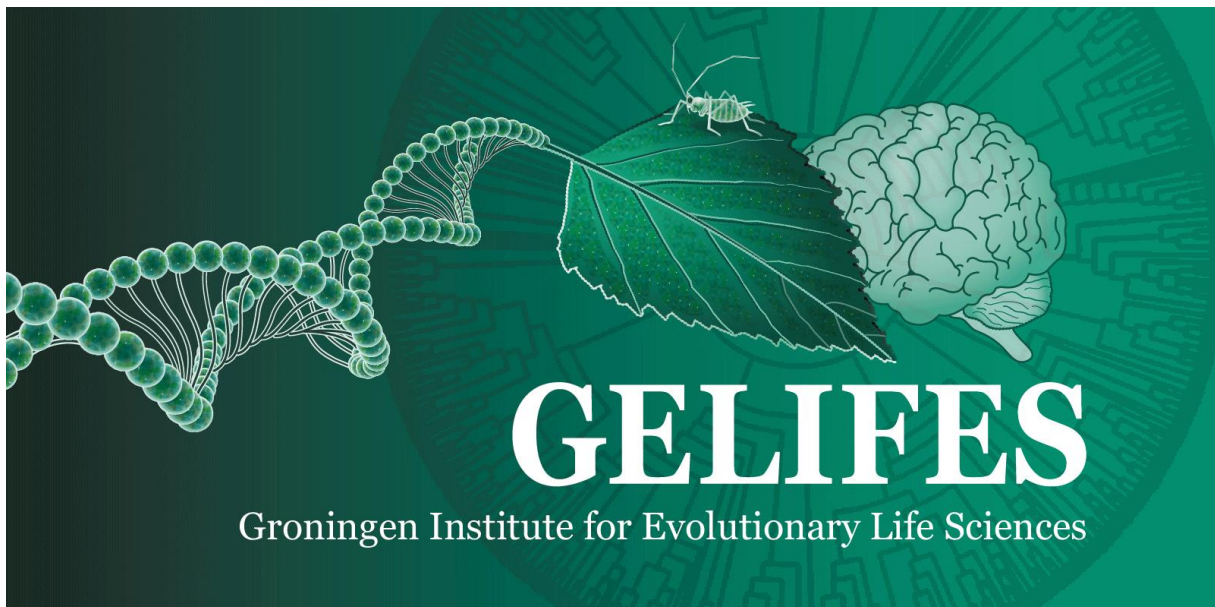


The effects of water availability on growth of *B.hybridum* facilitated and unfacilitated subpopulations

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Abstract

Climate change in the Mediterranean region has been projected to exceed global rates by 25%. Consequentially, droughts and flooding are expected to become more frequent in this area. Plant-plant interactions are known to be an important driver in plant population dynamics. Still, how plant-plant interactions drive plant population responses to environmental change is relatively unknown. In this research we hypothesize that facilitation (a positive plant-plant interaction) could be an important driver in response to environmental change. Facilitation is the interaction between two plants, in which one nurse plant can for example provide shade, increase water availability and reduce visibility to herbivores thusly impacting the local habitat for an associated benefactor plant. In this research we are using *B. hybridum*, which is an annual grass species that grows both facilitated (underneath a nurse shrub) and unfacilitated (outside a nurse shrub). Morphologically the facilitated subpopulation is different from the unfacilitated subpopulation. This difference in morphology may point toward a local adaptation of the facilitated subpopulation due to their differing microhabitat from their unfacilitated subpopulation. Given that this species grows in the Mediterranean and drought is projected to be occurring more often, we will investigate how this species physiologically reacts to water limitation during growth separated on their respective subpopulations. We used samples of the F2 generation of a wild population. This generation was put under several degrees of water stress ranging from low availability to high availability, and was separated based on subpopulation. Several leaf characteristics were measured during the experiment. While all parameters were significantly different due to watering treatment, most parameters did not show significant difference between the two subpopulations. Leaf water content however did show a significantly higher value for inside populations in comparison to outside populations. This may point to a differing adaptation regarding water storage for the facilitated population in comparison to the unfacilitated population. Further research is ongoing to test for differences in reproductive and physiological measurements, which may further point toward differing adaptations between unfacilitated and facilitated subpopulations.

Introduction

Climate change becomes an increasingly pressing problem for plant species in the Mediterranean area. Warming in the Mediterranean region is expected to exceed the global rate by 25%, heat waves will occur more frequently, intensity and frequency of drought will increase (Cramer et al., 2018). Water stress, in the form of either overabundance as well as lack of water, influences the plant's physiology, abundance and productivity (Manzaneda et al., 2015). Biotic interactions serve an important function in regulating community composition and ecosystems. Plant-plant interactions control the composition of the communities, by driving diversity both negatively (Grime, 1977) and positively (Callaway, 1992). But can also impact resource availability and habitat structure (Armas & Pugnaire, 2005; Brooker, 2006), through competition for resources (Craine & Dybzinski, 2013). Since the environment of the Mediterranean has been projected to undergo a relatively quick reduction in biodiversity and rainfall (Sala et al., 2000; Weiß et al., 2007), and the importance of the role of plant-plant interactions in controlling plant populations has been established, it is important to know how plant-plant interactions may drive plant population responses to environmental change.

Plant-plant interactions are often diffuse in the sense that there is not specific targeting of the effect of one species to another. These interactions can be both negative and positive. The

outcome of the interaction is dependent on many effects both biotic and abiotic that occur simultaneously, bidirectionally, directly between plants as well as indirectly via an organismic intermediary (Armas & Pugnaire, 2005; Brooker, 2006). One such negative interaction, competition, has been recognized as being one of the central drivers of the response of plant communities to environmental change (Connell & Slatyer, 1977). Competition has been broadly acknowledged as a key process in determining a plant community's ability to respond to environmental change drivers such as invasive species, climate change and nitrogen deposition (Brooker, 2006). While competition remains a focal point for plant ecology studies, facilitation, a positive plant-plant interaction is quite understudied. Interspecific facilitation is where a so called 'nurse plant positively affects spatially associated beneficiaries by ameliorating environmental conditions such as providing shade for the beneficiary plant (Segoli *et al.*, 2012), reducing visibility to herbivores (Smit *et al.*, 2007), affecting soil microbiota communities as well as increasing water availability (Brooker *et al.*, 2008; Michalet & Pugnaire, 2016). Several studies have shown that plants underneath nurse plants influence fitness related traits such as production of more flowers and earlier flowering (Halliday *et al.*, 1994), higher levels of fruit set and heavier seeds (Tirado & Pugnaire, 2003). As a landscape becomes more stressful, it is expected that facilitation plays an increasingly ecologically important role (Grime, 1977; Bertness & Callaway, 1994), as it has been found that microhabitat modification from the nurse plant can result in an expansion of the environmental niche of the beneficiary plant (Callaway, 1992; Maestre *et al.*, 2001). Despite the increasing knowledge of the importance of facilitation as a driver of plant community dynamics as well as its consequences for traits, we still know little of the evolutionary implications of facilitation.

Plants have evolved several strategies to maximize fitness in spatially and temporally variable environments. Adaptive plastic responses, where a genotype is able to produce a phenotype to respond to an environmental stressor, is one such strategy. Essentially, this means that on a short term an organism has a high phenotypic plasticity, i.e. the ability to physiologically respond to different stressors in the environment. The plasticity can be quick and reversible within one generation, which increases the likelihood that parents pass down traits which may increase fitness for a future generation (Geber & Griffen, 2003). An important factor of adaptive capacity is its ability to access greater genetic variability. As aforementioned, genetic diversity is essential for plant populations to adapt to changing environmental conditions (Hughes *et al.*, 2008). How facilitation impacts genetic diversity, and thus the adaptive capacity is thus far unclear.

