

NITROGEN FIXING SYMBIONTS: LEGUME  
SURVIVAL AND COEXISTENCE UNDER  
DROUGHT IN CALIFORNIA GRASSLANDS

by

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A THESIS

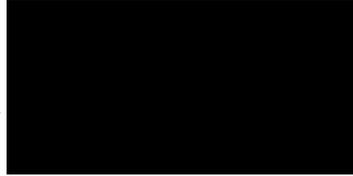
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## An Abstract of the Thesis of

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For the past 200 years, non-native species have been anthropogenically introduced in California grasslands, reducing native species abundance and diversity. *Trifolium hirtum* is a non-native legume commonly used for cattle fodder and cover cropping because of its ability to fix nitrogen via bacterial symbionts. *T. hirtum* currently coexists with California native legume *Trifolium willdenovii* in established communities, however, *T. hirtum* has the potential to outcompete *T. willdenovii* under increased environmental stress, such as that caused by climate change. Considering how symbiotic rhizobia contribute to these *Trifolium* species' ability to coexist or compete with other species, as well as how climate change alters these interactions, is essential for understanding potential impacts on native biodiversity and the response of important forage species to changing climate conditions.

I tested 1) how rhizobial symbionts contributed to the survival and growth of a native and non-native legume species. 2) How drought influences the competitive relationships of the two legumes. And 3) how each species responded under interspecific and intraspecific competition. For both non-native *T. hirtum* and native *T. willdenovii*, I measured rhizobia nodule mass, seeded background count, weed species counts, and weighed the aboveground biomass of individuals grown under drought and ambient precipitation treatments. On average, there were no differences in the mass of nodules by species, however there was an observed a negative correlation between the biomass and nodule mass of uninoculated background *T. willdenovii*. Drought positively impacted the biomass of background *T. hirtum* individuals, however drought treatments negatively affected focal *T. hirtum* biomass compared to ambient precipitation. Drought had no significant effect on focal *T. willdenovii* biomass.

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## Table of Contents

Introduction	1
Methods	4
Site Preparation	4
Data Collection	7
Data Analysis	7
Results	8
Discussion	11
Bibliography	14

## **Introduction**

For the past 200 years, non-native species that originate from Mediterranean regions have been anthropogenically introduced into California grasslands and successfully established due to California's similar climatic conditions (Brandt & Seabloom 2012). This phenomenon is relevant to modern coexistence theory, which describes when species will coexist together or be competitively excluded by characterizing differences in species niches and competitive abilities (Grainger, *et al.* 2019; Chesson 2000). Native Californian plant species are at risk of competitive exclusion due to the high rate at which invasive species have been introduced since European settlement (Catford, *et al.* 2018). Broader implications of this occurrence include increased loss of biodiversity and a decline in ecosystem services (such as nutrient cycling and carbon sequestration) as natural processes conducted by native plants are not replaced by non-native species (Liebhold, *et al.* 2017). In addition, the influence of climatic regimes has become increasingly important as a determinant of species survival due to global warming (Lopez 2017). Some non-native species, like *Trifolium hirtum*, are favored as forage for livestock due their persistence and tolerance of stressful environmental conditions, such as drought. Balancing the interests of agriculture and conservation in California grasslands is a key challenge that coexistence theory can begin to address.



Figure 1: Image of the non-native species *T.hirtum* (left) and the native species *T.willdenovii* (right).

Of particular interest to both groups of stakeholders are *Trifolium* species; native *Trifolium* species serve as a resource for local pollinators and bird species, while other *Trifolium* species are preferred as forage crop and to restore soil. *Trifolium* species are in the Fabaceae family (legumes), which associate with nitrogen fixing rhizobia bacteria in their root nodules (Wang 2019). Rhizobia bacteria stimulate cell division in legume roots resulting in the development and subsequent occupation of nodules known as bacteroids (Tewari & Arora 2013). Once nodules are formed, rhizobia fix atmospheric N with the enzyme nitrogenase, converting it to ammonia which is accessible to the host plant (Teng, *et al.* 2015). This symbiotic relationship may provide legumes with a competitive advantage as they are able to access nitrogen more readily. *Trifolium* may acquire their rhizobia bacteria by vertical or horizontal transmission (Wahdan, *et al.* 2021). To replicate pre-existing microbial communities, legume seeds are artificially inoculated with rhizobia, which has demonstrated increased nodule formation. In some cases, more nodules can

contribute to the greater fixation of nitrogen, which is also dependent on the nature of available nitrogen (Zhang *et al.* 2019).

