UNIVERSIDADE FEDERAL DE LAVRAS

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MICROPROPAGATION OF BLUEBERRY: STRATEGIES TO IMPROVE THE PRODUCTION OF SEEDLINGS OF THE SPECIES

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Agronomia/Fitotecnia, área de concentração em Produção Vegetal, para obtenção do título de Doutor.

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MICROPROPAGAÇÃO DE MIRTILEIRO: ESTRATÉGIAS VISANDO MELHORAR A PRODUÇÃO DE MUDAS

Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Agronomia/Fitotecnia, área de concentração em Produção Vegetal, para obtenção do título de Doutor.

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“Combati o bom combate, terminei a corrida, guardei a fé.” (Timóteo, 4:7)

RESUMO

O mirtilo (*Vaccinium* spp.) é uma frutífera da família *Ericaceae*. Nos últimos anos essa cultura vem se destacando, principalmente pelas características nutricionais, que despertam interesse da população. Sua propagação é realizada por meio de estacas, porém sua produção *in vitro* tem sido mais eficiente. Objetivou-se com este trabalho desenvolver alternativas e estratégias para melhorar as fases de propagação do mirtilo *in vitro*, a multiplicação, enraizamento até a aclimatização. Três cultivares da espécie (Bluegem, Climax e Woodard) foram submetidas a tratamentos com reguladores de crescimento nas fases de multiplicação e enraizamento, e a vedações distintas na fase de aclimatização. O comportamento das cultivares foi diferente para cada um dos parâmetros analisados (morfométricos e fisiológicos) e em cada uma das fases. Na fase de multiplicação houve interação entre as concentrações de sulfato de adenina e 2-ip, sendo observado aumento do número de rebentos e plantas mais uniformes favorecendo as fases subsequentes do processo de micropropagação. A inoculação inicial das microestacas com IBA e as concentrações do meio WPM, com ou sem carvão ativado, afetaram diretamente a fase de enraizamento das plantas de mirtilo. A cultivar Bluegem foi a que melhor respondeu aos tratamentos. A presença de carvão ativado no meio de cultura só foi necessária para a cultivar Climax. Em relação à percentagem de meio WPM, é possível determinar que 75% foi o melhor para a cultivar Bluegem, para as outras os resultados foram ainda muito variáveis, não sendo possível determinar uma concentração única. A utilização de diferentes tipos de vedações propiciou resultados distintos entre as cultivares, o uso de tampas com filtro melhorou o processo de micropropagação e aclimatização de mirtilo da cultivar Climax e uma maior sobrevivência das plantas da cultivar Bluegem, mas na cultivar Woodard o sistema não propiciou acréscimos relevantes.

Palavras-chave: Pequenos frutos. Cultivo *in vitro*. Reguladores de crescimento.*Vaccinium* spp.

ABSTRACT

The blueberry (*Vaccinium* spp.) is a fruit belonging to the Ericaceae family. In recent years, this crop has been gaining more and more prominence, mainly due to its nutritional characteristics, which have aroused public interest. It is often propagated by cuttings, but *in vitro* cultivation is proving to be more efficient. The aim was therefore to find alternatives and strategies to improve the propagation phases of blueberries *in vitro*, from multiplication to acclimatization. To this end, three cultivars of the species were used: Bluegem, Climax and Woodard, which were subjected to different treatments with growth regulators (multiplication and rooting phase) and different seals (acclimatization phase). As expected, the behavior of the cultivars was different in each of the parameters analyzed (morphometric and physiological) and in each of the phases, reinforcing the idea that each requires a different micropropagation protocol. In the multiplication phase, the interaction between the concentrations of adenine sulphate and 2-ip led to an increase in the number of shoots and more uniform plants, favoring the subsequent phases of the micropropagation process. The initial inoculation of the microcuttings with IBA and the concentrations of WPM medium, with or without activated charcoal, directly affected the rooting phase of the blueberry plants. The Bluegem cultivar responded best to the treatments. The presence of activated charcoal in the culture medium was only necessary for the Climax cultivar. With regard to the percentage of WPM medium, it can be determined that 75% was the best for the Bluegem cultivar; for the others, the results were still very variable and it was not possible to determine a single concentration. The use of different types of sealing provided different results between the cultivars. The use of filter lids improved the micropropagation and acclimatization process of blueberries of the Climax cultivar and greater survival of the plants of the Bluegem cultivar, but in the Woodard cultivar the system did not provide relevant increases.

Keywords: Small fruits. *In vitro* cultivation. Growth regulators. *Vaccinium* spp.

IMPACTOS SOCIAIS, TECNOLÓGICOS, ECONÔMICOS E CULTURAIS

A cultura de tecidos vegetais é uma ferramenta baseada no cultivo de células, tecidos, órgãos ou partes isoladas de uma planta matriz (explante) em meios artificiais sob condições totalmente assépticas e controladas (*in vitro*). Essa técnica possui uma aplicação indispensável tanto na agricultura quanto na indústria, fornecendo plantas necessárias para atender à crescente demanda mundial. Várias espécies são tradicionalmente produzidas através das técnicas de cultura de tecidos vegetais, como a banana, o morango, espécies ornamentais (orquídeas) e florestais (eucalipto), buscando atingir uma rápida frutificação, um número maior de mudas em um pequeno espaço de tempo e manter a identidade genética da planta. O mirtilo é uma espécie que vem ganhando notoriedade entre a população mundial principalmente por suas características nutricionais e benefícios que pode trazer para a saúde humana, possuindo altos níveis de compostos antioxidantes como flavonoides, fenólicos e taninos, carotenoides e antocianinas, vitaminas como ácido ascórbico e minerais. Diante disso, é necessário a busca por estratégias visando um aumento da produção dessa cultura e a expansão de sua área de produção e para tal, é necessário que haja uma produção de mudas de qualidade capaz de suprir a demanda global. A micropropagação se apresenta como uma ferramenta tecnológica extremamente importante para a expansão da cultura, produzindo mudas de qualidade, livre de vírus, de forma massal e durante o ano todo. Por isso, estudos visando melhorar a produção de mudas da cultura se tornam necessários, uma vez que esse fruto mostra um grande potencial para auxiliar nutricionalmente a população.

SOCIAL, TECHNOLOGICAL, ECONOMIC AND CULTURAL IMPACTS

Plant tissue culture is a tool based on the cultivation of cells, tissues, organs or isolated parts of a parent plant (explant) in artificial media under completely aseptic and controlled conditions (*in vitro*). This technique has an indispensable application in both agriculture and industry, providing the plants needed to meet the world's growing demand. Several species are traditionally produced using plant tissue culture techniques, such as bananas, strawberries, ornamental species (orchids) and forestry species (eucalyptus), with the aim of achieving rapid fruiting, a greater number of seedlings in a short space of time and maintaining the plant's genetic identity. The blueberry is a species that has been gaining notoriety among the world's population mainly due to its nutritional characteristics and the benefits it can bring to human health. It has high levels of antioxidant compounds such as flavonoids, phenolics and tannins, carotenoids and anthocyanins, vitamins such as ascorbic acid and minerals. In view of this, it is necessary to look for strategies to increase the production of this crop and expand its production area. To do this, it is necessary to produce quality seedlings capable of meeting global demand. Micropropagation is an extremely important technological tool for expanding the crop, producing quality, virus-free seedlings on a mass scale throughout the year. For this reason, studies aimed at improving the production of seedlings of this crop are necessary, since this fruit shows great potential for providing nutritional support to the population.

SUMÁRIO

PRIMEIRA PARTE

1 INTRODUÇÃO

Frutas vermelhas, mais precisamente o mirtilo, vem ganhando destaque mundial, isso ocorre principalmente pelas características nutricionais da cultura que vem despertando interesse da população por ser rica em nutrientes, vitaminas e minerais. Os Estados Unidos lideram a produção mundial da espécie, seguido do Canada, Chile e Peru, que juntos são responsáveis por 75% da área plantada no mundo (PÉREZ *et al.*, 2022).

A propagação do mirtilo é feita de forma vegetativa, por estacas. Neste formato há desafios como o enraizamento das estacas. O cultivo *in vitro* pode ser uma alternativa para a reprodução dessa espécie de forma mais rápida e em grande quantidade, além de melhorar a capacidade de enraizamento e o rendimento da cultura (WANG *et al.*, 2023). No entanto, os processos que envolvem a propagação diferem de acordo com as características de cada cultivar e cada uma necessita de um protocolo adequado para desenvolver melhor (LE *et al.*, 2023).

Existe, na literatura, estudos sobre a micropropação do mirtilo, porém há grande variação em relação aos meios de cultivo, reguladores de crescimento e condições adequadas para o crescimento e desenvolvimento da espécie (FIGIEL-KROCZYŃSKA *et al.*, 2022). Nas fases de enraizamento e aclimatização também há desafios a serem enfrentados, pois o sucesso da micropropagação está diretamente relacionado ao desenvolvimento radicular e aéreo que afetam a sobrevivência das plantas na aclimatização (TEJADA-ALVARADO *et al.*, 2022).

O objetivo deste trabalho foi avaliar alternativas para melhorar os processos de micropropagação da cultura do mirtilo em três cultivares (Bluegem, Climax e Woodard), desde a sua fase de multiplicação até a aclimatização, a fim de obter mudas em maior quantidade e qualidade comercial.

2 REFERENCIAL TEÓRICO

2.1 A Cultura do Mirtilo

O gênero *Vaccinium* tem cerca de 450 espécies vegetais, a maioria se localiza em ambientes declivosos nos trópicos, distribui-se em regiões subtropicais, temperadas e boreais predominantemente no hemisfério norte (WANG, 2015). Algumas espécies pertencentes à esse gênero são cranberry (*V. macrocarpon* Ait.), mirtilo (*Vaccinium* spp.) e lingonberry (*V. vitisidaea* L.), domesticadas no século XX e com grande importância econômica. As plantas podem ser de hábito epífito ou terrestre, encontradas em solos ácidos, arenosos, turfos ou com muita matéria orgânica (DEBNATH, 2007a).

O mirtilo tem como centro de origem a Europa e a América do Norte, nesses locais é onde essa espécie possuem maior mercado (TÂNIA *et al*., 2011), sendo uma planta importante nas industrias frutíferas dos Estados Unidos e Canadá (DEBNATH; GOYALI, 2020).Apesar de algumas espécies de mirtilo como highhbush (*V. corymbosum* L.), lowbush (*V. angustifolium* Ait.) e rabbiteye (*V. ashe* Reade) serem originárias da América do Norte, elas também são muito comercializadas em países da Europa, América do Sul, Ásia, Austrália e Nova Zelândia (STRIK; YARBOROUGH, 2005).

Os frutos de mirtilo são do tipo baga, carnudos, de pequeno a médio, com altos níveis de compostos antioxidantes como flavonoides, fenólicos e taninos, corantes de frutos como carotenoides e antocianinas, vitaminas como ácido ascórbico e minerais (SKROVANKOVA *et al*., 2015).

A reprodução do mirtilo acontece por vias sexuadas através de sementes ou assexuada através de estacas de caules ou raízes. Em viveiros predomina a propagação por estacas, sendo um processo fácil de ser realizado, mas demorado para ser feito em larga escala. A vantagem da estaquia é a passagem das características genéticas herdadas da planta mãe, ou seja, um clone que preserva as características consideradas vantajosas da planta matriz. As sementes são utilizadas em menor escala por ter como desvantagem a variabilidade genética (DEBNATH, 2007b; DEBNATH; GOYALI, 2020).Além do uso de sementes, outros fatores que são limitantes para a produção de mirtilio são as técnicas para o sistema de produção, dificuldade nutricional, sobrevivência das estacas pós enraizamento e métodos para rejuvenescimento de plantas adultas (TOMAZ *et al*., 2014).

2.2 Micropropagação

Os primeiros relatos quanto a pesquisas com a cultura de tecidos vegetais foi realizado por Gottlieb Haberlandt, utilizando a cultura de células individuais e a capacidade de totipotência dessas células (HABERLANDT, 1902). Muitas pesquisas foram desenvolvidas a partir desta descoberta, até que Murashige e Skoog (1962) descreveram um meio de cultura que é o mais utilizado no cultivo *in vitro* de plantas, sendo capaz de fornecer os nutrientes essênciais para o desenvolvimento de grande número de espécies (CARDOSO *et al*., 2018). Com o passar dos anos a cultura de tecidos se tornou uma técnica muito importante para a propagação de plantas que não conseguem se propagar bem da forma convencional, seja por sementes ou vegetativamente (BAHADUR; KRISHNAMURTHY, 2015).

A micropropagação ou propagação *in vitro*, é realizada em ambiente altamente controlado, em condições assépticas e com a inoculação de explantes de plantas que podem ser tecidos, células ou órgãos. O local utilizado para a propagação desses explantes é denominado meio de cultivo, ou seja, um meio artificial que possui macronutientes, micronutrientes, água, fonte de carbono como sacarose e glucose, vitaminas, agente geleificante e, em alguns casos, reguladores de crescimento como auxinas, citocininas e giberelinas, todos usados para o crescimento da planta (DEBNATH; MCRAE, 2021).

A propagação *in vitro* se baseia no aumento de brotos axilares e na capacidade que as células vegetais possuem de se regenerar e criar novos centros meristemáticos que originam novas plantas normais e com características semelhantes a planta matriz (DEBNATH; MCRAE, 2001). A regeneração dessas plantas pode ser feita através de três vias: (i) proliferação de rebentos axilares a partir de gemas apicais, (ii) organogênese, através da regeneração de órgãos ou de novas plantas e (iii) embriogênese somática, através de embriões somáticos (DEBNATH, 2007b).

Comparado à outros métodos de propagação a cultura de tecidos tem vantagens como o fornecimento de mudas de forma rápida e massal, geneticamente idênticas as plantas matrizes e livres de patógenos, rapidez para fornecer mudas para melhoramento genético, produção durante todo o ano, além da conservação de germoplasma (RANI; RAINA, 2000; BAHADUR; KRISHNAMURTHY, 2015). As limitações dessa forma de propagação são o alto investimento na construção de instalações, aquisição de maquinários, reagentes e necessidade de mão de obra especializada (DEBNATH, 2007a). Com relação ao sistema as limitações são a possível ocorrência de mutações, infecções internas, perdas nas fases de transferência do cultivo *in vitro* para a aclimatização, vitrificação, oxidação através da liberação de exsudados tóxicos, entre outros (LOBERANT; ALTMAN, 2010).

