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Development, integrative study and research prospects of *Deschampsia antarctica* collection

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Abstract: This review presents the results of multi-year efforts for the development and integral investigation of the collection of *Deschampsia antarctica* É. Desv. plants originated from the maritime Antarctic. To create the collection, we have optimized the procedures and conditions for germination of seeds and generation of aseptic plants *in vitro* together with their subsequent clonal propagation, long-term *in vitro* culture, and acclimatization of plants to pot culture, and finally produced fertile plants able to set viable seeds. The collection of plants cultivated *in vitro* and in pots is consisted of clones of 22 different genotypes originated from eight island populations from the Argentine Islands – Kyiv Peninsula region. Some of these clones have been successfully cultured for more than ten

years and were found to be genetically stable over this period. The collection was shown to be heterogeneous in a number of variables. The morphological and anatomical characteristics of the plants, as well as the data of karyological, molecular cytogenetic, molecular genetics, and biochemical analysis are presented. The created collection of *D. antarctica* plants can provide a valuable plant material with well-studied characteristics grown under controlled conditions to perform experimental research on the mechanisms for plant adaptation and resistance to various abiotic factors of Antarctic environment. Furthermore, development of the collection and its comprehensive characterization form the basis for further biotechnological developments focused on the use of this Antarctic plant as a source of biologically active substances, which can potentially be used for therapeutic and prophylactic purposes.

Keywords: Antarctic, *in vitro* plants, karyotype, genetic variability, nuclear DNA content, biologically active substances.

Introduction

Antarctica is located in the Southern hemisphere around the geographic South Pole, which occupies 10% of the Earth's surface. It is an isolated from other continents due to geographical distance, the presence of an oceanic polar frontal zone and being surrounded by the Antarctic Circumpolar Current (Smith 1984; Convey *et al*. 2008, 2011). Over 95% is covered with an ice sheet up to 3 000 m thick and is deprived of almost all life forms except for prokaryotes and fungi, as well as primitive aquatic animals living in subglacial reservoirs. Most terrestrial life forms are mainly distributed along the northern coastal territories and in adjacent islands.

The Antarctic biome is divided into three biogeographical zones: Subantarctic, maritime, and continental Antarctic zones (Smith 1984; Longton 1988; Ovstedal and Smith 2001; Convey 2005), but terrestrial vegetation is limited to Subantarctic and maritime Antarctic. In our study, we have focussed only on the Argentine Islands – Kyiv Peninsula region that is located in the centre of the maritime Antarctic, which includes the west coast of the Antarctic Peninsula with adjacent islands and located further north archipelagos consisting of the South Shetlands, the South Sandwiches and the South Orkneys (Longton 1979; Convey *et al*. 2008). Antarctic terrestrial communities usually are characterized by low biological diversity and a limited number of accessible habitats. Due to harsh environmental conditions, Antarctic terrestrial vegetation is restricted to ice-free areas that covers about 0.5% of the Antarctic surface (Singh *et al*. 2018). Low summer temperature, insufficient moisture, geographical isolation, and a short period of vegetation are main factors limiting the development of the terrestrial biota (Convey 2003).

Only two indigenous flowering plant species: *Deschampsia antarctica* É. Desv. (Poaceae) and *Colobanthus quitensis* (Kunth) Bartl. (Caryophyllaceae) occur in the maritime Antarctic. They are characterized by unique tolerance to the harshest environmental conditions in which the strong winds dry out the soil and vegetation, the temperature fluctuates rapidly and freeze-thaw cycles occur frequently, the average daily temperature during the growing season drops below zero, requiring effective cryoprotective mechanisms for plant survival, and ultraviolet radiation levels are high (Kyryachenko *et al*. 2005; Parnikoza *et al*. 2011; Matvieieva 2013; Cavieres *et al*. 2016).

Deschampsia antarctica, also known as Antarctic hairgrass, is a perennial, native to Antarctic terrestrial ecosystems grass. It grows from 0.5 to 25 cm tall. The young tillers are enclosed in a sheath, and leaves are sessile with linear leaf blades. *Deschampsia antarctica* may develop clones of regular shape with a life span of up to 35–40 years. Senescence and dieback of older plants are often observed in the central part of the clone, and the cycle of new clone formation begins when a plant is established in a new place (Moore 1970). *Deschampsia antarctica* forms minute, bisexual flowers gathered into a spikelet. The species is considered self-pollinating, thus flowers remain closed at the time of pollination (Moore 1970). However, there are reports on the formation of both cleistogamic and chasmogamic flowers in different environmental conditions that indicate the possibility of cross-pollination (Giełwanowska 2005; Yudakova *et al*. 2016). *D. antarctica* is capable of vegetative reproduction by the detached parts of a parental plant. Often, individual plants of this species form a very dense tussock, which can occupy a large area. The uprooted plant is able to root when relocated to other place in various ways including the transfer by birds when used as nesting material (Parnikoza *et al*. 2012). It was suggested that vegetative reproduction is an important mechanism of this species dispersal in the Antarctic (Parnikoza *et al*. 2012). In addition, the panicles of *D. antarctica* can be separated from the stem and carried away by the wind in the late summer.

Deschampsia antarctica is a tussock grass that lacks special adaptations for vegetative propagation. Nevertheless, the plant can propagate through the fragmentation of genets. Considering these features, the cultivation of *D. antarctica* plants on nutrient media and propagating them *in vitro* by dividing the parental plants into individual tillers is the optimal way to obtain the required amount of plant material. Previously developed method of micropropagation of *D. antarctica* using tissue culture techniques allowed rapid multiplication of mother plant with high genetic fidelity of regenerated plants (Cuba *et al*. 2005).

A number of responses and adaptations to abiotic stresses have been elucidated in Antarctic plants. These include the metabolic changes that affect the synthesis of carbohydrates and lipids, photosynthesis, respiration, and enzyme activity. Under low temperatures and high UV radiation conditions, *D. antarctica* was found to produce significant amounts of biologically active substances (BAS), in particular phenolic compounds (Perez-Torres *et al.* 2004; Ruhland

et al. 2005; Poronnik *et al*. 2014). The concentration of flavonoids in this species increased under UV-irradiation (Webby and Markham 1994; Day *et al.* 2001; Ruhland *et al*. 2005).

Despite several reports on the identification of genes involved in tolerance to various stresses, protective compounds synthesized in response to stress, as well as of physiological changes induced by the activation of the genes and accumulation of protective compounds (Parnikoza *et al*. 2011; Matvieieva 2013; Ozheredova 2015) the mechanisms of abiotic stress tolerance in Antarctic plants are not fully understood. A comprehensive characterization of Antarctic terrestrial ecosystems and fauna in general is essential for further applied research concerned the mechanisms of adaptation and resilience of Antarctic plants to harsh climatic conditions. In natural environments, such studies are hampered by the analysis of factors that act in complex and uncontrolled ways. This challenge can be overcome by using controlled conditions for this research, which may become possible with the development of collections of cultivated Antarctic plants. There are several studies of *D. antarctica* growing under controlled stress conditions (Lee *et al.* 2014), but the plants used in this study were taken from the field and then grown under greenhouse conditions. The development of the collections mentioned above allow avoiding the need to regularly transport directly from the Antarctic viable plant specimens required for the studies, thus reducing the burden on the environment to meet the requirements of international agreements on the Antarctic. Furthermore, it makes it possible to produce the required amount of plant material through *in vitro* culture and clonal propagation under controlled conditions throughout the whole year.

The use of plants *in vitro* enables the laboratory experiments to study the influence of individual stressors including various abiotic factors (*e.g.*, temperature, soil and air humidity, light, nutrient composition, exposure to heavy metals, lighting spectra, UV-radiation) and to identify their impact on genetic, physiological, and biochemical characteristics of plants, especially biomass growth, synthesis of photosynthetic pigments, storage substances, and other BAS or enzyme activity. The benefits of using plants grown under controlled conditions are particularly important for molecular biological studies because plants propagated *in vitro* are genetically homogeneous and contain less or even any foreign DNA, *e.g.*, viral or bacterial. The techniques of *in vitro* propagation of whole plants as well as the culture of isolated plant cells, tissues, and organs also make it possible to produce almost unlimited quantities of raw plant material of the desired genotype that can be useful for biotechnological applications (Kunakh 2005), as *D. antarctica* is a natural source of antioxidants, which can be used in the pharmaceutical and food industries, as well as in cosmetology (Gidegel *et al.* 2010, 2011). Phenolic compounds isolated from this plant species have been demonstrated to inhibit melanoma cell proliferation, to induce antitumor immunity against colorectal carcinoma and its metastasis to the

liver, as well as can be used to develop new drugs for the treatment of colorectal carcinoma (Gidegel *et al*. 2010; Malvicini *et al*. 2018).

