# The effect of growth regulators on the micro-shoots' regeneration of strawberries (*Fragaria* × *ananassa* Duch.) in in *vitro* culture

*O. V.* Matsneva*, and *L. V.* Tashmatova

Russian Research Institute of Fruit Crop Breeding (VNIISPK), 302530, Orel, Russian Federation

**Abstract.** The influence of various growth regulators on strawberry varieties' micro-shoots regeneration of Russian and foreign selection Bereginya, Tsaritsa, Urozhaynaya CGL, Frida, Asia, Kimberly, Honeoye, Marmolada has been established. The experiment involved micro-plants regenerated in in *vitro* culture. At the proliferation stage, 6-BAP, thidiazuron, kinetin, mival, succinic acid were added to the nutrient medium in specified concentrations to select the optimal growth regulator. The maximum response to the induction of additional shoots was obtained in explants cultivated on the control medium of Murashige and Skoog supplemented with 6-BAP. The positive effect of kinetin, thidiazuron, mival, succinic acid on the process of root formation was noted, which can accelerate the production of rooted plants *ex vitro*.

## 1 Introduction

In *vitro* micro-reproduction is the most popular technology for mass commercial production of valuable plant genotypes [1]. Apical dominance is removed under the action of cytokinins; the initiation of axillary buds and shoots is activated. In the process of micro-propagation, each culture imposes certain requirements on phytohormones and their concentrations. Cytokinins play a central role in the hormonal regulation of plant growth and development processes. Plants have an effective system for maintaining the endogenous level of biologically active forms of cytokinins. At the same time, the introduction of exogenous cytokinins into nutrient media causes the development of both the apical meristem and the micro-shoots' conglomerate [2-3].

The growth and development of plants in nature are controlled by endogenous growth regulators operating at very low concentrations [4]. Synthetic exogenous growth regulators used in in *vitro* culture often surpass natural compounds in their biological activity and effectiveness [5-6].

The most important factors affecting the effectiveness of introducing in *vitro* strawberry varieties into culture are the types of growth regulators used [7]. Cytokinin 6-benzylaminopurine (BAP) is most often used in the micro-reproduction of garden strawberries [8; 9]. In the experiments of K.A. Quiroz et al. [10], the use of BAP in nutrient media increased the average reproduction rate of strawberries to 3-6 shoots per plant. At the

* Corresponding author: mazneva@vniispk.ru

stage of strawberry proliferation, the most effective BAP amount in the medium was 1.0 mg/l [11]. In the research by H.K.S. Madumali and co-authors [12], successful regeneration of strawberry shoots of the Sweet Charlie variety was observed on a Murashige–Skoog (MS) base medium containing 0.5 mg/l BAP.

Currently, thidiazuron (TDZ) is widely used in tissue culture manifesting itself as auxin or cytokinin [13]. The addition of 6-benzylaminopurine (BAP) and thidiazuron affects the induction of microshoots to a greater extent [14]. The TDZ effect on the organogenesis induction in the culture of strawberry meristems has been studied on a limited number of varieties [10; 15].

Kinetin (6-furfurylaminopurin) induces cell proliferation and organogenesis of buds and shoots [16].

According to V.I. Demenko et al., the optimal ratio of exogenous growth regulators in the nutrient medium is very important for the industrial application of microclonal reproduction. All abnormal phenomena observed in plants in *vitro* are associated with changes in the content and activity of endogenous hormones. The microclonal reproduction effectiveness of garden plants depends on the regenerative capacity and can be increased by enhancing the growth of explant, reducing the vitrification of plants in *vitro* [17].

