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Research Article

Title: Effect of seed storing duration and sowing year on the seedling establishment of grassland species in xeric environments

Running head: Seedling establishment after seed storing

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KS, AM and KT conceived and designed the study; KS, AM, KH, AKJ and KT did the field work; AKJ, KS performed the statistical analyses; AKJ, KT, MH, KS, AM wrote and edited the paper. All authors contributed critically to the drafts and gave final approval for publication.

Abstract

There is limited availability of seeds of native species in many countries for grassland restoration therefore ex situ seed banks can gain importance as source of germplasm in the future. We tested the usability of seed accessions of the Pannon Seed Bank for reintroduction with the aim to restore sandy grassland in Hungary. Seeds of ten native sandy grassland species were seeded in the year of collection and after one or two years of storage. The establishment was estimated by counting seedlings along seeded transects for two vegetation seasons. This study produced the first numerical estimate we know about of native sand grassland species emergence in the field. A low establishment of the tested species was found, ranging from 0.002 to 8%. Within this range, *Dianthus serotinus* had the highest establishment, while *Festuca vaginata*, which was sown as matrix species, performed only medium establishment. The short-term storage (1 or 2 years) of seeds had no significant effect, except for *F. vaginata*, where the seed storage had positive effect on the reintroduction success. The year of seeding had the highest influence on recruitment. Four species were found to emerge over two years instead of only the first year. Based on our results, the weak seed yield of certain years and the low supply of native seeds in the market can be mitigated by using stored seeds. It is recommended to use multi-year, scheduled seeding to reduce the negative impacts of particularly dry years and to increase the restoration success.

Keywords:

establishment rate, sandy grassland, seed bank, seed introduction, short-term seed storage, year of seeding

Implications for Practice:

- Short-term storage of seeds does not reduce germinability and establishment of sandy grassland species, for one species (*F. vaginata),* the seed storage even increased reintroduction success.
- Seed banks can play a crucial role in overcoming seed limitation due to weak seed yields or low seed supply of the market.
- Species with scheduled seed emergence can survive drought years in the form of seeds, thus re-seeding can be superfluous.
- Gradual seeding in adjacent plots can minimize the risk of negative impacts of drought and increase restoration success.

Introduction

Natural and semi-natural grasslands are threatened due to fragmentation by human land use and the intensification of agricultural production (Pereira et al. 2012; Bond 2016; Török et al. 2018a). There is a great need for ecological restoration, which should be scaled up to large areas and extended from agricultural and semi-natural areas to urban and industrial sites to compensate for this large loss of natural areas (Aronson & Alexander 2013; Klaus 2013; Kövendi-Jakó et al. 2019). Spontaneous succession is often hindered by propagule limitation (Kiehl et al. 2014; Török et al. 2018b; Halassy et al. 2019). Therefore, the restoration success is substantially determined by seed introduction methods. These methods include direct seeding, diaspore transfer with substrate, hay or brush harvesting, slot seeding, plug planting (Hedberg & Kotowski 2010). To support extended restoration, the use of seeds of native species has to be enhanced and survival success has to be increased (Merritt & Dixon 2011). However, it is difficult to achieve these goals in the lack of sufficient amount of seeds (Merritt & Dixon 2011). The limited knowledge on necessary seed amounts can result in the wasting of seeds in the hope to ensure sufficient emergence and thus restoration success (Williams et al. 2002; Hedberg & Kotowski 2010; Merritt & Dixon 2011).

The in situ soil seed bank of degraded sites has usually low species number, and the seed content mostly consists of undesired species adapted to disturbance by forming a persistence seed bank (Thompson et al. 1997; Bossuyt & Honnay 2008; Kiss et al. 2016; Török et al. 2018b). In many cases, natural constituents (dominant grass species, protected dicotyledonous species) are completely missing or represented in very small numbers in the soil seed bank resulting from seed predation and loss of seed viability due to abiotic conditions (Halassy 2001; Kiss et al. 2016; Török et al. 2018b). In contrast, seed storage may be possible for several years or decades by providing the appropriate conditions in ex situ seed banks (Peti et al. 2015; Smith 2016; O'Donnell & Sharrock 2017; Chapman et al. 2019). The most efficient storage of dried seeds is under low temperatures (in a refrigerator, freezer, or liquid nitrogen) (ENSCONET 2009a; Peti et al. 2015). This storage method is only applicable for orthodox seeds, tolerant for moisture reduction and cooling (Hong & Ellis 1996). Nowadays the role of seed banking is increasing as an important form of ex situ conservation in botanic gardens e.g. the Royal Botanic Gardens (RBG) Kew's Millenium Seed Bank with 37,000 taxa (Chapman et al. 2019). Ex situ storage of collected seeds of native plant species can provide a basis for conservation and habitat restoration (Merritt & Dixon 2011; Török et al. 2016; Chapman et al. 2019). Storage in restoration seed banks will have a major role to provide appropriate seed quantity for extending restoration (Merritt & Dixon 2011). However, there is a lack of knowledge on the effect of seed storage on field establishment and survival.

The Pannon Seed Bank was established for the long-term storage of seeds of native species in Hungary (Peti et al. 2015; Török et al. 2016; Peti et al. 2017). Here we studied the effect of short-term storage of seeds on the in situ establishment of ten species of sandy grasslands in the frame of the Pannon Seed Bank Life project (LIFE08 NAT/H/000288). We tested the establishment success of plants after reintroduction from the seed accessions of the Pannon Seed Bank at an abandoned arable field. Our questions were: (1) How does the short-term storage of seeds affect field establishment of the seeded species? (2) How does the year of sowing influence field establishment? (3) How does the establishment of seeded species change in the two years following seeding?

