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Attempts to save a Spanish endemism *Astragalus devesae* Talavera, A. González & G. López (Fabaceae) from extinction

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Abstract: *Astragalus devesae* Talavera, A. González & G. López (Fabaceae) is a threatened Hispanic endemic species whose distribution is restricted to the center of Spain (the province of Ávila). It is classified as "Critically Endangered" in the 2010 List of Threatened Spanish Vascular Flora and as "Endangered" in the "Decree 63/2007" of Castile and Leon (administrative region in which Ávila is included). In this study, censuses of three existing populations were carried out and one of these that was found along the course of this work, was ecologically characterized. High interpopulation variability (0%, 26%, and 73%) was observed with respect to seed germination rates, obtained through germination tests using chemical scarification with sulfuric acid (H_2SO_4). In addition, successful experimental population reinforcement was carried out (40%–60% survival of individuals) using plants obtained from previously germinated seeds. The results of this work contribute positively to the conservation of this species, although further studies to understand its biology and to find new populations are required.

Key words: *Astragalus*, chemical scarification, endangered species, in situ and ex situ conservation, population reinforcement, seed germination

1. Introduction

The Iberian Peninsula is one of the most biologically diverse regions in the world (Lozano et al., 1996; Reyjol et al., 2007); however, the extinction rate of some species has intensified due to the global environmental crisis (Wilson, 1988; Fontaine et al., 2007). As a result of this fateful context, applied conservation works similar to the work presented here are fundamental for minimizing species loss. The genus *Astragalus* L. (Fabaceae) is comprised of 2500–3000 taxa with more than 250 sections and is one of the largest genera of vascular plants (Podlech, 1986; Maassoumi, 1998). In addition, *Astragalus* L. forms part of a noteworthy center of diversity within the Iberian Peninsula, with 42 species, nine of which are endemic (Podlech, 1999; Martinez-Fernandez et al., 2014). These species comprise the section termed Platyglottis and include: *Astragalus gines-lopezii* Talavera, Podlech, Devesa & F.M.Vázquez, endemic to the province of Badajoz (Extremadura, SW Spain), *A. nitidiflorus* Jiménez Mun. & Pau, endemic to Cartagena (Murcia, SE Spain), and *A. devesae* Talavera, A. González & G. López, endemic to Ávila (central Spain) (Martinez-Fernandez et al., 2014). In all three cases, these species live in very restricted areas and in small populations, and all of them were initially included within the same species *A. nitidiflorus* (Teresa et al., 2009a). However, detailed morphological studies differentiated them as separate species (Talavera and Salgueiro, 1999). In particular, *A. devesae* is distinguished by the presence of free stipules, and all have white hairs and a ventricular calyx, as opposed to the joined stipules, white and black hairs, and the tubular calyx of *A. gines-lopezii* and *A. nitidiflorus*. In addition, the fruits of *A. devesae* are larger. *A. gines-lopezii* in turn is distinguished from *A. nitidiflorus* by its retuse leaves blunt at the apex and an inflorescence containing 2–8 flowers, whereas *A. nitidiflorus* has 20–25 flowers. In addition, the chalice tube in *A. gines-lopezii* is 3.5–4.5 mm, shorter than that of *A. nitidiflorus* (Talavera and Salgueiro, 1999).

In the 2000 Red List of Spanish Vascular Flora (VV. AA, 2000), *A. devesae* appears as "Endangered" (EN) with the criteria of B1 + 2d (IUCN, 1994). Thanks to the new information available and according to IUCN's most recent criteria (IUCN, 2001), this species has been included within the greater threat level of "Critically

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Endangered" (CR), as recorded in the Atlas and Red Book of the Threatened Vascular Flora of Spain (Moreno, 2008; Bañares et al., 2010). Moreover, this species was also included in the category of "Endangered" in the June 14th "Decree 63/2007", which led to the creation of the Regional Catalog of Protected Flora of Castilla y León and the Figure of Natural Protected Areas called "Plant Micro-reserves" (Laguna-Lumbreras et al., 2016; BOCYL, 2007). Prior to this work, only two populations of *A. devesae* had been identified, in Padiernos and Collado del Mirón (1218 and 1180 m altitude, respectively), in the province of Ávila, occupying a total area of approximately 8000 m^2 (Martínez et al., 2004). The population located in Padiernos was the most extensive, while the population in Collado del Mirón was extremely small, with an occupation area of only 304 m^2 (Teresa et al., 2009a). The last two censuses of these two populations were carried out in 2008 (Teresa et al., 2009a), where 311 individuals in Padiernos and 22 in Collado del Mirón were recorded. These authors also suggested that an exhaustive exploration needed to be carried out in the Sierra de Ávila and adjacent territories, which could possibly lead to the discovery of new populations. Through this initiative, the Environmental Protection Agency of the Regional Government of Castile and Leon located a new population of *A. devesae* in the town of Avila in the spring of 2015. Here, we updated the censuses of known populations, carried out a detailed study of the occupation area, characterized the habitat, and created a census of the new population.

