Effect of colchicine on meristematic tissues of diploid apple varieties in in vitro culture

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Abstract. For apple tree selection, it is of great importance to transfer diploid varieties and hybrids with already known valuable properties to the polyploid level. Tetraploid apple varieties are of interest for breeding as donors of non-reduced gametes in the production of triploids. The purpose of these studies was to study the effect of colchicine on the viability of explants in in vitro culture and their further growth and development. The objects of the study were diploid apple varieties. Polyploidization was carried out by cultivation on an agarized nutrient medium containing colchicine in concentrations of 0.01% and 0.02% and by drip method. The study showed that prolonged exposure to colchicine causes the death of explants. The genotypic reaction of the varieties to the processing methods, the concentration of amitotic, and the duration of its exposure were noted. Explants of the Imrus variety turned out to be more sensitive to the action of colchicine.

1 Introduction

Polyploidy is a fold increase in the number of chromosomes, leading to the formation of new ones which are significantly different from the original forms. In polyploids, there is a change in the nature of inheritance, the degree of traits' severity, the plasticity of the form and its adaptive capabilities [1]. Traditionally, polyploid induction was performed on plants grown in the open ground by soaking roots or whole plants in colchicine solution, injection of colchicine into secondary buds or application of a cotton swab soaked in colchicine to the lateral buds. The bulk of induced polyploids of fruit and berry plants was obtained by polyploidization of young seedlings [2, 3, 4]. However, the low efficiency of these methods was noted due to the high occurrence frequency of chimaera [5, 6].

Modern research in the field of biotechnology has shown great potential for inducing tetraploid plants during in *vitro* cultivation. Using the in *vitro* method, tetraploid forms were obtained in several genera of ornamental crops, citrus fruits [7, 8, 9, 10, 11], banana varieties [12].

The effectiveness of mutation induction in in vitro culture is as follows: 1) a wide selection of source material for polyploidization (buds, tissues, cells, organs), 2) selection and reproduction of the obtained clones, 3) high phytosanitary conditions are maintained

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throughout the process, 4) work on the creation of new forms under controlled conditions, 5) keeping plant tissues with active cell division regardless of the season, 6) the ability to conduct a relatively fast cytological analysis and immediately propagate the selected forms in the required amount [13].

The most common compounds that cause mutations are acenaphthene and colchicine. However, the latter is the most effective. It has a pronounced selective effect on karyokinesis in the embryonic tissues' cells of animals and plants. As a chemical, colchicine has great resistance and does not decompose either when standing for a long time or when sterilized in an autoclave.

The polyploidy method in apple tree breeding has been used since the late thirties of the last century and remains relevant at the present time [14].

Among industrial varieties, diploid apple varieties occupy a significant place their somatic cells contain 34 chromosomes; however, due to their biological and economic features, polyploids (2n=51 and 2n=68) are of increasing interest. Due to the limited number of fruits formed during abnormal reduction division in the germ cells, triploid varieties do not have a pronounced periodicity of fruiting; therefore, a moderate harvest is established in triploids even in the most favorable years, which does not prevent the laying of generative buds for the next year's harvest [15, 16]. As a rule, triploid varieties have larger commercial fruits in comparison with diploid ones. In triploids, there is a manifestation of heterosis in relation to many traits [3, 17, 18]. Such triploid varieties as Rozhdestvenskoye, Vavilovskoye, Yablochny Spas, Yubilyar, Pamyat' Semakinu were created and zoned in VNIISPK [19].

Clones of Melba and Spartan varieties are known among tetraploid varieties. Welsey, Golden Delicious, Papirovka and others. However, tetraploid varieties do not have such economic significance as triploids. They are of interest for breeding as donors of non-reduced gametes in the production of triploids. However, a limited set of tetraploid forms is a significant deterrent in obtaining triploid varieties [20].

