Differences between ex-situ and in situ germination and seedling establishment in the case of the marsh gladiolus (*Gladiolus palustris*)

Különbségek ex-situ és in situ csírázásban és a csíranövények növekedésében a mocsári kardvirág (Gladiolus palustris) esetében

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Abstract: The marsh gladiolus (*Gladiolus palustris* Gaud.) is a species of community interest in the European Union, and it's strictly protected by law in Hungary. Survival of its populations is threatened by a number of factors, so thorough knowledge on the biology of the species is required for its conservation and long-time survival. To interpret the data collected in local populations, it is essential to examine the dynamics between age-stage categories and study germination rates of seeds in the populations. For this purpose, ex-situ and in-situ germination biology studies were set up to monitor the development of individuals starting from germination and establish a suitable age-stage categorization system for demographic purposes. During the autumn of 2023, seeds were sown in an open forest and in a meadow (in-situ), and seeds from these sites were sown in containers (ex-situ) at the same time. Germination rates were considerably higher in the ex-situ sowing than in the in-situ experiment for seeds of both origins. Moreover, there were significant differences between the average height (leaf length) of the seedlings. Under in-situ conditions average height was significantly larger in the open forest than in the meadows. There were significant differences between average height of seedlings in the in-situ and ex-situ experiments only during the first measurement, which differences became less pronounced later on.

Keywords: *endangered plants; recruitment; population biology; age-state; Gladiolus palustris*

Összefoglalás: A mocsári kardvirág (*Gladiolus palustris* Gaud.) az Európai Unióban veszélyeztetett, a hazai flóra fokozottan védett faja. Az állományok fennmaradását több tényező is veszélyezteti, így a faj biológiájának minél mélyebb ismerete rendkívül fontos a faj védelme és fennmaradása szempontjából. A hazai állományokban gyűjtött adatok értelmezéséhez elengedhetetlen a korállapot-kategóriák közötti átmenetek vizsgálata és a faj állományaiban a magok csírázóképességének megállapítása. Ennek érdekében ex-situ és in-situ csírázásbiológiai vizsgálatokat állítottunk be, hogy az egyedek fejlődését a csírázástól kezdve nyomon követhessük, illetve felállítsunk egy alkalmas rendszert az egyedeknek korállapot-kategóriákba való besorolásához. 2023 őszén két termőhelyen, egy nyílt erdőben és egy kiszáradó lápréten végeztünk in situ vetést, valamint az ezekről a termőhelyekről származó magokat ládákban is (ex situ) elvetettük. A csírázási arány az ex-situ kísérletben jelentősen magasabb volt, mint az in-situ vetésben mindkét termőhelyről származó magok esetében, továbbá a csíranövények magasságában (levélhosszában) is szignifikáns különbségeket tapasztaltunk. In situ körülmények között a magoncok átlagos magassága szignifikánsan nagyobb volt az erdőben, mint a réten. Az in situ és ex situ kísérletekben a magoncok átlagos magassága között csak fejlődésük kezdeti szakaszában figyeltünk meg szignifikáns különbséget, mely a későbbiekben mérséklődött.

***Kulcsszavak**: veszélyeztetett növényfaj; szaporodás; populációbiológia; korállapot-kategória; Gladiolus palustris*

1 Introduction

Marsh gladiolus or sword-lily (*Gladiolus palustris* Gaud.) is a 20–100 cm tall, perennial bulbous species, with 4–5 sword-shaped leaves. It has 3–6 bisexual rosy violet or magenta flowers that bloom in June–July in Hungary. *G. palustris* is native in Europe, its distribution ranges from eastern France to the southern Balkans (Vidéki & Máté, 2006). It has a declining population trend all across Europe and considered to be threatened in many countries (Bilz et al. 2011), and as a result, *G. palustris* became legally protected in the European Union (listed in Annexes II and IV of the Habitat Directive 92/43/EEC). It has been strictly protected by law in Hungary since 1982 (1/1982. (III. 15.) Annex 1 to the OKTH Regulation), conservation value of the species is HUF 250,000. According to Social Behaviour Types described by Borhidi (1995, it is categorized as a rare specialist.

While original habitats of this species may have been clearings and margins of open forests with varying water regime, today its largest populations live in meadows of anthropogenic origin. *G. palustris* prefers habitats alternately wet and dry, therefore in Hungary it occurs mostly on transitions between wet meadows and steppes in lowlands and hilly regions. In the Transdanubian region it has only two populations; one situated in an open birch forest and one in a *Molinia* meadow.