Regarding seed germination, there are few studies that relate the ecology of the germination of the endemic species of the Iberian Peninsula with their conservation (Albert et al., 2002; Giménez-Benavides et al., 2005; Copete et al., 2011; Fernández-Pascual et al., 2012). In this research approach, in situ conservation is complemented with ex situ conservation by storing the representative germplasm of the populations, allowing a better understanding of the anatomy and physiological characteristics of the stored material, as well as providing propagules for use in breeding programs and reforestation, reinforcement, reintroduction, or introduction projects (Conway, 1988; Bacchetta et al., 2008). In this sense, results of previous tests carried out at the Germplasm Bank of the University of Salamanca (BG-USAL) during the years of 2009 and 2011, have allowed the election of the optimal protocol for new germinative assays performed in our study.

The objectives of the present study were: 1) to evaluate the state of conservation of *A. devesae* by updating existing data on its chorology and to carry out new censuses on three known populations; 2) to determine the interpopulation variation of *A. devesae* with respect to the response of seeds to germination tests; and 3) to evaluate the experimental population reinforcement carried out using one of the populations.

2. Materials and methods

2.1. Study species

A. devesae is a multicauline perennial herbaceous plant with a woody base. The indument is dense and whitish, and formed of simple hairs. Stems can grow to 45 cm in length and are prostrate ascending or procumbent. The leaves are petiolate and imparipinnate, with 8–13 oblong–elliptic to ovate leaflet pairs with a glabrous upper leaf side and a villous underside. The stipules are widely ovate–acuminate and free, and the inflorescence is comprised of pale yellow flowers, sometimes slightly pink at the apex of the keel, present in loose clusters. The calyx is membranous, and the indehiscent leguminous fruit is cymbiform in shape and thickly covered with hairs, with a ribbed back and containing several kidney-shaped seeds (Teresa et al., 2009a) (Appendix A1). Plants flower between May and June and the fruiting period occurs between June and July. The flowers are insect pollinated, probably generalist, the fruits are dispersed whole at close range and seeds can be retained from one year to the next (Martínez et al., 2004; Teresa et al., 2009a).

Populations of this species are located within the vegetation of the supramediterranean range, characterized by a Mediterranean pluviestacional–oceanic bioclimate, with a dry ombroclimate. They grow in mixed soils made up of limestone and siliceous granitic sands, at altitudes that range from 1140 m to 1180 m (Martínez et al., 2004). The plants form a part of xerophytic pastures, which grow in sparse Mediterranean scrublands that develop under holm oaks (*Quercus ilex* L.subsp. *ballota* (Desf.) Samp. (= *Quercus rotundifolia* Lam.)). Only three populations are known, all of which are located in the province of Ávila (Figure 1), the town of Padiernos, Collado del Mirón, and municipal term of Ávila, and the distance between the three locations is less than 70 km (Martínez et al., 2004; Teresa et al., 2009a).

2.2. Censuses and ecological characterization of the habitat

In April 2017, censuses were carried out on the three populations and covered the total occupation area, in order to account for the total number of individuals per population. Data were georeferenced with GPS and the actual area of occupation of the species was then calculated using the ArcGIS 10.3.1® software "Calculate Geometry" function. In addition, data on habitat and any accompanying species were collected for the new population, and an assessment was carried out regarding their conservation status.

2.3. Seed collection and germination tests

The main objective of this work was to detect differences in the germination rates of the seeds among the known populations of *A. devesae* that could help us to explain

