion times in 0.5% sodium hypochlorite solution containing 0.1% (v/v) Tween 20, on surface sterilization of nodal segments on *in vitro* shoot culture in 'Delite' rabbiteye blueberry, using WPM culture medium.

Treatment	Percentage of uncontaminated explants %	Survival rate of the explants %	Percentage of explants with axillary shoot growth %
0 min	96.7 ± 3.3 a	90.0 ± 5.8 a	83.3 ± 3.3 a
5 min	96.7 ± 3.3 a	96.7 ± 3.3 a	83.3 ± 3.3 a
10 min	96.7 ± 3.3 a	86.7 ± 6.7 a	70.0 ± 11.5 a
20 min	93.3 ± 6.7 a	93.3 ± 6.7 a	73.3 ± 3.3 a
Mean	95.8	91.7	77.5
CV%	8.0	10.9	14.4

Results are presented as mean ± standard error (SE). Means in each column followed by the same letters do not differ significantly at 5% according to Tukey's multiple range test. Abbreviation: CV, coefficient of variation; WPM, Woody Plant Medium.

Table 2. Effect of different combinations of two cytokinins, zeatin (0, 2.5, and 5.0 μM) and 2iP (0 and 20.0 μM), in the establishment of nodal segments *in vitro* of 'Delite' rabbiteye blueberry, using WPM culture medium.

Zeatin	**2iP**		
	0 μM	20 μM	mean
Survival rate of the explants (%)			
0 μM zeatin	37.2 ± 11.6	43.1± 9.0	39.2 b
2.5 μM zeatin	92.3 ± 4.5	87.6 ± 5.9	89.9 a
5 μM zeatin	74.1 + 10.3	86.7 ± 8.2	80.4 a
mean	67.2 A	72.5 A	69.8
CV%:	24.6		
Percentage of explants with axillary shoot growth (%)			
0 μM zeatin	0.0 ± 0.0 bA	8.1 ± 4.9 bA	4.1
2.5 μM zeatin	84.5 ± 6.8 aA	60.5 ± 8.4 aB	72.5
5 μM zeatin	57.6 ± 10.2 aB	81.7 ± 10.7 aA	69.6
mean	47.4	50.1	48.7
CV%:	31.7		
Number of new shoots formed/explant (n.)			
0 μM zeatin	NA	1.0 ± 0.0	1.0 a
2.5 μM zeatin	1.0 ± 0.0	1.0 ± 0.0	1.0 a
5 μM zeatin	1.0 ± 0.0	1.0 ± 0.0	1.0 a
Mean	1.0 A	1.0 A	1.0
CV%:	0.0		
Mean length of shoots (cm)			
0 μM zeatin	NA	0.4 ± 0.1	0.4 b
2.5 μM zeatin	1.5 ± 0.32	0.5 ± 0.04	1.0 a
5 μM zeatin	0.5 ± 0.06	0.33 ± 0.03	0.4 b
mean	1.0 A	0.4 B	0.7
CV%:	47.2		
Number of leaves/shoot (n.)			
0 μM zeatin	NA	4.0 ± 2.0	4.0 b
2.5 μM zeatin	10.8 ± 1.4	9.6 ± 0.7	10.2 a
5 μM zeatin	6.3 ± 0.6	7.5 ± 1.0	6.9 ab
mean	8.5 A	7.6 A	8.0
CV%:	25		

Results are presented as mean ± standard error (SE). Means in each column followed by the same lowercase letters and means in each horizontal line followed by the same uppercase letters do not differ significantly at 5% according to Tukey's multiple range test. Abbreviation: 2iP, 2-isopentenyladenine; CV, coefficient of variation; NA, not available; WPM, Woody Plant Medium.

Table 3. Effect of different concentrations of zeatin (0, 2.5, 5.0, 7.5, and 10 μM) in the multiplication of shoots *in vitro* of 'Delite' rabbiteye blueberry, using WPM culture medium.

Treatment	Percentage of explants with shoot proliferation %	Number of new shoots formed/explant n	Number of leaves of the longest shoot n
0 μM zeatin	0.0 ± 0.0 b	NA	NA
2.5 μM zeatin	70.0 ± 5.7 a	1.0 ± 0.0 a	11.7 ± 0.4 a
5 μM zeatin	85.0 ± 5.0 a	1.0 ± 0.0 a	9.0 ± 0.5 a
7.5 μM zeatin	80.0 ± 0.0 a	1.0 ± 0.0 a	12.2 ± 1.6 a
10 μM zeatin	80.0 ± 0.0 a	1.0 ± 0.0 a	9.9 ± 1.0 a
Mean	69.40	1.00	10.70
CV%	11.00	0.00	18.10

Results are presented as mean ± standard error (SE). Means in each column followed by the same letters do not differ significantly at 5% according to Tukey's multiple range test. Abbreviation: CV, coefficient of variation; WPM, Woody Plant Medium.

Table 4. Effect of different explant orientations (vertical, 45º, and horizontal) in the multiplication of shoots *in vitro* of 'Delite' rabbiteye blueberry, using WPM culture medium.

Treatment	Survival of the explants	Percentage of explants with shoot proliferation	Number of new shoots formed/ explant	Mean length of new shoots formed	Mean number of leaves/ shoot formed	Shoot induction rate in the most basal bud (%)	Shoot induction rate in the most apical bud (%)
	%	%	n	cm	n	%	%
1st. Experiment replication							
Vertical	100.0 ± 0.0 a	100.0 ± 0.0 a	1.8 ± 0.1 ab	5.1 ± 0.3 a	12.7 ± 0.5 ab	46.4 ± 12.7 a	17.9 ± 7.1 b
45º	94.3 ± 3.7 a	94.3 ± 3.7 ab	1.7 ± 0.1 b	5.7 ± 0.6 a	10.9 ± 0.4 b	59.3 ± 5.7 a	37.9 ± 9.1 ab
Horizontal	90.7 ± 4.4 a	83.6 ± 4.3 b	2.2 ± 0.2 a	4.8 ± 0.6 a	14.2 ± 1.3 a	50.0 ± 12.7 a	69.1 ± 14.6 a
Mean	95.0	92.6	1.9	5.2	12.6	51.9	41.6
CV%	9.3	9.4	20.5	25.1	17.2	55.6	68.4
2nd. Experiment replication							
Vertical	96.4 ± 3.6 a	81.0 ± 7.4 a	1.6 ± 0.2 a	5.7 ± 0.5 a	13.1 ± 1.3 a	42.1 ± 9.9 a	21.7 ± 11.3 b
45º	90.7 ± 4.4 a	87.9 ± 6.2 a	1.6 ± 0.1 a	4.3 ± 0.5 a	11.9 ± 1.0 a	29.3 ±9.0 a	37.9 ± 12.1 ab
Horizontal	88.8 ± 5.5 a	86.0 ± 5.2 a	2.1 ± 0.2 a	5.1 ± 1.0 a	13.3 ± 1.6 a	26.4 ±6.8 a	76.7 ± 9.5 a
Mean	92.0	84.9	1.8	5.0	12.8	32.6	45.4
CV%	13.1	19.8	25.2	35.4	26.8	70.3	64.3

Results are presented as mean ± standard error (SE). Means in each column followed by the same letters do not differ significantly at 5% according to Tukey's multiple range test. Abbreviation: CV, coefficient of variation; WPM, Woody Plant Medium.

Table 5. Effect of different rooting environments (*In vitro* and *ex vitro*) combined with different IBA (indole-3-butyric acid) concentrations (0, 250, and 500 mg L^{-1}) on the rooting of micropropagated shoots of 'Delite' rabbiteye blueberry.

Environment	IBA			
	0 mg L^{-1}	250 mg L^{-1}	500 mg L^{-1}	mean
Survival rate (%)				
In vitro	97.5 ± 2.5	100.0 ± 0.0	100.0 ± 0.0	99.2 a
Ex vitro	95.0 ± 2.9	92.5 ± 4.8	77.5 ± 9.5	88.3 b
mean	96.3 A	96.3 A	88.8 A	93.8
CV%:	9.8			
Leaf maintenance rate (%)				
In vitro	97.5 ± 2.5	100.0 ± 0.0	100 ± 0.0	99.2 a
Ex vitro	95.0 ± 2.9	87.5 ± 7.5	77.5 ± 9.5	86.7 b
mean	96.3 A	93.8 A	88.8 A	92.9
CV%:	11.1			
Callus formation rate (%)				
In vitro	31.3 ± 17.1 aB	45.0 ± 6.5 aAB	77.5 ± 13.2 aA	51.3
Ex vitro	17.5 ± 2.5 aA	25.0 ± 6.5 aA	12.5 ± 6.3 bA	18.3
mean	24.4	35.0	45.0	34.8
CV%:	57.3			
Rooting rate (%)				
In vitro	8.8 ± 5.9	20.0 ± 7.1	37.5 ± 10.3	22.1 a
Ex vitro	0.0 ± 0.0	15.0 ± 2.9	12.5 ± 6.3	9.2 b
mean	4.4 B	17.5 AB	25.0 A	15.6
CV%:	80.1			
Number of roots/explant (n)				
In vitro	1.0 ± 0.0	1.6 ± 0.1	2.1 ± 0.7	1.8 a
Ex vitro	NA	2.1 ± 0.7	1.4 ± 0.4	1.7 a
mean	1.0 A	1.9 A	1.8 A	1.7
CV%:	59.6			
Length of the longest root (mm)				
In vitro	7.5 ± 5.5	16.2 ± 4.5	21.3 ± 2.7	16.5 a
Ex vitro	NA	6.0 ± 2.2	5.1 ± 1.8	5.6 b
mean	7.5 A	10.4 A	14.3 A	11.8
CV%:	44.6			

Results are presented as mean ± standard error (SE). Means in each column followed by the same lowercase letters and means in each horizontal line followed by the same uppercase letters do not differ significantly at 5% according to Tukey's multiple range test. Abbreviation: CV, coefficient of variation; IBA, indole-3-butyric acid; NA, not available.

Table 6. Effect of different *ex vitro* rooting substrates (Plantmax™ and vermiculite) combined with different mineral salts (WPM culture medium salts and Basacote™) on the rooting of micropropagated shoots of 'Delite' rabbiteye blueberry.

Substrate	Mineral salts		
	WPM culture medium	Basacote™	mean
Survival rate (%)			
Plantmax™	80.0 ± 7.1	28.7 ± 11.6	54.3 a
vermiculite	88.0 ± 5.8	32.0 ± 16.9	60.0 a
mean	84.0 A	30.3 B	57.2
CV%:	43.9		
Leaf maintenance rate (%)			
Plantmax™	80.0 ± 7.1	28.7 ± 11.6	54.33 a
vermiculite	86.0 ± 5.1	32.0 ± 16.9	59.0 a
mean	83.0 A	30.3 B	56.7
CV%:	43.9		
Bud induction rate (%)			
Plantmax™	10.0 ± 4.5 bA	2.2 ± 2.2 aA	6.1
vermiculite	50.0 ± 8.9 aA	12.0 ± 9.7 aB	31.0
mean	30.0	7.1	18.6
CV%:	85.0		
Callus formation rate (%)			
Plantmax™	4.0 ± 4.0 bA	0.0 ± 0.0 aA	2.0
vermiculite	76.0 ± 4.0 aA	8.0 ± 8.0 aB	42.0
mean	40.0	4.0	22.0
CV%:	49.8		
Rooting rate (%)			
Plantmax™	26.0 ± 11.2	0.0 ± 0.0	13.0 b
vermiculite	68.0 ± 8.0	20.0 ± 13.0	44.0 a
mean	47.0 A	10.0 B	28.5
CV%:	74.4		
Number of roots/explant (n)			
Plantmax™	2.8 ± 1.2	NA	2.8 a
vermiculite	4.9 ± 0.4	3.5 ± 1.6	4.4 a
mean	4.1 A	3.5 A	4.0
CV%:	46.1		
Length of the longest root (mm)			
Plantmax™	9.0 ± 3.3	NA	9.0 b
vermiculite	34.6 ± 5.6	35.7 ± 8.8	35.0 a
mean	25.0 A	35.7 A	28.0
CV%:	43.3		

Results are presented as mean ± standard error (SE). Means in each column followed by the same lowercase letters and means in each horizontal line followed by the same uppercase letters do not differ significantly at 5% according to Tukey's multiple range test. Abbreviation: CV, coefficient of variation; NA, not available, WPM, Woody plant medium.

LEGENDS TO FIGURES

Fig. 1. Initial establishment of nodal segments on *in vitro* shoot culture in 'Delite' rabbiteye blueberry, using WPM culture medium. (a–d) Effect of different immersion times in 0.5% sodium hypochlorite solution containing 0.1% (v/v) Tween 20, on surface sterilization of nodal segments. (a) 0 min. (b) 5 min. (c) 10 min. (d) 15 min. Abbreviations WPM, Woody Plant Medium. Scale bars = 1.0 cm.

Fig. 2. Initial establishment of nodal segments on *in vitro* shoot culture in 'Delite' rabbiteye blueberry, using WPM culture medium. (a–f) Effect of different combinations of two cytokinins, zeatin (0, 2.5, and 5.0 µM) and 2iP (0 and 20.0 µM), in the establishment of nodal segments *in vitro*. (a) Treatment 0 µM zeatin and 0 µM 2iP. (b) Treatment 2.5 µM zeatin and 0 µM 2iP. (c) Treatment 5.0 µM zeatin and 0 µM 2iP. (d) Treatment 0 µM zeatin and 20.0 µM 2iP. (e) Treatment 2.5 µM zeatin and 20.0 µM 2iP. (f) Treatment 5.0 µM zeatin and 20.0 µM 2iP. Abbreviations 2iP, 2-isopentenyladenine; WPM, Woody Plant Medium. Scale bars = 1.0 cm.

Fig. 3. Effect of different concentrations of zeatin (0, 2.5, 5.0, 7.5, and 10 µM) in the multiplication of *in vitro* shoots of 'Delite' rabbiteye blueberry, using WPM culture medium. (a) The differences among the five treatments in multiplication. (b) Simple linear regression graphic showing the effect of different zeatin concentrations (0, 2.5, 5.0, 7.5, and 10 µM) on the dependent variable shoot length (cm). ** statistically significant with p-value <=0.01. Abbreviations: WPM, Woody Plant Medium. Scale bar = 1.0 cm.

Fig. 4. Effect of different explant orientations (vertical, 45º, and horizontal) in the multiplication of shoots *in vitro* of 'Delite' rabbiteye blueberry, using WPM culture medium. Scale bars = 1.0 cm. (a, d, g, j, k) Vertical treatments. (b, e, h, I, m) 45º treatments. (c, f, i, n, o) Horizontal treatments. Abbreviations: WPM, Woody Plant Medium. Scale bars = 1.0 cm.

Fig. 5. Diagrammatic representation of the effect of different explant orientations (vertical, 45º, and horizontal) in the shoot induction rate (%) in the most apical bud and in the most basal bud, using *in vitro* shoots of 'Delite' rabbiteye blueberry, using WPM culture medium. The graphic on the left represents the 1st experiment and the graphic on the right represents the 2nd experiment. Means followed by the arrow with the same letters do not differ significantly at 5% according to Tukey's multiple range test. Abbreviations: WPM, Woody Plant Medium.

