Seed of the threatened annual legume, *Astragalus contortuplicatus*, can survive over 130 years of dry storage

Semena ohroženého jednoletého druhu *Astragalus contortuplicatus* přežijí přes 130 let skladování v suchu

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Molnár V. A., Sonkoly J., Lovas-Kiss Á., Fekete R., Takács A., Somlyay L. & Török P. (2015): Seed of the threatened annual legume, *Astragalus contortuplicatus,* can survive over 130 years of dry storage. – Preslia 87: 319–328.

Long-term seed viability is of crucial importance for short-lived species, since persistent seed banks can buffer the fluctuations in the establishment of plants from year to year. Temporarily flooded habitats are an unpredictable environment for plants, and for some species the only chance they have of surviving is the formation of persistent seed banks. *Astragalus contortuplicatus* is an annual species of periodically flooded habitats and is considered an endangered species in Hungary. Altogether 1993 seeds of this species were tested in a germination experiment: 1200 were freshly harvested and 793 were collected from herbarium specimens of various ages. Seed viability was tested using the germination method. The freshly harvested seeds were used for selecting the best out of seven frequently used dormancy-breaking methods for this species. The highest percentage of germination was recorded for the combined treatment of scarification and light. Thus, this method was used to test the viability of the seeds collected from herbarium specimens. The oldest seeds that germinated were 131 years old. Until now there are no records of seeds of herbaceous legumes germinating that are more than 100 years old. This record is the 9th oldest of all the literature records of viable seeds originating from biological collections. All the seeds that germinated developed into healthy, fertile plants, the seeds of which also readily germinated. Fitted linear regression showed a significant negative relationship between seed age and percentage germination. Based on this linear regression the calculated theoretical maximum viability is 309 years. Our results suggest that seeds of *A. contortuplicatus* stored in collections can be successfully used in this species' reintroduction for conservation purpose, to areas in which the plants were collected.

Keywords: biological collections, *Fabaceae*, hard seeds, herbarium, Hungary, physical dormancy, scarification, persistence, seed longevity, seed storage

Introduction

Long-term viability of seeds is crucially important for the maintenance of biodiversity in plant communities (Thompson 1997) because it enables plants to survive unfavourable abiotic and biotic conditions, especially in stressed or disturbed habitats (Thompson et al. 1998, Fenner & Thompson 2005). Long-term seed viability is especially important for short-lived species, since persistent seed banks can buffer yearly fluctuations in establishment and seed production, and enable the preservation of high genetic diversity both in space and time (Levin 1990, Meyer et al. 2006).

Knowledge of the persistence of seeds is also important for economic reasons: (i) plant breeders are interested in conserving the genetic resources of crop plants and their wild relatives because they can be used to increase crop production (Sallon et al. 2008) and (ii) the ability of weeds to persist in the soil seed bank is important for weed control (Kropac 1966, Gioria et al. 2012, Török et al. 2012). From a conservation perspective, seed persistence is important in the conservation of populations of rare and threatened species of plants (Bowles et al. 1993, Godefroid et al. 2011). Although seed banks are widely established worldwide, they mostly contain cultivars and landraces, and only a small percentage of them preserve seed of wild species of plants (Pritchard 2004).

Dry seed collections and herbaria can contain large numbers of viable seeds and can be effectively used in ex situ conservation of genetic material (Li & Pritchard 2009). Although storage conditions in natural history collections may be unfavourable for seed viability, several studies have shown that the seeds of herbarium specimens can remain viable for long periods of time (e.g. Milberg 1994, Godefroid et al. 2011), even as long as 237 years (*Nelumbo* sp.; Ramsbottom 1942). Consequently, herbaria can be used as resources for saving threatened or extinct plant species (Bowles et al. 1993, Godefroid et al. 2011). Moreover, rare and endangered species are usually overrepresented in herbaria, as they are of greater interest to collectors (e.g. Garcillán & Ezcurra 2011). On the other hand, using herbaria for such a purpose is limited by the fact that specimens of most taxa are usually collected at the flowering stage, except for taxa that can be reliably identified only by their fruit.

