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ANATOMICAL STRUCTURE OF SASKATOON BERRY (*AMELANCHIER* MEDIK.) LEAVES UNDER DIFFERENT CULTIVATION CONDITIONS

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Abstract

Amelanchier Medik. belonging to the family Rosaceae includes about 30 species with a high content of biologically active substances. The aim of the study was to determine the morpho-anatomical features of leaves at the adaptation and ontogenesis stages of Saskatoon berry variety 'Krasnoyarskaya' (*A. alnifolia*) and variety 'Prince William' (*A. canadensis*). Saskatoon berry leaves are characterized by hypostomatic and anomocytic, less often anisocytic stomata at all stages of ontogenesis. The study revealed in the average length of the polar axis and the stomata equatorial diameter, depending on the cultivation conditions and genotype. *A. canadensis* had a larger average stomatal area, a thicker epidermis, a greater height of the palisade chlorenchyma cells, and a higher coefficient of palisade, which made it more resistant to changes in atmospheric precipitation, at all stages of ontogenesis, in comparison *A. alnifolia*.

Introduction

The genus *Amelanchier* Medik. belongs to the family Rosaceae Juss. and includes 23 to 28 species (ITIS, 2023; POWO, 2023). The plant form of this genus is a deciduous shrub or a small tree growing in temperate regions of the Northern Hemisphere. Saskatoon berry fruits are characterized by a higher content of flavonoids (Mazza, 1982; Zatylny and St-Pierre 2003; Laksaeva and Sychev, 2013; Donno *et al.*, 2016; Szpadzik and Krupa, 2021; Asyakina *et al.*, 2022; Kolesárová, 2022), which allows them to be attributed to nutraceuticals that help reduce the risk of certain diseases in humans (WHO, 2023). This tree is cultivated on an industrial scale in certain regions of Canada and the USA (Template Business Plan for Manitoba Saskatoon Berry Producers, 2023; Saskatoon Berry Council of Canada, 2023). There are currently no large farms to produce planting material for Saskatoon berries in Russia. Using the sexual propagation method, Saskatoon berry was massively propagated in the Kudymkar nursery in the 1940, from where the seedlings were distributed to other regions of the Soviet Union. However, the seedlings obtained turned out to be highly heterogeneous, which reduced interest in this culture (Kuklina, 2006). Thus, despite the unpretentiousness of the cultivation, nutritional value, and high decorative qualities of the Saskatoon berry, it remains a rarely spread plant in Russia.

Saskatoon berry varieties are propagated only by vegetative methods such as grafting, cuttings, and clonal micropropagation (Hunková *et al.*, 2017). The last method is considered one of the most effective approaches to obtaining virus-free plants. Various studies have been carried out on the influence of mineral and hormonal compositions of the nutrient medium, as well as at the stage of adaptation, on some representatives of the genus *Amelanchier* (Pruski *et al.*, 1990; Hunková *et al.*, 2017; Yang and Du, 2018; Hunková and Gajdošová, 2019; Raeva-Bogoslovskaya *et al.*, 2021).

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In vitro culture is carried out under conditions of high relative humidity, which affects the anatomical structure of newly formed tissues and organs in the explant (Hazarika, 2006). This leads to a decrease in the ability of plants to maintain homeostasis under ex vitro conditions and, subsequently, reduces the number of adapted plants (Blanke and Belcher, 1989; Romano and Martins-Loução, 2001; Apóstolo *et al.*, 2005; Pospísilová *et al.*, 2007; Werner *et al.*, 2018; Mitrofanova *et al.*, 2018; Jagiełło-Kubiec, 2021). During the process of adaptation to *ex vitro* conditions, in vitro-derived plantlets are exposed to water stress. At this stage, the ability of plants to tolerate this stress depends on the genotype, leaf structure and stomatal apparatus (Sciutti and Morini, 1993; Pospísilová *et al.*, 2007; Cha-um *et al.*, 2010).

