ABSTRACT

BAGHERZADI, LALEH. Assessing Physiological Mechanisms to Elucidate the Slow-Wilting Trait in Soybean Genotypes (Under the direction of Dr. Randy Wells).

Water availability is one of the major limiting factors in crop growth and productivity. Soybean [Glycine max (L.) Merr.] is an important source of protein and oil, but its yields in dryland conditions are substantially lower. Thus, the development of drought tolerant soybean cultivars is an important objective of breeding programs. Traits such as slow-wilting, prolonged nitrogen fixation activity and limited transpiration rate under elevated dry environments play important roles in controlling the yield. The study investigated the effects of several parameters on slow wilting mechanism of a soybean genotype, PI 471938 in comparison to PI 416937. Although several mechanisms for PI 416937 have been reported, the main mechanisms for PI 471938 remain unknown. In the case of PI 416937 restrictions in transpiration rate under elevated atmospheric vapor pressure deficit and fibrous root morphology are found to be the key factors that affect expressing slow wilting phenotypes. To explore slow wilting mechanisms for PI 471938, the roles of water status and nitrogen fixation activity were examined.

In the first series of experiments, osmotic potential, elastic modulus of leaves and hydraulic resistance in the plant were investigated to determine whether greater water retention and rapid water redistribution were responsible. Next, the possibility that this genotype may express nitrogen fixation tolerance while maintaining a higher water potential under water deficit was explored. To evaluate the role of plants' above and belowground sections in maintaining nitrogen fixation activity reciprocal grafting technique was used. Third, the study investigated the effects of nitrogen stress, and restoration, on transpiration rate of these two genotypes and on possible adjustments in their response to increasing vapor pressure deficit (VPD). To avoid the use of subjective visual ratings, which depend on experience, a detailed wilting score was developed to assess slow and fast wilting genotypes in a controlled environment. Results suggest that PI 471938 maintains its nitrogen fixation activity under water deficit. Under progressive soil drying this genotype was able to maintain a higher water potential compared to other tested cultivars. However, no distinct differences were observed in osmotic potential and cell modulus of elasticity among the genotypes. PI 471938 also showed a difference in its leaf hydraulic conductance which might affect the delayed wilting of this genotype. Under nitrogen stress, both slow wilting genotypes showed a lower transpiration and stomatal conductance under VPD conditions. Furthermore, during recovery from nitrogen stress, the transpiration response reverted and became similar to the control plants. Results indicate that complex mechanisms might be responsible for slow wilting trait.

Assessing Physiological Mechanisms to Elucidate the Slow-Wilting Trait in Soybean Genotypes

by
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DEDICATION

This work is lovingly dedicated to my son, Sean-Araz Luca Shamey. His support, encouragement and constant love have sustained me throughout my life.

BIOGRAPHY

Laleh was born in Azerbaijan. She earned her bachelors' degree in Agronomy in Urmia University. She completed Masters of Science degree program in the Plant Biology department at the North Carolina State University. She was accepted into PhD program at North Carolina State University's department of Crop and Soil Sciences in 2012. During this period, she was working in the soybean physiology lab.

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CHAPTER 1

INTRODUCTION

Current Crop Status

There is a general agreement that the origin of soybean is from northeastern provinces of China (Qiu and Chang, 2010). Soybean first entered North America during the 18th century (Hymowitz et al., 1983). Seeds of soybean were distributed throughout both Europe and the United States, as botanists obtained soybean collections from China and Manchuria (Hymowitz, 1970). However, soybean was not processed industrially for edible oil and protein meal until the 1930s. Since then, soybean has become a major crop in the world, producing a large portion of world's supply of protein and oilseed (Gibson et al., 2005). The United States, Brazil and Argentina are the three largest exporters of soybean, accounted for over 90% of the world total of soybean exports, while China is the number one soybean importer and in 2005 accounted for 41% of the world total (Song et al., 2009).

Soybean Taxonomy

Soybean [*Glycine max* (L.) Merr.] is a member of the family Fabaceae, subfamily Papilionoideae and tribe Phaseoleae. Within the tribe Phaseoleae, there are 16 genera belonging to the subtribe Glycininae (Viviani et al., 1991, IT IS, 2015). The genus *Glycine* is divided into two subgenera *Glycine* (perennials) and *Soja* (annuals) with both subgenus having 2n chromosome number (2n=40). Soybean was cultivated from its closest relative, *Glycine soja* Sieb and Zucc (Joshi et al., 2013).

General Growth and Morphological Development

Soybean is an annual plant. The first leaves of the soybean are simple and opposite but all other leaves are alternate and trifoliolate (Fehr et al., 1971). All leaf axils give rise to flowers in which 0-5 pods per node, 1-5 seeds per pod is produced (Miksche, 1961). Soybean cultivars are categorized as either determinate or indeterminate in terms of developmental habit (Tian et al., 2010). Soybean determinate plants cease vegetative growth when stem terminates flowers and usually the height of the plant does not increase after flower initiation. Moreover, determinate plants have thicker stems, branch more extensively and less likely to lodge and flower over a shorter period of time. Indeterminate soybeans are taller and have more stem nodes, thinner stems and lodge more easily, less branched and flower over longer period of time. Plants continue to increase in height after flowering initiated. However, since indeterminate soybean have a longer flowering period, have more yield stability and drought tolerance. Determinate soybean is more preferred in the southern U.S. and indeterminate varieties are grown in the Northern U.S. because of the need for sufficient plant height (McWilliams et al., 1999). Soybean is a short day plant and flowers under short photoperiods (Kumudini, 2000).

Soybean is adapted to different latitudes and categorized into different maturity groups (MGs) ranging from 000 to VIII (McWiliams et al., 1999). Soybean varieties are classified for their day length, temperature requirement to initiate flower or reproductive development and their morphological growth habit. The classification for maturity is defined as the adaptability of a soybean to effectively utilize the growing season in a given region. Soybean varieties adapted to a particular region are given a group number from 00 to VIII; number 00 for the northern Minnesota and North Dakota, and VIII for the southernmost region such as Florida and southern parts of the Gulf coast states. Varieties in group V to VII are determinate growth habit (Boerma, 1979).

Stages of Development

Fehr et al. (1971) set the convention for soybean developmental stages. Soybean development has divided into vegetative (V) and reproductive (R) stages. Early subdivisions of the V stages are designated VE (emergence) and VC (cotyledon). Stages are labelled V1, V2, V3, through V(n), where V1 is the first vegetative node above VC and (n) represents the number for the last node which is determined by cultivar and environmental conditions that affect growth and development. Reproductive stages are subdivided into eight stages, these being R1, beginning bloom; R2, full bloom; R3, beginning pod; R4, full pod; R5, beginning seed; R6, full seed; R7, Beginning maturity; and R8, full maturity.

Drought Stress

Overall water deficit as a result of an extended period of scarce precipitation can cause decreased crop growth and reduced yield (Passioura, 2002). Water is a major limiting factor in plant growth. In Soybean, water deficit causes major yield reductions (Heatherly and Elmore, 1986, Sinclair et al., 2010). Soybean is sensitive to water deficit during the beginning of pod and seed filling (Liu et al., 2004). Irrigation can be used to minimize drought effect on soybean production, however, it is expensive and requires major investment in equipment and fuel. It has been reported that out of 51% U.S land used for Agriculture production, only 8% of this area is irrigated (Board and Kahlon, 2011), hence there is need to develop other strategies beside irrigation. One of the major goal in breeding program is to select and develop drought tolerant cultivars.

Drought Tolerance Mechanisms in Soybean

In general, the ability of the plants to adapt under drought conditions defines drought tolerance (Boyer 1996). Conservation of soil water and acquisition of water from deeper in the soil profile is a mechanism that could contribute in drought tolerance in soybean (Boyer, 1996). Similarly, adaptive traits such as prolong nitrogen fixation, slow canopy wilting, and limited transpiration under elevated vapor pressure deficits have been implicated in improving productivity under water deficit.

Traits related to drought tolerance

Slow Wilting

Drought tolerant soybean cultivars have been identified for their slow wilting trait and they display increased yield under drought conditions (King et al., 2009; Pathan et al., 2014). Slow wilting genotypes can conserve soil water through limiting transpiration under high atmospheric evaporative conditions or through deep rooting which would allow more access to the water in the deeper soil profile. Likewise, to avoid drought stress, slow wilting soybean genotypes have a prolonged nitrogen fixation activity under soil water deficit.

Breeding for improvement in yield under water deficit can be obtain by genotypes identified with the slow wilting trait. (Carter et al. 2016; King et al., 2009). Leaf wilting is considered as the first visible symptom under soil drying conditions (King et al., 2009) and it is a heritable trait and environmentally sensitive (Charlson et al., 2009). In soybean, visible leaf wilting can be characterized as rolling the top of the leaves, movement of the angle of the leaf and turgidity loss of the leaf.

Two soybean plant introductions (PI), PI 471938 and PI 416937, have been identified as a slow wilting and the progeny of these lines show higher yield under drought stress (Carter, 1999; Sadok, 2012). Delayed wilting was first observed in field screening trials in 1982-83 that examined exotic germplasm for improved drought tolerance (Carter et al., 2006). PI 416937 is a Japanese accession that expresses delayed wilting (Sloane et al., 1990). The mechanism behind the slow wilting of PI 416937 has been reported to be reduced leaf transpiration t attained through decreased stomata conductance under high vapor pressure deficit. Conversely, commercial cultivars possess transpiration rate that increase linearly as vapor pressure deficit intensifies (Fletcher et al., 2007). Another mechanism to explain slow wilting phenotype in PI 416937 is the fibrous rooting system (Sloane et al., 1990). This rooting morphology in PI 416937 could contribute to slow wilting trait thorough acquisition of water under water deficit conditions. However, the exact mechanism for slow wilting in PI 471938 still remain unknown. Devi and Sinclair, (2013) have reported the prolonged activity of nitrogen fixation in PI 471938 under soil drying. It was noted that ureide level in the PI 471938 was lower compared to the fast wilting genotype. In plant breeding programs aimed at drought tolerance, slow wilting plant introductions have been successfully used as parents (Carter et al., 2006, 2016).

Nitrogen Fixation

Nitrogen is a crucial nutrient for plants growth and development. Nitrogen is the component of proteins and amino acids, nucleic acids and energy molecules such as ATP (Wittenbach et al., 1980). Similarly, nitrogen is an essential element in chlorophyll and Rubisco (ribulose 1.5-bisphosphate carboxylase oxygenase) (Wittenbach et al., 1980). Chlorophyll and Rubisco play important roles in photosynthesis and it has been reported that nitrogen deficiency in soybean leads to reduce in yield (Hu and Schmidhalter, 2005).

Soybean seeds contain 40% protein and as a result, a large amount of nitrogen is required for soybean to maintain adequate protein in the seeds. Soybean relies on symbiotic nitrogen fixation to supply sufficient nitrogen from root nodule activity. Nodules are produced after infection by bacteria (*Bradyrhizobium japonincum)* and a symbiotic relationship is established between the host plant and the bacteria (Sprent, 1980). Through the nitrogen fixation process, free nitrogen gas (N_2) in the earth's atmosphere is converted into ammonia (NH_3) which is utilized by the host plant in return for carbohydrates (Newcomb, 1981). The carbohydrate supply (produced from photosynthesis) to the bacteria is used for ATP production and will be used to in the conversion of nitrogen into ammonia. Carbohydrates are also required for carbon skeletons used in amino acid and/or ureide production (Serraj, 2003).

Soybean plants initiate nodulation at early vegetative stage and the nitrogen fixation rate increases dramatically with a peak at about stage R5 and drops rapidly thereafter. It has been found that nitrogen fixation activity decreases under soil drying (Devi and Sinclair, 2013). Durand et al. (1987) stated that under water deficit conditions, nitrogenase activity decreased in a manner greater than photosynthesis. The main product of soybean nitrogen fixation consists of ureides, namely allantoin and allantoic acid which will be transported from the nodules to the shoot by xylem (McClure and Israel, 1979). It was reported that under drought conditions, ureide concentrations increased in the shoots (Serraj and Sinclair, 1996). Devi and Sinclair (2013) reported that accumulation of ureides in the leave of slow wilting soybean PI 471938 was less than fast witling Benning genotype. It was suggested that soybean genotypes that are able to maintain lower shoot ureide concentrations exhibit greater drought tolerant with regard to nitrogen fixation activity (Ladrera et al., 2007). The acetylene reduction activity (ARA) assay has been used for measurements of nitrogen fixation to study genotypic differences of drought tolerance in soybean. (Purcell et al., 1997). Sinclair and Ludlow (1986) have used fraction of transpirable soil water threshold in order to compare sensitivity of nitrogen fixation under soil drying among genotypes. Soybean genotypes with low ureide concentration in the shoot had a lower FTSW threshold at which ARA started to decline (Vadez and Sinclair, 2002). Furthermore, King et al, (2014) found different soybean genotypic responses in nitrogen fixation activity under water deficit. Drought tolerant soybean genotypes had a lower FTSW threshold for nitrogen fixation reductions compared with drought sensitive genotypes. Sinclair et al., (2010) used a soybean yield stimulation model in order to obtain a relationship between the soil water extraction capacity and crop yield improvement. Based on the results it was determined that drought tolerant nitrogen fixation and slow wiling are the most important traits for improving yield.

Limited transpiration trait

One of the key strategies in soybean drought research is to identify soybean genotypes that express increased stomatal sensitivity to high vapor pressure deficit (VPD). Considerable progress has been made in breeding programs to identify drought tolerant soybean based on slow wilting trait. The initial observation of slow wilting was made in PI 416937 (Carter and Rufty, 1993). The progeny of this line has relatively high yields under drought conditions (Gillen and Shelton, 2006). One of the key mechanism of PI 416937 appears to be expressing high sensitivity of stomata to high atmospheric vapor pressure deficit, in which transpiration is restricted at VPD around 2.1 kPa (Fletcher et al., 2007). Through the restriction of transpiration under high VPD, the effect of water deficit on plant tissues would be delayed and soil water is conserved. This would allow plants to access water later in the growing season. Purcell et al. (2006) suggested that slow wilting genotypes might transpire less during well-watered conditions, thus conserving soil water reserves for later use. the slow Conversely, fast wiling genotypes used more water during well-watered conditions and depleted water supplies prior to future drying cycles (Purcell et al. 2006).

Sinclair et al (2010), used a stimulationd model across the major growing regions in the USA using 50 years of weather data in order to evaluate the effectiveness of regulation of maximum transpiration rate in improving yields for soybean. It was reported that in regions, especially in the southern USA, restricting maximum transpiration was one of the more promising traits (Sinclair et al., 2010).

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CHAPTER 2

Assessing water-related plant traits to explain slow-wilting in soybean PI471938

ABSTRACT

Soybean [*Glycine max* (L.) Merr.] genotype PI 471938 expresses a slow-wilting phenotype in the field, and the progeny of this genotype have shown to have high yield under water-deficit conditions. However, the physiological basis for the slow-wilting trait in PI 471938 remains unclear and failure to understand the causal mechanism may limit future breeding efforts. This study investigated three primary hypotheses for trait expression that could explain slow-wilting trait in PI 471938: (1) a low osmotic potential in the leaves allowing greater water retention, (2) low elastic modulus of leaves resulting in delayed development of wilting, and (3) low hydraulic resistance allowing rapid water redistribution in the plants. Experiments included three other soybean genotypes as references for the results obtained with PI 471938. Surprisingly, the results for PI 471938 did not prove to be unique as compared to the other three tested genotypes for any of the three hypotheses. These negative results indicate that a hypothesis outside the usual candidates describing plant water transport, possibly anatomical features related to specific water transport properties, is required to explain slow-wilting in PI 471938.

INTRODUCTION

Soybean is a major source of protein and oil worldwide (Panthan et al., 2007), and drought has been identified as the main limiting factor in soybean yields (Burton et al., 2013). Hence, improvements in drought tolerance have been one of the main objectives of soybean breeding programs. There are, however, many challenges in breeding for improved soybean drought tolerance (King et al., 2009). One approach is to introduce exotic germplasm with drought-tolerant traits that might confer greater yields in progeny lines when compared with commercial lines under water-deficit conditions (Sloane et al., 1990). Two soybean plant introductions being exploited for drought tolerance are PI 416937 and PI 471938. These genotypes were initially screened on deep sandy soils and selected based on their delayed leaf wilting under water-deficit conditions compared with other soybean cultivars, and hence were labeled as having “slow-wilting”. Subsequently, both genotypes were confirmed to have to slow-wilting (King et al., 2009) on a soil with a traffic pan that resulted in virtually no root penetration below 30 cm (Slaton et al., 1990). Hence, deeper rooting seemed unlikely to be the basis of slow-wilting.

Water conservation appears to be the basic mechanism for the visual observation of delayed wilting in PI 416937 (Sinclair et al., 2008a). This genotype was able to conserve soil water by restricting transpiration rate under elevated atmospheric vapor pressure deficit (VPD) (Sinclair et al., 2008a). While PI 471938 was also identified visually as expressing the delayed-wilting phenotype, its transpiration rate continued to increase with increasing VPD (Fletcher et al., 2007), indicating no response of stomata at elevated VPD. In other experiments, the lack of a VPD response was confirmed, and there was no indication of an advantageous stomatal response, increased ability to maintain leaf water potential, or exceptional photosynthetic activity during soil drying (Sadok et al., 2012). Nonetheless, PI 471938 did maintain relatively high nitrogen fixation activity in soil-drying experiments (Devi et al., 2013). Thus, the question about the physiological mechanism for drought resistance and delayed wilting in PI 471938 remains unanswered.

Since PI 471938 has proved to be a very useful parent in developing high-yielding, drought-tolerant progeny lines (Devi et al., 2014), it is important to understand the basic processes that confer physiological advantage. Therefore, in this study additional possible physiological mechanisms beyond those investigated by Sadok et al. (2012) were explored that could be responsible for delayed wilting in soybean genotype PI 471938. Three specific additional hypotheses were studied as candidates to explain delayed wilting in PI 471938: (1) generation of osmotic potential in the leaves either under well-watered conditions or soil drying conditions that allow greater water retention by leaves, (2) an intrinsically low elastic modulus of leaves resulting in delayed wilting, and (3) an intrinsically low hydraulic resistance of leaves that allow rapid redistribution of water to and through leaves.

The first hypothetical mechanism is based on numerous reports of substantial genotype diversity for osmotic potential within species such as sorghum, wheat and cotton (Morgan, 1984). Further, water deficit has been found to induce lower osmotic potential, i.e. osmotic adjustment, in many species (Jones et al., 1978). Low osmotic potential contributes to maintenance of cell turgor as total water potential decreases (Brown et al., 1995). This, in turn, might help to delay visual leaf wilting and stomatal closure, prolong photosynthetic activity, growth, and potentially in higher yield under water-deficit conditions. Therefore, either a constitutively elevated amounts of osmoticum in the leaves or an induced increase in osmoticum under water deficit by PI 471938 could explain much of its response to soil drying.

The second hypothetical mechanism to explain the observations of drought response of PI 471938 is based on exceptional mechanical properties of its leaves as defined by modulus of elasticity (ε). The modulus of elasticity is a characteristic that reflects the rigidity of the cell walls, with a high modulus of elasticity indicating greater rigidity and, perhaps, an associated minimization of cell dehydration (Bartlett et al., 2012). Indeed, leaf modulus of elasticity has been linked to drought tolerance among species (Read et al., 2006), with occurrence of sclerophylly (prevention of wilting) being linked to leaf thickness and cell wall elasticity.

Thus, a constitutively high modulus of elasticity of the cell walls associated with leaf rigidity could result in the visual observation in delay of wilting of PI 471938.

The third hypothetical mechanism to be investigated results from rapid movement of water from the soil through the whole plant due to a high hydraulic conductance (K_{total}). A high hydraulic conductance would allow rapid movement of water throughout the plant and into leaves to replenish transpired water lost, and possibly a delay in leaf wilting. More specifically, it may be that high hydraulic conductance of leaves (K_{leaf}) allows rapid water redistribution in the leaves. Two components have been found that contribute to K_{leaf} with a more rapid water flux phase suggested for the pathway from vascular cells into the bundle sheath cells and epidermis, and thus feeding directly to the guard cells, and a slower phase representing water flux from the bundle sheath cells to the mesophyll cells (Zwieniecki et al., 2007). More rapid movement of water in leaves of PI 471938 in either water pathway as compared to other genotypes might result in an alteration in the timing of the visual appearance of leaf wilting.

The objective of this study was to examine each of the three hypothesized mechanisms and their possible role in delayed wilting in PI 471938. To allow comparisons with the characteristics of PI 471938, experiments included genotype PI 416937 and two fast-wilting genotypes.

MATERIAL AND METHODS

Plant material

Phenotypic response of PI 471938 was contrasted with PI 416937 (slow wilting), and two fast-wilting, commercial lines A5959 and Benning. Plants were studied in a controlled environment chamber and greenhouse in the Phytotron Facility at North Carolina State University, Raleigh, NC. Description of the selected genotypes for this study is shown in Table 1.

Leaf relative water content and osmotic potential under water deficits

Greenhouse and growth chamber studies were conducted to evaluate leaf relative water content (RWC) and rehydrated osmotic potential (OP) under well-watered and soil-drying conditions for PI 471938, and for comparison the other selected genotypes (PI 416937, A5959 and Benning). Plants were grown in 2.7-L pots in a sandy loam soil (69% sand, 18% silt and 12% clay) and randomly positioned on growth benches. Plants were grown in day/night temperatures regulated at 31±2 / 25±2 ºC and 28 / 24 ºC in greenhouse and growth chamber experiments, respectively. Air vapor pressure deficit (VPD) during the sampling time varied from 0.6 to 2.3 kPa for the greenhouse and from 0.5 to 1.0 kPa for the growth chamber experiment. In the growth chamber experiments, the plants were subjected to a 15-h light period with approximately 500 µmol m^{-2} s^{-1} photosynthetically active radiation.

