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ORIGINAL ARTICLE

Biotechnological approaches to the reproduction of remontant forms of red raspberry

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Rubus idaeus L. is one of the oldest and most widespread berry crops, which is cultivated for the excellent taste as well as for medical and dietary properties of berries. *Rubus* fruits contain a significant amount of vitamins A and C, anthocyanins, polyphenolic substances, which determines their high antioxidant activity. Remontant red raspberry forms are able to berry on annual shoots in the second half of summer, which extends the term of consumption of fresh berries by 1.5-2 months. However, many forms of remontant raspberry have a low potential for vegetative propagation compared to summer varieties, which makes them difficult to reproduce and to use in the breeding process. We investigated the possibility of increasing the efficiency of *in vitro* micropropagation of a remontant raspberry variety 'Biryulevskaya'. The effects of 6-benzylaminopurine (6-BAP) at concentrations of 0.5–3 mg l^{-1} and thidiazuron (TDZ) at concentrations of 0.05–0.2 mg l^{-1} as well as doubled and tripled iron chelate Fe-EDTA (Ferric ethylenediamine-tetraacetic acid) doses were studied. We found the adding 1.0 mg l^{-1} 6-BAP to the MS medium containing a triple dose of iron chelate, provided intensive proliferation of high quality adventitious shoots.

Keywords: Red raspberry; Remontant form; *In vitro* reproduction; Plant growth regulators; Iron chelate

Introduction

The raspberry (*Rubus idaeus* L.) belongs to the Rosaceae family. It is one of the oldest and most widespread berry crops, which is cultivated for the excellent taste as well as for medical and dietary properties of berries. Raspberry enters the fruiting season very early. The next year after spring planting, it already gives the first berries, and after another year, the yield is greatly increased. Berries are consumed fresh. They serve as valuable raw materials for the food and confectionery industry. In addition, they are used for drying and freezing. When frozen, raspberry fruits retain their taste, aroma and all useful properties. Rubus fruits contain a significant amount of vitamins A and C, anthocyanins, polyphenolic substances, which determines their high antioxidant activity (McGhie et al., 2002; Moyer et al., 2002; Çekiç and Özgen, 2010; Lee et al., 2012). Raspberry is an effective medicine against many diseases. Due to the valuable biochemical composition of the berry, it is successfully used for the treatment and prevention of cardiovascular, gastrointestinal, skin and other diseases, as well as vitamin deficiencies. In addition, it is shown that appreciable amounts of polyphenols and dietary fiber in red raspberry suggest metabolic benefits for people at risk for diabetes mellitus (Xiao et al., 2017).

Remontant raspberry forms are unique berry plants, which, unlike ordinary raspberry varieties, are capable of berrying on annual shoots in the second half of summer. Cultivation of remontant raspberry varieties, in addition to varieties of the usual type, allows us to extend the period of consumption of fresh berries by 1.5-2 months. When selecting varieties of different ripening periods, you can create a continuous conveyor of fresh raspberry berries from late June to October (Ivanova-Khanina, 2014). The best of modern varieties of remontant type have high yield, large-fruited, ecological adaptability, suitable for low-cost technologies of cultivation (Evdokimenko, 2009). However, many forms of remontant raspberry have a low potential for vegetative propagation compared to summer varieties, which makes them difficult to reproduce and to use in the breeding process. The using the method of clonal micropropagation allows to solve the problem of accelerated reproduction of valuable breeding material. Compared with traditional methods of raspberry reproduction by layering and bush division as well as root, leafy and green cuttings (Dziedzic and Jagła, 2013; Marchi et al., 2018) this method has a number of undoubted advantages. The main ones are the high reproduction rate and the possibility of improving planting material from a number of harmful microorganisms, including viral infection (Tsao et al., 2000).

