



*Michał Arciszewski**, *Magdalena Pogorzelec*,
Barbara Hawrylak Nowak, *Marzena Parzymies*, *Mateusz Piejak*

Towards successful reintroduction of *Salix myrtilloides*: the importance of monitoring plant physiological indicators during acclimatization

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Abstract: *Salix myrtilloides* L. is a relict species in Poland, considered to be particularly endangered, for which it is necessary to carry out active protection through reintroduction and the replenishment of existing populations. The aim of the study was to test the capacity of *S. myrtilloides* plants obtained in *in vitro* cultures for effective acclimatization (under both laboratory and field conditions) during the translocation process. In order to trace the response of individuals to changes in environmental conditions on their way from the laboratory to the intended natural habitat, research was carried out on the selected parameters of the functioning of the photosynthetic apparatus (chlorophyll *a* fluorescence, concentration of photosynthetic pigments in plant tissues), the response of plants to environmental stress (accumulation of reactive oxygen species; ROS) and the variability of biometric traits during their growth (rate of daily stem growth). At each stage of the study, verification was carried out on the number of *S. myrtilloides* plantlets that survived the successive stages of acclimatization and the 12-month growth period in the natural stand. The results of the study reveal that the physiological condition of *S. myrtilloides* regenerants developed in tissue culture is dependent on changes in environmental conditions during acclimatization, and that the first few days of each stage of this process are crucial and most difficult for the plants. An elevated accumulation of ROS in leaf tissue and lower photosystem II (PS II) efficiency may indicate increased level of stress. However, these changes may also serve as signals to initiate metabolic, physiological and developmental processes crucial for proper plant acclimatization. Analysis of the results provides important clues for optimising this process by varying the duration of the various stages.

Keywords: swamp willow, ex situ conservation, photosynthetic pigments, chlorophyll fluorescence, ROS

Addresses: M. Arciszewski, M. Pogorzelec, M. Piejak, Department of Hydrobiology and Protection of Ecosystems, University of Life Sciences in Lublin, Dobrzańskiego 37, 20-626 Lublin, Poland; MA  <https://orcid.org/0000-0002-1395-1169>, e-mail: michal.arciszewski@up.lublin.pl; MPo  <https://orcid.org/0000-0003-2584-2186>, e-mail: magdalena.pogorzelec@up.lublin.pl; MPi e-mail: piejak1111100@gmail.com;

B. Hawrylak-Nowak, Department of Botany and Plant Physiology, University of Life Sciences in Lublin, Akademicka 15, 20-950 Lublin, Poland;  <https://orcid.org/0000-0002-2935-1491>, e-mail: barbara.nowak@up.lublin.pl;

M. Parzymies, Institute of Horticulture Production, University of Life Sciences in Lublin, Głęboka 28, 20-612 Lublin, Poland;  <https://orcid.org/0000-0002-4835-3804>, e-mail: marzena.parzymies@up.lublin.pl

* corresponding author

Introduction

Among the most endangered species of flora, climate relics are very frequently mentioned, especially those whose populations function far from their range, in isolated habitats undergoing systematic and irreversible changes. Species inhabiting natural peatland ecosystems make up a large proportion of this group, and among these, boreal relics (Kruszelnicki & Gostyńska-Jakuszevska, 2014). In the course of their evolution many plant species associated with wetlands have developed unique adaptations to be able to function in these specific environmental conditions. However, a high degree of specialization also increases the potential risk of extinction, especially in the case of sudden or progressive changes taking place in the habitat (Minayeva & Sirin, 2012). The impoverishment of biodiversity which is occurring on a global scale and the climate crisis have created an urgent need for action to protect as many species and entire ecosystems as possible (Bartholomeus et al., 2011; Reiter et al., 2016).

Besides standard *in situ* and *ex situ* species conservation methods widely practiced by organizations and services associated with nature conservation, an alternative is to preserve the gene resources of endangered species by keeping a gene bank, renewing resources through the creation of tissue cultures, and introducing species to horticultural cultivation (Dias et al., 2011; Osório et al., 2013; Gonçalves et al., 2017; Volis, 2017). Native species, owing to the specific structural features, forms of growth, habitat preferences, and adaptability, are predisposed to function as elements of nature-friendly gardens which fit well into regional landscapes. The use of native flora to create naturalistic plantings is a very important chapter in the history of horticulture (Zachariasz, 2007). The introduction of native plant collections to professional as well as amateur cultivation is currently becoming popular (Heywood, 2003).

Wetlands and water bodies are habitats abundant in plant species with specific requirements, but also with ornamental potential and often medicinal properties as well. These include grasses and sedges, as perennials, but also trees and shrubs. Many of these species are currently under legal protection, because their population sizes have decreased significantly in recent years. Bringing them into cultivation provides the opportunity to preserve the gene pool but also to learn about and make use of their pharmacological properties as well as their ornamental value (Serafin et al., 2017; 2018; 2023).

