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Research Article

Micropropagation of *Vaccinium corymbosum* L.'Bluecrop' in Rocker Temporary Immersion System (TIS) Bioreactor

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Abstract: Blueberries are high-value fruits. The traditional method of propagation by cuttings cannot supply the modern market with large quantities of seedlings. The method of micropropagation of plants in vitro makes it possible to bring the production of blueberry seedlings to the highest level. Blueberries have not been sufficiently studied in in vitro culture, so the search for the simplest and most cost-effective methods of micropropagation remains relevant. The problem of accelerated micropropagation of blueberries can be solved using rocker-type bioreactors, which differ from other models in terms of simplicity of design and low cost. A study was carried out to evaluate the effectiveness of micropropagation of *Vaccinium corymbosum* 'Bluecrop' in rocker bioreactors. Two types of bioreactors were compared: the bioreactor of the Platform system and the TIS rocker bioreactor modified by the author. As a control, blueberries were grown on a semi-solid medium. The effectiveness of blueberry micropropagation was evaluated by the following indicators: multiplication coefficient, shoot length, and proportion of vitrified shoots. Experiments were conducted on WPM medium, with zeatin supplementation at a concentration of 1.0 mg/l, resulting in optimal results. It is shown that the rocker bioreactor is slightly inferior to the plantform bioreactor in micropropagation but outperforms the method of micropropagation on semisolid media. The rocker bioreactor can be fully utilized for production purposes. In order to reduce costs and increase technical reliability, the working principle of the mechanical drive of the author's model of a rocker-type bioreactor was changed.

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1. Introduction

Blueberries are highly nutritious, containing significant amounts of polyphenols and biologically active substances (Wang et al., 2019; Mengist et al., 2020). In recent years, blueberry planting areas and yields have rapidly increased worldwide. In 2016, the global blueberry planting area increased by 34.14% compared to 2012, reaching 110 928 hectares, and producing a total of 552 505 tons (Gallegos-Cedillo et al., 2018). Meanwhile, China's blueberry acreage reached 66 400 hectares in 2020, with a total harvest of 347 200 tons, making it the leading producer of blueberries in the Asia-Pacific region (Yang et al., 2022).

Traditionally, the propagation of blueberry varieties has been based on rooting cuttings. The formation of adventitious roots on blueberry shoots typically requires the exogenous application of auxins. Successful rooting occurs during a specific period of plant development (Litwinczuk et al., 2005; Debnath, 2007; Marino et al., 2014). The issue of obtaining large volumes of blueberry planting material can be resolved through the method of clonal propagation of plants under aseptic conditions. In vitro micropropagation protocols have been developed for numerous blueberry varieties. Initially, blueberry micropropagation was conducted on solid media. Subsequently, research emerged on the cultivation of blueberries in bioreactors (Fan et al., 2017; Wang et al., 2023). Nevertheless, reducing the expenses of blueberry micropropagation remains pertinent to this day.

Temporary immersion bioreactors (TIBs) are typically used for the micropropagation of blueberries. This method involves immersing explants in a liquid medium for limited time intervals rather than constant immersion (Georgiev, 2014; Welander et al., 2016). The process of periodic flooding of the culture is achieved in various ways, as described in relevant sources (Murthy et al., 2023). Temporary immersion systems (TIS) typically comprise multiple compartments or separate vessels within a container. The medium is transferred from the reservoir compartment/vessel to the compartment/vessel where the plants are cultivated using air pressure. Bioreactors such as RITA, PLANTFORM, and SETIS operate on this principle. However, these models may not always be accessible to blueberry producers. In addition, maintaining sterility in bioreactors of the aforementioned models can be challenging due to the interconnection of several containers. A simpler alternative is the Rocker system, which employs a mechanical platform to tilt individual plant containers at a fixed angle, facilitating the movement of the media. This system is easier to implement than the previous models. The BioMINT™ model (Patent No. PA/a/2004/003837, Centro de Investigación Científica de Yucatán (CICY), Yucatán, Mexico) is the most well-known. It reduces the risk of culture contamination by moving media within an individual container but does not effectively aerate the vessels.

