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ORIGINAL ARTICLE

Optimization of blackberry clonal micropropagation

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One of the modern methods widely used in the propagation of various garden crops is clonal micropropagation. Research in this direction is ongoing to optimize each stage of reproduction. The paper presents the results of a study of the clonal micropropagation of blackberry varieties Brzezina of Polish selection. For clonal micropropagation, sterile BlackBerry microshoots were used. In order to induce adventitious and axillary shoot formation, we tested various variants of culture medium: hormone-free medium, mediumsa with growth regulators (6-benzylaminopurine, β-indolyl-3-butyric acid, gibberellic acid). It was revealed that blackberry microshoots can take root on medium without growth regulators, but the resulting roots are thin, do not have root hairs. Such microshoots does not adapt to *ex vitro* conditions. A high reproduction rate (96%) was observed when rooting blackberry microshoots in a culture medium ½ MS + 2.0 μM IBA. Adaptation of regenerated plants to *ex vitro* conditions is more effective (97% survival rate) on a sand + vermiculite substrate (3: 1).

Key words: Blackberry, Multiplication, Rooting *in vitro*, Adaptation *ex vitro*

Introduction

Blackberry is a natural multivitamin complex (Hwida et al., 2018), which allows it to be widely used in the food industry, in medicine for the prevention and treatment of cancer and cardiovascular diseases (Eyduran et al., 2008; Muhammad et al., 2014; Radocaj et al., 2014). Blackberry is not of great industrial importance in the European Union, although it is bred as a market culture in America (Clark, Finn, 2014). At present, modern varieties of blackberries have been created, which are distinguished by their unshipability, high productivity, and large-fruited. Traditional methods of breeding blackberries in the Altai territory are limited by climatic features, so the use of alternative methods of production of planting material of blackberries is relevant. Clonal micropropagation of blackberries requires optimization of all stages of *in vitro* cultivation for each particular variety, as noted in many authors (Skovorodnikov et al., 2015; Pelizza et al., 2016; Muratova, 2017; Ivanova-Hanina, 2014, 2018; Makarov, 2019; Kefayeti et al., 2019). The purpose of our work was to optimize the clonal micropropagation of blackberries.

Materials and Methods

The studies were conducted in the laboratory of biotechnology and cytology of Federal Altai Scientific Centre of AgroBioTechnologies. The object of the study were sterile microshoots of the besshipnoy blackberry varieties Brzezina Polish selection, taken from previously cultured *in vitro* plants. The main method of blackberry clonal micropropagation was the induction of adventitious and axillary shoot formation. For the study, we have compiled various variants of culture media according to the recipe Murashige–Skoog (MS) (Murashige, Skoog, 1962) and Quoirin–Lepoivre (QL) (Quoirin, Lepoivre, 1977). As a control, the hormone-free MS medium was used (Table 1). Growth regulators were added to other culture media: 6-benzylaminopurine (BA), *β*-indolyl-3-butyric acid (IBA), gibberellic acid (GA_3). In each experiment, from 15 to 30 explants were used.

Table 1. Variants of the culture medium for blackberry clonal micropropagation.

№	The composition of the culture medium
Control	**MS**
1	MS+2 Fe+2.5 μM BA
2	MS+2 Fe+2.5 μM BA +0.5 μM IBA +1.0 μM ГК
3	MS+2.5 μM BA
4	MS+2 Fe +2.5 μM BA +0.5 μM IBA
5	MS+2 Fe+2.5 μM BA, replaced $CaCl_2$ on $Ca(NO_3)_2$
6	QL+2.5 μM BA

For rooting, the culture medium MS (full and half composition) with an IBA concentration of 0.5 to 2.0 μM was used (Table 2).

Table 2. Variants of the culture medium for rooting microshoots blackberry.