Temporarily flooded habitats with highly fluctuating waterline (such as shorelines and river banks) are a highly variable and unpredictable environment for plants. Seed banks play an especially important role in wetlands, since a high waterline results in the death of emergent plants and all species intolerant of high water levels. For some species, like *Alisma gramineum* (Hroudová et al. 2004) and *Elatine hungarica* (Takács et al. 2013), a persistent seed bank is the only way they can survive in such habitats (Thompson 2000).

It is well-known that most species of the *Fabaceae* have hard, water-impermeable seed coats, which enable them to retain their viability for a long period of time (Rolston 1978). For example, long seed dormancy associated with a hard seed coat is recorded for several species of the genus *Astragalus* (Baskin & Quarterman 1969, Townsend & McGinnies 1972, Duan et al. 2005, Eisvand et al. 2006, Patanè & Gresta 2006, Long et al. 2012). Bowles et al. (1993) obtained viable seeds from a 97 year old herbarium specimen of *A. neglectus*. Unfortunately, the ecological adaptations and requirements of most species of this genus are still poorly-known, though such knowledge is essential for planning proper conservation and management (Becker 2010).

Astragalus contortuplicatus L. is an annual species of temporarily flooded habitats on the floodplains of continental rivers. It is distributed throughout continental Eurasia, from China to central Europe (Roskov et al. 2005), appearing mostly sporadically as an ephemeral species (Podlech 2008). *Astragalus contortuplicatus* was considered "probably extinct" in Hungary by Podlech (2008), although previously two new occurrences were reported by Molnár V. & Pfeiffer (1999), and the species was considered endangered in Hungary by Király (2007). There are only 50 herbarium records of *A. contortuplicatus* collected in Hungary in the six herbaria studied (BP, BPU, CL, DE, EGR and SAMU), which were collected only in 25 years over a 164-year-long period (between 1849 and 2013). These facts underline the rarity of *A. contortuplicatus* in the Pannonian ecoregion. Because of the irregular temporal pattern in its appearance, we assumed that its seeds can remain viable for a long time.

Our aim was (i) to determine the best dormancy-breaking method for *A. contortuplicatus*, and (ii) to study how long the seeds can retain their viability in herbarium collections.

Materials and methods

To test the seed viability of *Astragalus contortuplicatus* we used the germination method. To obtain the highest dormancy-breaking and germination percentage we (i) tested seven dormancy-breaking methods on freshly harvested seeds and (ii) applied the most successful one to test the viability of seeds of variously aged herbarium specimens.

To obtain fresh seeds, field trips were conducted along the river Tisza (eastern Hungary) in 2011. To collect seeds of various ages, 44 specimens of *Astragalus contortuplicatus* preserved in the BP, DE and SAMU herbaria were searched for ripe fruit. We were able to collect seeds from altogether 18 specimens, which were collected in Hungary between 1835 and 2013 (Table 1). For the germination tests altogether 1993 seeds of *A. contortuplicatus* were used, of which 1200 were extracted from fruits of cultivated plants raised from the seed collected at Tiszaroff in 2011. In addition, 793 seeds were obtained from the 18 herbarium specimens of various ages.

The 1200 freshly harvested seeds were used to determine the most efficient treatment for inducing rapid germination. Seven dormancy-breaking methods, reported by Rolston (1978), were tested (Table 2, i–vii). We did not apply chemicals, radiation, percussion and pressure treatments, as the success of these treatments depends on the concentration of the chemical, the length of exposure etc., which are highly specific and not known for most species, including *A. contortuplicatus*. Moreover, in the case of *A. contortuplicatus* these may not be as important as the mechanical scarification or the effect of stratification by heat and frost. Each method was tested on 50 seeds in three replications, using also a control treatment (Table 2, viii) – altogether 8 treatments × 50 seeds × 3 replications = 1200 seeds in total. Following the physical treatments (scarification and/or temperature treatment) and one day of imbibing water the seeds were placed in Petri dishes on 1% agarose gel and kept for 30 days at room temperature (22±2 °C). There is a summary of the details of the light conditions used in this study in Table 2. After the tests on freshly harvested seed, the viability of seed originating from herbarium specimens (Table 1) was tested, using the above method of germination combined with the most efficient dormancy-breaking treatment.

