

Project Title: Selecting for improved water and nitrogen uptake by focusing on root characteristics.

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Abstract

Lettuce crops will need to be adapted to be grown with less nitrogen (N) fertilizers and water. Our lab has focused on improving both water and nitrogen use efficiency by mapping and selecting genotypes that have improved N uptake and assimilation. In addition to leaf characteristics that can limit water loss and improve photosynthetic efficiency, we have focused on root architecture and biomass to improve both NUE and WUE. In the trials discussed herein, we demonstrate a strong direct relationship between root biomass and root dry weight when grown under low N, suggesting that selection for root biomass will help ensure that the lettuce crop will make size and quality.

Objectives:

Objective 1. Determine root length and root biomass of a lettuce germplasm diversity panel consisting of cultivars and wild sexually-compatible *Lactuca* germplasm when grown under limiting and non-limiting N.

Objective 2. Determine leaf N concentration of the lettuce germplasm diversity panel when grown under limiting and non-limiting N.

Objective 3. Determine relationship between leaf N concentration and root biomass of genotypes grown in Objective 1 and 2.

Objective 4. Identify genetic lines that represent phenotypic extremes of root biomass when grown under non-limiting and limiting N.

Procedures:

Objective 1. Determine root length and root biomass of a lettuce germplasm diversity panel consisting of cultivars and wild sexually-compatible *Lactuca* germplasm when grown under limiting and non-limiting N.

To determine root length and biomass, diverse genotypes from a collection of *Lactuca* germplasm were screened in semi-controlled conditions (described below). The diverse germplasm included commercial cultivars, numbered accessions, plant introductions, sexually compatible *Lactuca* species and advanced breeding lines from previous NUE trials. Genotypes with the highest leaf N concentration were selected for follow-on studies of root growth. The long-term goal is to combine both high leaf N concentration with improved root biomass when grown under low N and water.

To minimize variation in soil moisture, soil texture and N levels commonly observed in field trials, plants were grown in a peat-based high porosity growing medium. Uniformity across cultural inputs (water, fertilizers) and soil texture serves to maximize the genetic signal and minimize the environmental component that influences root growth.

From the initial germplasm screening, six genotypes with high leaf N concentration were selected to be used to study root architecture and N uptake. For each of the selected genotypes, six plants of each genotype were grown in raised bed boxes (3m x 1m x 0.76 m; LxDxH) containing high porosity peat-based media (Pro-Mix® HP mycorrhizae). To gauge reproducibility and to more accurately determine root biomass plasticity in response to soil N availability, the entire experiment was replicated four times, with n=24 for each genotype across the four experiments.

Root growth and N uptake were determined from plants grown under two N treatments, a non-limiting N treatment (N100) equivalent to 280 kg N / ha (250 lbs N / acre) and a limited N treatment (N50), equivalent to 140 kg N / ha (125 lbs N / acre). Beginning at week five and at seven-day intervals thereafter, plants from both N treatments received a complete (major plus minor nutrients) water soluble fertilizer (15-5-25, Plant Marvel Laboratories, Inc.) distributed equally across four applications and applied through fertigation. Plants assigned to the non-limiting N treatment raised bed box received additional N (YaraVera® granular urea, 46% N) to a level of 280 kg N / ha, beginning at week five and thereafter at seven-day intervals, distributed equally across a total of four applications. For both N treatments, water was replaced at 130 % ETo to ensure that plants did not experience a water deficit stress. In summary, the only difference between treatments was the additional N in the non-limiting treatment.

To determine root length and biomass, plants (n=6 per genotype per experiment) were harvested at market maturity, approximately eight weeks after planting. Leaves and heads were cut off from each plant at soil level and the plant was partitioned into leaves or roots. To facilitate cleaning, the root ball of each plant was placed in five-gallon buckets filled with water and allowed to soak a minimum of 30 minutes. The growing media was carefully hand washed from the roots with a gentle water stream and hand agitation from the roots. Root length was measured, and digital photographs were taken for downstream evaluation of root architecture. After photographing, the roots were cut into small pieces, oven dried for 48 hours at 60 °C, and the dry weight recorded.