In the case of perennial herbaceous plants, such as *G. palustris*, it is difficult to determine the exact age of individuals, therefore age-state categorization is a more suitable approach for studying demographic characteristics of wild populations than the usage of actual age in years (Rabotnov, 1969; Harper, 1977; Silvertown et al., 1993; Vakhrameeva et al. 2008).

The potential 'age groups' or 'age-states' categories what were described by Rabotnov (1969) have been modified later in several ways by different authors, one of the better known being Gatsuk et al. (1980), who grouped age-states into ontogenetic periods.

In the case of *G. palustris*, the suitable age-state categorization has not been established yet, which makes it very difficult to interpret field data collected in order to support the protection of the species.

We started in-situ germination studies with the species in 2019 when we had sown seeds into plots, which we managed in different ways. When we set up our demography studies in 2023, has become necessary for the analysis and interpretation of our field data to define the characteristics of every age-state category applicable for *G. palustris*. In order to understand the life cycle of the species in detail, we carried out more detailed in-situ and ex-situ germination biology studies as well. Since in-situ germination experiments are carried out in the original habitats, these setups are suitable for population biology studies and age-state calibrations, but are difficult to manage and have uncertain outcomes, therefore we also started ex-situ germination experiments at the same time. In ex-situ experiments it is much easier to manipulate conditions and as we can almost freely choose location for the setups, it could also enable constant monitoring as well.

In-situ and ex-situ sowing was the first part of our population biology studies. Our goal was to study whether there were differences between germination of seeds and seedlings establishment originated from different environments (from an open forest and from and a hay meadow). Seeds originated from each site were sown in-situ and in containers (ex-situ) at the same time. Our short-term goal was to examine germination characteristics and our long-term goal was to follow the life history of the individuals developing from the seed sown.

We wanted to find answers to the following questions:

- Question 1: Are there differences in germination percentages between ex-situ and in-situ conditions?
- Question 2: Are there differences in germination percentages between seeds sown in forest and meadow habitats?
- Question 3: Are there differences in seedling heights between ex-situ and in-situ conditions?
- Question 4: Are there differences in seedling heights based on in-situ habitat conditions?
- Question 5.: Did seedlings that germinated earlier (so they had more time to grow) produce larger leaves?

2 Materials and Methods

As *G. palustris* is a strictly protected species in Hungary, therefore studying biology of the species required an official permission. This permission has been granted (File No: PE-KTFO/2595-13/2023.) prior the setup of our experiments.

2.1 Study areas and experimental garden of endangered species

Both in-situ sowing sites are located in the Balaton-felvidéki National Park. One woodland population was selected in Nyirádi Sár-álló, at the edge of an open birch forest with *Molinia caerulea* undergrowth, near the northern edge of a *Molinia* meadow, and one meadow population in the Pénzes-rét meadow near Raposka, where seeds were collected from a *Molinia* meadow habitat (*Succiso-Molinietum caeruleae*) (Figure 1).

Figure 1 *In-situ sowing sites at Nyirád (left), and at Raposka (right)*

In the experimental garden of Balaton-felvidéki National Park in Pécsely, the species has been grown in ex-situ conditions for several years (seeds originally sourced from Nyirád). The propagules produced here obliged by law to be returned to the original habitat. Therefore, we did not collect seeds in Nyirád (where the population is relatively small anyways), instead we used seeds and one-year old corms came from the experimental garden and we had to adapt the experimental setup to the amounts of propagules available.

2.2 In-situ sowing

Preliminary study

G. palustris corms were planted and seeds were sown at two sites in a *Molinia* meadow (not in the forest) at Nyirád in 2019. The distance between the original habitat and the two sites were 460 (Site 1) and 900 m (Site 2) respectively. The sites were receiving four different types of grassland managements as a part of an experiment: control (without treatment), mowed (cutting and removing the hay once in a year in July), topped (similarly as in 'mowing', but without collecting the hay) and burned (burned every 3 years in winter and topped in the other years). We set up sowing plots in every treatment type at both sites, so we had 2×4 sowing plots. The propagules were sown in 1 m × 1 m plots, each plot consisted of 5 rows. The distance between rows was 25 cm, the distance between seeds in a row was 2 cm.

