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CHARACTERIZING LOW NUTRIENT STRESS RESISTANCE IN CROP SUNFLOWERS (Helianthus annuus)

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CHARACTERIZING LOW NUTRIENT STRESS RESISTANCE IN CROP SUNFLOWERS (*Helianthus annuus*)

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by

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ABSTRACT

CHARACTERIZING LOW NUTRIENT STRESS RESISTANCE IN CROP SUNFLOWERS (*Helianthus annuus*)

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Historically agriculture has met global food production demands, but abiotic stresses are predicted to decrease crop yield in the context of climate change. In order to prevent losses in crop yield under conditions of increasing environmental stress and to reduce environmental damage from unsustainable farming practices, improvements must be made in crop breeding and bioengineering. However, these improvements require insight into the mechanisms of abiotic stress resistance. In this study, 60 different genetic sunflower (*Helianthus annuus*) lines were grown in the field under fertilized and unfertilized treatments to assess phenotypic traits associated with low nutrient stress resistance. Sunflowers were assessed for stress resistance using height, stem diameter, biomass, and root structure traits. Sunflowers responded to nutrient stress through a decrease in overall size and increased root width near the surface and steeper lateral root angles. Height, stem diameter, biomass, root mass, root area, and root density were found to be correlated with nutrient stress resistance. Overall plasticity of sunflowers was also found to be correlated with nutrient stress resistance. Root width allocation, root mass, root area, root density, and plasticity should be studied further for their potential to improve crop breeding and bioengineering efforts.

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CHAPTER I

INTRODUCTION

Ecological Impact of Chemical Fertilizers

Global agricultural production has historically met the increasing food demands resulting from exponential human population growth; however, modern high yields are unsustainably dependent on chemical fertilizers. In some areas, fertilizer use has artificially doubled the amount of nitrate and ammonium in the soil (Vitousek et al. 1997) and nitrogen fertilizer use has increased by 10 times globally (Robertson and Vitousek 2009). This increase in soil nitrogen can cause soil acidification (Juo et al. 1995, Matsuyama et al. 2005), which reduces crop yield or, in extreme cases, results in unarable soil (Delhaize and Ryan 1995, Hoegh-Guldberig et al. 2007). Soil acidification can also reduce phosphate accessibility to crops, thus requiring more fertilizer in an ongoing cycle of soil degradation (Blake et al. 1994).

Overuse of fertilizers can also cause ecological problems in natural systems (Vitousek et al. 1997, Carpenter et al. 1998, Orr et al. 2005, Feely et al. 2008, Hall-Spencer et al. 2008). Due to the mobility of nitrate, nitrogen from fertilizers can leach out of agricultural soils. Currently, less than half of the nitrogen added to crop systems globally is taken up and incorporated by crops, leaving the rest in the environment (Lassaletta et al. 2014). This nitrogen can increase the greenhouse gas nitrous oxide in the atmosphere (Vitousek et al. 1997), intensify acid rain (Vitousek et al. 1997), and reduce biological diversity (Tilman 1987). An increased flux of nitrogen from agricultural lands to marine systems causes eutrophication. The decomposition of resulting algal blooms

creates hypoxic environments known as “dead zones” (Diaz and Rosenberg 2008). These “dead zones” have increased exponentially over the last 50 years (Diaz and Rosenberg 2008).

Phosphorus leaching from agricultural soils also contributes to eutrophication (Carpenter et al. 1998). Only approximately 30% of applied phosphorus is taken up by plants (Carpenter et al. 1998). As a result, 22 kilograms per hectare of excess phosphorus is added to agricultural soils each year. This use of phosphorus fertilizers has caused many soils to approach saturation levels, resulting in increased phosphorus leaching into natural environments over time (Hooda et al. 2001).

Nutrient Stress in Agricultural Systems

The focus of modern agriculture on increasing food production through farming technologies, such as fertilizer and pesticide/herbicide use, as well as focused breeding for high yield crop varieties, has resulted in a decrease in many crop species’ natural low nutrient stress resistance (Donald 1968, Dawson et al. 2008, Lassaletta et al. 2014). As a result, in very low nutrient conditions crops will often produce lower yields than their wild relatives (Chapin et al. 1986). Crop dependence on fertilizers is becoming increasingly problematic for several reasons. Over the past 50 years crops from developed countries have shown a steady trend of decreasing nutrient use efficiency (Lassaletta et al. 2014). Also, mineral phosphorus supplies are running low in some regions (Vaccari 2009). Nitrogen and phosphorus fertilizers may not be readily available or are often too expensive to farmers in low-GDP countries. In fact, 85% of the global population lives in countries with little agricultural fertilization (Lynch 2005). Tropical

soils may respond poorly to fertilizers. For example, fertilizers often have negligible effects in sub-Saharan Africa (Steen 1998, Lynch 2007).

Lack of nutrient stress resistance in crop varieties is especially troubling as climate change threatens to increase these stresses in many parts of the world. Climate change is expected to increase nutrient and drought stress in tropical areas (McCouch et al. 2013) and, therefore farmers will need to turn to poorer soils for cultivation to maintain current crop yield levels (Young et al. 1998, Cassman 1999). Climate change is also projected to increase potential for soil erosion (Griffiths and Young 1994, Young et al. 1998), which would worsen nutrient stress for crops. Furthermore, the increase in atmospheric CO_2 will likely lower transpiration rates of plants. This reduced transpiration rate would inhibit the acquisition of nutrients normally taken up through mass flow, such as nitrate, sulfate, and calcium (Lynch and St. Clair 2004). It is clear that keeping pace with rising global food demand under climate change will require solutions to maintaining, even increasing, crop yield under nutrient stress. To this end, it is essential that agricultural research invests in improving our understanding of nutrient stress resistance in crop plants.

CHAPTER II

LITERATURE REVIEW

Plant Responses to Nutrient Stress

Crop yield is often limited by low nutrient stress (Von Liebig 1855, Boyer 1982, Vitousek and Howarth 1991). Nitrogen is the most common limiting nutrient in the majority of terrestrial environments (Tilman 1982, Tamm 1991, Vitousek and Howarth 1991), followed by phosphorus (Walker and Syers 1976). Nutrient uptake from the soil can take four main forms. Mass flow is the large passive movement of water soluble nutrients into the roots through symporter proteins (Comerford 2005). Diffusion is the relatively short travel distance of nutrients from a high to low concentration gradient (Comerford 2005). Microbial symbioses involve obtaining nutrients directly from nitrogen fixing bacteria or mycorrhizal fungi (Smith and Read 1997, Sprent 2005). Finally, nutrients can be acquired by root interception, which is when roots contact soil particles that contain nutrients and then absorb those nutrients (Barber 1995).

Changes in root architecture are one of the most important responses to nutrient stress for crop plants (Wang et al. 2010). Plant resource allocation has been studied for almost a century (Turner 1922, Crist and Stout 1929). It is well established that plants in nutrient deficient soils allocate more resources to their root versus shoot systems (Chapin 1980, Tilman 1988, Tilman and Wedin 1991). Studies on onion, clover, and rye showed a root to shoot ratio increase anywhere from 1.5 to 12 fold in response to low nutrient stress (Davidson 1969, Brewster et al. 1975, Chapin 1980). Increased allocation of carbon to roots under stress is one prediction of the functional equilibrium hypothesis, which

suggests that plants are continually balancing between carbohydrate synthesis and nutrient absorption through allocation of resources to shoots and roots (Brouwer 1962). More specifically, in their review Fitter and Stickland (1991) showed how nutrient absorption was more strongly correlated with root length in many plants. This suggests that root mass, shoot to root ratio, and root length should be included when studying nutrient stress resistance.

