RUST AND DROUGHT EFFECTS ON GENE EXPRESSION AND PHYTOHORMONE CONCENTRATION IN BIG BLUESTEM

by

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Abstract

While plants are typically exposed to multiple stressors in the field, studies of genome-wide gene expression and phytohormone responses in wild plant species exposed to multiple stressors are rare. Our objectives were to determine the effects of drought and rust stress on gene expression in *Andropogon gerardii*, the dominant grass in tallgrass prairie, and associated levels of phytohormone production. In a factorial design, plants experiencing drought or non-drought conditions were either inoculated with the rust pathogen *Puccinia andropogonis* or not inoculated. Gene expression was evaluated with maize microarrays. Drought-stressed plants significantly decreased expression of genes associated with photosynthesis and the hypersensitive response, while expression of genes associated with chaperones and heat-shock proteins increased. No significant differences in gene expression in response to the rust treatment were detected using a mixed model analysis of variance and false discovery rate protection, probably because of the low infection rate. Phytohormone production increased when both stresses were present. The rust treatment significantly increased benzoic acid (BA) production in the presence of drought, while the drought treatment alone significantly increased salicylic acid (SA) production. Leaf tips usually had higher levels of all phytohormones in all treatments and the leaf section evaluated had a larger effect on phytohormone level than did the treatments applied.

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Introduction

Plants are routinely subjected to multiple stresses simultaneously when grown in field or natural environments. The response to these combinations of stresses often differs from the response seen when only a single stress is applied (Mittler, 2006). For example, during heat stress, plants open stomata to cool the leaves through transpiration, whereas under drought stress plants close stomata to reduce water loss through transpiration. However, when heat stress is combined with drought stress, the stomata remain closed resulting in a higher leaf temperature (Mittler, 2006). Many insects are vectors for plant pathogens and when an insect feeds on a plant it may introduce a pathogen, where feeding and infection may result in the induction of different genes. Therefore, more complete knowledge of plant responses to stress is gained when the impact of multiple stresses are studied simultaneously rather than when the impact of each individual stress is studied alone.

Drought stress is one of the most common environmental factors limiting plant growth and yield. Plant resistance to drought typically has been grouped into three different strategies: escape, avoidance, and tolerance (Levitt, 1980). Plants may utilize various strategies to survive a period of low water availability. Plants with an escape strategy can complete their entire life cycle when water is available and can survive the dry, unfavorable conditions as seeds (Ludlow, 1989). The avoidance strategy is used by plants whose tissues are very sensitive to dehydration. These plants maintain high water potential by reducing transpiration to minimize water loss, or by increasing uptake of soil water to maximize water uptake (Ludlow, 1989). Some plants have traits that minimize

water loss such as stomatal closure, steep leaf angles, shedding older leaves, and a dense trichome layer. Other plants modify resource allocation to maximize water uptake (Chaves et al., 2003). Finally, plants with a drought tolerance strategy use osmotic adjustment, have smaller cells, or have cells with more rigid cell walls (Chaves et al., 2003). Osmotic adjustment is the ability of the cell to accumulate compatible solutes and lower water potential during periods of osmotic stress (Taiz and Zeiger, 2002). The tissues of plants utilizing the drought tolerance strategy can tolerate dehydration, but these responses are not as well developed as the responses that occur in plants with escape or avoidance strategies. Some of the responses in drought tolerant plants can actually promote water loss as a plant grows during drought stress (Ludlow, 1989).

The changes in a plant experiencing a water deficit vary with the level of water stress that occurs. The earliest responses to drought occur at the leaf level where stomatal closure and the inhibition of leaf growth happen regardless of the speed at which the water deficit develops (Chaves et al., 2003). Stomata close in response to low water potential or low leaf turgor and are more closely linked to soil moisture content than to leaf water status (Chaves et al., 2002). These experiments suggest that stomata close in response to chemical signals from roots experiencing dehydration while leaf water status is kept constant. Photosynthesis also is impaired when plants are drought-stressed, although there is disagreement on whether photosynthesis is limited by stomatal closure or by resulting changes in metabolic processes (Flexas, 2004). As the water deficit increases, stomatal guard cells lose turgor due to direct loss of water by evaporation, which causes the stomata to close (Taiz and Zeiger, 2002). Stomata also can close as a result of dehydration of whole leaves, root dehydration, decreased solute concentration in

the guard cells, or increased abscissic acid (ABA) production (Taiz and Zeiger, 2002). Stomatal closure restricts CO_2 uptake and thereby reduces photosynthesis. Photosynthesis also can be inhibited by metabolic processes that are altered during a water deficit, e.g. decreased ATP synthesis and RuBP (ribulose-1,5-bisphosphate) (Tezara et al., 1999).

The way cells respond to water stress also changes. These responses may include loss of turgor, changes in the plasma membrane and changes in water activity and solute concentration (Chaves et al., 2003). Cell turgor can be maintained by decreasing cell osmotic potential which also results in the production of solutes. The solutes produced during osmotic adjustment may function in protecting the cell membrane and metabolic processes during dehydration (Chaves et al., 2003). Osmotic adjustment typically is a slow process that is initiated only when a certain level of cell dehydration is reached.

Water potential and stomatal conductance measurements are commonly used to describe the water status of plants. The most popular method for measuring water potential is a pressure chamber (Kirkham, 2005). In a pressure chamber, the pressure is increased around an excised leaf until sap from the xylem is exuded from the cut end of the stem. The pressure required to push the xylem sap out is the negative pressure in the intact stem; the more negative the water potential, the more stressed the plants are. Stomatal conductance measures the change in water and carbon dioxide through the stomata, in and out of the leaf (Taiz and Zeiger, 2002). The most common method for measuring stomatal conductance is with commercially available diffusion porometers (Kirkham, 2005). These porometers measure the diffusion of water vapor from the leaf, and provide an alternative measure of plant water status.

Plants in the field also respond to biotic stresses, such as the presence of a pathogen resulting in disease. Disease severity may be enhanced or reduced in plants simultaneously experiencing drought. For example, Clover et al. (1999) found no interaction between drought stress in sugar beets and beet yellows virus (BYV) infection. The effects of the disease and the effects of drought occurred at different times of the day and of the season resulting in no interaction between drought and BYV. Viral infection reduced growth earlier in the season and its proportional effect decreased at the end of the season, unlike drought. Drought also reduced crop cover, which occurred later in the season than BYV. *Macrophomina phaseolina* is a fungal pathogen that causes disease in plants under hot, dry conditions and generally infects plants that are experiencing drought stress (Mayek-Perez, 2002). The combination of drought stress and *Macrophomina* infection caused the highest reduction in growth of common bean. When drought stress was imposed on bean plants it increased the negative effects of *Macrophomina* infection. Kackley et al. (1990) analyzed the effect of drought stress on the development of summer patch in Kentucky bluegrass caused by *Magnaporthe poae* and found that there was more disease in the non-drought-stressed plots than in the drought-stressed plots. The development of the disease was more severe when soil moisture was not limiting to the plant.

Research on the interactions of drought and rust infection is limited. Groundsel (*Senecio vulgaris*) plants infected with *Puccinia lagenophorae* were more adversely affected when exposed to water stress conditions (Paul and Ayres, 1987). The shoot water potential was lower in the rust-infected plants, and dry weight was correlated with

water potential, so the authors concluded that root systems of rust infected plants have reduced ability to compete for free water in the soil.

Exposure to drought also can predispose plants to disease and may result in more severe disease symptoms than would otherwise be observed. Predisposition processes include: altered photosynthate production, decreased protein synthesis, and decreased xylem water potential. Lower protein synthesis prevents the production of enzymes needed for resistance while decreased xylem water potential causes osmotic adjustment resulting in the availability of sugars, amino acids, and other solutes to the pathogen (Boyer, 1995). Tomato plants that experienced drought before inoculation with *Phytophthora parasitica* had significantly more disease with disease severity increasing if drought stress was reimposed after inoculation (Ristaino and Duniway, 1989). Mature tomato plants were more resistant to disease than were seedlings and symptom severity decreased on the roots of older tomato plants in the absence of drought, even under conditions that were conducive for disease development. Pre-inoculation water stress always increased disease symptoms on tomato plants, but post-inoculation water stress had little effect on older tomato plants, unlike younger seedlings.

One of the larger groups of plant pathogenic fungi is the rust fungi, which can infect a broad range of plants. Rusts occur worldwide but many species have specific climatic requirements for infection and survival. These pathogens are economically important and have been a major factor in agricultural productivity for many years causing millions of dollars of damage to crops (Littlefield, 1981). Their main mode of dispersal is through the production of spores. Their life cycle is one of the most complex amongst fungi, with the complete life cycle of the macrocyclic rusts containing five

different spore stages: basidiospores, pycniospores, aeciospores, urediniospores, and teliospores (Littlefield, 1981). However, many rusts lack one or more of these stages. Rusts may be autoecious, i.e. complete their life cycle on one host, or heteroecious, i.e. complete their life cycle on two hosts.

Rust infections can alter many aspects of the host plant's physiology. These changes may include: increased respiration, altered respiratory pathways, and decreased photosynthesis as the tissue becomes chlorotic. Photosynthate may be retained in the infected leaves while transport of materials such as carbohydrates and minerals are redirected from healthy to infected tissues (Littlefield, 1981). Rust infections can reduce transpiration rates through stomatal closure and redirect nutrients in the plant to the infected tissue, resulting in an increased supply of metabolites for the rust fungus (Littlefield, 1981). Wheat plants infected with leaf rust have higher stomatal resistance and higher evapotranspiration rates than do non-infected plants (Suksayretrup et al., 1982). The higher evapotranspiration rates resulted from water loss through pustules. Water potential in wheat leaves infected with *Puccinia recondita* f. sp. *tritici* decreased as the number of rust lesions increased; water potentials were similar in plants subjected to water stress only or to fungal stress only (Bethenod et al., 2001).

The physiological and biochemical response to these stresses is ultimately controlled at the molecular level when plant cells sense a change in the environment. Numerous genes aid in defense against abiotic and biotic stresses. These include resistance genes, genes encoding signal transduction proteins, and downstream defense genes among others (Reymond and Farmer, 1998). The defense genes PR-1 and PR-5 were expressed in maize plants infected with the rust *Puccinia sorghi* and both genes

were expressed in the compatible and incompatible interactions (Morris et al., 1998). In the incompatible interaction expression of the PR genes was at lower levels than observed in the compatible interaction or later in time. In the compatible interaction, PR gene expression levels were correlated with the maize resistance response. The higher the resistance, the faster defense-related gene expression was induced. These results are consistent with gene expression caused by pathogen infection in dicots (De Wit and van der Meer, 1986). In flax, expression of *fis1* increased in susceptible flax plants infected with rust (Roberts and Pryor, 1995). Homologues of the *fis1* gene also occur in other plants such as maize (*mis1*), barley (*bis1*), pearl millet, wheat (*wis1*), oats, sorghum, and rice, suggesting that the expression of this gene is a general response to rust infection in many plants (Ayliffe et al., 2002).

