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POLLEN VIABILITY AND TISSUE CULTURE INITIATION OF *Salix lapponum*, AN ENDANGERED SPECIES IN POLAND

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Abstract. The main aim of the study was to investigate the viability of pollen of a boreal relict, *Salix lapponum*, an endangered species in Poland as well as to evaluate the possibility to introduce the plant into a tissue culture. Two approaches were taken to estimate pollen viability: staining pollen with dyes and *in vitro* germination assay. The study results showed high pollen viability of the species, with regard to both fresh pollen and pollen stored for 12 months. The effectiveness of pollen tube growth was also found to be largely dependent on glucose content in a medium and thermal conditions. The study results have provided necessary information on the most optimal combinations for pollen germination of boreal willow in artificial conditions. The research on tissue culture initiation of *S. lapponum* was also undertaken. The most contamination free explants were obtained, when the shoot pieces had been soaked in a solution of fungicides, followed by the dip in 70% alcohol and surface disinfected in 2% NaOCl for 30 minutes. The most good quality shoots were obtained on the MS media supplemented with 0.1 mg·dm^{-3} BA and 0.01 mg·dm^{-3} IBA.

Key words: pollen viability, pollen germinability, seeds germination, shoot explants

INTRODUCTION

A subarctic willow *Salix lapponum* (a downy willow) is a small shrub, growing up to 1–2 m. It is a dioecious plant, primarily entomophilous and partially anemophilous. It has the potential for clonal as well as sexual reproduction. Its small seeds are characteristically enveloped in long, soft, silky hairs and dispersed by wind [Kruszelnicki 2001, Rutkowski 2006].

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S. lapponum grows separately or in small clusters in lowlands and submontane transitional peat bogs, in areas with significant hydration and a small amount of other shrubs. Everywhere it is associated with the occurrence of acidic peat soil [Kruszelnicki 2001].

In the face of the threat of extinction of *S. lapponum* in Poland, a multifaceted research was undertaken to show the most effective *in situ* and *ex situ* propagation possibilities, as one of the methods to preserve the species could be artificial replenishment of populations. The main problem to be solved is the origin of individuals to be introduced (obtained by *in vitro* fertilisation or by classical vegetative propagation methods). This study attempted to answer a number of questions on conditions for the effective pollen germination of *S. lapponum,* as well as asexual reproduction in tissue culture. In order to confirm that it is possible to ensure a proper sexual cycle and to exclude the probability of producing seeds only by pollination with foreign pollen (the studied species may potentially produce interspecific hybrids with *S. rosmarinifolia* and *S. cinerea* as well as with the species found in their natural stands in the study area) [Pogorzelec et al. 2014], laboratory methods were used to determine the viability of pollen grains of *S. lapponum*. The aim of the study was to verify the germination capacity of fresh and stored pollen that could also be used, if necessary, for *in vitro* fertilisation.

There are currently many laboratory methods used to verify the correctness of the processes of androgenesis and development of the male gametophyte which may be used for *in vitro* culture [Shivanna et al. 1991, Rodriguez-Riano and Dafni 2000, Kelly et al. 2002]. However, the main problem is the selection of appropriate parameters of physico-chemical factors that provide optimal conditions for the above-mentioned processes. Undoubtedly, one of the most important factor is the chemical composition of the medium, as it may have an enormous effect on pollen cell metabolism, while the second one, as shown by the research, may be the temperature at which the culture is carried out.

The other way to reproduce *S. lapponum* might be micropropagation. The tissue culture has been widely used to propagate different *Salix* species [Amo-Marco and Lledo 1996, Lyyra et al. 2006, Park et al. 2008]. There was only one research undertaken on micropropagation of *S. lapponum* and it concerned the specimens growing in a mountainous area [Skalova et al. 2012]. Micropropagation has been widely applied for propagation of different rare and endangered plants [Marszał-Jagacka et al. 2005, Sharma et al. 2008, Holobiuc et al. 2009]. It is especially useful when the plant taxa cannot be propagated by conventional methods or where there is a very low number of parent plants [Bunn et al. 2007].