The swamp willow (*Salix myrtilloides* L.) is a short shrub with small, blue-green leaves coated with a layer of wax. The main stem and side shoots are thin and straight, initially bare and yellowish brown or red, changing over time to grey or reddish-brown.

The species is dioecious, both insect- and wind-pollinated. The swamp willow is a sun-loving species which occurs naturally in flooded and unforested transitional peat bogs in lowlands and foothills; it prefers acidic peat with pH 3.5–5.5 as a substrate (Churski & Danielewicz, 2008; Serafin et al., 2015). *S. myrtilloides* is considered an endangered relic species in many countries of Central Europe and is found mainly in isolated stands (Kozel et al., 2022). Its populations have been systematically declining in recent decades (Pogorzelec et al., 2015). For this reason, attempts at active conservation of the species using translocations methods have recently been made (Arciszewski et al., 2023). The most important step was successful attempts at micropropagation of specimens from material taken from natural stands (Parzymies et al., 2023).

The acclimatization process of plants derived from tissue cultures is aimed at adapting them to the prevailing atmospheric and soil conditions, which significantly improves the chances of normal growth and development later on. The condition of plants determined on the basis of variation in certain morpho-physiological traits may indicate its critical points at each stage of acclimatization (Pogorzelec et al., 2022). Their precise determination can help to make the introduction in *ex situ* cultivation or reintroduction more successful and to ensure the long-term survival of the new populations. In addition, determination of the physiological condition of the species during translocation can help to estimate the ongoing risk associated with the effectiveness of the procedures. Certain physiological parameters of plants, such as changes in the content of photosynthetic pigments, the functioning and efficiency of the photosynthetic apparatus, or the accumulation of reactive oxygen species (ROS), can provide important information indicating early biochemical and physiological modifications even before significant changes in phenological and morphological parameters appear (Schönbeck et al., 2023; Carevic, 2016). Well-designed scientific experiments which include testing of research hypotheses improve the chances of perfecting protocols for the development of guidelines for carrying out specific procedures during the plant translocation process (Guerrant & Kaye, 2007; Kaye, 2008).

Two species of relic willows (*Salix myrtilloides* and *Salix lapponum*) occurring in Poland, despite their common origin, differ significantly not only in terms of morphology, but also in terms of tolerance ranges to various environmental factors (Kruszelnicki & Gostyńska-Jakuszevska, 2014). Specific requirements and related adaptive abilities certainly affect the efficiency of acclimatization and survival of individuals of both species in alternative locations to which they are transferred. Previous studies

conducted by Pogorzelec et al. (2022) allowed for the specification of the conditions of acclimatization of *Salix lapponum* plantlets produced in the process of micropropagation, both in laboratory and field conditions. In the case of *Salix myrtilloides*, it is equally important to develop effective methods and determine the optimal conditions for acclimatization of seedlings. In the case of plants threatened with extinction, it is necessary to develop precise procedures used during translocation to minimize the risk of losses of valuable plants.

The aim of the study was to test the capacity of *S. myrtilloides* plants obtained in *in vitro* cultures for effective acclimatization. Selected morpho-physiological traits of regenerants and their variability in different environmental conditions were used as diagnostic indicators to follow the phenomena taking place along the path travelled by *S. myrtilloides* from the laboratory to the target habitat during the reintroduction process. The following research hypotheses were put forth: (1) a change in the acclimatization conditions of *S. myrtilloides* affects the content of photosynthetic pigments and the functioning of the photosynthetic apparatus, particularly the activity of photosystem II (PSII); (2) accumulation of ROS in the leaves can be a useful indicator of the intensity of stress symptoms influencing the physiological condition of *S. myrtilloides* at various stages of acclimatization; (3) assessment of selected parameters of photosynthetic activity of PSII and the level of oxidative stress makes it possible to identify the period when homeostasis is established in plants and they are ready for the next stages of acclimatization.

Materials and Methods

The research object was *S. myrtilloides* regenerants obtained by micropropagation in the Tissue Culture Laboratory of the Institute of Horticultural Production, University of Life Sciences in Lublin. The studies were conducted in 2022–2023. The 100 plantlets used in the research were clones of plants from a natural population of the species, functioning in a stand at the Lake Bikcze (51°22'46.3"N; 23°2'35.17"E; Łęczna-Włodawa Plain, eastern Poland). The plants were obtained in the laboratory using micropropagation methods which had been developed and optimized for the species (Parzymies et al., 2023). After the plants had developed a root system in the growth substrate, they were transplanted to pots with a specially composed substrate and placed in a phytotron in controlled temperature (day/night 22 °C/20 °C), humidity (60%) and light conditions (35 $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ with the 16/8 hour light/dark cycle) (acclimatization in the laboratory, according to the procedure described for *S. lapponum*

by Pogorzelec et al. (2020). There they were pruned several times to stimulate the production of lateral shoots. To prepare the plants for growth in natural conditions, after about three months, in mid-May, the plantlets were transferred to cold frames with a substrate of sand and peat, with a net for protection against insects, where they spent another two months outside the laboratory (average temperature 20 °C, average precipitation 50 mm, average day length 15 h during field acclimatization, at the plant acclimatization station). The final stage of the acclimatization process was to transport the plants to the previously determined location and plant them in the natural stand, suitable for the studied species, which was a raised bog located in a protected area – Poleski National Park (51°27'8.96"N; 23°13'43.71"E). Conditions at the reintroduction site during first 14 days after planting the plantlets: average temperature 20 °C/12 °C – day/night, average precipitation 80 mm, average day length 16 h (the data comes from the meteorological station located at the plant acclimatization station; Fig. 1).

