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BIOCHEMICAL, PHYSIOLOGICAL, AND ANATOMICAL INSIGHTS INTO APHID-BIOENERGY SWITCHGRASS INTERACTIONS

Travis J. Prochaska
University of Nebraska-Lincoln

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by

Travis Joseph Prochaska

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Advisors: Tiffany Heng-Moss and Gautam Sarath

Switchgrass, *Panicum virgatum* L., a perennial, warm-season grass native to North America, is a candidate for development as a bioenergy crop. Previously, warm-season grasses were considered to be relatively pest free in their native habitats. However, recent studies using the hemipteran family Aphididae have shown phloem-feeding insects can lead to significant injury in switchgrass. The objectives of this research were to: 1) gain physiological, biochemical, and anatomical insights into insect-bioenergy switchgrass interactions to determine potential insect resistance mechanisms among susceptible and resistant switchgrass genotypes; and 2) to generate and evaluate diverse segregating populations of switchgrass, both resistant and susceptible, to assess for insect herbivory.

Recently, select aphids including greenbugs, *Schizaphis graminum* (Rondani), and yellow sugarcane aphids, *Sipha flava* (Forbes), have been identified as potential pests of switchgrass. However, limited research has been devoted to selecting insect-resistant switchgrasses and understanding the

physiological responses of susceptible and resistant switchgrasses to aphid feeding. Using a series of photosynthesis studies, differences in photosynthetic activity were detected among the switchgrasses evaluated in response to aphid feeding. Overall, the lowland ecotype (Kanlow) assimilated carbon dioxide more efficiently than the upland ecotype (Summer) and the hybrid KxS when exposed to aphid herbivory. These observations suggest the antibiotic population, Kanlow, has mechanisms similar to those observed in tolerant plant systems where changes in photosynthetic rates occurred in response to aphid herbivory.

Feeding by greenbugs and yellow sugarcane aphids on plants can elicit a number of stress-related responses. Our studies investigated the accumulation of reactive oxygen species (ROS) scavenging enzymes and defensive response genes in resistant and susceptible switchgrass populations using biochemical protocols along with gene expression studies. Genes of interest involving greenbug-switchgrass interactions were identified from previous Illumina® Solexa data, specifically RNAseq. These data provide valuable insight into the physiological, biochemical, and anatomical response of switchgrass when challenged by cereal aphids. Furthermore, continued screening of susceptible and resistant switchgrass germplasm will help researchers better understand the defensive systems operating in segregating switchgrass populations.

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2 Timothy 4:7

FIGHT the good fight, FINISH the race, and KEEP the faith.

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CHAPTER 1:

LITERATURE REVIEW

Switchgrass

Switchgrass, *Panicum virgatum* L., is a perennial, polyploid, warm-season grass that is native to the North American tallgrass prairie east of the Rocky Mountains (Vogel 2004, Mitchell et al. 2008, 2012). Along with indiangrass, *Sorghastrum nutans* (L.) Nash, and big bluestem, *Andropogon gerardii* Vitman, switchgrass is considered one of the 'big three' grasses, which together, makeup the largest percentage of the tallgrass prairie (Bouton 2008). Highly adapted to the prairie environment, switchgrass can be grown from 20° north latitude to 55° north latitude and east of the Rocky Mountains (Moser and Vogel 1995, Vogel 2004, Bouton 2008). Switchgrass can measure up to three meters in height, with many genotypes caespitose in appearance (they grow in dense clusters) producing short rhizomes (Vogel 2004, Bouton 2008). Switchgrass is characterized as an outcrossing species (Liu et al. 2013). Through time, switchgrass has evolved multiple, diverse populations that have led to significant variation and morphological diversity (Vogel et al. 2010, Zalapa et al. 2010, Lu et al. 2013). In switchgrass, the basic chromosome number is nine, although multiple ploidy levels exist with tetraploids (2n = 4x = 36) and octoploids (2n = 8x = 72) being the dominant ploidy level (Moser and Vogel 1995, Sanderson et al. 1996, Bouton 2008). Switchgrass ploidy values range from 2n = 2x = 18 to 2n = 12x = 108 (Church 1940, Nielsen 1944).

Switchgrass is identified by two diverse ecotypes, lowland and upland, which can be identified based on chloroplant markers (Hultquist et al. 1997, Young et al. 2012). Characteristically, lowland ecotypes are found to be coarser, taller, and have adapted for better growth in lowland areas that may be exposed to flooding (Vogel 2004). Upland ecotypes, on the other hand, are typically associated with drier environments, and tend to have smaller leaves and lower biomass yields, but are more suitable for cattle grazing (Vogel 2004, Bartley et al. 2013)

Looking Forward: Switchgrass as a Bioenergy Feedstock

Until relatively recently, research has been highly concentrated on the use of switchgrass as a rangeland forage crop with efforts mainly centered on improving forage value and forage yield (Vogel 2004). However, switchgrass has recently been identified as a principal candidate for the expansion of herbaceous bioenergy production as outlined by the U.S. Department of Energy (US-DOE) (Vogel 1996, Vogel et al. 2002, Sarath et al. 2008). Biomass feedstocks are used in ethanol production from starch- and sugar-rich crops, such as maize (*Zea mays* L.). However, negative environmental impacts may result by producing ethanol with row crops. Generally, crops such as maize, are grown in input-intensive agricultural environments that require high inputs of resources, such as nitrogen fertilization, which may lower the overall goal of reducing energy and carbon dioxide (CO_2) efflux within the agricultural system (Jakob et al. 2009). Other indirect factors, such as drought and loss of biodiversity may lead to further (negative) environmental complications (AGMRC 2015, Conca 2015). Within the

past few years, the rivalry between the ethanol and food industry has become a significant social issue. In 2000, more than 90% of the United States' corn supply went to feed the human population and livestock around the world, with only 5% used for ethanol production. By 2013, 40% of the U.S. corn supply was used to produce ethanol, 45% to feed livestock, with 15% used in food and beverage (AGMRC 2015). On average, one bushel of corn can be used to produce approximately 11.4 liters (3 gallons) of ethanol (AGMRC 2015, Conca 2015). Conca (2015) estimates the U.S. could use over 492 billion liters (130 billion gallons) of gasoline in 2015. On the other hand, ethanol can also be produced from other plant sources, including the fermentation of sugars from plant cell wall carbohydrate polymers, namely cellulose and hemicellulose. Forage crops, such as switchgrass, can yield high levels of plant cell walls (Vogel 1996). As a result, dedicated bioenergy feedstocks such as switchgrass, miscanthus and sorghum, show promise as a future renewable energy solution by establishing a more economical and sustainable energy resource based on lower annual input requirements and by promoting a positive energy environmental balance (Hill et al. 2006, Rooney et al. 2007, Heaton et al. 2008).

Switchgrass has become a top candidate for bioenergy production because of several desirable characteristics: low water and nutrient requirements, suitability for growth on marginal lands, high productivity yields across diverse environments, positive environmental benefits, and compatibility with modern farming practices (Sanderson et al. 1996, 2004, McLaughlin et al. 1999). In addition, because of its extensive and well developed root system,

switchgrass helps diminish soil erosion and runoff in marginal lands (i.e. waterways and terraces; McLaughlin and Walsh 1998). This in turn may help diminish the loss of soil nutrients, increase assimilation of soil carbon, and reduce grower dependence on agricultural chemicals (McLaughlin et al. 1994, Sanderson et al. 1996). With the use of herbaceous energy crops, like switchgrass, in agricultural systems, Hohenstein and Wright (1994) estimate a near 95% reduction in soil erosion when compared to traditional row crops. Along with reductions in soil erosion rates, data collected from across the U.S. suggest that soil texture and land quality do not appear to have a significant impact on switchgrass yield (Wullschleger et al. 2010).

Switchgrass yields can vary significantly between localities and cultivars with yields ranging from 10 to 14 Mg ha^{-1}; although yields approaching 40 Mg ha^{-1} have been observed in select localities with increased fertilizer inputs and precipitation (Wullschleger et al. 2010). With expanded research, it is expected that yields will continue to improve through breeding efforts that may also incorporate traits such as cold hardiness and insect resistance. Sustainability of bioenergy crops, including switchgrass, will depend not only on the biomass energy produced, but also by the energy spent with conventional growing practices and through the energy used to convert it to usable energy. Observations by Shaporui et al. (2003) have shown an average energy ratio of 1.34 (i.e., for every joule used to produce ethanol from maize, there is a 34% energy gain). Similar studies with switchgrass have shown as much as a 5-fold

net energy gain coupled with a 10-fold reduction in greenhouse gas emissions (McLaughlin and Walsh 1998, Schmer et al. 2008, Bartley et al. 2013).

Looking forward, biomass yields will continue to be a major focus. Simultaneously improving nutrient use will allow for increased yields while minimizing overall inputs including nitrogen and minerals. With biomass quality being essential for the conversion to ethanol, one of the key traits in breeding will revolve around maintaining biomass quality, along with other traits such as responses to biotic and abiotic stressors (emerging pests, pathogens, and climate changes) (Gustafson et al. 2003, Crouch et al. 2009, Grassini et al. 2009, Prasifka et al. 2009a, Prasifka et al. 2009b, Johnson et al. 2010, McIsaac et al. 2010, Kiniry et al. 2011, Bartley et al. 2013). In the short term, switchgrass improvement will continue to come from recurrent selection of superior germplasms. However, with the addition of new tools and technologies and advances in molecular resources for marker-assisted selections, these approaches will be better explored and utilized (Bartley et al. 2013).

Insect Pests of Switchgrass

Grasses (family *Poaceae*) serve as a host for a wide range of insect orders. Many foliage-feeding insects belong to the orders Coleoptera, Hymenoptera, Lepidoptera, Orthoptera, and Phasmatodea (Tscharntke and Greiler 1995). Important insect taxa include the stem borers, including members of the orders Coleoptera, Diptera, Hymenoptera, and Lepidoptera, along with the phloem-feeders within the orders of Hemiptera and Thysanoptera (Tscharntke and Greiler 1995). Arthropod surveys completed in managed Nebraska

switchgrass sites have recorded 12 arthropod orders and 84 insect families. Insects belonging to the orders Coleoptera, Hymenoptera, and Thysanoptera were the most abundant, representing nearly 80% of all arthropods collected (Schaeffer et al. 2011). Relatively few studies have been carried out on insects and their respective pest status. This is likely due to the fact that warm-season grasses, such as switchgrass, appear to be relatively pest free in their native habitats which has led to the assumption that few pest management practices will be needed (Moser et al. 2004, Parrish and Fike 2005, Prasifka et al. 2009a). Until recently, few reports have focused on phloem-feeding insects and their association with switchgrass. These include members of the hemipteran families Aleyrodidae (whiteflies), Delphacidae (planthoppers), Cicadellidae (leafhoppers) and Aphididae (aphids) that are sometimes referred to as the most damaging pests worldwide (Hilder et al. 1995). Recent studies in switchgrass, have focused primarily on members of the family Aphididae. Koch et al. (2014a) studied four aphid species including *Sipha flava* (Forbes), yellow sugarcane aphid (YSA); *Schizaphis graminum* (Randani), greenbug (GB); *Rhopallosiphum padi* (L.), bird cherry-oat aphid (BCOA); and *Diuraphis noxia* (Mordvilko), Russian wheat aphid (RWA). Overall, screening results indicated that switchgrass did not serve as a potential feeding and reproductive host for *R. padi* and *D. noxia* on switchgrass cultivars Kanlow, Summer, and two experimental lines, KxS (Kanlow male, Summer female) and SxK (Summer male, Kanlow female), which were derived by crossing Kanlow (K) and Summer (S) plants. However, these four switchgrass

cultivars were found to be feeding and reproductive hosts when challenged with *S. graminum* and *S. flava* (Koch et al. 2014a).

