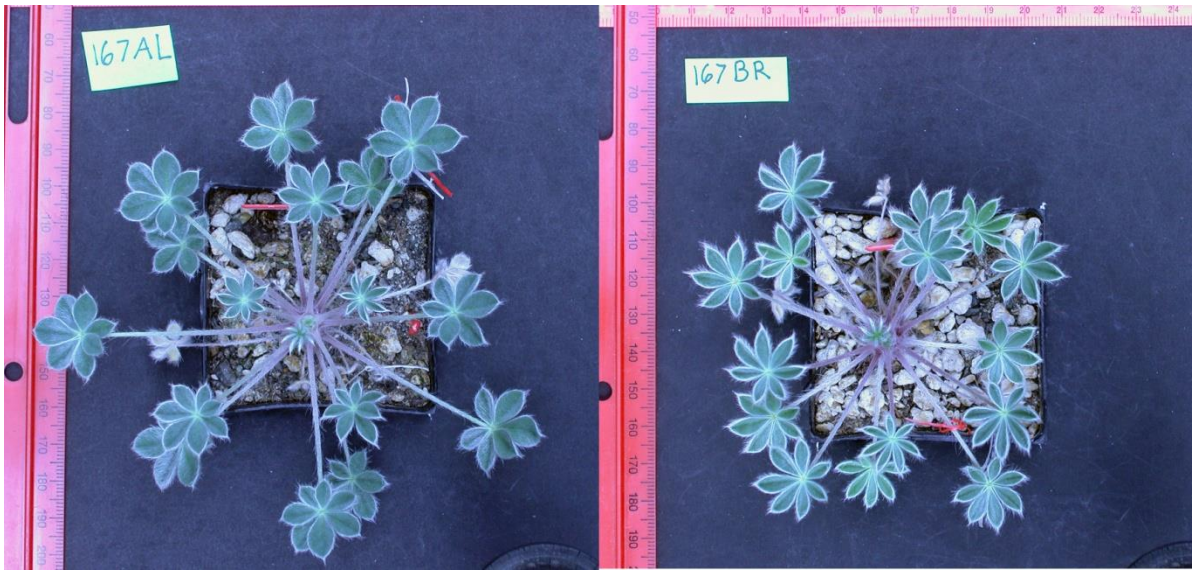
Propagation of Lassics lupine (*Lupinus constancei*): Investigations into the need for seed scarification for germination, and effects of soil inoculants on growth.



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by

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Propagation of Lassics lupine (*Lupinus constancei*): Investigations into the need for seed scarification for germination, and effects of soil inoculants on growth.

Abstract:

The central question of this study concerns the impact of naturally occurring soil symbionts on the growth of Lassics lupine (*Lupinus constancei*) a very rare and narrowly distributed plant endemic to serpentine barrens in the High North Coast Ranges of the California Floristic Province. Secondary questions have to do with the need or not, for scarification in germination, and the effects on plant growth of seed size and maternal parent.

Propagation of Lassic lupine is relatively straightforward, and this study revealed two rather simple procedures that enhance germination and growth rates. These data suggest that some form of artificial scarification greatly enhances germination rate rates, relative to those seeds not scarified (100% compared with 5% without scarification). Although the plants will grow in regular potting mix, plant size is consistently and significantly larger when a small portion of unsterilized native soil is added. Even a small amount of unsterilized native soil added to the potting mix apparently added some active biological agent, and is not a function simply of soil texture or water holding characteristics. The size differences were not apparent in early growth, when seed size appears to be the dominant determinant factor affecting plant size, and become increasingly larger as time progressed. The increasing divergence in sizes between those plants grown in soil that had been inoculated with unsterilized or sterilized native soil appears to be due to something living in the soil that did not survive sterilization.

Cover illustration:

The cover illustrations are examples of the type of images from which cover measurements were made. This pair are half siblings, and the one on the left (167 AL)was grown in medium that had naturally occurring soil that had NOT been sterilized, while the one on the right (167 BR) was grown in potting soil to which sterilized native soil had been added. These pictures, taken on 6 September, 2007, were chosen for use because each was very close to the mean size for plants grown in unsterilized and sterilized soil, respectively. They serve to illustrate the relative mean sizes to which plants grew under the different treatment regimes.

