EMP Grant - Final Report

Date: September 30, 2014

Project Tracking #: CNLM Acanthomintha

Grantee: Center for Natural Lands Management

Project: Species-specific Management: Genetic studies of San Diego thornmint (*Acanthomintha ilicifolia*) to inform restoration practices

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Project Summary

I. Tasks

This genetic study—the first for this species (San Diego thornmint, thornmint, SDTM)—was parsed into four tasks for the EMP grant purposes. All tasks, with the provision of this Final Report (part of Task 4), have been accomplished.

1. Task 1 (Population descriptions and leaf collections): This is the first step in both of the genetic studies. San Diego thornmint (SDTM) sites (representing 15-25 occurrences) will be visited and described, representing a large extent of the natural, ecological, and geographic range of the remaining populations of this species.

   Site visits will be timed to coincide with maximum or near-maximum vegetative growth but prior to seed maturity. This will allow collection of fresh vegetative material with as little impact on remaining plants as possible. A small amount of leaf material will be collected from several (depending on population size) plants per population. Collection guidelines have been developed. Vegetative material will be kept cool and transported quickly to the laboratory where the isozyme study will be conducted.

2. Task 2 (Seed collection and first genetic study – local adaptation): This genetic study is focused on determining local adaptations among a subset of the SDTM occurrences. This is the foundation for determining genetically appropriate restoration guidelines. We will include four to six occurrences, selecting those that are among the larger occurrences and that represent diversity in location and/or factors that may indicate adaptive differences (e.g., climate, soil type, elevation). Sites will be revisited when seeds are mature and a sample collected (that will not pose a risk to the donor occurrences). Seeds will be collected from several plants at suitable distances such as to obtain a representative sample of genetic diversity that is not biased by microsite or relatedness among plants. The study design includes two environments (two sites or two controlled-condition environments), and 3-4 replications. Traits of potential adaptive significance such as time-to-first-germination, time-to-mean-germination, rate of growth, root-to-shoot ratio, time to first flowering, time to seedset, etc. will be measured. Significant differences in any of these traits among the occurrences would suggest genetic differences, given the common environment(s) under which they would be grown.

3. Task 3 (Rangewide ploidy and isozyme analysis): The second genetic study will include the full set of occurrences sampled. Proteins will be extracted from the leaf material and an isozyme analysis conducted. This method was chosen due to the low risk, reasonable cost, and appropriate information derived. Other methods, such as RAPDs or microsatellites involve more risk, time, expense, and may result in less information (e.g., RAPDs reveal only the dominant allele; microsatellites would require marker development which would involve considerably higher expense and more time). Additionally, the larger number of plant studies that have been conducted with isozyme analysis provides a large and informative context in which to interpret results. Results from this study will reflect (selectively) neutral genetic diversity, providing information on the amount of (this kind of) genetic diversity within the species (relative to other species that are appropriate references) and genetic structuring within the species. Derived population-level statistics will also indicate levels of inbreeding/outcrossing. Information derived solely from this type of study (or RAPDs or microsatellites) is not an appropriate basis for restoration guidelines as this type of diversity may not reflect adaptive variation.

   Because there are many cases of polyploidy within this genus, it is a reasonable expectation that this species may be polyploid and it is prudent to look for evidence of polyploidy. Not only is the ploidy information needed for appropriate interpretation of the isozyme (or other) studies, but if there were determined to be multiple ploidy levels within the species, this would have consequences for appropriate restoration (seed source) practices. If there is evidence of tetraploidy, the isozyme results should still be interpretable (NB. any genetic method would be complicated by polyploidy, but the isozyme method can still be interpreted for tetraploids, as wells higher ploidy levels depending on the type of ploidy (e.g., autopolyploid, allopolyploid, etc.) and with appropriate inheritance studies.)

   The facility (National Forest Genetics Laboratory) and PI (Dr. Valerie Hipkins) conducting these analyses are very experienced in these methods and routinely perform such studies on native plant species, including herbaceous annual and perennials and woody perennials.

4. Task 4 (formerly Task 5) (Reports): Quarterly and annual reports will be prepared that indicate progress and accomplishments for Tasks 1-3, as appropriate.

CNLM designed the studies in general, collected seed and foliage, obtained regulatory approval, selected appropriate subcontractors, provide subcontractor coordination, and assisted in interpreting combined study results. The isozyme study was conducted by staff of the National Forest Genetics Laboratory of the USDA Forest Service. The common-garden study was conducted by staff in the Applied Ecology Division of the Institute for Conservation Research, San Diego Zoo Global.

