Assessing the impact of Clethodim on the Vigor, Seed Production and Seed Viability of the Endangered *Lupinus nipomensis*

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The Clethodim, grass-specific herbicide, experiment was conducted on 60 Nipomo lupine (*Lupinus nipomensis*) seedlings (10 replicates x 6 treatments). The goal of the research was to evaluate the potential impact of the field application of Clethodim over growing seedlings of the endangered Nipomo lupine through direct spray or aerial (helicopter) application as part of the Guadalupe Dunes Management team's efforts to eliminate *Ehrharta calycina* (perennial veldt grass) from the fragile dune habitat. Work is conducted under a CDFW Permit to Study the Effects of Clethodim Herbicide on Nipomo Mesa Lupine (*Lupinus nipomensis*) (permit No. 2081(a)-19-017-RP). The most effective time to apply the herbicide is early in the growing season of the grass (January) which coincides with the typical time for lupine germination. Spraying may also occur later in the winter or during the spring as perennial veldt grass continues its growth which coincides with the time when lupine may be flowering or fruiting. The experiment was designed to evaluate the effect of the herbicide on both stages of potted lupine seedlings to determine if Clethodim and associated surfactants affects the seedlings under either stage of growth. The goal is to more efficiently manage this invasive plant across the dune ecosystem by securing permit to allow for spraying within occupied Nipomo lupine habitat by both aerial methods (helicopter) and/or by ground crews. Past restrictions on management of veldt grass adjacent to the Nipomo lupine population centers has resulted in a dense cover of the invasive grass around the remnant lupine population. The results of this study indicate that there is no significant effect on vigor, lifespan, or seed production of either aerial (larger droplets) or direct spray application of the recommended herbicide and surfactant mixture on either newly leafing out seedlings or flowering individuals. In addition, seeds from the 6 treatment combinations were evaluated for their viability and we recorded the highest seed viability for all treatments (100%) relative to previous seed germination trials

Methods

The experiment was conducted at the University of California Santa Barbara Campus at the CCBER greenhouse and nursery facility. CCBER, the Cheadle Center for Biodiversity and Ecological Restoration, manages 340 acres of open space and restoration on the campus and conducts a variety of research and outplanting work with several endangered plants, including the Nipomo lupine. The work was conducted during the winter and spring of 2020 with 60 seedlings germinated at the greenhouse and nursery facility. Seeds collected from previous seed bulking work were used for this

experiment. Seeds were germinated in the winter after treatment with scarification and cold stratification. Seedlings germinated in the refrigerator and were planted in 2 gallon pots in a mixture of native sand from Nipomo and perlite at 50% volume, and grown out of doors in a raised, caged enclosure under 50% shade cloth (see image 1). Seedlings were monitored nearly daily and irrigated by hand every three days or so, as needed.

A staff member with his QAL conducted the treatment applications of herbicide and assigned seedlings to the treatment groups to balance out size and other factors and then randomly assigned a treatment to each group. The first treatment was conducted on April 10th on 30 newly leafing out seedlings and the second treatment was conducted on May 23rd on 30 flowering individuals. After treatment the numbered seedlings were returned to the raised, caged growing area without treatment labels where observers, unaware of which seedling had received which treatment, monitored vigor and collected pods as they were produced. Once all pods had been collected, seeds were extracted from the pods and classified as viable or non-viable based on size and color. Larger, darker seeds were considered viable and tiny, thin, pale tan seeds were considered inviable. A follow-up viability test of a subset of the seeds from each treatment will be conducted in October after a cold stratification period. We intend to germinate three replicates of 10 seeds each from each of the 6 treatments (180 seeds total).

Equipment: Two hand held sprayers, each containing 1 L of mixed herbicide were used to mimic each treatment type based on discussions with the State Parks applicators, advisors (Wilbur-Ellis Agribusiness), and product manufacturers. The sprayer nozzle for the aerial broadcast treatment was opened to its largest possible extent while maintaining a spray pattern. This was done to mimic the larger droplet size of an aerial broadcast spray. The sprayer nozzle for the direct ground spray treatment was tightened so that a finer mist in comparison to the Aerial treatment was produced. This was done to mimic the smaller droplet sizes produced by ground spray treatments.
All appropriate label-mandated PPE was worn during mixing and application.
Treatment areas: Two 5’ x 5’ application areas (25 square feet each) were designated to ensure the appropriate per acre applications could be administered for each treatment. The pots containing lupine seedlings were spaced evenly within the application areas (see image 2).
Herbicide Mix: The Aerial treatment called for a rate of 5 pints of Vaquero, 2.5 gallons of Renegade, and 20 ounces of Crosshair per 100 gallons of spray. In order to scale this down to the 1 L of mixture that was more manageable for experimental purposes, 6.25 ml Vaquero, 25 ml Renegade, and 1.5ml Crosshair were combined with water to comprise 1 L of mixed solution in the first hand-held sprayer. The table 2 treatment called for a rate of 4 pints of Vaquero, 2 gallons of Renegade, and 16 ounces of Crosshair

per 100 gallons of spray. In order to scale this down to 1 L of mixture that was more manageable for experimental purposes, 5 ml Vaquero, 20 ml Renegade, and 1.25ml Crosshair were combined with water to comprise 1 L of mixed solution in the second hand-held sprayer. Accurate measurements were ensured by using a graduated cylinder to measure the herbicide and adjuvants.