Fig. 6. Effect of different rooting environments (*In vitro* and *ex vitro*) combined with different IBA (indole-3-butyric acid) concentrations (0, 250, and 500 mg L^{-1}) on the rooting of micropropagated shoots of 'Delite' rabbiteye blueberry. (a, b, c) *In vitro* treatments (d, e, f) *Ex vitro* treatments. (a, d) 0 mg L^{-1} IBA. (b, e) 250 mg L^{-1} IBA. (c, f) 500 mg L^{-1} IBA. (g) Details on *in vitro* rooting. (h) Root details (i) Acclimatized plants in the greenhouse. Abbreviations: IBA, indole-3-butyric acid. Scale bars = 1.0 cm.

Fig. 7. Effect of different *ex vitro* rooting substrates (Plantmax™ and vermiculite) combined with different mineral salts (WPM culture medium salts and Basacote™) on the rooting of micropropagated shoots of 'Delite' rabbiteye blueberry. (a, b) Plantmax™ and WPM. (c, d) Plantmax™ and Basacote™. (e, f) Vermiculite and WPM. (g, h) Vermiculite and Basacote™. (i, j) Details on rooting. (i) Acclimatized plant in the greenhouse. Abbreviations: WPM, Woody Plant Medium. Scale bars = 1.0 cm.

FIGURES

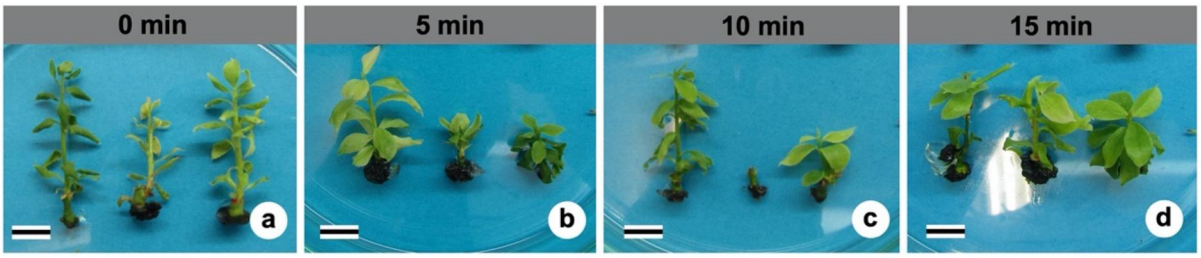


Fig. 1.

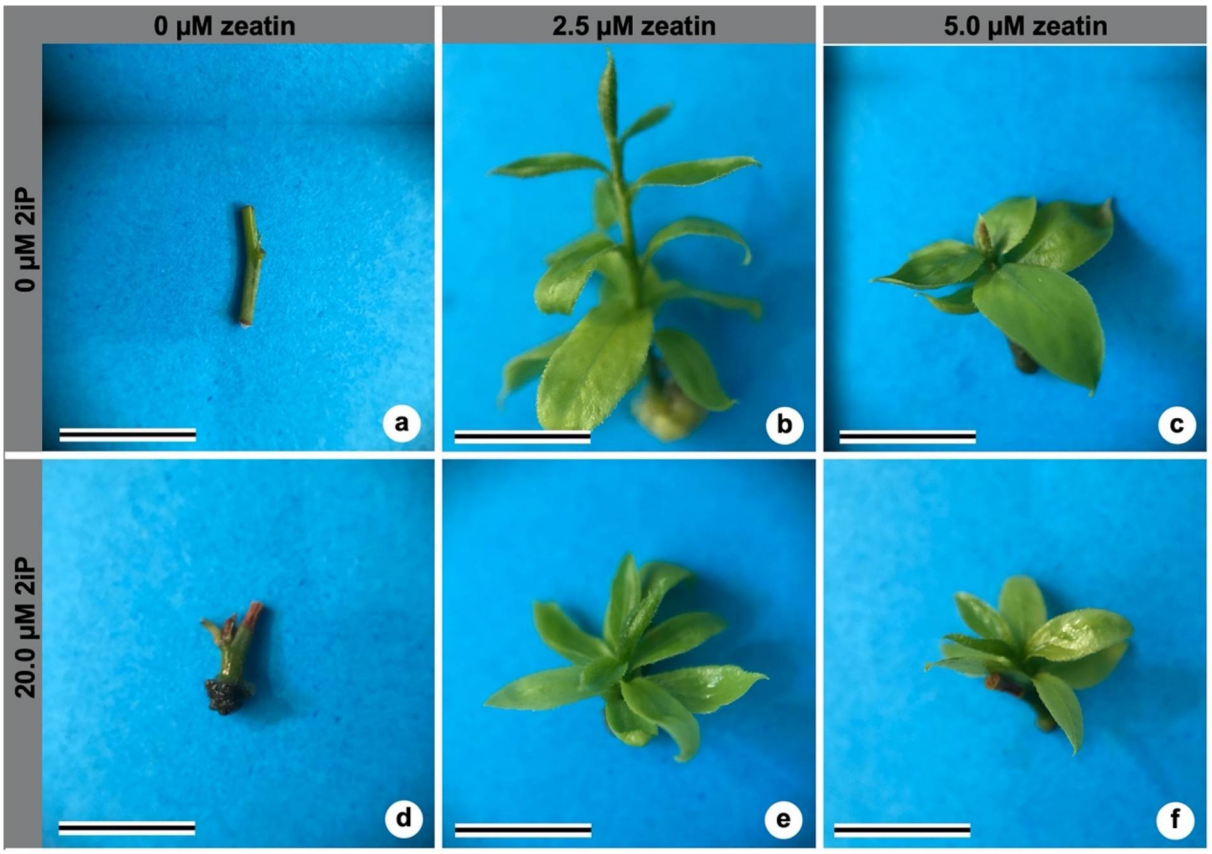


Fig. 2.

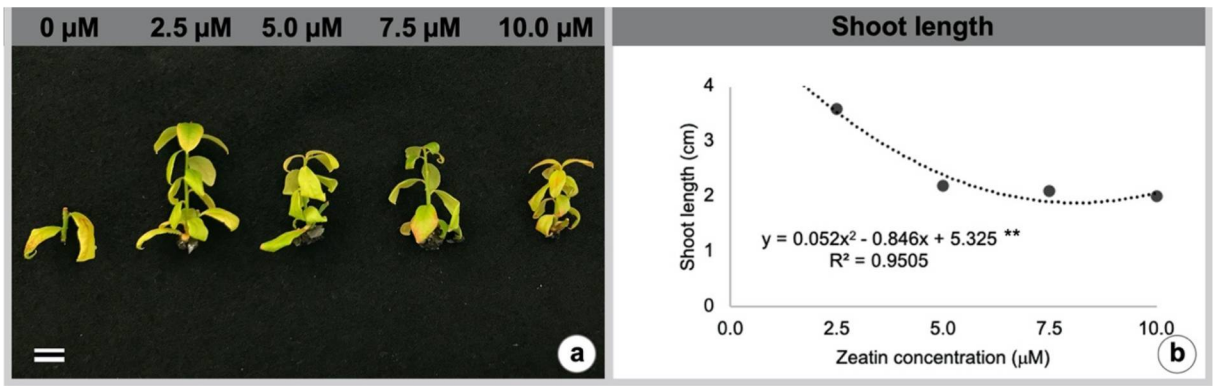


Fig. 3.

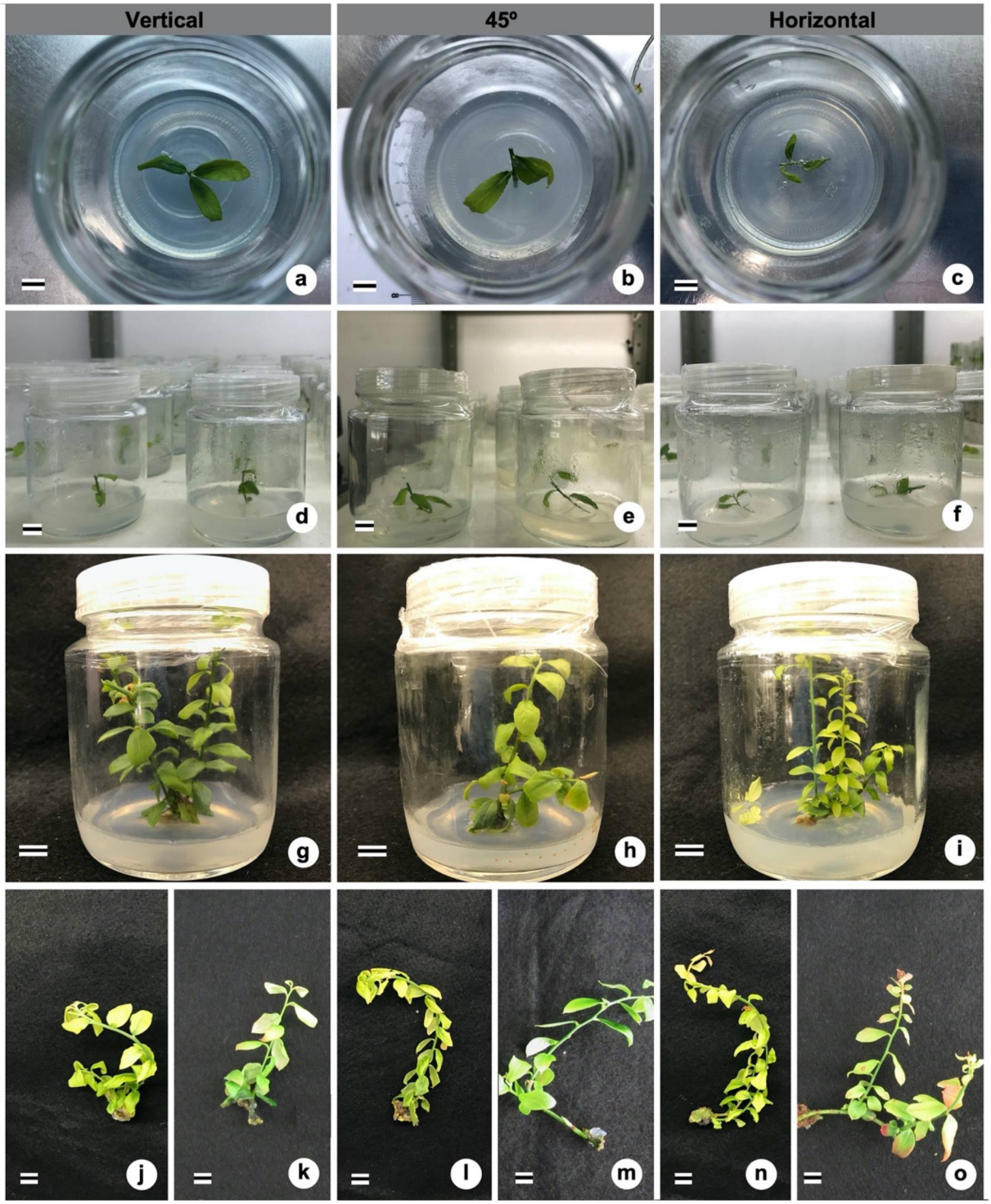


Fig. 4

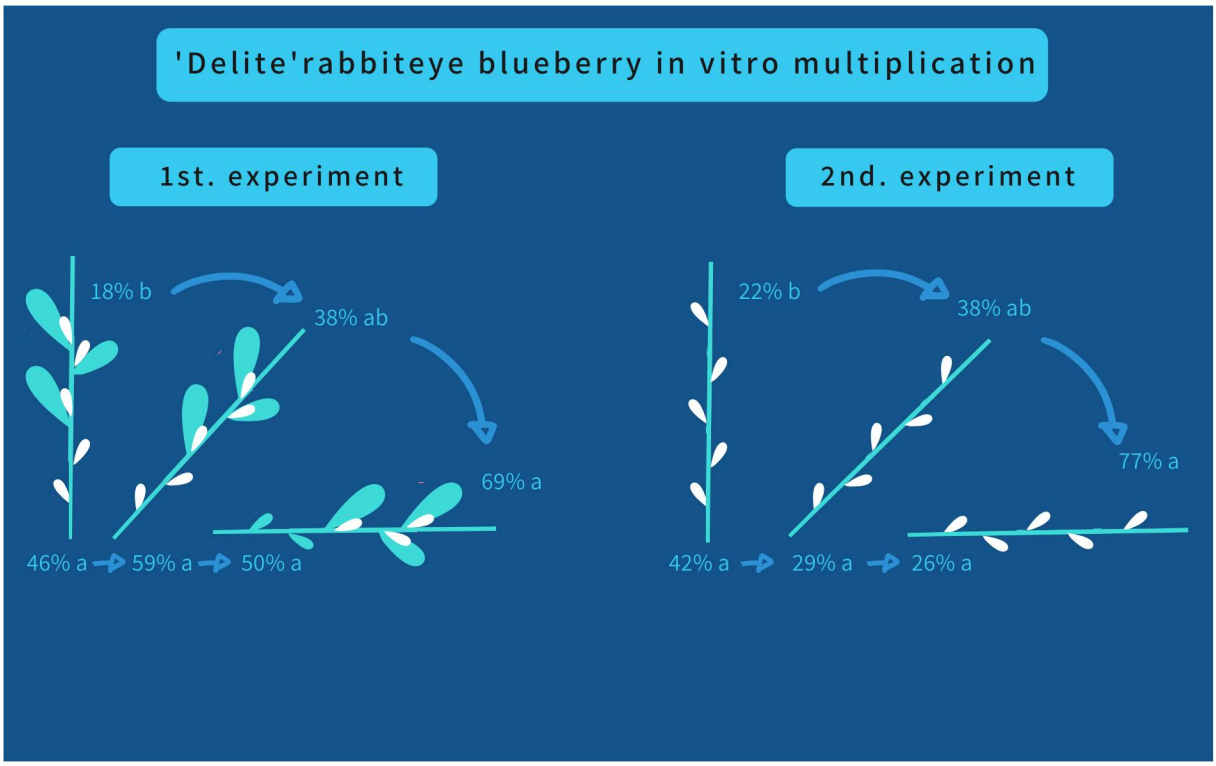

'Delite'rabbiteye blueberry in vitro multiplication
1st. experiment
2nd. experiment
18% b
38% ab
69% a
46% a
59% a
50% a
22% b
38% ab
77% a
42% a
29% a
26% a

Fig. 5


0 mg L-1 IBA
250 mg L-1 IBA
500 mg L-1 IBA
In vitro
Ex vitro
Rooting and acclimatization
a
b
c
d
e
f
g
h
i

