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**TARAN O.,** Cand. of Biological Sciences
*Taras Shevchenko National University of Kyiv*
**MATSKEVYCH V., FILIPOVA L.,** Cand. of Agricultural Sciences
*Bila Tserkva National Agrarian University*

# EXOGENOUS PHYTOHORMONES INFLUENCE ON THE BLACKBERRY (*RUBUS FRUTICOSUS L.)* REGENERATES DEVELOPMENT AND TOOLS OF THEIR CONTAMINATION REDUCTION IN POSTASEPTIC CULTURE

Досліджували вплив екзогенних фітогормонів на морфогенез та постасептичну адаптацію регенерантів ожини (*Rubus fruticosus* L.). Встановлено, що підвищений вміст ауксину у живильному середовищі в культурі *in vitro* сприяє приживленню регенерантів у постасептичних умовах, тоді як за підвищеного вмісту синтетичних цитокінінів регенераційна здатність рослин знижувалась. Застосування гібереліну та цитокініну при культивуванні рослин за умов *ex vitro* також знижує їх приживання, а внесення екзогенного ауксину значно підвищує показники регенерації. Контамінація регенерантів ожини у постасептичних умовах спричиняє загибель їх значної кількості. Ефективним заходом захисту є використання препарату Превікур Енерджі у дозі 1,5 г/л та розробка заходів щодо контролювання вологості повітря в культурі *ex vitro*.

**Ключові слова:** постасептична адаптація, ризогенез, фітогормони, контамінування *ex vitro*.

**Introduction.** Microclonal reproduction allows cultivating virus-free, genetically uniform plants on an industrial scale with the crop multiplication factor of 1: 1,000,000. However, after transferring the regenerants into *ex vitro* conditions, their amount may reduce tenfolds. This significant reduction of regenerants plant grafting is related primarily to their inability to adapt quickly to changing the cultivation conditions.

**Analysis of recent research and publications.** Mixotrophic, with the heterotrophic predominant, nutrition type is formed under the *in vitro* factor static conditions of the culture while under *ex vitro* conditions plant nutrition is of autotrophic type. Transferring into ex vitro conditions causes stress emergence of the regenerants, specifically through the tissues photosynthetic activity inhibition under in vitro conditions [1, 2]. Stress effect of the cultivation conditions in the postaceptic culture alters the plants phytohormonal balance [3, 4]. In addition, the stress impact reduces the defence reserve, and thus they are populated widely with both saprophytic and pathogenic organisms. Thus, regenerants adaptation to *ex vitro* conditions demands additional measures strengthening their survivability [5,6].

**The aim** of our research was to study the impact of exogenous phytohormones, the cuttings position and regenerants age on blackberry (*Rubus fruticosus L.*) postaseptic adaptation efficiency and the regenerants reproduction, as well as to develop the measures on plants contamination reduce under *ex vitro* conditions.

**Research methods.** Blackberry *Rubus fruticosus,* L. Reuben variety regenerants were cultivated in a nutrient medium according to Murasihe Skuha prescription [7] with the modifications according to the experiment variations. The basic version was that with 100 % mineral nutrient content of the medium according to the prescription. The phytohormones content made: adenine – 0,25 mg /l; BAP – 0.5 mg/l and IOA 0.1 mg/l. The modified culture medium contained 50 % mineral portion according to the prescription, the phytohormones content made: adenine – 0.016 mg/l; BAP – 0,032 mg/l; IOA – 1.5 mg/l. We took into account the morphometric parameters of the plants development and noted the impact of the regenerants cultivation duration under *in vitro* conditions regenerants as well as the origin of different areas stems of parent plants on their further healing under *ex vitro* conditions.

To study the regenerative ability of blackberries plants in postaseptic conditions we did the regenerants cuttings on perlite substrate adapted to these conditions, and, having analyzed the impact of the three exogenous synthetic phytohormones of different classes on the characteristics of morphogenesis cuttings *ex vitro*. We used GA (A3 hibereline acid) at a concentration of 1 mg/l, IOA – 2.5 mg/l, BAP – 0.6 mg/l. We also used a combination of these phytohormones: gibberellins (1 mg/l) + IOA (2.5 mg/l) and BAP (0.6 mg/l) + IOA (2.5 mg/l). The control was the culture medium without phytohormones. Cuttings from the medial part of the regenerants were used as there is a gradient of endogenous phytohormones along the plants stem [4].

