Cytoanatomical marker traits of the ploidy level in fruit and berry crops

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Abstract. For use in breeding work, the methods of complex accelerated cytological diagnosis of genotypes of fruit and berry crops with an altered ploidy level were optimized. The proposed diagnostic method was tested on polyploids of the genus *Malus*, *Fragaria*, *Ribes*, *Rubus*, rowan-pear hybrids and is recommended for wide scientific and practical application in the plant breeding and cytology. The effectiveness of this method is ensured by its availability and reliable statistical differences in accounting parameters. In a comprehensive cytological diagnosis of forms of fruit and berry crops with an increased level of ploidy, it is proposed to first study the morphoanatomical traits (sizes and proportions of stomatal guard cells, the number of chloroplasts in them, the diameter of pollen grains). This will significantly reduce the time of laboratory analysis and field assessment by deleting forms with unchanged indicator values.

1. Introduction
Experimental polyploidy is a great importance for the breeding of fruit and berry crops. It allows one to obtain forms with a new quantitative and qualitative expression of traits [1-4]. At the first stage, genotypes with an increased ploidy level are obtained (by a treatment with polyploidizing compounds or physical factors, through a somaclonal variability *in vitro* or *in vivo*, using a hybridization of heteroploid forms, etc.). A preliminary mass selection for ploidy is carried out among them, and genotypes with an unchanged ploidy level are rejected. Identified heteroploids are planted for further study.

The breeding of plant forms with a changed ploidy level has been developing for several decades; therefore, the basic principles of heteroploid isolation are known and optimized for many plant crops.

Direct counting of the number of chromosomes on stained cell micropreparations is the most reliable method for determining the ploidy level at plant genotypes, but it is difficult and time-consuming in the case of a large number of samples. In addition, most fruit and berry crops have very small chromosomes (about 1-3 microns) and a low mitotic index, which makes it difficult to obtain high-quality metaphase plates on a micropreparation and significantly increases the chromosome counting time. Therefore, indirect methods for diagnosing ploidy allow faster selection of polyploid forms, although each of them has certain disadvantages that slightly limit its use.

The most precise indirect method for determining ploidy level is flow cytometry assay. Its principle consists in the automated measurement of the chromatin amount in cell nucleus stained with a specific fluorescent dye with an affinity for DNA (Hoechst 33258, DAPI, PI, etc.). The fluorescence intensity of the stained nucleus is directly proportional to the amount of its chromatin and, accordingly, to the ploidy of the cell. This method is widely used in many countries to indirectly determine the ploidy level of...
human, animal and plant cells, including in plant breeding [5, 6]. Its serious disadvantage is the significant cost of a flow cytometer, so diagnostics is available to a limited number of researchers.

Modern identification and study of polyploidy plant forms using molecular genetic analysis is a high-resolution method that makes it possible to establish the unique biological characteristics of genotypes [7-9]. However, this method is also not always available when carrying out complex breeding studies of genotypes with different ploidy levels.

In the breeding of fruit and berry crops, general principles of indirect selection of heteroploids are also applicable, but they have a number of peculiarities. Due to the long time at which plants enter first fruiting, the application of the method of pollen analysis is significantly difficult for ploidy detection. In addition, distant polyploid hybrids are characterized by significant meiotic disturbances during microsporogenesis and morphologically heterogeneous pollen grains. Although, pollen analysis is an informative method for studying the generative sphere in genetically stable genotypes [10]. The most widespread methods of preliminary detection of ploidy genotypes are the quantitative analysis of stomatal guard cells and the study of their structural and anatomical features [11, 12].

The development of methodological techniques for a complex accelerated cytological diagnosis of genotypes of fruit and berry crops with an increased ploidy level is the urgent task. This will allow studying the minimum number of accounting parameters in order to speed up the breeding process and reduce labor costs in the analysis.

Experimental polyploidy is a promising method of plant breeding, allowing one to obtain genotypes with a new quantitative expression of traits of original forms. Certain successes in this direction have been achieved for fruit and berry crops [13, 14]. Detection of induced polyploids among unchanged genotypes can be a difficult problem in case of a large number of studied plant samples. The use of morphoanatomical markers of the ploidy level allows one to carry out preliminary express diagnostics of genotypes with less time for the presence of polyploid forms for the purpose of their further cytological analysis and chromosome counting. The least variability in many plants is characterized by cymotomorphological markers – the size and proportions of various plant cells (stomatal guard cells, epidermal cells, pollen grains), as well as their individual organelles.

The aim of the research was the analysis of morphoanatomical and cytological markers of the ploidy level in the genotypes of fruit and berry crops.

2. Materials and methods

Plants of the genus *Malus* (11 genotypes), *Fragaria* (15 genotypes), *Rubus* (10 genotypes), *Ribes* (12 genotypes), 4 rowan-pear hybrids with different ploidy levels were selected as biological objects of research.

For the analysis of stomatal guard cells, micropreparations of epidermal tissue of 10 mature leaves were prepared at the end of their growth period. The study of the size differentiation of pollen grains was carried out during their staining with acetocarmine [15]. The anthers were fixed from blossoming buds. The linear dimensions of stomatal guard cells and the diameter of pollen grains were measured on photographic images of preparations using the ImageJ software. For each genotype, 100 measurements of each trait were carried out.

Statistical processing of the obtained experimental data was carried out by the methods of descriptive statistics, regression and variance analyzes in the Microsoft Office Excel 2016.

3. The study of morphoanatomical marker features of the ploidy level in genotypes

*Influence of ploidy level on stomatal cell size*

The stomata are important structural formations of all plants, as they provide transpiration and gas exchange between the internal tissues and external environment. Stomata sizes depend on the ploidy level of the genotype in many plant cultures.

