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DEVELOPMENT OF THE MALE AND FEMALE GAMETOPHYTE, FERTILIZATION, AND ASSESSMENT OF GERMINATION AND REGULATION OF DORMANCY IN *Iris aphylla* L. SEEDS

Magdalena Śmigała-Lasota[1]✉, Kinga Dziurka[2], Agnieszka Dąbrowska[3], Krystyna Winiarczyk[1]

[1] Institute of Biological Sciences, Maria Curie-Skłodowska University, Akademicka 19, 20-033 Lublin, Poland

[2] Department of Biotechnology, The Franciszek Górski Institute of Plant Physiology, Polish Academy of Sciences, Niezapominajek 21, 30-239 Kraków, Poland

[3] Botanical Garden, Maria Curie-Skłodowska University, Sławinkowska 3, 20-810 Lublin, Poland

ABSTRACT

The observations of fruiting and formation of offspring through generative propagation presented in this study were aimed at determination of propagation potential in *Iris aphylla* L. The low percentage of germinating seeds largely limits its generative reproduction. The germination tests confirmed deep dormancy of the *Iris aphylla* seeds. The dormancy was disrupted by isolation of embryos from seeds, which were further cultivated *in vitro*. The germination of isolated embryos proved the localization of endogenous inhibitors of germinating seeds of *I. aphylla* in the endosperm. The comparison of phytohormonal profiles of seeds, endosperm and embryos of *I. aphylla* and *Iris sibirica*, i.e. a species with a high percentage of germinating seed, revealed that the deep dormancy of *I. aphylla* seeds was associated with the balance between abscisic acid and gibberellins (ABA/GA), a significant predominance of ABA and a massive accumulation of salicylic acid. Isolation of embryos from seeds and their germination in growing medium *in vitro* is recommended as the most efficient method for *I. aphylla* propagation. Results of this study can be applied in conservation efforts of this attractive and important native species.

Key words: *Iris aphylla*, seeds, *in vitro,* regulation of dormancy, seed germination, phytohormones

INTRODUCTION

Iris aphylla L. is a valued ornamental plant with decorative properties and medicinal use. A breeding program of a new cultivar must take into account the fertility of hybrids, which determines reproductive success [Ustuner et al. 2022]. *In vitro* breeding is considered an alternative, quick method of propagation if propagation by seed is difficult. The embryo development is closely correlated with the development of the endosperm. Any disturbances in the latter process have an impact on the development and viability of the embryo which ultimately determines the success of reproduction [Lafon-Placette and Köhler 2014]. Therefore, three important structures: the embryo, the endosperm, and the testa should be considered in the analysis of seed germination.

Seed dormancy in plants of temperate areas may be an adaptive function preventing seed germination in unfavorable seasons. Various types of seed dormancy

✉ magdalena.smigala@gmail.com

have been described in Angiospermae, e.g. mechanical dormancy [Tillich 2003], physiological dormancy [Morgan 1990], and morphological or morphophysiological dormancy [Coops 1995]. Dormancy can be released by treatment of seeds with exogenous hormones, in particular gibberellins. It has been found that the levels of endogenous gibberellins and cytokinins are very high in young developing embryos, but their levels decline drastically in subsequent development stages [Bewley and Black 1994]. Release of seed dormancy and initiation of seed germination are determined by a balance between the embryo growth potential and the constraint exerted by covering layers, i.e., the testa and the endosperm. An important role in this process is played by interactions between abscisic acid (ABA), gibberellins (GA), ethylene, brassinosteroids (BR), auxins, and cytokinins in the regulation of interrelated molecular processes [Kucera et al. 2005]. It has been shown that the application of GA3 not only strongly stimulates embryo growth but also has a positive effect on the number of germinating seeds [Zhang et al. 2019]. The delay in seed germination in plants is associated with intolerance of shoots to sub-zero temperatures, which is one of the evolution aspects in many plant species.

The objective of this study was to establish determinants of the inhibition of the metabolic activity and growth of *I. aphylla* seeds. Seed germination and seedling growth rates are crucial in competition with other species for microhabitats in natural environment. Many studies indicate that the decline in the *I. aphylla* population in Europe results from low competition with other plants in natural habitats. Since *I. aphylla* is protected in many European countries, it is important to determine factors responsible for deep seed dormancy of this species and develop effective methods for release of the dormancy state [Turis et al. 2014]. In addition, *I. aphylla* is an attractive rhizomatous perennial plant with great economic importance given its use in the pharmaceutical industry [Rudall 1994].

MATERIAL AND METHODS

Study area. *Iris aphylla* flowering and fruiting were observed in 2016–2018 in the Botanical Garden of Maria Curie-Sklodowska University in Lublin, central-eastern Poland (51°14'37.2"N, 22°32'25.3"E; 197 m a.s.l.). *I. aphylla* was cultivated in four approximately 1 m^2 plots located at a distance of 15–100 m. The plants were grown in three sunny locations and one shaded site with brown loess soil (26% fraction < 0.02 mm) with humus content of 2.75–5.37% and pH 7.76–7.94. The climate of the Lublin region is characterized by the influence of continental air masses. The vegetation period in Lublin lasts for approximately 215 days [Woś 1999]. The mean annual temperature in the study years was 10°C, and the mean annual rainfall was 543 mm. Seeds of *Iris sibirica* L. growing in the Botanical Garden of Maria Curie-Skłodowska University in Lublin were used for comparative analyses of hormone levels.

