Middle-Season Drought Tolerance in a RIL Population of Cultivated Peanut

by

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Abstract

Grown extensively in Asia, Africa, and the Americas, cultivated groundnut or peanut (*Arachis hypogaea* L.) is one of the world's most important food and oilseed crops. However, a vast majority of peanut crops are grown in nonirrigated conditions and are vulnerable to the effects of seasonal droughts. Methods of efficiently evaluating drought tolerance and understanding the inherent principles of traits related to drought tolerance are critical for the success of peanut breeding programs that aim to improve it. Therefore, the objectives of this research were to examine the consistency of traits related to drought tolerance, to examine their relationships with yield after drought stress, and to estimate their heritabilites. A RIL population of 149 runner peanut genotypes, resulting from the cross of 'Tifrunner' × 'C76-16' was examined for middle-season drought tolerance over two different growing seasons, using an augmented experimental design. Plants were grown in environmentally-controlled rainout shelters and phenotyped using specific leaf area (SLA), visual ratings, and infrared photography. SLA measurements were taken before drought, after drought, and after recovery. Results indicate that SLA measurements taken after recovery demonstrate the strongest correlation with yield for this population ($r = -0.23$, $p = 0.0027$) and that neither visual ratings nor infrared photography were statistically correlated with yield. Broad-sense heritability estimations were calculated for all traits studied and yield per se was calculated to be the most heritable. Top and bottom bulks from the population were identified for the highest and

lowest yielding genotypes across both years and treatments. These lines are also valuable materials for identifying the genes responsible for drought tolerance in peanut through the candidate gene expression approach.

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Through times of stress, discouragement, and anxiety about matters under the sun, I attribute any measure of perseverance to Jesus Christ, the Son of God, who continually reminds me that preserving my soul is more valuable than gaining even the entire world. In Him alone, my life has both perspective and purpose.

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List of Abbreviations

AFLP	amplified fragment length polymorphism
ANOVA	analysis of variance
ARS	Agricultural Research Service
bp	base pair
C	Celsius
CAP	cleaved amplified polymorphic sequence
CIM	composite interval mapping
cM	centiMorgan
DAE	days after emergence
DAF	DNA amplification fingerprinting
DAP	days after planting
DM	dry mass
EST	expressed sequence tag
ET	evapotranspiration
E-QTL	epistatic QTL
Gbp	giga base pairs
HI	harvest index
ICRISAT	International Crops Research for the Semi-Arid Tropics
IR	infrared

kg	kilogram
kPa	kilopascal
LA	leaf area
LG	linkage group
m	meter
MAB	marker-assisted breeding
MAS	marker-assisted selection
MS	mean square
MSE	mean square error
M-QTL	main effect QTL
nm	nanometer
NPRL	National Peanut Research Lab
PCR	polymerase chain reaction
PIC	polymorphic information content
PVE	phenotypic variation explained
RIL	recombinant inbred line
QTL	quantitative trait loci
RAPD	random amplified polymorphic DNA
RFLP	random fragment length polymorphism
SAS	Statistical Analysis Software
SCMR	SPAD chlorophyll meter readings
SLA	specific leaf area
SNP	single nucleotide polymorphism

SPAD	soil plant analysis development
SSR	simple sequence repeat
T_c	canopy temperature
TE	transpiration efficiency
USDA	United States Department of Agriculture
WUE	water use efficiency

Literature Review

Phenotyping and Genotyping for Drought Tolerance in Cultivated Peanut

Grown throughout the world, and extensively in Asia, Africa, and the Americas, cultivated groundnut or peanut (*Arachis hypogaea* L.) ranks as the world's 13th most important food crop and fourth most important oilseed crop. While its major producers are China, India, Nigeria, and the United States, over 45 million metric tons (in shell) are currently being produced on approximately 21.8 million hectares, on six different continents (FAO 2013). In addition to being a rich source of oil (44-50%), protein (20-35%), and carbohydrates (10-20%), peanut seeds contain vitamin E, niacin, falacin, calcium, phosphorus, magnesium, zinc, iron, riboflavin, thiamine, and potassium (Varshney et al., 2009).

Peanut (*Arachis hypogaea)* is a self-pollinated, annual, allotetraploid (AABB genome, 2n = 4x = 40), although most of its wild relatives are diploid species (Krapovickas and Gregory, 2007). A member of the Fabaceae family, the species itself is comprised of two subspecies (*A. hypogaea* ssp. *hypogaea* and *A. hypogaea* ssp. *fastigiata*), which are further classified into two (*hypogaea* and *hirsuta*) and four (*fastigiata*, *vulgaris*, *aequatoriana*, and *peruviana*) botanical varieties, respectively. While it has a large genome (2.8 Gbp), since cultivated peanut is believed to be monophyletic in origin, the germplasm shows significantly less genetic diversity than most other cultivated crops, making molecular marker studies for resistances to both biotic and abiotic stresses much more difficult to conduct (Kochert et al., 1996; Zhao et al., 2012).

Drought. Among the various abiotic stresses that peanut crops face, drought is undoubtedly one of the prime concerns, and arguably the most significant factor in limiting both production and quality (Hamidou et al., 2012; Nigam et al., 2005). Since about 90% of the world's peanuts are cultivated in tropical and semi-arid tropical regions, and approximately 65% of United States-grown peanuts are cultivated in dryland, rain-fed conditions, efficient water use is a global concern in peanut production (Hamidou et al., 2013). Losses due to water stress can be substantial, and as Hamidou et al. (2013) reported, can be compounded with increased temperature. When investigating this relationship, they found pod yield decreases of up to 72% in drought conditions, but no decreases in well-watered conditions at high temperatures.

In actuality, however, quantifying the specific effects of drought is far from simplistic or straightforward. Yield losses have been shown to be highly variable and dependent on a variety of factors including timing, intensity, and duration of the drought (Nigam et al., 2005). Additionally, while the exact relationship between preharvest aflatoxin contamination and late-season drought stress is not known, they are clearly and strongly correlated (Dickens et al., 1973; Holbrook et al., 2000; McDonald and Harkness, 1967; Pettit et al., 1971; Wilson and Stansell, 1983).

World peanut productivity is unquestionably limited by water availability. Hubick et al. (1986) have discussed the only three possible solutions to the problem. One solution would be to irrigate dry environments, but the extent to which that is feasible is limited economically (Boyer, 1982; Christiansen 1982). A second solution would be to farm humid areas more intensively, but water is usually limiting even in such humid areas, especially in the United States (McWilliam, 1986). This leaves the third solution-

to select and breed plants that require less water for growth, i.e. to increase water-use efficiency (WUE). In this context, WUE, defined as the ratio of dry matter production to water use, provides a means to compare the variation among genotypes in their ability to produce dry matter in water-limiting conditions, and thus to increase yield (Hubick et al., 1986). This concept has been summarized by Passioura (1977, 1986) for crops in water-limited environments with the following equation:

$$Y = \text{WUE} \times \text{Water used} \times HI,$$

where Y = yield and HI = harvest index.

Phenotyping for Drought-Tolerance. Plant breeding, a practice that spans millennia, was largely an art before the 20th century, and was practiced with little or no knowledge of genetic principles. Consequently, artificial selection for crop improvement has been carried out for centuries, based solely on the phenotypic expressions of the desired species (Stuber et al., 1999). In the past, peanut-breeding efforts for drought-resistance have followed an empirical approach, concerned almost exclusively with kernel yield, but due to the high variability of drought-induced yield losses, and the variety of contributing factors, progress in this endeavor has been slow and limited (Nigam et al., 2005). Ashraf (2010) admitted that several different drought-resistant cultivars/lines of several different crops have been developed over the last century using only conventional breeding approaches, providing a clear testimony to their effectiveness in producing plants with higher yields and abiotic stress tolerances. He then concluded, however, that most modern plant breeders agree that empirical breeding is highly time-consuming, and both cost- and labor-intensive.

In response to these concerns, Gutschick (1988) considered that selecting for yield alone may not be the best approach, but that it would be more successful and efficient to work towards improving growth and yield per resource use, instead (Nautiyal et al., 2002). Additionally, yield is a trait that is highly influenced by environment and genotype × environment interactions, making for highly unpredictable results if it is selected for exclusively (Chandra et al., 2003). Therefore, Chandra et al. (2003) proposed that identifying high-performance genotypes indirectly, according to associated traits that are less sensitive to these genotype-by-environment interactions may be more accurate. Wright et al. (1994) identified WUE as one such associated physiological trait to be targeted in breeding for drought tolerance because of its contributions to productivity when water resources are limited. However, as Wright et al. (1994) noted, WUE is not easy to measure directly in a large-scale breeding program because of practical limitations in measuring transpiration and root biomass in the field. As a result, several easily quantifiable surrogate traits of transpiration efficiency (TE), an important component of WUE, have been identified, among which are leaf carbon isotope discrimination, specific leaf area, and SPAD chlorophyll meter readings (Chandra et al., 2003).