Greenbug

The GB have been a recognized pest of small grains for more than 150 years (Nuessly and Nagata 2005). Currently, there are approximately 40 recognized species of *Schizaphis* worldwide with seven found in North America (Blackman and Eastop 1984, Nuessly and Nagata 2005). The first report of the GB was documented on wheat and barley in Virginia in the early 1880s (Webster and Phillips 1912, Nuessly and Nagata 2005). The first report of GB damage to sorghum was in Nebraska during the 1968 growing season (Harvey and Hackerott 1969, Nuessly and Nagata 2005). This report was before infestations spread throughout much of the grain production areas of North America (Harvey and Hackerott 1969, Nuessly and Nagata 2005). *Schizaphis graminum* are parthenogenetic in nature. Nymphs will pass through three instars before emerging as an adults in 7-9 days at temperatures between 60-80 °F. Each adult GB can produce one to five nymphs per day (Nuessly and Nagata 2005). GBs have been observed feeding on more than 70 graminaceous species including barley, bluegrass, maize, sorghum, wheat, and wheatgrass (Michela, Jr. 1986, Nuessly and Nagata 2005). In some of these host species, enzymatic activity in the GB's saliva can lead to degradation of cell walls and chloroplasts in susceptible plants (Al-Mousawi et al. 1983, Nuessly and Nagata 2005). Feeding by GBs initially causes yellow or red leaf spots with continued feeding resulting in general yellowing and reddening of the leaf along with leaf and root death. In

severe cases, GB feeding may result in plant death. Plant attributes such as size, yield, and survival can be greatly affected by GB herbivory on susceptible small grain cultivars (Nuessly and Nagata 2005). Furthermore, GBs can serve as virus vectors for plant viruses that include barley yellow dwarf (Murphy 1959), sugarcane mosaic (Ingram and Summers 1938), and maize dwarf mosaic virus (Nault and Bradley 1969). Overall, insecticides serve as the front line of defense against GBs among small grains (Hays et al. 1999), but the more economical long-term solution may be host plant resistance (Nuessly and Nagata 2005).

Yellow Sugarcane Aphid

The YSA, *S. flava*, is native to North America. It was first described in Illinois in 1884 (Forbes 1884, Nuessly 2005), with populations spreading throughout much of North America in the years to follow. *Sipha flava* are parthenogenetic. Nymphs pass through four instars before emerging as an adult in 8 days on sorghum and 18-22 days on sugarcane (Hentz and Nuessly 2004). On average, females produce one to five nymphs per day for approximately 22 days (Nuessly 2005). *Sipha flava* can be found on cultivated row crops, including maize, rice, sorghum, and sugarcane. YSAs are also pests on members of the genera Graminea, including *Hordeum, Oryza, Panicum, Sorghum,* and *Triticum* (Nuessly 2005). YSA feeding results in the yellowing and reddening of leaves, depending on host plant. Prolonged exposure to *S. flava* herbivory can lead to premature senescence of leaves and/or plant death. Yield reductions usually result from aphid damage to early plant growth stages which may include reduced tillering (Hall 2001). This aphid also serves as a vector of sugarcane

mosaic potyvirus (Blackman and Eastop 2000) and barley yellow dwarf virus (Garrett et al. 2004). Natural enemies, including 10 species of ladybird beetles (Coccinellidae), predacious ants, and young spiders also help reduce YSA infestations; however, this may not occur before plant damage has ensued. Heavy rainfall and temperatures above 95°F may help reduce YSA populations during the summer months (Nuessly 2005). Should natural enemies and other pest management tactics fail, insecticides are available that provide effective YSA control (Nuessly and Hentz 2002). Accurate timing of insecticide treatments is critical to avoid yield or stand loss. Many varieties of sorghum are susceptible to YSA feeding (Starkes and Mirkes 1979).

Plant Resistance

According to Smith (2005), plant resistance to arthropods is “the sum of the constitutive, genetically inherited qualities that result in a plant of one cultivar or species being less damaged than a susceptible plant lacking these qualities.” Smith (2005) describes susceptibility as “the inability of a plant to inherit qualities that express resistance to arthropods.” Overall, resistance of a plant is measured on a relative scale based on the degree of resistance compared to the susceptible control plant that is more severely damaged under identical experimental conditions. Further, the measurement of resistance should also be based on a resistant control with a known, predetermined level of resistance. These relative measurements are essential as resistance is “influenced by environmental fluctuations occurring over both time and space” (Smith 2005).

The plant resistance umbrella can be divided into three mechanisms of resistance: (1) antibiosis, (2) antixenosis, and (3) tolerance. These three mechanisms of resistance were initially described by Painter (1951), then more precisely redefined by Horber (1980) as functional categories. Antibiosis is observed when “the negative effects of a resistant plant affect the biology of an arthropod attempting to use that plant as a host” (Smith 2005). The effects of an antibiotic plant may range from mild to lethal and is the result of either chemical or morphological plant defenses. Lethal, acute effects often affect the larvae and eggs, while chronic effects can lead to mortality affecting older larvae and pre-pupae, which may fail to pupate (Smith 2005). Those (individuals) that survive the effects of antibiosis will often see reduced body size and biomass, reduced fecundity, and a prolonged developmental period in the immature stages (Smith 2005). Antixenosis, as described by Painter (1951), denotes “the presence of morphological or chemical plant factors that adversely alter arthropod behavior.” As a result, the arthropod may seek out an alternate host plant. Some plant characteristics that may adversely alter the arthropod’s behavior include thickened plant epidermal layers, waxy deposits on the leaves, deterrent compounds, or a change in trichome density on the leaf surface. Both antibiosis and antixenosis can impose selection pressure on arthropod pests, which could result in biotype development.

Biotypes can be defined as “populations within an arthropod species that differ in their ability to utilize a particular trait in a specific plant genotype” (Wilhoit 1992, Smith 2005). Although there is no agreed upon definition in the scientific

literature, there are currently more than 20 GB biotypes recognized, primarily for their ability to overcome different sources of plant resistance and their ability to utilize different cereal host plants (Nuessly et al. 2008, Bouktila et al. 2012). No recognized biotypes have been reported for the YSA (Hoelscher et al. 1997).

According to Smith (2005), tolerance is defined “by the ability to withstand or recover from damage caused by arthropod populations equal to those found on susceptible cultivars.” Observations have shown that tolerant plants produce a greater amount of biomass than susceptible cultivars under equivalent insect pressure (Smith 2005). Furthermore, there are six primary factors associated with plants possessing tolerance. These include (1) increased net photosynthetic rate, (2) high relative growth rate, (3) increased branching/tillering after apical dominance release, (4) pre-existing high levels of carbon found in the root system, (5) the ability to transfer stored carbon from the roots to the shoots, and (6) increased oxidative enzyme activity (Gawrońska and Kiełkiewicz 1999, Strauss and Agrawal 1999, Heng-Moss et al. 2004, Smith 2005, Franzen et al. 2007). Unlike the other two forms of plant resistance, tolerance has no direct impact on the insect’s development. As a result, tolerance imposes minimal, if any, selection pressure on the insect allowing the pest to remain avirulent (likely) to the plant resistant genes (Smith 2005).

A series of no-choice and choice studies documented the categories of switchgrass resistance to GBs and YSAs (Koch et al. 2014b). Two no-choice experiments were performed to determine antibiotic and tolerant responses and to determine relative levels of resistance among three switchgrass populations

(K-lowland ecotype, S-upland ecotype, and KxS) when challenged with GB or YSA. Koch et al. (2014b) found that the lowland ecotype K possessed resistance to both aphid species based on aphid survival, showing high levels of antibiosis. Cultivar KxS was observed to possess low-to-moderate levels of antibiosis to YSA, while plant loss indices indicated tolerance to be an important category when challenged with GB in cultivar S (Koch et al. 2014a). Finally, experimental results showed that the cultivar KxS lacked tolerance and antibiotic characteristics to GB, whereas, the cultivar S lacked tolerance and antibiotic characteristics to YSA (Koch et al. 2014b). Choice studies were used to evaluate the preference of GB and YSA on selected switchgrass cultivars. These studies documented a lack of antixenosis, with no preference by YSA for any of the switchgrasses evaluated (Koch et al. 2014c). On the other hand, at 24 hours after GB introduction, a preference for the switchgrass cultivar KxS was observed (Koch et al. 2014c).

Plant Response to Aphid Herbivory

Plant defenses in response to insect herbivory can be reflected in physical, biochemical, molecular, and physiological attributes and may involve hundreds of genes (molecular/transcript response) that are triggered by cellular disruption caused by the insect's mouthparts or toxins found in the insect's saliva (Miles 1999, Smith and Boyko 2007). These plant defenses can also be triggered by female oviposition or other cues. The specific defensive pathways and transcripts induced depend on the specific interaction between a particular insect and its host plant. Differential expression of genes between plants, both

susceptible (SUS) and resistant (RES), have been shown to include an oxidative burst, which serves as an early defense response involving the production of reactive oxygen species (ROS) as well as plant signaling and defense, and cell maintenance to piercing/sucking insects (Zhang et al. 2004, Park et al. 2005, Boyko et al. 2006, Gutsche et al. 2009a, Ramm 2014). Some ROS, such as H_2O_2, initially serve as an early signaling molecule, but if ROS accumulate and are not detoxified, they can result in plant injury and potentially cell death. Such scenarios have been observed in several plants such as barley, buffalograss, soybean, switchgrass, and wheat infested with aphids (Mittler et al. 1999, Kawano 2003, Apel and Hirt 2004, Heng-Moss et al. 2004, Kotchoni and Gachomo 2006, Franzen et al. 2007, Dowd and Johnson 2009, Gutsche et al. 2009a, Prasifka et al. 2009a, Gill and Tuteja 2010, Liu et al. 2010, Pierson et al. 2010a, Prochaska et al. 2015, Donze-Reiner *unpublished*).

Several examples of increased levels of oxidative enzymes in response to insect feeding have been reported in the literature. These include enzymes such as catalase (CAT), peroxidase (POX), lipoxygenase, superoxide dismutase, and polyphenol oxidase (Felton et al. 1994, Constabel et al. 2000, Chaman et al. 2001, Heng-Moss et al. 2004, Prochaska et al. 2015). Several defensive strategies, especially those that can distinguish SUS and RES (tolerant) plants have been studied.

Studies to understand plant tolerance have shown that several aspects of plant metabolism suggest the need to simultaneously change to overcome the negative effects of nutrient removal by piercing/sucking insects (and other

arthropods). Additionally, tolerant plants are able to compensate photosynthetically by avoiding feedback inhibition that occurs from insect feeding on leaves (Mabry and Wayne 1997, Strauss and Agrawal 1999, Franzen et al. 2007, Gutsche et al. 2009b). It has also been documented that tolerant plants are able to withstand greater levels of cellular oxidative stress through the up-regulation of enzymes (e.g., POX and CAT) that can efficiently reduce the concentrations of ROS (Heng-Moss et al. 2004, Garg and Manchanda 2009, Gutsche et al. 2009a, Gill and Tuteja 2010, Ramm 2014). Current research has shown that some tolerant plants have a higher basal level of detoxifying enzymes and proteins (verses SUS plants), thus reducing the plant's ability to protect itself from high ROS accumulations (Vleeshouwers et al. 2000, Ramm et al. 2013). Potentially, this may allow a tolerant plant to utilize a greater portion of available resources for growth rather than initiating a defensive response. However, plant defense responses can arise from multiple sources (Smith 2005, Kim et al. 2008), and tolerance can result from various combinations of these mechanisms. Modulations in photosynthesis, cellular redox control, and maintaining growth appear to be important attributes found within RES (tolerant) plant systems (Gawrońska and Kiełkiewicz 1999, Strauss and Agrawal 1999, Heng-Moss et al. 2004, Smith 2005, Franzen et al. 2007).

Photosynthetic studies on soybean have found physiological differences between aphid-infested and aphid-free plants. These have included photosynthetic rate reductions (Macedo et al. 2003) and changes in several variables associated with photosynthesis including photosynthetic capacity

(A_{max}), the regeneration of ribulose-1,5-bisphosphate, RuBP (J_{max}), the maximum rate of rubisco-mediated carboxylation (V_{Cmax}), gas exchange rates, and photosynthesis curves (Pierson et al. 2010a, 2010b).