We planted 22 corms in a mown area and 25 corms in a control area, sown 2×100 and 2×125 seeds in Site 1 and 4×125 seeds in Site 2. The 2 cm distance between seeds proved to be not enough; it was difficult to identify individuals over the years.

In all years after sowing (except 2022) we monitored the presence of individuals and measured their heights.

Sowing experiment

The sowing experiment was set up in the summer of 2023. One in-situ sowing area was designated on clearings in the open birch forest of Nyirádi Sár-álló. It was important that the species should be present in the surrounding habitat but absent in the close vicinity of the sowing plots.

Seeds collected from the Pécsely experimental garden were sown at four sites, 110 seeds overall in each plot (05.10.2023). The beginnings of the rows were marked with individually numbered nails and the end with unlabelled nails to simplify later retrieval. A distance of 20 cm was left between rows and 5 cm between seeds.

The other in-situ sowing area was set up near Raposka in a *Molinia* meadow. Seeds used for this setup were collected from the same meadow on the 17th of August in 2023. Seeds were sown not far from a *G. palustris* population, but the species was not present in the close vicinity of the plots, similarly as in Nyirád. 110 seeds were sown in each plot, in the same arrangement as in Nyirád, but with 6 replicates (16.10.2023).

2.3 Ex-situ sowing

Seeds were also sown in ex-situ conditions, in containers (24–25.10.2023), in total 150 seeds from the *Molinia* meadow near Raposka and 150 seeds from the Pécsely experimental garden. The containers were filled with soil from the original habitats (Raposka and Nyirád) and seeds were sown at a maximum depth of 0.5 cm and at a maximum distance of 3 cm between them. Throughout the study, the containers were kept in the open air, without controlling temperature and precipitation, but emerging weeds in the containers were removed.

2.4 Germination and seedling establishment

During the spring of 2024, we started following the germination and seedling establishment (Figure 2). In-situ sowings were examined twice (17.04.2024 and 23.05.2024), in both cases we checked the presence or absence of the seedlings and measured their height. Germination in the containers was checked on a weekly basis and the height of the seedlings was also measured on two occasions (13–14.04.2024 and 18.05.2024).

Figure 2 *Individuals germinated in spring 2024. Left: individual from in-situ sowing, right: from ex-situ sowing*

2.4 Data analysis

Procuring the experimental data was done with Microsoft Office Excel 2016 software, and analyses were done with R (version 4.3.1.). Normality of the datasets tested with Shapiro-tests, homogeneity of replicates tested with ANOVA, comparing the seedlings height were done with pairwise T-tests and connection between time of germination and final seedling length was examined with Pearson-correlation.

3 Results and Discussion

3.1 Preliminary study

In the preliminary study (Figure 3) corms were planted in 2 replicates, but data were collected successfully only from the mowed area, as the corms planted in the control area were destroyed by moles in 2020 and no plants have been found there since.

Figure 3 *Established plants arised from the preliminary study at the Nyirádi Sár-álló in 2023 (left) and in 2024 (right).*

During the 4 years of the preliminary study, we recorded 50% of the planted corms emerging at least once, while the rate of recoveries from sown seeds of the same condition was between 11 and 14,4%. Even in terms of all managed plots varied between 10.4 and 21.6% (Table 1). Therefore, corms had a noticeably higher survival rate than seedlings over the years.

Seedlings were found in almost all plots in the first year, most of which had disappeared later inlsl the majority of the plots, especially at Site 2 (Table 1). As numerous seeds could germinate in the first year but could not survive later, this may indicate that the species is highly habitat dependent: plants established better in Site 1 which is closer to the original habitat. Since from the control plot at Site 1 had all seedlings disappeared, we think that the litter (which accumulated in large amounts only in the control plots) also inhibited their survival. In actively treated areas we found developing plants 4 years after sowing, suggesting that seedlings prefer mown, topped and burnt areas instead of the undisturbed vegetation of control areas.

Table 1 Percentage of individuals recovered during the preliminary study. 100% means total number of seeds/corms sown at the site.