Fig. 6

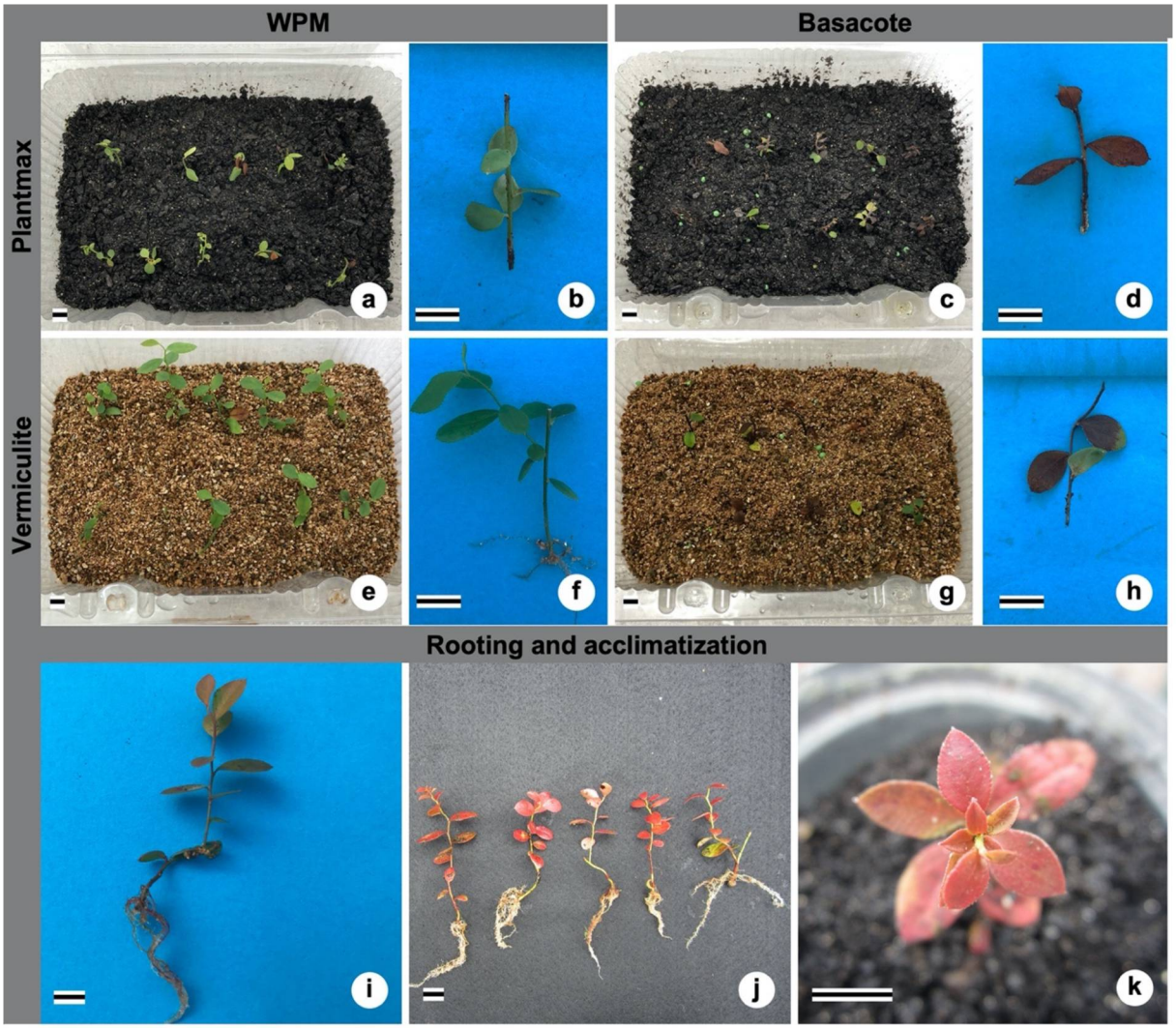

WPM
Basacote
Plantmax
Vermiculite
Rooting and acclimatization
a
b
c
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Fig. 7

SUPPLEMENTAL TABLES

SUPPLEMENTAL TABLE S1. Analysis of variance (ANOVA) of experiment 1, of surface sterilization evaluating the effect of different immersion times in 0.5% sodium hypochlorite solution containing 0.1% (v/v) Tween 20, using nodal segments on *in vitro* shoot culture in 'Delite' rabbiteye blueberry, with WPM culture medium. Abbreviations: DF, degrees of freedom; ms, mean squares, WPM, Woody Plant Medium

		Percentage of uncontaminated explants	Survival rate of the explants	Percentage of explants with axillary shoot growth
	DF	ms	ms	ms
Treatment	3	8.3	55.600	141.70
Residuals	8	58.3	100.000	125.00
Total	11			
p-value		0.9314 ns	0.6588 ns	0.3922 ns

* significant different with 0.05 > p-value > 0.01

** significant different with p-value <=0.01

ns, non-significant, p-value >= 0.05

SUPPLEMENTAL TABLE S2. Analysis of variance (two-way ANOVA) of the experiment 2, evaluating the effect of different combinations of two cytokinins, zeatin (0, 2.5, and 5.0 μM) and 2iP (0 and 20.0 μM), on *in vitro* nodal segments establishment in 'Delite' rabbiteye blueberry. Abbreviations: 2iP, 2-isopentenyladenine; DF, degrees of freedom; ms, mean squares, WPM, Woody Plant Medium.

	Source	DF	ms	p value
Survival rate of the explants (%)	Zeatin concentrations	2	5824.000	0.00003 **
	2iP concentrations	1	167.000	0.4600 ns
	Interaction (zeatin x 2iP)	2	159.000	0.5910 ns
	Error	18	294.000	
	Total	23		
Percentage of explants with axillary shoot growth (%)	Zeatin concentrations	2	11992.000	0.00000004 **
	2iP concentrations	1	45.000	0.6694 ns
	Interaction (zeatin x 2iP)	2	1200.000	0.0186 *
	Error	18	239.000	
	Total	23		
Number of new shoots formed/explant (n.)	Zeatin concentrations	2	0.000000	0.562 ns
	2iP concentrations	1	0.000000	0.317 ns
	Interaction (zeatin x 2iP)	1	0.000000	0.317 ns
	Error	13	0.000000	
	Total	17		
Mean length of shoots (cm)	Zeatin concentrations	2	0.774	0.0063 **
	2iP concentrations	1	1.381	0.0026 **
	Interaction (zeatin x 2iP)	1	0.681	0.0220 *
	Error	13	0.101	
	Total	17		
Number of leaves/shoot (n.)	Zeatin concentrations	2	39.770	0.0025 **
	2iP concentrations	1	0.010	0.9707 ns
	Interaction (zeatin x 2iP)	1	5.640	0.2575 ns
	Error	13	4.020	
	Total	17		

* significant different with 0.05 > p-value > 0.01

** significant different with p-value <=0.01

ns, non-significant, p-value >= 0.05

SUPPLEMENTAL TABLE S3. Analysis of variance (ANOVA) of the experiment 3, evaluating the effect of different concentrations of zeatin (0, 2.5, 5.0, 7.5, and 10 µM) in the multiplication *in vitro* of microshoots in 'Delite' rabbiteye blueberry, using WPM culture medium. Abbreviations: DF, degrees of freedom; ms, mean square.

		Survival rate	Shoot proliferation rate		Number of new shoots formed/explant	Shoot length	Number of leaves/shoot
	DF	ms	ms	DF	ms	ms	ms
Treatment	4	0.00	2848.50	3	0.00	2.30	8.81
Residuals	12	0.00	58.30	11	0.00	0.47	3.78
Total	16			14			
p-value		0.5681 ns	0.0000003 **		0.47367 ns	0.021309 *	0.13045 ns

* significant different with 0.05 > p-value > 0.01

** significant different with p-value <=0.01

ns, non-significant, p-value >= 0.05

SUPPLEMENTAL TABLE S4. Analysis of variance (ANOVA) of the experiment 4, evaluating the effect of different explant orientations (vertical, 45º, and horizontal) in the multiplication of microshoots *in vitro* of 'Delite' rabbiteye blueberry, using WPM culture medium. Abbreviations: DF, degrees of freedom; ms, mean square.

Treatment		Survival rate	Shoot proliferation rate (percentage of explants forming new shoots)	Number of new shoots formed/ explant	Mean length of new shoots formed	Mean number of leaves/ shoot formed	Shoot induction rate in the most basal bud (%)	Shoot induction rate in the most apical bud (%)
	DF	ms	ms	ms	ms	ms	ms	ms
1st. experiment replication								
Treatment	2	153.571	486.900	0.629	1.247	19.985	308.330	4657.900
Residuals	18	77.381	75.400	0.150	1.696	4.721	831.380	809.800
Total	20							
p-value		0.1664 ns	0.0077 **	0.0320 *	0.4932 ns	03112 *	0.695 ns	0.0117 *
2nd. experiment replication								
Treatment	2	110.040	89.011	0.586	3.444	4.112	490.480	5592.200
Residuals	18	145.360	281.601	0.196	3.182	11.773	525.640	851.800
Total	20							
p-value		0.4834 ns	0.733 ns	0.075 ns	0.3599 ns	0.7099 ns	0.412 ns	0.0072 **

* significant different with 0.05 > p-value > 0.01

** significant different with p-value <=0.01

ns, non-significant, p-value >= 0.05

SUPPLEMENTAL TABLE S5. Analysis of variance (two-way ANOVA) of the experiment 5, evaluating the effect of different rooting environments (*In vitro* and *ex vitro*) combined with different IBA (indole-3-butyric acid) concentrations (0, 250, and 500 mg L^{-1}) on the rooting of micropropagated shoots of 'Delite' rabbiteye blueberry. Abbreviations: DF, degrees of freedom; IBA, indole-3-butyric acid; ms, mean square.

	Source	DF	ms	p-value
Survival rate (%)	Environment (*in vitro* or *ex vitro*)	1	704.200	0.0099 **
	IBA concentration	2	150.000	0.1986 ns
	Environment x IBA	2	216.700	0.1053 ns
	Residuals	18	84.700	
	Total	23		
Survival rate (%)	Environment (*in vitro* or *ex vitro*)	1	937.500	0.0084 **
	IBA concentration	2	116.700	0.3571 ns
	Environment x IBA	2	200.000	0.1828 ns
	Residuals	18	106.900	
	Total	23		
Callus formation rate (%)	Environment (*in vitro* or *ex vitro*)	1	6501.000	0.0008 ***
	IBA concentration	2	851.000	0.1461 ns
	Environment x IBA	2	1564.000	0.0381 *
	Residuals	18	397.000	
	Total	23		
Rooting rate (%)	Environment (*in vitro* or *ex vitro*)	1	1001.000	0.0220 *
	IBA concentration	2	871.900	0.0139 *
	Environment x IBA	2	226.000	0.2679 ns
	Residuals	18	159.400	
	Total	23		
Number of roots/explant (n)	Environment (*in vitro* or *ex vitro*)	1	0.103	0.762 ns
	IBA concentration	2	0.586	0.594 ns
	Environment x IBA	1	1.234	0.307 ns
	Residuals	11	1.074	
	Total	15		
Length of the longest root (mm)	Environment (*in vitro* or *ex vitro*)	1	471.600	0.0016 **
	IBA concentration	2	112.500	0.0471 *
	Environment x IBA	1	30.900	0.3123 ns
	Residuals	11	27.500	
	Total	15		

* significant different with 0.05 > p-value > 0.01

** significant different with p-value <=0.01

ns, non-significant, p-value >= 0.05

SUPPLEMENTAL TABLE S6. Analysis of variance (two-way ANOVA) of the experiment 6, evaluating the effect of different *ex vitro* rooting substrates (Plantmax™ and vermiculite) combined with different mineral salts (WPM culture medium salts and Basacote™) on the rooting of micropropagated shoots of 'Delite' rabbiteye blueberry. Abbreviations: DF, degrees of freedom; ms, mean square.

	Source	DF	ms	p-value
Survival rate (%)	Substrate (Plantmax™ x vermiculite)	1	161.000	0.620047 ns
	Mineral salts (WPM x Basacote™)	1	14402.000	0.000202 ***
	Substrate x mineral salts	1	27.000	0.838038 ns
	Residuals	16	629.000	
	Total	19		
Leaf maintenance rate (%)	Substrate (Plantmax™ x vermiculite)	1	109.000	0.680242 ns
	Mineral salts (WPM x Basacote™)	1	13871.000	0.000225 **
	Substrate x mineral salts	1	9.000	0.906331 ns
	Residuals	16	619.000	
	Total	19		
Bud induction rate (%)	Substrate (Plantmax™ x vermiculite)	1	3097.600	0.00278 **
	Mineral salts (WPM x Basacote™)	1	2619.800	0.00507 **
	Substrate x mineral salts	1	1141.600	0.04787 *
	Residuals	16	248.700	
	Total	19		
Callus formation rate (%)	Substrate (Plantmax™ x vermiculite)	1	8000.000	0.0000004 **
	Mineral salts (WPM x Basacote™)	1	6480.000	0.000002 **
	Substrate x mineral salts	1	5120.000	0.000007 **
	Residuals	16	120.000	
	Total	19		
Rooting rate (%)	Substrate (Plantmax™ x vermiculite)	1	4805.000	0.00484 **
	Mineral salts (WPM x Basacote™)	1	6845.000	0.00127 **
	Substrate x mineral salts	1	605.000	0.26326 ns
	Residuals	16	450.000	
	Total	19		
Number of roots/explant (n)	Substrate (Plantmax™ x vermiculite)	1	5.499	0.234 ns
	Mineral salts (WPM x Basacote™)	1	4.070	0.301 ns
	Substrate x mineral salts			
	Residuals	8	3.325	
	Total	10		
Length of the longest root (mm)	Substrate (Plantmax™ x vermiculite)	1	1475.400	0.0131 *
	Mineral salts (WPM x Basacote™)	1	2.100	0.907 ns
	Substrate x mineral salts			
	Residuals	8	146.300	
	Total	10		

* significant different with 0.05 > p-value > 0.01

** significant different with p-value <=0.01

ns, non-significant, p-value >= 0.05

4 CHAPTER III

Morphological and anatomical insights into *de novo* shoot organogenesis of *in vitro* 'Delite' rabbiteye blueberries [3]

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ABSTRACT

Blueberries are valued for their taste and their high nutritional benefits, including their antioxidant and anti-inflammatory properties. *In vitro* culturing is an alternative method for clonal propagation, and has been used in many biotechnological studies. Most blueberry research is concentrated on highbush and lowbush taxa (*Vaccinium corymbosum* and *Vaccinium angustifolium* respectively), with only limited investigations of rabbiteye cultivars (*Vaccinium virgatum*) that are more suitable for subtropical climates and regions with warmer winters as a result of climate change. There is therefore a need to determine *in vitro* protocols for that species and group of cultivars. We examined here adventitious shoot regeneration in the 'Delite' rabbiteye blueberry cultivar. Leaf explants were cultured *in vitro* in Woody Plant Medium (WPM), and the effects of different thidiazuron (TDZ) concentrations, the orientation of the leaf (adaxial or abaxial surface in contact with the medium), and two portions of the leaf segment (basal or apical) were examined. *De novo* shoot development was studied using light and scanning electron microscopy. All concentrations of TDZ used showed similar survival and regeneration rates; 0.5 µM TDZ showed high efficiency in regenerating adventitious shoots (100%, with 57 adventitious shoots/explant), as did the adaxial surface in contact with the medium using either the apical or the basal portion of the leaf (97% shoot regeneration, 47.5 adventitious shoots/explant). Anatomical analyses showed direct and indirect organogenesis. The shoots developed leaf primordia with stomata, trichomes, and well-developed vascular tissues, with further elongation and rooting of the plants. We therefore describe here a high-efficiency regeneration method through *de novo* shoot organogenesis using TDZ in foliar explants of rabbiteye blueberry, with direct and indirect organogenesis.