The present study attempted to determine what conditions (medium composition, temperature, lighting) are the most optimal for growth of pollen tubes and initiation of *S. lapponum* shoots in tissue culture.

MATERIALS AND METHODS

The *S. lapponum* population existing in the peat bog on Lake Bikcze, located in the south-eastern part of the Łęczna-Włodawa Lakeland in the Polesie Lubelskie region (51°22' N longitude and 23°03' E latitude), was chosen for the research due to its size (more than 100 individuals – the largest population within the study area of E-S Po-

land), their optimal sexual structure (the ratio of male to female individuals nearly 1:1), and a large proportion of flowering individuals.

The pollen, which was used for research, was collected at full flowering (April 2014, air temperature 10–15°C). Two inflorescences (catkins) from every 25 male individuals of *S. lapponum* were sampled and put in a numbered paper bags. Half of the grains were used for analyses directly after collection (fresh pollen) and the other half was put for storage in the paper bags, in the temperature of 23°C for one year (stored pollen).

In the laboratory conditions, slide preperations, stained with 2% acetocarmine solution, were prepared [Ruebenbauer and Muller 1985, Nassar et al. 2000, Lyra et al. 2011] and after 1h they were analysed with the use of light microscopy. Fully stained pollen grains filled with cytoplasm were considered as viable, whereas unstained pollen grains or pollen grains only partially filled with cytoplasm were considered as non-viable. Pollen grains (300) were analysed from each of the 25 specimens of *S. lapponum*. The investigations were repeated in the year 2015 on stored pollen.

The germination test of fresh and stored pollen grains was performed on microscopic slides with 1% agar medium containing 1, 2.5, 5 or 7.5% glucose solution and the addition of 0.001% boric acid [Diaz and Garay 2007, Asma 2008, Beyhan and Serdar 2008], which were placed on glass rods in Petri dishes filled with filter paper soaked with distilled water. Four dishes with four slides with the same glucose concentration were placed in different temperature conditions (at room temperature ca. 23 and at 11°C – refrigerator). Additionally, one dish from each temperature was covered to prevent light. The slides were analysed under a light microscope and pollen grains that had germinated were counted in a given field of view. Additionally, a total of 400 pollen grains (4 × 100) were counted on each slide and the percentage of pollen grains with germinated pollen tubes was determined. Pollen grains with tubes of the same or greater length than their own diameter were considered to have germinated [according to Báez et al. 2002]. Observations were made after 3, 24 and 72 h.

The plant material for *in vitro* culture initiation were seeds and shoots fragments of *S. lapponum* obtained from mother plants growing in natural stands. The explants were surface sterilized with two methods (tab. 1) and placed individually in test tubes containing 10 ml of Murashige and Skoog (MS) [1962] mineral salts supplemented with thiamine 0.4 $mg \cdot dm^{-3}$, pyridoxine 0.5 $mg \cdot dm^{-3}$, nicotinic acid 0.5 $mg \cdot dm^{-3}$, glycine 2 $mg \cdot dm^{-3}$, myo-inositol 100 $mg \cdot dm^{-3}$, sucrose 30 $g \cdot dm^{-3}$. Shoot pieces were placed on the medium gelled with agar 6.75 $g \cdot dm^{-3}$, while seeds were placed on the medium gelled with the same amount of agar or on the surface of 3 layers of filter paper soaked in a liquid medium. The media were supplemented with the following growth regulators (in $mg \cdot dm^{-3}$): BA 0.1 + IBA 0.01, BA 1 + IBA 0.1, KIN 2 + IAA 0.2, 2iP 2 + IAA 0.2 (for shoots explants) and BA 0.1 + IBA 0.01 (for seeds). The medium pH was adjusted to 5.7 before autoclaving. The cultures were maintained in a culture room at a temperature of 22°C during the day and 20°C at night with 16-h photoperiod and irradiance of 35 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

The effectiveness of surface sterilization was also evaluated in relation to concentration of NaOCl (2 or 3%) and duration of its application (20 or 30 min).