№	The composition of the culture medium
Control	**MS**
1	½ MS+2.0 μM IBA
2	½ MS+1.5 μM IBA
3	½ MS+0.5 μM IBA

Growth regulators BA, IBA were added to the medium before autoclaving, GA - after autoclaving. Culture medium was autoclaved at 1 atmosphere for 20 minutes. The pH of the medium was measured before sterilization. The cultivation of blackberry microshoots was carried out under the following conditions: 16-hour photoperiod; illumination – 2000–3000 lux; constant temperature – 24±1°C. At the adaptation stage, two versions of a sterile substrate were used: 1) sand, 2) sand + fine fraction vermiculite in a ratio of 3:1. The substrate was autoclaved at 2 atmospheres for 1 hour. The cooled soil was placed in plastic glasses in which rooted regenerated plants were transplanted. To create 100% humidity, the plants were covered with caps. On the 45th day of cultivation, the number of shoots formed, the average length, the presence of roots and callus, and the morphological features of microshoots were taken into account. The rooting results were evaluated on the 7th, 14th and 21st day according to the following parameters: the number of microshoots (%), the number of roots, the average root length (mm), the presence or absence of root hairs, the presence or absence of callusogenesis, features morphological signs of shoots (discoloration, leaf size). The experimental data were statistically processed using the Microsoft Office Excel 2016 application software package. The reliability of the differences was determined by Student t-test at $p \leq 0.05$.

Results and Discussion

In the control variant, induction of adventitious and axillary shoot formation was not observed. A high breeding rate was obtained on medium №1 and №3. The number of shoots per explant ranged from 14 to 28, that was 1.5-2.0 times more than on medium № 6 (Figure 1).

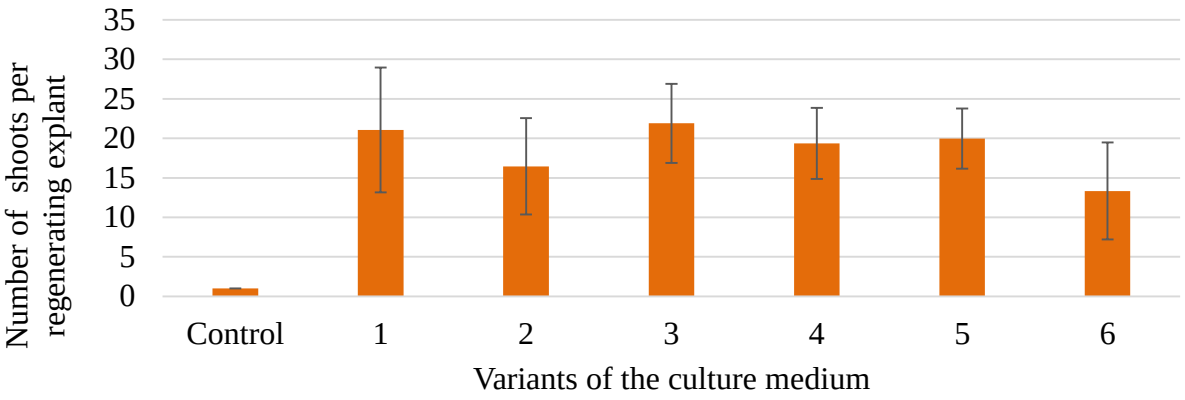


Figure 1. The influence of the composition of the culture medium on the adventive shoot formation of Brzezina blackberry.

There was no significant difference in the breeding coefficient of blackberries on medium №1 and №3, although they differed in iron content (2:1). In culture medium №5, we replaced $CaCl_2$ with $Ca(NO_3)_2$, which also did not contribute to an increase in the breeding coefficient of blackberries. The addition of IBA to culture medium №4 did not affect the reproduction rate. According to studies L.V. Ivanova-Hanina (2014), D.G. Shornikov et al., (2010), the inclusion of GA_3 in the culture medium has a positive effect on the reproduction rate of blackberries. Our studies showed that the addition of GA_3 to culture medium №2 and №4 significantly reduced the reproduction rate. According to some researchers (Solovyh, Muratova, 2011), blackberries are not too demanding on the mineral composition of the medium. In our studies, an analysis of the results obtained on culture medium differing in mineral composition revealed a preferable use of MS culture medium compared to QL. An important indicator at the stage of multiplication is the length of the shoots formed. For intensive growth of new microshoots, the apical stem meristem is removed or cytokinins are added to the culture medium. The interaction of phytohormones with cytokinins inhibits the growth of the apical kidney and stimulates the development of axillary kidneys (Makarov, Kuznecova, 2017). However, with an increased concentration of cytokinins, not only an increase in the multiplication coefficient can be observed, but also a decrease in the length of the shoots. To avoid this process, it is recommended to use low concentrations of cytokinin – 0,5–1,0 μM (Ivanova-Hanina, 2018). The shoots growing on the control medium were long, had roots, but there was no organogenesis (Figure 2). When reducing the nutrient base of MS by 2 times and adding auxin at a concentration of 0.5–1.5 μM (culture medium №2 and №3), the best results were obtained.

