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The karyotypes of interspecific grapevine hybrids (*Vitis vinifera* L. × *Vitis rotundifolia* Michx.) BC_4

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Abstract.

Aims*.** In this work, the results of cytological research, fertility, and biotic stress resistance of the genus *Vitis* L. interspecific hybrids between *V. rotundifolia* Michx. and *V. vinifera* L. are given. To better understand the nature of the characteristics of the hybrids between the *Euvitis* (*V. vinifera*) and *Muscadinia* (*V. rotundifolia*) subgenera, combining the quality of fruit from *V. vinifera* with disease resistance and environmental adaptation of muscadines (*V. rotundifolia*), for the completion of the grapevine genetic bank were carried out to study their metaphase chromosomes. ***Methods*.** The species *V. vinifera* (2*n* = 38), *V. rotundifolia* (2*n* = 40) and the interspecific hybrids BC_4 (BC_4-717, BC_4-718, BC_4-719, BC_4-720, BC_4-721, BC_4-754, BC_4-755, BC_4-756, BC_4-757, BC_4-758, BC_4-790, BC_4-591. BC_4-792, BC_4-793, BC_4-794) were included in the experiments. For counting the number of chromosomes in somatic cells the standard methods of cytological preparations were used. The interspecific hybrid grapevine specimens' metaphase chromosomes were calculated by the propion-lacmoid chromosome staining method, through which use, a contrasting image is obtained: the cytoplasm becomes colorless, and the chromosomes are stained red-brown. ***Results. Through the study of the karyotypes of interspecific grapevine hybrids, it was possible to count BC_4 chromosomes and select specimens with 2n = 38, which indicates the stability of the resulting genotypes. ***Conclusion.*** The quality and adaptability of the BC4 hybrids, and the stable number of chromosomes are essential to complement the genetic bank of grapevine, and select specimens will serve as the basis of the breeding programs effective.

Key words: biotic stress, chromosomes, *Euvitis* and *Muscadinia* subgenera, generation BC_4, distant hybrid, karyotype, species.

Каріотипи міжвидових гібридів винограду (*Vitis vinifera* L. × *Vitis rotundifolia* Michx.) BC4

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Реферат.

Мета. У роботі наведено результати цитологічних досліджень, фертильності та стійкості проти біотичних стресів міжвидових гібридів роду *Vitis* L. (*V. vinifera* L. × *V. rotundifolia* Michx.). Для кращого розуміння природи цінних для поповнення генетичного банку винограду гібридів між підродами *Euvitis* Planch. (*V. vinifera*) та *Muscadinia* Planch. (*V. rotundifolia*), що поєднують якість плодів *V. vinifera* зі стійкістю проти хвороб та екологічною адаптивністю винограду мускатного (*V. rotundifolia*), були проведені дослідження їхніх метафазних хромосом. ***Методи.*** Види *V. vinifera* (2*n* = 38), *V. rotundifolia* (2*n* = 40) та міжвидові гібриди BC_4 (BC4-717, BC4-718, BC4-719, BC4-720, BC4-721, BC4-754, BC4-755, BC4-756, BC4-757, BC4-758, BC4-790, BC4-591, BC4-792, BC4-793 та BC4-794) були включені в досліди. Для підрахунку кількості хромосом у соматичних клітинах використовували стандартні методи дослідження цитологічних препаратів. Метафазні хромосоми міжвидових гібридів винограду підраховували методом пропіон-лакмоїдного забарвлення хромосом, за допомогою якого отримують контрастне зображення: цитоплазма стає безбарвною, а хромосоми забарвлюються в червоно-коричневий колір. ***Результати.*** Внаслідок дослідження каріотипів міжвидових гібридів винограду вдалося підрахувати хромосоми рослин BC_4 й відібрати зразки з 2*n* = 38, що засвідчувало стабільність отриманих генотипів. ***Висновки.*** Якість і адаптивність гібридів BC_4, а також стабільна кількість хромосом є важливими для поповнення генетичного банку винограду, а відібрані зразки слугуватимуть основою вихідного матеріалу для ефективних селекційних програм.

Ключові слова: біотичний стрес, хромосоми, підроди *Euvitis* і *Muscadinia*, покоління BC_4, віддалений гібрид, каріотип, вид.

