Germination pretreatments to break hard-seed dormancy in *Astragalus cicer* L. (Fabaceae)

Joseph M. Statwick

2345 S Lowell Blvd, Denver, CO 80219 USA

jstatwick@gmail.com

University of Denver, Department of Biology

Denver, CO 80210, USA

and Denver Botanic Gardens, Department of Research and Conservation

Denver, CO 80206, USA

ABSTRACT

1   Conservationists often propagate rare species to improve their long-term population viability.

2   However, seed dormancy can make propagation efforts challenging by substantially lowering

3   seed germination. Here I statistically compare several pretreatment options for seeds of

4   *Astragalus cicer* L.: unscarified controls and scarification via physical damage, hot water, acid,

5   and hydrogen peroxide. Although only 30% of unscarified seeds germinated, just physical

6   scarification significantly improved germination, whereas one treatment, hot water, resulted in

7   no germination at all. I recommend that rare species of *Astragalus*, as well as other hard-seeded

8   legumes, be pretreated using physical scarification. Other methods may require considerable

9   optimization, wasting precious time and seeds.

INTRODUCTION

12   Propagating wild species in greenhouses and common gardens for their restoration or

13   reintroduction in native habitats can be an effective method of improving the size and viability of

14   rare or threatened populations (Maunder, 1992; Menges, 2008). Such *in situ* and *ex situ*

15   propagation techniques are beneficial, so long as these techniques are successful in establishing

16   additional reproductive adults in novel, degraded, or extirpated sites (Maunder, 1992; Menges,

17   2008). If, however, reintroduction is unsuccessful (which it usually is (Godefroid *et al.*, 2011)), it

18   accomplishes nothing more than wasting resources and even further threatening the species by

19   removing seeds that would have become the future seed bank.

20   At ~3270 species, *Astragalus* (Fabaceae) is the largest genus of flowering plants in the

21   world (Watrous and Kane, 2011). Though a few *Astragalus* are weedy, wide-ranging generalists,

22   specialization on uncommon and infertile soils seems to be a hallmark of the genus (Barneby,

23   1964). Unfortunately, this specialization appears to restrict many species to small geographic

24   ranges, making them more vulnerable to extinction. In the United States alone, the US Fish and

25   Wildlife service (2014) has listed 5 *Astragalus* species as threatened and 16 as endangered, with

26   an additional 5 as candidates for listing, and 3 more currently under review. Although the

27   International Union for the Conservation of Nature (IUCN) red list (2014), a global database to

28   track at-risk species, contains less than one half of one percent of known *Astragalus* species,

29   nearly 40 percent of those with sufficient data are considered "vulnerable" or worse (9

30   vulnerable, 12 endangered, 18 critically endangered, and 1 extinct). NatureServe (2014),

31   meanwhile, lists 100 vulnerable, 58 imperiled, and 31 critically imperiled species, which

32   combine to nearly a third of the 616 *Astragalus* species in its database.

33   *Astragalus* species, like most temperate legumes, as well as species of as many as 15

34   different plant families, have hard seed coats and physical dormancy, which often require

35   scarification or stratification to break (Baskin *et al.*, 2008; Long *et al.*, 2012). In particular, low

36   germination rate has been observed for several rare species of *Astragalus*, including *A.*

37   *nitidiflorus* (Vicente *et al.*, 2011), *A. bibullatus* (Albrecht & Penzagos, 2012), and *A. arpilobus*

38   (Long *et al.*, 2012). Physical dormancy is generally adaptive; it helps delay seedling emergence

39   until favorable environmental conditions, particularly in habitats with high seasonal or

40   interannual variation (Baskin *et al.*, 2008). Prolonged dormancy of the seed bank may also

41   contribute to the maintenance of genetic diversity in rare *Astragalus* such as *A. albens* by

42   resurrecting extirpated genotypes (Neel, 2007). However, this dormancy is counterproductive for

43   *ex situ* propagation efforts.

44   Many scarification treatments for various *Astragalus* species have been explored in the

45   literature, including dry heat (Albrecht & Penzagos, 2012; Chou *et al.*, 2012; Long *et al.*, 2012),

46   wet heat (Acharya *et al.*, 2006, Long *et al.*, 2012), stratification (Acharya *et al.*, 2006; Albrecht

47   & Penzagos 2012; Long *et al.*, 2012), physical scarification (Miklas *et al.*, 1987, Acharya *et al.*,

48   2006; Albrecht & Penzagos, 2012), acid (Miklas *et al.*, 1987, Acharya *et al.*, 2006; Long *et al.*,

49   2012) smoke water (Chou *et al.*, 2012), etc.