KEYWORDS

Ericaceae, scanning electron microscopy (SEM), light microscopy, organogenesis, *in vitro* regeneration, *Vaccinium virgatum.*

ABBREVIATIONS

2iP, 2-isopentenyladenine, 6-(γ-γ-dimethylallylamino)-purine); Ab, abaxial; Ad, adaxial; Ca, callus; CV, coefficient of variation; DF: degrees of freedom; Ex, explant; GC; guard cell; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; LP, leaf primordium; ms, mean squares; MS, Murashige and Skoog Medium; NA, not available; NAA, α -naphthaleneacetic acid; ns, non-significant; Os, ostiole; Pc, procambium; R, roots; SAM, shoot apical meristem; SE, standard error; SEM, scanning electron microscopy; Sh, shoot; TDZ, thidiazuron; Tr, trichome; VT, vascular tissue; WPM, woody plant medium.

INTRODUCTION

Blueberry is a perennial fruit crop of the Ericaceae family and genus *Vaccinium*. The fruits offer high nutraceutical benefits, and show antioxidant and anti-inflammatory properties [1]. Blueberries are rich in polyphenol compounds that can induce neurogenesis in adults [2] with anti-inflammatory activity [3]. Additionally, blueberries have high concentrations of anthocyanins, with beneficial effects against chronic diseases such as cancer, diabetes, neurodegenerative diseases, and cardiovascular disorders [4], and have high concentrations of vitamin C [5].

The fact that blueberries have several bioactive compounds related to health benefits, in addition to their good taste makes them attractive to consumers – and production has been steadily increasing, with the commercialization of fresh fruits as well as juices, and frozen and dried processed products [1].

Multiple species are involved in the commercial production of blueberries, with the vast majority composed of *Vaccinium corymbosum* L. (tetraploid highbush blueberry) and its hybrids and *Vaccinium angustifolium* Ait. (tetraploid lowbush blueberry), with lesser quantities of *Vaccinium virgatum* Ait. (hexaploid rabbiteye blueberry) [6]. Increased demand has led to increases in blueberry production in different regions beyond its native origin – demanding new cultivars adapted to different environments. In warmer regions, rabbiteye blueberries have been shown to be a noteworthy alternative, with lower demands for cold and chilling hours to grow and produce.

Blueberry crops are mainly propagated vegetatively through cuttings, which can lead to pathogenic infections. Therefore, for best blueberry production, vegetative propagation should employ methods that assure phytosanitary standards. *In vitro* culture therefore represents an important method for blueberry clonal propagation, as it can potentially produce large numbers of plants and propagate newly released cultivars [7]. There has been a good deal of previous research on *in vitro* blueberry culturing, although much of it has been related to cultivars adapted to temperate climates. Numerous studies have focused on highbush and lowbush cultivars [8–17], but only a few studies have focused on rabbiteye *in vitro* regeneration techniques [10].

The protocols already developed are specific to each genotype, and depend on suitable concentrations of growth regulators in the culture medium [8], indicating the importance of research into specific protocols for different genotypes. Therefore, specific techniques need to be developed for rabbiteye blueberry cultivars that are better adapted to warmer winter regions, with efficient *in vitro* regeneration protocols that could be used for mass propagation as well as for the development of other studies in biotechnology.

Different growth regulators used in culture media will elicit distinct morphogenic responses [8,18], and adventitious bud regeneration protocols for blueberries have employed cytokinins and auxins, such as IAA (indole-3-acetic acid), 2iP [2-isopentenyladenine; 6-(γ-γ-dimethylallylamino)-purine], TDZ (thidiazuron), NAA (α-naphthaleneacetic acid), zeatin [as reviewed in 18], and IBA (indole-3-butyric acid) [10].

Thidiazuron (TDZ) has been used in many *in vitro* culture protocols, and elicits effects similar to auxins and cytokinins [16]. It has been tested in the *in vitro* regeneration of some *Vaccinium* species [11,13,15,16,19–22], as well as other genera, such as *Billbergia* [23], *Melastoma* [24], *Brassica* [25], *Cucumis* [26], *Populus* [27], *Arachis* [28], *Ficus* [29–33], *Morus* [34], *Chenopodium* [35] and *Lotus* [36].

TDZ has been widely employed in many *in vitro* techniques, such as micropropagation, and has been found to induce axillary proliferation at low concentrations. It can also be used at high concentrations (greater than 1 μM) for callus formation, organogenesis, and somatic embryogenesis. The high activity of TDZ can be explained by its lower susceptibility to enzymatic degradation as compared to natural cytokinins, and it can be useful with genotypes that are

otherwise difficult to propagate, including woody species. Its use in high concentrations can lead to undesirable effects, however, such as reduced shoot elongation, hyperhydricity, and shoot fasciation. It is of significant importance to determine the optimal TDZ concentration (or combinations of TDZ with other growth regulators) required for efficient *in vitro* regeneration process [37].

We therefore sought to develop an efficient *in vitro* regeneration technique for 'Delite' rabbiteye blueberry through shoot organogenesis from leaf explants, to study the developmental process of the *de novo* formed shoots, and to address a number of questions: what TDZ medium concentration is most suitable for inducing adventitious shoot formation from leaf explants? Will leaf explant orientation and portions affect the results? Is organogenesis direct or indirect? Are *de novo* shoots well-formed?

MATERIAL AND METHODS

Plant material

Leaf explants of the 'Delite' rabbiteye blueberry cultivar were collected from *in vitro* plants growing on WPM [38] supplemented with Murashige and Skoog (MS) organic compounds [39], 2.5 µM zeatin, and 30 g L^{-1} sucrose. All media were jellified with 7 g L^{-1} agar (Vetec, Rio de Janeiro/Brazil) after the pH was adjusted to 5.2. The media were then autoclaved at 120 ºC and 1.0 atm for 20 min; the zeatin was sterilized through 0.22 µm filters and added to the cooled media. Cultures were maintained at 25 ± 2 ºC under cool daylight at 40 µmol m^{-2} s^{-1} with a 16-h photoperiod.

Experiment with different TDZ concentrations in WPM culture medium

This organogenesis experiment was conducted using a completely randomized design, with six treatments representing different TDZ concentrations (0, 0.5, 1.0, 1.5, 2.0 and 2.5 µM). The medium was prepared using WPM culture medium supplemented with MS organic compounds, 30 g L^{-1} sucrose, and different TDZ concentrations. All media were jellified with 7 g L^{-1} agar (Vetec) after the pH was adjusted to 5.2. The media were then autoclaved at 120 ºC and 1.0 atm and poured

into sterilized Petri dishes (15 mL/dish). Leaf explants were collected from *in vitro* plants and placed in the Petri dishes with their adaxial surfaces in contact with the medium. Cultures were maintained in the culture room as described above. Each treatment used four replications (ten leaf explants in each replicate, placed in one Petri dish), for a total of 40 explants per treatment and a total of 240 leaf explants. Leaf explants were evaluated under a stereomicroscope ten weeks later, and scored according to their survival rate (%), shoot regeneration rate (%) (percentage of explants showing adventitious shoots), number of new shoots formed per explant (total number), and number of new shoots formed per explant considering their sizes (large, medium, or small). The shoot sizes were classified as: large, if longer than 1 mm and held leaves; medium, if shorter than 1 mm and held leaves; or small, if less than 1 mm long and did not bear any leaves. Contaminated cultures (0-30% of the explants) were not included in the statistical analyses. After the first evaluation, the explants were placed in fresh media (as previously described), with no TDZ, and supplemented with 2.5 µM zeatin.

Experiment with two explant orientations (adaxial or abaxial), and two leaf portions (basal or apical)

In this experiment, a two-factor (2 x 2) arrangement and a completely randomized design were used, with factor 1 being the different explant orientations (adaxial or abaxial surface in contact with the medium) and factor 2 being the leaf portion (basal or apical), in a total of four treatments. The medium used was WPM supplemented with MS organic compounds, 30 $g.L^{-1}$ sucrose, and 1 µM TDZ. All media were jellified with 7 $g\ L^{-1}$ agar (Vetec) after the pH was adjusted to 5.2. The medium was then autoclaved at 120 ºC and 1.0 atm and placed in sterilized Petri dishes (15 mL/dish). Leaf explants were collected from *in vitro* plants and placed in the Petri dishes according to the arrangement of the different treatments: adaxial or abaxial surface in contact with the medium, and using the basal or apical portion of the leaf. The cultures were maintained in a culture room as previously described. Each treatment consisted of five replicates (ten leaf explants in each replication, placed in one Petri dish), for a total of 50 explants per treatment, and 200 total leaf explants. Ten weeks later the leaf explants were evaluated using a stereomicroscope, according to the same criteria mentioned earlier. There was no

contamination in this experiment. After the first evaluation, explants were placed in fresh media as previously described, with no TDZ and supplemented with 2.5 µM zeatin.

Experimental Design and Statistical Analysis

All of the experiments were conducted according to a completely randomized design. First, the means of the explants in each replication were calculated (evaluating all of the explants), and then the means of the four or five replicates in each treatment were calculated. Levene's test was performed to confirm the homogeneity of the variances among the treatments, and then analysis of variance (ANOVA) was performed to detect significant differences between treatments, and Tukey's multiple range test ($p<0.05$) was used to identify the superior treatments. The results are presented as the mean ± standard error in the tables. In the experiment with different TDZ concentrations, linear regression analyses were performed with the variables confirmed to have statistical significance in the analysis of variance of the regression. Those variables were "number of new shoots formed per explant (total number)" and "number of new shoots formed per explant (small sized)". All statistical analyses were performed using R software [40].

Morphoanatomical analyses

In these evaluations, the WPM culture medium was supplemented with MS organic compounds, 30 g.L-1 sucrose, and 1.0 µM TDZ. All media were jellified with 7 g L^{-1} agar (Vetec) after the pH was adjusted to 5.2. Subsequently, the media was autoclaved at 120 ºC and 1.0 atm, and cultures were maintained at 25 ± 2 ºC under cool daylight at 40 µmol m^{-2} s^{-1} with a 16-h photoperiod. Leaf explants were excised from *in vitro* plants and placed in Petri dishes containing 15 mL of culture medium. Each Petri dish contained ten leaf explants positioned with their adaxial surfaces in contact with the medium.

Ten leaf explants were collected at every stage weekly (from three- to seven-week-old culture), for a total of 70 explants. The developmental processes of *de novo* shoot organogenesis were observed using both light and scanning electron microscopy (SEM).

The aforementioned explants were observed, and photodocumentation was performed using a stereomicroscope. Samples were fixed in modified Karnovsky solution (2.5% glutaraldehyde and 10% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2) [41].

For SEM, the fixed samples (as previously described) were dehydrated in an ethylic series. Critical point drying was obtained using a Bal-Tec CPD 030 Critical Point Dryer. Samples were fixed onto aluminum stubs and gold coated. The images were obtained using a JEOL JSM 6360-LV scanning electron microscope.

In the light microscopy analyses, after fixation, the samples were dehydrated in an ethylic series and embedded in methacrylate (Historesin, Leica Microsystems, Nussloch/Germany). The solidified blocks were sectioned (8 μm thick) in a rotary microtome (Olympus CUT 4055), the slides stained with 5% (w/v) toluidine blue [42], and subsequently photographed under a light microscope (Olympus BX51).

RESULTS

De novo shoot organogenesis was achieved from blueberry leaf explants in WPM culture medium containing TDZ.

Experiment with different TDZ concentrations in WPM culture medium

In this experiment, the explant survival rates were higher than 93%. The treatments with TDZ showed 100% explant survival, superior to the treatment with no TDZ. All the treatments containing TDZ showed 100% of the explants with shoot regeneration (Table 1), while the treatment without TDZ showed no regeneration (Fig. 1). The analysis of variance is detailed in Table 2.

The numbers of new shoots formed per explant (total) were different between the treatments containing TDZ. A simple linear regression equation for that variable was statistically significant (Fig. 2), and describes that for each 1 μM increase in TDZ concentration in the medium, there was a decrease of 12.0 shoots per explant. Estimated values varied from 57 to 33 new shoots formed per explant increasing concentrations of TDZ, from 0.5 to 2.5 μM.

If we separate the results concerning the number of new shoots formed into large, medium, and small sized classes (as previously described), a similar pattern

can be recognized with small shoots. In this case, there was a decrease of 15.0 shoots at each increase of 1 µM of TDZ concentration (Fig. 2), with estimated values ranging from 50.1 to 20.2 small shoots per explant with increasing TDZ concentrations (from 0.5 to 2.5 µM).

However, observing the newly formed large and medium sized shoots, there were no differences between the TDZ treatments (Table 2), with values ranging from 1.3 to 4.3 (large sized) and 5.0 to 7.1 (medium sized), with means of 2.7 and 5.9 new shoots per explant (large and medium sized respectively) (Table 1).

In Fig. 1, *de novo* shoot formation through organogenesis can be observed in the six different treatments with TDZ (a to f) ten weeks after the initiation of culturing. Figure 1a shows an oxidized leaf explant and no shoot regeneration in the control treatment (without TDZ). Figures 1b–1f show the effects of different TDZ concentrations on leaf explants, regenerating small, medium, and large sized shoots.

Further shoot growth in fresh culture medium with 2.5 µM zeatin was observed 20 weeks after the first evaluations (Figs. 1g–1j), with subsequent *in vitro* rooting of the explants (Figs. 1h, 1j).

Experiment with two explant orientations (adaxial or abaxial), and two leaf portions (basal or apical)

Analysis of variance showed that there were interactions between the factors of explant orientation and leaf portion only for the variables of number of new shoots formed per explant (total) and number of new shoots formed per explant (small sized); there were no interactions between the factors in terms of the variables survival rate, shoot regeneration rate, number of new shoots formed per explant (large sized), and number of new shoots formed per explant (medium sized), (Tables 3 and 4).

The basal or apical leaf portion treatments showed no differences in their survival rate, with 91.0 and 97.0% of explants surviving respectively (Table 3). A difference was observed, however, between the adaxial and abaxial sides of the explant in contact with the medium, with the adaxial orientation achieving 100% survival, and the abaxial orientation only 88%.

The highest shoot regeneration rate occurred when the explant orientation was adaxial (97.0%), and the leaf portion apical (97.0%).