It has been observed that plants contain a smaller ratio of the naturally-occurring stable isotope ^{13}C to ^{12}C than the CO_2 of the air that surrounds them, due to a discriminatory effect that takes place when carbon is fixed in photosynthesis (Farquhar and Richards, 1984). However, since those C_3 plants which have the greatest WUE will fix the most carbon per unit amount of water transpired, Farquhar et al. (1982) predicted that they would also, by mass spectrometry, exhibit a decreased discriminatory effect,

and therefore, a higher ^{13}C /^{12}C ratio. After isotopic analyses of wheat plants, for which long-term accumulation of dry matter and water use was measured, this theory was claimed to be fully confirmed, demonstrating a strong negative correlation between WUE and carbon isotope discrimination (Δ) (r ranging from -0.88 to -0.92, $p < 0.01$) (Farquhar and Richards, 1984; Nageswara Rao, et al., 2001).

Specific leaf area (SLA) is defined by Wright et al. (1994) as the ratio of the leaf area of a leaf sub-sample to the oven-dry weight of the sub-sample. They, along with Nageswara Rao and Wright (1994), observed a strong negative relationship between WUE and SLA ($r^2 = 0.84$, $p < 0.01$), and a strong positive relationship between Δ and SLA (r = 0.90-0.93, $p < 0.01$). This indicates that genotypes with thicker leaves have greater WUE, leading to the conclusion that SLA can be used as a fast, relatively inexpensive method for identifying and selecting peanut genotypes with high WUE (low Δ). This positive relationship between SLA and Δ has been observed to be maintained when combined over both sites and treatments (Nageswara Rao and Wright, 1994), and has been interpreted to mean that SLA is a viable surrogate trait for Δ where mass spectrometry is either unavailable or economically impractical (Wright et al., 1994).

SPAD chlorophyll meter readings (SCMR) are based on the difference between light attenuation at 430 nm (the peak wavelength for chlorophyll *a* and *b*) and 750 nm (near-infrared) with no transmittance, thereby representing the chlorophyll concentration in the leaf (Nageswara Rao et al., 2001). The utilization of SCMR as a selection tool for SLA (and therefore TE) in peanut was first investigated by Nageswara Rao et al. (2001) as a rapid, low-cost, and non-destructive technique for screening large breeding populations. After standardizing SLA-measurement techniques to account for two

environmental variables (radiation and vapor pressure deficit), to consistently use turgid leaves from the second nodal position, and to account for diurnal variations, a significant negative correlation ($r = -0.80$, $p < 0.01$) was found, suggesting that SCMR can be used as a surrogate measure of SLA (Nageswara Rao et al., 2001). If it is accepted that SLA can be used as a surrogate measure for TE (and hence, WUE), then SCMR can be accepted as a surrogate measurement as well.

To be clear, however, the utilization of surrogate traits as a selection technique for peanut genotypes with high WUE is a topic filled with many conflicting studies and conclusions, especially in recent years. Many efforts have been made to improve selection of drought-tolerant genotypes based on physiological traits, but Devi et al. (2010) maintain that most of the attempts have failed due to large genotype × environment interactions for yield. There has been some discussion about deficiencies in the accuracy of the relationship between Δ and WUE, due mainly to the variation of microclimates in field canopies (as opposed to greenhouses) leading to potential differences in stomatal transpiration control (Cowan, 1988; Farquhar et al., 1988). It has also been noted that when plant water deficits are severe, there can be a breakdown in the relationship between WUE and Δ for peanut genotypes, possibly resulting from greater respiratory losses of carbon (Masle et al., 1990; Wright et al., 1993). A study by Condon et al. (2002) noted that Δ has several shortcomings in cereal crops due to an association with relatively slow growth rates, but then went on to point out strong implications that high growth rate and high WUE are more compatible in peanut; therefore making it feasible that consistent gains in yield may be achieved by breeding for higher WUE. After 2 years of phenotyping a RIL population, Krishnamurthy et al., (2007) found results

in stark contrast to several previous studies (Nageswara Rao et al., 2001; Nageswara Rao and Wright, 1994; Wright et al., 1994) regarding relationships between TE and SLA or SCMR, showing only occasional, poor, and stress level-dependent associations. These differing results were attributed to the limited number of genotypes evaluated in the previous studies. The same study found a poor relationship between TE and Δ, but suggested that this may be a result of their procedure of using all the leaves to evaluate Δ, without limiting the evaluations to those produced during the experimental period. Although SLA can be measured easily and cost-effectively, concerns center around the significant influence of factors such as time of sampling, leaf age, and the variable strength of correlations (r ranging from 0.71 to 0.94) between SLA and Δ that have been observed for a range of peanut genotypes and environments (Nageswara Rao et al., 2001). Devi et al.(2011) conducted additional experiments to investigate the relationships between TE and the surrogate traits in both well-watered and water-stressed conditions, with even different results, finding significant correlations between TE and SCMR and SLA, but only in the drought-stress treatment, and no correlation between TE and Δ whatsoever. In conclusion, these findings may mean that direct gravimetric evaluation of TE for phenotyping is more reliable, at least until the previously discussed surrogates (though widely accepted) are demonstrated to be consistently robust enough for selection purposes, and at the very least, they may indicate a need for further studies on the factors influencing Δ, SLA, and SCMR in peanut genotypes (Krishnamurthy et al., 2007; Nageswara Rao et al., 2001).

Since a major determinant of leaf temperature is the rate of evaporation or transpiration from the leaf, leaf temperature measurement using thermal infrared (IR)

sensing is used to study plant water relations. Because of this, thermal imaging is a well-suited screening device for observing differences in stomatal conductance among plants (Jones et al., 2009) and differences in canopy temperatures among crop cultivars are known to be related to drought avoidance characteristics (Garrity and O'Toole, 1995). Canopy temperature as a screening tool was reviewed by Blum as early as 1988 and claimed to be a useful technique when implemented with other practical measures, if care is taken in the measurements. Canopy temperature is a very appealing technique because it can be measured rapidly, nondestructively, and nondisruptively (Garrity and O'Toole, 1995).

Garrity and O'Toole (1995) assessed the canopy temperature response of a range of rice (*Oryza sativa*) germplasm in an attempt to develop a practical field screening system for reproductive phase drought resistance. To obtain canopy temperature (T_c) measurements, the researchers used a Teletemp AG-42 handheld infrared thermometer with an 8º field of view and equipped with a 10.5- to 12.5-µm bandpass filter. They observed the canopy from an oblique angle of about 30º above horizontal, from a distance of about 3 m from the plot center to the instrument to provide a target ellipse on the canopy surface of about 0.07 m^2. Measurements were taken on 12 dates in one trial and 8 dates in another trial, each at solar noon to minimize solar angle interactions with the viewing direction. With this setup, they observed mean T_c that increased from 28 to 37 ºC during the stress period. Additionally, visual drought tolerance scores ($r = 0.72$) and leaf rolling scores ($r = 0.68$) were correlated with mean canopy temperatures under moderate water stress, but not under severe stress ($p < 0.01$). With these findings, Garrity and O'Toole (1995) judged that infrared thermometry is well-suited to monitor the

progression of crop water stress development, and to aid in classifying cultivars for relative drought avoidance. However, the researchers warned that caution must be exercised to ensure proper application of the technique and proper data interpretation.

The utility of canopy temperatures for the identification of drought-resistant genotypes has not been well explored in peanut, but one study (Jongrungklang et al., 2008) did investigate the relationship of canopy temperature with WUE, among other phenotypic measures, using peanut germplasm of diverse origins. T_c measurements were taken from 3 plants for each plot at 12.00-14.00 am at 30, 60, and 90 DAE using an infrared thermometer (Testo 830-T1, Testo Inc., Germany). As expected, the study found that T_c measurements generally increased with drought conditions, noting that peanut genotypes with lower canopy temperatures are preferable due to their higher transpiration and therefore, higher CO_2 exchange rate compared to genotypes with higher canopy temperatures. In terms of the relationship between WUE and T_c, a correlation was found in a decreasing pattern in the negative direction, beginning with negative and significant correlation under well-watered conditions and becoming non-significant under severe drought stress.

Thermal imaging using IR is an established technology for phenotyping plants for differences in stomatal behavior, and as such possesses enormous potential for measuring plant response to water deficit, but as outlined in detail by Jones et al. (2009), the technology is not without its difficulties. Particular problems include the sensitivity of leaf temperature to temporal and spatial variation in absorbed radiation, with leaf temperatures varying up to 15 ºC between full sun and deep shade. However, the authors maintain that clear genotypic variation may be detected by using appropriate

normalization techniques to account for variation in soil moisture status or incident radiation in the field, and by using appropriate data analysis techniques. In all the studies discussed by Jones et al. (2009), thermal images were obtained using a Thermacam P25 (FLIR Systems, Danderyd, Sweden) long-wave thermal imager with a sensitivity of 0.08 ºC and accuracy within 2 ºC.

Genotyping for Drought-Tolerance. Controlled by many minor genes, called polygenes, drought-tolerance is not a simple response, and because of its complex interactions with the environment, correct selections based strictly on phenotype would prove very difficult to achieve (Collins et al., 2008). These polygenes have additive effects in their expression, and therefore occupy loci on chromosomes referred to as quantitative trait loci (QTL) (Ashraf, 2010). Ashraf (2010) concluded that the multiplicity of these genes, coupled with their additive effect and their interaction with the genes involved in yield potential, could be the reason for the limited progress that has been made in improving crop drought tolerance. The concepts of detecting QTL were devised 80 years ago (Sax, 1923), but the increased knowledge, in recent years, of their effects and numbers can give breeders the power to understand the genetic control of many traits of interest and how to develop more efficient methods of improving them (Broman and Speed, 1999).