50   Generally, physical scarification tends to be reliable for *Astragalus*, but other treatments

51   been successful in some circumstances (Acharya *et al.*, 2006). Long et al. (2012) found that the

52   germination of *Astragalus arpilobus* by hot water scarification was maximized at 100°C for 10

53   minutes of exposure, yet no amount of time at 90°C or below was sufficient to increase

54   germination significantly beyond controls. Fresh *Astragalus cicer* seeds, meanwhile, had

55   maximum germination rates at ≥15 rounds of alternating liquid nitrogen (-196°C for 5 minutes)

56   and steam (100°C for 5 minutes). *Astragalus* seeds treated with concentrated sulfuric acid (18M)

57   for 20 minutes have been shown to germinate very successfully (Miklas et al., 1987). Hydrogen

58   peroxide, which is cheaper and safer to use than acid, has been shown to marginally improve the

59   germination of *Ribes cereum* (Rosaceae) (Rostner et al., 2003), but does not appear to have been

60   tested in the literature for *Astagalus*.

61   Despite these successes, it is rare that the results of more than one or two treatments on

62   *Astragalus seeds* have been compared in the same study. Furthermore, because different species

63   and even collections within species vary in germination rate, (Miklas et al., 1987, Acharya *et al.*,

64   2006; Albrecht & Penzagos, 2012), the results of these studies are not directly comparable to one

65   another in order to determine the most effective scarification treatment. I therefore explored five

66   different pre-planting seed treatments to determine which would best promote germination in the

67   generalist forage crop, *Astragalus cicer* "Oxley".

68

69   METHODS

70   *Astragalus cicer* L. (cicer milkvetch) is an old-world native that was introduced to North

71   America as a hardy, palatable forage crop (Acharya *et al.*, 2006). "Oxley" is an ecotype that was

72   first collected in the former USSR and introduced to the United States in 1971 (Acharya *et al.*,

73   2006). Although *A. cicer* is not rare, it is a suitable model for rare species because it is readily

74   commercially available without threatening wild populations, and because it, like its rare

75   congenerics, is well known for its slow stand establishment, largely due to low germination rates

76   and prolonged seed dormancy (Acharya *et al.*, 2006).

77   I exposed a total of 250 *A. cicer* seeds (Granite Seed, Denver, CO) to each of five

78   different scarification treatments (n=50), starting March 15, 2013 at Denver Botanic Gardens

79 (DBG) in Denver, Colorado. The scarification treatments were physical damage, hot water,

80 hydrogen peroxide, acid, and a control. Control seeds were planted in 1 cm^2 cells in a plastic

81 germination tray, without scarification, on the surface of a seed starter mix, and covered with

82 approximately 3 mm of vermiculite. Treated seeds were planted in the same manner, after

83 scarification, in the same 288-cell tray as the control seeds.

84   For the physical scarification treatment, I cut the seed coat opposite the radicle with a pair

85 of infant nail clippers, being careful to not damage the endosperm or embryo. Because my

86 experiment was performed at ~1600m altitude where water boils at <95°C, I felt the hot water

87 treatment would require a more prolonged soak than is typical. Thus, the seeds were placed in a

88 thermos of boiling water, covered, and soaked for 20 hours before planting. The peroxide treated

89 seeds were soaked in pure ZeroTol, a commercial greenhouse fungicide/algaecide, (BioSafe

90 Systems, East Hartford, CT, 27% hydrogen peroxide) for one hour before planting, I chose a

91 more concentrated solution for a shorter duration than was effective for *Ribes* (4-8 hour soak in

92 3% hydrogen peroxide) because of the thicker, more recalcitrant seed coat in legumes and the

93 increasing seed rot observed with longer exposure times (Rostner et al., 2003). Acid treated

94 seeds were soaked in lab grade sulfuric acid (98%, 18M) for five minutes. This is a reduced

95 duration compared to previous studies because at least some seeds were rendered non-viable by

96 acid treatment, although admittedly "very few" (Miklas et al., 1987).

97   All seedlings were reared in a propagation greenhouse at DBG. The total number of seeds

98 germinated in each treatment was recorded approximately twice per week for one month. The

99 potting soil was checked daily and kept evenly moist by DBG horticulture staff. Plants were

100 exposed only to natural sunlight, which, given the date and latitude, ranged between

101   approximately 12 hours at the beginning of the trial and 13 hours and a half hours at the end of

102   the trial.