In arid ecosystems, facilitated plant populations are generally buffered from high temperatures and low water availability thus may have a decreased selection pressure when compared to unfacilitated plant populations from the same species. As the unfacilitated populations are specialized to growing in open patches and more highly adapted to the harsh dry landscape. Difference in fitness between these populations is then determined by the underlying cost of their respective adaptation. The facilitated (generalized) populations are expected to harbour the ability to respond positively to a wider array of stressors than specialized (unfacilitated) populations, resulting in a wider niche (Van Valen, 1965). The trade-off of the adaptation to a more stressful environment (unfacilitated) can result in genes which are favourable in one environment, but not in another. The stress-resistant (unfacilitated) plants have a more narrow ecological tolerance and may exhibit a reduced adaptive capacity to new stressors due to this trade-off. The facilitated population, being an ecological generalist population, could respond more favourably to a wider range of environmental conditions (Kassen, 2002).

In this study we will look at *Brachypodium hybridum* (Catalán *et al.*, 2012), a species of the *Brachypodium* genus. This annual grass grows in the semi-arid Mediterranean climate and is known to grow both in unfacilitated (open areas) environments as well as facilitated (under shrub, *Retama*, canopy) environments (Armas & Pugnaire, 2005). *Brachypodium hybridum* individuals that grow under shrub canopies show larger growth, produce more spikelets and more and heavier seeds as opposed to non-facilitated individuals from the same population (Korte *et al.*, 2022 *under review*). Furthermore, *B. hybridum* is primarily selfing with low dispersal. Given that this species grows in the Mediterranean and drought is projected to be occurring more often, we will investigate how this

species physiologically reacts to water limitation during growth separated on the fact whether the plant originates from a facilitated or unfacilitated population.

Water limitation, in all environments, is usually the main limit to plant productivity and survival (Boyer, 1982). In semi-arid areas such as the Mediterranean drought tolerant plants will maximize several physiological parameters in response to water restriction (Toscano & Ferrante, 2019). Water Use Efficiency (WUE), i.e. the physiological process that balances the trade-off between carbon assimilation and resulting loss of water, is an important physiological process (Dudley, 1996; Edwards *et al.*, 2012). For example, drought can promote the formation of smaller leaves, decrease stomatal conductance to water vapor thus increasing WUE. WUE reduces as water availability increases, and reduced WUE causes an increase in leaf area, photosynthetic activity and higher stomatal conductance (Rodrigues *et al.*, 1995; Chaves *et al.*, 2002;).

In our study we measured whether leaf physiological parameters of 30 genotypes from the F2 generation of facilitated and unfacilitated subpopulations of *B. hybridum* responded differently to 5 water treatments. We expected that the two groups regardless of heritage will show lower photosynthetic activity, specific leaf area, leaf water content and stomatal conductance as water availability decreases, WUE however is expected to increase. Between the two subpopulations we expected that the facilitated subpopulation would respond more positively to a wider range of water quantity. This is under the assumption that 1) the traits from the wild populations are heritable and 2) the facilitated subpopulation has undergone a more generalized selection in comparison to the unfacilitated subpopulation.

Material and Methods

Plant material and experimental setup

We collected mature *B. hybridum* plants in the spring of 2018 and 2019, in southern Spain near the town of Sorbas (lat; 37° 05' 51.40" N, long; -2° 07' 24.56" W). Seeds from these plants were used to produce an F1 generation in the greenhouse, summer of 2020, at the University of Groningen, in the north of the Netherlands. In the spring of 2021, we collected the seeds from this F1 generation from 30 plants (genotypes) of *B. hybridum*. The genotypes were separated into two groups of 15 based on whether the original plant originated from either a facilitated (F) subpopulation or an unfacilitated (NF) subpopulation. Facilitated in this case is, growing under nurse shrubs while unfacilitated is growing outside the canopy in open patches. The experiment has been staged across three time blocks, with 4 days between each group, to make the handling of plants when gathering data on the same day feasible. The collected seeds were cleaned and were placed in petri dishes on a damp filter. Then, they were moved to a refrigerator where they resided for 72 hours at 4.5°C. Thereafter, they were moved out of the refrigerator and placed in the greenhouse where they stayed uncovered for 5 days after which they were sown in pots. The plants were grown in autoclaved soil, containing a mixture of organic substrate, sand and perlite (2:1:1, v/v). For the 1st group (30-3-2021) all pots contained 1 seed, for the 2nd (2-4-2021) and 3rd (6-4-2021) groups 2 seeds from the same plant per pot were sown to control for germination time. Each group consisted of 5 treatments separated by percentage of maximum soil water content 100%, 80%, 60, 40% and 20%. To assess the maximum soil water content for these pots, we let pots with a similar weight to the ones used in the experiment saturate overnight using bottom watering. From this it was obtained that the maximum water content (MWC) of each pot was about ~100 ml (MWC-DW). Thus, for each of the 5 treatments we took consecutive percentages of the MWC

Within each treatment we placed 5 different F-genotypes and 5 different UF-genotypes, each with an additional 3 replicates across the 3 groups. We used 600 seeds in total. (20 plants per subpopulation (F & UF) per treatment 5 treatments per group (20*2*5*3)). The filled pots were

weighed for their dry weight (DW) as to fill each pot with similar amounts of soil. For reference of the experimental design see supplementary (Suppl, Fig. 1).

Initially for the first 7 days each group was treated similarly. Each group was bottom watered to 100% FC every other day. We assessed the amount of water necessary by randomly picking 3 pots from their respective treatment, assessed their WC and subtracting that from their MWC. As each treatment contained 40 pots, we multiplied the average differences between MWC and WC with 40. The watering treatments started for each group after the majority of seedlings in a plot had reached a height of at least 2.5 cm. For group 1 the treatment started 9 days after the seeds had been planted, and for groups 2 and 3, 11 days after planting. Every other day we bottom watered each plot to their respective percentage of MWC. After 26 days and 56 days each plant received 50 mL of fertilizer solution (1/2 Hoagland's solution) (0.5M KNO₃, 0.5M Ca(NO₃)₂, 0.1M KH₂PO₄, 0.2M MgCl₂, 0.25 ml/L micronutrient and 0.25 ml/L FE³⁺EDTA). For the 2nd and 3rd groups we took the smallest plant from the duplicates, and transplanted them to another pot within the same treatment to replace plants that failed to germinate. The greenhouse had an average temperature of ~25 °C and a relative humidity of ~40%. However, during the experiment we measured that the temperature had risen to peaks of 57 °C. On 10 separate days the temperature had risen to above 50 °C. While not physically detrimental to the health of the plants, we decided on day 44 of this experiment to move our samples to another greenhouse due to the possible stress of this extreme temperature on our samples. Since every plant had undergone these circumstances, we still believe the comparison between different treatments to be valid. As such we will not deal with the influence of these extreme temperatures on our samples in this study.