In order to determine the rate of flow from each sprayer, a calibration test was conducted to ensure that the appropriate amount of herbicide would be applied to each treatment area. The Aerial treatment called for an application of 20 gal of spray per acre which is equivalent to 43.5ml of spray per 25 sq ft. Using a graduated cylinder, a stop watch, and the first hand-held sprayer adjusted to the appropriate aperture size, it was determined that 44ml of spray would be applied in 6 seconds of spray time. The Direct Spray treatment called for an application of 25 gal of spray per acre which is equivalent to 54.25ml of spray per 25 sq ft. Using a graduated cylinder, a stop watch, and the second hand-held sprayer adjusted to the appropriate aperture size, it was determined that 54ml of spray would be applied in 14.5 seconds of spray time. A third sprayer (with no herbicide, but water only) was used to do separate dry runs for each application type (with nozzle adjusted in the same way as each treatment). These dry runs were conducted to ensure uniform coverage was achieved at the differing application speeds required (faster application in the case of the Aerial application, and slower in the case of the direct spray application) during the actual treatment, and were conducted in a separate 5’ x 5’ area.

Material - Table 1 (Aerial Broadcast)	**Rate per acre**	**Rate per 100 gal**	**Volume per acre**	**Rate per L**	**Volume per 25 sq ft**
1) Vaquero (clethodim)	1 pint	5 pints	20 Gal (total spray)	6.25 ml	43.5 ml (total spray)
2) Renegade-EA (activator-surfactant)	2 quarts	2.5 gallons		25 ml	
3) Crosshair (drift management agent)	4 ounces	20 ounces		1.5 ml	

Material - Table 2 (Directed ground spray)	**Rate per acre**	**Rate per 100 gal**	**Volume per acre**	**Rate per L**	**Volume per 25 sq ft**
1) Vaquero (clethodim)	1 pint	4 pints	25 Gal (total spray)	5 ml	54.25 ml (total spray)
2) Renegade-EA (activator-surfactant)	2 quarts	2 gallons		20 ml	
3) Crosshair (drift management agent)	4 ounces	16 ounces		1.25 ml	

Treatments:

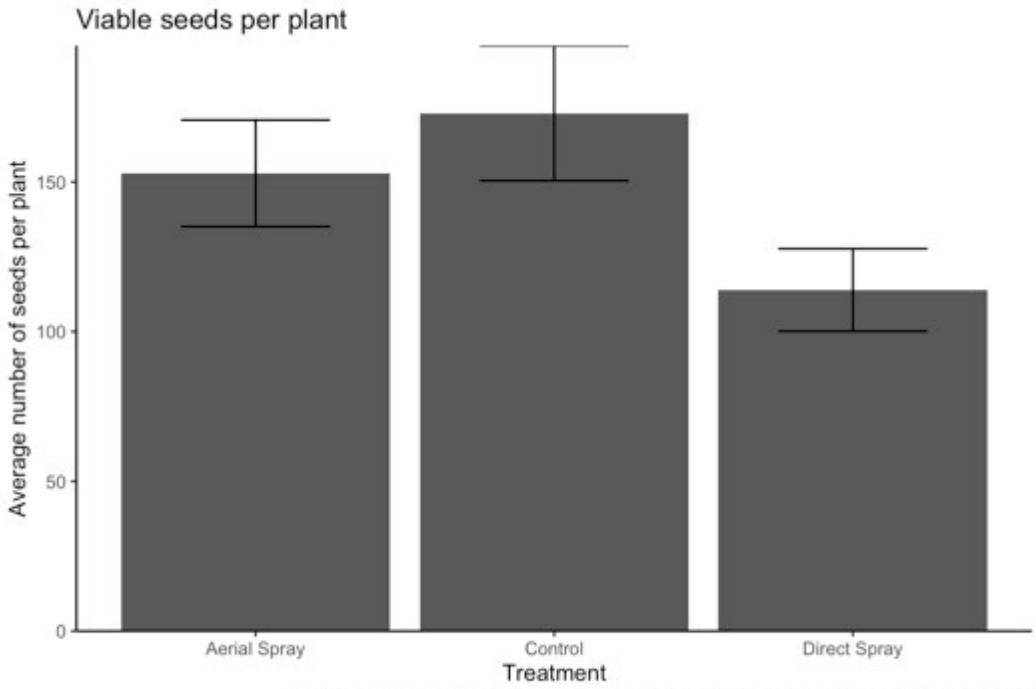
After the dry runs were complete, the Aerial and Direct spray treatments were conducted with the help of a stop watch to ensure proper timing and

uniform coverage over each treatment area. (**44 ml spray applied uniformly in 6 second to the 25 sq. ft. Aerial treatment area, and 54ml spray applied in 14.5 seconds to the 25 sq. ft. Direct spray treatment area**). When both treatments were complete, the pots containing the seedlings were placed in random order, in a secured growing area to be cared for and observed. The remaining herbicide formulation from the two hand sprayers was then applied to appropriate invasive grasses in CCBER management areas, and all equipment was cleaned and stowed. A re-entry interval of 24 hours was relayed and observed before any entry into the vicinity of the treatment area or the area where the plants were secured (without appropriate PPE) was done.