Material and methods

Study area

Our study area is located in the Kiskun LTER Fülöpháza site (46°52' N 19°23' E), in the Kiskunság region (in the centre of the Great Hungarian Plain) in Hungary. The climate is temperate with sub-Mediterranean and continental features (Kovács-Láng et al. 2000). The annual average temperature was 11-12°C and the annual mean precipitation was between 410-817 mm during the studied period (2011-2016, data from meteorological station in Fülöpháza). The historic landscape was characterized by inland sand dunes (with a dune height of 5 to 10 m). The dominant soil type is Calcaric Arenosol (IUSS Working Group WRB 2006) with high sand content (at least 90%), and little humus content (< 1%) (Kovács-Láng et al. 2000).

Due to the climatic and edaphic conditions, the natural vegetation is a xeric type of forest-steppe (Erdős et al. 2018a) where dune tops are covered with sandy grasslands, forest patches are usually small and have an open canopy, and marshlands are present in depressions. The most widespread habitat is open perennial sandy grassland (Festucetum vaginatae, Natura 2000 category: 6260, Pannonic sand steppes, a habitat of community importance in the European Union). This grassland has a grassland canopy cover around 40-70% and is dominated by two perennial bunchgrasses, *Festuca vaginata* and *Stipa borysthenica*, while typical subordinate perennial herb species include *Dianthus serotinus, Euphorbia seguieriana* and *Silene borysthenica* (Erdős et al. 2018b). At present, only remains of these semi-natural habitats (less than 20%) can be found within a mosaic of arable lands and tree plantations (Biró et al. 2013). Abandonment of arable lands is also observed starting from 1960s and 70s, due to the socio-economic change and the significant decrease of groundwater level (Biró et al. 2013). Abandoned fields are either left to spontaneous vegetation development (secondary grasslands) or used as tree plantations, mainly of alien species (*Robinia pseudo-acacia, Pinus sylvestris, P. nigra*).

We tested the applicability of seed accessions of the Pannon Seed Bank for reintroduction at a 11-ha abandoned field in Fülöpháza. This field was abandoned 10-15 years prior to the beginning of the experiment and now belongs to the Kiskunság National Park. Our reference habitat is the open perennial sandy grassland, which is a dominant habitat type in the neighboring Fülöpháza Sand Dune Area (Fig. 1).

Seed collection and handling

Seeds of species for reintroduction were collected from the open sandy grassland in the vicinity of the restoration area to sample populations genetically adapted to the local environmental conditions (Fig. 1). The species were chosen based on the following criteria: the selection should include both i) grasses and dicots; ii) dominant and subordinate species of sandy grasslands; iii) seeds should be orthodox, so capable to survive reduction of seed water content to 3-7% and subsequent storing at temperature of 0°C; and iv) seeds can be collected in the required quantity. For the chosen two grass and eight dicots see Table 1. Nomenclature followed Király (2009).

The seeds of target species were collected in 2011 and 2012. Due to the extreme drought in 2012, it was only possible to collect seeds in smaller amounts than in 2011. *Gypsophila arenaria* did not produce any seeds in 2012, therefore it was excluded from the seeding in 2012. Seed collection was done following the Seed Collection Guide (Zsigmond 2011; Peti et al. 2015), based on the ENSCONET (2009b) seed collection manual adapted for Hungary. Seed collection aimed to represent the genetic resources of the sampled population without endangering its survival. The collection affected up to 20% of total seed yield (10% for protected taxa) (ENSCONET 2009b; Zsigmond 2011; Peti et al. 2015).

Seed samples were transported to the Pannon Seed Bank (in Tápiószele) for cleaning, viability testing and storing or preparing for seeding. We used the methods of Rao et al. (2006) and ENSCONET (2009a) for seed cleaning and storing. Seeds were dried in drying chamber at 16 ± 1°C and at a relative humidity of 15-20%. Tests in germination chambers revealed very different level of germinability among species. *Centaurea arenaria*, *E. seguieriana* and *Onosma arenaria* had the lowest (0-8%), while *D. serotinus* and *S. borysthenica* had the highest

(40-82%) germination capacity (Table 1). Seeds were stored at 0-4°C in the active storage of the Pannon Seed Bank in Tápiószele.

Experimental design

The experiment was carried out in five replicates of 60 m x 65 m blocks (Fig. 1). The five blocks were divided to ten parcels (11 m x 28 m) for the treatments (Fig. S1). Each parcel received a different treatment, that is, the year of seed collection, the length of seed storage and/or the year of seeding was different. Species were seeded after 0, 1 or 2 years of storage (T0, T1, T2) in the seed bank in three consecutive years (2011-2014) (Table 2). Within a block, the treatments were assigned randomly to parcels. Only six out of ten treatments were used for this study (Table 2, Fig. S1); treatments had the same number of repetitions from every seed collection year.

The reintroduction blocks were partly infested with invasive common milkweed (*Asclepias syriaca*). To control milkweed, herbicide (8% of Medallon solution) was spot-sprayed on milkweed shoots in July 2011. Each parcel was mown, and the hay was removed only before seeding. Strip ploughing was applied by a rototiller in ten lines of 25 m (one meter apart from each other) at a 10-15 cm depth (Fig. S1). Seeds were seeded in the ploughed rows by hand on the soil surface and covered by 1-2 cm soil. Seeding density was different for each species (Table 1), but it was the same in every year. However, because of the low seed production due to the severe drought, only one single 25 m row was seeded instead of ten rows in treatments applying seeds collected in 2012. Seeds were sown in late September each year.