Evaluation of the decorative value of *I. aphylla* flowers. Based on the valuation scale used in the cultivation of ornamental plants, an analysis of decorative value was carried out in three periods: in May, June and July.

Pollen grain germination. The germination of pollen grains was analyzed on a medium prepared as described by Brewbaker and Kwack [1963] with the addition of 10% sucrose. The germination rate was observed using a stereomicroscope for 30 min.

Seed germination *in vivo* and *in vitro*. The *in vivo* *I. aphylla* seed germination test was carried out in a greenhouse and under field conditions. The greenhouse cultivation was carried out using filter paper, sand, vermiculite, and garden soil substrates, and generally recommended seed treatments, i.e., scarification and stratification. Some seeds were soaked in a 3% gibberellin solution for three days. Next, 30 seeds were placed on each of the substrates. Under field conditions, the experiment was carried out using a mixture of universal and leaf soil supplemented with chalk limestone. The experiment was conducted for a year in controlled substrate moisture conditions. The germination of seeds was also tested *in vitro*. The seed surface was disinfected with 96% ethanol for 1 min followed by 5% calcium hypochlorite for 15 min and rinsed five times with sterile water. Disinfected seeds were scarified by piercing the testa using a sterile dissection needle. Seeds were placed on a sterile MS medium (Murashige and Skoog 1962) solidified with 6% agar. Seed germination was carried out in an air-conditioned chamber at a 16 h photoperiod, a temperature of 20°C, and 240 µmol m^2s^{-1} irradiance.

Seed viability test (according to International Seed Testing Association ISTA Regulations). Fifty fresh iris seeds were collected for analysis and soaked for 24 h in distilled water at room temperature. Next, the testa was removed from the seeds and placed in a 1% tetrazolium solution, pH 6.5, for 4 h. The tetrazolium assay facilitates discrimination between viable and non-viable embryo tissues. It is based on the activity of dehydrogenase enzymes used as an indicator of the respiration rate and seed viability. Dehydrogenases react with substrates and release hydrogen ions into a solution of oxidized colorless tetrazolium salt, which is then converted to red formazan. Seed viability is interpreted based on the intensity of embryo tissue staining.

Preparation of seeds for observations using the transmission electron microscope (TEM). The seeds were fixed in 2% (v/v) glutaraldehyde in 0.05 M phosphate buffer, pH 7.2 at room temperature for 1 h and rinsed three times with phosphate buffer. The specimens were post-fixed in 1% (w/v) OsO_4 for 10 min, rinsed three times in distilled water, and then dehydrated in ethanol at room temperature. For transmission electron microscopy, capsules in 100% ethanol were slowly infiltrated with LR White or Spurr's resin (Electron Microscopy Sciences, Hatfield, PA, USA) at 25% increments over 8 days. The specimens were sealed in airtight gelatin capsules (LR White) or Beem capsules (Spurr's) and cured at 60°C for 7 days (LR White) or 3 days (Spurr's). Ultrathin sections (70–100 nm) were cut using a diamond knife on a Leica ultramicrotome (Leica Microsystems, Wetzlar, Germany), collected on 200-mesh nickel grids, and allowed to dry at room temperature for 2 h. The finished preparations were analyzed using a ZEISS LEO 912 AB electron microscope.

Preparation of seeds for observations in the scanning electron microscope (SEM). The seeds intended for SEM observations was prepared with the method developed by Talbot and White [2013]. Fresh seeds was fixed in methanol for 10 min and then rinsed in ethanol for 2 × 30 min. Next, it was dried in a CO_2 atmosphere, gold sputter coated, and viewed in a LEO1430VP scanning electron microscope with an acceleration potential of 15 kV. Photographic documentation was made using INCA-Mapping software (Billerica, MA, USA).

Determination of the content of plant regulators. Samples of whole seeds, mechanically isolated embryos, and endosperm were prepared for the analysis. The material was treated with liquid nitrogen and lyophilized. Samples were homogenized in a mixing mill (MM400, Retch, Germany). Phytohormones were extracted from 20 mg of lyophilized and ground plant material in an aqueous solution of formic acid in methanol [Dobrev and Kaminek 2002]. The determination of phytohormones was carried out as described by Dziurka et al. [2016]. The samples were analyzed using an ultra-high performance liquid chromatograph (UHPLC, Agilent 1260) with a tandem quadrupole mass spectrometry detector (Agilent 6410). The Ascentis Express RP-Amide 2.7 µm, 2.1 mm × 150 mm analytical column (Supelco, USA) was used for the analysis. Phytohormones were detected in the positive ion mode after electrospray ionization (ESI) at atmospheric pressure. The MRM (multiple reaction monitoring) technique was used for product ion monitoring. Results were referenced to a pure standard calibration curve, taking into account the internal isotopic standards: [$^{15}N_4$] kinetin (K-N15), [$^2H_5$] indole-3-acetic acid (IAA-D5), [$^2H_4$] salicylic acid (SA-D4), [$^2H_6$] *cis*, *trans*-abscisic acid (ABA-D6).