Seed Viability Assessment.
Three replicates of 10 seeds per treatment combination (30 x 6 treatments; 180 seeds) were germinated in petri dishes I the refrigerator after typical germination treatments of cleaning with dilute hydrogen peroxide, scarification and cold soak to evaluate whether the Clethodim treatment impacted seed viability.

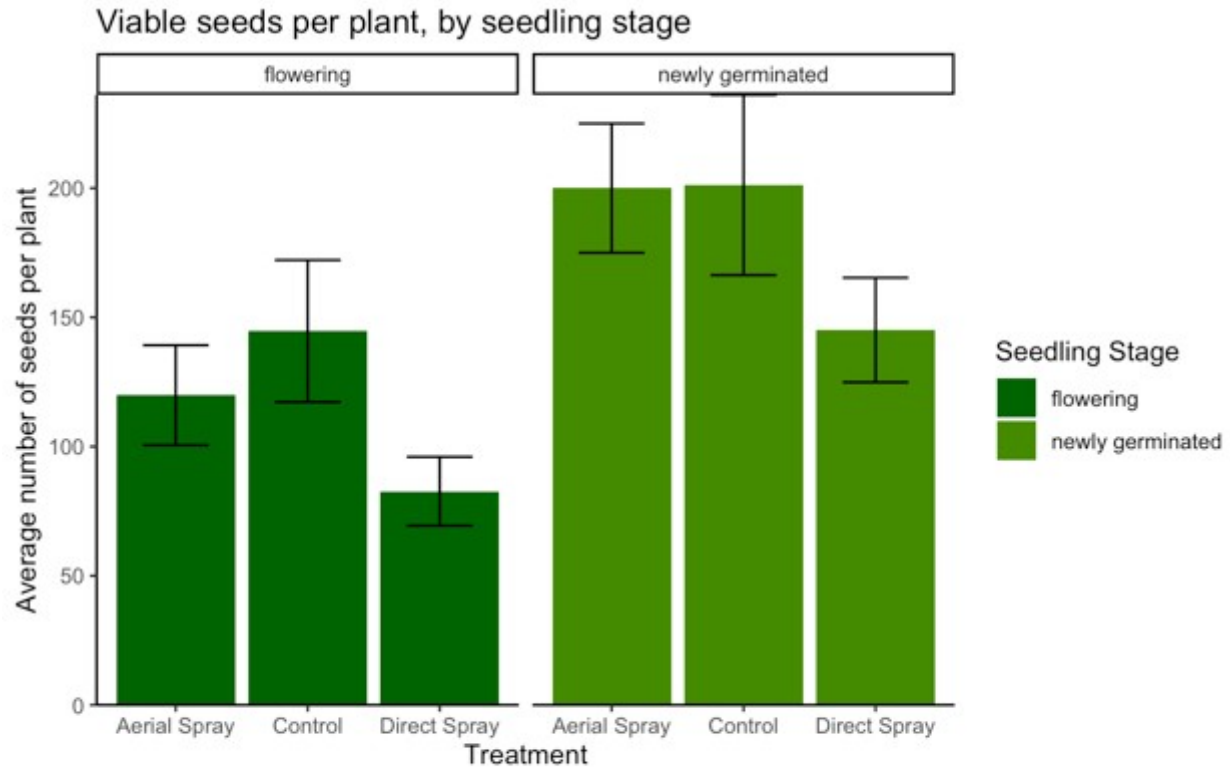
Results
An ANOVA analysis on number of pods per seedling, number of viable seeds per pod and viable seeds per plant (response variables) demonstrate that there was no significant effect of the treatments on seed production. Numerically, the seedlings in the direct spray treatment from both the seedling and the reproductive plant treatment had slightly lower seed pod production than either the control or the aerial treatments, but statistically the treatments were not different.

There were 34 excess Nipomo lupine seedlings which were germinated as part of the same cohort but because they germinated later, were not used as part of the experiment. Pods were collected from these seedlings as well. Interestingly, seed production of the excess seedlings was significantly lower than the seedlings in the treatment cohort. Their total pod production (22 pods/plant) was significantly below the production of pods by any of the Clethodim treatments (33 – 70 pods/plant). This provides additional evidence that the numerically lower seed pod production, though not significantly lower, in the sprayed groups versus the control group was not a result of the treatment.



