

Experimental reintroduction of the boreal species *Salix lapponum* L. to refuges at the southern limit of its range — short-term results

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Salix lapponum is a threatened species in locations outside its geographical range (N. Europe). In Poland, the number of populations has decreased within the last 10 years, which prompted us to reintroduce propagated plants into natural habitats. We describe our activities: from obtaining the plant material, through *in vitro* propagation, *ex situ* cultivation, to translocation and one-year monitoring. We found that plants produced in *ex situ* conditions are able to survive in natural habitats, but their condition depends on the production process. We noted that when the plant material was collected in May or July and the *in vitro* seedlings were cultivated in soil from the end of October or December and until introduction into the natural habitat at the end of May the following year, all plants survived. This contrasted the 43% of survival rate when explants were collected in September and the seedlings were cultivated in soil from February. The short-term results indicate that the morphology of plants intended for reintroduction is of great significance. This is particularly important for planning methods of plant production and acclimatization. Finally we can conclude that in the case of *S. lapponum*, an initial evaluation of reintroduction success is possible after just one year.

Introduction

Reintroduction is a multistage process combining *in situ* and *ex situ* species conservation (Ren *et al.*

2014). In the case of endangered species, ideally it should lead to the development of a resilient, self-sustaining and genetically diverse population that will enhance the species' chance for survival

in the wild (Guerrant and Kaye 2007; Godefroid et al. 2011).

We present the results of an experiment involving the reintroduction of an endangered boreal relict, *Salix lapponum* (downy willow) to refuges in Poland. The species is a dioecious winter-hardy shrub (nanophanerophyte), usually not exceeding 1 m in height. In habitats dominated by shrubs and woody vegetation, it can grow up to 2 m in height. The elliptical leaves of the downy willow are greyish green, covered with dense down and the underside is silvery grey. The stems are brown and shiny (Kaźmierczakowa et al. 2014; Zarzycki et al. 2002). The plant is usually found alone or in small clusters. The results of recent research conducted at dispersed population sites in Poland indicate that it reproduces mainly generatively (Pogorzelec et al. 2014c; Głębocka and Pogorzelec 2017), and not vegetatively, as previously described (Kaźmierczakowa et al. 2014). Within the central part of its range, the downy willow blooms in June and July and in Polish climate conditions from March to April (Pogorzelec et al. 2014c).

The range of occurrence of *S. lapponum* includes northern and north-eastern Europe, Scotland, the northern British Isles, Scandinavia, the Kola Peninsula, the Baltic states, northern Belarus and Siberia. There are also downy willow populations in isolated locations in the mountains of Central and Western Europe, i.e., the Sudetes, the Central Massif, the Pyrenees, and the mountains of Bulgaria (Kaźmierczakowa et al. 2014; Jalas and Suominen 1988). In Poland, the downy willow was still present as a glacial relic at over 60 sites in the 1950s, but today most of these locations have become historical. There are two populations of the species in the Karkonosze Mountains, growing in the subalpine altitudinal zone as a *Salicetum lapponum* community. Currently, in lowland sites of *S. lapponum*, it grows on the Łęczna-Włodawa Plain (in Polesie National Park and its vicinity, at five known sites), in Biebrza National Park, and in Knyszyn Forest (Kaźmierczakowa et al. 2014; Pogorzelec et al. 2014a). It prefers sunny or partially shaded areas in wetland peat bogs, mainly raised and transitional. *S. lapponum* grows on highly acidic and acidic substrates (pH: 4–6) (Serafin et al. 2015).

Since 1950, changes that have taken place in the natural environment of eastern Poland (due mainly to human activity) have contributed to a gradual decline in the downy willow population. In particular, wetland drainage has induced changes in habitat conditions, in terms of both abiotic and biocenotic factors. Progressive ecological succession is manifested by an increase in the share of woody plants and shrubs in phytocenoses (*Salix cinerea*, *S. aurita*, *Batula pendula* and *B. pubescens*), as well as expansive species (e.g. *Phragmites australis*). This is one of the most important reasons of the disappearance of the natural elements of peatland flora, especially those with specific preferences and narrow ecological tolerance ranges, such as *S. lapponum* (Pogorzelec et al. 2014b).