II. Regulatory approval

Permits were obtained for foliage and seed collections from the appropriate state and federal regulatory agencies as follows:

California Department of Fish and Wildlife Research and Management Permit No. 2081(a)-12-03-RP

US Fish and Wildlife Service Recovery Permit TE 221411-2 issued under section 10 (a)(1)(A) of the Endangered Species Act

III. Research Partners (SDTM owners/managers)

The following landowners and managers provided access to their SDTM populations for foliage and/or seed collections:

Back Country Land Trust
California Department of Fish and Wildlife
City of San Diego
Center for Natural Lands Mangement
County of San Diego
Endangered Habitats Conservancy
San Diego County Airports
San Diego Habitat Conservancy
The Nature Conservancy
USDA Forest Service (National Forests)
US Fish and Wildlife Service (National Wildlife Refuge)

IV. Isozyme study

For this study, sixteen thornmint populations, five of them with samples taken from two 'subpopulations' within them, were included, for a total of 21 sampled sites. The sites represented a broad range in latitude, longitude, and elevation within the extant range of the species (see Table 1). Between 11 and 30 plants were sampled per site (i.e., population or subpopulation) with the smaller sample sizes reflecting lower population sizes and appropriate limitations on collection of material so as to minimize risk of impact. Measures of genetic diversity indicated moderate genetic diversity within populations, on average, with up to 7 alleles observed at one locus in the entire collection, and an average of 3.5 over the study. Despite the variation, isozyme markers may have been insufficient to distinguish between all unique genotypes in San Diego thornmint (i.e., may have under-estimated the diversity).

Analysis of population differences indicated that the species has significant genetic structure and that differentiation among populations is consistent with gene flow decreasing as a function of geographic distance (Figure 1). The overall genetic differentiation observed in San Diego thornmint is slightly lower than mean values reported for endemic annuals, but higher than that reported for other members of the Lamiaceae. Populations that occur within a geographic region (ca. 20 km) were more genetically similar than populations separated by greater distances. This pattern indicates some level of gene flow may continue between populations, despite the limited potential for long-distance gene flow in this insect-pollinated ephemeral winter annual. Alternatively, these populations may have only recently become genetically isolated, and allele frequencies have not yet differentiated. These results also provide evidence for restricting seed dispersal among highly divergent populations Differentiation among populations appears to be most strongly related to longitude (and elevation) and less so to latitude. The Cleveland National Forest (CNF) sample was derived from plants grown out from a seed collection and thus it may not be directly comparable to other samples. However, it should be noted

that some 'private alleles' (i.e., not found in other populations) were discovered in the CNF sample. This population is of interest for further study.

The isozyme banding patterns observed are consistent with two, four, and six alleles per locus indicating diploid, tetraploid, and hexaploid plants, respectively, may be present in SDTM collections. The frequency of each ploidy level varies among populations, but the majority were observed to have a tetraploid banding pattern in at least one locus. For the populations sampled, the hexaploid banding pattern generally increases from west to east (Figure 2). This evidence of ploidy requires further confirmation through flow cytometry.

V. Common-garden study

Seeds were collected in the summer of 2012 from seven populations, and from two sub-populations for two of those. After seed were cleaned and assessed, five sampled sites were selected for inclusion in the common-garden study based on amount of (presumably filled and viable) seed. The two populations from which seed had been collected and that were not carried forward into the common-garden study were (CNLM's) Manchester and Rancho la Costa Preserves. The five study locations included in the common-garden are presented in Table 2. They cover considerable geographic and elevational range, as desired for some representation of the species in potential for local adaptation.

On February 6, 2013 fifty-five pots with 5 seed per pot were sown for each population for each watering treatment. Anderson Plant Bands AB58 (5" width by 8" depth and nine per tray) were used with a substrate of potting mix and washed sand (3:1). To achieve as much uniformity as possible, the two individuals did all the potting mix preparation, each doing the same steps and a single individual filled all the containers. All pots were watered in with 500ml of water and all plants were misted twice a week during the germination phase. Subsequent irrigations were 1000ml per pot. Plants were randomized within a tray and tray locations were randomized on benches. Benches were on raised platforms and received full sun. Plants in the high water treatment received water every 2 weeks while the lower water treatment was irrigated every 4 weeks. When the plants started to show signs of water stress, the frequency was adjusted to once a week for the higher water treatment and every 2 weeks for the lower water regime. Irrigation continued until all plants senesced and were harvested.