Over the past decades, numerous studies have been conducted in different countries to improve the method of clonal micropropagation in order to produce high-quality raspberry planting material. It is known that the coefficient of reproduction of plants in culture *in vitro* depends on the genotype, the composition of the nutrient medium, the physical conditions of cultivation, the stability of the reproduction process when subculturing shoots (Stoevska et al., 1995; Mezzetti et al., 1997; Tsao and Reed, 2002; Gajdosova et al., 2006; Zawadska and Orlikowska, 2006a; Clapa et al., 2008; Wu et al., 2009; Poothong and Reed, 2014, 2015; Hunková et al., 2016; Borodaeva et al., 2017). However, the biological features of the remontant forms of raspberries, associated with their complex interspecific origin, have led to the low effectiveness of the proposed biotechnological methods for the reproduction of raspberries at some stages of *in vitro* cultivation. In this regard, it became necessary to optimize the process of clonal micropropagation of remontant forms of raspberries. *In vitro* cloning involves several stages, the main of which are the introduction of explants into a sterile culture, micropropagation, *in vitro* rooting and adaptation of regenerants to *ex vitro* conditions. To increase the efficiency of the method, it is necessary to improve the technology for all of the above steps (James et al., 1980; Shornikov et al., 2010; Skovorodnikov et al., 2012; Clapa et al., 2013; Solovykh and Budagovsky, 2018; Lebedev et al., 2019). The aim of this work was to study *in vitro* growth and development of red raspberry microshoots at the stage of micropropagation.

Materials and Methods

The object of the study was the variety of red raspberry (*Rubus idaeus* L.) 'Biryulevskaya'. This variety was chosen due to their high yield, large-fruited, as well as the possibility of use in home gardens. Regenerant plants were placed on the Murashige and Skoog medium (MS) (Murashige and Skoog, 1962) with doubled and tripled iron chelate (FeEDTA) concentration supplemented with various growth regulators. The effects of cytokinins 6-benzylaminopurine (6-BAP) at concentrations of 0.5–3 mg l^{-1} and thidiazuron (TDZ) at concentrations of 0.05– 0.2 mg l^{-1} were studied. The control was a hormone-free medium with a single dose of iron chelate. Cultivation was carried out at 24 ± 1 ºC, in a photoperiod of 16/8 hours. The duration of the passage was 25-30 days. We estimated the number of shoots (pcs./explant); height of the shoot (mm); the number of leaves on the shoot (pcs.); presence of callus + \ -. The experiment was performed in 5 replicates. Five explants per each replicate were used. Mean values were compared according to least significant differences test (LSD) at P < 0.05. Statistical data processing was performed using the software package Microsoft Office Excel 2007. The studies were conducted in the Altai Center for Applied Biotechnology of the Altai State University (Barnaul, Russia).

Results and Discussion

The essential point of ensuring active proliferation of microshoots *in vitro* is the correct choice of cytokinin and its concentration. Previously, we found that 6-BAP and TDZ were the most effective growth regulators of the cytokinin series. In this experiment, we revealed that the MS medium with 1.0 and 1.5 mg l^{-1} of 6-BAP provided the highest reproduction rate of the 'Biryulyovskaya' variety (2.6 ± 0.3 and 2.7 ± 0.4 pcs./exp. respectively) (Table 1). The height of the shoot was 22.6 ± 2.4 and 21.7 ± 2.3 mm; the number of leaves on the shoot was 13.2 ± 0.9 and 12.9 ± 1.2 pcs. which was also the maximum result. Adding 6-BAP to the nutrient medium, regardless of the concentration, increased the number of shoots in comparison with the control variant. The use of higher doses of this cytokinin (2-3 mg l^{-1}) resulted in a decrease in the reproduction rate of remontant raspberry.