The aim of the study was to evaluate the feasibility of blueberry micropropagation in a rocker TIS bioreactor, intending to achieve maximum efficiency at minimal cost. The study results suggest that the rocker TIS bioreactor is a viable option for blueberry micropropagation. In this study, the working efficiency of PLANTFORM and the rocker type bioreactor developed for this study were compared. The control was blueberry culture on semi-solid agar medium.The approach discovered for more economical micropropagation of this variety is expected to be applied to other varieties of *Vaccinium corymbosum*.

2. Material and Methods

The research was done in 2022-2023 at the laboratory of the Botanical Garden of the Southern Federal University and Don State Technical University, Rostov-on-Don, Russia. The original *Vaccinium corymbosum* 'Bluecrop' mother specimen was acquired from a private collection in Rostov-on-Don. For all stages of in vitro micropropagation, McCown's Woody Plant (WPM) basal medium (McCown and Lloyd, 1981) was used. The stimulation of shoot formation was performed with zeatin (Sigma, USA).

2.1. Plant material and culture establishment

Shoots of *Vaccinium corymbosum* L. 'Bluecrop' the current year, no longer than 10 cm, were obtained from the mother specimen and washed in running water for 20 minutes. Subsequently, they were washed for an additional 15 minutes in running water with a drop of hand soap, followed by another 20-minute wash in running water. Further processing was conducted under sterile conditions in a laminar flow box. Surface sterilization was carried out in several processing stages. The cuttings were sectioned into 5 cm explants and then submerged in a 70% ethyl alcohol solution for 30 seconds using a 300 ml glass beaker. Subsequently, they were immediately transferred to a solution of 1% sodium hypochlorite (NaOCl) for 10 minutes in a beaker of the same volume. After the treatment, they were washed three times in sterile water for 15 minutes with 200 ml portions of water in the same glass. The explants were then divided into single-node segments of 1 cm and planted in 20 ml tubes with 7 ml of nutrient medium. To initiate shoots, WPM medium was used with the addition of 30 g/l sucrose, 6 g/l agar, and 0.5 mg/l zeatin. The zeatin solution was filtered using a 0.22 μm membrane filter before being added to the medium after autoclaving. The pH of the medium was adjusted to 5.0 using a 1 M HCl solution (Borsai et al., 2019). The medium was then autoclaved at 121°C for 20 minutes. The explant tubes were placed

in a growth room. Growth room conditions were maintained at 60% humidity, 25 ± 2 °C, 16 h photoperiod with PPFD 50 µmol m^{-2} s^{-1} using cold white fluorescent lamps (6 500 K).

2.2. Multiplication and elongation stages

After six weeks, sterile axillary shoots were selected from the culture and transferred to the medium for multiplication. For this, the WPM medium was used with the same composition as in the initiation step, but the zeatin concentration was increased to 1 mg/L. After 6 weeks, axillary and adventitious shoots obtained by propagation were transferred to a hormone-free WPM medium for shoot elongation. After 4 weeks of cultivation in the elongation step, the shoots were used in bioreactor experiments and also transferred to a semi-solid medium as a control of the experiment.

2.3. Bioreactor culture

Two types of TIS bioreactors were tested: the Plantform (Adelberg et al., 2004; Welander et al., 2014) and a rocker-type bioreactor in the author's modification. The Plantform bioreactors were purchased from the Plant Form Company (Ireland) and used without modification.

The rocker bioreactor was manufactured according to the principle diagram given in the work of Georgiev et al. (2014), with improvements. The culture box was constructed from a transparent polyethylene container measuring 180 mm × 160 mm × 90 mm. This was divided into two equal parts, with one containing explants and the other a liquid nutrient medium. The bioreactor lid was equipped with ventilation holes protected by foam filters. The culture box was fixed on a plastic plane, with the help of which the angle of inclination could be changed. This ensured that the explants were periodically flooded. To change the slope of the platform, a simple and reliable pneumatic device was developed, controlled by a timer.