The following features were evaluated during the research: a survival rate, a contamination rate and a number of regenerating shoots. All shoots with symptoms of contamination were removed, while sprouting shoots were placed on the fresh media to produce plant stock for further experiments.

Table 1. Disinfection methods of *Salix lapponum* explants

Successive treatments	Treatment description	Method I	Method II
1.	soaking in solution of Amistar (1 g·dm^{-3}) and Previcur (1 g·dm^{-3}) for 30 minutes		X
2.	rinsing in water with detergent 3 times × 20 minutes	X	X
3.	dipping in 70% ethanol alcohole for 5 seconds	X	X
4.	shaking in the solution of NaOCl (0.5% of chlorine) for 30 minutes	X	X
5.	rinsing in sterile distilled water for 3 times	X	X

Statistical analysis was performed for the pollen viability and germination capacity using three-way analysis of variance followed by Tukey's multiple comparisons. All computations were carried out in R environment, version 2.15.3 (2013-03-01).

RESULTS AND DISCUSSION

Pollen grain germination and pollen tube growth are very important processes in research on the morphology, physiology, ecology, biochemistry, and genetics of various plant species, primarily commercial plants. Pollen viability and germination capacity can be used as indicators of the correctness of sexual processes, but also of adaptive abilities of plants [Śnieżko 1991, Tangmitcharoen and Owens 1997, Bolat and Pirlak 1999, Báez et al. 2002, Dane et al. 2004, Khan and Perveen 2008, Skalova et al. 2010].

The required environment for pollen germination *in vitro* is related to the genetic factors and also to the quality and quantity of nutrient reserves of pollen, therefore the type of media (especially the sugar content) used for pollen germination depends on plant species [Baker and Baker 1979].

The investigation of the viability of pollen grains showed more than 90% proportion of viable pollen grains, both in fresh *S. lapponum* pollen (97.28% ±3.82) and in the samples of pollen grains stored for 12 months (94.84% ±4.62). Pollen stored for a certain period of time (depending on species and conditions) loses its viability in many cases. The reason for that is pollen desiccation, the use of reserve substances, and inactivation of enzymes responsible for the metabolic processes necessary for germination under both *in vivo* and *in vitro* conditions [Jaranowski 1965]. However, some data available in the literature refer to cases where stored pollen grains of different species do not lose their germinability [Shivanna et al. 199] even after 13-year storage [Sparks and Yates 2002]. The condition of storage, date of pollen collection, physiological and genetic factors influence the success of stored pollen germination. Temperature and ambient humidity during pollen storage have probably the most significant effect on pollen viability [Khan and Perveen 2008].

The pollen-tube germination test (*in vitro*) performed on fresh and stored *S. lapponum* pollen showed variations in germination effectiveness depending on the medium parameters as well as on temperature and light conditions.

The data analysis showed that the medium with 2.5% glucose solution deposited at 11°C, with the presence of light, was found (on the basis of the highest number of pollen tubes observed in the slides) to be the most beneficial combination of the examined

factors for the growth of pollen tubes in fresh *S. lapponum* pollen (phot. 1). The medium with 5% glucose solution deposited at 11°C in the dark proved to be optimal for pollen tube germination of stored pollen (fig. 1). In all experimental treatments, the highest amount of pollen grains in question germinated during the first three hours from the beginning of the experiment (both fresh and stored pollen). In the next time intervals (3–24, 24–72 h), the number of new pollen tubes that emerged decreased.