Table 1. Influence of cytokinins on *in vitro* reproduction, growth and development of microshoots of remontant red raspberry 'Biryulyovskaya'

Auxin	Concentration, mg l^{-1}	Number of shoots, pcs./explant	Height of the shoot, mm	The number of leaves, pcs./shoot	Callus
Control	0.0	1.2 ± 0.3^a	17.2 ± 2.1^a	6.1 ± 0.5^a	-
6-BAP	0.5	2.2 ± 0.2^b	19.5 ± 2.2^a	8.9 ± 0.9^b	-
	1.0	2.6 ± 0.3^c	22.6 ± 2.4^b	13.2 ± 0.9^c	-
	1.5	2.7 ± 0.4^c	21.7 ± 2.3^b	12.9 ± 1.2^c	-
	2.0	2.3 ± 0.1^b	22.4 ± 2.0^b	11.0 ± 1.3^c	-
	2.5	2.2 ± 0.2^b	18.4 ± 1.6^a	10.8 ± 0.9^c	-
	3.0	2.1 ± 0.1^b	17.6 ± 1.9^a	7.9 ± 0.6^a	-
TDZ	0.05	1.9 ± 0.2^a	15.4 ± 1.1^a	7.9 ± 0.6^a	-
	0.1	2.6 ± 0.4^c	18.8 ± 1.8^a	8.8 ± 0.7^b	+
	0.15	2.1 ± 0.2^b	19.3 ± 1.6^a	9.0 ± 0.8^b	+
	0.2	1.4 ± 0.1^a	19.1 ± 1.7^a	8.7 ± 0.8^b	+

Note: Data are in the form of mean ± SEM, and means followed by the same letter within the columns are not significantly different at P < 0.05.

In addition, when higher concentrations of 6-BAP were added to the nutrient medium (within 2.5–3.0 mg l^{-1}), we observed anomalous development of microshoots. Morphological disorders included the formation of twisted leaves, vitreous organs with signs of hyperwatering, shortened deformed stems. It is known that shoots with signs of vitrification during *in vitro* reproduction produce the same plants. They rarely take root and, as a rule, can only live *in vitro*. Even with a slight manifestation of the degree of vitrification, it is very problematic to transfer such plants to non-sterile conditions.

When used TDZ, almost the same multiplication rate was observed as with 6-BAP. The medium containing 0.1 mg l^{-1} of the hormone was the most productive. However, it was noted callus formation at the base of the shoot, which is undesirable during micropropagation, as it is difficult for nutrients to enter the plant. This is confirmed by a decrease in the height of the shoots and the number of leaves. Similar results were obtained by other authors (Kulkhanova et al., 2012). Zawadzka and Orlikowska (2006a) recommended TDZ at a concentration of 0.1 mg l^{-1} as the most effective cytokinin for direct regeneration of adventitious shoots from leaf explants. The authors found that at higher TDZ concentrations, the number of regenerated shoots did not increase, while excessive hyperhydricity and dying were observed.

One of the most important components of nutrient media is iron, because it participates in regulatory processes, in redox transformations and is a part of the coenzymes. Its role is extremely important for the chlorophyll biosynthesis. Therefore, iron deficiency strongly limits plant growth and provokes the development of chlorosis. It also changes the structure of chloroplasts and affects the efficiency of photosynthesis. Iron is most often used in the chelated form of Fe-EDTA (Ferric ethylenediamine-tetraacetic acid). Some early papers reported addition of a double amount of Fe-EDTA to MS or to Anderson medium (Anderson, 1980) to reduce chlorosis (Sobczykiewicz, 1984). Zawadzka and Orlikowska (2006b) used MS salts with $MgSO_4$ increased by 200% and Fe-EDTA increased by 50% as the basic medium for propagating various red raspberry varieties. In some varieties, these additives prevented chlorosis for up to 3-4 weeks. They also investigated the effect of Fe-EDDHA (Ethylenediamine di-2-hydroxy-phenyl acetate ferric) as an alternative to Fe-EDTA on the shoot multiplication and adventitious regeneration from leaf explants as well as on the rooting and acclimatization of 5 varieties of *R. ideaus.* The change in Fe-EDTA to Fe-EDDHA in the medium increased the availability of iron, which led to a decrease in tissue hyperhydration and an increase in the level of chlorophyll in the leaves, earlier and more abundant rooting, higher fresh and dry weights. As a result, they managed to significantly improve the quality of microshootings (Zawadzka and Orlikowska, 2006b, 2009).