Statistical analyses were performed using SigmaStat 3.5 program package (Systat Software Inc.). Germination percentages recorded in the seven treatments and the control germination tests were compared after ln (x+1) transformation using one-way ANOVA and Holm-Sidak tests. The relationship between age and viability of the seeds obtained from the herbarium specimens were analysed after a ln (x+1) transformation of age scores using linear regression (Zar 1999). Based on the equation of the fitted line we calculated the theoretical maximum viability of the seed of the species studied (indicated by where the fitted line crosses the axis x).

Table 1. – Age and origin of herbarium samples of *Astragalus contortuplicatus* used in germination tests and the results of the tests.

No.	Year of collection (age in years)	Country: locality & collector	Herbarium source	Number of seeds germinated (tested)	Germination percentage (%)
1	1835 (189)	Romania: Banat, Rochel A.	BP	0 (25)	–
2	before 1849 (> 165)	Hungary: Tiszabő, Sadler J.	SAMU	0 (77)	–
3	before 1849 (> 165)	Hungary: Tiszabő, Sadler J.	BP	0 (56)	–
4	1874 (140)	Hungary: Szarvas, Koren I.	BP	0 (54)	–
5	1875 (139)	Hungary: Békés county, Koren I.	BP	0 (75)	–
6	1883 (131)	Serbia: Bačka Palanka (‘Palánka’), Borbás V.	BP	29 (120)	24.2
7	1898 (116)	Serbia: Budva (‘Torontál, Beodva’), Thaisz L.	BP	0 (82)	–
8	1900 (114)	Hungary: Tiszaalpár, Wagner J.	BP	1 (71)	1.4
9	1908 (106)	Hungary: Szeged, Lányi B.	BP	0 (100)	–
10	1911 (103)	Serbia: Deliblat, Wagner J.	DE	16 (30)	53.3
11	1914–1915 (99)	Serbia: Bečej (‘Óbecse’), Kovács F.	DE	61 (160)	38.1
12	1918 (96)	Serbia: Novi Bečej (‘Törökbecse’), Boros Á.	BP	37 (90)	41.1
13	1918 (96)	Serbia: Novi Bečej (‘Törökbecse’), Boros Á.	BP	0 (30)	–
14	1927 (87)	Hungary: Kunszentmárton, Tamássy G.	BP	23 (79)	29.1
15	1943 (71)	Hungary: Szeged, Timár L.	BP	8 (100)	8.0
16	1950 (64)	Hungary: Tápé, Timár L.	DE	3 (30)	10.0
17	1952 (62)	Hungary: Szolnok, Csapody V.	BP	0 (20)	–
18	2011 (3)	Hungary: Tiszaroff, Lovas-Kiss Á. & Sramkó G.	DE	18 (32)	56.3
19	2013 (1)	Hungary: Tiszaroff, Lovas-Kiss Á. & Molnár V. A. (cultivated)	DE	148 (150)	98.7

Table 2. – Details of the treatments of the freshly harvested seed of *Astragalus contortuplicatus*. Scarification was done using Bosch red, Woodeco P60 120/1305 sandpaper. Light during germination was 14 hours per day with a light intensity of 30 μmol·m^{-2}·sec^{-1}.

ID	Scarification	Temperature treatments			Light during germination
		Temperature	Duration of temperature treatment	Handling conditions during treatment	
i	+	–	–	–	+
ii	+	–	–	–	–
iii	–	–	–	–	+
iv	–	100 °C	10 minutes	in 25 ml water	+
v	–	80 °C	10 minutes	in paper bag	+
vi	–	–20 °C	10 days	in paper bag	+
vii	–	–20 °C	10 days	in 25 ml water	+
viii (control)	–	–	–	–	–

Results

In the case of freshly harvested seeds the highest percentage germination was recorded after scarification and exposure to light during germination (Table 2: treatment "i"), and significantly lower percentage germinations were recorded in all other treatments (Fig. 1). Consequently, treatment "i" was used for inducing the seeds of herbarium specimens to germinate.