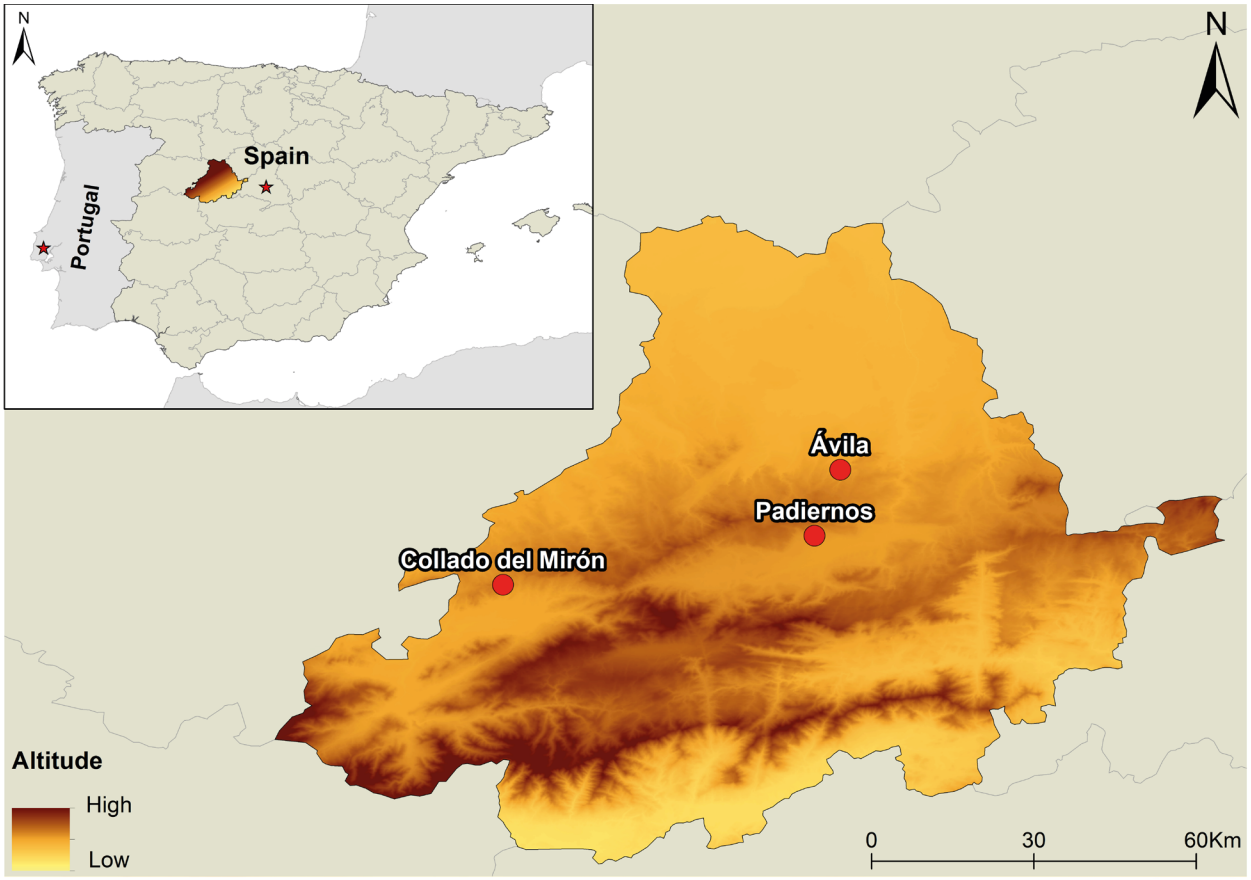

Figure 1. Location of the three known populations of *Astragalus devesae*, province of Ávila, Spain.

their conservation status. Since the seed cover of most Fabaceae is quite hard, being responsible for its physical latency (Baskin and Baskin 1998; Zeng et al.; 2005; Kimura and Islam 2012), the following preliminary treatments, as recommended by ISTA (ISTA, 2004), were carried out between 2009 and 2011 in the BG-USAL with *A. devesae* seeds: 1) cold room stratification at 5 °C for two months; 2) physical scarification with boiling water; 3) mechanical scarification using sandpaper, and 4) chemical scarification with sulfuric acid (H_2SO_4) (96%) for different periods of time (30, 45, 60, and 75 min). Among the various treatments, the last one produced the highest germination rate (69%), which included the seeds being imbibed in H_2SO_4 for 60 min, followed by five washes with distilled water. Germination took place in a chamber with a controlled alternating temperature of 22/10 °C, with 12 h of light during the high temperature and 12 h of darkness during the low temperature (Sánchez-Agudo et al., 2011; Sánchez-Duran et al., 2011). From the previous result, we chose the optimal germination protocol. Due to reduced seed availability and the need to obtain live plants, no other treatments were tested.

The seeds for this study were collected manually in July 2015 (Padiernos and Ávila) and July 2016 (Collado del Mirón). Approximately 325 seeds from 14 specimens were collected from the population located in Padiernos, approximately 600 seeds from 20 individuals in Ávila, and 310 seeds from 6 specimens in Collado del Mirón. Seeds were stored at room temperature (approximately 23 °C) for a postmaturation period of one month, a step recommended by Bacchetta et al. (2008). Germination tests were carried out in August 2015 with the seeds collected from Padiernos and Ávila, and in August 2016 for the seeds collected in Collado del Mirón. For each population, ten replicates of 30 seeds were plated out onto Petri dishes containing presterilized 1% agar. Light photoperiod was provided using cool white fluorescent tubes with an irradiation of 35 µmol m^{-2} s^{-1}. The seeds were checked every two days for a period of 30 days, and the germinated seeds (with a radicle length of more than 2 mm) were counted. The seedlings were transplanted into soil and housed and maintained in a greenhouse at the Spanish–Portuguese Institute for Agricultural Research (CIALE) of the University of Salamanca.

2.4. Experimental reinforcement of the Padiernos population

Seventy plants obtained from the seeds collected (2015) in the Padiernos population were used for experimental population reinforcement, which was carried out in two steps. Firstly, in the spring of 2016 three cores were established (I, II, and III) within the total occupation area of the population (Figure 2). In order to evaluate the survival rate of the plants per core, each population was visited four times in different seasons: in June and July, dry period, November 2016, and finally in February 2017. In April 2017, a new reinforcement was carried out, and four more cores were established (IV, V, VI, and VII) (Figure 2). All cores were monitored in two more visits, carried out in July and October of 2017. In each core, 10 specimens were planted, marked, and labeled, respecting a minimum distance of 1.5 m between each plant. In Collado del Mirón population, no reinforcement was done due to lack of germinated seeds. Finally, in the case of the Ávila population, no reinforcements were carried out since the population was located on private property and we did not have the appropriate permission.

2.5. Statistical analysis

A statistical analysis was performed to evaluate the interpopulation difference in the tests carried out in the same year (2015, Padiernos and Ávila). The final percentage of accumulated germination (mean value ± standard error) and the mean germination time (MGT, mean value in days ± standard error) for each population were calculated. The MGT was determined according to the following formula: $MGT = \Sigma DN / \Sigma N$, where D is the number of days counted from the date of sowing and N is the number of seeds germinated on day D (Ellis and Roberts, 1981). The values for the final percentage of germination were transformed into an arcsine square root and then subjected to a two-way analysis of variance using the Student's t-test (data not shown transformed into Table 2). Subsequently, the Mann–Whitney U test was applied to MGT values. All statistical analyses were performed using the R program (R Core Team, 2016).