Legumes in specific can be largely impacted by variations in weather, particularly following prolonged periods of either drought or rainfall (Suttle *et al.* 2007). This has implications for how grasslands are managed, because grasslands comprise 75% of livestock forage in California (Eviner 2016), and legumes are a predominant source of forage. The use of non-native legume species (such as *T.hirtum*) over native species (such as *T.willdenovii*) has increased the distribution of non-native species (Figure 2); with 88% of California grasslands under private ownership (California Native Grasslands Association, 2011), this spread is largely dictated by human activity. As *T.hirtum* has demonstrated the ability to coexist with California native legumes (California Invasive Plant Council, 2017), one goal of this study is to predict how drought conditions may affect this coexistence.

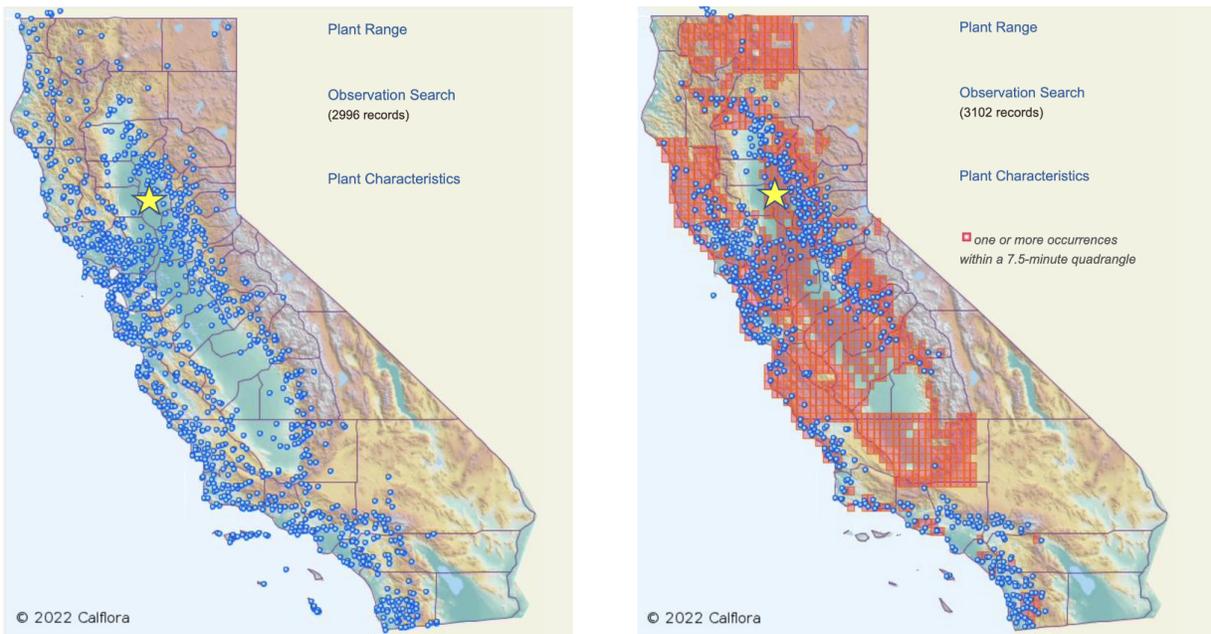


Figure 2: A comparison of current *T.hirtum* (left) and *T.willdenovii* (right) ranges across California (Califlora 2022). The yellow star indicates the location of the Sierra Foothill Research and Extension Center, located in Browns Valley, California.

**Hypothesis I:** Inoculated *Trifolium* individuals will outcompete uninoculated individuals under drought conditions because increased access to nitrogen via rhizobial symbionts reduces stress on plant vital functions.

**Hypothesis II:** *T.willdenovii* will be less impacted by drought conditions than *T.hirtum* due to local adaptations to climate and seasonal drought.

**Hypothesis III:** *Trifolium* individuals will perform better when there are less background competitors and weeds present due to greater access to resources.