Introduction. Cultivated and wild grapevines belong to the genus *Vitis* L. from the grape family *Vitaceae* Juss. The genus *Vitis* contains two subgenera: *Euvitis* Planch., bunch grapes, and *Muscadinia* Planch., muscadine grapes (Rahemi et al., 2022; Walker et al., 2019).

V. vinifera L. (subgenus *Euvitis*) originated around the Mediterranean basin and the Middle East. It is a woody species, which played an essential role in human history (Vignani & Scali, 2024). *V. vinifera* is considered the predominant grape species grown worldwide for fresh or processed fruits, both for wine and table grapes/dried grapes production (Alston & Sambucci, 2019; Dudić et al., 2024). The desirable quality traits include superior aroma and flavor characteristics: thin and tender skin, meaty pulp, large berries, high sugar, low pH content, and soft or delicate flavor (Fortes & Pais, 2016; Maia et al., 2021).

V. rotundifolia Michx. (subgenus *Muscadinia*) is native to the South-Eastern United States and was the first muscadine grape species to be cultivated. The native range of *V. rotundifolia* extends from Delaware to central Florida and along the Gulf of Mexico to eastern Texas (Buck & Worthingt, 2022; Conner & Worthington, 2022). *Muscadinia* grapes are distinguished essentially from the *Euvitis* species genetically, anatomically, physiologically, and in taste that they should be considered a separate fruit. The major problem for gaining wider acceptance of muscadine grapes is the relatively low fruit qualities compared to the excellent fruit of *V. vinifera*, but they are characterized by high disease and pest resistance among *Vitis* species, in the first-place phylloxera or gall-louse (*Daktulosphaira vitifoliae* Fitch) is a worldwide pest of grapevines (Granett et al., 2001; Walker et al., 2019; Rispe et al., 2020). Therefore, the *Muscadinia* may be the source for resistance genes to phylloxera, nematodes, and a number of fungal diseases (Burger et al., 2009).

The *V. rotundifolia* plants have the chromosome number $2n = 40$, i.e., $2x = 2n = 40$, in the somatic cells, and are characterized by fruit borne in many clusters. Their smooth, thin bark, which is tightly attached to the young wood and separated from the old wood by scales, has unbranched tendrils, dense wood, and solid flesh. In contrast, *Euvitis* grapes have 38 somatic chromosomes ($2x=2n=38$), branched tendrils, many berries in a fruit cluster, no dieback zone between the cluster and the stone, striped bark that peels off in strips on the old wood, less dense wood than *Muscadine*, and flesh interrupted by diaphragms at the nodes (Buck & Worthington, 2022).

A long-standing goal of *Euvitis* and *Muscadinia* breeding programs has been developing hybrids between species of these subgenera, combining fruit quality from *V. vinifera* with disease and pest resistance and environmental adaptation of muscadines. The long intercontinental distance separation between *Euvitis* and *Muscadinia* and chromosomal differences between *V. vinifera* and *V. rotundifolia* past prevented the movement of genes between the subgenera species (Guzmán-Ardiles et al., 2023).

The works on synthesis of new genome of grapevine was initiated by Peter Wylie (1871). He pollinated two *V. vinifera* varieties with pollen of a male muscadine. Seedlings derived were highly sterile and considered true hybrids. Hybrids *muscadinia–euvitis* were later reports by Alexis Millardet (1901), Thomas Munson (1909) and Charles Dearing (1917). The most extensive controlled crosses between the two subgenera were made by Louis Detjen (1919). The hybrids, obtained from the female muscadine pollinated with bunch grape pollen, were later proven to be straight muscadine derivatives. Hybrids from *Muscadinia* and *Euvitis* crosses were successfully produced by Robert Dunstan (1964) in North Carolina, G. I. Patel and H. P. Olmo (1955) at the University of Carolina, and Alain Bouquet (1980) in France. Now thanks to of wild–to–crop introgression through repeated backcrossing has introduced disease and pest resistance from *V. rotundifolia* to *V. vinifera* and ameliorated the general grapevine tolerance (Conner & Worthington, 2022; Foria et al., 2022).

The metaphase chromosomes of the hybrids between the *V. vinifera* and *V. rotundifolia* study will help us better understand the genetic determinism of their resistance to disease, pests, and abiotic stresses and outline the challenges and opportunities that the grapevine breeder faces.