The pots were initially watered daily to avoid water deficit. In the growth chamber experiments, a complete nutrient solution (Saravitz et al., 2009) was applied to the pots one week after sowing. In the greenhouse experiment, a solution of 17.0 mM N, 4.0 mM P, and 9.0 mM K (MaxiGro, General Hydroponics, Sebastpol, CA) was applied to the pots on alternate days for the first 10 d after sowing.

Four-week old plants were subjected to a soil dry-down treatment. Prior to the experiment, pots were over-watered and allowed to drain. Pots were then sealed in plastic bags to avoid evaporation from the soil surface, and the pots were split between two treatments. Three plants were maintained as well watered (WW) throughout the experiment by daily watering to return their pot weight to 100 g less than their initial weight measured at the beginning of the experiment. Five plants of each genotype were assigned to the water-deficit treatment (WD) and allowed to dry progressively over two weeks as a result of transpiration water loss. To avoid a rapid decrease of soil water content, these pots were watered if needed so that the daily decrease in soil water content was no more than 70 g.

The daily transpiration ratio of each WD pot was calculated by dividing its transpiration rate by the average transpiration rate of the well-watered pots of the same genotype. To obtain normalized transpiration rate (NTR) for each WD pot, an average transpiration ratio of each WD pot when it was still under well-watered conditions was calculated and divided into each daily transpiration ratio. Therefore, the NTR of each drying pot during its initial stages while still in the well-watered phase was centered around a value of 1.0.

In this soil-drying experiment, fraction of transpirable soil water (FTSW) was used to define the water status of the soil for each pot. FTSW was determined by calculating the difference between current pot weight and final pot weight divided by the total transpirable soil water (difference between the initial weight of the pot and the weight of the pot when daily NTR of that pot was 0.11 or less).

Measurements of RWC and OP were made every day during the course of the dry-down experiments in the growth chamber and greenhouse described above. RWC and OP were determined on fully expanded leaves (expanded trifoliolate leaf number 3, 4, 5, & 6). Samples were collected between 09:00 am and 11:00 am EST for the greenhouse experiment and pre-dawn in the growth chamber experiment. RWC and OP were determined on leaf discs collected using a hole punch (6-mm diameter). The fresh weight of each leaf disc was obtained usually within 150 s of collection. The turgid weight of each leaf disc was obtained by placing each disc in a mini-zip plastic bag (Fisher Scientific) filled with deionized water and stored in a dark refrigerator (approximately 4^{o}C). Initial tests showed that after 4 h, no further increase in leaf disc weight occurred. Consequently, after 4 h, the discs were removed from the bag and blotted gently between filter paper, and turgid weight was measured. Each leaf disc was then oven dried at 70 ^{o}C for 24 h to obtain dry weight. Relative water content was calculated using the following equation:

$$\text{RWC} = (\text{Fresh wt} - \text{dry wt}) / (\text{Turgid wt} - \text{dry wt})$$

Separate leaf discs were collected for determination of leaf OP. Samples were placed in the mini-zip plastic bags filled with deionized water immediately after being taken from the leaf and stored in a dark refrigerator (approximately 4^{o}C). After four hours, leaf discs were blotted dry and immersed in liquid nitrogen and then stored in a –80 ^{o}C freezer. Prior to osmotic measurement, leaf discs were removed from the freezer and allowed to thaw. Samples were analyzed using a vapor pressure osmometer (Model 5500, Wescor Inc., Logan, UT).

Leaf mechanical properties

The intrinsic mechanical properties of leaves collected from well-watered plants were determined by measuring pressure-volume response curves (Turner, 1988). In this experiment, only two soybean genotypes PI 471938 and Benning (a fast-wilting genotype) were selected and grown in a growth chamber at day/night temperature 28/24 ^{o}C during 15-h light period with approximately 500 µmol m^{-2} s^{-1} photosynthetically active radiation. Air VPD was about 1.5 kPa. Seeds were sown into a sandy loam soil (69% sand, 18% silt and 12% clay) in a 2.7-L size pot.

Fully expanded trifoliolate leaf numbers 6 and 7 were selected from four replicate plants from each genotype for the determination of the pressure-volume curves. Leaf pressure-volume data were obtained using the bench-drying technique described by Koide et al. (1989). Leaves were cut from well-watered plants after enclosing the lamina in a plastic bag, and the petioles were immediately immersed in deionized water. The harvested leaves were taken to the laboratory for water potential measurements using a pressure chamber (Model 1000, PMS instrument Company, Albany, OR, USA). The weight of leaves for the pressure-volume analysis was taken as the average weight measured on a balance (± 0.0001 g) immediately before and after water potential was measured. After measurement, the leaves were allowed to dry briefly on a bench top (VPD of the room was about 1.5 kPa). Leaf weight and pressure measurements were repeated 10 to 14 times during drying of each leaf. At the end of measurement, leaves were oven-dried at 70 ^{o}C for 24 hours and dry mass was determined.

Water relation parameters for each of the sampled leaves were determined based on the pressure-volume analysis of the water potential and decrease in leaf volumetric water content. The bulk leaf modulus of elasticity (ε) was calculated from data in the range where turgor pressure was positive. The estimate of ε was calculated as the slope of the turgor pressure against the decrease in leaf volumetric water content. The number of data in these regressions was 7 to 10 and the R^2 of the regression was greater than 0.95 in all cases.

Hydraulic conductance

Soil-plant conductance

In this experiment, variation in soil-plant conductance among the four genotypes was examined by measuring changes in transpiration rate after pressuring the rhizosphere of well-watered, intact plants (Choudhary et al., 2014). Calculation of soil-plant conductance was based on the measurements of transpiration rate and water potential gradient. Twelve plants were each grown in 1.35-L pots (3 replicates per each genotype). Seeds were sown in a mixture of 50% sand, 25% peat, and 25% vermiculite. Plants were watered every day and the experiment was done under well-water conditions. The pots were constructed from PVC pipe (7.6-cm diameter, 29-cm length) fitted with a round end cap attached to its bottom end. The end cap had a threaded opening to allow water drainage during plant growth, and application of pressure during hydraulic conductance measurements. Plants were grown in a growth chamber with 31^{o}C day and 26^{o}C night temperature and 16 h photoperiod. The air VPD in the growth chamber was maintained at approximately 2.5 kPa. Plants were exposed to approximately 400 µmol m^{-2} s^{-1} photosynthetically active radiation. The pots were kept well-watered and the experiment was conducted when plants had six fully expanded leaves.

The day prior to pressurization, the pots were overwatered. After the pots stopped dripping, the pots were sealed using a specially constructed lid with weather-stripping and parafilm around the plant stem to ensure an air tight seal. The next day, after two hours of acclimation, plants were weighed in the morning (~09:00 am) on a balance with a resolution of 0.1 g (Model SI-8001, Denver Instrument, Denver, CO, USA), and reweighed 120 min later to establish an initial transpiration rate. Transpiration rate was calculated based on the weight change over the 120 min period. After the second weighing, positive pressure (~0.2 MPa) was applied to each pot to increase the hydrostatic water potential in the leaf xylem. After being subjected to the pressurized conditions for 120 min, pots were again weighed to determine transpiration rate during the pressurized period. Increased transpiration rate (ITR) was then calculated as the ratio of transpiration before and after pressure treatment for each plant.

Whole soil-plant conductance (K_{total}) for well-watered plants was calculated from the transpiration rate and the water potential gradient between the soil and a top leaf. Transpiration per unit leaf area was calculated based on total plant leaf area measured using an area meter (Model LI-3100, Li-Cor, Lincoln, NE). So that the estimate of K_{total} reflected much of the water pathway in the plant, the second youngest fully expanded leaf from each plant was sampled the water potential measurement (Ψ_L). The leaf to be excised was sealed in a plastic bag while plants were still under pressure. Leaf water potential was measured by using a pressure chamber. The value of K_{total} (mg H_2O s^{-1} MPa^{-1} m^{-2} leaf area) was calculated by dividing the transpiration rate (TR, mg H_2O s^{-1} m^{-2} leaf area) of the plant by water potential difference between the soil (pressure applied) and leaf.

$$K_{\text{total}} = \text{TR}/\left(\Psi_{\text{soil}} - \Psi_{\text{leaf}}\right)$$

Leaf rehydration kinetics

The leaf rehydration method has been used to study the movement of water in leaves (Boyer, 1985). Leaf rehydration-kinetic experiments were conducted to test the hypothesis that there are differences in the intrinsic hydraulic conductance in the leaves of PI 471938 as compared to the other three genotypes (PI 416937, A5959 and Benning). Rehydration kinetics was measured by using a ‘reverse Polish guillotine’ (Zwieniecki et al., 2007). The measurement involved cutting the petiole of an excised leaf and attaching it to a water-filled tube in a single motion performed under water (i.e. limiting the possibility of embolism formation and allowing for immediate determination of water uptake). Leaves were measured from individual plants from each of four genotypes. Seeds were sown in 1.5-L pots and grown in a greenhouse with a mean temperature of 24 ^{o}C (UC-Davis, CA). Plants were kept well-watered and the experiment began when plants had 7-8 fully expanded leaves. Overall, leaf rehydration was measured on one to three leaves per plant and six plants per genotype.

Plants were watered 18 h before the experiment began and the plants were not re-watered again so that a slight deficit developed in the leaves with water potential ranging from -0.2 to -1.0 MPa. This watering regime prevented major water deficit but still allowed sufficient deficit to develop in the leaves so that water uptake by leaves in the rehydration test occurred readily. The leaf petiole was prepared for initiation of the rehydration test by wrapping it in Blu-tack (Bostik, Pty. Ltd., Notting Hill, VIC, Australia) plus parafilm to ensure a tight holding spot near the area of the petiole of the leaf that was to be cut in the guillotine apparatus. In cutting the petiole with the blade, the petiole was also tightly connected to a water-filled tube. The tube was linked to a water reservoir placed on a balance. The whole system was immediately put inside a glass container filled with water to stop leaf evaporation. The container was positioned above the balance used to track weight changes. This positioning of the balance meant that any leakage resulted in water movement from the tube attached to the leaf back into the balance would be easily detected. Water uptake was recorded every 1 s and provided a continuous recording of water-time function of water flow. The data from the curve were recorded by a computer.

A double exponential function of weight plotted against time was used to describe the data (Zwieniecki et al., 2007).

$$f(\mathrm{t}) = a\,(1-e^{-\mathrm{t}/b}) + c\,(1-e^{-\mathrm{t}/d})$$

This model describes leaf water uptake in two phases: fast phase and slow phase. The coefficients obtained from regression analysis (a plot of water uptake vs. time) included the half times (b and d). Variables a and c represent two separate water volumes. SigmaPlot 10 (Systat Software, Inc. Point Richmond, CA, 2006) software package was used for the regression analysis.

Data Analysis

Data among genotypes were subjected to one-way analysis of variance (ANOVA) using the Tukey test to identify significant differences among the genotypes using R package ‘RStudio’ (R Development Core Team, 2013). The maximum accepted *P* value for significance was 0.05. Relative water contents and rehydrated osmotic potential values were plotted against FTSW and analyzed using two-segment linear regression analyses (GraphPad Prism 2.0, GraphPad Software Inc., San Diego, CA, 1996). The breakpoint values at which there was a decrease from the early plateau segment and linearly decreasing segment at low FTSW was an output from the regression analysis. For the comparisons of the threshold values, the 95% confidence interval generated by Prism 2.0 was used.

RESULTS

Transpiration rate, osmotic potential and leaf relative water content

PI 471938 and the reference genotypes Benning and PI 416937 were subjected to soil drying over about a 2-wk period. Results for normalized transpiration rate (NTR), rehydrated osmotic potential (OP) and relative water content (RWC) regressed against FTSW are shown in Figures 1, 2, and 3, respectively. All data were well represented by the two-segment linear regression as shown in regression results given in Table 2. The threshold for the decline in NTR by PI 471938 was 0.27 in the greenhouse experiment and 0.39 in the growth chamber experiment. These thresholds were in the range commonly reported for soybean and were not different within each experiment from the other two genotypes.

Rehydrated OP values (Fig. 2) were stable for much of the dry-down with average values for PI 471938 of -1.0 MPa in the greenhouse experiment and -0.7 MPa in the growth chamber experiment. The well-watered OP of PI 471038 was not different from what was observed in the two reference genotypes. There was no change in OP at the FTSW levels were NTR had not decreased in each of the experiments. In fact, there was no decrease in OP until values reached 0.10 FTSW in the greenhouse experiment and 0.25 FTSW in the growth chamber experiment. Again, there was no difference in these low observed thresholds for OP decrease in PI 471938 as compared to the two reference genotypes. RWC had a high stable value in PI 471938 over a wide range of FTSW (Fig. 3). The RWC through the “wet phase” of the soil dry down was about 0.9 in both experiments. These RWC were consistent with the two reference genotypes. The threshold for the decrease in RWC was at 0.12 FTSW in the greenhouse experiment and 0.13 FTSW in the growth chamber experiment. These thresholds were not different from that of the two reference genotypes.

Leaf mechanical properties

Data to determine cell wall modulus of elasticity were obtained from pressure-volume data obtained by dehydrating leaves in a cycle of water loss and pressure measurements (Table 3).

Consistent with the direct measures of OP and RWC in PI 471938, the results of the pressure-volume analysis resulted in values for the turgor loss at a potential of -0.84 MPa and RWC of 0.94. The estimate of the rehydrated osmotic potential for PI 471938 was -0.62 MPa. There were no differences in these values between PI 471938 and Benning. Similarly, there was no difference in the estimate of the apoplastic fraction of 0.11 for PI 471938 and 0.10 for Benning. The leaf modulus of elasticity was 10.7 MPa for PI 471938 and 11.6 MPa for Benning, and was not significantly different between genotypes. Thus, there was no difference in cell elastic properties between the two genotypes.

Hydraulic conductance of soil-plant system

Comparisons of hydraulic properties as measured by increased transpiration rate (ITR) and whole soil-plant conductance (K_{total}) were determined for soybean plants grown in well-watered conditions in a controlled environment chamber. The mean ITR for PI 471938, Benning, and A5959 was 63.9% (s.e. 10.0), 64.2% (s.e. 14.1), and 65.9% (s.e. 13.0), respectively. The ITR value of PI 416937 was 49.8% (s.e. 10.6) but not significantly lower than the other three genotypes.

The mean K_{total} for PI 471938 was 12.2 ± 3.8 mg H_2O m^{-2} s^{-1} MPa^{-1}. This value was comparable to K_{total} for Benning (10.0 ± 3.9 mg H_2O m^{-2} s^{-1} MPa^{-1}) and A5959 (13.4 ± 5.2 mg H_2O m^{-2} s^{-1} MPa^{-1}). The K_{total} for PI 416937 (5.6 ± 3.2 mg H_2O m^{-2} s^{-1} MPa^{-1}) appeared lower than the other three genotypes but the differences were not significant.

Leaf rehydration kinetics

The hydraulic properties of leaves were compared using leaf rehydration kinetics. A representative rehydration curve of water uptake vs. time is shown for one leaf of PI 471938 in Fig. 4. From the rehydration curve of each leaf, the half time for the rapid and slow phases compartments were calculated (Fig. 5 A, B). The average half times for the fast-phase and slow-phase rehydration of leaves of PI 471938 was 20.5 ± 0.5 s and 192.9 ± 12.7 s, respectively. These values are consistent with those of the fast-wilting genotype A5959 (19.6 ± 1.3 s and 184.2 ± 22.5 s, respectively). The half-times were slightly longer for Benning (23.6

± 2.1 s and 200.5 ± 26.0 s). Slow-wilting PI 416937 had the longest half times of 24.9 ± 0.9 s for the fast phase and 229.4 ± 39.3 s for the slow phase). However, the differences among genotypes for the slow-phase were not significant.

DISCUSSION

Genotype PI 471938 has proven to be a very useful parent in developing soybean lines that have increased yields in water-deficit environments (Devi et al., 2014). However, studies thus far have been unable to resolve the basic mechanism that accounts for the expression of delayed wilting of this genotype, and the possible link of the mechanism to yield increase. This study examined three hypotheses as possible explanations.

The first hypothesis was that PI 471938 has a lower OP by accumulation of solutes in leaves, and that the lower OP would result in turgor maintenance during soil drying resulting in delayed leaf wilting. Measurements of OP while plants were in the well-watered state showed no difference between PI 471938 and the other tested genotypes. Also, there were no differences in OP among genotypes in the stress phase of the soil-drying experiment. In fact, there was no osmotic adjustment until substantial water deficit had developed in the soil and transpiration rate had substantially decreased.

The pattern of decline in normalized transpiration rate during the soil drying experiments resembled that observed previously for soybean (King et al., 2009; Sadok et al., 2012) with a threshold of FTSW for all genotypes in the range of 0.27 to 0.43. RWC also exhibited a response to soil drying with the two linear segments as RWC decreased from about 98% to 55%. The threshold between the two segments describing RWC decrease was much lower than the threshold for NTR decrease with values for the RWC threshold in the narrow range of 0.10 to 0.13 FTSW in all genotypes.

The absence of differences in OP between PI 471938 and other three genotypes is generally consistent with previous observations of stability in OP across a wide range of soil water levels for soybean. Examinations of osmotic adjustment in leaves of field-grown plants indicated no genotypic differences as drought stress progressed (Turner et al., 1978, Cortes et al 1985). An exception to these observations was found with field grown PI 416937 and cultivar Forrest (Slone et al., 1990). In that case, PI 416937 maintained higher relative water content and a more negative OP, and differences became more obvious in late afternoon in comparison with Forrest, which was not included in the current experiments.

The second hypothesis examined was that PI 471938 might have a constitutively higher cell wall modulus of elasticity, which could result in delayed wilting due to the maintenance of leaf rigidity at the onset of water deficit. The results from the pressure-volume experiment showed that the turgor loss point and cell modulus of elasticity were not significantly different between PI 471938 and Benning. As a point of comparison, Sloane et al. (1990) also found that modulus of elasticity was similar between PI 416937 and Forrest in well-watered and drought-stressed soybean leaves from the field. In using the pressure-volume approach, however, it is not possible to dismiss the possibility that PI 471938 might develop a higher cell modulus of elasticity than other genotypes under severe drought conditions. Some studies have observed genotypic differences in cell wall elasticity when plants were under severe water-limited conditions (e.g. Edelman et al., 1995; Schultz et al., 1993).

The third hypothesis was that PI 471938 had unique hydraulic properties in well-watered plants that could contribute to maintenance of leaf turgor. In this study, two indicators of hydraulic conductance were measured. The first was the increase in transpiration rate in response to rhizosphere pressurization and the second was an estimate of whole-plant hydraulic conductance. PI 471938 showed no significant difference in these two parameters as compared to A5959 and Benning. There was a tendency of PI 416937 to have a lower ITR and a lower conductance as reported by Sinclair et al. (2008a), but the difference among genotypes was not significant in these experiments. In any event, these results do not indicate any unique property in PI 471938 in its whole-plant hydraulic conductance.

A leaf-kinetic experiment was conducted to investigate whether faster water movement between water pools in the leaf could be detected in PI 471938. The results here for the four soybean genotypes using the kinetic-rehydration approach confirmed the existence of two distinct rehydration pools as proposed by Zwieniecki et al. (2007). For the fast rehydration pool, there was a lower time constant for PI 471938 as compared to PI 416937 indicating more rapid water movement during rehydration. No significant difference was observed, however, in the comparison of PI 471938 with A5959 and Benning. A longer time constant for the fast phase with PI 416937 compared with A5959 was reported previously (Sinclair et al., 2008b). No significant differences were found among genotypes in the slow-phase of rehydration. Hence, there is little support that the mechanism explaining delayed wilting in PI 471938 is related to difference in leaf hydraulic conductance as measured in these experiments.

Overall, none of the hypotheses tested in this study can clearly explain the mechanism(s) underlying the slow-wilting trait in PI 471938. These results are rather surprising and might indicate that the basis of the slow-wilting trait in PI 471938 is much more physiologically subtle than expected. Nevertheless, the importance of PI 471938 in soybean breeding continues to demand a better understanding of the mechanisms underlying its delayed phenotype. Some evidence of avoiding embolism and cavitation has been reported for crops under drought conditions (Sack et al., 2006); this would allow for maintenance of water potential and hydraulic conductance, which would result in water availability for the cells. Consequently, vascular anatomy of PI 471938 might be the next useful focus to explain the unique properties of this genotype under soil drying conditions.

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FIGURE CAPTIONS

Figure 1. Normalized transpiration rate (NTR) plotted against FTSW (fraction of transpirable soil water) for genotypes PI 471938 (A,B), Benning (C,D), and PI 416937 (E,F) for both greenhouse and growth chamber experiments. The two sets of symbols represent replicate experiments in each case. The breakpoint (BP) is given for each case.

Figure 2. Rehydrated osmotic potential (OP) plotted against FTSW (fraction of transpirable soil water) for genotypes PI 471938 (A,B), Benning (C,D), and PI 416937 (E,F) for both greenhouse and growth chamber experiments. The two sets of symbols represent replicate experiments in each case. The breakpoint (BP) is given for each case.

Figure 3. Relative water content (RWC) hydrated osmotic potential (OP) plotted against FTSW (fraction of transpirable soil water) for genotypes PI 471938 (A,B), Benning (C,D), and PI 416937 (E,F) for both greenhouse and growth chamber experiments. The two sets of symbols represent replicate experiments in each case. The breakpoint (BP) is given for each case.

Figure 4. Representative rehydration kinetic curve for PI 471938 obtained by plotting water uptake vs. time since cutting of petiole.

Figure 5. Halftimes for (A) fast-phase compartment and (B) slow-phase compartment for the rehydration kinetics of the four tested soybean genotypes. Values are Mean ± SEM (n=6). Bars with different letters are significantly different ($p < 0.05$) resulting from one-way analysis of variance.

Table 1. Description of characteristics of soybean genotypes used in experiments.