Isolation and *in vitro* cultivation of embryos. The seeds were surface disinfected by application of 96% ethanol for 1 min, followed by a treatment with a 5% calcium hypochlorite solution for 15 min, and rinsing five times in sterile water. Disinfected seeds were scarified by piercing the testa with a sterile dissection needle. After two days, embryos were isolated from sterile seeds and placed on the MS medium. The embryo germination was evaluated one week after the isolation, and the percentage of emerging seedlings was calculated after 2 weeks.

Seeds from 2019 were placed in 8 dishes with 8 seeds in each (64 seeds). Embryos were isolated from half of the seeds and the other half served as a control. Seeds from 2017 and 2020 were placed on 4 plates with 8 seeds in each (32 seeds). As in the other variant, embryos were isolated from half of the batch, and the other half served as a control. The morphological and biomechanical features of iris seed capsules were presented by our research team previously [Śmigała et al. 2021].

RESULTS

Iris aphylla begins to grow in early spring (from the third decade of March to the second decade of April, depending on meteorological conditions). The full bloom phase takes place in May. Its fruits (capsules) are fully developed at the end of June. Seed maturity and the beginning of capsule cracking are noted in the "late summer" phenological period when most plants enter the fruiting phase. The level of generative reproduction is reflected in plant fertility expressed in the mean number of produced fruits and seeds. During the observation period, one plant usually produced from 1 to 3 capsules (exceptionally 4 or 5) with on average 35 seeds per capsule.

Evaluation of the decorative value of *I. aphylla* flowers. During the observation of the plant in the Botanical Garden, attention was paid to its attractiveness. Based on the rating scale, in May iris received the highest score of 4.7 for flower color, flowering date, flowering abundance and length (Fig.1A). In June, the color and shape of leaves, the uniformity of growth and the appearance of seed capsules were assessed (Fig. 1B) and the iris received 3.7 points for these features. During the fruiting period (July), the plants received the lowest score of 3.2. Iris flowers received a high rating and aroused the interest of visitors.

Gynoecium. The observations of the female gametophyte structure started in the full anthesis phase in flowers with a developed embryo sac in the ovules.

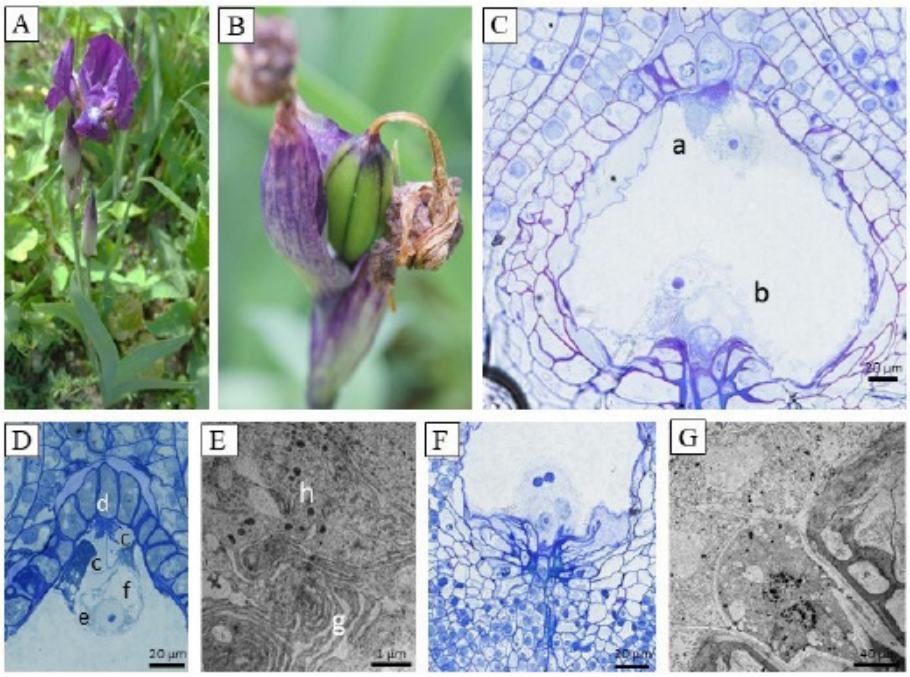

Fig. 1. A. *Iris aphylla* in May; B. seed capsule in June; C. longitudinal section of an ovule with a developed embryo sac: (a) egg apparatus with an egg cell with a large cell nucleus (arrow), (b) antipodes. Semi-thin slide stained with toluidine blue, LM; D. longitudinal section of the micropylar part of the ovule with a visible egg apparatus: (c) two synergids with (d) a highly stained filiform apparatus; below: (e) an egg cell with a nucleus at the chalazal pole and (f) a large vacuole in the micropylar part. Semi-thin slide stained with toluidine blue, LM; E. a fragment of the synergid cytoplasm with a branched system of endoplasmic reticulum membranes in the form of long linear systems of cisterns, (g) small vesicles, (h) small plastids. TEM; F. antipodes in the embryo sac on the eighth day after anthesis showing signs of cytoplasm degeneration and cell wall disintegration. Semi-thin slide stained with toluidine blue, LM; G. ultrastructure of one antipode in the anthesis stage with two cell nuclei. TEM [phot. by M. Śmigała-Lasota]