Content of photosynthetic pigments

At each stage of the acclimatization process the content of chlorophyll *a* and *b* and carotenoids was determined in the leaves of 10 randomly selected specimens. Plant material was analysed: (i) four times in conditions of laboratory acclimatization, at weekly intervals from the start of acclimatization; (ii) twice during field acclimatization, 1 day and 14 days after the plants were transferred from the laboratory; and (iii) twice in natural stands, after 14 days and 12 months.

Leaf from the middle part of the shoots were homogenized in 96% ethanol for pigment extraction. The homogenates were then placed in a water bath for 5 min, after which the liquid phase was separated from the solid phase using a centrifuge (9 279 × g). A spectrophotometer (SPECORD 40, Analytik Jena GmbH, Germany) was used to measure the absorbance of the solutions at three wavelengths (470, 649 and 665 nm). The content of pigments was calculated using formulas given by Lichtenthaler & Wellburn (1983).

Selected parameters of chlorophyll *a* fluorescence

To assess the effect of variable habitat conditions on the physiological response of *S. myrtilloides*, chlorophyll *a* fluorescence was measured at each stage of acclimatization to monitor changes in the efficiency of photosystem II (on leaves located in the middle of the shoot in 10 randomly selected specimens).

Measurements were made on plants (i) transferred from *in vitro* cultures to the soil substrate (5 weeks and 3 months after transplantation; due to the small size of young plantlets there was no physical possibility to take measurements before the 5th week of their growth); (ii) in the group of plantlets undergoing field acclimatization (after 1 and 14 days and after 1, 1.5 and 2 months of growth in cold frames); and (iii) on plants transferred to the natural habitat (14 days and 12 months after planting at the sites). In the case of plants growing in the laboratory, at the acclimatization station and those introduced to

natural sites, measurements were carried out in the morning, from 9 to 11 a.m.

Chlorophyll fluorescence was measured using a fluorimeter (Handy PEA fluorimeter, Hanstech Instruments, UK). The following parameters of chlorophyll *a* fluorescence were analysed: the minimum (F_0) and maximum (F_m) possible level of chlorophyll fluorescence and the maximum quantum yield of PS II (F_v / F_m , where $F_v = F_m - F_0$). Prior to the measurements, special clips eliminating access to light for 15 minutes were placed on the leaves.