The objective of this paper is to review and summarize the data from our previous research on the development and integral analysis of the collection of *D. antarctica*. We review the procedures and conditions for the germination of seeds, generation of aseptic plants *in vitro* with their subsequent clonal propagation, long-term *in vitro* culture, and acclimatization of plants to pot culture. We present the morphological and anatomical characteristics of the plants, as well as the data of karyological, molecular cytogenetic, molecular genetic, and biochemical analysis, and demonstrate that the collection of *D. antarctica* plants can provide a valuable background for further studies in various fields of plant biology and biotechnological developments.

Material

Collection sites. — The collection of *D. antarctica* plants was developed from the seeds collected during seasonal Ukrainian Antarctic expeditions (Table 1, Fig. 1). They were collected from the plants growing on rock terraces, slopes, or shores with northern exposition, except for Skua and Winter Islands, where the exposition was northern-western, and at altitudes ranging from 5 to 18 m above sea level. The exception were Rasmussen Cape and Berthelot Island at the altitude 60 m and 62 m, respectively. Most of the sites of seed collection on Galindez and Winter Islands were in the zone of kelp gull activity, and the site of G/D1-1 collection was near penguin rookeries. The total vegetation cover (TVC) ranged from 1% to 60%, except for Rasmussen Cape and Berthelot Island, where it was about 90%. In most cases *D. antarctica* grew together with *Colobanthus quitensis* (Kunth) Bartl. (<1% of TVC) and Bryophytes (0.5–89% of TVC).

Clonal propagation and cultivation *in vitro*. — To obtain plant material of *D. antarctica*, a protocol was developed to generate aseptic plants from seed, followed by clonal propagation *in vitro* (Zagrychuk *et al*. 2012). First the seeds were surface sterilized with 15% hydrogen peroxide solution (H_2O_2) for 40 min (sterilization efficiency was 100%). Due to the low toxicity of H_2O_2 and its spontaneous decomposition under exposure to light, the resulting aseptic plants were morphologically normal as well as displayed high vitality and intense growth. Two factors were used to stimulate the germination of *D. antarctica* seeds: exposure to low positive temperatures (+5ºC over 1–40 months) and treatment with gibberellic acid (GA_3). It was found that cold exposure for 21 months increased seed germination up to 55%. GA_3 treatment also increased the percentage of germination. Among the tested GA_3 concentrations (100, 300, 400 and 600 mg/L) and durations of treatment (from 12 to 24 hours), the optimum results were achieved when the seeds were treated with 600 mg/L GA_3 for 22 hours (Zagrychuk *et al*. 2012). The seeds were then placed in Petri dishes with

Table 1.

Characteristics of *Deschampsia antarctica* genotypes used to develop the collection of cultured plants.

No.	Genotype	Seed sampling season	Year of introduction to *in vitro* culture	Origin	Location (65°S, 64°W)
1	G/D1-1	2013/2014	2016	Galindez Island	14.686', 15.348'
2	G/D4-1	2012/2013	2014		14.916', 14.293'
3	G/D6-1	2016/2017	2017		14.880', 14.556'
4	G/D9-1	2013/2014	2018		14.847', 15.172'
5	G/D9-2	2013/2014	2017		14.847', 15.172'
6	G/D11-1	2016/2017	2017		14.757', 14.900'
7	G/D12-1	2013/2014	2014		14.845', 15.156'
8	G/D12-20	2006/2007	2009		14.845', 15.156'
9	G/D12-2a	2006/2007	2009		14.845', 15.156'
10	DAR 12	2006/2007	2009	Darboux Island	23.707', 12.905'
11	DAR 13	2006/2007	2009		23.707', 12.905'
12	R30	2004/2005	2008	Rasmussen Cape	14.819', 5.156'
13	R35	2004/2005	2008		14.819', 5.156'
14	S16	2009/2010	2010	Skua Island	15.302', 16.493'
15	S22	2007/2008	2008		15.300', 16.465'
16	Y62	2004/2005	2009	Great Yalour Island	14.039', 09.761'
17	Y66	2004/2005	2009		14.039', 09.761'
18	Y67	2004/2005	2009		14.039', 09.761'
19	L57	2009/2010	2010	Lahille Island	33.167', 23.249'
20	L59	2009/2010	2010		33.167', 23.249'
21	W1	2013/2014	2014	Winter Island	14.851', 15.487'
22	BE1	2013/2014	2015	the largest of the Berthelot Islands	19.440', 08.436'

the Murashige and Skoog medium (Murashige and Skoog 1962) with half content of macro- and micronutrients (MS/2) without phytohormones, before they were germinated at a temperature of 16–18ºC and 16 h light/8 h dark photoperiod at a light intensity of 6500 lux. The seed germination time was on average about four months (Fig. 2A). For clonal propagation, aseptic plants were used, which were cultured on MS and B5 media (Gamborg and Eveleigh 1968) supplemented with different concentrations of phytohormones, *e.g.*, 1-naphthylacetic acid (NAA), indoleacetic acid and kinetin (Fig. 2B). Propagation was achieved by dividing the original plant into fragments (Zagrychuk *et al.* 2012).

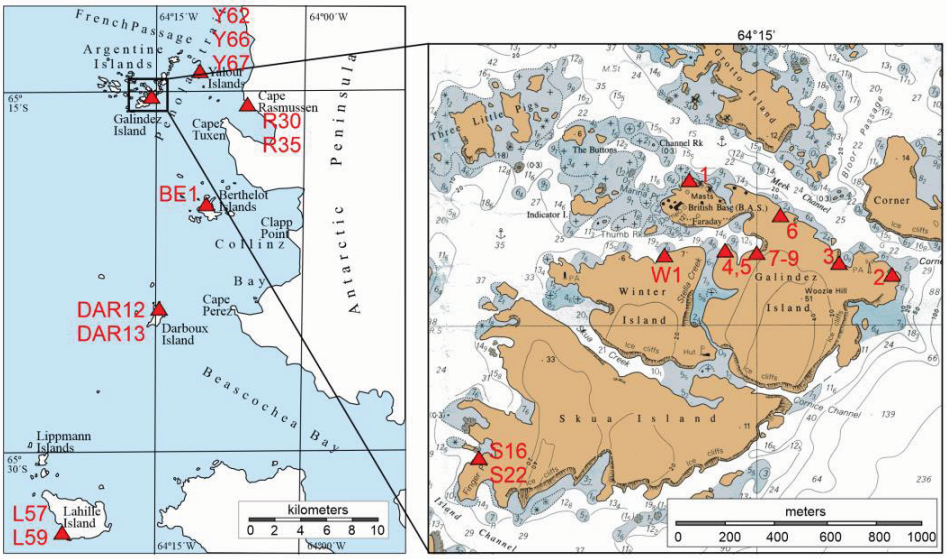

Fig. 1. Distribution of *Deschampsia antarctica* seed collection sites, from which the plants of the collection are originated. The markers on the maps indicate the origins of corresponding genotypes except for the plants from Galindez Island, where: 1 – G/D1-1; 2 – G/D4-1; 3 – G/D6-1; 4 – G/D9-1; 5 – G/D9-2; 6 – G/D11-1; 7 – G/D12-1; 8 – G/D12-20; 9 – G/D12-2a.

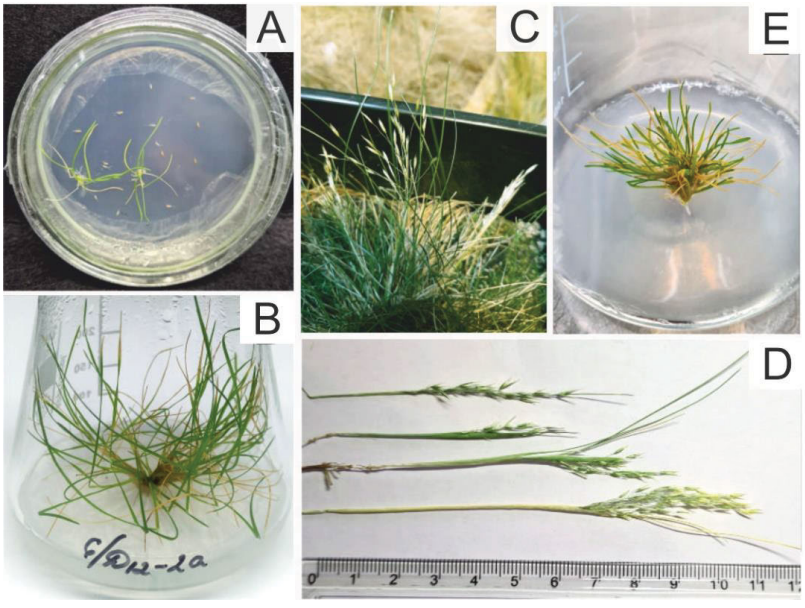

Fig. 2. *Deschampsia antarctica* plants in culture *in vitro* and in a pot: **A** – plants generated from seeds germinated *in vitro*; **B** – *in vitro* plant; **C** – plant with spikelets in a pot; **D** – collected spikeles; **E** – F1 plant generated from the collected seeds.