Salix lapponum has the status of a critically endangered species in the Polish Red Book of Plants. It is also included on the "Red List of Plants and Fungi in Poland". The International Union for Conservation of Nature (IUCN) places it in the EN category (endangered — very high risk of extinction). In Polesie National Park, active protection of the downy willow involves enlarging existing populations and creating new ones, as well as protecting its habitats (Głębocka and Pogorzelec 2017; Pogorzelec et al. 2014a).

Inventories carried out during the 1950s and 2010s showed that the downy willow population has drastically decreased in eastern Poland (Fijałkowski 1958, Pogorzelec et al. 2014a, 2014b). Thus, there is an increasing risk of *S. lapponum* extinctions, which prompted us to undertake active conservation measures of this species. Our actions were based on the knowledge of the ecology and biology of this species gained during 10 years of research on the condition of this species in eastern Poland (Pogorzelec et al. 2014a, 2014b, 2014c; 2015; Serafin et al. 2015, Głębocka and Pogorzelec 2017). In order to choose the most suitable habitat for reintroduction, we placed special focus on habitat studies, which primarily involved analysis of selected environmental abiotic factors in the acrotelm water, i.e., pH, electrolytic conductivity ($\mu\text{S}\cdot\text{cm}^{-1}$), and concentrations of N_{tot} , NO_2 , NO_3 , $\text{NH}_4\text{-N}$, P_{tot} , and PO_4 (mg dm^{-3}), as well as verification of data on biocenoses with past or

present populations of the species (Pogorzelec *et al.* 2014b; Serafin *et al.* 2015; Pogorzelec *et al.* 2020). We conducted observations and analyzed interactions, especially the importance of herbivore pressure, which can significantly affect the condition of *S. lapponum* and its survival prospects (Kmieć *et al.* 2018). The results of our study revealed the habitat preferences specific for this species with regard to physico-chemical factors, as well as the threats arising from changes taking place within the study area, which affect the plant cover of the entire peatland (Serafin *et al.* 2015).

Based on the knowledge of *S. lapponum* habitat preferences (Pogorzelec *et al.* 2014b; Serafin *et al.* 2015) and the recommendation of Godefroid *et al.* (2011) that species should be reintroduced in protected areas, we chose one suitable location in Polesie National Park for restoration of the downy willow population. We decided to translocate *ex situ* cultivated plants obtained by micropropagation from plants derived from the most genetically diverse natural population in the study area (Głębocka and Pogorzelec 2017). In case of the species endangered with extinction, there is usually a limited number of existing specimens. In order to obtain a large number of new, healthy and good quality offsprings it is advised to use *in vitro* propagation, because it makes it possible to obtain many plants with no bigger harm to donor plants (Bunn *et al.* 2007; Slazak *et al.* 2015). Tissue cultures have been used in case of propagation of various rare or endangered plant species (Sharma *et al.* 2008; Holobiuc *et al.* 2009). The micropropagation have been also used to multiply *S. lapponum* plants, however, there is no information on the use of the species (Skalova *et al.* 2012).

In practice, it is difficult to determine the success of reintroduction, as it includes many elements that cannot be measured objectively. Most researchers dealing with this issue consider the survival of individuals and normal reproductive processes to be a measure of reintroduction success (Maschinski and Duquesnel 2006; Godefroid *et al.* 2011; Ren *et al.* 2011). The best measure of reintroduction success seems to be confirmation of seedlings recruitment, which indicates that the population can be considered self-sustaining.

Godefroid *et al.* (2011) indicate factors affecting the success of active conservation. They suggest that we can only estimate the success of reintroduction after an adequate period of time which has passed and it depends on the plant species and the changes taking place in the environment. In general, reintroduction success can be measured in the short or long term (Ren *et al.* 2014). A species should be able to complete its entire life cycle at the reintroduction site and to increase its numbers through reproduction, while seeds should be able to disperse and form a population at a different location. Another indicator of successful reintroduction is the integration of the species in the ecosystem, which requires multilevel research on both the population and its habitat.

The aim of this study was to create a procedure for reintroducing *S. lapponum* into natural habitats at the southern limit of this species' range and to test the short-term results of this procedure, as well as to answer whether: 1) *ex situ* cultivated plants are able to survive and grow in the wild over a 12-month cycle; and 2) the morphological features of the offspring determine their adaptation in the natural environment.