Genotype	MG	Type of germplasm/Origin	Description
PI 416937[a]	V	Plant Introduction/ Japan	Slow- wilting in field; prolific rooting; constant TR at high VPD
PI 471938[b]	V stress	Plant Introduction/ Nepal	Slow-wilting in field; high yield under High WUE; increasing TR at high VPD
Benning[c]	VI	Hutcheson x Coker 6738/ USA	Fast-wilting; constant TR at high VPD
A5959[d]	V	Monsanto /USA (Commercial cultivar)	High-yield; Fast wilting; constant TR at 30°C, linear TR at 35°C

[a] Sloane et al. (1990), Pantalone et al. (1999), Fletcher et al. (2007)

[b] King et al. (1990), Hufstetler et al. (2007), Sadok et al. (2012), Gilbert et al. (2011)

[c] Boerma et al. (1997), Gilbert et al. (2011)

[d] Fletcher et al. (2007), Sinclair et al. (2008), King et al. (1990), Seversike et al. (2012)

[†] WUE, water use efficiency (g DM kg^{-1} H_2O)

Table 2. Fraction of transpirable soil water threshold values for normalized transpiration rate (NTR), relative water content (RWC), rehydrated osmotic potential (OP) and confidence limit of selected soybean genotypes in Phytotron and Green-house experiments under progressive soil dry down due to two segmental regression analysis.

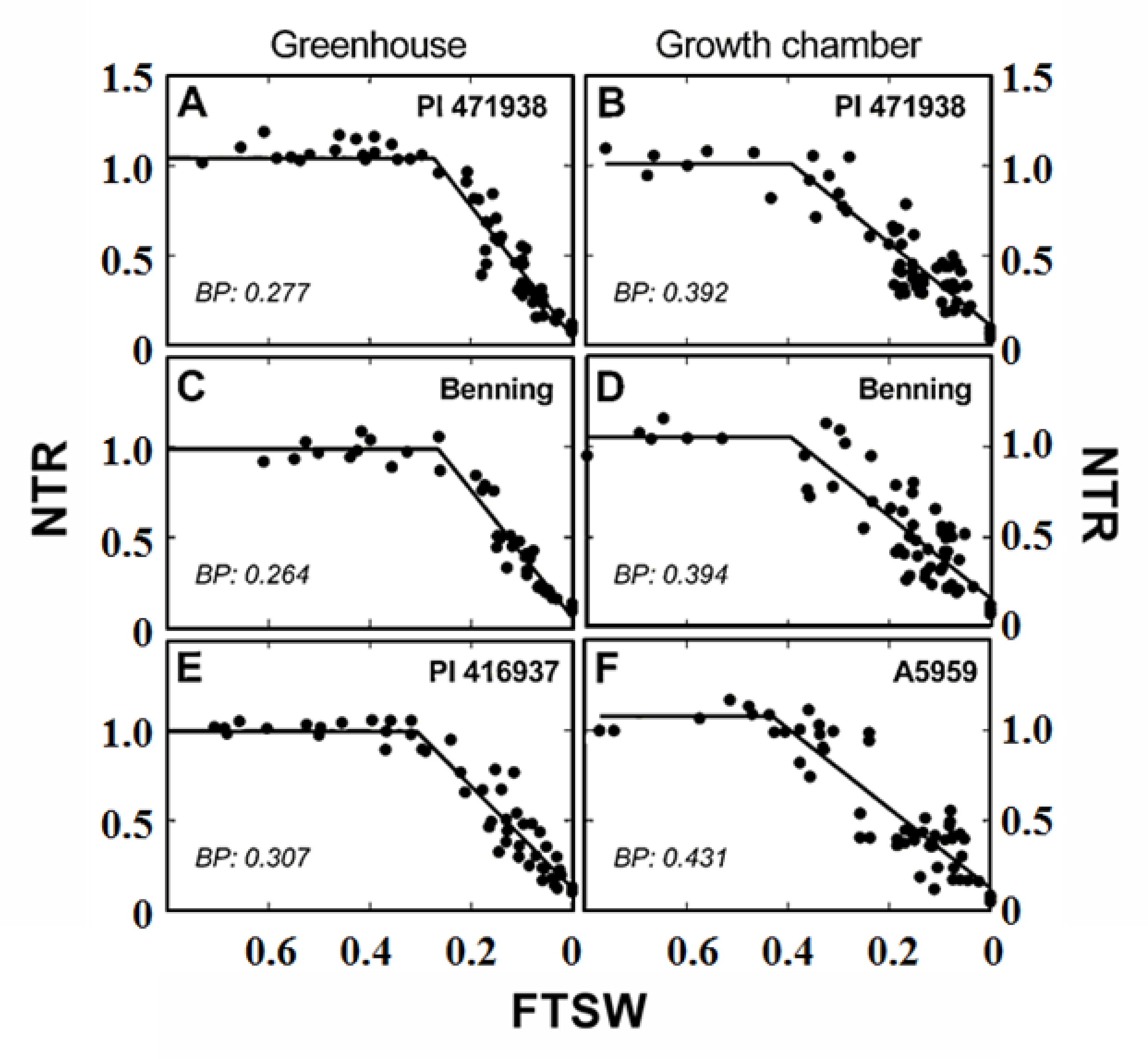
Experiment	FTSW Threshold NTR	Confidence Limits	FTSW Threshold RWC	Confidence limits	FTSW Threshold OP	Confidence limit
Greenhouse						
Benning	0.26a	0.24 to 0.28	0.13a	0.10 to 0.16	0.08a	0.05 to 0.1
PI 471938	0.27a	0.24 to 0.29	0.12a	0.10 to 0.14	0.10a	0.06 to 0.1
PI 416937	0.30a	0.27 to 0.33	0.13a	0.11 to 0.15	0.05a	0.02 to 0.09
Growth chamber						
Benning	0.39a	0.31 to 0.46	0.11a	0.06 to 0.16	0.24a	0.1 to 0.38
PI 471938	0.39a	0.33 to 0.44	0.13a	0.07 to 0.19	0.25a	0.16 to 0.33
A5959	0.42a	0.36 to 0.48	0.10a	0.06 to 0.13	0.23a	0.13 to 0.32

[†] The genotypes represented by similar alphabet are not significantly different from each other (comparing threshold values) based on the confidence limit.

Table 3. Average mean of Turgor Loss Point (ψ_{TLP}), Relative Water Content at turgor loss point (RWC_{TLP}), Bulk Elasticity Module (ε) and Osmotic Potential at full turgor (Π_{100}) from two selected soybean genotypes, PI 471938 and Benning subjected to the vapor pressure deficit (1.5 kPa).

	ψ_{TLP} (MPa)	RWC_{TLP}	ε (MPa)	Π_{100} (MPa)
Benning	-0.84 ± 0.23a	0.94 ± 0.23a	10.7 ± 1.5a	-0.62 ± 0.17a
PI 471938	-0.88 ± 0.24a	0.94 ±0.26a	11.6 ± 1.0a	-0.69 ±0.20a

[†] Data were statistically analyzed using analysis of variance analysis (ANOVA, *p*= 0.05) and Tukey test was used for mean comparison. Means within a column followed by the same letter are not significantly different at p=0.05.



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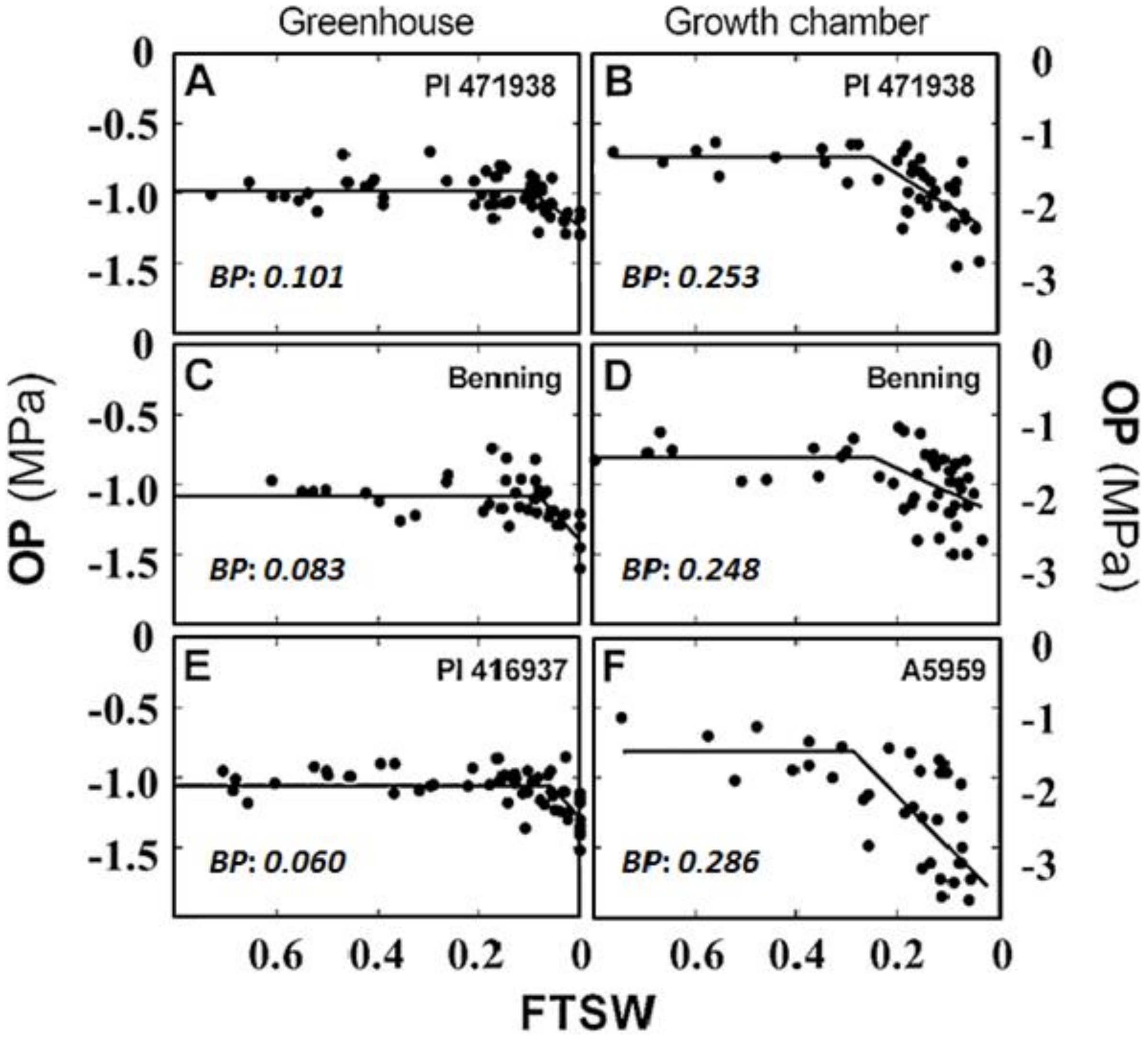
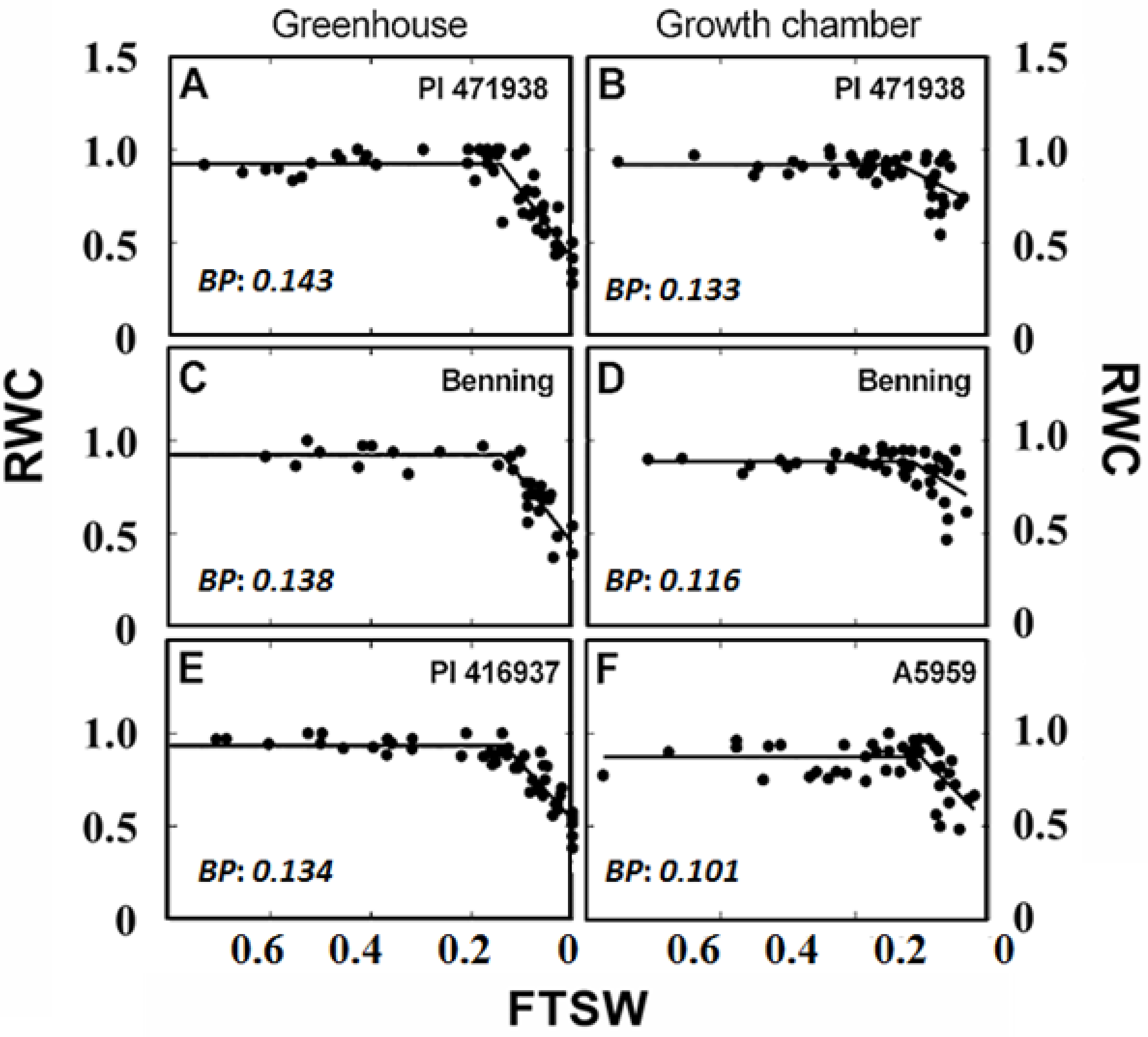


Figure 2. Rehydrated osmotic potential (OP) plotted against FTSW (fraction of transpirable soil water) for genotypes PI 471938 (A,B), Benning (C,D), and PI 416937 (E,F) for both greenhouse and growth chamber experiments. The two sets of symbols represent replicate experiments in each case. The breakpoint (BP) is given for each case.



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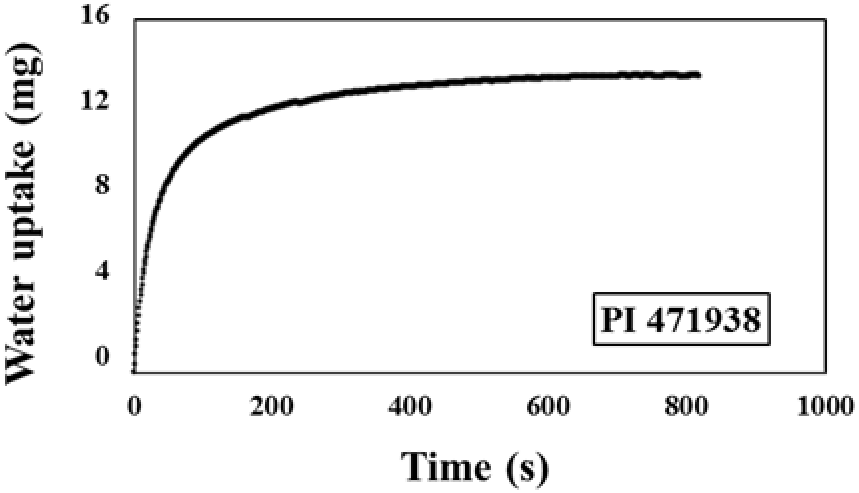

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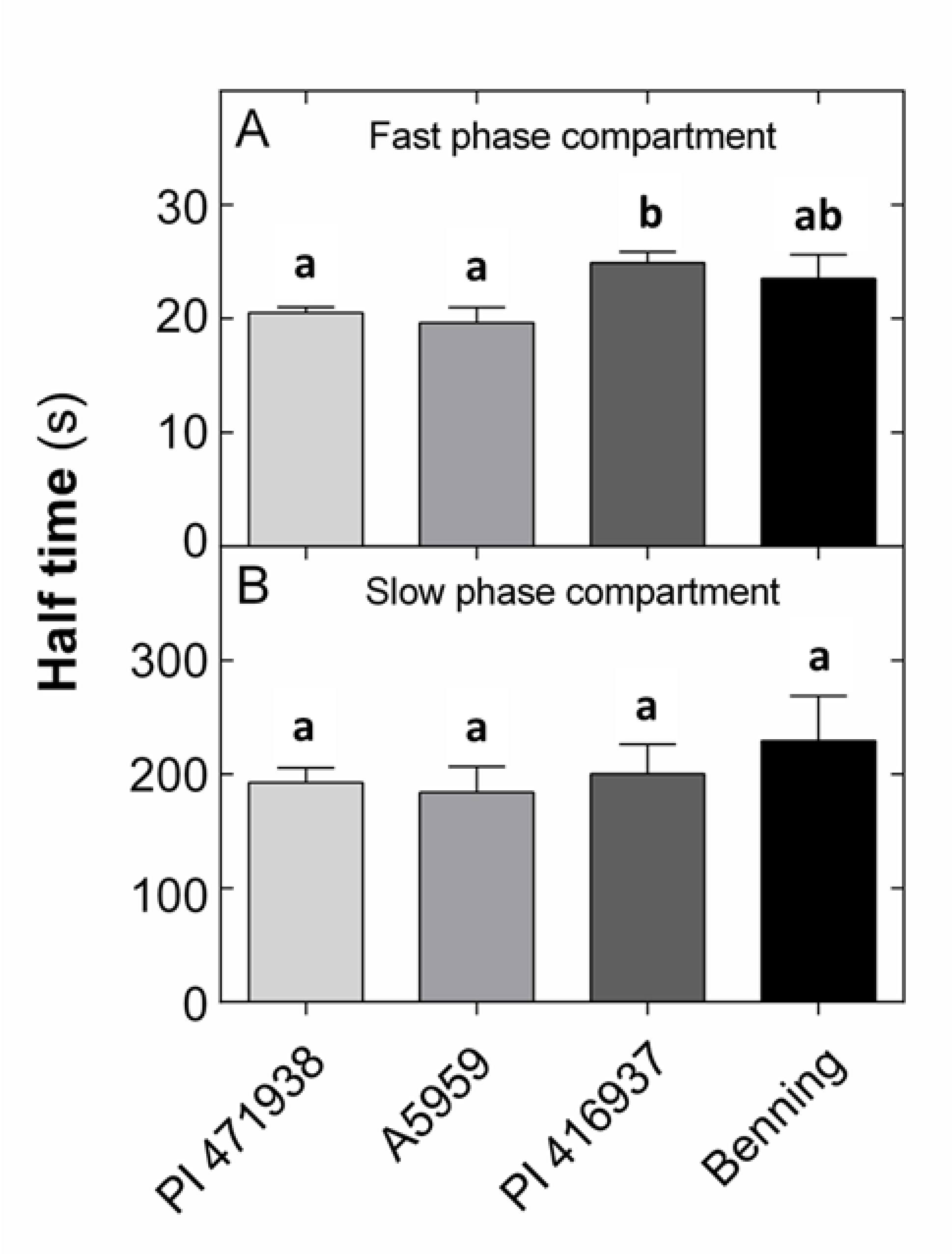

Figure 5. Halftimes for (A) fast-phase compartment and (B) slow-phase compartment for the rehydration kinetics of the four tested soybean genotypes. Values are Mean ± SEM (n=6). Bars with different letters are significantly different ($p < 0.05$) resulting from one way analysis of variance.

CHAPTER 3

Assessing Nitrogen Fixation Activity under Water Deficit Conditions in "Slow Wilting" PI 471938 Soybean

ABSTRACT

Drought results in limitation in crop yield. Many plant traits are being explored for overcoming the effect of water deficit on crop productivity and yield. Soybean genotype PI 471938 expresses “slow-wilting” phenotype. This genotype has been evaluated as a useful drought trait in breeding program. However, physiological mechanisms explaining the slow-wilting trait of PI 471938 still remain unknown. In this study the possibility that this genotype may maintain nitrogen fixation under water deficit was being explored. For evaluating the contribution of root and shoot genotypes in maintaining nitrogen fixation activity under water deficit condition reciprocal grafting technique was used. Moreover, nitrogen fixation activity as well as leaf water potential of slow-wilting PI 471938 compared to fast-wilting genotypes. Greenhouse experiment confirmed the possibility of PI 471938 nitrogen fixation tolerance. Measurements of water potential of PI 471938 as compared to other fast-wilting genotypes showed higher maintaining of leaf water potential during soil drying. However, no distinct difference was observed in grafting experiment when nitrogen fixation activity of PI 471938 compared to Hutcheson genotype.