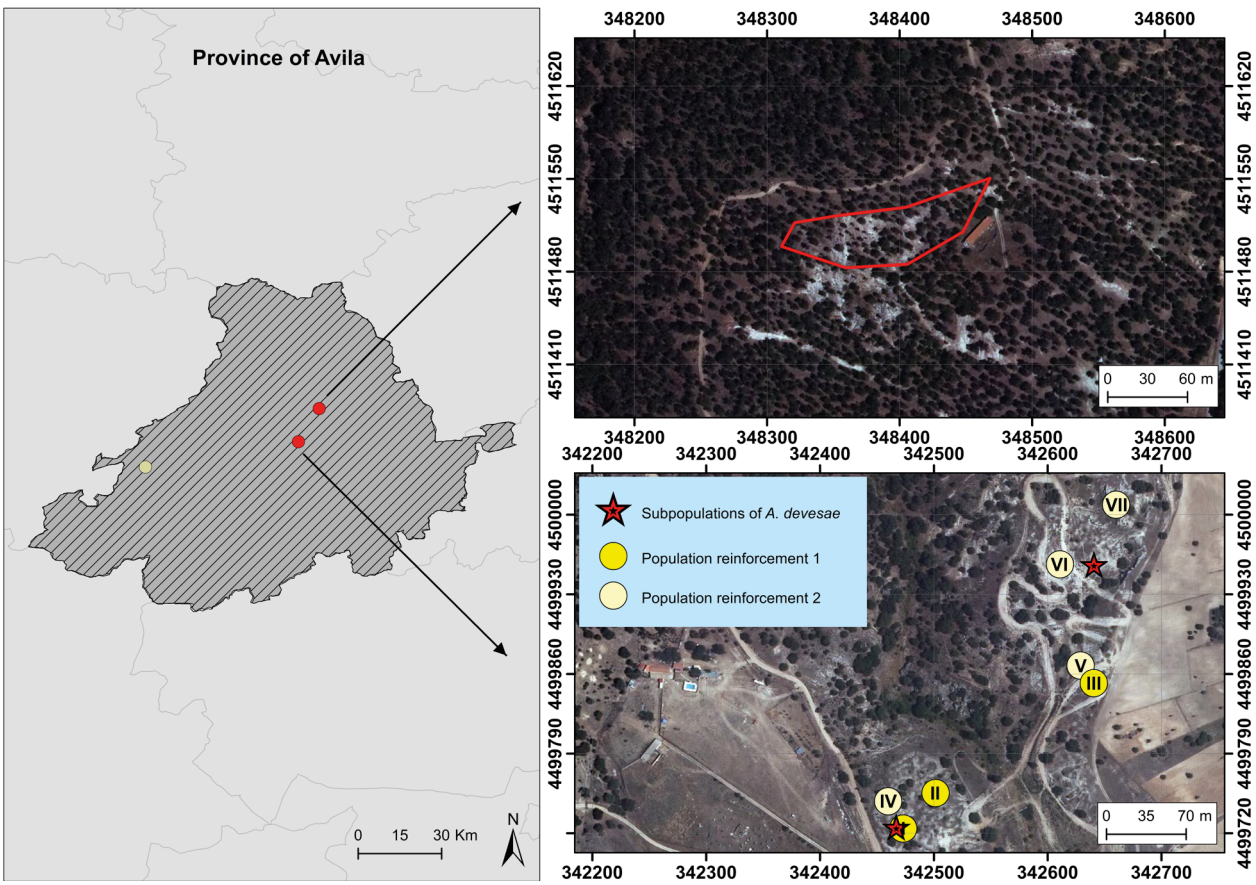

Figure 2. (Above) Area of occupation (5176 m^2) of *Astragalus devesae* in the new population of Ávila. (Below) Approximate location of the two subpopulations of *Astragalus devesae* indicated with a triangle; circles show the location of population reinforcements performed in two steps.

3. Results

3.1. Census

The total number of plants recorded in 2017 for each population is shown in Table 1. Considering the three populations, the occupation area is around 13,500 m^2. The Padiernos population was comprised of 317 individuals distributed in two subpopulations (Figure 2). The state of the small population of Collado del Mirón was even more critical in that year, since only one individual plant was found. This means that more than 90% of the population (according to the census of 2009) had been removed as a result of extractive activities (Appendix A2). The new population located in Ávila was made up of 234 individuals within a total occupation area of 5170 m^2 (Figure 2). This population was located in a pasture with holm oaks (agrosilvopastoral system) that was being grazed by cattle. In addition, the following accompanying species were observed: *Astragalus alopecuroides* L., *Eryngium campestre* L., *Carthamus lanatus* L., *Agrostis castellana* Boiss. & Reut, *Cytisus multiflorus* (L'Hér.) Sweet, and *Thymus mastichina* L. The pressure caused by livestock grazing on this population seems to be low. Therefore, taking into account the high number of individuals and their general good condition, it can be said that the state of conservation and protection of the *A. devesae* population in Ávila was adequate.