The genotype has a significant effect on the morphogenetic potential of cultured tissues [18]. Continuous improvement of the technology is required due to the changing assortment of garden strawberries, the genotypic features of which are not yet known when grown in *vitro* [19]. The establishment and growth of explants depends not only on the salt composition of the nutrient medium, but also on growth regulators - mainly cytokines and auxins. The effect of growth regulators is determined by their ability to penetrate plant cells. It is often difficult to choose the optimal ratio due to the quality diversity of explants. Therefore, it is necessary to search for other growth regulators that promote the formation of adventitious shoots [17]. Positive results were obtained with the introduction of organosilicon compounds into the nutrient medium, which showed an auxin-like effect [20]. Succinic acid is used at the stages of micro-reproduction, rhizogenesis, and adaptation of plants to *ex vitro* conditions [21]. In the experiments of L. L. Buntsevich et al. [22], the stimulating effect of succinic acid on drawn micro-shoots of Stanley plum varieties in *vitro*, their leafage, a decrease in the level of tissue vitrification, leaf chlorosis, a positive effect on the effectiveness of rhizogenesis and shoots' rooting was found; it makes it possible to accelerate the production of rooted plants.

Since the morphogenetic potential of cultivated tissues largely depends on the genotype, the development of an effective and reproducible regeneration system under the influence of various growth regulators under in *vitro* conditions for the studied strawberry varieties is an urgent task [18].

**Research objective**: to develop a successfully reproducible in *vitro* regeneration protocol for a group of important commercial strawberry genotypes.

## 2 Materials and methods

The objects of research were the most popular industrial varieties of strawberries. The studies were conducted according to generally accepted methods [23-25]. The stage of the actual in *vitro* micropropagation consisted of 4 passages. In each passage, strawberry conglomerates were divided into separate micro-plants and were then planted on a fresh nutrient medium. For cultivation, MS nutrient medium [26] was used with the addition of 0.8 mg/l (BAP); 2.0 mg/l kinetin; 0.2 mg/l thidiazuron (TDZ); 10.0 mg/l mival; 4.0 mg/l succinic acid. Growth regulators were introduced into the nutrient medium. The interval between passages was 4 weeks.

Cultivation conditions: temperature 23-25 ° C, 16-hour photoperiod of 16/8 hours, illumination – 2.0-3.0 thousand lux.

The following indicators were considered: the number of micro-rosettes formed per explant, shoot height, the percentage of plants with roots, the number of roots per explant. All rosettes from one test tube were considered to determine the multiplication coefficient. Its value was defined as the average value for four passages.

Statistical processing of experimental data was carried out according to the method by B.A. Dospekhov [27] and using the Microsoft Excel 2016 computer program.

The influence degree of the genotype and growth regulators on the morphometric parameters of strawberries in *vitro* was evaluated using the indicator's coefficient of variation (CV). The value of CV ≤ 10% is a weak variability of the indicator, 10% ≤ CV≤ 20% – average, CV over 20% – high.

## 3 Research results

To improve the quality of strawberry shoot formation at the multiplication stage, various growth regulators were introduced into the MS nutrient medium: 6-BAP, kinetin, thidiazuron, mival, succinic acid. The maximum response to the induction of additional shoots was obtained in explants cultivated on a control medium of Murashige and Skoog supplemented with 6-BAP (0.8 mg/l) (Table 1).

**Table 1.** Influence of growth regulators on the multiplication coefficient of strawberry varieties, pcs./explant.

| Variety (Factor A) | Growth regulators (Factor B) | | | | | $LSD_{05}$ by factor A |
|---|---|---|---|---|---|---|
| | 6-BAP (control) | kinetin | TDZ | mival | succinic acid | |
| Frida | 2.6 ± 0.4 | 1.2 ± 0.1 | 1.8 ± 0.5 | 1.4 ± 0.2 | 1.3 ± 0.2 | 0,7 |
| Bereginya | 3.2 ± 0.5 | 1.4 ± 0.2 | 1.4 ± 0.2 | 1.3 ± 0.1 | 1.3 ± 0.2 | 0,4 |
| Kimberly | 3.2 ± 0.4 | 2.6 ± 0.3 | 2.6 ± 0.4 | 1.5 ± 0.1 | 1.6 ± 0.2 | 1,1 |
| Asia | 3.7 ± 0.4 | 2.0 ± 0.4 | 3.2 ± 0.4 | 1.4 ± 0.2 | 1.4 ± 0.2 | 1,1 |
| Honeoye | 3.1 ± 0.4 | 1.7 ± 0.3 | 1.9 ± 0.3 | 1.1 ± 0.2 | 1.4 ± 0.2 | 0,7 |
| Tsaritsa | 3.2 ± 0.5 | 1.3 ± 0.2 | 1.7 ± 0.3 | 1.3 ± 0.1 | 1.3 ± 0.2 | 0,6 |
| Urozhaynaya CGL | 5.3 ± 0.8 | 1.3 ± 0.4 | 1.4 ± 0.2 | 1.5 ± 0.4 | 1.2 ± 0.1 | 1,1 |
| Marmolada | 2.6 ± 0.4 | 1.1 ± 0.1 | 1.3 ± 0.4 | 1.5 ± 0.1 | 1.2 ± 0.2 | 0,3 |
| $LSD_{05}$ by factor B | 1,1 | 0,5 | 0,8 | 0,5 | 0,5 | |