To determine differences among genotypes in root length and biomass, an analysis of variance with treatment as main effects was used. Determinations were made from six biological replications of each genotype and the mean value was used for ranking and pairwise comparison. Pairwise differences were used for each genotype to determine if root biomass or root length were affected by N treatment.

Objective 2. Determine leaf N concentration of the lettuce germplasm diversity panel when grown under limiting and non-limiting N.

Using the same plants as in Objective 1, leaf and root N and C concentration of roots and leaves was determined at market maturity. That is, root and leaf biomass were obtained as a matched

pair from the same plant. C and N concentrations of root and leaf (separately) were determined in the Still lab using an Elementar vario Max C/N analyzer.

To determine if N treatment impacted leaf and root C and N concentration, the data were subjected to an analysis of variance using N treatment as the main effect.

Objective 3. Determine relationship between leaf N concentration and root biomass of genotypes grown in Objective 1 and 2.

Previously, we observed that some genotypes selected for high leaf N concentrations did not have the ability to increase root biomass when grown under low N. Our goal is to make selections that have increased leaf N concentration and increased root biomass when grown under low N. We sought to determine if there is a tradeoff between high leaf N concentration and the ability to add root biomass? That is, do lettuce plants maximize one at the expense of the other?

The data for objectives 1 and 2 were compared using Pearson's correlation coefficient (r). We explicitly tested the hypothesis that there is an association between the two variables (leaf N concentration and root biomass, and head weight). If the hypothesis is not supported, it suggests that the two traits are not closely correlated, and they are under different genetic control.

Objective 4. Identify genetic lines that represent phenotypic extremes of root biomass when grown under non-limiting and limiting N.

Genotypes were ranked by leaf N concentration and root biomass. Parental lines were chosen for a follow-on project to try to combine both traits (high root biomass and high leaf N concentration), to develop mapping populations and from which to make selections to develop germplasm with improved root biomass.

Objective 5. Assessment of water use efficiency and resistance to water deficit.

Plant materials developed with partial support from the Leafy Greens Board, were planted at the Maricopa Ag Center to assess growth and development under water deficit. Genotypes from the Cool Guard x Western Red Leaf recombinant inbred line population segregating for $\Delta 13C$ discrimination were grown under two water treatments (limiting and non-limiting).

Seed was sown in December 2021 and plants harvested in mid-March 2022. A total of 85 genotypes were planted that segregated at two alleles associated with $\Delta 13C$ discrimination, one on linkage group (LG) 4, the other on LG 6. Genotypes were assigned at random to each 3-meter plot with each genotype replicated three times in a randomized complete block design. At market maturity, the leaves/heads were harvested and the fresh weight obtained from three representative plants from the center of each plot. Leaf N and C concentration were obtained from three different plants of each genotype as previously described.

Results and Discussion:

Our long-term goal is to develop lettuce germplasm that requires less water and nitrogen while still making size and quality. While breeding objectives can be achieved without understanding what physiological mechanisms and genes (alleles) of the plant were changed in the improved genetic lines, understanding how this was accomplished improves breeding efficiency and advances science. Our lab employs genetic mapping which enables development of DNA-based markers that can be used in breeding programs. This approach generally does not allow insight into mechanisms and genes. To provide insight into the mechanistic underpinnings, we have employed physiological studies using genotypes from our mapping populations that are phenotypically dissimilar. Using both approaches, we can identify genes and understand the mechanism(s) the plant is using to improve water and nitrogen use efficiency.

In nitrogen and water use efficiency field trials we have observed significant variation in soil N, soil water and texture, making it difficult to accurately assess the genetic potential of a plant. Previously, we have shown that leaf N concentration is a key physiological trait which can be used to improve NUE. We have used leaf N concentration as a proxy for NUE because it integrates N uptake by the roots, the transfer of N from the roots to leaves, and assimilation of the N into organic forms more readily available for use by the plant. We have reported genetic variation in leaf N concentration, noting that genotypes with higher leaf N concentration have higher carbon assimilation rates (i.e., photosynthesis) and a commensurate higher biomass compared to genotypes with lower leaf N concentration. From these observations we hypothesized that lettuce plants with high leaf N concentration when grown under low N would likewise have greater root biomass.