Microarrays allow studies of gene expression for genes in all or a portion of an organism's genome. These studies can be used to compare transcription profiles among individuals exposed to different treatments. Cross-hybridizations between species also are possible with cDNA from the species of interest being hybridized to a microarray containing cDNA of genes from a related species (Moore et al., 2005). There are numerous studies of plant responses to individual stresses. However, less is known about the molecular mechanisms underlying the response of plants to a combination of stresses. In a study examining gene expression in peanuts in response to drought and *Aspergillus* infection, the expression of 52 genes increased only in the drought treatment while the expression of 42 genes increased in response to drought and fungal infection (Luo et. al., 2005).

Phytohormones such as salicylic acid (SA) and jasmonic acid (JA) also may help protect the plant against biotic and abiotic stresses. Many defense-related genes are regulated by pathways involving JA or SA (Reymond and Farmer, 1998). When SA is produced by the plant, phytochemicals other than those associated with the plant defense response are produced. SA is a crucial signaling molecule in the induction of systemic resistance and regulates pathogenesis-related (PR) gene expression (Reymond and Farmer, 1998). Basal levels of SA can vary between different plant species with rice producing much larger amounts of SA than any other species (Raskin et al., 1990). The production of JA also results in phytochemical and protein production, with some products known to increase protection against insects and pathogens (Thaler et al., 2002).

This study examines the response of big bluestem (*Andropogon gerardii*) to combinations of drought stress and infection by *Puccinia andropogonis*, the causal agent of leaf rust in *A. gerardii*. *Puccinia andropogonis* is a member of the largest genus of rust fungi, being comprised of about 3,000-4,000 species (Littlefield, 1981). This rust is heteroecious and macrocyclic, producing all five spore types on two different hosts. The aecial host for this species of rust varies across the range of *A. gerardii*. For example, buckeye (*Aesculus glabra*) is apparently the most common aecial host found in Kansas but comandra (*Comandra umbellata*) is a common aecial host in Minnesota (Barnes et al., 2005). *Andropogon gerardii* occurs naturally from Mexico to Canada and is the dominant grass found on the tallgrass prairie. It reproduces asexually through rhizomes and sexually by outcrossing. It is in the same tribe (Tribe Andropogonae) as corn. The leaves of *A. gerardii* are hypostomatal and typically fold in response to low leaf water potential.

Travers et al. (2007) analyzed gene expression in *A. gerardii* by using maize microarrays to evaluate differential expression in response to simulated precipitation change. Plots receiving the ambient treatment had the same rainfall amount applied immediately after the event. Plots receiving the altered treatment had the same amount of rainfall applied at an interval that was increased by 50% between rainfall events. Expression of numerous genes changed significantly, with, for example, decreased expression of genes involved in photosynthesis and increased expression of genes involved in stress response and signaling in response to altered precipitation. Travers et al. (2007) also found that expression of a putative hypersensitive-induced response (HIR1) gene, involved in the hypersensitive response was decreased significantly in response to altered rainfall patterns. This study led to more questions for the current study, such as in a more controlled environment would similar genes be expressed or would different genes be discovered that were not present in the field study? How does drought affect the severity of disease when the two stresses are present together and how do the effects of drought stress and rust infection compare in relation to gene expression? How does phytohormone production change when the plant is exposed to drought stress or pathogen infection? What is the relation between gene expression and phytohormone concentration? The present study provides a more complete analysis of the changes that occur in a plant subjected to two stresses simultaneously. The objectives of this study were: (1) to determine gene expression and phytohormone levels in response to drought and infection by *P. andropogonis*, and (2) to determine the effect of drought on disease severity. Water potential and stomatal conductance measurements were made along with soil water measurements to determine plant water status for comparison to field

conditions. While many studies of plant responses to stresses emphasize a single stress event, e.g. a single infection event, this experiment includes two drought and two infection events prior to sampling to more closely approximate the repeated stresses experienced by plants in field conditions.

Materials and Methods

Plant Materials

Andropogon gerardii seeds were supplied by the USDA Plant Materials Center, Manhattan, Kansas. Seeds were sown in vermiculite then transplanted when they were approximately 10 cm tall to cones 12 cm in length and 2 cm in diameter containing soil collected at Konza Prairie Biological Station (KPBS), Manhattan, Kansas. Seedlings were tested for susceptibility to a mixture of local isolates of *Puccinia andropogonis* when the plants were ~16 cm tall by inoculation and evaluation as described below. Thus, all plants had a leaf that was exposed to the rust fungus, but typically this exposure took place at least 90 days before sampling of a much younger leaf. Susceptible plants were transplanted to three gallon pots containing soil from KPBS with five plants per pot. The planting and sampling of each block was staggered by at least one week, so each block experienced a slightly different environment. Pots were randomized within a block each week. Soil moisture was monitored and one liter of water was added to each pot when pots were at approximately 24% soil moisture. Plants were grown in the greenhouse with a 16 hour light cycle and a day/night temperature of 28°C/22°C.

Drought and Rust Treatments

Drought and rust treatments were applied in a factorial design, with presence or absence of two intervals of drought stress as one treatment and presence or absence of two rust inoculations as the other. When plants were approximately 40 cm tall, pots were arbitrarily assigned to drought or non-drought conditions. Soil moisture was measured by using Echo probes (Decagon Devices, Inc., Pullman, WA) 20 cm in length; readings were taken daily. One probe was buried in each pot and probes were read with a Campbell Scientific datalogger (CR10, Campbell Scientific, Logan, UT).

Drought-stressed plants experienced two drought cycles with water being withheld until the leaves began to fold. Pots were watered on the third day of leaf folding, ending the first drought cycle. Once the plants were watered, water was withheld again to begin the second drought cycle. Leaves were sampled on the third day of leaf curling in the second drought cycle.

Plants receiving the rust treatment were inoculated twice with *P. andropogonis*, the first time when the plants were brought out of the first drought cycle and again approximately three days before sampling. Leaves of plants not receiving the rust treatment were mock-inoculated by rubbing in the same manner as described below for inoculation, and were always processed first to ensure no spores were accidentally introduced. Pots were separated in the mist chamber so that none of the leaves were touching to avoid any accidental infection. Accidental infection was rare and occurred only on other leaves of the inoculated plants.

Rust Maintenance

Infected leaves were collected from the USDA Plant Materials Center in Manhattan, Kansas, from the same population of *A. gerardii* from which our seeds were collected. Leaves were dried at room temperature for 2-3 days and stored at -80°C. Since rust fungi are obligate pathogens, they are maintained on plant tissues. Rust fungi were multiplied and maintained on plants distinct from the experimental plants. Frozen leaves were cut into small pieces and placed in 10-15 ml Soltrol oil (Chevron Phillips Chemical Co., Houston, TX) and shaken for approximately 10 minutes. The resulting spore suspension was sprayed onto *A. gerardii* plants using an air compressor operated at 100 psi. The plants were placed in a mist chamber overnight and sprayed with distilled water once the oil on the leaves evaporated. Experimental plants were inoculated differently to ensure a higher probability of infection in the leaves to be sampled. Spores were rubbed from an infected leaf of a non-experimental plant directly onto the leaf surface of an experimental plant, while maintaining leaf wetness by using distilled water.

Physiological Measurements

Leaf water potential and stomatal conductance measurements were taken at the time of tissue sampling on the leaves nearest to the leaf sampled for gene expression. Stomatal conductance then was measured with a leaf porometer (model SC-1) from Decagon Devices, Inc. (Pullman, WA). The clip was placed at the widest part of the leaf and conductance was measured for 30 seconds on the bottom of the leaf. Measurements were averaged between leaf samples within a pot and 8-10 leaves were sampled from each pot. Each of the leaves used for stomatal conductance measurements were then cut

and leaf water potential was measured in a pressure chamber (PMS Instrument Co., Corvallis, OR). Nitrogen gas filled the chamber until the first water drop appeared at the cut end of the leaf. In each pot 8-10 leaves were sampled for water potential and these measurements were averaged across all leaf samples from the same pot.

RNA Extraction

Efforts were made to sample plants at the same time of day for all blocks, usually within a span of approximately four hours (7:30-11:30) in the morning. Leaves collected for gene expression analyses were sampled first. These leaves were cut off at the base, frozen in liquid nitrogen and stored at -80°C. RNA was extracted from each leaf separately after which the extracted RNA from leaves in the same pot was bulked into one sample. This improved extraction efficiency for each leaf. Leaves were ground to a fine powder with liquid nitrogen and suspended with 3-5 ml Trizol (Invitrogen, Carlsbad, CA). Leaf tissue from *A. gerardii* is tougher than tissue from agricultural species typically studied, so they were ground in liquid nitrogen for a longer period of time for extractions (approximately 10 minutes) and the whole leaf was used to ensure that sufficient RNA was recovered for microarray analysis. Samples were centrifuged (max speed, 10 min, room temp), the aqueous phase collected and 200 µl chloroform added. Samples were vortexed and centrifuged again (max speed, 15 min, 4°C). The aqueous phase was collected and 250 µl of both isopropanol and a high salt solution (8 M Na citrate + 1.2 M NaOH) were added to precipitate the RNA. RNA was precipitated by centrifugation (max speed, 10 min, 4°C) and the resulting pellet was washed with 75% ethanol, dried and resuspended with RNase-free water. Each sample was purified

immediately following extraction. Purification for each sample was carried out according to the protocol in the RNeasy Mini Kit (Qiagen, Maryland).

Phytohormone analysis

Leaves were separated into a tip section and a base section at sampling. The sampled leaf was folded in half and the leaf was cut about one third of the way from the bottom, such that the tip and the base of the leaf were cut off. The resulting middle section was cut in half with the half closer to the tip labeled "tip" and the half closer to the base labeled "base." Samples were frozen in liquid nitrogen immediately after sampling and stored at -80°C until extracted. Extraction procedures were carried out according to Schmelz et al. (2004). Tissue was ground in liquid nitrogen and poured into 1.5 ml FastPrep tubes containing about 1 g of yttrium cubic zirconium beads (Zirmil® beads, 1.1 mm; Saint-Gobain ZirPro, Mountainside, NJ, USA). Extraction buffer (300 µl of 1-propanol:H_2O:HCl, 2:1:0.005, v/v/v) was added to the samples and stored at -80°C until phytohormone extraction. A mixture of internal standards (100 ng of each phytohormone in 5 µL EtOH: $^{13}C_6$-BA (benzoic acid), $^{2}H_6$-SA (salicylic acid), $^{2}H_5$-CA (cinnamic acid), dhJA (trans/cis jasmonic acid), $^{2}H_5$-IAA (indole acetic acid), and $^{13}C_{18}$-linolenic acid) was added at the time of extraction and the tissue was pulverized by homogenizing (FastPrep FP 120 Bio 101 instrument, Qbiogene, Carlsbad, CA). After homogenizing for 10 sec, 1 ml dichloromethane (CH_2Cl_2) was added to each sample and the samples were re-homogenized and centrifuged (3 min, room temperature, 12,000 rpm). For the derivatization, the bottom layer of CH_2Cl_2:1-propanol was transferred to a 4 ml glass vial by pipetting. Twenty µl of 200 mM trimethylsilyldiazomethane was then

added. The sample was vortexed and incubated for 30 minutes at room temperature to allow for methyl ester formation. Once methyl ester formation was complete, 20 µl of 2.0 M acetic acid in hexane was added to quench the sample; the samples were vortexed and then incubated for 30 minutes at room temperature to quench the reagent. A vapor phase extraction was performed on the samples. Super Q filters (Alltech Associates, Inc., Deerfield, IL, USA) were used to collect the phytohormones. The Super Q filter was placed in the high temperature septa of the vial. A needle supplying a stream of nitrogen gas was inserted into the other septa and a vacuum line was connected to the Super Q filter. The vial was placed in a 70°C heating block until the solvent evaporated. The dry vial was transferred to a heating block at 200°C for 2 minutes to recover less volatile compounds. The Super Q filters were eluted into a sample vial with 150 µl of CH_2Cl_2 and analyzed by gas chromatography-mass spectrometry (GC-MS, Agilent Technologies, Wilmington, DE). The internal standards added at the beginning of the extraction are used to control the GC-MS procedure and quantification. Phytohormones analyzed for each sample included jasmonic acid (JA), salicylic acid (SA), benzoic acid (BA) and oxophytodienoic acid (OPDA). OPDA was quantified using the $^{13}C_{18}$-linolenic acid internal standard.