When the basal portion of the leaf was cultured, the variables of number of shoots formed per explant (total) and number of shoots formed per explant (small sized), using an adaxial placement, were found to be superior (59.2 total shoots, and 46.1 small shoots) to an abaxial orientation (22.8 total shoots, and 16.2 small shoots). When the apical portion was used, no differences were observed between the adaxial or abaxial orientations in terms of the variables of: number of shoots formed per explant (total number) and number of shoots formed per explant (small sized) (Table 3). In the treatments using the adaxial side in contact with the medium, there was no difference between apical and basal portions in terms of the total number of shoots and the number of small shoots per explant.

The adaxial positioning of the leaf on the medium resulted in larger numbers of large shoots formed per explant (4.3 shoots) than the abaxial orientation (1.8 shoots), although no differences were observed between the basal and apical leaf portions.

No differences were observed between the numbers of new medium sized shoots formed per explant, with an overall mean of 6.4 (Table 3).

In Fig. 3, regenerating shoots can be seen forming over the leaf explant in the four treatments (Figs. 3a–3d), and, ten weeks later, the shoots can be seen growing in the WPM medium supplemented with 2.5 µM zeatin but without TDZ (Figs. 3e, 3f).

Morphoanatomical analyses

De novo shoot organogenesis can be observed in Figs. 4, 5, and 6 after three to seven weeks of culture. Three-week-old cultures show leaf explants with shoots (Figs. 4a–4c), followed by four-week-old cultures (Figs. 4d–4f), five-week cultures (Figs. 4g–4i), six-week cultures (Figs. 5a–5c), and seven-week cultures (Figs. 5d–5f).

The adaxial surface of the leaf explant can be seen with many adventitious small, medium, and large shoots in Figs. 4a, 4d, 4g, 5a, 5d. The *de novo* shoots formed appear green when observed under a stereomicroscope, a feature indicative of the presence of chloroplasts in the epidermal cells.

Shoot organogenesis can be observed on the leaf explants, with recognizable leaf primordia – many of them already bearing glandular trichomes

(Figs. 4b, 4e, 4h, 5b, 5e). Figure 5e presents a top view of a forming shoot with many leaf primordia. The oldest leaf primordia are located along the outermost region of the shoot, while the youngest leaf primordia formed are located along the inner region of the shoot.

The development of adventitious shoots with leaf primordia can be observed in Figs. 4c, 4f, 4l, 5c, 5f. Figure 4 c shows an adventitious shoot with leaf primordium being formed, and those shoots already show vascular tissue. Figures 4f and 4i highlight the dome-shaped shoot apical meristem with meristematic characteristics. That region could be recognized in histological observations by its small isodiametric cells with dense cytoplasm and large nuclei (Figs. 4f, 4i, 6g, 6h, 6i, 6l)

Indirect organogenesis is evidenced by shoot formation from callus cells (Fig. 5c), with disorganized aspects and green staining by toluidine blue. In Figure 5f, on the other hand, direct organogenesis is confirmed by the observation of shoot formation directly from the explant, with no callus cells. Additionally, the connections between the vascular tissue of the leaf explant with the adventitious shoot indicate direct organogenesis (Fig. 5f).

Details of SEM images show (Figs. 6a–6f) of three- to seven-week-old leaf explant cultures, with newly formed adventitious shoots easily visible (Figs. 6a, 6b). Fig. 6b shows the abaxial surface of the leaf primordia. More advanced stages are shown of the adaxial and abaxial surfaces of the leaf primordia, with numerous stomata on the abaxial surface and well-formed trichomes (Fig. 6c). Detailed views of the stomata formed on the abaxial surface of the leaf primordium (Fig. 6d) show opened ostioles surrounded by guard cells. The absence of stomata on the adaxial surface of the leaf primordium indicated that blueberry leaves are hypostomatic (Fig. 6e). Glandular trichomes on blueberry leaves with evident secretory heads can be seen in Fig. 6f.

Adventitious shoots, with details such as the shoot apical meristem and leaf primordium formation can be seen after three weeks of culturing (Fig. 6g), with recognizable callus. Fig. 6h shows details of the adventitious shoot with leaf primordium, evidence of a shoot apical meristem at four weeks of culturing, and tissue arrangements.

Figure 6i shows an adventitious shoot with leaf primordia, shoot apical meristem, procambium, vascular tissue, and trichomes on the leaf primordia. Figure 6j shows a shoot with leaf primordia. Fig. 6k sows an adventitious shoot with the

formation of vascular tissue after four weeks of culture. Fig. 6I shows an adventitious shoot with an apical meristem, procambium, vascular tissue, and leaf primordia with protoderm, after four weeks of culture.

DISCUSSION

This study describes *de novo in vitro* shoot formation from leaf explants of 'Delite' rabbiteye blueberry, in the development of an important in vitro culture technique (Fig. 7). We described the morphological and anatomical aspects of the developing shoots of blueberry based on light microscopy and SEM images. *De novo* shoot organogenesis is an example of a dedifferentiation process, where mature plant cells are capable of undergoing a reversible process from a mature and differentiated state to a meristematic stage [43].

Adjusting plant growth regulators in culture media is one of the most common approaches used in developing regeneration protocols such as somatic embryogenesis [44,45] and shoot proliferation [46,47]. TDZ is a potent cytokinin-like growth regulator that also shows auxin-like activity [16], and is a powerful plant growth regulator for establishing regeneration protocols.

We were able to regenerate shoots by incorporating TDZ into the culture medium, and found that the low concentration of 0.5 µM proved to be effective in adventitious shoot formation in 'Delite' blueberry. Other studies of *Vaccinium* reported that concentrations higher than 0.5 µM TDZ were more effective, with 0.5 mg L^{-1} (2.27 µM) TDZ inducing the highest number of shoots in the blueberry cultivar 'Duke' as compared to the lowest concentrations tested [0.1 mg L^{-1} (0.45 µM) and 0.2 mg L^{-1} (0.91 µM)] [8]. In another study with lowbush blueberry (*V. angustifolium*), the use of 2.3 to 4.5 µM TDZ allowed adventitious bud differentiation and shoot formation [13].

Some authors have examined organogenesis in blueberries using combinations of TDZ and other growth regulators (zeatin, zeatin riboside, and NAA), or even without TDZ, in studies of adventitious regeneration in different blueberry cultivars [15], and concluded that the optimum combinations of growth regulators were cultivar-dependent. In a study [10] with 'Bluejay' (highbush, *V. corymbosum*), 'Pink Lemonade' (rabbiteye derivative hybrid, *V. virgatum*), 'Sunshine Blue' (highbush, *V. corymbosum*), and 'Top Hat' (highbush x lowbush cross) cultivars,

adventitious shoots were regenerated in culture media supplemented with different combinations of zeatin and IBA. A study with cranberry (*Vaccinium macrocarpon*) reported maximum regeneration rates in medium containing 10.0 µM TDZ with 1.0 µM NAA [19]. Another study with cranberry (*V. macrocarpon*) found that 10.0 µM TDZ with 5.0 µM 2iP was effective in initial adventitious regeneration [20].

The use of TDZ whenever possible as an alternative growth regulator to substitute the more commonly used zeatin will have the benefit of lowering the costs of *in vitro* blueberry culture [8].

We observed that *de novo* shoot cultures formed with TDZ in 'Delite' did not continue growing unless they were transferred to fresh medium supplemented with zeatin. It is known that TDZ can inhibit shoot elongation [19,37], so in order to assure shoot regeneration in lowbush blueberry (*V. angustifolium*), cultures initiated in TDZ must be transferred to a new medium containing zeatin (2.3 to 4.6 µM) to allow shoot elongation [13].

Leaf orientation, and the portion of it that is used, have been studied in *in vitro* organogenesis. A study with lowbush blueberry (*V. angustifolium*) found that basal leaves with their adaxial surface in contact with the medium proved to be most effective [12], and shoot apices were likewise found to form from the adaxial surfaces of leaf explants of 'Aurora' highbush *V. corymbosum* [11].

We found that both the apical and basal portions of the leaves generated high numbers of shoots per explant, but that the adaxial surface in contact with the medium resulted in higher survival and shoot regeneration rates, and great numbers of large shoots formed per explant. In evaluating the differences between adaxial and abaxial surfaces, considering the use of the basal leaf portion and the variable numbers of new shoots formed (total number and small sized shoots), higher yields were observed with the adaxial leaf surface in contact with the medium as compared to the abaxial surface. The observation that the adaxial surface in contact with the medium produced more shoots could be related to the fact that the abaxial side does not settle and completely enter in contact with the medium (as much as the adaxial treatments), due to its concave curvature.

Most of the shoots formed in our work appeared on the adaxial surface of the leaf explant, as was also reported with 'Aurora' (*V. corymbosum*) [11] and cranberry (*V. macrocarpon*) adventitious regeneration [20].

Both direct and indirect organogenesis were observed in this study, as shoots could originate directly from the leaf tissue of the explant with no apparent or histological evidence of callus formation, giving rise to direct organogenesis (Fig. 5f), with connections between the vascular tissue of the new shoot with that of the leaf explant with no callus tissue being observed. The shoot formed in Fig. 5c, on the other hand, originated from callus tissue, in a process of indirect organogenesis.

Callus proliferation is a process of unstructured cell division and enlargement, usually initiated from parenchymatous cells, and the cell walls typically contain secondary metabolites such as suberin, lignin, or phenolics [43]. In work with 'Troyer' citrange shoot regeneration, callus cells were found to evidence some lignification in cases of either direct or indirect organogenesis [48].

Callus tissue could be recognized in our work by its disorganized aspect, with a certain disaggregation and green staining with toluidine blue, generally indicating phenolic compounds in the cells [49]. Feder and O'Brien (1968) reported that toluidine blue will stain polyphenol containing cells a green color [50]; two studies with *Spondias dulcis* likewise reported that accumulations of phenolic compounds in the cells were stained green by toluidine blue [51,52], and the same staining was observed in a study with *Brassica oleracea* [53].

Various studies of *Vaccinium* adventitious shoot regeneration have reported either direct and/or indirect organogenesis; shoot apices of 'Aurora' highbush (*V. corymbosum*), were observed to form directly from parenchyma cells on the surface of leaf explants [11], and histological studies showed organogenesis without callus formation that initiated in sub-epidermal cells in highbush blueberry (*V. corymbosum*) [54].

Indirect organogenesis has been observed in 'Bluejay' (highbush, *V. corymbosum*), 'Pink Lemonade' (rabbiteye derivative hybrid, *V. virgatum*), 'Sunshine Blue' (highbush, *V. corymbosum*), and 'Top Hat' (highbush x lowbush cross) cultivars, with callus being induced from the explants, followed by adventitious shoot regeneration [10]. Callus formation was also observed in a somatic embryogenesis study with blueberry cultivars (*V. corymbosum* x *V. angustifolium*), with embryo development without the callus phase in a culture medium containing TDZ [16].

Similar to what we observed with the 'Delite' cultivar, direct and indirect organogenesis was obtained from leaf explants using 'Duke' highbush blueberry [8].

Additionally, in a study with lowbush blueberry (*V. angustifolium*), adventitious bud and shoot formation was observed with or without an intermediary callus phase [13].

Among other morphoanatomical characteristics, we identified dome-shaped shoot apical meristems under light microscopy with diameters varying from 120 to 200 µm, similar to the description of the shapes and sizes of shoot apical meristems in highbush field-grown blueberry (approximately 120 µm) [55].

Additional meristematic characteristics observed here, such as protoplasts strongly stained by toluidine blue, are in accordance with the literature [50].

We observed that leaf primordia were initiated along the flanks of the shoot meristem, which is in agreement with other studies [43]. Blueberries have simple leaves that are arranged alternately along the stem [55,56]. SEM images provided here show some details of leaf primordia formation (Fig. 5e).

A study of the leaf anatomy of field-grown *V. corymbosum* showed their leaves to be bifacial, with all the stomata on the abaxial side of the leaf (hypoestomatic) [57]. An anatomical study of highbush blueberry leaves (*V. corymbosum*, cv. 'Bluetta') reported that stomata were present only on the abaxial surfaces of field-grown leaves, but they were observed on both surfaces of *in vitro* leaves [58] – differing from our findings with rabbiteye 'Delite' *in vitro* organogenesis, where only the abaxial surfaces of the leaf primordia held stomata. Therefore, the leaves of the shoots formed in our work demonstrated characteristics similar to those of field-grown plants, with their stomata restricted only to the abaxial surface – a common feature in blueberry plants.

We did not observe any signs of tissue hyperhidricity, which represents an essential achievement of our tissue culture work. Hyperhidricity is always a concern in *in vitro* culture, as it can limit subculturing and acclimatization survival, and represents a serious problem for tissue culturing, including for propagation, germplasm conservation, and plant breeding [59]. Hyperhidricity represents an alteration of the plant's normal morphophysiological state, with high water accumulation in the tissues and the formation of abnormal organs with water-soaked appearances [60]. Hyperhydric plants show discontinuous epidermal development, irregular stomatal formation, decreased stomatal density, intercellular spaces in the mesophyll, and reduced chlorophyll contents [59]. Blueberry cultivars (*Vaccinium* spp.) cultivated *in vitro* and showing hyperhydricity have a glassy aspect with translucent stems and leaves that are shortened and brittle, with deformed glandular

trichomes, rough and crinkly epidermal, damaged stomata guard cells, enlarged mesophyll, disintegrated cell contours, deformed nuclei, and more intercellular spaces [59].

Morphological and anatomical analyses of the *de novo* shoots produced here showed them to be well-developed and with indicators of high viability, such as the green color of their shoots, well-developed and un-deformed stomata, glandular trichomes, shoot apical meristems, and leaf primordia, and cells with regular contours and well-delimited intercellular spaces. During the processes of sample preparation for microscopic examination the cells did not hardly dehydrate (the opposite of what would be expected with hyperhydric tissues), and the shoots were not glassy or translucent. Additionally, when the shoots were transferred to fresh medium with zeatin and without TDZ, they were able to survive, elongate, and form roots.

It is important to note that the morphogenic pathway observed here was of *de novo* shoot organogenesis from somatic cells in the leaf explant, developing a unipolar structure, and somatic embryogenesis (bipolar structure) was not observed. According to a study in *Passiflora* [61], changes of the auxin/cytokinin ratios can trigger those different developmental pathways; the authors observed both routes, but concluded that *de novo* shoot organogenesis generally occurred with exposure to a high cytokinin-to-low auxin ratio, or with cytokinin alone. Our study used only the cytokinin TDZ in the culture medium (although that growth regulator possibly have auxinic activity).

Adventitious shoot development stages are described here, showing that 'Delite' blueberry can demonstrate either direct or indirect organogenesis, with well-developed shoot apical meristems and leaf primordia. The leaf primordia of *de novo* shoots showed laminar shapes and a green color, with well-developed stomata and trichomes; adventitious shoots, and epidermal, parenchymatic, and vascular tissues were observed, with eventual shoot elongation, root formation, and the development of the whole plants.

