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ORIGINAL RESEARCH PAPER

Pollen viability of *Salix myrtilloides* L. – an endangered species in Poland

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Abstract
Salix myrtilloides L. (swamp willow) is the most endangered species among the boreal *Salix* species in Poland. The number and size of its population have been decreasing constantly since the 1990s. The main aim of the study was to determine the viability of collected *S. myrtilloides* pollen and optimal conditions for its in vitro germination. The pollen of *S. myrtilloides* was collected from 25 male individuals from a population growing in the mid-forest peat bog Dekowina (Sobibór Landscape Park) in May 2014. Two methods were applied to estimate the viability of fresh and stored pollen: staining pollen with 2% acetocarmine solution and in vitro germinability. Various temperature (11°C, 23°C) and light conditions as well as different concentrations of glucose (1%, 2.5%, 5%, or 7.5%) were tested for the optimization of in vitro germination. We documented relatively high *S. myrtilloides* pollen viability. Pollen tube growth was found to be largely affected by both glucose content in the medium and thermal conditions during germination. Fresh pollen germinated most effectively on the medium with 2.5% glucose (stored pollen – in 5% glucose), at 23°C and in the presence of light. We conclude that pollen viability of *S. myrtilloides* does not seem to be a limiting factor for reproductive success. Moreover, the pollen is not sterile even after storage for 12 months. The *S. myrtilloides* individuals from the Dekowina peat bog produce viable pollen grains that are able to germinate and therefore it can be used to pollinate other populations present in the Polesie Lubelskie region for gene pool enrichment.

Keywords
Salix myrtilloides; pollen viability; pollen germination; Polesie Lubelskie

Introduction

Many plant species in natural peat bog ecosystems in Poland are threatened with extinction. These are often relict species whose proper functioning depends on specific habitat conditions in the stands. They are characterized by a narrow range of ecological tolerance and high sensitivity to environmental change. Peatland ecosystems, which are refuges for relict species, are constantly under direct or indirect pressure of human activity. Due to changing values of the physicochemical factors, and thus also the biocenotic ones, many species that are strongly bound to wetland habitats withdraw from their natural stands. Habitat fragmentation adversely affects the proper functioning of single populations, primarily through their spatial isolation. The lack of gene flow among populations may lead to impoverishment of the gene pool and thus reduce the adaptation of individuals.

Salix myrtilloides L. (swamp willow) is a species strongly bound to raised and transitional peat bog habitats. It has been under full protection in Poland since 1983 and is included in the *Red list of plants and fungi in Poland* as a critically endangered species (category of threat – E) and in the Polish red data book of plants (*Polska Czerwona Księga Roślin. Paprotniki i rośliny kwiatowe*) as an endangered species (category of threat – EN) [1–4]. The main threats to populations of *S. myrtilloides* are indirect or direct human activity, habitat eutrophication, drainage of peat bogs, and expansion of other woody species [3–5].

Salix myrtilloides is a Euro-Siberian species. Its range covers Eastern and Central Europe as well as almost the whole of Siberia. The western limit of its range runs through the Polish regions of the Sudetes, Tatras, and South Carpathians. Isolated stands of *S. myrtilloides* can be found in the Swiss Alps, in the mountains of southern Bavaria, and in the Don River valley [2,4,6,7]. In Poland, *S. myrtilloides* is considered to be a glacial relict. In the 1950's, it was reported in about 90 localities, but most of these stands do not exist any longer [8–10].

Among the 14 populations that had been present in the Polesie Lubelskie region, six were determined to be extinct and five were not confirmed [11].

Currently, there are three known *S. myrtilloides* populations: near Moszne Lake in the Polesie National Park, in the peat bog near Bikcze Lake, and in the mid-forest Dekowina peat bog in the Sobibór Landscape Park. The observations indicate that the populations present on Bikcze Lake and in the Dekowina peat bog are the most vulnerable to changes in their habitats (unpublished data). In both locations, a significant acceleration of the process of ecological succession is observed, manifested primarily by invasion of expansive tree and shrub vegetation. Additionally, the survival of *S. myrtilloides* near Bikcze Lake is seriously threatened due to the lack of males in the population. Since 2010, no seedlings have been found there, despite that flowering females have been noted each year in all the known populations [12].

As there is a need for protection of the remaining populations of *S. myrtilloides* in the Polesie Lubelskie region, in order to plan and implement their conservation, a study to explore different aspects of the biology and ecology of the species was undertaken.