Physiological measurements

Leaf characteristics

Photosynthetic activity (Pn), stomatal conductance (C), and vapor pressure deficit (Vpd) of the leaf were all measured using a portable photosynthesis measurement system (CI-340; CID, Inc., Camas, WA, USA). Furthermore, intrinsic water use efficiency (iWUE) was calculated as a ratio of photosynthetic activity to stomatal conductance. Measurements started for each treatment 44 days after the first harvest had been planted. The measurements lasted for 2 weeks. To ensure that variability between measurements due to effects of temperature and light availability was controlled, all measurements were done between 12 and 2pm. The measurements were done 3 times in a ten minute time span for one genotype, resulting in an average Pn, C, E and Vpd for each single genotype. As the rectangular shaped leaf of *B. hybridum* did not fully cover the area of the chamber (6.25 cm²) with which we measured the leaf characteristics, we had to estimate the proportion of the covered leaf area using ImageJ (Schneider et al., 2012). This was done by taking a picture with a smartphone of the leaves in the leaf chamber; afterwards the proportion of leaf area to chamber area was calculated. Multiplying the proportion with the gathered data from the photosynthetic system provided the actual result of the leaf characteristics. Specific leaf area (SLA) was calculated at 2 months. To assess the SLA we took the third leaf from the main stem of each genotype (2 replicates). Wet weight (WW) was assessed using a 5 decimal scale (Sartorius ME235S Genius). The leaf area (A) was estimated using ImageJ. We scanned in the leaves with a 1 cm² square as reference area. Dry weight (DW) of the leaves was assessed using the same scale as the wet weight measurements; this was done after the leaves had been left in a stove for approximately 65 hours at 60 °C. SLA was then calculated by dividing the area over dry weight (A/DW). Additionally the

leaf water content (LWC) was determined (WW-DW).

After approximately 50 days from the initial sowing of the seeds, leaves started to senesce. We rated the leaf wilting based on a scale of zero (no observable wilting) to three (more than half of leaves on the plant have wilted). This was done for each sample (every genotype, 4n).

Data Analysis

The analysis was done in Rstudio v 4.0.0. (Rstudio, 2019). Before the statistical analysis we transformed all data to account for normality. Using a Shapiro test we found that Vpd, SLA and LWC did not show a normal distribution. However, the residuals for these parameters did look normal. Lastly, we accounted for homogeneity of variances using Barlett's test.

Leaf characteristics

The leaf characteristics contained only samples from watering-treatments 60% to 100%, we have no samples for 20% and 40% as the leaves were too small for the photosynthesis measurement system. We tested whether water treatment, time-group and inside or outside subpopulation resulted in differences in iWUE, Pn, and C by using a one-way ANOVA from the *rstatix* (Kassambara, 2021) package. For Vpd we used a non-parametric Kruskal-Wallis ANOVA, as its data did not show normality for the Shapiro test. Post-hoc analysis was done for the results of the ANOVA test using a pairwise comparison.

To test whether water treatment and subpopulation predicted iWUE, Pn, C and Vpd, we used linear mixed models from the *lme4* (Bates *et al*, 2015) package. The initial model we used was $\text{lme}(\text{response variable} \sim \text{Treatment} + \text{I.O} + \text{Treatment} * \text{I.O} + (1 | \text{Group}))$. Response variable is defined as one of the logistically transformed aforementioned parameters (iWUE, Pn, C and Vpd), our fixed effects treatment and I.O are defined as the watering treatment and subpopulation respectively, and time-group was utilized as a random effect. To test for the significance of the fixed effects, we used backwards elimination of the model reducing it to only the significant fixed effects. Accordingly, the effect size and p-values of the reduced models are mentioned in our paper. Overall, for the leaf characteristics we had 89 samples. That is one sample of the 100%, 80% and 60% treatments for each genotype minus one outlier. There are no replicates for the genotypes within a treatment.

SLA and LWC

We tested whether water treatment, time-group and inside and outside subpopulations resulted in differences in SLA and LWC by using a non-parametric Kruskal-Wallis ANOVA test. Post-hoc analysis was done for the results of the ANOVA test using a pairwise comparison.

To test whether water treatment and subpopulation predicted SLA and LWC we used linear mixed models from the *lme4* (Bates *et al*, 2015) package. The initial model we used for SLA and LWC was $\text{lme}(\text{response variable} \sim \text{Treatment} + \text{I.O} + \text{Treatment} * \text{I.O} + (1 | \text{Group}))$. Beforehand we compared this model with a model which included genotype as a random factor next to group, however this proved to be a lesser fit for our data, thus we went with the aforementioned model. The initial model we used for LWC was $\text{lme}(\text{response variable} \sim \text{Treatment} + \text{I.O} + \text{Treatment} * \text{I.O} + (1 | \text{Group}))$, as it was a better fit than the full model with genotype included as a random effect. To test for the significance of the fixed effects, we used backwards elimination of the model reducing it to only the significant fixed effects. Accordingly, the effect size and p-values of the reduced models are mentioned in our paper. In total 295 samples were analysed. That is, 2 samples for all 5 watering-

treatments, and for each genotype 2 replicates. The genotypes that did not have a replicate were excluded from analysis.

Results

Overall the experiment went well. However, time of growth until flowering exceeded our expectation. During the experiment we found that growth correlated with watering treatment, as the plants receiving the most water grew largest, and plants receiving the least grew smallest. Coinciding, the plants receiving the least water had a lower survival rate until flowering than the plants receiving more water. Data for growth and survival rate will not be discussed however. Due to the smaller leaves in treatments 20 and 40, we did not measure their leaf characteristics. In contrast to the leaf characteristics, we were able to measure SLA and LWC for all watering treatments. Lastly, we find that the interaction term Treatment*I.O shows significance with all leaf characteristics, SLA and LWC. However, these differences are mainly attributed to differences between I and O between different treatments, not within the same treatments. Thus they were omitted from visualization, as the data does not tell anything of significance.