## **Methods**

### Site Preparation

The Hallett Lab's Mega-Competition Experiment experiment was conducted at the Sierra Foothill Research and Extension Center (SFREC) in California. The site's soil was initially sterilized with herbicides to remove prior established species and seed banks. It should be noted that despite sterilization some accidental *Erodium botrys* individuals did establish in test plots. In addition, drought blocks were irrigated once and ambient blocks were irrigated once a week during winter months due to the severity of drought. Lab members selected twenty species for this experiment, to test relationships between native and nonnative plant species coexistence. We planted each species at three background densities: a control (0g/m<sup>2</sup>), low density (4g/m<sup>2</sup>), and high density (8g/m<sup>2</sup>). Within each background, we planted three seeds of each other species to obtain one focal individual. Seeds were planted in a triangular pattern to ensure there was no competition for resources between focal individuals. We designed the experiment to include ten

4.9m x 15.24m test blocks, subdivided into 46 plots. Out of the 46 plots, 42 plots were 1m x 2m plots and four plots were 0.5m x 0.5m. We planted focal individuals using 1m x 1m PVC quadrats divided into 25 subplots with fishing line. It was decided a 0.45m around the block would account for edge effects. For my sub-experiment, two species were selected for observation with four seed types utilized: uninoculated *Trifolium willdenovii*, inoculated *Trifolium willdenovii*, uninoculated *Trifolium hirtum*, and inoculated *Trifolium hirtum*. Seeds were purchased from Pacific Coast Seeds, Inc (Tracy, CA), to ensure uniformity of seed quality. Uninoculated *T. hirtum* seeds were collected at SFREC as they were unable to be purchased.

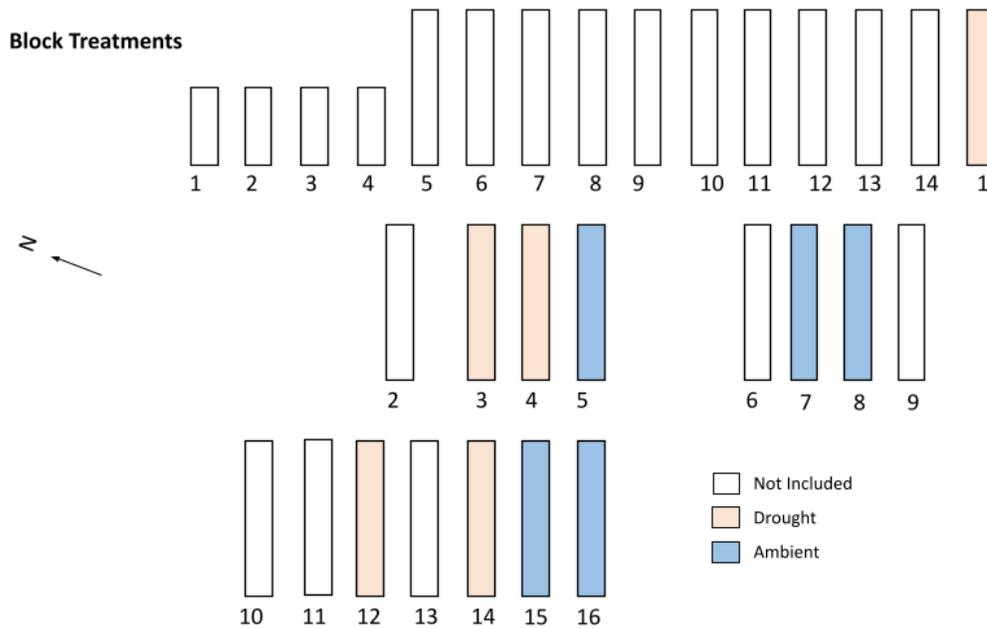


Figure 3: An aerial view of block arrangements highlighted by treatment type. Only blocks containing the *Trifolium* sub-experiment are colored; five blocks for each treatment were selected.