Data recording and analyses

The monitoring of seedlings and established individuals took place twice a year, in late May and in early September from 2012 to 2016. The number of seedlings and young adults of each seeded species was counted in contagious 0.5 m^2 quadrats along two 25 m seeding lines per parcel (only one line in parcels seeded in 2012) for two consecutive years after seeding (Fig. S1). In total we used 1,125 quadrats along 45 seeding lines for monitoring. The average establishment rate was obtained as the ratio of number of seedlings counted and number of seeds sown for each species per experimental block. The first-year establishment is defined by mean establishment rates from first May and first September sampling. The second-year establishment is the mean establishment rates from second May and second September monitoring. Since the individuals were not tracked, second year data includes both new seedlings and live young adults from previous year germination. Csapody (1968) was used to help identify the seedlings.

Because *Echinops ruthenicus* and *G. arenaria* emerged only rarely, therefore their data were excluded leaving eight species altogether for the analyses. We evaluated seedling establishment at two levels: establishment rates pooled for all species and at species level. Only *F. vaginata* and *D. serotinus* had sufficient establishment rates for species level analyses. We used generalized linear mixed models (glmm) of the package *"glmmTMB"* with zero-inflated option (Brooks et al. 2017) of the R 3.3.1 statistical environment (R Core Team 2019). The use of zero-inflated option was necessary for establishment rate based analysis, because our data contained many zeros.

The first group of models was built to check the impact of storage and seeding year on seedling establishment. The response variables were the first-year establishment rate pooled for the eight seeded species and that of *F. vaginata* and *D. serotinus* in three separate models. The length of storage (freshly seeded seeds (T0), seeds stored for one (T1) or two years (T2)) and the year of seeding (2011-2014) were included as fixed factors. To consider potential dependence of sampling units within blocks and treatments, we allowed for a random intercept for each block/treatment, year (2012-2015) and date of monitoring (first May and first September) for all models, plus species for the pooled data. The establishment rates for pooled data and for *F. vaginata* were cube root transformed and for *D. serotinus* were square root transformed, respectively, to approximate the assumptions of normality and homoscedasticity.

The second group of models were built to assess the change of establishment with elapsed time after seeding. The May and September establishment rates of both the first- and second-year after seeding of the eight seeded species pooled, and of *D. serotinus* and of *F. vaginata* were used as the response variables in three separate models. The elapsed time after seeding (first May, first September, second May, and second September) was used as fixed factor. We used for a random intercept for each block/treatment, and year (2012-2016) of monitoring for all models, plus species for the pooled data. In this model the establishment rate approximated the assumptions of normality and homoscedasticity best with cube root transformation for pooled data and for *F. vaginata*, square root transformation for *D. serotinus*.

For each model the "*dharma*" package (Hartig 2019) was used to check model correctness. For multiple comparisons, the *emmeans* test was applied using the "*emmeans*" package (Lenth et al. 2019) of the R 3.3.1 statistical environment (R Core Team 2019).

Results

Effect of seed storage length

The first-year establishment rate was not influenced significantly by the length of seed storage for the pooled data of all studied species (X^2= 0.105 *df*= 2 *p*= 0.949) (Fig. 2).

The establishment rate of *D. serotinus* was not significantly influenced (X^2= 2.09, *df*= 2, *p*= 0.352), but *F. vaginata* was significantly influenced by the short-term seed storage (X^2= 9.689, *df*= 2, *p*= 0.008). Based on pairwise comparisons of *F. vaginata,* two-years stored seeds produced significantly different seedling emergence from fresh and one-year stored seeds (T0-

T2: t= -2.6, p= 0.032; T1-T2: t= -3.049, p= 0.01). Fresh seeds and two-years stored seeds of *D. serotinus* had similar establishment rates (1.65%; 1.82%) contrary to one-year stored seeds, which had the highest establishment rate (5.05%). Fresh seeds of *F. vaginata* had similar low establishment rate (1.24%) contrary to one and two-years stored seeds, which performed higher establishment rates (1.77%; 1.77%).

Effect of year of seeding

The first-year establishment rate was significantly influenced by the year of seeding for the pooled data (X^2= 47.898, df= 3, p< 0.001). Based on the results of pairwise comparisons, 2013 was significantly different from the other seeding years in their resulting establishment rate (2011-2013: t= -4.005, p< 0.001; 2012-2013: t= -4.272, p< 0.001; 2013-2014: t= 5.191, p< 0.001). The highest establishment rate averaged for all species was detected in 2013 (1.73%), while the lowest number was found in 2011 (0.15%) (Fig. 3).

The year of seeding had significant effect on the first-year establishment rate also at the species level (*D. serotinus:* X^2= 62.308, df= 3, p< 0.001; *F. vaginata:* X^2= 51.41, df= 3, p< 0.001). Based on pairwise comparisons of *D. serotinus* data, 2011 and 2014 were significantly different from 2012 and 2013 (2011-2012: t= -4.898, p< 0.001; 2011-2013: t= -4.885, p< 0.001; 2012-2014: t= 2.718, p= 0.044; 2013-2014: t= 5.854, p< 0.001). In case of *D. serotinus* there was no or limited establishment (0% and 0.01%) after seeding in 2011 and 2014, the highest establishment (5.26%) was found after seeding in 2013. Based on the results of pairwise comparisons of *F. vaginata*, each year of seeding was significantly different from 2014 (2011-2014: t= 3.577, p= 0.004; 2012-2014: t= 4.486, p< 0.001; 2013-2014: t= 7.126, p< 0.001). There was a very low establishment of *F. vaginata* in 2014 (0.11%) and an increasing establishment from 2011 to 2013 (from 1.04% to 2.52%).