Several wild species of the genus *Fragaria* L. form a natural polyploid series with a base number of chromosomes $x = 7$. We have studied the sizes of stomatal guard cells in the most used species in breeding – *F. orientalis* Los. (tetraploid, $2n = 4x = 28$), *F. moschata* Duch. (hexaploid, $2n = 6x = 42$),
In strawberry genotypes of different ploidy levels, the interclass interval of local maxima in the length of stomatal guard cells is 4 μm. The difference between the trait of tetraploid and hexaploid is 1.17 times, hexaploid and octoploid – 1.12 times, tetraploid and octoploid – 1.32 times (Figure 1). The relationship between the ploidy level and the length of stomatal guard cells is directly proportional, described by the linear regression equation $y = 2.53x - 26.25$ (determination coefficient $R^2 = 0.66$).

**Figure 1.** Variability (a) and mean value (b) of the length of stomatal guard cells in different ploidy genotypes of the genus *Fragaria* L.

A significant increase in the dimensional differentiation of cells is observed at the experimental induction of polyploidy of vegetative cells. It is associated with the disturbances of mitotic divisions due to the complete and partial blocking of the spindle of division by the influencing factors of polyploidization. Emerging hyper- and hypo-ploid cells contribute to a significant expansion of the interval of manifestation of the trait and the dispersion of its mean value. For example, in the black
currant variety Cherny Zhemchug (Black Pearl) during shoot polyploidization, the range of stomatal guard cell lengths increased 4.1 times, and dispersion - 7.4 times. The variation curve shows several local peaks associated with the presence of groups of cells with several ploidy levels, which arise as a result of the long-term polyploidizing action of amitotic (Figure 2).

![Length of stomatal guard cells](image)

**Figure 2.** Change in the length of stomatal guard cells in black currant variety Cherny Zhemchug under experimental induction of polyploidy

In fruit and berry crops, positive correlation at very high level was found between the width and length of stomatal guard cells \( r = 0.92\ldots0.97 \); therefore, in order to reduce time and labor costs, it is more rational not to measure the width of the cells (Figure 3).

![Width and length of stomatal guard cells](image)

**Figure 3.** Dependence of the length and width of stomatal guard cells in fruit and berry crops of different ploidy levels (apple, pear, rowan-pear hybrids, strawberry, blackberry).

**Influence of ploidy level on the number of chloroplasts in stomatal cells**

In experimental mitotic and meiotic polyploidization, it is also necessary to take into account the indices of their organelles, which are additional diagnostic features, in addition to analyzing the size of
cells in morphologically altered tissue. Thus, in the studied heteroploids of fruit and berry crops, the number of chloroplasts in stomatal guard cells positively highly correlates with stomata length – in the ranges of 0.71 ... 0.80 in different ploidy genotypes of the genus *Malus* Mill.; *Rubus* L. \( r = 0.90 \); distant rowan-pear hybrids \( r = 0.92 \) (Figure 4).

**Figure 4.** Dependence of the number of chloroplasts in guard cells and the length of stomata in genotypes of fruit and berry crops with different ploidy levels (apple, pear, rowan-pear hybrids, strawberry, blackberry).

**Influence of ploidy level on pollen size**

A number of quantitative morpho-anatomical indicators of pollen grains are marker traits in the diagnosis of the ploidy level. However, it should be borne in mind that disturbances of meiosis during microsporogenesis determine the morphological heterogeneity of pollen. Therefore, pollen analysis may give unreliable results in plant forms with meiotic anomalies caused by natural (genetic, biochemical) or induced (physicochemical, climatic) factors [16]. The analysis of the size variability of pollen grains is most objective for genetically stable forms growing in places with favorable soil and climatic conditions.

For plants of the genus *Fragaria* L., forming a natural polyploid series with a base number of chromosomes \( x = 7 \), differences in the average diameter of pollen grains were revealed with partial overlapping of the ranges of their variation curves (Figure 5). Thus, pollen of hexaploid *F. moschata* \( (2n = 6x = 42) \) is larger by 6.9% than the pollen of the tetraploid *F. orientalis* \( (2n = 4x = 28) \) and by 10.2-14.6% than the octoploids *F. ovalis* and *F. virginiana* \( (2n = 8x = 56) \).

In our opinion, in express diagnostics of the ploidy level of fruit and berry crops, the analysis of the size of their pollen grains should be used not as an independent methodological method, but in combination with the cytological study of vegetative tissues. In addition, a comparative analysis of vegetative cells of genotypes with different ploidy level can be carried out starting from the seedling, while their generative period begins much later.
Figure 5. Variability (a) and mean value (b) of the diameter of pollen grains in genotypes of the genus *Fragaria* L. with different levels of ploidy.

4. Conclusion
The efficiency of preliminary selection of polyploids of fruit and berry crops by cytoanatomical traits was shown. In a complex cytological diagnosis of forms with increased ploidy level, it is proposed to study first the size of stomatal cells and the number of chloroplasts in them, and then direct chromosome counting only in selected polyploids. Evaluation of the size and quality of pollen in the studied genotypes of different ploidy levels is possible only after the onset of the flowering period and more reflects their suitability for use in artificial crosses.

The use of preliminary diagnostics of polyploids of fruit and berry crops by cytoanatomical characteristics will speed up their selection. The proposed diagnostic method has been tested on polyploids of the genus *Malus, Fragaria, Ribes, Rubus*, rowan-pear hybrids and is recommended for
scientific and practical application in the plant breeding and cytology. The effectiveness of the proposed analysis is ensured by its availability and reliable statistical differences in accounting traits. This makes it possible to reduce the costs of selecting polyploid genotypes due to the optimization of labor costs.

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