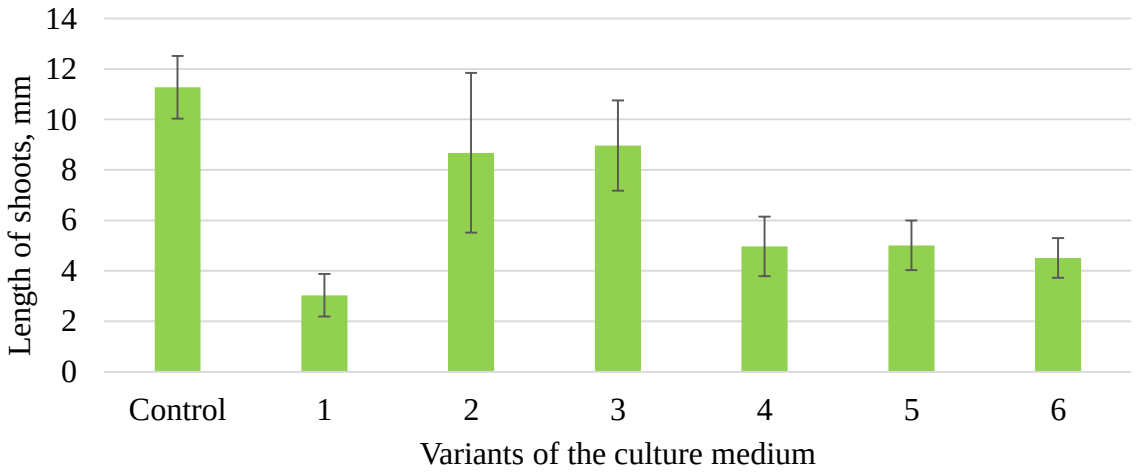


Figure 2. The effect of medium composition and IBA concentration on the length of blackberry regenerants in culture *in vitro*.

Modification of the mineral base of culture medium for certain plant species can give good results. For example, for raspberries and raspberry-blackberry hybrids, it was effective to double the content of chelated iron in the culture medium (Solovyh, Muratova, 2011). According to some authors (Shornikov et al., 2010; Muratova, 2017), such modifications contribute to the active growth of blackberry shoots and provide high breeding efficiency. The increased iron content stimulates the formation of well-developed shoots with large dark green leaves. In our experiments, a twofold increase in the concentration of iron on culture medium №1 inhibited the growth of shoots by a factor of 3. Regenerants had short shoots with small leaves unsuitable for rooting (Figure 3). In 50% of shoots at the base, growth of dense callus was observed.

№1 (MS+2 Fe+2.5 μM BA) №3 (MS+2.5 μM BA)

Figure 3. The influence of the composition of the culture medium on the development of blackberry microprobe at the stage of multiplication.

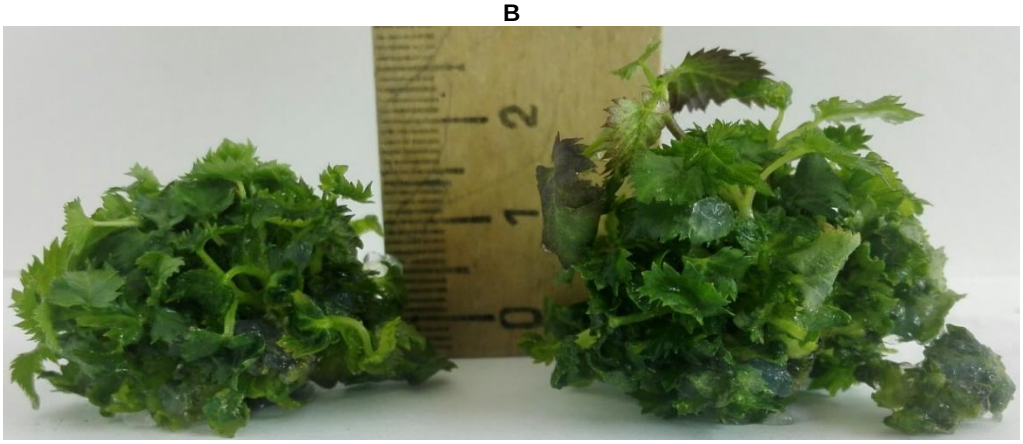
Further modification of the culture medium with the addition of auxin or auxin with GA_3 together with BA positively affected the results of clonal micropropagation of blackberries. Thus, the presence of IBA in medium №2 and №4 (Figure 4A) and the replacement of $CaCl_2$ with $Ca(NO_3)_2$ in medium №5 (Figure 4B) contributed to a 1.5-fold increase in the average length of microprobe compared to medium №1. The presence of GA_3 in nutrient medium №2 also had a positive effect on the growth of blackberry microshoots.