Effect of elapsed time after seeding

The establishment rate was significantly influenced by the elapsed time after seeding for the pooled data (X^2= 30.517, *df*= 3, *p*< 0.001). Post hoc test proved that establishment rate of the second September was significantly higher than at the other survey times (1 May - 2 Sept: *t*= -4.630, *p*< 0.001; 1 Sept – 2 Sept: *t*= -4.921, *p*< 0.001; 2 May - 2 Sept: *t*= -3.895, *p*< 0.001). The lowest establishment averaged for all species was detected in the first September (0.78%), while the highest establishment was found in the second September (1.2%) (Fig. 4).

The effect of elapsed time after seeding had significant effect on the first- and second-year establishment rate also at the species level (*D. serotinus:* X^2= 13.513, *df*= 3, *p*= 0.004; *F. vaginata:* X^2= 66.464, *df*= 3, *p*< 0.001). Based on pairwise comparisons the first May significantly differed from the second vegetation season for *D. serotinus* (1 May – 2 May: *t*= 3.454, *p*= 0.004; 1 May – 2 Sept: *t*= 2.827, *p*= 0.028). In the first vegetation season *D. serotinus* had an establishment rate of 3.24%, the highest of all species, and establishment rate decreased with time (2.45%, 1.5%, 1.61% for consecutive years). Based on pairwise comparisons of *F. vaginata* the first May significantly differed from the other survey times (1 May – 1 Sept: *t*= 7.862, *p*< 0.001; 1 May – 2 May: *t*= 5.471, *p*< 0.001; 1 May- 2 Sept: *t*= 5.392, *p*< 0.001). Similarly, to *D. serotinus, F. vaginata* had the highest establishment rate in the first May survey (2.52%) that decreased by September (0.67%) and remained low (0.78%; 0.74%) in the following surveys.

Discussion

Our study proved that short-term seed storage in seed bank does not reduce the viability of seeds of the studied native species. Seed viability and storability is of crucial importance for seed-based restoration. Germination tests are carried out in laboratories based on different protocols, but these protocols do not include testing of germination and establishment in the field that would be the most relevant for restoration (James et al. 2011; Larson et al. 2015). Therefore, the knowledge gained during this study is valuable with new data on the target species.

Although all sown target species are present in the study region, they had negligible cover (0.01 – 0.02%) in the study sites by spontaneous establishment, contrary to other studies that reported higher cover of spontaneous establishment of target species e.g. *D. serotinus*, *E. segueriana*, *S. borysthenica* in old-fields (Albert et al. 2014). A low establishment of the tested species was found, ranging from 0.002 to 8% per treatment. Within this range, *S. borysthenica* had the lowest establishment (0.002%) and *D. serotinus* had the highest establishment (8%), while *F. vaginata*, which was sown as matrix species, performed only medium establishment (4%). It is easy to understand the lower establishment success in the field in comparison to controlled laboratory germination tests, as seed predation, pathogens, abiotic conditions, competition etc. can hinder emergence and survival in the field (Larson et al. 2015). Seeding methodology (depth, time etc.) can also influence recruitment success that this study did not aim for to test. Further studies could search for other effective seeding methods and experiment with field germination (Larson et al. 2015).

Establishment of seeded species differed greatly, also among years, but most species could establish after one or two years of storage, sometimes even better than freshly collected seeds (e.g. *F. vaginata*). This provides an excellent opportunity for ecological restoration in that the weak seed yield of certain years and the low supply of native seeds in the market can be overcome by using stored seeds for reintroduction (Merritt & Dixon 2011). The lack of significant impact of storage for most studied species might be due to the high variability of intra-species establishment data (e.g. *D. serotinus* establishment ranged from avg. 1.65 to 5.05%). The significant increase in the establishment of *F. vaginata* after seed storing might be a year effect; due to the high establishment rate in 2013 that coincided with one or two years of storing. We assume if the study had started a year later, this effect would have been different. Seed storage, seeding years, collection years, and time elapsed after seeding are biologically interdependent, therefore they cannot be interpreted separately in our study. We confirmed that the tested species have orthodox seeds, tolerant to dry and cool storage (Hong & Ellis 1996). Storage of seeds in seed banks can be suggested in countries with insufficient market seed supply and for species with orthodox seeds (Peti et al. 1995; Merritt & Dixon 2011). According to the study of Haslgrübler et al. (2015) the harvested seed material should be stored under cool conditions and used within 2 years. Viability decreases over long time as demonstrated e.g. by Molnár V. et al. (2015), who reported negative correlation between seed age and germination percentage for *Astragalus contortuplicatus* over a long-term storage (>100 years), but short-term storage is usually adequate for restoration purposes. This experiment focused on a three-year seeding period, so only testing of one or two years of storage was feasible, but further studies could be planned with longer storage.