Materials and Methods. In our study, the species *V. vinifera* ($2n = 38$), *V. rotundifolia* ($2n = 40$) and the interspecific hybrids BC_4 (BC_4-717, BC_4-718, BC_4-719, BC_4-720, BC_4-721, BC_4-754, BC_4-755, BC_4-756, BC_4-757, BC_4-758, BC_4-790, BC_4-591. BC_4-792, BC_4-793, BC_4-794) were included in the experiments. For counting the number of chromosomes in somatic cells, the standard methods of cytological preparations were used. The interspecific hybrid grapevine specimens' metaphase chromosomes were calculated by the propion-lacmoid chromosome staining method, through which use, a contrasting image is obtained: the cytoplasm becomes colorless, and the chromosomes are stained red-brown (Ivasishin, 2022).

This method consists of the following steps: in a few micro-tubes (5 × 40 mm) pour 0.5 ml of dye, where 5–6 small portions of meristem are introduced and collected from the top of the shoots with active growth, then cleaned of scales and cut hairs or embryo root tips respectively. The apical meristem is cut with the blade into small portions to facilitate the deep penetration of the dye. After the fixation–staining time elapsed (12–24 hours), the study material was placed on the slide. Excess dye is removed with filter paper, and then 1–2 drops of 40% propionic acid are pipetted to reduce cytoplasm staining. Subsequently, the material on the blade is ground with a glass stick and subjected to heat treatment on the alcohol lamp for about 30 seconds, to completely macerate the material. After boiling, drip 1–2 drops of 40% propionic acid, then cover the mixture with a cover glass. A strip of filter paper is placed on the slide and the study material is pressed with the end of the tweezers (it is advisable to obtain a layer of cells). The temporary "squash" preparation is completed for viewing after about 15 minutes from the moment of preparation, and already after 12 hours, it begins to dry and discolor. To avoid damage to the preparations, the edges of the cover glass were treated with a special mixture. To prepare this mixture, 50 ml of distilled water + 30 g of gum arabic (food additive E414) + 16 ml of glycerin + 200 g of chloral hydrate. That, so storing the preparations is possible for 2–3 months in the refrigerator at a temperature of about + 4° C. However, the preparations gradually discolor even if these measures are taken (Ivasishin, 2022).

Results and Discussion. Studies of the causes of sterility of the interspecific grapevine hybrids aided by the microscope revealed the prevailing failure of the pollen tube to reach the embryo sac. Consequently, most hybrids have been sterile, but a few have some levels of fertility. Our results agreed with the previous findings that hybrids were extremely difficult to produce when muscadine grapes were used as the female parent and pollinated by some *V. vinifera*. The advantage to using muscadine as the female parent is also that fresh *V. vinifera* pollen can be used for pollination of muscadines the same season, since *V. vinifera* grapes always bloom a few weeks earlier than *V. rotundifolia* (Patel & Olmo, 1955; Coito et al., 2019). Absolute sterility of male gametophytes and high or partial one of the female gametophytes, specific to distant hybrids of BC_2 and BC_3 has remained intact.

In addition to standard breeding techniques, tissue culture, and protoplast fusion methods were being employed with the hope of discovering fruitful hybrids through backcrossing programs to develop both *V. vinifera* and *V. rotundifolia* cultivars (Derman, 2020).

The results obtained when examining 50 distant hybrids demonstrated that all BC_4 hybrids possess the same diploid number of chromosomes 2n=38, and they were fertile, but both gametophytes (female and male) of first-generation hybrids (F_1) were completely sterile. The male gametophytes of F_2 hybrids also were sterile, but the female gametophytes functions normally. The F_3 hybrids represent obtained from backcrosses of the DRX-55 hybrid with the species *V. vinifera* and *V. rotundifolia*, the ‘Soiaki’ and ‘Seyve Villard’ were varied by fertility. Some of them were sterile after both gametophytes; sterile after the male gametophyte; partially fertile; and self-fertile with fertility completely restored similar to the European hermaphrodite grapevine cultivars.