A

№ 2 № 4

MS+2 Fe+2.5 μM BA+0.5 μM IBA+1.0 μM GA_3 MS+2 Fe +2.5 μM BA+0.5 μM IBA

B



№ 1 № 5

MS +2 Fe+2.5 μM BA (MS+2.5 μM BA+replacement $CaCl_2$ with $Ca(NO_3)_2$)

Figure 4. The influence of the composition of the culture medium on the development of blackberry microshoots at the stage of multiplication.

A comparative analysis of the influence of the mineral composition of the medium (№3 and №6) on the growth and development of blackberry shoots showed that the length of shoots on MS medium was 2 times longer than on QL medium (Figure 5). In addition, on the MS medium, the formed shoots had large leaves, at the base of the shoots, a dense roller with numerous apical meristems was observed. On QL medium, microshoots were shorter, had small leaves, and a dense callus formed at the base. Separate shoots formed single roots 25–35 mm long. Thus, at the stage of multiplication on culture medium №3 (MS+2.5 μM BA), regenerants were obtained in the optimal ratio of quantity/quality.

№ 6 (QL+2.5 μM B) № 3 (MS+2.5 μM BA)

Figure 5. The influence of the composition of the culture medium on the development of blackberry microshoots at the stage of multiplication.

In vitro microclonal propagation of plants is carried out in several stages. After receiving a sufficient number of shoots, they are rooted. At the rooting stage, it is necessary to stimulate the process of rhizogenesis, since the adaptation of regenerants to growth in conditions *ex vitro*. Root formation is induced by a change in the hormonal and mineral composition of the nutrient medium. Blackberries can root in hormone-free environments, but auxins must be used to obtain a good root system (Demenko et al., 2014). The choice of auxin and its concentration affect the morphological features of the root system of blackberry microshoots. Many authors point to the effectiveness of using IBA as an inducer of rhizogenesis of blackberry microshoots (Tavartkiladze, Vechernina, 2007; Shornikov et al., 2010; Matushkin, Yarmolenko, 2017; Muratova, 2017). The results of rooting the blackberry microshoots were evaluated on the 7th, 14th and 21st days. After a week, only a single microshoots formed 1–2 roots. The data obtained on the 14th and 21st days are presented in Figure 6.