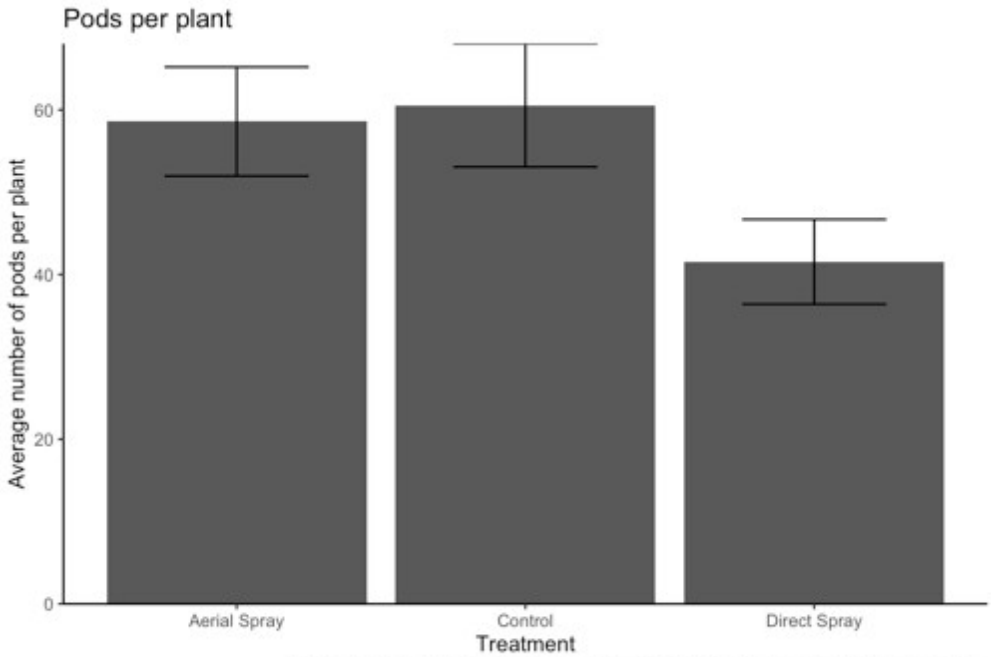
Graph of average number of pods per plant, with standard error represented as error bars.
No significant difference between treatments (ANOVA F = 2.6, p = 0.084)

This graph shows the average viable seeds per plant and combines seedling and flowering stage cohorts to compare treatment impacts across all 60 seedlings. No significant difference in seed production were found, $P > 0.05$; $p = 0.0843$.



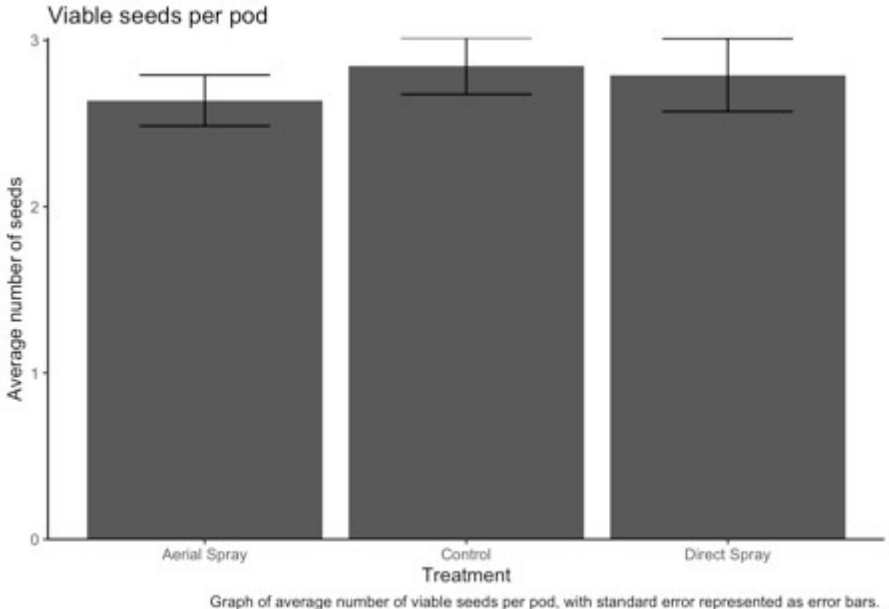
Graph of average number of viable seeds per plant, with standard error represented as error bars. No significant difference between treatments for early germinating seedlings (ANOVA F = 1.2, p = 0.31) or for flowering seedlings (ANOVA F = 2.2, p = 0.13)

This figure separates the two treatment cohorts (flowering and early germinating seedlings) and is also non-significant. The p-value for this assessment exceeds 0.1 (Viable seeds per plant -- flowering: p = .13; Viable seeds per plant -- early germinating: p = .31). It is likely that the newly germinating seedlings had higher seeds per plant because they germinated earlier than the seedlings in the flowering treatment and so had a longer period of good environmental conditions to produce flowers and pods before the summer heat developed and natural senescence began.