Mapping QTL in plants conventionally begins with generating a population (F_2, backcross, recombinant inbred, etc.) from a biparental cross, genotyping the individuals with genetic markers that span the genome, phenotyping the individuals for the trait(s) of interest, and then finally analyzing the results via linkage mapping to pinpoint the loci

controlling the trait (Ashraf, 2010; Asins, 2002; Flint-Garcia et al., 2005). It is only by analyzing both the segregation of marker genotypes and the phenotypic values of individuals together that it is possible to detect and locate the loci affecting QTL (Asins, 2002). Once the segregating progeny of differing parental lines are analyzed, and the QTL are linked to known DNA markers, breeders can use this information to make selections (at least partially) on genetic information acquired through molecular markers, in a method called marker-assisted selection (MAS) (Asins, 2002). MAS, the core component of marker-assisted breeding (MAB), provides the capability to investigate the usefulness of thousands of genomic regions of a crop germplasm under drought stress, a previously-impossible process that can enhance plant breeding efforts, enabling accelerated cultivar creation (Ashraf, 2010; Asins, 2002).

To examine how stress tolerance is inherited in QTL, polymorphic molecular markers must be observed among the population being studied. Ashraf et al. (2008) list a variety of DNA markers that have been utilized, including RFLPs, RAPDs, CAPS, InDels, AFLPs, microsatellites (SSRs), and SNPs. Using these different types of markers, QTL mapping for drought tolerance, specifically, has been done in a variety of crops including maize, wheat, barley, cotton, sorghum, and rice (Bernier et al., 2008; Quarrie et al., 1994; Sanchez et al., 2002; Saranga et al., 2001; Sari-Gorla et al., 1999; Teulat et al., 1997). Ravi et al. (2011) pointed out additional studies that have reported QTL for drought tolerance in different crops, including soybean, in which 5 QTL were identified for WUE in an F_2 population with 14-20% phenotypic variation explained (PVE) (Mian et al., 1998), wheat, in which 5 QTL were identified for drought tolerance

with 13-34% PVE (Dashti et al., 2007), and rice, in which 47 QTL were identified for different plant stress indicators with 5-59% PVE.

Before QTL mapping and subsequent MAS for any trait can be accomplished, peanut breeders and geneticists require access to a sufficient number of polymorphic genetic markers to identify high-value makers that are closely linked to traits like drought tolerance (Wang et al., 2012). Because cultivated peanut, unlike many other polyploid crops, is believed to be monophyletic in origin, its germplasm exhibits significantly less molecular genetic variation than most other cultivated crops, resulting in the identification of much fewer polymorphic DNA markers among parental lines (Kochert et al., 1996; Zhao et al., 2012). Consequently, the application of biotechnology for improving peanut has been significantly limited by an inability to visualize genetic variation in germplasm lines (Ferguson et al., 2004).

Since cultivated peanut exhibits substantial morphological and physiological diversity (Stalker, 1992), and the potential benefits of identifying polymorphic molecular markers has long been recognized (Sax, 1923), over two decades of work have gone into searching for discernible DNA variation among its genotypes. However, much of the research of the early 1990s found no DNA variation at all among genotypes using either random amplified polymorphic DNA (RAPD) or restriction fragment length polymorphism (RFLP) approaches (Halward et al., 1992; Kochert et al., 1991; Paik-Ro et al., 1992). Finding polymorphisms with a total of 45 primers, He and Prakash (1997) were the first to report DNA variation in cultivated peanut, using DNA amplification fingerprinting (DAF) and amplified fragment length polymorphism (AFLP) approaches to discover 63 and 111 polymorphic markers, respectively.

The DAF approach is essentially a variation of the RAPD technique that has been shown to be relatively more informative because of altered reaction conditions, shorter primers, and silver staining (Williams et al., 1990; Caetano-Anollés et al., 1991). He and Prakash (1997) used six different genotypes of cultivated peanut from three botanical varieties. After screening 559 different DAF primers using PCR and separating the DNA fragments using vertical polyacrylamide-based vinyl polymer electrophoresis, they found that 17 of the primers they screened displayed polymorphisms, producing an average of 3.7 polymorphic bands per primer, and 63 total polymorphic markers (He and Prakash, 1997).

In the same study, AFLP was presented as a new procedure that not only detects a large number of polymorphic DNA markers quickly, but also more reliably and rapidly than RAPD markers, and more easily than the RFLP technique. Using the same plant materials and basic procedures from the DAF portion of their study, He and Prakash (1997) tested eight primers corresponding to the *Eco*RI adapter and eight corresponding to the *Mse*I adapter in 64 possible combinations. Doing so, they found DNA polymorphism in 28 of the 64 AFLP primer pairs tested, 3.96 polymorphic bands per primer pair, and 111 cumulative polymorphic loci. This AFLP approach detected DNA polymorphism in peanut more efficiently, as 43% of the primer pairs that were tested identified polymorphism, as compared to 3% of the DAF primers; however, both approaches yielded similar levels of polymorphism once an informative primer pair or primer was identified (He and Prakash, 1997).

Later, Bhagwat et al. (1997) screened peanut mutants obtained by X-ray irradiation of cv. Spanish Improved seeds with 42 decamers and one octamer in RAPD

analysis, amplifying a total of 1182 fragments and finding 65 polymorphic bands (5.5%) and a ratio of 1.51 polymorphic bands/primer. Subramanian et al. (2000) also found DNA polymorphisms with the RAPD approach by screening 70 peanut genotypes that exhibited a broad spectrum of phenotypic traits. Using 48 oligonucleotide primers that produced 408 bands, amplified fragments with polymorphism were observed for 7 primers (14.6%) in 27 bands (6.6%) (Subramanian et al., 2000). Building on these findings and to assess genetic diversity in *Arachis hypogaea*, Dwivedi et al., (2001) screened 26 accessions with 8 primers in RAPD analysis. Of 939 amplified fragments, this study found 176 (18.74%) to be polymorphic, an average of 4.51 polymorphic bands/primer, and an average genetic similarity of 86.2% (Dwivedi et al., 2001). Other work has been done with different molecular markers such as RFLP and isozymes, but very little genetic variation has been detected (Stalker and Mozingo, 2001).

In response to reports of simple sequence repeat (SSR) markers being more variable than RFLPs or RAPDs, and their widespread use in human genetics studies, Hopkins et al. (1999) investigated their usefulness in studying DNA variation in peanut. A library screening method was implemented with a diverse array of 22 single plant accessions (including 19 accessions of cultivated peanut) that found five polymorphic markers from 26 primer pairs (19%) (Hopkins et al., 1999). Although a total of only six polymorphic SSRs (one from a search of publicly-available DNA sequences) were identified in the study, it was surmised that these markers detected more variation in cultivated peanut than all other molecular markers studied thus far, representing 17 unique genotypes among the 19 accessions tested.

SSRs, also known as microsatellites, are motifs of one to six bases that are arranged in simple internal repeat structures that are scattered frequently and randomly throughout eukaryotic genomes. Abundant and co-dominant, SSRs comprise the molecular markers with the highest polymorphic information content (PIC) because of their high mutation rate (Krishna et al., 2004; Tang et al., 2007). These features, coupled with added benefits such as high reproducibility have made SSR markers to be considered the markers of choice in crop breeding operations, and among the most widely used in recent years (Gupta and Varshney, 2000; He et al., 2003). Polymorphisms are created when slippage mutations, which cause variation in the number of repeating units, occur during DNA replication. Using primers designed from the conserved DNA sequences flanking the SSR, different alleles of a given locus can be detected by PCR (Mace et al., 2006).

Traditionally, screening genomic libraries by hybridizing with SSR probes and sequencing the hybridized positive clones is the process by which microsatellite markers have been obtained, though it is both labor-intensive and costly (He et al., 2003). He et al. (2003) used an improved technique for SSR development in cultivated peanut by enriching the AFLP or specific adaptor-amplified DNA fragments. After converting the genomic DNA into AFLP fragment assembly, the AFLP fragments were then amplified using selective primers and hybridized with SSR probes. Using SSR markers derived from this method, He et al. (2003) observed polymorphism in 19 of 56 primer pairs (34%), and were able to detect polymorphism among 24 peanut genotypes.

Ferguson et al. (2004) designed 226 total primers from two 27,648-clone libraries (*Pst*I and *Sau*3AI/*Bam*HI) for investigation of SSRs in a diverse set of 24 peanut

accessions. Of these 226 primers, 110 pairs (48.7%) revealed polymorphism, at 123 loci (a possibility because of the presence of two genomes in allotetraploid peanut), 35 derived from the *Pst*I library and 74 derived from the *Sau*3AI/*Bam*HI library. Significant findings included ATT and GA as the most frequent repeat motifs identified (29% and 28%, respectively), and that of the amplifiable primers, 81% of ATT and 70.8% of GA repeats were polymorphic in the cultivated peanut test array (Ferguson et al., 2004).