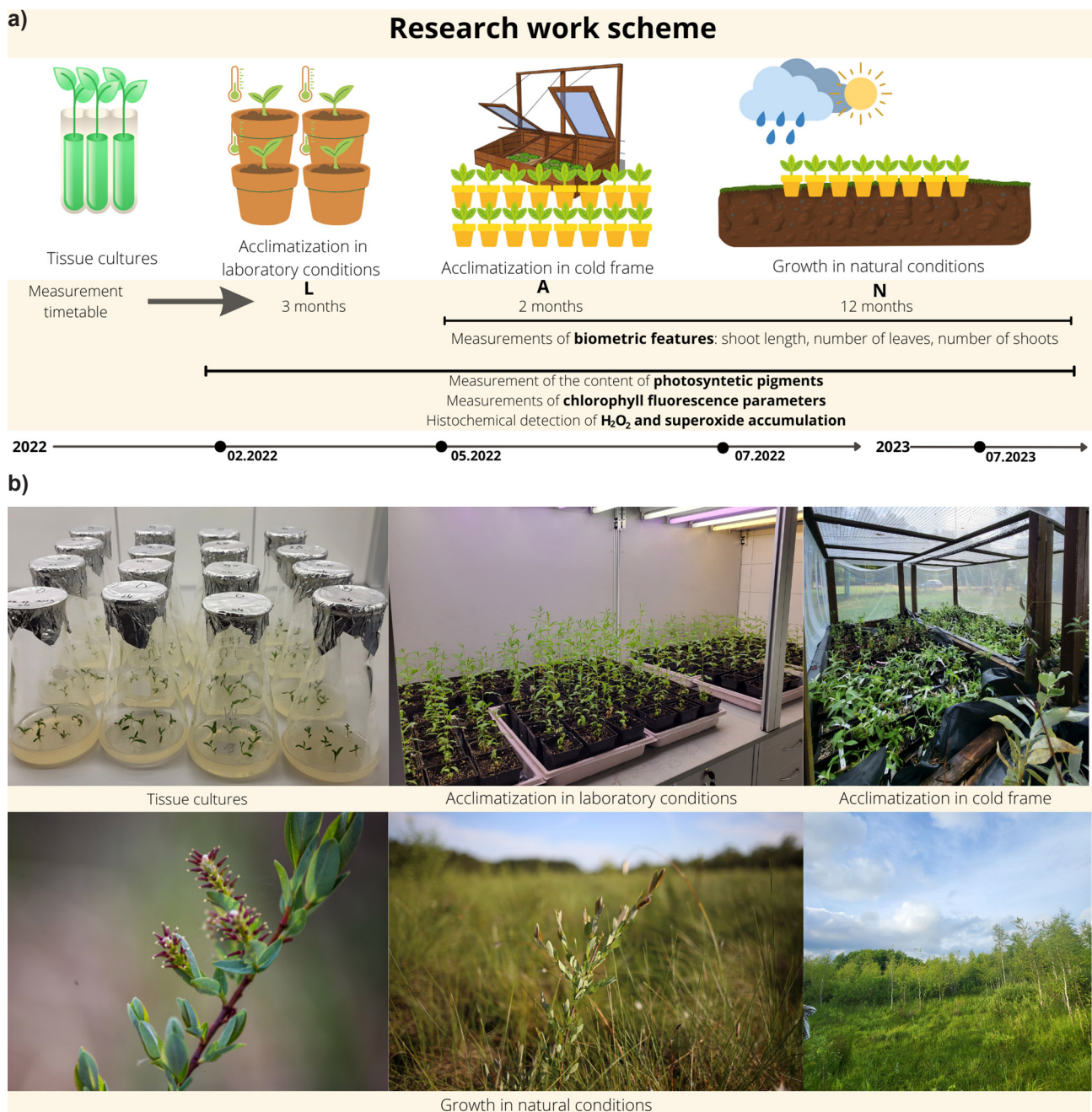


Fig. 1. Research design, taking into account the stages of *Salix myrtilloides* acclimatization and plant analyses (a). Photographic documentation of the reintroduction process (b)

Histochemical detection of H₂O₂ and superoxide anion

Histochemical analyses of the accumulation of H₂O₂ and O₂^{•-} in the tissues of *S. myrtilloides* leaves were carried out according to the method described by Kumar et al. (2014). Leaves were taken from the middle part of the shoot from plants at specific stages of acclimatization: (i) after 3 months of acclimatization in the laboratory, (ii) after the beginning of acclimatization using cold frame and after 1 month of growth in the cold frame, and (iii) 2 weeks and 12 months after transfer to the natural stand (the same times as for chlorophyll fluorescence measurements, environmental sampling took place in the morning, between 9 and 11 a.m.). Then the plant material was placed in test tubes filled with distilled water and immediately taken to the laboratory. H₂O₂ detection was carried out using 3'3-diaminobenzidine (DAB). The leaves were immersed in a DAB solution (1 mg/mL) for 10–12 h and placed in the dark at room temperature. After that they were immersed in boiling water and then in hot 80% ethanol to remove chlorophyll from the tissues and visualize the brown product of DAB polymerization taking place in the presence of H₂O₂.

Nitrotetrazolium blue chloride (NBT) was used to visualize O₂^{•-} in the leaf tissues. The oxidized form of NBT (yellow) is reduced by endogenous O₂^{•-} to the diformazan form (bluish purple). The leaves were immersed in 0.2% NBT solution in phosphate buffer (50 mM, pH = 7.5) and placed in the dark for 10–12 h. Then they were decolorized in hot 80% ethanol until the reduced, bluish-purple form of the pigment became visible.

About 10 leaves were stained for each of the experimental combinations, and 3–4 of the most representative leaves were chosen for photographic documentation.

Selected morphological traits of plants

To determine the daily growth rate of the plant shoots, their height was measured with a ruler (from the base of the plantlet to the top of the shoot). The total number of leaves and the number of side shoots was also determined on individual specimens. The measurements were initially made on the group of plantlets in the field acclimatization stage for a period of seven weeks, at seven-day intervals. In the next stage, the plants were divided into two groups: the first was transferred to a natural stand, while the other was left in the cold frames so that the growth rate of the two groups could be compared. Measurements of the previously determined morphological traits were made four times at 14-day intervals for plants from both groups.

Verification of the success of the translocation procedures

The number of *S. myrtilloides* plantlets which survived each stage of acclimatization and the 12-month period of growth at the natural stand was determined. Their percentage share was determined according to the following formula:

$$\text{Survival rate (\%)} = \frac{\text{number of surviving plantlets}}{\text{number of plantlets reintroduced}} \times 100$$

Statistical analyses

The Shapiro-Wilk test was used to check the normality of the numerical data distribution. Then one-way analysis of variance (ANOVA) was carried out to identify statistically significant differences between groups. In case the differences were significant, the post-hoc Tukey test was performed. All analyses were carried out for a significance level of $\alpha = 0.05$ using STATISTICA 13.3 software (TIBCO Software Inc. 2017, Palo Alto, CA, USA).