Selecting for root growth is challenging because it is a highly plastic trait that responds to environmental signals. In general, plants increase root biomass when soil N levels are low. During trials, we discovered that while most the lettuce germplasm follows this general observation, many high leaf N concentration genotypes did not. Therefore, root growth of each genotype must be evaluated under low N to verify that root biomass increases under low N. Further, evaluation of root growth using field trials is challenging because recovering an intact root system from soil is difficult and relatively few plants can be evaluated in a day. Growing plants in a peat-based media served to minimize the environmental variation inherent to field evaluation, improve throughput, and increase the accuracy of root biomass data. Since it does not represent field conditions, we sought to determine if peat-based media accurately and reproducibly, matched results from field trials in terms of leaf N concentration.

A recombinant inbred line population derived from a cross between cultivars ‘Diplomat’ and ‘Margarita’ was grown under low N (140 kg N/ha), harvested at market maturity, and the leaf N concentration obtained. The genotypes were ranked, and the phenotypic extremes of leaf N concentration were identified using the 97.5 and 2.75 percentiles as cutoff thresholds. Those genotypes with the lowest N concentration (2.75 percentile or below) were defined as low leaf N concentration phenotypes, and those at or above 97.5 percentile were defined as high leaf N concentration. These genotypes were grown in a peat-based media with no added N, harvested at market maturity, and the leaf N concentration determined. Photosynthesis (i.e., CO₂ uptake) was measured on recently expanded leaves and plotted against leaf N concentration (Figure 1).

The leaf N concentration phenotypes observed of plants grown under peat-based media were entirely consistent with those observed in the field (Figure 1). Over multiple experiments using a variety of genetic materials, we observed concordance in leaf N concentration between field-grown and peat-based grown plants, suggesting that the physiological, metabolic and morphological mechanisms controlling leaf N concentration of plants grown in the peat-based media is consistent with those from field-grown plants. We conclude that for the purposes of screening lettuce germplasm for leaf N concentration, the peat-based media accurately represents field data.