A cultura de tecidos pode ser usada em diferentes áreas da agricultura e silvicultura, ajudando na propagação de plantas ornamentais, medicinais, frutíferas, olerícolas, florestais entre outras. Além disso a micropropagação se divide nos seguintes etágios: (i) Estágio 0: fase preparativa ou condicionamento da planta matiz, (ii) Estágio I: estabelecimento do cultivo *in vitro*, (iii) Estágio II: multiplicação do material vegetal, (iv) Estágio III: alongamento e enraizamento e (v) Estágio IV: aclimatização (BAHADUR; KRISHNAMURTHY, 2015).

A expansão da cultura do mirtilo poderá ocorrer caso a produção de mudas seja mais eficiente e isso pode acontecer através do aprimoramento de técnicas de micropropagação, acelerando o processo de produção de mudas (ARRUDA *et al.*, 2017). A fase mais crítica no cultivo *in vitro*, para o mirtilo, é a fase de estabelecimento *in vitro* do material propagado, sendo necessários mais estudos afim de determinar metodologias para o estabelecimento *in vitro* dessa espécie (SCHUCH; TOMAZ, 2019).

2.3 Reguladores de Crescimento Vegetal

Originário do grego o termo hormônio significa “estimular”. Os hormônios vegetais também conhecidos como fitohormônios, atuam com fatores externos, no processo de crescimento e diferenciação, e ajudam no desenvolvimento da planta, no transporte vegetal e na atividade metabólica (BOTIN; CARVALHO, 2015).

No cultivo *in vitro* são utilizadas algumas substâncias conhecidas como reguladores de crescimento que ajudam a controlar a produção de rebentos e raízes, através dos níveis hormonais produzidos internamente nas plantas (KUMARI *et al.*, 2018). Os hormônios vegetais ocorrem naturalmente nas plantas e os reguladores de crescimento são seus análogos sintéticos. De uma forma geral os reguladores de crescimento são utilizados para proporcionar respostas fisiológicas, explorar os mecanismos de controle bioquímico, controlar processos de crescimento, germinação, senescência, enraizamento, maturação, pós colheita e também para estimular a produção de metabolitos secundários (BASRA, 2000; JAMWAL *et al.*, 2018).

O equilíbrio existente entre os reguladores de crescimento e as fases que ocorrem a aplicação dos mesmos *in vitro*, são responsáveis pela regulação da desdiferenciação do tecido vegetal em calos, na formação de novas raízes, brotações, na embriogenese somática e multiplicação (LOBERANT; ALTMAN, 2010).

Os principais reguladores de crescimento usados nas plantas são a citocinina e a auxina. As citocininas são substâncias que contribuem para o crescimento das plantas por estimular a divisão celular, além disso, ajudam a regular a síntese de proteínas, inibem o crescimento da parte aérea e do sistema radicular. As principais citocininas sintéticas são o BAP (6-Benzilaminopurina), 2ip (N6-D2-isopentenil adenina) e o TDZ (thiadizurom (N-fenil-N-1,2,3-tidiazol-5-tiuréia)) (CAMARGO *et al*., 2007). As auxinas são responsáveis por estimular a divisão, diferenciação e alongamento celular, além disso, são responsáveis pela dominância apical. As auxinas mais utilizadas são o ANA (ácido naftaleno acético), AIA (ácido indol acético) e AIB (ácido indol butírico) (GOELZER *et al*., 2019). As giberelinas, GA3 (ácido giberélico), o etileno, os jasmonatos como o metil jasmonato e o ácido jasmônico, são outros reguladores de crescimento que podem ser utilizados (JAMWAL *et al*., 2018).

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SEGUNDA PARTE – ARTIGOS*

ARTICLE 1 - *In vitro* MULTIPLICATION OF BLUEBERRY: EFFECT OF 2-ISOPENTENYLADENINE AND ADENINE SULFATE

ABSTRACT

Blueberry is a fruit belonging to the Ericaceae family that can be propagated by cuttings or *in vitro* cultivation. Therefore, the objective of this work was to improve the *in vitro* multiplication phase of the culture with the aim of providing greater uniformity and number of shoots, aiming at mass propagation, as well as obtaining better quality materials. For this purpose, stem segments of blueberries (1.5 cm long) were transferred to test tubes containing 15 mL of WPM (Wood Plant Medium) with concentrations of 2-ip (0 and 2.5 mg L^{-1}) and adenine sulfate (0. 10, 20, and 40 mg L^{-1}) that remained in the growth room. After 60 days, phytotechnical analyses, photosynthetic pigment content, water activity X, and flow cytometry were performed. Three experiments were carried out, one for each cultivar (Bluegem, Climax, and Woodard). The experimental design was a 2 x 4 factorial scheme with 20 replicates for each treatment. A significant difference was observed between treatments in all parameters analyzed for the three cultivars. Treatment with 2.5 mg L^{-1} 2-ip and 10 mg L^{-1} adenine sulfate provided a greater number of shoots in Bluegem and Climax cultivars. The treatment with 2.5 mg L^{-1} 2-ip and 20 mg L^{-1} adenine sulfate allowed for a greater number of shoots in the Woodard cultivar. The interaction between the concentrations of adenine sulfate and 2-ip, regardless of the blueberry cultivars analyzed, led to an increase in the number of shoots as well as more uniform plants, favoring the subsequent stages of the micropropagation process.

Keywords: Micropropagation. *Vaccinium* spp. Regulators. Seedlings

Abbreviations: cv: cultivar, CV: Coefficient of variation, 2-ip: 2-isopentenyladenine, WPM: Woody Plant Medium.

1 INTRODUCTION

The blueberry is a small fruit belonging to the Ericaceae family and the genus *Vaccinium*. The centers of origin are in Europe and North America, places where the fruits have greater economic appeal (TÂNIA *et al.*, 2011). Due to its great nutritional potential, the blueberry has gained considerable prominence over the years, which is why world production has been expanding rapidly (FAN *et al.*, 2017).

Blueberries are normally propagated vegetatively by means of cuttings. However, this technique has not proven to be a very effective method since it is not possible to quickly increase the number of propagated materials, making it impossible to introduce new commercial cultivars to the market (FAN *et al.*, 2017).

However, as a way to solve this problem, in vitro propagation or micropropagation can be used, as it allows obtaining a new regenerated plant that is identical to the matrix plant, all under aseptic conditions (DEBNATH; GOYALI, 2020). In the case of blueberries, micropropagation is used to obtain virus-free plants as well as propagated throughout the year. In addition, many fruit trees have been propagated through tissue culture techniques, which allow the maintenance of the genetic identity of the material as well as its rapid fruiting (AHMAD; FAISAL, 2018).

Aiming at improving the blueberry *in vitro* multiplication phase, the culture medium can be incremented with growth regulators, such as adenine sulfate and 2-isopentenyladenine, which are responsible for determining the *in vitro* plant growth process. However, the concentration used and the balance between the regulators are what determine the development of the aerial part of the plant, roots or callus, but may vary according to the plant species (SUMAN, 2017).

Therefore, the objective of this work was to improve the *in vitro* multiplication phase of the culture, with the aim of providing greater uniformity and a greater number of shoots, aiming at mass propagation, as well as obtaining better quality materials.

2 MATERIALS AND METHODS

2.1 Experiment location

The experiment was carried out at the Plant Tissue Culture Laboratory of the Department of Agriculture (DAG) of the Federal University of Lavras (UFLA), Lavras, Minas Gerais, Brazil (44º57’50’’ W, 21º13’40’’ S, 919 m altitude).

2.2 Obtaining the material

Blueberry plants, cultivars Bluegem, Climax, and Woodard, already established *in vitro*, were transferred four times in WPM (LLOYD; MCCOWN, 1980), with 2.5 mg L^{-1} 2-ip, at intervals of 30 days. The material was kept in a growth room with artificial lighting provided by white LED lamps and a mean irradiance of 49.4 µmol m^2 s^{-1}, photoperiod of 16 hours, and temperature of 25 ± 2 ºC.

2.3 Installation of the experiment

Stem segments of the three blueberry cultivars, approximately 1.5 cm long, were transferred to tubes (x) containing 15 mL of WPM medium containing concentrations of 2-ip (0 and 2.5 mg L^{-1}) and adenine sulfate (0, 10, 20, and 40 mg L^{-1}). The material was kept in a growth room with the same characteristics previously mentioned for 60 days.

2.4 Analysis

2.4.1 Morphometric parameters

The shoot length, internode length, number of leaves, leaf length, number of shoots, shoot length and fresh and dry mass of plants were evaluated using 12 samples. To measure the shoot length, internode, leaves and shoots, a digital caliper was used. A precision analytical balance was used to weigh fresh and dry mass. Subsequently, the plants were taken to a forced air circulation oven at a temperature of 40 °C for 48 h.

2.4.2 Photosynthetic pigments

Fresh leaves (0.025 g) of blueberries were transferred to Falcon tubes containing 5 mL of 80% acetone for photosynthetic pigment extraction. The tubes were wrapped in aluminum foil in order to avoid contact of the sample with light and possible degradation of chlorophyll. After 24 h in a refrigerator at ± 4 °C, the absorptivity of the samples wasmeasured in an Elisa Multiskan GO spectrophotometer (Thermo Fisher Scientific) at wavelengths of 470, 645, 652, and 663 nm (SCOPEL *et al.*, 2011). The analysis wasperformed in triplicate, with four replicates per treatment, using Skanit Software 5.0 for Microplate Readers RE version 5.0.0.42. Chlorophylls *a*, *b*, total, and carotenoids, were determine by equations (LI; TANG AND; XU, 2013).

2.4.3 Relative water content

For the evaluation of the relative water content (RWC), two leaf discs with an area of 28.27 mm^2 each were extracted from the two young and completely expanded leaves. These disks were weighed to quantify the fresh mass (FM). The discs were immersed for

24 hours in tubes containing deionized water. After this period, the disks were weighed again to determine the turgid mass (TM). The disks were dried in an oven with forced air circulation at 70 °C for 48 hours. Subsequently, the disks were weighed to obtain the dry mass (DM). For the evaluation of TRA, six replicates were used, with each plant corresponding to one. The RWC was calculated according to the equation proposed by Barrs and Weatherley (1962) (Equation 1):

$$\mathrm{RWC}\ (\%) = \frac{(\mathrm{FM}-\mathrm{DM})}{(\mathrm{TM}-\mathrm{DM})} \times 100 \quad \text{(Equation 1)}$$

Being:
RWC = Relative water content (%)
FM = Fresh mass (g)
MT = Turgid mass (g)
DM = Dry mass (g)

2.4.4 Extravasation of electrolytes

Six leaf discs with an area of 28.27 mm^2 were taken from the young and completely expanded leaves, and placed in a test tube containing 15 mL of deionized water, for a total of six tubes per treatment. The tubes were placed on a shaking table for 24 hours at room temperature (25 ± 2 ºC). After this period, the free electrical conductivity (FEC) of the solution was measured. The tubes were placed in a bain-marie for 1 hour at 100 °C to measure the total electrical conductivity (TEC) of the solution. The determination of the extravasation rate of electrolytes was calculated based on the equation 2 (SHI *et al.*, 2006):

$$\mathrm{EE}(\%) = \frac{\mathrm{FEC}}{\mathrm{TEC}} \times 100 \quad \text{(Equation 2)}$$

Being:
EE = Extravasation of eletrolytes (%)
FEC = Free electrical conductivity (dS m^{-1})
TEC = Total electrical conductivity (dS m^{-1})

2.4.5 Membrane integrity

Membrane integrity (MI) was estimated through extravasation of electrolyte, measured by a conductivity meter. Six leaf discs with an area of 28.27 mm^2 of fully expanded leaves were used.

The disks were kept for 24 hours in tubes immersed in 30 mL of deionized water to measure the free electrical conductivity (FEC), and after 1 hour in a water bath at 100 °C, the total electrical conductivity (TEC) was measured.

Membrane integrity was estimated using equation 3 (AZEVEDO *et al.*, 2008):

$$\mathrm{MI}(\%) = \left(1 - \left(\frac{\mathrm{FEC}}{\mathrm{TEC}}\right)\right) \times 100 \quad \text{(Equation 3)}$$

Being:

MI = membrane integrity (%)

FEC = free electrical conductivity (dS m^{-1})

TEC = total electrical conductivity (dS m^{-1}).

2.4.6 Flow cytometry

For the determination of DNA content, samples containing 50 mg of blueberry leaf tissue were minced with a scalpel in a Petri dish containing 1 mL of Marie buffer (MARIE; BROWN, 1993) for nuclei extraction. The samples were aspirated using a Pasteur pipette and filtered through a 50 µm mesh. The suspension was stained with 25 µL of propidium iodide solution (1 mg mL^{-1}). In each sample, at least 10,000 nuclei were analyzed for fluorescence emission. Samples were read in triplicate.

The analysis was performed using a BD FACSCalibur 4-color flow cytometer (Becton Dickinson). Histograms were obtained and analyzed using Cell Quest software.

The reference standard used was the species *Vicia faba* cv. Inovec (26.90 pg). Nuclear DNA content (pg) was estimated using the equation: DNA content (pg) = (sample G1 peak position/*V. faba* G1 peak position) x 26.90.

2.5 Experimental design and statistical analysis

The experiment was installed separately using three cultivars (Bluegem, Climax, and Woodard) in a 2 x 4 factorial scheme with 20 replicates for each treatment, using two concentrations of 2-ip (0 and 2.5 mg L^{-1}) and four concentrations of adenine sulfate (0, 10, 20, and 40 mg L^{-1}).

The data were submitted to analysis of variance and, subsequently, to the Scott-Knott test at 5% probability using the R studio software.

3 Results

Plants of blueberry cultivars Bluegem (Figure 1a), Climax (Figure 1b) and Woodard (Figure 1c) at different concentrations of 2-ip and adenine sulfate.

Figure 1- Plants of blueberry cultivars Bluegem (a), Climax (b), and Woodard (c) under the effect of different concentrations of 2-ip and adenine sulfate.

Source: From the author (2024).

3.1 Morphometric parameters

A significant difference was observed between treatments for Bluegem (Table 2), Climax (Table 3), and Woodard (Table 4) cultivars.

3.1.1 Bluegem cultivar

Regarding the Bluegem cultivar, there was a significant difference between the treatments for plant length, internode length, and number of shoots (Table 2). A positive highlight occurred for the treatments involving the lowest concentrations of adenine sulfate for shoot and internode lengths. However, there was no significant difference when using 2-ip. As for the number of shoots, there was a positive highlight for treatments T6, T7, and T8. For number of leaves, length of leaves and shoots, and fresh and dry mass, no significant difference was observed between treatments.