The plants were cultivated *ex vitro* at a temperature of 24 ± 2 $C^0$ in containers with adjustable air humidity and gas composition. For this purpose a necessary mixture of air, water and carbon dioxide was prepared in a tank and conveyed in a vessel [8]. The microflora inseminating the regenerats *ex vitro* was defined using a stereomicroscope with 1x40 increasing according to [9]. 1.5 mg/l of AgNO3 was added into the nutrient solution to prevent bacterial contamination. Fungicides application conditions are shown in Table 1.

Table 1 – **Scheme and terms of using drugs in the experiment**

| Variants | Preparation | Application terms |
|---|---|---|
| 1 | Control | Without treatment fungicides |
| 2 | Fundasolum 0,5 g/l | Adding to the nutrient medium and spraying the plants in three days during the cultivation |
| 3 | Topsine M 0,5 g/l | Spraying the plants in three days during the cultivation |
| 4 | Topsine M 0,75 g/l | Spraying the plants in five days during the cultivation |
| 5 | Previcure Energy 0,375 g/l | Spraying the plants in two days during the cultivation |
| 6 | Previcure Energy 0,75 g/l | Spraying the plants in three days during the cultivation |
| 7 | Previcure Energy 1,5 g/l | Spraying the plants in three days during the cultivation |

The accounted number of plants was 240 pieces with a fourfold experiment repetition. Statistical analysis of the data was performed using ANOVA applied programs of MS Excel package.

**Results and discussion**. It is known that rhizogenesis inducement takes place through balancing the contents of phytohormones in a nutrient medium under auxin predominance over cytokinins in accordance with the Skuh-Miller rule [3]. Only a small amount of regenerants – 6.10 % – took root under postaseptic conditions on the basic reproduction medium with cytokinins predominance (Table 2).

Table 2 – **Rhizogenesis inducement in blackberry in vitro regenerants in postaseptic conditions**

| Variants | Roots length, mm | Roots number, pieces | Plant height, mm | Scions number in a conglomerate, pieces | Taking roots under ex vitro, %. |
|---|---|---|---|---|---|
| Basic nutrient medium | 3±2 | 2±2 | 129±7 | 4,7±0,3 | 6±4 |
| Modified nutrient medium | 17±4 | 6±2 | 86±7 | 1,5±0,2 | 91±8 |

The roots length and number increased as well, indicating auxin higher concentrations positive effect on the development of the root system under *in vitro* conditions. Overall, the regenerants cultivated under *in vitro* conditions on a modified medium, when moved into postaseptic conditions, adapted much better since the plants taking roots increased by 81-89 % compared with the base variant.

In addition, it was found out that taking roots in postaseptic conditions was affected by the plants and regenerants age. Thus, 86-91 % of the plants took roots in 15-days old plants, while the taking roots in regenerants cultivated under *in vitro* conditions for 30 days under *ex vitro* conditions was 91-99 %. Further increase in the age of the regenerants transferred to postaseptiv conditions reduced their taking roots. Thus, taking roots made 88-94 6 % in 45-days-old regenerants, 84-9 % in 60-days-old ones, and 63-74 % of the regenerants cultivated under *in vitro* conditions for 90 days took roots.

To investigate the regenerative ability of blackberry plants under postaseptic cuttings we studied the effect of the following factors: 1) exogenous phytohormones and 2) the cuttings position on the mother plant. The latter factor can be very important for a successful micropropagation of blackberry, since it is known that the areas proximal to the tip of the stem have higher proliferative activity than distal ones [2].

It has been found out that in the control variant without application of exogenous phytohormones rooting in the cuttings started on the 27$^{th}$ day of cultivation, while in the variant with the IOA application it started on the 14$^{th}$ day (Table 3). Application of GA in combination with auxin and that of BAP combined with the IOA, as well as independently, prolonged the rooting period. The highest indices of root development were recorded in the variant with using indoliloil acid since on the 45$^{th}$ day of the plants cultivation in this embodiment, they exceeded twice the roots length of the control plants. The formed roots number per regenerant was 1.8 times higher.