Viability of pollen grains is extremely important for sexual reproduction of plants as reproduction success largely depends on pollen dispersal and effective pollination within and between populations [13–16]. These are key elements guaranteeing population continuity (including seed germination and seedling survival) and connection between them in fragmented habitats [17]. The present study was carried out to test the pollen viability of *S. myrtilloides*. Moreover, we attempted to determine optimal conditions (medium composition, temperature, light) for in vitro germination of pollen tubes. The results are supposed to help to answer two questions important in the aspect of planning active conservation of the studied species in the Polesie Lubelskie region: (***i***) is pollen viability a limiting factor for the sexual reproduction process of *S. myrtilloides* and (***ii***) could pollen collected from *S. myrtilloides* individuals from the Dekowina peat bog be used for hand pollination of plants in other populations and hereby help to enrich the gene pool?

Material and methods

The *S. myrtilloides* population which exists in the mid-forest Dekowina peat bog (51°26.689′ N, 023°31.368′ E), located in the eastern part of the Łęczna-Włodawa Lake District (Sobibór Landscape Park) in the Polesie Lubelskie region, was chosen for the research due to its size (more than 100 individuals in the population), optimal sexual structure (the ratio of male to female individuals nearly 2:1), and a large proportion of flowering individuals [12].

The pollen was collected at full flowering (May 2014, air temperature 20°C). We selected male individuals of *S. myrtilloides* ($N = 25$) and then two inflorescences (catkins) from each were sampled and put in paper bags. For the analyses, we used (***i***) pollen grains directly after collection (fresh pollen) and (***ii***) pollen kept in the paper bags at 23°C for one year (stored pollen).

Two methods were used to evaluate *S. myrtilloides* pollen functionality: (***i***) pollen viability and (***ii***) pollen germination. To assess pollen viability, the pollen was stained with 2% acetocarmine solution [18–20] and within 1 h analyzed under a light microscope (Olympus BX40). 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. The pollen (300 grains) was analyzed in each of the 25 samples of *S. myrtilloides*. The same investigations were repeated in 2015 for stored pollen.

Pollen in vitro germination was tested under three-factorial crossed combinations: different light conditions, temperature, and glucose concentrations. 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 [21–23]. They were placed on glass rods in Petri dishes (10 cm, glass) 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°C and at 11°C in a refrigerator). Additionally, light vs. dark conditions were created for each temperature. The slides were analyzed under a light microscope and pollen grains that had germinated were counted in a given field of view. To optimize the germination protocol, different durations were tested (3 h, 24 h, and 72 h). Additionally, a total of 400 pollen grains (4 × 100) were counted on each slide and the percentage of pollen grains with pollen tubes was determined. According to Báez et al. [24], the pollen grains with tubes of the same or greater length than their own diameter were considered as germinated.

Statistical analysis was performed for the pollen viability and germinability with 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

The pollen viability of *S. myrtilloides* was high. The average number of *S. myrtilloides* pollen grains with stained protoplasts was 89.82% (±6.42) for fresh pollen, and 79.15% (±11.52) for stored pollen (Fig. 1). However, the above results were not confirmed by the germination tests, although 16 different combinations of in vitro conditions were tested.

The analysis of the data concerning *S. myrtilloides* pollen proved that fresh pollen germinated most effectively on the medium with 2.5% glucose, at 23°C and in the presence of light. The medium with 5% glucose, room temperature conditions, and the presence of light is the most optimal for pollen tube germination of stored pollen (Fig. 2, Fig. 3).

In all experimental treatments, the highest number of pollen grains germinated during the first 3 hours (for both fresh and stored pollen). The number of new pollen tubes decreased after 3 to 24 h and after 24 to 72 h (Fig. 4).

The main results of four ANOVA tests for pollen germination in three-factorial crossed combinations test are summarized in Tab. 1. All significant values are reported at $p < 0.05$. Fig. 5 and Fig. 6 provide graphical summaries of Tukey's HSD tests.

The ANOVAs showed that all the main factors as well as the interactions between them, except for the glucose-light interaction, were significant. The post-hoc difference test for glucose concentrations demonstrated that the application of glucose at a concentration of 2.5% significantly increased the germination percentage of fresh pollen. Considering the results for stored pollen, it was noted that the two highest germination values were recorded when glucose was used in concentrations of 5% or 2.5%, and they were regarded as statistically equal (Fig. 5).