Traits related to root area (Bottrill et al. 1970, Trubat et al. 2006), lateral root growth (Bonser et al. 1996, Ge et al. 2000, Liao et al. 2001, Ho et al. 2005, Zhu et al. 2005), higher root area per mass (Nielson et al. 1998, 2001, Lambers et al. 1992), root depth (Zhu et al. 2005), and lateral root angles (Lynch 2007) may also inform how plants respond to nutrient stress. However, many of these experiments were carried out in greenhouses and growth chambers. Few field studies include extensive root data due to the amount of time and effort it takes to assess roots (Lynch 2007). Recently, with the development of the Digital Imaging of Root Traits (DIRT) program (Das et al. 2015), root data from large field studies is easier to obtain. Detailed root architecture can now be examined using DIRT in conjunction with yield estimates in the field in order to better elucidate how root architecture contributes to nutrient stress resistance in crop plants (Lynch 1995, Aerts and Chapin 1999).

Genetic Basis of Nutrient Stress

Once target root architecture traits related to low nutrient stress resistance have been identified, the next step is to identify genes for these traits to improve crop engineering efforts. Studies have shown that some genes for abiotic stress resistance

work independently of yield, allowing improved stress resistance and yield to occur simultaneously (Chapin and Pugnaire 1993, Reich et al. 2003, Munns 2011, Sadras and Richards 2014) and root architecture is a good candidate for yield independent genes (Lynch 2007). Research relating to root architecture and nutrient stress has been carried out for several crops. Many studies have identified genes related to root architecture in corn (Li et al. 2011), rice (Dai et al. 2012), and soybean (Guo et al. 2011) that maintain or increase yield. A gene (ZmPTF1) was identified and cloned from corn by Li et al. (2011) that affects root development, root mass, and lateral root growth, while increasing kernel size in low phosphate conditions. A gene (OsMYB2P-1) discovered by Dai et al. (2012) was shown to increase low phosphate tolerance in rice and Arabidopsis by decreasing primary root length while increasing lateral root length. Finally, a gene (GmEXPB2) was identified in soybeans by Guo et al. (2011) that increases root hair production that increased its tolerance to low phosphate. These are only a few examples of the many genes recently discovered that have the potential to improve yield through root architecture traits (Li et al. 2016). Mapping of genetic traits involved in phenotypic responses to nutrient stress will be important for informing future crop breeding efforts.

Sunflower as a Model for Crop Nutrient Stress Resistance

Most recent crop nutrient stress studies generally focus on the root structures of major crops including corn, wheat, rice, and soybean. Corn, for example, has been shown to have higher nitrogen use efficiency when larger and deeper root systems are produced (Yu et al. 2015). In contrast, soybeans show higher phosphorus efficiency when they

allocate a higher proportion of their roots near the soil surface (Zhao et al. 2004). Much less in known about nutrient stress resistance in other crops including oilseeds.

Helianthus annuus (common sunflower) is a good model for studying nutrient stress tolerance in oilseed crops for several reasons. Sunflower is one of the five most highly produced oilseed crops worldwide (Ash 2012). The *Helianthus* genus is naturally stress resistant. Wild species of sunflower have been documented in extremely stressful environments, including sand dunes in Arizona and Utah and salt marshes in west Texas and New Mexico (Rieseberg et al. 2003). Despite resistance to abiotic stress in natural populations of the same species, crop sunflower yield is limited under abiotic stresses (Fereres et al. 1986, Andrich et al. 1996). Loss of stress resistance in crop sunflower is likely due to trade-offs with yield resulting from selective breeding for higher oil content (Smith 1989, Rieseberg and Seiler 1990).

This thesis reports a detailed study of root and shoot architecture and yield in a crop sunflowers grown under nutrient stress. My specific objectives were to 1) describe how nutrient stress (unfertilized conditions) affects crop sunflower above and below ground traits, 2) characterize variation across lines for these traits, 3) estimate resistance to nutrient stress, and 4) examine how these traits associate with resistance to low nutrients. These results may improve our understanding of how crop plants allocate resources to resist nutrient stress and inform breeding efforts aimed toward more stress resistant oilseeds.

CHAPTER III

METHODS

Study System

This study utilizes a previously established sunflower association mapping (SAM) population (Mandel et al. 2011). The full SAM population consists of 288 inbred *H. annuus* lines that represent almost 90% of the genetic variation segregating in cultivated sunflowers (Mandel et al. 2011). Minimal linkage disequilibrium found during 10k single nucleotide polymorphism (SNP) array studies suggests a high independence of gene segregation in the SAM population that will facilitate genetic mapping of phenotypic traits (Mandel et al. 2011). The full 288 SAM lines were grown under fertilized and unfertilized treatments in a greenhouse study at University of Georgia, Athens, GA in 2016 (unpublished data). Difference in biomass at the R4 stage (immediately pre-flowering) between treatments was used to rank lines for their resistance to nutrient stress (NDSU 2017). From these data, we excluded lines that did poorly in both treatments and then chose 20 high-resistant, 20 mid-resistant, and 20 low-resistant SAM lines for our field experiment. These 60 lines represent a continuum of cultivated sunflower resistance to low nutrient stress that ranges from the least to most resistant SAM lines as determined by the greenhouse experiment.

Study Site

The 60 SAM lines were grown to maturity under fertilized and unfertilized treatments at a garden site on the Central Washington University campus, in Ellensburg, WA (47°00’49”N, 120°31’25”W) during May to October, 2017. Ellensburg has an

average temperature of 8.3 degrees Celsius, and an average annual rainfall of 22.6 cm (WRCC 2018). The field site was a 15.8 m by 21.3 m fenced-in plot that had been previously used for sunflower research. Results from nine surface soil samples taken evenly across from the field one month prior to planting showed low soil nitrate, 5 to 8 ppm (MidWest Laboratories 2017). Phosphorus was 37-66 ppm (medium to high), and potassium was 203-335 ppm (high to very high). The field did not exhibit a cline in soil nutrients. The soil series is an Opnish Ashy Loam, categorized as a Vertisol.

Field Irrigation

The field was irrigated using 14 drip hoses running the entire length of the field. The watering regime started at 30 minutes every 12 hours at planting, but based on continued observations of soil saturation the regime was consistently reduced biweekly finishing the season with a rate of 30 minutes of watering every 72 hours. The irrigation probes were placed at a depth of 25.4 cm with a horizontal orientation with the edges of the probe faced vertically to prevent the probe from trapping water flowing through the soil. Soil moisture and conductivity data were monitored twice weekly for the duration of the field season (Spectrum Technologies 2017).

Planting and Emergence

On May 5th, 2017 four replicates for each of the 60 lines were planted in each of four sub-blocks (2 fertilized sub-blocks, 2 unfertilized sub-blocks). The seeds were planted in paired rows on either side of each irrigation hose. Plants were spaced 30.5 cm apart along the row and across the hose. Hoses were spaced 107 cm apart. Border

sunflowers were planted along the outside of each sub-block following the same design. These border plants helped to reduce edge effects and prevent nutrients from leaching between sub-blocks. Emergence was recorded every 3-4 days starting two weeks after planting. Recording of emergence was completed three weeks later.

Sub-blocks 1 and 4 were treated with fertilizer on June 15th, as 90% of seedlings had reached the 4 leaf stage. Osmocote Blend 20-8-4 (Osmocote 2017) was added at a rate of 12.5 pounds per block using a Scott's hand spreader.