Many other studies have been done with various peanut plant materials to discover increasing numbers of polymorphic SSR markers. Krishna et al. (2004) screened 48 Valencia peanut genotypes with 18 primer pairs that detected polymorphism, amplifying 119 polymorphic loci. Cuc et al. (2008) screened a diverse set of 32 genotypes with 104 primer pairs that were designed from a microsatellite-enriched library constructed from the genotype TMV2. Forty-six (44.2%) of these primers showed polymorphism, detecting an average of 2.44 alleles per locus and 112 total alleles.

In an effort to increase the number of microsatellite markers for peanut to a point at which linkage maps may be constructed, Moretzsohn et al. (2005) implemented a combination of techniques to develop markers from SSR-enriched genomic DNA libraries, from ESTs, and by data mining published *Arachis* spp. sequences. A single *A. hypogaea* cv. Tatu plant's genomic DNA was digested to construct two different libraries based on the dinucleotide repeat motifs TC and AC, from which 121 primers were designed. After screening 2,740 EST sequences for SSRs, 81 (3.0%) were found suitable for primer design. Finally, 69 additional primers were designed after sequences from GenBank were screened for repeats, making for a total of 271 new SSR markers that were confirmed to be unique, using the Staden-TROLL module and a BlastN search

against a database of all published *Arachis* SSR-containing sequences. These 271 new SSR markers were then screened against six cultivated peanut samples, 234 amplified well, and 66 (28.2%) of these remaining 234 were found to be polymorphic. Sixty-two polymorphic markers were used for the detection of allelic diversity in a sample of 16 *Arachis hypogaea* accessions, finding 2 to 12 alleles at each of the 62 polymorphic loci analyzed, with an average of 5.87 alleles per locus.

Moretzsohn et al. (2005) then used their 271 newly developed SSR markers along with 162 previously-published SSR markers for map construction. After all 433 markers were screened against the progenitors (*A. duranensis*, accession K7988 and *A. stenosperma*, accession V10309), 204 (46.8%) were found to be polymorphic, with 170 segregating codominantly. Of these 170 markers, 80 appeared undistorted from the expected 1:2:1 segregation ratio ($p < 0.05$) and were used to establish the linkage groups (LGs), initially. Other markers (distorted and dominant) were further included in the map. The results mapped into 11 LGs, covering 1,230.89 cM of total map distance. Thirty-six of the 170 loci placed on the map are ESTs or characterized genes. Twenty-five of the other 134, developed from genomic sequences, gave BlastX hits, meaning that about 61 of the mapped microsatellites most likely represent genes. This study was estimated to cover 70.6% and 86.4% of the total genome, with the constructed framework map and total map, respectively. By publishing the first microsatellite-based and gene-rich linkage map for *Arachis*, this study represents a significant improvement in the ability to map useful genes and to pursue MAS effectively. Thusly, this opened up the possibility of using other mapping populations to construct a consensus map for *Arachis*,

map QTLs, and implement MAS strategies as increasing numbers of markers linked to resistance genes are identified (Moretzsohn et al., 2005).

A genetic map based on a cross of a synthetic amphidiploid (TxAG-6) and a US cultivar (Florunner) was developed in the past using RFLP loci by Burow et al. (2001), but as has been stated earlier, RFLP is labor-intensive and reveals relatively less polymorphism than SSR, making it unsuitable for widespread use in breeding programs or MAS. The map generated by Moretzsohn et al. (2005) was innovative, and created from SSRs, but it was only a diploid *Arachis* AA genome map, based on a cross of the most probable AA genome donor to cultivated peanut, *A. duranensis*, with a closely related species. Arguably most noteworthy for MAB, however, Varshney et al. (2009) genotyped a RIL population to construct the first ever genetic map based only on cultivated peanut.

Upon screening the 1,145 SSR markers that were either available in the public domain or presently unpublished, Varshney et al. (2009) found that 144 (12.6%) of them showed polymorphism among the progenitors they were studying. All 144 of these were used to genotype a population of 318 F_8 RILs derived from the cross ICGV 86031 × TAG 24, producing segregation data for a total of 150 SSR loci. Ninety-three of these loci showed the expected 1:1 segregation ratio ($p < 0.05$) and were therefore used to establish LGs, initially. In all, 135 loci were integrated into a total of 22 LGs, covering 1,270.5 cM of total map distance. Importantly, this study produced the first genetic map of cultivated peanut, giving subsequent genetic maps a point of comparison, and demonstrating a vision for future cultivated peanut mapping using SSR markers (Varshney et al., 2009).

After gathering phenotypic data in all 318 RILs for transpiration, TE, SLA, and SCMR for two consecutive years at ICRISAT, this data was analyzed with genotypic data for mapping QTLs by using the composite interval mapping (CIM) method using WinQTL Cartographer, version 2.5. In doing so, Varshney et al. (2009) also produced the first report of identification of QTLs for drought-related traits in peanut, finding 2-5 QTLs for each trait mentioned above and explaining 3.5-14.1% phenotypic variation. Though admitting that none of these identified QTLs demonstrated a high enough PVE for MAB, Varshney et al. (2009) predicted that higher phenotypic variation in the mapping population and higher marker density genotyping data in the future could identify the major QTLs with higher PVE for drought tolerance-related traits.

To saturate the framework map that Varshney et al. (2009) developed, Ravi et al. (2011) first phenotyped a RIL mapping population of 318 $F_8/F_9/F_{10}$ lines (derived from a cross TAG 24 × ICGV 86031) for transpiration, TE, SLA, leaf area (LA), SCMR, Δ, biomass, canopy conductance, total dry matter, dry weight, pod weight, seed weight, and stalk weight for 2-3 seasons. Yield was also monitored for the mapping population in the field under both water-stressed and well-watered conditions. After screening the two parents with the same 1,145 SSRs that Varshney et al. (2009) used and 2,070 additional SSRs (developed at the University of Georgia or genomic) for a total of 3,215 SSR markers, Ravi et al. (2011) obtained segregation data for 215 marker loci. Segregation data for 65 of these loci were obtained directly in this study and were attempted to be integrated into the framework map of 135 loci that Varshney et al. (2009) constructed. Fifty-six of the 65 loci were evenly distributed into 17 of the 22 linkage groups, using Mapmaker ver. 3.0, bringing the present map up to a total of 191 loci, covering 1785.4

cM, with an average of 9.34 cM between loci among the linkage groups. This produced the most comprehensive genetic map of cultivated peanut to date, that is based only on a single mapping population from cultivated tetraploid genotypes (Ravi et al., 2011).

To identify candidate QTL regions for drought component traits, Ravi et al. (2011) used two types of trait mapping: (a) interval mapping to identify main effect QTLs (M-QTLs) and (b) epistatic interaction analysis (EIA) to identify epistatic interactions between different QTL regions (epistatic QTLs, or E-QTLs). To initially find the most likely locations of QTLs and their genetic effects, a CIM technique was implemented using the WinQTL Cartographer, version 2.5. Since QTL identification is a statistical approach, Ravi et al. (2011) admit that there is a possibility of identifying false positive and false negative QTL for the thresholds and mapping approaches used (McElroy et al., 2006; Mackay and Powell, 2007). Thus, to enhance the reliability of the QTLs identified, a second software, QTLNetwork, was used for comparison. Using these methods, Ravi et al. (2011) identified a large number of M-QTLs for several drought component traits (105 with 3.48-33.36% PVE using QTL Cartographer; 65 with 1.3-15.01% PVE using QTLNetwork). Of the M-QTLs observed by the two programs, there were 53 in common, leading to the identification of 117 unique M-QTLs, distributed on 17 of the 22 LG (Ravi et al., 2011).

To look for interactions between different loci, EIA was implemented with Genotype Matrix Mapping (GMM) software, ver. 2.1, which tested for two and three loci interactions. Additionally, QTLNetwork, ver. 2.0, was also used to identify E-QTLs. In total, Ravi et al. (2011) identified not only 186 three-loci interactions with 8.54-44.72% PVE and 63 two-loci interactions with 7.11-21.13% PVE using GMM, but also 8 E-QTL

interactions with 1.7-8.34% PVE using QTLNetwork. After identifying such a large number of QTLs for drought and component traits, with a considerable amount of phenotypic variation remaining unexplained, it was concluded that drought tolerance in peanut is controlled by a large number of M-QTLs and E-QTLs, each producing a small phenotypic variation. The identification of few major, many minor M-QTLs and QTL × QTL interactions (E-QTLs) in this study is evidence of the complex and quantitative nature of drought tolerance in peanut, suggesting that marker-assisted recurrent selection or genomic selection instead of marker-assisted backcrossing will be more efficient for the selection of these numerous small-effect QTLs in the future (Ravi et al., 2011).