In our study we found significant effect of seeding year on the establishment of target species supporting the results of Vaugh & Young (2010), who highlight that ecological field experiments have usually rare temporal replication despite several studies have proved the strong influence of the initial years. In our study, we experienced good and bad years for reintroduction, however, we did not experience 'forb years' and 'grass years' as in the study of Stuble et al. (2017). Comparing the different year of seeding, we found that establishment rates were the highest after the 2013 sowing. Our results can be explained by the weather of the year after sowing (2014), which was characterized by high annual precipitation (817 mm) and high average annual temperature (12°C). The role of precipitation and temperature on field establishment and survival is supported by other studies (Khurana & Singh 2001; Bakker et al. 2003). The lowest establishment rate of fresh seeds can be explained by the effect of lower annual precipitation in both 2011 and 2012 (410 mm and 439 mm, respectively), than the long-term average of 550 mm (Szitár et al. 2014, 2018). The significance of drought is supported by other studies (Stampfli & Zeiter 2008; James et al. 2013) as well. Because of our sandy target species do not form large soil seed bank in abandoned arable lands (Halassy 2001), seeds of these species should be stored and preserved for longer term. In order to minimize the loss of seeds due to a drought year, reintroduction should be gradual. This will gain even more importance in the foreseen decades with climate change and more frequent drought years (Bede-Fazekas & Szabó 2019). Drought not only impacts species establishment but can also cause mortality of adult plants (Tilman & El Haddi 1992). Mojzes et al. (2018) reported that severe manipulated summer drought strongly reduced the cover of dominant perennial grasses, which provided an opportunity for a winter annual grass to increase its performance and abundance in an open sandy grassland. Sandy grasslands have adapted to midsummer dry conditions due to the low water retention capacity of the soil (Kovács-Láng et al. 2000) but are not adjusted to recently experienced extreme droughts which can result in serious damage. Our study highlights the importance of the protection and restoration of this habitat type.

We found that the effect of time elapsed after seeding years was significant. The establishment rates of *D. serotinus* and *F. vaginata* were the highest in the first May than in the other survey times, implying dieback. In contrast, the establishment of *C. arenaria, K. glauca, S. ochroleuca* and *S. borysthenica* performed a steady rise during the sampling period. These results can be explained by the different germination behavior, different type of seed dormancy of the studied species (Baskin & Baskin 2004). The two major types of behavior revealed (mainly first year germination, two species; versus more gradual emergence, four species) have implications for restoration planning. Gradual seeding over years should be planned for first year germinating species to avoid wasting seeds in drought years (Vaughn & Young 2010), but this is not necessary for those that emerge naturally over more years. Gradual emergence implies that if a drought year causes low emergence, re-seeding might not be needed. Besides scheduled seeding, spatial planning in parcels can also help overcome drought impacts, or less effective years by seeding in adjacent plots. This way, parcels may operate as colonization windows in good years, similarly to those reported by Valkó et al. (2016), and target species later have the opportunity to establish in the unsuccessful parcels as well.

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Tables

Table 1. Species selected for reintroduction, code, germination rate in the laboratory and sowing rate in the field. Seed germination method according to Peti et al. (2017). Abbreviations: pt- pretreatment, t- temperature, dgt- duration of germination temperature, l- light condition

Species	*Code*	*Mean germination in chamber (%)*	*Germination condition*	*Sowing seed density (number/m)*	*Conservation status*
Centaurea arenaria	cenare	5%	pt: cold-stratification, t: 20-30 °C, dgt: 16 h (20°C) - 8h (30°c), l: 24 h light	2.1	
Dianthus serotinus	diaser	64%	pt: cold-stratification, t: 20-30 °C, dgt: 16 h (20°C) - 8h (30°c), l: 24 h dark	39.9	protected
Echinops ruthenicus	echrut	24%	pt: cold-stratification, t: 20-30 °C, dgt: 16 h (20°C) - 8h (30°c), l: 24 h light	3	protected
Euphorbia seguieriana	eupseg	3%	pt: cold-stratification, t: 20-30 or 15°C, dgt: 16 h (20°C) - 8h (30°c), l: 24 h light	20	
Festuca vaginata	fesvag	32%	pt: cold-stratification, t: 20-30 °C, dgt: 16 h (20°C) - 8h (30°c), l: 24 h light	837.7	
Gypsophila arenaria	gypare	74%	pt: cold-stratification, t: 20°C, dgt: 24 h, l: 24 h light	10.2	protected

Koeleria glauca	koegla	47%	pt: cold-stratification, t: 20-30 °C, dgt: 16 h (20°C) - 8h (30°c), l: 24 h light	168.9	
Onosma arenaria	onoare	0.3%	pt: cold-stratification, t: 20-30 °C, dgt: 16 h (20°C) - 8h (30°c), l: 24 h light	1.8	protected
Scabiosa ochroleuca	scaoch	40%	pt: cold-stratification, t: 20-30 °C, dgt: 16 h (20°C) - 8h (30°c), l: 24 h dark	13	
Silene borysthenica	silbor	78%	pt: cold-stratification, t: 20-30 °C, dgt: 16 h (20°C) - 8h (30°c), l: 24 h light	209.6	

Table 2. The table shows with the letter S the six seeding treatments: seeds collected in 2011 and 2012 were seeded in years 2011-2014 after a different storage duration. The letter x indicates the surveying years.

Collection year	*Storage duration (years)*	*Seeding year*					
		2011	*2012*	*2013*	*2014*	*2015*	*2016*
2011	0 (T0)	S	x	x	-		
	1 (T1)		S	x	x		
	2 (T2)			S	x	x	
2012	0 (T0)	-	S	x	x		
	1 (T1)			S	x	x	
	2 (T2)				S	x	x

Figures

Figure 1. Map of the seed collection area, the reintroduction area and the experimental blocks.