Leaf characteristics

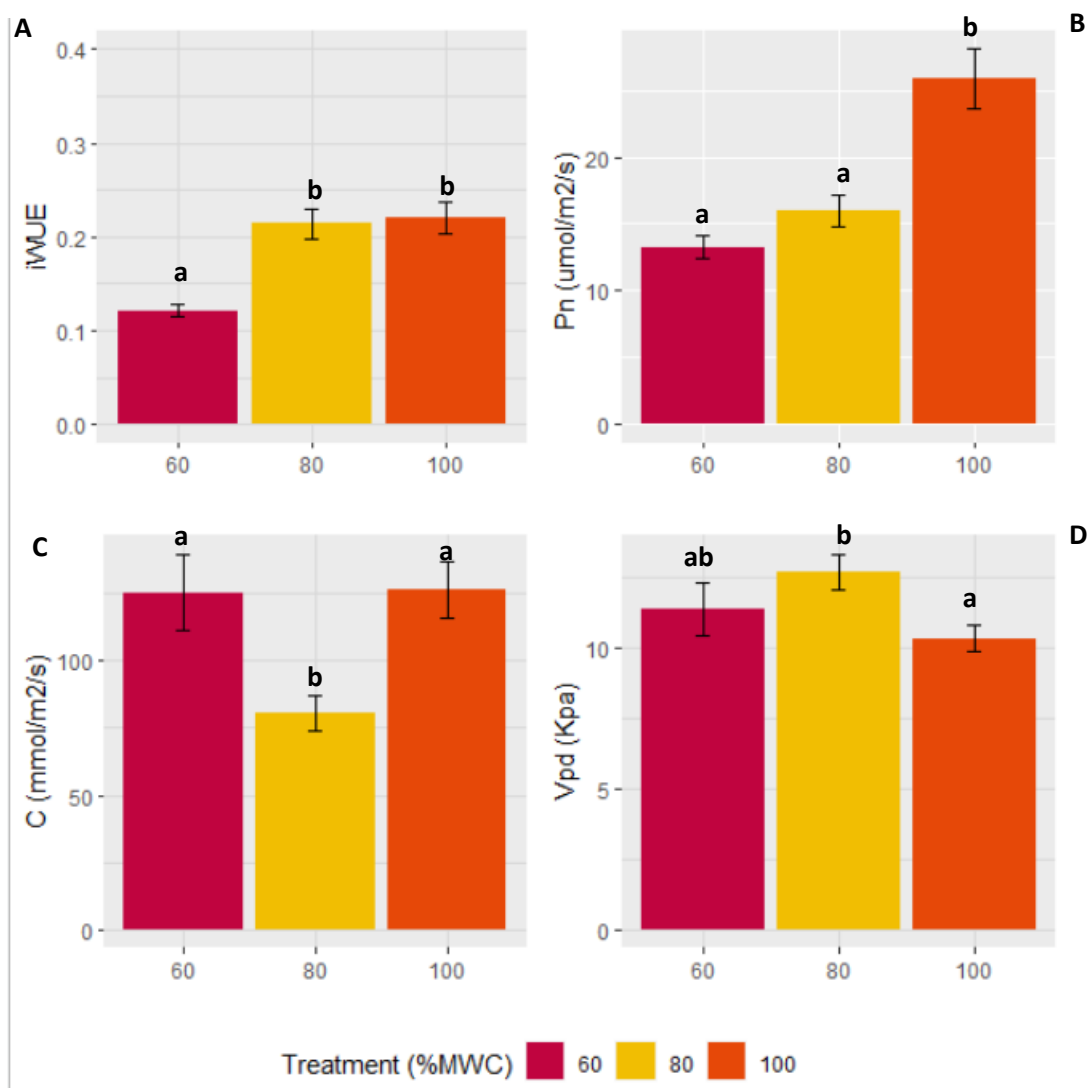


Figure 1. Barcharts for our physiological measurements. a) intrinsic water use efficiency(iWUE),b)photosynthetic activity(Pn), c) stomatal conductance(C) and d) vapour pressure deficit(Vpd). Coloring is based on treatment. Treatments that show similar values are assigned the same letter, treatments that are significantly different are assigned a different letter.

iWUE

We found that watering treatment affected water use efficiency (iWUE) significantly. However, watering treatment did not affect iWUE between subpopulations. For iWUE the differences between treatments significant ($p < 0.0001$, table 1), but differences between I (facilitated) and O (unfacilitated) were not ($p = 0.1814$, table 1). Post-hoc pairwise comparisons showed that iWUE in treatment 60% FC, was significantly lower than both treatment 80% & 100% (Fig. 1, a) ($p < 0.0001$). Furthermore, based on lmm modelling we found that watering treatment predicts iWUE (Table 2). Overall we find that iWUE is increasing with water availability (Fig. 2a).

Photosynthetic activity

Similar to iWUE, photosynthetic activity (Pn) was significantly different between watering treatment ($p < 0.0001$, table 1), but not between subpopulations (p . Pn was significantly lower in the 60% and 80% treatment compared to the 100% treatment (Fig. 1b). Lmm modelling showed that watering treatment predicts Pn as well. Lmm was controlled for a random intercept as well as for group. Pn showed a similar trend as water use efficiency, both increased with water availability (Fig. 2b).

Stomatal conductance

For stomatal conductance (C) we found that watering treatment caused significant difference ($p = 0.00196$, table 1), while subpopulation did not have a significant effect (0.2017, table 1). Post-hoc analysis showed that treatment 80% is significantly lower than 60% and 100% (Fig.1c). We see that stomatal conductance seems to dip between treatment 60% and 80% but increases in the 100 % FC treatment. Lmm modelling did not show any prediction for C in regards to watering treatment or subpopulation.

Vapour pressure deficit

Significant differences in vapour pressure deficit were found based on watering treatment ($p = 0.02686$, table 1), but no differences were found based on subpopulation although it was nearing significance ($p = 0.0505$, table 1). Treatment 80% showed a significantly higher vpd than treatment 100% (Fig 1d). Lmm modelling showed that watering treatment did not predict vpd. It did however show that subpopulation (I.O) predicted vpd (table 2). Overall it seems that Vpd is pretty level between treatments, but treatment 80% seems to have a slightly larger Vpd compared to treatment 100%.

Table 1 ANOVA results for all predictors. Predictors were analyzed separately rather than in one model. In some cases a one-way ANOVA was used due to a lack of homogeneity in variances. This is the case for; SLA & LWC Note here that treatment is handled as a factor rather than a continuous variable. Data was logistically transformed before analysis. Significant p values are indicated with a *

Log(Factor)	Predictors final model	df	F	p
Pn	<i>Treatment</i>	2	15.65	1.57·10 ^{-6*}
	<i>I.O</i>	1	1.814	0.1814
	<i>Treatment*I.O</i>	5	7.483	7.545·10 ^{-6*}
C	<i>Treatment</i>	2	6.701	0.00196*
	<i>I.O</i>	1	1.655	0.2017
	<i>Treatment*I.O</i>	5	3.374	0.007948*
Vpd	<i>Treatment</i>	2	3.772	0.02686*
	<i>I.O</i>	1	3.933	0.0505
	<i>Treatment*I.O</i>	5	2.524	0.0353*
iWUE	<i>Treatment</i>	2	20.563	4.684·10 ⁻⁸
	<i>I.O</i>	1	5·10 ⁻⁴	0.982
	<i>Treatment*I.O</i>	5	8.0119	3.225·10 ^{-6*}
SLA	<i>Treatment</i>	4	13.04	4.34·10 ^{-9*}
	<i>I.O</i>	1	3.05	0.0818
	<i>Treatment*I.O</i>	9	7.163	3.308·10 ^{-8*}
LWC	<i>Treatment</i>	4	64.51	2.2·10 ^{-16*}
	<i>I.O</i>	1	11.904	0.00064*
	<i>Treatment*I.O</i>	9	33.693	2.2·10 ^{-16*}

Table 2 Lmm results for leaf characteristics. Displayed are the final model, the AIC and the F- and p-values of the final model. Parameters without a significant fixed effect are excluded from the table

Factor	Final model	AIC	Fixed effects final model	F	p
Pn	Lme(Log_APN ~ Treatment + (1 Group))	141.53	<i>Treatment</i>	29.17	5.573·10 ⁻⁷ *
Vpd	Lme(Log_Avpd ~ I.O + (1 Group))	69.838	<i>I.O</i>	5.13	0.02606*
iWUE	Lme(Log_iWUE ~ Treatment + (1 Group))	122.87	<i>Treatment</i>	32.97	1.38·10 ⁻⁷ *
SLA	Lme(Log_SLA ~ Treatment + (1 Group))	-79.883	<i>Treatment</i>	22.98	2.728·10 ⁻⁶ *
LWC	Lme(Log_LWC ~ Treatment + I.O (1 Group))	151.53	<i>Treatment</i> <i>I.O</i>	207.85 8.53	2.2·10 ⁻¹⁶ * 0.006813*

