

Morphology, anatomy, ploidy level and genetic similarity investigation of the *Prunus persica* (L.) Batsch ‘Dostoynyi’ *ex situ* and *in vitro*

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Abstract. The article discusses the structure and genetic stability of the *Prunus persica* ‘Dostoynyi’ microshoots, culturing *in vitro* on the Gamborg and Eveleg (B5) nutrient medium compared with the shoots of the mother plants *ex situ*. Conventional methods were applied to study plant morphology, anatomy, ploidy level and genetic similarity. Statistical analysis was performed using the Past software. According to the results, common leaf morphology and its structure *in vitro* were preserved, but several quantitative and qualitative changes were revealed, except for rearrangements. The analysis of the ploidy level of the nuclei isolated from the leaf tissue cells of the microshoots *in vitro* revealed no changes. Similar results were obtained with ISSR-PCR and genetically material *in vitro* was stable. Short-term cultivation of *Prunus persica* ‘Dostoynyi’ microshoots on a B5 nutrient medium with optimal concentrations of 6-BAP promoted morphogenesis without significant deviations, and the absence of the ploidy level changes and genetic variations under *in vitro* conditions.

1 Introduction

Fruit crops are grown throughout the world mainly as a source of food. The peach (*Prunus persica* (L.) Batsch.) is one of the most valuable woody plants because it fruits contain sugars, vitamins, microelements, organic acids, enzymes, and other biologically active substances, which serve as an indispensable part of high-quality nutrition [1]. Traditional ways for peach reproduction are seedlings or re-grafting. Micropropagation is an effective method for the conservation of important germplasms, which can produce large-scale plantlets within a short time frame. At the same time woody species are difficult to establish *in vitro* due to internal contamination, premature explant death, secondary metabolites like phenols, vitrification etc. which complicate *in vitro* culturing [2]. Peach is one of the most recalcitrant species with regard to micropropagation [3]. Despite these

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difficulties, efficient regeneration of *P. persica* microshoots [4], *in vitro* micropropagation stage [1, 5] and clonal micropropagation protocols had been developed [6].

It is postulated that clones obtained via buds or microshoots are genetically identical. At the same time under *in vitro* culture conditions there is possibility of physiological and anatomical disorders, which can interfere negatively after *ex vitro* transfer [2], as well as somaclonal variations. Due to that *P. persica* is the most difficult crop to propagate under *in vitro* conditions [4], data about microshoots' anatomy, ploidy level and genetic identity are very limited. Therefore, the aim of our investigation was to study leaf structure of the *P. persica* microshoots *in vitro*, its ploidy and genetic similarity compared to mother plants *ex situ*.

2 Materials and methods

Prunus persica (L.) Batsch. (L.) Batsch 'Dostoyiniy' cultivated *ex situ* at the collection plot of Nikita Botanical Gardens (NBG) was used. An *in vitro* introduction and culturing on a B5 medium were performed according to protocol developed early [1].

Organs of plants *ex situ* and microshoots *in vitro* were placed in a Petri dish or on a glass slide and its morphology was examined using SMZ 745T stereomicroscope (Nikon, China) equipped with a digital camera DC29111251 (View Solutions, China) and ImageView v. software. x64, 3.7.10121.20171030.

For leaf anatomical investigation, *ex situ* and *in vitro* material was fixed in FAA solution (formalin, alcohol, acetic acid and water (1:5:0.5:3.5 with one drop of Tween-20)) during 1–2 days, dehydrated in graded alcohol series and embedded into paraffin. Sections (8–10 µm) were made on a microtome Rotmic 2A (Orion Medic, Russia), stained with a 0.005% solution of methylene blue, mounted into 60% sucrose.

Obtained microslides were investigated under light microscope CX-41 (Olympus, Japan) with a digital camera SC 50 (Olympus, Germany) and CellSens image processing software version 1.17. A total of 10 leaves and 30 cells were analyzed.

Ploidy level analysis was carried out on fresh material. Fragments of six *ex situ* and *in vitro* leaves of *P. persica* 'Dostoyiniy' were immersed in a modified WPB buffer with 2% polyvinylpyrrolidone (K10) supplemented with propidium iodide (50 µg/ml), RNase (50 µg/ml) and β-mercaptoethanol (0.3%), grinded up with a safety razor blade. The obtained samples were passed through 30 µm CellTrics® filter (Partec, Germany) [7]. The analysis was performed using CyFlow® Ploidy Analyzer (Sysmex, Partec, Germany). *Prunus persica* 'Red Haven' and *Ficus benjamina* L. plants were used as an external control [8]. The measurements were carried out on the same analyzer settings with at least 5.000–10.000 nuclei.