Material and methods

We collected *S. lapponum* plant material from the largest population in eastern Poland (over 300 individuals), located in a peat bog by Lake Bikeze, (51°22'53.89'N 23°02'40.00'E). The chosen population has a satisfactory female to male sex ratio (3:1), high genetic variation, and no clonal individuals, which indicated its ability to reproduce sexually (Głębocka and Pogorzelec 2017).

We collected 5 cm shoot fragments three times: in May, July and September 2016 (we marked them as A, B and C, respectively), from mother plants which we had been previously numbered and whose sex had been verified. We collected fragments from different individuals separately. Each time, we surface-disinfected the pieces in the following steps: 1) rinsing three times in tap water with a drop of detergent; 2) shaking for 30 minutes in a fungicide



Fig 1. *In vitro*-derived *Salix lapponum* plantlet before planting into soil.

(azoxystrobin 250 g dm⁻³) solution at a concentration of 2 ml dm⁻³; 3) shaking for 10 seconds in 70% ethanol; 4) disinfection for 30 minutes in 1% hypochlorite solution (10% of the active chlorine); 5) rinsing three times in sterilized distilled water.

We prepared the growing medium according to Murashige and Skoog MS medium (1962) with the addition of growth regulators, BA (benzyladenine) at a concentration of 1 mg dm⁻³ and IBA (indolebutyric acid) at 0.1 mg dm⁻³, and activated charcoal (AC) at 2 g dm⁻³. We set the pH of the medium at 5.5 using 1N NaOH and 1N HCl. We solidified the medium with Microbiological Lab-Agar at a concentration of 7 g dm⁻³, poured it into tubes, and autoclaved it for 21 minutes at 121°C and 1 hPa.

We cut the disinfected shoots into single node 2 cm long explants, placed them individually in tubes on 10 ml of solidified MS medium in a laminar flow chamber, and then cultivated the explants in a growing room, at 22°C during the day and 20°C at night, with a 16-hour photoperiod and light intensity of 35 μmol m⁻² s⁻¹. We

used all obtained shoots for further multiplication, subculturing them at 6-week intervals on the same medium until we had enough plants for acclimatization. After three subcultures, we removed all shoots with symptoms of contamination and began to cultivate the shoots in 500 ml jars, with 60 ml of solidified media. We placed 12 plants in each jar. The cultivated shoots rooted spontaneously, so we omitted the *in vitro* rooting step (Fig. 1).

We transferred the rooted plants, which were 7–10 cm long, from *in vitro* cultures to *ex vitro* conditions, according to the terms of plants collection and cultivation, as follows: plants obtained from shoots collected in May were planted into soil at the end of October, those collected in July were planted into soil at the beginning of December and those collected in September were planted into soil at the beginning of February the next year (Table 1). For planting, we removed the plants from the jars, washed the roots with tap water to remove agar residues, shortened the roots to 2 cm, and planted them in 1 dm³ boxes containing a mixture of acidic peat (pH: 3.5–4.5), deacidified peat (pH: 5.5–6.5), washed river sand and perlite in equal volume proportions. We planted 10 plants per box at random. We placed the boxes with plants in a glass aquarium and covered them tightly with plastic foil to maintain high humidity. The plants were cultivated in the same conditions as during *in vitro* cultivation. After two weeks we began hardening the plants by gradually removing the foil, and after three weeks we removed the cover completely. After two months of growth in soil, we

Table 1. The *ex situ* cultivation protocol for each date of collection of *Salix lapponum* explants from plants growing in the wild.

Cultivation steps	Terms of activities for each collection date		
	A	B	C
Explants collection	Beginning of May	End of Jul.	Mid-Sep.
Culture initiation	May	Jul.	Sep.
Passages to fresh medium	Jun., Jul., Sep.	Aug., Oct.	Sep., Oct., Dec.
Planting in substrate	End of Oct.	Beginning of Dec.	Beginning of Feb.
Transplantation to P9 pots	End of Nov.	Mid-Jan.	Beginning of Mar.
Cutting	Jan., Feb., Apr.	Feb., Apr.	Apr.
Reintroduction	End of May	End of May	End of May



Fig 2. *S. lapponum* young plants in cold frames.