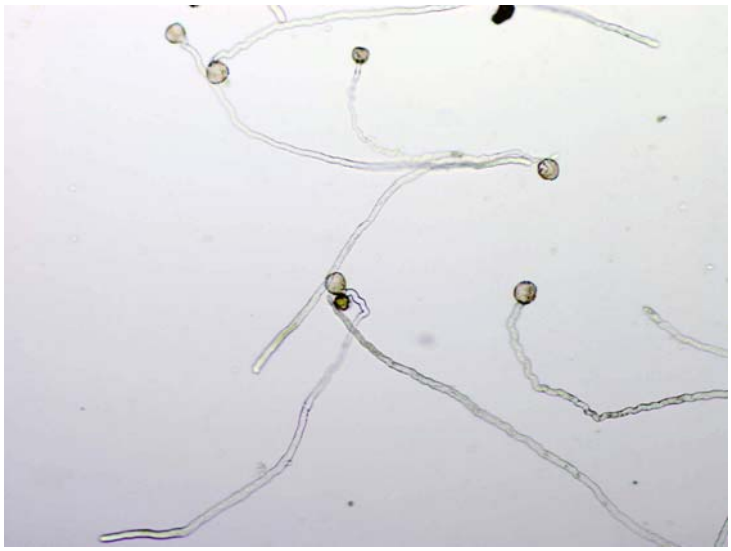

Photo 1. *Salix lapponum* pollen grains germinated in the medium supplemented with 2.5% of glucose solution, deposited at 11°C, in presence of light

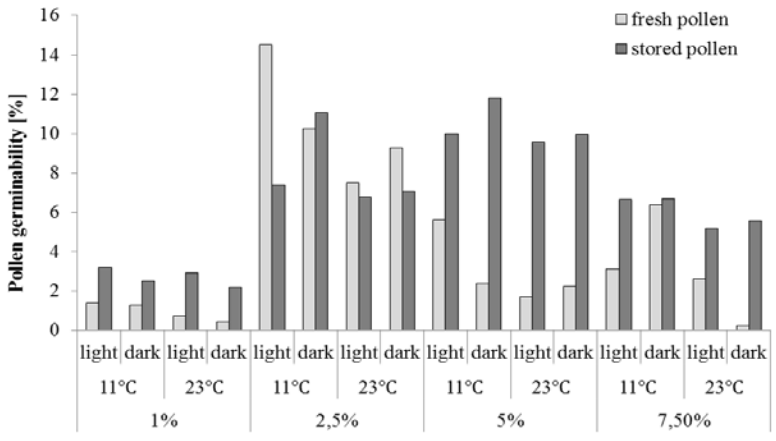

Fig. 1. A comparison of *S. lapponum* pollen germinability under various experimental conditions

In the present study, a relatively low sugar content in the medium was used (from 1 to

7.5%), and the study results revealed varying glucose demand for the development of pollen tubes in fresh and stored pollen. Thereby, this study confirmed the thesis presented by Jaranowski that the older pollen is, the higher the sugar concentration must be to stimulate germination. In case of *S. lapponum*, stored pollen germinated more effectively in a medium with higher concentration of glucose than fresh pollen.

The pollen germinability of vascular plants in artificial conditions have been usually studied regarding the sugar content in media. 5, 10, 15, 20, and even 25 up to 40% sugar solutions in the medium have been used most frequently. Most research results have confirmed a clear relationship between the amount of sugar and effectiveness of pollen tube germination under *in vitro* culture conditions in case of various plant species. The optimal sugar content in media has been found to be species-specific [Bolat and Pirlak 1999, Dane et al. 2004, Beyhan and Serdar 2008, Lyra et al. 2011].

The results of the study showed that not only the sugar concentration in the medium, but also temperature influence effective pollen tube germination. A relatively low ambient temperature had a positive effect on the number of germinating pollen grains of *S. lapponum.* The tendency for activity of cultured pollen grains in low temperature is probably reflected in the conditions in which this process takes place in natural stands. *S. lapponum* is a species which, in the centre of its range, flowers and produces seeds during the period from June to July, whereas in the conditions of Poland from March to May when air temperature in its natural stands, peat-bog ecosystems, does not usually exceed 20 and 15^{o}C during the full flowering period (at the turn of March and April). A dependence of the studied phases of the reproductive cycle and the impact of environmental factors can also be clearly seen in this case.