The bioreactors were filled with 2-3 node segments cut from shoots that had undergone the elongation stage in vitro. The composition of the medium for the cultivation of blueberries in bioreactors was taken from the protocol of Clapa et al. (2022) and included the following components: WPM salts, 100 mg/l myo-inositol, 2 mg/l thiamine, 1 mg/l pyridoxine, 1 mg/l nicotinic acid, 1 mg/l zeatin, 30 g/l sucrose. The pH of the medium was adjusted to 5.0. Autoclaving mode: 121 degrees for 20 minutes. 510 ml of medium was poured into Plantform bioreactor culture boxes and 60 explants were placed. 170 ml of medium was poured into rocker bioreactor culture boxes and 20 explants were transferred. Growth room conditions were maintained at 60% humidity, 25 ± 2 °C, 16 h photoperiod with PPFD 100 µmol m^{-2} s^{-1} using cold white fluorescent lamps (6 500 K).

After 8 weeks of cultivation, the results of plant growth and development were assessed, in particular shoot length, number of nodes per shoot, and multiplication rate. The multiplication rate was calculated by dividing the number of shoots produced by the number of explants inoculated.

In order to obtain the maximum effect from the operation of the bioreactors, it was necessary to find the optimum immersion mode for each model. To this end, three immersion modes were tested: 2, 4, and 6 times a day for 2 minutes. The efficiency of the bioreactors was evaluated after 8 weeks of cultivation using the following indicators: number of shoots per explant, and degree of their hyperhydricity. The degree of vitrification was determined visually. Both versions of the experiment were carried out on a medium with the same composition as above. In the control version of both experiments, the node segments of blueberry shoots were cultivated on a medium with 6 g/l agar of the same composition.

2.4. Ex vitro rooting and acclimatization

Shoots obtained on a semi-solid medium after the elongation stage and in both types of bioreactors were rooted ex vitro. In this way, the rooting phase was combined with the phase of acclimatization. The shoots were planted in 100 ml containers filled with a mixture of peat and perlite in a ratio of 5:1. The cultures were maintained in a climatic greenhouse at 100% humidity, 25 ± 2 °C, 16 h photoperiod with PPFD 250 µmol m^{-2} s^{-1} using cold white fluorescent lamps (6 500 K). After 4 weeks of rooting, the humidity in the greenhouse was gradually reduced until the plants had adapted to room conditions. After 8 weeks of rooting combined with acclimatization, the percentage of rooted acclimatized shoots out of the total number of shoots planted was determined.

2.5. Statistical analysis

All data obtained during this study were statistically analyzed using the Past 3.16 software package (Hammer et al., 2001) and an online software resource. Website www.socscistatistics.com [accessed 20 December 2023].

Each experiment was performed in triplicate as an independent experiment. 20 explants or shoots were used for each treatment. Normality was tested using the Shapiro-Wilk test. For multiple group comparisons, an ANOVA test was performed and significant differences between means were calculated using Tukey's HSD test.

3. Results

Optimal bioreactor operation mode: In an experiment to determine optimal bioreactor operation, flooding blueberry shoots for 2 minutes every 6 hours was found to be best. The multiplication rate was 8.9 and the vitrification rate was 2.4% on average. Flooding the shoots every 4 hours gave a multiplication rate of 7.5 and a vitrification rate of 3.5%. A further increase in the frequency of flooding (every 2 hours) resulted in an increase in vitrification (6.1%) and a slight decrease in the multiplication factor (7.3). The differences in reproduction coefficients in the last two versions of the experiment are not statistically significant at $p > 0.05$ (Figure 1).