103   Germination data were analyzed with a Cox proportional hazards analysis using JMP v10

104   (SAS, Cary, NC, USA). This analysis type is well suited to germination data in that it is intended

105   for time series datasets composed of binary data in which each observation is a replicate (i.e.

106   each seed has germinated or not germinated), and compares observed and expected frequencies

107   with a χ distribution. It calculates a pairwise risk ratio (RR) between treatments, where a RR

108   greater than one means higher relative germination and a RR less than one means lower relative

109   germination. Alternatively, the RR can be interpreted as the likelihood that a random individual

110   from one treatment will reach the endpoint (i.e. germinate) before a random individual from the

111   other treatment (Spruance *et al.*, 2004). Seeds that did not germinate during the entire treatment

112   period were right-censored, while all other individuals were interval censored. The statistical

113   significance of post-hoc comparisons was assessed at a Bonferroni-corrected alpha of 0.005.

114   Repeated measures ANOVA was not used because calculating the variance of proportions based

115   on grouped binary data is inappropriate in that the proportions are both ordinal and bounded

116   between 0 and 1.

117

118   RESULTS

119   Seed treatment was an exceptionally strong predictor of seed germination success

120   (χ^2=67.6, P<0.0001, df=4, n=250). Physically scarified seeds germinated most quickly, and were

121   more than twice as successful as any other treatment (Table 1), with a final germination rate of

122   74% over 33 days (Figure 1). Statistically similar percentages of unscarified, acid scarified, and

123   peroxide scarified seeds germinated (30%, 34%, and 26%, respectively) (Table 1). No seeds

124 from the hot water scarification treatment germinated. Across all treatments, the bulk of

125 germination occurred within the first 2 weeks, with virtually no germination after that point

126 (Figure 1).

127

128   DISCUSSION

129   Although many scarification treatments have been attempted for *Astragalus* species, my

130 data show that not all treatments are equal in efficacy. In fact, only one treatment, physical

131 scarification, was significantly better than the control, and the hot water treatment was

132 significantly worse than the control, resulting in no germination at all.

133   Based on my data, I recommend that propagation efforts involving rare *Astragalus*

134 species use physical scarification as the primary method for breaking seed dormancy. The major

135 disadvantage of using physical scarification, the labor-intensive nature of individually damaging

136 the seed coat with sandpaper, a razor blade, or nail clippers, can be overcome with batch

137 scarification methods. These include abrasive-lined drums or vane polishers for relatively small

138 lots, or commercial seed polishing, hulling, or scarifying equipment for larger lots, albeit at the

139 cost of slightly greater seed loss from damage (Acharya *et al.*, 2006). However, the 10/10 rule of

140 wild seed collection (take no more than 10% of the seeds from no more than 10% of the

141 reproductive plants) (Guerrant *et al.,* 2013) severely limits the number of seeds available from

142 rare species, which may have only dozens or hundreds of reproductive individuals within a given

143 year. Because seed numbers from these collections are likely limited to the hundreds, the time

144 required to scarify individually is minimal, whereas the higher seed loss with batch scarification

145 equipment would be unacceptable. If individual scarification is impractical because a species is

146 more common or has been propagated *ex situ*, I suggest performing additional optimization trials

147   specific to the type and model of scarification equipment, according to the manufacturer

148   recommendations.

149   Although *Astragalus cicer* is a relative generalist that would likely not require the sorts of

150   atypical scarification techniques that might be necessary for strongly specialized lineages, to my

151   knowledge, there are no reports of physical scarification being ineffective in *Astragalus*.

152   Nonetheless, *Astragalus* as a genus has a very broad range of morphological and physiological

153   variation, with species that are annual or perennial, endemic to mineral or humic soils, etc. Thus,

154   care should be taken in extending these results across the entire range of *Astragalus* species.

155   Still, whereas other studies have demonstrated that methods involving cold, heat, acid,

156   etc., can improve germination over controls, I recommend against their widespread use in

157   *Astragalus,* as the studies comparing different durations and intensities (i.e., temperature and

158   concentration) of these treatments have found a relatively narrow range of optimal conditions

159   (Albrecht & Penzagos, 2012; Chou *et al.*, 2012; Long *et al.*, 2012). Treatments of insufficient

160   duration or intensity appear to be incapable of breaking seed dormancy, whereas treatments of

161   excessive duration or intensity damage not only the seed coat, but the embryo as well, causing a

162   loss of viability (Albrecht & Penzagos, 2012; Chou *et al.*, 2012; Long *et al.*, 2012). Even when

163   such treatments are better than controls, I have found no reported instance for an *Astragalus*

164   species in which they are more effective than physical scarification, and they are sometimes still

165   worse (Miklas *et al.*, 1987; Acharya *et al.*, 2006). In addition, some treatments, particularly those

166   that involve concentrated acid, liquid nitrogen, fire, or other reactive substances, could be

167   hazardous and are best avoided unless absolutely necessary.