In vitro collection includes *D. antarctica in vitro* plants of 22 genotypes, which are cultured in solid B5 medium supplemented with 0.1 mg/L NAA, at a temperature of 16–18ºC, 16 h light/8 h dark photoperiod at a light intensity of 6500 lux, and relative humidity of 55–65% (Fig. 3A). Plants are subcultured every 2–3 months to maintain the collection *in vitro*, as well as to obtain the required amount of plant material for research.

Cultivation of potted plants. — To acclimate plants to the conditions of pot culture, plants cultivated *in vitro* were transferred into pots containing a mix of

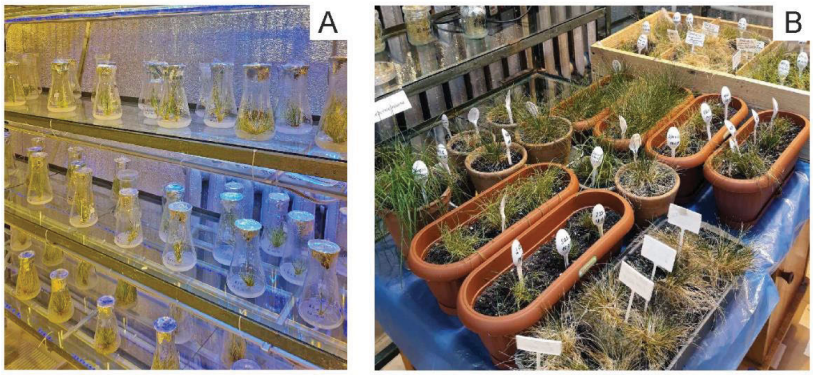

Fig. 3. *Deschampsia antarctica* plants cultivated *in vitro* (**A**) and in pots (**B**).

soil, vermiculite, and peat (3:1:1). Every two weeks, the plants were treated with nitrogen fertilizers (a mixture of NH_4NO_3, KNO_3, and $(NH_4)_2SO_4$), because Antarctic soils are reported to be rich in nitrogen (Parnikoza *et al*. 2017). Plants cultivated in pots were grown under the same conditions as *in vitro* plants (temperature, light intensity, humidity, and duration of daylight) (Fig. 3B). Inflorescence formation was observed in some of the plants cultivated in pots for 3–4 years. The spikelets with mature seeds were collected, and dry seeds were stratified in the refrigerator at 5°C for five months. Then, the seeds of DAR12 genotype were germinated to produce the plants of the F_1 generation, and were cultivated in B5 medium supplemented with 0.1 mg/L NAA (Fig. 2C–E). Currently, the established collection of *D. antarctica* included plants from the maritime Antarctic cultivated *in vitro* and in pots for 7–12 years.

Results and discussion

Morphometric and anatomical characteristics. — *Deschampsia antarctica in vitro* plants exhibited typical growth forms as wild plants from the same habitats (Poronnik *et al*. 2017). The leaf length of plants cultivated *in vitro* ranged from 1.5 to 18 cm. Most of the *in vitro* plants formed tufts with erect stems, which varied in height from 6 to 15 cm (Fig. 4A–D). The plants of L57

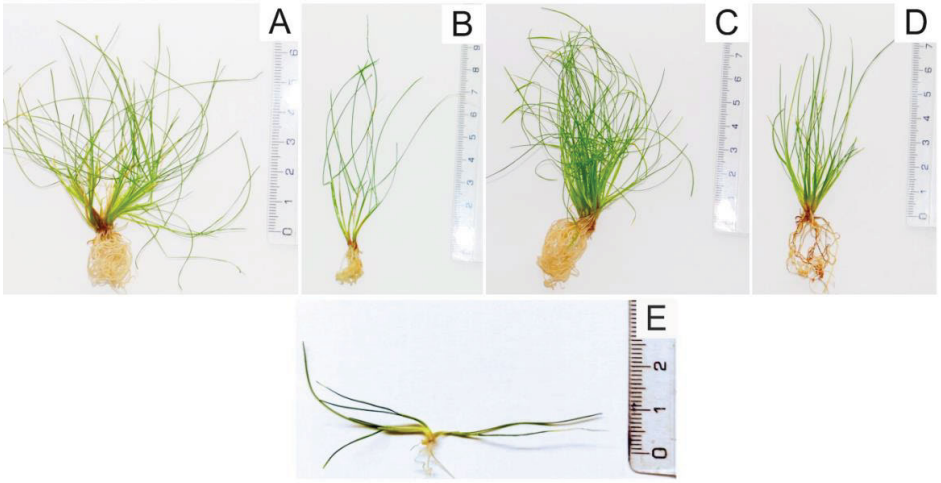

Fig. 4. Morphology of *Deschampsia antarctica* plants of different genotypes *in vitro*. Erect form: **A** – G/D12-2a; **B** – DAR12; **C** – R35; **D** – S22; prostrate form: **E** – L57.

and L59 genotypes differed from other genotypes from the collection by the growth form, especially because they had prostrated stems 3–15 cm long spread out on the surface of the media (Fig. 4E). These plants revealed the morphological traits typical for the wild plants in natural habitats from where the seeds were collected. In nature, *D. antarctica* plants have a wide range of growth forms that differ in morphological traits and the development of certain anatomical structures (Parnikoza *et al*. 2013). In elevated areas with soil moisture deficit located far from the coastline, Antarctic hairgrass develops small clumps 0.5–1.5 cm in height, which form 2–3 small leaves during the growing season that soon turn yellow and dry. This morphology was typical of *in vitro* plants of G/D4-1, G/D9-1, and G/D9-2 genotypes. In humid habitats, rich in organic compounds, plants grow more actively and form 4–6 deep green radially located leaves during the vegetation. Plant tufts in natural conditions are 10–12 cm tall (Giełwanowska 2005). This was characteristic of S16, S22, Y62, Y66, Y67, and W1 *in vitro* plants. R30 and R35 plants were distinguished by their brightly coloured leaves. These traits correspond to the two morphological forms of *D. antarctica* previously described from the Argentine Islands, *e.g.*, xerophytic growing in the drier environment and mesophytic growing in wetter habitats (Parnikoza *et al*. 2013).

Leaf anatomical structure may reflect the plant adaptation mechanisms to various abiotic stresses. Nuzhyna *et al*. (2019, 2021) have studied the leaf anatomical structure in wild *D. antarctica* plants sampled from natural environments as well as from the plants cultivated *in vitro*. Leaves sampled from *D. antarctica* plants cultivated *in vitro* as well as from wild plants shared a similar anatomical structure of the amphistomatic leaf, which was covered with a single layer of epidermis with thick cuticle, spongy mesophyll; in the

interbundle region there were big bulliform or motor cells which are involved in folding of leaf blade inwards toward the adaxial side (Fig. 5). The vascular system was mainly composed of three collateral bundles located in the central part of each rib (Nuzhyna *et al*. 2019, 2021). The variation among *D. antarctica in vitro* plants of different genotypes in leaf anatomical traits such as stomatal

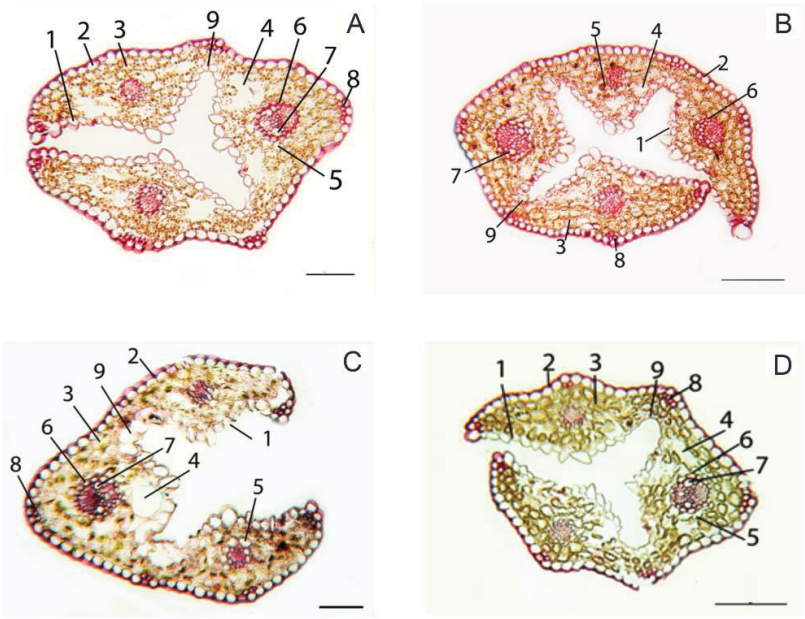

Fig. 5. Cross-sections of the leaves of *Deschampsia antarctica in vitro* plants of four different genotypes: **A** – W1; **B** – S22; **C** – DAR12; **D** – Y66. 1 – adaxial epidermis, 2 – abaxial epidermis, 3 – mesophyll, 4 – intercellular space, 5 – exterior sheath of the vascular bundle, 6 – interior bundle sheath, 7 – metaxylem, 8 – sclerenchyma, 9 – motor cells. Scale bars = 100 µm (after Nuzhyna *et al.* 2021).

