Seed germination and seedling development of two rare *Astragalus* species (*Fabaceae*)

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Received: November 04, 2009 ▹ Accepted: March 10, 2010

Abstract. In this preliminary study the authors have tested the seed germination process, seedling development and the *ex-situ* seedling behaviour of *Astragalus alopecurus* and *Astragalus dasyanthus,* both rare and threatened species. The mature seeds of the test plants germinated well within several days, if the seed coat was scarified with sandpaper. Seedling survival was rather poor. This confirms the necessity to preserve the native habitats of both species and to ensure their ability for *in situ* propagation.

Key words: *Astragalus alopecurus*, *Astragalus dasyanthus*, Bulgaria, seed germination, seedlings

Introduction

Astragalus alopecurus Pall. (subgenus *Calycophysa*) (Kozhuharov 1992) was known earlier as *A. centralpinus* Braun-Blanq. (Chater 1968; Vulev 1976; Stanev 1984). This plant is a tall hairy perennial, with alternately compound pinnate leaves and about 20–30 pairs of leaflets, and lanceolate stipules. It bears several fertile stalks with several multifloral and dense flower heads on each stalk, with yellowish flowers and an ovoid legume. It is known from only one site in the territory of Bulgaria, in the area of Beglika, Western Rhodopi Mts. This plant species occurs rather restrictedly elsewhere: in Asia – China, the former USSR, Middle East (Turkey); and South Europe – France, Italy (South Alps) (Vulev 1976). *Astragalus alopecurus* is a glacial relict listed in the *Red Data Book of PR Bulgaria* (Stanev 1984) as a rare species and, according to the latest IUCN assessment, it is *Critically Endangered* (Sopotlieva 2009). The propagation is by seeds (Stanev 1984). Even young plants send down long tap roots and the ability for vegetative propagation is restricted. This species depends on bumblebees for pollination and seed production, as the flowers are primarily incapable of spontaneous self-pollination: 99.6 % of flowers excluded from pollinators do not set fruit (Kožuharova & Firmage 2007).

Astragalus dasyanthus Pall. (subgenus *Astragalus*) is a 20–30 cm tall hairy perennial, with alternately compound pinnate leaves and numerous odd leaflets. The stipules are triangular, lanceolate, and acuminate at the top. There are about 16–18 pairs of leaflets. The plant bears several fertile stalks, with several dense inflorescences on each stalk. It has bright lemon-yellow flowers and an ellipsoid legume, swollen in the middle, with a long, terminal beak (Chater 1968; Vulev 1976). The species is of a rather ancient origin, with a primitive karyotype (Pavlova 1988). It is a Pannonian–Pontian-Balkan geoelement and a rare species for the Bulgarian flora, protected by the *Biological Diversity Act* of Bulgaria (2002). Its IUCN category is *Critically Endangered,* according to Stoyanov & Tzonev (2009). The populations of *Astragalus dasyanthus* occur rather restrictedly elsewhere in South and East Europe, extending northwards to Hungary and Central Russia (Chater 1968; Vulev 1976). This species propagates by seed and the observations and field experiments reveal its dependency on bumblebees for pollination and seed production. Vegetative propagation has low probability of success for this species even in a laboratory environment (Ermolaev 2007). Fruit set as a result of spontaneous self-pollination is low (less than 11 % of the flowers excluded from pollinators mature to fruits). As a result of free pollination, 45.8 % of the flowers produce legumes and about half of their ovules mature to seeds. The other flowers either remain unpollinated, or the fruit or seeds are damaged by insects: *Bruchidae* (*Coleoptera*) and *Chalicoidea* (*Hymenoptera*) (Kožuharova & Firmage 2009).

Many *Astragalus* species are used for remedial purposes, being rich in flavonoids and saponins (Nikolov & Benbassat 1997; Krasteva & al. 2000; Dimitrov & al. 2003; Krasteva 2005). *Astragalus dasyanthus* is a particularly well known medicinal plant (Khoron'ko & Glyzin 1973; Skakun & al. 1988).

Vulnerability of these two astragals in their native habitats and their importance as medicinal plants is a good reason to investigate their germination potential, *ex situ* propagation, and possibilities for cultivation or reintroduction.