The pollen germination was also tested under crossed combinations of three treatments in different conditions of light and temperature as well as various levels of glucose concentration. Two separate data sets for each species were subjected to three-way ANOVA and Tukey's post hoc HSD tests. Double arcsine transformation was applied to the germination percentages to normalize the data (tab. 2). All significant values are reported at $p < 0.05$ (figs 2–3).

The ANOVA test detected significant variations between temperature conditions and glucose levels both for fresh and stored pollen. The effects of light conditions were significant only for fresh pollen. The ANOVA test revealed a significant glucose-temperature interaction and a three-way interaction for fresh pollen. The other interaction factors were found to be statistically insignificant (fig. 2). In total, Tukey's post-hoc analysis shows that means for levels of glucose differed significantly, except for the pairwise comparison 5–7.5% for fresh pollen.

Tukey's comparison for fresh pollen concludes that the mean germination percentage for the 2.5% level is significantly higher than the means associated with the 5 and 7.5% levels. By contrast, the 5% glucose concentration results in significantly the highest germination percentage for stored pollen.

Post hoc results of Tukey's HSD test for comparing the means for crossed combinations between glucose and temperature levels (c = cold 11°C, h + hot 23°C) are shown in Fig. 3. Germination of fresh pollen in the treatment combination 2.5%:c (glucose concentration: temperature) proved to be significantly greater than those for any other treatments. Further, the combinations 5% : c, 5% : h, 2.5% : c appeared to be the best

treatment conditions for germinability of stored pollen. These treatment combinations were found to be not significantly different from each other.

Table 2. The ANOVA table (df – degrees of freedom, F statistic and p-value) for *S. lapponum* pollen germinability

Source	df	*Salix lapponum*			
		fresh pollen		stored pollen	
		F	Pr (> F)	F	Pr (> F)
G	3	171.231	< 2e–16 ***	68.705	< 2e–16 ***
L	1	6.897	0.00919 **	0.925	0.33716
Temp	1	108.359	< 2e–16 ***	8.912	0.00313 **
G : L	3	0.318	0.81246	1.672	0.17367
G : temp	3	5.001	0.00221 **	0.742	0.52793
L : temp	1	0.032	0.85872	1.278	0.25935
G : L : temp	3	19.629	2.05e–11 ***	0.832	0.47745
Residuals	240	–	–	–	–

Signif. codes: *** p<0.001; ** p<0.01
G – glucose solution, L – lighting conditions, Temp – temperature

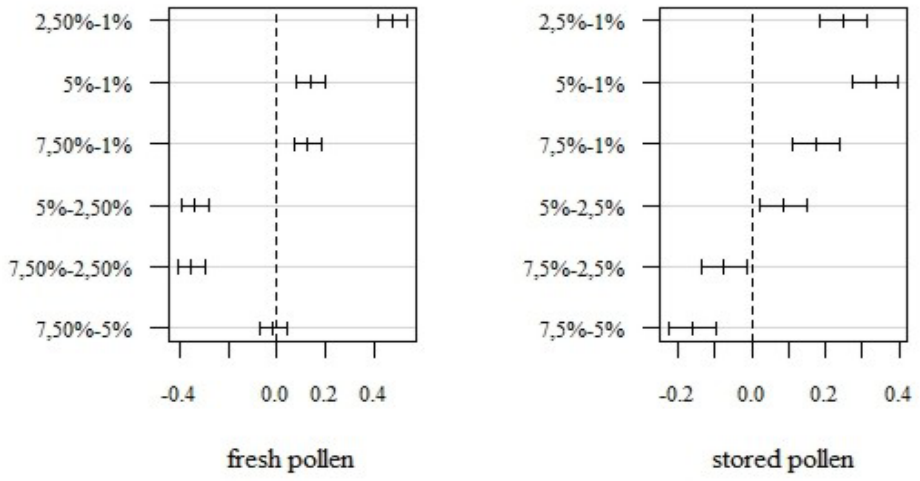

Fig. 2. 95% confidence intervals for the differences between the means of glucose solution levels for the *S. lapponum* pollen germinability data. Pairwise comparisons are pictured at a vertical position on the left hand axis. The corresponding interval estimates are marked with horizontal lines. Comparisons having intervals that do not overlap the vertical dashed line $x = 0$ are found significantly different