	Site	No. of seeds/corms sowed/planted	Germination rate (%)				
			2020	2021	2023	2024	total
Mowing Corms	1	22	31.82	0.00	22.73	18.18	50.00
Control Corms	1	25	0.00	0.00	0.00	0.00	0.00
Mowing Seeds	1	100	6.00	2.00	1.00	4.00	11.00
	2	125	14.40	0.00	0.00	0.00	14.40
Control Seeds	1	100	2.00	0.00	0.00	0.00	2.00
	2	125	12.80	6.40	0.00	1.60	17.60
Topped Seeds	1	125	14.40	5.60	2.40	4.80	21.60
	2	125	10.40	0.00	0.00	0.00	10.40
Burned Seeds	1	125	11.20	3.20	6.40	1.60	14.40
	2	125	13.60	0.00	0.80	0.00	13.60

3.2 Germination

At the in-situ site at Nyirád no seedlings were found during the first survey (17th April). During the second measurement (23rd May) 7 seedlings were found, resulting in a total of 1.59% germination. At Raposka we surveyed 102 seedlings during the first measurement. On the second occasion 22 new seedlings appeared, but 31 previously recorded individuals were not found, thus we surveyed 93 seedlings during the second survey altogether. A total of 124 seedlings were found at Raposka, which results in a 18.79% overall germination (Table 2), however, it cannot be ruled out that despite a careful search for seedlings, some still might be overlooked at both sites due to close resemblance between *Gladiolus* seedlings and young blades of several grass species.

Table 2 Number of individuals germinated in the first and second measurement and germination percentage.

area	seeds sown	no. of seedlings					germination percentage (%)
		1st survey	2nd survey			total	
		total	new	missing	total		
Raposka	6x110	102	22	31	93	124	18.79
Nyirád	4x110	0	7	-	7	7	1.59

In the ex-situ experiment 31.33% of seeds from Raposka and 42.00% of seeds from Nyirád germinated in the first year. In both containers the first seedlings emerged during the week before 15th March and this initial period was also the peak in terms of germination rate during the experiment (Figure 4). After this initial peak the number of seedlings germinated per week decreased gradually until 12th April.

Our results show that in the case of *G. palustris* germination percentages are tend to be higher in meadows than in forest habitats, and germination percentages are higher in ex-situ than in in-situ conditions.

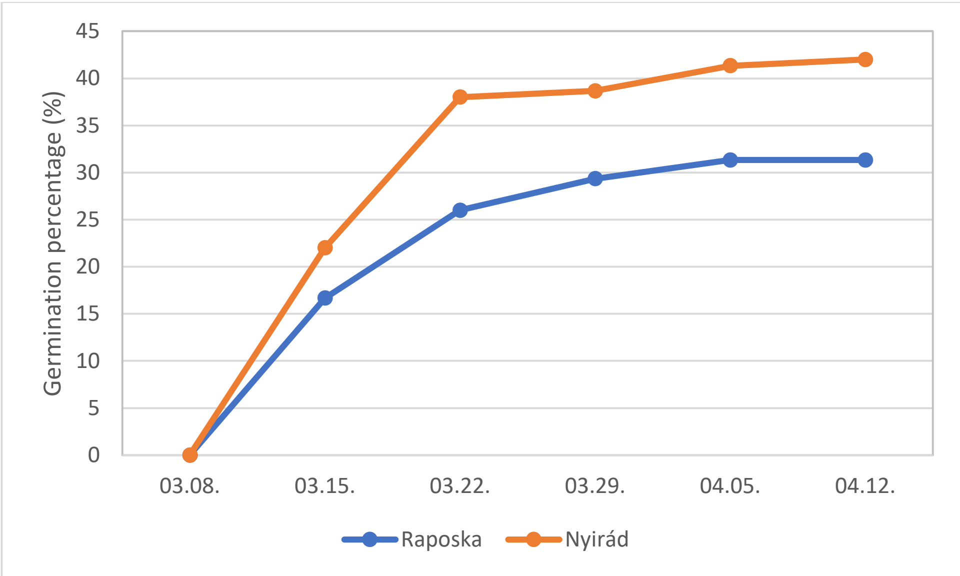

Figure 4 *Germination percentages of* G. palustris *seeds of different origin over the spring of 2024 in the ex-situ experiment.*

3.3 Seedling height (leaf length)

At Nyirád, seedlings emerged only in two from four plots. There was no significant difference in average seedling height between these two replicates. At Raposka there were no significant differences in the average height of the 6 replicates during the first survey. During the second survey, there was significant difference ($p=0.006$) only between the plot with the lowest (3rd rep) and the highest mean (4th rep), which we found still acceptable as one cohort with slight in-between differences and we used all 6 replicates combined for the comparisons with the data collected at Nyirád (Table 3).