3.2. Germination tests and interpopulation variability

Regarding the population of Collado del Mirón, although its seeds appeared healthy and with optimum conditions for maturation, none of the seeds germinated. For the two remaining populations, Padiernos and Ávila, significant differences ($P = 1.06^{E-08}$) were found with respect to the final percentages of germination observed (73% and 25.66%, respectively) (Table 2). Also, there were differences ($P > 0.01$) between the MGT values of the two populations, which would point to a slightly earlier germination in the Ávila population. The seedlings used for the reintroduction experiment in Padiernos were obtained from these germination tests.

It is necessary to highlight the occurrence of fungal contamination of the agar plates with seeds at the end of the 30-day monitoring period. Fungal contamination was common in all the analyzed populations; this was the reason why it was not possible to carry out viability tests on nongerminated seeds. Further studies are needed to evaluate the causes and effects of the fungi on seeds of *A. devesae*.

3.3. Experimental reinforcement in Padiernos

Table 3 shows the number of dead specimens and the survival rate per core and day of evaluation. ***Population reinforcement 1:*** It was obvious for us that summer was the critical period for the establishment of *A. devesae*, where a mortality rate of 60% was observed in core II and 30% in core I. Cores I and III were located on soils similar to that of the two natural *A. devesae* subpopulations. Overall, the final survival rate was 70% in core I, 10% in core II, and 80% in core III, and the total survival rate

Table 1. Census data, altitude, latitude, and longitude coordinates, and surface occupied related to the three known populations of *Astragalus devesae*.

Population	Altitude (m a.s.l.)	Latitude	Longitude	Surface (m^2)	Census 2017
Collado del Mirón	1180	40°33N	–5°21N	304	1
Padiernos	1218	40°38N	–4°51N	8000	317
Ávila	1099	40°44N	–4°47N	5176	234

Table 2. Final germination percentage and mean germination time (MGT, days) of chemical scarified seeds from *Astragalus devesae* populations. Results after 30 days of incubation at 22/10 °C under a 12-h light photoperiod.

Population	Germination (% ± SE)			MGT (days ± SE)		No. of seeds tested
Collado del Mirón	0			0		300 (6 plants)
Padiernos	73 ± 8.95			8.94 ± 1.58		300 (14 plants)
Ávila	25.66 ± 10.42			6.48 ± 2.84		300 (20 plants)
t Table / U Table	t	df	P	W	P	-
Pop. (Padiernos x Ávila)	9.8902	18	***	82	0.01469	-

***: $P < 0.000$

Table 3. Number of dead plants and survival rate observed for *A. devesae* by date in each of the seven cores of population reinforcement carried out in the population of Padiernos.

Date	Core	No. of dead plants	Survival rate (%)
Population reinforcement 1			
Jun/2016	I	0	100
	II	0	100
	III	0	100
Jul/2016	I	0	100
	II	2	80
	III	0	100
Nov/2016	I	3	70
	II	8	20
	III	2	80
Feb/2017	I	3	70
	II	9	10
	III	2	80
Population reinforcement 2			
Jul/2017	IV	0	100
	V	0	100
	VI	6	40
	VII	2	80
Oct/2017	IV	6	40
	V	5	50
	VI	10	0
	VII	4	60

Core: n = 10

was 53.3%. ***Population reinforcement 2:*** Only one core (VI) had a low survival rate, with six dead plants as soon as the first evaluation visits in July 2017. Unfortunately, in the second and last evaluation visits the plants of that core had disappeared. This circumstance would be associated with the presence of wild animals, since the soil was quite removed and the stones were also moved (Figure 3). In the remaining cores, the survival rate was satisfactory, with an average of 93% in the first visit and 50% in the second (Table 3). Also, we observed plants with flowers in cores I and III during the first visit to evaluate the survival rate of the second reinforcement (July 2017; Figure 3). Figure 4 shows the average survival rate of all plants per stage (reinforcement 1 and reinforcement 2) and per evaluation visit.

4. Discussion

One of the main objectives of germination tests is to develop a specific protocol for each taxon that makes it easy to get plants into the laboratory for subsequent use in plans to reintroduce or reinforce populations in their natural habitat (Conway, 1988; Bacchetta et al., 2008). In this context, the first stage carried out in the BG-USAL took place between 2009 and 2011, in which a viable germination protocol was designed. Later on, between 2015 and 2017, our work included in vitro germination, population reinforcement, and population monitoring. Therefore, the present study is a practical case for evaluating the effort required to carry out ex situ conservation studies applicable and complementary to in situ conservation. It was observed that *A. devesae* maintained high germinative capacity over time; our results (73% germinative rate) are similar to those obtained in previous studies that compared the same treatments (Sánchez-Agudo et al., 2011; Sánchez-Duran et al., 2011).