The 2.5% glucose significantly increased the germination of fresh pollen in comparison to any other treatment (Fig. 6). The 2.5% glucose at the higher temperature and 5% glucose at the same temperature caused significantly higher germinability of stored pollen.

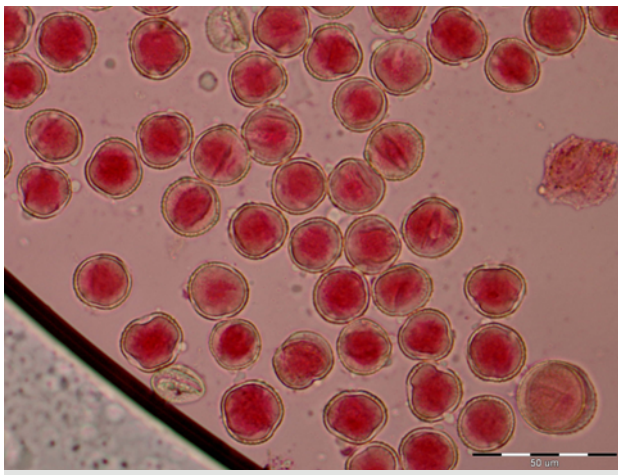

Fig. 1 Fresh pollen grains of *S. myrtilloides* stained with 2% acetocarmine.

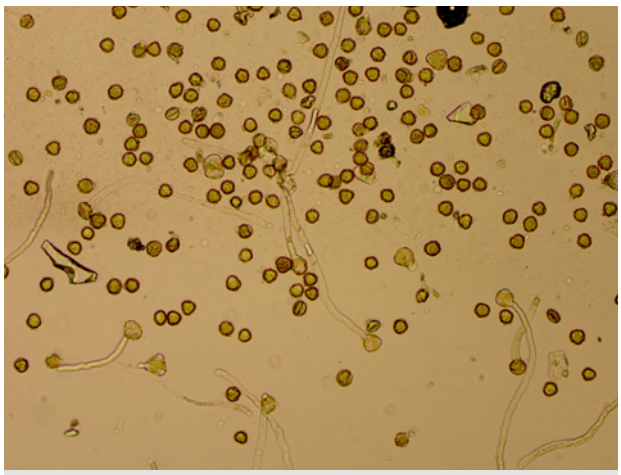

Fig. 2 Pollen grains germinated on the medium with 2.5% glucose, at 23°C, in the presence of light.

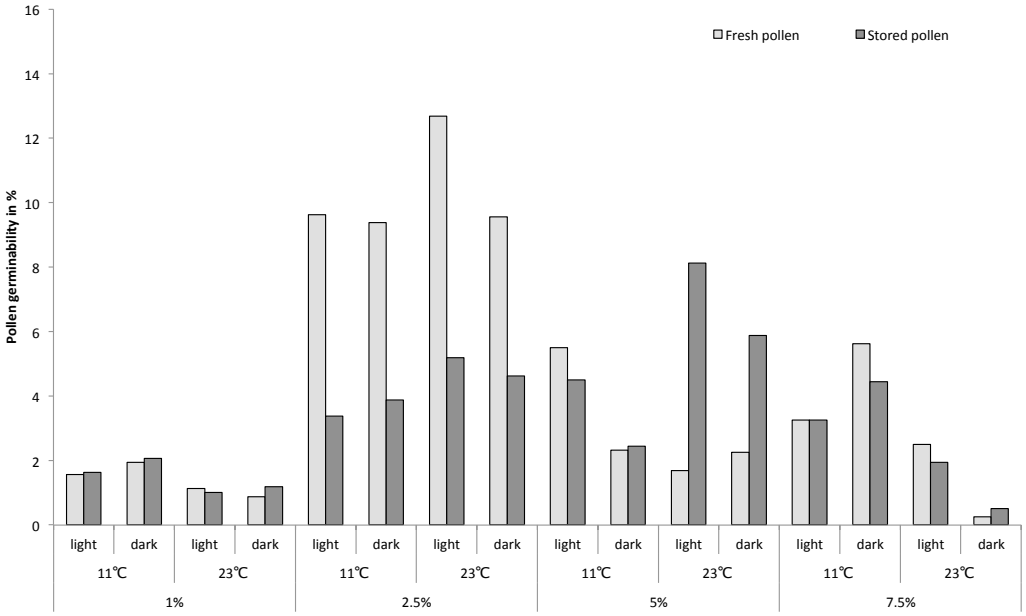

Fig. 3 Germination of *S. myrtilloides* pollen under various experimental conditions.