Germination was noted in early March and number of seedlings per pot was monitored once a week from March 12 through March 26, 2013. The maximum number of germinants per pot was recorded. Following completion of germination, seedlings were thinned to one per pot. Note that from germination onward, there was some herbivory by snails and insects that affected both the number of surviving seedlings and the extent of foliage on some seedlings, until a treatment was performed around and under the bench area. Extent of herbivory appeared to be similar regardless of population. Plants were checked for senescence 5 days per week starting in late April. The first plants senesced on May 2 and continued through July. When plants completed their life cycle, data were taken on plant height, plant width and number of inflorescence whorls. The top of the plant was harvested and dried to obtain dry biomass data. The roots were so fine that it proved impossible to separate them from the potting mix, so it was not possible to collect data on root biomass. Seed production data was obtained by processing twenty-five dried tops per population per watering treatment (for a total of 300 samples) to

extract the seed. Seed was separated into dark, light and damaged fractions and the number of each was recorded. Total number of seed produced and total weight of seed produced per plant were also recorded.

In general, differences among populations in reproductive traits (e.g., number of inflorescence whorls, days to flowering) were greater and more consistent than those in vegetative traits (e.g., total plant height, plant above-ground biomass). See Figures 3-6. For reproductive traits, in general, inland and higher elevation populations were often different from coastal populations.

VI. General conclusions and recommendations

The genetic studies conducted within this project comprise the first direct genetic information for San Diego thornmint. There is evidence of moderate levels of genetic diversity within the species, and substantial genetic differentiation among populations, with differences increasing in general with distance. However, longitudinal and elevational distance is more strongly related to genetic differentiation than is latitudinal distance. There is evidence of local adaptation for some traits in some populations, with the reproductive traits showing more dramatic and consistent differences than vegetative traits. A cautious interpretation is that coastal populations are distinguished from more inland and higher elevation populations, but the common-garden study was strongly constrained by a fairly small sample of populations and extrapolation to the species level is not recommended without further study. The discovery of evidence of multiple ploidy levels although not entirely unexpected based on other species within the genus, has implications for management and restoration activities and should be confirmed.

General recommendations pertaining to protecting genetic integrity and diversity for this species:

- Support natural regeneration in large populations with adequate genetic diversity
- For recently small populations, support rapid increase in population size (through management)
- For historically small populations, provide enhancement of population size with collections from same population
- For genetically depauperate populations, supplement with genetic materials from nearby populations, favoring longitudinal and elevational similarities
- Record and maintain records of any movement of plant material among populations (i.e., especially important for collections related to restoration or research activities)
- Design any seed collections to maximize the genetic diversity within a population (i.e., general principles)
- Keep seed collections separate (and identified) at least by EO/site and by smaller units if collecting within large populations that cover substantial longitudinal or elevational distance

VII. Additional activities outside scope of project

A poster was prepared by NFGEL with input from CNLM staff and presented at the joint Botany (Botany 2014: New Frontiers in Botany) meetings July 26-30, 2014 in Boise, Idaho. This poster, concerning ploidy, including only the ploidy data/aspect of the SDTM genetic study. Poster title: Inferring ploidy variation in natural populations, and its implications for species conservation. J. DeWoody, V.D. Hipkins, J.K. Nelson, and D.L. Rogers. Online: http://www.2014.botanyconference.org/engine/search/index.php?func=detail&aid=254

D.L. Rogers, J. DeWoody, and B. Endress (formerly with San Diego Zoo Global, now at the University of Oregon) continue to analyze data from these studies to better establish the patterns, if significant, between the adaptive and selectively neutral genetic diversity. The intent is to prepare and submit a paper for publication in an appropriate peer-reviewed journal.

VIII. Recommended further activities (with the exception of #1, the studies are not listed by priority):

1. Confirmation, through flow cytometry, of the polyploid nature of the species for which evidence was obtained in the current (isozyme) study. This will involve resampling selected populations and with a specific design geared towards determining any geographic or morphological correlations with cytotype.

2. Extension of the species-wide isozyme study to include more populations—particularly those that extend the elevational, latitudinal, or longitudinal range of the study, and to include direct sampling from Cleveland National Forest (if permission is granted).