The A_{max} is the net CO_2 assimilation at saturating intercellular CO_2 (Caemmerer and Farquhar 1981, Farquhar and Sharkey 1982). Lower A_{max} values suggest aphid herbivory is negatively impacting photosynthetic responses. J_{max}, the ability of a plant to regenerate RuBP, is calculated using the saturated point of the AC_i curve (assimilation rate (A) plotted against intercellular CO_2 concentation (C_i); Caemmerer and Farquhar 1981, Farquhar and Sharkey 1982, Wullschleger 1993). Where values are lower in the infested plants compared to the control plants, the infested plants may express a decreased ability to regenerate RuBP. Pierson et al. (2010a) documented that tolerant soybean aphid-infested soybean plants of variety KS4202 had higher J_{max} values as compared to the SUS infested Asgrow plants. Overall, these results indicated an increased ability to regenerate RuBP in the tolerant soybean, perhaps as a mechanism to compensate for soybean aphid feeding. The V_{Cmax} is computed using data points at the lower/linear end of the photosynthesis curve and the maximum rate of rubisco-mediated carboxylation (Caemmerer and Farquhar 1981, Farquhar and Sharkey 1982, Manter and Kerrigan 2004). Lower V_{Cmax} values may suggest impaired rubisco activity within a plant. No differences were observed in control and aphid-infested treatments among V_{Cmax} values due to soybean aphid herbivory.

Franzen et al. (2007) reported similar characteristics when looking at

physiological responses of RES (Halt and Prairie Red) and SUS (TAM 107) wheat to injury by RWA. Observations from this study indicated that RES plants subjected to RWA feeding were able to maintain or compensate for aphid injury by altering leaf senescence pathways, while SUS plants appeared to have an accelerated senescence pattern. Other studies on wheat, suggested that antibiosis in plants comes with a cost; reduced photosynthetic capacity for defense and photosynthetic compensation in the tolerant line when exposed to aphid feeding (Haile et al. 1999).

Physiological studies by Macedo et al. (2009), using 4 strains of wheat and two different aphids, RWA and BCOA, indicated that visible symptoms of injury associated with aphid feeding can be highly species specific, and that photosynthetic reduction is a common physiological response in wheat to aphid herbivory.

Similar results were also found in the physiological responses of barley to RWA (Gutsche et al. 2009b). AC_i curves showed RWA feeding negatively affected the photosynthetic capacity of both cultivars (RES ‘sidney’ and SUS ‘otis’), although a more negative impact was found in the SUS genotype. Differences observed in carbon assimilation curves between control and infested plants showed that RWA herbivory affected the dark reaction, specifically the rubisco activity and RuBP regeneration. Overall, these studies indicated that the resistance mechanisms found within the RES barley genotype may be connected to the plant’s ability to maintain or elevate RuBP and Rubisco activity.

A number of studies have reported increases among oxidative enzymes

such as lipoxygenase, superoxide dismutase, CAT in response to insect feeding (Constabel et al. 2000, Chaman et al. 2001, Hiraga et al. 2001, Ramm et al. 2013). Ni et al. (2001), studied biochemical changes in RES and SUS cereals in response to infestation by BCOA and RWA. In this study, BCOA herbivory did not elicit any changes of POX activity among the cereals (wheat, barley, oat). Previous studies by Riedell and Kieckhefer (1995) concluded BCOA was asymptomatic. However, RWA herbivory resulted in increased POX activity in the RES Halt wheat and the SUS Morex barley. Overall, RWA herbivory led to a nine-fold increase in POX activity on Morex barley and three-fold on Halt wheat 9 days after infestation (DAI) when compared to uninfested control plants. These findings suggest that RWA feeding likely leads to oxidative stress in plants. Moderate increases of POX activity were found in the RES Halt when compared with SUS Arapahoe wheat. Differential expression of these enzymes may contribute to the response found in wheat, barley, and oats to RWA and BCOA feeding, suggesting these cereals have different mechanisms of aphid resistance. Franzen et al. (2007) documented similar results for POX activity between infested and control plants among three cultivars in wheat (RES ‘Halt’ and ‘Prairie Red’ and SUS ‘TAM 107’). RWA feeding resulted in the up-regulation of POX activity in RES cultivars, but not in SUS plants. Overall, Franzen et al. (2007) results compare favorably with the studies performed by Pierson et al. (2010a), indicating increased photosynthetic capacity and increased POX activity in plants found to be RES, and decreased rates in the more SUS genotypes.

Ramm et al. (2013) investigated buffalograss-chinch bug interactions in

two cultivars of buffalograss, Prestige (tolerant) and ‘378’ (SUS), and found changes in POX activity when challenged by herbivores (Heng-Moss et al. 2002, 2003, 2004). These studies confirmed observations by Heng-Moss et al. (2004) and Gulsen et al. (2010) who observed increased levels of POX activity and a loss of CAT activity when plants were challenged with chinch bugs. Overall, their findings suggested that increased POX levels in the tolerant buffalograss may help the plant detoxify ROS, such as H_2O_2 that accumulate within plants as a result of stress.

To more effectively query global plant responses to aphid feeding, it is possible to utilize microarrays and next generation sequencing (NGS) technology. Microarrays have been regularly used to study plant responses to insect herbivory (Reymond et al. 2000, Voelckel et al. 2004, Park et al. 2005, Smith and Boyko 2007, Li et al. 2008, Gutsche et al. 2009a).

Studies performed by Couldridge et al. (2007) found transcriptional changes as a result of green peach aphid, *Myzus persicae* (Sulzer), feeding on *Arabidopisis*. These studies reported several defense-related genes to be differentially expressed in response to aphid feeding. Cytochrome P450s were induced; consistent with a plant defense response to aphid stylet penetration activity. A gene coding for an esterase family protein was up-regulated two hours post-infestation. Esterases often play a role in detoxification. Significant increases in esterase activity have been reported in barley in response to RWA herbivory. This was hypothesized by Ni and Quisenberry (2003) to be related to toxic and oxidative stress inflicted by the aphid feeding.

Gutsche et al. (2009a) reported 909 differentially expressed genes in tolerant barley in response to RWA feeding. Of the 909 differentially expressed genes, many were assigned to specific metabolic categories, including several associated with plant defense and scavengers of ROS. Two POX genes (HvPRXA1 and HvPRXA2) were up-regulated in response to RWA feeding on tolerant barley plants, indicating that specific POXs could be important for the tolerance in barley. These findings by Gutsche et al. (2009a) provided evidence that the ability to elevate and sustain levels of ROS-scavenging enzymes, like POXs, could play an important role in the tolerant response in barley and potentially other plant systems.

NGS performed (Prochaska et al. 2015) on tolerant (KS4202) and SUS (K03-4686) soybeans (Chandran 2011) found significant genotypic differences when transcriptomes from aphid uninfested plants (day 0 tolerant vs. day 0 SUS) were compared. Select genes have been implicated in plant defense responses to abiotic and biotic stresses (Heng-Moss et al. 2004, Gutsche et al. 2009a, Pierson et al. 2010a, Studham and MacIntosh 2012, Ramm et al. 2013, Prochaska et al. 2015). These results indicate that tolerant/RES plants may already be predisposed to tolerate insect feeding. Similar trends with regard to constitutive resistance have also been documented in wheat, barely, and buffalograss (Delp et al. 2008, Han et al. 2009; Ramm et al. 2013).

Overall, observations among a variety of plant systems showcase some interesting trends between RES and SUS plants when exposed to phloem-feeding insects. Physiologically, several studies have shown that resistance

mechanisms found within tolerant plants (wheat, barely, soybean) may be connected to the ability of these plants to maintain or elevate RuBP and rubisco activity when challenged by insect pests. Similarly, studies documenting POX and CAT activity have shown similar patterns of increased levels of these oxidative enzymes within more RES plants. NGS technology confirmed these findings between these two oxidative enzymes along with several other defense related transcripts within *Arabidopsis*, buffalograss, and soybean. Furthermore, research has shown constitutive resistance may be in play in RES plants (soybean and buffalograss) as several defensive related genes were found to be up-regulated, even before the onset of insect herbivory. However, defense related genes were not up-regulated until after the onset of insect herbivory in SUS plants. Overall, these trends may provide a starting point documenting the response system associated with bioenergy feedstock-insect interactions.

CHAPTER 2:

PHOTOSYNTHETIC RESPONSES OF SELECT SWITCHGRASS, *PANICUM VIRGATUM* L., CULTIVARS TO GREENBUG, *SCHIZAPHIS GRAMINUM* (RONDANI), HERBIVORY

Introduction

Switchgrass, *Panicum virgatum* L., is a perennial, polyploid, warm-season grass that is native to the tallgrass prairies throughout North America, east of the Rocky Mountain region (Vogel 2004, Mitchell et al. 2008, 2012). Based on chloroplast markers, switchgrass can be differentiated into lowland and upland ecotypes (Hultquist et al. 1997, Young et al. 2012). Generally, lowland ecotypes are coarser, taller, and have adapted for better growth in lowland areas that maybe exposed to flooding (Vogel 2004). Upland ecotypes are typically associated with drier environments, and tend to have smaller leaves and lower biomass yields. They are more suitable for cattle grazing (Vogel 2004, Bartley et al. 2013).

Among the possible feedstocks, switchgrass has become a leading candidate for bioenergy use because of its low water and nutrient requirements, suitability for growth on marginal lands, high yield returns across diverse environments, positive environmental benefits, and compatibility with modern farming standards (Sanderson et al. 1996, 2004, McLaughlin et al. 1999).

Photosynthetic comparisons of upland and lowland switchgrass, under abiotic stress, indicated that two lowland ecotypes, Alamo and Kanlow (K), maintained superior photosynthetic rates under low nitrogen (N) regimes.

However, a lower photosynthetic response was found in low moisture conditions for upland ecotypes (Stroup et al. 2003). Overall, this study found lowland ecotypes were better able to adjust to environmental stressors, like drought and low N, with little change in plant productivity when compared to upland ecotypes.

Similar characteristics were observed by Barney et al. (2009) when lowland and upland ecotypes were exposed to high soil moisture. Of the four cultivars evaluated (lowland switchgrasses: Alamo and K; upland switchgrasses: Blackwell and Cave-In-Rock), net photosynthetic rates differed only in K, which had nearly a 30% higher rate when compared to the other three cultivars. Net photosynthetic rate and photosynthetic water use efficiency were 17% and 34% higher in lowland ecotypes over upland ecotypes. Although no other differences were found between the ecotypes, results indicated that lowland ecotypes typically outperformed upland ecotypes (Barney et al. 2009). Similar results have been reported in comparisons of other upland and lowland plants (Gesch and Johnson 2010).

Changes in photosynthetic rates or compensation under insect infestation has been reported in many systems (Ryan et al. 1987, Baldwin and Preston 1999, Haile et al. 1999, Heng-Moss et al. 2006, Franzen et al. 2007, Gutsche et al. 2009a, Pierson et al. 2010a), and appear to be among the first set of plant responses impacted, especially to phloem feeding arthropods (Gutsche et al. 2009a, Haile et al. 1999, Franzen et al. 2007, Pierson et al. 2010a). Additionally, resistant (RES) and susceptible (SUS) plants can exhibit a differential modulation of photosynthesis in response to feeding by piercing-sucking insects (Miller et al.

1994, Haile et al. 1999, Franzen et al. 2007, Smith and Boyko 2007, Gutsche et al. 2009a, Pierson et al. 2010a).

Recent studies with switchgrass have shown that upland, lowland and a hybrid between upland x lowland (KxS) plants can serve as hosts for greenbugs, *Schizaphis graminum* (Rondani) (GB) and the yellow sugarcane aphid, *Sipha flava* (Forbes), (YSA) (Koch et al. 2014a). The lowland cultivar K has resistance to both aphid species, showing high levels of antibiosis and antixenosis to GB (Koch et al. 2014b, 2014c). Cultivar Summer (S) plants were categorized as tolerant when challenged with GB, but lacked resistance to YSA. In contrast, KxS plants were found to possess low-to-moderate levels of antibiosis to YSA, but lacked tolerance and antibiosis to GB (Koch et al. 2014b). Plant productivity was also impacted differentially in these plant x aphid interactions (Koch et al. 2014a).