The mature ovule had a thin nucellus and was covered by a single-layered epidermis. The embryo sac was pear-shaped (wider in the chalazal part and narrower in the micropylar part). The longitudinal section showed its polarization in the chalazal-micropylar axis. The egg apparatus was located at the micropylar pole of the sac, the secondary nucleus was visible in the central part, and the antipodes were situated at the chalazal pole (Fig. 1C). The egg apparatus consisted of two synergids and a large egg cell. The egg cell had a large cell nucleus at the chalazal pole and a large vacuole on the micropylar side (Fig. 1D). The synergid cytoplasm contained numerous small vesicles and plastids as well as a branched system of endoplasmic reticulum membranes forming a system of long tubules, branched tubes, and vesicles. The filiform apparatus in the synergids formed a massive network of radially arranged tubular projections (Fig. 1E).

Three antipodal cells were located at the chalazal pole of the embryo sac. The cytoplasm of these cells in the anthesis stage was dense and had numerous small vacuoles. On the chalazal side, somatic ovule cells with thick walls strongly stained with toluidine blue adhered to the antipode. Occasionally, two cell nuclei were present in the antipodes (Fig. 1F). The embryo sac had partially located endosperm nuclei linked via cytoplasmic bands (Fig. 1G).

Androecium. The *I. aphylla* flowers had three large stamens adjacent to the lobate branches of the style. The three stamens developed simultaneously and had the same size. The anther did not change its size during the maturation of the perianth, whereas the filaments were light purple initially but later turned dark purple (Fig. 2A).

Iris aphylla pollen grains had a shape of a slightly flattened sphere and sizes ranging from 74.5 to 81.2 µm. They were heteropolar and unicolpate, and the colpus on the distal side was smooth, wide open, long, and rounded at the edges (Fig. 2B). The sporoderm layer covering the pollen grain had reticulate sculpture with lumina of various shapes and sizes (2–10 µm). The lumina exhibited the presence of circular sporopollenin structures. The ultrastructural analysis showed that the cytoplasm of the *I. aphylla* pollen grain was filled with starch grains and numerous lipid bodies. The male gametophyte was composed of two cells: a larger vegetative cell and a smaller generative cell. The nuclei of the vegetative and generative cells were surrounded by a strongly undulating nuclear membrane. The vegetative cell had dense cytoplasm and a large nucleus with a nucleolus (Fig. 2C). The pollen grain was surrounded by a layered cell wall.

The Alexander staining-based analysis of the viability of pollen grains was carried out to estimate the number of viable and degenerated pollen grains in the anther. The viability of the pollen grains varied in the study years. In 2019, there were 69% of smaller, green, non-viable grains, which were unable to germinate. In turn, 31% of the grains stained red, which indicated their viability. In 2020, 58% of pollen grains were viable and able to develop further, while 42% were non-viable. The first bulges in the pollen grain porus were observed after 3 min of incubation (length > 20 µm) – Figure 2D. Further growth of the pollen tube to a size of about 35 µm and accumulation of dense cytoplasm were observed over the next few minutes. In the 10th minute of incubation, the number of germinating pollen grains increased significantly from 28% to 46%. After 15 min of observation, 51% of pollen grains developed a 400-µm long pollen tube. Incubation for another 15 min did not enhance the intensity of this process. The rate of pollen tube growth on the medium was estimated at 26.7 µm/min. At 72 h post anthesis, the pollen tube was observed to penetrate into the ovule tissues through the micropylar canal (Fig. 2E). Three weeks after the anthesis, paraffin preparations showed a globular embryo surrounded by an amorphous endosperm tissue in the embryo sac. The embryo proper was composed of a group of small cells differing in their anatomy from the adjacent somatic cells of the ovule. These cells with strongly stained walls had dense cytoplasm arranged in regular layers with clearly stained cell nuclei (Fig. 2F).

Tetrazolium assays of seeds. On average, there were approx. 35 seeds in one *I. aphylla* capsule (Fig. 3A). A thick testa (350 ±29 µm) surrounded the endosperm with the centrally located embryo (Fig. 3B). The tests conducted in a sample of 20–40 seeds indicated 71% viability.

Seed germination in field and *in vitro* conditions. Treatments facilitating germination, i.e., soaking, scarification, and stratification, were applied to the seeds first. The application of all recommended pre-sowing treatments did not bring the expected results, as the