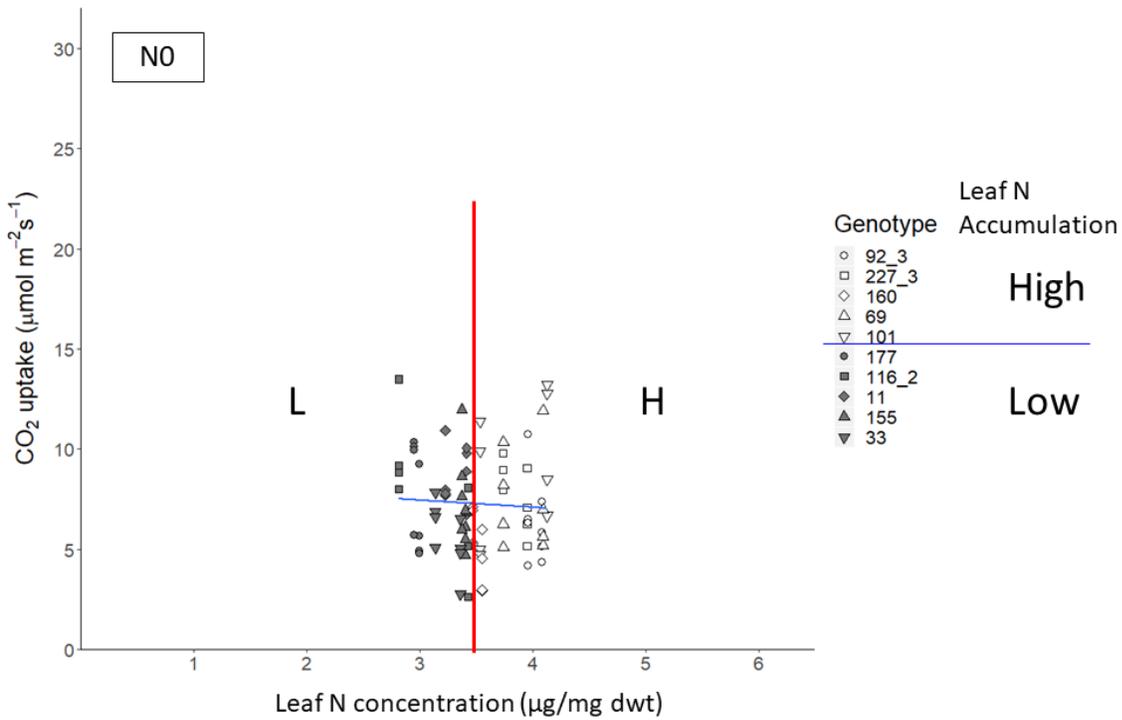


Figure 1. Leaf nitrogen concentration and CO₂ uptake (photosynthesis) of genotypes grown in a peat-based potting medium with no additional N added. In previous trials, genotypes were grown in the field and ranked by their leaf N accumulation, and subsequently categorized as either having a high or low leaf N accumulation phenotype. Selecting plants exhibiting high or low leaf N concentration phenotypes, the plants were then grown under low N using a peat-based medium and after taking photosynthesis measurements the leaves were harvested and the leaf N concentration determined. The red vertical line separates Low leaf N concentration and High leaf N concentration phenotypes. This relationship between field and peat-based grown plants was observed across multiple experiments, suggesting that trials conducted in peat-based media are valid for the purposes of screening lettuce germplasm for leaf N concentration.

In previous experiments two RIL populations were screened to facilitate mapping of NUE and WUE. Multiple QTL associated with WUE and NUE were identified, and selections were made

for further trials to help confirm the loci-phenotype associations and to help fine-map the causative genes. Because biparental RIL lines have limited genetic variation, we expanded trials to include a more diverse germplasm pool. This increases the probability that the variation in root biomass and growth is captured and allowed us to determine the correlation between root biomass, leaf biomass, and leaf N concentration. We selected genotypes with high leaf N concentration and high photosynthetic capacity to serve as parental lines for new RIL lines and from which to make additional selections for improving NUE and WUE traits. From these evaluations, six genotypes were selected for further characterization, which included Cibola, D221, DF7, W28, W34 and Western Red Leaf (WRL). Notably, W28 and W34 are accessions of *L. virosa* and *L. serriola*, respectively, and have 20-30% higher photosynthetic rates compared to other high leaf N genotypes.

When grown under the low N treatment (N50), root biomass (as measured by root dry weight) differed among genotypes (Prob > F = 0.0001), with genotypes W28 and W34 (3 g and 2.5 g, respectively) having the greatest weight and the longest roots (> 40 cm). Conversely, the cultivars ‘Cibola’ and ‘WRL’ (both 1.4 g) and had the lowest root dry weight and shortest roots (Figure 2). Similar trends in root biomass and length were observed when grown under high N, with W28 and W34 having the highest root biomass and longest root lengths and ‘Cibola’ and ‘WRL’ having the lowest root biomass and root length (data not presented).