Table 3 – **Phytohormones influence on the features of blackberries postaseptic cuttings propagation *ex vitro*** (45th day of cultivation)

| Variants | Start, days | | Root system development | | Leaves forms | Leaf plate length, mm |
|---|---|---|---|---|---|---|
| | rhisogenesis | Scion development from buds | length, mm | roots number, pieces | | |
| Control, without phytohormones | 27±4 | 12±1 | 19±4 | 4±1 | simple juvenile | 5-8 |
| GA | 43±6 | 7±4 | 5±3 | 2±1 | trifoliate | 3-5 |
| GA+ IOA | 38±6 | 9±5 | 9±3 | 5±2 | trifoliate | 4-6 |
| IOA | 14±3 | 12±4 | 38±6 | 7±3 | simple juvenile | 15-20 |
| BAP | 47±7 | 9±3 | - | - | | 7-9 |
| BAP + IOA | 31±4 | 11±3 | 7±4 | 9±3 | | 10-14 |

Exogenous gibberellin presence in the nutrient solution predetermined newly formed shoots pulling out and their thickness reduction. The plants leaves in this variant were small, trifoliate, indicating juvenility loss. Subsequent cuttings of these scions shoots resulted in the death of about 80 % of the cuttings during the regeneration. However, formation of 2-4 micro scions was often observed in the variant with BAP using (Fig. 1), the scions rooted well under their placing on the medium with IOA (2.5 mg/l).

In the variant with addition of IOA the plants had the most developed root system, which apparently provided the optimal phytohormonal balance. This, in turn, resulted in the leaf plates better development.

Under repeated cuttings of blackberry plants rooted cuttings *ex vitro* (passage 2) we have found a significant difference in the regeneration rate between the cuttings originated from different parts of the parent plant shoot (apical or medial cuttings) (Fig. 2, Table. 4).

Significantly larger plants were regenerated from the apical cuttings. Adding IOA into the nutrient solution improved the plants formation from both apical and medial cuttings. The largest regenerants were obtained in the variant with apical cuttings and IOA adding. The average weight of plants in the 30$^{th}$ day of cultivation in this variant was twice as large as in the plants from the relative variant without auxin, and was 5.3 times higher than in the plants from the variant with medial cuttings and the use of exogenous auxin (Table 4).

Fig. 1. **Rooted blackberries cutting grown on a nutrient solution with BAP and IOA adding.**


Fig. 2. **Effect of the cuttings origin and IOA contents in the nutrient solution on plants development:**
1 - apical cutting, IOA; 2 - apical cutting without IOA;
3 - medial cutting, IOA; 2 - medial cutting without IOA.

Table 4 – **Effect of explants type on blackberry plants regeneration *ex vitro* under repeated subcultivation in the 30th day of cultivation**

| Cutting type | IOA presence in the nutrient vedium | Plants taking roots, % | Root system development | | Plant weight, g |
|---|---|---|---|---|---|
| | | | length, mm | roots number, pieces | |
| apical | + | 96±3 | 68±7 | 10±2 | 11,73±3,12 |
| apical | - | 92±4 | 53±5 | 6±2 | 5,76±0,67 |
| medial | + | 71±10 | 31±6 | 4±2 | 2,22±0,34 |
| medial | - | 9±6 | 5±3 | 1±1 | 0,94±0,18 |

Only a small number of medial cuttings in the version without exogenous auxin took roots in the cassettes, and adding IOA to the nutrient solution provided increase in taking roots rate by 71 %. 92 and 96 % of regenerants originated from apical cuttings took roots in the variants without IOA and under the presence of auxin in the solution respectively. Thus, blackberry plants rooting and development from the regenerants cuttings under postaseptic adaptation are controlled by auxin which should be considered in microclonal propagation of this culture.

While studying the composition of microflora, which bacterize the regeneranta *ex vitro* we found out that the contaminants were represented mainly with fungi, specifically with the representatives of the genus *Aspergillus.*

Fungicide decontaminative activity with regard to these microorganisms depended on the air humidity, on the drug and the way it was used (Table. 5).