Propagation of Lassics lupine (*Lupinus constancei*): Investigations into the need for seed scarification for germination, and effects of soil inoculants on growth.

Introduction

Lassics Lupine (*Lupinus constancei* TW Nelson and JP Nelson 1983) is a very rare, narrowly distributed endemic plant species, known only from two occurrences, one each on Red Lassic Mountain and Signal Peak, in adjacent Humboldt and Trinity counties, California. Both occurrences are found on serpentine barrens in the High North Coast Ranges of the California Floristic Province (Hickman, 1993). Lassics lupine is a California Native Plant Society (CNPS) List 1B.2 species (List 1B indicates that it is rare, threatened, or endangered in California, and the 0.2 denotes that it is "fairly endangered in California".) In the Natural Heritage system, it is ranked G1 (Critically imperiled because of extreme rarity, or because it is somehow especially vulnerable to extinction or extirpation. In quantitative terms, G1's have fewer than 6 occurrences OR fewer than 1,000 individuals OR less than 2,000 acres.). Lassics lupine is not, however, on either the state or federal lists of threatened or endangered species.

This report addresses the germination and propagation requirements of *Lupinus constancei*, an herbaceous, iteroparous (i.e. polycarpic) perennial. The central question addressed in this report has to do with the impact on plant growth of potential naturally occurring soil symbionts. Does the addition of even small amounts of native soil to potting soil mix increase probability that plants will either survive, or grow faster/larger than without such addition? Three secondary questions include 1.) is seed scarification required for germination, 2.) to what degree, if any, is seed mass correlated with germination and/or growth rate (and ultimately, reproduction), and 3.) do any of the above factors have a genetic (or at least maternal) basis?

Materials and Methods

A total of 527 seeds were collected by David Imper (USFWS) in July, 2005,. A total of 64 seeds were used for the work described here, two seeds each from 32 maternal lines collected in the field, and an additional 74 seeds were banked (two seeds each from 37 maternal plants). The remaining 389 seeds were returned by the USFWS to the habitats from which they were collected (Imper, pers. com.) In addition, David Imper collected ca 6.9 lbs (air dried at room temperature) soil from around parent plants.

Each seed was weighed individually, on a Mettler AG104 microbalance (to the nearest 0.0001 gram), and arbitrarily labeled either A or B. Each seed was placed in its own Petri dish, on three sheets of germination paper in, and moistened with distilled water on 22 Feb, 2007. The dishes were placed in a controlled environment germination chamber maintained at a fluctuating temperature 10ºC/20ºC (16 hrs dark / 8 hrs light). None of the seeds were originally scarified, and each was visually judged visually and tactilely, originally on a daily basis, as to whether it had taken up moisture or not. Seeds that had not imbibed at the end of 4 days after being moistened, were mechanically scarified, by nicking the seed coat with a scalpel under a dissecting scope.

The A samples were placed in a soil mixture that contained native soil that had NOT been sterilized, and the B samples were placed in the soil mixture that contained native soil that had been sterilized.

The soil came in several separate polyethylene bags, which were mixed thoroughly and combined into one batch for use. This single sample was divided into two samples, each ca 3.45 lbs. in weight. One was sterilized for 30 minutes in a pressure cooker at 17 psi, and cooled before use. Upon germination, seeds were placed into pots containing potting soil mix (3:3:2 Pumice (screened to ¼ inch): sand: "Pro Gro brand Professional Blend potting soil), to which native soil was added. Half of the pots had native soil that had been sterilized, and half had native soil added that had not been sterilized. Native soil (sterilized or not) was added to the potting soil mix in a ratio 1:20, and thoroughly mixed, and put in its own 5 gallon container with lid, to be used as needed. The purpose of adding either sterilized or unsterilized native soil to the potting mix was to control for any texture and water holding effects the native soil itself might have on the final mix. Seedlings were placed individually in 2 5/8" x 2 5/8" x 5" deep black plastic band pots, with metal window screen in the base to prevent soil from falling out from the bottom of the pots.