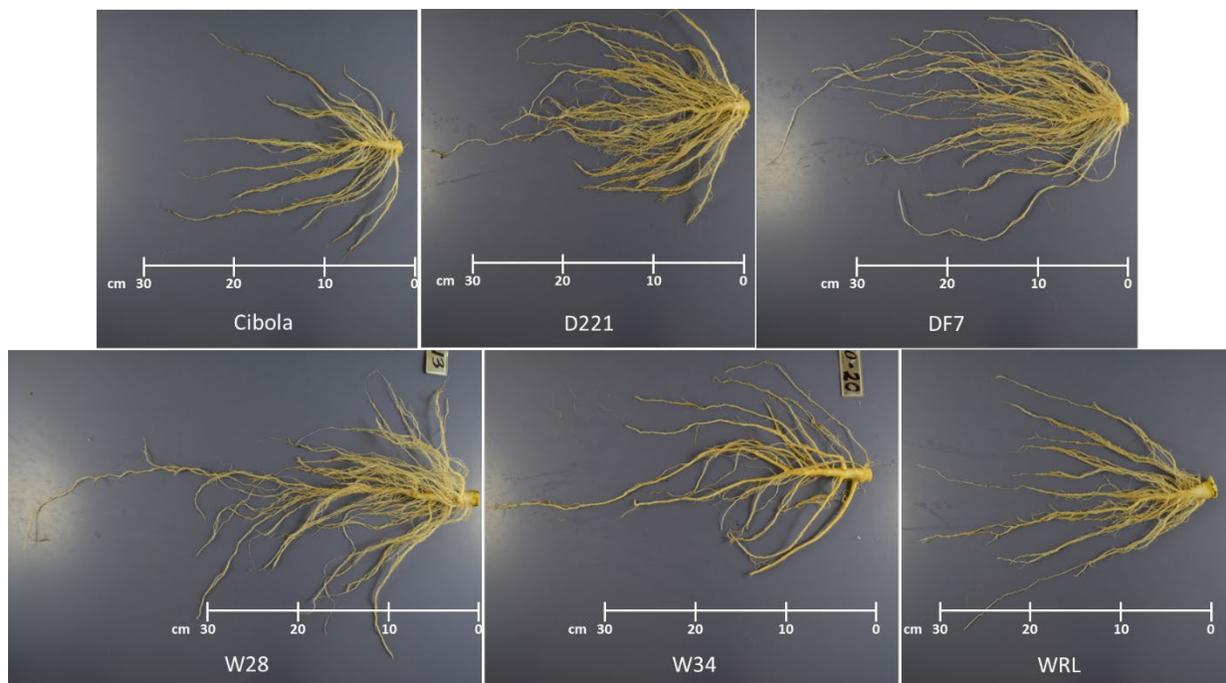


Figure 2. Root architecture of genotypes selected for leaf N concentration and root biomass. Lettuce genotypes grown under the low N (125 lbs N/acre) treatment and the genotypes with the highest leaf N concentration were selected. These genotypes served as parental lines to generate

populations for selecting both high leaf N concentration and increased root biomass when grown under low N. Genotypes include ‘Cibola’, ‘D221’, ‘DF7’, ‘W28’ (*Lactuca virosa*), ‘W34’ (*Lactuca serriola*) and ‘WRL’ (Western Red Leaf). Each genotype was selected for their N uptake and assimilation ability and contrasting root architecture.

Regression analysis indicated that root dry weight was highly predictive of head weight when plants were grown under low N (N50), with an average R^2 value of 0.84 (range = 0.65 to 0.93; Table 1). Similar results were observed for plants grown under high N (N100), with an average R^2 value of 0.82 (range = 0.66 to 0.93; Table 1). Note the 23-to-25-point difference in the coefficient of determination between N50 and N100 for genotypes D221 and DF7.

For each genotype except for genotype W28, the root dry weight was greater in plants grown under low N (N50) than when grown under high N (Figure 3). Root dry weight was used as a metric of root biomass, and it accurately portrays biomass since it is not affected by the plant’s relative hydration level. It does not, however, provide an indication of rooting depth or other aspects of root architecture that might affect water and N uptake. Since N is dissolved in the soil water, N uptake only occurs when plants transpire, and only when roots are growing where soil water can be found. Under field conditions, water is often available at lower depths (18-24”) than that observed closer to the surface (6-12”). Thus, our goal is to increase root biomass toward lower depths. In that regard, we predict that selections from populations with W28 and W34 as parental lines will be useful.