Mapping additional QTLs will require further analysis of polymorphic molecular markers, and as has been discussed earlier, SSRs have emerged as the markers of choice. To organize a comprehensive database of polymorphic SSR markers, Zhao et al. (2012) scanned scientific publications from various research groups around the world, determining that there have been a total of 9,274 SSRs reported in cultivated and wild peanut species to date. Among these, 5,949 were determined to be EST-SSRs and 3,328 were determined to be genomic SSRs, from which 603 and 740 were confirmed to be polymorphic at frequencies of 10.1% and 22.2%, respectively. Among these 1,343 polymorphic SSR markers, dinucleotide and trinucleotide repeats were found to be predominant, with 1,508 in total. Of these 1,508 markers, the motifs AAG and AG were most abundant in EST sequences (21.1% and 20.9%, respectively), and the motifs AG, AT, AC, and AAT were the most abundant in genomic sequences (46.7%, 13.6%, 12.3%, and 12.0%, respectively). The information from this database not only facilitates better understanding of the nature of SSRs in the peanut genome, but also provides valuable

tools for conducting additional genetic and genomic studies to improve this crop. With a total of 1,343 polymorphic markers available and organized now, Zhao et al. (2012) asserted that construction of a higher density linkage map with ~500 SSR loci in the cultivated peanut is feasible, which would greatly aid in further molecular research, QTL mapping, and marker-assisted selection for peanut improvement.

Objectives of Research

Means of efficiently evaluating drought tolerance and understanding the inherent principles of traits related to drought tolerance are critical for the success of peanut breeding programs that aim to improve it. Therefore, the objectives of this research were to examine the consistency of traits related to drought tolerance, to examine their relationships with yield after drought stress, and to estimate their heritabilites. This research seeks to provide valuable information for the importance of traits related to middle season drought-tolerance and to produce information that will give breeders crucial knowledge for the advancement of marker-assisted breeding in the future. Additionally, unique genotypes with desirable drought-resistant characteristics should be identified, which can subsequently be used for peanut cultivar improvement.

Phenotyping a RIL Population for Middle-Season Drought Tolerance in Cultivated Peanut

Introduction

Insufficient water at some point in the growing season is often the limiting factor in the production of a peanut crop. Since about 90% of the world's peanuts are cultivated in tropical and semi-arid tropical regions, and approximately 65% of United States-grown peanuts are cultivated in dryland, rain-fed conditions, efficient water use is a global concern in peanut production (Hamidou et al., 2013). Additionally, the effects of drought can be economically devastating when it occurs at critical growth stages (Rivero et al., 2007). While several researchers have reported that the crop's sensitivity to water deficit stress is dependent on the timing of the stress (Klepper, 1973; Martin and Cox, 1977; Pallas et al., 1979), more recent research indicates that early season drought may actually increase yields due to changes in root growth during the vegetative development (Jongrunklang et al., 2011). This is believed to occur because of the peanut plants' ability to recognize drought conditions early enough for plant development to facilitate adaptation by the root system (Songsri et al., 2008), or at the molecular level, for a cascade of responses to become activated by transcription factors through induction of gene-expression (Dang et al., 2012). For reasons including these, the drought stresses that have been shown to be most detrimental to yield are those that occur in the middle of the growing season (Rucker et al., 1995). Water deficit during fruiting (50 to 80 DAP) is known to reduce flowering, pod formation, and yield more than at any other stage.

To minimize drought-related yield losses and to ensure food production for an ever-growing global population, there is an increasingly urgent need to develop better

adaptive agricultural strategies, including the improvement of drought-related traits through direct or indirect selection (Chen et al., 2013). Consequently (and also due to a lack of information on the phenotypic response of crop genotypes), a strong interest has developed in the investigation of traits associated with drought tolerance, to be used in plant breeding programs (Chen et al., 2013). These surrogate traits, including specific leaf area (SLA) are important because of their relationship with water-use efficiency (WUE), which is defined as the ratio of dry matter production to water use (Hubick et al., 1986) and is a component of yield for crops in water-limited environments, as summarized by Passioura (1977, 1986) with the following equation:

$$Y = \text{WUE} \times \text{Water used} \times HI,$$

where Y = yield and HI = harvest index (which was calculated on mature plants as a ratio of pod yield over aboveground biomass plus pod yield). In order to make progress in breeding plants that require less water for growth, it is important to understand the strength of the correlation between WUE and its more easily-measured surrogate traits in a population of plants.

A strong negative relationship between WUE and SLA has been reported in several peanut studies, indicating that genotypes with thicker leaves have greater WUE, and leading to the conclusion that SLA can be used as a fast, relatively inexpensive method for identifying and selecting genotypes with high WUE (Wright et al., 1994; Nageswara Rao and Wright, 1994; Nigam et al., 2005). Additionally, SLA has been estimated as highly heritable by Songsri et al. (2008), who found estimates ranging from 0.81 to 0.95 under drought stress and non-drought stress conditions, respectively.

Infrared imaging possesses enormous potential for measuring plant response to water deficit; however its utility as a surrogate trait for WUE or as a screening tool for drought resistance in peanut has not been extensively explored. To our knowledge, only one study to date (Jongrungklang et al., 2008) has investigated the relationship of canopy temperature with WUE in peanut. As expected, using peanut germplasm of diverse origins, the study found that canopy temperature (T_c) measurements generally increased with drought conditions, noting that peanut genotypes with lower canopy temperatures are preferable due to their higher transpiration and therefore, higher CO_2 exchange rate compared to genotypes with higher canopy temperatures. In terms of the relationship between WUE and T_c, a correlation was found in a decreasing pattern in the negative direction, beginning with negative and significant correlation under well-watered conditions and becoming non-significant under severe drought stress.

Worthy of consideration is the fact that several studies have identified correlations between WUE and SPAD, SLN, or SLA, or between carbon-13 isotope discrimination and the latter three measurements. Despite such strong correlations, however, accessions with the highest yields frequently do not manifest the highest of these surrogate measures, and those individuals with the highest surrogate measures often have only mediocre yields (Wright et al., 1988; Puangbut, 2010). This has resulted in some studies concluding that these surrogate traits are *not* associated with yield, and suggests that they may not have as much predictive power as thought (Hamidou et al., 2012), that their utility may be highly dependent on specific growing environments, or that other factors provide significant contributions to yield.

While WUE is commonly understood to be a component of the equation for crop yields in water-limiting environments (Passioura, 1977; 1986), this research was aimed to investigate the correlations between three phenotypic traits (SLA, visual ratings, and T_c) and yield under middle-season drought stress in a recombinant inbred line (RIL) population of runner peanuts. Additionally, the heritability of these traits was calculated and unique genotypes with desirable drought-resistant characteristics were identified for use in future peanut cultivar improvement.

Materials and Methods

Development of RIL population: A recombinant inbred line (RIL) population of 149 individual runner peanut genotypes was generated from the cross 'C76-16' ×'Tifrunner.' Since 'C76-16' is a drought-tolerant cultivar and 'Tifrunner' is less drought-tolerant, these cultivars were chosen as parents for their contrasting phenotypes, with the intent of generating a segregating population for both phenotyping and genotyping research. The initial cross was made in 2008 and the progeny has been advanced to the $F_{6:8}$ generation through the method of single seed descent.

Rainout Shelter Experiment. A total of 149 RILs were used in this experiment. Additionally, both parental lines and two more cultivars, 'AP-3' and 'Georgia Green',were also included for the purpose of implementing an augmented experimental design. The study was conducted for two growing seasons (2013 – 2014) at the USDA-ARS National Peanut Research Lab (NPRL) in Dawson, GA, USA. All plants were grown in environmentally controlled rainout shelters (5.5 m × 12.2 m), which are

equipped with sensors to close at the first drop of rain, a controlled irrigation system installed under the shelters, and heating cables installed underground for the purpose of controlling soil temperature and simulating drought (Figure 1).

The RIL population was planted in single-row plots of 15 × 80 cm at a rate of 20 seeds m^{-1} in an augmented experimental design with four control cultivars in three replications. All plots were irrigated before planting to provide uniform germination. Irrigation treatments were designed as two regimes: full irrigation (control) and middle-season drought (experimental) (Figure 2). Control plots were fully irrigated throughout the growing season, based on evapotranspiration (ET) replacement for peanut as described by Stansell et al. (1976). Watermark moisture sensors (Irrometer, Riverside, CA) were placed at two different depths (10 cm and 20 cm) and read every 4^{th} day. Irrigation was triggered when the average measurement of both sensors was below -60 kPa. Experimental plots were subjected to drought stress 60 Days After Planting (DAP) when water was totally withheld for 3 weeks, after which they were allowed to go through a re-irrigated recovery stage until harvest. With the exception of the irrigation treatments, agronomic management inputs were applied according to the University of Georgia best management practices for peanut.

In the 2014 growing season, SLA measurements were taken from both control and experimental plots on three separate occasions: before drought, after drought, and after recovery. In the 2013 growing season, SLA measurements were taken from both control and experimental plots after drought and after recovery; however only experimental plots were measured before drought because they were assumed to be the same as the controls. For each measurement, 3 fully-expanded third nodal leaves from

main stems were randomly selected within plots, beginning at about 7:30 AM EDT. Freshly collected leaves were immediately placed into plastic bags and put on ice in coolers until collection was completed (~ 60 mins). Immediately following complete collection of the entire population, each leaf was placed into an individual Petri dish, fully submerged in deionized water, and placed under white light lamp for 2 hours to ensure tissues were completely turgid. Afterwards, leaves were blotted dry and leaf area (LA) was immediately measured using a LI-3100 area meter (LI-COR Biosciences, Lincoln, NE, USA). Leaves were then placed into a 65 °C oven for 2 days to ensure complete dryness and subsequently weighed to obtain the leaf dry mass (DW). Finally, SLA was calculated as the ratio of leaf area to leaf dry mass (LA/DW) for each leaf measured.