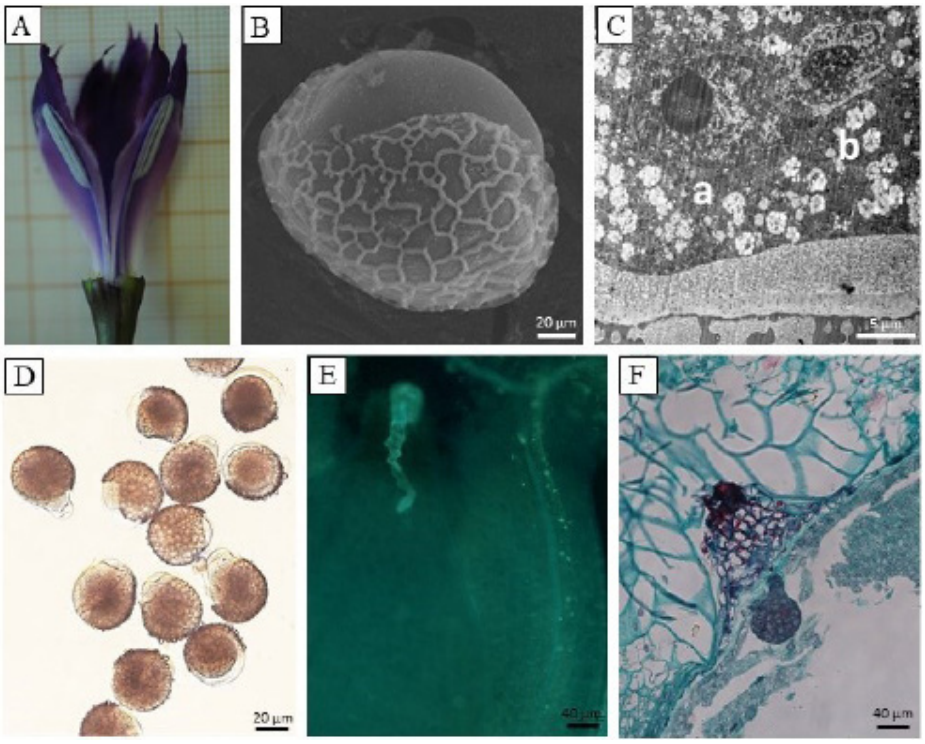

Fig. 2. A. stamens *Iris aphylla* adjacent to the lobate branches of the style; B. mature pollen grain. Equatorial view with reticular sculpture of the pollen grain wall with a large smooth colpus surface. SEM; C. fragment of the cytoplasm with visible vegetative (a) and generative (b) nuclei. TEM; D. germination of pollen grains after 3 min of incubation; E. growth of the pollen tube into the ovule tissues the through the micropylar canal. Image from a fluorescence microscope. Preparation stained with aniline blue; F. globular embryo three weeks after anthesis. At the micropyle of the embryo sac, a group of small cells with strongly stained cell walls is visible, and amorphous endosperm is visible inside the sac. Paraffin preparation stained with safranin and light green. LM [phot. by M. Śmigała-Lasota]

I. aphylla seeds did not germinate in the greenhouse conditions, and only 18% of seeds sown in the field germinated after 15 months (Tab. 1).

In the next stage of the study, whole *I. aphylla* seeds and embryos isolated in sterile conditions were transferred onto MS medium plates. Three weeks after the establishment of the culture, all the embryos developed into young plants (Fig. 3C). After an approximately two-month acclimatization process, the plants were transplanted into pots filled with garden soil (Fig. 3D). In June 2020, the plants were transferred to plots in the UMCS Botanical Garden. Plants obtained *in vitro* with isolated embryos developed properly and were suitable for additional cultivation.

The whole seeds (not devoid of the testa and endosperm) placed on the medium did not germinate, which suggested that the inhibitor of seed germination was contained in the endosperm. Since the whole *I. aphylla* seeds did not germinate on the medium, the next stage of the study involved analysis of endogenous phytohormones contained in the seeds. *Iris sibirica* seeds, which are characterized by an 87% germination rate, were used as a control in this stage of the study. The presence of phytohormones in whole seeds, embryos, and endosperm was analyzed.

The hierarchical tree diagram shows the percentage content of groups of phytohormones: auxins, cytokinins, gibberellins, and abscisic and jasmonic acids

Table 1. Germination of *Iris aphylla* seeds in greenhouse and field conditions

Seed collection date	Sowing date	Cultivation site	Substrate	Germination date	Germination percentage
2019	May 2020	greenhouse	filter paper	no germination	0%
			sand	no germination	0%
			vermiculite	no germination	0%
			garden soil	no germination	0%
2020	April 2021	field	soil mixed with chalk limestone	July 2022	18%