A

B

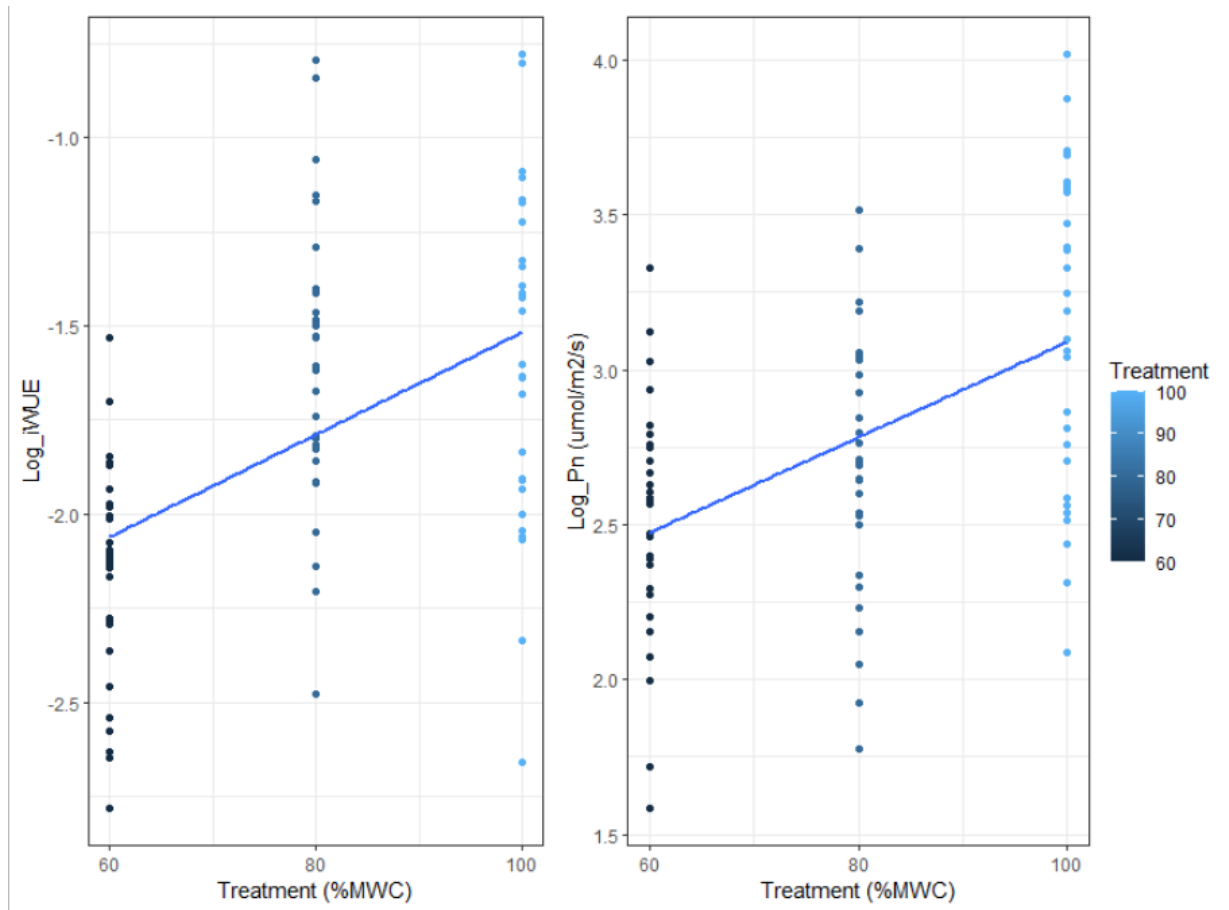


Figure 2. Linear mixed models for both the logistically transformed a) *iWUE* and b) *Pn*. Along the x-axis the treatment is displayed.

Specific Leaf Area & LWC

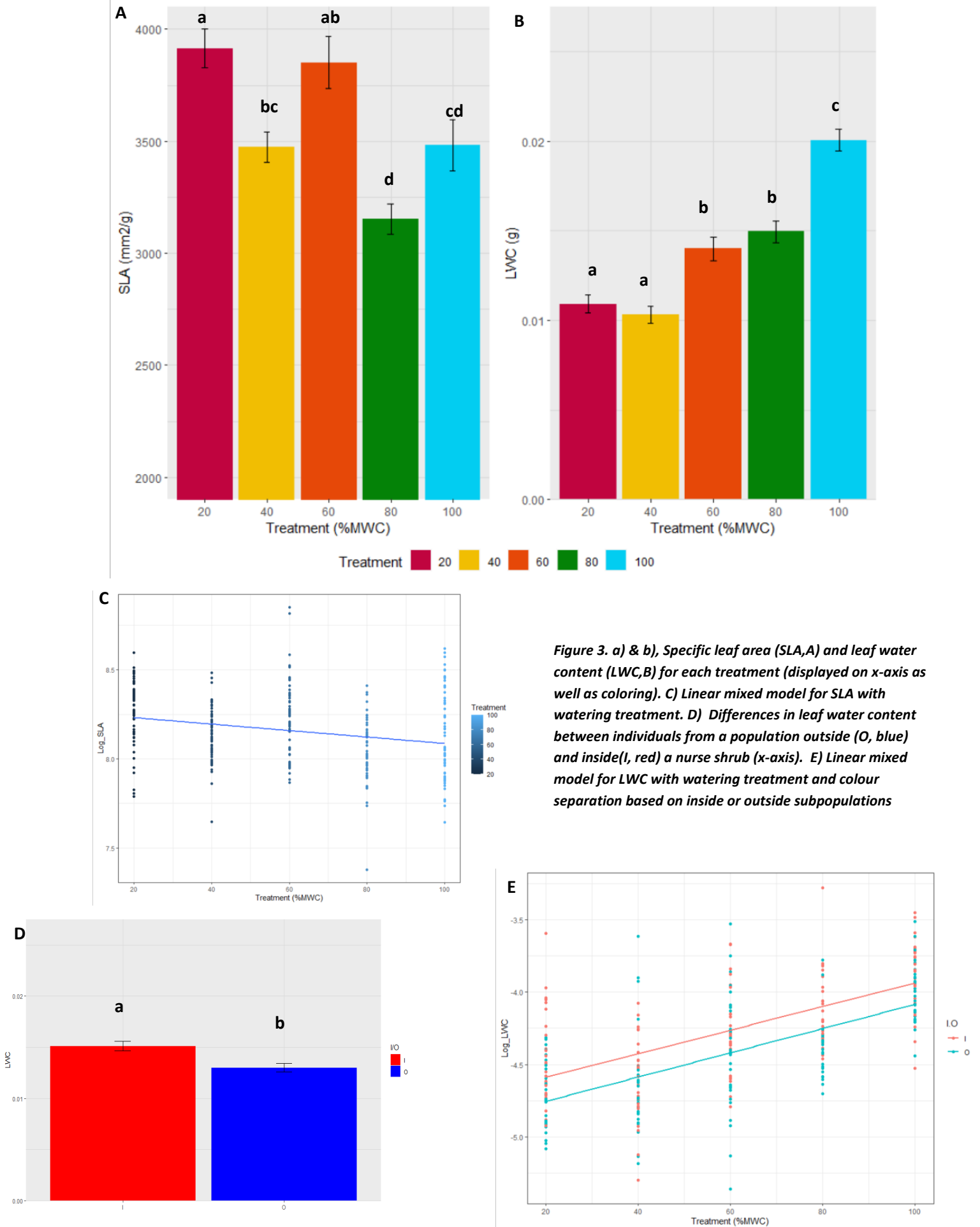


Figure 3. a) & b), Specific leaf area (SLA,A) and leaf water content (LWC,B) for each treatment (displayed on x-axis as well as coloring). C) Linear mixed model for SLA with watering treatment. D) Differences in leaf water content between individuals from a population outside (O, blue) and inside (I, red) a nurse shrub (x-axis). E) Linear mixed model for LWC with watering treatment and colour separation based on inside or outside subpopulations