Flowering Date and Chlorophyll Content

To ensure self-pollination, the apical inflorescence (API) of each individual was covered using a pollination bag (Delnet 2017) when it reached the R3 stage (some yellow pollen may be visible but the inflorescence is closed) (NDSU 2017). Bagged inflorescences were self-pollinated by hand by rubbing a clean paper towel across the flowers every other day. Flowering date (see Table 1 for detailed trait descriptions) was recorded for each individual at the R5.1 stage (first open flower visible), and plants were no longer hand-pollinated once they reached the R6 stage (flowering is completed). Chlorophyll content was measured using a MC-100 Chlorophyll Concentration Meter (Apogee Instruments 2017) during peak flowering (50% of individuals at R5.1), July 30 - August 2. Two measurements were taken (one on each side of the mid-vein) on the most recent fully expanded leaf (MRFEL) and averaged.

Table 1 Trait descriptions. Trait names, abbreviations used in text, and definitions.

Type	Trait Name	Abbreviation	Definition
Other	**Days to Flower**		Days from planting day to first flower formation
	Chlorophyll Content		Average of two leaf light reflectance readings given by MC-100 meter
Size	**Height**		Centimeters from base of stem to base of API
	Stem Diameter		Diameter of stem measured at 3 cm above soil line
	Stem Mass		Mass of dry main stem
	Leaf Biomass		Mass of dry leaves including petioles
	Apical Inflorescence Mass	API Mass	Mass of dry API
	Leaf Mass Fraction	LMF	Leaf biomass divided by total plant mass
	Specific Stem Length	SSL	Height divided by stem mass
Size and Root	**Root Mass**		Dry mass of whole root system
Root	**Root Mass Fraction**	RMF	Mass of root system divided by mass of entire plant
	Root Area		Number of pixels belonging to root (DIRT)
	Root Density		Ratio of root pixels to background pixels within root shape (DIRT)
	Median Root Width		Median width of root system calculated horizontally from first to last root pixel (DIRT)
	Accumulated Root Mass Above 20% Length	ARW20	Percentage of width accumulation at 20% depth (DIRT)
	Median Root Tissue Angle	Median RTA	Median angle between lateral roots and tap root (DIRT)
	Minimum Root Tissue Angle	Min RTA	Minimum angle between lateral roots and tap root (DIRT)
	Root Tip Paths	RTPs	Overall number of root tips detected (DIRT)
	Tap Diameter		Tap root diameter estimated over the detected tap root region as the average of diameters of medial circles (DIRT)

Harvest and Trait Measures

Over 90% of plants had reached stages R7 - R9 (mostly developed seeds to senescence) and were harvested September 16 -19. The remaining 10% were harvested on October 6th, immediately prior to first frost and below freezing temperatures at the field site. For all individuals, height was measured from soil level to the base of the API (Table 1). Stem diameter was measured 3 cm above the soil level. All above ground parts of plants were dried in paper bags for 48 hours at 37°C. Plants were then separated into API, branch flowers, stem, leaves, and branches. API was the largest flowering head and was removed directly at the base of the flower where the API attached to the stem. Branch flowers were any other flower besides the API. The branch flowers were detached where the flowering headed attached to the peduncle. The stem was defined as the main branch that connected the roots to the API. No other leaves or branches were included. The leaves were made up of any leaf or petiole tissue on the plant. Finally, the branches were any other part of the plant that was not part of the sub-sections mentioned above. Each sub-section was massed using a digital scale.

Detailed root architecture data was collected using the DIRT protocol (Das et al. 2015) on a subset of one plant per line per sub-block, or two individuals per line and treatment, 238 total individuals. A brief summary of the DIRT protocol includes digging up the roots with a radius of 15 cm from the stem on each side, and 15 cm deep. The roots were cut from the stem three centimeters above the soil line. The roots were then washed to remove any dirt. A side view picture was taken of each root with a fixed, black background and scale marker. The pictures were then uploaded and calibrated to a threshold value of 10 on the DIRT website (http://dirt.iplantcollaborative.org). The DIRT

program was then used to calculate nine root traits related to root architecture. These nine root traits were chosen based on previous work indicating a connection between these root traits and nutrient stress resistance (Turner 1922, Fitter 1991, Bonser et al. 1996, Lynch and Brown 2001, Zhu et al. 2005, Lynch 2007). The roots were then dried for 48 hours at 37 degrees Celsius and massed using a digital scale.

Data Analyses

Days to flower was calculated as the number of days from the planting day (May 5th, 2017) to the date of first flower formation. LMF was calculated as the plants leaf biomass (grams) divided by the total plant mass (grams). SSL was calculated as the height (cm) of the plant divided by the stem mass (grams). RMF was calculated at root mass (grams) divided by the total plant mass (grams). Table 1 shows every trait analyzed, the type of trait it is, any abbreviations for the trait, and the definitions of each trait.

Data analysis was carried out using R statistical software (R Core Team 2012). Final sample sizes were ~800 for chlorophyll content and days to flower, ~600 for harvest traits taken on all individuals (height, stem diameter, stem mass, leaf biomass, API mass, LMF, and SSL), and 218-227 for root traits. A general linear mixed model (LMM) was used to test the effects of block, treatment, line, and line × treatment on each trait (Kuznetsova et al. 2015). Block was defined as either the northern (block 1) or southern (block 2) two sub-blocks of the field in order to account for possible water variability differences due to the elevation gradient running downhill from north to south. Block and treatment were fixed factors and line and line × treatment were random. Treatment was crossed within block.

Resistance was defined as the model residuals taken from regressing mean API mass for each line for the unfertilized treatment as a function of the API mass for each line under the fertilized treatment. A line with a higher residual value was defined as more resistant to nutrient stress, whereas a lower residual value indicated less resistance. Pearson correlations were then used to test relationships between each trait in each treatment and resistance.

In order to examine how shifts in trait values between treatments affect resistance to nutrient stress, plasticity was calculated for each trait by subtracting the mean trait value for each line in the fertilized treatment from its value in the unfertilized treatment and then dividing this by the unfertilized value. Plasticity values for all traits, excluding API mass as that was used to determine resistance, were then included in a principal components analysis (PCA). The first principal component (PC1) from this analysis was correlated with resistance.

CHAPTER IV

RESULTS

Trait Responses to Fertilization Treatments

Days to flower, chlorophyll content, and the majority of the size traits differed significantly by treatment (Tables 2, 3). Of the ten size and other traits, eight were significantly different by treatment, whereas only three of ten root traits differed significantly by treatment (root mass is both a size and root trait). For all size traits with significant treatment effects, plants were larger in the fertilized treatment (Table 2). Plants also flowered earlier, had more chlorophyll in their MRFEL, and had lower values for ARW20 and min RTA in the fertilized than the unfertilized treatment.

Line effects were significant for most size and other traits, with the exception of leaf biomass, indicating genetic variation across the 60 SAM lines for these traits regardless of treatment (Table 3). For the root traits, line effects were only significant for root tip paths and marginally significant for minimum root tissue angle. Lines differed in their response to treatment (significant line × treatment effects) for stem mass and SSL (Figure 1). For example, some lines produced larger stems in the fertilized than the unfertilized treatment and other lines did the opposite. Several lines also showed little difference between treatments. Although line × treatment effects were not significant for any other traits, reaction norms between treatments were highly variable across lines (Figure 1). Root density and ARW20 are good examples of traits that were highly variable in their response to treatment between lines (Figure 1).

Table 2 Trait means and standard errors in each treatment. Carets (^) indicate traits assessed on a population subset. Asterisks (*) indicate traits differed significantly ($P < 0.05$) by treatment. Full model results are in Table 3.