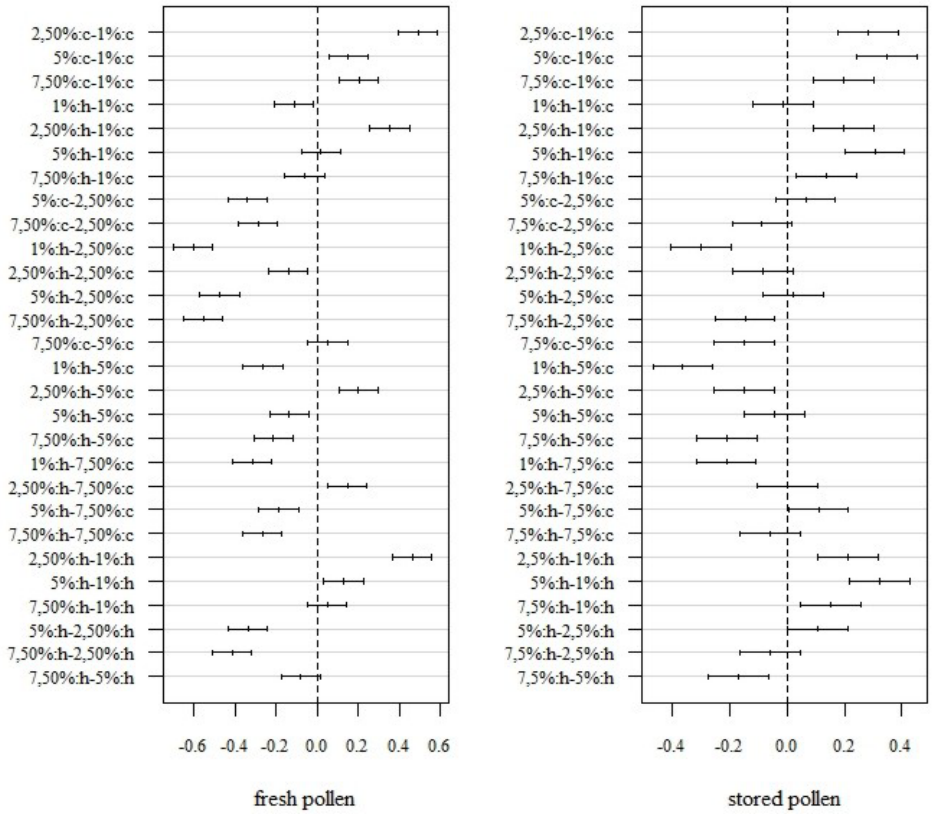

Fig. 3. Graphic representation of Tukey's HSD comparison results for the *S. lapponum* pollen germinability data for the interaction of glucose solution (1, 2.5, 5, 7.5%) and temperature (c – 11°C, h – 23°C)

The research on the *S. lapponum* tissue culture initiation was also undertaken. The method of surface sterilization had an influence on the percentage of healthy explants in tissue culture. The most explants without the symptoms of contamination were obtained when the explants were soaked in the solution of Amistar 1 g·dm^{-3} and Previcur 1 g·dm^{-3} (16%), in comparison to the disinfection method without the use of fungicides (4%). Such high rate of contamination was probably due to the fact that the mother plants grow in wet and isolated peat bog (high humidity and temperature and no wind). The other Authors also reported contamination problems with *Salix lapponum* disinfection. The best result obtained so far was 80% of clean cultures after but mother plants used as a source of explants grew in dry mountainous area [Skalova et al. 2012].