Legume seeds are known for their rigid seed coat. They possess physical dormancy: a primary reason for lack of germination is the impermeability of seed coats to water (Baskin & Baskin 2001). Scarification is often applied for increasing the germination of seeds of *Astragalus, Oxytropis, Hedysarum,* and *Lupinus* (Pavlova 1988; Burton & Burton 2001; Crowder 2006; Platikanov & al. 2006; Kholina & Voronkova 2008; Voronkova & al. 2008). Germination increase can reach up to 100 % (Kaye 1997). Even the seeds of the annual *A. hamosus* need such treatment and almost full and rapid germination is achieved by hand scarification with sandpaper, thus demonstrating that dormancy is exclusively imposed by seed coat impermeability (Patane & Gresta 2006; Platikanov & al. 2006). Scarification is often performed with a blade but there is a possibility of embryo damage by this procedure (P. Daniel Schlorhaufer unpubl.). Scarification with sandpaper has proven to be an efficient method (Kaye 1997; Platikanov & al. 2006; Patane & Gresta 2006). Also treatment with concentrated H_2SO_4 for 15 min is an effective method for rendering the seed coats of *A. bibullatus* permeable to water, while cold treatment is unnecessary (Morris & al. 2002). Peak germination percentage for *A. australis* var. *olympicus* occurred at alternating temperatures of 15/25 °C and at moisture availability with distilled water combined with scarification (Kaye 1999). Platikanov & al. (2006) reported that the seeds of four *Astragalus* species, (*A. hamosus, A. corniculatus, A. ponticus,* and *A. alopecurus*) would germinate at any temperature (13–34 °C), if scarified. The mountaine *A. alopecurus* required lower temperatures to complete germination, as compared to the other three species. Its maximum percentage of germination occurred at 13 °C. On the other hand, the mean time for completing germination was shortest between 26 to 34 °C – about three or four days, in contrast to the lower temperatures, when germination took longer – about 10 to17 days. The authors concluded that the major germination of these four species probably begins in the spring, after some natural scarification phenomena in the autumn and winter.

Astragalus dasyanthus has difficulties in reproduction and propagation in Moldova. Many conditions have to be met for seeds to germinate in nature, namely: moisture content and amount of nutrients in the soil and sufficient sunlight. Furthermore, seeds of *A. dasyanthus* are subject to fungal and insect larvae attacks. Three germination tests were accomplished for *A. dasyanthus* seeds collected from populations in Moldova, each involving 50 seeds at 25 °C and 100 % humidity, in a germination chamber with no access to light. The percentage of germination was determined by the number of germinated seeds. The first treatment was made right after collection, the second after drying the material to a humidity content of 5 % and the third after cold storage. Germination rate before treatments was 88 %, after drying 76 % and after cold storage 74 % (Ermolaev 2007).

The aim of this preliminary study is to test seed germination and development of the seedlings of *A. dasyanthus* and *A. alopecurus*. Raising seed in cultivation of these two rare and threatened plants is the first step towards their reintroduction in nature, whenever necessary.

Material and methods

Seeds were collected according to the accepted protocol (Hunt & Moore 2003), e.g. when the seed pods started cracking, and the pods were harvested by hand. We collected a small sample of seeds, bearing in mind that these are strongly vulnerable species and their population balance *in situ* should not be disturbed. The seeds were kept at room temperature (16–21 °C).

Germination procedures took place from 3rd to 15th April 2008, in a laboratory of the Faculty of Pharmacy in Sofia, using a natural light–dark photoperiod, at room temperature (18–21 °C). The sets of seeds, each of 10, were processed as follows: two sets of *A. alopecurus* seeds collected in October 2003, six sets of seeds *A. alopecurus* collected in October 2005, and two sets of *A. dasyanthus* seeds collected in August 2007. Also two seeds of *A. dasyanthus* produced by spontaneous self-pollination were processed for germination (self-pollination occurs rather seldom). Scarification of the seeds was done with fine sandpaper. The seeds were placed in Petri dishes on wet filter paper.

Once the seeds had germinated, the seedlings were transferred individually to plastic pots filled with soil. At first watering was from below (pots stood in 3 cm of tap water). The experiment was terminated when the seeds that failed to germinate began to go mouldy.

When seedlings were at the 2–6 true leaf stage, they were transported to the experimental botanical garden of the Faculty of Pharmacy in the centre of Sofia. The altitude of the experimental plot was 550 m a.s.l. The native population of *A. alopecurus* was situated at 1670–1680 m a.s.l., in a spruce forest belt (Kožuharova & Firmage 2007). The native population of *A. dasyanthus* was situated at 500–575 m a.s.l., in the Struma Valley, where Mediterranean influence is obvious (Kožuharova & al. in press). Artificial watering of the seedlings was applied periodically. Summer temperatures in the three places are given in Table 1. While the average July temperatures in Sofia and Boboshevo are rather similar, it is much cooler in Beglika.