Differences in observed photosynthetic parameters, e.g., photosynthetic rates, photosynthetic capacity (Stroup et al. 2003, Barney et al. 2009, Gesch and Johnson 2010), and responses to aphid injury reported for different switchgrass strains suggest that upland and lowland plants could have unique physiological adaptations to aphid herbivory. In this study, potential physiological differences were investigated in a variety of switchgrass plants through a series of gas exchange studies. Additionally, several photosynthetic variables were evaluated to observe possible changes in photosynthetic capacity as a result of GB herbivory.

Materials and Methods

Plant material. Photosynthetic evaluations were conducted on three tetraploid switchgrasses: (1) Kanlow (K), a lowland-tetraploid switchgrass cultivar originally derived from plants collected near Wetumka, Oklahoma (Alderson and Sharp 1994, Vogel and Mitchell 2008); (2) Summer (S), an upland-tetraploid switchgrass cultivar, collected from plants near Nebraska City, Nebraska (Alderson and Sharp 1994, Vogel and Mitchell 2008); and (3) KxS, an experimental line derived by mating randomly selected Kanlow (male) x Summer (female) plants, which, hereafter is referred to as KxS, was developed by Dr. Kenneth Vogel (USDA-ARS, Lincoln, NE) (Martinez-Reyna and Vogel 2008, Vogel and Mitchell 2008).

Experimental Studies. Two gas exchange studies were conducted. The first examined the differences among three plants randomly selected from three different switchgrass populations (K, S, and KxS) to observe basal (aphid-free) differences in gas exchange responses among these populations. Additionally, three plants from each population that had been previously categorized as SUS or RES to GB (Koch et al. 2014a) were examined for differences in photosynthetic parameters in an aphid-free state. Both AC_i curves (assimilation rate (A) versus intercellular CO_2 concentration [C_i]) and survey measurements were taken. In study 2, three plants from each of the three switchgrass cultivars previously identified as RES or SUS to GB (Koch et al. 2014a) were tested (e.g., gas exchange, AC_i curves) to observe photosynthetic parameters in the absence (control; aphid free) and in the presence of aphids. These experiments are described in greater detail below.

Plant Growth. Plants were grown in SC-10 Super Cell Single Cell Cone-tainers (3.8 cm diameter by 21 cm deep; Stuewe & Sons, Inc., Corvallis, OR) containing a Fafard Growing Media (Mix No. 3B; Conrad Fafard, Awawam, MA). Cone-tainers were maintained under greenhouse conditions at 25 ± 7°C with light supplemented by a 400-W metal halide lamps to produce a 16:8 hour (L:D) photoperiod. Upon emergence, plants were thinned down to one plant per cone-tainer. Plants were fertilized twice a month with a soluble (20:10:20 N-P-K) fertilizer. For study 2, plants were clonally propagated from ramets.

Insect colonies and infestation. GB (biotype I) were obtained from Dr. John D. Burd, USDA-ARS in Stillwater, OK. The colony was maintained on a constant supply of the SUS sorghum cultivar ‘BCK60’ plants maintained in a plant growth chamber at a temperature of 25 ± 2°C and a 16:8 hour (L:D) photoperiod.

For study 2, switchgrass plants were infested with five apterous, adult aphids with a fine paintbrush and caged using a tubular plastic cage (4 cm diameter by 46 cm in height) with vents covered in organdy fabric to confine aphids to their respective plants. Uninfested (control) plants were maintained similarly. Infested and control uninfested plants (caged, with no aphids) were maintained in a greenhouse at 25 ± 7°C with light and a 16:8 hour (L:D) photoperiod. Aphid numbers were determined daily for 8 days. Visual damage ratings were also recorded daily using a 1-5 scale. Overall, damage ratings served as a visual evaluation of injury resulting from GB herbivory (Smith et al. 1993). The damage rating scale used was adopted by Heng-Moss et al. (2002)

and refined by Koch et al. (2014a) where a damage rating of 1 = 10% or less of the total leaf area damaged, 2 = 11-30% of leaf area damaged, 3 = 31-50% of leaf area damaged, 4 = 51-70% of leaf area damaged, and 5 = 71% or more of leaf area damaged with the plant approaching death from the result of GB herbivory. Eight days following aphid introduction, aphids were removed with a fine bristle paintbrush from infested plants and photosynthetic parameters (e.g., survey measurements and AC_i curves) using the LiCor 6400 photosynthesis system were recorded for control and infested plants.

Gas exchange measurements.

Gas Exchange Parameters. Plants selected for photosynthetic survey measurements and AC_i curves were moved outdoors each morning (approximately 6:30 am) and allowed to adapt to the new light and temperature conditions for a minimum of 90 minutes. Measurements were taken from the upper-most fully expanded (collared) leaf from three replications. Survey measurements were taken at the 400 µM intercellular CO_2 concentration and 1800 µmol photons $m^{-2}s^{-1}$ light intensity using the portable LI-6400 photosynthesis system (Li-Cor Biosciences, Lincoln, NE) with a CO_2 injector and light source (for making measurements at stable light and CO_2 concentrations). For studies 1 and 2, survey measurements were performed on three biological replications from each treatment combination. Gas exchange rates within each treatment were analyzed using mixed model analysis (PROC MIXED, SAS

Institute 2011). Where appropriate, means were separated using Fisher's least significant differences (LSD).

AC_i curves were performed using the LI-6400 to gain a better understanding of the basal gas exchange responses among the three switchgrass populations and document the effect of GB herbivory on plant gas exchange. Rates were measured at 1800 µmol photons $m^{-2}s^{-1}$ light intensity and reference IRGA CO_2 concentrations ranging from 100 to 1000 ppm. Automated programs of the LI-6400 were used to gather data for each AC_i curve.

As previously described (Farquhar and Sharkey 1982), stomatal and non-stomatal components of photosynthesis were calculated. Stomatal limitation can be determined by comparing A at a C_i of 400 µL L^{-1} CO_2 to A at the C_i corresponding to a C_a (Intracellular CO_2) of 400 µL L^{-1} CO_2 (Ryan et al. 1987) where C_a is the CO_2 concentration outside of the leaf (e.g., CO_2 concentrations within the leaf chamber). The following equation describes stomatal limitations (SL) is as follows:

$SL = A(C_i = 400\ \mu L\ L^{-1}) – A(C_a = 400\ \mu L\ L^{-1}) / A(C_i = 400\ \mu L\ L^{-1})$

The AC_i curve was used to calculate carboxylation efficiency (CE, the slope from the linear portion of the AC_i curve) and changes in net CO_2 assimilation at saturating C_i (A_{max}). Through further analysis of the AC_i response curve, the maximum carboxylation velocity of rubisco (V_{cmax} – derived from the linear portion of the curve, µmol CO_2 $m^{-2}s^{-1}$) and the maximum potential rate of electron transport contributing to ribulose-1,5-biphosphate (RuBP) regeneration (J_{max} – µmol electrons $m^{-2}s^{-1}$) were determined. Values were calculated using the

estimation utility developed by Sharkey et al. (2007). For each treatment, response curves from three biological replicates were measured to calculate Amax, Vcmax, and Jmax for each curve.

Results

Plant damage.

Significant differences were observed for plant damage between treatments within two of the three switchgrass populations. Overall, S (control vs. infested) had a mean damage rating of 2.25 ± 0.17 (P=0.0005) and KxS (Control vs. Infested) had a mean damage rating of 2.00 ± 0.52 (P=0.0042). However, no significant differences were detected for the K infested vs. K aphid-free treatments (P=0.1318; control damage: 1.00 ± 0.00; infested damage: 1.33 ± 0.17). No differences were found among the switchgrass populations (P<0.05); however, trends indicated S and KxS may be more damage prone when compared to K.

All three populations were found to surpass 600 cumulative aphid days (CADs) 8 days after aphid introduction. K accumulated 627.8 ± 76.4 aphid-days, KxS accumulated 687.9 ± 71.6 aphid-days, while S accumulated 971.5 ± 272.1 aphid-days.

CO_2 assimilation rates and AC_i Curves.

To better understand population-specific differences in assimilation rates, three plants from each of the three switchgrass populations and plants that had

been identified as either SUS or RES to GB from each population were evaluated.

Significant differences were found for CO_2 assimilation rates at the population level. Plants from the lowland population (K) had the highest net assimilation rates of 16.2 ± 0.7 μmoles CO_2 m^{-2} s^{-1} at a fixed CO_2 level of 400 μmoles (P=0.0012). This value was ~30 % greater and significantly different than those observed for plants from the upland (S) and hybrid KxS populations (Table 1). AC_i curves were also generated to better understand the overall trends of CO_2 assimilation over a range of CO_2 levels. Again, plants from the lowland population (K) had the highest assimilation rates at each CO_2 level, respectively (Figure 1).

To better understand if there were CO_2 assimilation rate differences between RES and SUS plants from the three populations, plants that had been previously identified as either SUS or RES to GB from each population were evaluated. No significant differences were found for CO_2 assimilation rates at the population level, when comparing GB RES and GB SUS plants at a fixed CO_2 level of 400 μmoles (Table 2).

Plants from the lowland population K (Figure 2) and the hybrid population (KxS; Figure 3) showed trends suggesting higher assimilation rates at each CO_2 level, between the RES and SUS plants in an aphid-free state. In both instances, RES plants appeared to better assimilate CO_2 over their SUS counterparts. No changes in CO_2 assimilation rates were observed between RES and SUS plants from S (Figure 4).

When challenged by GB, no significant differences in survey measurements by treatment were observed between aphid-infested and aphid-uninfested treatments from the populations K, KxS and S (data not shown). However, a preliminary study documented significant differences in assimilation rates between aphid-infested and aphid-control treatments for the population KxS when challenged by GB ($P<0.0001$; SAS Institute 2011).

GB herbivory decreased net CO_2 assimilation nearly 37 % to a value of 27.6 ± 2.2 μmoles CO_2 m^{-2} s^{-1} at a fixed CO_2 level of 400 μmoles (Table 3). No significant interactions were observed between aphid-infested and uninfested control plants randomly selected from the populations K and S (Table 3). However, CO_2 assimilation was enhanced by approximately 26 % in GB-infested Ka plants as compared to the uninfested controls. These data suggest that K plants could be compensating photosynthetically when challenged by GB. Although not statistically significant, a 16 % reduction in CO_2 assimilation was observed in S infested plants as compared to uninfested S control plants (Table 3).

AC_i curves were also developed to help better understand the overall trends of CO_2 assimilation over a range of CO_2 levels in GB-infested and uninfested control plants. Plants from the lowland population (K) had the highest assimilation rates at each CO_2 level, respectively, when compared with S and KxS (Figure 5). When AC_i curves were compared between aphid-infested and aphid-free control treatments, a net gain in CO_2 assimilation in infested K plants was observed (Figure 5). No discernable trends were observed between GB-

infested and uninfested KxS plants (Figure 6). Finally, no significant differences were found in the AC_i curves between the S-infested and -uninfested control plants, however, GB infestation appeared to increase CO_2 assimilation rates (Figure 7).

Carboxylation efficiency

No significant differences were observed for any of the switchgrass experiments in Study 1 (basal; $P<0.05$; SAS Institute 2011). Similar slopes were observed between the switchgrass basal AC_i curves and between RES-SUS treatments within each population (Figures 1-4). These results imply that carboxylation efficiency in aphid-infested plants were comparable to that of aphid-free plants (within their respective treatments), suggesting that rubisco activity was not different between RES and SUS plants. Study 2 experiments showed similar trends between aphid infested and aphid-free treatments within each population ($P<0.05$; Figures 5-7). However, there was a significant main effect of population ($P=0.0327$; S: 0.3713 ± 0.07 μmoles CO_2 m^{-2} s^{-1}, $P=0.0027$; KxS: 0.5383 ± 0.10 μmoles CO_2 m^{-2} s^{-1}, $P=0.0001$; K: 0.7912 ± 0.10 μmoles CO_2 m^{-2} s^{-1}, $P<0.0001$). This suggests that carboxylation efficiency is higher in the antibiotic population of K (Koch et al. 2014b) with a 2-fold increase over the population S.