We studied the effect of different doses of iron chelate on the effectiveness of clonal micropropagation of remontant red raspberry 'Biryulyovskaya'. Single, double and triple concentrations of this substance were added to the nutrient medium MS containing 1.0 and 1.5 mg l^{-1} of 6-BAP. The maximum multiplication rate was achieved with a combination of 1.0 mg l^{-1} cytokinin with a triple dose of iron chelate (Figure 1). The number of microshoots per explant averaged 4.2, varying from 3 to 5. Reproduction of remontant raspberry 'Biryulyovskaya' on MS medium with a triple dose of iron chelate and 1.5 mg l^{-1} 6-BAP was also successful, but somewhat inferior to the previous version. We managed to get an average of 3.6 microshoots per explant. It should be noted that with the increased iron content, well-developed shoots with large leaves of dark green color were formed (Figure 2). Shornikov et al. (2010) found that for raspberries and raspberry-blackberry hybrids a similar effect can be achieved by doubling the content of iron chelate in MS medium, while iron deficiency provoked the development of chlorotic shoots.

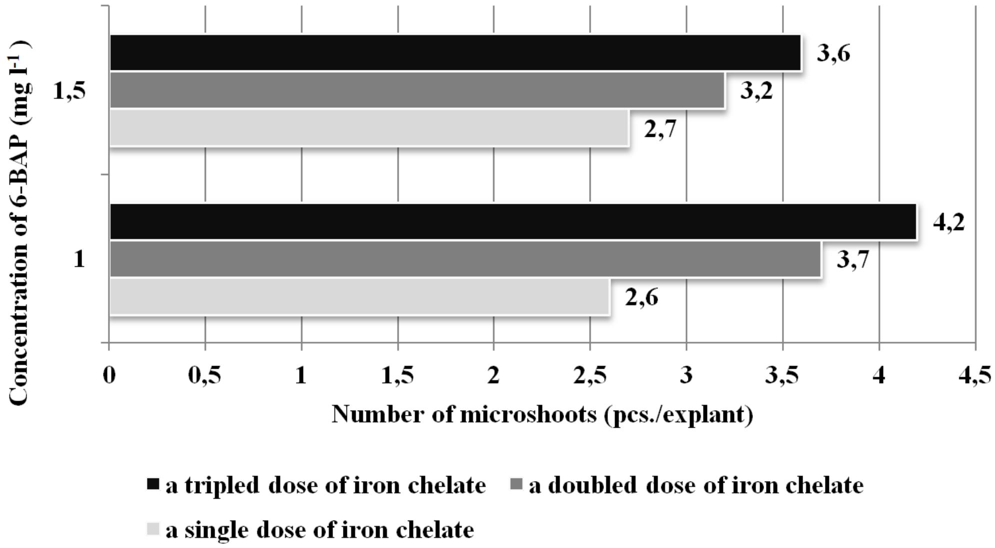

Figure 1. The influence of the concentrations of Fe-EDTA and 6-BAP in MS medium on *in vitro* reproduction of remontant red raspberry 'Biryulyovskaya'.

Figure 2. *In vitro* multiplication of remontant red raspberry 'Biryulyovskaya' on the MS medium with a tripled (left) and a single dose (right) of Fe-EDTA supplemented with 1 mg l^{-1} 6-BAP.

Conclusion

So we found the adding 1.0 mg l^{-1} 6-BAP to the MS medium containing a triple dose of iron chelate Fe-EDTA, provided intensive proliferation of high quality adventitious shoots of remontant red raspberry 'Biryulyovskaya'. The use of higher doses of this cytokinin (2–3 mg l^{-1}) resulted in a decrease in the reproduction rate of the variety. The medium containing 0.1–0.2 mg l^{-1} of TDZ induced callus at the base of the shoots decreasing their height and the number of leaves.

Conflict of Interest

The authors declare that they have no conflict of interests.

References

Anderson, W. C. (1980). Tissue culture propagation of red raspberries and black raspberries, *Rubus idaeus* and *R. occidentalis*. Acta Hort., 112, 13-20.