Table 2 – Plant length (PL, mm), internode length (IL, mm), leaf number (LN), leaf length (LL, mm), number of shoots (NS), shoot length (SL, mm), fresh (FM, g) and dry mass (DM, g) of blueberry plants cv. Bluegem in different concentrations of 2-ip and adenine sulfate

Treatments	PL (mm)	IL (mm)	LN	LL (mm)	NS	SL (mm)	FM (g)	DM (g)
T1	57.89 a	4.25 a	18.25 a	6.22 a	0.00 c	0.00 b	0.09 a	0.02 b
T2	53.29 a	4.05 a	19.25 a	5.97 a	0.00 c	0.00 b	0.08 a	0.02 b
T3	46.17 a	3.82 a	17.25 a	6.03 a	0.00 c	0.00 b	0.08 a	0.02 b
T4	36.66 b	3.45 b	13.75 a	6.12 a	0.00 c	0.00 b	0.11 a	0.01 b
T5	33.87 b	3.01 b	17.25 a	4.17 a	2.75 b	19.73 a	0.12 a	0.02 b
T6	36.46 b	3.01 b	16.08 a	4.88 a	4.25 a	17.54 a	0.16 a	0.03 a
T7	31.74 b	2.42 c	17.58 a	3.59 a	3.66 a	13.72 a	0.14 a	0.03 a
T8	36.10 b	3.23 b	19.33 a	4.00 a	3.41 a	16.37 a	0.18 a	0.03 a
Means	41.52	3.40	17.34	5.12	1.84	16.84	0.12	0.02
CV (%)	32.76	24.29	27.72	45.66	27.72	35.33	37.82	42.95

*Means followed by the same letters in column do not differ according to the Scott-Knott test at 5% probability.

Source: From the author (2024).

3.1.2 Climax cultivar

For the Climax cultivar, no significant difference was observed for plant length , internode length, leaf length, number of shoots, shoot length and fresh mass (Table 3). However, a positive highlight was observed for treatments T2, T3, and T4 for shoot length; T3 for internode size; and T1, T2, and T3 for leaf length. Greater increments in the number of shoots were observed in treatments T6 and T7. For shoot length, better results occurred in treatments T2, T3, and T4. While in the T6 treatment, the greatest increment in dry mass was observed. However, it is noteworthy that there was no significant difference between treatments for number of leaves and dry mass.

Table 3 – Plant length, internode, leaf number, leaf length, number of shoots, shoot length, fresh mass and dry mass of the Climax cultivar in different treatments.

Treatments	Plant length (mm)	Internode (mm)	Leaf number	Leaf length (mm)	Number of shoots	Shoot length (mm)	Fresh mass (g)	Dry mass (g)
T1	27.29 a	2.65 b	11.50 a	6.39 a	0.83 c	15.03 b	0.07 b	0.02 a
T2	27.35 a	3.38 b	12.00 a	6.44 a	0.75 c	21.49 a	0.09 b	0.02 a
T3	30.99 a	4.55 a	10.75 a	6.51 a	1.00 c	18.00 a	0.08 b	0.02 a
T4	29.16 a	3.31 b	11.58 a	5.14 b	1.75 c	18.74 a	0.07 b	0.01 a
T5	19.94 c	2.84 b	10.00 a	3.01 c	3.25 b	12.79 b	0.06 b	0.01 a
T6	22.96 b	2.84 b	10.25 a	4.04 c	5.83 a	12.17 b	0.15 a	0.01 a
T7	23.46 b	3.33 b	10.91 a	3.89 c	4.91 a	13.06 b	0.10 b	0.02 a
T8	24.11 b	3.14 b	12.00 a	4.00 c	3.16 b	14.03 b	0.10 b	0.02 a
Means	25.65	3.25	11.12	4.92	2.68	15.66	0.09	0.01
CV (%)	15.60	27.58	18.41	24.67	43.12	31.52	45.58	47.34

*Means followed by the same letters in column do not differ according to the Scott-Knott test at 5% probability.

Source: From the author (2024).

3.1.3 Woodard cultivar

For the Woodard cultivar, a significant difference between treatments was observed for internode length, leaf length, number of shoots, shoot length, and fresh and dry mass (Table 4). However, there was no significant difference in shoot length and leaf number. Treatments T1 and T2 provided the best results for internode length. Treatments T1, T2, T3, and T4 were responsible for the greatest leaf lengths. A greater increase in the number of shoots was observed in the T7 treatment. Greater shoot lengths were obtained in treatments T1 and T4. A greater accumulation of fresh mass was observed in treatment T7, while for dry mass, the accumulation was greater in treatments T7 and T8.

Table 4 – Plant length, internode, leaf number, leaf length, number of shoots, shoot length,

fresh and dry mass of the Woodard cultivar in different treatments.

Treatments	Plant length (mm)		Internode (mm)		Leaf number		Leaf length (mm)		Number of shoots		Shoot length (mm)		Fresh mass (g)		Dry mass (g)	
T1	25.99	a	3.35	a	14.33	a	4.84	a	0.33	c	17.36	a	0.05	b	0.01	b
T2	28.31	a	3.16	a	13.58	a	5.18	a	1.16	c	12.20	b	0.08	b	0.02	b
T3	23.37	a	2.45	b	12.58	a	5.68	a	0.08	c	1.00	c	0.05	b	0.01	b
T4	27.82	a	2.63	b	14.75	a	4.95	a	0.50	c	18.28	a	0.07	b	0.01	b
T5	23.94	a	2.14	b	15.33	a	3.79	b	1.91	b	6.62	b	0.08	b	0.01	b
T6	20.31	a	2.22	b	14.83	a	3.03	b	2.75	b	9.92	b	0.08	b	0.01	b
T7	23.70	a	2.15	b	12.75	a	3.72	b	6.16	a	11.06	b	0.23	a	0.03	a
T8	27.29	a	2.09	b	17.58	a	4.21	b	2.33	b	12.45	b	0.12	b	0.02	a
Means	25.09		2.52		14.46		4.42		1.90		12.55		0.09		0.01	
CV (%)	28.36		26.53		25.97		26.78		59.56		33.53		43.19		39.52	

*Means followed by the same letters in column do not differ according to the Scott-Knott test at 5% probability.

Source: From the author (2024).

3.2 Photosynthetic pigments

The content of photosynthetic pigments clorophylls *a*, *b*, total and carotenoids were differenteamong treatments, regardless of the cultivar (Table 5).

Table 5 – Chlorophyll *a* (CHLa), *b* (CHLb), total (CHLt) and carotenoids (Car) of blueberry plants cvs. Bluegem, Climax and Woodard in different concentrations of 2-ip and adenine sulfate.

Cultivars	Treatments	CHLa	CHLb	CHLt	Car
		------------------------------------- $mg.g^{-1}$			-------------------------------------
Bluegem	T1	0.26 c	0.21 c	0.48 c	0.10 d
	T2	0.52 a	0.42 a	0.94 a	0.16 b
	T3	0.37 b	0.29 b	0.67 b	0.13 c
	T4	0.58 a	0.46 a	1.05 a	0.20 a
	T5	0.25 c	0.21 c	0.47 c	0.10 d
	T6	0.34 b	0.30 b	0.65 b	0.14 c
	T7	0.24 c	0.21 c	0.46 c	0.10 d
	T8	0.38 b	0.32 b	0.70 b	0.13 c
Means		0.36	0.30	0.67	0.13
CV (%)		18.55	19.08	18.76	16.76
Climax	T1	0.23 a	0.20 a	0.43 a	0.12 a
	T2	0.17 a	0.13 a	0.30 a	0.06 b
	T3	0.05 c	0.04 b	0.10 b	0.02 c
	T4	0.13 b	0.10 a	0.24 a	0.04 c
	T5	0.19 a	0.15 a	0.34 a	0.06 b
	T6	0.18 a	0.14 a	0.32 a	0.06 b
	T7	0.20 a	0.16 a	0.36 a	0.07 b
	T8	0.18 a	0.13 a	0.32 a	0.07 b
Means		0.16	0.13	0.30	0.06
CV (%)		36.09	45.26	39.45	36.37
Woodard	T1	0.44 a	0.46 a	0.91 a	0.17 a
	T2	0.30 b	0.34 b	0.65 b	0.16 a
	T3	0.27 b	0.33 b	0.60 b	0.16 a
	T4	0.31 b	0.35 b	0.67 b	0.15 a
	T5	0.26 b	0.31 b	0.58 b	0.12 b
	T6	0.43 a	0.46 a	0.90 a	0.17 a
	T7	0.29 b	0.33 b	0.63 b	0.11 b
	T8	0.32 b	0.37 b	0.69 b	0.14 a
Means		0.32	0.36	0.70	0.14
CV (%)		29.85	21.97	25.65	21.96

*Means followed by the same letters in column do not differ according to the Scott-Knott test at 5% probability.

Source: From the author (2024).

In the Bluegem cultivar, greater increments of chlorophyll *a*, *b*, and total wereobserved in treatments T2 and T4, while for carotenoids, the greatest increment was observed in T4. In the Climax cultivar, greater increases in chlorophyll a occurred in treatments T1, T2, T5, T6, T7, and T8. For chlorophyll *b* and total, treatments T1, T2, T4, T5, T6, T7, and T8 showed the highest values, but in relation to carotenoids, the highest value was observed in treatment T1. As for the Woodard cultivar, treatments T1 and T6 showed the highest values of chlorophyll *a*, *b*, and total. However, treatments T1, T2, T3, T4, T6, and T8 allowed the greatest increments of carotenoids (Table 5).

3.3 Water analysis

3.3.1 Relative water content

The relative water content was different among treatments only for the plants of blueberry cv. Woodard with T6 and T8 being the lowest values.

Table 6 – Relative water content (RWC) of blueberry plants cvs. Bluegem, Climax and Woodard in different concentrations of 2-ip and adenine sulfate

Treatments	-------------------Cultivars-------------------		
	Bluegem	Climax	Woodard
	-------Relative water content (%)-------		
T1	50.92 a	47.20 a	45.40 a
T2	40.77 a	47.93 a	46.46 a
T3	48.25 a	45.00 a	59.49 a
T4	54.57 a	45.37 a	51.89 a
T5	52.99 a	45.63 a	51.55 a
T6	47.59 a	40.63 a	30.66 b
T7	56.22 a	69.44 a	54.11 a
T8	47.07 a	66.49 a	31.54 b
Means	49.49	50.96	46.38
CV (%)	21.46	38.57	26.39

*Means followed by the same letters in column do not differ according to the Scott-Knott test at 5% probability.

Source: From the author (2024).

3.3.2 Extravasation of electrolytes

For the extravasation of electrolytes, there was no difference between treatments, regardless of the cultivars (Tabel 7).

Table 7 – Extravasation of electrolytes of blueberry plants cvs. Bluegem, Climax and Woodard in different concentrations of 2-ip and adenine sulfate

Treatments	-------------------Cultivars-------------------		
	Bluegem	Climax	Woodard
	-------Extravasation of electrolytes (%)-------		
T1	55.63 a	45.46 a	50.83 a
T2	66.06 a	54.62 a	54.22 a
T3	54.14 a	64.49 a	64.24 a
T4	59.45 a	41.18 a	64.75 a
T5	56.73 a	64.10 a	86.41 a
T6	55.21 a	58.76 a	63.20 a
T7	53.24 a	72.84 a	53.52 a
T8	47.04 a	68.89 a	78.06 a
Means	55.93	58.83	64.40
CV (%)	37.97	33.80	43.68

*Means followed by the same letters in column do not differ according to the Scott-Knott test at 5% probability.

Source: From the author (2024).

3.3.3 Membrane integrity

The membrane integrity was observed a difference between treatments for blueberry plants of cvs. Bluegem and Climax. The plants of T1 for Bluegem and T1 and T4 for Climax were the largest. No difference was observed between the treatments of the cv. Woodard (Table 8).

Table 8 – Membrane integrity relative of the Bluegem and Climax cultivars in different treatments.

Treatments	------------------- Cultivars -------------------		
	Bluegem	Climax	Woodard
	------- Membrane integrity (%) -------		
T1	12.00 a	5.36 a	3.06 a
T2	2.43 b	3.56 b	2.53 a
T3	2.43 b	2.65 c	1.41 a
T4	3.16 b	5.31 a	2.46 a
T5	3.11 b	1.11 c	0.95 a
T6	3.61 b	1.23 c	1.26 a
T7	3.61 b	1.78 c	1.86 a
T8	2.58 b	2.01 c	1.18 a
Means	4.11	2.87	1.83

CV (%)	50.77	46.63	7.92

*Means followed by the same letters in column do not differ according to the Scott-Knott test at 5% probability.

Source: From the author (2024).

3.4 Flow cytometry

Flow cytometry analysis was performed in order to determine the DNA content of the blueberry cultivars used in the study. Therefore, no alteration was observed in the DNA of the plants (Table 8), allowing us to infer that they are genetically stable, according to the histograms obtained (Figure 2).

Table 9 – DNA content in blueberry cultivars grown *in vitro*.