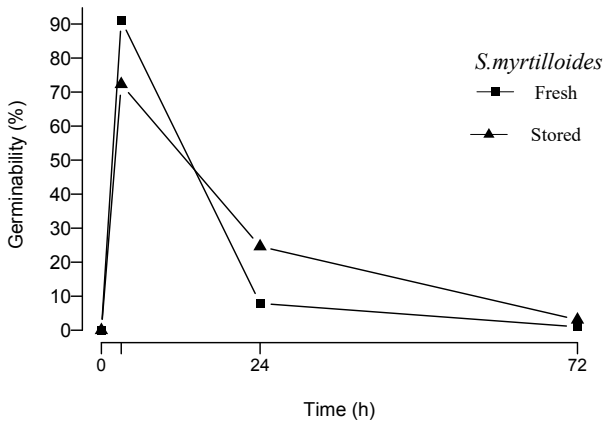

Fig. 4 Germination of *S. myrtilloides* pollen grains in relation to time. The graph shows the pollen germination efficiency under optimal conditions regarding temperature, light, and medium composition (refer to Fig. 1).

Tab. 1 The ANOVAs (*df* – degrees of freedom, *F* statistic, and *p* value) for *S. myrtilloides* pollen germinability.

		Salix myrtilloides			
		fresh pollen		stored pollen	
Source	*df*	*F*	*p* (>*F*)	*F*	*p* (>*F*)
G	3	153.094	<2e−16***	43.486	<2e−16***
L	1	9.056	0.0029**	3.935	0.0484*
Temp	1	45.665	1.05e−10***	1.810	0.1798
G:L	3	1.318	0.2692	3.862	0.0100*
G:Temp	3	14.840	6.78e−09***	28.274	1.08e−15***
L:Temp	1	5.816	0.0166*	4.506	0.0348*
G:L:Temp	3	14.779	7.32e−09***	2.230	0.0853
Residuals	240				

Significance levels codes: $p < 0.001$***; $p < 0.01$**; $p < 0.05$*; $p < 0.01$. G – glucose solution; L – light conditions; Temp – temperature.

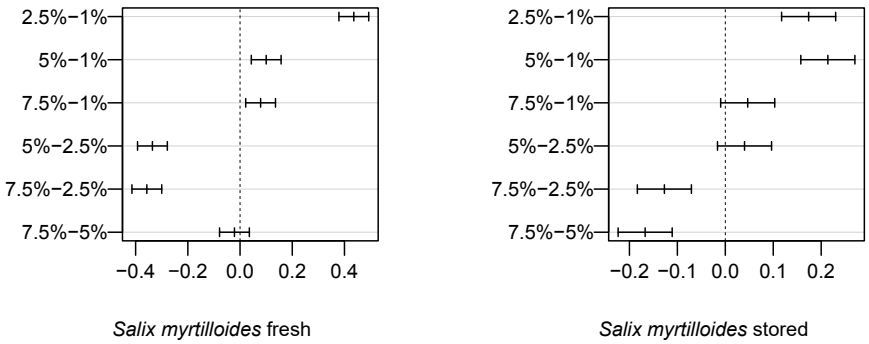

Fig. 5 95% confidence intervals of the differences between the means of glucose concentrations for the *S. myrtilloides* pollen germinability data. Pairwise comparisons are pictured at a vertical position on the left hand axis. The corresponding interval estimates are marked with horizontal lines. Comparisons with intervals that do not overlap the vertical dashed line $x = 0$ are found significantly different.

Discussion

The destruction of habitats and their fragmentation cause a reduction in population size of many plants species. Increasing habitat isolation decreases migration and gene flow among populations. To survive the changes in the environment, local populations depend on phenotypic plasticity and adaptive genetic differentiation [25]. These features are conditioned by the effectiveness of sexual propagation to a substantial degree, which in case of dioecious plants is determined by the presence of males in a population and their ability to produce viable pollen. Pollen viability and germination capacity may be used as indicators of the correctness of sexual processes and adaptive abilities of plants, which is used in planning active conservation of plant species [24,26–31].