The leaves are the most flexible organs because of their individual structures that are associated with certain features. Therefore, the type of stomatal apparatus and stomata size, the shape and height of the epidermal cell, and the structure of the mesophyll can be not only taxonomic characteristics, but also characterize the plant adaptability to various cultivation conditions (Akhkubekova and Tamakhina, 2020).

The available studies on the structure of *Amelanchier* leaves *in vitro* and *ex vitro* are few and mostly reflect the structure of the epidermis and stomatal apparatus (Ganeva and Uzunova, 2010; Bošnjak Mihovilović *et al.*, 2020). Therefore, the aim of our study is to determine the morphological and anatomical structures of *Amelanchier* leaves at various stages of ontogenesis.

Materials and Methods

Plant materials and experimental conditions

The research was carried out in the Laboratory of Plant Biotechnology of the Tsytsin Main Botanical Garden of the Russian Academy of Sciences in 2022–2023. The objects of this study are two varieties: 'Prince William' developed from species *A. canadensis* (L.) Medik. and 'Krasnoyarskaya' derived from *A. alnifolia* (Nutt.) Nutt. *ex* M. Roem. The natural habitat *of A. canadensis* is Canada from Newfoundland west to southern Ontario and USA from Maine south to Alabama, and *A. alnifolia* is native to Alaska, most of western Canada, as well as the western and north–central regions of the United States.

Comparative analysis of the anatomical structure and development of the stomatal apparatus was carried out on mature leaves, which were selected from different stages:

1. From plantlets rooted in Murashige and Skoog's 1962 nutrient medium supplemented with 1.0 mg/l of IBA. Culture was inducted at 25 ± 2°C under 16/8 (light /dark light conditions) with a light intensity of 3,000 lux.
2. From plants after 30 days of adaptation under greenhouse conditions (at a temperature of 25°C, the photoperiod of 16 hrs of light, 8 hrs of darkness and 2000 lux light intensity).
3. Leaves of the middle formation from 3-year-old plants obtained by clonal micropropagation and grown in the open ground at the collection site.

Observation of the stomatal apparatus

Stomata were analyzed using the replica method (Hilu and Randall, 1984). The samples were prepared by applying a thin layer of nail polish to the middle area of the leaf blade. After drying, the varnish was removed with transparent adhesive tape and attached to the object-glass. Morphometric traits of stomata (the length of the polar axis and the equatorial diameter) were measured using a digital microscope (Keyence VHX-1000E) in at least 5 fields of view at magnification x1500. Stomatal shape was calculated as the ratio of the polar axis (L) to the

equatorial axis length (D). Stomata are often considered round if L/D is in the range from 1.0 to 1.4, and elliptical if this proportion ranges from 1.5 to 2.0.

The stomatal area was calculated using a following formula (Vinogradova *et al.*, 2019):

$S=\pi*L*D/4$,

Where 'S' is the area of one stoma, 'L' is the length of the polar axis of the stoma, 'D' is the equatorial diameter of the stoma.

Anatomical structure of leaves

All materials were fixed in 70% ethanol. Cross-sections slices through the middle part of a leaf blade were made. The slices were made on a sledge microtome MS-2 (Tochmedpribor, Kharkiv, Ukraine) with an attached freezing table OMT-2802E (KB TECHCOM LLC, Yekaterinburg, Russia) at a temperature of -10°C, the thickness of the slices was – 80 µm for samples from in vitro culture and 20 µm for leaves from open ground. photographs of leaf cross-sections were obtained using an Olympus CX41 light microscope (Olympus Corporation, Tokyo, Japan) and a Canon 7D Mark II digital camera attached to it (Canon Incorporated, Tokyo, Japan). At this stage, the following parameters were assessed: leaf thickness, palisade cell height, spongy chlorenchyma thickness, leaf lower epidermis thickness, and upper epidermis thickness.