The 32 pots for each treatment were aggregated into larger, draining trays, and placed in trays that would hold water. This was to facilitate bottom watering of the plants when they were small. As the plants grew and were able to withstand watering from above, that was how it was done. To prevent cross contamination, the soil treatments were kept separate, and their position in the greenhouse regularly moved to reduce the potential for location effects on plant growth within the greenhouse.

Data were initially gathered daily, to monitor imbibitions, germination and early growth rates, and the intervals were eventually lengthened as the plants got larger. This was done, in part, to allow us to distinguish early effects of soil differences from potentially early acting differences in seed weight or differences in maternal lines.

Length measurements were taken using a vernier caliper, at a resolution of 0.1 mm. Area measurements were made from digital images using Image J 1.37v image analysis software, which is available in the public domain from the National Institutes of Health at http://rsb.info.nih.gov/ij.

Results.

Germination:

The day after seeds were moistened, and before any seeds were mechanically scarified, only, only 3 of 64 (0.05) seeds had imbibed water. Even after four days, none of the other 61 seeds had imbibed water, at which time they were individually mechanically scarified by nicking the seed coat with a scalpel. By the day after mechanical scarification, 45 of the 61 (0.74) scarified seeds showed signs of having imbibed water (i.e. had increased dramatically in size, and become soft to the touch, and did not make a noise when tapped with a metal probe). The 16 that had not imbibed were re-scarified. Of those 16, 15 (0.94) had imbibed by the next day, the

one that had to be scarified a third time had imbibed by the following day. Ultimately, all 64 seeds germinated.

All 3 seeds that had imbibed without scarification had germinated by the next time they were monitored, 3 days later. Of the remaining 61 seeds that were mechanically scarified, all but one germinated the day after imbibing water, and it germinated two days after imbibition.

Growth:

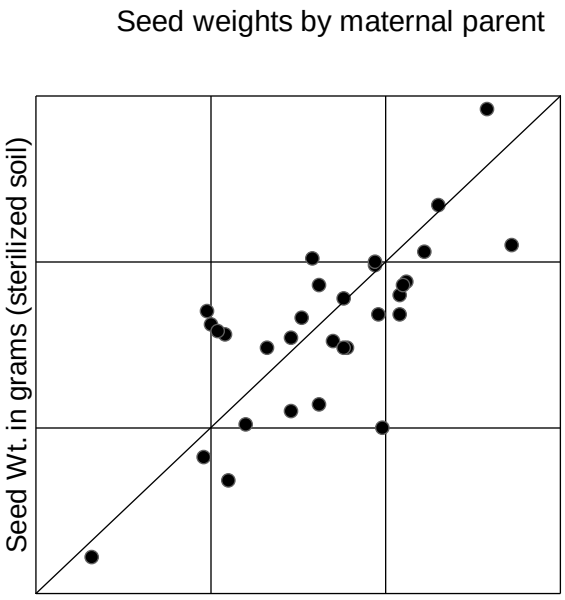
Having two seeds each from 32 maternal plants allowed an experimental design that was able to separate between genetic or at least maternal effects and the presence or absence of soil symbionts on later growth. Each parent contributed one seed each to the two soil treatment types. Seed size is known to affect germination, growth and survival prospects, especially early in life, so all seeds were individually weighed.

The effects of maternal parent were examined using ANOVA tests to characterize the extent to which maternal parent had an effect on seed weight and a series of size measurements, and the degree to which these effects might be statistically significant (Table 1). Maternal parent had a substantial impact on all variables considered (i.e. relatively high squared multiple R values), but the differences are significant only on seed weight, where the differences in seed weight as a function of maternal parent are highly significant. Indeed, maternal line explained almost 85% of the variation seen in seed size. Descriptive statistics for each of the 32 parents for the various parameters compared in Table 1 are presented in Appendix 1.

Table 1 Summary results of a series of ANOVA tests using maternal parent as the independent variable. Two seeds each from 32 parent plants were available for use, yielding 31 degrees of freedom. The squared multiple R statistic, which can vary between zero and one, indicates the proportion of the total variation explained by each variable, and the p value the significance of any effect.