SLA

Specific leaf area (SLA) shows significant differences between watering treatments, but not by subpopulation. Treatment 20% has a significantly higher SLA than treatment 40% ($p = 0.014$), 80% ($p < 0.0001$), and 100% ($p = 0.003$). Treatment 40% has a higher SLA than treatment 80% ($p = 0.045$). Treatment 60% has a significantly higher SLA than treatment 80 ($p < 0.0001$) and treatment 100 ($p = 0.025$) (Fig. 3a). Overall, as the watering treatment increases, the SLA decreases. This is also confirmed by the linear mixed model. As watering treatment increases, our model predicts a decrease in SLA (Table 2; Fig 3c)

LWC

Leaf water content (LWC) was significantly different between watering treatments ($p < 0.0001$, table 1), as well as between subpopulations ($p < 0.001$, table 1). Pairwise comparisons show that treatments 60%, 80% and 100% have a significantly higher LWC than treatments 20% (60; $p = 0.0003$, 80 & 100; $p < 0.001$) and 40% (60, 80, 100; $p < 0.0001$). Furthermore, treatment 100% is higher than treatment 60% and 80% ($p < 0.0001$). Over all treatments, plants that stemmed from inside a nurse shrub have a significantly higher LWC than plants that stemmed from outside a nurse shrub (Fig. 3d). Lastly, our linear mixed models shows that both watering treatment as well as subpopulation predict that LWC increases with watering treatment and is generally higher for inside populations in comparison to outside populations (Fig. 3e).

Discussion

The main goal of this study was to find physiological differences in adaptation to water limitation between *B.hybridum* individuals which stemmed from either facilitated (inside) or non-facilitated (outside) subpopulations. We did this by subjecting the two populations to differing amounts of soil moisture over time. We found that differences in adaptation between individuals which stemmed from facilitated and non-facilitated subpopulations were minimal, as they mostly showed similar trends.

We did find however that for leaf water content (LWC), differences between facilitated and unfacilitated subpopulations were significant. Regardless of treatment, leaf water content is higher in plants from facilitated subpopulations than non-facilitated subpopulations (Fig. 3,c). This is quite unexpected. Plants that are ancestors of populations growing outside nurse shrubs, were expected to be more adapted to dry environments. Hence, we'd expect that these plants would store larger quantities of water, as plants from places undergoing increased drought generally possess the capacity to store more water (Grime, 1977; Wang *et al.*, 2021). However, the inside (facilitated) population had a larger leaf area than the outside population (Suppl, Fig. 3), thus harbouring more space to store the water. Perhaps this indicates that inside populations had a reduced response to drought stress. As in general an increased drought should decrease leaf area (Banakar & Soltani, 2011). Furthermore, although not yet investigated, the plants that descended from facilitated populations may be larger than the ones that descended from an unfacilitated subpopulation (Korte *et al*, 2022 under review). Still, as we see the difference in LWC between the F2 generation of facilitated and unfacilitated subpopulations, it may point toward an inheritance of the trait from the original population.

Intrinsic water use efficiency (iWUE), stomatal conductance(C), photosynthetic activity (Pn) and vapour pressure deficit (Vpd) were all unaffected by whether the plant originated from a

facilitated or unfacilitated subpopulation. However, all of them were significantly affected by watering treatment. Surprisingly, for iWUE we find that as the water availability decreases, the iWUE decreases as well. This is surprising as in general iWUE increases as the water deficit increases (Des Marais *et al.*, 2017; Manzaneda *et al.*, 2015; Monclus *et al.*, 2005). The iWUE is calculated as P_n over C (P_n/C). As C is high in treatment 60 but P_n is low, we find a low iWUE. A low P_n in lower water availability is not out of the ordinary for grasses of the *Brachypodium* family. *B. hybridum*'s sister species *B. distachyon* has been shown to have reduced chlorophyll fluorescence under dry circumstances (Luo *et al.*, 2011), indicating reduced photosynthetic efficiency (Marais & Juenger, 2015). Other woody and herbaceous species from the Mediterranean show similar trends (Chaves *et al.*, 2002). A relatively high stomatal conductance (C) in treatment 60 is unexpected. In general C decrease as water availability decreases (Laffray & Louguet, 2014). This is caused by an increased production of the metabolite Abscisic acid (ABA) (Anosheh *et al.*, 2016, Chapter 3). *B. hybridum* and its sister species *B. stacei* have been observed to significantly increase ABA production in drought conditions (Martínez *et al.*, 2018). In our experiment we did not measure the ABA contents at the time of measuring the stomatal conductance, although this may have provided insight into our findings of high C. Besides the production of ABA, stomatal conductance responds to changing irradiance. As irradiance increases, stomatal conductance increases (Davey *et al.*, 2016). While we changed the position of our plots at random during the experiment, it could be that at the time we measured stomatal conductance our 60 treatment was in a place that was relatively sunny. Still, the cause for a high stomatal conductance, and consequentially a low iWUE, under our moderately dry circumstances remains unclear, for example; the inconsistency of the measurements taken by the photosynthesis measurement system may have also caused the relatively high stomatal conductance.

Another relatively unexpected finding is that specific leaf area (SLA) seems to decrease as water availability increases. It is common for grasses in the Mediterranean systems to show a decreased SLA as drought occurs (Wellstein *et al.*, 2016). Coincidentally, we would expect something similar in our experiment. The SLA depends on the ratio between the dry weight of the leaf and leaf area (leaf area/ dry weight). Dry weight seems to increase relatively more in comparison to leaf area as water availability increases, possibly explaining why we find that SLA is lower in higher water availability. Although it is still up to investigation, this may point toward a strategy in which an increase in leaf thickness is preferred over leaf area as water availability increases. Also, a high SLA value is usually associated with a high potential in carbon acquisition (Díaz *et al.*, 2004), with consequentially a low drought survival time (Lopez Iglesias *et al.*, 2014). Perhaps the relatively high SLA points toward a drought avoidant strategy for the plant with lower water availability. In which rapid growth and flowering is favoured over loss of water. Flowering data and survival data is still lacking however.

Concluding, the initial aim of this research was to find differences in physiological adaptations to water stress between descendants of a facilitated subpopulation and unfacilitated subpopulation of *B. hybridum*. This research was done in the light of climate change in the Mediterranean area, where drought is expected to increase over the coming years. We wanted to know how plant communities in this area will respond to these changes, thus it is important to know how potentially differentiated subpopulations within the same species would react to differing water availabilities. Mainly, we found that water treatment influences all physiological parameters significantly, albeit in some unexpected ways. Furthermore, the differences in LWC between the two subpopulations seem to point toward different adaptations regarding water storage between

subpopulations. From this we could state that there were few differences between facilitated and unfacilitated subpopulations in physiological adaptation, at least from the parameters that we measured. However, preliminary results of stem length and leaf count point toward differences in adaptation between the two subpopulations. Including these results with further physiological measurements on biomass and reproductive measurements on flowering and seed count may tell us more on how the different subpopulations adapt to differing water availabilities.