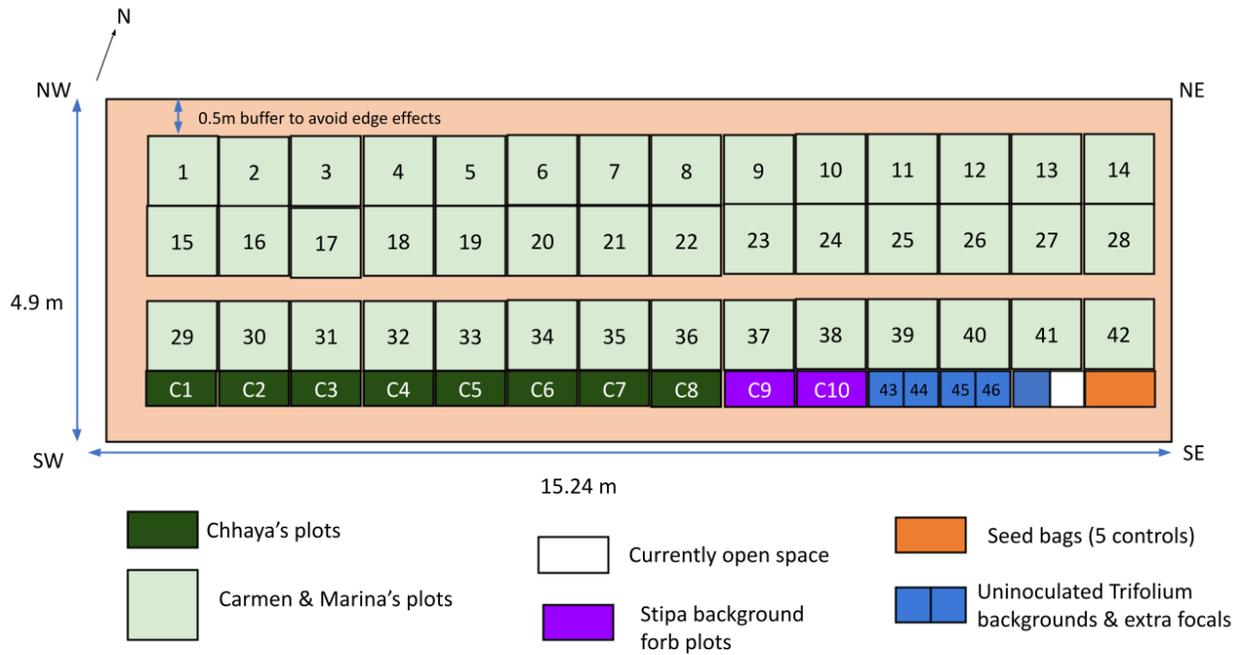


Figure 4: A layout of one block; subplots colored blue to indicate where the *Trifolium* sub-experiment was located in the context of the greater Mega-Competition experiment.

## Sub-plot Layout

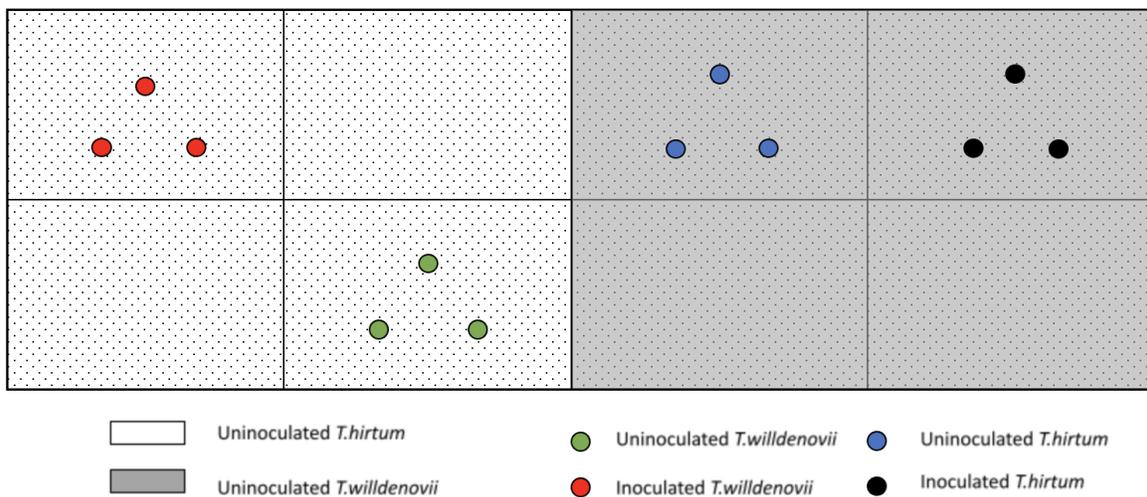


Figure 5: An example of two subplots, separated by a background species and divided into quadrants. Circles depict the location of seeded focal individuals. The black spot pattern depicts the background seeds. Empty subplots were seeded with focal individual species that are not included in this study.