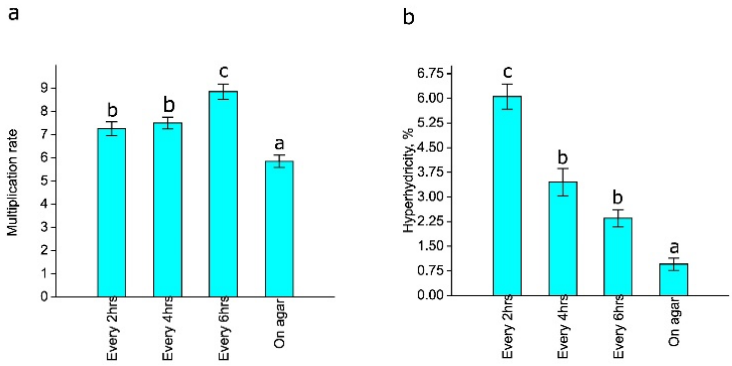

Figure 1. Comparison of rocker bioreactor operating modes for multiplication rate (a) and hyperhydricity (b). The control is a shoot culture on agar. All data are presented as mean ± standard error of the mean (SEM). Different letters indicate significant differences by Tukey's test at $p < 0.05$.

Comparison of bioreactor efficiency: After 8 weeks of blueberry cultivation in two types of bioreactors, the highest number of nodes was formed in the Plantform bioreactor (19.8). In the rocker bioreactor, an average of 16.1 nodes were formed per shoot, which was significantly lower ($p < 0.01$). In the control variant of the experiment on a semi-solid medium, an average of 9.9 nodes were formed per shoot, which is significantly lower than in the previous two variants ($p < 0.001$) (Figure 2a). The average shoot length in the Plantform bioreactor (6.5) was higher than in the rocker bioreactor (5.4) at $p < 0.05$. Compared to the control (3.7), the average shoot length was significantly higher in the Plantform system ($p < 0.001$), while in the rocker bioreactor, this indicator was not significantly different from the control ($p > 0.05$) (Figure 2b). The highest multiplication rate was observed in the Plantform bioreactor (12.5), while it was significantly lower (8.8) in the rocker bioreactor ($p < 0.001$). On the agar medium, this indicator was significantly lower (5.9) than in both types of bioreactors ($p < 0.001$) (Figure 2c).

Rooting together with acclimatization for 8 weeks under ex vitro conditions showed excellent results. After all in vitro cultivation methods, about 90% of the shoots were rooted and acclimatized with $p > 0.05$ (Figure 2d).

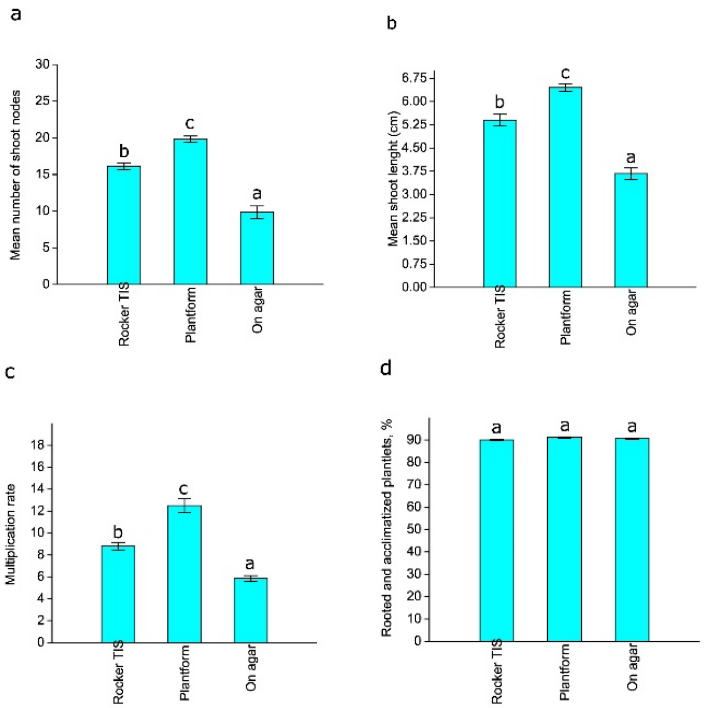

Figure 2. Comparison of the operation of a rocker bioreactor and a platform bioreactor according to the following indicators: the number of nodes of the shoots formed (a), the length of the shoots (b), the multiplication rate (c), the proportion of rooted and acclimatized regenerants obtained in different ways (d). All data are presented as mean ± standard error of the mean (SEM). Different letters indicate significant differences by Tukey's test at $p < 0.05$.