Infrared photographs were taken of all 162 experimental plots and 54 of the control plots at the end of the drought treatment in 2014. A single photograph was taken of each individual plot with a FLIR T640 infrared camera (FLIR Systems, Wilsonville, OR, USA) from approximately 1.5 m above the leaf canopy at solar noon (Figure 3). This was done to ensure that the sun was at its highest point in the sky, at the hottest time of the day, and to minimize the effects of shadows in the images. Images were analyzed using FLIR Tools software to measure and record average leaf canopy temperatures.

Visual ratings were also utilized to assess the apparent drought tolerance of each of the 153 genotypes in the experimental treatment, throughout the course of the middle-season drought. Ratings were made at the end of the drought period each growing season. Ratings were based on the following 5 point scale: 1 = not wilted, 2 = 20% wilted, 3 = 40% wilted, 4 = 60% wilted, and 5 = 80% wilted. Finally, at the completion

of each growing season, every plot was harvested based on the hull scrape maturity profile and its yield was recorded and adjusted on the basis of 10% moisture content yield.

Data Analysis. All data analysis was performed with SAS (version 9.3) with PROC GLM, using the augmented method. Experimental error was estimated by treating the control genotypes (i.e., 'C76-16', 'Tifrunner', 'AP-3' and 'Georgia Green') as if they were treatments in a randomized block design. The mean square errors (MSE) of both the individual genotypes and the rainout shelters were then used to estimate the significance of their respective contributions to yield, SLA measurements, and visual ratings within each treatment, within each year.

Next, data from each trait (yield, three SLA measurements, and visual ratings) was analyzed separately using analysis of variance (ANOVA) of the entire dataset, both experimental treatments combined, both control treatments combined, and each of the four individual treatment/year combinations. For this, and all statistical analyses, the three sub-samples of each SLA measurement were analyzed as a single mean value for each collection, for a total of three values (before drought, after drought, and after recovery), which will be referred to as "mean 1," "mean 2," and "mean 3". Finally, Pearson correlation coefficients and their corresponding *p*-values were obtained for all trait datasets.

To identify top and bottom bulks (i.e., the highest and lowest yielding genotypes across both experimental and both control treatments), yield data from each genotype was compiled from each treatment/year combination. Each genotype was ranked from 1 to

153 for yield within each treatment/year combination and also within an overall mean of the four yield measurements. Final rankings were determined based on two criteria: top and bottom bulks must be ranked within the top or bottom third of each environment, and within the top or bottom 10 percent of the overall mean, respectively. In other words, only the top 50 and bottom 50 yielding genotypes from each of the four environments were considered for the bulks, and from those genotypes, only those in the top or bottom 10 percent of the overall mean qualified for the final designation.

From ANOVA, broad-sense heritability estimates were made for each of the traits measured in both years (i.e., yield, mean 1, mean 2, and mean 3), for each environment (drought and irrigated). Broad-sense heritability (H^2) was estimated as:

$$H^2 = \sigma_g^2 / [(\sigma_e^2/re)+(\sigma_{ge}^2/e)+\sigma_g^2],$$

where σ_g^2 is variance for genotype, σ_e^2 is error variance, σ_{ge}^2 is variance for genotype x environment, r is number of replications, and e is number of environments (Fehr, 1987).

Results and Discussion

Augmented Design. Analysis of the variance of yield in each treatment of this augmented design demonstrated that there was no block effect in the drought treatment in 2014, the irrigation treatment in 2014, or the irrigation treatment in 2013 (*p*-values = 0.17, 0.37, 0.07, respectively) (Table 1; some data not shown). In the drought treatment in 2013, however, a statistically significant block effect was discovered ($p = 0.04$) (Table 1). In part, this block effect may be explained by two different factors that occurred in the drought period of the 2013 growing season. From October 1 through 16, 2013, the United States federal government entered into a shutdown period when neither legislation

appropriating funds for fiscal year 2014 nor a continuing resolution for the interim period was enacted in time. Since this experiment was conducted at a USDA research laboratory, this resulted in the indefinite furlough of all employees and access to the research plots being restricted. This delayed harvest, affected some genotypes more than others, and led to the observed block effect. In addition, severe leaf spot was observed in the shelters of the drought treatment in 2013. The areas of the rainout shelters that were particularly infected produced markedly reduced yields, which also contributed to the observed block effect. For these reasons, the most significant conclusions from this study are those that can be drawn from the 2014 growing season, due to the demonstrated absence of a block effect by the augmented experimental design.

Analysis of the variance of yield in each treatment also demonstrated that there was no significant genotype effect for either the irrigation treatment in 2014 or the irrigation treatment in 2013 ($p = 0.30$ and 0.19, respectively) (Table 1). However, genotype was considered significant for both the drought treatment in 2014 and the drought treatment in 2013, at p-values of 0.076 and 0.067, respectively (Table 1). These results indicate a greater segregation for yield in drought conditions, as compared to irrigated conditions for this RIL population. Analysis of the variance of yield across both years demonstrated that genotype and year were both highly significant in the drought treatments ($p = 0.002$ and $p < 0.001$, respectively) and in the irrigation treatments ($p < 0.001$). Yield across all four environments was also statistically affected by genotype, experimental treatment, and year ($p < 0.001$) (Table 1).

In summary, these results indicate that genotype was a statistically significant factor in the yield of the drought treatments in both years, which is an appropriate

situation for correlation with surrogate traits. However, due to the block effect in 2013, the 2014 test provides a better representation of genotypic contribution and is less confounded by the variance of the blocks. For this reason, the yield data of the 2014 drought experiment will be considered the most accurate and meaningful for all conclusions.

Specific Leaf Area. When yield data was analyzed as one entire dataset (both experimental treatments and both years combined), SLA showed no consistent correlation with yield whatsoever at any of the three collections (r = -0.096 to 0.14) (data not shown). When only the irrigated treatments from both years were analyzed together, however, it is clear that there is a particularly weak correlation within the irrigated treatments, as compared to the dataset as a whole (r = -0.029 to 0.026). This can be explained by the uniformity of the plants in the irrigated treatments and their failure to segregate for SLA under well-watered conditions. Since, for this reason, there are no significant correlations between SLA and yield in the irrigated treatments, it is important to consider possible correlations when the plants are under drought stress.

As was previously discussed, the drought treatment in 2013 experienced a statistically significant block effect for yield. Therefore, the most appropriate yield correlations that can be drawn from the SLA data are those that exist in the dataset from the drought-stressed treatment in 2014, where there was no block effect for yield. Within the 2014 drought treatment, SLA before drought (mean 1) was not correlated with yield at all (r = -0.019, p = 0.81) (Table 2). This is consistent with SLA measurements throughout the irrigated treatments and supports the conclusion that SLA does not

strongly segregate without drought stress. Within the 2014 drought treatment, SLA after drought (mean 2) was also uncorrelated with yield (r = 0.025, p = 0.75) (Table 2). This may be representative of all leaves in the drought period experiencing similar effects from the drought stress simultaneously, without any meaningful differences between genotypes. However, as depicted in Figure 4, the strongest correlation between any SLA and yield measurement in this study was found to be in the 2014 drought treatment, with SLA measured after the recovery stage (mean 3) (r = -0.23, p = 0.0027) (Table 2).

While the magnitude of this correlation coefficient is smaller than what has been reported between SLA and WUE in previous literature (Wright et al., 1994; Nageswara Rao and Wright, 1994; Nigam et al., 2005), it is consistent with previous research in its negative value. This is believed to be a result of thicker leaves having higher WUE, which confers greater drought tolerance to the plant. Significantly, the only SLA measurement found to have any noteworthy correlation with yield in this study was the SLA measured at the conclusion of the recovery stage. This may be explained by the lack of phenotypic segregation in either of the previous two measurements. As has been discussed above, genotypes did not segregate for yield in the irrigated treatments. Since mean 1 was measured before any drought stress took place, it is reasonable that SLA measured at this time would not segregate either, and would therefore be uncorrelated with yield after stress. SLA measured after the drought stress (mean 2) may be uncorrelated with yield for a similar reason if adverse conditions suddenly affected all genotypes relatively equally before they had a chance to adequately adapt. Lower SLA measurements after recovery (mean 3), however, may be representative of genotypes with a greater ability to recover from drought stress quickly, and this study, it was this

trait that was most correlated with yield. Therefore, in this RIL population of peanuts, the most predictive measure of a genotype's yield was its SLA measurement after it underwent middle-season drought stress and was allowed a re-irrigated recovery phase.