With its application, the largest number of newly formed micro-rosettes was noted in all genotypes. BAP was more active than other growth regulators in removing apical dominance and stimulating the formation of additional shoots. From the second passage, an undesirable vitrification phenomenon of plant tissues of individual plants was observed, which can be explained by the constant presence of high BAP concentrations. Such rosettes were not suitable for further reproduction, which disrupted the technological in *vitro* cycle. The research results show that regeneration strongly depended on the genotype. Sequential cultivation of strawberries for four passages showed that the variety Urozhaynaya CGL in *vitro* has the greatest shoot formation (up to 5.3 pcs./explant).

The addition of kinetin and TDZ to the nutrient medium did not significantly affect the formation of additional shoots in most of the strawberry varieties under study. Exception were Asia and Kimberly varieties; for those, the use of kinetin and TDZ at the stage of active reproduction can be recommended. The reproduction coefficient of the Asia variety averaged to 2.0 (kinetin) and 3.2 (TDZ) pcs./exp., Kimberly variety - 2.6 pcs./exp. Studies by Fatemeh

et al. [15] confirm that strawberry varieties with different genetic backgrounds can give different reactions to regeneration after TDZ application.

At the same time, the formed shoots turned into normal mature plants; the general condition of strawberry in *vitro* micro-rosettes improved. Active formation of young roots was observed. The plants were strong, dark green, and had at least 5-6 leaves. Vitrification of plant tissues was absent.

The effect of mival and succinic acid synthetic growth regulators on the reproduction coefficient was minimal and did not exceed 1.6 pcs./explant. However, their positive effect on the root formation process was noted, which can accelerate the production of rooted plants *ex vitro,* bypassing the in *vitro* rooting stage according to generally accepted technology.

Statistical results' processing of growth regulators' application on the reproduction coefficient showed the reliability of differences in factor B only when using kinetin ($F_f$ =11.0$F_t$ =6.9).

According to factor A, the reliability of differences was noted in all the studied varieties ($F_{fF\ t}$)

The height of regenerating plants varied significantly across the experimental options, which is associated with the constant new buds' set and different ages of the micro-plants formed (Table 2).