Data analysis

A completely randomized design (CRD) was used to conduct the experiments. To study the anatomical traits, 10 leaves were selected from in vitro culture and at the adaptation stage, and 20 leaves from three-year-old plants grown in the open ground. The quantitative data were analyzed using Microsoft Office Excel 2016 and PAST 3.2 (Pale-ontological Statistical) using the methods of descriptive statistics, and one-way analysis of variance (ANOVA). Significant variances between treatments were subsequently tested by the t-test at a value of $p<0.05$.

Results and Discussion

Stomatal apparatus

Successful adaptation of in vitro-derived plants can be affected by the structural and functional features of the plant's various parts. In our study, it was noted that a hypostomatic leaf type was noted in both varieties, regardless of environmental conditions (*in vitro* or adapted plant): stomata were located only on the abaxial (lower) side of the leaf blade. The walls of the epidermal cells are coarsely wavy (Fig. 1A). All samples were characterized mainly by the anomocytic stomata type, where the guard cells of the stomata were surrounded by an indefinite number of subsidiary cells that did not differ from the other epidermal cells (Fig. 1B). However, the selected leaves from *in vitro* conditions also had anisocytic stomata type (Fig. 1C), one cell of which is smaller than the other subsidiary cells.

To find the most resilient plant species, it is important to assess the resistance of plants to adverse environmental factors (Semenyutin, 2000). Our observations agreed with other studies: a hypostomatic leaf type and an anomocytic stomatal type are characteristic of this taxon (Ganeva and Uzunova, 2010; Bošnjak Mihovilović *et al.*, 2020). During the process of clonal micropropagation, rejuvenation of explant tissue occurs, which is characterized mainly by morphological and anatomical criteria (Putenikhin and Farukshina, 2007). The appearance of anisocytic stomata in leaves under in vitro conditions may be associated with this phenomenon. Thus, Pautov *et al.* (2015) observed a change in the types of stomatal apparatus in the leaves of

Exbucklandia populnea (R.Br. *ex* Griff.) R.W.Br. at different ages: in juvenile leaves, the predominant types are lateracytic and paracytic, and in adults, encyclocytic type.

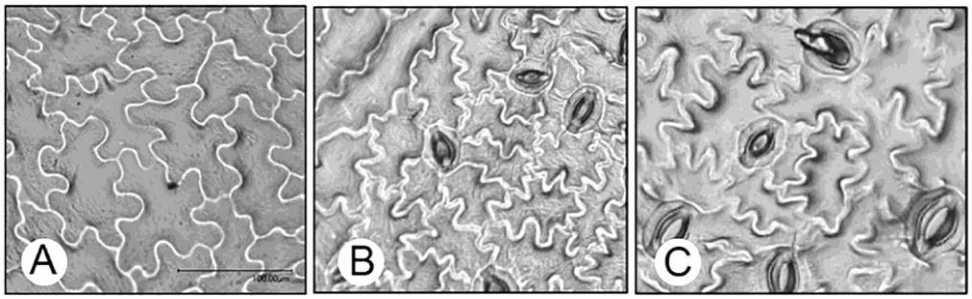

Fig. 1. Leaf epidermis of Saskatoon berry in *in vitro* culture: A. adaxial side; B. anomocytic stomata type, abaxial side; C. anisocytic stomata type, abaxial side.

The number, size, and shape of stomata significantly affect the regulation of gas exchange and photosynthesis in plants (Semenyutina, 2000; Vieira *et al.*, 2015). Significant differences in the equatorial diameter and polar axis of stomata, both between studied varieties and between the plants of the same variety, were noted under different cultivation conditions (Fig. 2).