Results and Discussion

In the face of increasingly unpredictable changes in habitat conditions induced by climate change (both natural and anthropogenic) and deliberate human interference in natural ecosystems, the capacity of organisms for acclimatization and adaptation are crucial for their survival in the natural environment. In the case of endangered species, analysis of processes related to acclimatization on morphophysiological traits can provide valuable information on adaptation strategies and potential (Anderson & Song, 2020). A variety of physicochemical factors (light, soil moisture, etc.), and biocenotic factors (intra- and interspecific interactions) together create a complex mosaic of conditions which individual specimens encounter in their natural environment and which are confronted with the body's specific reaction to stress. The success of conservation procedures is to a great extent determined by basic knowledge as well as in-depth knowledge of the factors limiting plant growth and development, both in the natural environment and in *ex situ* conditions (Asgher et al., 2020; Liu et al., 2006; Gailite et al., 2023; Bose et al., 2022).

The results of ecophysiological tests conducted for the first time on *S. myrtilloides* regenerates obtained in *in vitro* conditions and later grown and acclimatized *ex vitro*, provided a broader picture of the response of this species to the variable conditions of the surrounding environment during the long process from the laboratory to the natural habitats.

The measurements of photosynthetic pigment content in the *S. myrtilloides* leaves, performed over four weeks in laboratory acclimatization conditions, revealed a systematic increase in the concentrations of chlorophyll *a* and *b* in the plant tissues. However, no significant changes were noted in the content of carotenoids (Fig. 2). This may have been due to inhibition of dynamic growth stimulated in *in vitro* conditions by the components of the growth medium. The plants most likely begin intensive photosynthesis at that time, which may be linked to enhanced production of photosynthetic pigments (Guan et al., 2008).

The change in conditions from laboratory acclimatization to field acclimatization also influenced the content of pigments in the species. Following transfer from the phytotron to the field acclimatization station, a statistically significant decrease in Chl *b* level was observed between the plantlets in the fourth week of laboratory acclimatization and those at the beginning of acclimatization in cold frame. In consequence, the Chl *a/b* ratio increased as well. A similar response of plants at this stage of acclimatization was observed in grapevine (*Vitis vinifera*) and wild olive (*Olea madarensis*). It has been suggested that the change in light conditions and exposure of plants to high intensity of this factor can lead to the phenomenon of photoinhibition or even photooxidation of chlorophyll (Amâncio et al., 1999; Brito et al., 2009). After two weeks of field acclimatization, a significant increase in the concentration of all photosynthetic pigments was noted in the *S. myrtilloides* leaves in comparison to the initial period. This was followed by a marked decrease in the concentrations of pigments in the plants transferred to the natural habitat. Under natural light conditions, despite a decrease in individual pigments, the Chl *a/b* ratio gradually

increases, reaching its highest value after 12 months. This may indicate that the plant is fully adapted to intense natural light, where the dominance of chlorophyll *a* allows maximum photosynthetic efficiency in the full spectrum of sunlight (Nyongesah et al., 2015; Fig. 2).

Depending on the conditions in which they function, plants efficiently adjust the content of photosynthetic pigments in the leaf tissues in order to optimize the course of photosynthesis (Grzeszczuk et al., 2018). A change in environmental conditions often induces stress responses in plants, which can disturb chlorophyll synthesis or the quantitative relationships between groups of pigments (Mane et al., 2010). The change in light conditions (from constant light in laboratory conditions to the natural photoperiod in field acclimatization) and the acceleration of the growth rate (increase in shoot length) observed at this stage of acclimatization may have been factors influencing the content of photosynthetic pigments in *S. myrtilloides*. During field acclimatization plants are exposed to frequent and unpredictable changes in temperature, light intensity, and substrate hydration. These factors can significantly modify both the biosynthesis/degradation of pigments and the intensity of photosynthesis (Osório et al., 2013).

In a normally functioning photosynthetic system, the F_v/F_m index indicating the maximum quantum yield of the PSII photosystem should range from 0.800 to 0.830 in most plant species (Murchie & Lawson, 2013). Analysis of parameters of chlorophyll fluorescence in the *S. myrtilloides* specimens in laboratory acclimatization conditions revealed that five weeks after the plants had been transferred from *in vitro* cultures to a soil substrate, the F_v / F_m value approach the optimal level. However, it was only after three months that the value exceed the threshold of 0.800. After this time, there was a statistically significant decrease in the minimum fluorescence value (F_0), and slight reduction in the maximum fluorescence (F_m ; Fig. 3). It can be concluded that extending the acclimatization period for *S. myrtilloides* in the phytotron to three months may increase the photosynthetic efficiency of the plantlets and thereby positively influence their overall condition at later stages of translocation.