The concentration and duration of NaOCl application also influenced the contamination rate of willows explants. It was observed that the least contaminated explants were obtained when 3% NaOCl was used for 30 minutes (89% of contamination free explants) but 100% had symptoms of necrosis. The best result was obtained when NaOCl was applied in concentration of 2% for 30 minutes (26% of contamination free explants and 29% of growing shoots).

The composition of the culture media affected the regeneration of shoot explants. The highest regeneration was observed on the media supplemented with BA in concentration of 0.1 mg·dm^{-3} and IBA in concentration of 0.01 mg·dm^{-3} (9%). Higher concentration of the cytokinins (BA 1 + IBA 0.1) enhanced growth of 3% of *S. lapponum* shoots. Explants placed on the media containing KIN or 2iP did not regenerate and had the symptoms of necrosis.

The MS media was used for micropropagation of other *Salix* species [Bhojwani 1980, Amo-Marco and Lledo 1996]. Brandova et al. [2011] used MS media supplemented with 0.01 mg·dm^{-3} BA and 0.01 mg·dm^{-3} IBA. They obtained 80% of contamination free explants with double sterilization and 28% of regeneration. Skalova et al. propagated *Salix alba*, *S. matsudana*. *S. lapponum* and *S. hastata* on MS media and *S. alba* and *S. matsudana* on WPM media. They obtained 20% of contamination free explants with 20% of growing shoots of *S. lapponum* on MS media supplemented with 1 mg BA·dm^{-3}, although the shoots were of poor quality. The best media for initiation of *S. lapponum* shoots was MS containing BA and IBA in concentration of 0.01 mg·dm^{-3}.

The seeds of *S. lapponum* germinated in 30%. Each seedling reaching 2 cm was transferred into a fresh media. None of the seedlings survived longer than two weeks. The further research on micropropagation of downy willow through seeds will be conducted.

In vitro culture of endangered species (pollen and tissues) will become in the near future one of key stages of active conservation of this species, thereby making it possible to preserve gene resources of the already sparse populations that exist within the area of eastern Poland. The results of the present study are the first one of this type and provide information on the combination of some parameters of environmental factors that are of key importance for the success of pollen storage and use for the propagation of boreal willow species under artificial conditions.

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ŻYWOTNOŚĆ PYŁKU I INICJACJA KULTUR TKANKOWYCH WIERZBY LAPOŃSKIEJ, GATUNKU ZAGROŻONEGO W POLSCE

Streszczenie. W pracy przedstawiono badania dotyczące zagrożonego w Polsce gatunku reliktowego wierzby lapońskiej (*Salix lapponum*). Głównym celem badań było określenie żywotności pyłku badanego gatunku w warunkach sztucznych oraz zweryfikowanie możliwości inicjacji kultur tkankowych. Żywotność pyłku *S. lapponum* szacowano na dwa sposoby, metodą barwienia oraz kiełkowania ziaren pyłku w łagiewkę. Na podstawie uzyskanych wyników badań wnioskuje się wysoką żywotność pyłku, zarówno świeżego jak i przechowywanego przez 12 miesięcy. Kiełkowanie ziaren pyłku uzależnione było od stężenia glukozy w pożywce i temperatury. Wyniki badań dostarczyły informacji na temat optymalnych warunków kiełkowania ziaren pyłku wierzby lapońskiej w warunkach laboratoryjnych. Podjęto również badania nad wprowadzeniem *S. lapponum* do kultur tkankowych. Najwięcej eksplantatów bez objawów zakażenia uzyskano, gdy fragmenty pędów moczone były w mieszaninie fungicydów, zanurzane w 70% roztworze alkoholu i odkażane powierzchniowo 2% NaOCl przez 30 min. Najwięcej dobrej jakości pędów uzyskano na pożywce MS, uzupełnionej 0,1 mg·dm^{-3} BA i 0,01 mg·dm^{-3} IBA.

Słowa kluczowe: żywotność pyłku, zdolność kiełkowania pyłku, kiełkowanie nasion, explantaty

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