The blueberry culture obtained in different bioreactors and on semi-solid medium is shown in Figure 3.

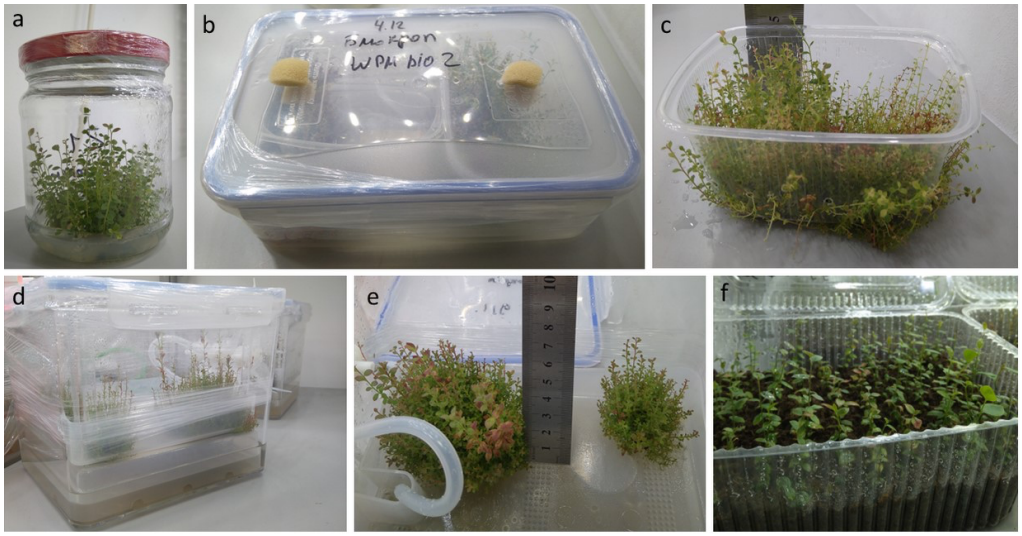

Figure 3. Culture of *Vaccinium corymbosum* 'Bluecrop' after 8 weeks of cultivation: on agar medium (a), in a rocker bioreactor (b, c), in a Plantform bioreactor (d, e), during rooting and acclimatization (f).

4. Discussion

The high efficiency of in vitro plant cultivation using temporary immersion bioreactors has been demonstrated by many researchers (Ahmadian et al., 2017; Jing et al., 2024; Sereda et al., 2024). Mass

micropropagation of berry crops was significantly faster in liquid media in bioreactors than in semi-solid media (Debnath, 2017; Bošnjak et al., 2021; Le et al., 2023). In studies with cranberries, the authors showed that after 8 weeks of cultivation, shoot reproduction was 2-3 times higher in liquid medium than in semi-solid medium (Arigundam et al., 2020). Clapa et al. (2022) reported that the highest reproduction rates of Duke blueberries were recorded in the TIS bioreactor compared to cultivation in semi-solid media. A wide variety of bioreactor models are available today. The choice of model depends on the biological crop and the end goal of cultivation (Murthy et al, 2023).

Obviously, to achieve the maximum effect of blueberry propagation in bioreactors, it is necessary to use the most economical TIS with the minimum risk of loss of the resulting shoots. A reduction in shoot quality can usually be caused by contamination of bioreactor parts and components or by vitrification of the shoots. The risk of shoot contamination increases with the complexity of the bioreactor design. A complex aeration system consisting of filters and air supply pipes, as well as the presence of additional media reservoirs, can lead to infection. This ultimately increases the cost of the plant material. On the other hand, simplification of the design due to the aeration system can lead to an increase in the number of vitrified shoots, as poor aeration of the culture vessels often leads to vitrification of the shoots (Sanyürek et al., 2021; Polivanova and Bedarev, 2022).