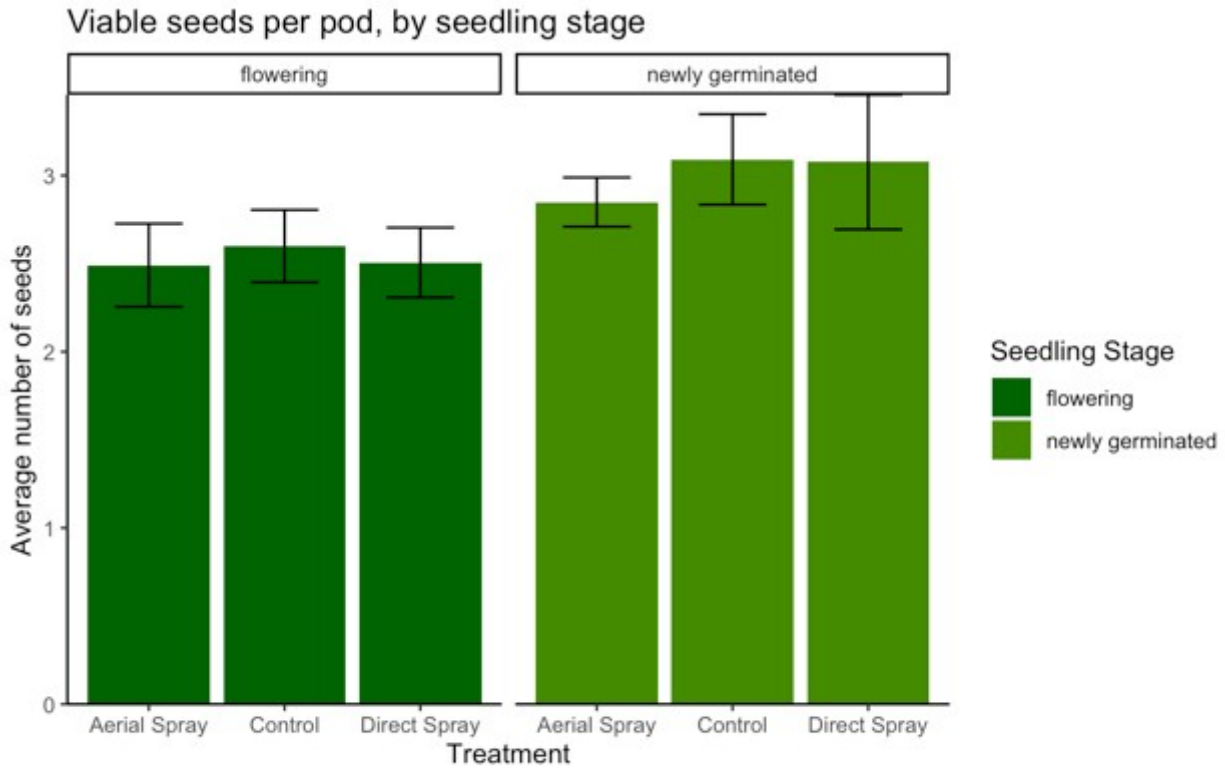
Graph of average number of pods per plant, with standard error represented as error bars.
No significant difference between treatments (ANOVA F = 2.0, p = 0.15)

The average number of pods per plant across all treatments ranged from 40 to 58 and was also not significant to even the 0.1 level and is significantly higher than later germinating seedlings of Nipomo lupine that were not part of the experimental group but which had an average of 22 pods per plant across more than 30 seedlings.



Graph of average number of viable seeds per pod, with standard error represented as error bars.
No significant difference between treatments (ANOVA F = 0.88, p = 0.42)

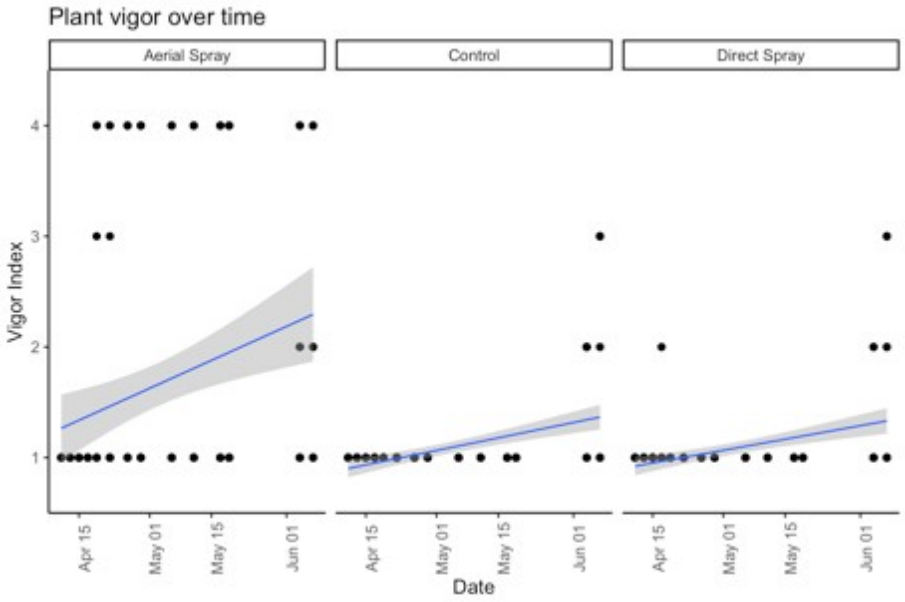
All treatments produced between 2 and 3 viable seeds per pod.