Microarray methods

RNA from *A*. *gerardii* was hybridized at 50°C to maize cDNA microarrays from the Schnable Laboratory at Iowa State University (www.plantgenomics.iastate.edu/maizechip/). Purified RNA was converted to cDNA with the Array 900 3DNA detection kit (Genisphere, Hatfield, PA). For each microarray,

2 μg of sample was used. Samples were paired across drought treatments on half of the microarrays and the remaining samples were paired across rust treatments on the other half of the microarrays with both samples on an array hybridized with a different dye (Figure 1). Microarrays were scanned using a GenePix 4000B scanner (Molecular Devices Corp, Sunnyvale, CA). After scanning, each microarray was assessed for quality control, where spots that hybridized poorly were deleted and only spots with good hybridization and a strong signal were used for analysis.

Statistical analyses

The statistical analysis of gene expression was similar to that in Travers et al. (2007), using the general approach of Wolfinger et al. (2001). Prior to analysis, the data were log transformed then SAS Proc Mixed was used to evaluate treatment effects in an analysis of variance. The data was first normalized using the following mixed model:

$$y_{ij}=\mu+A_i+D_j+(AxD)_{ij}+e_{ij}$$

where y_{ij} is the log fluorescence intensity from the cDNA on the *i*th array labeled with the *j*th dye, μ is the sample mean, A_i is the effect of the *i*th array (i=1-16), D_j is the effect of the *j*th dye, $(AxD)_{ij}$ is the array-dye interaction and e_{ij} is the error term. The residuals (*r*) were then analyzed using a second mixed model on a gene-by-gene basis:

$$r_{ijlmn}=\mu+A_i+W_l+D_j+P_n+R_m+e_{ijlmn}$$

where W_l is the effect of the *l*th precipitation treatment, R_m is the effect of the *m*th rust treatment and P_n is the effect of the *n*th block (random effect). The experiment-wise false discovery rate was controlled by computing Q values using the QVALUE software (Storey and Tibshirani 2003).

Data for phytohormone analysis and disease severity were log transformed and were also analyzed using Proc Mixed with block as a random effect. The model used for phytohormone analysis was the following:

$$y_{lmn} = \mu + R_m + W_l + S_n + (RxW)_{lm} + (RxS)_{mn} + (WxS)_{ln} + (RxWxS)_{lmn} + e_{lmn}$$

where R_m is the effect of the *m*th rust treatment, W_l is the effect of the *l*th precipitation treatment and S_n is the effect of the *n*th leaf section. Water potential and stomatal conductance data were also analyzed with Proc Mixed in order to detect any effects of the rust treatment on the physiology of the adjacent leaves.

Results

Water Potential and Stomatal Conductance

Water potential (Figure 2) was higher in the leaves of plants experiencing water stress and lower in the leaves of well-watered plants, as expected. The drought treatment had a significant effect on water potential measurements ($p < 0.0001$), however, rust inoculation did not have a significant effect on water potential in the adjacent leaves ($p = 0.98$). Stomatal conductance measurements (Figure 3) showed similar responses. Stomatal conductance measurements were higher in the leaves of well-watered plants and

lower in the leaves of drought-stressed plants, as expected. The rust inoculation did not have a significant effect on stomatal conductance measurements in the adjacent leaves ($p = 0.18$), but the drought treatment was significant ($p < 0.0001$).

Disease Severity

From block to block, disease severity was quite variable, with a significant block effect ($p = 0.055$). Overall, disease severity (Figure 4) was higher for plants experiencing drought in six of the seven blocks, with a corresponding average increase in disease severity under drought stress ($p = 0.072$).

Gene Expression

1894 genes (Figure 5) were significantly differentially expressed in response to the drought treatment, with a Q-value of 0.05 or less. Of these, expression of 1225 increased and expression of 669 decreased. Expression of genes involved in disease resistance, e.g. hypersensitive-induced response genes and PR-5, and photosynthesis, e.g. those involved with photosystems or various cytochromes, consistently decreased. Several heat shock proteins and molecular chaperones also were significantly differentially expressed in response to drought. Expression of heat shock proteins such as Hsp70 and sHsp17.9 consistently increased and expression of others such as Hsp90 and Hsp81-1 consistently decreased.

No significant differences in gene expression in response to the rust treatment, or the interaction between rust and drought, were detected using the mixed model analysis of variance if either Q-values ($Q < 0.05$) or a Bonferroni correction was used to establish

the criterion for significance. Of the 100 genes with the lowest *p*-values, expression of 60 genes decreased and expression of 40 genes increased. Genes for which expression decreased included genes involved with actin, translation initiation factors and a cytochrome gene. Genes in which expression increased included a sucrose-phosphate synthase, a gibberellin gene and a gene similar to a seven transmembrane Mlo4.

Only one of significant genes in the drought treatment was found for the four phytohormones analyzed and none in the top 100 rust genes, based on annotation. The only gene with annotation to indicate a role in SA or JA pathways whose expression was altered significantly in response to the drought treatment was a coumarate Co-A ligase-like protein, whose expression increased. This gene may be involved in the salicylic acid pathway.

Phytohormones

Four phytohormone concentrations were determined (Figures 6-9). The section of leaf analyzed was the most significant predictor for all phytohormones (p = 0.0066, p = 0.0001, p = 0.011, and p = 0.0057 for JA, BA, OPDA and SA, respectively). Rust infection was generally more severe towards the tip of the leaf which may contribute to the significance of the leaf section. There was a significant rust effect for BA (p = 0.074) and a significant drought effect for SA (p = 0.068). The interaction of drought and section was significant for SA (p = 0.032) and the three-way interaction between rust, drought and section was significant for JA (p = 0.021) and BA (p = 0.052). Typically the tip of the leaf had higher levels of all phytohormones in all treatments except for the

drought treatment alone, for which the base of the leaf had a higher average phytohormone concentration than the tip for SA. Blocks were highly variable.

Discussion

Drought

Drought can have many adverse effects on plants including changes in water status, gene expression and disease severity. The effects of drought stress on water potential and stomatal conductance in this study were as expected; drought stressed plants had more negative water potentials and lower stomatal conductance. Plants infected with rust have lower water potentials (Bethenod et al., 2001) and higher stomatal conductance (Suksayretrup et al., 1982). In the early stages of a rust infection, plants will lose less water because the stomata will be closed as a result of the infection. In later stages, the rust ruptures the epidermis and cuticle water loss through transpiration increases greatly (Littlefield, 1981). However, the rust treatment in this experiment had no significant effect on either water potential or stomatal conductance. This lack of effect may have been due in part to the low rust infection rates. In all blocks, rust severity rate was never greater than 10% in either the watered or the drought treatments and that level was observed for only two plants. Water stress measurements may also have been unaffected by the rust treatment because measurements were taken from uninfected leaves directly above and below the inoculated leaf.

Environmental conditions experienced by the plants in the greenhouse were similar to those observed on the prairie, based on results from the Rainfall Manipulation Plots (RaMPs) experiment at Konza Prairie, studied by Travers et al. (2007). The RaMPs

are a set of undisturbed plots containing native prairie plants under a fixed rainout shelter. The shelters exclude natural rainfall from the plots but a collection system is in place to collect the rainfall and apply it at a later time. The plots also are surrounded by a barrier 1.2 m deep to limit lateral water movement (Fay et al., 2002). *A. gerardii* individuals in the greenhouse experienced drought cycles and recovery periods based on soil water content (Figures 10 and 11) that was similar to the cycles seen with soil water content measured in the RaMP experiment (Fay et al., 2002). Water potential and stomatal conductance patterns for both drought-stressed plants and watered plants were similar to plants observed on the prairie (Fay et al., 2002). The water potential of *A. gerardii* plants in the RaMP experiment range from approximately -15 to -20 bars, while the range of stomatal conductance was approximately 30-90 mmol/m^2s.

Rust Infection

The severity of rust infection in *A. gerardii* plants was higher in plants experiencing drought stress than plants with an adequate water supply. However, the response of disease severity to different soil moisture regimes is highly dependent on the pathogen being studied. Some studies have shown an increase in disease severity under drought stress (Mayek-Perez, 2002; Ristaino and Duniway, 1989), no change in disease severity (Clover et al., 1999), or a decrease in disease severity with drought stress (Kackley et al., 1990). The increase in disease severity in the presence of drought could be caused by a change in the way the plant uses its resources. The plant may be putting more of its resources towards surviving the drought and thus less energy is available for disease resistance. Expression of genes encoding proteins that would be used for plant

protection from invading pathogens will show decreased expression and gene expression for genes involved in photosynthesis, ATP production, increased water movement, or seed production will increase to allow the plant to survive the drought stress it is experiencing. Some of the genes found in the drought-stressed plants whose expression significantly decreased were genes involved in the hypersensitive response, a cell death-related protein and a gene similar to PR-5. These results are consistent with the higher disease severity observed in drought-stressed plants.

Natural infection rates by *P. andropogonis* differed from those obtained in the greenhouse. Infection rates at Cedar Creek Natural History Area in Minnesota were relatively higher. Barnes et al. (2005) noted that all big bluestem plants observed in every year of their study were infected with rust, although the severity levels varied from year to year. The mean rust severity in the four years of their study was 5.50%, 3.38%, 3.93%, and 4.43% in 1998, 1999, 2000, and 2001, respectively. Rust infection levels at Konza Prairie are comparable to those observed at Cedar Creek (Garrett, personal communication). The low rust infection rate on the plants in the greenhouse may be due to the differences in wet periods and dry periods that the plants experienced. In a comparison between stripe rust (*P. striiformis*) and leaf rust of wheat (*P. recondita* f.sp. *tritici*), de Vallavieille-Pope et al. (1995) found that infection efficiency decreased for both rust species as the length of the dry period increased. Spores that began to germinate could not complete the process in the dry period and died. Only spores that had not begun to germinate were able to infect during a second wet period. They also found that the inoculum loss was greater for leaf rust than it was for stripe rust because of

the different infection techniques. Headrick and Pataky (1986) found that the dew period had a significant effect on urediniospore germination of *P. sorghi* on sweet corn.