Visual Ratings. ANOVA indicated that there was no statistically significant genotype effect for visual ratings in the 2013 drought treatment ($p = 0.40$), but a significant genotype effect was found in the 2014 drought treatment ($p = 0.08$) (data not shown). Accordingly, the correlation coefficients between visual ratings and yield were statistically insignificant in the 2013 drought treatment ($p = 0.11$), but statistically significant in the 2014 drought treatment ($p = 0.04$) (Table 2; some data not shown). However, although the correlation coefficient was statistically significant in the 2014 drought treatment, it was extremely weak ($r = -0.16$) and determined to be poorly correlated with final yield measurements (Table 2). While these results may be counterintuitive, they are highly meaningful, since they indicate that visual ratings may be an unreliable indicator of drought's effect on yield, even when the ratings are made at the conclusion of a significant drought event. Upon further reflection; however, these results are reasonable when the anatomy and physiology of a peanut plant is considered. Since peanuts produce their crop underground, only the vegetative parts of the plant above ground can be observed and rated. In times of drought, it may be possible that plants are able to make physiological adjustments that preserve their eventual yield, at the expense of the visual appearance of their foliage. While visual ratings may demonstrate some correlation with yield, for these reasons, it is proposed that the most significant drought-tolerance responses are simply not visually observable.

Infrared Photography. After ANOVA of the single treatment that infrared photography data was collected for (drought in 2014), preliminary results indicate that there is no statistically significant genotype effect for canopy temperature at the 0.05 significance level. Accordingly, there was also no statistically significant correlation found with yield (data not shown).

Interestingly, a very significant correlation was found between canopy temperature and visual rating ($r = 0.34$, $p < 0.001$) (data not shown). This indicates that the visual drought stress observed in this study was a function of increased leaf canopy temperatures, and it also supports the accuracy of the visual ratings made. However, since neither canopy temperature nor visual rating demonstrated any strong correlation with yield, neither trait is recommended as an accurate predictor of yield under drought stress for this population.

Top and Bottom Bulks. Of high importance for research following this project is the identification of top and bottom bulks (i.e., the highest and lowest yielding genotypes across both experimental and both control treatments). Nine genotypes were considered to be the top bulk and 10 genotypes were considered to be the bottom bulk. The 9 genotypes in the top bulk had overall yield means between 6512 and 7284 kg/ha, and all ranked above the drought tolerant check, 'C76-16', which had an overall yield mean of 6230 kg/ha and ranked number 25 (Table 3). The 10 genotypes in the bottom bulk had overall yield means between 1838 and 3570 kg/ha, and all ranked below the drought

susceptible check, ‘Tifrunner’, which had an overall yield mean of 4453 kg/ha and ranked number 114 (Table 3).

Identification of these bulks provides valuable information for further research by distinguishing genotypes with the most consistent drought-tolerant or drought-susceptible responses. Of particular note are genotypes with the highest yields in both drought and irrigated environments, since the goal of breeding for drought tolerance is to improve yield under drought conditions without compromising yield when drought is not a factor. By further studying the genotypes identified here, more precise conclusions regarding phenotypic indicators of drought tolerance may be established, and superior resistant lines may be developed.

Heritability. Since heritability estimates were made from two years with no replications within years, the values reported here are very rough estimates of broad-sense heritability. However, yield appears to be the most highly heritable trait of the traits considered, especially in non-stressed, irrigated conditions. The heritability estimates of yield were determined to be 0.36 in the irrigated treatments and 0.25 in the drought treatments, and the heritability estimates of the various SLA measurements in the two environments ranged from 0.03 to 0.23 (Table 4).

As compared to previous reports, these broad-sense heritability estimates of SLA seem low and variable since Chen et al. (2013) found estimates consistently ranging from 0.73 to 0.80, across three nonirrigated environments and dropping to only 0.31 in a fourth. Similarly, Songsri et al. (2008) found SLA heritability estimates ranging from 0.81 to 0.95 under drought stress and non-drought stress conditions, respectively. The

heritability estimates of yield, however, appear to be more consistent with Chen et al. (2013), who found estimates ranging from 0.12 to 0.65 and Mohammed et al. (1978), who found estimates ranging from 0.16 to 0.21. Although Mohammed et al. (1978) used F_2 and F_3 generations of two crosses between a Virginia and two Spanish lines, Chen et al. (2013) used 15 runner genotypes, and this study used a RIL runner population, it is noteworthy that all three studies arrived at broad-sense heritability estimates within a similarly moderate range. Therefore, we conclude that early generation selection for yield under drought stress would be an effective component for improving drought tolerance during cultivar development.

Research Implications. Phenotyping peanuts for drought tolerance is a challenging endeavor, due to complex physiological and biological responses that are still not well understood, and due to the inherent difficulty of quantifying a somewhat subjective designation. The most important component of drought tolerance from an agronomic standpoint, however, is a plant's ability to produce a high yield despite water-limiting conditions, so the most important trait to be evaluated for drought tolerance in cultivated peanut is still yield under drought stress.

Since simple visual rating was observed to be a poor predictor of yield under drought stress, the need for reliable phenotyping techniques has been emphasized. In this study, it was determined that compared to visual rating, SLA measured after a recovery from middle-season drought has potential to be a superior predictor of yield. This is significant because it suggests that the highest yielding peanut genotypes in seasons with middle-season drought stress are not the ones that simply tolerate the stress better, but the

ones that recover from it more quickly. Additionally, since peanut is considered to be a fairly drought-tolerant crop already, the greatest opportunity for improvement may be in the enhancement of the recovery phase (in speed or efficiency). However, due to inconsistent results of SLA from year to year (2013 test vs. 2014 test), at least one more year of phenotyping research is recommended.

Further research with this RIL population will not only continue phenotyping, but will also progress into genotyping, with the eventual identification of QTL underlying drought tolerance in peanut. These QTL may then be applied in breeding programs with marker-assisted selection. The lines identified here for the most tolerant and susceptible yield responses to drought stress will be valuable for studying the most phenotypically contrasting individuals and better understanding their differing drought responses. These lines are also valuable materials for identifying the genes responsible for drought tolerance in peanut through the candidate gene expression approach. As this research continues, these findings will help pave the way for the future development of peanut cultivars improved for tolerance to middle season drought stresses.

Table 1. Analysis of variance of yield in the 2014 and 2013 drought treatments[a]

2014

Source of variance	df	Type III SS	Mean squares	F value	Pr > F
genotype	152	187390589	1232832.8	3.1	0.0756
block	2	1933555.3	966777.6	2.43	0.1685

2013

Source of variance	df	Type III SS	Mean squares	F value	Pr > F
genotype	152	203574310	1339305	3.26	0.0673
block	2	4941745.1	2470873	6.01	0.0369

2013 and 2014 combined

Source of variance	df	Type III SS	Mean squares	F value	Pr > F
genotype	152	258753257	1702323.4	2.32	0.0276
year	1	23236793	23236793	31.71	< 0.0001
genotype x year	152	151788611	998609.3	1.36	0.2439

[a] Data are presented by year as a significant genotype by year interaction was not detected ($p = 0.2439$)

Table 2. Pearson correlation coefficients among five measured traits in 2014 drought treatment[a]

	Yield	Visual Rating[b]	SLA mean 1[c]	SLA mean 2[d]	SLA mean 3[e]
Yield	1	-0.16319	-0.01882	0.02548	-0.23464
		0.0386	0.8127	0.7484	0.0027
Visual Rating	-0.16319	1	-0.07105	0.07224	-0.03776
	0.0386		0.3704	0.3625	0.6344
SLA mean 1	-0.01882	-0.07105	1	0.00489	0.12368
	0.8127	0.3704		0.9509	0.118
SLA mean 2	0.02548	0.07224	0.00489	1	-0.0236
	0.7484	0.3625	0.9509		0.7663
SLA mean 3	-0.23464	-0.03776	0.12368	-0.0236	1
	0.0027	0.6344	0.118	0.7663	

[a] Bottom number of each cell is the *p*-value at the 0.05 significance level.

[b] Visual ratings were made on a scale from 1 to 5, with 1 being the least wilted and 5 being the most wilted.

[c] SLA mean 1 is the average of three whole-leaf subsamples measured before drought.

[d] SLA mean 2 is the average of three whole-leaf subsamples measured after drought.

[e] SLA mean 3 is the average of three whole-leaf subsamples measured after recovery.