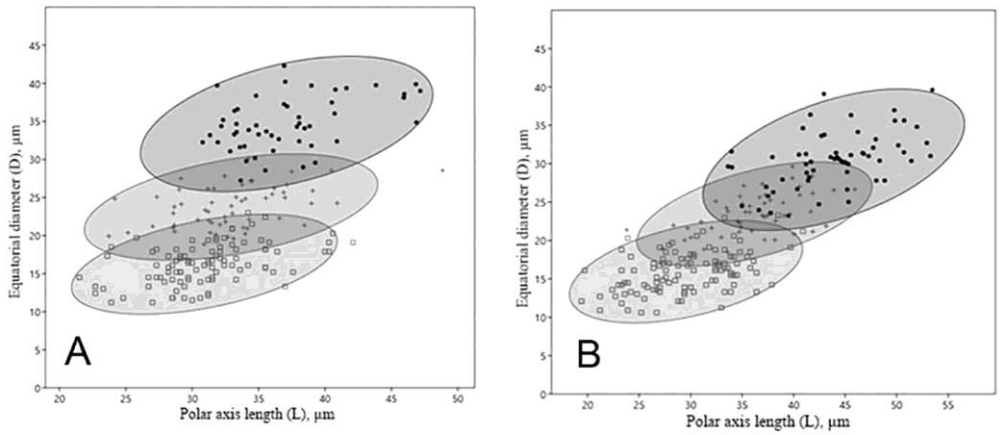

Fig. 2. Polar axis length and equatorial diameter of stomata in different Saskatoon berry varieties: A. *A. alnifolia* 'Krasnoyarskaya'; B. *A. canadensis* 'Prince William' under various cultivation conditions: Dot – *in vitro*; Plus – 30 days after planting under *ex vitro* conditions; Square – from open ground.

Under *in vitro* conditions, the stomata were characterized by a high average length of the polar axis and the equatorial diameter (L 40.8±0.5 µm; D 32.4±0.4 µm). While the average length of the polar axis and the equatorial diameter of the stomata decreased under *ex vitro* conditions (L 34.5 ± 0.5 µm; D 23.3 ± 0.3 µm). Consecutively, the stomata of the leaves taken from the open ground were characterized by the smallest length of the polar axis and the equatorial diameter (L 30.2±0.3 µm; D 16.1 ± 0.2 µm).

After the adaptation stage, the average length of the polar axis decreased by 13% in the variety 'Krasnoyarskaya', and by 21% in 'Prince William'. While the average length of the polar axis of stomata in open-ground cultivated plants decreased by 7% in the variety 'Krasnoyarskaya',

and in the variety ‘Prince William’ by 23%. Thus, the area of stomata of the *A. canadensis* variety from open ground is 49% smaller than stomata from *in vitro* culture, and the stomata of the *A. alnifolia* variety is 20% smaller.

The stomata of Saskatoon berry leaves formed *in vitro* vary in shape from those formed *ex vitro*. Under *in vitro* conditions, the stomata were characterized by a round shape (L/D ranged from 1.0 to 1.4). This is mainly due to the stomatal apertures, which are wide open under high humidity conditions (Ziv, 1991; Pautov *et al.,* 2015). Whereas, in *ex vitro* conditions, elliptical stomata were formed (L/D ranges from 1.5 to 2.0). It was found that in the variety of *A. alnifolia*, the shape of the stomata changed due to a decrease in the equatorial diameter, and in the variety of *A. canadensis* both the equatorial diameter and the polar axis of the stomata. The change in the length of the polar axis and equatorial diameter of the stomata in cultivars of the same species, depending on the ecological zone, was noted by Babosha *et al.* (2020) on the varieties of *Malus domestica* Borkh. With decreasing altitude above sea level and moving to more arid regions, the length of the polar axis and the equatorial diameter of the stomata become smaller. One of the distinctive features of *in vitro* culture conditions in the high air humidity of up to 90%. After transferring regenerants to the greenhouse, they begin to adapt to the conditions of lower humidity. The decrease in morphometric parameters of *A. canadensis* stomata may be due to the same mechanisms as in *Malus domestica*.