### Data Collection

After a five month period, I returned to the study site in April to collect samples between *T.willdenovii* and *T.hirtum*'s observed flowering period. I counted the number of focal individuals present in each subplot, then conducted neighborhood counts around the individual to estimate potential competition from background species and weed species. For these counts I used an 18x18cm wire square, which is a standard size for measuring the neighborhood of these annual plant species (Lanuza *et al.* 2018; Levine & HilleRisLambers 2009). After the neighborhood census, I collected the focal individuals. For background individuals, I collected between 4-23 stems depending on the amount of nodules present on each individual. Biomass was dried for 48 hours at a temperature of 60 degrees Celsius. After drying, I weighed stem biomass, root mass, nodules and soil mass. Mean survival was calculated by dividing the total number of surviving stems by three.

### Data Analysis

The data collected was limited to first year growth, therefore my observations may only reflect which species have an initial advantage, however this remains relevant to determinants of coexistence outcomes. In addition, direct comparisons between *Trifolium* species biomass could not be made due to the difference in phenology. *T.willdenovii* flowered early in the spring season, and was senescing at the time of collection, thus the weight of biomass and nodules were likely reduced (Zhou, *et al.* 2021), while *T. hirtum* was collected prior to flowering. Therefore, for each

species I divided the data into the categories of focal (experiencing interspecific competition) and background (experiencing intraspecific competition). The focal dataset included comparisons of inoculation, precipitation treatment, background species count, and weed counts on the biomass of focal individuals. The background dataset included the effects of treatment on the biomass and nodule weight of uninoculated *Trifolium* individuals. Nodule weight was only included in background individual counts due to the lack of ample nodule formation on focal individuals.

I conducted all data analysis on the platform RStudio Version 4.0.3. I used a two-way Analysis of Variance (ANOVA) to determine if treatment, inoculation, background species counts, or weed counts significantly impacted focal individual biomass (focal dataset), and a one-way ANOVA to test if treatment had a significant impact on uninoculated background individuals nodule mass and biomass (background dataset). In the two-way ANOVA I first tested for interactions between treatment and inoculation, but as these were not significant I examined the additive effects of the two variables. I also used a linear model to test for a linear relationship between two continuous variables, biomass and nodule mass (background dataset).

## **Results**

Overall, drought treatment reduced *T.hirtum* biomass and had no significant effect on *T.willdenovii* biomass under interspecific competition (Figure 6a). Based on the ANOVA, treatment had a marginally significant effect on *T.hirtum* biomass (mean = 0.29 , sd = 0.22, p-value = 0.0887). Inoculation was not a significant determinant of biomass, however, it appeared to change the effect of precipitation treatment on biomass for *T.hirtum*, with less

biomass under drought for uninoculated individuals and more biomass under drought for inoculated individuals (Figure 6b). Under intraspecific competition (Figure 6c), the average *T.hirtum* and *T.willdenovii* background individual's biomass had greater biomass under drought, the opposite response from focal individuals under interspecific competition (Figure 6a). The effect of treatment on the biomass of uninoculated *T.hirtum* background individuals was significant (mean = 4.4 , sd = 1.5, p-value = 0.0284) (Figure 6c).