Trait	Fertilized Mean (Standard Error)	Unfertilized Mean (Standard Error)
Days to Flower	89.04 (0.531)*	91.10 (0.610)
Chlorophyll Content ($\mu mol/m^2$)	13.74 (0.184)*	11.67 (0.162)
Height (mm)	1201.57 (20.57)*	1137.92 (21.82)
Stem Diameter (mm)	15.92 (0.331)*	14.31 (0.308)
Stem Mass (g)	53.63 (6.26)*	32.41 (3.85)
Leaf Biomass (g)	22.21 (1.64)*	15.94 (1.13)
API Mass (g)	59.66 (3.23)*	39.15 (2.37)
Leaf Mass Fraction	0.1361 (0.004)	0.1551 (0.004)
Specific Stem Length (cm/g)	6.17 (0.287)	8.38 (0.486)
Root Mass^ (g)	26.97 (3.91)*	11.83 (1.51)
Root Mass Fraction	0.1229 (0.006)	0.1139 (0.004)
Root Area^ (pixels)	6506.71 (496.97)	5867.33 (442.34)
Root Density^ (pixels root/pixels background)	2.36 (0.099)	2.19 (0.088)
Median Root Width^ (pixels)	52.86 (4.24)	50.42 (4.44)
ARW20^ (proportion)	0.3880 (0.007)*	0.4109 (0.008)
Median Root Tissue Angle^ (°)	41.21 (0.749)	42.73 (0.853)
Minimum Root Tissue Angle^ (°)	8.36 (0.981)*	9.49 (0.910)
Root Tip Paths^ (root tips)	38.61 (2.74)	34.06 (2.07)
Tap Root Diameter^ (mm)	3.50 (0.071)	3.46 (0.066)

Table 3 Result of general linear mixed models fit to several of the response variables for each predictor variable. Below are the F statistics or Chi^2 values for each variable. Sample sizes are also shown.
Significance: ^$P < 0.1$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

	Days to Flower	Chlorophyll Content	Height	Stem Diameter	Stem Mass	Leaf Biomass	API Mass
Block (F Statistic)	0.0196	1.29	0.4762	2.62	6.11*	3.97*	1.41
Treatment (F Statistic)	26.43***	98.28***	20.06***	24.98***	39.15***	28.92***	47.98***
Line (Chi^2)	137.73***	52.00***	99.34***	59.30***	78.61***	79.51	69.66***
Line by Treatment (Chi^2)	0	0.104	0.1819	0.2557	7.07**	0.9717	1.27
Sample Size	671	780	569	570	578	578	576

	Leaf Mass Fraction	Specific Stem Length	Root Mass	Root Mass Fraction	Root Area	Root Density	Median Root Width
Block (F Statistic)	0.2397	0.5889	7.40**	0.3753	8.01**	5.02*	7.06**
Treatment (F Statistic)	12.51	5.58	27.67***	0.1113	0.4383	2.79^	0.5864
Line (Chi^2)	15.91***	69.86***	41.96***	2.03	39.55***	24.44***	16.84***
Line by Treatment (Chi^2)	0.0496	6.18*	0.739	4.40	0.62	0	0.1629
Sample Size	226	567	227	227	219	219	219

	ARW20	Median Root Tissue Angle	Minimum Root Tissue Angle	Root Tip Paths	Tap Root Diameter
Block (F Statistic)	7.02**	1.07	3.64^	20.44***	3.56^
Treatment (F Statistic)	5.41*	2.18	3.99*	0.7339	0.1007
Line (Chi^2)	0	0.6268	3.15^	30.51***	0
Line by Treatment (Chi^2)	0	0	0	0	0
Sample Size	219	219	219	219	218

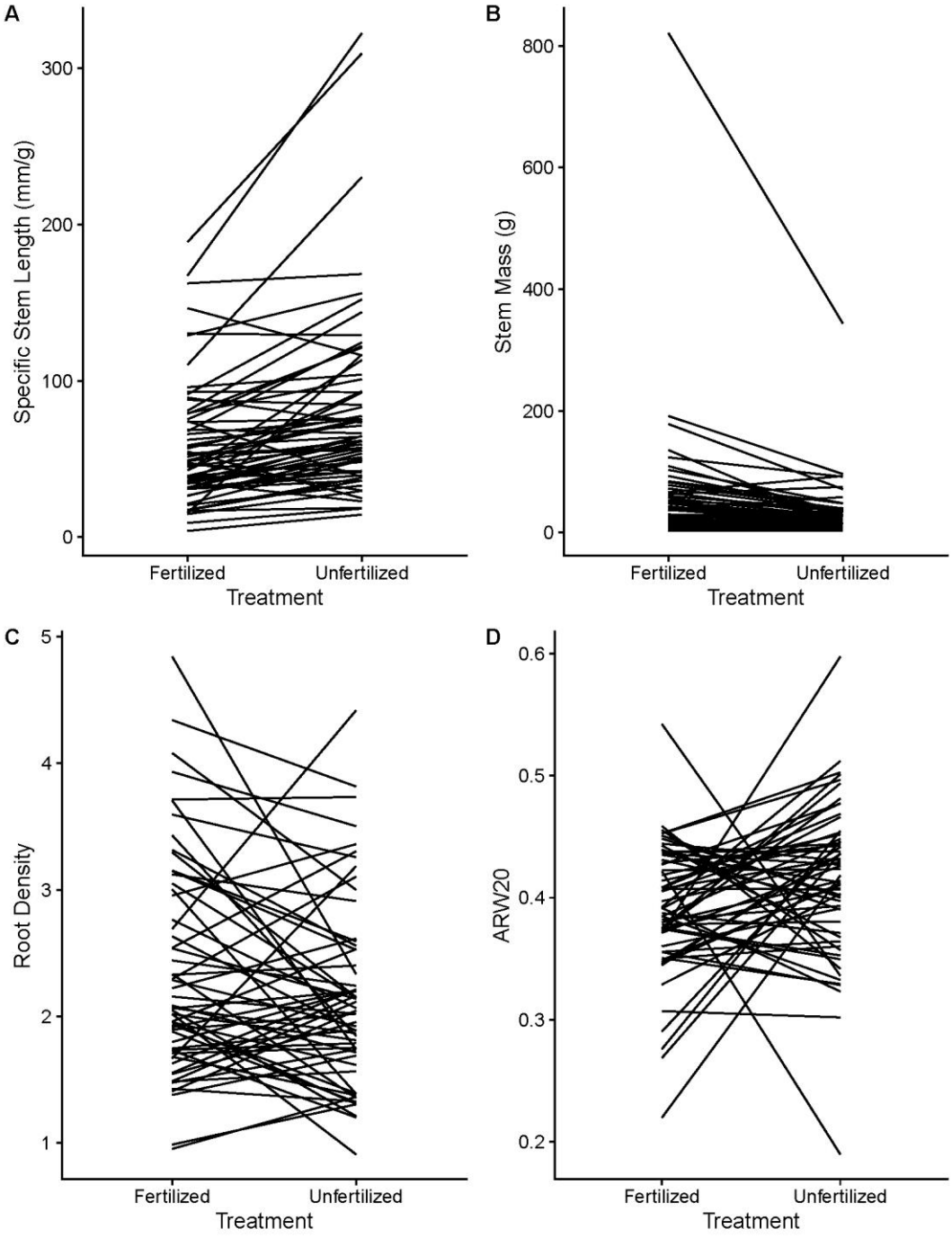

Figure 1 Representative reaction norm plots. These plots show average specific stem length (A), stem mass (B), root density (C), and ARW20 (D) for each of the 60 SAM lines in the fertilized and unfertilized treatments. Each black line represents a SAM line. These plots are representative of most traits.