Results and discussion

Seed germination and seedling development of *A. alopecurus*

The mature seeds of the test plants germinated within a couple of days, if the seed coat was scarified (Fig. 1). A total of seven seeds (35.0 %) germinated from the sample collected in 2003 (N_{seed} = 20). A total of 17 seeds (28.3 %) germinated from the sample collected in 2005 (N_{seed} = 60). Most of the seeds germinated during the 4th and 5th day after seed treatment (Fig. 1.). Of all seeds that germinated, 14.4 % of those produced in 2003 and 58.8 % of those produced in 2005 germinated on day four, while 71.4 % of those produced in 2003 and 11.8 % of those produced in 2005 germinated on day five. The seeds which matured in 2003 maintained high germination ability for five years (Fig. 1).

Of the 24 seeds that germinated, a total of 11 seedlings survived to be planted in the experimental garden. These were as follows: four seedlings from seeds matured in 2003 (57.2 % survival rate) and seven seedlings from seeds matured in 2005 (41.2 % survival

Table 1. Climate specifics of the *in situ* and *ex situ* study sites.

Locality	Annual temperature	Average temperature in January	Average temperature in July	Spring arrival and the start of vegetation season
Beglika	Average 3.7°C absolute min −35.7°C absolute max 33.7°C	−5.7° C Winter rainfall 244 mm Sustainable snow cover 110 cm that lasts on the average 108 days	12.7°C Summer rainfall 255 mm	Cold spring the real spring comes at the end of May Spring rainfall 250 mm Warm autumn Autumn rainfall 189 mm
Boboshevo	Average 11.5°C absolute min −17.0°C absolute max 36.0°C	0.5° C	22,0°C 206 days with temperature above 10°C	Last snow cover end of March Late spring frosts Average annual rainfall 600 mm The vegetation season is 7 months Long dry summer periods
Sofia	Average 10.0°C absolute min −31.5°C absolute max 40.0°C	−2.0°C	19.7°C	the real spring comes in mid-April (last spring frost) Artificial watering in the garden

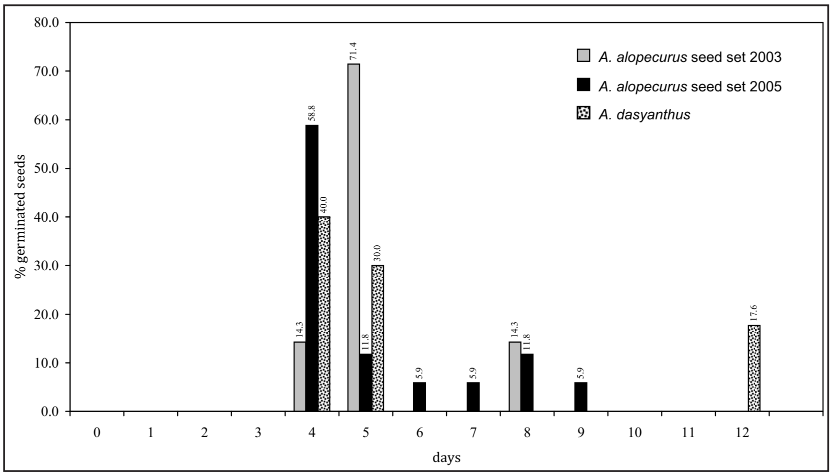

Fig. 1. Seed germination dynamics. Percentage of all germinated seeds during each day of the experiment.

rate). Of these, only three survived until the end of the summer (all originating from the seeds matured in 2003). They wintered successfully and produced vigorous stems (Plate I, figs 1 & 4): the first seedling – a total of six stems (three flowering stems with 1–3 flower heads per stem), the second seedling – a total of three stems, and the third seedling – a total of six stems (four flowering stems with 1–3 flower heads on each stem). The flowering phenology was several weeks earlier, as compared to the native population: end of May – early June *ex situ* (the garden in Sofia), versus the first two decades of July *in situ* (in the mountains, Beglika). This is not surprising, considering the fact that in Beglika the spring comes later in comparison to Sofia and it is much cooler in the summer (Table 1). Also, the thick snow cover in Beglika is an efficient insulation against the winter frosts, while in Sofia frosts without snow are the main cause of the high mortality of many mountain plants introduced into gardens. The flowering phenology of individual flowers and flower heads was similar to the wild population (Plate I, figs 2 & 3). The flowering of a head was accomplished in approximately a little over a week during our study of the native population, as the hot and dry weather speeded up the process that year (Kožuharova & Firmage 2007).

By the end of the second season of garden growth two plants died. The first one, with only three vegetative stems, dried up by mid-July. The other, with six stems, four of which were generative ones, dried up by mid-September, after a successful fruit set of those few flowers from its flower heads that were pollinated. The underground parts of both plants dried up for some reason.