Table 3. The superior lines identified for most tolerant and susceptible responses to drought stress based on yield (kg/ha) across all tests

		Drought 2014		Irrigation 2014		Drought 2013		Irrigation 2013		Overall[a]	
Genotype		Yield	Rank[b]	Yield	Rank	Yield	Rank	Yield	Rank	Mean	Rank
587	Tolerant	4499	40	11618	2	5425	20	7595	13	7284	1
582	Tolerant	4777	31	8837	24	4882	48	10579	1	7269	2
580	Tolerant	4616	37	12957	1	4882	48	6239	43	7174	3
543	Tolerant	6456	2	9459	10	4882	48	7324	17	7030	4
539	Tolerant	5256	15	9757	7	5154	32	7866	10	7008	6
431	Tolerant	5755	5	8405	38	5696	13	6781	27	6659	10
586	Tolerant	4615	38	8012	50	5425	20	8409	5	6615	11
565	Tolerant	5588	8	9171	16	5154	32	6239	43	6538	13
426	Tolerant	4723	34	8849	23	4882	48	7595	13	6512	15
C76-16	Tolerant Check	4079	66	8452	55	4973	45	7414	38	6230	25
472	Susceptible	1955	151	6358	119	3526	113	2441	155	3570	138
393	Susceptible	1960	150	4637	153	3797	102	3526	134	3480	139
499	Susceptible	3159	111	3661	159	2441	152	4611	104	3468	140
459	Susceptible	2696	135	5114	147	2984	132	2712	153	3376	141
592	Susceptible	2374	139	4481	154	1899	156	3526	134	3070	144
517	Susceptible	1508	159	5479	140	1627	158	3526	134	3035	145
483	Susceptible	1894	154	4825	151	1627	158	1627	160	2493	146
458	Susceptible	1487	161	2393	161	2712	145	2984	144	2394	147
491	Susceptible	1927	153	3803	157	1085	161	2441	155	2314	148
506	Susceptible	2202	144	3251	160	1627	158	271	161	1838	149
Tifrunner	Susceptible Check	3214	105	5465	141	5244	41	3888	121	4453	114
$LSD_{0.05}$		177.0		354.4		179.9		344.0		426.0	

[a] Overall means and rankings were obtained from both years and both treatments combined.

[b] Yield rankings from 1 to 153 were determined for each treatment/control group.

Table 4. Estimated broad-sense heritability values for selected traits in irrigation and drought treatments

Trait	Irrigation	Drought
Yield	0.36	0.25
SLA mean 1[ae]	-	0.10
SLA mean 2[b]	0.03	0.22
SLA mean 3[c]	0.23	0.12
Visual rating[de]	-	0.14

[a] SLA mean 1 is the average of three whole-leaf subsamples measured before drought.

[b] SLA mean 2 is the average of three whole-leaf subsamples measured after drought.

[c] SLA mean 3 is the average of three whole-leaf subsamples measured after recovery.

[d] Visual ratings were made on a scale from 1 to 5, with 1 being the least wilted and 5 being the most wilted.

[e] SLA mean 1 was not measured in the 2013 irrigation treatment and visual ratings were not made in either the 2013 or 2014 irrigation treatment.

Figure 1. Rainout shelters at the National Peanut Research Laboratory, Dawson, GA. Shelters were equipped with sensors to close at the first drop of rain, additional controlled irrigation system is installed under the shelters, and heating cables are installed underground to control soil temperature and simulate drought

Figure 2. Side-by-side view of a drought treatment block (left) and an irrigated control block (right) during the 3 weeks of drought stress.

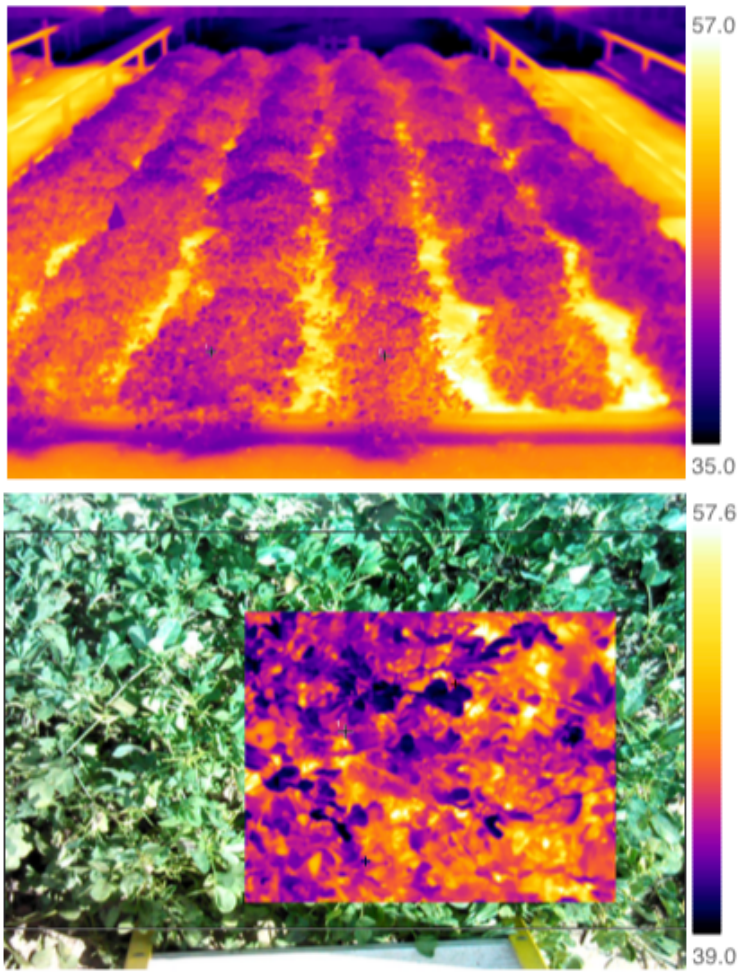

Figure 3. Infrared images captured with FLIR T640 infrared camera. Top image depicts an entire shelter in the 2014 drought treatment. Note the varying canopy temperatures among different genotypes. Bottom image depicts a single plot within the 2014 treatment, with an average T_c of 46.3 °C.

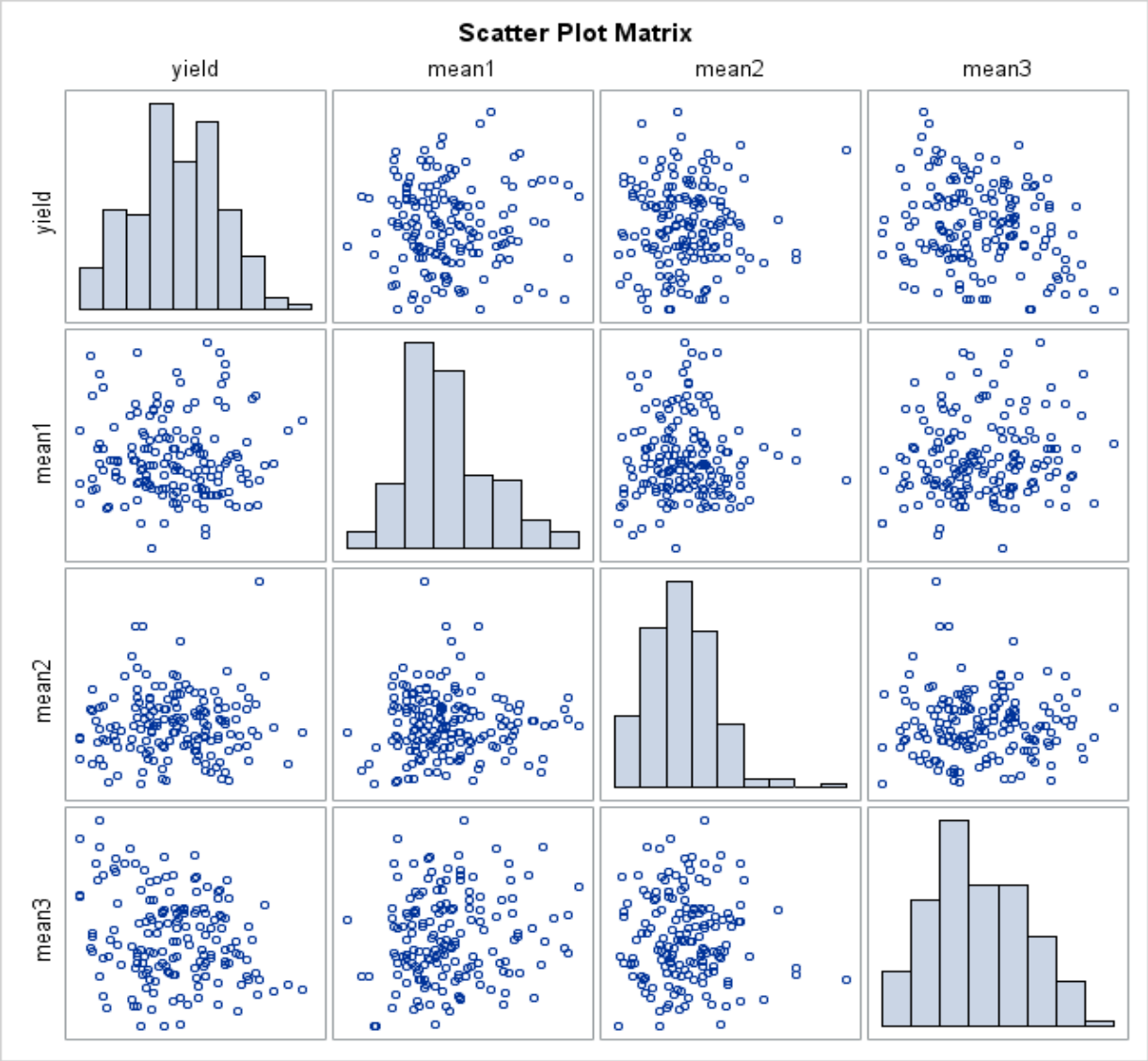

Figure 4. Scatter plot matrix showing the correlations of each of the three SLA measurements with each other and yield in the 2014 drought treatment.

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