Since the waterproof layer of the seed seems to be responsible for the physical latency of many Fabaceae species (Baskin and Baskin, 1998; Baskin and Baskin, 2004; Patanè and Gresta, 2006; Pérez-García, 2009; Kimura and Islam, 2012), one of the best techniques to overcome this latency is to use mechanical scarification, which involves the creation of physical scars on the surface of the seed to increase water absorption (ISTA, 2004; Kimura and Islam, 2012). In contrast to our previous results, Martínez-Fernández et al. (2014) achieved a germination rate of up to 99% by applying this technique to *A. gines-lopezii*, which, as already mentioned, is one of the closest species to *A. devesae*. Likewise, scarification treatments also provided the best results for *A. nitidiflorus* (Carrión et al., 2007). Patanè and Gresta (2006) observed that mechanical scarification with sandpaper was the best treatment to break the physical latency of *A. hamosus* L. seeds (the percentage of germination of nonscarified seeds was less than 10%, whereas 100% of the scarified seeds germinated). The effectiveness of sandpaper scarification was also reported for *A. sinicus* L. by Kim et al. (2008). In the study carried out by Ramos et al. (2010) for *A. gines-lopezii*, two scarification treatments were applied (physical and chemical treatments using 96% sulfuric acid), as well as a third protocol with no previous treatment and different light regimes (photoperiod and darkness). These results differ from those found by Martínez-Fernández et al. (2014) and ours, since these authors showed that nonscarified seeds had the highest percentage of germination (80%) and that scarified seeds with sulfuric acid had the lowest one (2.5%). This contradiction might be due to divergent conditions of seed storage or light regimes applied in these studies.

The physical latency of seeds implies that not all mature seeds produced by the same plant germinate at the same time (Baskin and Baskin, 1998). As a result, the risk of an extreme event damaging the entire generation

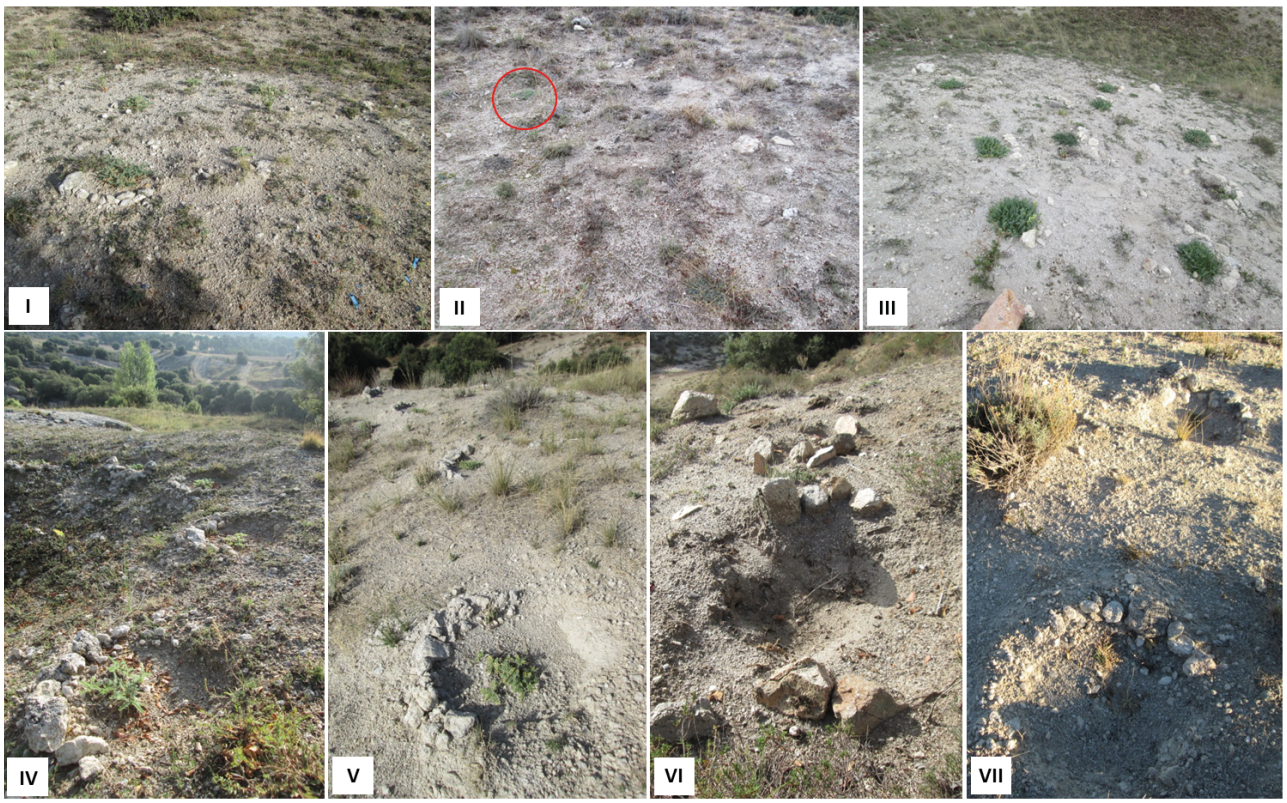

Figure 3. General view of the seven cores of population reinforcements in Padiernos, Ávila. Failure of establishment and survival of plants in core II (red circle indicates the only surviving plant) and in core VI is observed.