Jmax

Significant differences were not detected for Jmax between GB-infested

and control plants among the three switchgrass populations, however, trends were observed in study 2. Infested K plants showed a 15.6 % increase in Jmax when compared to the control (Figure 5) plants of 104.0 ± 12.0 and 90.0 ± 11.8, respectively. Jmax values for aphid-infested KxS plants (82.3 ± 14.9; Figure 6) were found to be similar to the control plants (87.3 ± 33.8). Finally, Jmax values for S infested plants (80.3 ± 19.3; Figure 7) were increased relative to the S control plants (50.0 ± 12.1). Although not significant, Jmax values in the infested-K and -S plants were higher than values in the control plants, which may suggest an increased ability, albeit slight, to regenerate RuBP to compensate for aphid feeding. However, due to variation within the observations, further investigation is needed. For infested KxS plants, lower Jmax values were observed in GB-infested plants as compared to the uninfested control plants, suggesting that aphid-infested KxS plants may have a decreased ability to regenerate RuBP.

Amax

There were no differences between Amax values of the infested and control plants for each population; however, trends were present. There was a 28% increase in Amax values when comparing the infested K plants (18.7 ± 2.6; Figure 5) to control plants (14.6 ± 2.1). Amax values were also found to be increased in S infested (14.7 ± 3.0) when compared to the control treatment (9.5 ± 1.7; Figure 7). No differences were observed between the infested and control KxS plants.

Vcmax

No differences were found for Vcmax measurements between treatments, infested and control plants, among any switchgrass population, however, trends were noticed. Infested K and S plants had a small increase in Vcmax when compared to the control plants (Figure 5 and 7).

Stomatal limitation

No significant differences were observed in stomatal limitation between switchgrass populations or treatments (Studies 1 and 2; data not shown).

Discussion

Overall, basal AC_i curves between the three switchgrass populations of K, KxS, and S displayed some interesting trends. K and KxS plants had superior net CO_2 assimilation rates when compared to plants from the cultivar S. These data support other studies that have shown that lowland ecotypes (K) can photosynthetically outperform upland ecotypes. This study represents the first report on photosynthetic responses of KxS to aphid herbivory.

Differences in carbon dioxide assimilation rates among the three populations were not as pronounced, when compared to the population specific data, for plants that had been previously selected as RES or SUS to GB herbivory (Koch et al. 2014a) and clonally propagated in the greenhouse. It remains unclear if cultural practices and differences in environmental conditions at the time measurements were taken influenced the results.

Overall, the AC_i curves indicated reduced photosynthetic capacity in S and

increased photosynthetic capacity in K. This was a commonality found among all studies. Reduced Amax values could negatively impact the overall process of photosynthesis; however, Amax values were not significantly different. On the other hand, lowered Jmax in GB-infested KxS plants could indicate a small decrease to regenerate RuBP as a compensatory mechanism during aphid feeding. Vcmax and carboxylation efficiency were similar in all studies for K and S. However, KxS plants had lower Vcmax values when challenged by aphid feeding. This may indicate that rubisco activity is negatively impacted due to aphid herbivory, but variation among observations suggest that further clarifying experiments may be needed. No differences were found among Amax and Jmax statistics in populations K and S.

Photosynthetic studies performed on several other plant species have found physiological differences between aphid-infested and aphid-free plants. These have ranged from reductions in net assimilation rates (Macedo et al. 2003) to other compensatory mechanisms, especially in plants with demonstrated tolerance to a specific aphid species (Franzen et al. 2007, Gutsche et al. 2009a, Pierson et al. 2010a). Collectively, these studies indicate that compensatory mechanisms found in tolerant or RES genotypes may be connected to the ability to maintain or elevate RuBP and rubisco activity.

Haile et al. (1999), predicted that plant physiological responses, like that of photosynthesis, could contribute to plant tolerance to RWA injury. These studies indicated that aphid-injured wheat seedlings had lower light-saturation points, which suggest less efficient use of light energy compared with control seedlings.

Once aphids were removed, aphid injury was shown to reduce photosynthetic rates in all lines, with the antibiotic cultivar showing the lowest photosynthetic rate overall. Three days following aphid removal, the tolerant wheat line began to recover, with full photosynthetic recovery seven days following aphid removal. This same response was not observed in the SUS and antibiotic lines. This study indicates that tolerant plants compensated photosynthetically to arthropod injury. Haile et al. (1999) hypothesized that antibiosis can come at a cost, with reduced photosynthetic capacity for physiological defense. However, our studies indicate that GB herbivory does not induce the same fitness costs on antibiotic K in switchgrass as suggested by increased by photosynthetic capacity.

Conclusions

These studies suggest that the antibiotic cultivar K has mechanisms similar to those observed in tolerant soybean, wheat and barley plants with changes in photosynthetic rates occurring in response to aphid feeding (Franzen et al. 2007, Gutsche et al. 2009a, Pierson et al. 2010a). It is possible that in switchgrass, antibiosis may not induce the same fitness costs observed in wheat (Haile et al. 1999). This could be important in understanding the overall plant defense mechanisms of cultivar K. Furthermore, switchgrass S does not appear to efficiently alter photosynthetic rates or gas exchange rates in response to GB herbivory. Additional studies are needed to determine the degree to which RuBP regeneration and rubisco activity are affected by aphid injury in the defense response of these switchgrass populations and within their respective genotypes.

The relative contributions of the parental population in the defense responses of the hybrid also needs to be studied at greater depth. A cultivar derived originally from KxS hybrids has recently been released (Vogel et al. 2014).

Table 1. Survey measurements of carbon dioxide assimilation for aphid-free switchgrass plants including K, KxS, and S using a constant 400 µM intercellular CO_2 concentration and 1800 µmol photons $m^{-2}s^{-1}$ light intensity. Values are the means ± SE (n = 3)**.**

Cultivar	Mean ± SE	P-Value
Kanlow	16.15 ± 0.67	0.0012 a
K*S	11.28 ± 1.08	<0.0001 b
Summer	11.75 ± 1.36	<0.0001 b

*Means significantly different at $P \leq 0.05$ by least significant difference

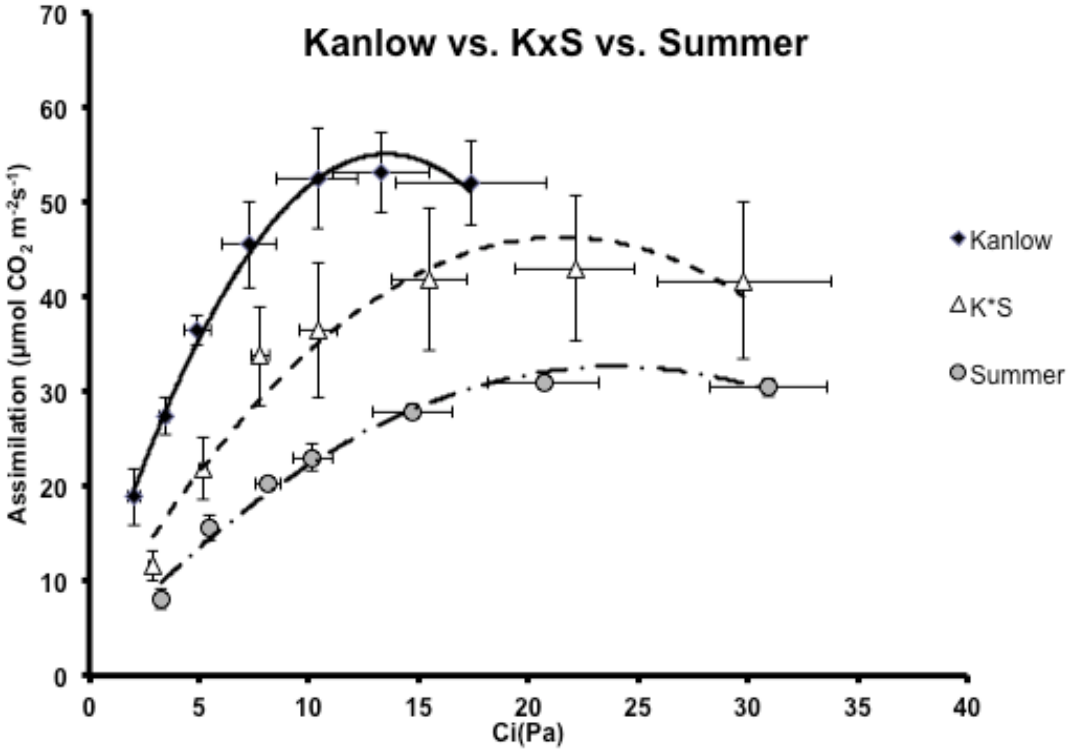

Figure 1. AC_i curves of aphid-free switchgrass plants including K, KxS, and S using a constant 1800 µmol photons $m^{-2}s^{-1}$ light intensity over a range of CO_2 levels (100, 200, 300, 400, 600, and 1000 µmoles). Values are the means ± SE (n = 3).

Table 2. Survey measurements of carbon dioxide assimilation for aphid-free switchgrass plants from RES and SUS genotypes of K, KxS, and S using a constant 400 µM intercellular CO_2 concentration and 1800 µmol photons $m^{-2}s^{-1}$ light intensity. Values are the means ± SE (n = 3).

Cultivar	Susceptible	Resistant	P-Value
Kanlow	9.40 ± 3.05	7.82 ± 1.71	0.67 a
K*S	9.30 ± 1.44	11.63 ± 3.33	0.54 a
Summer	10.47 ± 3.67	10.24 ± 1.19	0.95 a

*Means significantly different at $P \leq 0.05$ by least significant difference

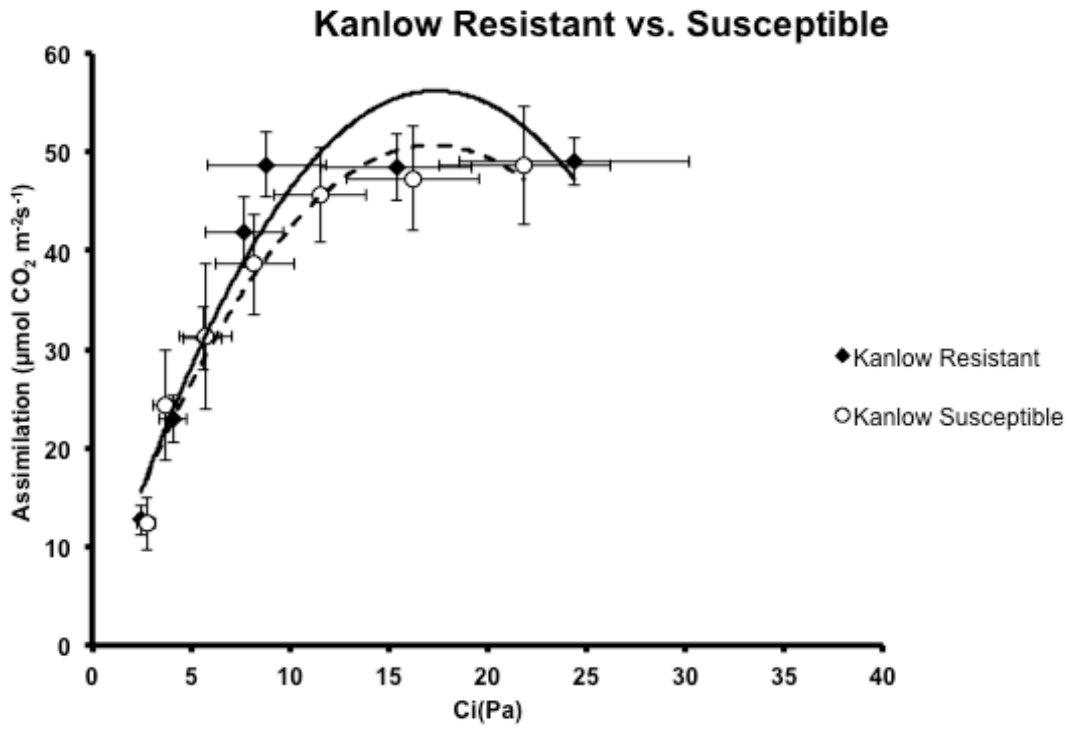

Figure 2. AC_i curves using RES and SUS genotypes of aphid-free K switchgrass using a constant 1800 µmol photons $m^{-2}s^{-1}$ light intensity over a range of CO_2 levels (100, 200, 300, 400, 600, and 1000 µmoles). Values are the means ± SE (n = 3).