168

169   CONCLUSIONS

170   Physical scarification is a simple, safe, and reliable way to improve germination rates in

171 *Astragalus* species with hard seed dormancy. I advise that, particularly for rare species for which

172 seeds are limited, attempting to optimize other techniques is an unnecessary waste of resources

173 unless physical scarification has been demonstrated to be ineffective.

174

175 *Acknowledgements.* - I would like to thank Anna A. Sher for her help with data analysis and

176 Jennifer Neale for her comments on the manuscript and facilitation of use of the DBG

177 greenhouse. I also thank the DBG horticulture staff, particularly Mike Bone and Katy Wilcox,

178 for permission to use valuable greenhouse space and planting materials, and for their invaluable

179 aid and expertise. I thank Elizabeth Pilon-Smits for advice on cultivating *Astragalus* species.

181   REFERENCES

182   Acharya, S.N., Kastelic, J.P., Beauchemin, K.A. & Messenger, D.F. (2006) A review of research

183   progress on cicer milkvetch (Astragalus cicer L .). *Can J Plant Sci*, 1, 50–62.

184   Albrecht, M.A. & Penzagos Z, J.C. (2012) Seed germination ecology of three imperiled plants of

185   rock outcrops in the southeastern United States. *J Torrey Bot Soc*, 139, 86–95.

186   Barneby, R.C. (1964) Atlas of North American Astragalus. The New York Botanical Garden,

187   Bronx, NY.

188   Baskin, J.M., Baskin, C.C. & Li, X. (2000) Taxonomy, anatomy and evolution of physical

189   dormancy in seeds. *Plant Spec Biol*, 15, 139–152.

190   Chou, Y.-F., Cox, R.D. & Wester, D.B. (2012) Smoke Water and Heat Shock Influence

191   Germination of Shortgrass Prairie Species. *Rangeland Ecol Manag*, 65, 260–267.

192   Guerrant, E.O., Havens, K., Maunder, M., Raven, P.H. & Conservation, C.P. (2013) Ex Situ

193   Plant Conservation: Supporting Species Survival In The Wild. Island Press.

194   Godefroid, S., Piazza, C., Rossi, G., Buord, S., Stevens, A.-D., Aguraiuja, R., Cowell, C.,

195   Weekley, C.W., Vogg, G., Iriondo, J.M., Johnson, I., Dixon, B., Gordon, D., Magnanon, S.,

196   Valentin, B., Bjureke, K., Koopman, R., Vicens, M., Virevaire, M. & Vanderborght, T. (2011)

197   How successful are plant species reintroductions*? Biol Conserv*, 144, 672–682.

198   Long, Y., Tan, D.Y., Baskin, C.C. & Baskin, J.M. (2012) Seed dormancy and germination

199   characteristics of Astragalus arpilobus (Fabaceae, subfamily Papilionoideae), a central Asian

200   desert annual ephemeral. *S Afr J Bot*, 83, 68–77.

201   Maunder, M. (1992) Plant reintroduction: an overview. Biodiversity and Conservation, 1, 51–61.

202   Menges, E.S. (2008) Restoration demography and genetics of plants: when is a translocation

203   successful? *Aust J Bot*, 56, 187.

204   Neel, M. (2008) Patch connectivity and genetic diversity conservation in the federally

205   endangered and narrowly endemic plant species Astragalus albens (Fabaceae). *Biol Conserv*,

206   141, 938–955.

207   Rosner, L S, J T Harrington, D R Dreesen, and L Murray. 2003. "Hydrogen Peroxide Seed

208   Scarification of New Mexico Collections of Ribes Cereum." *Seed Science and Technology*

209   31: 71–81.

210   Spruance, Spotswood L, Julia E Reid, Michael Grace, and Matthew Samore. 2004. "Hazard

211   Ratio in Clinical Trials." *Anitmicrobia Agents and Chemotherapy* 48 (8): 2787–2792.

212   doi:10.1128/AAC.48.8.2787.