Independent Variable Maternal parent			PROOFED	ANOVA
Dependent variable	**d.f**	**N**	**Squared Mult R**	**P value**
Seed Wt	31	64	0.84351	**<0.00001**
Cotyledon Max Len in mm	31	64	0.53531	0.31417
Lf 1 Maximum Len mm	31	63	0.64748	**0.04781**
Lf 3 Max Length in mm	31	62	0.59606	0.16597
Leaf 10 Max Length in mm	31	62	0.58350	0.20348
Cover area June 14	31	61	0.57283	0.27097
Cover area June 28	31	62	0.49514	0.55767
Cover area July 13	31	61	0.47482	0.67685
Cover area July 25	31	62	0.50638	0.50874
Cover area Sept 6	31	58	0.46663	0.79646

Figure 1 is a graphical representation of the seed weights of the 32 parent plants, with the horizontal and vertical axes representing the weights of seeds arbitrarily assigned to one of two soil treatments. The diagonal line indicates location where both seeds would have identical weights.



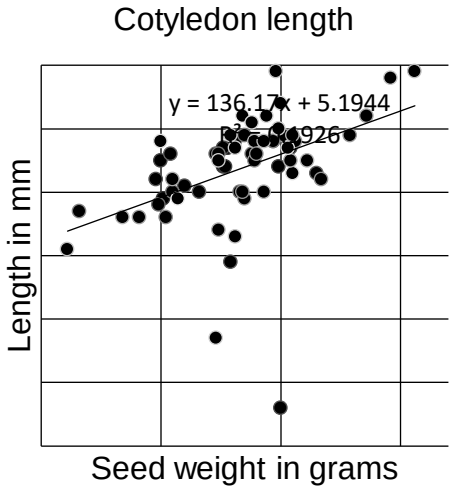
Inde	D
	value
Coty	29
Lf 1	01
Lf 3	60
Leaf	57
Cove	51
Cove	45
Cove	10
Cove	76
Cove	57

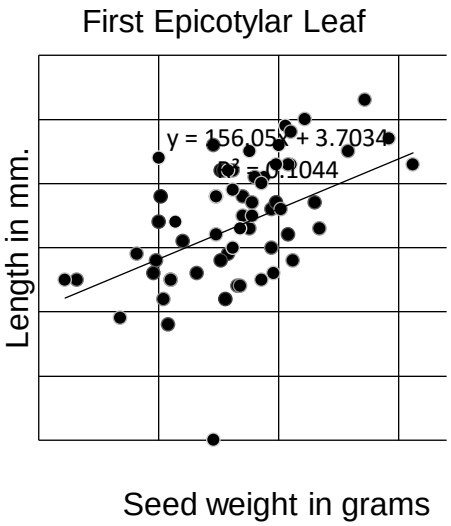
Figure 1 Graph showing the absolute and relative sizes of seeds from each of the 32 parent plants used in the study. Each symbol represents the two seeds used from each parent, arbitrarily assigned to one soil treatment or the other.

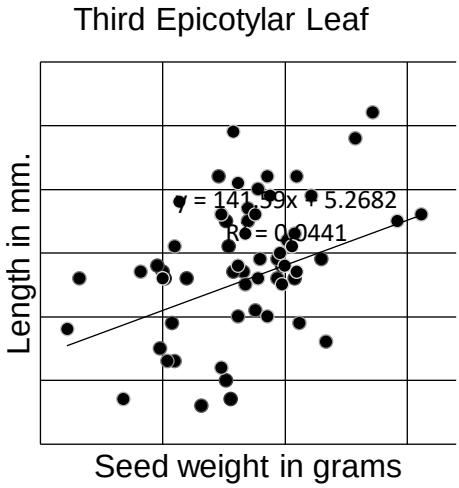
Table 2 provides the results of a least squares regression analysis on the same set of dependent variables described in Table 1 (except seed weight), as a function of seed weight. Seed weight had a significant effect on cotyledon size, and the size of the first and third epicotylar leaves, but not on the size of the tenth epicotylar leaf, or on plant rosette area. Figures 2 - 10 graphically illustrate the relationship of the various characters described in Table 2, to seed weight.