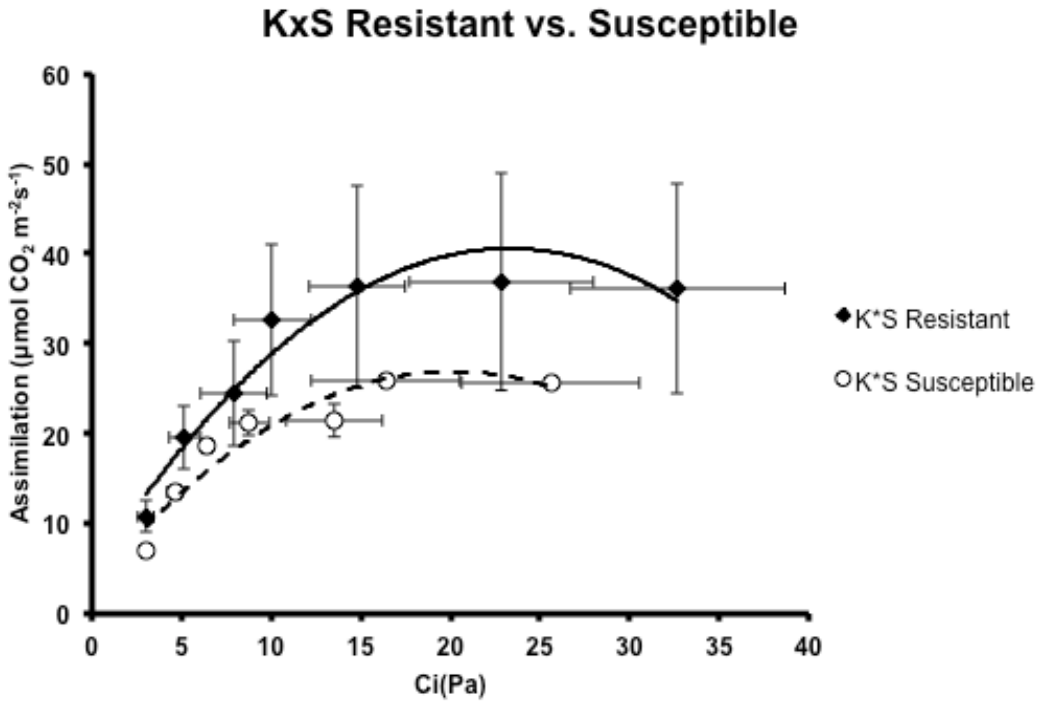

Figure 3. AC_i curves using RES and SUS genotypes of aphid-free KxS switchgrass using a constant 1800 µmol photons $m^{-2}s^{-1}$ light intensity over a range of CO_2 levels (100, 200, 300, 400, 600, and 1000 µmoles). Values are the means ± SE (n = 3).

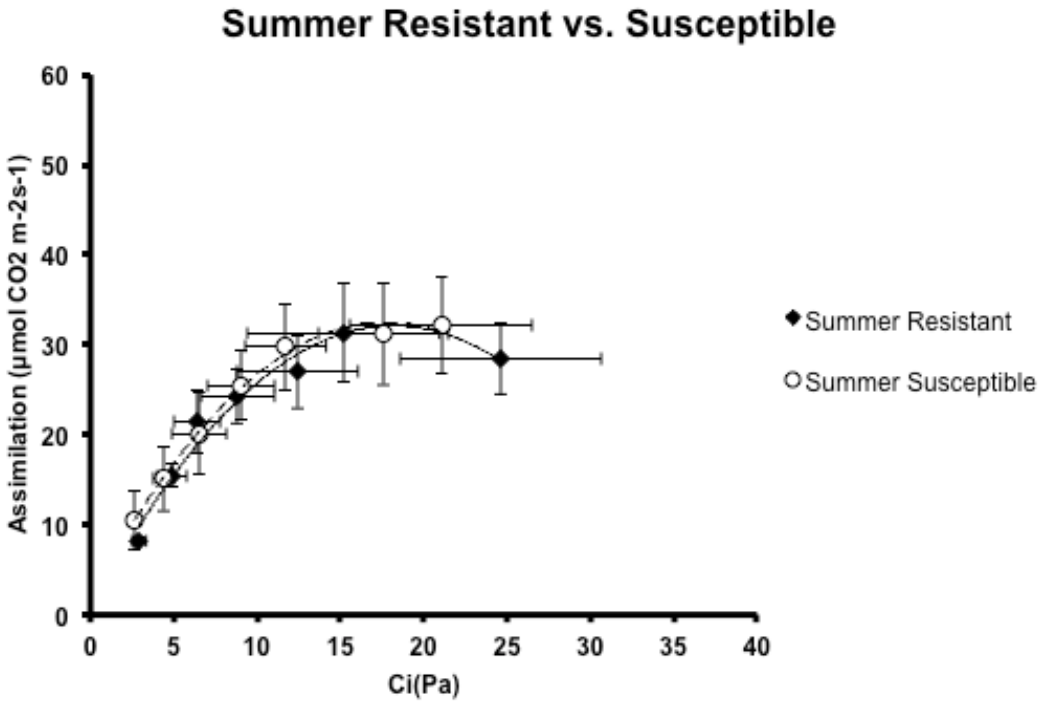

Figure 4. AC_i curves using RES and SUS genotypes of aphid-free S switchgrass using a constant 1800 µmol photons $m^{-2}s^{-1}$ light intensity over a range of CO_2 levels (100, 200, 300, 400, 600, and 1000 µmoles). Values are the means ± SE (n = 3).

Table 3. Survey measurements of carbon dioxide assimilation for aphid-infested and aphid-free (control) 8 DAI in switchgrass plants from K, KxS, and S using a constant 400 µM intercellular CO_2 concentration and 1800 µmol photons $m^{-2}s^{-1}$ light intensity. Values are the means ± SE (n = 3).

Cultivar	Control	Infested	% Reduction	P-Value
Kanlow	27.33 ± 0.30	34.30 ± 1.18	-25.50%	0.11 a
K*S	43.95 ± 4.39	27.58 ± 2.15	37.25%	<0.0001 b
Summer	27.20 ± 1.47	22.82 ± 1.68	16.10%	0.31 a

*Means significantly different at $P \leq 0.05$ by least significant difference

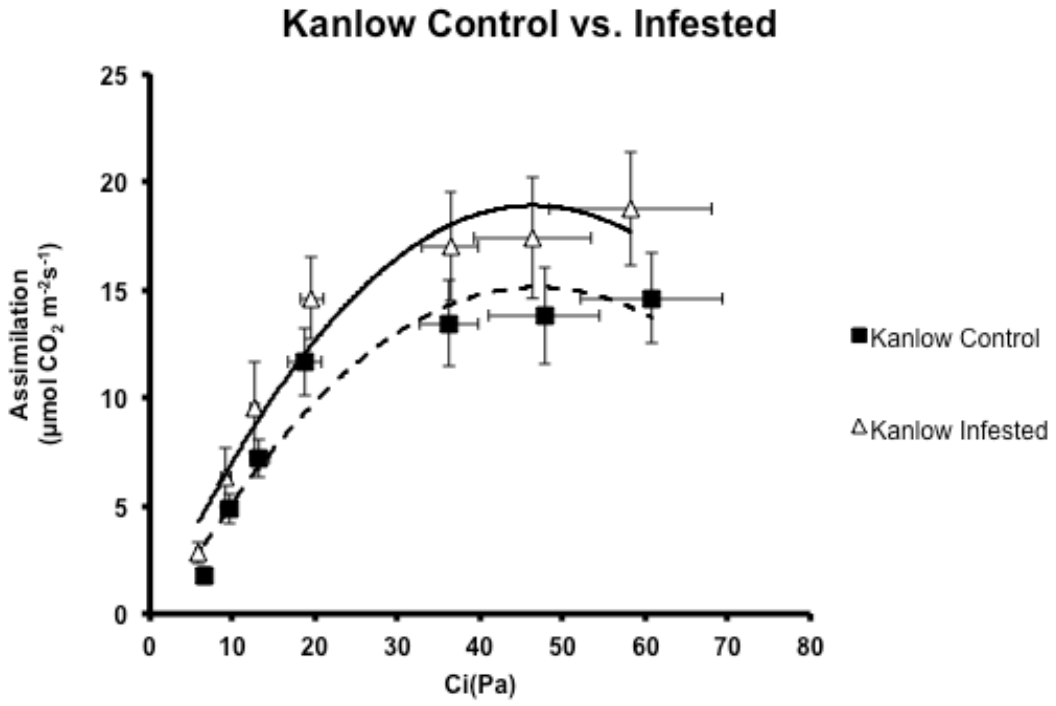

Figure 5. AC_i curves for aphid-infested and aphid-free (control) plants of K 8 DAI using a constant 1800 µmol photons $m^{-2}s^{-1}$ light intensity over a range of CO_2 levels (100, 200, 300, 400, 600, and 1000 µmoles). Values are the means ± SE (n = 3).

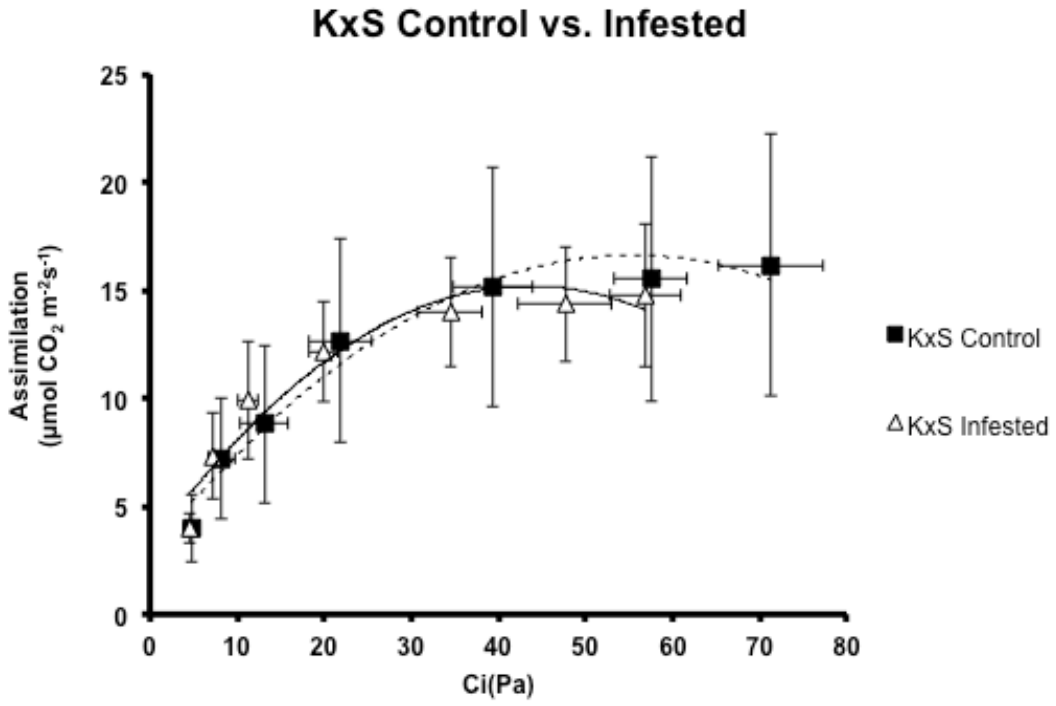

Figure 6. AC_i curves from aphid-infested and aphid-free (control) plants of switchgrass hybrid KxS 8 DAI using a constant 1800 µmol photons $m^{-2}s^{-1}$ light intensity over a range of CO_2 levels (100, 200, 300, 400, 600, and 1000 µmoles). Values are the means ± SE (n = 3).