CONCLUSION

The results presented here contribute to a better understanding of the *in vitro* organogenesis process in 'Delite' rabbiteye blueberry, and indicated a TDZ concentration of 0.5 µM in the WPM medium, using either the apical or the basal

portions of the leaf and its adaxial surface orientation in contact with the medium. Both direct and indirect organogenesis were observed in that cultivar. The adventitious shoots showed the development of normal leaf tissues, and they grew and developed into rooted plants. Due to the high rate of regenerating explants and high numbers of shoots formed per explant, the techniques we describe here could be used for *in vitro* clonal propagation once genetic stability is confirmed. Additionally, it is expected that this research can help elucidate *in vitro* organogenesis regeneration process of 'Delite' rabbiteye blueberry plants, and contribute to further developing the biotechnology of blueberry cultivation.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

AUTHORS CONTRIBUTIONS

Carolina Schuchovski: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper. Bruno Francisco Sant'Anna-Santos: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper. Raquel Cristina Marra: Performed the experiments; Contributed reagents, materials, analysis tools or data. Luiz Antonio Biasi: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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TABLES

Table 1. Effects of different TDZ concentrations on *in vitro* shoot organogenesis in 'Delite' rabbiteye blueberry, using WPM culture medium.

Treatment	Survival rate	Shoot regeneration rate	Number of new shoots formed/explant (large sized)	Number of new shoots formed/explant (medium sized)
	%	%	n.	n.
TDZ 0 µM	93.3 ± 3.3 b	0.0 ± 0.0 b	NA	NA
TDZ 0.5 µM	100.0 ± 0.0 a	100.0 ± 0.0 a	1.3 ± 0.6 a	5.4 ± 1.9 a
TDZ 1 µM	100.0 ± 0.0 a	100.0 ± 0.0 a	3.0 ± 0.2 a	5.0 ± 0.7 a
TDZ 1.5 µM	100.0 ± 0.0 a	100.0 ± 0.0 a	2.4 ± 1.1 a	5.4 ± 1.4 a
TDZ 2 µM	100.0 ± 0.0 a	100.0 ± 0.0 a	2.4 ± 1.0 a	7.1 ± 1.5 a
TDZ 2.5 µM	100.0 ± 0.0 a	100.0 ± 0.0 a	4.3 ± 1.5 a	6.9 ± 1.8 a
Mean	99.1	85.7	2.7	5.9
CV%	2.1	0.0	70.8	50.6

The results are presented as mean ± standard error (SE). Means followed by different letters in the same column differ statistically at 5% of the Tukey's multiple range tests. Abbreviation: CV, coefficient of variation; NA, not available; TDZ, thidiazuron; WPM, woody plant medium.

Table 2. Analysis of variance (ANOVA) of the experiment evaluating the effect of different thidiazuron (TDZ) concentrations on *in vitro* shoot organogenesis in 'Delite' rabbiteye blueberry. Abbreviations: DF, degrees of freedom; ms, mean squares, ns, non-significant; TDZ, thidiazuron.

		Survival rate	Shoot regeneration rate	n	Number of new shoots formed/ explant (total)	Number of new shoots formed/ explant (large sized)	Number of new shoots formed/ explant (medium sized)	Number of new shoots formed/ explant (small sized)
	DF	ms	ms	DF	ms	ms	ms	ms
Treatment	5	22.8	5142.9	4	572.5	4.8	3.239	697.7
Residuals	15	4.4	0.000	13	127.3	3.6	8.947	105.7
Total	20			17				
p-value		0.0060 **	< 2.2e-16 **		0.0169 *	0.3190 ns	0.8313 ns	0.0040 **

* significant different with 0.05 > p-value > 0.01

** significant different with p-value ≤ 0.01

ns, non-significant, p-value ≥ 0.05

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Table 3. Effects of two explant orientations (adaxial or abaxial side in contact with the medium) and two leaf portions (basal or apical) on *in vitro* shoot organogenesis in 'Delite' rabbiteye blueberry, using WPM culture medium supplemented with TDZ.

Explant orientation	**Leaf portion**		
Survival rate (%)	basal	apical	mean
adaxial	100.0 ± 0.0	100.0 ± 0.0	100.0 a
abaxial	82.0 ± 10.3	94.0 ± 4.5	88.0 b
mean	91.0 A	97.0 A	94.0
Shoot regeneration rate (%)			
adaxial	94.0 ± 4.5	100.0 ± 0.0	97.0 a
abaxial	74.0 ± 12.0	94.0 ± 4.5	84.0 b
mean	84.0 B	97.0 A	90.5
Number of shoots formed/explant (total)			
adaxial	59.2 ± 15.4 aA	35.8 ± 3.8 aA	47.5
abaxial	22.8 ± 3.1 bA	41.3 ± 9.6 aA	32.0
mean	41.0	38.6	39.8
Number of shoots formed/explant (large sized)			
adaxial	4.1 ± 0.7	4.5 ± 1.5	4.3 a
abaxial	2.5 ± 0.5	1.1 ± 0.2	1.8 b
mean	3.3 A	2.8 A	3.1
Number of shoots formed/explant (medium sized)			
adaxial	9.0 ± 1.1	6.4 ± 1.1	7.7 a
abaxial	4.0 ± 0.6	6.1 ± 1.8	5.1 a
mean	6.5 A	6.3 A	6.4
Number of shoots formed/explant (small sized)			
adaxial	46.1 ± 15.3 aA	24.9 ± 4.3 aA	35.5
abaxial	16.2 ± 3.0 bA	39.5 ± 7.6 aA	27.9
mean	31.2	32.2	31.7

The results are presented as mean ± standard error (SE). Means followed by different lowercase letters in the same column and by different uppercase letters in the same horizontal line differ statistically at 5% of the Tukey's multiple range tests. Abbreviation: TDZ, thidiazuron; WPM, woody plant medium.

Table 4. Analysis of variance (two-way ANOVA) of the experiments evaluating the effect of explant orientation (adaxial or abaxial side in contact with the medium) and the leaf portion (basal or apical) on *in vitro* shoot organogenesis in 'Delite' rabbiteye blueberry. Abbreviations: CV, coefficient of variation; DF, degrees of freedom; ms, mean squares.

	Source	DF	ms	p value
Survival rate (%)	Explant orientation	1	720.00	0.0289 *
	Leaf portion	1	180.00	0.2476 ns
	Interaction (explant orientation x leaf portion)	1	180.00	0.2476 ns
	Residuals	16	125.00	
CV: 11.9%	Total	19		
Shoot regeneration rate (%)	Explant orientation	1	845.00	0.0484 *
	Leaf portion	1	845.00	0.0484 *
	Interaction (explant orientation x leaf portion)	1	245.00	0.2667ns
	Residuals	16	185.00	
CV: 15.0%	Total	19		
Number of shoots formed/explant (total)	Explant orientation	1	1196.60	0.0844 ns
	Leaf portion	1	29.40	0.7766 ns
	Interaction (explant orientation x leaf portion)	1	2199.10	0.0239 *
	Residuals	16	353.40	
CV: 47.3%	Total	19		
Number of shoots formed/explant (large sized)	Explant orientation	1	31.25	0.0053 **
	Leaf portion	1	1.36	0.5100 ns
	Interaction (explant orientation x leaf portion)	1	3.93	0.2696 ns
	Residuals	16	3.00	
CV: 56.5%	Total	19		
Number of shoots formed/explant (medium sized)	Explant orientation	1	34.45	0.0312 *
	Leaf portion	1	0.26	0.8407 ns
	Interaction (explant orientation x leaf portion)	1	27.31	0.0516 ns
	Residuals	16	6.17	
CV: 39.9%	Total	19		
Number of shoots formed/explant (small sized)	Explant orientation	1	289.18	0.3561 ns
	Leaf portion	1	5.63	0.8962 ns
	Interaction (explant orientation x leaf portion)	1	2478.21	0.0133 *
	Residuals	16	320.16	
CV: 56.5%	Total	19		

* significant different with 0.05 > p-value > 0.01

** significant different with p-value ≤ 0.01

ns, non-significant, p-value ≥ 0.05

FIGURES

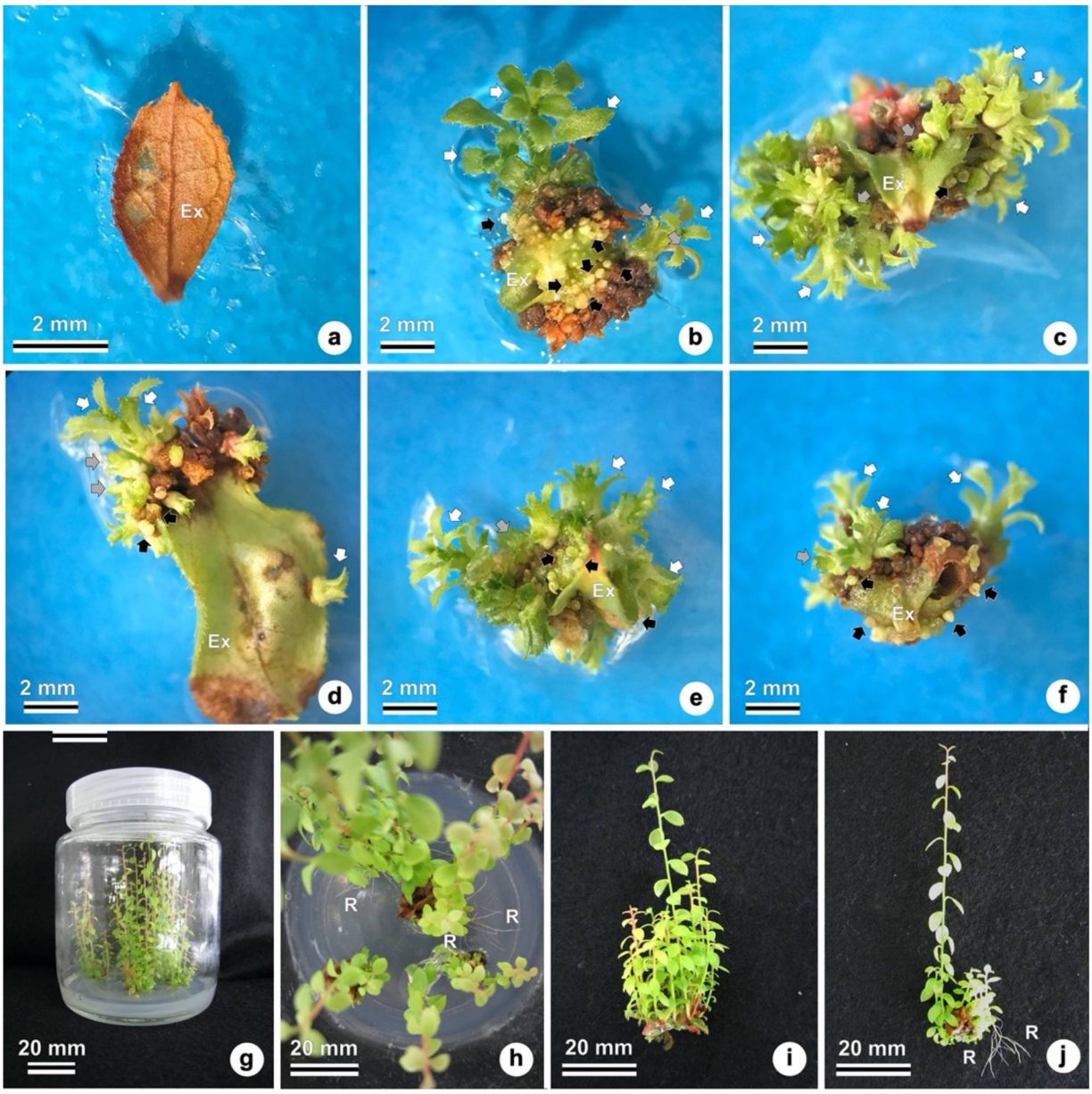

Fig. 1. Effects of different TDZ concentrations on *in vitro* shoot organogenesis in 'Delite' rabbiteye blueberry using WPM culture medium. Different views of explants and shoots under a stereomicroscope (a-f) and digital camera (g-j). (a) TDZ 0 µM, showing an oxidized leaf explant. (b) TDZ 0.5 µM with many small (black arrow), medium (gray arrow), and large (white arrow) shoots. (c) TDZ 1.0 µM, with small (black arrow), medium (gray arrow), and large (white arrow) shoots. (d) TDZ 1.5 µM, with small (black arrow), medium (gray arrow), and large (white arrow) shoots. (e) TDZ 2.0 µM, with small (black arrow), medium (gray arrow), and large (white arrow) shoots. (f) TDZ 2.5 µM, with small (black arrow), medium (gray arrow), and large (white arrow) shoots. (g-j) Development of the shoots at 20 weeks after the first evaluation in fresh culture medium with 2.5 µM zeatin. Details of *in vitro* rooting in (h) and (j). Abbreviations: Ex, explant; R, roots; TDZ, thidiazuron; WPM, woody plant medium.

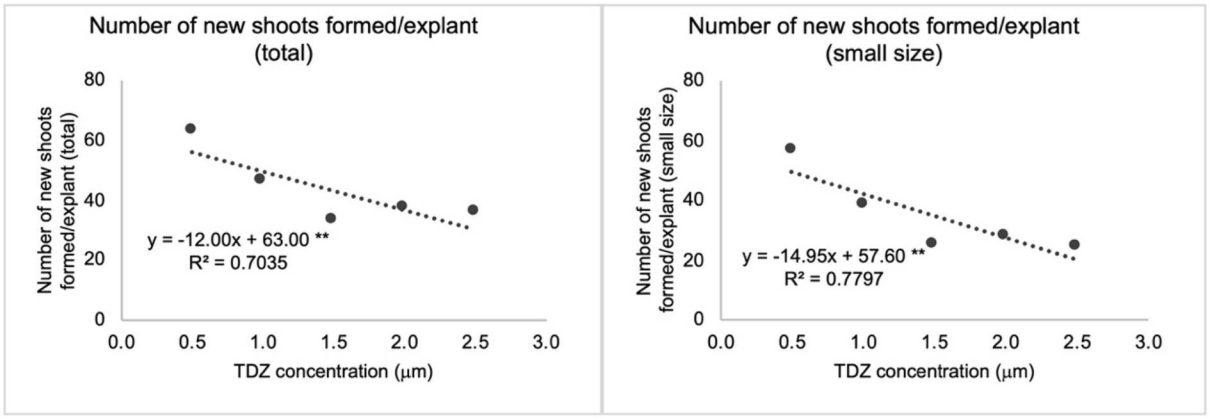

Fig. 2. Effects of different TDZ concentrations on *in vitro* shoot organogenesis in 'Delite' rabbiteye blueberry using WPM culture medium. Simple linear regression graphics showing the dependent variables number of new shoots formed/explant (total) on the left, and the number of new shoots formed/explant (small size) on the right. ** statistically significant with p-value ≤0.01. Abbreviations: TDZ, thidiazuron; WPM, woody plant medium.