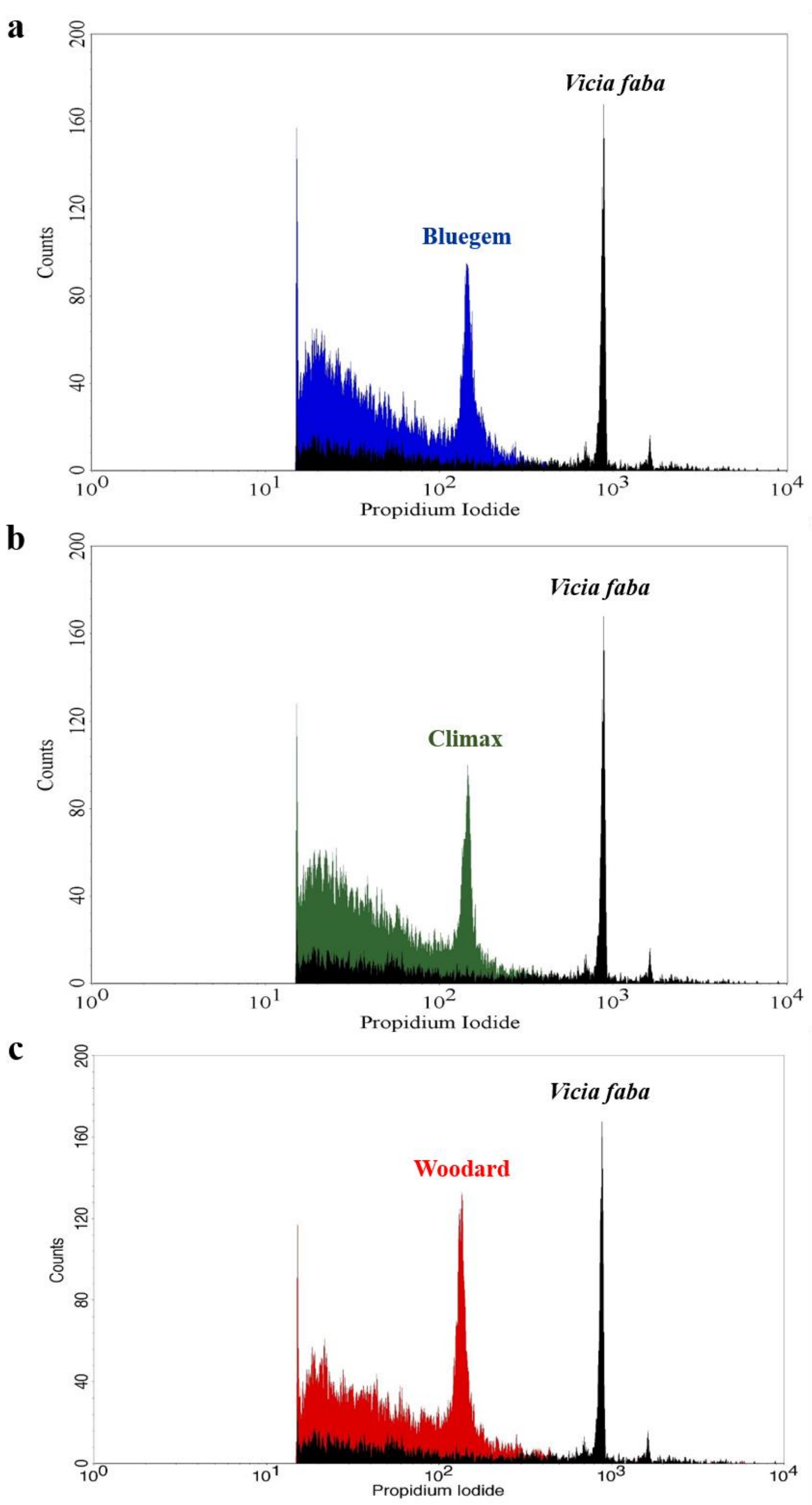
Cultivars	DNA content (pg)
Bluegem	4.43 a
Climax	4.65 a
Woodard	4.38 a
Means	4.49
CV (%)	3.75

*Means followed by the same letters in column do not differ according to the Scott-Knott test at 5% probability.

Source: From the author (2024).

Furthermore, no significant difference was observed in the study between treatments with different concentrations of adenine sulfate and 2-ip, regardless of the analyzed cultivars.

Figure 2 – Histograms obtained via flow cytometry in blueberry cultivars. a) Bluegem; b) Climax, and c) Woodard.



Source: From the author (2024).

4 DISCUSSION

In the blueberry *in vitro* multiplication phase, the most common cytokinins used are zeatin and 2-ip. Several studies point to zeatin as the growth regulator that provides a greater number of shoots (REED; ABDELNOUR-ESQUÍVEL, 1991).

Adenine sulfate, on the other hand, is a growth regulator that has been used to obtain a greater number of shoots *in vitro*, as it provides effects similar to those promoted by cytokinins. Ribeiro *et al.* (2014) report that when adenine sulfate is used in the culture medium in combination with other cytokinins, the proliferation of shoots becomes possible, as well as the homogeneity of plants. Therefore, in the present work, 2-ip was used combined with adenine sulfate, seeking a greater number and size of shoots.

Cüce and Sökmen (2017) evaluated different combinations of hormones aiming at the in vitro production of buds of *Vaccinium uliginosum* L., and observed that the highest bud production (3.73) occurred in medium containing 1.0 mg L^{-1} zeatin, 0.1 mg L^{-1} IBA, and 0.2 mg L^{-1} GA3. In the present work, the production of sprouts was similar for the Bluegem cultivar when the medium with the combination of 2-ip and adenine sulfate was used. However, values higher than this were obtained in the Climax and Woodard cultivars. This fact allows for demonstrating the effectiveness of these two growth regulators acting together.

Higher values for the number of shoots were also observed in the present study for the three cultivars compared to the results obtained by Schuchovski and Biasi (2022). These researchers sought to analyze the micropropagation of the species *Vaccinium virgatum* ‘Delite’, where they analyzed different concentrations of zeatin (0, 2.5, 5.0, 7.5, and 10 μM), which correspond to 0.5, 1.0, 1.5, and 2 mg L^{-1}. This allowed for one sprout per plant in all treatments.

In addition, most studies point to zeatin as a promoter of higher multiplication rates compared to the results obtained with 2-ip, but it was clear in this study that the combination of adenine sulfate with 2-ip favored the development of a greater number of shoots and homogeneity in size, which may favor the next stages of the micropropagation process.

On the other hand, it is known that photosynthetic pigments are important indicators of the physiological state of a plant and that they are directly related to their good development. These pigments are responsible for promoting good absorption, transformation, and transmission of light energy throughout the plant system (PARK *et* *al.*, 2018).

Yavorska and Vorobets (2019), in studies involving photosynthetic pigments in shoots of *Vaccinium corymbosum* L. (cv. Elliott), observed in the multiplication phase values such as 0.16 mg g^{-1} of chlorophyll *a*, 0.08 mg g^{-1} of chlorophyll *b*, 0.25 mg g^{-1} of total chlorophyll and 0.03 mg g^{-1} of carotenoids. All cultivars involved in the present study showed results superior to those observed by these authors.

Water is a vital and fundamental factor for the good growth and development of plants, so it is necessary to verify whether these indices were negatively affected by the use of growth regulators. The relative water content was not affected in the Climax and Bluegem cultivars, while for the Woodard cultivar, greater water storage was observed in some treatments, demonstrating its absorption efficiency.

The extravasation of electrolytes is directly related to the integrity of the cell membrane. The reduction of this integrity can compromise the functioning of the membrane, leading to its dysfunction (CHATTOPADHYAY *et al.*, 2011). As observed in the present study, the blueberry cultivars analyzed did not show significant differences in terms of electrolyte leakage, demonstrating that 2-ip together with adenine sulfate concentrations did not affect the cell walls of the plants to the point of breaking and leaking electrolytes. It was even noted through the integrity of the membrane that for the Bluegem and Climax cultivars, some treatments showed greater integrity, that is, the walls were practically not affected.

The water analyzes were extremely important in the present study since they showed that the use of 2-ip and the concentrations of adenine sulfate did not affect the process of absorption and distribution of water, corroborating the good in vitro growth of the plant.

Flow cytometry is a technique applied in several areas, including plant breeding, and can be applied in different ways, such as the determination of DNA content, ploidy, and genetic recombination. In addition, it has the advantage of faster and easier sample preparation (BREEDING, 2022).

Flow cytometry has also been widely used to verify the genetic stability of materials of interest due to its great precision and efficiency (SLIWINSKA; THIEM, 2007; ZAFAR *et al.*, 2019).

The peaks of the histograms of the three cultivars cultivated *in vitro* and exposed to the concentrations of adenine sulfate and 2-ip were similar to the peaks obtained in the samples of plants considered controls, which demonstrates that the DNA content was not altered. This factor is extremely important to guarantee the integrity of the analyzed material and that the initial characteristics of the plants were maintained, guaranteeing genetically identical plants to the original plants.

5 CONCLUSION

The interaction between the concentrations of adenine sulfate and 2-ip, regardless of the blueberry cultivars analyzed, led to an increase in the number of shoots as well as more uniform plants, favoring the subsequent stages of the micropropagation process.

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ARTICLE 2 - *In vitro* ROOTING OF BLUEBERRY: CONCENTRATIONS OF WPM MEDIUM AND ACTIVATED CHARCOAL

ABSTRACT

Micropropagation is a technique that makes it possible to speed up the process of obtaining quality seedlings. However, the species is woody and has difficulty rooting *in vitro*. Therefore, the objective of this work was to improve the rooting phase of the *in vitro* culture in order to facilitate the acclimatization process of the species. To this end, stem segments (1.5 cm long) from three blueberry cultivars (Bluegem, Climax, and Woodard) were extracted from plants already established *in vitro* and transferred to tubes containing 15 mL of medium containing water, 5.5 g L^{-1} agar and 20 mg L^{-1} IBA and kept in a growth room for 48 h. Then, the stem segments were transferred to tubes containing 15 mL of WPM culture medium (0, 25, 50, 75, and 100%) with and without activated charcoal. Phytotechnical evaluations, photosynthetic pigments, and *ex vitro* plant survival were carried out after 60 days. The initial inoculation of microcuttings with IBA and the concentrations of WPM medium, with or without activated charcoal, directly affected the rooting phase of blueberry plants in the three cultivars analyzed. The Bluegem cultivar in general was the one that responded best to the treatments. The presence of activated charcoal in the culture medium was only necessary for the Climax cultivar. Regarding the percentage of WPM medium, it is possible to determine that 75% was the best for the Bluegem cultivar; for the others, the results were still very variable, and it was not possible to determine a single concentration.

Keywords: Growth regulator, *Vaccinium* spp., micropropagation.

Abbreviations: Cv: cultivar, IBA: indole-3-butyric acid, PA: length of the aerial part, CR: length of roots, NR: number of roots, NF: number of leaves, CF: length of leaves, MF: fresh mass, MS: dry mass, WPM: Woody Plant Medium.

1 INTRODUCTION

Furthermore, for the blueberry crop to expand successfully, it is necessary to produce quality seedlings, and this can occur through micropropagation. This technique allows you to accelerate the process of obtaining seedlings that develop more slowly, both *in vitro* and *ex vitro*, when compared to other fruit species (ARRUDA *et al.*, 2017).

The rooting phase of the species is essential for successful *in vitro* propagation. Root regeneration can vary between species and their cultivars, and several factors are associated with this process, such as the sensitivity of plants to rooting factors, as well as the rooting techniques adopted. In vitro cultivation, most species require an ideal balance between growth regulators and mineral components of the medium for cell differentiation and the formation of the root meristem. However, the response of plants to these stimuli varies with the plant's ability to absorb and metabolize the compounds (ERST *et al.*, 2018b).

In the case of blueberries, *in vitro* rooting a acclimatization are major challenges that need to be overcome. For this reason, during the *in vitro* root formation process, it is very important that the explants develop in the best possible way, since the development of plants considered abnormal hinders the transition to the *ex vitro* environment (TEJADA-ALVARADO *et al.*, 2022). For this reason, it is so important to evaluate characteristics that can improve this phase, such as the effect of growth regulators, activated charcoal, and concentrations of the culture medium.

Based on the above, the objective of this work was to improve the rooting phase of the *in vitro* culture in order to facilitate the acclimatization process of the species.

2 MATERIALS AND METHODS

2.1 Experiment location

The experiment was carried out at the Plant Tissue Culture Laboratory of the Agricultural Sciences School of Lavras in the Federal University of Lavras, located in Lavras, Minas Gerais, Brazil.

2.2 Obtaining the material

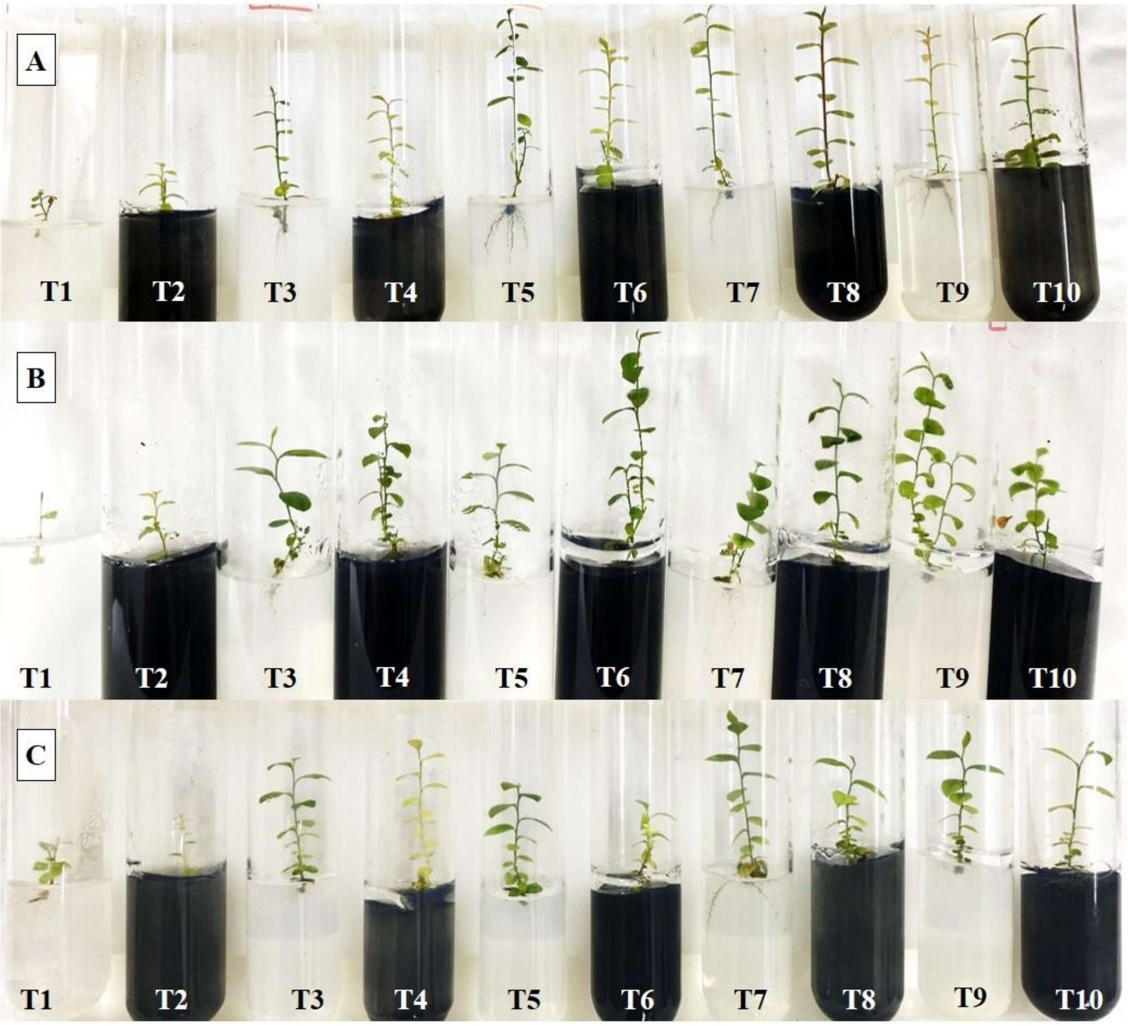
Three blueberry cultivars were used (Bluegem, Climax, and Woodard). Plants already established *in vitro* were transplanted into WPM medium (LLOYD; MCCOWN, 1980) containing 2.5 mg L^{-1} 2-ip. Subsequently, they were stored for 30 days in a growth room with artificial lighting provided by white LED lamps and an average irradiance of 49.4 µmol m^{-2} s^{-1}, photoperiod of 16 h and temperature of 25 ± 2 ºC.