According to Dafni and Firmage [32], there is no single method that would be the best test to examine pollen viability in vitro. All known methods have advantages and disadvantages and therefore it is recommended to use a few at the same time. The advantages of staining methods (vital stains) are quickness and simplicity. They also correlate with in vitro germination tests, which measure pollen germinability

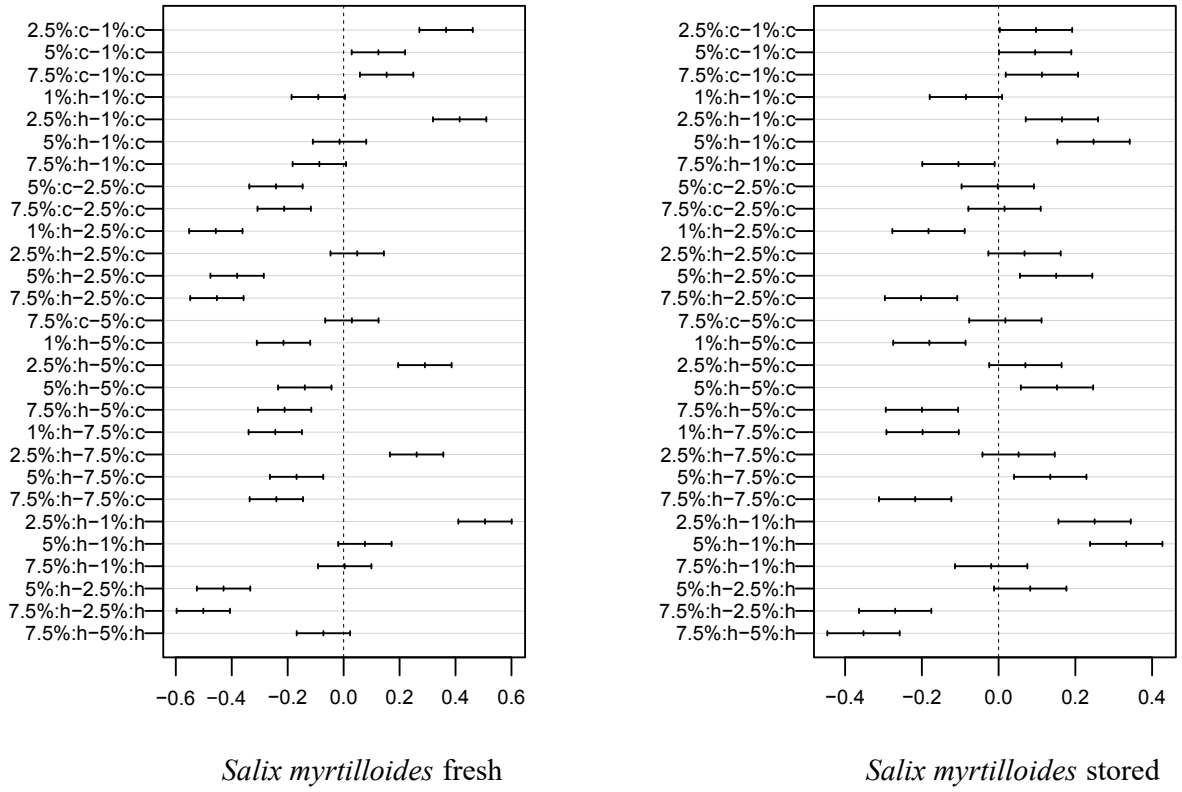

Fig. 6 Graphic representation of Tukey's HSD comparison results for the *S. myrtilloides* pollen germinability data for the interaction of glucose concentrations (1%, 2.5%, 5%, 7.5%) and temperature (c – 11°C; h – 23°C).

under specific conditions of the medium and temperature. They are rapid, reasonable, simple, and fully quantitative [33].

In our study, two methods were used to evaluate the viability of *S. myrtilloides* pollen. In case of the in vitro germination test, 16 combinations of germination conditions were used, which differed in the concentration of carbohydrates in the media as well as in thermal and light conditions. The ability of pollen to germinate into tubes was evaluated in different time intervals, from the moment of starting the experiment up to 72 hours. According to Stanley and Linskens [34: p. 33–36], results can vary enormously depending on the composition of the medium, temperature, and duration of the test.

Effectiveness of pollen tube growth in vitro is related to the amount of sugar in media and it is considered to be species-specific [20,23,28,29]. As in the case of the study on *Salix lapponum* [35], a relatively lower concentration of glucose was used in the present research. However, it was found that the optimal content of sugar in the medium was within the range of 1% and 70%. It was also confirmed that the older the pollen, the higher sugar concentration is needed to stimulate pollen germination [36].