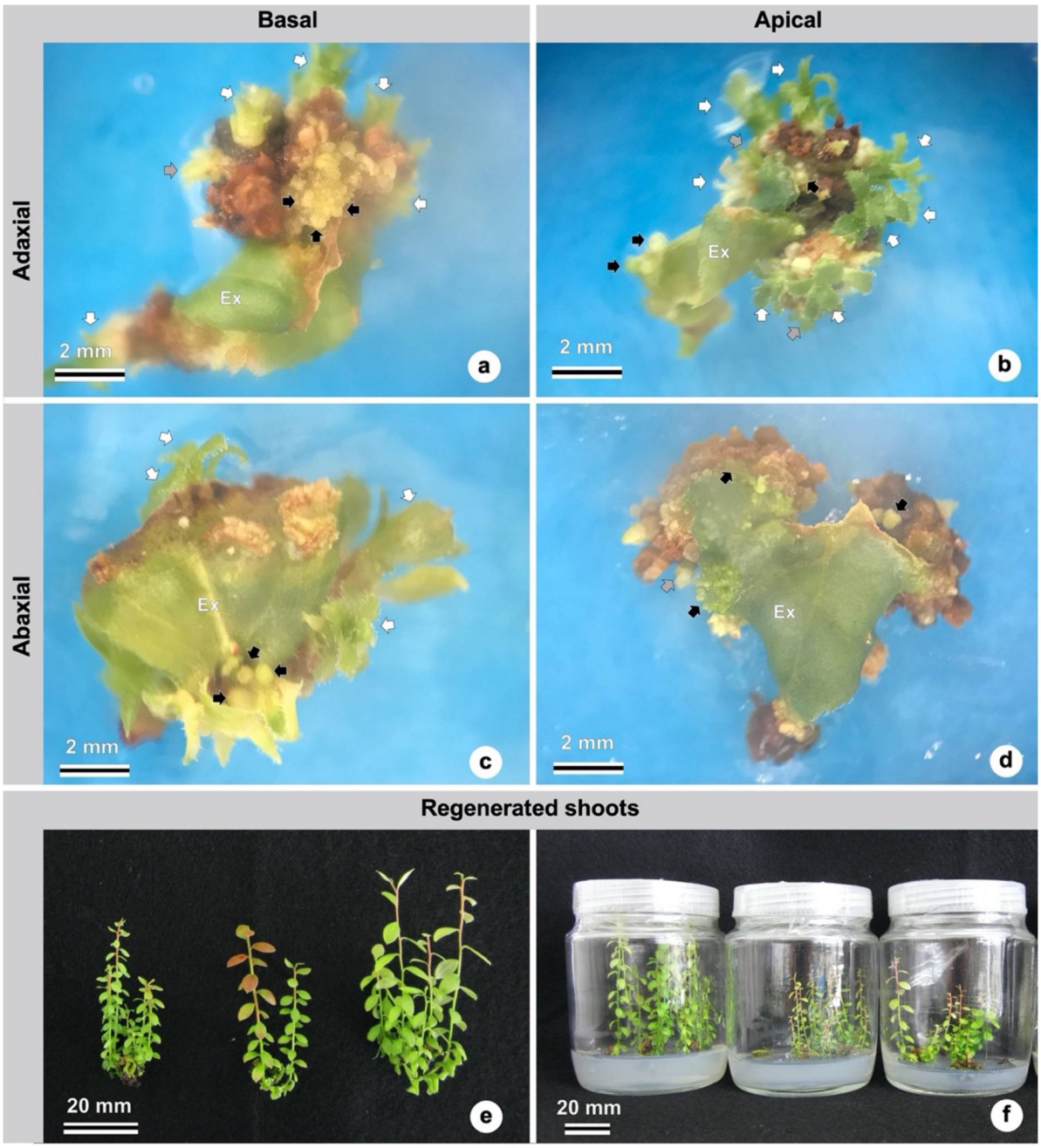

Fig. 3. Effects of two explant orientations (adaxial or abaxial side in contact with the medium) and two leaf portions (basal or apical) on *in vitro* shoot organogenesis in 'Delite' rabbiteye blueberry using WPM culture medium supplemented with TDZ. Different views of explants and shoots under a stereomicroscope (a-d) and digital image capturing (e-f). (a) Adaxial x Basal, with many small (black arrow), medium (gray arrow), and large (white arrow) shoots. (b) Adaxial x Apical, with many small (black arrow), medium (gray arrow), and large (white arrow) shoots: (a, b) Those two adaxial treatments showed the best efficiency in regenerating shoots. (c) Abaxial x Basal with small (black arrow) and large (white arrow) shoots. (d) Abaxial x Apical, with small (black arrow) and medium (gray arrow) shoots. (e) Regenerated shoots after adventitious organogenesis. (f) *In vitro* regenerated shoots after adventitious organogenesis, subcultured into WPM culture medium supplemented with 2.5 µM zeatin, pictured ten weeks later. Abbreviations: TDZ, thidiazuron; WPM, woody plant medium.

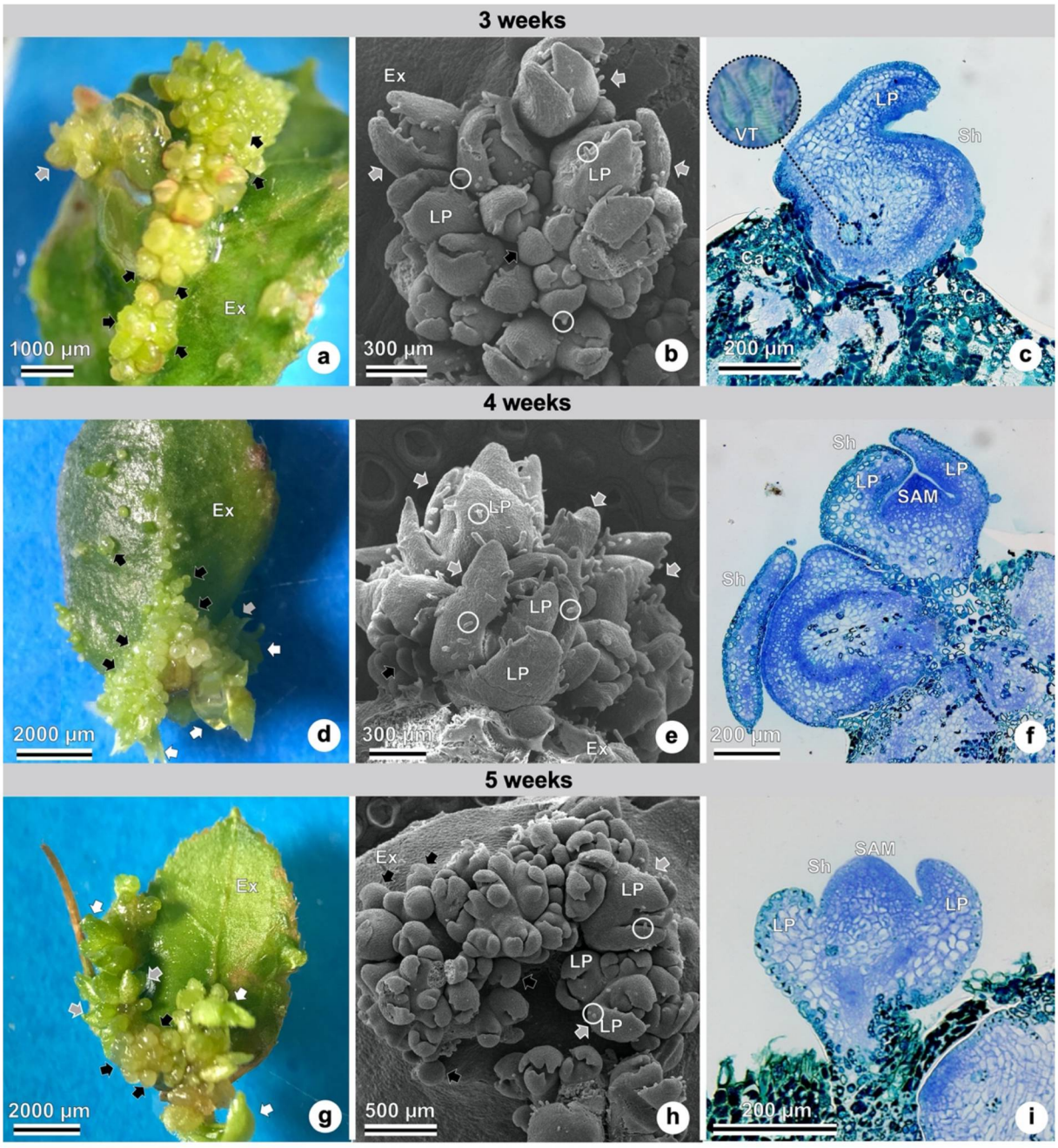

Fig. 4. *In vitro* shoot organogenesis in 'Delite' rabbiteye blueberry using WPM culture medium supplemented with TDZ at different times (three- to five-week culture). Different views of explants and shoots under a stereomicroscope (a, d, and g), scanning electron microscope–SEM (b, e, and h), and light microscope (c, f, and i). (a) Three weeks of culture, leaf explant with small (black arrow), medium (gray arrow), and large (white arrow) shoots. (b) Three weeks of culture, leaf explant with small (black arrow), and medium (gray arrow) shoots, showing leaf primordia with trichomes (white circle). (c) Three weeks of culture, leaf explant with shoot, leaf primordium, vascular tissue (in detail), and callus formation. (d) Four weeks of culture, leaf explant with small (black arrow), medium (gray arrow), and large (white arrow) shoots. (e) Four weeks of culture, leaf explant with small (black arrow), and medium (gray arrow) shoots, showing leaf primordia with trichomes (white circle). (f) Four weeks of culture, shoots with leaf primordia, and shoot apical meristem. (g) Five weeks of culture, leaf explant

with small (black arrow), medium (gray arrow), and large (white arrow) shoots. (h) Five weeks of culture, leaf explant with small (black arrow), and medium (gray arrow) shoots, showing leaf primordia with trichomes (white circle). (i) Five weeks of culture, shoots with shoot apical meristem and leaf primordia. Abbreviations: Ca, callus; Ex, explant; LP, leaf primordium; SAM, shoot apical meristem; Sh, shoot; TDZ, thidiazuron; VT, vascular tissue; WPM, woody plant medium.

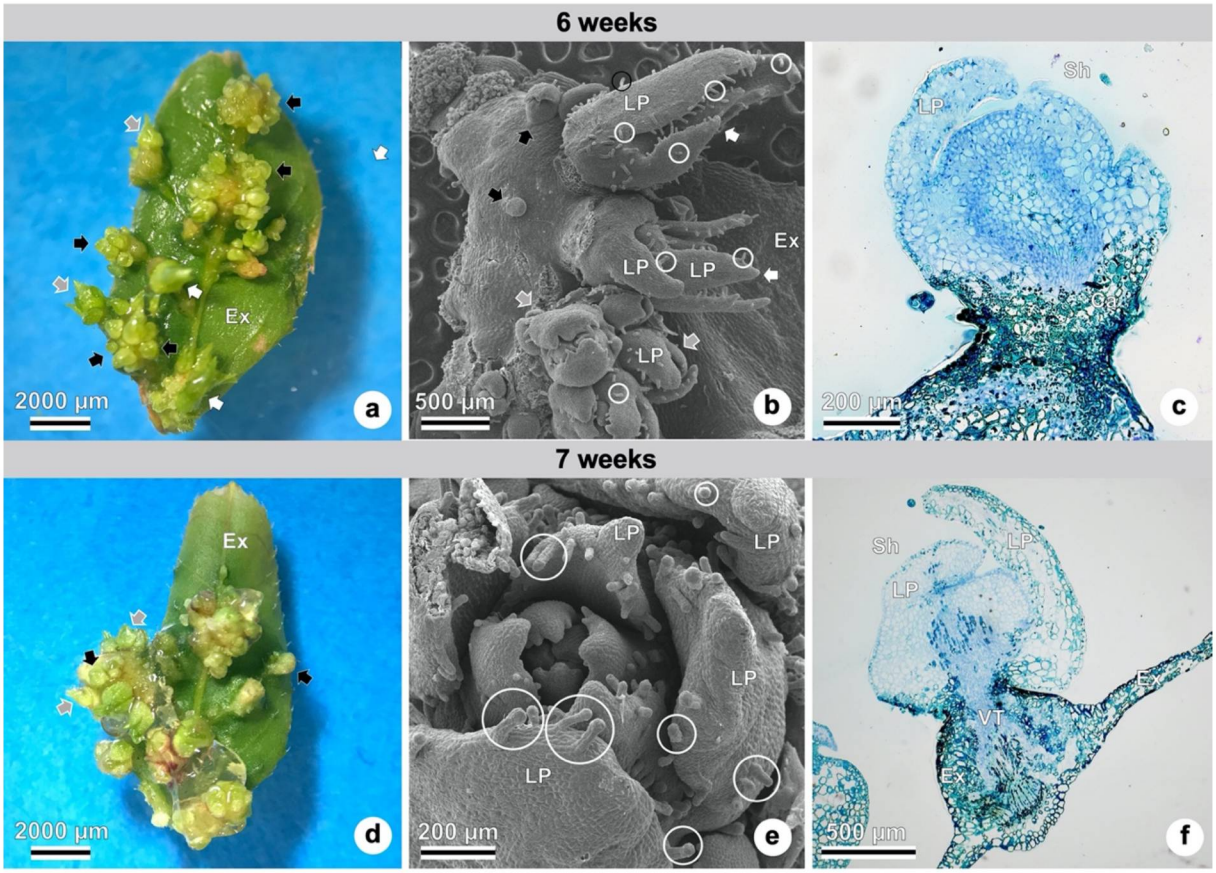

Fig. 5. *In vitro* shoot organogenesis in 'Delite' rabbiteye blueberry using WPM culture medium supplemented with TDZ at different times (six and seven-week-old cultures). Different views of explants and shoots under a stereomicroscope (a and d), scanning electron microscope–SEM (b and e), and light microscope (c and f). (a, b, and c) Six-week-old culture. (d, e, and f) Seven-week-old culture. (a) Adaxial surface of the explant with small (black arrow), medium (gray arrow), and large (white arrow) shoots. (b) Explant with small (black arrow), medium (gray arrow), and large (white arrow) shoots, and leaf primordia with trichomes (white circle). (c) Shoot with leaf primordium, in indirect organogenesis: shoot formation originated from callus. (d) Adaxial surface of the explant with small (black arrow) and medium (gray arrow) shoots. (e) Shoot with several leaf primordia, showing trichomes (white circle). (f) Transversal view of the explant, with longitudinal view of the shoot formation through direct organogenesis, connecting to the explant vascular tissue. Abbreviations: Ca, callus; Ex, explant; LP, leaf primordium; Sh, shoot; TDZ, thidiazuron; VT, vascular tissue; WPM, woody plant medium.