Fluctuations in access to light, ambient temperature, humidity, and other environmental factors may affect the ability of plants to carry out photosynthesis and its efficiency (Brito et al., 2009). Measurements of chlorophyll fluorescence in the leaves of *S. myrtilloides* plants transferred from laboratory conditions to field acclimatization showed that the most favourable values for this parameter were noted after the plants had been in the cold frames for one month. In the case of the species *S. lapponum*, studied previously, the maximum quantum yield of PSII (F_v / F_m)

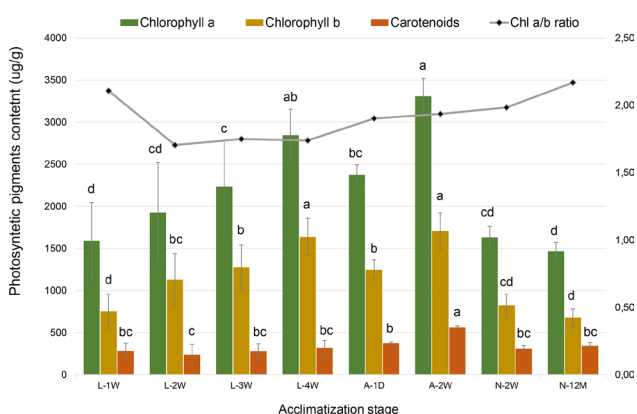


Fig. 2. Concentrations of photosynthetic pigments in *Salix myrtilloides* leaves at specific stages of plant acclimatization (L – laboratory acclimatization, A – acclimatization in cold frame, N – acclimatization in natural stand; D – day, W – week, M – month). Mean values (\pm standard deviation; $n = 10$) for each class of pigments marked with different letters are significantly different ($p < 0.05$)

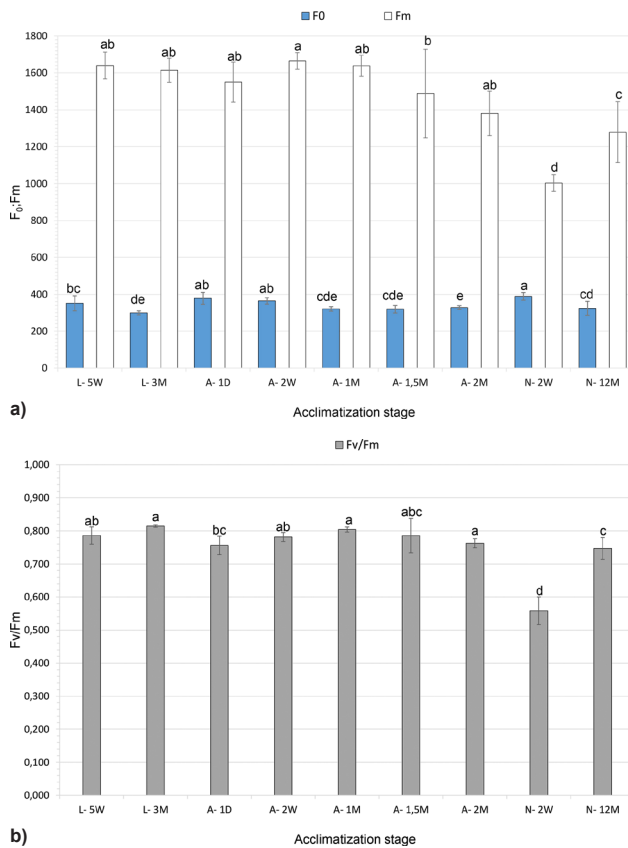


Fig. 3. Selected parameters of chlorophyll *a* fluorescence: (a) minimum fluorescence (F_0) and maximum fluorescence (F_m) value (b) maximum quantum yield of PSII photochemistry (F_v / F_m) in *Salix myrtilloides* leaves at each stage of acclimatization (L – laboratory acclimatization, A – acclimatization in cold frame, N – acclimatization in natural stand; D – day, W – week, M – month). Mean values (\pm standard deviation; $n = 10$) marked with different letters are significantly different ($p < 0.05$)

was noted after about 1.5 months of growth in cold frames (Pogorzelec et al. 2022). Extending the acclimatization time for *S. myrtilloides* resulted in an F_v / F_m value below the expected minimum, and after 1.5–2 months at the acclimatization station their photosynthetic efficiency began to decrease slightly, showing a downward trend (Fig. 3).

In the case of *S. myrtilloides*, as noted previously in *S. lapponum*, 14 days after the plants had been transferred to natural stands, the parameters of maximum fluorescence (F_m) and F_v / F_m values chlorophyll fluorescence had deteriorated (Fig. 3). Since chlorophyll fluorescence is related to the state of photosynthesis and thus to the chlorophyll *a* content (Gan et al., 2023), the observed decrease in PS II efficiency may be caused by significant decrease in chlorophyll *a* concentration during this period. This changes can unquestionably be linked to the dramatic change in environmental conditions, where an increase in F_0 , accompanied by a decrease in F_m , is usually associated

with stress responses (Mathur et al., 2011). Similarly, Percival (2005) observed that the oak species' response to heat stress resulted in significant damage to PSII, as evidenced by a marked decrease in F_v / F_m values along with an increase in F_0 , indicating that such physiological responses might be a common reaction of plants to sudden environmental changes. The results of monitoring of the condition of *S. myrtilloides* specimens that had been in the natural stand for 12 months showed significant improvement in chlorophyll fluorescence parameters. However, as previously noted for *S. lapponum* (Pogorzelec et al., 2022), their efficiency of PSII was lower than in the specimens growing in conditions of laboratory and field acclimatization. This confirms the tendency towards substantial intensification of physiological processes associated with plant growth during their acclimatization.