Seeds of half of the 18 herbarium specimens tested were viable. The highest viability (56.3%) was recorded for the youngest, 3-year-old samples, but surprisingly, a similary high viability (53.3%) was recorded also for the 103-year-old sample. The oldest viable seeds were 131 years old (Table 1). Healthy plants developed from each of the seeds that germinated (Fig. 2), which produced flowers and ripe fruit without pollination, i.e. autogamously. Seed from these plants also germinated readily. Voucher specimens of these cultivated mature plants were deposited in the P, DE, HAL, PRC, SAMU, SAV and W herbaria.

Linear regression revealed a significant negative correlation between percentage germination and the age of the seed ($r = -0.643$, $P < 0.001$, $n = 18$). Based on this equation the maximum of viability of the seed of this species is 309 years.

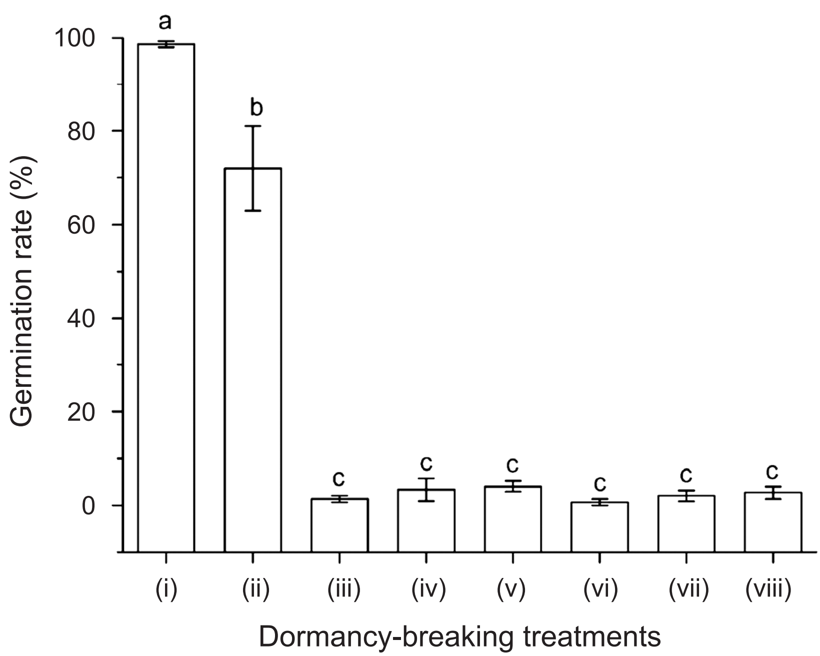

Fig. 1. – Percentage germination of fresh seed of *Astragalus contortuplicatus* recorded in the different treatments. Treatments were as in Table 2. Significant differences are indicated by different lower case letters (one-way ANOVA and Holm-Sidak test, $P < 0.001$, $F_{7,23} = 131.608$).