Figure 2. First-year establishment rate of sown species after 0, 1 or 2 years of storage in seed bank (T0, T1, and T2, respectively). Eight out of ten species are shown which performed greater than 0.01% mean establishment rate. Species codes are shown in Table 1. One data (33.3 % establishment rate of *O. arenaria*) was removed as an outlier from the figure for better representation of the data.

Figure 3. The effect of year of seeding on the first-year establishment rate of seeded species. Eight out of ten species are shown which performed greater than 0.01% mean establishment rate. The codes are shown in Table 1. One data (33.3 % establishment rate of *O. arenaria*) was removed as an outlier from the figure for better representation of the data.

Figure 4. The effect of elapsed time after seeding on the first- and second-year establishment rate of the seeded species. Eight out of ten species are shown, which performed greater than 0.05% mean establishment rate. The codes of species are shown in Table 1. Abbreviations: 1May- establishment rate in first May, 1Sept- establishment rate in first September, 2May- establishment rate in second May, 2Sept- establishment rate in second September. One data (33.3 % establishment rate of *O. arenaria*) was removed as an outlier from the figure for better representation of the data.

Figure 1.

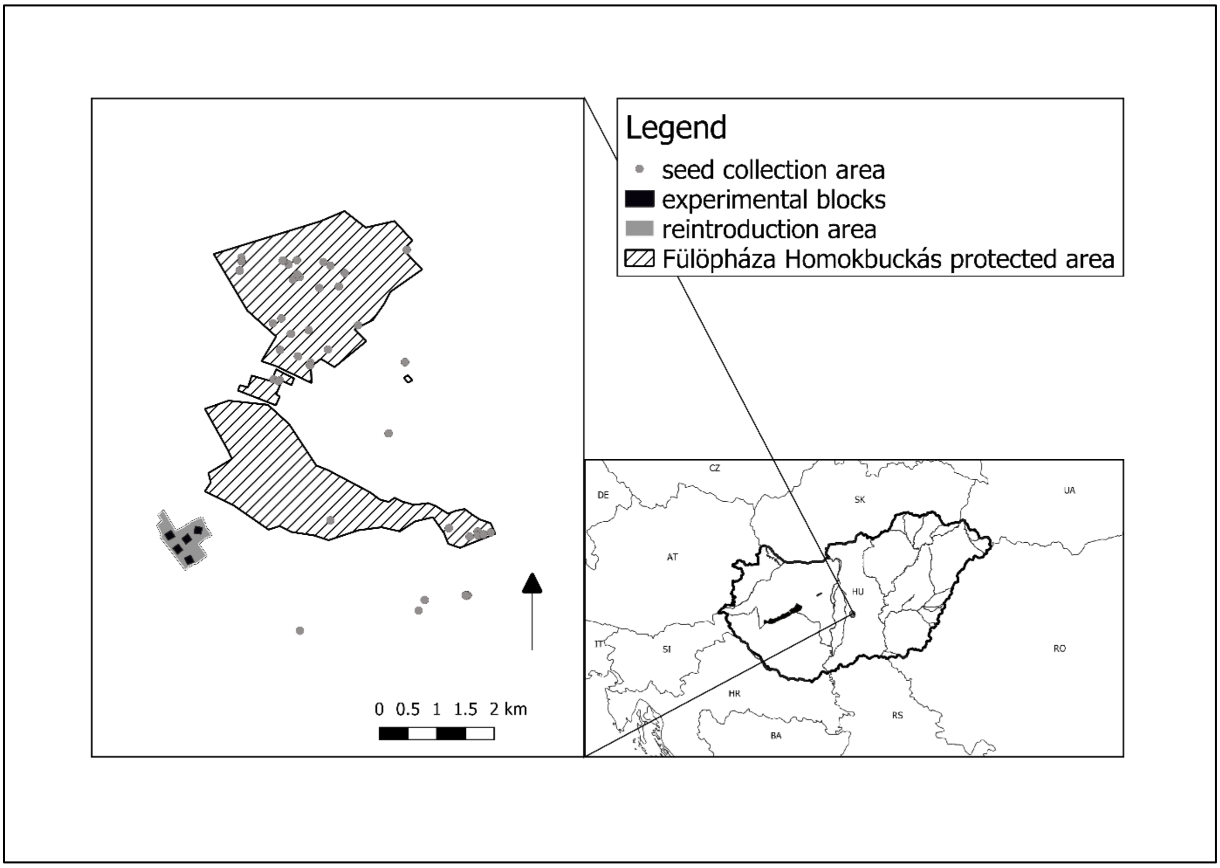


Figure 2.