Stomatal density plays an important role in the adaptation of plants to environmental conditions (Mizutani and Kanaoka, 2018; Goremykina *et al.*, 2018). This characteristic is flexible and may change depending on temperature, humidity, light, and other factors (Goremykina *et al.*, 2018). In our study, the number of stomata varied from 143 to 199 per 1 m^2, which suggests an average density of stomata location (Trukhmanova, 2014). Stomatal density in leaves selected from under *in vitro* conditions and in plants grown on open ground did not show any significance.

Anatomical structure of leaves

Adaptation to cultivation conditions is a complex process. Mesophyll tissue, like the stomatal apparatus, is sensitive to changes in the microclimate (Timonin and Notov, 1993). Leaf blades of Saskatoon berry varieties under *in vitro* conditions showed dorsoventral anatomy, were poorly differentiated, and had only one layer of palisade chlorenchyma overlying four layers of spongy chlorenchyma. The palisade cells were not densely grouped and were characterized by a slightly elongated shape. The cells of spongy chlorenchyma had large intercellular spaces and irregular shapes. It was found that the cells of the upper and lower epidermis did not have cuticles, and the upper epidermis was thicker than the lower epidermis (Fig. 3).

Most tree crops, when cultivated *in vitro*, obtain a similar leaf structure (Mitrofanova, 2018; Sarropoulou *et al.,* 2023). This is probably largely due to the increased humidity and reduced light conditions during cultivation. Despite the similarity of qualitative characteristics, the varieties of *Amelanchier* species differed in quantitative parameters: The variety ‘Prince William’ (*A. canadensis*) was characterized by a higher height of epidermis cells and palisade chlorenchyma under in vitro conditions in comparison with the variety ‘Krasnoyarskaya’ (*A. alnifolia*).

After one month of plantlet cultivation under *ex vitro* conditions, no changes were observed in the number of palisade layers of chlorenchyma. However, it was noted that the formation of cuticles on the adaxial leaf side was an insignificant increase in the height of the spongy chlorenchyma and epidermis cells. This is consistent with the results of other studies on different species (Shekhawat *et al.,* 2021 on *Santalum album* L. and Tevik *et al.,* 2017 on Canna × hybrida hort. *ex*-Backer). In these studies, it was noted that in both species, the number of mesophyll cells increased after adaptation to *ex vitro* conditions, and they began to be grouped more densely than in *in vitro* conditions.

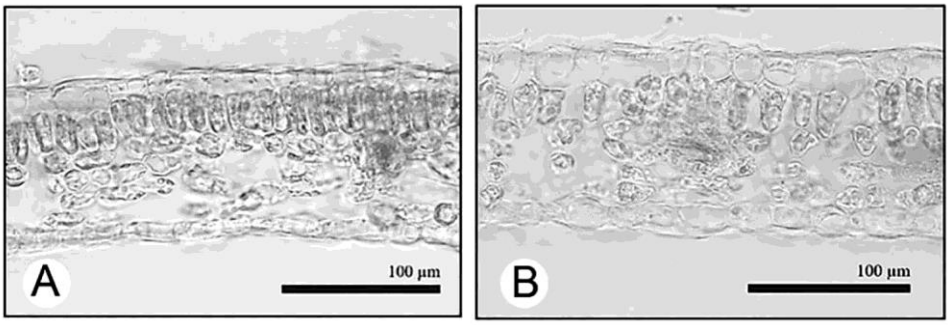

Fig. 3. Leaf cross-sections of Saskatoon berry plantlets selected from *in vitro* culture: A. 'Krasnoyarskaya' (*A. alnifolia*); B. 'Prince William' (*A. canadensis*).