N. V. Lisnev [2] obtained genetically diverse plants with a tetraploid set of chromosomes as a result of exposure to apple seedlings with 0.5% colchicine solution. The disadvantage of this technique is the difficulty of predicting what useful properties the resulting seedlings will possess. For breeding, it is of great importance to transfer specific varieties and hybrids that already have known valuable qualities to the polyploid level. This makes the breeding process more focused. Due to that it is necessary to act with amitotic only at a certain stage of plant development, namely at the moment of the greatest cell division, the polyploidization process can be carried out all year round only in laboratory conditions [21, 22]. Another problem is obtaining valuable plants from polyploid tissues.

In this regard, the purpose of this research was to study the effect of colchicine on the viability of explants in in *vitro* culture and their further growth and development.

2 Materials and methods

The research was carried out in the laboratory of Biotechnology of the Russian Research Institute of Fruit Crop Breeding (VNIISPK), (Orel). Their objects were diploid apple varieties selected by the FSBSI VNIISPK: Bolotovskoe, Imrus, Veteran, Kandil Orlovsky, Girlyanda. The studies were conducted in accordance with the methodological recommendations by O. V. Matushkina et al. [23], V. G. Trushechkiv, V. A. Vysotsky [24]. L. V. Tashmatova et al. [25]. Optimization of in *vitro* technology for polyploidy induction in apple varieties (2n=34) was carried out in several stages. At the first stage, the varieties were introduced into culture during the active growth period and propagated on a Quorin-

Lepoivre (QL) nutrient medium [26] containing nicotinic acid – 0.5mg/l, thiamine – 0.4mg/l, pyridoxine – 0.1mg/l, ascorbic acid – 1.5mg/l, 6-benzylaminopurine – 1.0 and 2.0mg/l.

At the second stage, lateral buds, growing tops of shoots with a length of 5 mm and single-bud shoots' cuttings in *vitro* were taken as material for research. Colchicination was carried out in two ways: by the introduction of amitotic into the nutrient medium and by the drip method.

In the first case, the concentration of amitotic was 0.01 and 0.02%. Processing time: 24 hours, 48 hours, 72 hours, 120 (5 days), 144 hours (6 days), 168 hours (7 days), 216 hours (9 days), 240 hours (10 days), 288 hours (12 days), 336 hours (14 days), 456 hours (19 days), 528 hours (22 days). After the exposure time, the explants were placed on a nutrient medium without colchicine with 6-BAP 0.5 mg/l. The material for polyploidization was immersed in the QL nutrient medium enriched with vitamins and containing 6-BAP at a concentration of 0.5mg /l and colchicine at the above concentrations. After treatment with colchicine, the plant material was planted on the same nutrient medium without amitotic.

In the second case, the concentration of colchicine was 0.1 and 0.2%. A 1% dimethyl sulfoxide (DMSO) solution was used as a solvent. The material for amitotic treatment was placed on the surface of the QL nutrient medium. A solution with colchicine was applied on the explant using a pipette. Exposure time: 24 hours, 48 hours, 72 hours, 120 hours (5 days), 144 hours (6 days), 168 hours (7 days), 216 hours (9 days), 240 hours (10 days), 288 hours (12 days), 336 hours (14 days), 432 hours (18 days). The explants were then washed in sterile distilled water and placed on a new QL medium. After 3 weeks of cultivation, the survival rate of explants treated with colchicine was evaluated. Then they were transplanted to the QL nutrient medium containing 6-BAP at a concentration of 1.0mg/l. After 3-4 cultivations, the survival rate of explants, development, and further ability to reproduce in *vitro* were recorded. Each explant was a separate clone line.

Explants were cultivated in a light culture room at illumination intensity of 3.5-4.0 thousand lux, temperature of 25 ± 1 °C with a photoperiod of 16 hours/day, 8 hours/night.

To obtain rooted colchicinated plants, a micro-graft method for seed rootstocks was used considering the recommendations of L. V. Tashmatova, V. A. Vysotsky, V. E. Dzhafarova [27].

Buds' fixation for cytological analysis was carried out according to the method by V. A. Rybin [28] and Z. P. Pausheva [29] in modified Carnoy solution. The number of chromosomes of apple genotypes was calculated by direct cytological method in the meristematic tissues of the buds at the "green cone" stage.