**Table 2.** The effect of growth regulators on the height of strawberry varieties' micro-plants, mm

| Variety (Factor A) | Growth regulators (Factor B) | | | | | $LSD_{05}$ by factor A |
|---|---|---|---|---|---|---|
| | 6-BAP (control) | kinetin | TDZ | mival | succinic acid | |
| Frida | 6.0 ± 0.5 | 10.3 ± 0.8 | 8.5 ± 0.7 | 8.1 ± 0.7 | 9.5 ± 0.9 | 2,1 |
| Bereginya | 11.1 ± 0.7 | 9.2 ± 0.9 | 9.4 ± 0.6 | 7.5 ± 0.9 | 9.6 ± 0.9 | 2,4 |
| Kimberly | 5.7 ± 0.2 | 6.4 ± 0.8 | 5.5 ± 0.4 | 7.9 ± 0.6 | 7.5 ± 0.5 | 0,9 |
| Asia | 9.1 ± 0.4 | 6.7 ± 0.6 | 5.9 ± 0.4 | 6.6 ± 0.5 | 7.7 ± 0.6 | 0,9 |
| Honeoye | 6.1 ± 0.4 | 7.9 ± 0.9 | 6.9 ± 0.5 | 8.3 ± 0.7 | 9.1 ± 0.8 | 1,1 |
| Tsaritsa | 9.0 ± 0.7 | 8.0 ± 0.6 | 6.5 ± 0.7 | 7.9 ± 0.5 | 8.3 ± 0.7 | 2,5 |
| Urozhaynaya CGL | 10.4 ± 0.5 | 11.0 ± 0.9 | 9.8 ± 0.8 | 8.3 ± 0.8 | 10.6 ± 0.9 | 1,9 |
| Marmolada | 6.9 ± 0.6 | 6.3 ± 0.6 | 7.3 ± 0.6 | 7.6 ± 0.5 | 9.1 ± 0.9 | 2,0 |
| $LSD_{05}$ by factor B | 1,9 | 1,5 | 1,7 | 1,4 | 1,6 | |

In general, the plants in the option with succinic acid were characterized by the highest height indicators, which promotes the micro-shoots' drawing. As a weaker cytokinin, kinetin also caused shoots' elongation. This property can be used to increase the proportion of strawberry plants in *vitro* suitable for rooting.

The addition of growth regulators had various effects on plant height depending on the genotype. The maximum growth in all experimental options was observed in the Urozhaynaya CGL variety. The height of Asia, Bereginya, Tsaritsa varieties' plants did not exceed the control for all treatment options.

Statistical results' processing of the growth regulators' application on the height of micro-shoots showed the reliability of differences in factor B when using 6-BAP ($F_f$=10.3$F_t$=6.9), kinetin ($F_f$=12.8$F_t$=6.9) and TDZ ($F_f$=7.9$F_t$=6.9).

According to factor A, the reliability of differences was noted in varieties Asia ($F_f$=16.0$F_t$=6.9)., Kimberly ($F_f$=16.0$F_t$=6.9)., Honeoye ($F_f$=9.0$F_t$=6.9).

The negative auxins' effect on the process of root formation was overcome using growth-regulating substances that promote the establishment and growth of roots (Table 3).

**Table 3.** Influence of growth regulators on root formation of strawberry varieties' micro-plants in *vitro*, %

| Variety | 6-BAP (control) | kinetin | TDZ | mival | succinic acid |
|---|---|---|---|---|---|
| Frida | 0 | 61 | 80 | 69 | 66 |
| Bereginya | 14 | 66 | 45 | 61 | 75 |
| Kimberly | 0 | 8 | 0 | 56 | 67 |
| Asia | 21 | 44 | 29 | 63 | 79 |
| Honeoye | 24 | 72 | 40 | 52 | 69 |
| Tsaritsa | 11 | 47 | 21 | 24 | 59 |
| Urozhaynaya CGL | 10 | 81 | 42 | 35 | 91 |
| Marmolada | 10 | 31 | 56 | 35 | 74 |

The percentage of rooting increased in all four treatment options compared to the control. The addition of kinetin, TDZ, mival, and succinic acid often enhanced root induction. The most significant effect on the formation and development of the strawberry root system was the use of succinic acid, the rootability increased 3-9 times compared to the control depending on the genotype. The use of mival also contributed to a significant increase in the proportion of plants with roots. These results are consistent with the conclusions of V.I. Demenko et al. [20]; they studied the effect of the organosilicon compound mival on the regenerative ability of strawberries in *vitro* and established its auxin effect.

In the TDZ option, short strong roots were formed, vitrification was absent, plants were planted in the ground bypassing the rooting stage. There was no root formation in Kimberly variety plants.

The addition of kinetin, TDZ, mival, and succinic acid to the nutrient media stimulated the formation of more roots than in the control option (Table 4).