Graph of average number of viable seeds per pod, with standard error represented as error bars. No significant difference between treatments for early germinating seedlings (ANOVA F = 0.93, p = 0.41) or for flowering seedlings (ANOVA F = 2.2, p = 0.93)

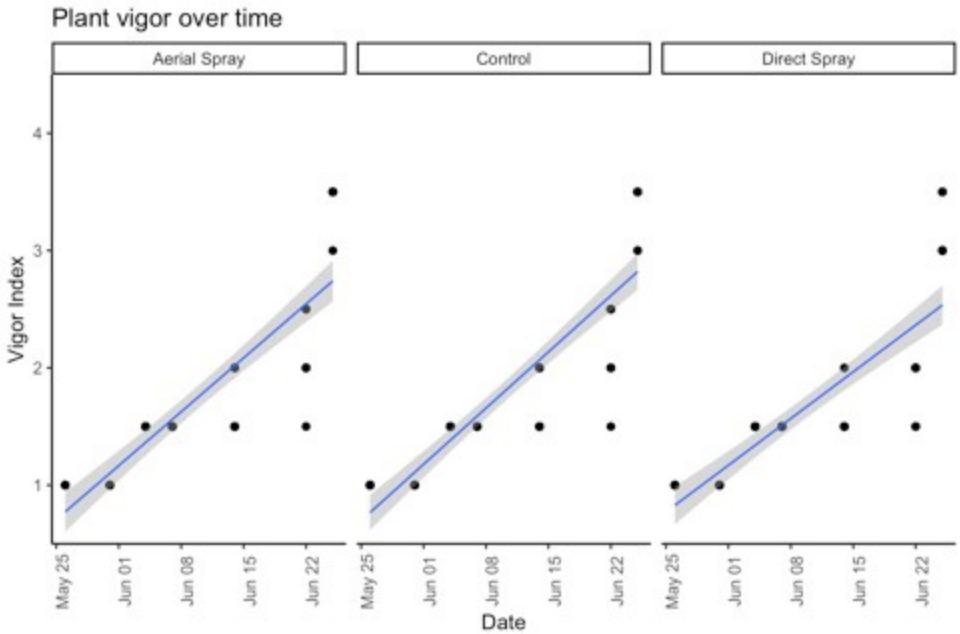
By cohort, the statistics support this finding of no significant difference in seeds per pod with viable seeds per pod in the flowering treatment: p = .928; and viable seeds per pod -- early germinating: p = .4.

All seedlings were observed for vigor over time. Vigor was assessed according to four stages of vigor: **high vigor** (1) No chlorosis, wilting or other evidence of stress; **medium vigor** (2) Slight wilting, herbivory or chlorosis; **low vigor** (3) Wilted, brown; and **dead** (4) Dried and unable to recover. The changes in vigor were associated with one herbivore (caterpillar and/or mouse) that got into the treatment area and consumed three of the early germinating seedlings in the aerial treatment soon after germination and to natural senescence in mid-May of these annual plants. Annual plants like the Nipomo lupine, naturally die after producing seed and/or when conditions dry out.



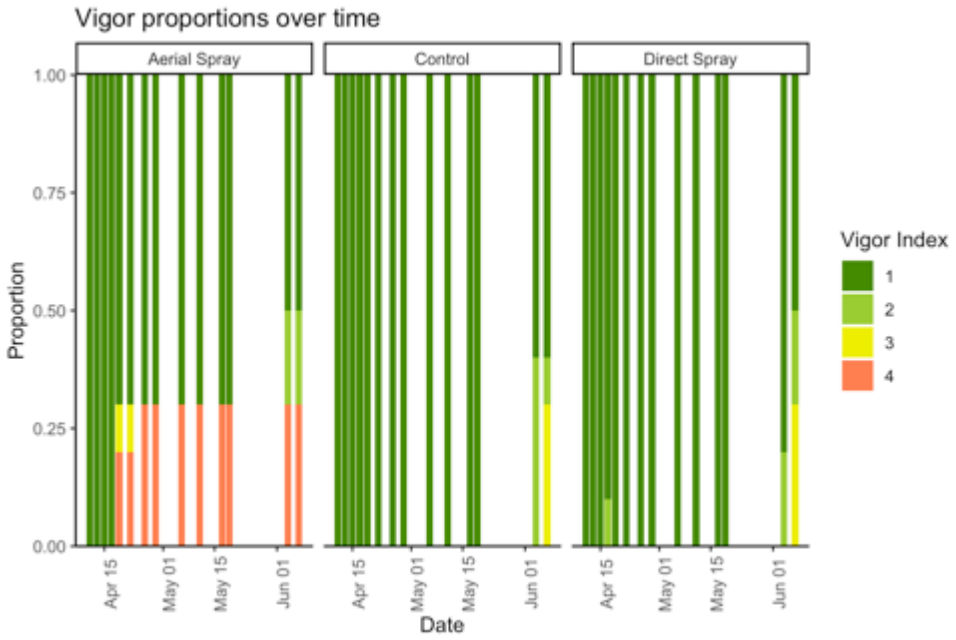
Graph shows general linear model of plant vigor index (ranging from 1 = healthy to 4 = dead) over time for early germinating seedlings

The cohort of treated newly germinating seedlings had longer to grow between treatment and sensesence – from April 10 to mid June and thus the slope of the line reflects a longer period in the high vigor condition (1).