3 Results and discussion

When inducing polyploidization of meristematic tissues, an important task is to choose the treatment method, the type of amitotic, the concentration, and exposure of the treatment. It is important to study the effect of polyploidogenic substances on the viability of explants and their further growth and development. It is believed that colchicine is more toxic to plants than, for example, acenaphthene or trifluraline [1, 30]; however, it gives a higher percentage of the plants' yield with altered ploidy: in pomaceous - up to 32%, in stone fruit – up to 18.3%

At the second stage of research, a large difference was found in the toxicity manifestation degree of colchicine for apple explants with two methods of exposure. On average, the viability of explants in both treatment options varied from 100 to 0%

depending on the exposure option. The concentration of colchicine and the duration of treatment had a great influence.

Studies have shown that when cultivating Bolotovskoye variety explants on a nutrient medium with amitotic, their survival rate was more dependent on the concentration of the latter. An increase in the content of colchicine led to a sharp decrease in the number of regenerating explants. The maximum yield of viable explants was obtained in the option with 0.01%, 240 hours. The complete death of explants was observed in the option with 0.02%, 528 hours. When applying a colchicine solution to the apical points, results were obtained showing that the viability of explants was more influenced by the treatment time. Moreover, the number of explants suitable for further generation was higher at a concentration of 0.2%. With an increase in the exposure period, the survival rate dropped sharply. These data are reflected in Figure 1. Prolonged exposure to amitotic resulted in the explants' death either during treatment (0.1%, 168 hours) or with further micro-propagation without colchicine (0.2%, 264 hours).

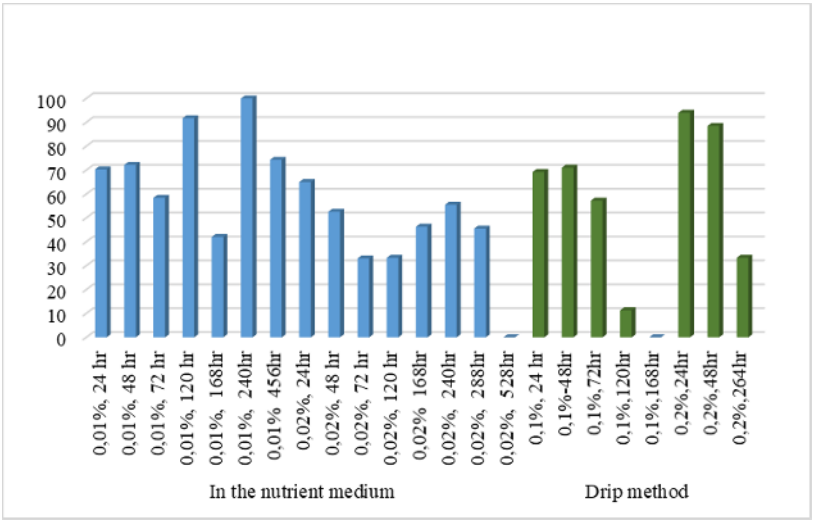

Fig. 1. The effect of various colchicine treatment options on the survival of Bolotovskoe variety's apical points.

Colchicine had the most toxic effect in the Imrus variety, which affected the viability of explants. In general, there was no pattern in the explants' reaction to the concentration, exposure time, or method of exposure to colchicine. However, the average viability value was quite low and averaged to 45-60%. The survival rate was 100% on only some options as shown in Figure 2.