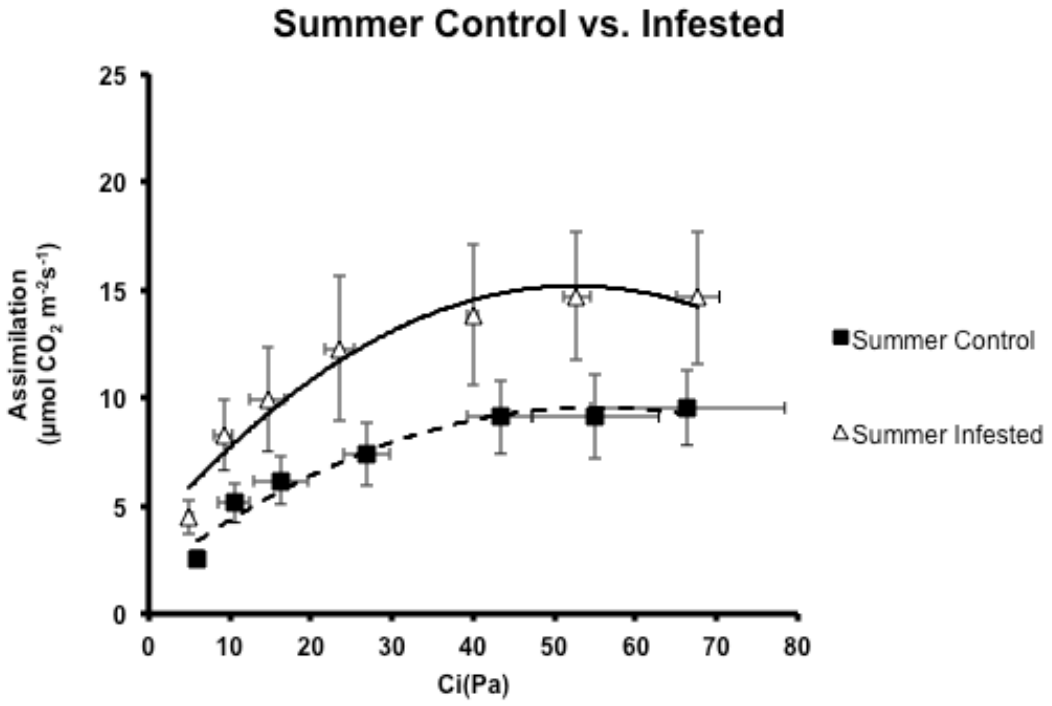

Figure 7. AC_i curves from switchgrass aphid-infested and aphid-free (control) plants of S 8 DAI using a constant 1800 µmol photons $m^{-2}s^{-1}$ light intensity over a range of CO_2 levels (100, 200, 300, 400, 600, and 1000 µmoles). Values are the means ± SE (n = 3).

CHAPTER 3:

BIOCHEMICAL RESPONSES OF SWITCHGRASS, *PANICUM VIRGATUM* L., TO APHID HERBIVORY

Introduction

Switchgrass (*Panicum virgatum* L) is a promising crop for the biofuel industry (Vogel et al. 2014; Vogel et al. 2011). It has several desirable characteristics including high biomass yields across a variety of diverse environments, positive environmental benefits, and is adapted to environments with minimal water and nutrient requirements (Sanderson et al. 1996, 2004, McLaughlin et al. 1999).

However, recent research has shown that switchgrass can be a suitable host for diverse array of insect pests including aphids (Dowd and Johnson 2009, Prasifka et al. 2009a, Nabity et al. 2011, Dowd et al. 2012, Koch et al. 2014a, 2014b, 2014c) . Studies by Koch et al. (2014b, 2014c) have shown that switchgrass plants have different categories of resistance to two aphid species, greenbugs (GB), *Schizaphis graminum* (Rondani), and yellow sugarcane aphids (YSA), *Sipha flava* (Forbes). Upland tetraploid plants from cultivar Summer (S) were tolerant to GB and SUS to YSA. Plants from the lowland cultivar Kanlow (K) were usually antibiotic to both aphids, whereas plants derived by crossing Kanlow x Summer (KxS) plants were SUS to GB and tolerant to YSA. These studies suggest that switchgrass plants have divergent physiological defense responses to aphid herbivory.

Oxidative responses of plants to environmental stressors such as insect or pathogen attack, have garnered considerable attention. Many studies have shown a shift in the oxidative status in plants resulting from stress on the host plant (Bi and Felton 1995, Moran et al. 2002, Bruce et al. 2007, Kempema et al. 2007, Khattab 2007, Holopainen and Gershenzon 2010). Reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), appear to be an essential component of a plant's response to stress, with low concentrations of H_2O_2 required for triggering a number of changes within cells (Zurbriggen et al. 2010, Baxter et al. 2014). However, in higher concentrations, ROS species are highly toxic to the plant if not efficiently detoxified by enzymatic and non-enzymatic mechanisms (Apel and Hirt 2004, Zurbriggen et al. 2010, Baxter et al. 2014). Oxidative enzymes, such as ascorbate peroxidase (APX), catalase (CAT) and peroxidase (POX), aid in the detoxification of ROS in plants (Bi and Felton 1995, Moran et al. 2002, Heng-Moss et al. 2004, Zhu-Salzman et al. 2004, 2005, Khattab 2007, Maffei et al. 2007, Smith and Boyko 2007, Gutsche et al. 2009a, Prochaska et al. 2015).

Numerous studies have provided evidence of increased levels of POX in switchgrass and other plants when exposed to insect herbivory (Bi and Felton 1995, Stout et al. 1997, 1999, Moran 1998, Chaman et al. 2001, Allison and Schultz 2004, Heng-Moss et al. 2004, Franzen et al. 2007, Gutsche et al. 2009a, Pilon-Smits et al. 2009, Gulsen et al. 2010, Pierson et al. 2010a, Ramm et al. 2013, Saathoff et al. 2013). Similar patterns have been observed for CAT and

APX, in *Miscanthus sinensis* (cv Giganteus) and buffalograss (*Buchloë dactyloides* (Nuttall) Engelmann) (Heng-Moss et al. 2004, Gulsen et al. 2010).

Studies by Ramm et al. (2013) found significant differences in transcripts for genes encoding two POX and one CAT between the chinch bug-free control and chinch bug infested genotypes of buffalograss. Additionally, basal expression levels of POX and CAT were found to be up-regulated in the tolerant buffalograss, suggesting these oxidative enzymes may be an effective defensive strategy in buffalograss against chinch bug herbivory and other biotic stressors.

APX is another enzyme crucial for detoxifying excess cellular H_2O_2 (Ishikawa and Shigeoka 2008, Gill and Tuteja 2010). APX genes were enhanced in tobacco and soybean in response to arthropod herbivory, specifically that of phloem-feeding insects (Mittler et al. 1999, Prochaska et al. 2015).

The overall focus of this study was to investigate changes in the relative oxidative stress in different cultivars of switchgrass in response to aphid herbivory. Investigations were completed using biochemical protocols to observe changes in POX, APX, and CAT concentrations when challenged by cereal aphids. Additionally, gene expression studies were used to gain a better understanding of changes occurring in gene expression before and during the onset of YSA.

Materials and Methods

Plant material. Three different cultivars of switchgrass, K, S, and KxS which was derived by crossing K (male) x S (female) plants, (Koch et al. 2014a,

2014b, 2014c, Vogel et al. 2014, Prochaska et al. *unpublished*) were evaluated. Plants previously scored as resistant (RES) or susceptible (SUS) to either GB or YSA (Koch et al. 2014b, 2014c) were clonally multiplied from ramets (3 clones each) in cone-tainers. All plants were maintained in a greenhouse setting at 25 ± 7°C with light supplemented by a 400-W metal halide lamps to produce a 16:8 hour (L:D) photoperiod. Plants were fertilized bimonthly with a soluble (20:10:20 N-P-K) fertilizer. New tillers that emerged were allowed to reach the second leaf stage prior to the introduction of aphids.

Insect colonies. GB (biotype I) and YSA were provided by Dr. John D. Burd, USDA-ARS in Stillwater, OK. Colonies of both aphids were established and maintained on a constant supply of the susceptible sorghum cultivar 'BCK60.' GB colonies were housed in a plant growth chamber with a temperature of 25 ± 2°C and a 16:8 hour (L:D) photoperiod. YSA colonies were housed in the greenhouse with a temperature of 25 ± 7°C and a 16:8 hour (L:D) photoperiod.

Plants were infested with 10 apterous adult aphids with a fine bristle paintbrush and caged using a tubular plastic cage (4 cm diameter by 46 cm in height) with vents covered in an organdy fabric to confine aphids to their respective plants. Uninfested (control) plants were maintained similarly. The experimental design was a completely randomized design with a 3x2x2x2 factorial (3 cultivars: S, KxS, K; 2 genotypes: RES and SUS; 2 time points: day 0 and day 10; 2 treatments: aphid-infested and aphid control) and 5 replications per treatment combination.

Plants were harvested 10 days following aphid introduction. Aphids were removed using a paintbrush and aerial portions of the plants flash frozen with liquid N_2. Samples were ground to a fine powder in liquid N_2, then stored at -80 °C until analyzed.

Aliquots of ground plant tissue were weighed (~100 mg) into 1.5 mL centrifuge tube, 300 µL of buffer (0.1 M sodium phosphate) and 10 µL of protease inhibitor cocktail (Sigma P9599) were added to each sample. Samples were sonicated twice for 7 sec using a Branson Digital Sonicator (amplitude: 20%), and centrifuged at 14,000 x *g* at 4 °C for 10 minutes. A 20 µL aliquot from each sample was added to microcentrifuge tubes containing 1 mL of acetone for protein determination (see below), and tubes were mixed by inversion and placed in a -20°C freezer for 1 hour to precipitate proteins.

The remaining sample was transferred to a new micro-centrifuge tube and centrifuged for an additional 3 minutes using the above settings. The homogenate was collected and prepared for POX, APX, and CAT assays. All enzymatic activities were measured using a spectrophotometer (BioTek Synergy; Winooski, VT).

Total Protein Content. After 1 hour at -20°C freezer, samples were removed and centrifuged at 14,000 x *g* at 4 °C for 10 minutes. The liquid portion was then discarded and the pellet allowed to air dry. The pellet was then dissolved in 40 µL of 25 mM NaOH by repeated pipetting. Triplicate 10 µL aliquots were analyzed for proteins using the BCA protein assay (Thermo-Fisher) following the manufacturer-supplied protocols. Bovine serum albumin was used as a standard.

Peroxidase. POX activity was measured using a previously published protocol (Heng-Moss et al. 2004, Pierson et al. 2010a) by observing the increase in absorbance (470 nm) for 2.5 minutes. Five microliter samples were placed in a 96 well microtiter plate containing 20 µL of 200 mM HEPES-NaOH buffer (pH 6.0), 75 µL of 18 mM guaiacol, and 75 µL of distilled water. Enzymatic reactions were initiated by adding 25 µL of 3% H_2O_2 (0.1 mM) to each well. Assays were performed at 30°C. Specific POX activity was determined using a molar absorptivity of 26.6 x 10^3 M^{-1} cm^{-1} for guaiacol at 460 nm (Heng-Moss et al. 2004, Pierson et al. 2010a) and was defined as µmol min*mg^{-1}.

Ascorbate peroxidase. APX activity was determined as described by Murshed et al. (2008) with the following modifications. Triplicate 10 µL sample aliquots were placed into a 96 well microtiter plate containing 18 µL of 0.5 mol/L sodium phosphate buffer, 5 µL of 200 mM H_2O_2, 2 µL of ascorbic acid, and 165 µL of distilled water in each well. Assays were performed at room temperature. Changes in absorbance were recorded at 290 nm for 15 min. The best-fit slope was determined for each sample and used in calculations and the statistical analysis. Control reactions without ascorbate or without plant extracts were run in unison for each treatment. One unit of enzyme activity was defined as µmoles min^{-1} mg^{-1} protein.