Borodaeva, Zh. A., Muratova, S. A., Kulko, S. V., & Tokhtar, L. A. (2017). Influence of various sources of carbohydrate nutrition on rhizogenesis of microcrops of berry crops under the *in vitro* conditions. Belgorod State University Scientific Bulletin. Biological sciences, 41, 25 (274), 21-35. (In Russian)

Çekiç, Ç., & Özgen, M. (2010). Comparison of antioxidant capacity and phytochemical properties of wild and cultivated red raspberries (*Rubus idaeus* L.). Journal of Food Composition and Analysis, 23, 540-544.

Clapa, D., Fira, A., & Joshee, N. (2013). An efficient *ex vitro* rooting and acclimatization method for horticultural plants using float hydroculture. Hortscience 48(9), 1159-1167.

Clapa, D., Fira, A., & Pacurar, I. (2008). The *in vitro* propagation of the raspberry cultivar Citria. Bulletin UASVM, Horticulture 65(1), 99-103.

Dziedzic, E., & Jagła, J. (2013). Micropropagation of *Rubus* and *Ribes* spp. Lambardi, M., Ozudogru, E. A., Jain, S. M. (eds.). Protocols for micropropagation of selected economically important horticultural plants. Humana Press: Totowa, NJ, USA.

Evdokimenko, S. N. (2009). Use of the productivity potential of remontant forms of raspberry in breeding. Russian Agricultural Sciences, 35(3), 160-162.