density, epidermis thickness, and outer cell wall thickness for both adaxial and abaxial surfaces was revealed (Nuzhyna *et al*. 2021). An inverse relationship between the stomata density and their size was apparent. The adaxial epidermis had the largest thickness in DAR12 plants, and the abaxial epidermis had the largest thickness in R35 plants. Y62 and S22 plants were found to have a relatively thin epidermis and outer cell wall on both leaf sides. The L59 plants had the largest thickness and cross-sectional area of the leaf blade. Unicellular non-glandular spine-shaped trichomes were found above the veins on the adaxial surface of the leaf of W1 plants (Nuzhyna *et al*. 2021). In L59 plants, the leaf thickness and cross-sectional area were significantly larger compared to other *in vitro* plants. The leaf cross-sectional area was directly related to the number of leaf ribs. In most of the *in vitro* plants, leaves had three ribs and were symmetrical. In contrast, in S22 plants, individual leaves with four ribs were observed, while L59 plants had five-rib symmetrical leaves.

D. antarctica plants grown in greenhouse conditions and in the Antarctic tundra (400 m from the coastline) had three-rib leaves, whereas 4–5-ribs leaves were more common in plants grown in more humid habitats, which were located 30 m from the coastline (Giełwanowska *et al.* 2005). Romero *et al*. (1999) has emphasized that the wild plants growing in the Antarctic have higher stomata density than the laboratory plants. However, the differences in stomata density between the plants propagated *in vitro* correspond to those between wild plants sampled from the same habitats (Nuzhyna *et al*. 2019, 2021).

The mechanical tissue in all *in vitro* plants was poorly represented and consisted of sclerenchymal bundles of two to five fibers under the abaxial epidermis above the vascular bundles and between the ribs. In the central part of each leaf rib, the vascular bundle was observed, which was surrounded by two sheaths: an inner sheath of lignified sclerenchyma cells and an outer sheath of parenchymatous cells with chloroplasts. It was reported that plants from wet habitats (growing close to the coastline) typically had a well-developed bundle sheath of parenchymatous cells, while in plants from drier habitats, such as the Antarctic tundra, the bundle sheath was less developed (Romero *et al*. 1999). Parenchymatous sheath development is also associated with high insolation (Pyykko 1966) and low temperature, possibly as a prerequisite for solar radiation tolerance (Romero *et al*. 1999). The metaxylem vessels were found to be better developed in the vascular bundles of all studied plants *in vitro* compared to wild plants from the same habitats (Nuzhyna *et al*. 2019, 2021). This may be caused by higher temperatures of growth in the laboratory compared to natural environments. Vessels with small apertures are common for plants growing under low-temperature conditions because water freezes slowly in smaller area vessels (Romero *et al*. 1999).

Cytogenetic characteristics. — Cytogenetic analysis showed that most of *D. antarctica* plants from the collection, particularly 19 of 22 genotypes, have a chromosome number 2*n*=26, which is typical of this species (Navrotska *et al*. 2014, 2017, 2018; Amosova *et al*. 2015, 2017). The plants of other genotypes include diploid plants with B-chromosomes (DAR12, 2n=26+0–3B), mixoploid with Robertsonian translocation (Y66, with chromosome numbers ranging from 26 to 54 and a near-triploid modal class of 36–39), and mixoploid (Y67, with chromosome numbers ranging from 13 to 38 and a diploid modal class of 26) (Fig. 6). The latter chromosomal forms of the species were previously unknown and discovered in our studies (Navrotska *et al*. 2014, 2017, 2018; Amosova *et al*. 2015, 2017). Karyotyping revealed four groups of chromosomes differed in centromere position and morphology, and the karyotype formula of the species was 2n=26=2(6m+2sm+3st+2t) (Fig. 7) (Amosova *et al*. 2015) compared to the earlier described one of 2n=26=2(5m+3sm+4st+t) (Cardone *et al*. 2009; Gonzalez *et al*. 2016). This discrepancy may be attributed to the use of chromosome-specific markers for more precise identification of homologous pairs in our studies. C-banding of *D. antarctica* chromosomes demonstrated

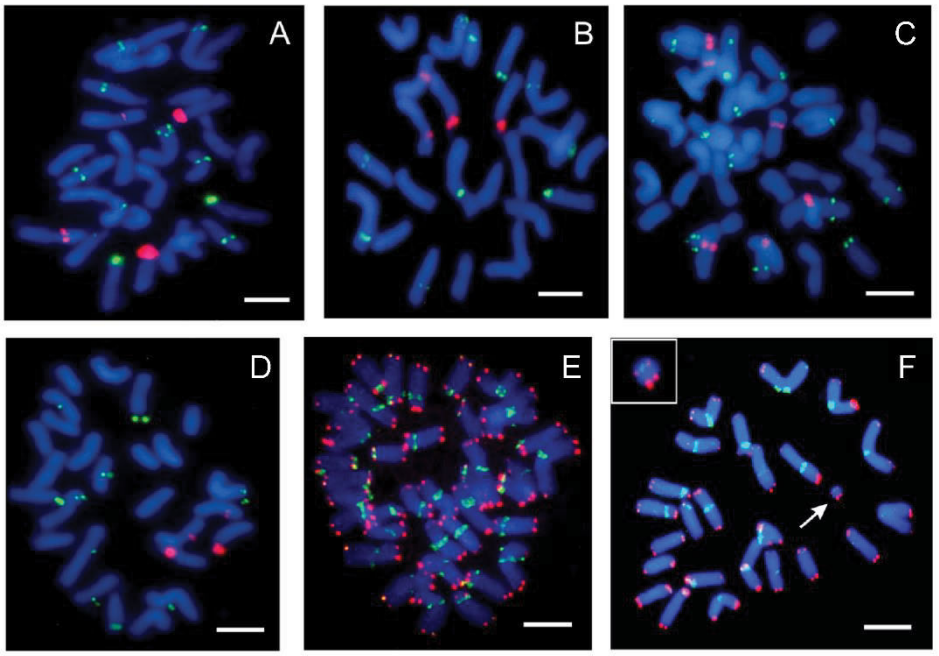

Fig. 6. DAPI stained metaphase plates from root apical meristem and localization of 5S rDNA (green) and 45S rDNA (red) loci on metaphase chromosomes of *Deschampsia antarctica* plants of different genotypes: **A** – DAR12 (2n=26); **B** – G/D12-2a (2n=26), **C** – Y66 (2n=36), **D** – S22 (2n=26). Localization of *Arabidopsis thaliana* (L.) Heynh. telomeric repeats HT100.3 (red) and *Brachypodium sylvaticum* (Huds.) P. Beauv. centromeric repeats CCS1 (green) in: **E** – Y66 plant (3n=39) and **F** – DAR12 plant (2n=26+1B). An arrow indicates a supernumerary chromosome. Scale bars = 10 µm (after Navrotska *et al.* 2018).

a similar distribution of C-bands in karyotypes of different plants: large C-bands were located in the sub-centromeric and telomeric regions of chromosomes, while a number of small and weak C-bands were revealed in the interstitial chromosome regions (Fig. 7) (Amosova *et al*. 2015).

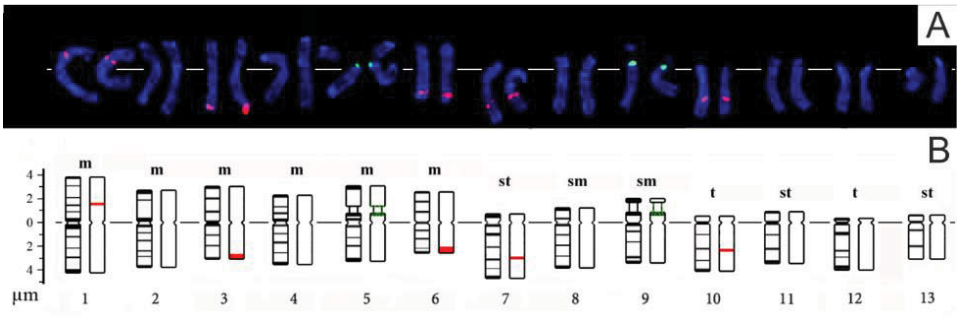

Fig. 7. Karyogram (**A**) and idiogram (**B**) of chromosomes of *Deschampsia antarctica* diploid plants. DAPI staining with localized signals of 5S rDNA (red) and 45S rDNA (green) (after Amosova *et al*. 2015).