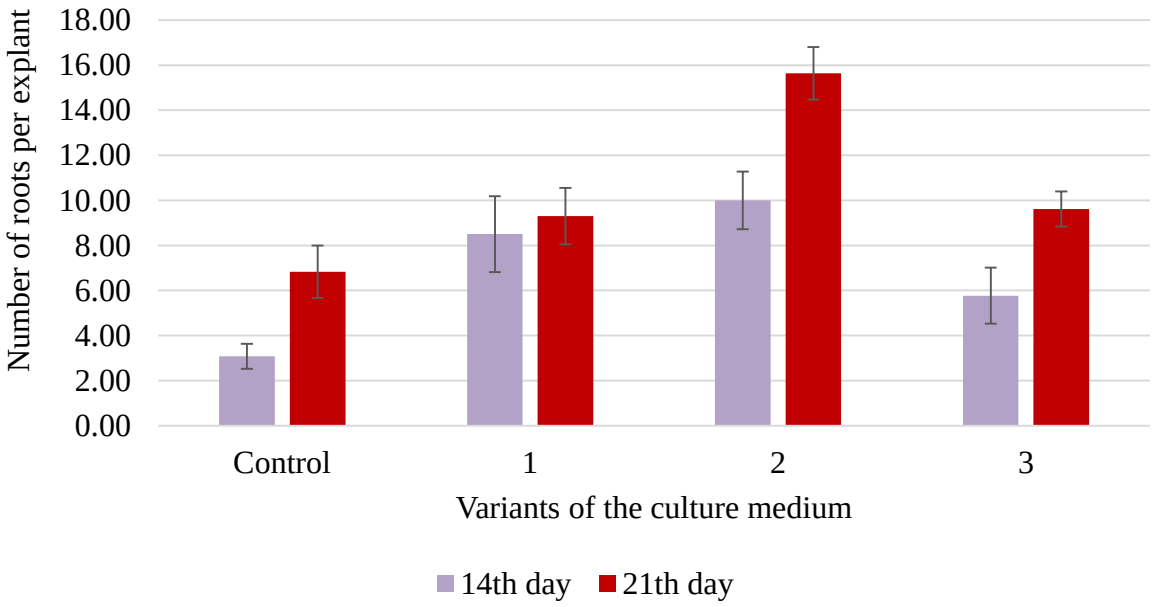


Figure 6. Effect of IBA concentration on blackberry rooting in culture *in vitro.*

In the control hormone-free variant, after 14 days, up to 85% of microshoots took root. On each microshoot 3-4 roots were formed, 8–9 mm long, without root hairs. In the control hormone-free variant, after 14 days, up to 85% of microshoots took root. On each microshoot run, 3-4 roots were formed, 8–9 mm long, without root hairs (Figure 7). On medium №1 18% less shoots took root than in control. On each microshoot, 8–9 roots 4–6 mm long were formed. Root hairs were present in 50% of the roots. A dense callus formed on unrooted microshoots. A decrease in the amount of auxin in the composition of the culture medium by 0.5 μM increased the induction of rhizogenesis to 96%. All microshoots had 10–12 roots (11–12 mm long) with root hairs.

Figure 7. Blackberry regenerants on culture medium at the rooting stage (14th day)

A 1 μM decrease in auxin concentration inhibited microshoots rhizogenesis by 5%. Rhizogenesis was observed in 91% of microshoots. The number of roots on the microshoots decreased by 2 times, root hairs were present in 30% of the roots (Figure 8).

Figure 8. Blackberry regenerants on culture medium №3 at the rooting stage (14th day)

On the 21st day in the control, the number of roots increased 2 times (Figure 6), and their length increased 5-6 times. The roots were thin, long, dark in color, without root hairs. Such roots are not able to actively absorb and conduct water with minerals, which was the reason for the death of regenerants at the stage of adaptation in conditions *ex vitro*. On medium №1, 9–10 roots 9–10 mm long were formed on each microshoot (Figure 9). Root hairs were present in 65% of the roots. The formation of dense callus at the base of unrooted shoots was noted. On medium № 2, 15–16 roots 16–17 mm long were formed on each microshoot. Root hairs were present on all roots. On medium № 3, 9–10 roots 13–14 mm long were formed on each microshoot. Root hairs were found only in 33% of the roots, the shoot height was the lowest.

Figure 9. Blackberry regenerants after cultivation on different culture media for rhizogenesis (№ 1–3 from left to right, 21th day)

Thus, the maximum efficiency of rhizogenesis in microclones of blackberry varieties Brzezina was obtained on a culture medium ½ MS + 1.5 μM IBA. The maximum yield of rooted shoots with optimal root length (16–17 mm) and root hairs was 96%.

Adaptation to *ex vitro* conditions is the final stage of the clonal micropropagation process. Successful adaptation depends on a number of factors: on the type of plant, the physiological state of the plant; composition, sterility and humidity of the substrate, the intensity and frequency of lighting; humidity and room temperature (Skovorodnikov et al., 2012). To date, there is no universal technology for the adaptation of regenerants of different plant species to non-sterile conditions. It is very important to create optimal conditions under which the aboveground and underground parts would develop successfully (Zlenko et al., 2005). A large role is played by the choice of substrate, which should be moisture-absorbing and breathable. In plants cultivated *in vitro*, the activity of the stomatal apparatus is impaired, since they are in conditions of 100% humidity. During *ex vitro* adaptation, the relative humidity should be reduced gradually (Vechernina, 2009). For adaptation, we used 2 variants of a sterile substrate: 1) sand, 2) sand+vermiculite (ratio 3:1). A total of 60 regenerated plants were planted (30 plants on each type of substrate). For 10 days, a constant humidity of 85±5% was maintained by small-droplet spraying. Then the humidity gradually decreased to 70%.