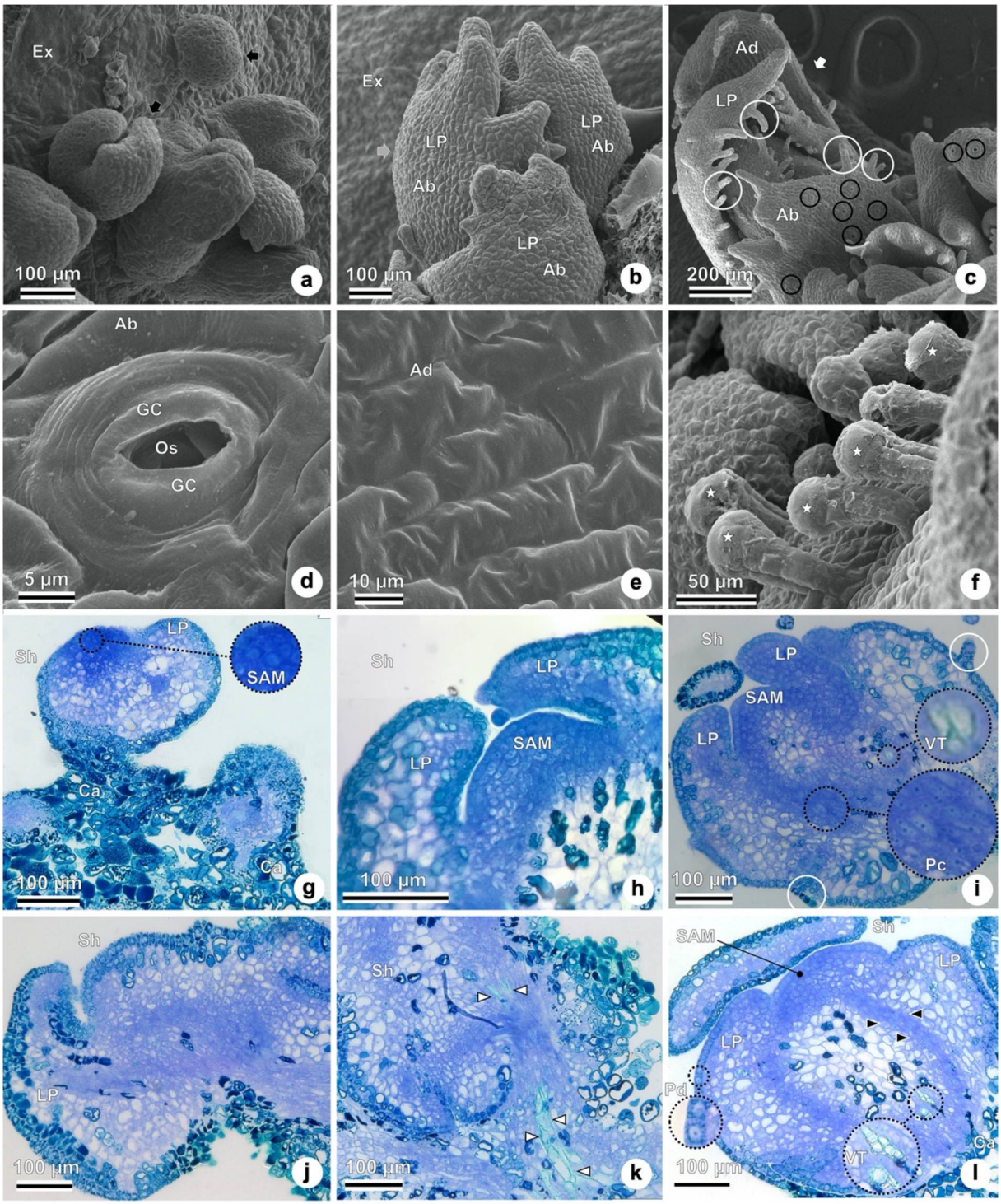

Fig. 6. Details of *in vitro* shoot organogenesis in 'Delite' rabbiteye blueberry using WPM culture medium supplemented with TDZ. Different views of shoots and details under scanning electron microscopy–SEM (a–f) and light microscopy (g–l). (a) Newly formed adventitious small shoots (black arrow) on the surface of the three-week-old leaf explant culture. (b) Adventitious shoot (gray arrow) on a four-week-old leaf explant culture, showing leaf primordia (abaxial side visible). (c) Adventitious shoot (white arrow) on a four-week-old leaf explant culture, showing foliar primordia with stomata (black circle) and trichomes (white circle). Abaxial and adaxial surfaces of the leaf primordia visible. (d) Stomata on the abaxial surface of the leaf primordium: ostiole surrounded by guard cells. (e) Adaxial

surface of the leaf primordium showing no stomata. (f) Trichomes on the leaf primordium (stars). (g) Adventitious shoots at three weeks of culture: shoot apical meristem (in detail), leaf primordium, and callus. (h) Adventitious shoot with leaf primordium and shoot apical meristem at four weeks of culture. (i) Detail of the adventitious shoot, showing shoot apical meristem, leaf primordium, procambium (detail), vascular tissue (detail), and trichomes (white circles) at four weeks of culture. (j) Leaf primordium formation at four weeks of culture. (k) Adventitious shoot showing the formation of vascular tissue (white arrowhead) at four weeks of culture (l) Adventitious shoot with shoot apical meristem, leaf primordia with protoderm (detail), procambium (black arrowhead), vascular tissue (detail) at 4 weeks of culture, and callus tissue. Abbreviations: Ab, abaxial; Ad, adaxial; Ca, callus; Ex, explant; GC, guard cell; LP, leaf primordium; Os, ostiole; Pc, procambium; Pd, protoderm; SAM, shoot apical meristem; Sh, shoot; TDZ, thidiazuron; Tr, trichome; VT, vascular tissue; WPM, woody plant medium.

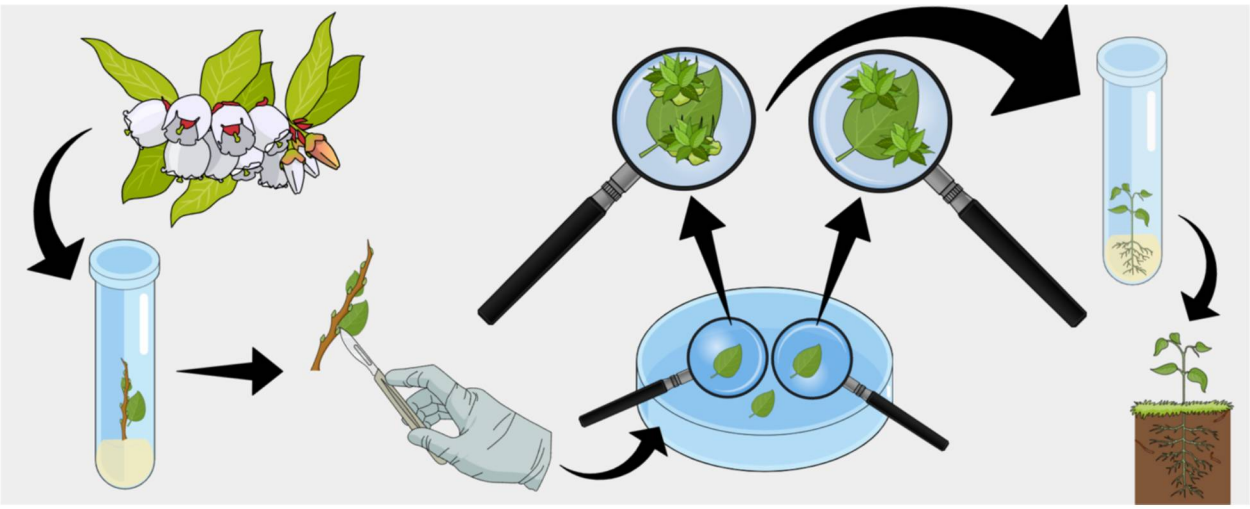

Fig. 7. Diagrammatic representation of *de novo* shoot organogenesis in *in vitro* rabbiteye blueberry from leaf explants.

3 GENERAL CONCLUSIONS

The results presented in this study contribute for establishing protocols of *in vitro* propagation and organogenesis and providing a better understanding of these *in vitro* process in 'Delite' rabbiteye blueberry (*V. virgatum*).

The first part of the research (chapter I) is a study of the effects of different cytokinins in different concentrations and different nitrogen salt ranges on *in vitro* establishment. This initial step is a critical stage in blueberry *in vitro*, usually with low rates of establishment. We could overcome these problems and presented an efficient technique for *in vitro* plant establishment in this species. One of the most important variables is the use of adequate growth regulators and a balance of mineral salts in a suitable concentration. Focusing on an efficient strategy for *in vitro* establishment in 'Delite' rabbiteye blueberry, we recommend the lowest concentration of zeatin tested, 2.5 µM, 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 new shoots of 1.3, with 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 results than the original medium. This initial study with 'Delite' can be the basis for further experiments with different combinations of salts, 2iP, and ZEA.

In chapter II we propose a micropropagation protocol of a rabbiteye cultivar 'Delite' (*V. virgatum*) testing *in vitro* establishment multiplication, rooting, and achieving well-developed and acclimatized plants. The establishment of cultures was achieved using nodal segments with surface sterilization in ethanol (30 min at 70% solution) and sodium hypochlorite (immersion of 5 min in 0.5% solution). Initial cultures can be supplemented with 2.5 µM zeatin in the WPM culture medium, as well as the multiplication stages, also with 2.5 µM zeatin, using nodal segments in the vertical position. Moving to the rooting stage, *in vitro* rooting can be achieved with explants immersion in 500 mg L^{-1} IBA solution, or else, choosing direct *ex vitro* rooting in vermiculite, and irrigation with a solution of WPM mineral salts. This would result in 96.7% of uncontaminated explants with the surface sterilization procedure, 92.3% survival rate in the initial establishment and 84.5% of explants with axillary shoot growth. In the multiplication stage the survival rate was 100%, and 70% of

explants with shoot proliferation, forming one new shoot per explant, with 3.6 cm and 11.7 leaves. Assuming this multiplication rate, this new shoot formed could be divided in 6 new two-node segments, at every 3 months. In one year, a two-node segment would multiply in 1296 new two-node segments. Opting for *in vitro* rooting, we obtained 100% survival of microcuttings, with 100% of maintenance of leaves and 37.5% rooting rate. Or else, opting for *ex vitro* rooting, 88% survival, 86% of microcuttings with leaf maintenance, 50% of microcuttings with new buds induction, but with a higher rooting rate (68%).

The results presented in chapter III contribute to a better understanding of the *in vitro* organogenesis process in 'Delite' rabbiteye blueberry, and developed a high-frequency regeneration method through *de novo* shoot organogenesis, using the addition of 0.5 µM TDZ in WPM medium as an efficient concentration (100% explants with shoot regeneration and 57 adventitious shoots formed per explant); and using the apical or the basal portion of the leaf and the adaxial surface orientation of the leaf touching the medium (97% explants with shoot regeneration and 47.5 adventitious shoots formed per explant). Anatomical analyses showed direct and indirect organogenesis in this cultivar. The adventitious shoots were green and showed the development of shoot apical meristem, vascular tissue, procambium, and leaf primordium with protoderm, glandular trichomes and well-developed stomata. These shoots were well-formed, with organized tissues and cells, with no signs of hyperhydricity. They elongated, grew and developed into rooted plants. Due to the high rate of regenerating explants and to the high number of shoots formed per explant, these results could also be applied to *in vitro* clonal propagation, once the genetic stability is confirmed.

All these results can help to better understand the *in vitro* culture of 'Delite' rabbiteye blueberry, providing basic knowledge for further experiments in rabbiteye blueberry tissue culture, and contributing to the development of *in vitro* plant propagation, germplasm conservation, and other biotechnological studies in blueberries.

4 RECOMMENDATIONS FOR FUTURE RESEARCH

Future studies could be developed aiming to advance and expand the research presented here. In the establishment, the salts composition in the culture medium could be expanded to the multiplication stage.

Some other concentrations of zeatin (lower than 2.5 µM) could be tested during multiplication step, and even other approaches combining zeatin to other cytokinins, auxins and gibberellins, aiming to develop more shoots per explant in the multiplication stage, as well as longer shoots, and trying to reduce the concentrations of zeatin to a minimum and decreasing the costs of the process.

Other studies in bioreactors using 'Delite' blueberry or either rabbiteye cultivars would also be interesting for mass propagation.

Observing the rooting process, we would suggest that the explants were immersed for a longer time in the auxin solution. We used only 10 seconds, and this seemed to be a reduced amount of time for the explant get in contact with the auxin. Or even, we could suggest the use of auxin in powder, instead of the solution, to assure the explant will have the necessary amount of auxin for rooting.

For adventitious regeneration studies, other cytokinins could be tested, to improve the length of shoots. We suggest less time in TDZ medium, moving the explants faster to a fresh medium with different growth regulators, or no growth regulators at all. Other explants could also be tested, such as internodal segments and roots. The morphological and anatomical evaluations could start earlier than three weeks, to investigate the earlier process of organogenesis, including day zero.

The process of organogenesis could have a further investigation on the genetic stability of the new shoots formed, once this uniformity is confirmed, the organogenesis could become an important technique in clonal propagation in this cultivar.

We suggest another study comparing more blueberry cultivars investigating the differences in the organogenesis process, structuring the eventual differences among the cultivars. Some of the research in organogenesis in blueberry is not complete, and only some of them present morphoanatomical evaluations. In some cases, the pictures presented do not show the process in detail.

Also, based on these results in blueberry organogenesis we suggest further evaluation on the somatic embryogenesis pathway of morphogenic development. At

the best of our knowledge, only one research was published evaluating this process in blueberries, and these investigations could offer a variety of options for clonal propagation.

To conclude, a review article could be organized with all the tissue culture research and biotechnological techniques related to *in vitro* culture in blueberries.

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Carolina Smanhotto Schuchovski was born in Curitiba, Brazil, and after studying the elementary, middle and high school at Colégio Nossa Senhora de Sion, she accomplished her undergraduate and Master degree at Universidade Federal do Paraná, in Agronomy - Plant Production, focused on plant biotechnology and morphogenesis and studying blackberry micropropagation under the orientation of Prof. Dr. Luiz Antonio Biasi.

She finished her PhD at Universidade Federal do Paraná, in Agronomy - Plant Production with the orientation of Prof. Dr. Luiz Antonio Biasi. She worked with blueberry (*Vaccinium virgatum*) *in vitro* culture on her thesis and most of her research was focused on the steps of micropropagation and *de novo* shoot organogenesis in blueberries, studying a protocol that could contribute to a better development of this species *in vitro*. Part of her studies was developed in morphoanatomical analyses of the *in vitro* organogenesis, using light and scanning electron microscopy.

Another research during the PhD was developed in field-grown blueberry plants, evaluating the dynamics of the floral buds dormancy in four blueberry cultivars, evaluating the chilling requirement for these crops in mild winter climate.

During six months of her PhD, she developed an international experience during a doctoral internship at the Department of Horticulture and Crop Science at The Ohio State University (USA), under the supervision of Prof. Dr. Jonathan Fresnedo-Ramírez. There, she developed a research studying the floral and inflorescence development in rubber dandelion (*Taraxacum kok-saghyz* - TK), an important renewable alternative crop to produce natural rubber. The floral organogenesis and the transition from the vegetative meristem to the inflorescence meristem were described using scanning electron microscopy.