In diploid karyotypes, 10 sites of 5S rDNA and 4 sites of 45S rDNA were revealed using fluorescence *in situ* hybridization (FISH) analysis (Fig. 7). 5S rDNA sites were mapped to the proximal regions of six chromosomes and to the terminal regions of four chromosomes. The 5S rDNA loci were smaller and more polymorphic in size than the 45S rDNA loci. The 45S rDNA loci were found close to the centromeres of two chromosomes and in the terminal regions of two other chromosomes. Using Ag-NOR staining specific to transcriptionally active NORs (Miller *et al*. 1976, 1977), the NORs of both SAT chromosomes in *D. antarctica* were shown to be functionally active.

In DAR12 plants, karyotypes of root apical meristem cells were found to contain one to three supernumerary or B-chromosomes (Fig. 6F). The frequency of metaphases with B-chromosomes was low (less than 10%), and metaphases with one B-chromosome were the most frequent. B-chromosome-containing cells were found in 25% of the studied roots. B-chromosomes in the karyotype of DAR12 plants had distinct heterochromatin bands in telomeric regions, which were intensely stained by C-banding and displayed a condensed dark-stained appearance in interphase nuclei that is specific for B-chromosomes. Two morphological types of B-chromosomes could be distinguished based on the position of heterochromatin blocks and the presence of 5S rDNA sequences (Amosova *et al*. 2015; Navrotska *et al*. 2018). Weak 5S rDNA sites were identified in the subtelomeric region of one of the two types of B-chromosomes of the DAR12 karyotype (Amosova *et al*. 2015). The supernumerary chromosomes were significantly smaller than other chromosomes and showed clear hybridization signals of centromeric and telomeric repeats in the centromeric and both terminal telomeric regions, respectively. This finding indicates the structural integrity of B-chromosomes in DAR12 karyotype (Navrotska *et al*. 2018). It should be noted that the plants of another genotype DAR13 originating from Darboux Island were typical diploids.

We initiated callus culture from root explants of the DAR12 plant and induced regeneration after several passages. A few plants were regenerated, and cytogenetic analysis of one of these regenerated plants (DAR12-R1) showed the absence of B-chromosomes in its karyotype. DAR12-R1 regenerated plant was found to be diploid. This fact demonstrates the unstable inheritance of B-chromosomes in *D. antarctica* plants propagated through *in vitro* culture.

In the root apical meristem of Y66 plants, cells containing 37 and 38 chromosomes were found in addition to diploid (2n=26) and triploid (3n=39) cells (Fig. 6E) (Navrotska *et al*. 2014; Amosova *et al*. 2015). The triploid nature of Y66 plants was confirmed by FISH with probes containing 5S rDNA, 45S rDNA, centromeric and telomeric repeats (Fig. 8), as well as by flow cytometry-based measurement of nuclear DNA content (see Section Nuclear DNA content) (Navrotska *et al*. 2018). In the Y66 plant, fourteen 5S rDNA loci and six 45S rDNA loci were identified. 5S rDNA loci were found in the proximal regions of eight chromosomes and in the terminal regions of six chromosomes.

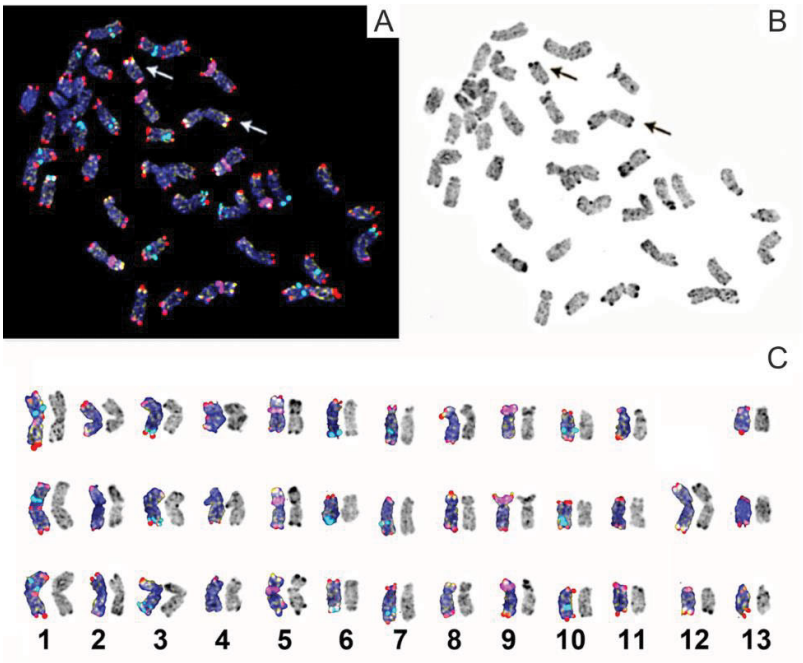

Fig. 8. Molecular cytogenetic analysis of the karyotype of Y66 plant: **A** – FISH with *Arabidopsis thaliana* telomeric repeats (red), 45S rDNA (purple), and 5S rDNA (light blue) followed by GISH analysis with genomic DNA of *Deschampsia caespitosa* (yellow); **B** – inverted image of DAPI/C-banded chromosomes. Arrows indicate chromosome 12 and a Robertsonian translocation between two homologous chromosomes of the 12-th pair; **C** – karyograms of metaphase chromosomes after FISH analysis (left) and DAPI/C-banding (right). Scale bar = 5 μm (after Amosova *et al*. 2015).

45S rDNA signals were localized in the proximal regions of three chromosomes and in the terminal regions of three other chromosomes (Fig. 6C, 8). Identified differences in the number of rDNA loci are probably caused by an increase in the number of chromosomes sets in the triploid karyotype of the Y66 plant. Telomeric sequences formed clear sites at the terminal ends of all chromosomes, and centromeric sequences hybridized to the region of primary constriction of all chromosomes (Fig. 6E, F) (Amosova *et al*. 2015; Navrotska *et al*. 2018).

The FISH analysis did not reveal any chromosomal markers associated with the rearranged chromosome in the karyotype of the Y66 plant. Therefore, genomic *in situ* hybridization (GISH) analysis was performed using labelled genomic DNA of the related species *Deschampsia caespitosa* (L.) P.B. as a probe. The bright hybridization signals were detected in the subtelomeric regions of the short arms of 5, 8, and 12 chromosomes (Fig. 8). These findings indicate that Robertsonian translocation or fusion of homologous chromosomes of the 12-th pair, or so-called Robertsonian translocation t(12; 12) (Amosova *et al.* 2015). In addition, tetraploid metaphases (4n=52) were observed in Y66 plants. Moreover, two chromosomal translocations t(12; 12) with four haploid

chromosome sets were found in one metaphase with 54 chromosomes (4n=52+2t (12; 12)). This rearrangement was observed in most of the cells of the Y66 plant (Amosova *et al.* 2015).

In Y67 plants, a modal chromosome number was 26; in addition, cells with 13 and 38 chromosomes were also found. Thus, the data of molecular cytogenetic analysis suggest that the karyotype variation in the plants *in vitro* from the collection may be the result of genomic instability induced by the impact of stress factors in a severe Antarctic environment. The finding of an intercalary site of telomere repeats in the largest chromosome 1 indirectly supports the hypothesis that the unusual for cereals chromosome number in this species may be caused by ancestral chromosome fusion.

Molecular genetic analysis. — *In vitro* cultivation can induce significant stress, which affects the plant as a whole and can trigger genetic changes (Kunakh 2005). To assess the genetic stability of long-term *in vitro* cultivated *D. antarctica* plants, cytogenetic analysis and PCR analysis with 10 ISSR primers was applied. In particular, *in vitro* propagated plants of five genotypes (G/D12-2a, G/D12-20, DAR12, DAR13, S22) were studied at different stages of cultivation (Spiridonova *et al*. 2016).

The DNA was isolated from dried leaves with the modified CTAB protocol (Doyle and Doyle 1987) adapted for small amounts of plant material. ISSR-analysis of plants at the initial (1–8 passages) and advanced (24–26 passages and later) stages of cultivation *in vitro* did not reveal molecular genetic differences neither between the clones of common origin nor between the clones and their parental plant. Furthermore, the cytogenetic analysis did not identify any karyotype rearrangements in long-term cultivated *in vitro* (49–79 passages) diploid plants. However, karyotype variation was found in DAR12 plants with B-chromosomes studied at the 49-79 passages of cultivation *in vitro*, which included a decrease in the percentages of cells bearing B-chromosome and aneuploid cells with hyperdiploid chromosome numbers (27, 28, and 29 chromosomes). Therefore, the findings of ISSR-analysis and cytogenetic analysis indicated the stability of molecular genetic and cytogenetic characteristics of *D. antarctica* plants propagated *in vitro* for a long period (5–7 years). In general, the obtained data confirmed that the methods and procedures of growing plants from seeds and their subsequent clonal propagation *in vitro* ensure the genetic stability of *D. antarctica* plants during long-term cultivation *in vitro*, which provided a basis for developing the collection of *D. antarctica* plants (Spiridonova *et al*. 2016).