Table 5 – **Efficiency of fungicides application under blackberry plants postaceptic growing in *vitro* in the 30th day of cultivation**

| Variants | Plants contamination, % | | Plants height, mm | |
|---|---|---|---|---|
| | 70 % air humidity | 100 % air humidity | 70 % air humidity | 100 % air humidity |
| 1 | 86±4 | 100 | 47±3 | - |
| 2 | 82±4 | 100 | 48±4 | - |
| 3 | 69±5 | 95±5 | 36±3 | 9±4 |
| 4 | 51±5 | 93±6 | 34±2 | 9±3 |
| 5 | 36±8 | 57±10 | 51±6 | 26±5 |
| 6 | 7±3 | 21±9 | 57±6 | 31±5 |
| 7 | 2±1 | 11±4 | 59±5 | 33±6 |

It has been noted that in the control without the fungicides application the plants destruction with their further death was 86 % under the low humidity while under high humidity all the plants died. In the variant with fundazol contact fungicide application the plants loss under both levels of humidity were close to the control. Також по усіх варіантах пригнічувався ріст при 100 % вологості повітря. Application of Topsin M fungicide, versus the control, reduced the number of affected plants to 17-24 % depending on the application method. 5-7 % of plants survived in the variant with the abovementioned fungicide under 100 % humidity. We observed plants growth inhibition in these variants, specifically of the scions sizes were smaller. Also, in all the variants the growth was suppressed at 100 % humidity. We assume that this is due to metabolic processes activation under anaerobic respiration of plants. Previkur Energy preparation proved to be the most effective at a dose of 1.5 g/l under spraying within five days of the cultivation, since almost 98 % of the regenerants took roots in this variant under low humidity, and 79-85 % – under high humidity.

**Conclusions.**

1. Auxin content increase to 1.5 mg/l in the nutrient medium and the cultivation under *in vitro* conditions for no longer than 30 days increased the blackberries regenerants taking roots in postaseptic culture up to 91-99 %.

2. Auxin plays a leading role in the blackberry plants regenerants cuttings taking roots and development under postaseptic adaptation conditions since the highest rates of reproduction and development regenerants were obtained inder exogenous indoliloil acid. This should be considered when planning microclonal propagation of this crop.

3. It has been established that plants contamination in postaseptic culture takes place under Aspergillus genus fungi participation mostly. Protective measures for blackberries regenerants should include applying Previkur Energy preparation at a dose of 1.5 g/l, as well as the measures on air humidity reduction which improves the plants performance and reduce their contamination.

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**Влияние экзогенных гормонов на развитие регенерантов ежевики (*Rubus fruticosus* L.) и мероприятия снижения их контаминирования в асептической культуре**

**О.П. Таран, В.В. Мацкевич, Л.М. Филиппова**

Исследовали влияние экзогенных гормонов на морфогенез и постасептическую адаптацию регенерантов ежевики (*Rubus fruticosus* L.). Установлено, что повышенное содержание ауксина в питательной среде в культуре *in vitro* способствует приживаемости регенерантов в постасептических условиях, тогда как при повышенном содержании синтетических цитокининов регенерационная способность растений понижалась. Приминение гиббереллина и цитокинина при культивировании в условиях *ex vitro* также снижает их приживаемость, а внесение екзогенного ауксина повышает показатели регенерации. Контаминирование регенерантов ежевики в постасептических условиях приводит к гибели их весомого количества. Эффективным средством защиты есть использование препарата Превикур Енерджи в количестве 1,5 г/л и мероприятий контролирования влажности воздуха в культуре *ex vitro*.

**Ключевые слова:** постасептическая адаптация, ризогенез, фитогормоны, контаминирование *ex vitro*.

**Exogenous phytohormones influence on the blackberry (*Rubus fruticosus* L.) regenerates development and tools of their contamination reduction in postaseptic culture**

**O. Taran, V. Matskevych, L. Filipova**

We researched the impact of exogenous phytohormones on morphogenesis and postaseptic adaptation of blackberry regenerants (*Rubus fruticosus* L.). We have ascertaine, that increased auxin's content in the nutrient medium of culture *in vitro* facilitates taking roots under postaseptic conditions, while regenerative ability of plants decreased under high content of synthetic cytokinins.

The use of gibberellins and cytokinins in plant's cultivation under conditions *ex vitro* also decreases their healing, as well as adds to exogenous auxin increase regeneration's indexes.

Blackberry regenerants' contamination cause deaths of a significant number under postaseptic conditions. An effective way of protection is using Previcure Energy at a dose of 1,5 g/l and develops the measures on controlling humidity under *ex vitro* conditions.

**Key words:** postaseptic adaptation, rhisogenesis, phytohormones, contamination *ex vitro*.

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