INTRODUCTION

Crops require adequate water during their growth and physiological development in order to achieve optimum productivity and yield. Lack of soil moisture greatly impacts crop production and causes yield loss (Kramer 1983). Soybean [Glycine max (L.)] is a major crop that is utilized for protein and oil (Grieshop et al., 2001, Dornbos and Mullen, 1982). Symbiotic nitrogen fixation is a key process that supports soybean growth and yield. Maximal nitrogen fixation activity occurs during early reproductive growth (Zapata et al., 1987) and decreases in activity may lead to reductions in yield. Soybean is susceptible to yield loss under drought stress. Sensitivity of nitrogen fixation to water deficit has been implicated as an important factor in yield loss (Serraj et al., 1999). It has been shown that nitrogenase activity declines under water deficit through a shortage of carbon and ATP, oxygen limitation, or sensitivity to volumetric phloem flow (Serraj et al., 1999). Studies have reported that nitrogen fixation is even more sensitive than gas exchange in response to soil water deficit (Sinclair, 1986; Sinclair et al., 1987). Under soil drying, the infection process by the bacterium (Graham, 1992), nodule number, and nodule growth (Mahler and Wollum, 1981) are all inhibited by water deficits. Nodules receive water and other compounds through the phloem and phloem hydrostatic pressure may be inhibited and hence negatively influence nodule physiology (Walsh et al., 1989).

Soybean cultivar differences have been observed in response to nitrogen fixation activity under water deficit conditions (Sinclair et al., 2007; Purcell et al., 2000). Sinclair et al., (2007) found different genotypic responses to soil drying among soybean lines. Greenhouse-based screening with imposed drought stress identified 17 lines out of 100with reduced negative responses. These lines were derived from a cross between two cultivars showing high yield and nitrogen fixation tolerance to water deficit. Among the seventeen lines tested for higher yield, two lines were found to have higher yields compared to commercial cultivars grown in the same study. The two lines also showed higher nitrogen fixation activity under lower soil water content. In another study, there was substantial variation in ureide accumulation among soybean lines in field experiments. It was reported that, soybean lines in which transport N as amides rather than ureides showed substantially increased N_2 fixation rates (Devi and Sinclair, 2013; King and Purcell, 2005).

Soybean (*Glycine max* (L.) Merr.) genotype PI 471938, a plant introduction from Nepal, has been shown to be a slow-wilting phenotype under field soil water deficit conditions (Solane et al., 1990). The genotype possessed a more prolific, fibrous rooting characteristic yet the true mechanism associated with the slow wilting trait remains unknown. This genotype failed to express four different drought-related traits when tested under water-deficit conditions (Sadok et al., 2012). From our previous studies, this genotype also failed to express both higher osmotic adjustment and higher relative water content when tested under drought conditions. Under well-watered conditions, this genotype also did not show substantial difference in cell wall modulus of elasticity when compared with Benning (a fast wilting genotype) (Bagherzadi et al., 2017).

Nitrogen fixation activity is very sensitive to a decrease in the fraction of transpirable soil water (FTSW) (Sinclair et al., 2000). Devi and Sinclair (2013) explored the possibility that PI 471938 may have markedly tolerant N fixation activity in response to water deficit. Based on the greenhouse studies, PI 471938 indeed showed higher nitrogen fixation activity under soil drying. Similarly, this genotype had lower accumulation of ureides in its leaves compared to the fast wilting cultivar Benning. In addition, the water status of PI 471938 was maintained at a greater leaf turgor pressure than Benning during soil drying.

The hypothesis of this study was that PI 471938 has unique water deficit tolerance mechanisms that result in slow leaf wilting and a concurrent maintained level of nitrogen fixation activity. Sustained water potential in the leaves under water deficit might slow leaf wilting, thereby maintaining superior photosynthetic activity and greater volumetric phloem flow. These consequences would in turn result in a reduced sensitivity of nitrogen fixation activity. Hence, the objective of this study was to investigate the characteristics of the slow wilting trait and the concomitant elevated nitrogen fixation rate under water deficit conditions in PI 471938. These measurements utilized reciprocal grafting techniques to contrast genotypic differences in nitrogen fixation and leaf water potential under water deficit conditions.

MATERIALS AND METHODS

Plant material

Two greenhouse experiments examining nitrogen fixation activity were conducted at North Carolina State University, Raleigh, NC. In the first experiment, two genotypes were used, PI 471938 and Hutcheson, which are slow- and fast wilting, respectively. A reciprocal grafting technique was used to determine the effects of the scion (shoot) and rootstock from both PI 471938 and Hutcheson on nitrogen fixation. In the second experiment, four genotypes were used. Two genotypes, PI 471938 and PI 416937, were slow-wilting, while the other two genotypes, Benning and A5959, were fast-wilting. These genotypes were contrasted for transpiration, nitrogen fixation and leaf water potential in response to water deficit.

In both experiments, seeds were sown in polyvinyl chloride pots (10 cm diameter wide and 30 cm tall). Eight pots for each genotype were used. Soil used in both experiments contained 69% sand, 18% silt and 13% clay. The pots were inoculated with Rhizobium bacteria (*Bradyrhizobium,* Nitragin, Inc.). Plants were grown under well-watered conditions. Incandescent lamps used to extend the day length in all experiments to 16 hours to prevent initiation of reproductive growth.

Grafting technique

Soybean seeds were sown in the 1.5 L pots and seedlings were grafted two weeks after sowing. Graft combinations include self-grafted PI 471938 and Hutcheson and reciprocal grafts (each scion-to-root combination). Grafting was done with seedlings that had two trifoliolate leaves. The grafting method was after Bezdicek et al. (1972). Plastic drinking straws (5 mm in diameter) and 3 mm rubber band was used. Straws were cut into 1.2 cm lengths and cut lengthwise using a razor blade. The rootstock section of the hypocotyl was slit from the cut end downward about 1.5 cm (rootstock was vertically split). The scion portion of the hypocotyl sliced to form a wedge, (V-shaped stem base). The scion was then inserted into the split portion of the rootstock and the grafted area was secured by placing the straw portion around it and held in place by a rubber band. White plastic cups were placed on top of the plants to maintain high humidity and increase survival rate of the grafted seedlings. Grafted seedlings were kept in the shade away from direct sunlight. Survival rate was 90%. After one week, plants were transferred to 1.5 L polyvinyl chloride pots (10 cm diameter and 30 cm tall).

Acetylene reduction activity measurement

For measuring nitrogen fixation activity, acetylene (C_2H_2) reduction activity (ARA) was measured (Rice and Paul, 1971). Water was withheld when plants had 4 to 5 trifoliolate leaves. Prior to the water limitation, pots were overwatered, drained overnight and constituted the maximal water holding capacity (MWHC). The following morning, a two-piece lid was placed on the pots to render them air-tight and preventing soil evaporation. The pots containing the lids were weighed early in the morning (the fully watered pot weight at this point considered as the initial weight). Pots were weighed again daily during mid-afternoon (at the same time every day) and the difference in daily weight was used to calculate daily loss due to transpiration. Water was added daily to well-watered pots (WW) to return them to 100 g less than the MWHC (to prevent over saturation of the pots). Three pots were used as well-watered throughout the experiment. The water deficit plants (WD) were dried progressively with water only added if necessary to limit the daily decrease in soil water to no more than 70 g per pot. Five pots per genotype were used for the water stressed treatments.

Pots were measured for their daily nitrogen fixation activity during the soil drying cycle. Each day (before noon), a 10% acetylene to air mixture was passed through the pots at a rate of 1 L/min. The mixture flowed for 15 min through the inlets attached to the bottom of the pots. Gas samples were collected using 1 ml syringes from the outflow in the lid. After the sample collection, the ambient air flowed through the pots for one hour to purge the acetylene. Samples were analyzed for ethylene concentration by using flame ionization gas chromatography (Shimadzu Model 5710A; Hewlett-Packard Crop.), as shown in Figure 1.

Mean ARA was calculated for well-watered plants per genotype once per day. For drought stressed plants, a ratio was calculated between the mean ARA of well-watered plants and the value of ARA of drought stressed plants (first normalization). In all experiments, double normalization used for acetylene reduction activity values (Ray and Sinclair, 1997). The first normalization used to correct for any differences in activity among individual plants within a genotype. The second normalization used to correct for fluctuations in the acetylene reduction activity for well-watered plants among days of measurements. The normalization was obtained by taking the mean ARA ratio prior to any appreciable soil drying. As a result, normalized ARA (NARA) data were valued at 1.0 when the soil was under well-watered conditions.

Double normalization was also used for normalizing transpiration rate (NTR). Normalized transpiration rate was calculated for each drought stressed plant. The experiment was terminated for each pot when daily NTR ratio reached to 0.1, reflecting the fact that transpiration of drought stressed plants reached to 10% of the well-watered plants.

For water deficit plants, data was plotted against soil water content expressed as FTSW (fraction of transpirable soil water). The FTSW was calculated from the difference between current pot weight and final pot weight divided by the total transpirable soil water, which is the difference between the initial weight of the pot and the weight of the pot when daily NTR of that pot reached to 0.1.

Leaf water potential measurements

In experiment 2, leaf samples were collected for water potential analysis from four genotypes (PI 471938, PI 416937, Benning and A5959). Daily, pre-dawn leaf samples were taken from well-watered (WW) and water deficit (WD) plants. Leaf samples were immediately placed in a closable plastic bags for transport and water potential measurements were taken using a pressure chamber (Model 1000, PMS instrument Company, Albany, OR, USA).

Data analysis

The FTSW was plotted against NARA and NTR by using two-segment linear regression analysis. A threshold (Xo) for each genotype was analyzed at which ARA began to decrease using GraphPad Prism 2.0 software (GraphPad Prism 2.0 Software Inc., San Diego, CA, 1996). The regression model for the analysis consist of two intersecting linear regressions. The first slope value is equal to 1.0 as a result of the normalization of the data when the soil water content was still at pre-stress levels. The breakpoint value (Xo) for NARA and NTR calculated where there was a decrease from the early plateau segment and linearly decreasing segment at low FTSW. Genotypes were compared for their breakpoint values using 95% confidence interval.

RESULTS

The relationships of NTR and NARA to FTSW under soil water deficit in both experiments were described by two segment linear regression (Figures 2, 3, 4 and 5). The R^2 values for individual genotypes for the relationship of NTR to FTSW were greater than 0.80. In addition, the R^2 values for relationship of NARA to FTSW was approximately 0.50 or greater. The FTSW thresholds for the decrease in NTR for both experiments ranged from 0.26 to 0.38 (Table 1). The results demonstrated that the decline observed for each soybean genotype in NTR is the same range in both experiments.

The FTSW threshold values for NARA ranged from 0.12 to 0.44 for experiment 1 and 2 respectively (Table 1). In experiment 1, FTSW threshold values at which NARA started to decline for two genotypes were very similar (Figure 3). Self-grafted PI 471038 and Hutcheson had a FTSW threshold 0.13 and 0.12, respectively. The reciprocal grafted PI/H (PI 471938 scion on the Hutcheson root) had a FTSW threshold value of 0.13 and H/PI (Hutcheson scion on the PI 471938 root) had a value of 0.15. For comparing genotypes for their FTSW thresholds, confidence intervals used and genotypes showed similar threshold values for NARA (Table 1).

Additionally, in experiment two, the FTSW threshold at which NARA started to decrease was 0.23 for PI 471938 and was the lowest value in the four genotypes tested (Figure 5). Genotypes PI 416937 and A5959 had thresholds of 0.44 and 0.42, respectively. Genotype Benning had an intermediate threshold of 0.33. In experiment 2, PI 471938 found to have a lower threshold than other tested genotypes.

There was a general decline in morning leaf water potential as soil water deficits intensified in all tested genotypes (Figure 6). The well-watered control plants maintained their water potential during the course of the experiment. The initial water potential was approximately -0.38 MPa for all the genotypes and decreased to -3.7 MPa for PI 416937 at the end of the experiment (day 9). The leaf water potential of the genotypes was similar until the soil started to dry to a FTSW of about 0.3 (data not shown). At the sixth day, PI 416937 and A5959 displayed leaf water potentials of approximately -2.4. On this same day PI 471938 and Benning had leaf water potentials of -0.35 and -0.90, respectively. All genotypes displayed reductions in leaf water potential greater than PI 471938 after day one. At day seven, water stressed genotypes had a lower value of morning leaf water potential compared to control plants, however no significant deference was observed among the genotypes.

DISCUSSION

In this study, the possibility that either above or below ground plant metabolism is involved in maintenance of nitrogen fixation in PI 471938 was tested. Water potential of the leaves of selected genotypes was also determined under drying soil to explore the association of water status to nitrogen fixation. Devi and Sinclair, (2013) reported PI 471938 had less sensitivity of nitrogen fixation to soil drying when compared to another slow wilting genotype (PI 416937) and fast a wilting genotype (Benning). The threshold for the decrease in N_2 fixation with the soil drying for PI 471938 reported around 0.26 whereas Benning exhibited a value of 0.49. The results are consistent with the study herein with PI 471938 exhibiting a lower threshold compared to other tested genotypes, however, no differences were observed in the grafting experiment.

Delayed decreases in nitrogen fixation activity in response to soil drying could be an advantage for PI 471938. The prolonged nitrogen fixation ability could result in increased yield especially during seed filling (Sinclair et al., 2004). While maintenance of greater nitrogen fixation could be a beneficial trait for PI 471938, it is not known whether such an advantage arises from the shoot, the root or both. The grafting experiment allowed for the separation of the above and below ground involvement in transpiration rate and nitrogen fixation rate under soil dying. In contrast, Hutcheson possesses a fast-wilting trait and an earlier decline in transpiration rate in response to vapor pressure deficits when compare to slow wilting genotypes (Devi et al., 2015).

Neither the N_2 fixation threshold nor transpiration rate displayed differences in response to self and reciprocal grafts. Differences were observed in transpiration rate in the reciprocal grafts H/PI and H/H plants. The decrease in normalized transpiration rate with soil drying for H/H grafted plants were around 0.26, while mix grafted H/PI showed the threshold value of 0.34. The results may indicate some benefit from the PI 471938 rootstock in the H/PI graft combination. This response indicates more control of stomata closure under soil drying possibly due to lower root conductance (higher resistance of water movement in the root system) or lower activity of aquaporin (Devi et al., 2016, Carpentieri-Pipolo et al., 2012).

No intact, non-grafted plants were available for the nitrogen fixation measurements due to experimental constraints (40 pots). Comparisons of nitrogen fixation and transpiration rate were only performed on reciprocal grafted and self-grafted plants. As a result, any differences caused by the grafting technique could not be detected. The present results are consistent with other reported studies for transpiration rate under soil dying. As previously reported the threshold decline for transpiration rate for soybean genotypes ranged from 0.4 to 0.3 (King et al., 2009, Devi et al., 2014). These previously reported values suggest that grafting did not affect stomatal behavior. However, there was a shift in threshold values between grafted and intact soybean genotypic nitrogen fixation rates in experiment 2. The threshold values ranged from 0.12-0.15 in experiment 1 and 0.23-0.44 in experiment 2.

Pervious work reported a higher threshold values in both transpiration rate and nitrogen fixation activity for Hutcheson than PI 471938 under soil drying (Cerezini et al., 2016). However, in our grafting experiment no differences were observed in threshold values of the two genotypes. Future experiments should evaluate the effects of grafting on plant performance under stress conditions and non-grafted controls should be included.

Soybean genotypes with tolerance in nitrogen fixation to water deficit have been identified (Sall and Sinclair, 1991). Based on their initial study of 28 cultivars, nitrogen fixation tolerance cultivar Jackson exhibited tolerance. It was reported that Jackson had a decreased threshold for nitrogen fixation of around 0.35. In general, the decline thresholds had a range of 0.11 to 0.28 for FTSW. Based on the present study, plant 471938 falls into this range (Table 1). Although differences in nitrogen fixation activity among genotypes were reported (Sinclair et al., 2000), no clear differences in the threshold for the decline in N_2 fixation activity with soil drying was observed. This lack of trend in decreased threshold was also observed for transpiration rate. Sadok and Sinclair, (2009), have shown no clear difference of NTR threshold comparing five cultivars with PI 471938. Such early decreases in stomata conductance (higher FTSW threshold) under soil drying could lead to lower water usage. However, no link between stomata response and nitrogen fixation has been observed in the presence of water deficit.

Likewise, studies have shown that decreased nitrogen fixation during soil drying could be linked to differences in ureide and amino acid concentration. It has been reported that the concentration differences of organic nitrogen speciation could involve genotypic differences in response of nitrogen fixation to water deficit (Serraj et al., 1999; Purcell et al., 2000). Ureide depletion in response to decreased nitrogen fixation in the leaves or nodules could further cause feedback inhibition of nitrogen fixation activity (Vadez et al., 2000).

While ureide level was not measured in the present study, leaf water potential was measured as well as nitrogen fixation in selected genotypes. The comparison of leaf water potential of the slow wilting PI 471938and PI 416937 and the fast wilting Benning and A5959 showed association between the leaf water potential and nitrogen fixation activity. Although there was no difference between leaf water potential among the well-watered genotypes, the leaf water potential decreased the least in PI 471938 compared to the other genotypes.

Nitrogen fixation has been recognized as being sensitive to water stress (Sinclair et al., 1987; Sinclair, 1986). Water stress can cause decreased nitrogen fixation due to the decrease in photosynthetic supply or can lead to reduction in O_2 flux into the nodule cell (Serraj et al., 1999). Maintaining the integrity and turgidity of the cell plays an important role in mediating the flux of O_2 into the nodule cells. Water potential reflects water status of the plant and can be useful in identifying plant tolerant to water stress (Boyer, 1969). As a result, maintaining water potential of the whole plant could play important role in delaying reduction in nitrogen fixation under drought. The present study demonstrated that water stress reduced genotypic leaf water potential and nodule nitrogen fixation activity. Leaf water potential was maintained in PI 471938 longer than any other genotype. The lower threshold for decreased nitrogen fixation in PI 471938 compared with other genotypes supports the idea that regulating and preserving water potential could be a key factor in maintaining N_2 fixation activity.

Although there is no clear mechanism for maintenance of water potential and fixation in response to water deficit, phloem flow and feedback inhibition effects on nitrogen fixation could be important mechanisms. Serraj et al, (1999) suggested that the transport of water and carbohydrates to the nodules and nitrogenous compounds from nodules occurs through the phloem. Phloem flow rate is sensitive to any variation in leaf water potential. As a result, any variation in phloem flow through the changes in water status could cause change in the activity and physiology of the nodules. Likewise, the feedback inhibition hypothesis might be closely related to the water economy of the nodules. Even small decreases in water potential can cause decreased phloem flow rates and cause increased accumulation of nitrogenous products in the nodules. This build-up could trigger feedback inhibition of nodule activity (Serraj et al., 1999).

In summary while the grafting experiment showed some linkage between root system of PI 471938 and decreased NTR threshold values under soil drying, no particular differences were observed in the NARA values. Furthermore, it was found that PI 471938 has more nitrogen fixation tolerance under soil drying than slow-wilting and fast-wilting genotypes. Maintaining leaf water potential in this genotype could be a key factor in regulating of water economy of the plant and nodules. Resolving the basis for the unique turgor characteristics of PI 471938 is an important topic for future study.

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FIGURE CAPTIONS

Figure 1. As shown the mixture of acetylene-air flushes through the pots through the inlet attached at the bottom of the pots. Escaping as was sampled with syringes from the outlet connected to the top of the pots. Samples were subsequently analyzed for ethylene concentration utilizing gas chromatography.

Figure 2. Normalized transpiration rate data plotted against FTSW (fraction of transpirable soil water). The four combinations of grafting included, self-graphed PI/PI and H/H ((same genotype used for scion and root stock) and reciprocal graphed H/PI and PI/H (mixed grafts of PI 471938 and Hutcheson) with either used as a scion or rootstock).

Figure 3. Normalized acetylene reduction activity values of self and reciprocal-grafted soybean genotypes plotted against FTSW. Grafted combinations are self-grafted PI 471938 and Hutcheson (PI/PI and H/H) and reciprocal-grafted PI 471938 and Hutcheson (H/PI and PI/H).

Figure 4. Normalized transpiration rate data plotted against the fraction of transpirable soil water (FTSW). Selected soybean genotypes are two slow wilting plant introductions (PI 471938 and PI 416937) and two fast wilting genotypes (Benning and A5959).

Figure 5. Normalized Acetylene reduction activity values plotted against the fraction of transpirable soil water (FTSW). Selected soybean genotypes are two slow wilting plant introduction (PI 471938 and PI 416937) and two fast wilting cultivars (Benning and A5959).

Figure 6. Total water potential of predawn leaf samples of the slow wilting genotypes (PI 471938, PI 416937) and fast wilting cultivars (Benning and A5959) during soil drying cycle. Samples were collected every day for each genotype and plotted against days.

Table 1. Fraction of transpirable soil water (FTSW) and threshold values for normalized transpiration rate (NTR) and normalized acetylene reduction activity (NARA). The confidence limits are for selected soybean grafts (Experiment 1) and genotypes (Experiment 2) under progressive soil desiccation due to two segmental regression analysis.

	FTSW Threshold NTR	Confidence Limits	FTSW Threshold NARA	Confidence Limits
	Nitrogen fixation / Grafting Experiment 1			
PI/PI	0.38a[†]	0.29 to 0.37	0.13a	0.09 to 0.18
H/H	0.26a	0.21 to 0.31	0.12a	0.07 to 0.16
PI/H	0.30a	0.27 to 0.33	0.13a	0.09 to 0.17
H/PI	0.34a	0.31 to 0.37	0.15a	0.10 to 0.19
	Nitrogen fixation / WP Experiment 2			
PI 471938	0.34a	0.31 to 0.37	0.23a	0.11 to 0.35
PI 416937	0.32a	0.29 to 0.34	0.44a	0.24 to 0.64
Benning	0.32a	0.29 to 0.34	0.33a	0.15 to 0.51
A5959	0.30a	0.27 to 0.34	0.42a	0.18 to 0.66

† - The genotypes represented by the same letter are not significantly different from each other (comparing threshold values) based on the confidence limit.