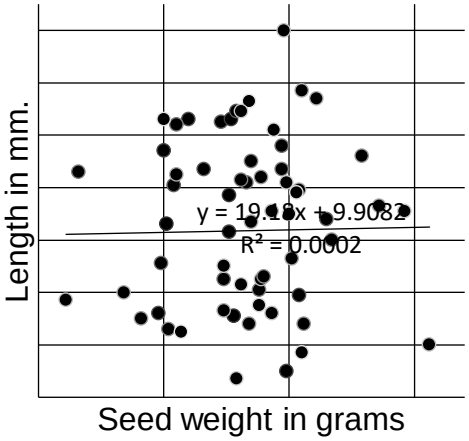
Table 2 Results of least squares regression analysis of various dependent variables as a function of seed weight.

Figures 2- 5 show the relationship between seed weight on the one hand, and the maximum lengths of the cotyledons, first, third and tenth epicotylar leaves on the other.

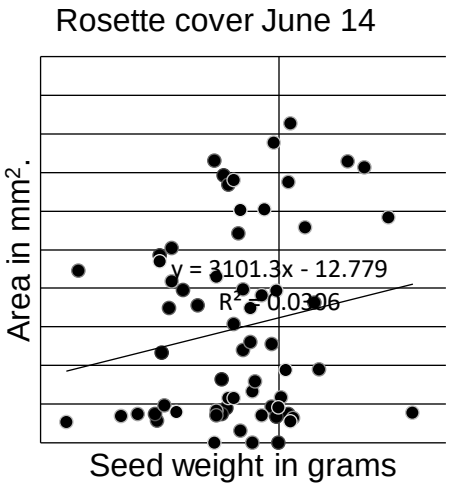


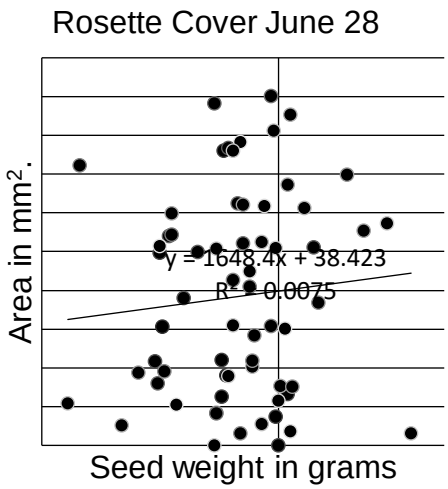


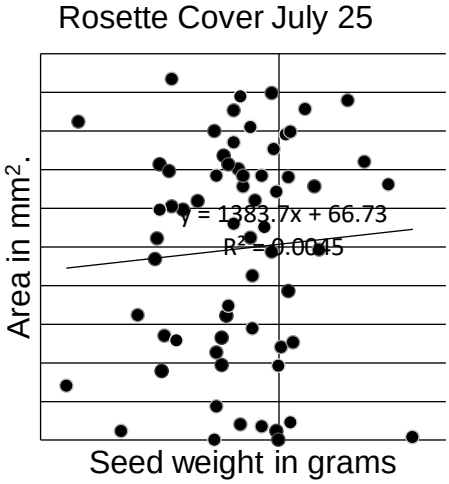




Figures 6 – 9 show the relationship between seed size and subsequent rosette area on four dates 25).







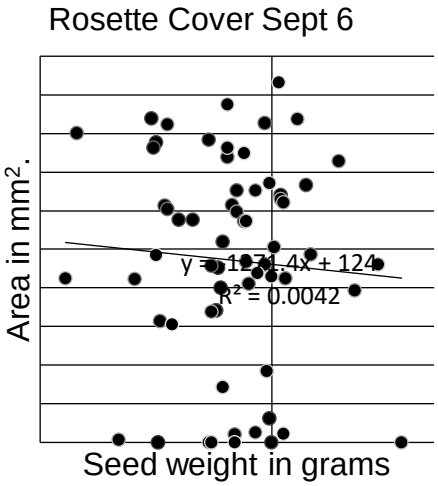

Rosette Cover Sept 6
Area in mm2.
y = -1274.4x + 124
R² = 0.0042
Seed weight in grams

Table 3 provides summary descriptive statistical data (N, mean and standard error) for seed weights, maximum lengths measured of cotyledon, and the first, third and tenth epicotylar leaves, and rosette areas on the five dates, as a function of soil treatment, as well as the results of ANOVA tests for effects on these parameters due to soil treatment.