3. Further analysis of (current and to-be-collected) genetic data to determine relationships, if any, with additional site factors and demographic data (e.g., population size).

4. Further pollination (and fertilization, if possible) studies to better characterize the reproductive biology of the species and to determine if pollinators are limiting population size or contributing to population variability on some or all sites.

TABLES and FIGURES

Table 1. Sampled populations for the isozyme study.

Occurrence	Abbrev.	Date coll.	No. subpops.	Elevation (m)	N
Los Penasquitos Canyon	EO19	4/26/2013	1	26	16
McGinty Mountain (southwest slope)	EO21	4/8/2013	1	420	30
McGinty Mountain (summit and ridgeline)	EO22	4/8/2013	1	612	30
Lux Canyon (east), Manchest. Avenue Mitigation Bank	EO28	3/30/2013	2 (A & B)	95	42
Sycamore Canyon	EO32	4/11/2013	2 (1 & 2)	317[2]	40
Mission Trails Regional Park	EO33	3/30/2013	1	204	21
Sabre Springs (west) - Penasquitos	EO36	4/26/2013	1	86	14
Emerald Pointe	EO58	3/30/2013	1	62	11
Wright's Field (north)	EO63	4/15/2013	1	567	30
Wright's Field (south)	EO63	4/15/2013	1	564	30
Palomar Airport Road	EO70	3/30/2013	2 (A&B)	84[2]	42
La Costa Greens	EO82	3/30/2013	1	73	17
Rancho Jamul ER - Hollenbeck Canyon	EO86	4/10/2013	1	483	30
South Crest	EO72	4/10/2013	2 (1 & 2)	398	40
San Diego National Wildlife Refuge	EO87	4/8/2013	2 (lower & upper)	377	60
Cleveland National Forest	EO75	2012	1		30[3]

[1]Number of distinct genotypes observed in all samples.
[2]Average of min and max reported.
[3]EO75 assessed by 30 seedlings germinated from a bulk seed lot collected in 2012

Table 2. SDTM locations included in the common-garden study.

Location	EO	Longitude	Latitude	Elevation
Carlsbad N #1	70	Coast	N	Low – 250 '
Carlsbad N #2	70	Coast	N	Low – 159 '
Sycamore Canyon #2	32	Intermediate	Intermediate	Mid - 1120 '
Mission Trails RP	33	Inland	S	Low – 459 '
SDNWR	87	Inland	S	High – 2150 '
Cleveland NF	75	Inland	S	High – 2310 '

Figure 1. Significant isolation by distance in San Diego thornmint: genetic differentiation [FST/(1-FST)] increases as a function of geographic distance. Each point represents one pair of populations. Correlations were examined through Mantel Tests.