Fig 3. *S. lapponum* individuals planted in the peat bog mat.

transferred the plants individually to P9 pots (9 cm³), which was done at the end of November for May-collected shoots, in the mid-January for July collections and at the beginning of March for September collections. A month later, when the plants reached about 25 cm, we cut them to a height of 10 cm to stimulate lateral shoot formation. This treatment was repeated at 1.5–2-month intervals. The number of treatments depended on the planting date and therefore, on the length of cultivation in soil (Table 1).

At the beginning of May 2017, we transported 40 young rooted plants (20 male and 20 female) from each date of plant material collection: May, July and September (denoted as A, B and C, respectively) to the acclimatization site in Polesie National Park. We placed all plants in P9 pots in cold frames protected with insect screens and left them for three weeks (Fig. 2). Then we transferred the plants to a natural site where downy willow populations had been recorded in the past — on the midforest peat bog complex (raised and transitional) named Blizionki (51025'41.69''N 23004'05.39''E) in

Polesie National Park. We chose the most suitable location for reintroduction (using the methods presented in the publication by Pogorzelec *et al.* 2020) on the basis of previous studies of habitat conditions (Serafin *et al.* 2015). We labeled all plants (with group A–C, sex and individual number) and inserted them randomly into holes in the bog mat together with the substrate, at 20–100 cm spacing (Fig. 3). We planted a total of 120 plants at the end of May 2017.

From April to September 2018, we monitored the number and condition of individuals introduced into this habitat from *ex situ* cultivation. We determined the following major parameters: number of individuals which began to grow during the growing season, percentage of flowering individuals, plant height, and number of leaves of each individual.

The data obtained on the survival rate and selected morphological features of the reintroduced plants were analyzed by one-way ANOVA using Statistica ver. 13.1 (StatSoft). The significance of differences between means was determined using Tukey intervals at the 5% level of significance.



Fig 4. *S. lapponum* plants after one year of growth in natural habitat.

Results

The results of our experiment on the reintroduction of *S. lapponum* indicate that future success is possible, as a significant percentage (over 80% in total) of individuals survived longer than 12 months in the new habitat (Table 2). It is particularly significant that the plants used in the experiment were cultured *in vitro* and successfully underwent both acclimatization (100%

of individuals) and transfer to harsh natural conditions. The number of plants that survived this period, recognized as critical in the case of *ex situ* grown plants, is promising for the future.

The *S. lapponum* plants obtained in the tissue culture acclimated well in the soil, but the plants differed morphologically depending on the date of explant collection what is unequivocal with the length of cultivation both *in vitro* and in soil as well as with the number of cuttings done. We noted that the plants obtained from explants collected in May, then cultivated *in vitro* until the end of October and planted into soil at the end of October, which were cut 3 times (A), characterized with a woody stem and 4–5 lateral shoots. The plants propagated from explants collected in July (B), cultivated *in vitro* until planting into soil in the beginning of December and cut two times, also had a woody stem and 2–3 lateral shoots. Plants obtained from explants collected in September (C), which were cultivated for the shortest time and cut only once, had a herbaceous stem and one lateral shoot.

We observed that 81% of all plants survived in natural conditions for longer than 12 months (Table 2, Fig. 4). However, this depended on the morphological structure of the plants resulting from the length of cultivation. In the case of plants with woody stems and lateral shoots, obtained from material excised in May and July (A and B), all specimens survived and some of them flowered (18% and 15%, respectively). Among plants derived from tissue cultures set up

Table 2. Survival rate of reintroduced plants and their morphological features after 12 months of growth in natural habitat depending on *ex situ* cultivation time.

Plant group Unit	A*		B		C		Total	
	No.	%	No.	%	No.	%	No.	%
Number of initial shoots	40	100	40	100	40	100	120	100
12 months after reintroduction into natural habitat								
Number of surviving plants	40A**	100	40A	100	17B	43	97	81
Number of flowering plants	7	18	6	15	0	0	13	11
Number of fruiting plants	0	0	0	0	0	0	0	0
Mean plant height (cm)	45.3A	—	43.1B	—	30.2C	—	39.5	—
Mean number of leaves	15.53A	—	13.20A	—	7.53B	—	11.6	—

*Offspring propagated from pieces collected in May (A), July (B) and September (C)

**Means followed by the same letter in rows do not differ significantly at $p = 0.05$.

in September (C), 43% survived and none flowered. The morphological features of the specimens also varied depending on the cultivation process. Plants propagated from pieces collected in May (A) had the longest shoots (45.3 cm) and more leaves (15.53 cm) than those propagated in September (30.2 cm and 7.53 pcs, respectively). Plants propagated from pieces collected in July had longer shoots (43.1 cm) and more leaves (13.20 pcs) than those set up in September (30.2 cm and 7.53 pcs, respectively) (Table 2).