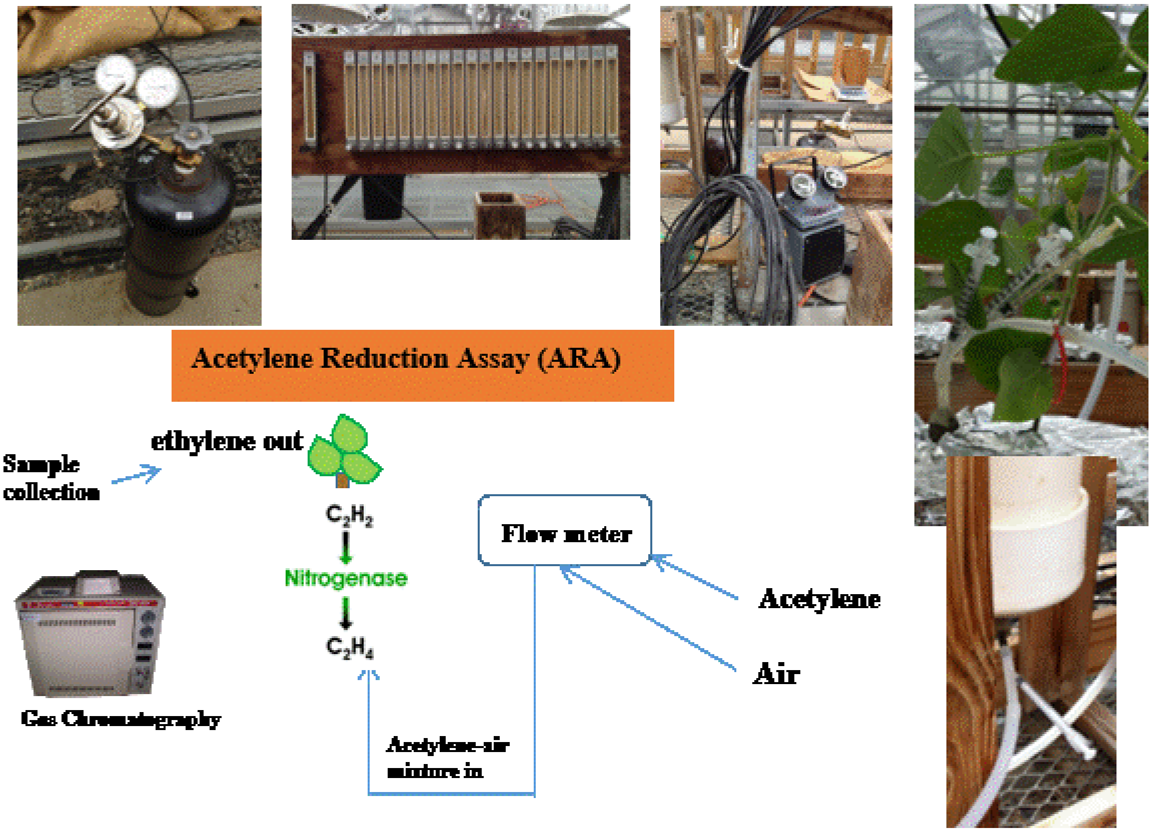

Figure 1. As shown, the mixture of acetylene-air flushes through the pots through the inlet attached at the bottom. Escaping gas was collected with syringes from the outlet at the top of the pot. Samples then were subsequently analyzed for ethylene concentration using gas chromatography.

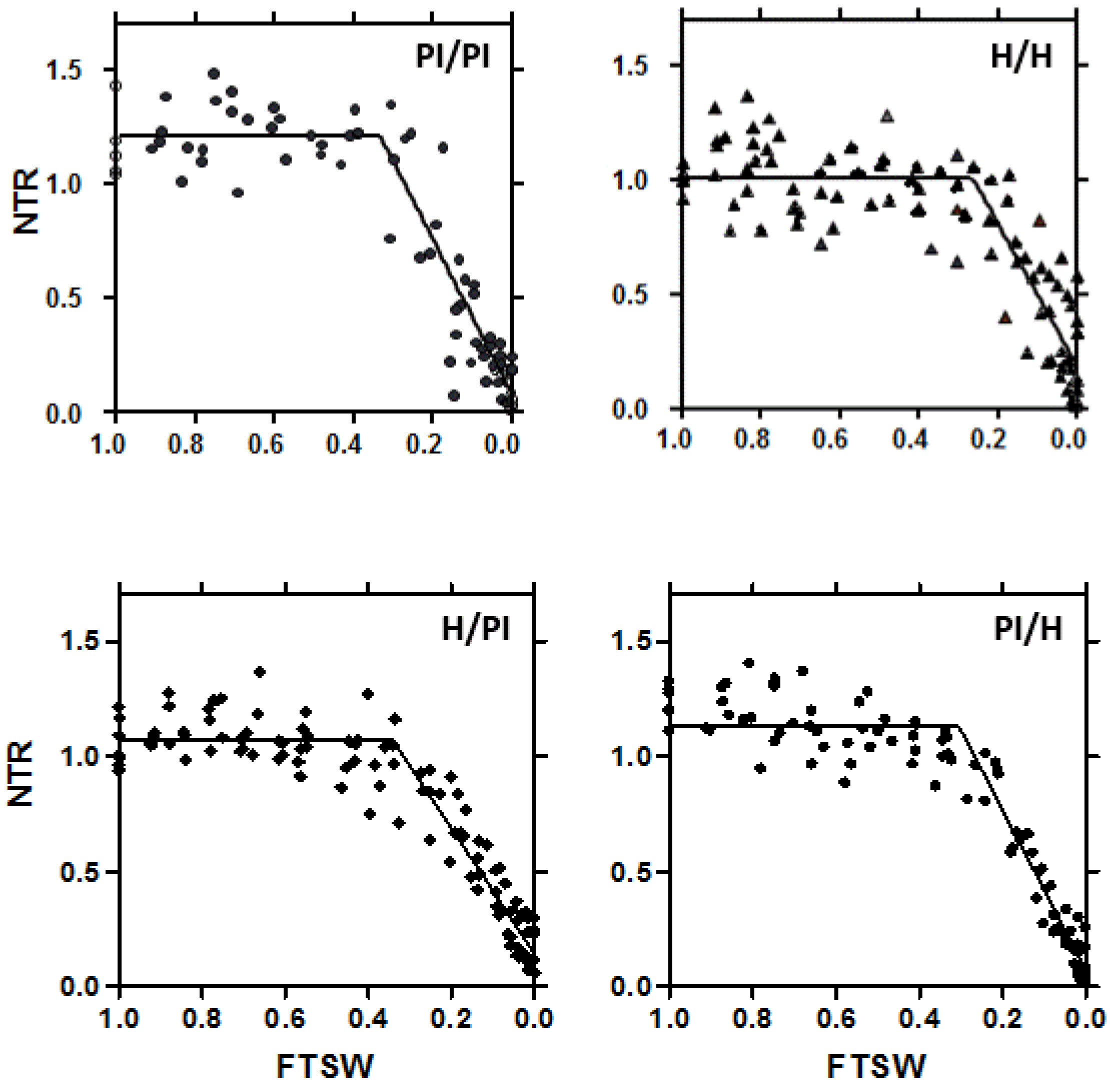

Figure 2. Normalized transpiration rate data plotted against FTSW (fraction of transpirable soil water). The Four combinations of grafting include, self-graphed PI/PI and H/H (self-grafted PI 471938 and Hutcheson, (same plants genotype used for scion and root stock), and reciprocal graphed H/PI and PI/H (mixed grafted, of PI 471938 and Hutcheson) with either used either as a scion or root stock). Confidence interval ranged from 0.21- 0.37.

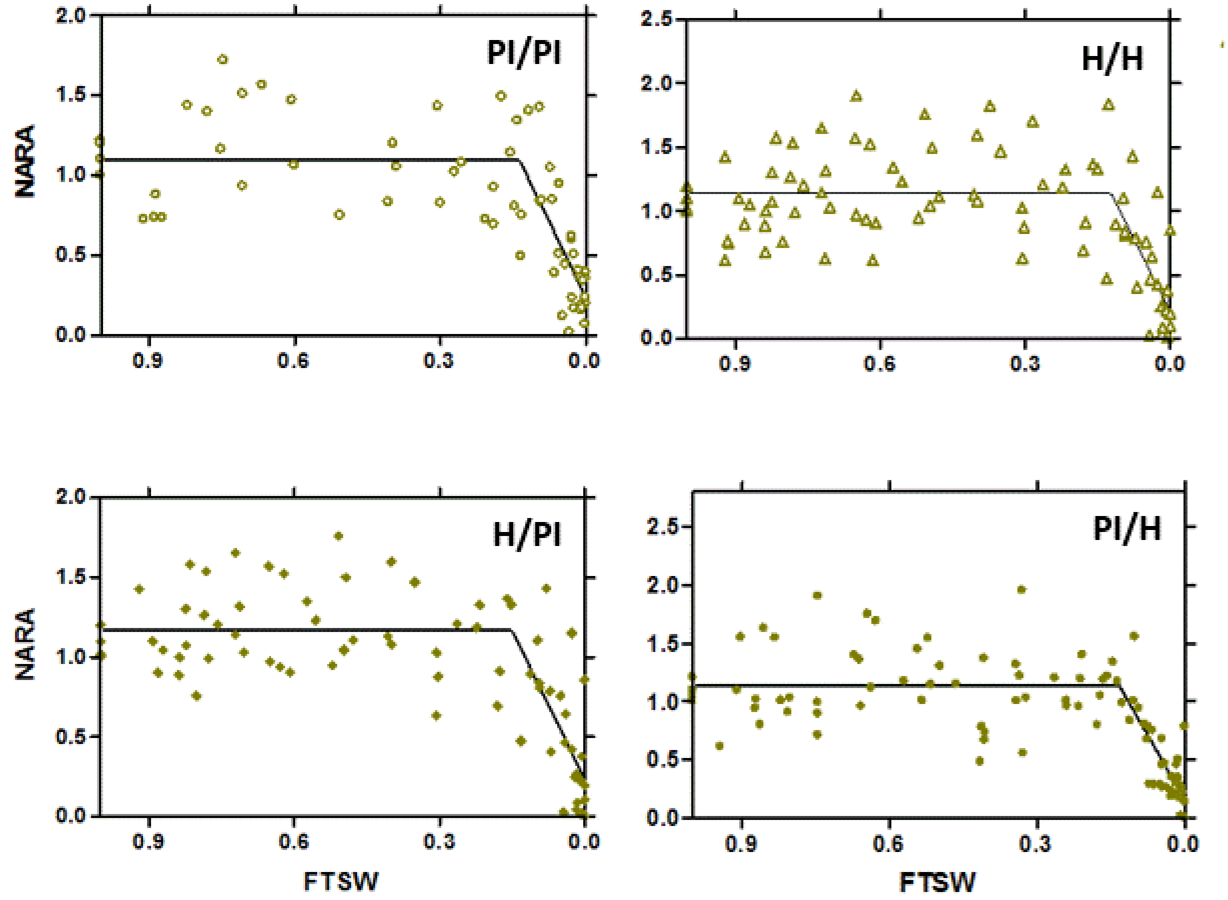

Figure 3. Normalized acetylene reduction activity values of self and reciprocal -grafted soybean genotypes plotted against FTSW. Grafted combinations are self-grafted PI 471938 and Hutcheson (PI/PI and H/H) and reciprocal-grafted PI 471938 and Hutcheson (H/PI and PI/H). Confidence interval ranged from 0.09-0.19.

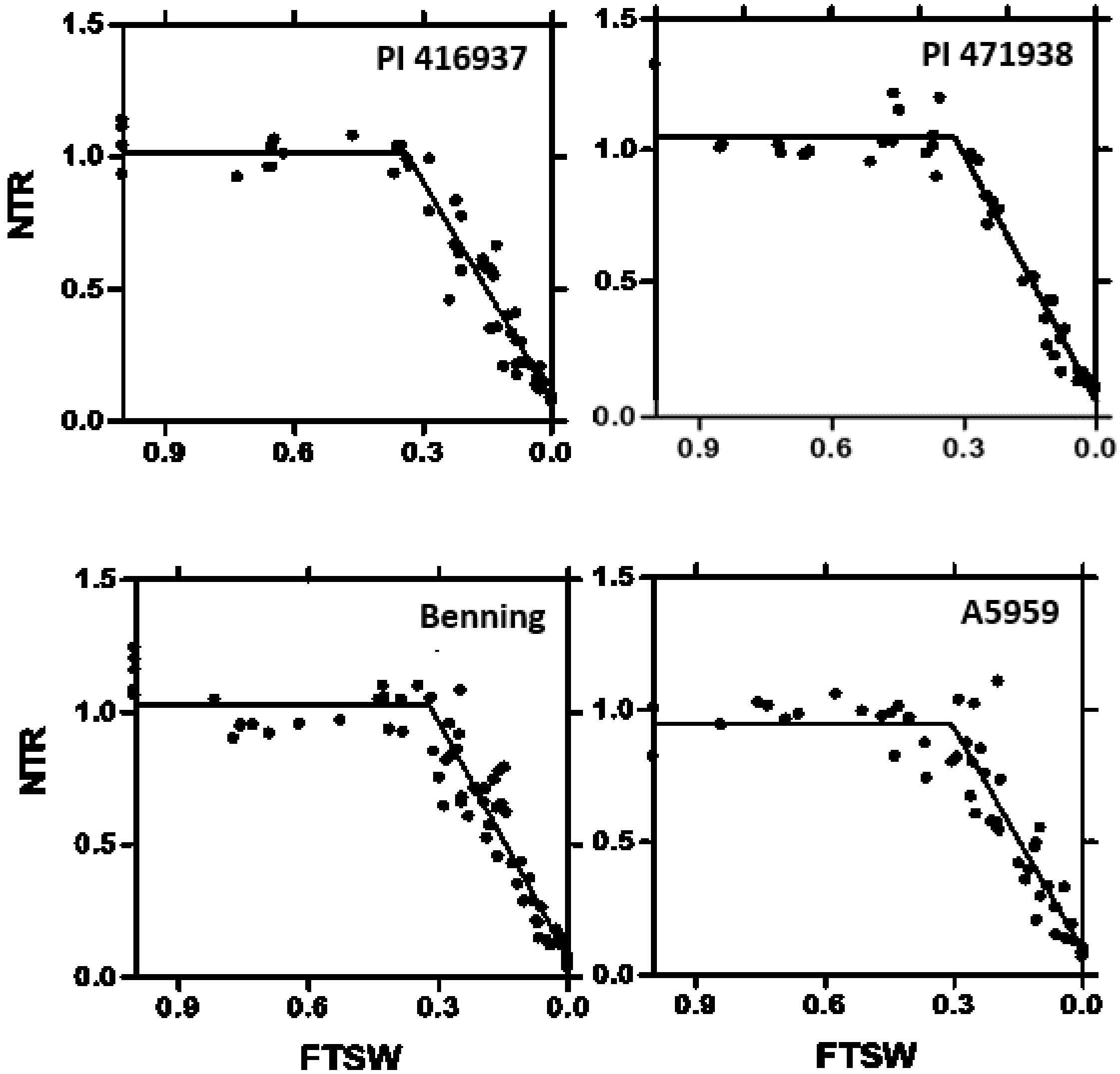

Figure 4. Normalized transpiration rate data plotted against FTSW the (fraction of transpirable soil water (FTSW). Selected soybean genotypes are two slow wilting plant introductions (PI 471938 and PI 416937) and two fast wilting genotypes, (Benning and A5959). Confidence interval ranged from 0.27-0.37.

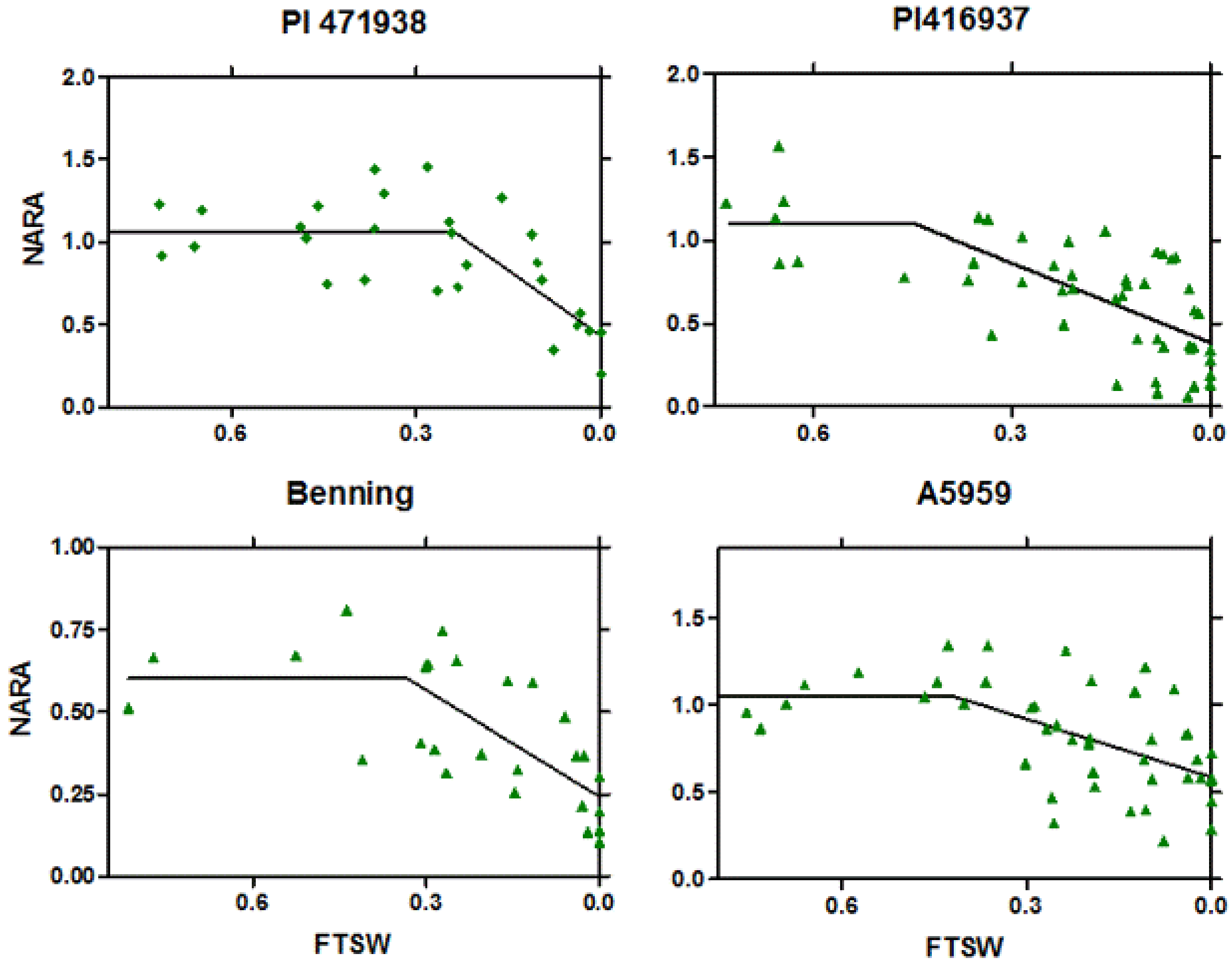

Figure 5. Normalized Acetylene reduction activity values plotted against FTSW (fraction of transpirable soil water (FTSW). Selected soybean genotypes are two slow wilting plant introduction (PI 471938 and PI 416937) and two fast wilting genotypes, (Benning and A5959). Confidence interval ranged from 0.11-0.66.

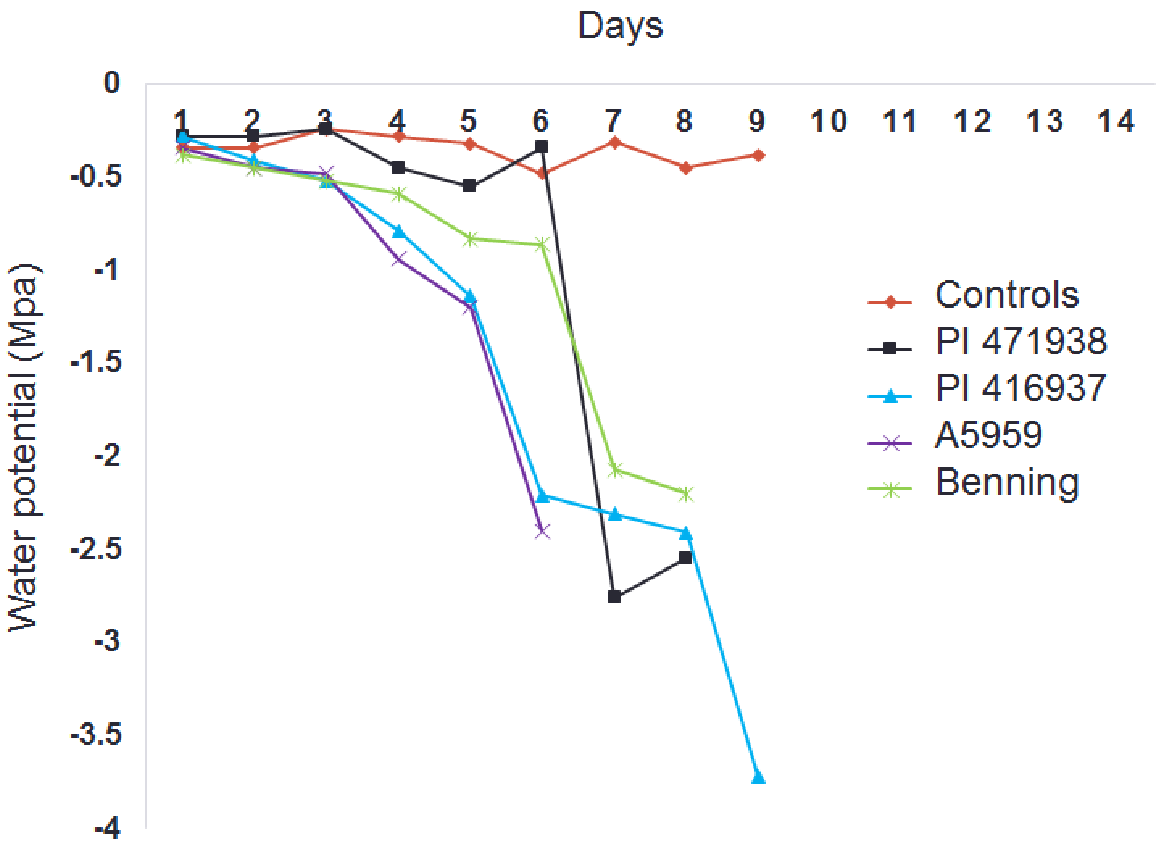

Figure 6. Total water potential of predawn leaf samples of the slow wilting genotypes, (PI 471938, PI 416937) and fast wilting cultivars (Benning and A5959) during soil drying cycle. Samples were collected every day for each cultivars genotype and plotted against days.