Gajdosova, A., Ostrolucká M. A., Libiaková, G., Ondrušková E., & Šimala D. (2006). Microclonal propagation of *Vaccinium* sp. and *Rubus* sp. and detection of genetic variability of culture in vitro. Journal of Fruit and Ornamental Plant Research, 14(1), 103-119.
Hunková, Ju., Libiaková G., & Gajdošová A. (2016). Shoot proliferation ability of selected cultivars of *Rubus* spp. as influenced by genotype and cytokinin concentration. Journal of Central European Agriculture, 17(2), 379-390. DOI: 10.5513/JCEA01/17.2.1718
Ivanova-Khanina, L. V. (2014). Optimizing conditions for introduction of raspberry and blackberry into cultivation *in vitro.* Scientific Journal of KubSAU, 101(07), 1-12. (In Russian)
James, D. J., Knight, V. H., & Thurbon, I. J. (1980). Micropropagation of the red raspberry and the influence of phloroglucinol. J. Hort. Sci., 12, 313-319.
Kulkhanova, D. S., Plaksina, T. V., & Borodulina, I. D. (2012). Remontant raspberry varieties' propagation in vitro. The News of Altai State University, 3-2(75), 42-45. (In Russian)
Lebedev, V., Arkaev, M., Dremova, M., Pozdniakov, I., & Shestibratov, K. (2019). Effects of growth regulators and gelling agents on *ex vitro* rooting of raspberry. Plants, 8, 3, 1-10. doi:10.3390/plants8010003
Lee, J., Dossett, M., & Finn, C. E. (2012). Rubus fruit phenolic research: The food, the bad, and the confusing. Food Chem., 130, 785-796.
Marchi, P. M., Antunes, L. E. C., Pereira, I. S., Höhn, D., & Valgas, R. A. (2018). Vegetative propagation of raspberry from leafy cuttings. Rev. Bras. Frutic., Jaboticabal, 40, 5, 1-5.
McGhie, T. K., Hall, H. K., Ainge, G. D., & Mowat, A. D. (2002). Breeding *Rubus* cultivars for high anthocyanin content and high antioxidant capacity. Acta Hort, 585, 495-500.
Mezzetti, B., Savini, G., Carnevali, F., & Mott, D. (1997). Plant genotype and growth regulators interaction affecting *in vitro* morphogenesis of blackberry and raspberry. Biologia Plantarum, 39(1), 139-150. 10.1023/A:1000381612029.
Moyer, R. A., Hummer, K. E., Finn, C. E., Frei, B., & Wrolstad, R. E. (2002) Anthocyanins, phenolics, and antioxidant capacity in diverse small fruits: *Vaccinium*, *Rubus* and *Ribes*. Journal of Agricultural and Food Chemistry, 50(3), 519-525. DOI: 10.1021/jf011062r
Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth and bioassays with tabacco tissue culture. Physiol. Plant, 15, 473-497.
Poothong, S., & Reed B. M. (2015). Increased $CaCl_2$, $MgSO_4$, and KH_2PO_4 improve the growth of micropropagated red raspberries. *In Vitro* Cell. Dev. Biol. – Plant, 51, 648-658 DOI 10.1007/s11627-015-9720-y
Poothong, S., & Reed, B. M. (2014). Modeling the effects of mineral nutrition for improving growth and development of micropropagated red raspberries. Scientia Horticulturae, 165, 132-141. doi:10.1016/j.scienta.2013.10.040
Shornikov, D. G., Bryukhina, S. A., Muratova, S. A., Yankovskaya, M. B., & Papikhin, R. V. (2010). *In vitro* conditions improvement for berry and ornamental plants micropropagation. TSU Bulletin, 15(2), 640-645. (In Russian)
Skovorodnikov, D. N., Kazakov, I. V., Evdokimenko, S. N., & Sazonov, F. F. (2012). Application of diphenylurea derivates in clonal micropropagation of primocane fruiting raspberry and black currants. Acta Hort. ISHS, 946, 135-138.
Sobczykiewicz, D. (1984). Mass production of raspberry plantlets through micropropagation and rooting them directly in sand-peat mixture. Fruit Sci. Rep., XI(2), 73-77.
Solovykh, N. V., & Budagovsky A.V. (2018). Stimulation of risogenesis of red and black raspberry *in vitro* with use of coherent radiation. Agrarnaya nauka Evro-Severo-Vostoka, 66(5), 64-68. doi: 10.30766/2072-9081.2018.66.5.64-68 (In Russian)
Stoevska, T., Trifonova, A., & Karadocheva, D. (1995). Micropropagation of raspberries (*Rubus idaeus*). Biotechnology & Biotechnological Equipment, 9(2-3), 27-30. DOI: 10.1080/13102818.1995.10818837
Tsao, C. W. V., Postman, J. & Reed, B. M. (2000). Virus infections reduce *in vitro* multiplication of 'Malling Landmark' raspberry. *In Vitro* Cellular & Developmental Biology. – Plant, 36. 65-68. 10.1007/s11627-000-0015-5.
Tsao, C. W. V., Reed, B. M. (2002). Gelling agents, silver nitrate, and sequestrene iron influence adventitious shoot and callus formation from *Rubus* leaves. *In Vitro* Cell. Dev. Biol. – Plant, 38, 29-32.
Wu, J. H., Miller, S. A., Hall, H. K., & Mooney, P. A. (2009) Factors affecting the efficiency of micropropagation from lateral buds and shoot tips of *Rubus*. Plant Cell, Tissue and Organ Culture, 99 (1). 17-25. DOI: 10.1007/s11240-009-9571-5
Xiao, D., Huang, Y., Park, E., Edirisinghe, I., & Burton-Freeman, B. (2017). Red raspberries and insulin action: understanding the role of red raspberry consumption on postprandial metabolic indices. FASEB Journal, Bethesda, 31(1) 973-979.
Zawadska, M., & Orlikowska, T. (2006a). Factors modifying regeneration *in vitro* of adventitious shoots in five red raspberry cultivars. Journal of Fruit and Ornamental Plant Research, 14, 105-115.
Zawadzka, M., & Orlikowska, T. (2006b). The influence of Fe-EDDHA in red raspberry cultures during shoot multiplication and adventitious regeneration from leaf explants. Plant Cell Tiss Org Cult, 85, 145-149.
Zawadzka, M., & Orlikowska, T. (2009). Influence of FeEDDHA on *in vitro* rooting and acclimatisation of red raspberry (*Rubus ideaus* L.) in peat and vermiculite. J. Hort. Sci. Biotechnol., 84, 599-603.

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