The low infection rates I observed in this study could result from the long dry periods that the plants experienced after the inoculation period. *A. gerardii* plants were inoculated with *P. andropogonis* and placed in a mist chamber overnight (approximately 14-16 hours) after which they were placed on a greenhouse bench until the second inoculation. So after the initial wet period, the plants remained dry for 10-14 days, which may have resulted in the death of any spores that had begun germinating. Plants in the field experience dew formation in the mornings, which allows more spores to continue germinating and could increase the infection rate. After being in the mist chamber, the plants in the greenhouse did not have any comparable periods in which the leaves had water on them that would allow spore germination to continue. This dryness could have contributed to the low infection rates seen on the plants in the greenhouse.

There are two different components of infection related to drought that could have an effect on the infection rates. First, the presence of moisture on the leaf, even though the plant may be drought-stressed, can be critical for successful infection by the rust. The humid environment will cause the stomata to open and allow urediniospores to enter the plant. Water droplets formed on the leaf can move spores around as the water drop runs down the leaf, potentially increasing the leaf area infected. The response of the plant to drought stress also can have an effect on rust infection. Expression of genes encoding proteins used for plant protection from invading pathogens, e.g. disease resistance genes or hypersensitive response-related genes will have decreased expression, as observed in the gene expression analyses, potentially resulting in higher infection probabilities.

Gene Expression

When a plant is infected by an obligate pathogen, e.g. rust fungi, many changes occur at the cellular level. Genes involved in plant defense, e.g. PR genes, HR genes, or resistance (R) genes, tend to be expressed at higher levels. Along with the increased expression of resistance genes, expression of secondary messengers and transcription factors also tends to increase (Heitefuss, 2001). In cells of susceptible plants cytosolic calcium levels increase, electron-generating activity in mitochondria that are close to the invading fungi decreases, oxidative activity decreases, and hydrogen peroxide generation decreases (Heath, 1997).

A. gerardii samples exposed to the drought treatment exhibited ~1900 differentially expressed genes. Previously, Shinozaki and Yamaguchi-Shinozaki (1997) divided gene products produced as a result of gene expression into two groups. The first group contained proteins that function in stress tolerance, e.g. sugars, LEA proteins, chaperones, ubiquitin, glutathione S-transferase, and ascorbate peroxidase. The second group included protein factors involved in the continuation of signal transduction and proteins that function in stress responses, e.g. protein kinases, transcription factors, and phospholipase C. Microarray analyses showed many of the genes in these two groups were differentially regulated in *A. gerardii* individuals that received the drought treatment.

The responses to drought stress play an important role in protecting plants against this stress and regaining cellular homeostasis (Wang et al., 2004). Heat shock proteins and chaperones interact to regulate the folding of specific proteins. Microarray analyses

for *A. gerardii* showed both genes whose expression decreased and genes whose expression increased from different families of heat shock proteins (Hsp) and molecular chaperones. Of the heat shock proteins, expression of Hsp70 and sHsp17.9 was consistently increased while expression of Hsp81-1 and Hsp90 was consistently decreased. Hsp70 is involved in modulating signal transducers and may be critical for regulating the expression of downstream genes in signal transduction pathways during stressed conditions (Wang et al., 2004). The sHsps are heat shock proteins that have low molecular mass and constitute a more diverse family than Hsps. The sHsps respond to a wide variety of environmental stresses and there may be a correlation between sHsp accumulation and plant tolerance to stress (Wang et al., 2004). In maize, mitochondrial sHsps improve electron transport when plants were exposed to salt stress, by protecting NADH:ubiquinone oxidoreductase activity (Wang et al., 2004). Hsp90 plays an essential role in protein folding, signal transduction networks, cell-cycle control, protein degradation and protein trafficking (Wang et al., 2004). However, it also requires ATP to function properly (Wang et. al, 2004). The decrease in expression of Hsp90 in *A. gerardii* could be due decreased levels of ATP. Decreased ATP synthesis can result during a water deficit (Tezara et al., 1999).

Genes involved in photosynthesis had decreased expression which is consistent with previous studies (Flexas et al., 2004) although the mechanism by which decrease in expression occurs is not settled (Flexas et al., 2004; Tezara et al., 1999). Decreased photosynthesis in plants experiencing drought may result from stomatal closure or from reduced ATP synthesis (Flexas et al., 2004).

Drought effects on gene expression in the experiments reported here were similar to those of Travers et al. (2007) who evaluated plants sampled from Konza Prairie. In their study, they found that expression of photosynthesis-related genes and a hypersensitive response gene was significantly decreased and expression of heat shock proteins was significantly increased. The gene lists between the two experiments are very similar as well. Many of the genes identified by Travers et al. (2007) also were identified as significant in this study and their expression is equivalent to those previously reported. However, a clear link between gene expression and phytohormone concentration could not be made. A preliminary search for genes related to phytohormone production for each of the four phytohormones resulted in a single gene, a coumarate Co-A ligase-like protein, which is an enzyme involved in the SA pathway. We are evaluating other types of statistical analyses that may have more power for detecting the effects of infection on gene expression.

Phytohormone Production

Phytohormones analyzed in this experiment such as JA and SA play an important role in abiotic stress and in the expression of defense related genes in plants. Natural levels of JA and SA differ throughout the plant. In soybeans JA levels are highest in the hypocotyl hook, a zone of cell division, and in young plumules (Creelman and Mullet, 1997). Meristematic cells are localized to the leaf base in monocots. Therefore, if monocot leaves are similar to soybean hypocotyls, higher concentrations of jasmonates should be found in the leaf base and lower concentrations should be found in the mature cells at the leaf apex (Creelman and Mullet, 1997). This relationship could explain why

the leaf section was a significant factor in the phytohormone analysis. In contrast, SA is ubiquitous in many agronomic species with rice having much higher amounts of SA than any other crop species and corn having the lowest amount (Raskin et al., 1990).

Synthesis of JA and SA in response to pathogens is well documented (Creelman and Mullet, 1997; Bostock, 2005; Reymond and Farmer, 1998; Shah, 2003). SA is an important signaling molecule involved in local defense as well as systemic acquired resistance (SAR) and regulates the expression of many pathogenesis-related (PR) genes (Reymond and Farmer, 1998; Shah, 2003). JA also plays an important role in insect and disease resistance but regulates induced systemic resistance (ISR), which is not associated with major changes in gene expression, unlike SAR (Bostock, 2005). A precursor to JA is oxophytodienoic acid (OPDA) which has been found to increase resistance to aphids and fungal and bacterial pathogens in a mutant that overexpresses OPDA (Bostock, 2005).

Phytohormone levels also can change as a result of abiotic stress, e.g. drought. Responses to abiotic and biotic stress may be similar (Bostock, 2005). Increased levels of both JA and SA correspond to changes in the levels of abscissic acid (ABA) and cytokinins (CK) which occur as a result of water stress (Bostock, 2005). As levels of ABA and CK increase, the levels of JA and SA also increase. The levels of JA and SA in *A. gerardii* only appeared to increase in one section of the leaf when drought-stressed and watered treatments were compared. The tip of the leaf had slightly more JA in the drought treatment than in the watered treatment (Figure 6b), whereas the level of SA in the base of the leaf of a non-stressed plant was much higher than that in the base of a leaf from a drought-stressed plant (Figure 7b). The concentration changes between the tip

and the base of the leaf could be due to the difference in rust severity. Rust infection usually is heavier at the tip of the leaf, e.g. as seen in maize infected with *P. sorghi* (Mahindapala, 1978). When comparing the rust treatment to the uninoculated control, the changes in SA and JA levels both increase in the tip section of the leaf (Figures 6b and 7b). On average however, SA concentrations increased in the rust treatment, but JA levels decreased slightly (Figures 6a and 7a). The concentrations of all phytohormones increased when both the drought and the rust treatments were applied.

There has been abundant research (Bostock, 2005; Thaler et al., 2002; Jalali et al., 2006) showing crosstalk between the phytohormones produced in different pathways. Crosstalk involves a network of signal interactions, common to multiple stresses, which results in a positive, negative, or neutral outcome (Bostock, 2005). The production of SA has an inhibitory effect on JA levels (Reymond and Farmer, 1998). This effect may be due to the plant using its resources and energy to produce pathogen-related genes, induced by SA, and, in doing so, suppressing the expression of wound-related genes, which are induced by JA (Reymond and Farmer, 1998). However, there are other studies that have documented an additive relationship between JA and SA (Bostock, 2005). Although SA levels in *A. gerardii* may be naturally higher than JA levels, these results could also suggest that SA may have an inhibitory effect on JA. On average, *A. gerardii* plants exposed to different treatments have higher SA levels than JA levels in both sections of the leaf.

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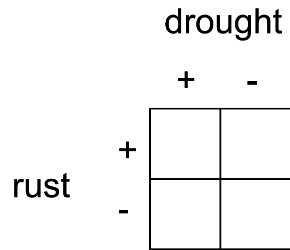
Figures



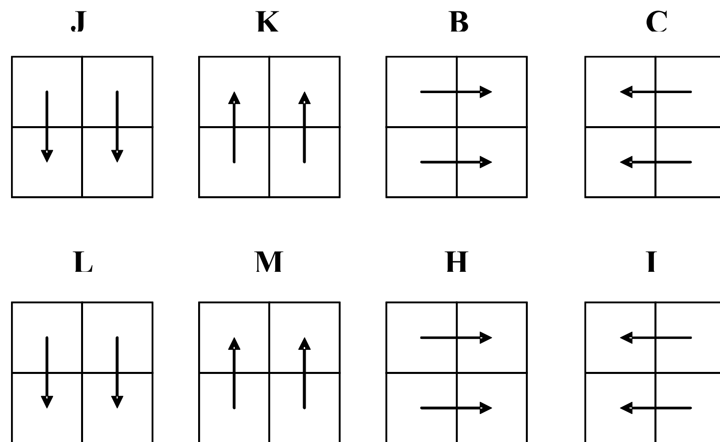


Figure 1. Microarray Pairing

Samples were paired on microarrays according to the following diagram. The head of the arrow represents the Cy3 dye and the tail of the arrow represents the Cy5 dye.