Salix myrtilloides blooms from May to July, after the development of leaves [4], when the air temperature in Poland often exceeds 20°C. Our results show that the higher temperature used in the experiment (23°C) is more appropriate for in vitro germination of swamp willow, similarly as in the case of *S. lapponum*, which blooms at lower temperatures in natural conditions and pollen needs lower temperatures to germinate in vitro [35].

The obtained results of the research on *S. myrtilloides* pollen viability evaluated with different methods were divergent, although a high percentage of viable pollen grains able to germinate was observed. Even though many authors consider staining methods as subject to risk of overestimating pollen viability in comparison with other methods or staining old or dead pollen [37,38], the problem of male sterility of the studied plants may be excluded.

Similar results were obtained for the pollen of *S. lapponum* (a related species that is also threatened in the area of eastern Poland). The discrepancy between pollen

viability tested with the staining method and the percentage of pollen that germinated into a tube was clearly visible. It is worth mentioning that viability of *S. lapponum* pollen was significantly higher than for *S. myrtilloides*, ca. 97% for fresh pollen and around 94% for stored pollen. A similar tendency was also observed in the case of germinating pollen grains (ca. 15 %) [12].

The viability of *S. myrtilloides* pollen is slightly higher in the case of plants growing in the Dekowina peat-bog in comparison to those growing on Moszne Lake (84.17%) [12].

The same tendency is observed in case of pollen germinability. The population of *S. myrtilloides* which is present in the Bikcze peat bog consists only of female individuals. A trial to use *S. myrtilloides* pollen from other populations, like the one in Dekowina, for hand pollination in order to support sexual propagation might be undertaken. Measures should be taken to ensure that effective pollination takes place in spite of the fragmentation of *S. myrtilloides* natural habitats. In the first stage of conservation of the species, such activities could be more effective than reinforcement of male individuals from other populations or obtained ex situ.

Conclusions

Our results on pollen viability have implications for species conservation and future research. The pollen viability of *S. myrtilloides* does not seem to be a limiting factor for reproductive success in terms of potential fertilization and seed production. *Salix myrtilloides* individuals from the Dekowina population produce viable pollen grains which are able to germinate in in vitro, and therefore they could be used to pollinate individuals from other populations in Polesie Lubelskie in order to enrich and diversify the gene pools.

Acknowledgments

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Żywotność pyłku wierzby borówkolistnej (*Salix myrtilloides*) L. – gatunku zagrożonego w Polsce

Streszczenie

Salix myrtilloides L. (wierzba borówkolistna) jest najbardziej zagrożonym gatunkiem spośród borealnych gatunków z rodzaju *Salix* występujących w Polsce. Liczba i wielkość populacji *S. myrtilloides* maleje nieprzerwanie od 1990 roku. Głównym celem pracy było określenie żywotności oraz optymalnych warunków dla kiełkowania ziaren pyłku *S. myrtilloides*, zarówno świeżych jak i przechowywanych przez okres 12 miesięcy. Pyłek *S. myrtilloides* pozyskano z 25 osobników męskich, z populacji rosnącej na śródleśnym torfowisku Dekowina (w Sobiborskim Parku Krajobrazowym) w maju 2014 roku. Potencjał pyłku do zapylenia określono na podstawie (***i***) żywotności pyłku określanej metodą barwienia 2% roztworem acetocarminu oraz (***ii***) na podstawie zdolności kiełkowania. Zdolność kiełkowania testowano in vitro w zróżnicowanych warunkach termicznych (11°C, 23°C) świetlnych, oraz na pożywkach z 1%, 2.5%, 5% i 7.5% stężeniem glukozy. Udokumentowano stosunkowo wysoką żywotności pyłku *S. myrtilloides*. Stwierdzono, że na wzrost łagiewki pyłkowej w znacznym stopniu wpływa zarówno zawartość glukozy w pożywce jak i temperatura. Świeży pyłek kiełkował najlepiej na pożywce z 2.5% roztworem glukozy (pyłek przechowywany – z 5% roztworem glukozy), w temperaturze 23°C i w obecności światła. Żywotność pyłku nie powinny być czynnikiem ograniczającym sukces reprodukcyjny osobników *S. myrtilloides*. Pyłek nie był sterylny, nawet po okresie przechowywania go przez 12 miesięcy. Osobniki *S. myrtilloides* rosnące na torfowisku Dekowina wytwarzają żywotne ziarnka pyłku, które są zdolne do kiełkowania. Pyłek ten może być wykorzystany do sztucznego zapylania osobników z innych populacji na Polesiu Lubelskim, w celu wzbogacania ich pul genowych.