It was observed that the leaves of plants from the open ground were fully differentiated, and the formation of a secondary palisade layer of chlorenchyma was noted. The thickness of the leaf blade, total thickness of palisade chlorenchyma, and spongy chlorenchyma thickness, in comparison with the leaves selected after 30 days of *ex vitro* adaptation, increased by 3, 4.5, and 1.5 times, respectively. At the stages of in vitro cultivation and adaptation, plantlets of the 'Prince William' variety were characterized by a higher leaf thickness compared to the 'Krasnoyarskaya' variety, as well as a greater thickness of the palisade layers of chlorenchyma, and the upper and lower layers of the epidermis. In open-ground conditions, the leaves of 'Prince William' plants were characterized by a greater thickness (249.63 ± 3.6 µm^2) in comparison with the 'Krasnoyarskaya' variety (243.4± 2.2 µm^2). Significant differences in the thickness of secondary palisade chlorenchyma and spongy chlorenchyma were noted. The 'Krasnoyarskaya' variety was characterized by a dense arrangement of columnar-shaped cells, their high length, and, in some places, the rudiments of the third layer. At the same time, the second layer of columnar cells in the 'Prince William' variety were loosely packed with air spaces in between, and the height of the cells is smaller in comparison with the 'Krasnoyarskaya' variety (Fig. 4).

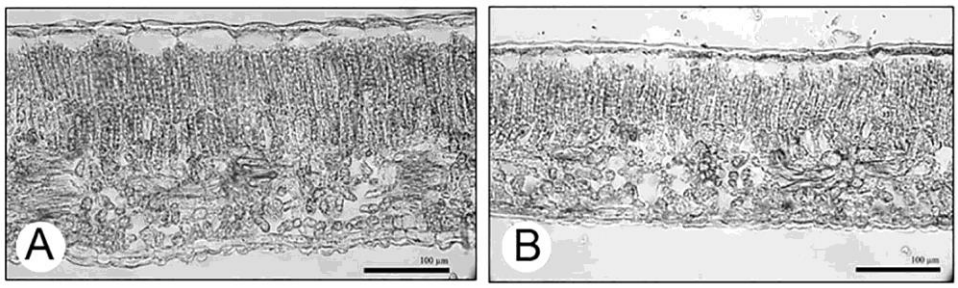

Fig. 4. Leaf cross-sections of Saskatoon berry plants selected from open ground: A. 'Krasnoyarskaya' (*A. alnifolia*); B. 'Prince William' (*A. canadensis*).

The studied varieties of the genus *Amelanchier* can be attributed to the group of mesophytes with some traits of xerophytic plants, since their leaf blade is differentiated, characterized by two layers of palisade chlorenchyma, and the thickness of the leaf ranges from 200 µm to 250 µm (Ivanova, 2014).

The present study of the anatomical features of the genus *Amelanchier*, both *in vitro* and *ex vitro*, is the first of its kind. The result showed that plantlets of *Amelanchier* species do not differ in the characteristics of the stomatal apparatus under *in vitro* conditions as the leaves of both studied species are hypostomatic. The type of stomatal apparatus is mainly anomocytic, anisocytic type is rarely observed and the shape of the stomata is rounded. When cultivating plants in an open-ground environment the stomatal elongation and an eventual shift to an oval shape occur. In the 'Krasnoyarskaya' variety (*A. alnifolia*), cells in the second palisade layer are densely arranged and share a similar shape with those in the first layer. Conversely, in the 'Prince William' variety (*A. canadensis*), the intercellular spaces of the second palisade layer are larger than those in the first layer, and the cells are less elongated. Notably, 'Prince William' exhibits a thicker epidermis, greater palisade chlorenchyma height, and a higher coefficient of palisade development compared to 'Krasnoyarskaya' at all stages of cultivation.

Several structural changes in leaf development have been identified during the ontogenesis of Saskatoon berry plants, including an increase in leaf thickness by three times and the height of the first palisade layer by 2.5 times. A second palisade layer is formed, which is 44% thinner than the first. The height of the spongy chlorenchyma increases by 1.5 times; and the epidermal thickness increases by 38%.

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