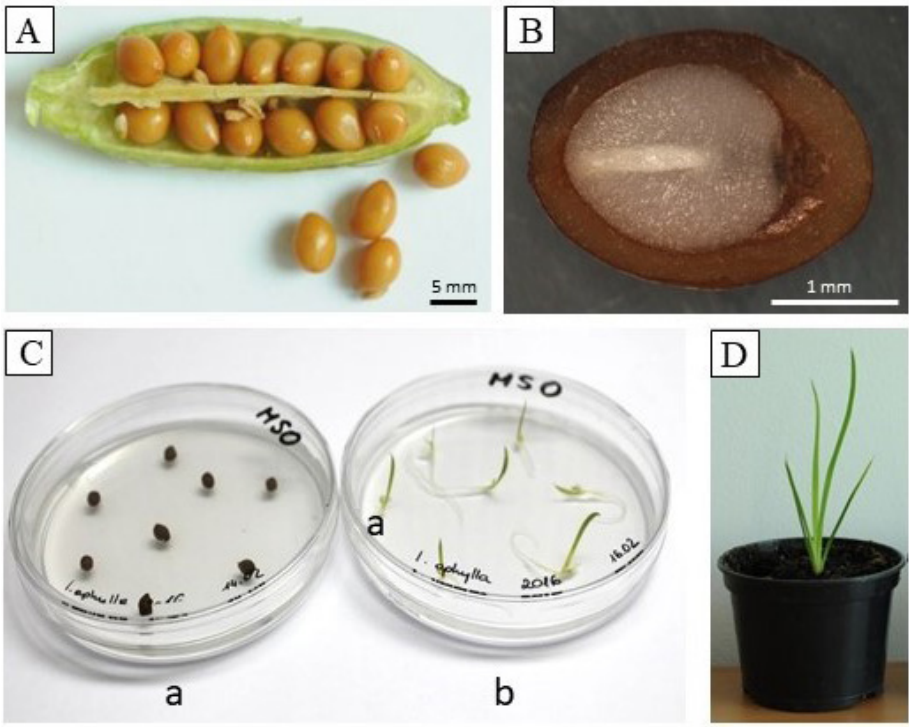

Fig. 3. A. structure of an *I. aphylla* fruit: ripe seeds before rupture of the seed capsule; B. longitudinal section through a seed with the embryo located centrally in the endosperm and a thick seed coat (testa); C. whole seeds on MS medium after three weeks of cultivation (a), dissected seed embryos on MS medium after three weeks of cultivation (b); D. young iris plants in laboratory conditions [phot. by M. Śmigała-Lasota]

in the total pool (Fig. 4). The levels of accumulation of the groups of phytohormones varied depending on the species and the element of the seed structure. Both the seeds and the endosperm of *I. aphylla* were characterized by similar percent content of the phytohormone groups. In turn, the *I. sibirica* seeds exhibited greater similarity to the embryos than to the endosperm in terms of the percent content of the groups of phytohormones. In the *I. sibirica* endosperm, gibberellins were the most abundant active phytohormones. In addition to salicylic and benzoic acids, the embryos of both species mainly accumulated active forms of

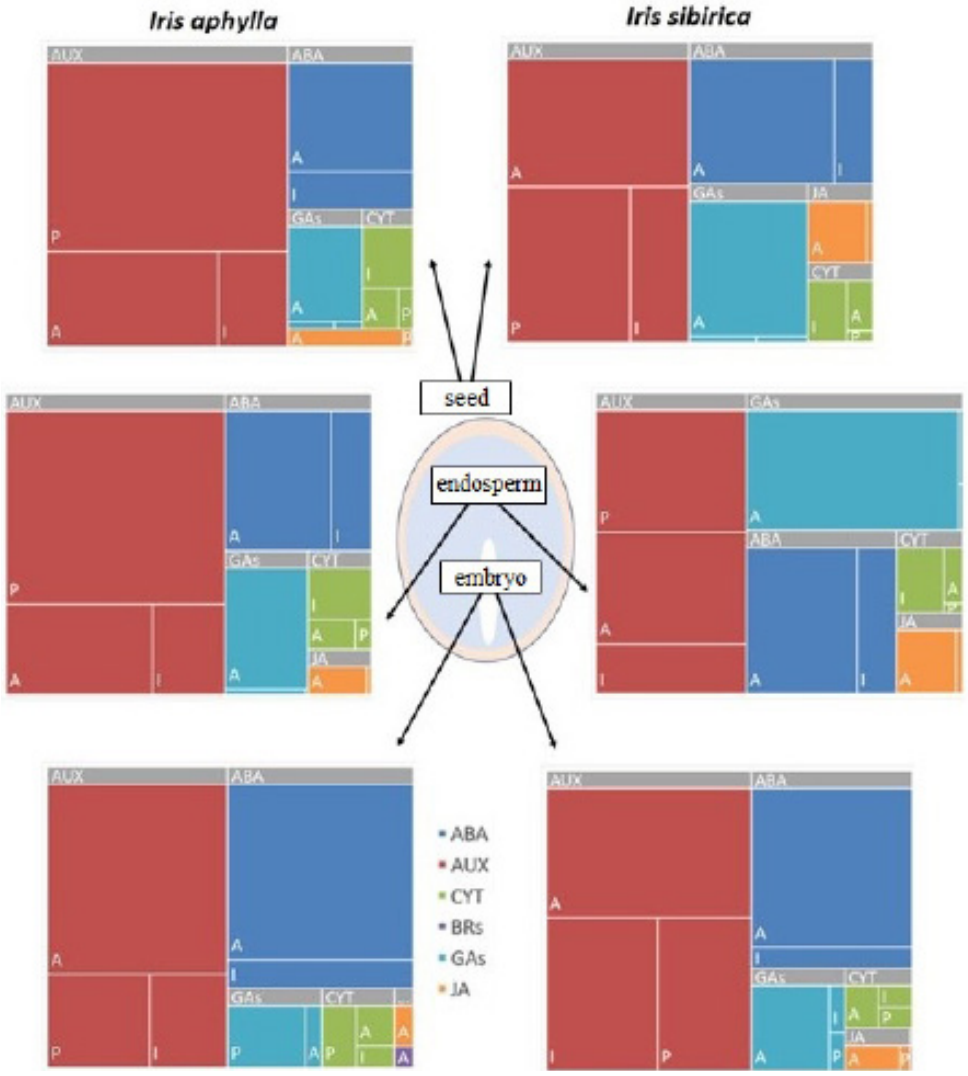

Fig. 4. Hierarchical tree map of phytohormone content in the whole seed, endosperm, and embryo of *I. aphylla* and *I. sibirica*. The size of individual rectangles reflects the level of accumulation of a given group of phytohormones (AUX – auxins, ABA – abscisic acid, GAs – gibberellins, CYT – cytokinins, JA – jasmonic acid, BRs – brassinosteroids marked with colors), active (A), precursor (P), and inactivated (I) [prepared by K. Dziurka]