By performing 2 cross combinations with pollen collected from the hybrids DRX-M3-90 × S.V.20-366 and DRX-M3-232 × 12 S.V.20-309, the synthesis process was stimulated and the F_4 generation was created. The diversity of distant hybrids in the *F*4 generation (BC3) consisted of self-fertile hybrid forms — DRX-M4-502, DRX-M4-504, DRX-M4-508, DRX-M4-536, DRX-M4-542, DRX-M4-545, DRX-M4-560, DRX-M4-564, DRX-M4-567, DRX-M4-583, DRX-M4-658, and functional female hybrids — DRX-M4-634, DRX-M4-649, DRX-M4-661, for which the causes of sterility of the male gametophyte, morphological variations of the pollen grain and the variable number of chromosomes were directly established (2*n*=39, 2*n*=38 etc.). Unlike previous research on the F_3 and F_4 generations, which highlighted the causes of absolute and partial sterility in distant hybrids (Topale & Roychev 2021), the present study identified the presence of the 2*n*=38 chromosome set in BC_4 hybrid metaphases (Fig. 1).

This confirmed the elimination of the odd chromosome from the karyotype, generating the appearance of the character of high productivity and resistance to diseases, pests, and external factors in distant hybrids at the level of Eurasian standard cultivars, which allows their direct use as parents in breeding programs.