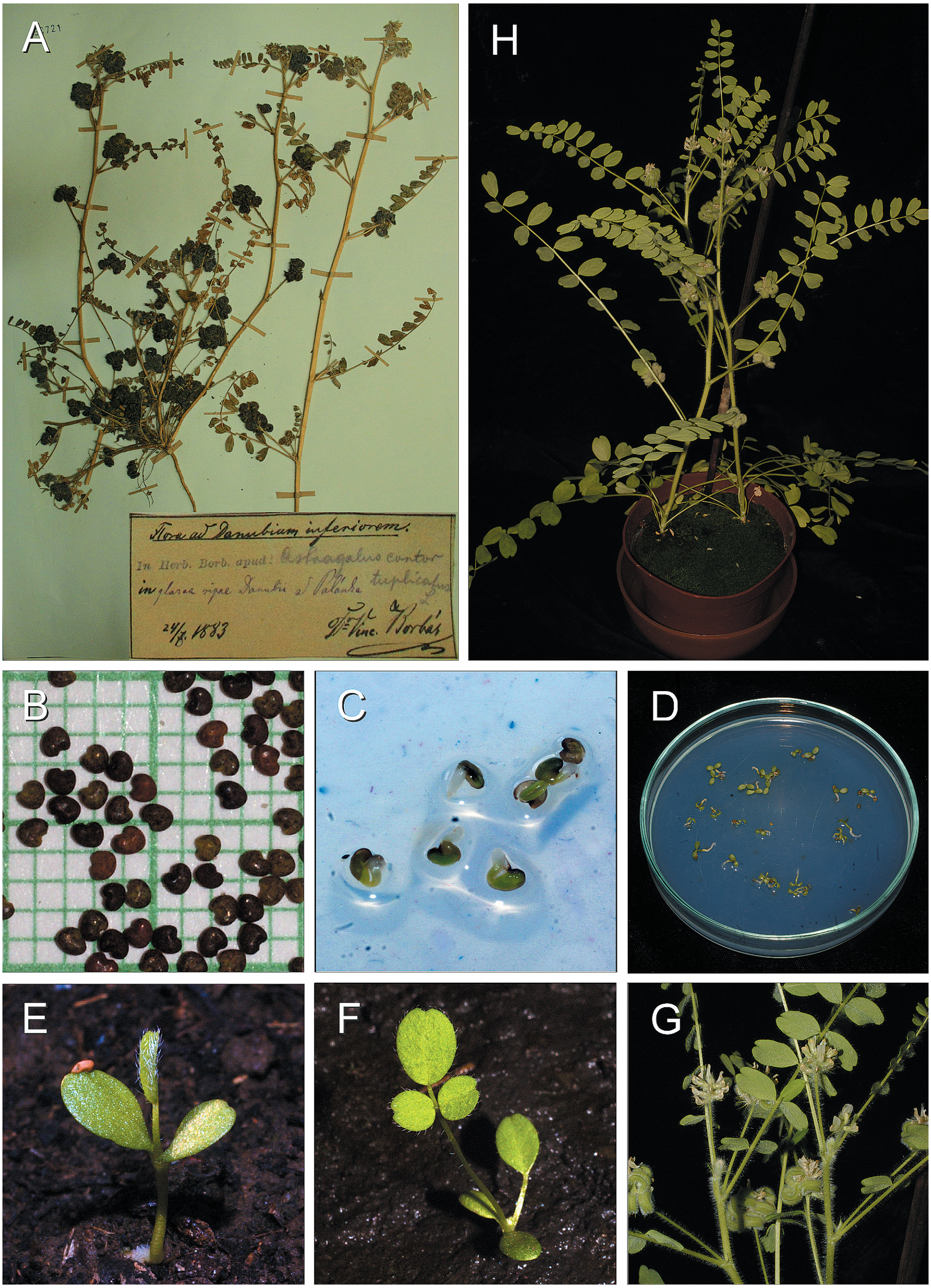

Fig. 2. – (A) Herbarium specimen of *Astragalus contortuplicatus* collected by Borbás in 1883 near Bačka Palanka ('Palánka') and its offspring, (B) seeds, (C–D) germinating seeds in Petri-dish, (E-F) transplanted seedlings, (G) detail of a flowering and fruiting specimen, (H) flowering specimens. Photo: A. Molnár V.

Discussion

Viability of more than 100-year old seed stored in biological collections has been recorded several times previously, for 23 species of 19 genera belonging to eight plant families (Table 3). Of these 23 species 12 belong to the *Fabaceae* and most of them are tropical or subtropical shrubs and trees. However, there appears to be no records in the literature of seed of herbaceous legumes more than 100 years old germinating. Our results indicate that the seed of *Astragalus contortuplicatus* can remain viable for a very long time and of the published records of seed from biological collections it is the 9th longest. In addition, we showed that species has a high selfing ability. The theoretical viability of 309 years predicted by the linear regression indicates that even older viable seed may be found in other herbaria.

All samples of seed from DE, even the oldest, showed some degree of viability, but only five of the 13 samples from BP germinated, and there were even younger samples from BP which were not viable. The reason for this difference could be the different storage conditions in these two herbaria. Temperature, humidity and chemicals used against insects can have considerable effects on the viability of stored seed (Godefroid et al. 2011). Duplicate specimens from the BP herbarium were traced in one case (no. 12 and no. 13, Table 1.). Interestingly, the seeds from the first sheet were highly viable, but those from the other sheet were not, probably because the two sheets were previously stored under different conditions in different herbaria. This also underlines the importance of the storage conditions in herbaria.