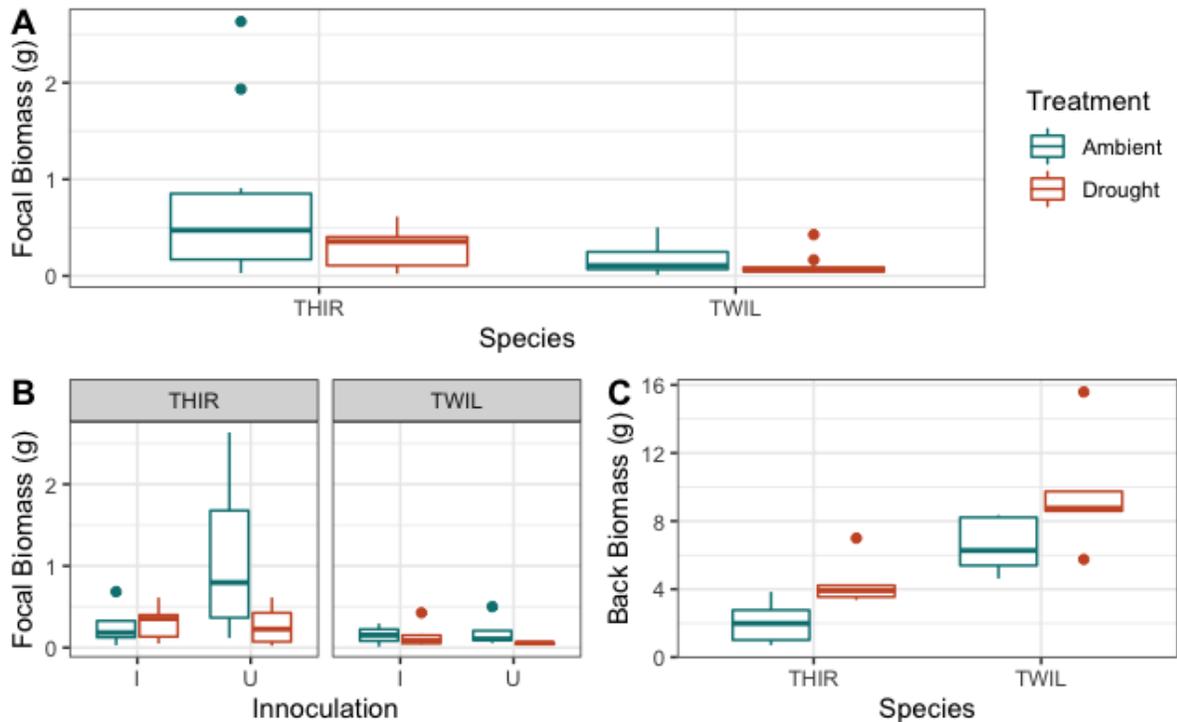


Figure 6: (a) Comparison of overall focal biomass weight per species and treatment. THIR = *T. hirtum*, TWIL = *T. willdenovii*. Error bars represent one standard error above and below the mean. Drought decreased the biomass of *T.hirtum* (p-value = 0.0887) and slightly decreased the biomass of *T.willdenovii*, although this effect was not significant. (b) Comparison of focal biomass weight per species; species are further subcategorized by inoculation and treatment. I = inoculated seeds, U = uninoculated seeds. Drought appeared to decrease the biomass of uninoculated *T.hirtum*, increase the biomass of inoculated *T.hirtum*, and slightly decrease the biomass of *T.willdenovii*. (c) Comparison of background biomass weight per species and treatment. There was a significant negative relationship for the effect of drought on uninoculated *T.hirtum* (p-value = 0.0284).

Weed counts and background species counts did not have a significant effect on *T.hirtum* or *T.willdenovii* biomass. However, both species reflected a change in survival based on inoculation and drought. *T.hirtum* survival increased under drought and inoculation, and *T.willdenovii* showed an increase in survival under drought (Figure 7).