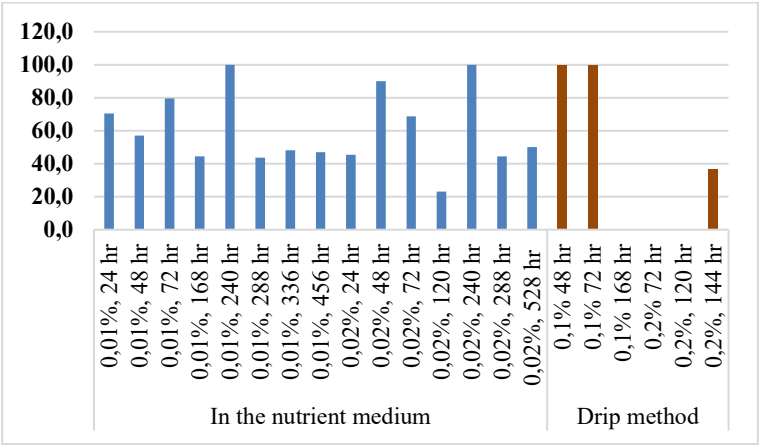

Fig. 2. The effect of various colchicine treatment options on the survival the Imrus variety's apical points.

For the Veteran variety, colchicine turned out to be less toxic, especially when added to the nutrient medium. The survival rate of the apical buds was in the range of 53-100%. However, after prolonged cultivation on a nutrient medium with colchicine at a concentration of 0.02%, explant death was observed during subsequent passaging. With the drip method, an increase in concentration to 0.2% turned out to be disastrous with prolonged exposure. This data is shown in Figure 3.

During micro-shoots' cultivation of the Veteran variety, it was found out that this variety has a high ability to regenerate lateral shoots when removing apical dominance; therefore, lateral buds on single-bud cuttings were also used as the source material for polyploidization in *vitro*. As a result of exposure with 0.2% colchicine solution, 45.7% of the buds took roots and regenerated within 240 hours.

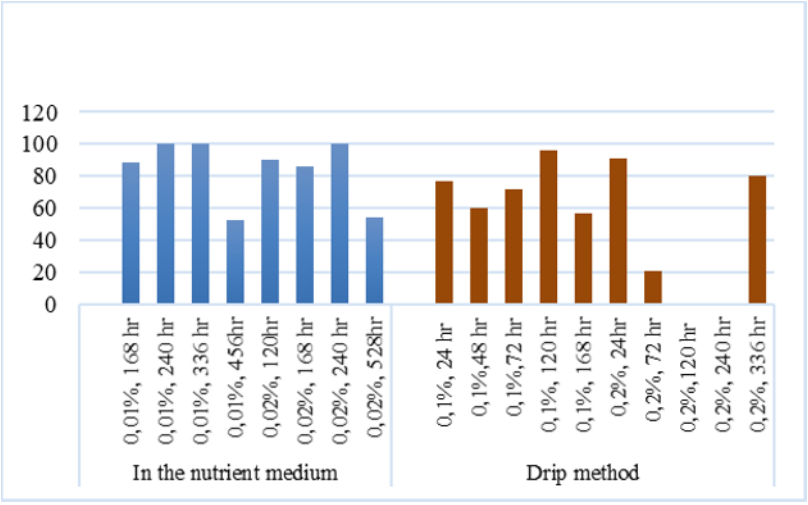

Fig. 3. The effect of different treatment options with colchicine on the survival of the Veteran variety's apical points.

For colchicine treatment of the Kandil Orlovsky variety, as well as for the Veteran variety, two types of buds were taken – apical and lateral. Cultivation of the latter did not

give positive results, since there was no shoots' regeneration due to the removal of apical dominance and the explants died.

The treatment of the tops with a drip method showed good viability in this variety. When using 0.1% solution, 89% of explants took roots and regenerated; when using 0.2% solution (168 h and 240 h), the survival rate was 49% and 61%, respectively.

After treatment with 0.2% colchicine of the growing shoots' tops of the Girlyanda variety for 6, 7, 9, 12 and 18 days, the survival rate fluctuated, but did not depend on the time of exposure. The concentration of polyploidogen 0.2% did not have a toxic effect on the explants of the Girlyanda variety. However, the study showed that prolonged exposure to amitotic (432 hours) leads to the complete death of explants. With an exposure of less than 18 days, the survival rate ranged from 30.0% to 77.4%. This is reflected in Figure 4.