DNAs were isolated from leaves of *ex situ* plants and microshoots *in vitro* by classical methods with trimethylammonium bromide (2×CTAB) and 2% polyvinylpyrrolidone (PVP). PCR were carried out using BioMaster HS-Taq PCR (2×) kit (Biolabmix, Russia), primers UBC807 5'-(AG)8T-3', UBC818 5'-(CA)8G-3', UBC836 5'-(AG)8YA-3', GR215 5'-(CA)6GT-3', HB12 5'-(CAC)3GC-3', X10 5'-AGC(ACG)5C-3'(ISSR) and DNAs (20 ng) in the GeneExplorer™ GE-96S (Bioer, China) thermal cycler. The reaction conditions were as follows: initial denaturation at 95°C for 5 min, denaturation at 95°C for 15 s, annealing at 45°C for 20 s, extension at 72°C for 1 min, and with a final extension step at 72°C for 10 min (35 cycles). The Step 100 Long (Biolabmix, Russia) was used as the molecular marker. The amplified fragments were analyzed by horizontal electrophoresis in 1.7% agarose gel with 0.5×TBE buffer at 60 V during 1 h using universal power supply PowerPac™ (BioRad, Singapore). The agarose gel was imaged with E-box documentation system (VilberLourmat, France) and analyzed using Image Lab™ Software Version 6.0 (Bio-Rad, USA).

Statistics was carried out using the Past software v. 4.03 [9]. Samples were checked for normality of distribution, and either the t-criterion or the U-criterion was used ($p \leq 0.05$).

3 Results and discussion

The *P. persica* ‘Dostoyiny’ leaf blade morphology and structure *ex situ* were analyzed. The leaves of the *ex situ* plants were 8–15 cm long and 2–4 cm wide, light or dark green, lanceolate, with acuminate apex, cuneate base, and serrated edge (Fig. 1). The stomata were located on the abaxial side. Trichomes were sparsely located along central and lateral veins of different orders as well as leaf margins. Hydathodes were formed at the ends of the leaf blade denticles.



Fig. 1. *Prunus persica* ‘Dostoyiny’ leaf morphology *ex situ*: (a) – leaf top, (b) – leaf base, (c) – leaf margin, (d) – leaf adaxial side in the midrib, (e) – leaf abaxial side in the midrib.

A single-row epidermis was identified on the cross-section in the midrib area (Fig. 2, a) consisting of small rectangular-rounded cells with thickened walls covered by the cuticle outside. Under the upper and lower epidermis, there were collenchyma layers. The vascular bundle was collateral, horseshoe-shaped, and large rounded xylem elements diverging fan-shaped to the abaxial side. The phloem elements were adjacent to the xylem in the lower part. Parenchyma cells were also differentiated.

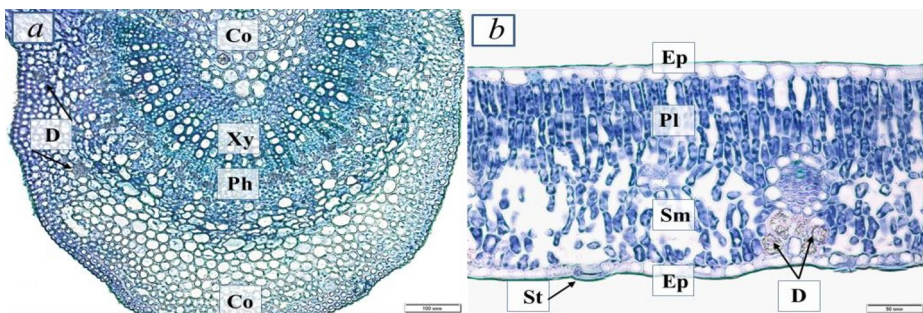


Fig. 2. *Prunus persica* ‘Dostoyiny’ leaf structure *ex situ* in the midrib (a) and lateral part (b): Co – collenchyma, D – dryse, Ep – epidermis, Pl – palisade, Ph – phloem, Sm – spongy mesophyll, St – stomata, Xy – xylem (light microscopy, methylene blue staining).

In the lateral part of the leaf blade (Fig. 2, b), a single-layer epidermis consisted of rectangular-rounded cells covered by the cuticle outside. Upper epidermis cells were larger compared to the lower ones. Palisade (2–3 layers) had greater development than spongy

mesophyll and was represented by cells elongated in the radial direction relatively tightly adjacent to each other however intercellular space presence was established. The druses were also identified in the cells of the leaf tissues.