**Table 4.** The effect of growth regulators on the number of roots in micro-rosettes of strawberry varieties, pcs./explant.

| Variety (Factor A) | Growth regulators (Factor B) | | | | | $LSD_{05}$ by factor A |
|---|---|---|---|---|---|---|
| | 6-BAP (control) | kinetin | TDZ | mival | succinic acid | |
| Frida | 0 | 3.3 | 2.9 | 3.1 | 5.0 | 1,1 |
| Bereginya | 2.3 | 3.7 | 2.7 | 3.0 | 4.6 | 1,1 |
| Kimberly | 0 | 1.8 | 0 | 3.8 | 4.0 | 2.8 |
| Asia | 1.1 | 2.6 | 2.3 | 3.2 | 3.6 | 0,9 |
| Honeoye | 1.7 | 3.2 | 3.5 | 3.2 | 4.8 | 1,1 |
| Tsaritsa | 1.5 | 3.1 | 2.3 | 3.5 | 3.2 | 1.3 |
| Urozhaynaya CGL | 1.5 | 3.0 | 2.9 | 1.5 | 5.2 | 1,8 |
| Marmolada | 1.7 | 3.5 | 1.9 | 2.6 | 3.8 | 2.2 |
| $LSD_{05}$ by factor B | 1,0 | 1.3 | 0,9 | 1,5 | 1.3 | |

The maximum number of roots per plant in all genotypes was observed on a nutrient medium with the addition of succinic acid - 3-5 times greater than in the control. The largest average number of roots per plant was formed by micro-plants of the varieties Urozhaynaya CGL and Frida (5.2 and 5.0 pcs./explant, respectively).

Statistical results' processing of growth regulators' application on the number of roots showed the reliability of differences in factor B when using TDZ ($F_f$=14.1$F_t$ =6.9).

According to factor A, the reliability of differences was noted in Asia varieties ($F_f$=10.1$F_t$=6.9), Urozhaynaya CGL ($F_f$=7.6$F_t$=6.9), Honeoye ($F_f$=8.8Ft=6.9), Frida ($F_f$=24.6F t=6.9), Bereginya ($F_f$=9.5$F_t$=6.9).

## 4 Conclusion

The highest multiplication coefficient for all varieties was obtained when exposed to 6-BAP. Sequential cultivation of strawberries for four passages showed that the variety Urozhaynaya CGL in *vitro* has the greatest shoot formation (up to 5.3 pcs./explant). The constant presence of high BAP concentrations in the nutrient medium caused vitrification of plant tissues, which disrupted the technological cycle and reduced the yield of plants.

Kinetin and TDZ can be recommended for the multiplication of Kimberly and Asia varieties; the multiplication coefficient amounted to 2.6 and 2.0-3.2 pcs./explant, respectively.

The effect of mival and succinic acid synthetic growth regulators on the reproduction coefficient was minimal and did not exceed 1.6 pcs./explant. The variety-specific reaction nature of the effect of growth regulators on the shoots' elongation was noted. The height of strawberries' micro-rosettes of the varieties Frida, Honeoye, Kimberly with the addition of succinic acid was 1.5 times higher (9.5; 7.5; 9.1 mm, respectively) compared to the control. The Asia variety had a lower height than in the control in all options.

The positive effect of kinetin, TDZ, mical, and succinic acid on the root formation process was noted, which can accelerate the production of rooted plants *ex vitro.* The percentage of rooting increased in all four treatment options compared to the control. The maximum number of roots per plant in all genotypes was observed on a nutrient medium with the addition of succinic acid. The presented regeneration scheme of commercially important strawberry varieties with the use of growth regulators kinetin, TDZ, mival, succinic acid can be used in the mass production of seedlings in the in *vitro* system.