auxins and abscisic acid. The analysis of the content of individual phytohormones in the material revealed that the *I. aphylla* embryos exhibited lower contents of most of the determined cytokinins, auxins, gibberellins, and abscisic and jasmonic acids and simultaneous seven-fold higher content of salicylic acid than the *I. sibirica* embryos (42,63 and 5,95 pmol/embryo, respectively). In turn, the endosperm and seeds of *I. aphylla* contained eight- and ten-fold higher levels of salicylic acid, respectively, compared to *I. sibirica*. They also accumulated similar amounts of active cytokinins (t-Z, c-Z, DH-Z, K), similar or lower amounts of most gibberellins (except GA1 and GA3), and lower amounts of active auxins (IAA, IAA-Met) as well as abscisic and jasmonic acids. In comparison with *I. sibirica*, the *I. aphylla* whole seeds and endosperm

accumulated two-fold larger amounts of the precursors of auxin I3CA and gibberellin GA5 and several-fold higher levels of cytokinin precursor forms (DH-Z-R, IPD, t-Z-R, K-R).

DISCUSSION

The double fertilization process takes place at different rates in different taxonomic groups. Studies conducted in various Iridaceae species have shown that fertilization occurs 16 h after pollination in *Iris mandshurica* Maxim., 24 h in *Iris sanguinea* Donn ex Hornem, and 50 h in *Iris bloudowii* Bunge [Fan et al. 2019]. Both the male and female gametophytes in *I. aphylla* were fully developed in the full anthesis stage. The microgametophyte, i.e., the pollen grain, consisted of three cells: a vegetative cell and two sperm cells. As shown by the assays, the viability of the pollen ranged from 58 to 69%. The *I. aphylla* megagametophyte development corresponded to the Polygonum type, which was also described in other species of the genus *Iris*, e.g., *Iris tanax* Douglas [Riley 1942]. There were fundamental differences in the arrangement of elements in the filiform apparatus of the *I. aphylla* embryo sac in comparison with this structure described in *I. tanax* [Wilson 2001]. As reported by the authors, the apparatus in *I. tanax* is an unstable structure appearing in the late flowering phase. The filiform apparatus in this species developed immediately before or at the time of degeneration and disappearance of synergids after pollination [Pande and Singh 1981]. These findings differ essentially from our data, as we documented that the filiform apparatus in *I. aphylla* appeared as early as on the day of flowering and persisted until 14 days post anthesis. The filiform apparatus in the synergids formed a large network of radially arranged tubular projections with a heterogeneous structure visible in the cross section. The role of the filiform apparatus is to transport sperm cells to the egg cell and the central cell. Thus, our observations indicate quite a long time of functioning of this structure in *I. aphylla*, which ensures readiness of the embryo sac to accept the genetic material of the other partner in the generative reproduction process. The penetration of the pollen tube into ovule tissues through the micropylar canal was observed after 72 h post anthesis. Three weeks after anthesis, a globular embryo surrounded by an amorphous endosperm was present in the embryo sac. The description of structures involved in the double fertilization process and the embryo development rate in *I. aphylla* has been described in this study for the first time.

The embryo development is preceded by earlier development of the endosperm, which contains reserve substances and phytohormones regulating the metabolic activity of seeds. Endosperm hydrolysis products and phytohormones are transported to the developing embryo. This is accompanied by cessation of the action of auxins and gibberellins and increased activity of abscisic acid, which slows down metabolism in seeds and induces seed dormancy [Goyal et al. 2005]. Seed dormancy in plants growing in temperate areas may be an adaptive function preventing seed germination in unfavorable seasons [Crocker and Barton 1953].

Methods recommended for breaking dormancy in irises involve different types of stratification: cold, hot [Morgan 1990], chemical [Sun et al. 2006], and physical [Blumenthal et al., 1986]. One of the oldest and most commonly used methods for breaking seed dormancy is stratification, i.e., storage of seeds in moist conditions at low temperatures followed by exposure thereof to high temperatures. *Iris versicolor* seeds responded positively to exposure to a temperature range of 33–50°C for 75 days. Germination of *I. tenax* and *Iris virginica* L. seeds was achieved through application of both types of stratification breaking their deep morphophysiological dormancy [Morgan 1990, Jones and Kaye 2014].