2.3 Induction of *in vitro* rooting

Blueberry microcuttings (1.5 cm long) were transferred to tubes containing 15 mL of medium containing water, 5.5 g L^{-1} agar, and 20 mg L^{-1} IBA for 48 h in a growth room.

The IBA concentration and time period were determined through a pre-test carried out previously (data not shown). After 48 hours, the explants were transferred to tubes containing 15 mL of WPM medium concentrations (0, 25, 50, 75, and 100%), without (0.0 g L^{-1}) or with (2.0 g L^{-1}) activated charcoal (Figure 1). The pH of the medium was 5.7 ± 0.3 and autoclaving at 121 ± 1 °C and 1.05 atm pressure for 20 min. The tubes were placed again in a growth room for 60 days.

Figure 3- Plants of blueberry cultivars [Bluegem (A), Climax (B) and Woodard (C)] in WPM mediuns, with and without activated charcoal after 60 days



Source: From the author (2024).

2.4 Analyses

2.4.1 Morphometric parameters

The phytotechnical analyses were carried out at the Tissue Culture Laboratory (Department of Agriculture/UFLA). To this end, length of aerial part (mm) and roots (mm), number of roots (mm) and leaves, length of leaves (mm), and fresh and dry mass of plants (g) were evaluated. For the analysis, eight replicates were used. A millimeter ruler was used to measure the length of the aerial part and roots, and a precision analytical balance was used to analyze fresh and dry mass. The dry mass of the plants were taken to the forced air circulation greenhouse at a temperature of 40 ºC until they reached constant weight.

2.4.2 Photosynthetic pigments

Fresh blueberry leaves (0.025 g) were transferred to Falcon tubes containing 5 mL of 80% acetone for the extraction of photosynthetic pigments. The tubes were wrapped in aluminum foil to avoid contact of the sample with light and possible degradation of chlorophyll. After 24 h in a refrigerator at ± 4 °C, the absorptivity of the samples wasmeasured using an Elisa Multiskan GO spectrophotometer (Thermo Fisher Scientific) at wavelengths of 470, 645, 652, and 663 nm (SCOPEL *et al.*, 2011). The analysis wasperformed in triplicate, with four replicates per treatment, using Skanit Software 5.0 for Microplate Readers RE version 5.0.0.42. Equations were used to calculate the concentration of chlorophylls (*a*, *b*, and total) and carotenoids (LI *et al.*, 2013).

2.4.3 Percentage of survival

Blueberry plants from *in vitro* cultivation of the three cultivars were transplanted into trays filled with commercial substrate. The trays were placed in a greenhouse with a micro-sprinkler and the survival rates of each cultivar were assessed 30 days after transplanting.

2.5 Experimental design and statistical analysis

The experiment was installed using three blueberry cultivars (Bluegem, Climax and Woodard), using 20 mg L^{-1} IBA obtained through pre-test with IBA concentrations, 5 WPM medium percentages (0, 25, 50, 75, and 100%), and without or with (2.0 g L^{-1}) the presence of activated charcoal.

The experimental design was completely randomized, in a 5x2 factorial scheme, with five percentages of WPM medium and two concentrations of activated charcoal.

The data were subjected to analysis of variance and the means were compared using the Scott-Knott test at 5% with the statistical software SISVAR (FERREIRA, 2019).

3 RESULTS

Plants of blueberry cultivars Bluegem (a), Climax (b), and Woodard (c) under the effect of IBA and the concentrations of WPM.

Figure 4 – Plants of blueberry cultivars [Bluegem (A), Climax (B) and Woodard (C)] in WPM mediuns with IBA

Source: From the author (2024).

3.1 Morphometric parameters

Significant interaction was observed between the percentages of WPM medium and the cultivars analyzed for all phytotechnical variables performed (Table 2).

Regarding the length of the aerial part, it can be observed that among the cultivars, the best result was found for the Bluegem cultivar. When we compare each cultivar individually, it is possible to notice that the best results for both the Bluegem and Woodard cultivars were the treatments with 75 and 100% WPM, while the Climax cultivar, with the exception of the treatment with 0% WPM, had similar results.

In terms of length of roots, the cultivars Bluegem and Climax were the positive highlights when we observed the interaction between the three cultivars. Separately, both the Bluegem and Woodard cultivars did not show significant differences within the different medium concentrations. Within the Climax cultivar, the percentages of 25, 50, and 75% stood out positively.

Taking into account the number of roots presented when comparing the cultivars analyzed, Climax stood out positively compared to the others. Within each of the cultivars, different results were found; in Bluegem, the best results were presented by the treatments that had 25, 75, and 100% of the medium; in the Climax cultivar, with the exception of 0%, all the others presented similar results; as for to Woodard, there was no difference between treatments.

The number of leaves varied according to the percentage of culture medium and the cultivar analyzed. Comparing the cultivars in the treatments containing 0% and 25% of medium, Woodard presented the best results, and in the others, the Bluegem cultivar. In Bluegem, the highest number was presented when using 50% of the WPM medium; in Climax, the treatment was 100%; and in Woodard, the results were similar for all treatments except the treatment without the culture medium.

Observing the length of leaves, it is possible to say that there was not much difference when comparing the interaction between the three cultivars. Separately, all showed significant differences within the treatments, the best results in the Bluegem cultivar were with the 50, 75, and 100% treatments; in Climax the lengths were similar for all treatments except the treatment without the culture medium; and the Woodard treatments with 75, and 100% of WPM medium were superior.

A significant difference was observed in the interaction between cultivars for fresh mass analysis, among which the Bluegem cultivar presented the best results. Individually, there was also a difference between the treatments for each of them, with highlights being the treatments with 75 and 100% of WPM in the Bluegem cultivar. Within Climax the best results were found for 50, 75, and 100% of the medium, and for the Cultivate Woodard treatments with 100% of the culture medium.

The existing interaction for dry mass analysis demonstrated that the Bluegem and Woodard cultivars were similar in all analyzed data, and the worst results were observed in the Climax cultivar. Analyzing each cultivar individually, there was also a significant difference between treatments. The treatment containing 100% of the medium presented a higher dry mass for the Bluegem and Woodard cultivars, while in the Climax cultivar, the results were similar for all treatments except for treatment without the culture medium.

Table 2 – Effect of the interaction between cultivars and culture medium concentrations on length of the aerial part (LAP), length of roots (LR), number of roots (NR), number of leaves (NL), length of leaves (LL), fresh mass (FM) and dry mass (DM).

Variables	**Cultivar**	**%WPM**				
		0	**25**	**50**	**75**	**100**
LAP (mm)	Bluegem	15.08 Ad	33.42 Ac	43.94 Ab	51.68 Aa	50.74 Aa
	Climax	16.75 Ab	31.21 Aa	34.53 Ba	34.79 Ba	37.53 Ba
	Woodard	16.91 Ac	27.49 Ab	27.56 Cb	31.05 Ba	33.88 Ba
	CV (%)	26.07				
LR (mm)	Bluegem	15.37 Aa	21.06 Aa	26.08 Aa	23.19 Aa	18.45 Aa
	Climax	14.48 Ab	23.96 Aa	24.01 Aa	22.41 Aa	16.89 Ab
	Woodard	13.35 Aa	14.06 Ba	18.51 Aa	10.51 Ba	12.37 Ba
	CV (%)	34.11				
NR	Bluegem	2.65 Ab	4.30 Aa	3.25 Bb	5.15 Aa	3.85 Aa
	Climax	1.65 Ab	4.40 Aa	6.05 Aa	4.80 Aa	4.00 Aa
	Woodard	1.60 Aa	1.90 Ba	2.35 Ba	2.20 Ba	2.95 Aa
	CV (%)	44.92				
NL	Bluegem	5.20 Bd	9.85 Bc	15.35 Aa	13.10 Ab	13.45 Ab
	Climax	5.55 Bc	9.70 Bb	11.10 Bb	11.25 Ab	13.30 Aa
	Woodard	7.45 Ab	11.70 Aa	10.15 Ba	12.35 Aa	11.15 Ba
	CV (%)	26.24				
LL (mm)	Bluegem	4.77 Ab	6.21 Ab	7.18 Aa	8.22 Aa	8.26 Aa
	Climax	3.22 Ab	7.27 Aa	6.76 Aa	5.86 Ba	6.84 Aa
	Woodard	3.62 Ac	6.23 Ab	6.41 Ab	8.23 Aa	7.46 Aa
	CV (%)	35.30				
FM (g)	Bluegem	0.02 Ac	0.04 Ab	0.06 Ab	0.10 Aa	0.12 Aa
	Climax	0.01 Ab	0.02 Bb	0.03 Ba	0.03 Ca	0.04 Ca
	Woodard	0.01 Ac	0.05 Ab	0.05 Ab	0.06 Bb	0.09 Ba
	CV (%)	31.76				
DM (g)	Bluegem	0.00 Ad	0.01 Ac	0.01 Ac	0.01 Ab	0.02 Aa
	Climax	0.00 Ab	0.01 Aa	0.00 Ba	0.00 Ba	0.01 Ba
	Woodard	0.00 Ac	0.01 Ab	0.01 Ab	0.01 Ab	0.02 Aa
	CV (%)	39.37				

*Averages followed by the same letter, uppercase in the column and lowercase in the row, do not differ from each other using the Scott-Knott test at 5% probability.

Source: From the author (2024).

A significant interaction was observed between the presence or absence of charcoal in the culture medium and the cultivars analyzed (Table 3). When comparing the cultivars, it was observed that Bluegem presented the best results in all analyzed parameters (except for length of leaves) in a medium without activated charcoal. With the presence of activated charcoal, the Bluegem cultivar presented the best results for leaf length and fresh and dry mass, while Climax presented the best results for length of roots and number of roots.

For the length of the aerial part of the plants, no significant difference was observed between the Bluegem and Climax cultivars. Furthermore, in relation to the number of leaves, no significant difference was observed between the three cultivars.

Table 3 – Effect of the interaction between cultivars and the presence of activated charcoal in the culture medium on length of the aerial part (LAP), length of roots (LR), number of roots (NR), number of leaves (NL), length of leaves (LL), fresh mass (FM) and dry mass (DM).

Variables	**Cultivar**	**Activated charcoal**	
		Without	**With**
LAP	Bluegem	**42.63 Aa**	35.32 Ab
	Climax	28.16 Bb	**33.65 Aa**
	Woodard	**29.48 Ba**	25.35 Bb
	CV (%)	26.07	
LR (mm)	Bluegem	**23.29 Aa**	18.37 Bb
	Climax	11.39 Bb	**28.96 Aa**
	Woodard	12.55 Ba	14.96 Ba
	CV (%)	34.11	
NR	Bluegem	4.80 Aa	2.88 Bb
	Climax	3.08 Bb	5.23 Aa
	Woodard	2.18 Ba	2.21 Ba
	CV (%)	44.92	
NL	Bluegem	12.26 Aa	10.52 Ab
	Climax	9.91 Ba	10.43 Aa
	Woodard	9.67 Bb	11.41 Aa
	CV (%)	26.24	
LL (mm)	Bluegem	6.35 Bb	7.51 Aa
	Climax	6.14 Ba	5.84 Ba
	Woodard	7.40 Aa	5.42 Bb

	CV (%)	35.30	
FM (g)	Bluegem	0.08 Aa	0.06 Aa
	Climax	0.03 Ca	0.02 Ca
	Woodard	0.06 Ba	0.04 Bb
	CV (%)	31.76	
DM (g)	Bluegem	0.01 Aa	0.01 Aa
	Climax	0.00 Ba	0.01 Ba
	Woodard	0.01 Aa	0.00 Cb
	CV (%)	39.37	

*Averages followed by the same letter, uppercase in the column and lowercase in the row, do not differ from each other using the Scott-Knott test at 5% probability.

Source: From the author (2024).

Individually, it is possible to observe that the best results for the Bluegem and Woodard cultivars were presented without the presence of activated charcoal in the culture medium. For the Climax cultivar, treatments using activated charcoal together with WPM stood out.

A significant interaction was observed between activated charcoal and the percentage of WPM medium, regardless of the cultivars used (Table 4). In the treatment with 0% WPM, no significant difference was observed in the analyzed variables, regardless of the presence or absence of activated charcoal in the culture medium. The percentages of 25 and 50% of culture medium, with the addition of activated charcoal, provided the best results for length of roots. However, there was no significant difference regarding the presence or absence of activated charcoal in the medium in the other variables analyzed (Table 4).

Table 4 – Effect of the interaction between the percentage of WPM and the activated charcoal in the culture medium on length of the aerial part (LAP), length of roots (CR), number of roots (NR), number of leaves (NL), length of leaves (LL), fresh mass (FM) and dry mass (DM).