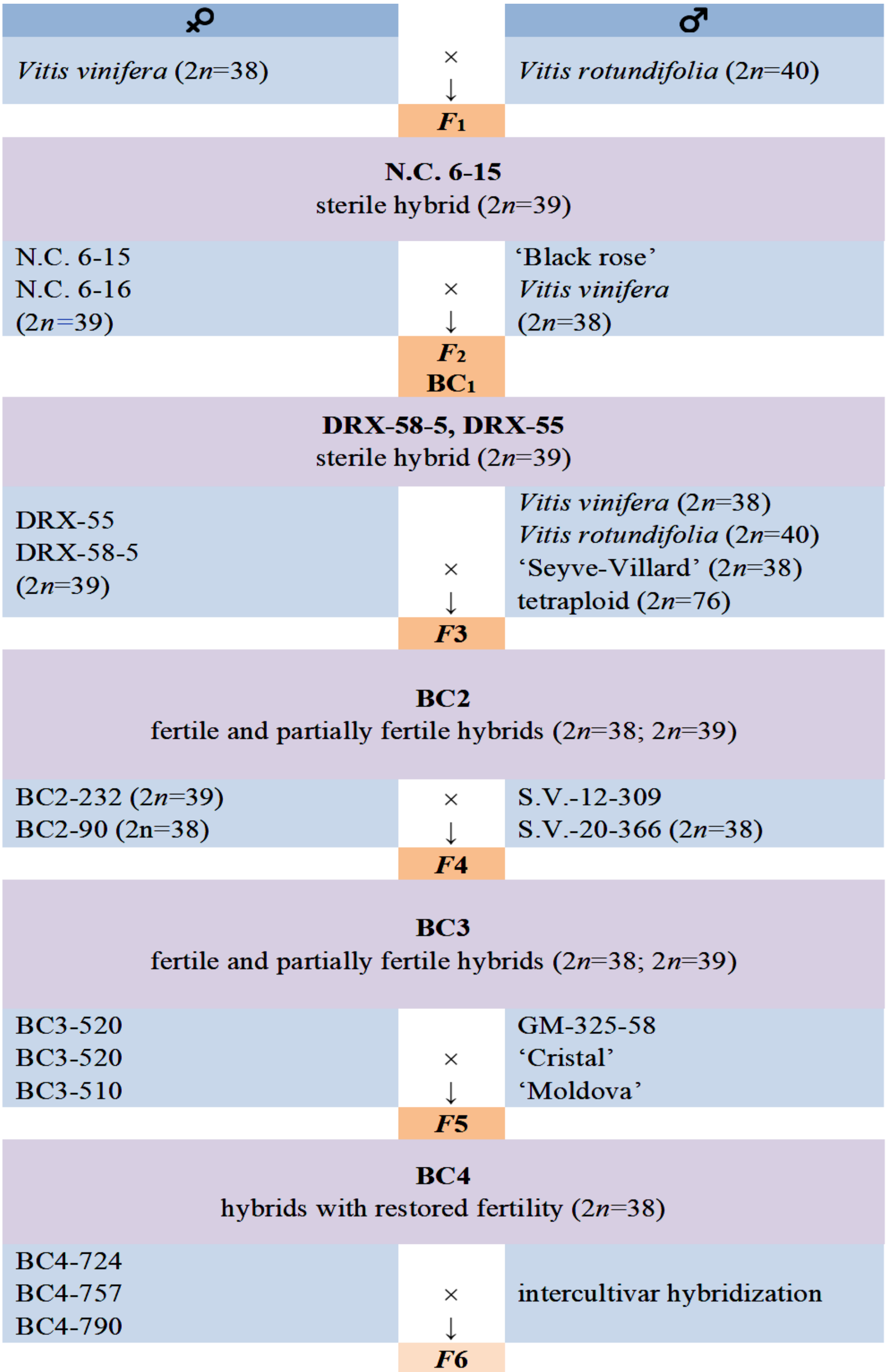


Figure 1. The scheme of distant crossings of grapevine, according to Ş. Topală with modifications.

The selected self-fertile hybrids with 2n = 38 were clustered into three groups. The plants in these groups differed in phenotype and some utilitarian characteristics. Model hybrids (BC_4-721; BC_4-757 and BC_4-790) for cytological studies were selected from each group (Fig. 2).

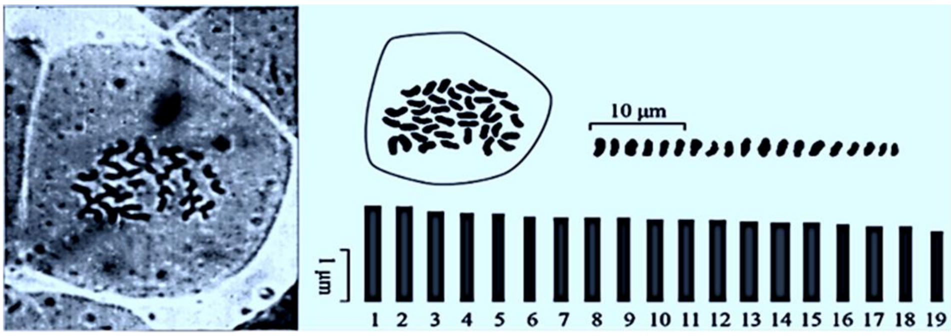
A ***B*** ***C***

Figure 2. The model distant grapevine hybrids of the three groups of genotypes:
A — BC4-721; ***B*** — BC4-757; ***C*** — BC4-790

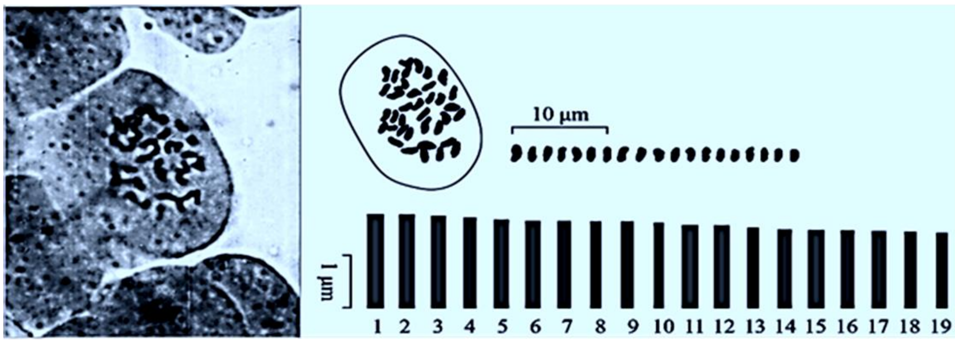
Analyzing the three groups of genotypes, we note that the karyotype of the first group, which was a cross of the F_4 generation with the 'Bianca' and was characterized by genetic stability (Fig. 3). Overall, the chromosomes were small. The maximum length of the chromosome was 1.94 µm, and the minimum — 1.17 µm. The maximum thickness of the chromosomes was 0.88 µm, and the minimum — 0.52 µm. The average size of a chromosome reached the size of 1.58 × 0.74 µm. The total length of the chromosomes of the diploid set was equal to 60.03 µm.