Table 1. Regression analysis of root dry weight versus head weight for six lettuce genotypes selected for high leaf N concentration and increased root biomass under low N (N50). The coefficient of determination indicates the amount of variation in head weight varied from 65% to 93%, depending on genotype and treatment. N=24 for each genotype.

Genotype	N50		N100	
	Prob > F	R^2	Prob > F	R^2
Cibola	<0.0001	0.84	<0.0001	0.81
D221	<0.0001	0.89	<0.0001	0.66
DF7	<0.0001	0.65	<0.0001	0.90
W28	<0.0001	0.89	<0.0001	0.83
W34	<0.0001	0.81	<0.0001	0.93
WRL	<0.0001	0.93	<0.0001	0.79

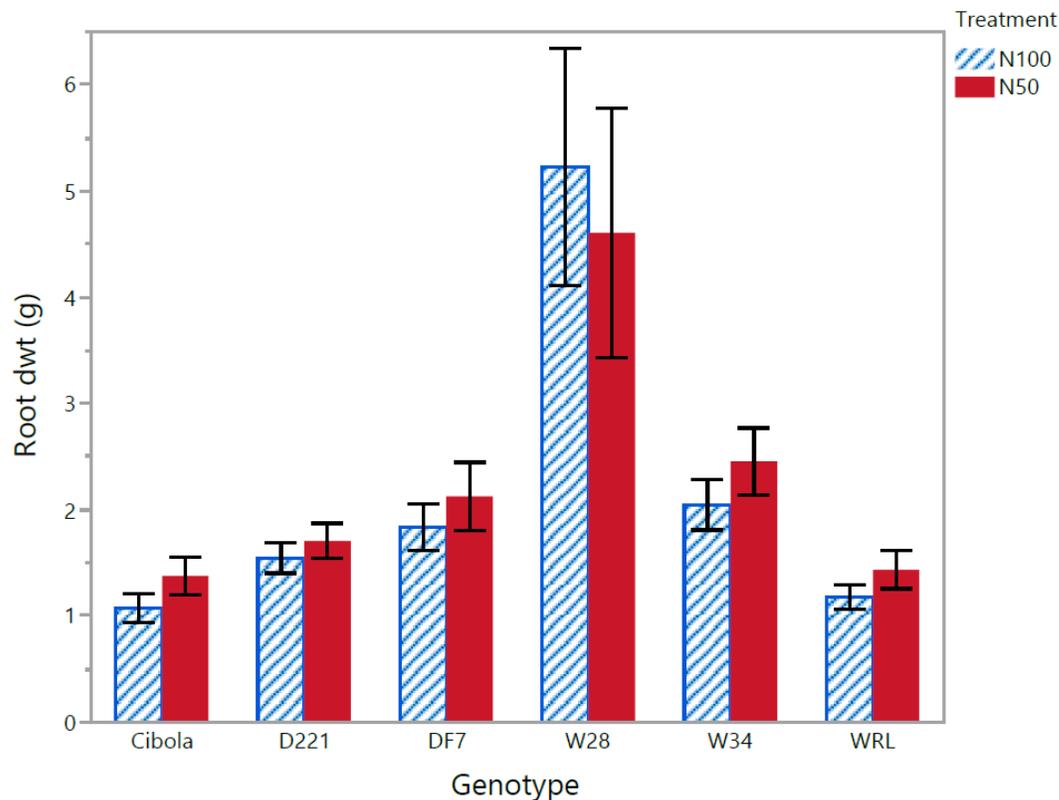


Figure 3. Root dry weight of high leaf N genotypes used as parental lines. Root dry weight was used as a proxy of root biomass. Data are the means of four independent experiments, with bars representing mean standard error. Note that with the exception of genotype W28, root biomass was greater in plants grown under low N than when grown under high N.