The leaf blade structural organization of the *P. persica* ‘Dostoyiny’ microshoots *in vitro* on a B5 nutrient medium containing 1.0 mg/l 6-BAP and 0.025 mg/l IBA was studied. According to obtained data, general morphological (Fig. 3) and anatomical features (Fig. 4) were preserved, in comparison with those *ex situ*.

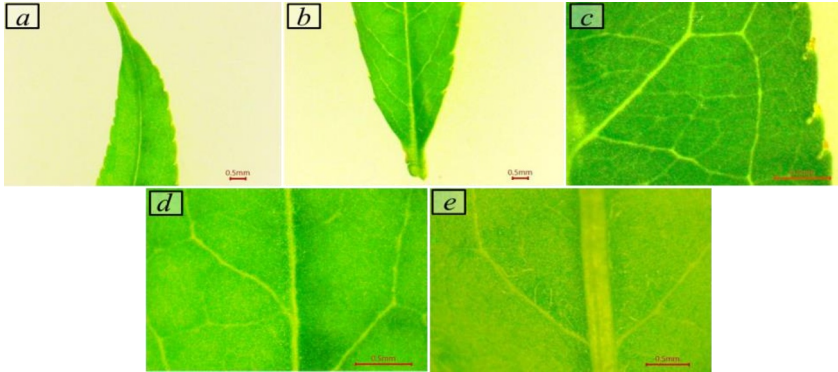


Fig. 3. *Prunus persica* ‘Dostoyiny’ leaf morphology *in vitro*: (a) – leaf top, (b) – leaf base, (c) – leaf margin, (d) – leaf adaxial side in the midrib, (e) – leaf abaxial side in the midrib.

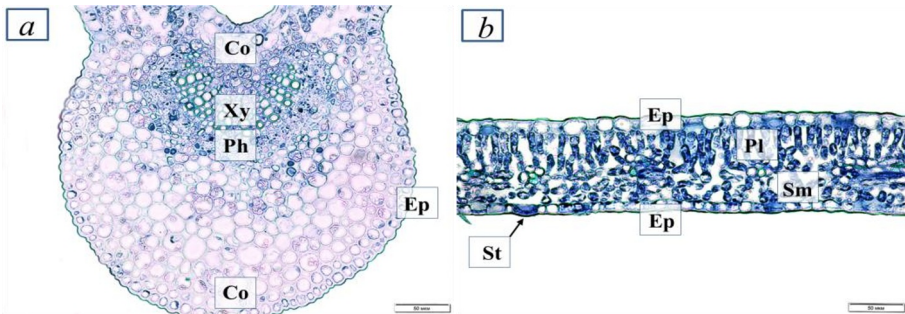


Fig. 4. *Prunus persica* ‘Dostoyiny’ leaf structure *in vitro* in the midrib (a) and lateral part (b): Co – collenchyma, Ep – epidermis, Pl – palisade, Ph – phloem, Sm – spongy mesophyll, St – stomata, Xy – xylem (light microscopy, methylene blue staining).

Among the qualitative changes, palisade less compactness and the absence of druses in the cells were noted. Leaf blade quantitative parameter analysis *in vitro* revealed a decrease in midrib height and width, leaf lateral part height, spongy mesophyll, the total number of leaf cell layers, as well as an increase in the palisade and spongy mesophyll cell wide, lower epidermis height compared with those *ex situ* (Table 1).

Table 1. Quantitative anatomical parameters of *Prunus persica* ‘Dostoyiny’ leaf blades *ex situ* and *in vitro*.

Parameter	<i>Ex situ</i> leaf	<i>In vitro</i> leaf
Height of the leaf along the midrib	592.02±45.16	487.92±43.05

Height of the conductive bundle	251±59.81	80.75±7.68*
Width of the conductive bundle	534.51±35.50	259.47±26.62*
Leaf height, lateral part	198.24±19.75	123.48±9.33*
Cell height, upper epidermis	18.93±0.58	19.59±0.46
Cell width, upper epidermis	25.32±1.20	24.05±1.46
Cell height, lower epidermis	13.49±0.44	17.05±0.89*
Cell width, lower epidermis	15.79±1.03	16.09±0.99
Cell height, palisade mesophyll	24.87±0.97	22.96±1.61
Cell width, palisade mesophyll	7.94±0.33	11.23±0.62*
Cell height, spongy mesophyll	14.03±0.48	14.34±0.49
Cell width, spongy mesophyll	12.03±0.68	14.95±0.73*
Palisade mesophyll layer number	2.5±0.17	2.2±0.13
Spongy mesophyll layer number	4.7±0.33	3.6±0.22*
Mesophyll layer total number	7.2±0.25	5.4±0.37*

* – statistically significant differences between the values in the line.