The seeds scarified with sandpaper in this experiment did not germinate as well, as in our previous tests. All test seeds ($N_{seed} = 10$) matured in 2003 that were kept for four years at room temperature and were scarified carefully with a needle, germinated within a couple of days (Kožuharova & Firmage 2007). At the beginning, these seedlings developed well. However, only one survived to be planted in a garden (experimental plot at the foothill of the Pirin Mts at 900 m a.s.l., with environmental conditions similar to the native habitat). No seedling survived until the first winter. The needle versus sandpaper scarification tests revealed that the hard seed coat is better scratched with the needle. Only the seedlings' further successful development is dubious and perhaps the need to break through a more intact seed coat is beneficial to the emerging seedling.

All these tests showed that the seeds of *A. alopecurus* preserve their viability for several years but the seedlings are vulnerable and show low tolerance of the *ex situ* environment.

Seed germination and seedling development of *A. dasyanthus*

A total of 10 seeds (50.0 %) germinated. Most of the seeds germinated during the 4th day (40.0 % of all germinated seeds) and 5th day (30.0 % of all germinated seeds) after the seed treatment (Fig. 1). Seven seedlings survived to be planted in the botanical garden. Both seeds produced from self-pollination germinated, but only one of them survived to be planted in the botanical garden. By the end of the season, three plants survived: two produced by seeds from free pollination (Plate II, fig. 1 plants "a" and "b"), and one by self-pollination (Plate II, fig. 1 plant "c"). They wintered successfully and one of these plants produced a flowering stem with three flowering heads (Plate II, figs 2–6, plant "a").

The flowering phenology was a bit later, as compared to the native population. In the wild population near Boboshevo, it took place during the entire month of June and early July (Kozuharova & Firmage 2009), while in the experimental garden it was in the first decade of July. By the end of the second season of vegetation all plants were alive.

Conclusion

One of the reasons for the vulnerability of both studied rare *Astragalus* species is obviously connected to the peculiarities of their reproductive biology. Pollination, fruit and seed set in their native populations are more or less sufficient (Kožuharova & Firmage 2007, 2009). The preliminary observations on seed germination and survival of seedlings have indicated obstacles at the second stage. The low survival of seedlings may be genetically determined, but also there was a low tolerance of the *ex situ* environment provided for the seedlings. This was particularly true of the specimens of *A. alopecurus,* whose native habitat is at higher altitudes in the mountains. Obviously, a set of factors has their say: soil water balance, insufficient/excess of soil moisture, heat, dust and gas air pollution may have suppressed the seedlings.