The *I. aphylla* seeds had a normal structure, and the tetrazolium assay showed a high degree of their viability. Nevertheless, the seeds germinated at a very low level of several percent. Literature data indicate serious problems with seed germination in many species of the genus *Iris*, hence the decline in the populations of these species in natural habitats [Arditti and Pray 1969]. As a part of the study, we applied all treatments recommended in the literature to break the dormancy of *I. aphylla* seeds. Unfortunately, no treatment brought the expected effect, and the percentage of germinating seeds was very low. However, we obtained positive results through isolation and transfer of embryos to the *in vitro* culture medium. After 2 weeks of the experiment, regeneration was observed in 100%

of the embryos, and all embryos underwent further development. After several months of acclimatization, young plants were transferred to the greenhouse and then to Botanical Garden flower beds. This result of the experiment clearly suggests that the germination of *I. aphylla* seeds is blocked by inhibitors located in the endosperm, as the removal of this element ensured further development of the embryos without any disturbances. The endosperm is a reservoir of reserve substances for the developing embryo. It contains substances that control its physiological state; they are responsible for the resting period length and regulate the time of the dormant state. Therefore, it can be confirmed that the endosperm not only nourishes the embryo but also controls the physiology of the seed through endosperm-embryo interactions [Lafon-Placette and Köhler 2014].

Germination of seeds of flowering plants, especially seeds obtained as a result of hybridization, is sometimes problematic. For example, obtaining new varieties of olives (*Olea europaea* L.) is very difficult due to the low percentage of seed germination. In this species, *in vitro* culture of embryos is used. This method can significantly reduce the time needed to carry out the initial assessment of breeding progeny. Our results indicate that *in vitro* cultures of isolated embryos may increase the efficiency of germination and developing new varieties of flowering plants in breeding programs [Acebedo at al. 1997].

It has been shown that ABA is a positively correlated with dormancy induction and a negatively correlated with germination. In turn, GA breaks seed dormancy and supports germination, thereby abolishing the effect of ABA. A similar effect is exerted by ethylene and brassinosteroids (BR), which not only support seed germination but also counteract the impact of ABA [Kucera et al. 2005]. To detect compounds responsible for *I. aphylla* seed dormancy, we conducted an analysis of phytohormonal profiles. For comparative studies, we used *I. sibirica*, as its seeds do not exhibit problems with germination. We found differences in the content of phytohormones between *I. aphylla* and *I. sibirica* embryos, indicating different physiological states of seeds from which they were isolated. However, these differences are not crucial for the ability to germinate, as evidenced by the successful *in vitro* germination of *I. aphylla* embryos isolated on agar medium without the addition of growth regulators. Therefore, the causes of the inhibition of *I. aphylla* seed germination might be primarily associated with the endosperm. The balance between ABA and GA is the most important determinant of seed dormancy and germination [Tuan et al. 2018]. Disruption of dormancy and resulting seed germination are accompanied by increased GA synthesis. Gibberellins in endosperm aleurone cells induce the expression of the α-amylase gene, thus activating storage materials accumulated in the endosperm [Zhang et al. 2004]. We showed that, regardless of the seed component of *I. aphylla* (seed, endosperm, embryo), the level of abscisic acid was substantially higher than that of gibberellins. In turn, in the seeds and especially in the endosperm of *I. sibirica*, the ABA/GA balance was shifted in favor of GA, in comparison with *I. aphylla*. Active GA_4 was the most abundant gibberellin determined in the *I. sibirica* seeds and endosperm. Its content was three-fold higher than in the seeds and endosperm of *I. aphylla*. Its level in the embryos was thousand-fold higher. Similarly, Li et al. [2016] reported that the amount of bioactive gibberellin GA_4 was greater in brown seeds of *Suaeda salsa* (L.) Pall., which germinate at a higher rate than black seeds of this species. Based on the present results and literature data, we believe that a high ABA/GA ratio in the endosperm is the main cause of the poor germination of *I. aphylla* seeds. An additional factor that inhibits *I. aphylla* seed germination is the accumulation of salicylic acid. As shown by Xie et al. [2007], SA inhibited the expression of the α-amylase gene (an enzyme responsible for starch decomposition in the endosperm) induced by GA, and thus had a negative impact on the germination of barley seeds.

The readiness for germination of *I. sibirica* seeds is associated with a shift in the ABA/GA balance in favor of GA. Additionally, we observed a higher concentration of jasmonic acid in *I. sibirica* tissues than in *I. aphylla*. As reported by Nguyen et al. [2022], although JA is not a key regulator of the germination process, it is involved in the process of dormancy breaking through changes in the ABA/GA balance. Disruption of dormancy in *Triticum aestivum* (L.) cv. AC Domain seeds was associated with increased expression of the gene encoding a hydrolase enzyme which releases IAA from conjugates and with higher

levels of IAA in seeds during imbibition [Liu et al. 2013]. We detected higher content of both active IAA and its conjugates (IAA-ASA, IAA-GLUT) in the whole seeds, endosperm, and embryos of *I. sibirica* in comparison with *I. aphylla*. These differences were particularly visible in the embryos, which exhibited thirty-fold higher content of IAA and fifteen-fold higher content of conjugates.

CONCLUSION

The structure of the male and female gametophyte and the analysis of the fertilization process in *I. aphylla* were typical and did not indicate any problems with further development of the new iris generation. The lack of germination of properly formed seeds in natural conditions indicated physiological disorders. The examination of the phytohormonal profile of the seeds allowed to indicate the location of endogenous germination inhibitors in the endosperm of the iris, which effectively prevents its reproduction and places it in the classification of a plant threatened with extinction.

Based on results of this study, it can be concluded that isolation of embryos from seeds and further cultivation *in vitro* is the most effective method of propagation of *I. aphylla*. This recommendation seems reasonable since the occurrence of this species in Europe is seriously endangered. It is important to take action to protect it.

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