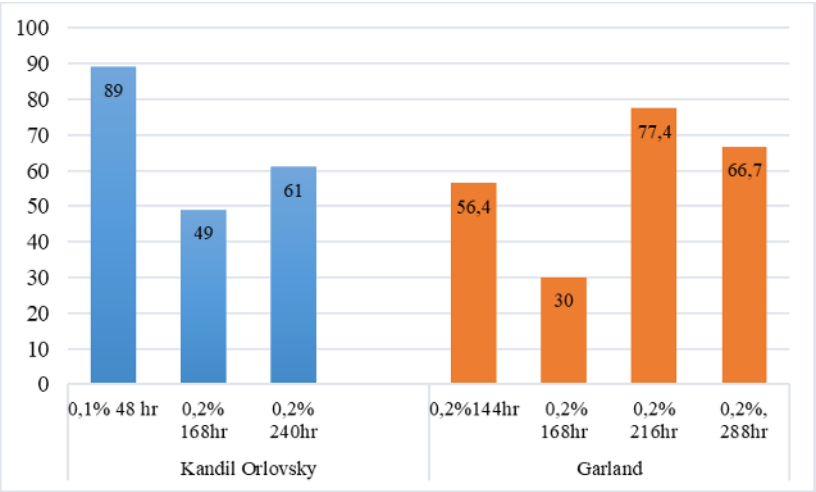

Fig. 4. The effect of colchicine on the survival of Kandil Orlovsky and Girlyanda varieties' apical points.

In the Girlyanda variety, the use of lateral buds on single-bud cuttings as a source material for polyploidization also did not bring positive results since they did not regenerate shoots and died as a result of necrosis.

Further reproduction of micro-shoots obtained from the treated tops and buds was carried out on a QL nutrient medium containing 1.0 mg/l 6-BAP. The formation of conglomerates consisting of shoots of various lengths was observed (Figure 5). Depending on the processing method and the concentration of amitotic, the reproduction coefficient in the Bolotovskoye variety ranged from 1.7 to 4.9, in the Imrus variety - from 1.2 to 4.8, in the Veteran variety - from 1.1 to 4.6, in the Kandil Orlovsky variety - from 1.6 to 1.8, in the Girlyanda variety - from 1-3 to 3.0.

Fig. 5. Micro-shoots' development of Veteran apple trees formed from colchicinated buds (drip method, 0.1% solution of colchicine, 48h)

Micro-grafting was carried out due to the poor rootability of apple trees in tissue culture, where micro-shoots of colchicinated forms were used as grafts. Fixation was carried out in the open ground in April. Direct chromosomes' counting in forms obtained after cultivation on a nutrient medium with colchicine at exposure of 24, 48 and 72 hours did not reveal the presence of tetraploids.

Further cultivation of micro-shoots after treatment with colchicine was carried out on a QL nutrient medium against a background of 6-BAP 1.0 mg/l. The reproduction coefficient ranged from 1.1 to 3.0 (Table 9).

4 Conclusion

The viability of explants exposed to colchicine depends on the concentration of the amitotic, the method of exposure and varietal characteristics. In the option of including colchicine in the nutrient medium, the maximum exposure period at which explants remain viable is 22 days. With the drip method, the maximum processing time ranges from 12 to 14 days depending on the variety. Treatment of apple explants with colchicine for 24-72 hours does not cause ploidy disturbance.

References

1. R. V Papikhin., S. A Muratova, Agricultural biology, **5**, 63-68 (2011)
2. V. N. Lisnev, Tr. Novosibirsk Fruit and Berry Experimental Station named after I. V. Michurin, **2**, 3-9 (1975)
3. V.N. Lisnev, Fruit and vegetable farming, **11**, 21-23 (1985)
4. G. A. Bavtuto, Collection: Apple breeding in the USSR, **3**, 186-191 (1977)
5. X. S. Pei, *Polyploidy Induction and Breeding. Shanghai Science and Technology Press*, 95–98 (1985)
6. X. M. Yang, Z. Y. Cao, L. Z. An, Y. M. Wang, X. W. Fang, Euphytica, **152**, 217–224 (2006) DOI 10.1007/s10681-006-9203-7
7. S.L. Gao, B.J. Chen, D.N. Zhu.Plant Cell Tissue Organ Cult, **70**, 289–293 (2002) DOI:10.1023/A:1016577002039
8. K. K. Petersen, P. Hagberg, K. Kristiansen Plant Cell Tissue Organ Cult, **73**, 137–146 (2003) DOI:10.1023/A:1022854303371
9. J. B. Rose, J. Kubba, K. R. Tobutt, Plant Cell Tissue Organ Cult, **63**, 127–132 (2000) DOI:10.1023/A:1006472101185