Apparently, seed dormancy in *Astragalus contortuplicatus* can be considered as a physical dormancy (Baskin et al. 2000, Baskin & Baskin 2004) in which a hard, water-impermeable seed coat prevents water uptake. In this type of dormancy some kind of mechanical or chemical scarification is required for germination (Spurný 1954, Finch-Savage & Leubner-Metzger 2006). For the congeneric *A. siliquosus*, Eisvand et al. (2006) also conclude that the impermeability of the seed coat to water is responsible for about 95% of the dormancy. Baskin & Quarterman (1969) conclude that seeds of *A. tennesseensis* can germinate only when both the hard outer and the inner seed coat is abraded and this may happen in nature by stratification or the action of microorganisms. We suppose that the hard seed coat of *A. contortuplicatus* may be broken down by abrasion caused by alluvial sediments during floods, or in the digestive tract of waterfowl, by the mechanical effect of grit in their gizzard or the chemical effect of digestive enzymes (Á. Lovas-Kiss et al., unpublished). Another important factor can be the effect of temperature fluctuations and low winter temperatures (Baskin & Baskin 2001), but as temperature treatments proved to be ineffective, these are not considered important in this case. Based on its habitat preference and the ‘rather abnormal distributional pattern’ of *A. contortuplicatus*, a similar hypothesis is suggested by Ali (1977), in terms of the role of migratory birds.

From a conservation point of view, the extremely long-term viability of its seed means that the conservation of *A. contortuplicatus* can be effectively supported by seeds stored in collections. Our results suggest that seed from herbarium specimens can be successfully used in ex situ conservation for subsequent reintroduction and population reinforcement. Plants that originate from seeds of herbarium specimens can be reintroduced into the area where the herbarium plants were collected, ensuring that the reintroduced populations are well-adapted to local environmental conditions. Our results also suggest that herbarium collections should be analysed in this respect, especially for other species with hard-coated seed.

Table 3. – Literature records of 100 or more years old seed stored in biological collections (dry seed collections or herbaria) that was viable. Abbreviations: n.d. – no data; Ph – phanerophyte, H – hemicryptophyte, Th – therophyte, TH – hemitherophyte, Hy – hydrophyte.

Species	Family	Raunkiær plant life-form	Age (year)	Number of seeds that germinated (no. tested)	References
Nelumbo sp.	*Nelumbonaceae*	Hy	215–237	1 (1)	Ramsbottom 1942, Barton 1961
Acacia sp.	*Fabaceae*	Ph	≥ 203	1 (2)	Daws et al. 2007
Liparia sp.	*Fabaceae*	Ph	≥ 203	16 (25)	Daws et al. 2007
Leucospermum sp.	*Proteaceae*	Ph	≥ 203	1 (8)	Daws et al. 2007
Acacia melanoxylon R. Br. ex Ait. f.	*Fabaceae*	Ph	151	1 (20)	Leino & Edqvist 2010
Acacia farnesiana (L.) Willd.	*Fabaceae*	Ph	151	2 (37)	Leino & Edqvist 2010
Albizia julibrissin Durazz.	*Fabaceae*	Ph	147	several (n.d.)	Ramsbottom 1942
Bupleurum tenuissimum L.	*Apiaceae*	Th	144	1 (50)	Godefroid et al. 2011
Astragalus contortuplicatus L.	*Fabaceae*	Th	131	29 (120)	Present study
Geranium bohemicum L.	*Geraniaceae*	Th	129	3 (10)	Milberg 1994
Bupleurum tenuissimum L.	*Apiaceae*	Th	125	2 (33)	Godefroid et al. 2011
Hordeum vulgare L.	*Poaceae*	Th	125	n. d.	Aufhammer & Simon 1957
Avena sativa L.	*Poaceae*	Th	125	n. d.	Aufhammer & Simon 1957
Senna bicapsularis (L.) Roxb.	*Fabaceae*	Ph	115	4 (10)	Becquerel 1934
Astragalus contortuplicatus L.	*Fabaceae*	Th	114	1 (71)	Present study
Vaccaria hispanica (P. Mill.) Rauschert	*Caryophyllaceae*	Th	110	9 (11)	Steiner & Ruckenbauer 1995
Sinapis arvensis L.	*Brassicaceae*	Th	110	11 (34)	Steiner & Ruckenbauer 1995
Sinapis alba L.	*Brassicaceae*	Th	110	1 (3)	Steiner & Ruckenbauer 1995
Lolium temulentum L.	*Poaceae*	Th–TH	110	2 (2)	Steiner & Ruckenbauer 1995
Hordeum vulgare L.	*Poaceae*	Th	110	47 (52)	Steiner & Ruckenbauer 1995
Avena sativa L.	*Poaceae*	Th	110	321 (400)	Steiner & Ruckenbauer 1995
Agrostemma githago L.	*Caryophyllaceae*	Th	110	1 (5)	Steiner & Ruckenbauer 1995
Hovea heterophylla A. Cunn. ex Hook.f.	*Fabaceae*	Ph	105	2 (12)	Ewart 1908
Goodia latifolia Salisb.	*Fabaceae*	Ph	105	2 (26)	Ewart 1908
Astragalus contortuplicatus L.	*Fabaceae*	Th	103	16 (30)	Present study
*Bupleurum tenuissimum*L.	*Apiaceae*	Th	101	5 (50)	Godefroid et al. 2011
Lotus uliginosus Schkuhr	*Fabaceae*	H	100	4 (600)	Youngman 1951
Trifolium pratense L.	*Fabaceae*	H	100	4 (600)	Youngman 1951