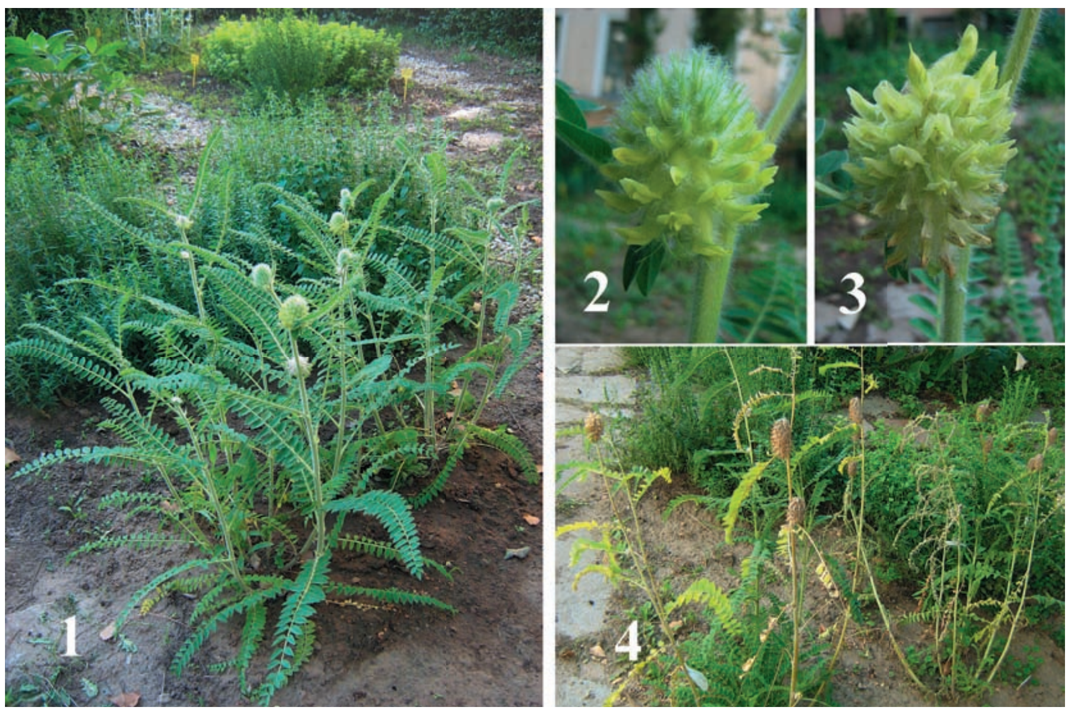

Plate I. Development of *A. alopecurus* seedlings in the summer of 2009: **1**. Phenological stage at 28th May 2009 of the three plants developed from seed in 2008; **2.** Phenological stage at 28th May 2009 of a middle flower head of the frontal plant; **3.** Phenological stage at 3rd June 2009 of the same middle flower head of the frontal plant; **4.** Phenological stage of the plants on 23rd July – only two of them survived by that time.

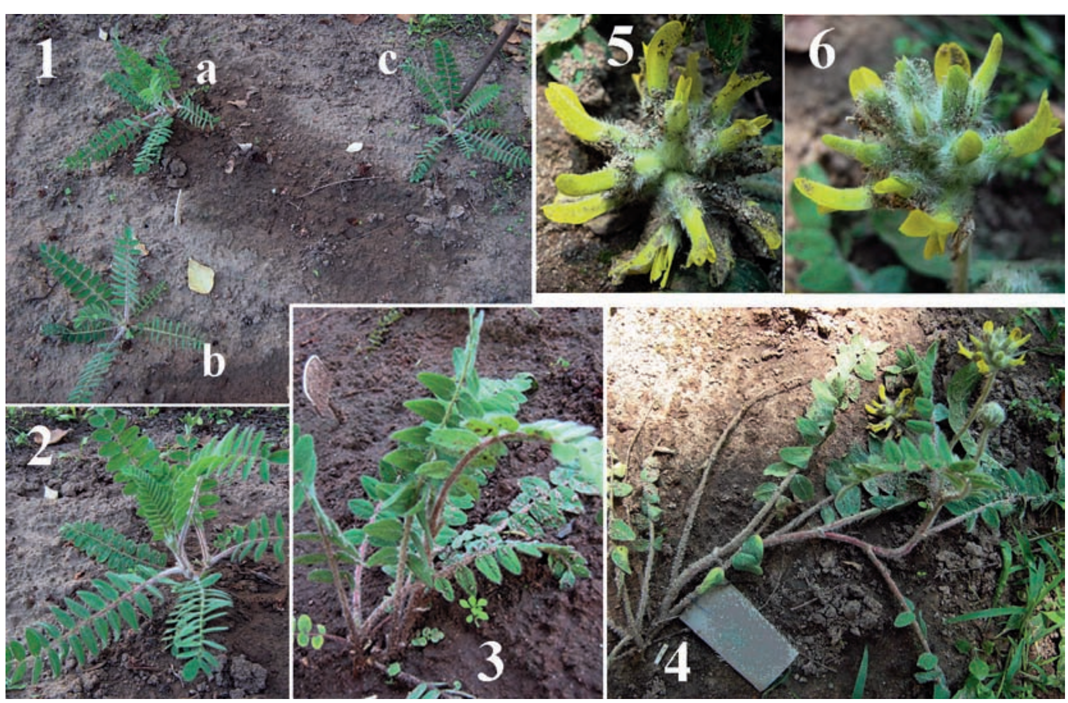

Plate II. Development of *A. dasyanthus* seedlings in the summer of 2009: **1.** Phenological stage at 28th May 2009 of the three plants developed from seed in 2008; **2.** Plant “a” on 28th May 2009; **3**. Plant “a” on 3rd June 2009; **4.** Plant “a” on 5th of July 2009; **5.** Phenological stage of first/base flower head of plant “a” on 5th of July 2009; **6.** Phenological stage of second/middle flower head of plant “a” on 5th of July 2009.

We assume that the necessity of scarification of the seeds is the reason for both *A. alopecurus* and *A. dasyanthus* to occupy such sandy and stony places and to need bare ground to start their development from seed.

The observed difficulties with the seedlings confirm the necessity to preserve the native habitats of both species and to ensure possibility for their *in situ* propagation.

Acknowledgements. This study was conducted with the financial support of the National Science Fund, Ministry of Education, Youth and Science, R Bulgaria, Project B–1540/05. The authors are indebted to the anonymous reviewer for the valuable suggestions.

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