Trait Responses to Block

Block accounted for more variation between DIRT-derived traits than either treatment, line, or line x treatment. For root traits, root mass, root area, root density, median root width, ARW20, and root tip paths were all significantly different by block. Out of all other traits only stem mass and leaf biomass showed significant differences by block. Water content data taken during the first two months of the experiment showed the average water content for blocks 1 and 2 were 36.9 and 45.2 (percent saturation), respectively (Figure 2). This difference was highly significant ($p = 8.26\text{x}10^{-9}$).

Defining Resistance

We defined resistance as the residuals from a model regressing each line's API mass in the unfertilized treatment on its API mass in the fertilized treatment (Figure 3). The regression line fell further below the one to one line (i.e., API was the same for both treatments) as API mass increased. The five lines that deviated the most above the regression line, and had the highest residuals, were defined as the most resistant to nutrient stress. The most resistant SAM lines were: 75, 187, 86, 84, and 48. The five lines that fell the furthest below the regression line, and had the lowest residuals, were defined as the least resistant (or most susceptible) to nutrient stress. The least resistant SAM lines were: 170, 182, 39, 268, and 206. All 10 most and least resistant lines were in the top half of all lines for average API mass in the fertilized treatment. The difference was that the most resistant lines were able to maintain their API mass in the unfertilized treatment, while the susceptible lines lost API mass. All of the most resistant lines fall very close to the one to one line, some are even slightly above it. These lines were able to fully maintain their fertilized API mass in the unfertilized treatment.

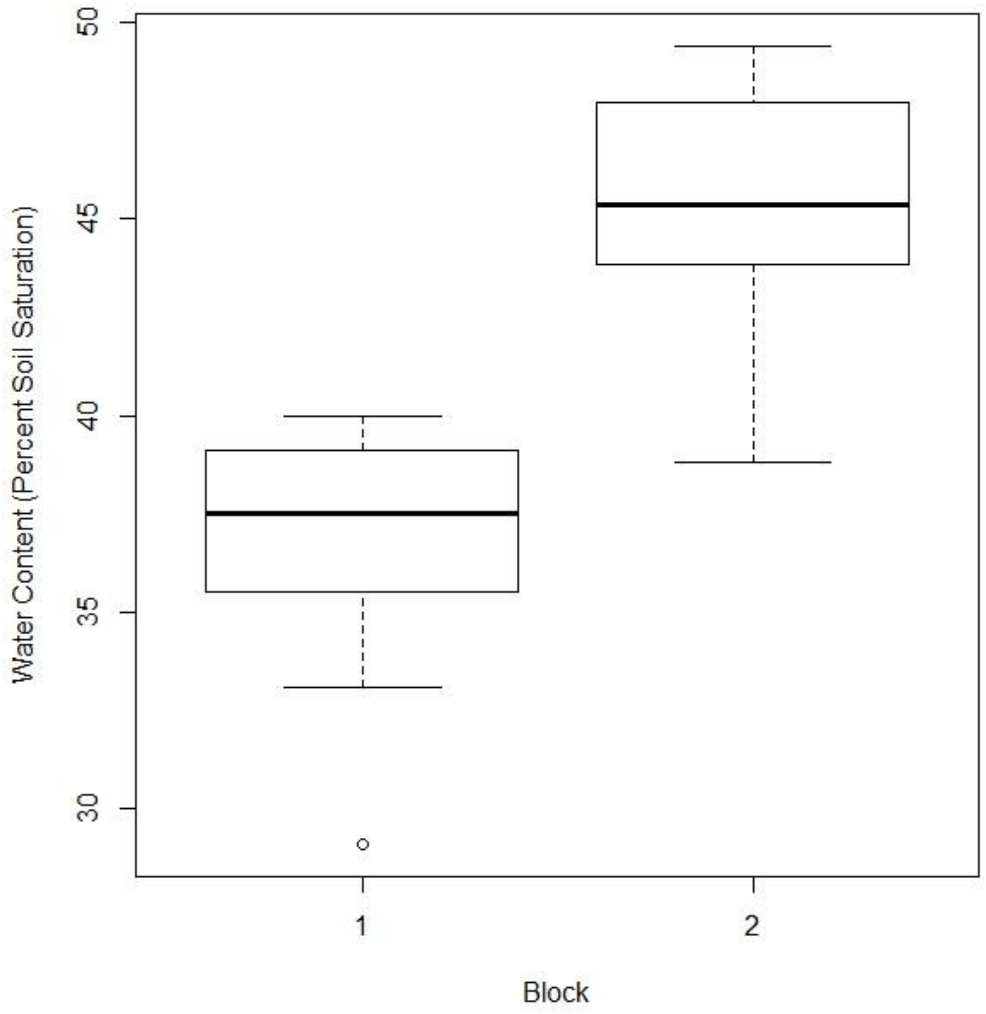

Figure 2 A boxplot showing the differences in water content between blocks 1 and 2. This difference is statistically significant ($p = 8.26 \times 10^{-9}$).