Table 3. Summary descriptive statistical data (N, mean plus and minus standard error) for seed weights, maximum lengths measured of cotyledon, and the first, third and tenth epicotylar leaves, and rosette areas on the five dates, as a function of soil treatment, as well as the results of ANOVA tests for effects on these parameters as a function of soil treatment.

Independent Variable **Soil treatment**		Soil NOT Sterilized	Soil Sterilized	ANOVA PROOFED		
Dependent var	N	Mean ± s.e.	Mean ± s.e.	d.f	Squared Mult R	P value
Seed Wt	64	0.0235 ± 0.0005 g	0.0231 ± 0.0005 g	1	0.00664	0.52201
Cotyledon Max Length	64	8.42 ± 0.14 mm	8.31 ± 0.16 mm	1	0.00419	0.61120
1st epicotylar leaf len	63	7.40 ± 0.19 mm	7.51 ± 0.14 mm	1	0.00319	0.66012
3rd epicogtylar leaf len	62	8.94 ± 0.19 mm	8.74 ± 0.18 mm	1	0.00995	**0.00995**
10th epicotylar leaf ln	62	11.73 ± 0.56 mm	9.65 ± 0.52 mm	1	0.10870	0.10870
Cover area June 14	61	77.02 ± 9.22 cm^2	48.13 ± 7.42 cm^2	1	0.09232	**0.01729**
Cover area June 28	62	96.87 ± 8.85 cm^2	61.67 ± 8.54 cm^2	1	0.12015	**0.00578**
Cover area July 13	61	117.06 ± 8.85 cm^2	74.93 ± 9.84 cm^2	1	0.14600	**0.00238**
Cover area July 25	62	121.70 ± 7.58 cm^2	82.56 ± 10.52 cm^2	1	0.13181	**0.00373**
Cover area Sept 6	58	124.37 ± 6.35 cm^2	82.53 ± 9.10 cm^2	1	0.20591	**0.00035**

These same descriptive data presented in Table 3 are presented graphically in Figures 10 and 11. Figure 12 illustrates the mean number of leaves on surviving plants in each soil treatment as a function of time. Note how the difference increases over time.

Figure 10. Histogram of mean maximum lengths (plus and minus the standard error of the mean maximum value), of cotyledons, and of the first, third and tenth epicotylar leaves

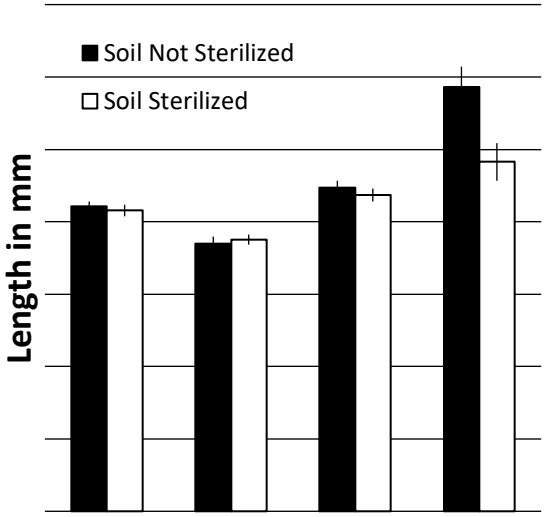

Soil Not Sterilized
Soil Sterilized
Length in mm

Figure 11. Mean plant size, as judged by cover area estimate done on digital images with the use of Image J image analysis software, over a series of five dates, from June 14, 2007 through September 6, 2007.