Most TIS bioreactors have a complex aeration and nutrient delivery system. Combining dozens of culture boxes in one system also increases the risk of contamination. One such model is the Plantform bioreactor. Undoubtedly, the TIS Plantform is a highly effective system for in vitro plant micropropagation, which has been proven by many works (Almusawi et al., 2017; Aka Kaçar et al., 2020). However, it was decided to test a rocker bioreactor, which is a simple and reliable system, in the modification to reduce the economic cost and the risk of contamination. Analysis of literature data shows that rocker bioreactor systems, such as BioMint, are used much less frequently than other systems. However, the application of this TIS has yielded good results (Etienne and Berthouly, 2002; Robert et al., 2006; Belo-Belo, 2010; Peña-Ramírez et al., 2010). It is believed that the diffusion of this type of bioreactor is hindered by a complex system of inclined platforms driven by an electric motor. In this study, the electric drive system was replaced by a simple pneumatic system that varied the inclination of the platform and was programmed with a household timer.

An experiment with different operating modes of a rocker-type bioreactor showed that with a flooding frequency of every 6 hours for 2 minutes, the maximum number of shoots is formed at a low level of vitrification. In this case, the value of the multiplication coefficient is higher than in the control with a semi-solid medium and decreases with increasing frequency of flooding. In addition, the degree of shoot vitrification directly depends on the frequency of flooding. The water content of the shoots is associated with insufficient aeration of the culture boxes, which leads to an excessive increase in humidity and the accumulation of ethylene and CO_2. As a result, a number of physiological parameters decrease (photosynthesis, amino acid synthesis, etc.), which has been repeatedly reported in the relevant literature (Kevers et al., 2004; Gao et al., 2018; Polivanova and Bedarev, 2022). The frequency and duration of flooding also depend on the type of digester and crop. In studies with blueberries grown in Planform TIS, the authors chose an optimal frequency of flooding every 4 hours for 1 minute, with forced aeration every hour for 4 minutes. At the same time, the authors did not note the vitrification of the shoots and obtained high rates of reproduction (Clapa et al., 2022).

The number of nodes per shoot and the shoot length are crucial indicators in the micropropagation process of blueberries. Cloning occurs in segments consisting of several nodes, each about 2 cm in length (Wang et al., 2023). A long, multi-node shoot can be divided into many segments, increasing the productivity of the entire propagation cycle. The number of shoot nodes in Rocker bioreactors was lower than in Plantform bioreactors but higher than in semisolid media. This difference can be attributed to the inadequate aeration in rocker-type bioreactors, as with the multiplication rate.

Blueberry rooting is typically conducted as a separate in vitro stage using auxins and activated carbon (Ruži et al., 2012). The combination of rooting and acclimatization processes under ex vitro conditions maximizes the yield of rooted and acclimatized regenerants (Osrolucka et al., 2007; Vescan et al., 2012). The experiment results indicate that the method of propagation did not affect the degree of rooting and acclimatization of regenerated plants. Around 90% of shoots obtained in a rocker-type bioreactor were successfully adapted to ex-vitro conditions.

Conclusion

The micropropagation of blueberries using a rocker-type bioreactor represents a promising approach for the rapid production of high-quality planting material. This method has been demonstrated to be more productive than growing on a semi-solid medium, as evidenced by higher reproduction rates, average number of nodes per shoot, and average shoot length. By comparison of the operational characteristics of a rocker bioreactor and a Plantform, bioreactor was observed that the micropropagation efficiency of a rocker bioreactor is inferior to that of the Plantform system. This is due to insufficient aeration, which increases the degree of vitrification of shoots. However, the slower rate of reproduction in the rocker bioreactor is compensated by the significantly lower cost of this system. In addition, the study demonstrated that shoots from a rocking-type bioreactor are capable of taking root and acclimatizing ex vitro in a single operation, with up to 90% of seedlings ready for planting in open ground. Future research will be conducted to enhance the efficiency of blueberry micropropagation in rocker-type bioreactors to implement this technology in mass production.

Ethical Statement

Ethical approval is not required for this study because given that only plant specimens were involved.

Conflict of Interest

The author declare that there are no conflicts of interest.

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Author Contributions

The author confirms sole responsibility for the following: study conception and design, data collection, analysis and interpretation of results, and manuscript preparation.

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