Each week, adaptable plants were irrigated with complex mineral fertilizers. Regenerant plants were measured on day 30 (Figure 10). The survival rate of microshoots on the sandy substrate was 70%, and on the substrate sand+vermiculite – 97%. This can be explained by the fact that the sandy substrate dries quickly and requires frequent watering. Vermiculite holds water well, promotes aeration of the substrate, and inhibits the development of molds. It is resistant to high temperatures, does not rot and improves the effect of fertilizers. S.A. Muratova (2008) notes that microplants obtained *in vitro* culture have a special growth dynamics. In the first 14–20 days after planting in *ex vitro* conditions, regenerated plants experience severe stress; there is no growth process during this period. Further, the growth rate increases, new leaves begin to form.

А **Б**

Figure 10. Adaptation of blackberry regenerated plants to conditions *ex vitro* (30th day):
А – sand, **Б** – sand+vermiculite (3:1)

In our experiments *ex vitro*, regenerated plants on different substrates after 30 days did not differ in height (31–32 mm) (Figure 11).

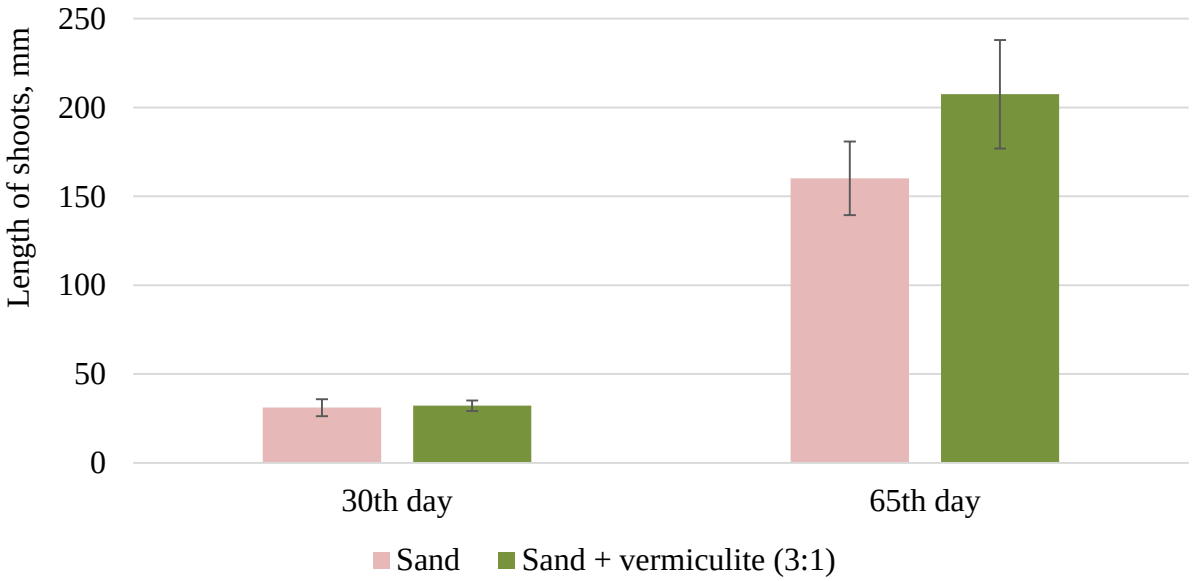


Figure 11. The length of the blackberry shoots at the adaptation stage (30th day and 65th day):
А – sand, **Б** – sand+vermiculite (3:1)

On day 65, the length of plant shoots on the sand + vermiculite substrate was 40–50 mm longer (Figure 12).

А **Б**

Figure 12. Blackberry regenerant plants in conditions *ex vitro* (65th day): **А** – sand, **Б** – sand + vermiculite (3:1)

Thus, studies on the adaptation of Brzezina blackberry microplants to non-sterile conditions showed that the use of a two-component substrate (sand + vermiculite) in a 3:1 ratio contributed to an increase in the survival rate, intensive growth and development of plants.

Conclusion

1. In the case of clonal micropropagation of Brzezina blackberry, the highest reproduction rate (21.90 ± 2.39) and the longest shoot length (8.96 ± 0.86 mm) were observed on the culture medium MS + 2.5 μM BA.
2. The maximum rooting of microshoots (96%) was observed on a culture medium ½ MS + 1.5 μM IBA. On each micrun run, an average of 15.64 ± 0.56 roots with a length of 16.02 ± 0.54 mm was formed.
3. To adapt regenerated plants to *ex vitro* conditions, it is better to use sand + vermiculite in a ratio of 3: 1 as a substrate for Brzezina blackberry (microshoot survival rate is 97%, shoot height is 207.44 ± 15.58 mm).

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