The ploidy level of nuclei isolated from the *P. persica* ‘Dostoyiniy’ microshoot leaves cultivated *in vitro* for a short-time (three and six months) on a B5 nutrient medium with 1.0 mg/l 6-BAP and 0.025 mg/l IBA, was studied. According to histograms of *in vitro* samples compared with those *ex situ* and external control such as *P. persica* ‘Red Haven’ and *Ficus benjamina*, no changes were detected, the nuclei were diploid (Fig. 5).

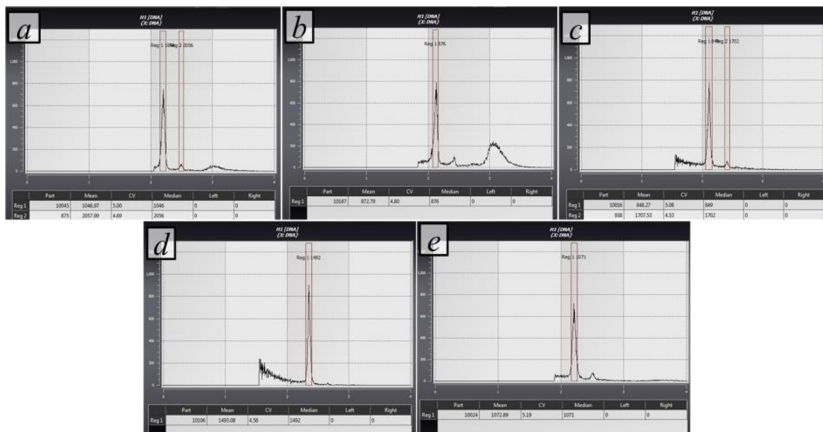


Fig. 5. Histograms of the ploidy level of the analyzed *Prunus persica* ‘Dostoyiniy’ material: (a) – external control (*Prunus persica* ‘Red Haven’), (b) – *Prunus persica* ‘Dostoyiniy’ *ex situ*, (c) – samples of nuclei from leaves of microshoots *in vitro* (B5 medium, three months of cultivation), (d) – external control (*Ficus benjamina*), (e) – samples of nuclei from leaves of microshoots *in vitro* (B5 medium, six months of cultivation).

Investigation of plant genetic similarity was performed between *ex situ* plants and *in vitro* microshoots. Six ISSR primers were used. Our results show that the primers produced amplicons, which were monomorphic for investigated *ex situ* and *in vitro* samples and no polymorphism was detected (Fig. 6).

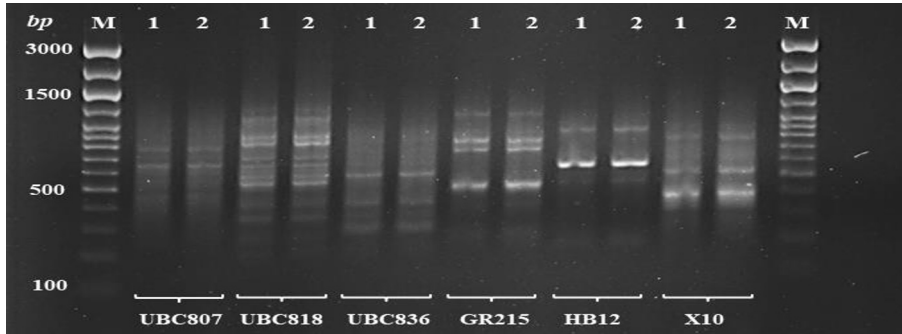


Fig. 6. Electrophoregram of the amplified products with ISSR primers and DNA isolated from leaves of *ex situ* plants (1) and *in vitro* microshoots (2) of *Prunus persica* 'Dostoyiny'. M – marker, bp – base pairs.

The morphology, anatomy and physiological parameters of plants depend on the factors affecting them [10]. The micromorphological development of plants *in vitro* is influenced by cultivation conditions, the composition of the nutrient medium, the source of carbohydrates and the duration of exposure to these factors [11]. Thus, for *Ficus carica* L., *Lavandula angustifolia* Mill., *Lavandula × intermedia* Emeric ex Loiseleur, *Prunus persica* (L.) Batsch., *Chrysanthemum × morifolium* Ramat. and *Chrysanthemum × hybrida* showed preservation of the morphological characteristics of leaf blades of microshoots *in vitro*, in comparison with those *ex situ* [12]. In contrast, an *in vitro* leaves of *Passiflora foetida* L. were thin, small, ovoid, pale green in color and had an indistinct midrib [11].