Table 3 Number of seedlings, average height and the corresponding standard deviation, minimum and maximum values during the two in-sit surveys.

Study area	Code	count1	mean1 (mm)	sd1	min1 (mm)	max1 (mm)	count2	mean2 (mm)	sd2	min2 (mm)	max2 (mm)
Rap	1	23	33.70	10.80	9	58	21	59.70	14.50	29	93
Rap	2	8	31.20	5.65	24	41	6	57	19.20	31	81
Rap	3	17	28.50	10.30	9	44	19	52.40	12.70	25	69
Rap	4	18	30.80	6.90	18	42	18	68.10	10.30	49	88
Rap	5	20	27.70	6.00	18	40	17	62.60	13.90	35	78
Rap	6	16	32.60	9.01	18	48	12	62.30	11.40	40	78
Nyir	3	0	-	-	-	-	3	67.30	5.03	62	72
Nyir	4	0	-	-	-	-	4	76.50	13.70	58	91

In the in-situ experiment at Nyirád no seedlings were found for the first time (17th of April, 5 months after sowing). At the same time at Raposka average height of seedlings was 30.8±8.7

mm. During the second survey (23rd of May, 6 months after sowing) average height of seedlings at Raposka was 60.5±13.9 mm and 72.6±11.2 mm at Nyirád.

During the first measurement (14th of April) of ex situ containers, when the seedlings were about 3 weeks old, their average height was 48.1±22.2 mm from Nyirád and 45.5±21.0 mm from Raposka. During the second measurement (18th of May), when the seedlings were about 8 weeks old, the average height of seedlings from Nyirád was 70.8±20.6 mm and 64.1±21.8 mm from Raposka (Figure 5).

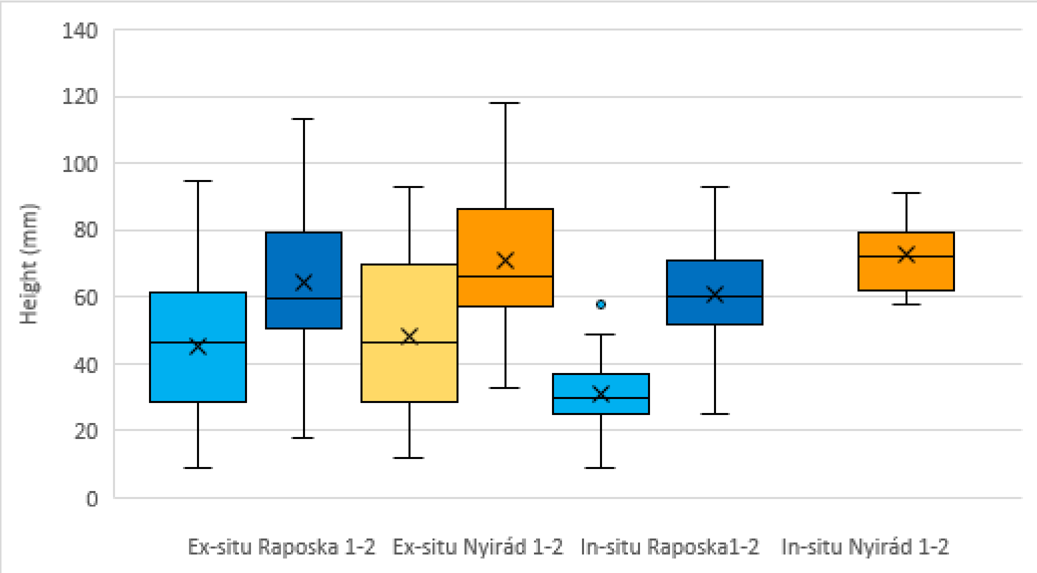

Figure 5 *Average height of seedlings in both ex-situ and in-situ sowings at the time of the two measurements.*

In the ex-situ experiments there was no significant difference between average height of seedlings from Nyirád and Raposka in either during the first and second height measurement. In the in-situ experiments average height of seedlings at Nyirád was significantly higher than seedlings at Raposka (Table 4). These results show that the average height of seedlings in the open forest is higher than in meadow populations.