To assess the genetic diversity of 22 *D. antarctica* plants of the collection, PCR analysis with 7 ISSR and 3 IRAP primers was used (Table 2). These primers were successfully applied in previous population genetic analysis of *D. antarctica* from the maritime Antarctic (Andreev *et al*. 2022). In total, 147 PCR fragments were generated with 10 primers, among which 44 (30.6%) were polymorphic. An example of polymorphic band patterns produced by PCR is shown in Fig. 9A.

Table 2.

Primers used for PCR-analysis of genetic polymorphism of *Deschampsia antarctica in vitro* plants, sequence, and the number of bands obtained per primer. B – total number of produced bands, P – the number of polymorphic bands, and P% – the proportion of polymorphic bands.

Primer	Sequence, 3'-5'	B	P	P%
ISSR 03	$(AC)_8TT$	12	4	33.3
ISSR 04	$(AC)_8AG$	15	3	20.0
ISSR 23	$(AC)_8TA$	14	4	28.6
ISSR 807	$(AG)_8T$	21	4	19.0
ISSR 811	$(GA)_8C$	15	0	0
ISSR 836	$(AG)_8YA$	10	2	20.0
ISSR 840	$(GA)_8YT$	16	9	56.3
IRAP 642	TTTGAAAACTGGCGGCAACG	15	3	20.0
IRAP 1651	TGACCAAGGGCGCGTATCGTG	14	7	50.0
IRAP 1861	ATACCTCGGAGGCGCTGCACCTG	15	9	60.0
	Total bands	147	45	30.6

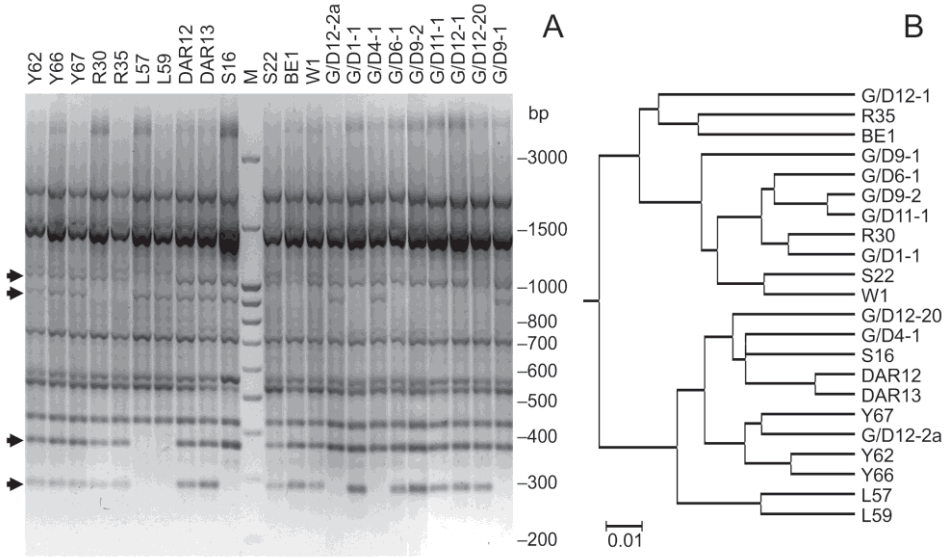

Fig. 9. Genetic diversity of *Deschampsia antarctica in vitro* plants from the collection. **A** – agarose gel electrophoresis profile of PCR products amplified with IRAP1861 primer and DNA isolated from *D. antarctica in vitro* plants from the collection. M –100 bp DNA ladder. **B** – dendrogram illustrating genetic relationships among *D. antarctica in vitro* plants from the collection, which was generated by cluster analysis using the unweighted pair-group method with arithmetic average (UPGMA) based on the matrix of Jaccard's pairwise genetic distances between plants.

The indices of genetic variability (Shannon Index) of *D. antarctica* plants from the collection calculated from the PCR data using GenAlEx ver. 6.503 (Peakall and Smouse 2006), were as follows: Shannon diversity index (I) = 0.163 ± 0.022, Nei's gene diversity (expected heterozygosity, He) = 0.111 ± 0.015, and Jaccard's pairwise genetic distances (Djav) between individuals ranged from 0.016 to 0.187 with an average value of 0.104. Based on Jaccard's genetic distances matrix calculated with FAMD software (Schlüter and Harris 2006), the UPGMA dendrogram was generated to assess the genetic relationships among *D. antarctica* plants from the collection (Fig. 9B). The values of genetic diversity indices of *in vitro* plants from the collection are generally comparable with those of wild plants collected from *D. antarctica* populations in the Argentine Islands region (Andreev *et al*. 2021). This finding suggests that the collection of *D. antarctica* plants may to some extent represent populations from this part of the maritime Antarctic.

Nuclear DNA content. — Haploid DNA content per nuclei or C-value is an important biological characteristic of an organism. Each species has a typical number of nucleotide pairs in the nuclear genome or nuclear DNA content, which is generally a constant value (Swift 1950). Bennett *et al*. (1982) have reported the nuclear DNA content of *D. antarctica*. The nuclear DNA content of *D. antarctica in vitro* plants from the collection was measured by flow cytometry (Fig. 10) and proved that the average nuclear DNA content of diploid plants was 10.88 pg/2C (Navrotska *et al.* 2018). The nuclear DNA content in DAR12 plant bearing B-chromosomes was 10.86 pg/2C (Fig. 10J) and fell within the range of the values of diploid plants. The nuclear DNA content of the Y66 myxoploid plant was 16.46 pg/2C, thus being 1.5 times higher than the average value obtained for diploid plants (Fig. 10K). The flow cytometry data confirmed the myxoploid nature of the Y66 plant with triploid modal chromosome number, which was previously identified by molecular cytogenetic analysis (Navrotska *et al.* 2018).

The findings of nuclear DNA content measurement in *D. antarctica in vitro* plants from the collection were consistent with the previous results (Bennett *et al*. 1982). In this study, 2C DNA content determined using Feulgen staining was 9.95 pg/2C in *D. antarctica* from Galindez Island, and 17.10 pg/4C in the tetraploid form of *D. caespitosa* from the British Islands of the maritime Antarctic. In addition, nuclear DNA content was determined for other species of the genus and the measured 2C values were as follows: 10.43 pg for *D. caespitosa* (2n=26), 11.05 pg for *D. chapmanii* (2n=26) and 10.07 pg for *D. tenella* (2n=26) from New Zealand (Murray *et al.* 2005). Recently, another study was published (Pascual-Díaz *et al*. 2020) that reported an average 2C value of 10.63 pg/2C for *D. antarctica* plants sampled in several populations from the Antarctic Peninsula, and it is very close to the figures obtained in our study. Our findings also indirectly indicate the constancy of nuclear DNA content in *D. antarctica* plants propagated *in vitro*.