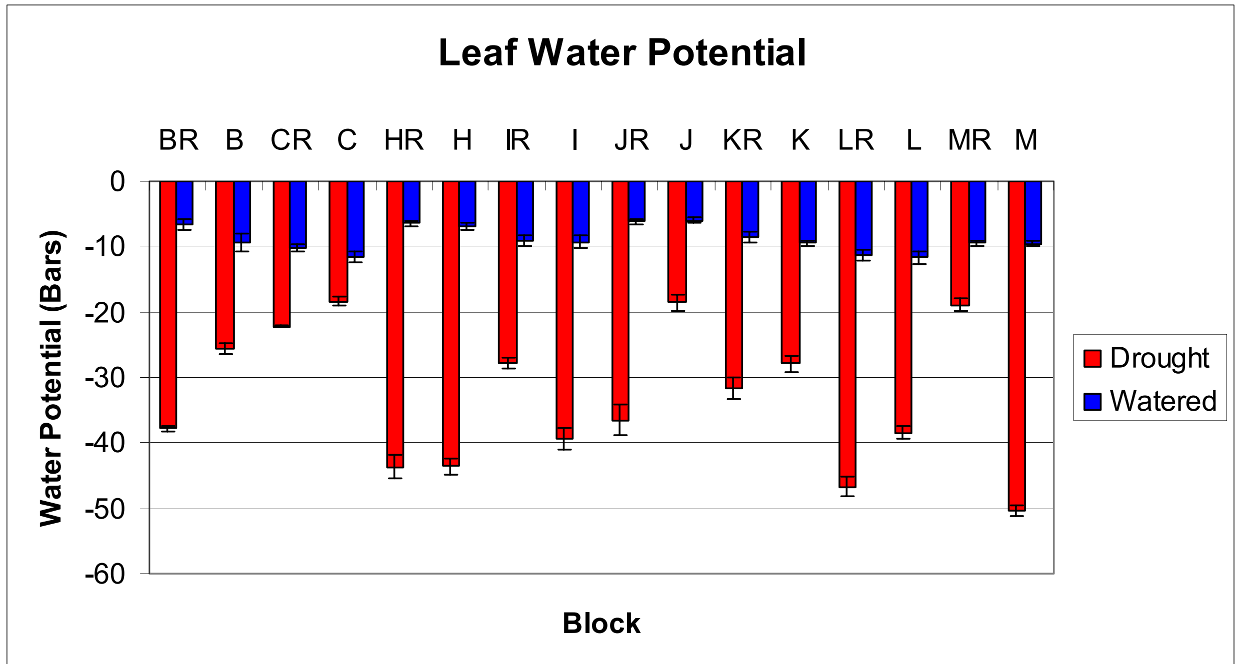


Figure 2. Leaf Water Potential

Water potential was averaged within pots for each of the eight blocks with the letter abbreviations of blocks on the x-axis and the average water potential, measured in bars, on the y-axis. The letter "R" denotes those pots with rust infection. Letters lacking the "R" represent non-inoculated pots from the same block. The rust treatment did not have a significant effect ($p = 0.98$) but the drought treatment did ($p < 0.0001$). Error bars are +/- 1 SE.

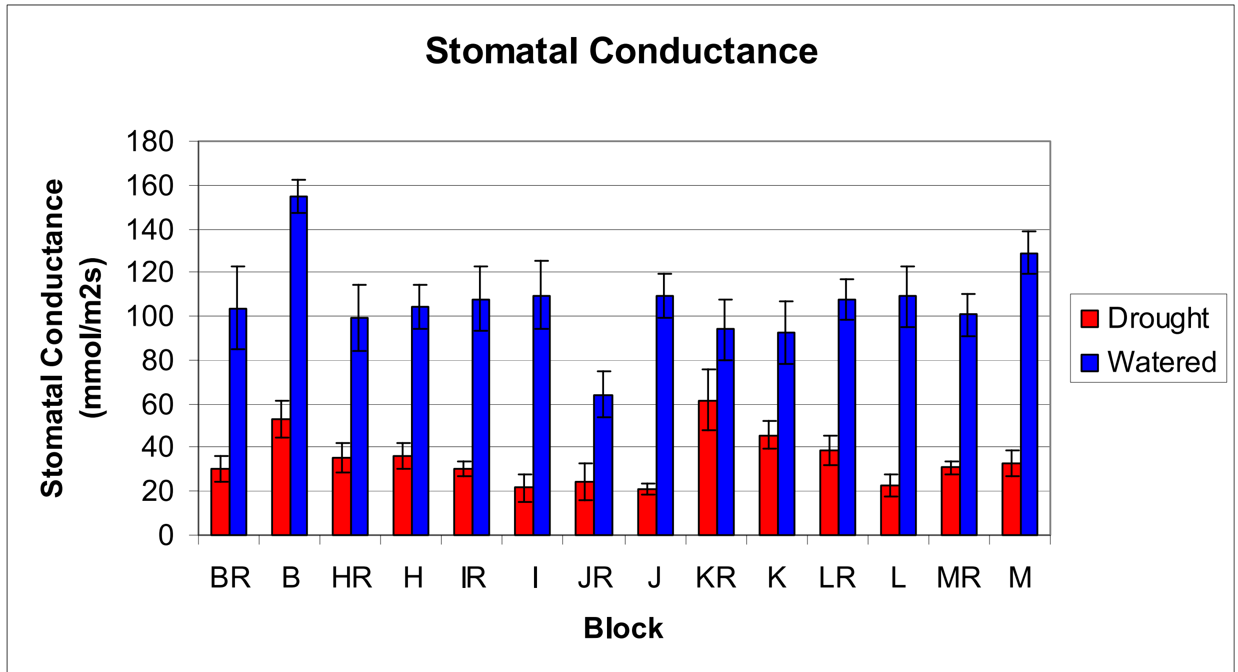


Figure 3. Stomatal Conductance

Stomatal conductance for seven of the eight blocks with the letter abbreviations of blocks on the x-axis and the average stomatal conductance, measured in mmol/m^{2}s, on the y-axis. Block C is missing because of a malfunction in the porometer. The letter "R" denotes those pots with rust infection. Letters lacking the "R" represent non-inoculated pots. The rust treatment did not have a significant effect ($p = 0.18$) but the drought treatment did ($p < 0.0001$).Error bars are +/- 1 SE.

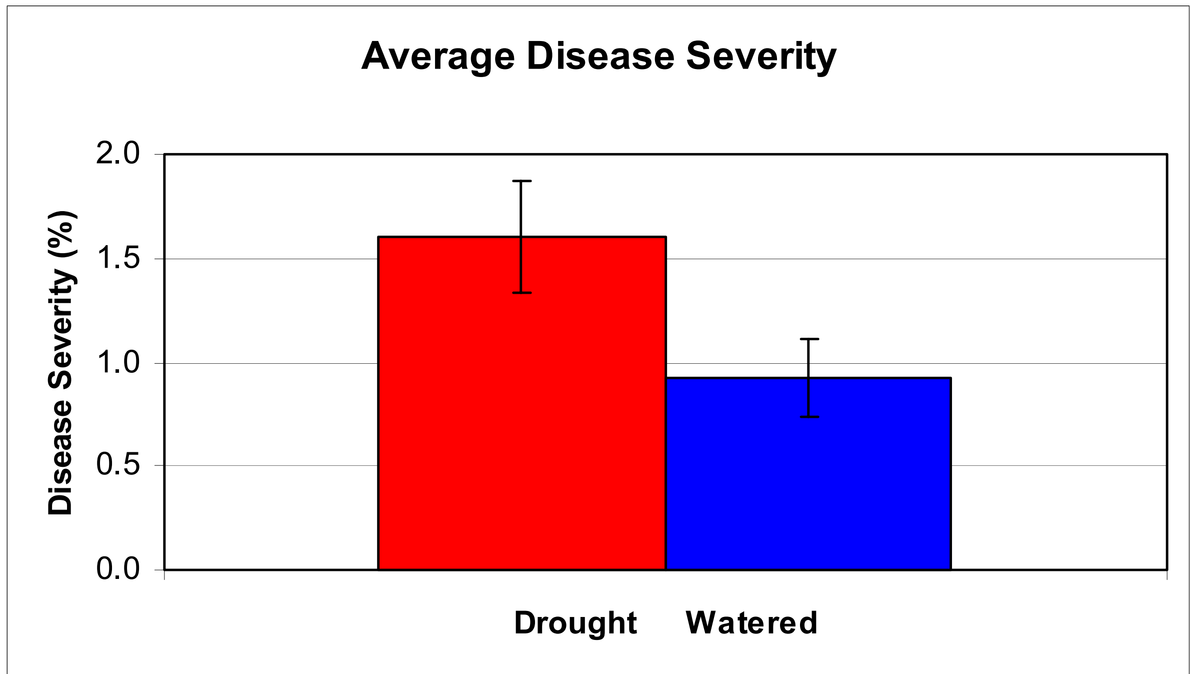


Figure 4. Disease Severity

Disease severity was measured as percent of leaf covered with disease and the measurements were averaged between all plants in the same pot for each block. The drought treatment and block both had significant effects ($p = 0.072$ and $p = 0.055$, respectively). Error bars are +/- 1 SE.

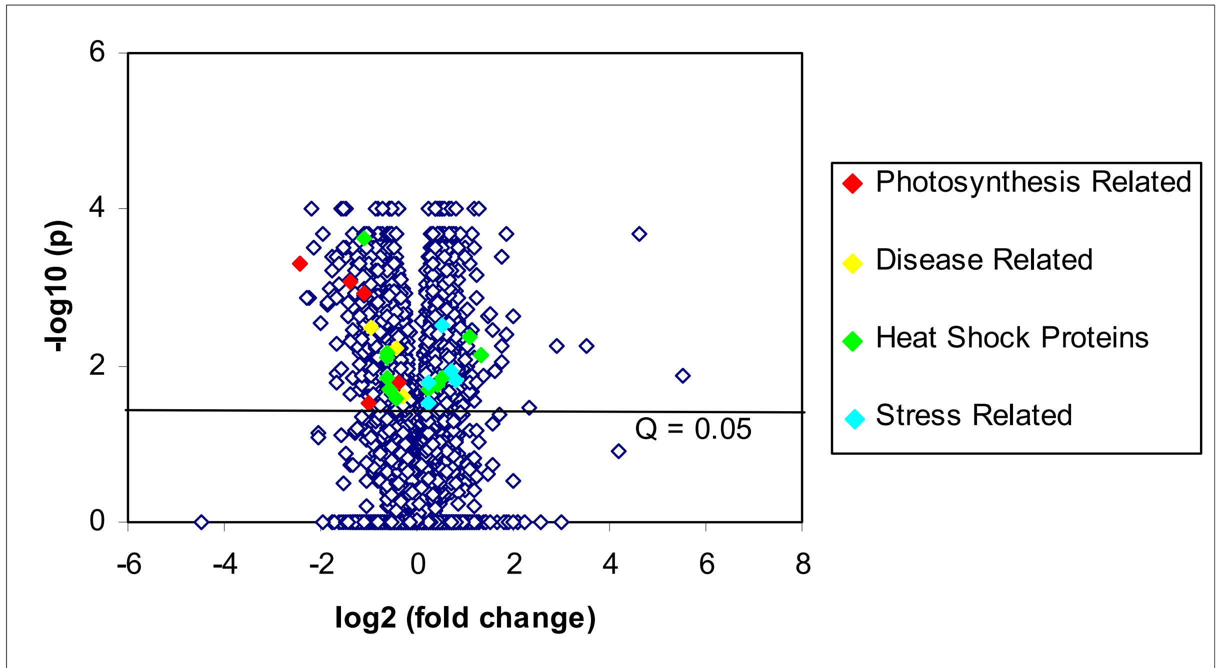


Figure 5. Gene Expression Fold Changes

Volcano plot of gene expression in drought-stressed vs. watered plants. The *x*-axis is log-transformed fold change in expression levels of drought-stressed vs. watered plants for each gene and the *y*-axis is the corresponding statistical significance level. Each spot represents a different gene. The false discovery rate was protected by calculating Q-values. Spots above the line representing Q = 0.05 indicate genes for which differential expression was statistically significant.