CHAPTER 4

Soybean Transpiration Response to Vapor Pressure Deficit under Nitrogen Stress and Subsequent Recovery

ABSTRACT

This study investigated the effects of nitrogen deficiency and subsequent recovery on transpiration rate in soybean and possible adjustments in their response to increasing vapor pressure deficit (VPD). Two soybean genotypes [Glycine max (L.) Merr.] were studied, PI 471938 and PI 416937. Both are currently used as key germplasm in soybean breeding programs. Non-nodulated soybean plants were grown in sand culture in a controlled environment with a four-week treatment duration. A complete nutrient solution with 2.0 mM nitrogen was supplied during establishment and to control plants throughout. Nitrogen was withheld from N-deprived plants for an initial 10-14 days and then resupplied with 2.0 mM nitrogen during a 10-day recovery. Plant transpiration was continuously measured using balances. Periodically during the treatment period, plants were exposed to a range of VPD conditions to determine their ability to control transpiration as VPD increased. In nitrogen-stressed plants, leaf expansion and transpiration per unit leaf area were markedly inhibited in both genotypes. Under all conditions, PI 416937 maintained the ability to limit transpiration at high VPD, with a breakpoint for lower transpiration remaining at about 1.75 kPa. The PI 417938 also had lower transpiration at high VPD in both nitrogen treatments, but the breakpoint did not occur until about 2.3 kPa. Both genotypes under nitrogen stress showed lower transpiration and stomatal conductance under vapor pressure deficit conditions. Furthermore, during recovery from nitrogen stress, the transpiration response reverted and became similar to control plants.

INTRODUCTION

In legumes, nitrogen required for growth may be obtained from either mineral nitrogen available in the growth medium or through biological nitrogen fixation (George and Singleton, 1992). The proportion of nitrogen derived from each source depends on environmental factors (i.e. mineral nitrogen availability, abiotic stress, rhizobia bacterium availability, developmental stage and genotypic variability (Wery et al., 1986). However, in soils with modest nitrate levels, as much as 50% of the nitrogen content may be derived from symbiotic fixation (Di Ciocco et al., 2011).

The potential for large interactions between nitrogen assimilation and water relations in crops is substantial. Water and nitrogen are the main limiting factors in crop growth and production. However, both N_2 fixation and nitrate uptake are sensitive to drying conditions (Serraj et al. 1999; Cramer et al. 2009). Nitrogen fixation decreases as water availability diminishes. This effect could be due to reduced photosynthesis and subsequent reductions in photosynthate, decreased transport of nitrogen products from the nodules and limited aeration in the nodule (Serraj et al., 1999). Water deficit also reduces nitrogen assimilation, distribution and demand (Gonzalez-Dugo et al., 2005). Further, nitrogen absorption and uptake by crops is reduced under soil drying conditions. Under water deficit plant growth rate diminishes causing decreased N demand by plants (Gonzalez-Dugo et al., 2010).

Transpiration and leaf initiation and expansion are highly influenced by the availability of nitrogen (Tolley-Henry and Raper, 1986). These researchers reported that leaf initiation and expansion as well as photosynthetic activity in nitrogen-stressed soybean were severely restricted. Upon resupply of nitrogen, leaf expansion rate increased, leaf initiation and photosynthetic rate was stimulated and retuned to values similar to those of non-stressed soybean leaves (Tolley-Henry and Raper, 1986). Further, substantial evidence indicates that lowering stomatal conductance (limited transpiration trait) at high atmospheric vapor pressure deficit (VPD) is an important mechanism in drought avoidance through decreased water loss (Devi et al., 2014). Water conservation, especially early during soil drying, reduces the onset of water stress and can help the crop avoid severe consequences of drought (Sinclair et al., 2010). Such experiments, however, have always been conducted with adequate N nutrition and no determination of the interactions between water and N availability were possible.

Nitrogen-deficient conditions reduced transpiration and hydraulic conductivity in cotton plants (Radin, 1990, Radin et al., 1991). Further, nitrogen-stressed cotton plants showed an enhanced sensitivity of stomata to water deficit. Thus, the hypothesis tested in the present study is whether nitrogen stress accompanying water stress alters the hydraulic response of soybean [Glycine max (L.) Merr.] to high VPD. Further, the objectives of this study with soybean were (i) to investigate the effects of nitrogen stress and subsequent restoration of nitrogen on transpiration rate and leaf expansion, (ii) to assess adjustments in these transpiration responses to increasing VPD and (iii) evaluate the stability of limited transpiration trait under nitrogen stress and recovery.

MATERIAL AND METHODS

Experimental conditions

Soybean seeds were germinated on moist paper towels in the dark at 24 C^o (Rufty et al., 1982). Seedlings with longer radicles transplanted into sand culture in 2.5 L pots made of polyvinyl chloride with no *Rhizobia* inoculum. Plants were located in a controlled-environment growth chamber in the North Carolina State University Phytotron. The growth chamber was 9 m^2 in area and had 2.13 m of vertical clearance. It was programmed for day/night temperatures of 30/26^{o}C and photosynthetic photon flux density (PPFD) of 500 umol $m^{-2}s^{-1}$, with a 12h photoperiod and a 3h night interruption to repress flowering.

In the growth chamber, the pots were watered and nutrient added every day. Pots were allowed to drain. Two pots were randomly placed on each cart (0.37 m^{-2}). Balances were used to continuously record pot weights to determine transpiration. Due to the limitation in the number of available balances, thirteen plants were used in each experiment. Plants were assigned to two treatments, six plants for control and seven plants for nitrogen stress treatment. Two genotypes PI 471938 and PI 416937 were used in each experiment; a single genotype was tested during an experiment. Each experiment was repeated twice.

Nitrogen treatment application:

All plants received complete nutrient solutions with 2.0 mM NO_3^- until the 4th or 5th trifoliolate leaf had expanded. Thereafter, N was withheld from plants assigned to the N-stress treatment. After 10 to 14 days of stress, plants were re-supplied with NO_3^- for a ten-day recovery period. Initial concentrations of nutrients in culture solution were 2.0 mM NO_3^-, 0.5 mM H_2PO^-, 1.1 mM K^+, 1 mM Ca^{2-}, 1.0 mM Mg^{2+}, 1mM SO_4^{2-}, 17 uM B, 3.0 uM Mn, 2.2 uM Cl, 0.3 uM Zn, 0.1 uM Cu, 0.04 uM Mo, and 1.0 mg Fe L^{-1} as Fe-EDTA (Tolley-Henry and Raper 1985). The complete culture solution contained $Ca(NO_3)_2$ for the nitrate treatment, but this was replaced with CaCl2 in the nitrogen-deficient treatment. All other nutrient concentrations were the same in both solutions. Over the course of experiment, nutrient solutions were made fresh daily and added to each pot. The amount of solution was added to each pot based on the calculated transpiration water loss on that day per each pot. Every week, pots were flushed out with distilled water to avoid nutrient and salt build-up in the pots.

Chamber VPD experiments:

Data loggers (EL-USB-2-LCD+, Lascar Electronics Ltd., Erie, PA) were used to record air temperature and relative humidity. Data loggers recorded temperature and humidity over 1-minute intervals and the values were used to calculate the mean VPD. (Figure 1). Three target VPD levels (1 to 2, 2 to 3, and 3 to 4 kPa) were maintained by manipulating relative humidity through use of ultrasonic humidifiers (Model V5100NS, Vicks P&G Cincinnati, OH). Two separate VPD experiment were performed, one during the N deficiency phase (10 days after N stress began) and one during the N recovery phase (5 days after N recovery began).

Transpiration measurements:

Pots were placed on individual balances and plant transpiration was continuously measured **(**Figure 1**)**. The balances had a 6000g capacity with a precision of 0.01 g (Model SP-6001, Scout Pro, Ohaus Crop., Parsippany, NJ). The balances were connected to a computer via USB connections and pot weights were recorded in 1-minute intervals. WinWedge data collection software (TAL Technologies Inc., Philadelphia, PA) was used to obtain readable output from each balance.

Weights recorded each minute showed considerable variation, possibly due to air circulation in the growth chamber. To overcome this issue, spline regression was applied to weight data using Proc TPSPLINE procedure in SAS 9.4 (SAS institute Inc., NC). The spline regression was used to obtain weights for one-hour intervals. Transpiration rate per unit leaf area was expressed as a function of VPD, with daily leaf area calculated from terminal leaflet length (Bakhshandeh et al., 2011). Ratio of leaf area was calculated taking the ratio of average of all stress plants to the average of control plants per that day. Alterations in transpiration rate at increasing VPD were determined using two segmented linear regressions (Graph pad prism

2.01; Graph pad Software Inc., San Diego, CA, 1996). Final leaf area was also determined with a LI-3100C portable area meter (LI-COR Biosciences, Nebraska, USA).

RESULTS

Leaf area

Significant decreases in growth for both PI 471938 and PI 416937 were observed in response to nitrogen deficiency (Figure 2). This outcome was observed through reductions in both leaf number and leaf expansion in response to 0.0 mM NO_3^- compared with plants continuously fed 2.0 mM NO_3^-. Leaf number and leaf expansion rate began to decrease a few days after initiation of N deficiency (data not shown). After exogenous NO_3^- was resupplied in the nutrient solution at around 14 days after treatment (DAT), leaf area ratio halted its decline. Addition of nitrogen prevented the decrease of leaf area ratio however, it did not reach an equivalent value as non-stress plants in either genotype (Figure 2). The decrease in leaf area ratio for PI 416937 was less than observed for PI 471938. The decrease at the time of N resupply was approximately 20 and 50% for these respective genotypes. In addition, the cessation of the decline upon N resupply was later for PI 471938 than PI 416937.

Transpiration rate

Transpiration rate per unit leaf area remained constant during the course of the experiment for non-stressed soybean genotypes (Figure 3). However, imposing nitrogen stress caused transpiration rates to decline within 2 to 3 days following NO_3^- withdrawal. The decrease was greater for PI 471938 than PI 416937 with maximal reductions of transpiration of approximately 55 and 35%, respectively. The decline of transpiration rates per unit leaf area was halted quickly after NO_3^- was resupplied in both genotypes. The response of PI 471938 was a linear increase approaching 140% of the control response at 21 DAT. The response of PI 416937 was more meandering yet reaching approximately 145% of the control plants by 22 DAT (Figure 3).

Transpiration water loss in response to vapor pressure deficit

Hourly transpiration was measured for the genotypes while exposed to varying VPD levels during the nitrogen stress and recovery phases (Figure 4). Overall, mean normalized transpiration rate increased in response to intensified VPD. However, the amount of water loss (transpiration increase) differed substantially between nitrogen stressed and non-stressed plants in both genotypes. Maximal mean transpiration for PI 471938 at the greatest VPD values was approximately 26 and 16 $gH_2O/m^2/10min$ per unit leaf area for non-stressed and stressed plants, respectively. For PI 416937, the maximal mean transpiration for non-stressed and stressed plants was considerably higher with approximate values of 40and 30 $gH_2O/m^2/10min$, respectively.

The two genotypes exhibited breakpoints (BP) in transpiration responses to increased VPD. The BP is the point where transpiration transitions to a more moderate increase following initial responses with greater slopes. Values for the breakpoints are shown in Table 1 for two separate experimental runs. Break points ranged from 1.7 to 2.3 and 2.1 to 3.1 kPa during nitrogen stress for experiments one and two, respectively. The R^2 for the two-segment regression for each of these two genotypes ranged from 0.6-0.91 during nitrogen stress phase overall for both experiments (Table 1). The BP for PI 416937 was lowest (1.7 kPa) during the N-stress phase for both the control and the N-deficit plants (Table 1, Figure 4).

The slope of the linear regression below the BP (S1), ranged from 9.1 to 38.7 $gH_2O/m^2/10min/kPa$ for experiment one and were generally larger than experiment two which ranged from 7.5 to 13.5 $gH_2O/m^2/10min$. The slope of the transpiration response to VPD above the BP (S2) was much less than S1. The value of S2 did not vary among the genotypes during nitrogen stress phase. The range was from 0.8-5.7 $gH_2O/m^2/10min/kPa$ overall for both experiments.

During the nitrogen recovery phase, nitrogen supply was re-established to the nitrogen-stressed plants. During that period, the relationship of transpiration rate to VPD response was followed for five days. The same trend was observed during the nitrogen recovery phase; VPD increases resulted in increased transpiration rate in both genotypes. The mean transpiration for the high

VPD treatment was approximately 26 and 37 for PI 471938 and PI 416937, respectively (Figure 4).

During the nitrogen recovery phase the effect of prior nitrogen status on transpiration was less evident (Figure 4). Nitrogen recovery plants showed same rate of water loss compared to control plants after resupply of nitrogen. The range in BP was much reduced to 2.0 to 2.3 and 2.1 to 3.1 kPa for experiments one and two, respectively. The TR response during recovery of PI 471938 was much reduced compared with that of PI 416937. At the highest VPD, PI 471938 and PI 416937 displayed TR values of about 22 and 35 g H20/m2/10 min, respectively.

DISCUSSION

Drought is the main limiting factor in crop growth and production (Boyer, 1982). Zipper et al. (2016) studied drought sensitivity of US maize and soybean production from 1958 through 2007. Drought sensitivity was associated with 13% of yield variability especially in the central and southeastern US and that sensitivity has become more susceptible to drought over time (Zipper et al., 2016). Identifying traits such as limited-transpiration response to water deficit would likely lead to water conservation (Gholipoor et al., 2010). This trait has been identified in various crop plants and shows yield advantages through water conservation under high levels of atmospheric VPD (Sinclair et al., 2016). One critical question is whether the limited transpiration trait persists in variable in growing conditions. For example, does this trait remain engaged under nitrogen stress? Sinclair et al, (1975) noted that availability of nitrogen within the plant could be a huge factor in increased yield in soybean. The main question of this study is to find whether the limited- transpiration trait holds up under low-nitrogen conditions.

Genotypes PI 471938 and PI 416937 were collected from Nepal and Japan, respectively and have been key germplasm resources in soybean breeding programs aimed at increasing drought tolerance. It has been shown that progeny of these genotypes possess drought tolerance and higher yields (Carter at al., 2016). Soybean genotype PI 416937 possesses a slow-wilting trait in the field as well as limited transpiration above a VPD breakpoint of 2 kPa. Reports based on the stimulation studies have shown that the limited-transpiration trait in soybean could result in yield increase of approximately 65% at higher VPD levels (Sinclair et al., 2005). As a result, identifying and developing commercial soybean cultivar with limited transpiration rate at higher VPD is an important goal for conserving water and increasing yield. Carter at al. (2016) released USDA-N8002 in September 2015 and it is the first North American soybean cultivar exhibiting drought resistance and high yield potential. This cultivar exhibits delayed canopy-wilting, lasting N fixation during drought stress, and limited-transpiration when exposed to greater VPD.

In the present study plants showed sensitivity to nitrogen availability. The results indicate nitrogen-stressed plants displayed markedly reduced transpiration and leaf expansion in both genotypes. Both genotypes showed immediate decreasing trends transpiration rate upon nitrogen limitation (Figure 3) with PI 471938 decreasing to its lowest rate of approximately 50% at 12 (DAT). Genotype PI 416937 decreased to approximately 60% at 12 DAT. Transpiration rates of N-deficit plants returned to the level of control plans within one week after resupply of nitrogen. Transpiration rate eventually reach levels above those seen for the non-stressed plants at 5 and 8 DAT for PI 471938 and PI 416937, respectively. Likewise, resupply of nitrogen in both genotypes resulted in a return of leaf area production in the N-stressed plants. The decrease in transpiration and leaf area is in agreement with both Radin et al. (1982) and Radin (1990) who found lower transpiration and conductance with nitrogen stress in cotton. It was noted that nitrogen deficiency in cotton led to stomata closure through a decrease in water potential and a production of abscisic acid (Radin et al., 1982). The authors hypothesized that nitrogen nutrition altered pH gradients and ABA levels were increased causing stomatal closure. Furthermore, Tolley-Henry and Raper (1986) demonstrated that nitrogen stress severely restricted leaf expansion and when nitrogen concentration in the leaves reached to 9 mg dm^{-2}. In addition, shoot growth and photosynthetic rate decreased with accompanying shift in dry matter partitioning from shoot to root.

The more common response in crops plants is that as the VPD increases (atmospheric air becomes drier) transpiration increases linearly causing plans loose large amount of water (Sadok and Sinclair, 2009). The limited-transpiration trait has been identified in soybean genotypes and they therefore expend less soil water under deficit condition and they use water more efficiently (Sinclair et al., 2008). Genotype PI 416937 has limited transpiration rates when the vapor pressure deficit (VPD) of the atmosphere exceeds about 2 kPa (Fletcher et al., 2007). Breakpoint values for PI 471938 were similar under nitrogen stress and recovery phases with values greater than two. These results however, are not consistent with the measurements of Fletcher et al. (2007) on greenhouse grown plants of PI 471938. It was shown that there was no expression of an increasing limitation on plant transpiration at high vapor pressure deficit. This difference could be due to the different soil type used in the studies. In the present study sandy soil was used; lower water retention capacity of sandy soil might cause closer of stomata and limitation of transpiration rate under higher VPD in PI 471938.

On the other hand, PI 416937 exhibited breakpoints under nitrogen stress of 1.7. Under nitrogen deficiency, nitrogen stressed plants overall show lower transpiration rate however, the breakpoints value remains the same as control plants. Transpiration and stomata conductance responses during the recovery phase returned to similar levels seen in the control plants. With these soybean genotypes, the ability to limit transpiration at high VPD (i.e. the two-segmented increase) is retained under nitrogen stress and displayed BP values similar to the non-stress plants.

In summary, nitrogen deficits caused decreased TR in both genotypes and a return to non-stressed levels is attainable. Further, the TR of PI 471938 was lower than PI 416937 in response to increased VPD in both N-deficit and recovery phases and confirms the TR-limited trait of this genotype. In addition, the present data indicate that the limited-transpiration trait is stable and recoverable upon renewed nitrogen availability. Future genetic enhancement of drought tolerance in soybean may well depend on this trait. This trait can be included in soybean cultivar development and yield advantages can be observed under drought conditions and under nitrogen stress (Carter et al., 2016). Improved soybean germplasm expressing limited water use under either water stress or nitrogen stress would allow more water to be available later in the growing season to complete crop growth and increase soybean yield.

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FIGURE CAPTIONS

Figure 1. A) PVC pots are positioned on balances allowing transpiration to be measured on balances from weight changes in one minute intervals, B) Differences in VPD were established using humidifiers. C) Vapor pressure deficits were calculated from temperature and relative humidity data.

Figure 2. The percentage of leaf area of the N-stressed and subsequent resupply plants compared with the non-stressed plants (set at 100%) for PI 471938 and PI 416937. Non-stressed plants received 2.0 mM NO_3^- continuously while stressed plants received 0 mM NO_3^- until 12 to 14 days after treatment. Stressed plants then received 2.0 mM NO_3^- until termination of the experiment. Each point represents the mean leaf area of seven nitrogen stressed (-N) and resupplied plants (+N) and the mean of six plants for non-stressed plants.

Figure 3. The percentage of transpiration (TR ratio) of the N-stressed and subsequent resupply plants compared with the non-stressed plants (set at 100%) of PI 471938 and PI 416937. Non-stressed plants received 2.0 mM NO_3^- continuously while stressed plants received 0 mM NO_3^- until 12 to14 days after treatment. Stressed plants then received 2.0 mM NO_3^- until termination of the experiment. Each point represents the mean leaf area of seven nitrogen stressed (-N) and resupplied plants (+N) and the mean of six non-stressed plants.

Figure 4. The relationship of transpiration (TR) to vapor pressure deficit during both nitrogen stress (A,B) and nitrogen recovery (C,D) for PI 417938 and PI 416937. Each data point is the average of seven nitrogen stress plants and six control plants.

Table 1. Two segment equations for two experiments examining the relationship of transpiration to vapor pressure deficit during both nitrogen stress and nitrogen recovery for PI 417938 and PI 416937. Results of experiment one are shown in Figure 4.

		Nitrogen Stress Phase		Nitrogen Recovery Phase	
		2 mM NO_3^-	0 mM NO_3^-	2 mM NO_3^-	0 mM NO_3^-
		Experiment 1			
PI 471938	S_1[†]	13.1	10.4	9.1	9.3
	Bp[‡]	2.3	2.2	2.1	2.3
	S_2	5.1	2.9	6.1	6.0
	R^2	0.80	0.60	0.70	0.67
PI 416937	S_1	38.7	20.7	28.1	25.4
	Bp	1.7	1.7	2.0	2.1
	S_2	5.7	5.4	2.2	0.01
	R^2	0.85	0.72	0.92	0.70
		Experiment 2			
PI 471938	S_1	10.4	4.7	6.9	4.2
	Bp	3.0	3.0	3.0	3.1
	S_2	2.3	0.8	3.5	0.21
	R^2	0.80	0.80	0.77	0.40
PI 416937	S_1	13.5	7.9	7.5	6.9
	Bp	2.3	2.1	2.7	2.1
	S_2	3.0	2.8	3.1	2.6
	R^2	0.91	0.84	0.81	0.81

† - Slopes of the first (S1) and second (S2) line segments.