In general, plants display a high degree of root plasticity in response to environmental conditions. When soil water is limited or minerals (especially N) are low, root biomass typically increases relative to leaf biomass. Similarly, root biomass typically increases in plants grown under low N compared to high N. The dry root biomass ratio of plants grown under N50 to N100 (i.e., N50:N100), ranged from 1.1 to 1.3, for D221 and Cibola, respectively, but in W28, the N50:N100 ratio was 0.6. Even under carefully controlled conditions and highly uniform growing conditions in the peat-based media, plant-to-plant variation was substantial. While the trends were consistent, they did not rise to the level of significance in any of the pairwise N50 to N100 comparisons within a genotype. This again, illustrates the plasticity of root growth and the need to lower environmental variation in trials to increase genetic signals and reduce the likelihood of selecting false positives for advancement in a breeding program.

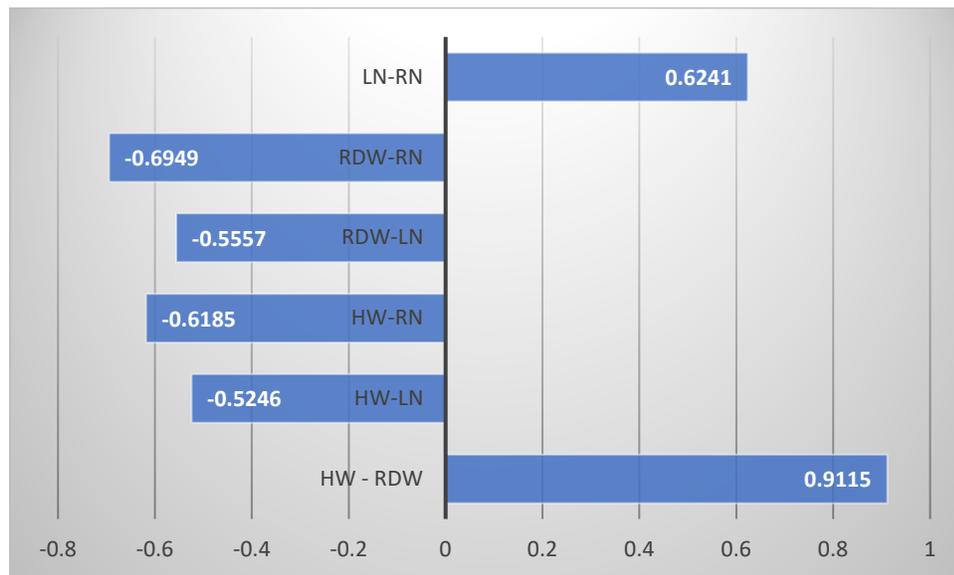


Figure 4. Mean Pearson's correlation coefficient (r) averaged across six genotypes grown under the low N treatment across four independent experiments. A positive coefficient displayed within the blue bar indicates a positive linear relationship between two variables, while a negative number indicates a negative correlation between two variables. Note that the probability level for each is 0.0001. HW - head weight; RDW – root dry weight; LN – leaf nitrogen; RN – root nitrogen. For each genotype, $n=24$.

Growing plants in conditions where soil uniformity, texture, water availability, and fertilizer availability are controlled, the reproducibility of the trials is more likely. While plant-to-plant variation was still observed, it is likely due to physiological variation and plasticity in root growth. Despite the plasticity in both root and leaf growth, and strong differences among genotypes in plant growth and form, a few strong correlations emerged from the data. The strongest positive relationships were observed between head weight and root dry weight ($r = 0.9115$) and between leaf N and root N ($r = 0.6241$; Figure 4). Thus, a large root system develops in plants to support large above ground biomass, suggesting that conditions that limit root growth will also limit head weight and size. A negative relationship was found between head weight and both root N and leaf N. A negative relationship was found between root dry weight and both root N and leaf N. This suggests that N is highly mobilized to support tissue growth. However, note that the absolute value of the correlation coefficients is of lower magnitude than that observed for the head weight – root dry weight, with an average absolute value of r of 0.5984 vs. 0.9115, respectively. This indicates the presence of other unknown factors that work to modulate N metabolism and plant growth.