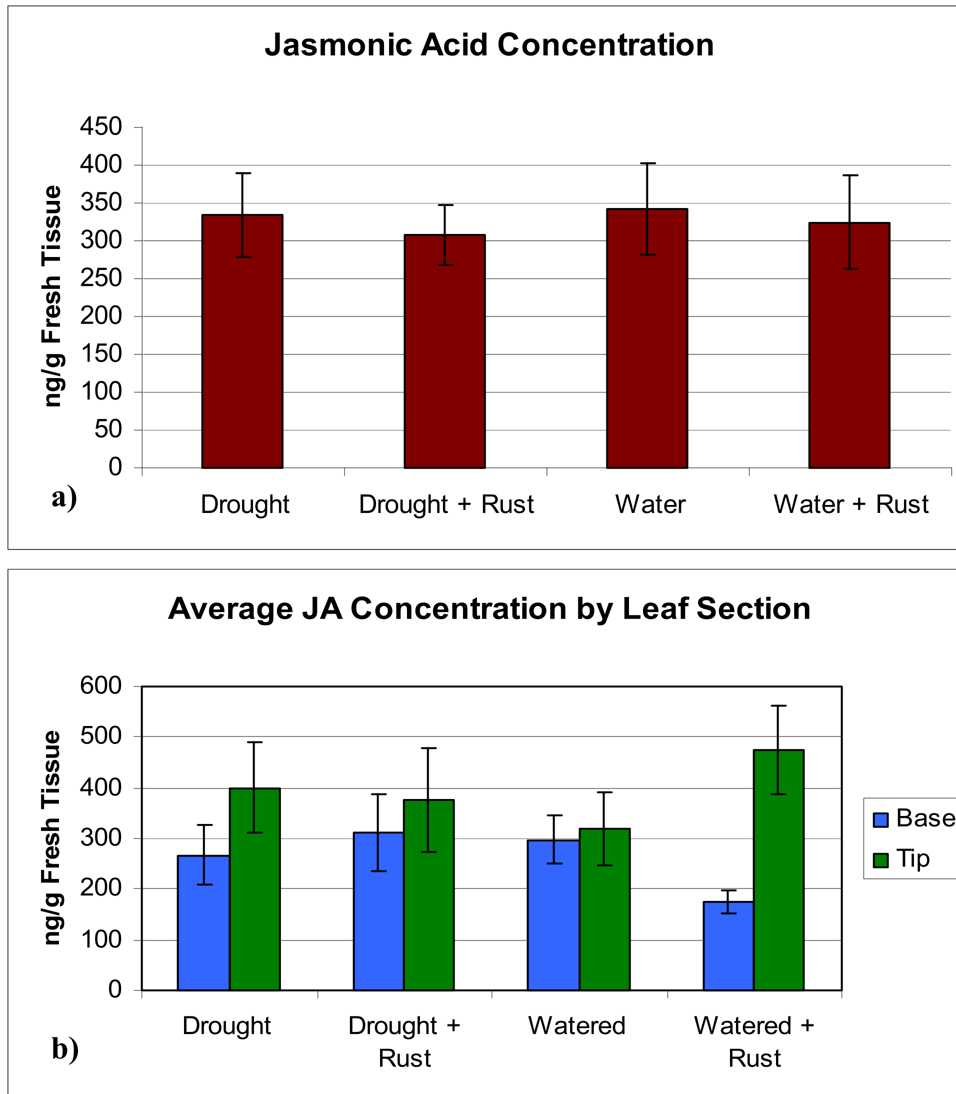


Figure 6. Average JA Concentration

Average concentration for JA in each treatment (6a) and average concentration for JA by each leaf section (6b). The section effect and three-way interaction were significant (p = 0.016 and p = 0.085, respectively). The rust effect, drought effect, rust*drought interaction, drought*section interaction, rust*section interaction, and the three-way interaction were not significant (p = 0.964, p = 0.724, p = 0.799, p = 0.434, p = 0.244, respectively). Error bars are +/- 1 SE.

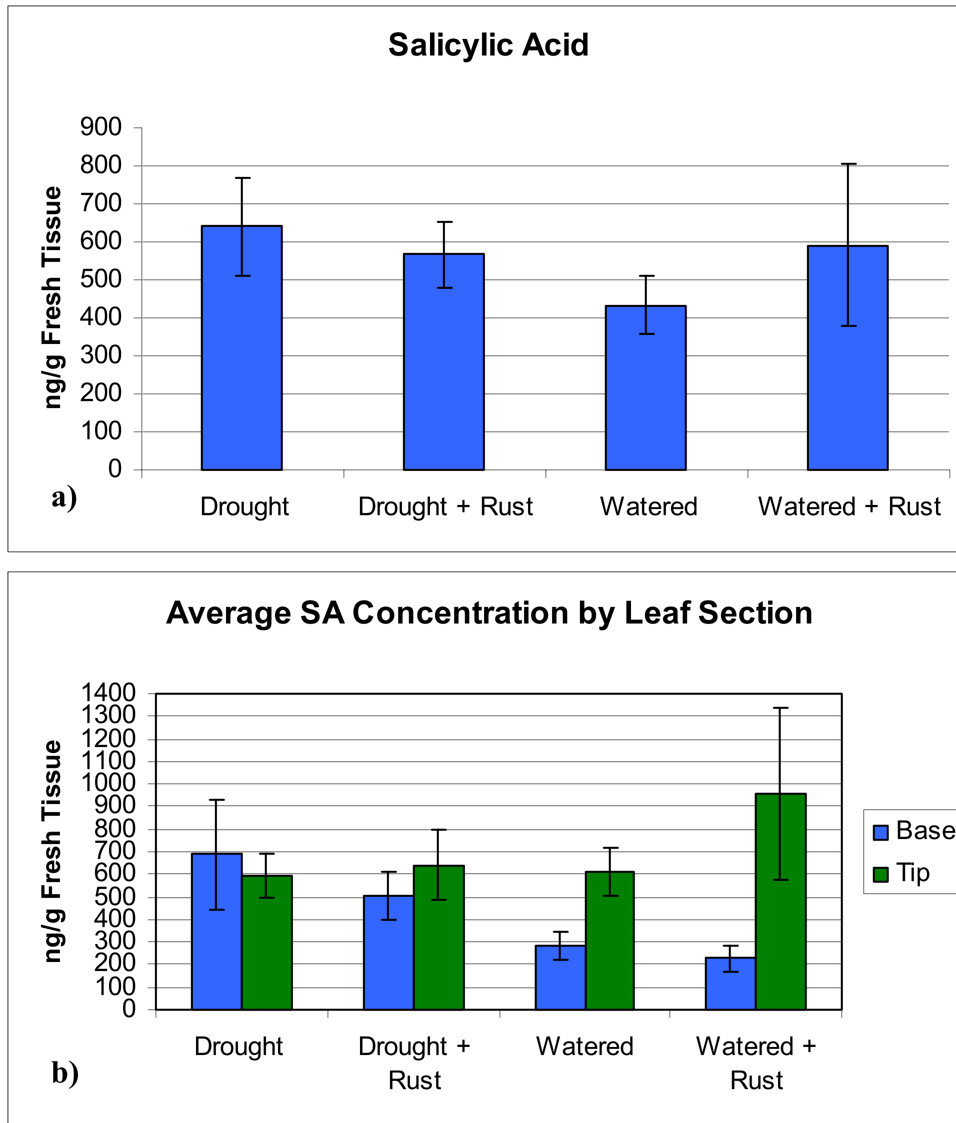


Figure 7. Average SA Concentration

Average concentration for SA in each treatment (7a) and average concentration for SA by each leaf section (7b). The drought effect, section effect and drought*section interaction were significant (p = 0.063, p = 0.006, p = 0.033, respectively). The rust effect, rust*drought interaction, rust*section interaction, and the three-way interaction were not significant (p = 0.733, p = 0.963, p = 0.457, p = 0.903, respectively).Error bars are +/- 1 SE.

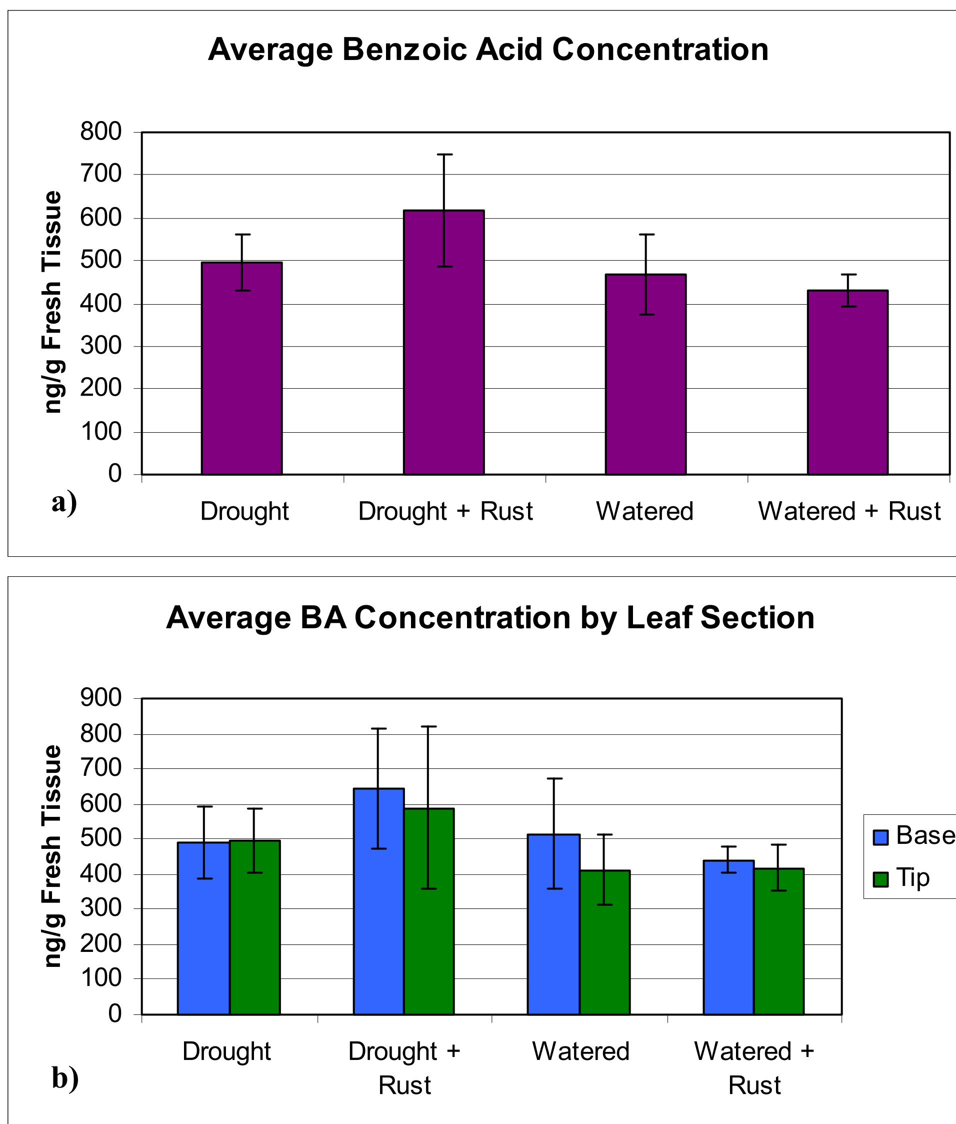


Figure 8. Average BA Concentration

Average concentration for BA in each treatment (8a) and average concentration for BA by each leaf section (8b). The rust effect, section effect and three-way interaction were all significant ($p = 0.072$, $p < 0.0001$, and $p = 0.056$, respectively). The drought effect, rust*drought interaction, drought*section interaction and rust*section interaction were not significant ($p = 0.103$, $p = 0.540$, $p = 0.453$, and $p = 0.601$, respectively). Error bars are +/- 1 SE.