‡ - Breakpoint (Bp) between the two line segments over the range of VPD tested.

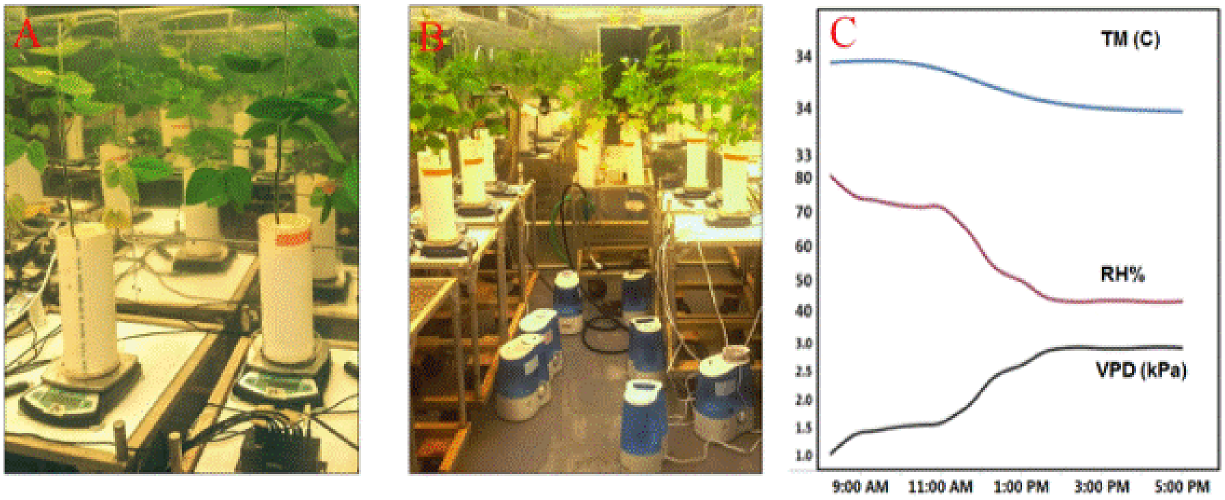

Figure 1. A) PVC pots are positioned on balances allowing transpiration to be measured on balances from weight changes in one minute intervals, B) Differences in VPD were established using humidifiers. C) Vapor pressure deficits were calculated from temperature and relative humidity data.

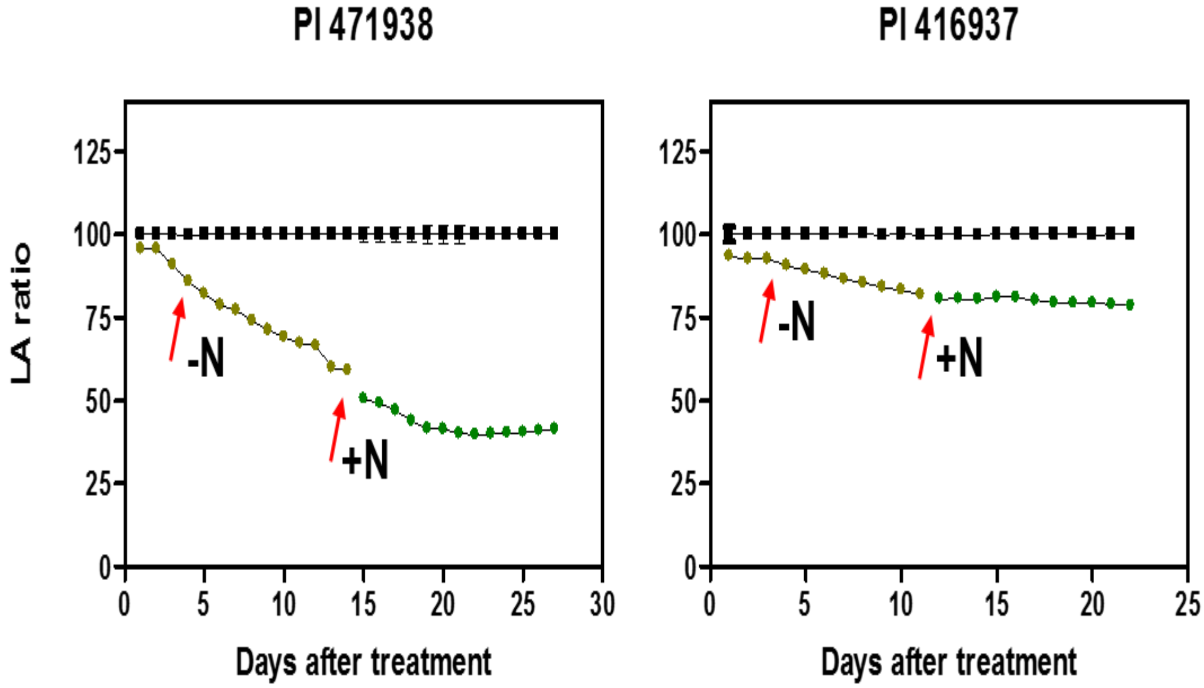

Figure 2. The percentage of leaf area of the N-stressed and subsequent resupply plants compared with the non-stressed plants (set at 100%) for PI 471938 and PI 416937 plotted against days of experiment. Non-stressed plants received 2.0 mM NO_3^- continuously while stressed plants received 0 mM NO_3^- - until 12 to 14 days after treatment. Stressed plants then received 2.0 mM NO_3^- until termination of the experiment. Each point represents the mean leaf area of seven nitrogen stressed (-N) and resupplied plants (+N) and the mean of six plants for non-stressed plants.

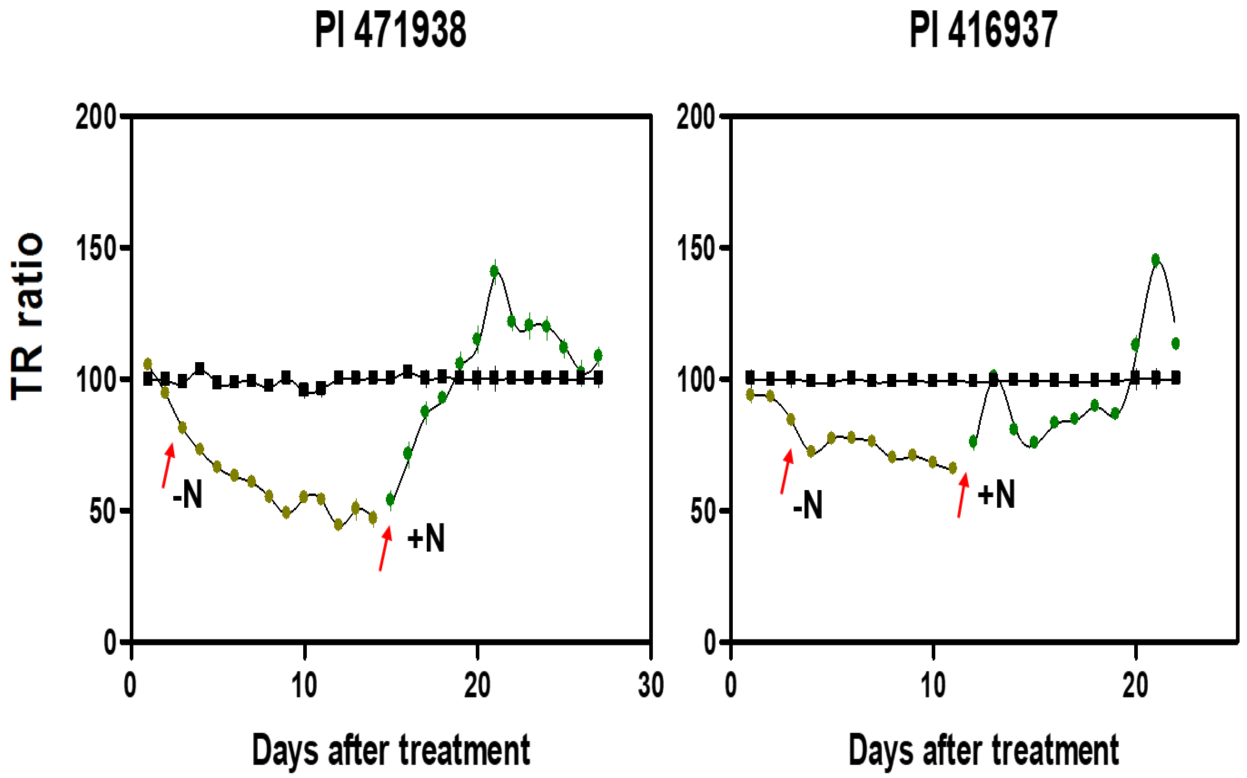

Figure 3. The percentage of transpiration (TR ratio) of the N-stressed and subsequent resupply plants compared with the non-stressed plants (set at 100%) of PI 471938 and PI 416937 over the course of the experiment. Non-stressed plants received 2.0 mM NO_3^- continuously while stressed plants received 0 mM NO_3^- until 12 to 14 days after treatment. Stressed plants then received 2.0 mM NO_3^- until termination of the experiment. Each point represents the mean leaf area of seven nitrogen stressed (-N) and resupplied plants (+N) and the mean of six non-stressed plants.

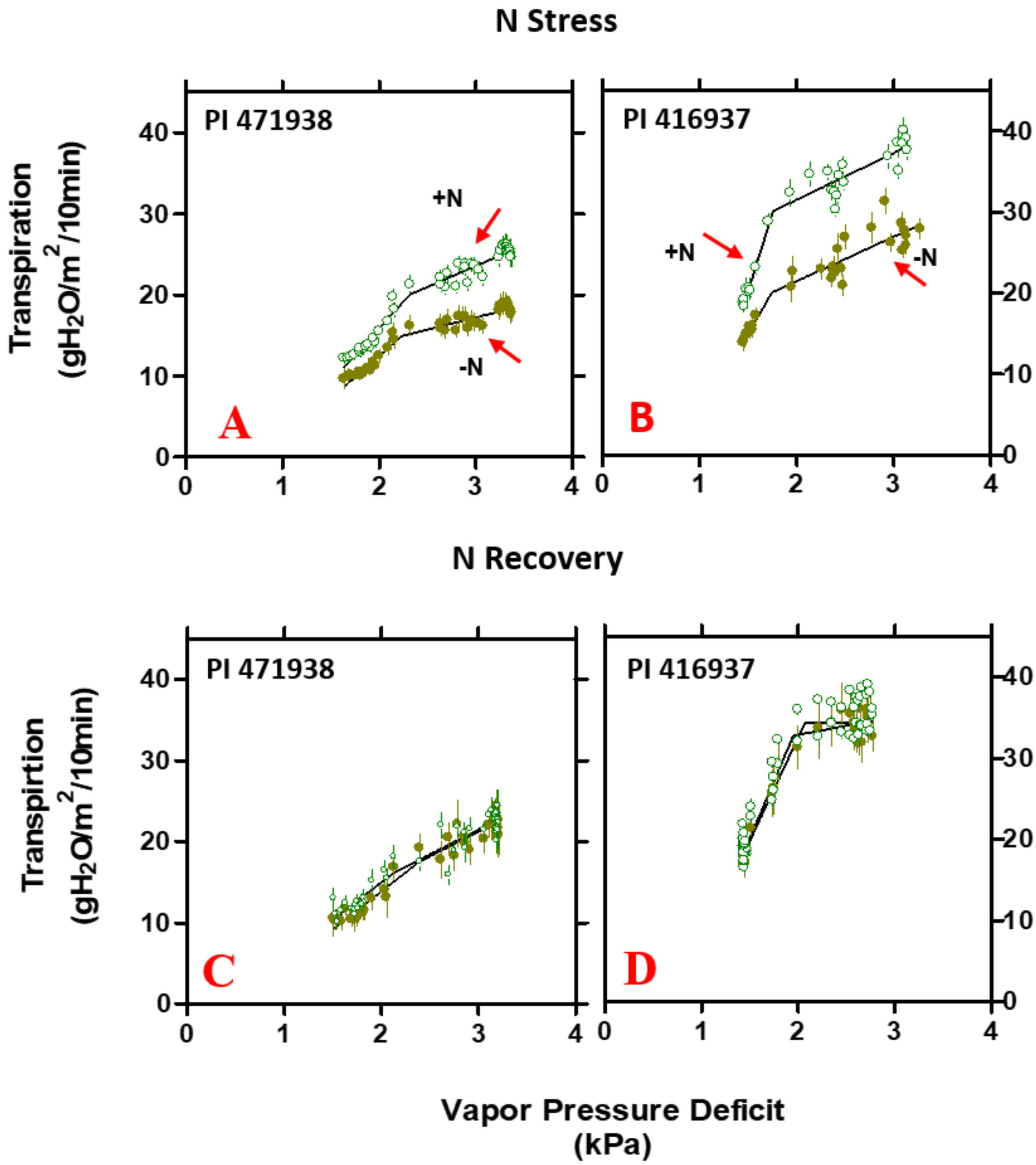

Figure 4. The relationship of transpiration (TR) to vapor pressure deficit during both nitrogen stress (A,B) and nitrogen recovery (C,D) for PI 417938 and PI 416937. Each data point is the average of seven nitrogen stress plants and six control plants.

CHAPTER 5

Assessing Nitrogen Fixation Activity under Water Deficit Conditions in "Slow Wilting" PI 471938 Soybean

ABSTRACT

In order to reduce yield loss in soybean under drought conditions genetic improvement for drought tolerance promising, especially due to the higher cost and availability of irrigation. Slow-wilting soybean genotypes has been evaluated for their drought tolerance trait and it has resulted lessened yield reductions under drought. Soybean genotype PI 471938 has been identified under water deficit as being a slow leaf wilting phenotype but all previous determinations of leaf wilting have been qualitative. The research herein was conducted to improve the soybean leaf wilting scoring system and to examine wilting in a controlled environment. Two soybean genotypes, PI 471938 (slow-tilting) and Benning (fast wilting) were scored for leaf wilting during the stress period using digital cameras. A scale of 1 to10 was developed where, 1 represent fully turgid leaves and 10 represent completely dead leaves. In growth chamber study, transpiration declined similarly for fast and slow-wilting genotypes in response to soil drying. Wilting response to progressive soil drying was also the same for slow-wilting PI 471938 and fast-wilting Benning indicating that complex mechanisms might be responsible for slow wilting trait and leaf wilting is environmentally sensitive.

INTRODUCTION

The legume species soybean [Glycine max (L.) Merr.] originated in China and is a good source of vegetable oil (19%) and protein (36%) (Liu, 1997). The United States, Brazil, Argentina, China and India are the main producers of soybean (Kanchana et al., 2015). Based on the 2015 USDA crop production summary, US soybean production averaged approximately 48.0 bushels per acre (3,225 kg ha^{-1}) total production was 3.93 billion bushels (USDA-NASS, 2016).

Plant growth and development are greatly affected by water deficits through alterations of molecular, physiological and morphological processes (Anjum et al., 2011). Drought tolerance therefore is considered a complex trait. Drought especially during the reproductive stage of soybean causes reductions in flower survival, pod number, seed per pod and seed size, leading to reduced yield (Abdel-haleem et al., 2012). It has been reported that 39-45% yield decrease can occur when there are four days of visible drought stress during the period of the second through the fourth week of seed fill (Wright et al. 2012). As a result, identifying drought tolerant cultivars will be a main goal in any breeding program seeking increased crop water use efficiency and yield (Lauer et al. 2014).

Leaf wilting and leaf rolling is a common plant response under water deficit conditions. Likewise, leaf wilting has been widely used as an indicator of tolerance to water stress (Blum 2011). In soybean, the more obvious sign of drought stress is leaf rolling and/or flipping. A rolled leaf reflects more sunlight and conserves water by reducing temperature by exposing the silver-green, abaxial surface of the leaf (Casteel, 2012). Two soybean plant introductions, PI 416937 and PI 471938, have been described as possessing drought tolerance (Devi et al., 2014). Under water deficit conditions, PI 416937 showed delayed wilting in the field compared with elite cultivars. Soybean genotypes differ in their wilting rate during water stress and the delayed expression of the trait has been reported to lead to an increase in productivity and yield improvement (King et al., 2009). Both PI 416937 and PI 471938 have been widely used to improve soybean productivity and yield (Carter et al., 2016; Devi et al., 2014). Abdel-haleem et al., (2012) identified several QTL in PI 416937 associated with the slow canopy wilting trait. However, it was noted that five QTL were identified on different chromosomes, implying that canopy wilting trait is a complex trait. In addition, QTL identified for canopy wilting were suggested to be associated with morpho-physiological traits in soybean as these putative QTL were co-localized with other QTL that have been related to abiotic stresses in soybean (Abdel-haleem et al., 2012).

While many studies have reported slow canopy wilting in field studies, no wilting data has been published for the selected genotypes PI 471938 and Benning utilizing plants grown in controlled environments. Field environments have been utilized for germplasm improvement in response to water deficit. Due to the unpredictability and harshness of the field environment, exploring the underlying morpho-physiological and genetic traits of drought tolerance is extremely difficult. For this reason, experimental approaches utilizing controlled growth environments would be desirable. It is not known, however if the slow wilting trait is expressed in controlled environments in a manner similar to field observations. The objective of this research was to evaluate canopy wilting of two soybean genotypes, PI 471938 (slow wilting) and Benning (fast wilting) for transpiration and wilting responses to water content under controlled environments. In doing so, detailed ranking of wilting will be performed by both monitoring cameras and visual rankings.

MATERIAL AND METHODS

Plant material

Experiments were conducted in a controlled environment chamber at the North Carolina State University Phytotron in Raleigh, NC. Leaf canopy wilting of 'PI 471938' and 'Benning' were evaluated under soil-drying conditions. A description of the genotypes is shown in Table 1. Plants were grown in 2.7-L pots in a sandy loam soil (69% sand, 18% silt and 12% clay) and randomly positioned on rolling carts. Plants were grown in 28/24 ± 2 ^{o}C day/night temperatures. The average air vapor pressure deficit (VPD) during the experiment was 2.5 kPa. Plants were subjected to a 15-h light period with approximately 500 µmol m^{-2} s^{-1} photosynthetically active radiation. The pots were initially watered daily to avoid water deficit and supplied a complete nutrient solution for the first 10 days after sowing (Saravitz, Downs, and Thomas 2009).

Soil desiccation

Prior to the experiment, pots were watered to saturation and subsequently allowed to drain and weighed. Pots were sealed with plastic bags to avoid soil evaporation. Thereafter, water was withheld starting when the plants were five weeks old. Two treatments, well-watered (WW) and water deficit (WD), were imposed at that time. Well-watered treatments had daily watering to return their pot weight to 100 g less than their initial weight. Water deficit plants were allowed to dry progressively over two weeks as a result of transpiration water loss. In order to avoid excessive soil drying and decreases in soil water content, pots were watered to maintain the daily decrease in soil water content of no more than 70 g. Three plants of each genotype under WD and the control were monitored.

Pots were placed on a separate balances and plant transpiration was continuously measured. The balances had a 6000g capacity a precision of 0.01g (Model SP-6001, Scout Pro, Ohaus Crop., Parsippany, NJ). The balances were connected to a computer via USB connections and pot weights were recorded in one minute intervals. WinWedge data collection software (TAL Technologies Inc., Philadelphia, PA) was used to obtain readable output from each balance.

Weights recorded each minute showed considerable variation, possibly due to air circulation in the growth chamber. To overcome this issue, spline regression was applied to weight data using Proc TPSPLINE procedure in SAS 9.4 (SAS institute Inc., NC). The spline regression was used to obtain weights for one-hour intervals.

Fraction of transpirable soil water (FTSW) was used to define the soil water status of each pot. The FTSW was calculated using the following relationship;
$\mathrm{FTSW} = (\mathrm{PDW}_{c} - \mathrm{PDW}_{f}) / \mathrm{PDW}_{i} - \mathrm{PDW}_{\mathrm{NTR0.11}})$, where PDW_{c}, PDW_{f}, PDW_{i} and $\mathrm{PDW}_{\mathrm{NTR0.11}}$ is the current pot dry weight (PDW), final PDW, initial PDW and PDW with a daily NTR of 0.11%, respectively.

Assessment of wilting score

Visual ratings of leaf rolling and wilting were recorded on a 1 to 10 scale (Table 2). Briefly leaf rolling and leaf angle for leaves numbered 4-10 (basipetal and upwards)) were used as follows: 0-2 (Rolled in the corner of the leaf); 2-3 (rolled and flipped more); 3-4 (rolled half way); 4-5 (rolled almost fully); 5-6 (rolled all the way). Lamina angle was compared to the horizontal line and scores were given as follows: 1 (if the angle is less than 45^{o}); 2 (if the angle is between $45\text{-}60^{o}$); 3 (if the angle is between $60\text{-}90^{o}$). Values then were combined and new wilting score (0-10) were generated from leaf rolling and leaf lamina angle. The visual wilting score was plotted against FTSW and analyzed using two-segment linear regression analysis (GraphPad Prism 2.0, GraphPad Software Inc., San Diego, CA, 1996). The breakpoint value at which there was a decrease from the early plateau segment and linearly decreeing segment at low FSWT were an output from the regression analysis. Analysis of wilting score (leaf angle and leaf rolling analysis) was performed using the software ImageJ analysis (Schneider et al., 2012).

In addition, to the visual wilting score, leaf wilting was also estimated utilizing digital images produced with a digital DSLR camera (Alpha 65, Sony Corp). Lateral views of each plant were used for digital analysis with four digital cameras. Each camera captured images of two plants and images were acquired and evaluated every 2 hours for 14 days from the onset of the experiment.