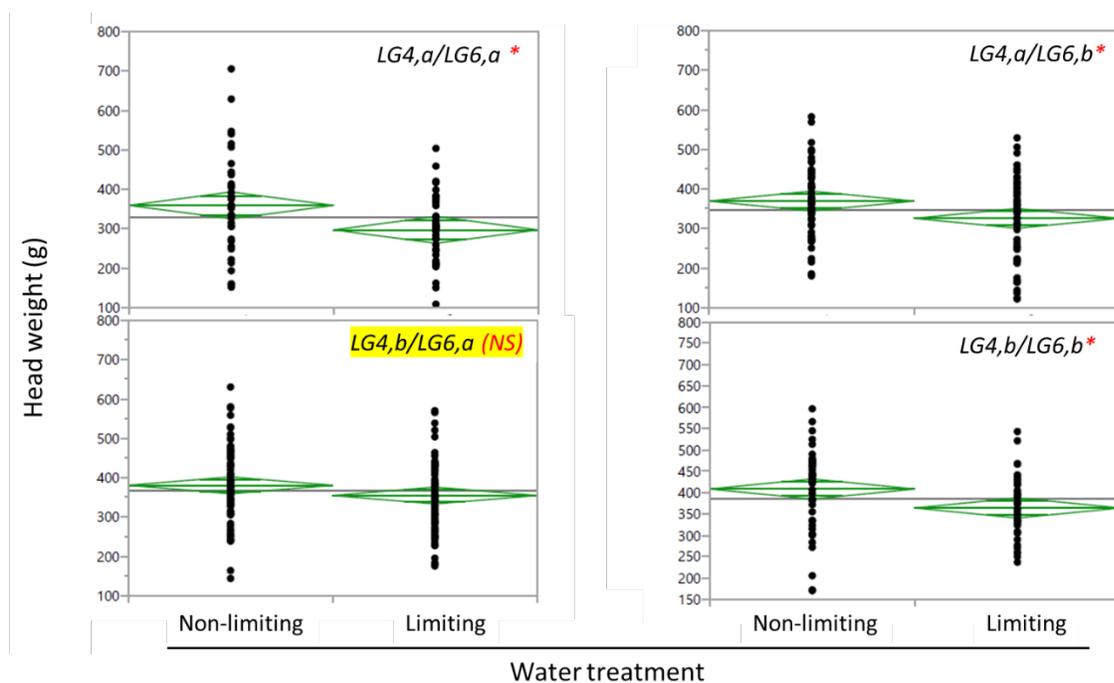


Figure 5. Head weight of genotypes segregating for loci associated with water use efficiency. genotypes included in the trial were previously grown in field trials and selected for resistance to water deficit, using $\Delta^{13}\text{C}$ values as an integrating metric. Loci associated with $\Delta^{13}\text{C}$ values were detected on linkage group 4 (LG4) and LG6. Note that when grown under limiting water, only genotypes with the combination *LG4,b/LG6,a* had head weights equivalent to those grown under the non-limiting water treatment. Cooperators for these trials included Richard Michelmore, Duke Pauli, Maria Truco, and Dean Lavelle.

From previous trials, we selected genotypes with contrasting water use efficiency were identified from their $\Delta^{13}\text{C}$ values and planted them at the University of Arizona Maricopa Ag Center. After thinning stage, water was restricted for the plants in the limiting water treatment plots. At market maturity, plants were harvested and the head weight determined. The selections were segregating for two loci associated with $\Delta^{13}\text{C}$, one on linkage group (LG) 4 and the other on LG6. Of three allele combinations, water limited plants had significantly lower head weight, while only the plants with *LG4 b/LG6 a* alleles did not differ. This indicates that these plants did not experience stress associated with water deficit to the degree the plants that lacked this allelic combination. We can speculate that the favorable allelic combination allowed these plants to continue to photosynthesize while those lacking could not, and by doing so accumulated greater leaf biomass. Several factors, including root architecture and/or leaf characteristics, could be responsible.

In summary, the important breeding message from these trials is that when grown under limiting water or N, selection for root biomass will help ensure that the lettuce crop will make size and quality.