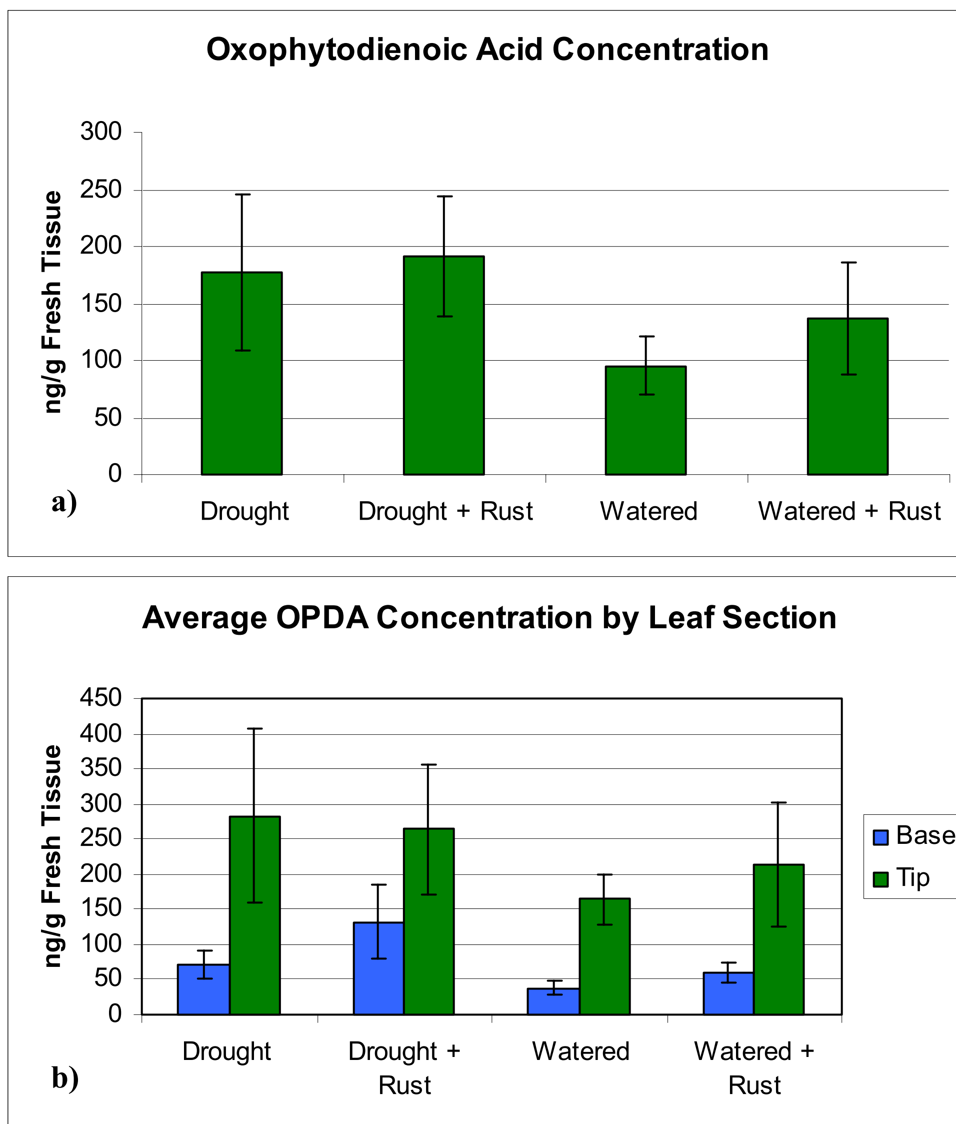


Figure 9. Average OPDA Concentration

Average concentration for OPDA in each treatment (9a) and average concentration for OPDA by each leaf section (9b). The section effect was significant ($p = 0.011$). The rust effect, drought effect, rust*drought interaction, drought*section interaction, rust*section interaction and the three-way interaction were not significant ($p = 0.228$, $p = 0.318$, $p = 0.883$, $p = 0.321$, $p = 0.707$, and $p = 0.518$, respectively). Error bars are +/- 1 SE.

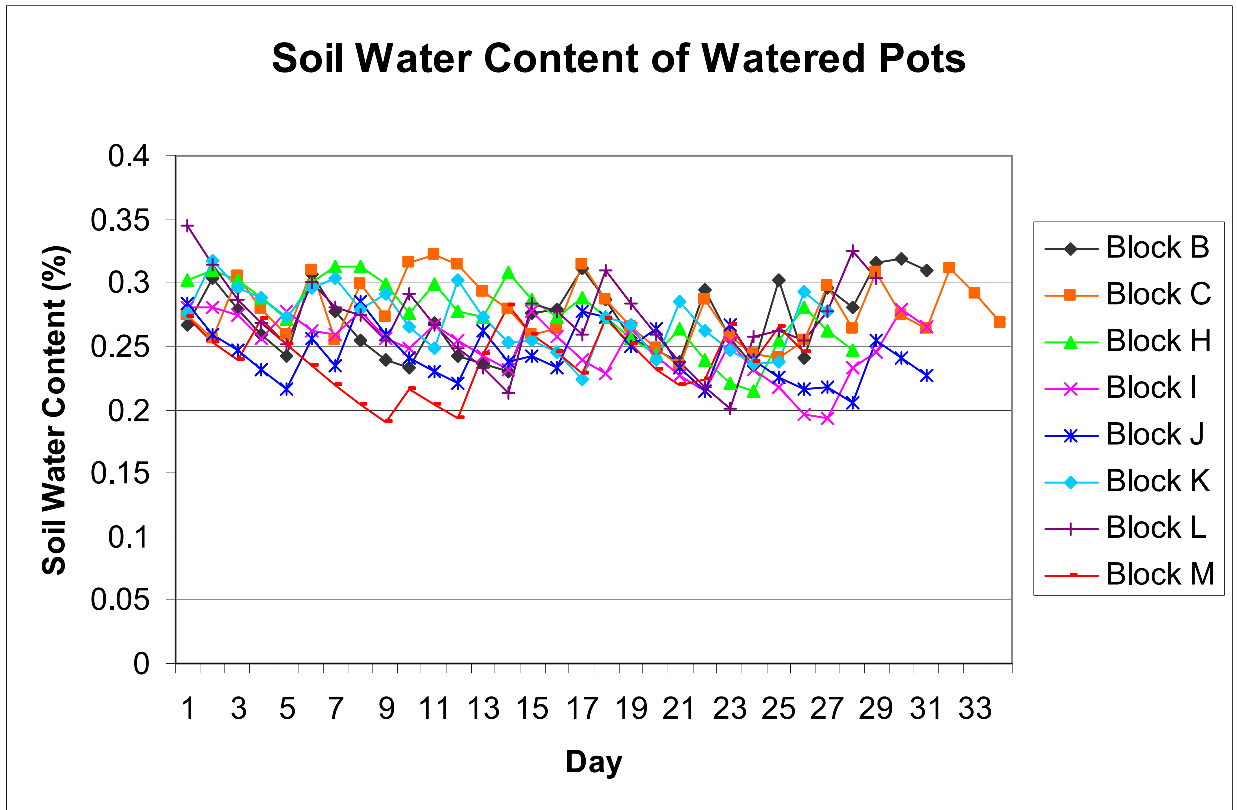


Figure 10. Soil Water Content of Watered Pots

Soil water content was averaged between watered pots in each block. Plants receiving the watered treatment were inoculated on the same day the plants receiving the drought treatment in the same block were inoculated.

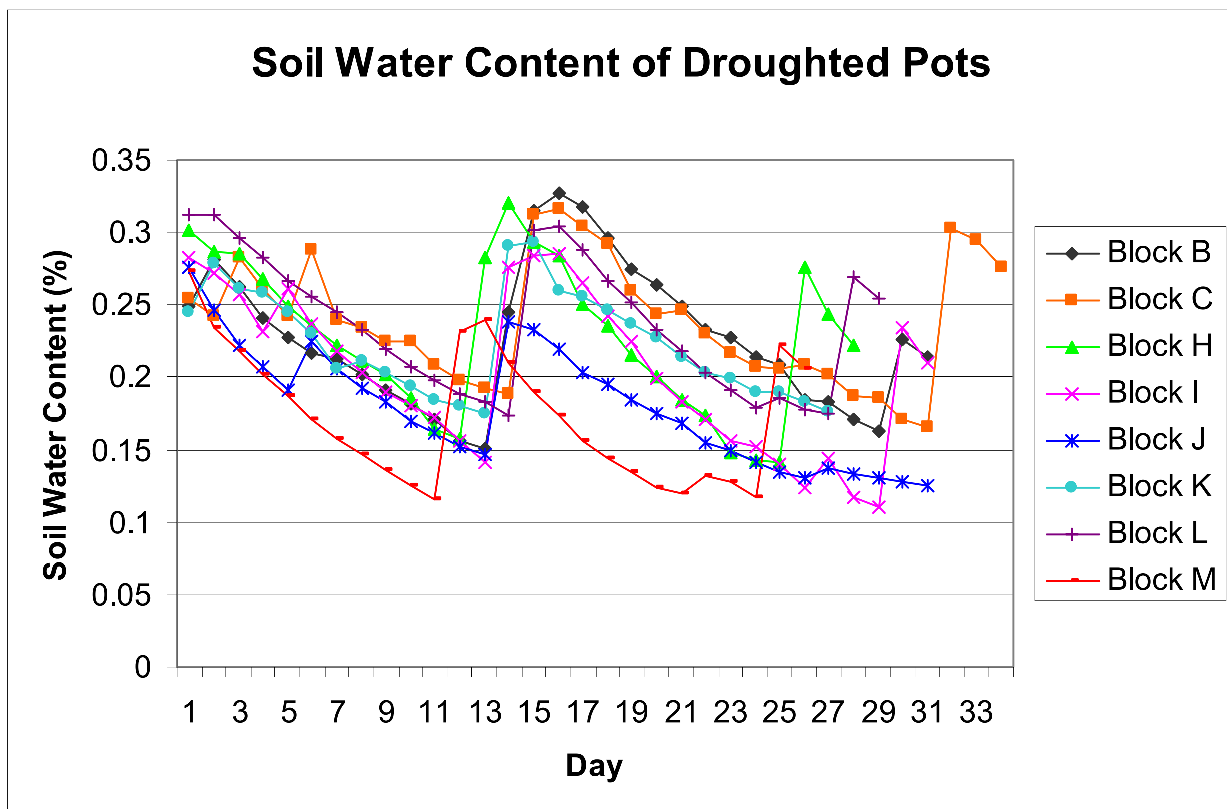


Figure 11. Soil Water Content of Drought-Stressed Pots

Soil water content was averaged between drought-stressed pots in each block. Plants were inoculated the first time at approximately day 14 following the first drought cycle. Soil water content was allowed to decrease again for the second drought cycle. Plants were inoculated for the second time at approximately 3 days before sampling (which occurred at the second lowest peak between Days 23-31).