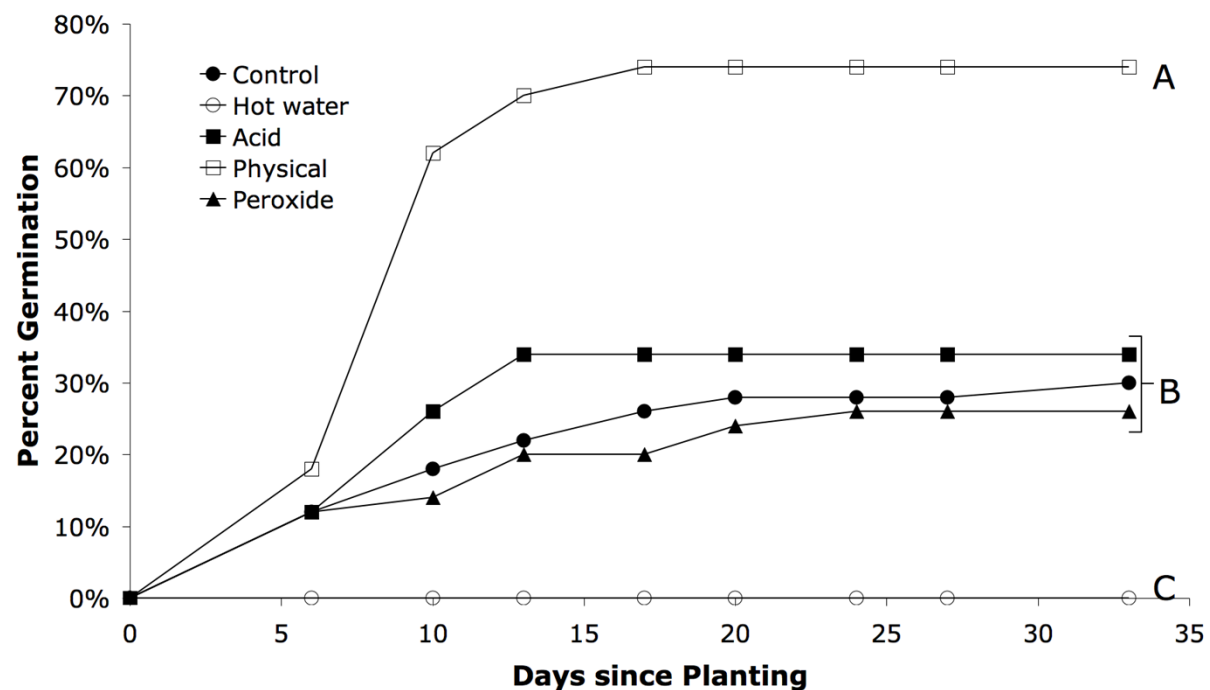
213   Vicente, M.J., Segura, F., Aguado, M., Migliaro, D., Franco, J. a. & Martínez-Sánchez, J.J.

214   (2011) Genetic diversity of Astragalus nitidiflorus, a critically endangered endemic of SE Spain,

215   and implications for its conservation. *Biochem Syst Ecol*, 39, 175–182.

216   Watrous, K.M. & Cane, J.H. (2011) Breeding Biology of the Threadstalk Milkvetch, Astragalus

217   filipes (Fabaceae), with a Review of the Genus. *Am Midl Nat*, 165, 225–240.

219   FIGURES

220

221   Figure 1: Germination rates over time for different scarification treatments for *Astragalus cicer.*.

222   Letters indicate statistically different treatments via proportional hazards analysis.

224   Table 1: Pairwise risk ratios for treatments, expressed as the ratio of the germination success of

225   the row relative to the column. For example, the risk ratio of controls relative to nail clippers was

226   0.32 (32% as likely to germinate), while the risk ratio of nail clippers relative to controls was

227   3.17 (317% more likely to germinate). n=50 for each treatment. Asterisks (*) represent statistical

228   significance at the P<0.001 level. All other post-hoc comparisons were not significant.

Treatments	Control	Hot Water	Sulfuric Acid	Nail Clippers	Hydrogen Peroxide
Control	1	>100*	0.85	0.32*	1.17
Hot Water	<0.01*	1	<0.01*	<0.01*	<0.01*
Sulfuric Acid	1.17	>100*	1	0.37*	1.38
Nail Clippers	3.17*	>100*	2.69*	1	3.72*
Hydrogen Peroxide	0.85	>100*	0.72	0.27*	1

229

230