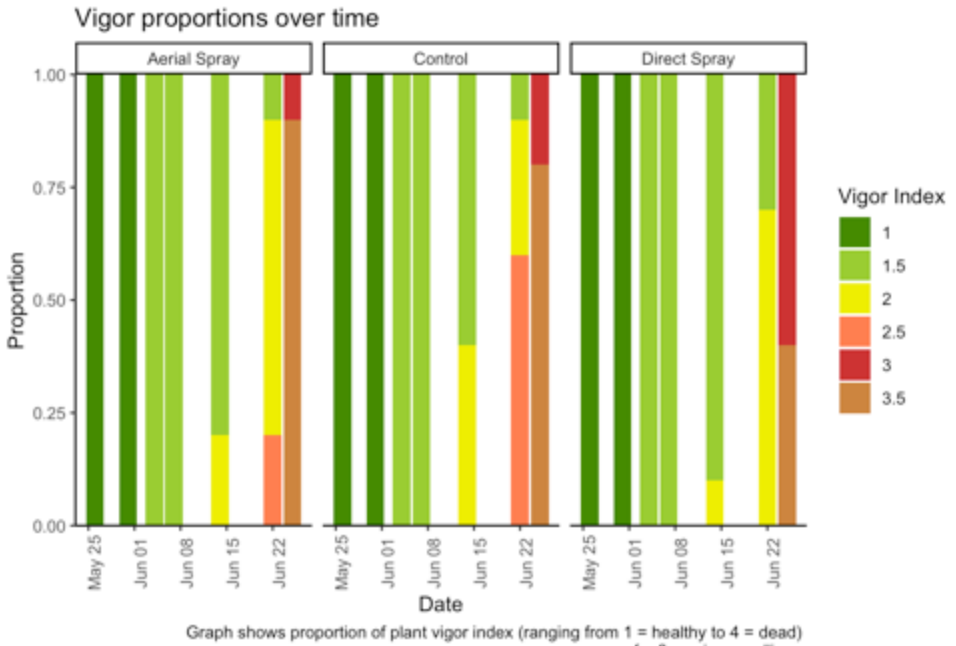
Graph shows general linear model of plant vigor index (ranging from 1 = healthy to 4 = dead) over time for early flowering seedlings

This graph shows the cohort treated after they had started flowering which was conducted May 23rd and follows the seedlings through late June when they had completed their seed formation and naturally senesced.



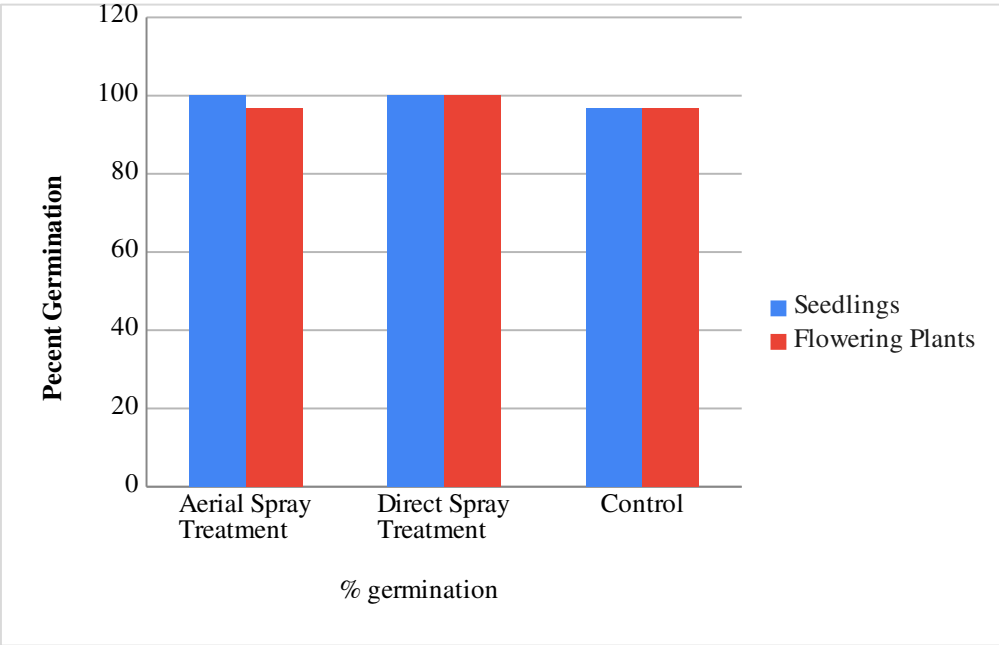
Graph shows proportion of plant vigor index (ranging from 1 = healthy to 4 = dead)
for early germinating seedlingss (chi-squared = 9.5, p = 0.15)

These graphs show the proportions of seedlings in each treatment cohort and treatment group within each vigor category. The red (dead) seedlings in the aerial treatment has to do with a herbivore getting in the cage and not with the aerial treatment of herbicide as you can see from the multiple observations of high vigor before they died (see photos below).