The second group of distant hybrids was the result of selection in the crossing of hybrids of the F_4 × 'Cristal' (SV 12-375 × 'Alifeld-100', Hungary). The plants of this group have characteristic chromosomal dimensions, similar to Eurasian cultivars. For the purpose of their karyotype study, the distant hybrid BC_4-757 was selected (Fig. 3). Overall, its chromosomes were of small dimensions, with small deviations, unlike the first group of hybrids. The maximum length of the chromosome was 2.12 µm, and the minimum — 1.19 µm. The maximum thickness of the chromosomes was 0.88 µm, and the minimum — 0.52 µm. The average size of a chromosome reached 1.67 × 0.78 µm. The total length of the chromosomes of the diploid set was equal to 65.11 µm.

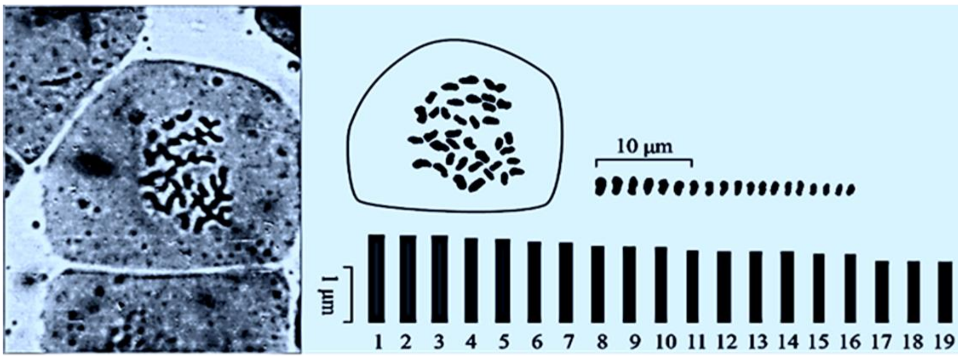
The third group of distant hybrids, consisting of backcrossing of F_4 generation hybrids, has similar characteristics to the first studied groups of hybrids. Plants of this group were characterized by the same genetic stability (as plants of the first two groups), with minor deviations in chromosome sizes. For their karyotype features study, the distant hybrid BC_4-790 was selected (Fig. 3).



BC_4-721



BC_4-757



BC_4-790

Figure 3. The metaphase boards and karyograms of the model distant grapevine hybrids *V. vinifera* × *V. rotundifolia* (×1770)

Overall, its chromosomes were small. The maximum length of the chromosome was 2.17 µm, and the minimum — 1.39 µm. The maximum thickness of the chromosomes was 0.93 µm, and the minimum — 0.67 µm. The average size of a chromosome reached the size of 1.75 × 0.78 µm. The total length of the chromosomes of the diploid set was equal to 66.42 µm.

It is necessary to mention that because the satellite filament is very thin and short, it is the first element to discolor during the study process, which is why in most temporary and permanent cytological preparations the satellite is difficult to observe. In addition, the satellite filament spirals, and consequently, the satellite is strongly attracted to the chromosome body, becoming practically inaccessible for investigations. Thus, at the level of chromosome length and thickness, the three groups taken into study are attested to be close in size with minimal deviations.

During the study of the chromosome morphology of the model distant grapevine hybrids were identified: metacentric chromosomes with centromere in the middle and even and short shoulders (four pairs); metacentric chromosomes with equal and long shoulders (six pairs); submetacentric chromosomes with centromere in the submedial region, due to which one shoulder is shorter than the other (eight pairs) and one pair of acrocentric chromosomes with centromere in the terminal region.

The performed studies of somatic chromosomes of distant grapevine hybrids (*Euvitis* and *Muscadinia*) obtained as a result of BC_4 crosses with *V. vinifera* cultivars confirmed that they are similar to Eurasian cultivars by the investigated parameters.

Conclusions. The genomes of the distant hybrids *V. rotundifolia* × *V. vinifera* stabilization and restoration of their normal fertility were regained after a fourth backcross with *V. vinifera* (in generation BC_4). The BC_4 plants were characterized by universal-intermediate type inheritance of paternal characters with obvious evident tendencies towards maternal. The use of distant backcrosses BC_4 in breeding programs of new grape cultivars combining resistance to biotic and abiotic stress factors environmental with high qualities of Eurasian cultivars can contribute to the development of new improved cultivars adapted to current climate changes in the Republic of Moldova and adjacent regions.

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