Although the success of plant reintroduction can only be confirmed after a much longer period than 12 months, this short-term experiment provides answers to the questions posed in this study. *S. lapponum* plants cultivated *ex situ* are able to survive and grow in the natural environment over a 12-month cycle, provided that the plants used for reintroduction have appropriate structural characteristics, i.e., a woody main stem and lateral shoots, as this likely determines the survival of plants and their growth in new conditions. Individuals with the required morphological structure can be obtained *ex situ* after 9–12 months, provided that appropriate treatments are performed, such as cutting to induce lateral shoots.

Discussion

It was possible to collect the plant material and initiate the tissue cultures of *Salix lapponum* both in spring and autumn. Then they were cultivated *in vitro* until acclimatization in soil and then cultivated until introduction into natural habitat. The time of tissue initiation determined the length of cultivation of plants. Obviously, the longer cultivation process, the higher costs of plants production. It was noted that the length of cultivation determined the quality of plants, as the longer they were grown, the older cuttings were produced, which is characterized with more woody stem and more lateral shoots thanks to more cutting treatments. There was no difference in the terms of survival rate in the case of spring and summer collections, but the plants cultivated the shortest — from the September collection, survived only in 43%. At the moment, a substantial number of scientific studies draw

attention to high cost of tissue culture production and they try to find methods to lower the costs of plant production *in vitro* (Datta *et al.* 2017; Ogero *et al.* 2012). Therefore, the fact that the *Salix lapponum* tissue cultures may begin in the early summer allows to lower the costs of plant cultivation and production.

There are many scientific reports on the treatments influencing the quality of propagated plants, however they mainly concern agricultural or horticultural plant production for yield or sale purposes and therefore different features are considered and described. In the case of willow species, the treatments that affect the survival rate of cuttings are mainly based on fertilization or soil type (Kozlov *et al.* 1999; Schaff *et al.* 2003).

Plants obtained during *in vitro* cultivation are exposed to stress caused by sudden changes of temperature, light and air humidity conditions, which might also influence their survival ability. According to Hampe and Jump (2011), in case of relict species, young plants (mainly seeds and seedlings) are the most sensitive to temperature or humidity fluctuations. Adult specimens, properly developed, are more resistant to any changes of abiotic parameters of the environment (Niinemets 2010). The probability of the success of the species reintroduction is then higher if the plants used to rebuild or set up a population are well developed and of good quality (Tojibaev *et al.* 2019). It is also possible, that time and acclimatization conditions play an important role in the survivability of plants in the natural habitat (Soares *et al.* 2020). That part of the reintroduction process is incredibly important in case of relict plant species, which are especially sensitive to the climate changes — which for them are the biggest threat in nature (Garfi and Buord 2012).

The question of the survival and flowering of male and female individuals of the species in new habitat conditions requires further study. Hroneš *et al.* (2019) and Hughes *et al.* (2010) reported a strong female-biased sex ratio in most naturally occurring populations of *S. lapponum* in Sweden and the Czech Republic. However, they do not rule out the possibility that the actual sex ratio of the population is different, because a large group of plants in the study populations were non-reproductive individuals of unknown

sex (plants that did not flower during the study). They also noted morphological differences in the structure of males and females (males were generally taller than females). Further observations of reintroduced plants may contribute to knowledge of the adaptability of individuals depending on their sex.

Conclusions

The short-term results of this reintroduction experiment indicate that in the case of *S. lapponum*, the morphology of plants intended for reintroduction is of great significance. This is particularly important for planning methods of plant production and acclimatization. We can conclude from this study that in the case of *S. lapponum* we can conduct an initial evaluation of reintroduction success after just one year. After one year, the plants that survived (81%) were able to grow and flower. However, ongoing monitoring on the number and condition of reintroduced individuals is required, and the population must be supplied with new plants as needed until *S. lapponum* seedlings appear naturally in the environment, providing evidence of reintroduction success. This monitoring should also cover the habitat, both abiotic and biocenotic factors, as these directly or indirectly affect plant growth and development and thus the success of active conservation of the species.

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