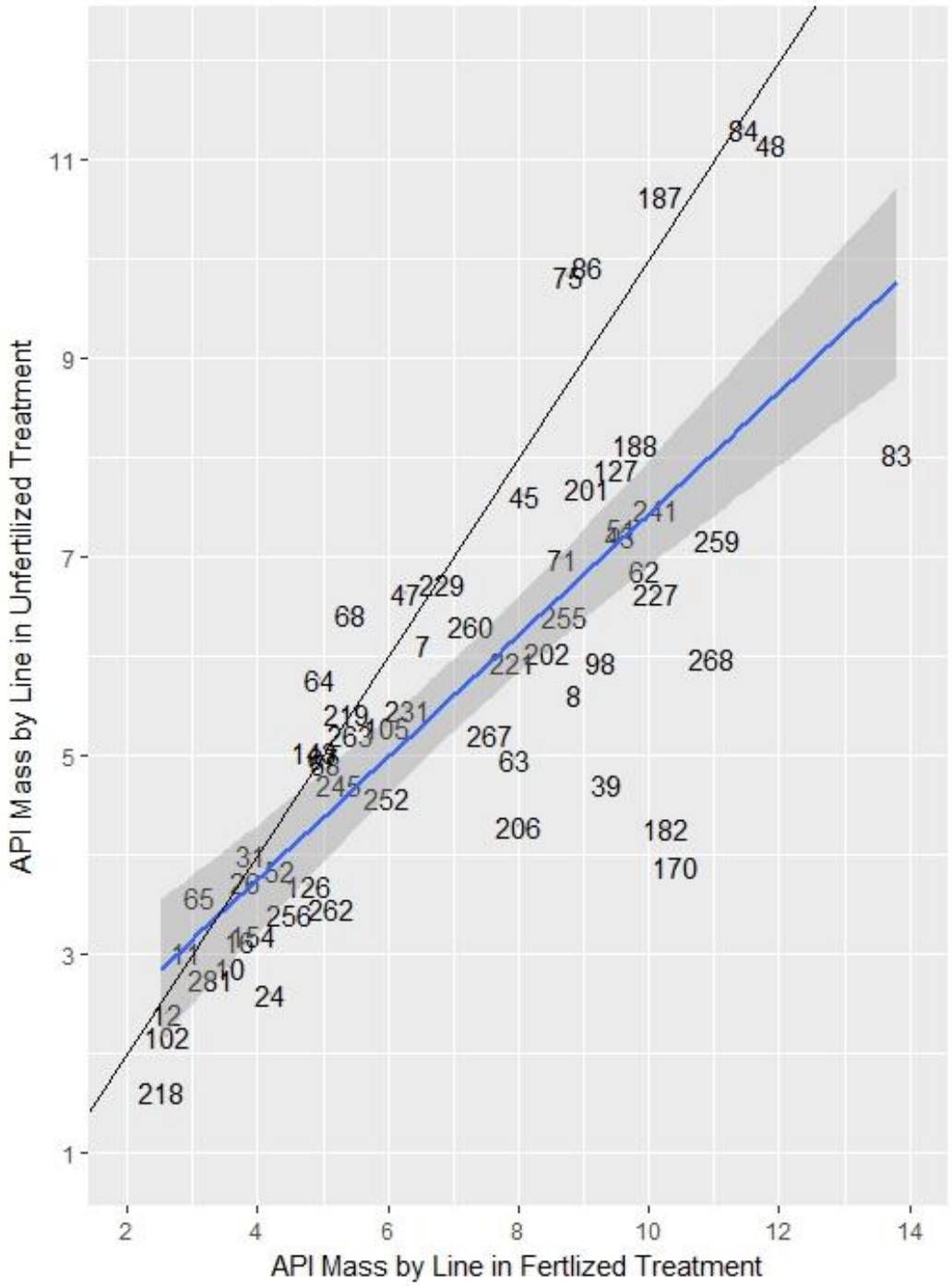

Figure 3 A scatter plot showing transformed (API^0.1) mean API mass for each line (numbers shown) in the fertilized treatment by the transformed mean API mass for each line in the unfertilized treatment. The black, one to one line has a slope of 1. The blue line is the best fit line for the regression. The higher above the blue line each genetic line is, the more resistant it is. The further below the blue line the more susceptible the genetic line is. The gray area indicates the 95% confidence intervals for the regression.

Correlations with Resistance

Size traits from the unfertilized treatment were always significantly correlated with resistance. Generally, plants that were larger in size in the unfertilized treatment were also more resistant. The correlations were negative for LMF and SSL, indicating that lines with less leaf mass relative to overall mass and more stem mass relative to height were more resistant to nutrient stress. Root area, root density, median root width, and root tip paths in the unfertilized treatment were also significantly or marginally significantly, positively correlated with resistance (Table 4). No correlations with resistance were significant for the fertilized treatment.

Principal Component Analysis of Plasticity

The traits that loaded the most highly on PC1 were size traits, as all these traits except LMF, which is a relative size trait, had a loading above 0.25 or below -0.35 (Figure 4 and Table 5). The root traits, root area and root tip paths also loaded above this threshold. The remaining root traits loaded between 0.1 and -0.1. Also worth noting was SSL, which was the only trait to have a large, negative loading onto PC1. PC1 accounted for 32% of the total variation for the plasticity PCA. PC1 was also significantly correlated with resistance ($R^2 = 0.41$, $p = 4.43 \times 10^{-8}$) suggesting a strong relationship between plasticity of these traits and resistance to nutrient stress (Figure 5).

Table 4 Correlations between each trait and the resistance measure separated by treatments. *R* values are shown. Significance: ^*P* < 0.1; **P* < 0.05; ***P* < 0.01; ****P* <0.001.

	Days to Flower	**Chlorophyll Content**	**Height**	**Stem Diameter**	**Stem Mass**	**Leaf Biomass**
Correlation (Unfertilized)	-0.11	0.11	0.30*	0.42***	0.43***	0.43***
Correlation (Fertilized)	0.002	-0.03	0.03	0.02	0.03	0.02
	Leaf Mass Fraction	**Specific Stem Length**	**Root Mass**	**Root Mass Fraction**	**Root Area**	**Root Density**
Correlation (Unfertilized)	-0.27*	-0.45***	0.46***	0.08	0.29*	0.28*
Correlation (Fertilized)	0.09	-0.01	0.06	0.08	0.05	0.05
	Median Root Width	**ARW20**	**Median Root Tissue Angle**	**Minimum Root Tissue Angle**	**Root Tip Paths**	**Tap Root Diameter**
Correlation (Unfertilized)	0.24^	0.18	-0.09	-0.07	0.25^	0.18
Correlation (Fertilized)	0.08	0.01	0.13	-0.19	0.02	0.03

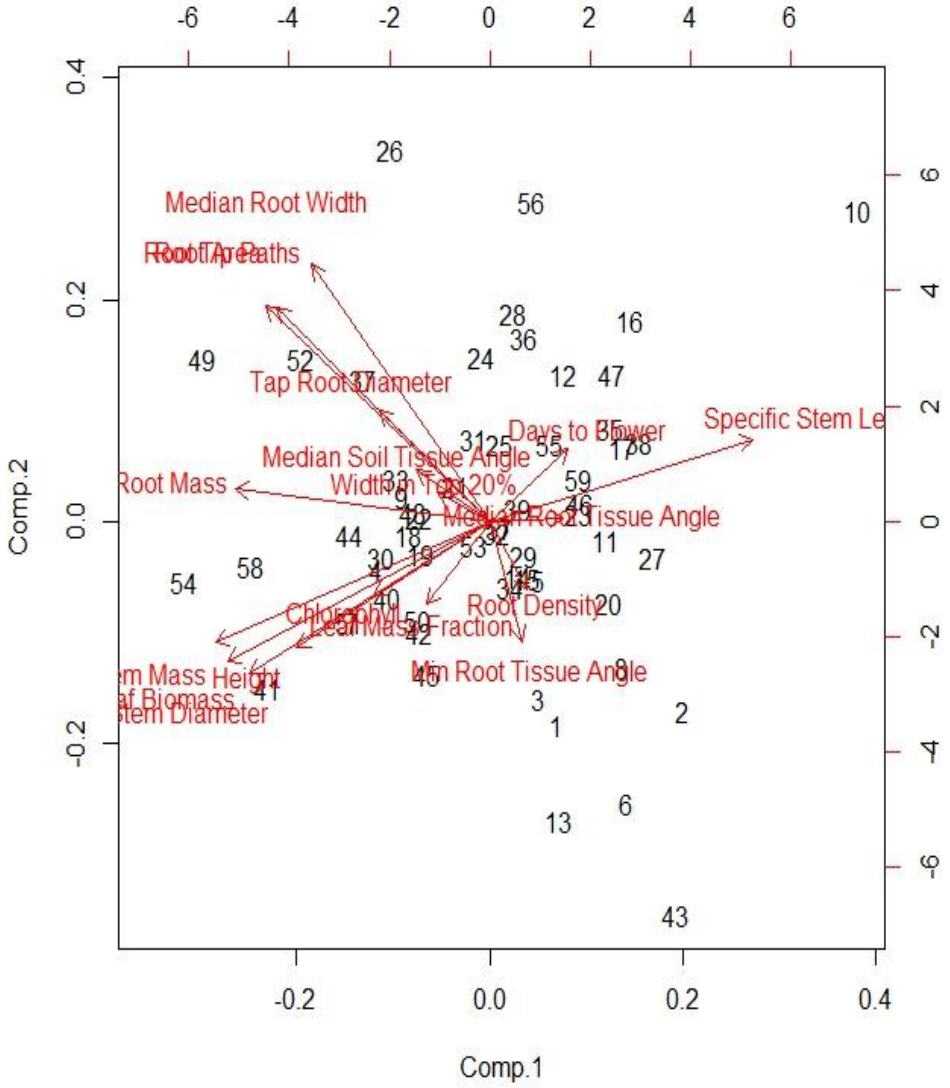

Figure 4 Biplot from the principal component analysis on the trait plasticity values. The longer the red arrow, the higher the loading for that trait's plasticity. Lines that are more horizontally orientated load higher on the first principal component, whereas more vertical lines load higher on the second component.