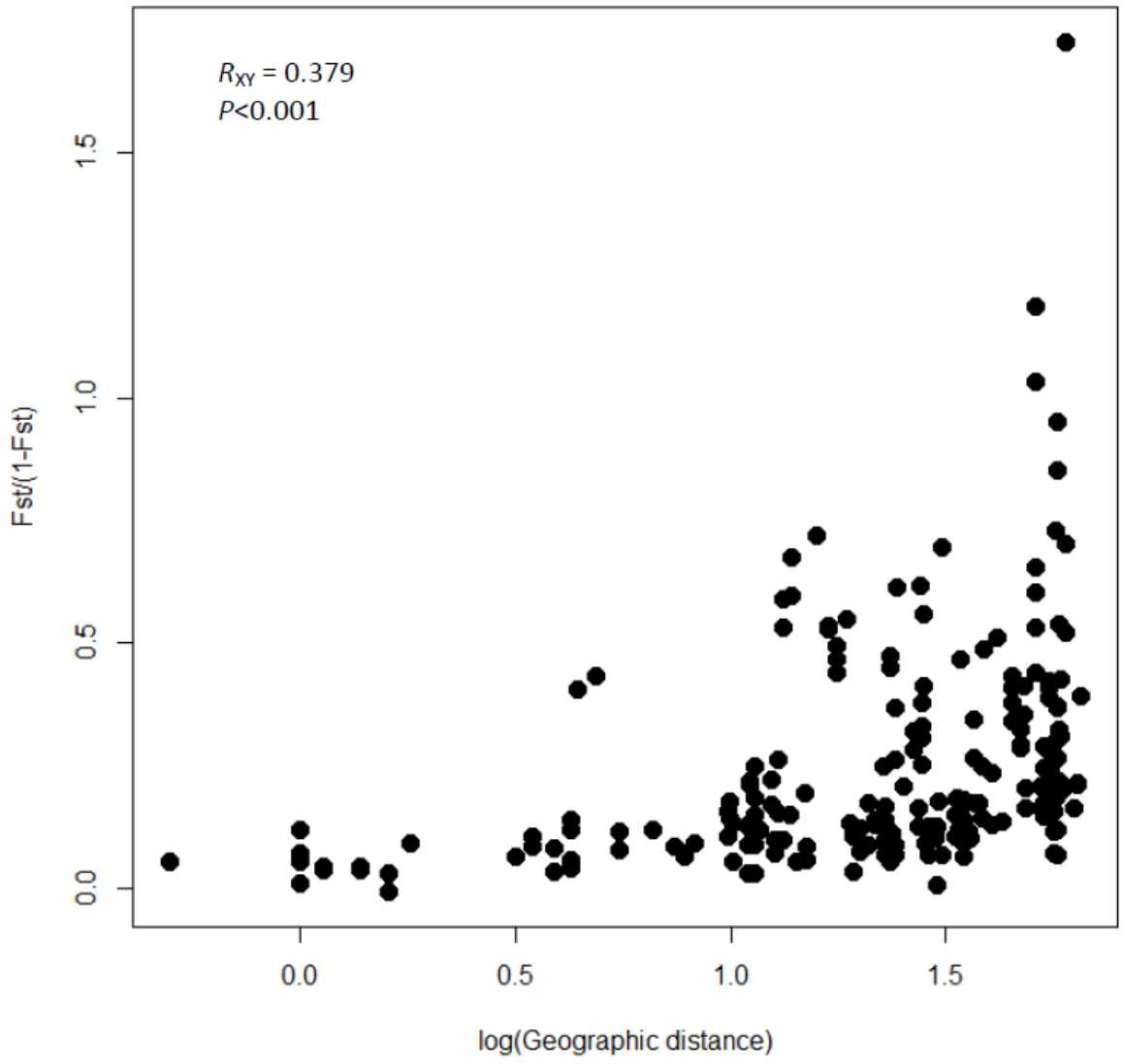

Figure 2. Three levels of ploidy (diploid, tetraploid, and hexaploid) were identified in San Diego thornmint from isozyme banding patterns. Ploidy tends to increase with latitude (from west to east) across the sampling range, which also roughly corresponds with increasing elevation. Note: EO75 was assessed as seedlings from a bulk seed lot and may include related individuals. Also, some EO's may need to be revised (see Table 1).