Graph shows proportion of plant vigor index (ranging from 1 = healthy to 4 = dead)
for flowering seedlings
Significant effect of direct spray treatment on final vigor index proportion (chi-squared = 6.7 p = 0.04)

Seed Viability Results
Within one week of sowing the seeds in cold water in a refrigerator, 100% of the seeds (except one seed) had germinated (see figure below). This is the highest germination observed for Nipomo lupine in all of our years of working with this species. The result reflects improved strategies for germination and the uniform viability of the seeds from these plants despite treatment with the grass-specific herbicide, Clethodim.



Conclusions:

There were no significant impacts of applying aerial or direct spray of Clethodim herbicide on the vigor, lifespan, seed production or viability of produced seed of the Nipomo lupine.

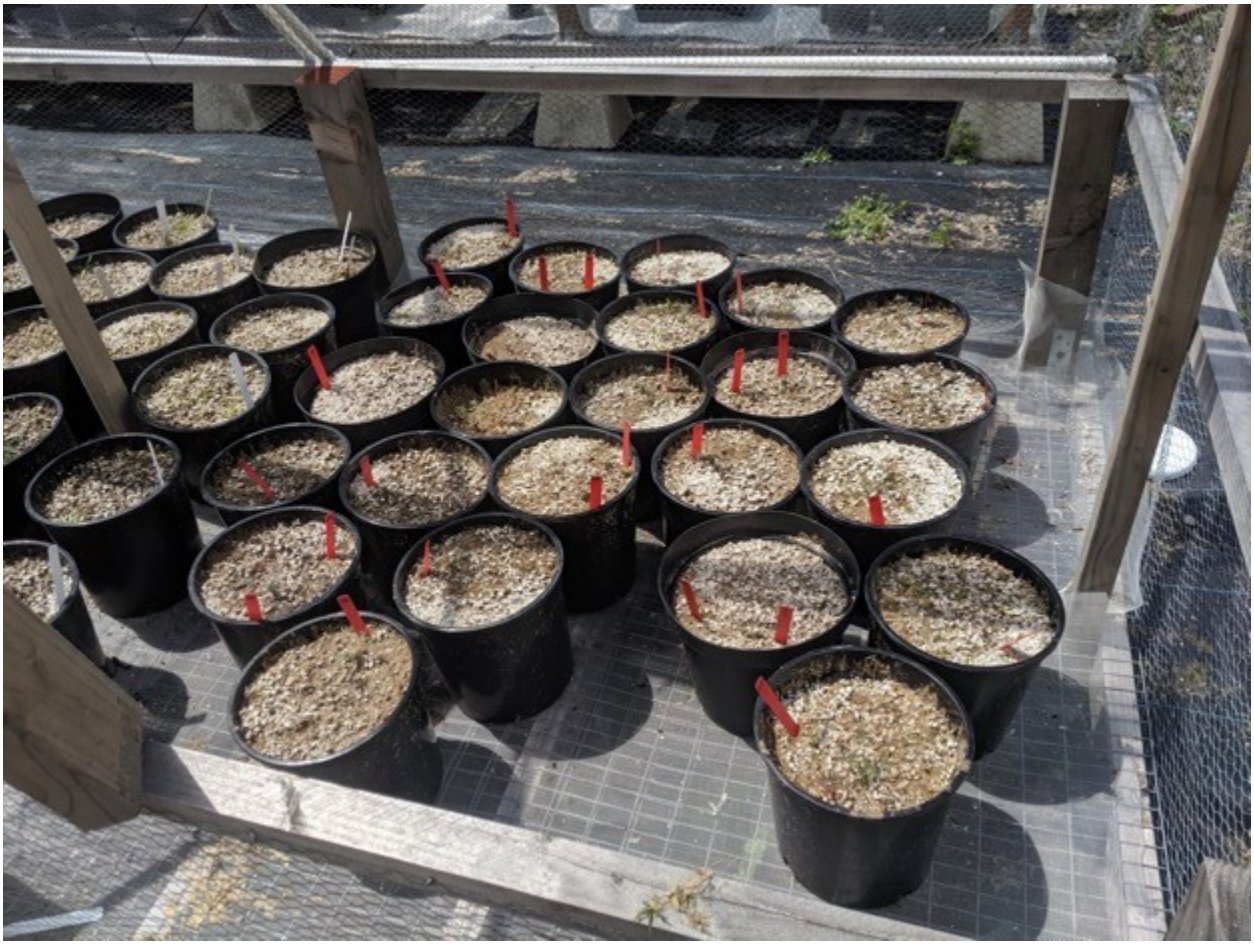


Image 1. Growing facility at CCBER and newly germinating seedlings.

Image 2. Treatment application site for even dispersal of spray.

Image 3. Typical newly leafing out seedling before spray treatment

Image 4. Newly flowering/fruiting seedling in second treatment period cohort.

Image 5. May 17th – first treatment seedlings thriving post-treatment

Image 6. Mouse or caterpillar damage unrelated to treatments

Image 7. Second plant from first cohort with missing leaves due to herbivory.

Image 8. May 17th, vigorous seedling cohort post treatment.