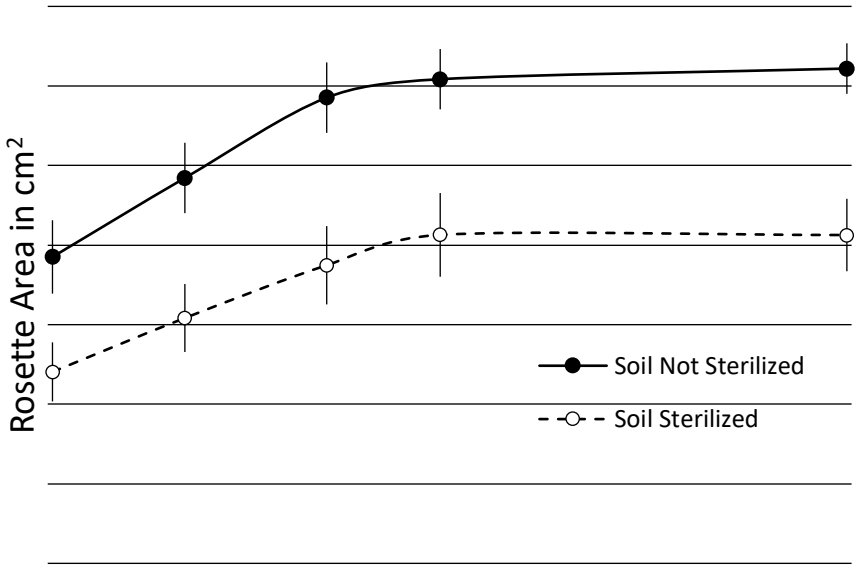
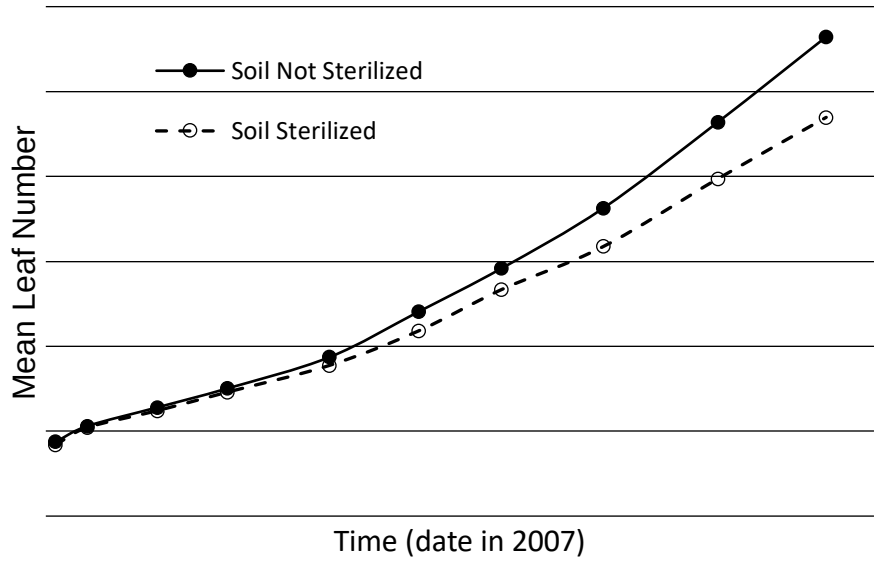


Figure 12 shows the mean number of leaves on plants growing in medium that includes unsterilized soil as compared with plants growing in soil that had had sterilized native soil added to the potting mix.



Reproduction:

Only one plant of the 62 that grew beyond the cotyledon stage produced mature flowers, and one other appeared to have at least initiated an inflorescence that did not go on to develop. Both were from the same parent plant (SB2005-0194), and no other parent plant produced any plants that flowered. The one plant that produced mature flowers was grown in the soil mix with the native soil that had not been sterilized.

Neither of these plants was consistently larger than others in multiple size estimates. Neither came from particularly large seeds (ranking 19th and 33rd out of 64 total.) The one that flowered did have the largest measurement for leaf 10 of any of the plants (its sibling had the 4th largest), and the second and fourth largest plant sizes on June 14 and 28 (compared with 11th and 5th) after which the one that did not mature its inflorescence continued to stay relatively large, while the one that did produce mature flowers declined in size.

Mortality:

On October 24, 2007, plants were visually examined and the survivors moved from the greenhouse to an outdoor cold frame, where the pots were sunk into a sand bed to within an inch of their tops. Of the original 64 seedlings, 56 plants survived until October 24, the last time they were monitored.