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SUPPLEMENTARY

I/O-Treatment	Leaf characteristic	Mean	±SE
O-60%	<i>Pn</i>	14.31	1.14
	<i>C</i>	141.97	23.67
	<i>Vpd</i>	12.38	1.67
	<i>iWUE</i>	0.12	0.011
O-80%	<i>Pn</i>	14.92	1.17
	<i>C</i>	76.48	6.67
	<i>Vpd</i>	13.04	0.96
	<i>iWUE</i>	0.21	0.020
O-100%	<i>Pn</i>	29.16	3.20
	<i>C</i>	143.94	17.34
	<i>Vpd</i>	11.33	0.78
	<i>iWUE</i>	0.23	0.026
I-60%	<i>Pn</i>	12.23	1.40
	<i>C</i>	108.65	15.25
	<i>Vpd</i>	10.41	0.92
	<i>iWUE</i>	0.12	0.0059
I-80%	<i>Pn</i>	17.10	2.09
	<i>C</i>	84.60	10.70
	<i>Vpd</i>	12.38	0.81
	<i>iWUE</i>	0.22	0.025
I-100%	<i>Pn</i>	22.50	2.96
	<i>C</i>	108.63	10.81
	<i>Vpd</i>	9.36	0.38
	<i>iWUE</i>	0.21	0.024

Table 1. Analysis of the leaf characteristics per treatment and associated I/O factor. The table showcases the mean and associated standard error per leaf characteristic.

I/O - Treatment	Leaf characteristic	Mean	±SE
O-20%	<i>SLA</i>	3898.27	120.18
	<i>LWC</i>	0.0095	0.00044
O-40%	<i>SLA</i>	3535.64	107.09
	<i>LWC</i>	0.013	0.0028
O-60%	<i>SLA</i>	3879.66	119.96
	<i>LWC</i>	0.013	0.00098
O-80%	<i>SLA</i>	3203.02	120.29
	<i>LWC</i>	0.013	0.00058
O-100%	<i>SLA</i>	3688.30	146.35
	<i>LWC</i>	0.019	0.00073
I-20%	<i>SLA</i>	3828.37	175.23
	<i>LWC</i>	0.012	0.00078
I-40%	<i>SLA</i>	3409.24	81.14
	<i>LWC</i>	0.010	0.00051
I-60%	<i>SLA</i>	3838.38	209.15
	<i>LWC</i>	0.015	0.00085
I-80%	<i>SLA</i>	3091.09	78.07
	<i>LWC</i>	0.017	0.00098
I-100%	<i>SLA</i>	3283.01	172.14
	<i>LWC</i>	0.021	0.00088

Table 2 Analysis of SLA (mm²/g) and LWC(g) per treatment and associated I/O factor. The table showcases mean and standard error of both SLA and LWC..

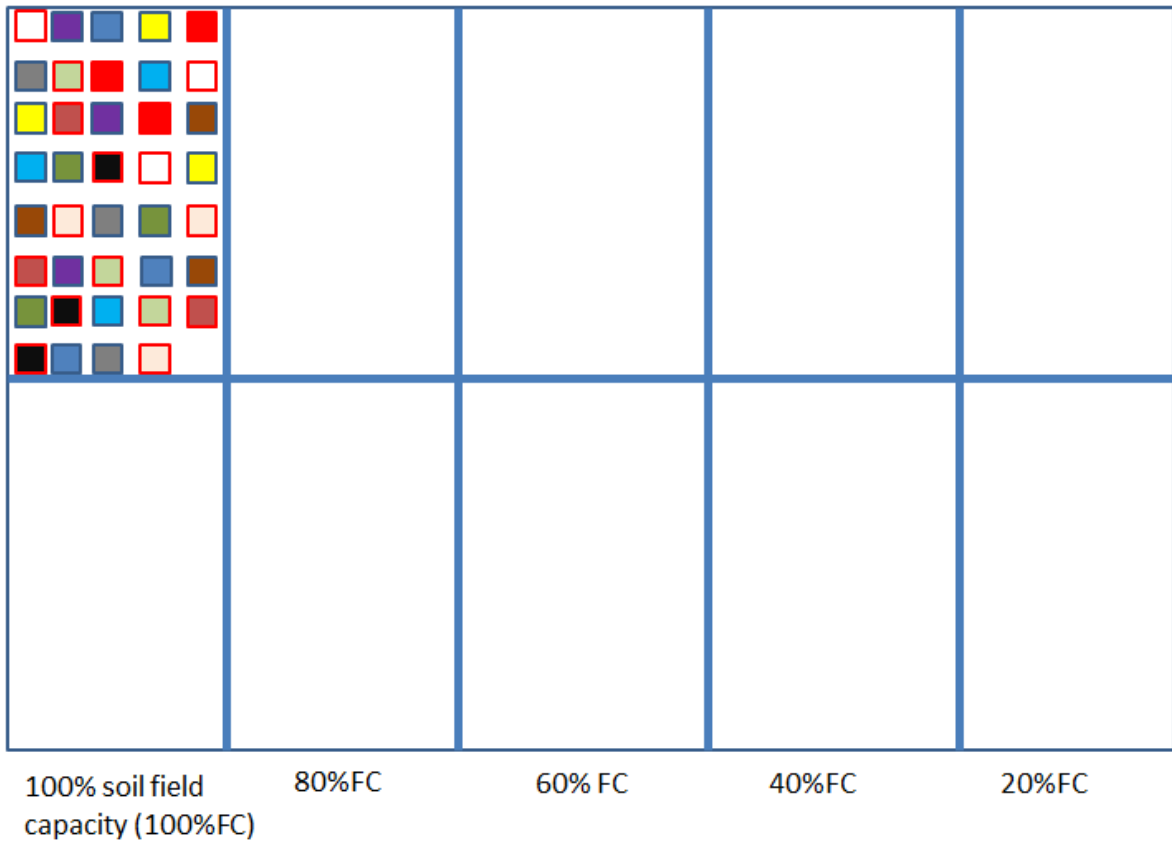


Fig 1. Sketch of the experimental design. Only 1 block is filled in. Essentially, we replicated the filled block for all other blocks as well.