Appendices

Appendix 1: SAS Code written with Zhongwen Tang

Analysis of Water Potential

```
data potential;

input block $ pot potential rust $ trt $;

cards;

B       13      37.8            R       D

B       9       25.6667         NR      D

C       18      22.1667         R       D

C       19      18.3889         NR      D

H       1       44.4375         R       D

H       5       43.5625         NR      D

I       15      27.75           R       D

I       14      39.3125         NR      D

J       28      36.5            R       D

J       26      18.55           NR      D

K       33      33.125          R       D

K       31      27.8571         NR      D

L       42      46.6667         R       D

L       35      38.4            NR      D

M       47      18.8889         R       D

M       45      50.4375         NR      D

B       11      6.625           R       W

B       10      9.35714         NR      W

C       17      10.25           R       W

C       16      11.625          NR      W

H       4       6.45            R       W

H       2       6.95            NR      W
```

```
I       16      9.15            R       W
I       13      9.2222          NR      W
J       24      6.1875          R       W
J       25      5.95            NR      W
K       30      8.5             R       W
K       32      9.45            NR      W
L       39      11.25           R       W
L       41      11.6875         NR      W
M       48      9.4444          R       W
M       46      9.5             NR      W
;
proc print;
proc mixed;
class block rust trt;
model potential=rust trt;
random block;
proc univariate plots normal; var potential;
run;
```

Analysis of Stomatal Conductance

```
data stomatal;
input block $ pot stomatal rust $ trt $;
cards;
B       13      30.45           R       D
B       9       52.7444         NR      D
H       1       35.45           R       D
H       5       36.3125         NR      D
I       15      30.19           R       D
I       14      21.675          NR      D
```

```
J       28      24.1714      R       D

J       26      21.05        NR      D

K       33      61.7875      R       D

K       31      45.7429      NR      D

L       42      38.93        R       D

L       35      22.7125      NR      D

M       47      30.8444      R       D

M       45      32.9375      NR      D

B       11      103.725      R       W

B       10      154.825      NR      W

H       4       99.19        R       W

H       2       104.24       NR      W

I       16      107.96       R       W

I       13      109.722      NR      W

J       24      64.3         R       W

J       25      109.32       NR      W

K       30      93.86        R       W

K       32      92.82        NR      W

L       39      107.513      R       W

L       41      109.1        NR      W

M       48      100.67       R       W

M       46      129.06       NR      W

;

proc print;

proc mixed;

class block rust trt;

model stomatal=rust trt;

random block;

proc univariate plots normal; var stomatal;
```

```
run;
```

Disease Severity

```
data dissev;
input block $ pot logdissev trt $;
cards;
H    3    0.09691    D
H    10   0.525045   D
I    18   -0.16273   D
I    12   0.146128   D
J    23   -0.60206   D
K    29   -0.60206   D
L    37   0.591065   D
L    36   0.157608   D
B    12   0.176091   D
C    20   0.146128   D
H    9    -0.12494   W
H    7    -0.42597   W
I    20   -1.14618   W
I    21   -0.52288   W
J    27   -1.30103   W
K    34   0.40654    W
L    40   0.278754   W
L    38   0.151278   W
B    15   -0.1549    W
C    22   -0.09691   W
;
proc print;
proc mixed method=type3;
```

```
class block trt;
model logdissev=trt;
random block;
proc univariate plots normal; var logdissev;
run;
```

Phytohormone Analysis

```
data logphytohormone;
input block $ rust $ drought $ pot section $ JA BA OPDA SA;
cards;
;
proc mixed;
class block rust drought section;
model JA=rust drought rust*drought rust*section drought*section
section rust*drought*section/ddfm=Satterth;
random block block*rust*drought;
proc mixed;
class block rust drought section;
model BA=rust drought rust*drought rust*section drought*section
section rust*drought*section/ddfm=Satterth;
random block block*rust*drought;
proc mixed;
class block rust drought section;
model OPDA=rust drought rust*drought rust*section drought*section
section rust*drought*section/ddfm=Satterth;
random block block*rust*drought;
proc mixed;
class block rust drought section;
```

```
model SA=rust drought rust*drought rust*section drought*section
section rust*drought*section/ddfm=Satterth;
random block block*rust*drought;
proc univariate plots normal; var JA BA OPDA SA;
run;
```

Gene Expression

```
/*---read in the data; assumes files are stored as text files
     with names sudarsanam1.txt - sudarsanam12.txt; change the
     datafile= pathname below to match directory---*/
%macro readdata;



%do a = 1 %to 16;


   proc import out=s
      datafile="c:\Erin\Iowa2006stacked&a..txt"
      dbms=tab replace;
      getnames=yes;
   run;


   data dsn&a;
      set s;
              ID = upcase(ID);
            value = upcase(Value);
            flags = upcase(Flags);
            dye = upcase(Dye);
            trt = upcase(Treatment);
```

```
            pot = upcase(Pot);
            block = upcase(Block);
            chip = upcase(Chip);
        if flags < 0 then delete;
        if (value > 0) then logi = log2(value);
            else logi = log2(1);
   run;

%end;

%mend;

%readdata
run;

data s;
      set dsn1 dsn2 dsn3 dsn4 dsn5 dsn6 dsn7 dsn8 dsn9 dsn10 dsn11
dsn12 dsn13 dsn14 dsn15 dsn16;
run;
```

Microarray Normalization 5

```
libname h 'C:\Erin';
run;

/*---overall normalization---*/
proc mixed data=s;
   class dye chip;
   model logi = dye /
```

```
        outp=h.Frank(keep=chip
        ID DYE TReatment block RESID);
        random chip/G;
        ods output G=G;
run;

proc sort data=h.frank;
        by id;
run;

data frank;
        set h.frank;
        if treatment="DD" then do; drought="d"; rust="n"; end;
        else if treatment="DR" then do; drought="d"; rust="y"; end;
        else if treatment="WW" then do; drought="w"; rust="n"; end;
        else if treatment="WR" then do; drought="w"; rust="y"; end;
run;

ods listing close;
proc mixed data=frank;
        class drought rust block dye chip;
        model resid=drought rust drought*rust dye;
        random chip block;
        by id;
        lsmeans drought rust drought*rust;
        ods output tests3=tests3 lsmeans=lsmeans;
run;
ods listing;
```

```
%macro trt(trt);
      data &trt;
            set lsmeans;
            where upcase(effect)=upcase("&trt");
            keep id &trt estimate;
      run;

      proc transpose data=&trt out=&trt;
            by id;
            id &trt;
            var estimate;
      run;
%mend;

%trt(drought);
data droughtfold;
      set drought;
      fold=d-w;
run;

%trt(rust);
data rustfold;
      set rust;
      fold=y-n;
run;

data int;
      set lsmeans;
      where upcase(effect)=upcase("drought*rust");
```

```
      keep id drought rust estimate;
run;

proc transpose data=int out=int2;
      by id;
      var estimate;
run;

data int3 (rename=(col1=dn col2=dy col3=wn col4=wy));
      set int2;
run;

data intfold;
      set int3;
      dnfold=dn-wn;
      dyfold=dy-wn;
      wyfold=wy-wn;
run;

proc export data=intfold outfile="C:/erin/intfold.csv"
dbms=csv replace;
run;
```

Appendix 2: Phytohormone Data

Block	Trt.	Sample	Tube	fresh weight	JA trans	me-BA	OPDA	me-SA
H	D + R	1-1-B	1	0.2052	222	295	39	404
H	D + R	1-4-T	2	0.128	298	429	97	549
H	W	2-2-B	3	0.1655	335	282	40	281
H	W	2-5-T	4	0.1357	319	317	34	589
H	W + R	4-4-T	5	0.058	380	874	229	1819
H	W + R	4-5-B	6	0.2409	149	208	30	144
H	D	5-2-B	7	0.1241	323	404	41	1883
H	D	5-3-T	8	0.075	289	655	234	793
I	W	13-4-T	9	0.1357	283	354	172	304
I	W	13-5-B	10	0.0838	336	639	34	306
I	D	14-4-T	11	0.0768	557	837	123	499
I	D	14-5-B	12	0.0956	218	636	166	630
I	D + R	15-3-T	13	0.1212	141	523	47	425
I	D + R	15-5-B	14	0.1399	246	437	52	200
I	W + R	16-1-T	15	0.142	293	383	66	143
I	W + R	16-2-B	16	0.1688	219	363	41	175
J	W + R	24-1-B	17	0.2066	185	328	34	166
J	W + R	24-5-T	18	0.0382	584	1441	126	607
J	W	25-4-T	19	0.0946	0	587	0	294
J	W	25-5-B	20	0.1938	200	286	55	156
J	D	26-1-T	21	0.1097	370	451	857	506
J	D	26-4-B	22	0.1827	181	339	48	475
J	D + R	28-2-T	23	0.0406	469	1506	560	671
J	D + R	28-5-B	24	0.081	265	738	148	478
K	W + R	30-2-T	25	0.0555	822	1259	200	2422
K	W + R	30-5-B	26	0.2323	135	211	43	205
K	D	31-3-T	27	0.0842	111	692	356	307
K	D	31-4-B	28	0.2776	130	249	28	207
K	W	32-4-T	29	0.1189	206	440	164	916
K	W	32-5-B	30	0.2599	127	170	36	143
K	D + R	33-1-T	31	0.1116	247	456	270	346
K	D + R	33-2-B	32	0.1184	382	512	36	448
L	D	35-4-T	33	0.0745	740	501	129	968
L	D	35-1-B	34	0.1755	222	184	63	381
L	W + R	39-1-T	35	0.0797	516	478	629	327
L	W + R	39-2-B	36	0.1411	248	269	81	172

L	W	41-4-T	37	0.0889	595	465	242	747
L	W	41-5-B	38	0.1195	445	252	0	265
L	D + R	42-2-B	39	0.0733	643	500	378	962
L	D + R	42-3-T	40	0.0685	731	545	345	1216
M	D	45-3-B	41	0.0905	532	344	84	546
M	D	45-4-T	42	0.0697	332	652	0	488
M	W	46-2-B	43	0.1214	343	282	64	570
M	W	46-5-T	44	0.0651	189	472	210	489
M	D + R	47-3-B	45	0.0801	114	379	136	540
M	D + R	47-4-T	46	0.0545	452	481	0	0
M	W + R	48-2-B	47	0.07	113	475	127	505
M	W + R	48-3-T	48	0.0638	245	523	31	413

Highlighted rows were not included in the statistical analysis.