The results of the statistical analysis indicate differences in F_v / F_m value when the stage of acclimatization changed from laboratory to field and after the plants were transferred from cold frames to natural habitats. This may indicate that the initial period (a few days), just after the change in the conditions of the growth environment, is a critical point of acclimatization, when significant changes are noted not only in the chlorophyll fluorescence, but also in the level of oxidative stress, which is confirmed by the histochemical analyses (visualization of ROS – H_2O_2 and $O_2^{\cdot -}$). In the leaves of plants acclimatized in the laboratory for about three months, increased accumulation of $O_2^{\cdot -}$ was noted over the entire surface of the leaf. On the other hand, H_2O_2 accumulated in small amounts only within the main vascular bundles (Fig. 4), which may have been due to mechanical damage to the leaf petioles when they were cut from the parent plant and the associated secondary oxidative stress. In the second stage, ROS accumulation in plants transferred from the phytotron to field acclimatization was visualized. The results obtained at this stage of the study indicate that after one day in the cold frames severe oxidative stress takes place in the plants, associated in particular with excessive accumulation of $O_2^{\cdot -}$ within the entire leaf blade. Local, point accumulation of H_2O_2 was observed as well. After a month of acclimatization in cold frames, oxidative stress subsided, and the accumulation of both ROS forms tested was much lower than after one day (Fig. 4), which was also confirmed by the improved parameters of chlorophyll fluorescence (Fig. 3).

ROS in the leaves of plants growing in natural conditions was visualized among specimens two weeks after reintroduction and also after 12 months in those that had survived the winter. Two weeks after transfer from the cold frames to the natural stand, there was significant ROS accumulation indicating increased oxidative stress. In the specimens that

survived the winter, accumulation of $O_2^{\cdot-}$ and H_2O_2 decreased, as indicated by the less intensive blue (in the case of $O_2^{\cdot-}$) or brown (H_2O_2) colour of the leaf blades. Visualization of ROS in *S. lapponum* specimens examined at the same stages of the acclimatization process as *S. myrtilloides* shows substantial similarity in accumulation of $O_2^{\cdot-}$ and H_2O_2 in the leaf tissues. Marked differences were noted only at the first stage of the process – in laboratory conditions increased accumulation of $O_2^{\cdot-}$ was observed over the entire surface of the leaf blade in *S. myrtilloides* (Fig. 4), while in *S. lapponum* it accumulated only in the main vascular bundles (Pogorzelec et al., 2022).

Here it is important to highlight the special function of ROS in plants as ubiquitous signalling and regulatory molecules with a significant metabolic function, which are also involved in acclimatization processes; their role in the early stages of signal transduction initiated by environmental stimuli and stress factors is well documented (Waszczak et al., 2018). For this reason, the substantially increased accumulation of these compounds in the cells in response to the initially drastic change in living conditions during individual stages of acclimatization, despite the induction of oxidative stress, may allow the plants to activate coordinated signalling networks regulated by ROS, so that the homeostasis can be restored

Plant growth conditions	$O_2^{\cdot-}$	H_2O_2
L-3M		
A-1D		
A-1M		
N-2W		
N-12M		

Fig. 4. Visualization of reactive oxygen species (ROS) in *Salix myrtilloides* leaf tissues at each stage of acclimatization (L – laboratory acclimatization, A – acclimatization in cold frame, N – acclimatization in natural stand; D – day, W – week, M – month)

as quickly as possible and the plants can adapt and become acclimatized to the surrounding conditions (Dietz et al., 2016; Devireddy et al., 2021).

During the observations made for this publication, the length of the main shoots of *S. myrtilloides* plantlets growing in cold frames, increased on average by 5 cm. The greatest daily increase was noted in the second month of field acclimatization and amounted to 1.74 mm/day. This confirms the intensification of the processes determining growth which take place during field acclimatization. In the weeks after the plantlets had been separated into two groups, the growth rate of the plants was lower, in the case of both plants left in the cold frame (1.67 mm/day) and those growing in the natural stands (on average 0.57 mm/day; Fig. 5). No significant changes were observed in the number of side shoots in the two groups. The average number of leaves underwent changes during the study, but these were associated mainly with the death of old organs and the appearance of young ones (data not shown).