Table 5 Loadings for the first principal component (PC1) from a principal components analysis of the plasticity of each of the below traits. Loadings are listed from high to low. Signs were inversed for ease of explanation, which is why Figure 4 is the mirror image of these loadings. Only loadings of > 0.1 or < -0.1 are listed.

Plasticity Trait	PC1 Loadings
Stem Mass	0.369
Leaf Biomass	0.353
Root Mass	0.342
Stem Diameter	0.324
Root Area	0.302
Root Tip Paths	0.287
Height	0.260
Median Root Width	0.240
Chlorophyll Content	0.157
Tap Root Diameter	0.148
Days to Flower	-0.105
Specific Stem Length	-0.353

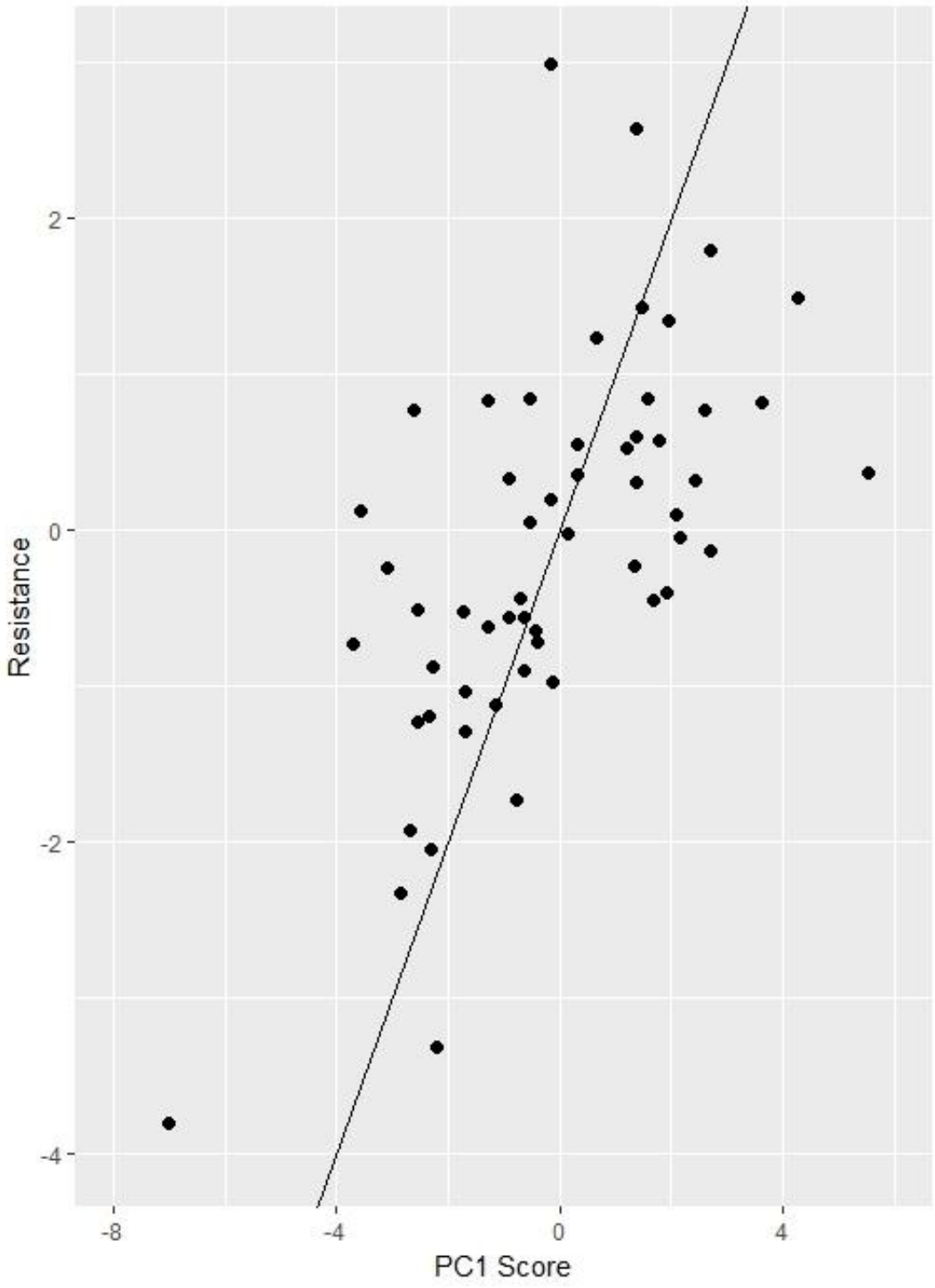

Figure 5 A scatterplot showing the correlation of resistance and PC1 for each line. Lines are indicated using black dots and the best fit line is shown. $R^2 = 0.41$ and $p = 4.43 \times 10^{-8}$.

CHAPTER V

DISCUSSION

General Trait Responses to Nutrient Stress

Nutrient stress of the unfertilized treatment resulted in smaller plants that flowered later and had less leaf chlorophyll. These patterns are consistent with expectations because nitrogen limitation can limit production of enzymes related to photosynthesis and this limitation will reduce the production of photosynthates necessary for plant growth (Rabbinge et al. 1990). Plants that were limited by nitrogen (unfertilized) would be limited in their ability to produce chlorophyll (Schertz, 1921, 1929, Evans 1989, Rabbinge et al. 1990). However, it is common for crops to "bolt" in low nutrient environments. These plants flower quicker to produce seeds before they die. For example, *Arabidopsis thaliana, Perilla frutescens*, *Lemna paucicostata,* and *Pharbitis nil*, low nitrogen or low sugar concentration has been shown to accelerate flowering (Tanaka et al. 1991, Kolár and Seňková 2008, Wada and Takeno 2010). This response contradicts our results. However, a mechanism for delayed flowering with low nutrients has been shown (Jeong et al. 2015).

Few DIRT-derived traits exhibited significant differences between treatments. One explanation for these data is that sample sizes for the root traits were less than half as much as for the majority of other traits, as we were only able to obtain roots for a subsample of the population. However, root mass had a low sample size and differed greatly between treatments, suggesting that sample size is not the only reason the traits measured by DIRT appeared to have little response to treatment. Unlike overall size traits, components of root architecture may respond differently to nutrient stress (Lynch

and Van Beem 1993, Zobel 1996). For example, a plant could improve nutrient uptake efficiency in the unfertilized treatment by increasing root density in pockets of soil with high nutrients, but overall root density could still decrease (Drew 1975). The DIRT system cannot measure variation in root density within individuals. Another example would be median RTA. Plants could have high RTAs in the top soil to take advantage of high nutrient concentrations (Lynch and Brown 2001, Zhu et al. 2005), but could have lower RTAs for deeper roots to take up water (Zhu et al. 2005). These plants could have similar median RTA to plants that do not show these responses making differences in median RTA difficult to detect by the DIRT program.

Lack of an ability to measure more detailed root architecture may also contribute to why some DIRT root traits did not exhibit significant line effects. We expected to observe line effects for most traits as our 60 lines represent the majority of genetic variation (for nutrient resistance) segregating in the entire 288 SAM population. All size traits met this expectation except for leaf biomass. Root area, root density, median root width, and root tip paths also differed significantly by line as would be expected because high within-species variability in root architecture has been shown in other crops (O'Toole and Bland 1987, Lynch and Van Beem 1993, Izumi et al. 1996). RMF, ARW20, the two root angle traits, and tap root diameter had almost no detectable line effects, suggesting that there is little genetic variation for these traits. If that is the case, it will be difficult to use genetic mapping to identify underlying causal loci for these traits. It is also possible that line differences for these traits are not detectable from a two dimensional image.