Discussion and Conclusions

The basic questions addressed in this study concern the need for seed scarification for germination, and more centrally for propagation the degree to which, if any, naturally occurring soil symbionts might be necessary for or at least facilitate plant growth, survival and reproduction. Given the limited sample size of seeds available for study, and that we had two seeds each from 32 different parent plants with which to work, we were able to design a study that would not only look at the need for scarification for germination and the effect of soil symbionts on subsequent growth, but also examine the effects of seed size differences (and to a lesser degree genetic or at least maternal effects) on germination and growth rates.

Germination:

Like many legumes, *Lupinus constancei* exhibits a form of seed dormancy known as Physical Dormancy (Baskin and Baskin, 1998). This means that there is some physical barrier to moisture entering the seed, thus preventing germination in the presence of conditions otherwise suitable for germination (e.g. adequate moisture and appropriate temperature). There is no evidence to suggest the presence of any inherent, physiologically mediated dormancy mechanism at work in this species.

The great majority of *L. constancei* seeds tested (ca 95%) appear to have required some sort of scarification - in this case mechanically nicking the seed coat with a scalpel - before they would imbibe water. The great majority of seeds germinated within a day or so of imbibing

water, and all germinated within three days of imbibing water. Once the seeds had taken up water, they readily germinated in the fluctuating (10ºC/20ºC) temperature regime used in this study. Ultimately, all 64 seeds germinated.

Three of 64 seeds, approximately 5%, however, did take up water without having been mechanically scarified. This may be due to their having been accidentally scarified or otherwise physically damaged before being moistened. Alternatively, a small fraction of seed may naturally not have the ability to prevent water from crossing the seed coat into the embryo. The moisture barrier in the seed coat does appear to be relatively robust, as many seeds required more than one experimental attempt to damage it sufficiently to allow moisture to enter the seed.

Size, Growth and Flowering:

Size differences among growing plants appear to be due to a variety of causes, which change as the plant matures. Differences in seed sizes, which themselves appear strongly affected by maternal parent (due to genetic and/or maternal effect reasons), provide the best statistical accounting of plant size differences in the earlier growth stages (sizes of the cotyledons and first and third epicotylar leaves.) Although the magnitude of the effect on early leaf size is relatively modest as judged by the adjusted squared multiple R statistic, the differences were highly significant. As the plants grew older, as measured by the size of the 10th epicotylar leaf and of plant cover across a series of dates from June 13 through September 6, seed size differences did not exert either a large or significant effect on plant size during later stages measured. In contrast, soil treatment, which did not have a significant effect on plant size during these earlier stages, did have a significant effect on plant size as the season progressed and the plants got larger and more mature.

The basic propagation question investigated in this study, the central question, has to do with degree to which the presence of naturally occurring symbiotic organisms in the native soil affect plant growth. The possible answers form a continuum from being absolutely necessary for growth, to having no effect at all. Not surprisingly, the data reveal something in the middle. Plants can grow in the absence of naturally occurring soil symbionts, but they did on average grow larger and at least one plant did flower and produce seeds when grown in soil having been inoculated with a small amount of unsterilized native soil.

That the only two plants to produce even early developmental stage inflorescences were the only two seeds that came from that particular maternal plant, and no other maternal plants produced any inflorescences. This suggests

In summary, propagation of Lassic lupine is relatively straightforward, and this study revealed two rather simple procedures that enhance germination and growth rates. These data suggest that some form of artificial scarification greatly enhances germination rate (100% compared with 5%) rates, relative to those seeds not scarified. Although the plants will grow in regular potting mix, plant size is consistently and significantly larger when a small portion of unsterilizled native soil is added. This presumably adds some active biological agent, and is not a function simply of

soil texture or water holding characteristics. The differences were not apparent in early growth, when seed size appears to be the dominant determinant factor affecting plant size, and become increasingly larger as time progressed. This initial similarity and later size divergence due to the effect of native soil organisms on Lassic lupine, became increasingly more clearly expressed as the whole plant cover measurements were begun in mid June. This was after the plants had become established and produced a in the range of 12-15 epicotylar leaves.

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