Variables	**Activated Charcoal**	**%WPM**				
		0	**25**	**50**	**75**	**100**
LAP (mm)	Without	14.37 Ad	30.74 Ac	34.73 Ac	40.10 Ab	**46.23 Aa**
	With	17.89 Ac	30.67 Ab	35.96 Aa	38.25 Aa	35.20 Ba
	CV (%)			26.07		
LR (mm)	Without	12.83 Aa	15.10 Ba	16.69 Ba	17.64 Aa	16.52 Aa
	With	15.77 Ab	24.29 Aa	29.04 Aa	19.76 Ab	15.29 Ab
	CV (%)			34.11		
NR	Without	1.96 Ab	3.93 Aa	3.43 Aa	3.70 Aa	3.70 Aa
	With	1.96 Ab	3.13 Aa	4.33 Aa	4.40 Aa	3.50 Aa
	CV (%)			44.92		
NL	Without	5.53 Ad	9.80 Ac	12.06 Ab	11.43 Bb	13.96 Aa
	With	6.53 Ac	11.03 Ab	12.33 Aa	13.03 Aa	11.30 Bb
	CV (%)			26.24		
LL (mm)	Without	3.78 Ab	6.95 Aa	6.88 Aa	8.13 Aa	7.21 Aa
	With	3.95 Ac	6.19 Ab	6.68 Ab	6.74 Bb	7.83 Aa
	CV (%)			35.30		
FM (g)	Without	0.01 Ad	0.04 Ac	0.05 Ac	0.07 Ab	0.09 Aa
	With	0.01 Ad	0.03 Ac	0.04 Ab	0.05 Ab	0.07 Aa
	CV (%)			31.76		
DM (g)	Without	0.00 Ad	0.01 Ac	0.01 Ac	0.01 Ab	0.02 Aa
	With	0.00 Ac	0.01 Ab	0.01 Ab	0.01 Ab	0.01 Ba
	CV (%)			39.37		

*Averages followed by the same letter, uppercase in the column and lowercase in the row, do not differ from each other using the Scott-Knott test at 5% probability.

Source: From the author (2024).

The greatest lengths of roots were observed in medium with concentrations of 25 and 50% and in the presence of activated charcoal. The lowest number of roots was observed in plants grown in 0% medium, regardless of whether the medium contained activated charcoal or not. The greatest increase in the number of leaves was observed in culture medium with 75%, and activated charcoal. On the other hand, in the absence of activated charcoal, there was a greater increase in the number of leaves in the medium with a concentration of 100%. Furthermore, greater lengths of leaves were observed in

100% medium, regardless of whether or not there was activated charcoal.

Regarding the length of the aerial part, the best result was observed in medium without activated charcoal with concentration of 100%. When activated charcoal was added to the medium, the best results were observed with concentrations of 50, 75 and 100%.

Greater increases in fresh and dry mass occurred in mediums containing 100% concentration. However, the best results for fresh mass were provided by the absence of activated charcoal.

3.2 Photosynthetic pigments

Significant interaction was observed between cultivars and percentages of WPM medium in the three cultivars studied (Table 5).

Table 5 – Effect of the interaction between the cultivars Bluegem, Climax and Woodard and the concentrations of WPM medium on the content of chlorophyll (*a*, *b*, total) and carotenoids.

Pigments	**Cultivar**	**%WPM**				
		0	**25**	**50**	**75**	**100**
Chlorophyll *a* (mg g^{-1})	Bluegem	0.21 Ad	0.22 Ad	0.42 Bb	0.55 Aa	0.30 Bc
	Climax	0.09 Bb	0.27 Aa	0.10 Cb	0.12 Cb	0.23 Ba
	Woodard	0.08 Bd	0.15 Bc	0.63 Aa	0.35 Bb	0.39 Ab
	CV (%)			38.21		
Chlorophyll *b* (mg g^{-1})	Bluegem	0.14 Ad	0.17 Bd	0.32 Bb	0.47 Aa	0.24 Ac
	Climax	0.13 Ab	0.26 Aa	0.10 Cb	0.12 Cb	0.18 Bb
	Woodard	0.02 Bd	0.10 Cc	0.45 Aa	0.25 Bb	0.28 Ab
	CV (%)			41.17		
Total chlorophyll (mg g^{-1})	Bluegem	0.35 Ad	0.40 Bd	0.74 Bb	1.03 Aa	0.54 Bc
	Climax	0.23 Bb	0.54 Aa	0.21 Cb	0.24 Cb	0.42 Ba
	Woodard	0.11 Bd	0.25 Cc	1.08 Aa	0.60 Bb	0.67 Ab
	CV (%)			39.35		
Carotenoids (mg g^{-1})	Bluegem	0.06 Ad	0.07 Ad	0.12 Bb	0.16 Aa	0.09 Cc
	Climax	0.03 Bb	0.07 Aa	0.02 Cb	0.02 Cb	0.07 Ba
	Woodard	0.04 Bc	0.06 Ac	0.18 Aa	0.12 Bb	0.13 Ab
	CV (%)			33.46		

*Averages followed by the same letter, uppercase in the column and lowercase in the row, do not differ from each other using the Scott-Knott test at 5% probability.

Source: From the author (2024).

At 0% concentration, the Bluegem cultivar presented the better results compared to other cultivars, regardless of the pigments analyzed. On the other hand, at a concentration of 25%, Climax showed the highest increases in chlorophyll *a*, *b* and total. But regarding carotenoids, at a concentration of 25%, no significant difference was observed between the three cultivars. At concentrations of 50 and 100% of medium, the Woodard obtained the best results. In a medium with 75%, the Bluegem showed the largest increases in all pigments analyzed.

In relation to photosynthetic pigments, a significant interaction was observed between cultivars and activated charcoal in the three cultivars studied (Table 6). However, the absence of activated charcoal provided better results for chlorophyll *a* and carotenoids in the Bluegem cultivar and for chlorophyll *b* and total in the Woodard cultivar. The presence of activated charcoal provided better results in all variables analyzed in the Woodard cultivar (Table 6).

Table 6 – Effect of the interaction between the cultivars Bluegem, Climax and Woodard and the presence of charcoal in the culture medium on the content of chlorophyll (*a*, *b* and total) and carotenoids.

Pigments	**Cultivar**	**Activated charcoal**	
		Without	**With**
Chlorophyll *a* (mg g^{-1})	Bluegem	0.46 Aa	0.22 Bb
	Climax	0.14 Ca	0.19 Ba
	Woodard	0.25 Bb	0.39 Aa
	CV (%)	38.21	
Chlorophyll *b* (mg g^{-1})	Bluegem	0.36 Ba	0.18 Bb
	Climax	0.15 Ba	0.17 Ba
	Woodard	0.16 Ab	0.28 Aa
	CV (%)	41.17	
Total Chlorophyll (mg g^{-1})	Bluegem	0.82 Ba	0.40 Bb
	Climax	0.29 Ca	0.36 Ba
	Woodard	0.42 Ab	0.66 Aa
	CV (%)	39.35	
Carotenoides (mg g^{-1})	Bluegem	0.13 Aa	0.07 Bb
	Climax	0.04 Cb	0.05 Ca
	Woodard	0.09 Bb	0.12 Aa
	CV (%)	33.46	

*Averages followed by the same letter, uppercase in the column and lowercase in the row, do not differ from each other using the Scott-Knott test at 5% probability.

Source: From the author (2024).

In the Bluegem cultivar, regardless of the pigments analyzed, better results were observed in the absence of activated charcoal. In the Climax cultivar, there was no significant difference in relation to pigments, with the exception of the carotenoid content, in which a greater increase was observed in the medium with activated charcoal. For the Woodard cultivar, regardless of the variable, the presence of activated charcoal provided the best results.

A significant interaction was observed between the percentages of WPM medium and activated carbon in photosynthetic pigments (Table 7).

Table 7 – Effect of the interaction between the cultivars Bluegem, Climax and Woodard and the presence of charcoal in the culture medium on the content of chlorophyll (*a*, *b* and total) and carotenoids.

Pigments	**Activated Charcoal**	**%WPM**				
		0	**25**	**50**	**75**	**100**
Chlorophyll *a* (mg.g^{-1})	Without	0.18 Ad	0.25 Ac	0.51 Aa	0.39 Ab	0.10 Be
	With	0.07 Bd	0.18 Bc	0.25 Bb	0.29 Bb	0.52 Aa
	CV (%)			38.21		
Chlorophyll *b* (mg.g^{-1})	Without	0.14 Ad	0.19 Ac	0.38 Aa	0.31 Ab	0.09 Be
	With	0.06 Bd	0.16 Ac	0.20 Bc	0.25 Bb	0.37 Aa
	CV (%)			41.17		
Chlorophyll total (mg.g^{-1})	Without	0.32 Ad	0.45 Ac	0.90 Aa	0.71 Ab	0.18 Bd
	With	0.13 Bd	0.34 Bc	0.46 Bb	0.54 Bb	0.90 Aa
	CV (%)			39.35		
Carotenoids (mg.g^{-1})	Without	0.06 Ac	0.07 Ac	0.14 Aa	0.11 Ab	0.04 Bd
	With	0.03 Bd	0.06 Bc	0.07 Bb	0.09 Bb	0.16 Aa
	CV (%)			33.46		

*Averages followed by the same letter, uppercase in the column and lowercase in the row, do not differ from each other using the Scott-Knott test at 5% probability.

Source: From the author (2024).

Concentrations of 0, 25, 50, and 75% allowed the best results in the absence of activated charcoal in the culture medium, but the 100% concentration allowed the best results in the medium with activated charcoal, regardless of the variables analyzed. However, it was observed that in the absence of activated charcoal, the best results were seen at a concentration of 50%. In a medium with activated charcoal, the best results were observed in WPM medium at a concentration of 100% (Table 7).

3.3 Survival

In the present study, the efficiency of the *in vitro* rooting process of the seedlings when transferred to the acclimatization phase was observed (Table 8).

Table 8 – Survival (%) of plants rooted *in vitro* when acclimatized.

Treatment	**Bluegem**	**Climax**	**Woodard**
T1	60	0	80
T2	20	80	60
T3	80	60	80
T4	0	80	20
T5	40	80	80
T6	0	100	20
T7	100	80	80
T8	100	100	0
T9	80	80	80
T10	40	80	60
Means	52	74	56

Source: From the author (2024).

As can be seen, survival rates varied according to the cultivars analyzed. Treatments T7 and T8 showed 100% survival of the Bluegem cultivar, standing out from the others. In the Climax cultivar, treatments T6 and T8 demonstrated the highest efficiency among the others. For the Woodard cultivar, the best values were found were in treatments T1, T3, T5, T7, and T9 (Table 8).

4 DISCUSSION

To verify the success of *in vitro* rooting of plants, it is necessary to observe some important characteristics, such as the number and length of roots, as well as their quality, in addition to the *ex vitro* rooting rate (FIGIEL-KROCZYŃSKA *et al.*, 2022). The auxin most used in the rooting process in blueberry crops is IBA as it is considered a stable molecule (COSTA JUNIOR *et al.*, 2018). And in the present work, the concentration of growth regulator used together with the percentages of culture medium were effective.

Erst *et al.* (2018a) carried out *in vitro* rooting of *Vaccinium uliginosum* in a similar way to what was done in the present study. However, the shoots were treated with 30 mg

L^{-1} IBA for 4 h and were subsequently transferred to medium 50% of Anderson (1975) without growth regulator. The researchers observed an average of 5.9 roots per explant. This number is similar to that found in the Bluegem cultivar, but lower than that found in the Climax cultivar and higher than that in the Woodard cultivar. The difference between the number of leaves was also large in *V. uliginosum*, averaging 7.9, but lower than the values obtained in the three cultivars studied (ERST *et al.*, 2018a).

Figiel-Kroczyńska *et al.* (2022) studied the rooting of *Vaccinium corymbosum* L. involving three cultivars (Elizabeth, Meader, and Liberty), and observed the number of roots varying around 2.14, with the largest root measuring 0.77 cm, being the highest number observed in the Elizabeth cultivar, in WPM medium supplemented with 0.1 mg L^{-1} zeatin and 1.0 mg L^{-1} IAA. Even so, the values they found were lower than those found in the present study for the three cultivars analyzed.

For the good development of roots *in vitro* and for subsequent survival *ex vitro*, activated charcoal is one of the most used and important inputs, as it favors root growth, helps to reduce inhibitory substances that may occur in the culture medium, and also releases natural substances that can help with the in vitro growth of cultures (MENEGUZZI *et al.*, 2020). However, despite all these positive characteristics of activated charcoal, the present study reinforces the idea that each plant and/or cultivar presents a different behavior in relation to the inputs used. As occurred in the present work, two of the three cultivars managed to develop better in treatments where there was no charcoal.

Tejada-Alvarado *et al.* (2022) observed divergent results in the Bluecrop and Biloxi cultivars, where the rhizosphere development capacity was improved when the culture medium contained activated charcoal. In another study involving *Vaccinium corymbosum* L., El-Dis *et al.* (2018) observed that the addition of activated charcoal to the culture medium made it possible to increase the rooting rate of plants in vitro and, consequently, better acclimatization of the seedlings.

Chlorophylls and carotenoids are extremely important pigments for capturing light by plants, as well as in the direct or indirect induction of photosynthetic processes. The existence of a high-quality and more vigorous root system allows plants to better absorb fundamental nutrients from these molecules (SILVA *et al.*, 2021). This relationship can be found in this study in two cultivars analyzed (Bluegem, and Woodard). The best values presented relate to both the morphometric parameters and the pigment analyses, demonstrating that a greater number of roots enabled an increase in the production of photosynthetic pigments.

Furthermore, in the present study, it is also evident how the development of quality roots can affect the acclimatization phase. *Ex vitro* survival is an extremely important parameter to measure the success of the *in vitro* rooting process, as already mentioned. The three cultivars analyzed showed high levels of *ex vitro* survival, which corroborates the success of the technique developed.

These factors demonstrate how each blueberry cultivar responds in different ways to the rooting stimuli caused by IBA in the presence or absence of activated charcoal and in different doses of the culture medium, showing the difficulty of developing a standard protocol for the culture including in the phase of *in vitro* rooting of the species, at the same time, we observed that it is possible to have quality *in vitro* rooting in blueberry plants.

5 CONCLUSION

The initial inoculation of microcuttings with IBA and concentrations of WPM medium, with or without activated charcoal, directly affected the rooting phase of blueberry plants in the three cultivars analyzed. The Bluegem cultivar in general was the one that responded best to the treatments. The presence of activated charcoal in the culture medium was only necessary for the Climax cultivar. Regarding the percentage of WPM medium, it is possible to determine that 75% was the best for the Bluegem cultivar; for the others the results were still very variable, and it was not possible to determine a single concentration.