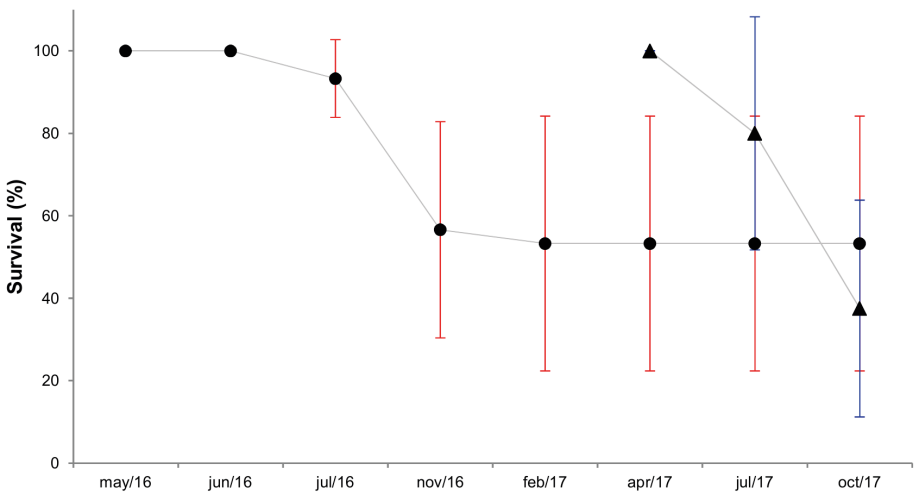

Figure 4. Average survival rate and SD of all plants per stage (circles represent reinforcement 1 and triangles represent reinforcement 2) and per evaluation visit (days of execution included); population of Padiernos, province of Ávila.

of new seedlings diminishes. Therefore, physical latency is an adaptive characteristic that has been developed by numerous species and has been generalized in various plant families throughout their evolution (Cistaceae, Leguminosae, or Malvaceae) (Baskin and Baskin, 1998). Despite the high germination potential observed in the present study on seeds from the Padiernos population (73%), this population is noticeably decreasing. Prior to

this study, in the census carried out on this population, Martínez et al. (2014) counted 478 individuals in 2004. In 2008, Teresa et al. (2009a) counted 311, revealing a 35% decrease in the number of individual plants. Moreover, Teresa et al. (2009a, 2009b) associates the circulation of motocross vehicles within the exact occupation area of *A. devesae* as the main threat to this population. However, the closure of this circuit in 2008 and the implementation of a microreserve (Teresa et al., 2009b) seem to have been good strategies for the protection and maintenance of this species, since we counted 317 individuals in our study, a number similar to that of 2009.

In many cases, seeds with waterproof protection have a longer life under natural conditions and accumulate in soil seed banks (Baskin and Baskin, 2000). This relationship between hard seed and seed bank dynamics has been reported for different annual legumes (Russi et al., 1992; Zeng et al., 2005). This hypothesis could explain the low germination rate of the seeds found in the Ávila population (25.6%) and the significant difference observed in the MGT between the two populations. The same difference in germination rates between populations had been found by Martínez-Fernández et al. (2014) for *A. gines-lopezii*. However, for the MGT, these authors did not find significant differences between populations, which differs from our results. Thus, more germination improvement studies are required, using the seeds produced over the course of several years.

In relation to the absence of germination for the seeds from the Collado del Mirón population, several studies involving different species confirm a reduced seed germination potential in small and isolated populations (Menges, 1991; Buza et al., 2000; Bellusci et al., 2009; Sletvold et al., 2012). This is indeed the case for this small population, located approximately 65 km away from the other two known populations. The fact that only one specimen was observed in the last census highlights the need for the implementation of legal measures to protect this population, which has suffered greatly due to the mechanical removal of soil on which this species is found. Perhaps the existence of a seed bank could allow its survival; in a recent visit to this population in October 2017, we observed five new individuals, which indicate that their seed bank is active. However, due to its low genetic variability, this population will probably become extinct, a common circumstance to other endangered species (Raijmann et al., 1994; Newman and Pilson, 1997; Buza et al., 2000; Luijten et al., 2000; Zoro Bi et al., 2003). Inbreeding depression may have been the main cause of the decline of the Collado del Mirón population, which is currently even more noticeable. Teresa et al. (2009a) pointed out that this population is so small that any perturbation could lead to its rapid disappearance, a statement which has been proven in our study.

The preliminary data presented here in this first reinforcement experiment for *A. devesae* can be considered successful according to the criteria of Godefroid et al. (2011). About 50% of the cultivated plants have survived more than a year after sowing, and flowering plants were observed in the last visit. The ability of transplants to flourish and fructify is a key qualitative measure on the final destination of populational restoration (Menges, 2008; Rita and Cursach, 2013). Recruitment is considered the most reliable parameter that indicates the success of a populational restoration because it reflects the life cycle processes, including survival and plant vigor, flower production, pollination, and the presence of suitable areas for establishment of new seedlings (Guerrant and Pavlik, 1998; Godefroid et al., 2011). The monitoring of the survival rates presented in this work, together with the recorded flowering data, indicate that some reinforcement cores have real potential to persist over time. On the other hand, the presence of wild animals was associated with the failure of the establishment of some cores. For example, the main reason for the failure of the reintroduction in core II (only 10% of the final survival rate) was probably the presence of rabbits (*Oryctolagus cuniculus* L.) and wild boars (*Sus scrofa* L.) that could remove the plants (Figure 3).

In conclusion, in addition to other measures for the protection of *A. devesae*, new studies on reproductive biology and genetic variability of this species are necessary. For example, ecological niche modelling (MNE) is being carried out right now to try to find new populations. Thus, the main contribution of this study is the design of an efficient germination method that allows the production of seedlings to be used to reinforce populations, which so far have been found to be successful. Finally, in order to improve the results of new reinforcements, we recommend surrounding the cores with a fence to avoid the entrance of animals, since it was the major factor for plant mortality in our experimental reinforcement.