10. A. T. Roy, G. Leggett, A. Koutoulis, Plant Cell Rep, **20(6),** 489–495 (2001) DOI:10.1007/s002990100364
11. J. H. Wu, P. Mooney, Plant Cell Tissue Organ Cult, **70**, 99–104 (2002) DOI:10.1023/A:1016029829649
12. J.H Wu, P. Mooney. Plant Cell Tissue Organ Cult, **70**, 99–104 (2002)
13. R.V. Papikhin., S.A. Muratova., D.O. Gorlov, Current state, problems, and prospects for the development of agricultural science: materials of the IV International scientific and practical conference. Scientific editor V.S. Pashtetsky, 234-236 (2019)
14. V. E. Jafarova, Modern scientific research, - Concept. ART 53091., URL: http://e-koncept.ru/article/464 / - State Reg. V. 1. E-mail No. FS 77- 49965. - ISSN 2304-120X (2013)
15. A.S. Tuz, A.Ya. Lozitsky, Genetics, **9**, 41-50 (1970)
16. L.I. Dutova, *Selection of apple trees to improve the quality of fruits*. Orel, 202-206 (1985)
17. V.V. Ponomarenko, Selection of apple trees to improve the quality of fruits. Orel, 163-168 (1985)
18. E.N. Sedov., G.A. Sedysheva, M.A. Makarkina., Z.M. Serova, Vavilov Journal of Genetics and Breeding, **21(2),** 207-213 (2017) DOI 10.18699/VJ17.238
19. E. N. Sedov, G. A. Sedysheva, Z. M. Serova, E. V. Ulyanovsk, Horticulture, and viticulture, **6**, 35- 41 (2011)
20. G. A. Sedysheva, E. N. Sedov, Z. M. Serova, *Selection and variety breeding of garden crops: Collection – Orel*, 40-45 (1998)
21. V. K. Shcherbakov, Polyploidy in plants, **5**, 110-120 (1962)
22. R. V. Papikhin, S. A. Muratova, S. V. Luchnikova, *Problems of agroecology and adaptability of varieties in modern horticulture of Russia:* mat. all-Russian scientific method. conf. (July 1-4, 2008, Orel) 213 – 217 (2008)
23. O.V. Matushkina, I.N. Pronina, Technology of clonal micropropagation of apple and pear trees: method. rec, 32 (2008)
24. V.G. Trushechkin., V.A. Vysotsky, O.A. Leontiev-Orlov, Methodological guidelines for clonal micro-reproduction of rootstocks and apple varieties, Moscow: VASHNIL, 19 (1985)
25. L. V. Tashmatova., O. V. Matsneva, T. M. Khromova, V. V. Shakhov, Creation of homogeneous tetraploids of apple varieties in vitro, as well as tetraploids and chimeras from colchicinated apple seedlings, method. rec. 36 (2020)
26. M. Quorin, P. Lepoivre. Improved media for in vitro culture of Prunus sp. Acta Hortic, **78(4),** 437–442 (1977)
27. L.V. Tashmatova., V.A. Vysotsky., V.E. Dzhafarova, Clonal micropagation and deposition of pears in viro method. rec, 18 (2015)
28. V. A. Rybin, Cytological method in fruit breeding, 216 (1967)
29. Z. P. Pausheva, Practicum on plant cytology, 304 (1980)
30. O. V. Mochalova, D. A. Gusev, Achievements of science and technology, **30(9),** 36-39 (2016)