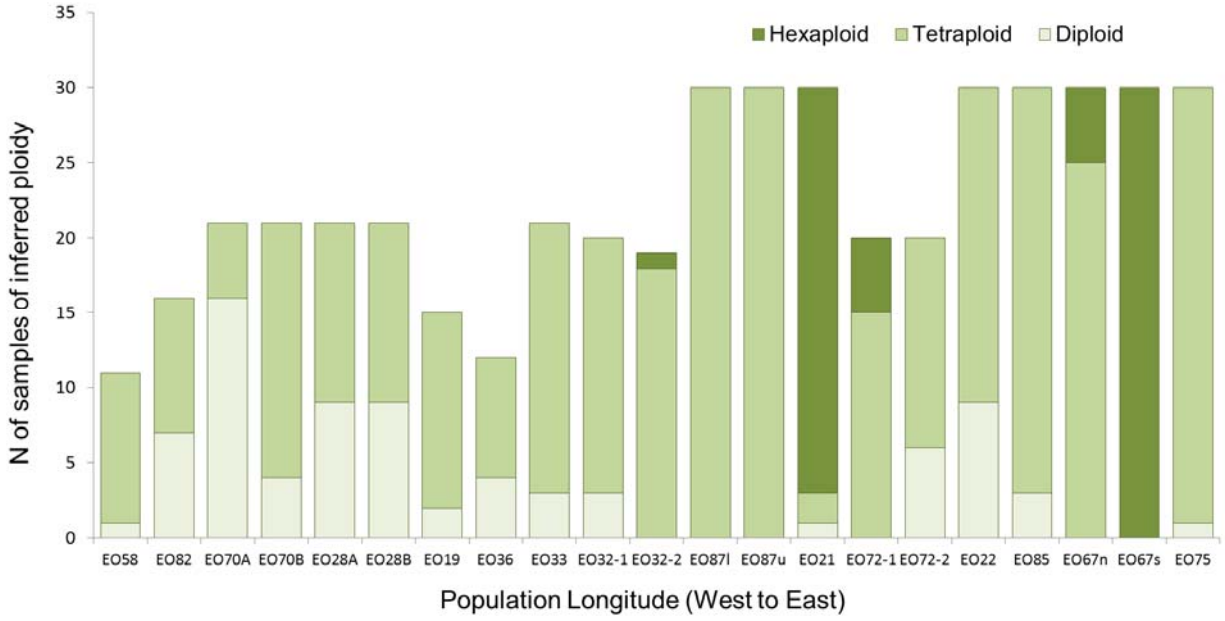

Figure 3. Days to flowering x population (per watering treatment)

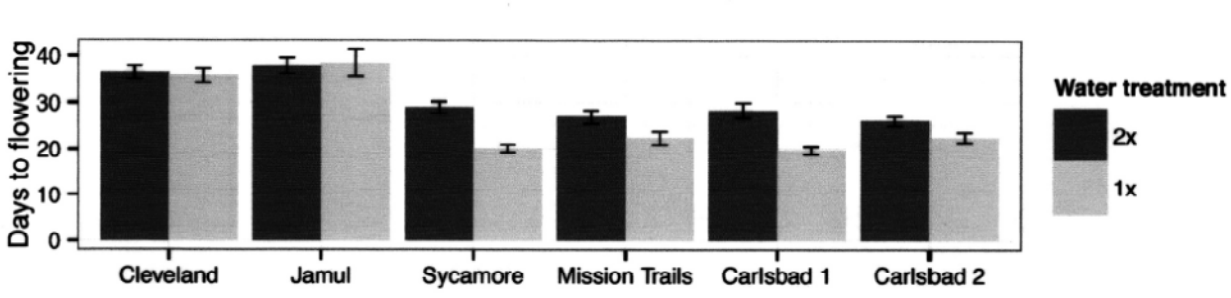

Figure 4. Number of inflorescence whorls x population (per watering treatment)

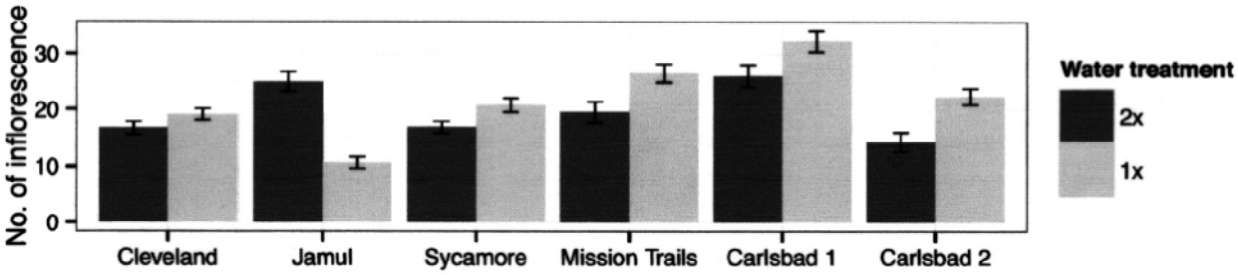

Figure 5. Plant biomass x population (per watering treatment)

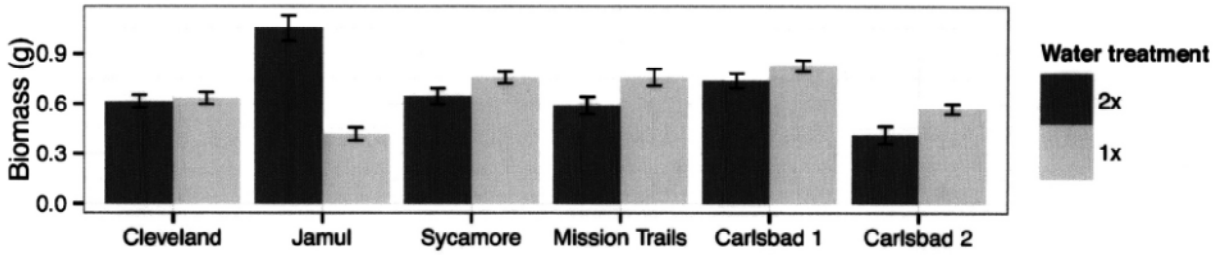

Figure 6. Plant height x population (per watering treatment)