Our studies showed general morphological characteristics' preservation of the *P. persica* 'Dostoyiny' leaves *in vitro*. It is noted that under specific *in vitro* conditions, plants retain their morphological and biological characteristics inherent in a particular genotype, while some new features may also appear. When studying the ecological and anatomical variability of plants *in vitro*, researchers came to the conclusion that predominantly quantitative changes occur [12]. Thus, in an *in vitro* culture of *P. persica* 'Dostoyiny' we found a decrease in the leaf blade thickness, that is also consistent with published data [11, 12, 13, 14]. An *in vitro* leaf photosynthetic tissues structure investigation of ornamental, essential oil, and fruit crops revealed the presence of differentiated mesophyll. One-, two-row palisade and four-row sponge mesophyll were established. In some cultivars of *F. carica* and *L. angustifolia* L. × *intermedia*, the formation of isopalisade mesophyll was observed [12]. For *in vitro* leaves of *P. persica* 'Dostoyiny', the presence of differentiated mesophyll was shown. At the same time, the palisade layer smaller closeness was noted, that can be manifested under *in vitro* culturing [15, 16]. According to quantitative characteristics, a greater roundness of palisade and spongy mesophyll cells also were established.

Changes in the midrib structure are also characteristic of *in vitro* culture. For example, for *Cynara scolymus* L., limited development of a vascular bundle consisting of several elements was shown [15]. In *Vitex negundo* L., a concave vascular bundle was described, with a small amount of xylem and phloem elements. The lateral veins crossing the mesophyll were represented by inconspicuous collateral bundles surrounded by weakly defined endoderm [17]. According to our analysis, the conductive bundles were characterized by decreasing in height and width, while the conducting elements were sufficiently developed and clearly differentiated on the cross-sections. The change in their proportions most likely occurs due to correlative relationships during the development of the leaf blade, depending on its linear parameters.

It is believed that introduction into *in vitro* culture destabilizes the genetic and epigenetic program of intact plant tissue and can lead to variations at the level of chromosomes and DNA sequences [18]. There are chances of somaclonal variations in tissue culture raised plants with the increase in passages of subculture which can restrict the implementation of *in vitro* propagation for clonal multiplication [19]. So, the plants grown *in vitro* require assessment of their genetic stability using the ploidy level and/or DNA content analysis, molecular markers, or both methods [20].

In our work, ploidy level was checked by flow cytometry method after three and six months of *P. persica* ‘Dostoyiniy’ microshoot culturing *in vitro*. Our results showed no changes. Genome stability was also demonstrated under clonal micropropagation of sour cherry cultivar ‘Čačanski Rubin’ (*P. cerasus* L.) and cherry rootstock Gisela 5 (*P. cerasus* × *P. canescens*) [21]. *Populus trichocarpa* × *deltoides* [22], Dezful cultivar of olive (*Olea europaea* subsp. *europaea* L.) [23] under short-term cultivation. Also, the absence of polyploid nuclei was for hybrid larch (*Larix* × *eurolepis* Henry) for a year *in vitro*, and confirmed that this plant belong to the group of genetically stable conifers, by authors' opinion. [24]. In the case of direct regeneration, a reduction in the activity of genomic rearrangements, associated with prolonged tissue cultivation in the presence of plant growth regulators was marked [25].

Genetic stability of some Rosaceae representatives can be preserved during clonal micropropagation *in vitro*. For example, when Jaspi rootstocks were cultured *in vitro* for four weeks, they showed monomorphic bands obtained using ISSR markers [26]. Similar results were observed for *Prunus mume* Siebold & Zucc. with ISSR markers [27] and *Prunus armeniaca* L. using RAPD-PCR after all stages of clonal propagation [28]. It should be noted, that identifying the degree of cellular, tissue, organs, and plant parts sensitivity *in vitro* of all possible genotypes, variants of their morphogenetic potential realization, and responses of tissues and organs that are within the normal developing and deviating forms are of interest.

4 Conclusion

Morphological and anatomical studies of the leaf blade of *P. persica* ‘Dostoyiniy’ microshoots *in vitro*, cultivated on a B5 nutrient medium containing 1.0 mg/l BAP and 0.025 mg/l IBA, showed the preservation of their general structure, differentiation of tissues inherent cultivar in the presence of variations in quantitative parameters. The ploidy level did not change. The genetic similarity investigation revealed no shifts according to the markers' used. Both genomic and genetic analysis confirmed *P. persica* ‘Dostoyiniy’ microshoots' stability during short-time cultivation *in vitro*.

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