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ARTICLE 3 - *In vitro* FLASKS SEALS INFLUENCE THE ACCLIMATIZATION OF BLUEBERRY PLANTS

ABSTRACT

Blueberries are a fruit belonging to the Ericaceae family and the genus *Vaccinium* spp. Strategies such as micropropagation have been used to optimize the species' seedling production process. Therefore, the objective was to improve the acclimatization process of the species using different types of flask seals during *in vitro* cultivation. For this purpose, three blueberry cultivars (Bluegem, Climax, and Woodard) were used. The explants were induced to root (water, 5.5 g L^{-1} agar, and 20 mg L^{-1} IBA), transferred to flasks containing WPM medium with charcoal, and placed in a growth room for 30 days using three types of seals: normal lid, plastic film, and lid with filter (Samavidros® commercial model). After 30 days, the explants were transferred to polyethylene trays containing commercial substrate and kept in a greenhouse with an intermittent nebulization system (microspray) for 60 days. Morphometric, histological, survival, and photosynthetic pigment analyzes were carried out in the experiment. The results differed according to the cultivars analyzed. In *vitro* cultivation, no significant difference was observed between the morphometric data in the three cultivars, but divergent results were observed in the survival rates and in the morphometric and pigment parameters at the end of the acclimatization processes. In blueberries, the use of filter covers made it possible to improve the micropropagation and acclimatization process of the Climax cultivar. Furthermore, the filters enabled greater plants survival in the Bluegem cultivar, but in the Woodard cultivar, the system did not provide relevant additions for micropropagation and acclimatization.

Keywords: Tissue culture, *Vaccinium* spp., seedling production.

Abbreviations: Cv: cultivar, IBA: indol-3-butyric acid, LAP: length of aerial part, LR: length of roots, NR: number of roots, NL: number of leaves, LT: leaf thickness, FM: fresh mass, WPM: Woody Plant Medium, LB: leaf blade, AbET: abaxial epidermis thickness, AdET: adaxial epidermis thickness, TPR: thickness of the palisade parenchyma, TSP: thickness of the spongy parenchyma.

1 INTRODUCTION

Blueberries are a berry-type fruit belonging to the Ericaceae family and the genus *Vaccinium* spp., produced mainly in the United States and Europe. Furthermore, it has been presenting itself as a crop experiencing great global expansion, mainly because it is a nutritionally attractive fruit with low caloric content, high polyphenol content, and a pleasant flavor (BRAZELTON; YOUNG, 2017).

The propagation of this species is generally carried out through cuttings; however, this method has a low percentage of rooting, which makes it difficult for producers to have seedlings available. Thinking about optimizing the production of seedlings and making them available quickly and in large quantities, new production strategies have been studied, and among them is micropropagation. This technique enables the effective production of seedlings *in vitro* in an aseptic and fully controlled environment, quickly and on a large scale (FIGIEL-KROCZYŃSKA *et al.*, 2022).

However, one of the main challenges faced in *in vitro* cultivation is acclimatization, as it is the process by which plants adapt to the external environment. During this process, many plants can be lost or damaged, and this occurs due to the controlled conditions that are offered to plants when they are still *in vitro*, such as stable nutrition, absence of pathogens, adequate temperature, humidity, and photoperiod. Due to these factors, plants go through a stressful situation during acclimatization since their photosynthetic system is not yet active and prepared for autotrophic nutrition, their roots are more fragile, and their functionality is compromised (AKIMOVA *et al.*, 2022).

Among the factors that can influence the germination, growth and development of a plant material *in vitro* is the microenvironment in which it is inserted, as it can vary according to the bottles and the types of seals used (RIBEIRO *et al.*, 2022).

Generally, it is conventional in tissue culture that flasks and tubes are sealed in order to avoid loss of water and contamination. However, these lids do not have ventilation systems, and this makes gas exchange impossible. For this reason, plants have limited photosynthetic capacity, fewer functional chloroplasts, and a high rate of respiration, especially during the night (OLIVEIRA *et al.*, 2021).

However, in mixotrophic cultivation, there is the possibility of gas exchange between *in vitro* plants and the environment through gas-permeable membranes (commercial or not). On the other hand, in the heterotrophic system, polypropylene lids are used to seal the flasks, and this can restrict gas exchange between the plants and the environment, in addition to providing a more humid environment for plant growth (OLIVEIRA JUNIOR *et al.*, 2022).

Therefore, the objective was to improve the acclimatization process of the species using different types of flask seals during *in vitro* cultivation.

2 MATERIALS AND METHODS

2.1 Experiment location

The experiment was conducted at the Tissue Culture Laboratory of the Department of Agriculture (DAG) of the Federal University of Lavras (UFLA), located in Lavras, state of Minas Gerais, Brazil (44°57’50” W, 21°13’40” S, 919 m altitude).

2.2 Obtaining the material

Blueberry plants from three cultivars (Bluegem, Climax, and Woodard) were used in the experiment.

Plants were maintained in Woody Plant Medium (WPM) (LLOYD; MCCOWN, 1980) containing 2.5 mg L^{-1} 2-ip. Successive subcultures were carried out in order to multiply the material. The material was stored for 30 days in a growth room with artificial lighting provided by 18 W white LED lamps (ECP®) and an average irradiance of 49.4 µmol m^2 s^{-1}, with a photoperiod of 16 h and temperature of 25 ± 2 ºC.

2.3 Induction of *in vitro* rooting and acclimatization in a greenhouse

Blueberry plants were segmented into 1.5 cm sizes and transferred to tubes containing 15 mL of medium containing water, 5.5 g L^{-1} agar, and 20 mg L^{-1} IBA. The segments remained for 48 h in the same growth room presented before. Like the IBA concentration, the time was determined through a pre-test carried out previously. After 48 h, the induced explants were transferred to flasks containing 50 mL of WPM culture medium (50%) with the addition of activated charcoal (2%). The pH of the medium was adjusted to 5.7 ± 0.3 before autoclaving at 121 °C and 1.05 atm pressure for 20 min. Three types of seals were used in the bottles (normal lid, plastic film, and lid with filter) (Figure 1). The lid has a Samavidros® commercial-model filter, the BioSama model. After 30 days, the explants were transferred to polyethylene trays containing commercial Carolina soil® substrate and kept in a greenhouse with an intermittent nebulization system (micro sprinkler) for 60 days.

Figure 5 – Bottles used in the installation of experiment.

Source: From the author (2024).

2.4 Analyzes

2.4.1 Morphometric parameters

The morphometric parameters were analyzed at the Tissue Culture Laboratory (DAG/UFLA). Length of aerial part (mm), root (mm) and leaves (mm), number of leaves, and fresh and dry mass of plants (g) were evaluated. To measure the length of aerial part and the length of root, a digital caliper was used and for the analysis of fresh and dry mass, a precision analytical balance. The dry mass of the plants were taken to the forced air circulation greenhouse at a temperature of 40 ºC until they reached constant weight.

2.4.2 Histological and morphometric analyzes

Histological and morphometric analyzes were carried out at the Applied Botany Laboratory (Department of Biology/UFLA). For the histological analysis of the leaves, the samples were initially fixed in a 70% FAA solution (70% ethyl alcohol, 15% formaldehyde, and 15% acetic acid) for 72 h and then preserved in 70% alcohol. The plant material was dehydrated in an increasing series of ethyl alcohol concentrations every 2 h (70, 80, 90, and 100%). Subsequently, the samples were stored for 12 hours in 100% ethanol + activated resin (resin + activating powder) in a 1:1 ratio in a freezer in order to be filtered. The activated resin was prepared using 50 mL of liquid resin and 1 sachet of activating powder. After 24 h, the ethanol + activated resin solution were removed, and then only activated resin was added to the samples, which will remain in the freezer for 5 days.

For blocking, 3×5 mm rectangles were sectioned in the middle of the leaf blade of blueberry plants in vitro, using three samples from each treatment. They were placed in histomold wells and infiltrated with inclusion solution (60 µL of hardener + 400 µL of activated resin for each mold). Subsequently, they were left to polymerize in an oven at 37 ºC for 24 h.

After the material hardened, the blocks were sectioned on a bench microtome, setting the section thickness at 10 µm. The transverse sections were spread over a slide with water; after drying, they were stained with toluidine blue, and 24 h later, transparent varnish was added to fix the coverslip.

The slides were analyzed using a ZEISS light microscope with an attached digital camera system. Morphometric evaluations were carried out using the ImageJ image analysis program bundled with 64-bit Java 8.

The parameters measured were the tissue thickness of the adaxial and abaxial epidermis (µm), of the palisade and spongy parenchyma (µm), and of the leaf blade (µm). Measurements were carried out on 12 replicates of leaf photomicrographs from each treatment.

2.4.3 Percentage of survival

Blueberry plants from in vitro cultivation of the three cultivars were transplanted into trays filled with commercial substrate. The trays were placed in a greenhouse with a micro-sprinkler and the survival rates of each cultivar were assessed 60 days after transplanting.

2.4.4 Photosynthetic pigments

Photosynthetic pigments 0.025 g of fresh plant material, which was placed in Falcon tubes containing 5 mL of 80% acetone to extract the pigments. The tubes were wrapped in aluminum foil to prevent the sample from coming into contact with light, preventing chlorophyll degradation, and remained in a refrigerator at ± 4 °C for 24 h. The absorptivity of the samples was then measured using a spectrophotometer at wavelengths of 470, 645, 652, and 663 nm (SCOPEL *et al.*, 2011). The analysis was performed in triplicate, with four replicates per treatment. Equations were used to calculate the concentration of chlorophylls (*a*, *b*, and total) and carotenoids (LI *et al.*, 2013)..

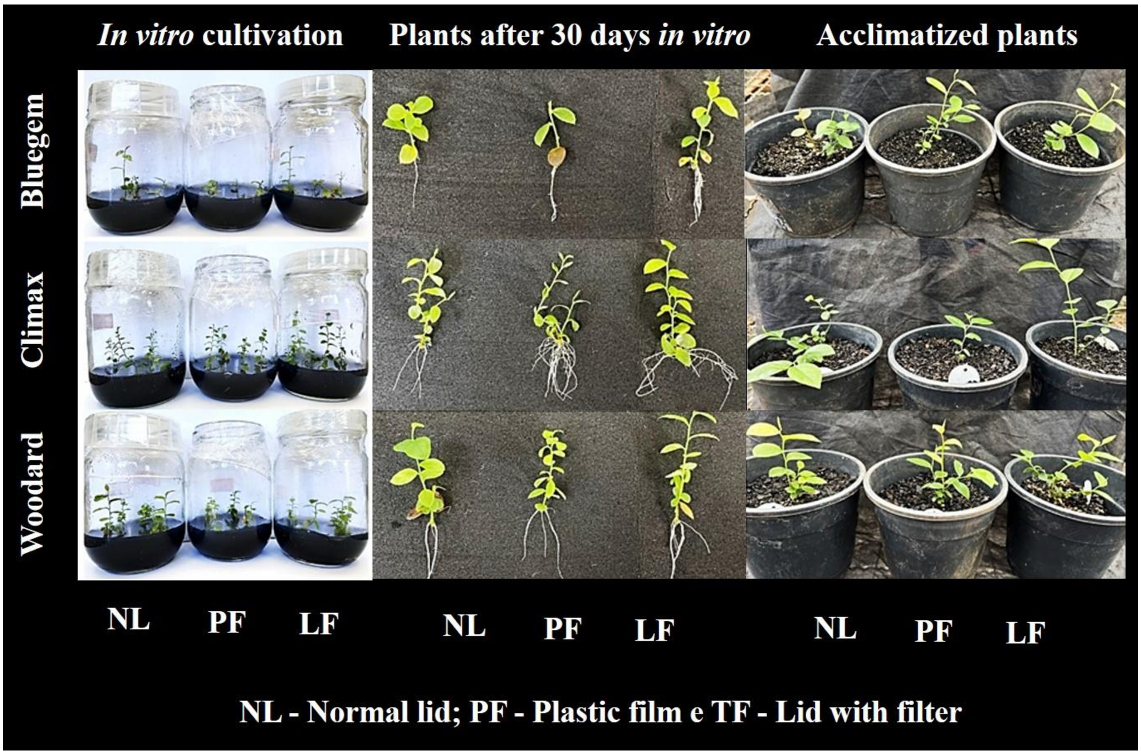
2.5 Experimental design and statistical analysis

The experiment was set up separately using the three cultivars (Bluegem, Climax, and Woodard) in a completely randomized design (CRD), with 3 treatments with 40 replicates per treatment, with each plant considered 1 replicate. The data were subjected to analysis of variance and, subsequently, the Scott-Knott test at 5% probability using the R studio software.

3 Results

The blueberry cultivars Bluegem, Climax, and Woodard in different types of seals after 30 days *in vitro* and during the acclimatization process are showed below (Figure 2).

Figure 6 – Visual appearance of the different types of seals in *vitro* cultivation in the three cultivars analyzed (Bluegem, Climax and Woodard), after 30 days of *in vitro* cultivation and during the acclimatization process.



Source: From the author (2024).

3.1 *In vitro* morphometric parameters

A significant difference was observed between treatments in the Woodard cultivar in the parameters of root length, number of leaves, and fresh mass. For the Bluegem and Climax cultivars, there was no significant difference between the treatments (Table 1).

Table 1 – Results of the morphometric parameters: Length of aerial part (AP), length of roots (LR), number of roots (NR), number of leaves (NL), length of leaves (LL) and fresh mass (FM) presented by the three cultivars analyzed with the different types of seal.