Root traits derived from DIRT were also more affected by block than by treatment. The only non-root traits different by block were stem mass and leaf biomass. Based on water content data taken during the first two months of the experiment it is hypothesized that the southern part of the field (block 2) had a higher water table than the northern part (block 1) and, therefore, had more access to water. All plants in our experiment were irrigated, so it is unlikely that water availability was a limiting factor that affected size traits. However, root growth can be affected by water availability through hydrotropism (Dietrich 2018).

Specific Trait Responses to Treatment

Minimum root tissue angle and ARW20 were the two root traits that differed significantly by treatment. The lowest angled lateral roots of unfertilized plants were more horizontal than the fertilized plants on average. Some root angle traits like root steepness have been shown to respond to low nutrients (Bonser et al. 1996, Trachsel et al. 2013). However, in this experiment unfertilized plants did not show significantly steeper (lower) root tissue angles.

Unfertilized plants had a higher ARW20 than fertilized plants even though the fertilizer had been top dressed. Top soil foraging has been documented as response to low nutrient stress (Lynch and Brown 2001, Zhu et al. 2005) but to our knowledge it has not been previously shown in sunflowers. Typically, there are more nutrients in the top soils, so nutrient stressed plants will allocate more of their root width to explore the top layers of soil (Lynch and Brown 2001, Zhu et al. 2005). Our results suggest that crop sunflowers also exhibit this allocation behavior.

The only two traits with detectable genetic variation by treatment were related to stems, stem mass and SSL. However, all size and other traits except leaf biomass, LMF, and SSL were significant by both treatment and line. This indicates that there was genetic variation and detectable, consistent responses to treatment for these traits.

Resistance to Nutrient Stress

We proposed a model to estimate resistance based on each line's relative ability to maintain head mass (API) under low nutrient conditions. This identifies the most and least resistant lines in the experimental population for further, more detailed phenotypic study and genetic analysis. The lines most resistant to nutrient stress were 75, 187, 86, 84, and 48, whereas the most susceptible lines were 170, 182, 39, 268, and 206. These lines warrant a more detailed comparison to detect commonalities among lines that are resistant that differ from lines that are susceptible. About a third of the lines fell within the 95% confidence intervals around the model best fit line, suggesting that twice as many had resistance values outside what would be expected. If the most feeble lines (those that have a fertilized API mass of < 5 and an unfertilized API mass of < 4) are discounted, the majority of remaining resistant (positive residual values) and susceptible (negative residual values) lines have relatively close mean and range values for fertilized API mass. Susceptible lines had a mean fertilized API mass of 9.4 versus the resistant line's mean fertilized API mass of 7.5 (transformed API masses). The masses ranged from 6.0-13.8 and 4.9-11.9 (transformed API masses), respectively for susceptible and resistant lines. However, the resistant lines are able to maintain that API mass in the unfertilized treatment, whereas the susceptible lines lose mass. API mass was the best

estimate of overall seed yield from this study. However, seed mass or oil content should be used for calculating resistance when available.

Traits and Resistance

All size traits from the unfertilized treatment were significantly correlated with resistance. This is not surprising given that we defined resistance using a size trait (API mass). The plants with the largest heads in the unfertilized treatment were the most resistant, and large plants produce large inflorescences. It is more interesting that lines that produced less leaf mass per body size and less dense stem tissue in the unfertilized treatment were more resistant to nutrient stress. One explanation for these results is that sunflowers in our experiment were not limited by sunlight or CO_2 so investing in leaf biomass did not provide an increase in overall plant size and API mass. Only extremely low light and CO_2 levels make increased LMF necessary (Poorter et al. 2012). At the time of flowering, sunflower leaves contain a majority of the plant's nitrogen, so if nitrogen is limiting it may be advantageous to reduce LMF (Hocking and Steer 1983). Similarly, investing in height relative to stem mass produced no benefit and lowered resistance. Interestingly, both height and stem mass are significantly positively correlated with resistance. Yet the ratio of the two traits creates an even stronger negative correlation. This implies that a lower stem density is the best predictor (besides root mass) of a line's resistance.

Resistance was also related to increased root mass, root area, and root density. Root mass and, less directly, these other root traits, are likely correlated with resistance because larger and more resistant plants also produced larger roots. The fact that RMF

was not correlated with resistance adds further evidence that the correlation between root mass and resistance is due to root mass being a size trait. Root area and density may also contribute more directly to resistance by increasing the plant's reach to soil nutrients under nutrient stress. Even though these data don't demonstrate this, studies have shown that nutrient stressed plants will allocate more resources to their roots (Chapin 1980, Tilman 1988, Tilman and Wedin 1991, Nielson et al. 1998). However, it has also been shown that increasing root area makes several crops more efficient at nutrient uptake (Trubat 2006), and that crop plants have increased root density in response to low nutrients **(**Lynch and Brown 2001, Zhu et al. 2005**)**. The data from this study supports the latter two responses. Greater root density increases the volume of soil that the roots come in direct contact with allowing access to more soil nutrients and higher nutrient stress resistance **(**Lynch and Brown 2001, Zhu et al. 2005). Root mass, area, and density warrant future study in crop species under nutrient stress and may be able to improve stress resistance without decreasing yield (Lynch 2007).

Plasticity and Resistance

We also examined the relationship between resistance and trait plasticity between the nutrient environments. After excluding our resistance measure (API mass), all traits were included in a principal components analysis and the first principal component (PC1) was strongly correlated (R^2 = 0.41*)* with resistance. Our model posits that traits that load heavily on PC1 contribute the most to nutrient stress resistance. The traits that loaded the most on PC1 (above 0.25 or below – 0.24) were stem mass, leaf biomass, SSL (only large negative loading), root mass, stem diameter, root area, root tip paths, and height. These

data support the correlation results and further suggest that plants that are able to maintain or even improve body mass (of shoots and roots) under nutrient stress are also able to maintain yield. More resistant plants were also able to increase their root area and root tip path number under nutrient stress. This could be due to the increased volume and absorption points to uptake nutrients allowing for more resources to put into a larger API.

CHAPTER VI

CONCLUSION

In an attempt to describe how crop sunflower responds to nutrient stress and show the genetic variation in these responses, this study showed that size traits, chlorophyll content, and days to flower were more often different by treatment effect than by block or line differences. However, increased ARW20 should be studied further as a possible response of sunflowers to nutrient stress. As expected, most of the analyzed traits showed significant genetic differences, although root traits showed fewer genetic differences than size traits.

After defining resistance as the resulting residual value from a regression of unfertilized API mass as a function of fertilized API mass, it was shown that most size traits were significantly correlated with resistance. Due to their significant correlation with resistance, root mass, root area, root density and the maintenance or increase of many traits in the unfertilized treatment should be studied further as they may be have potential to improve future crop breeding and engineering efforts.

Future studies should increase the number of roots destructively sampled to increase statistical power. This may lead to more detectable responses to nutrient stress. Also, resistance should be defined using better yield traits, such as seed mass or oil content. These traits will give better insight into crop resistance to nutrient stress and be more interesting to crop breeding efforts. Finally, a field site should be chosen that has more consistent water availability. Although I believe our models controlled for these differences in water availability it is possible that more root responses to nutrient stress will be detectable without this extra factor.

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