RESULTS

In order to evaluate the response of wilting trait to soil dying, wilting data was plotted against FTSW for each genotype (two plants each). Wilting score was zero at the beginning of the experiment (not stressed) then increased and reached 10 (very stressed) as water content reached to zero. As shown in Figures 1 and 2, each leaf per plant were analyzed for their wilting score in response to water stress. Overall, the leaves of PI 471938 leaves started to wilt at lower FTSE threshold than Benning. For PI 471938, leaf number 4, 5, 6 and 7 started to wilt at threshold value of 0.2 or below, whereas leaf number 8 and 9 started to wilt at a FTSW threshold value between 0.2 and 0.4. Wilting increased as soil water content declined below this FTSW value. For Benning, the FTSW threshold varied among different leaves. Leaf number 8 and 9 started showing wilting symptoms at threshold between 0.3 and 0.5 while leaf numbers 4, 5, 6 and 7 wilted at a threshold value below 0.2.

Based on the normalized transpiration rate (NTR) values no differences were observed between genotypes. The FTSW threshold where NTR began to decline was around 0.4 (Data are not shown). Water deficit plants started to decrease in transpiration at three days after initiation of water stress (Figure 3). The capacity to recover from the water deficit diminished as the diurnal cycles progressed. Water loss through transpiration fell from a high of approximately 5 g day^{-1} for water deficit plants at the beginning of the experiment to 2.5 g day^{-1} at the last measurement. The decline in transpiration of the water deficit plants is quite striking during the light period with and equally noticeable recovery upon re-watering. Minimal transpiration rates of the water deficit plants just prior to re-watering decreased from 4.5 to 1.0 g on days one and six, respectively.

The FTSW values were also plotted against days for each genotype (Figure 4). The FTSW decreased approximately 50 % during the first day of the experiment for all genotypes. This initial decrease was followed by a generally linear decline to zero values. The number of days to attain zero FTSW ranged from 11 to 13 days for the genotypes studied.

DISCUSSION

Canopy wilting in soybean has been reported to be the first visible symptom in response to the progression of soil water deficit (King et al., 2009). Severity of wilting has been observed in soybean genotypes in the field in response to water deficit (Solane et al., 1990). Dr. Tommy Carter has conducted field experiments for over thirty years with the goal of identifying slow wilting genotypes (Carter, Jr., T.E., personal communication). Carter et al., (2006) identified two soybean plant introductions as “slow-wilting” in their response to field soil water deficits. These genotypes showed delayed wilting of a few days compared with commercial genotypes. Major traits associated with slow canopy wilting include sustained nitrogen fixation under drought, increased water use efficiency, deeper taproots, fibrous lateral roots, osmotic adjustment and antioxidant capacity (Manavalan et al. 2009, 2010). Earlier reports concerning canopy wilting have been field-based. Alternatively, this study assessed wilting in the controlled growth chamber. In doing so, the interaction between environment and the genotypic slow wilting trait in a controlled environment could be observed.

Previous studies relied solely on the human ability to assess wilting severity (Carter et al., 2006). In this preliminary study, we analyzed wilting using digital camera generated images to score leaf wilting. Digital images may allow a more precise and reproducible wilting assessments than possible by the human eye. In this preliminary study, the use of digital cameras to document leaf wilting has proved promising. However, the number of available cameras should prove to be a limiting factor since only two plants per genotype could be documented with a given camera and plant-to-plant variation would require multiple cameras.

It still remains to be determined if cameras would supplant an experienced visual rating due to the limitation of sample size.

Generally, vast genotypic differences were not observed. Wilting responses showed relatively small differences compared with observations in field environments (Carter et al., 2006). The same was true of transpiration and the attainment of zero FTSW responses with no differing genotypic trends of note. There is a large difference between growth chamber and field environments including soil volume, PPFD levels, wind, diurnal temperature changes, humidity and ultra-violet radiation levels. The fact remains that experiments in controlled environments do not successfully mimic field conditions. Raper and Downs, (1976) approximated the field environment under growth chamber conditions for tobacco culture. However, the conditions implemented were many and complex.

In summary, the slow-wilting trait failed to appear in controlled environment of the growth chamber. Further, the experimental approach utilizing digital cameras should be examined in greater detail than in this preliminary study. However, the use of digital cameras to document leaf wilting in controlled environments presents some interesting challenges. The first is the number of cameras required to have a sufficient experimental design to test a hypothesis is considerable. The required cost is difficult to justify against a zero expenditure for human evaluations and the fact that visual evaluations in the field have been effective in breeding efforts targeted at drought responses (Carter et al., 2016). The second is the mere fact that controlled growth environments are completely alien from the field due to environmental factors that are irreproducible in the controlled setting (i.e. soil volume, PPFD levels, wind, diurnal temperature changes, humidity and ultra-violet radiation).

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FIGURE CAPTIONS

Figure 1. Visual wilting scores for PI 471938 in response to the fraction of transpirable soil water (FTSW) in a growth chamber study. Fully expanded leaves, (4 to 9) ranked for their wilting score in response to water deficit.

Figure 2. Visual wilting score for Benning in response to the fraction of transpirable soil water (FTSW) in a growth chamber study. Fully expanded leaves, (4 to 9) are ranked for their wilting score in response to water deficit.

Figure 3. Diurnal transpiration (g H_2O) between well water and water deficit plant during the course of the experiment. As the soil water deficit increases, transpiration water loss in water stressed plants decreases compared to control plants.

Figure 4. Fraction of transpirable soil water (FTSW) was plotted against days during the course of the experiment for slow (PI 471938) and fast (Benning) wilting soybean genotypes.

Table 1. Description of the soybean genotype characteristics

Genotype	MG[†]	Origin	Phenotypic description
PI 417938[‡]	V	Plant introduction/Japan	Slow wilting in field
Benning[¶]	VI	Hutcheson x Coker 6738/USA	Fast wilting

[†] - Maturity group
[‡] - Identified by Sloane et al, 1990
[¶] - Boerma et al., 1997

Table 2. Visual wilting scores for leaf anatomical changes (leaf rolling and degrees from a perpendicular leaf presentation) in response to water deficit.

Rolled in corner of the leaf: 0, Angle below (45^{o}): 1 = wilting score 0
Rolled in corner of the leaf: 1, Angle below (45^{o}): 1 = wilting score 0.5
Rolled and flipped more: 2, Angle below (45^{o}): 1 = wilting score 1.0
Rolled half way: 3 Angle below (45^{o}): 1 = wilting score 1.5
Rolled almost fully: 4, Angle below (45^{o}): 1 = wilting score 2.0
Rolled all the way: 5 Angle below (45^{o}): 1 = wilting score 2.5
Rolled all the way: 6 Angle below (45^{o}): 1 = wilting score 3.0

Rolled in corner of the leaf: 0, Angle between (45 to 60^{o}): 2 = wilting score 3.2
Rolled in corner of the leaf: 1, Angle between (45 to 60^{o}): 2 = wilting score 3.5
Rolled and flipped more: 2, Angle between (45 to 60^{o}): 2 = wilting score 4.0
Rolled half way: 3 Angle between (45 to 60^{o}): 1 = wilting score 4.5
Rolled almost fully: 4, Angle between (45 to 60^{o}): 2 = wilting score 5.0
Rolled all the way: 5 Angle between (45 to 60^{o}): 2 = wilting score 5.5
Rolled all the way: 6 Angle between(45 to 60^{o}): 2 = wilting score 6.0

Rolled in corner of the leaf: 0, Angle between (60 to 90^{o}): 3 = wilting score 6.2
Rolled in corner of the leaf: 1, Angle between (60 to 90^{o}): 3 = wilting score 6.5
Rolled and flipped more: 2, Angle between (60 to 90^{o}): 3 = wilting score 7.0
Rolled half way: 3 Angle between (60 to 90^{o}): 3 = wilting score 7.5
Rolled almost fully: 4, Angle between (60 to 90^{o}): 3 = wilting score 8.0
Rolled all the way: 5 Angle between (60 to 90^{o}): 3 = wilting score 9.0
Rolled all the way: 6 Angle between (60 to 90^{o}): 3 = wilting score 10.0

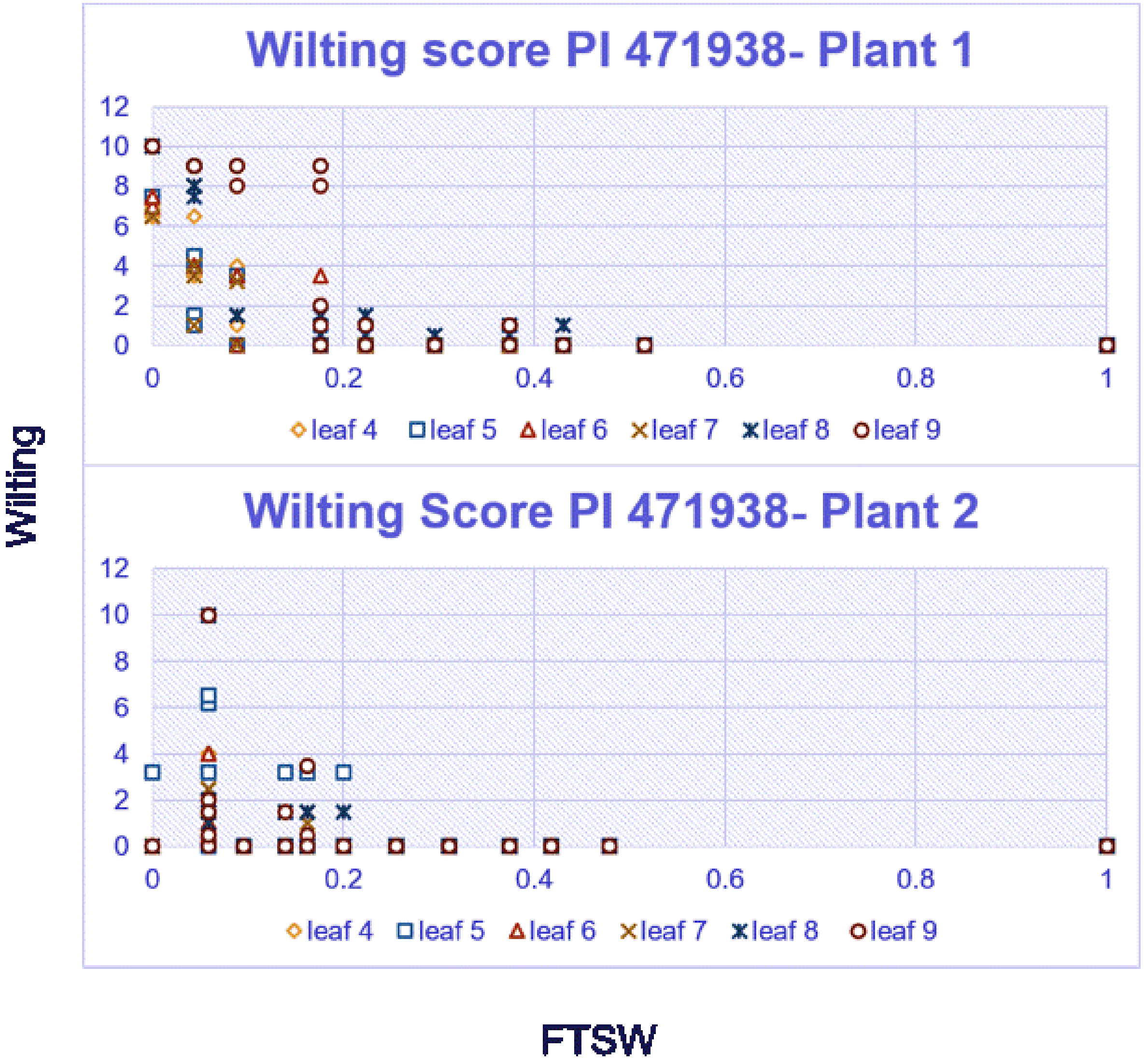

Figure 1. Wilting scores for PI 471938 in response to the fraction of transpirable soil water (FTSW) in a growth chamber study. Fully expanded leaves, (4 to 9) ranked for their wilting score in response to water deficit.

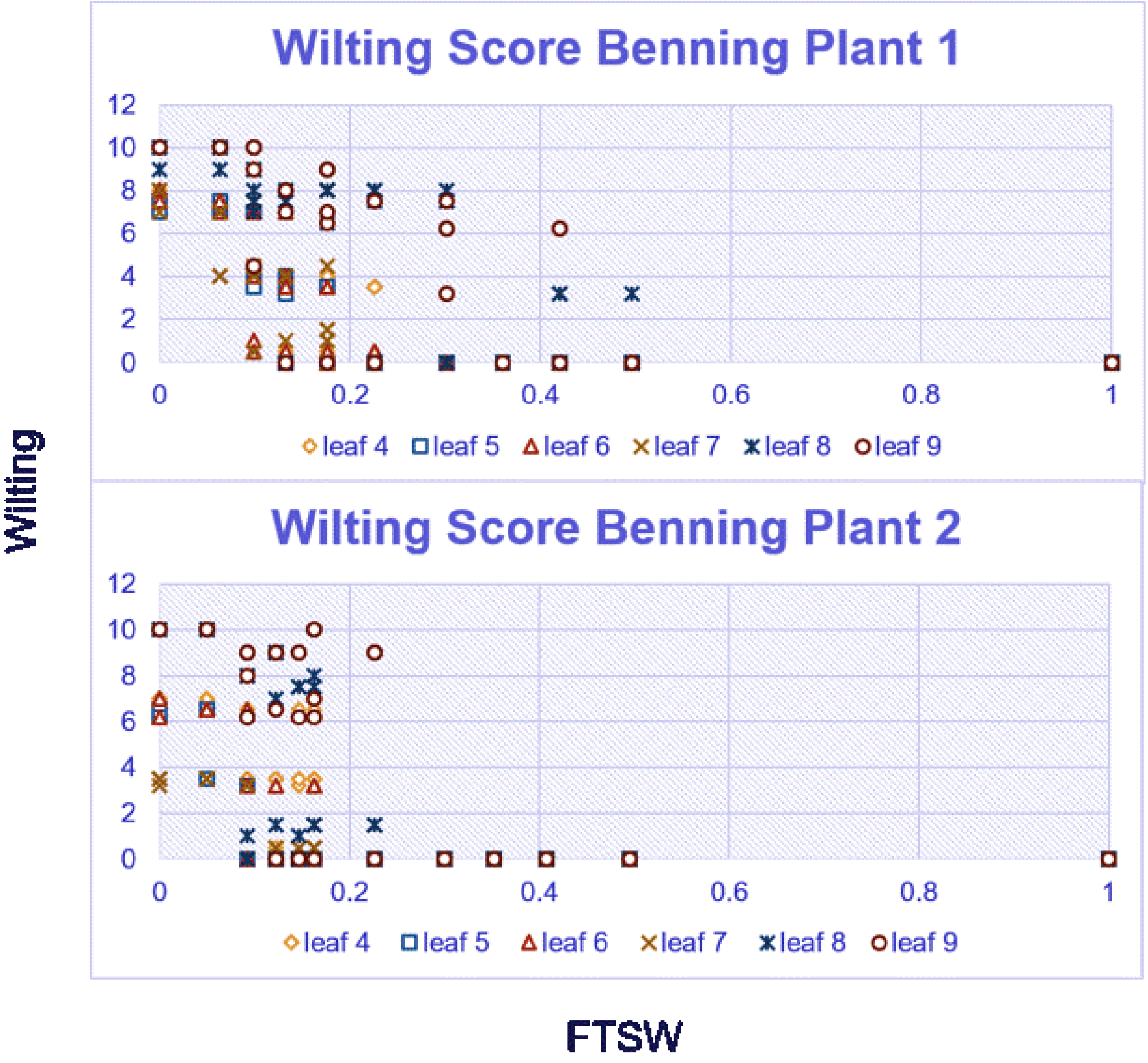

Figure 2. Wilting score for Benning in response to the fraction of transpirable soil water (FTSW) in a growth chamber study. Fully expanded leaves, (4 to 9) ranked for their wilting score in response to water deficit.

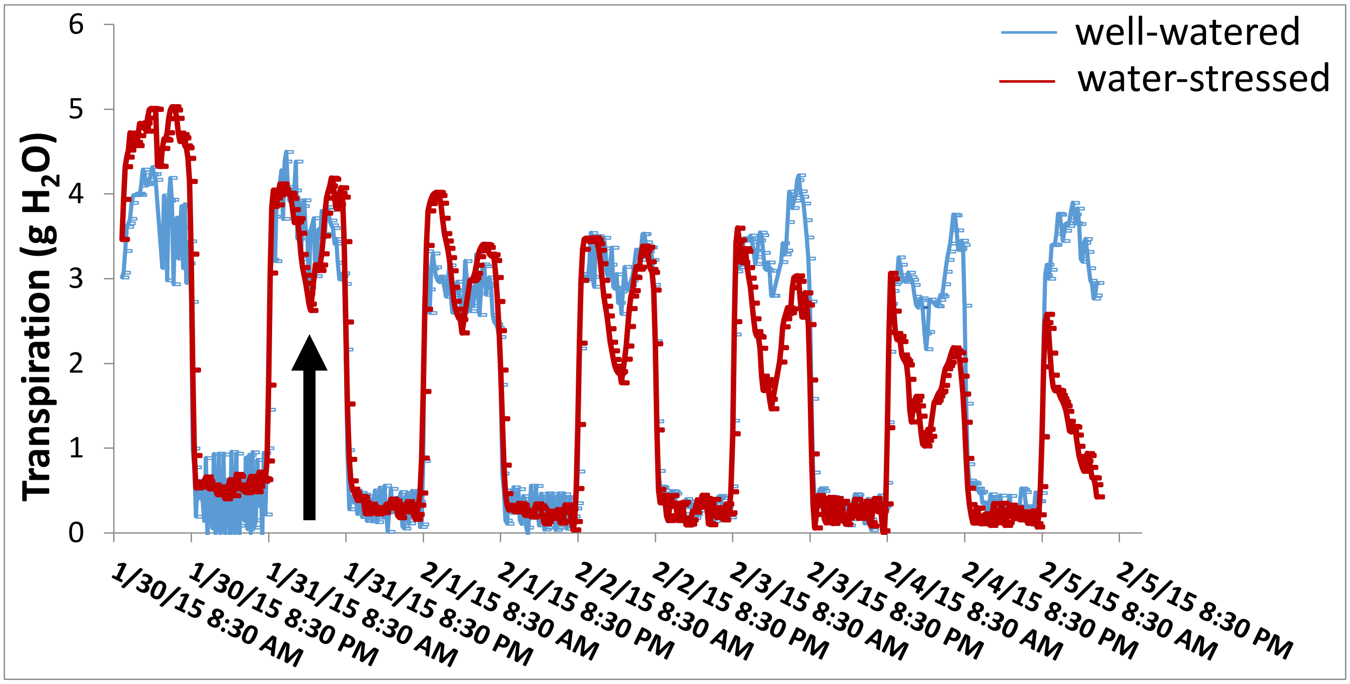

Figure 3. Diurnal transpiration (gH_2O) between well water plant and water deficit PI 471938 soybean genotype during the course of the experiment. The arrow indicates when re-watering of the pots occurred during the light period and is present for each diurnal cycle.

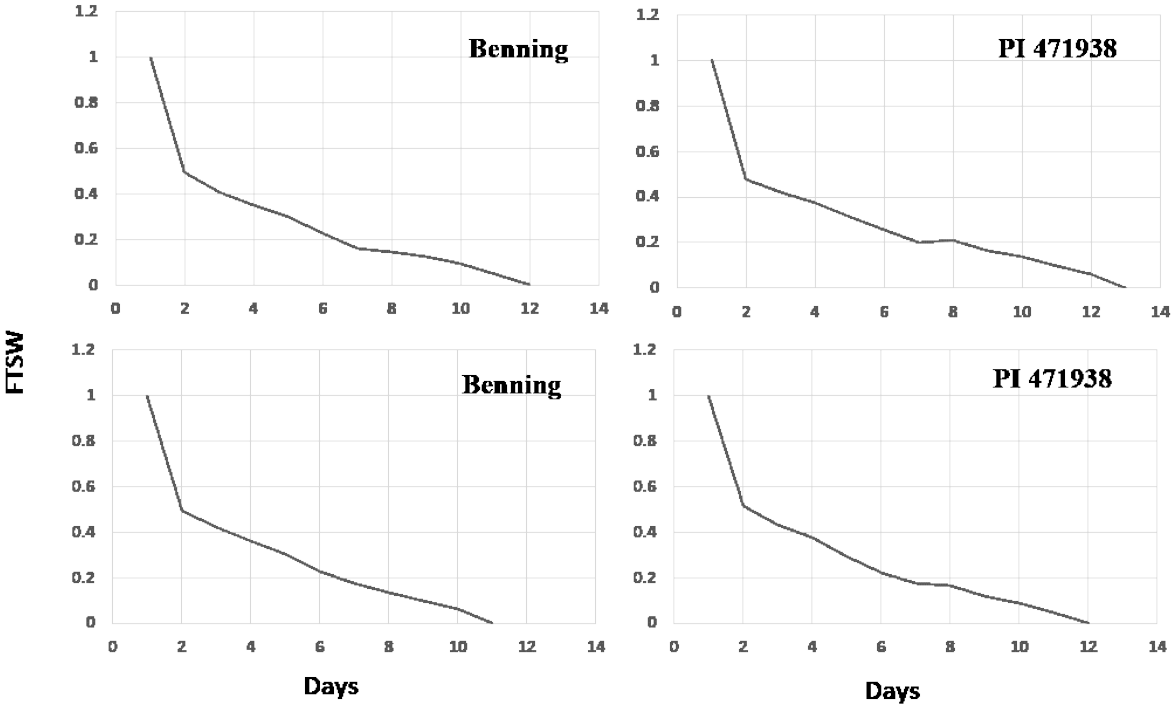

Figure 4. Fraction of transpirable soil water (FTSW) was plotted against days during the course of the experiment for slow (PI 471938) and fast (Benning) wilting soybean genotype