The average height of ex-situ seedlings originated from Raposka was significantly higher than the in-situ seedlings in Raposka during the first measurement, but we found no such difference on the second measuring. Also, no statistically justified difference was found between average heights of in-situ and ex-situ seedlings of Nyirád (Table 4).

Table 4 Significance levels of differences between sites and in-situ/ex-situ experiments at the time of the two surveys. * means significant difference.

		Time of measurement	p-value
Between sites / origin of seeds	ex situ	1	0.586
		2	0.189
	in situ	1	-
		2	0.029*
Between in situ and ex situ	Nyirád	1	n/a
		2	0.746
	Raposka	1	<0.001*
		2	0.379

We also investigated whether there is a correlation between the number of days elapsed between germination and the second measurement and the height of individuals measured for the second time. We found almost no correlation ($R=0.007$, $p=0.95$) between these two factors, thus, seedlings that had germinated earlier and had more time for development did not produce larger leaves.

4 Conclusions

To interpret the data collected in local populations, it is essential to examine the dynamics between age-stage categories and study germination rates of seeds in the populations. The seedling 'age-state' category is a critical phase as several factors alone can cause high mortality. The examination of seedlings is complicated by the fact that seedlings are often present aboveground for a short time and are difficult to detect, especially in a dense vegetation. To monitor development of individuals starting from germination, ex-situ and in-situ germination studies were set up. During the autumn of 2023 seeds were sown in an open forest and in a meadow (in-situ), and seeds collected from these sites were sown in containers (ex-situ) at the same time.

Germination rates were considerably higher in the ex-situ sowing than in the in-situ experiment for seeds of both origins. Although the germination rate was low in open forest habitats, seeds originated from the same population germinated well in ex-situ conditions. There were significant differences between the average height (leaf length) of the seedlings. Under in-situ conditions average height was significantly larger in the open forest than in the meadows. There were significant differences between average heights of seedlings in the in-situ and ex-situ experiments only during the first measurement, which difference disappeared with the subsequent growth of the plants.

Successful germination and survival of recruitment are generally very important factors in long-time survival of a species. Nevertheless, we have very few information about the germination biology of *Gladiolus* species.

Several germination experiments were done previously with the genus *Gladiolus*, but these experiments mostly carried out in laboratory conditions.

Ramzan (2010) examined the effect of priming with potassium nitrate (KNO_3) on seed germination percentage and germination time on seeds of *G. alatus*. He achieved the highest germination success was with distilled water.

Amişculesei (2022) studied the effect of length of stratification and different temperature regimes on germination percentages of three *Gladiolus* species (*G. imbricatus*, *G.× byzantinus* and *G. tristis*) in a germination chamber. In the case of *G.× byzantinus*, stratification had a much stronger influence on germination than temperature conditions. On the contrary, temperature had a major influence on germination time and percentage of *G. tristis*. In the case of *G. imbricatus*, the duration of stratification has a marked impact on seed germination.

Fernández (2005) investigated the effect of temperature and light conditions on germination of *G. illyricus*, *G. italicus* and *G. tristis*. In the case of *G. illyricus* and *G. tristis* seeds, the highest germination percentages were obtained at 13–15ºC. The germination percentage of *G. italicus* was almost zero in all treatments, probably as a result of seed dormancy.

Dutt et al. (2000) studied the effect of soaking in gibberellic acid (GA_3) solution on the germination of 10 different *Gladiolus* hybrids. All concentrations of GA_3 significantly increased germination percentages and reduced the number of days required for germination compared to water.

Our knowledge on the germination of *G. palustris* is rather incomplete. Brunzel (2010) found that the germination percentages of *G. palustris* in pot cultures kept outdoors were lower than in the laboratory (which was over 90%). His findings confirm our results as he demonstrated in the outdoor sowing experiments that significantly higher number of seedlings were recorded in plots where the vegetation and litter were removed prior. This indicates the potential importance of small-scale vegetation-free areas in the germination success of *G. palustris.*

After a one-year study we have a better understanding of the germination and seedling establishment of *G. palustris,* however, there are still many questions that we will only be able to answer during the following years. For example, there was a high disappearance among the in-situ seedlings, for which we do not yet know whether they went into dormant phase early or perished.

Our long-term goal for the next years is to establish a suitable age-stage categorization system for demographic purposes. We hope to have answers to our additional questions in the following years to get closer to providing detailed information on the conservation methods of a species of Community importance.

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