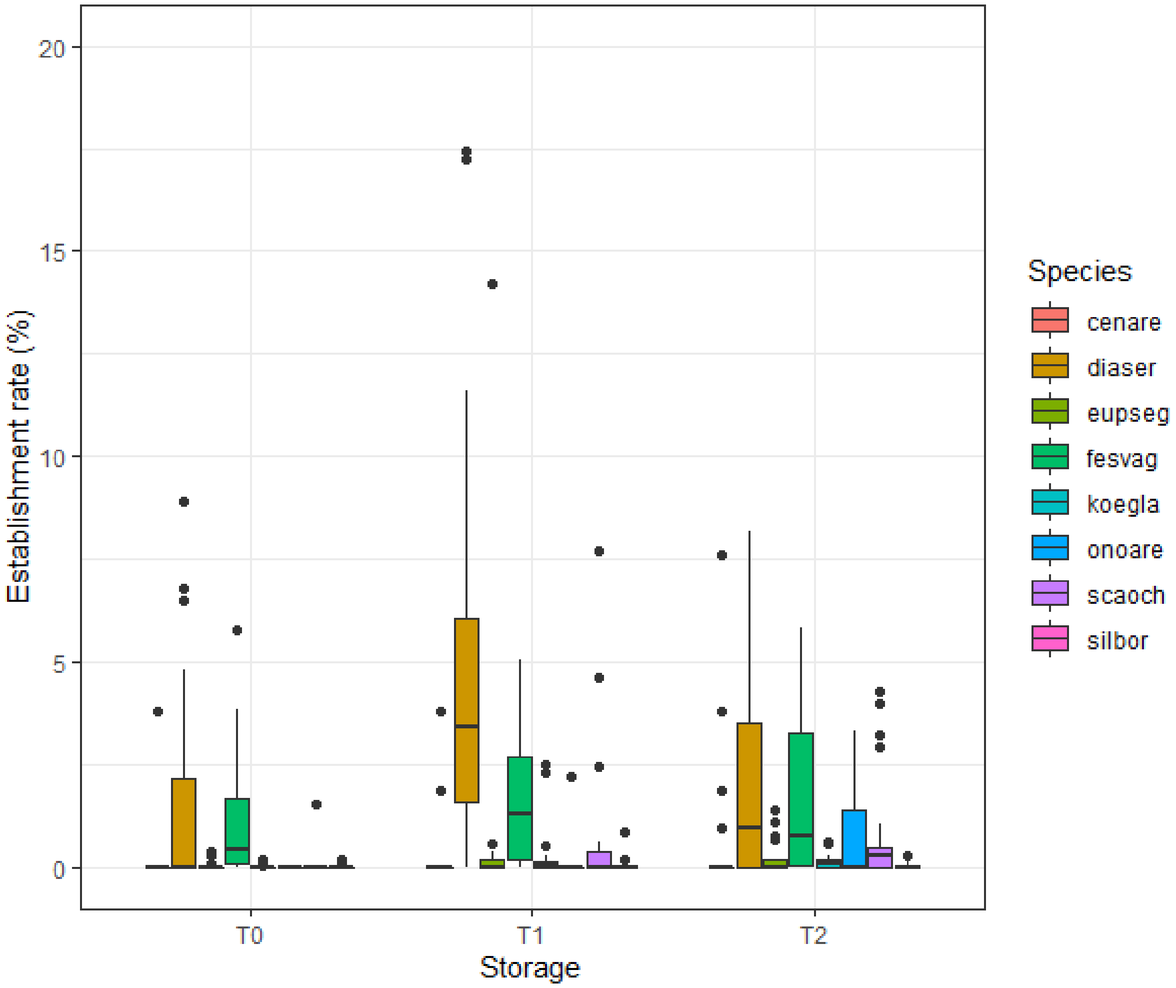

Establishment rate (%)
20
15
10
5
0
T0
T1
T2
Storage
Species
cenare
diaser
eupseg
fesvag
koegla
onoare
scaoch
silbor

Figure 3.