Cultivar	**Treatment**	**LAP (mm)**	**LR (mm)**	**NR**	**NL**	**LL (mm)**	**FM (g)**
Bluegem	Normal lid	26.94 a	23.11 a	3.57 a	6.58 a	7.34 a	0.04 a
	Plastic film	22.59 a	20.25 a	3.00 a	5.16 a	7.45 a	0.03 a
	Lid with filter	27.69 a	19.62 a	2.80 a	6.91 a	8.01 a	0.04 a
	Mean	25.74	20.99	3.12	6.21	7.60	0.03
	CV (%)	50.77	26.11	55.38	48.09	18.65	30.11
Climax	Normal lid	34.84 a	17.75 a	3.50 a	12.33 a	6.05 a	0.03 a
	Plastic film	30.97 a	21.03 a	3.12 a	10.41 a	5.67 a	0.03 a
	Lid with filter	32.35 a	19.33 a	3.44 a	11.25 a	6.27 a	0.04 a
	Mean	32.72	19.37	3.35	11.33	5.99	0.03
	CV (%)	33.67	27.91	52.01	25.88	27.19	48.27
Woodard	Normal lid	27.06 a	19.77 a	1.85 b	8.41 a	7.15 a	0.04 a
	Plastic film	25.06 a	17.51 a	1.50 b	10.50 a	4.65 b	0.03 b
	Lid with filter	29.11 a	21.49 a	3.28 a	10.08 a	6.13 a	0.04 a
	Mean	27.07	19.59	2.21	9.66	5.97	0.03
	CV (%)	21.25	33.71	42.43	28.75	30.63	37.56

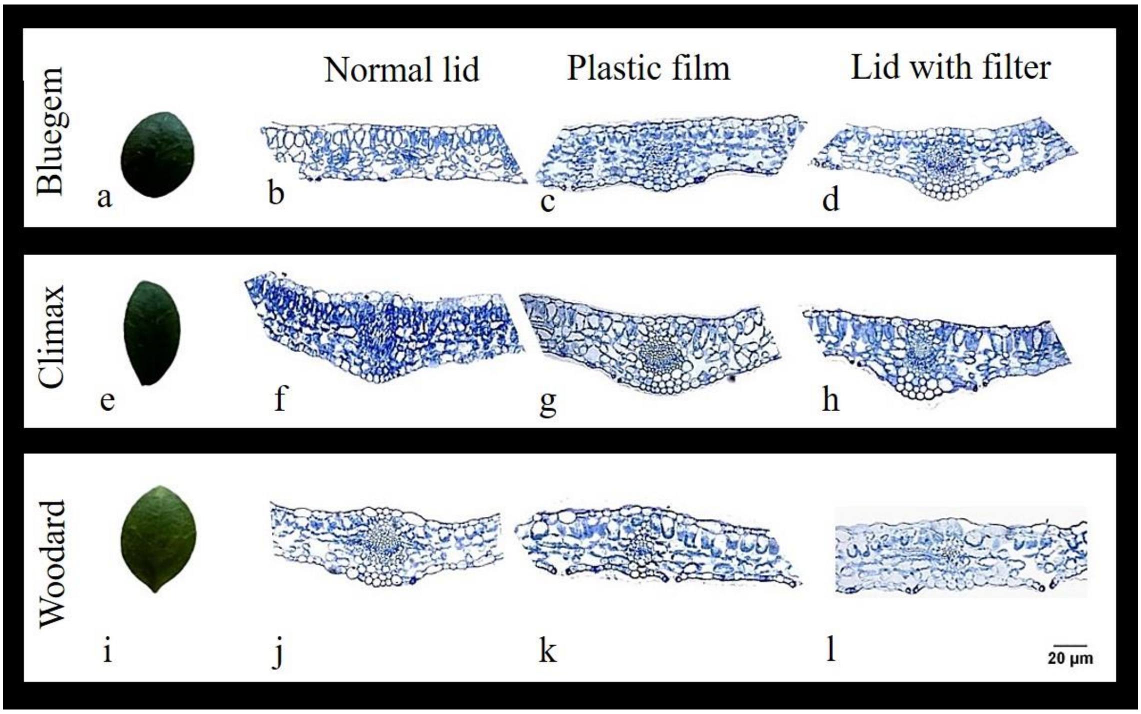
*Means followed by the same letters do not differ from each other using the Scott-Knott test at 5% probability.

Source: From the author (2024).

3.2 Histological and morphometric analyzes

Below, it is possible to visually observe the anatomical difference of the cultivars Bluegem, Climax, and Woodard (Figure 3) in the different types of seals.

Figure 7 – Anatomical difference between the different types of seals in the cultivars Bluegem, Climax and Woodard.



Source: From the author (2024).

There was a significant difference between treatments in all morphometric parameters analyzed for the three cultivars, except for the parameters length of the adaxial epidermis and length of the abaxial epidermis in the Climax cultivar (Table 2). In the Bluegem cultivar, the treatment with the normal lid stood out positively, while in the Climax cultivar, the treatments with plastic film and filter lid stood out. As for the Woodard cultivar, the lid with filter allowed better results for the leaf blade, length of the palisade, and spongy parenchyma.

Table 2 – Morphometric analyzes of the anatomical sections: leaf blade (LB), abaxial epidermis thickness (AbET), adaxial epidermis thickness (AdET), palisade parenchyma thickness (PPT) and spongy parenchyma thickness (SPT), carried out on the three cultivars after *in vitro* cultivation.

Cultivar	**Treatment**	**LB**	**AbET**	**AdET µm**	**PPT**	**SPT**
Bluegem	Normal lid	83.09 a	8.76 a	9.97 a	21.99 a	39.85 a
	Plastic film	61.22 b	6.17 b	6.72 b	11.89 c	31.60 b
	Lid with filter	64.27 b	7.56 a	10.88 a	14.24 b	28.52 b
	Mean	69.52	7.49	9.19	16.04	33.32
	CV (%)	5.91	26.81	19.42	12.6	12.44
Climax	Normal lid	56.72 c	7.34 a	8.49 a	15.50 b	22.56 b
	Plastic film	59.95 b	6.90 a	9.68 a	21.15 a	36.27 a
	Lid with filter	69.12 a	7.32 a	7.59 a	21.57 a	24.38 b
	Mean	61.93	7.18	8.58	19,4	27.73
	CV (%)	4.59	18.92	26.23	17.94	19.04
Woodard	Normal lid	55.26 b	8.76 a	9.97 a	14.44 b	27.05 a
	Plastic film	46.25 c	4.73 c	7.75 b	15.85 a	19.15 b
	Lid with filter	60.77 a	7.03 b	7.62 b	16.94 a	28.96 a
	Mean	54.09	6.84	8.44	15.74	25.05
	CV (%)	7.36	24.73	22.01	12.88	13.34

*Means followed by the same letters do not differ from each other using the Scott-Knott test at 5% probability.

Source: From the author (2024).

3.3 Survival

The values presented in Table 3 demonstrate the efficiency of the rooting process of seedlings in the laboratory (*in vitro*) when passed to the acclimatization phase (*ex vitro*). For the Bluegem and Climax cultivars, the lid with filter was better, while for the Woodard cultivar, the highest percentage of survival was observed with the use of the normal lid.

Table 3 – Survival percentage of blueberry explants of the three cultivars analyzed after sixty days of the acclimatization process.

Treatment	Bluegem	Climax	Woodard
Normal lid	50.00	66.66	91.66
Plastic film	25.00	75.00	83.33
Lid with filter	66.66	91.66	66.66
Mean	47.22	77.77	80.55

Source: From the author (2024).

3.4 *Ex vitro* morphometric parameters

A significant difference was observed between treatments for all cultivars analyzed in the parameters length of aerial part, number of leaves, and length of leaves (Table 4). For the Bluegem cultivar, treatments with plastic film provided the best results. For the other Climax and Woodard cultivars, better results were observed using filter lids. However, it is important to highlight that the parameters of number of roots and root length were not measured due to the continuity of the species' development process aimed at closing the cycle.

Table 4 – Results of the morphometric parameters: length of aerial part (LAP), number of leaves (NL) and length of leaves (LL) presented by the three cultivars after the acclimatization process.

Cultivar	Treatment	LAP (mm)	NL	LL (mm)
Bluegem	Normal lid	44.51 b	8.50 a	10.36 b
	Plastic film	96.48 a	8.50 a	17.09 a
	Lid with filter	69.71 b	10.4 a	13.61 b
	Mean	70.23	9.13	13.68
	CV (%)	27.25	25.24	18.39
Climax	Normal lid	63.90 b	6.80 a	16.37 b
	Plastic film	57.26 b	7.74 a	14.09 b
	Lid with filter	100.62 a	9.28 a	21.52 a
	Mean	73.92	19.05	
	CV (%)	24.13	19.05	17.33
Woodard	Normal lid	68.06 b	9.25 a	18.14 b
	Plastic film	80.55 a	9.57 a	19.03 b
	Lid with filter	84.12 a	8.20 a	23.62 a
	Mean	77.57	9.00	20.26
	CV (%)	12.67	15.08	16.88

*Means followed by the same letters do not differ from each other using the Scott-Knott test at 5% probability.

Source: From the author (2024).

3.5 Photosynthetic pigments

A significant difference was observed between treatments for the content of photosynthetic pigments (Table 5). For the Bluegem cultivar, the best results were presented by the treatment using the lid with filter. As for the Climax and Woodard cultivars, the normal lid stood out positively in all analyzed parameters.

Table 5 – Results of the analysis of photosynthetic pigments chlorophyll *a*, chlorophyll *b*, total chlorophyll and carotenoids presented by the three cultivars after the acclimatization process.

Cultivar	**Treatment**	**Chlorophyll *a***	**Chlorophyll *b***	**Chlorophyll *total***	**Carotenoids**
		mg.g^{-1}			
Bluegem	Normal lid	0.38 a	0.35 b	0.74 b	0.18 a
	Plastic film	0.30 b	0.27 b	0.58 b	0.13 b
	Lid with filter	0.46 a	0.47 a	0.93 a	0.20 a
	Mean	0.38	0.36	0.75	0.17
	CV (%)	24.33	23.19	23.58	22.48
Climax	Normal lid	0.85 a	0.75 a	1.61 a	0.30 a
	Plastic film	0.59 b	0.53 b	1.13 b	0.19 b
	Lid with filter	0.51 b	0.52 b	1.03 b	0.20 b
	Mean	0.65	0.60	1.26	0.23
	CV (%)	25.10	24.60	24,67	28.09
Woodard	Normal lid	0.78 a	0.75 a	1.53 a	0.28 a
	Plastic film	0.54 b	0.59 b	1.14 b	0.25 a
	Lid with filter	0.57 b	0.49 b	1.07 b	0.22 b
	Mean	0.63	0.61	1.24	0.25
	CV (%)	15.82	21.56	18.48	12.37

*Means followed by the same letters do not differ from each other using the Scott-Knott test at 5% probability.

Source: From the author (2024).

4 DISCUSSION

The literature shows us that the types of seals used in *in vitro* cultivation directly interfere with the morphology, anatomy, and physiology of micropropagated plants, and gas exchange can provide negative or positive effects depending on the plant species. For this reason, environmental control becomes essential to obtaining quality propagation material (BARBOSA *et al.*, 2021).

Plants grown *in vitro* are found in an environment where humidity is high and there are reduced possibilities for gas exchange. This fact directly interferes with the acclimatization phase of plants since plants have reduced photosynthetic capacity in addition to being able to present morphophysiological disorders (FORTINI *et al.*, 2021).

The use of gas permeable membranes in a system known as mixotrophic allows for an increase in plant growth, improving physiological and anatomical aspects, and also the ability to adapt and develop in an acclimatization process (ŠEVČÍKOVÁ *et al.*, 2019). In the present study, this fact was confirmed, as can be seen in the anatomical analyzes and growth rates through the morphometric parameters of the plants after the acclimatization process involving the Climax and Woodard cultivars.

The *in vitro* development of *Jacaranda cuspidifolia* microcuttings, for example, was also affected by the membrane sealing system; in addition, greater gas exchange enabled an increase in the rooting rate, length of roots, and growth of the aerial part of the plants. when compared to the traditional sealing system (FERREIRA *et al.*, 2022).

Studies involving the micropropagation of *Caesalpinia spinosa* using a ventilation system presented results similar to those found in this study, mainly in relation to the morphometric parameters of the *in vitro* plants. However, for the species in question, there was no significant difference between the height of the shoots using this resource (NÚÑEZ-RAMOS *et al.*, 2021).

In the *in vitro* cultivation of *Acca sellowiana*, the number of regenerated shoots did not undergo considerable changes under the ventilation system; on the other hand, the propagation rate of the species increased (GUANAIS *et al.*, 2022).

The use of the permeable membrane enabled a significant increase in plant survival in the Bluegem and Climax cultivars. Therefore, this increase may have occurred due to the fact that the lids with filter allow greater gas exchange within the flask, reducing the accumulation of gases and increasing the perspiration flow. This increases the probability of plant survival when transferred to the ex vitro environment (BARBOSA *et al.*, 2021).

It is known that different levels of gas exchange can also directly affect the leaf anatomy of plants grown *in vitro*, directly interfering with their acclimatization process (FORTINI *et al.*, 2021). This fact was confirmed in the present study, although treatments with the permeable membrane did not present the best results.

Contrasting results were observed in studies carried out by Oliveira Junior (2022), where bottles with a ventilation system significantly favored the leaf anatomy of *Croton lechleri*, palisade parenchyma, and a larger leaf blade when compared to treatments using only PVC film as a seal. The thickness of the spongy parenchyma and the adaxial and abaxial epidermis were not affected by the different treatments.

The increase in the concentration of gases, such as CO_2, can favor leaf expansion, which directly interferes with photosynthesis and the production of photosynthetic pigments (GAO *et al.*, 2017). Contrary to expectations, plants from the cultivars Climax and Woodard had better pigment production when grown with standard covers. This may have occurred because the two cultivars used the sucrose present in the medium as their main source of energy, not requiring an increase in chlorophyll content (NÚÑEZ-RAMOS *et al.*, 2021).

5 CONCLUSIONS

The use of different types of seals directly interfered with the development of the explants. Systems that enable gas exchange are important and efficient for improving the quality of micropropagation processes in several works already carried out. However, answers vary depending on the species and cultivar of interest.

The use of filter lids made it possible to improve the micropropagation and acclimatization processes of blueberries from the Climax cultivar. Furthermore, the filters enabled greater plant survival in the Bluegem cultivar, but in the Woodard cultivar, the system did not provide relevant increases.

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TERCEIRA PARTE

CONSIDERAÇÕES FINAIS

O sucesso da micropropagação de plantas se deve a junção de diversos fatores, tais como meio adequado, balanço de reguladores de crescimentos, condições as quais os explantes são expostos e, principalmente, a particularidade de cada espécie que ficou ainda mais evidente no estudo realizado, observando que cada cultivar de mirtileiro reagiu diferente às condições aqui apresentadas.

Na fase de multiplicação *in vitro*, a combinação de 2-ip e sulfato de adenina proporcionou melhora considerável na produção de brotos da espécie nas três cultivares. Partindo para o enraizamento, foi possível determinar a porcentagem ideal de meio WPM para o enraizamento da cultivar Bluegem. Por fim, os diferentes tipos de vedações influenciaram de maneira diferente em cada uma das cultivares trabalhadas. Já os filtros melhoraram o processo de aclimatização da cultivar Climax e a sobrevivência da cultivar Bluegem.

Sendo assim, esta pesquisa proporcionou informações capazes de auxiliar ainda mais na obtenção de plantas micropropagadas de mirtileiro e evidenciou como o estudo de cada espécie e cultivar individualmente é importante para melhorar a eficiência dos sistemas de produção de plantas em geral.