Acknowledgements

We are very grateful to Lajos Balogh (Szombathely) for providing seeds from SAMU herbarium. We gratefully acknowledge the help of János Csiky (JPU), Tamás Deli (Békéscsaba) and Andrea Dénes (PECS). This research was supported by TÁMOP-4.2.4.A/2-11/1-2012-0001 and TÁMOP-4.2.2.B-15/1/KONV-2015-0001 programs. Instrumental and infrastructural support of OTKA K108992 Grant to AMV and OTKA PD 100 192 to PT is also gratefully acknowledged.

Souhrn

Produkce semen, která zůstávají životaschopná po dlouhou dobu, je pro krátce žijící druhy důležitou vlastností, protože se s pomocí vytrvávající půdní semenné banky mohou vyrovnat s výkyvy prostředí. Na dočasně zaplavovaných stanovištích jsou některé rostliny schopny přežívat pouze díky tomu, že takovou semennou banku vytvářejí. *Astragalus contortuplicatus* je jednoletý druh periodicky přeplavovaných stanovišť a v Maďarsku je považován za ohrožený. V naší studii byla testována klíčivost celkem 1993 semen tohoto druhu; 1200 z nich bylo sebráno v přírodě a 793 z herbářových položek různého stáří. Čerstvě sebraná semena byla využita ke zjištění nejúčinnější metody přerušení dormance, nejlepších výsledků bylo dosaženo kombinací skarifikace a vystavení světlu. Pomocí této metody pak byla testována životaschopnost semen z herbářových položek. Nejstarší životaschopná semena pocházela z položky staré 131 let, což představuje nejdelší zjištěnou dobu pro bylinnou vikvovitou rostlinu; u této skupiny dosud nejstarší údaje nepřekročily 100 let. Údaj je zároveň devátou nejstarší hodnotou mezi literárními údaji o životaschopných semenech pocházejících z biologických sbírek. Ze všch klíčivých semen vyrostly zdravé plodné rostliny, jejichž semena byla také klíčivá. Pomocí lineární regrese stáří semene na délce skladování jsme odhadli, že teoretická nejdelší doba skladování, po níž by bylo možno ještě získat životaschopná semena, je 309 let. Výsledky naší studie ukazují, že semena *A. contortuplicatus* z herbářových sbírek by bylo možno použít k reintrodukcím tohoto ohroženého druhu do oblastí, z nichž rostliny pocházejí.

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Received 16 March 2015
Revision received 2 June 2015
Accepted 4 June 2015