Biometric traits and their modifications over time may be indicative of the condition of plants, which changes under the influence of environmental conditions. In the case of *S. lapponum*, as in the case of *S. myrtilloides*, the growth rate was also highest in the second month of field acclimatization, but was as high as 6 mm/day. Differences in leaf morphology were also clearly evident in *S. lapponum*, while the characteristic traits of the species (shape, size, and tomentum) did not become visible until after several months of growth in natural conditions (Pogorzelec et al., 2022). This phenomenon was not observed in the *S. myrtilloides* specimens.

In the study carried out in 2022–2023 on *Salix myrtilloides*, the percentage share of plants that survived each stage of acclimatization was determined. During three months of growth in the laboratory, 5% of all plantlets died. No losses were recorded during

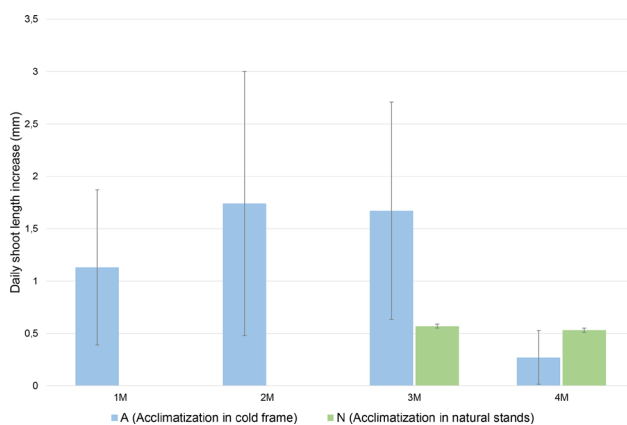


Fig. 5. Daily shoot length increase of *Salix myrtilloides* individuals during successive stages of acclimatization. (M – month). Mean values (\pm standard deviation; $n = 9$)

the two-month field acclimatization or in the group of plants that remained in the cold frames for another eight weeks. After 12 months of growth in the natural stand, the survival of 79% of specimens from the group of 48 plants was noted. Literature data indicate that if even 50% of specimens survive the first growing season after translocation the result is considered satisfactory, or even very good in the case of woody plants (Fenu et al., 2019).

It should be stressed that ecophysiological study of the species *S. myrtilloides* has not previously been conducted, although its biology and elements of its population ecology are fairly well known, especially at the edge of the species' range. The first attempts at conservation of the species by translocation (reintroduction) in combination with *ex situ* cultivation have been made as well (Arciszewski et al., 2023; Zalewska et al., 2019; Pogorzelec et al., 2015; 2016; Parzymies et al., 2023; Serafin et al., 2015). The results of the current study, carried out specifically for this publication in 2022–2023 significantly expand knowledge of the responses of plants to stress associated with a change in living environment, but will also influence the planning of individual stages of conservation procedures for the species in the future.

Conclusion

The results of the study showed that the physiological condition of acclimatized *S. myrtilloides* specimens depended on changes in the conditions of the plant environment. Indicators of oxidative stress and photosynthesis efficiency as well as the biometric traits of plants changed during each stage of their transition from laboratory conditions to the natural habitat. The values for the parameters noted during the study provide important pointers for optimizing the acclimatization process of *S. myrtilloides* regenerants obtained in tissue cultures. For example, they suggest that extending the acclimatization period in the phytotron to three months and shortening field acclimatization to one month may increase the photosystem II efficiency of plants and restore ROS homeostasis, thereby positively influencing their overall condition at further stages of translocation. It was also noted that the first few days after the change in environmental conditions at each stage of growth are crucial and the most difficult for plants. This is manifested in a decline in efficiency of PSII, but also accumulation of ROS in the leaf tissues, which on the one hand indicates increased oxidative stress, but on the other hand may be essential for efficient signalling and activation of numerous metabolic, physiological and developmental processes crucial to acclimatization. The intensive response of plants to stress factors during this period may allow the plants

to activate mechanisms aimed at restoring homeostasis and to undertake growth and development processes.

An optimized, multi-stage acclimatization process guarantees the normal growth and development of plants in the conditions of natural habitats. This is evidenced by the final outcome of the study carried out in 2022–2023 study, i.e. the survival of as many as 79% reintroduced *S. myrtilloides* individuals from *ex situ* cultivation for a period of 12 months.

Author Contributions

Conceptualization, M.A. and M.P. (Magdalena Pogorzelec); methodology, M.A. M.P. (Magdalena Pogorzelec) B.H.-N.; validation, B.H.-N. and M.P. (Marzena Parzymies); formal analysis, M.A., M.P. (Mateusz Piejak); investigation, M.A., M.P. (Magdalena Pogorzelec), B.H.-N. M.P. (Marzena Parzymies), M.P. (Mateusz Piejak); data curation, M.A.; writing—original draft preparation, M.A.; writing—review and editing, M.P. (Magdalena Pogorzelec), B.H.-N., M.P. (Marzena Parzymies), M.P. (Mateusz Piejak); visualization, M.A. and B.H.-N.; supervision, M.A. All authors have read and agreed to the published version of the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

Founding

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