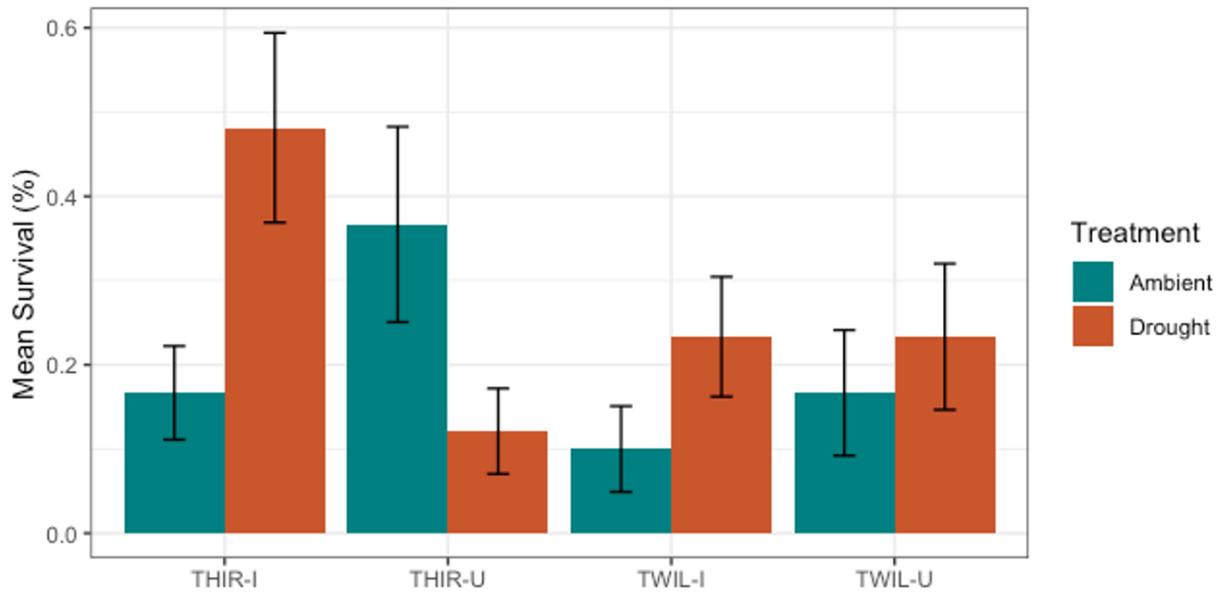


Figure 7: Effect of treatment on survival of each species, categorized by inoculation/uninoculation.

There was a significant (p-value = 0.04129) negative relationship between the biomass and nodule mass of *T.willdenovii* (Figure 8). However, there was no significant relationship between the biomass and nodule mass of *T.hirtum*.

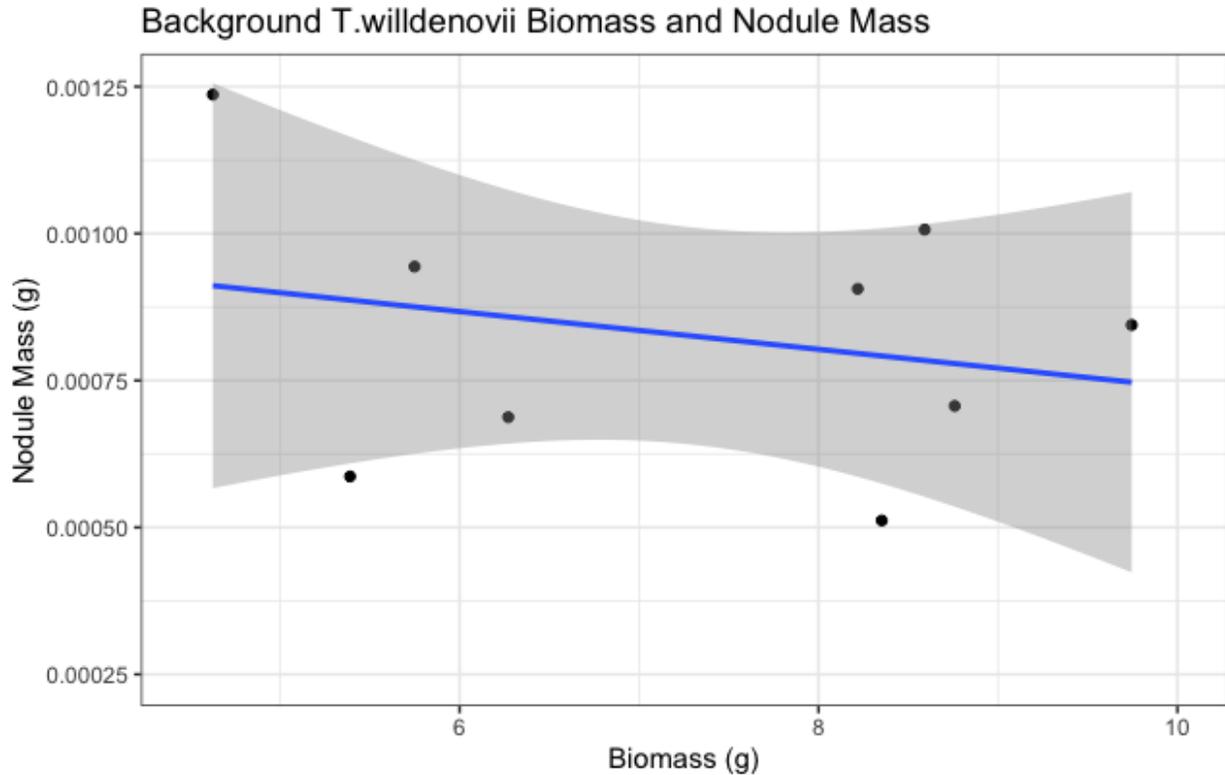


Figure 8: Comparison between the biomass and nodule mass of uninoculated background *T.willdenovii* individuals. The blue line demonstrates a significant negative relationship between biomass and nodule mass (p-value = 0.04129).