## References

1. M.E. Kane, Boca Raton. Florida: CRC Press LCC, 145-158 (2005)
2. A.M. Avalbayev, K.A. Somov, R.A. Yuldashev, F.M Shakirova, Biochemistry, **7**, 12, 1621-1630 (2012)
3. N.V. Kukharchik, Science and innovation, **6**(196), 17-21 (2019)
4. H-H. Chung, H-Y. Ouyang, Plants, **10**(1), 67 (2021)
5. A. Varshney, M. Anis, New Delhi: Springer, 116 (2014)
6. D.S. Muraseva, T.I. Novikova, A.A. Erst, Siberian Ecological Journal, **6**, 909-919 (2015)
7. E.V. Ambros, E.I. Chertenkova, S.Y. Tolusakova, E.G. Trofimova, T.I. Novikova, News of universities. Applied chemistry and biotechnology, **11**, 4. (2021) DOI: https://doi.org/10.21285/2227-2925-2021-11-4-549-560
8. M. Ashrafuzzaman, S.M. Faisal, D. Yadav, D. Khanam, F. Raihan, Bangladesh J. Agril. Res., **38**, 3, 467-472. (2013) DOI:10.3329/bjar. v38i3.16973
9. A.V. Polyakov, T.A. Linnik, Bulletin of Moscow State University, **3**, 35-41 (2014)
10. K.A. Quiroz, M. Berríos, B. Carrasco, J.B. Retamales, P.D.S. Caligari, Garcia-Gonzales R., Biol. Res., **50,** 20 (2017) https://doi.org/10.1186/s40659-017-0125-8
11. M.V. Karpushina, M.V. Vinter, E3S WEB of Conference "International Scientific and Practical Conference "Fundamental and Applied Research in Biology and Agriculture:

Current Issues, Achievements and Innovations" «FARBA 2021), EDP Sciences (2021) DOI: 10.1051/e3sconf/202125404004

12. H.K.C. Madumali, P.D. Abeythilakarathna, T.H. Seran, Sri Lanka Journal of Food and Agriculture, **5**(1), 41-48. (2019). DOI: http://doi.org./10.4038/sljfa.v5i1.69
13. L. Landi, B. Messetti, Plant. Cell. Rep., **25**, 281-288 (2006)
14. S.K. Assem, M.M. Samsam, B.A. Hussein, E.H.A. Hussein, African Journal of Biotechnology, **13**, 36, 3672-3681 (2014) DOI: 10.5897/AJB2014.13924
15. H. Fatemeh, A.A. Mehran, S. Ghizan, A.R. Azmi, K. Hussain, Hort. Science, **45**, 3, 453–456 (2010), DOI: 10.21273/HORTSCI.45.3.453
16. S.N. Timofeeva, Yu.V. Smolkina, N.V. Apanasova, O.I. Yudakova. Technologies of micro-multiplication in vitro: studies. - method. Stipend, Saratov, 38 (2016)
17. V.I. Demenko, Bulletin of TSKHA, **2**, 48-58. (2005)
18. E.V Ambros., Yu.G. Zaitseva, A.A. Krasnikov, T.I. Novikova, Flora of Asian Russia, **4**(28), 73-80 (2017), DOI:10.21782/RMAR1995-2017-4(73-80)
19. M.V. Karpushina, M.A. Vinter, Scientific works of SKFFNTSSVV, **31**, 108-113 (2021), DOI: 10.30679/2587-9847-2021-31-108-113
20. V.I. Demenko., K.A. Shestibratov, V.G. Lebedev, Bulletin of TSKHA, **1**, 73-85 (2010)
21. A.A. Zmushko, T.A. Krasinskaya, Fruit growing, **31**, 288-292 (2019)
22. L.L Buntsevich., E.N. Besedina, M.A. Kostyuk, Technologies of food and processing industry. Agro-industrial complex – healthy food products, **4**, 64-69 (2015)
23. R.G. Butenko, Science, 272 (1964)
24. F.L. Kalinin, V.V. Sarnatskaja, V.E. Polishchuk, Kiev, Naukova dumka, 488 (1980)
25. O.V. Matsneva, L.V. Tashmatova, T.M. Khromova, Biotechnological techniques for optimizing microclonal reproduction and adaptation of strawberry genotypes (Fragaria × ananassa Duch.), Orel, VNIISPK, 24 (2021)
26. T. Murashige, F. Skoog, Physiol. Plant, 1962, **15**, 4, 473-497
27. B.A. Dospekhov, Moscow, Agropromizdat, 351 **(**1985)