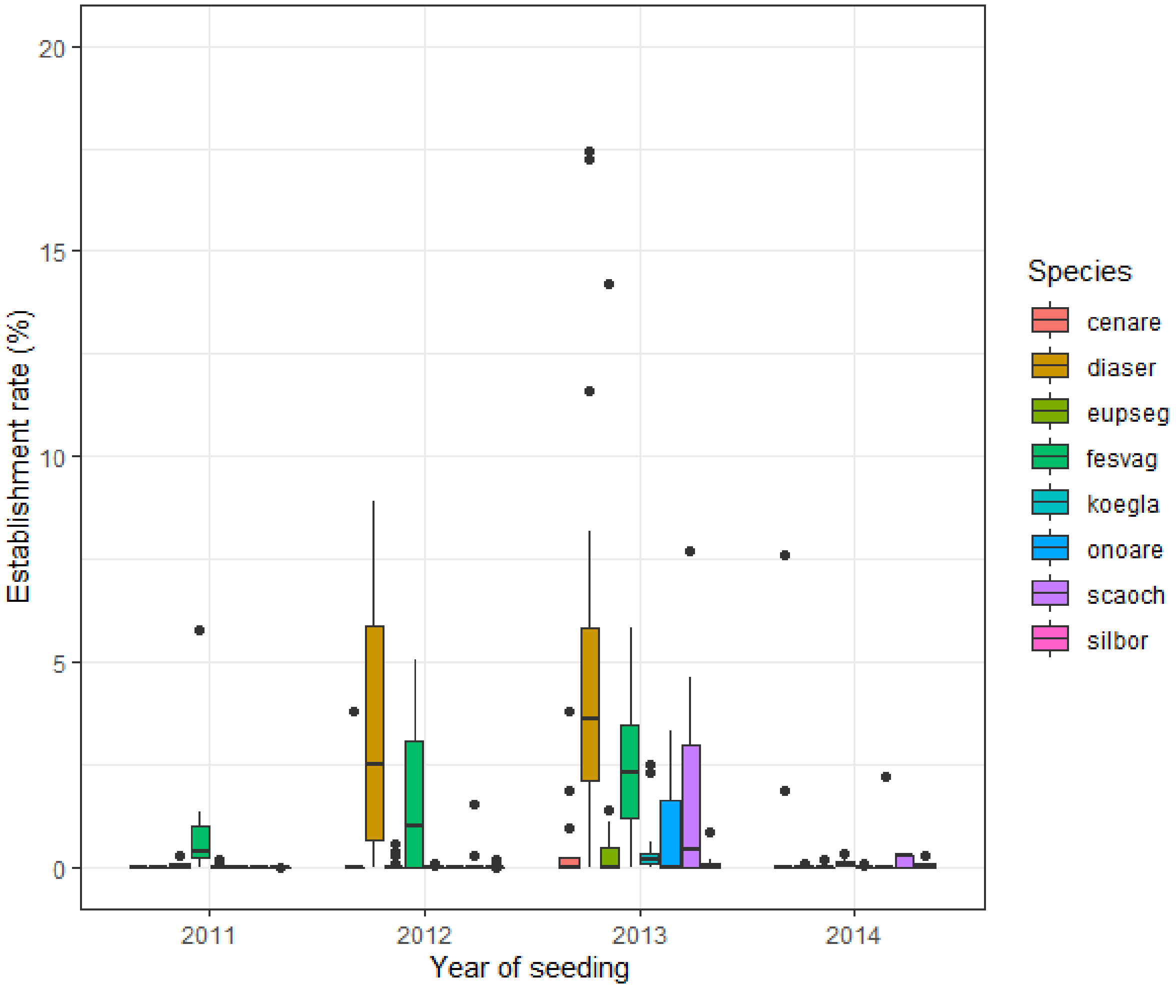
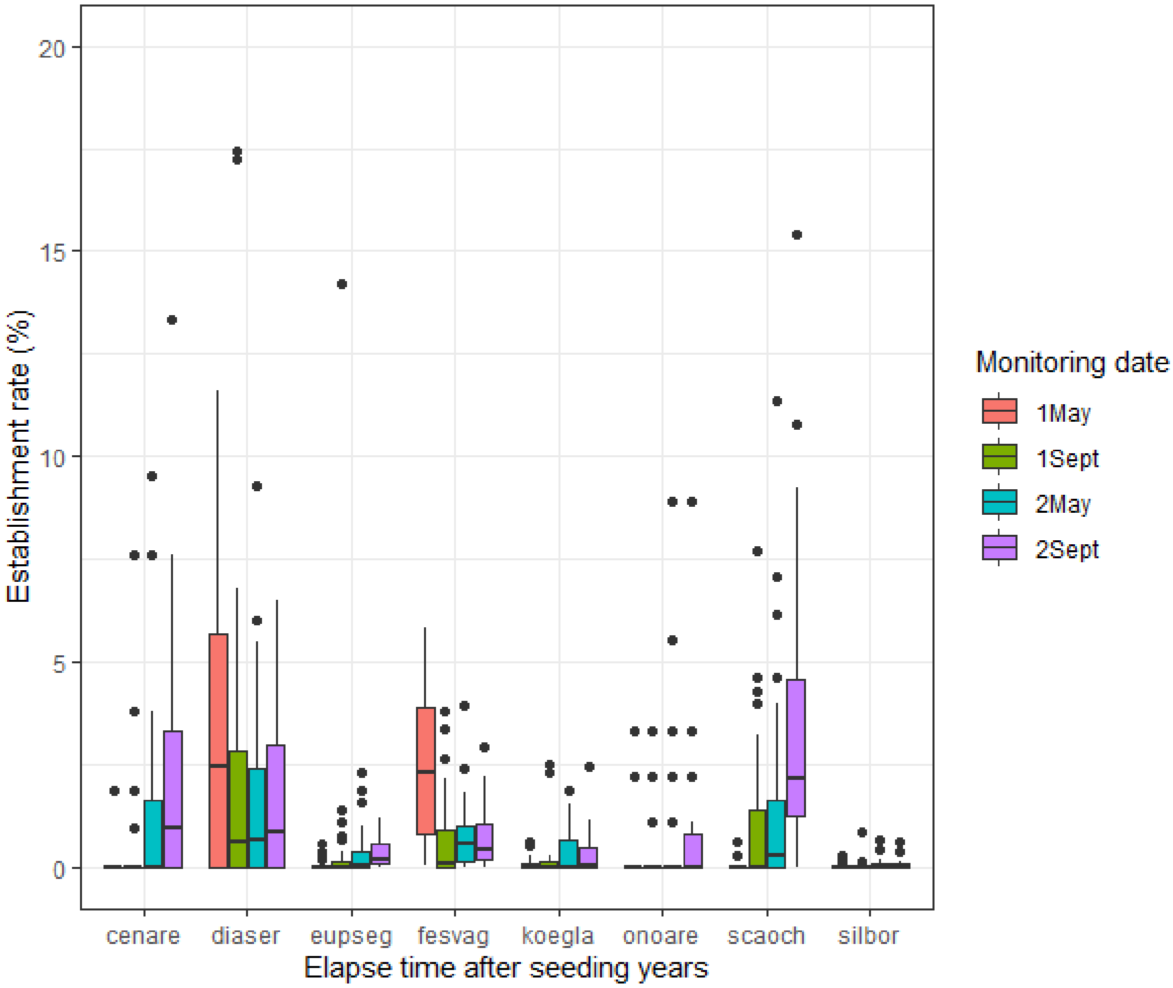

Establishment rate (%)
0
5
10
15
20
2011
2012
2013
2014
Year of seeding
Species
cenare
diaser
eupseg
fesvag
koegla
onoare
scaoch
silbor

Figure 4.



Supporting Information

The following information may be found in the online version of this article:

Figure S1. Presentation of an experimental block (60 m x 65 m) and an experimental parcel (28 m x 11 m).