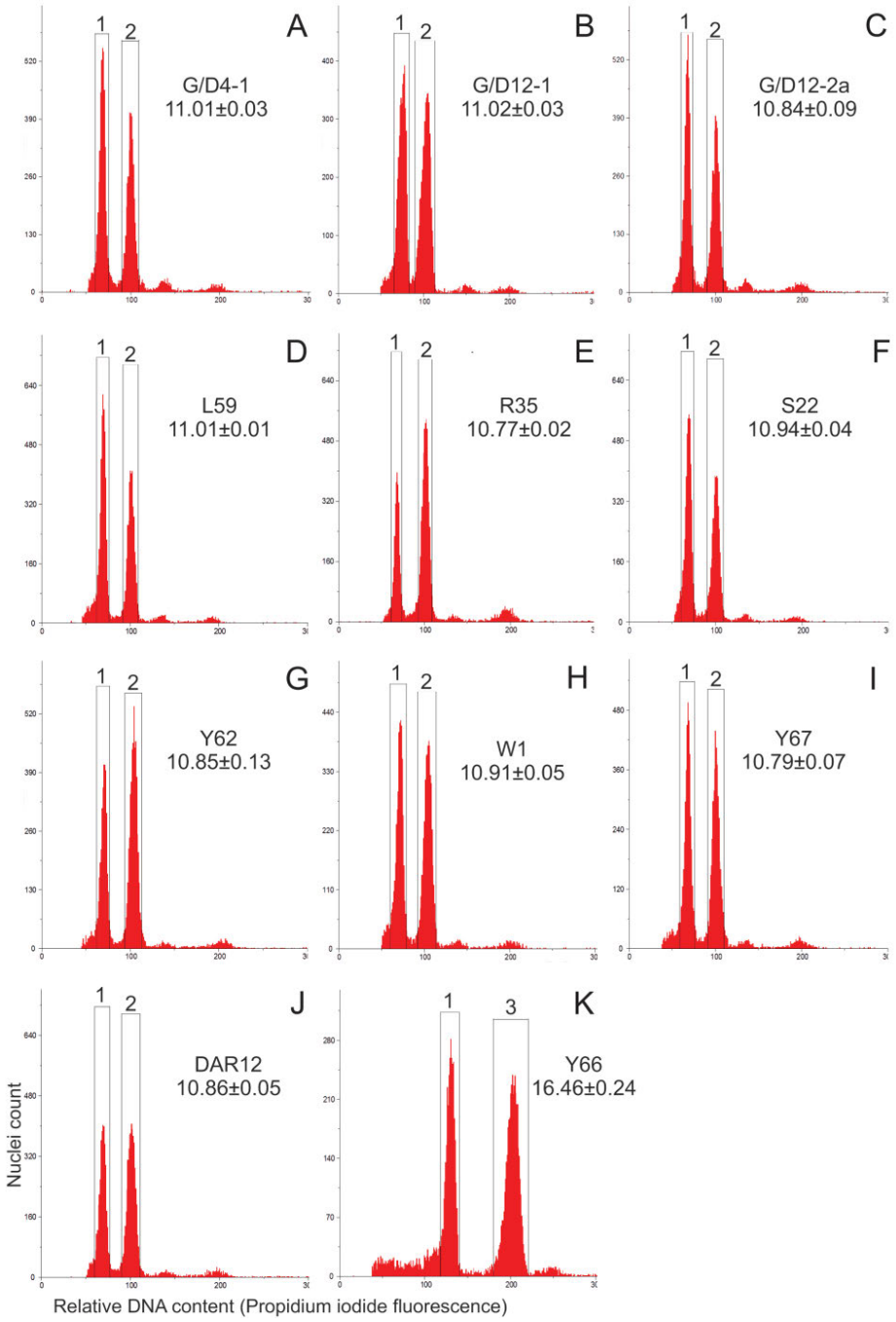

Fig. 10. Flow cytometry measurements of nuclear DNA content (2C-value) in *Deschampsia antarctica in vitro* plants (1). *Secale cereale* subsp. *cereale* (16.01 pg/2C, GeneBank Gatersleben AcNo: R737) (2) and *Vicia faba* cv 'Tinova' (26.21 pg/2C, GeneBank Gatersleben AcNo: FAB602) (3) were used as an internal control (after Navrotska *et al.* 2018).

Quantification and qualitative analysis of phenolic compounds and flavonoids. — The studies of biologically active substances from *D. antarctica in vitro* plants included three stages: (*i*) measurement of the content of BAS in alcoholic extracts of *in vitro* plants cultivated under different growth conditions (Twardovska *et al.* 2020, 2021); (*ii*) qualitative mass spectrometry analysis to identify specific groups of BAS in extracts of wild-grown and *in vitro* plants; and (*iii*) evaluation of antioxidant/reducing activity of extracts of wild-grown and *in vitro* plants (Ivannikov *et al.* 2021a, 2021b). The measurement of the content of BAS in in vitro plants was carried out jointly with the Institute of Cell Biology and Genetic Engineering of the NAS of Ukraine; MALDI MS studies and evaluation of antioxidant/reducing activity was performed by researchers of the Chuiko Institute of Surface Chemistry of the NAS of Ukraine.

The total content of phenolic compounds was 7.3–16.5 mg/g in leaves and 2.1–14.1 mg/g in roots of plants *in vitro*; and the total content of flavonoids was 2.3–21.3 mg/g and 1.9–17.4 mg/g, respectively. In general, the total content of these secondary metabolites was higher in leaves compared to roots of *in vitro* plants (Twardovska *et al*. 2021). The content of these substances in leaf and root tissues were comparable only in a few of *in vitro* plants. The lowest number of phenolic compounds was found in G/D12-2a and L57 plants. When comparing Y67 and Y66 mixoploid plants originating from Great Yalour Island, the total content of phenolic compounds in Y67 plant was slightly larger, but the difference was not statistically significant at $p<0.05$. Plants of different genotypes originating from Darboux Island (DAR12 and DAR13) also contained almost the same amounts of phenolics (Twardovska *et al*. 2021).

The content of flavonoids was found to differ significantly among the plants *in vitro* of different genotypes. The highest content of these secondary metabolites was observed in DAR12 *in vitro* plants, and DAR12 and G/D12-2a regenerated plants, while the smallest amount of flavonoids was in the L57 plant. When comparing *in vitro* plants originated from Darboux Island, DAR12 contained 2.5 times more flavonoids than DAR13. DAR12 *in vitro* plants and regenerated plants had very similar content of flavonoids. In contrast, the flavonoid content in G/D12-2a regenerated plant was almost twice as much as that in G/D12-2a *in vitro* plant. Y66 and Y67 *in vitro* plants originating from Great Yalour Island did not differ significantly in flavonoid content (Twardovska *et al*. 2021). Figure 11 shows a typical profile of phenolics and flavonoids in the alcoholic extract of *D. antarctica* leaf tissue fractionated by high-performance liquid chromatography (HPLC) and analysed at 318 nm. Alcoholic leaf extracts obtained from *in vitro* plants were found to contain the five most abundant phenolic compounds. Quercetin, kaempferol, or luteolin were not identified in the extracts. In some samples, residual amounts of rutin and small amounts of chlorogenic acid were detected. The most abundant was currently unidentified substance 3 (Fig. 11). One of the main peaks was identified as orientin (luteolin-8-C-glucoside). The content of orientin was highest in regenerated plants (2.7 and 4.8 mg/g), while in the other *in*

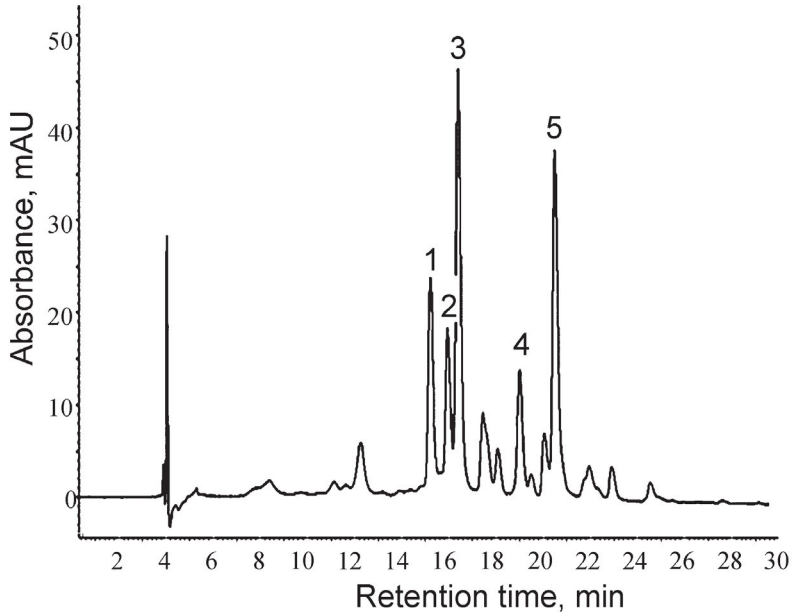

Fig. 11. HPLC chromatogram of phenolic compounds from *Deschampsia antarctica* leaf extract. The detection wavelength was 318 nm. 1 and 3–5 – unidentified substances, 2 – orientin (after Twardovska *et al*. 2021).

vitro plants it ranged from 1.0 to 2.5 mg/g (Twardovska *et al.* 2021). The presence of this substance in the samples of *D. antarctica* was also previously reported by other researchers (Webby and Markham 1994; Staaij *et al*. 2002).

The biochemical analysis did not reveal significant differences between the chromosomal forms of *D. antarctica* in the content of phenolic compounds and flavonoids (Navrotska *et al*. 2018; Twardovska *et al*. 2020, 2021). Slight quantitative differences were observed only for individual peaks in the profiles of the myxoploid Y66 plant compared to the diploid ones (Navrotska *et al*. 2018).