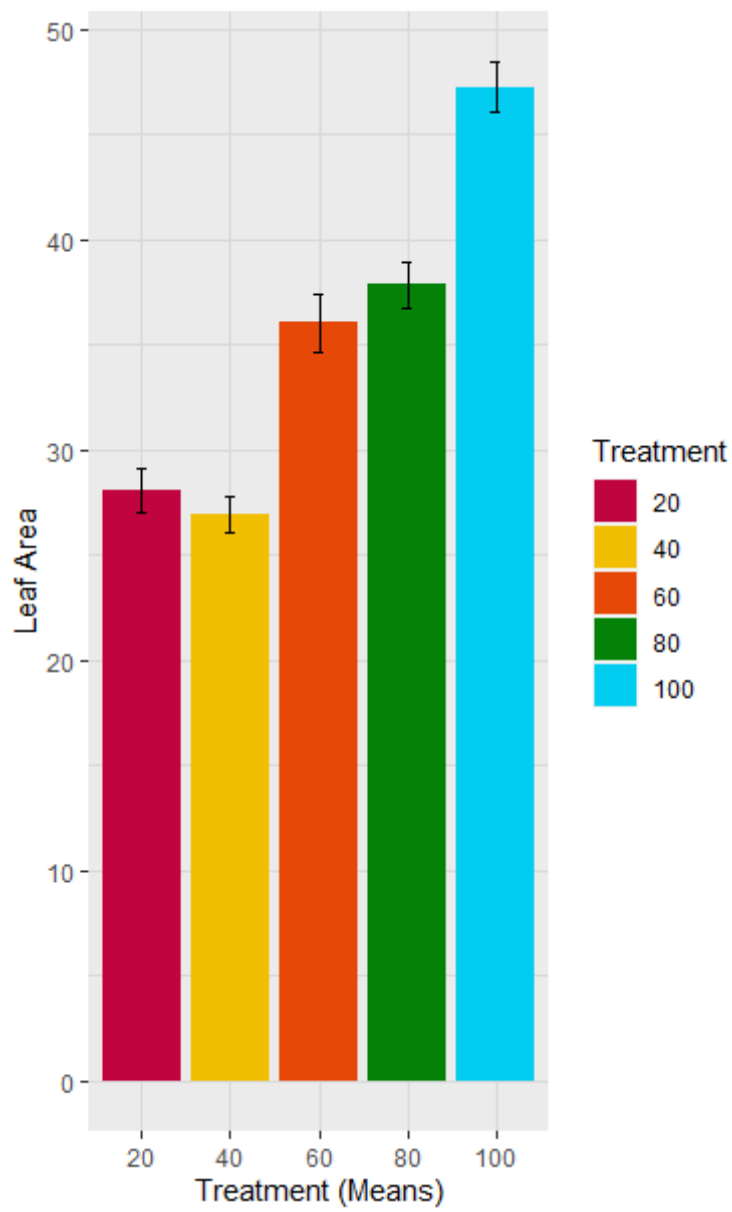
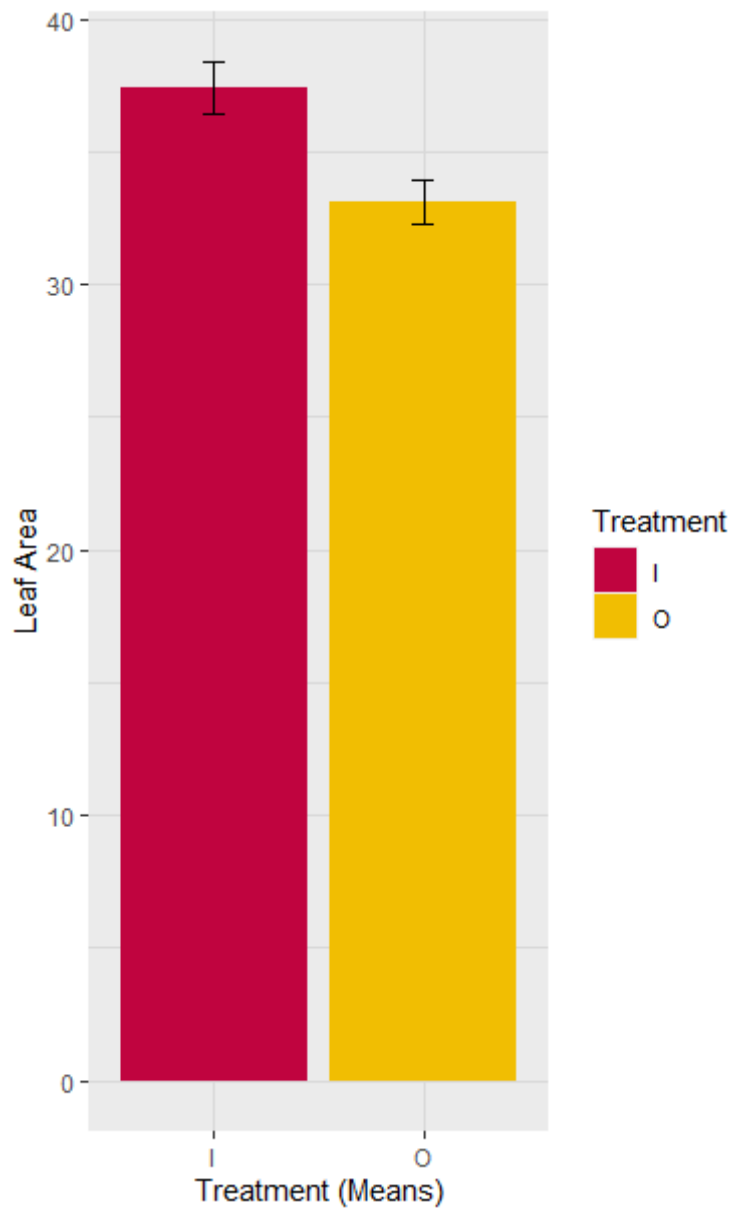
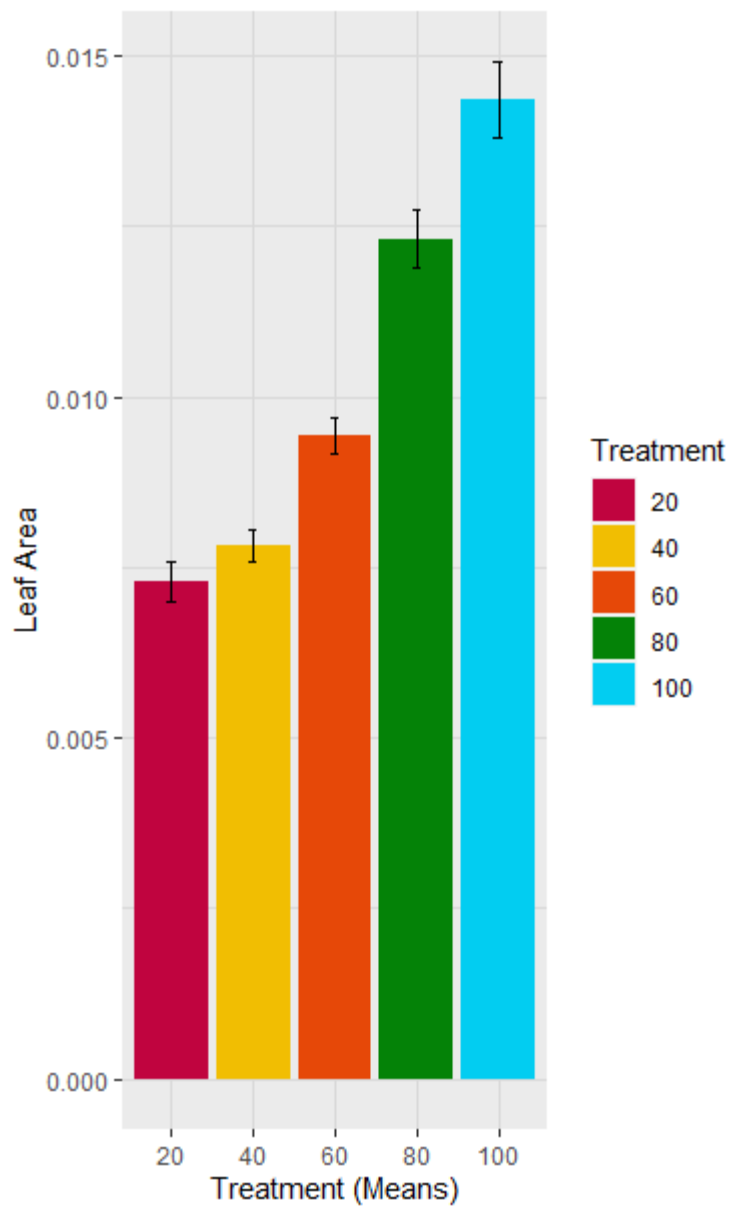


Fig 2. Leaf area for all treatments



Suppl Fig 3. Leaf area for both subpopulations.



Suppl fig 4. Dry weight measurements of the leaf for all treatments.