## Discussion

A recurring theme throughout my results, in support of my Hypothesis II, was the impact of precipitation treatment on *T.hirtum*, and the lack of an effect on *T.willdenovii*. In the instance with focal *T.hirtum* individuals, drought caused a decrease in biomass, which could be attributed to reduced soil moisture (Mackie *et al.* 2018), as non-native species have been shown to require more water than native species (Cavaleri, M. & Sack, L. 2010). The contradictory results from the background dataset, which show an increase in *T.hirtum* biomass under drought conditions, could indicate that growing with intraspecific neighbors provides a facilitative effect on *T.hirtum* productivity and survival. One possible explanation is that *T.hirtum* is highly competitive for soil

moisture (California Invasive Plant Council, 2017) and had an established nitrogen pool to rely on (Nölke, *et al.* 2022), individuals were able to persist under drought conditions. Similar results were found in a study (Gerakis, *et al.* 1975), in which drought conditions decreased the biomass and higher densities increased the biomass in a relative non-native clover species, *Trifolium subterraneum*. Another explanation is that *T.hirtum* had not reached a density at which there was maximum yield (Gerakis, *et al.* 1975; Black 1960), thus the effects of intraspecific competition were minimized. In regard to *T.willdenovii*, the insignificant changes in survival or biomass under drought may be due to its adaptations to the local climate; additionally, more energy may have been spent on the dissemination of seeds than accumulation of biomass (LaForgia, *et al.* 2018).

Although background counts, weed counts and inoculation did not demonstrate any significant effect on the productivity or survival of either *Trifolium* species (thereby rejecting my Hypothesis III), there was still a discernable pattern. Uninoculated *T.hirtum* biomass and survival decreased under drought, which could be explained by the lack of access to nitrogen due to the absence of rhizobial inoculant. This partially supports my Hypothesis I, because inoculation appeared to increase *T.hirtum*'s survival, but did not increase *T.willdenovii*'s survival. The insignificant results could also partially be attributed to the time at which data was collected. The aforementioned factors may have made a larger impact at varying stages of *Trifolium*'s life stage, or demonstrate that precipitation is the most important determinant of *Trifolium*'s performance. In addition, senescence may have reduced the nitrogenase activity and ultimately the cell structure of *T.willdenovii*'s nodules (Zhou *et al.* 2021), thus further studies on the impact of inoculation on biomass and nodule mass are required. However, the significant negative

relationship between the biomass and nodule mass of *T. willdenovii* indicates there may be some impact of belowground dynamics on establishment (including aspects that were not sampled such as root length and the microbial communities in the soil and nodules). Additional studies on the role of inoculation and nitrogen fixation are necessary to better understand the role of symbiotic bacteria in the success of *Trifolium* species, and allow for a direct comparison between species to make greater claims regarding coexistence.

The results of my study serve as a preliminary basis for understanding how future drought conditions may impact the survival of both native and non-native clover species in California grasslands. Given *T. hirtum* is inoculated for agricultural purposes, it will likely persist in currently invaded areas. *T. hirtum* could potentially invade wild areas if drought conditions are recurring because drought appears to increase its survival rate and biomass. This potential invasion is also likely, given it can withstand stressful moisture conditions, spread readily and establish in disturbed areas. Consequently, *T. hirtum* may outcompete *T. willdenovii* for resources in wild areas. *T. willdenovii*, which has adapted to California's seasonal drought, could potentially persist without intervention if not for the threat of other non-native species. However, as non-native species have few obstacles limiting population growth (Vasquez, *et al.* 2010), native species like *T. willdenovii* may require active management in order to preserve biodiversity. This responsibility falls largely to the private property owners of grasslands; improving the accessibility of research that details the impacts non-native species have on local biodiversity can help inform management decisions that will become of increasing importance as drought conditions worsen under climate change.

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