Identification of biologically active substances in *D. antarctica* extracts. — Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI MS) analysis of alcoholic extracts of *D. antarctica* wild plants sampled in the Antarctic and plants cultivated *in vitro* showed that the most intense peak in the mass spectrum of the wild plant extract (Fig. 12A) corresponded to orientin, which was identified also by HPLC with UV-detection (Fig. 11). The spectrum also contained the signals, that can be referred to luteolin and luteolin derivatives, such as luteolin trimethyl ether, isoswertiajaponin, isoswertiajaponin 2"-*O*-*β*-arabinopyranoside, orientin 2"-*O*-*β*-arabinopyranoside, and isoswertiajaponin 2"-*O*-*β*-arabinopyranoside acylated. Thus, seven of 17 signals can be referred to luteolin or luteolin derivatives, with three of those being among the most prominent in the mass spectrum. The rest of the signals corresponded to two

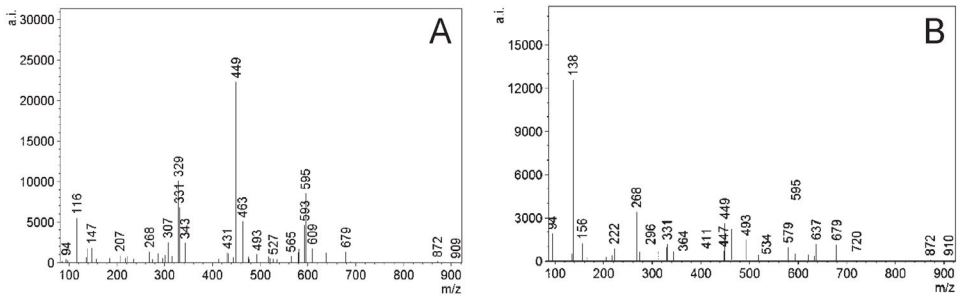

Fig. 12. MALDI mass spectra of the extracts of *Deschampsia antarctica* wild plants sampled in the maritime Antarctic (**A**) and plants cultivated *in vitro* (**B**) (after Ivannikov *et al.* 2021a).

apigenin derivatives and four other flavonoids/flavonoid derivatives. The presence of hydroxybenzoic acids and caffeic acid glucoside was also confirmed (Ivannikov *et al*. 2021a, 2021b). The main peak in the MALDI mass spectrum of extracts of *in vitro* plants (Fig. 12B) corresponded to the ions of hydroxybenzoic acids, four other intense peaks belonged to hydroxycinnamic acids derivatives, several other moderately intense signals were luteolin derivatives, and two peaks corresponded to apigenin and apigenin derivatives. The data also showed the presence of several other flavonoids/flavonoid derivatives. Thus, the extracts from *D. antarctica in vitro* plants were found to contain high amounts of phenolic compounds including hydroxybenzoic acids, hydroxycinnamic acids, and flavonoids, as well as the derivatives of the above compounds. Luteolin and luteolin glycosides were the most common compounds of the flavonoids group, while hydroxybenzoic acids prevailed among phenolic low-molecular-weight compounds. The extracts of wild plants and *in vitro* plants were found to have a similar composition but differed in the concentrations of individual groups of phenolic compounds (Ivannikov *et al*. 2021a).

It was reported that *D. antarctica* extracts contain the following compounds: 2-*O*-*β*-galactopyranosylorientin, 2"-*O*-*β*-arabinopyranoside orientin, orientin, isowetiajaponin (7-*O*-metilorientin 2"-*O*-*β*-arabinopyranoside), isowertijaponin (7-*O*-metilorientine 2"-*O*-*β*-arabinopyranoside), and luteolin. However, these compounds were found in extracts from *D. antarctica* wild-grown plants or plants subjected to 4°C for 72 hours, but they were not detected in plants grown *in vitro* at 13°C (Gidekel *et al*. 2011). In contrast to this study, we found that aerial parts of *D. antarctica* plants cultivated *in vitro* at 18°C contain about 2–4.8 mg/g d.w. biomass of orientin.

Antioxidant activity of extracts. — All the *D. antarctica* extracts under study had a very high content of polyphenols (*ca.* 300–900 mg/L) (Ivannikov *et al*. 2021*a*). Therefore, all of them showed high activity in the 2,2′-diphenyl-1-picrylhydrazyl radical (DPPH) assay. Under standard assay conditions, instantaneous discoloration of the reaction mixture was observed that indicates a complete quenching of the radicals. The 10 times diluted extracts also inhibited 50–90% of DPPH radicals during 30–60 min (Fig. 13). However, no strong

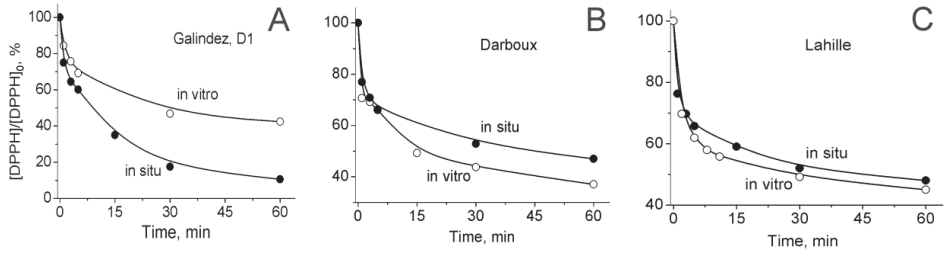

Fig. 13. Inhibition of DPPH radicals by the extracts of *Deschampsia antarctica* wild plants sampled from the maritime Antarctic and plants cultivated *in vitro*. (after Ivannikov *et al*. 2021a).

correlation was found between the total phenol content and radical scavenging activity of the extracts (Ivannikov *et al*. 2021a, 2021b).

To illustrate, the extract of *in vitro* plant from Lahille Island had the highest phenol content, but its activity in the DPPH assay was one of the lowest. There was no clear pattern when comparing extracts of wild plants and plants cultivated *in vitro* originating from the same locality. However, extracts from both wild-grown plants and plants *in vitro* exhibited free-radical scavenging activity in the DPPH assay, which ranged from 52 to 78% (Fig. 13A).

The antiradical activity of the extracts was assessed in the assays with NO• and OH• radical scavenging. These radicals are considered the most common reactive oxygen and nitrogen species, which are produced during normal cellular metabolism and play an important role in the pathogenesis of several oxidative stress-related diseases (Pavithra and Vadivukkarasi 2015). It was found that 10-fold diluted extracts from both wild-grown plants and plants *in vitro* scavenged 20–40% of NO• radicals and 40–60% of OH• radicals (Ivannikov *et al.* 2021a).

In conclusion, it should be emphasized, that *D. antarctica* plants *in vitro* can serve a valuable source of phenolic compounds, which are effective radical scavengers of natural origin, and that the amount and content of phenolic compounds in plants cultivated *in vitro* and regenerated plants are similar to those of wild plants that give grounds to consider *D. antarctica* plants cultured *in vitro* as a prospective raw material for obtaining these BAS.

Conclusions

The established collection of *D. antarctica* from the maritime Antarctic includes plants of 22 genotypes cultivated *in vitro* and in pots. To establish the collection, the optimal conditions for the germination of *D. antarctica* seeds and the generation of aseptic plants *in vitro* were determined. Methods and procedures were developed for clonal propagation and long-term cultivation *in vitro* of aseptic plants grown *in vitro* from seeds, as well as for plant acclimatization to pot culture. The relevance of developed methods and

procedures was confirmed by cytogenetic and molecular genetic studies, which established the genetic stability of propagated and long-term cultivated *in vitro* plants.

The established collection of *in vitro* plants is heterogeneous in a number of parameters. *Deschampsia antarctica* plants of different genotypes were shown to have different morphometric epidermal parameters, which may be caused by the differences in the microenvironmental conditions of wild plant habitats. Molecular cytogenetic analysis along with nuclear DNA content measurement demonstrated that most plants in the collection are diploids. However, the collection includes also other chromosomal forms of the species, which were found for the first time in the Antarctic region: DAR12 diploid with B-chromosomes (2n=26+0–3B), Y66 mixoploid (with chromosome numbers ranging from 26 to 54, a near-triploid modal class of 36–39, and Robertsonian translocation) and Y67 myxoploid (with chromosome numbers ranged from 13 to 38 and a diploid modal class of 26). It was assumed that the karyotype variability identified in *in vitro* plants from the collection may reflect genome instability induced by the harsh environmental conditions of the maritime Antarctica. Molecular genetic analysis demonstrated that the genetic diversity of the collection is comparable to that of the sample of wild plants from the Argentine Islands region, which was studied previously. Thus, created collection to some extent may represent populations from this part of maritime Antarctica.

In addition to the identification of morphological, anatomical, karyological, molecular cytogenetic, and molecular genetic characteristics, we conducted biochemical analysis and determined the composition of two main groups of biologically active substances, *i.e.,* phenolic compounds and flavonoids. Our results indicate the variation in the amounts of phenolic compounds and flavonoids contained in *D. antarctica* wild and *in vitro* grown plants of different genotypes that may be a manifestation of genetic variation at the species level, thus necessitating further biochemical studies and careful selection of plants for the use as a possible source of BAS. Furthermore, the antioxidant activity of extracts of *in vitro* plants was evaluated and was shown to be comparable to that of wild plants from the Antarctic.

The established collection of *D. antarctica* plants provides a valuable plant material with well-studied characteristics grown under controlled conditions to perform experimental research on the mechanisms for plant adaptation and resistance to various abiotic factors of the Antarctic environment. Furthermore, the development of the collection along with its comprehensive characterization make the baseline for further biotechnological developments focused on the use of Antarctic plants as a source of BAS, and could be used for therapeutic and prophylactic purposes in pharmaceutical and food industries, as well as in cosmetology. Furthermore, the obtained data also provide a baseline for genetic and biotechnological applications and can be useful for breeding cold tolerance in crops.

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