Acknowledgments

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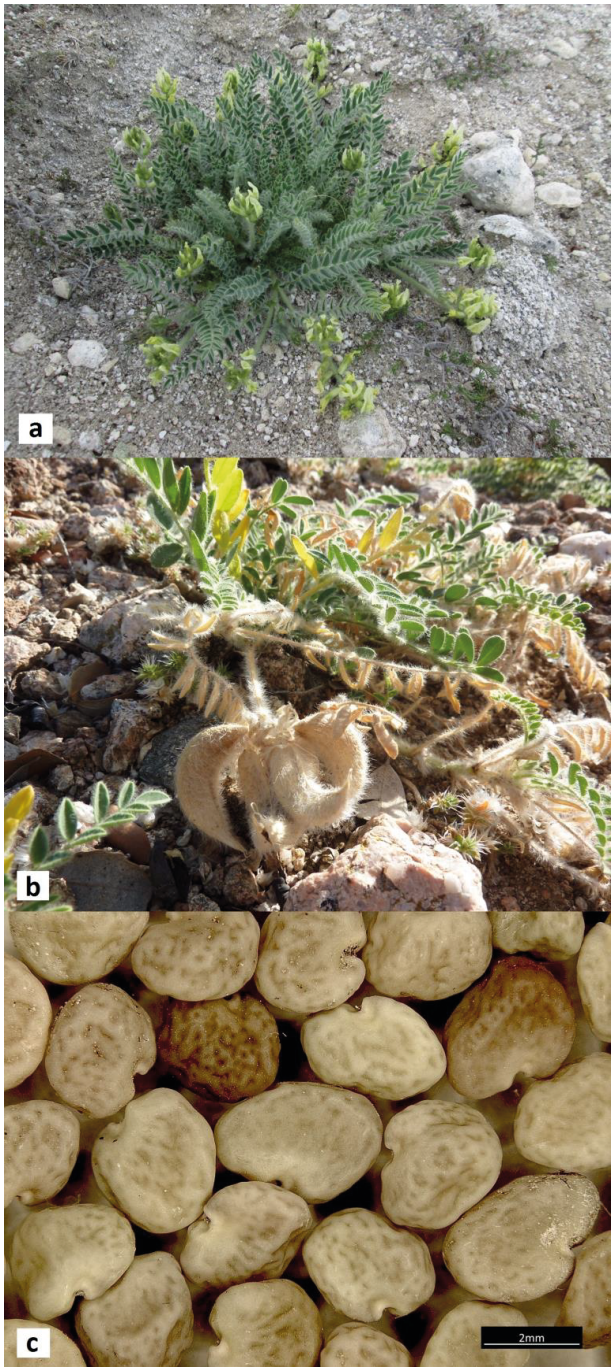
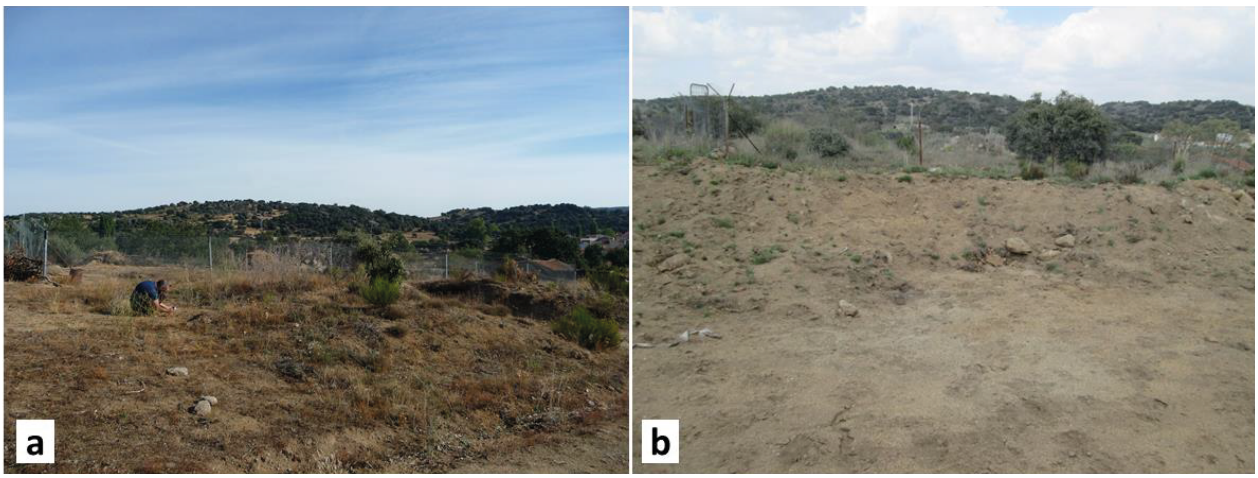


Appendix A1. (a) Specimen of the *Astragalus devesae* from the population of 2 Padiernos; (b) Detail of the fruits of a specimen in the population of Avila; (c) 3 *Astragalus devesae* seeds microscope view.



Appendix A2. (a) Population of Collado del Mirón in July / 2016, when six individuals 2 were observed and the seeds collected. (b) Population in April / 2017, when we 3 observed the mechanical removal of soil and only one individual of *A. devesae* was 4 found.