Catalase. CAT activity was measured through recorded changes in absorbance at 240 nm. Triplicate 20 µL aliquots of samples were placed into wells of a 96 well microtiter plate containing 16 µL of 0.5 mol/L sodium phosphate buffer, 5 µL of 200 mM H_2O_2, and 159 µL of distilled water to each well. Plates

were read at room temperature. Changes in absorbance were recorded for 15 minutes. The best-fit slope was determined for each sample and used in calculations and the statistical analysis. Control reactions without H_2O_2 or without plant extracts were run in unison for each treatment. One unit of enzyme activity was defined as μmoles min^{-1} mg^{-1} protein.

H_2O_2 determination using Amplex Red. H_2O_2 levels were detected with Amplex red using a protocol modified from Estavillo et al. (2011) and Oh et al. (2012). Approximately 50 mg of ground plant sample was mixed by repeated inversion with 300 μL 10 mM Tris buffer pH 7.5 containing 0.1 M NaCl, and 0.1 mM EDTA and placed on ice for 5 minutes. Samples were sonicated for 7 sec (as described above), and centrifuged for 20 minutes at 14,000 x *g* at 4°C, and the supernatant was collected. Triplicate 50 μL aliquots from each sample were placed into individual wells of a 96 well microplate. Next, 50 μL of a solution containing 0.01 mM Amplex red and 0.2 units mL^{-1} horseradish peroxidase in Tris buffer were added to each well, and plates were placed in the dark for 30 min at room temperature. Absorbance was subsequently measured at 560 nm using a BioTek Instruments plate reader equipped with Gen5 software. A standard curve was constructed using a range from 0 to 20 nmoles H_2O_2 / μL.

Data from Amplex red, total protein, POX, APX, and CAT activities were analyzed using SAS Version 9.4 mixed model analysis PROC GLIMMIX procedure (SAS Institute 2011, Cary, NC) to identify statistical differences.

RNA extraction. Total RNA was extracted from three biological replications of tissues collected at Day 0 and Day 10 (aphid infested and non-infested) for

only the YSA experiment using TRIzol reagent following the manufacturer protocol (Invitrogen, Carlsbad, CA, USA). RNA samples were purified using the RNeasy MinElute Cleanup Kit and associated manufacturer protocol (Qiagen, Valencia, CA, USA). The integrity of extracted RNA was verified using gel electrophoresis.

RT-qPCR. RT-qPCR was performed using RNA extracted from switchgrass plant samples, using 2.5 µg of total RNA treated with RNase-free DNase I (Life Technologies, Rockville, MD). First strand cDNA synthesis was completed using the ThermoScript RT-PCR system (Life Technologies) according to manufacturer's protocol. All qPCR was performed on a 7500 fast realtime PCR platform (Applied Biosystems) using Bio-Rad SsoAdvanced SYBR Green (Bio-Rad Laboratories, California, USA) following manufacturer's protocol which consists of 95°C for 30 sec, then 40 cycles of 95°C for 5 sec and 60°C for 30 sec. Genes analyzed by RT-qPCR and primers used for analyses are provided in Table 1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was included as an endogenous control (FWD: 5'-TCTTCGGTGAGAAGCCGGT-3'; REV: 5'-CATAGTCAGCGCCAGCCTC-3').

Results

Damage ratings. GB: Significant differences were observed for damage between treatments within one of the three switchgrass cultivars: S (control vs. Infested: Summer resistant (S^{RES}), P=0.0101, Summer susceptible (S^{SUS}), P=0.0010; Figure 1). However, no significant differences in damage were

detected between KxS and K plants when comparing infested vs. control plants (Figure 1). Overall, Kanlow resistant (K^{RES}) had a mean aphid number of 4.30 ± 1.04, while Kanlow susceptible (K^{SUS}) had a mean aphid number of 2.71 ± 0.62 GB. KxS resistant (KxS^{RES}) had 3.44 ± 1.03 aphids, whereas KxS susceptible (KxS^{SUS}) had 12.67 ± 5.45 GB. S^{RES} had 4.60 ± 1.48 GB and S^{SUS} 4.50 ± 2.06 GB. No aphids were placed on control plants. Mean aphid numbers were not significantly different between aphid infested and aphid-control, likely because aphid numbers were relatively low.

YSA: Damage ratings in S^{RES} were found to approach significance, when comparing control and infested treatments, with significant differences observed in S^{SUS} comparisons (S^{RES}: P=0.06, S^{SUS}: P=0.007; Figure 2). No significant differences were observed in damage ratings for KxS and K plants. A mean of 11.56 ± 3.79 (K^{RES}), 30.10 ± 4.45 (K^{SUS}), 8.60 ± 1.90 (KxS^{RES}), 6.1 ± 1.20 (KxS^{SUS}), 28.50 ± 8.31 (S^{RES}), and 50.30 ± 7.24 aphids (S^{SUS}), respectively were observed at the conclusion of this experiment. Mean aphid numbers were not significantly different for the aphid-infested and aphid-control comparisons within each genotype (RES and SUS).

Amplex Red. Amplex red was used to determine the concentration of H_2O_2 content in samples. GB infested KxS^{RES} plants had significantly lower H_2O_2 levels when compared to infested KxS^{SUS} plants (P=0.0006). No significant differences in H_2O_2 content were observed between infested K^{RES}-K^{SUS} or S^{RES}-S^{SUS} plants. Infested S^{SUS} and KxS^{RES} plants had a small increase in the levels of H_2O_2 (Figure 3; Supplement Table 1A).

For YSA, infested S^{RES} plants had significantly lower levels of H_2O_2 when compared to their respective controls. In infested S^{SUS} plants, H_2O_2 content was significantly greater (Figure 4; Supplement Table 1B). There were no significant differences for the KxS plants, although KxS^{SUS} plants had significantly higher levels of H_2O_2 at day 0 (Figure 4; Supplement Table 1B). For K^{RES}, infested plants had higher peroxide levels 10 DAI when compared to the uninfested 10 DAI controls. No differences were observed for the K^{SUS} samples (Figure 4; Supplement Table 1B).

Total Protein. At 10 days after aphid introduction (DAI), there were no significant differences in total protein content between aphid-infested and aphid-free control treatments among the six switchgrass genotypes for either aphid species (data not shown).

Peroxidase activity. No significant differences occurred for S^{RES} or S^{SUS} GB infested plants 10 DAI, although there was an increase in POX activity when compared to the Day 0 activities (Figure 5), suggestive of a possible developmental change occurring within the plant. These (possible) developmentally associated increases in POX activities were also noticed for the KxS and K plants (Figure 5; Supplement Table 2A). As observed for the S plants, no significant infestation effect was evident, although POX activities were elevated in the aphid-infested treatments for K and KxS plants relative to their respective control plants (Figure 5; Supplement Table 2A).

Similar developmental trends in POX activity were found in the YSA study. Again, trends were apparent among the three switchgrass cultivars. Within S^{RES},

plants having greater levels of POX activity as compared to the S^{SUS} plants (Figure 6; Supplement Table 2B). In KxS, there were no apparent differences in the KxS^{RES} plants 10 DAI (infested vs. control), whereas POX activity was elevated in the infested KxS^{SUS} plants relative to their controls. For K, POX activity was higher in the infested K^{RES} over the K^{SUS} plants, although this was not statistically significant (Figure 6; Supplement Table 2B).

Ascorbate peroxidase. All plants had an increase in APX activity 10 DAI regardless of the genotype (Figure 7). Within S^{RES} plants, there was no difference in APX, although in infested plants, APX activity was moderately elevated relative to uninfested controls. For S^{SUS} plants, APX activity was significantly reduced 10 DAI as a result of aphid herbivory (P=0.02; Figure 7; Supplement Table 3A). APX activity was significantly increased 10 DAI in aphid-infested KxS^{RES} plants (P=0.009; Figure 7, Supplement Table 3A). APX was lower in the infested KxS^{SUS} plants, although not significantly (P=0.4; Figure 7, Supplement Table 3A). No significant differences were found in APX activities 10 DAI in either K^{RES} or K^{SUS} plants. However, APX activity was somewhat elevated in GB infested plants relative to their respective controls (Figure 7; Supplement Table 3A).

Observed APX activities were lower in infested S^{RES} and S^{SUS} plants although the difference was not significant (P=0.8; Figure 8, Supplement Table 3B). In infested KxS^{RES} plants, APX activity was elevated, albeit slight, 10 DAI when compared to the control (Figure 8; Supplement Table 3B). APX activity was depressed in infested K^{RES} plants, but elevated in the infested K^{SUS} plants (Figure

8; Supplement Table 3B). Overall, it appears that APX activity may also change in response to plant development especially in the KxS and K plants.

Catalase. No significant differences were observed for CAT activities in either the GB or YSA treatments, but there was an increase in CAT activity in all plants 10 DAI as compared to the Day 0 (Figure 9 and 10). However, CAT activity was elevated in GB-infested S^{SUS}, KxS^{RES} and K^{RES} and K^{SUS} plants (Figure 9; Supplement Table 4A). Ten days after YSA introduction, CAT activity was elevated primarily in KxS^{RES} and KxS^{SUS} and K^{SUS} plants, but remained unchanged for the other comparisons (Figure 10; Supplement Table 4B).

RT-qPCR analysis. RT-qPCR analysis was only performed for the YSA study. A total of ten genes were selected for analysis based on published (Studham and MacIntosh 2012, Ramm et al. 2013, Prochaska et al. 2015) and unpublished data (Donze-Reiner *unpublished*). A reference gene, GAPDH, was used as a reference across all reactions (Czechowski et al. 2005). The relative expression of the ten select genes across the entire data set is shown in tables 2-4. In S^{RES} plants, three genes, including two encoding for POXs and one encoding a pathogenesis related (PR) protein, were significantly up-regulated in infested plants 10 DAI when compared to the uninfested controls (Table 2). A total of three genes were differentially expressed in comparisons between S^{SUS} infested vs. S^{SUS} control (Table 2). One gene was differentially expressed in KxS^{RES} infested vs. KxS^{RES} control while no genes were differentially expressed in KxS^{SUS} genotypes (Table 3). Six total genes were significantly different in K^{SUS} infested vs. K^{SUS} control and two total genes were significantly different between

K^{RES} infested vs. K^{RES} control (Table 4). The genes reported here have been previously identified as defense related genes and include a POX, a WRKY, and a pathogenesis related protein (Studham and MacIntosh 2012, Ramm et al. 2013, Prochaska et al. 2015).

Gene expression studies were also performed using RNA extracted from day 0 basal switchgrass plants in the YSA experiment. Transcript abundance of select genes determined to be differentially expressed were analyzed in the basal SUS and basal tolerant switchgrasses (Table 5). One gene was found to be differentially expressed between K^{SUS} (day 0 uninfested) vs. K^{RES} (day 0 uninfested). This gene, which encoded for a Pathogenesis-related protein (*Pavir.Cb00592*), was observed to be up-regulated (~48 fold, P=0.03, PROC TTEST; Table 5) in the K^{RES} genotype at day 0.

Four genes were found to be differentially expressed in S^{SUS} vs. S^{RES} (day 0) comparisons (PROC TTEST; $P<0.05$). All four genes were up-regulated in the SUS genotype of cultivar S. This included a POX (P=0.01; *Pavir.Ba00166*), a NB-ARC domain (LRR) resistance protein (P=0.003; *Pavir.J28677*), a gene encoding for terpene synthesis (P=0.01, *Pavir.J11635*), and a gene of unknown function (P=0.02, *Pavir.J10977*) (Table 5).

Finally, KxS exhibited changes in gene expression. Analyses comparing day 0 KxS^{RES} and KxS^{SUS} plants identified two differentially expressed POX encoding genes. One of the POXs, *Pavir.Ba00166*, was up-regulated (P=0.006; Table 5) in the RES plant by nearly 11 fold. A second POX, *Pavir.Ba00168*, was also up-regulated in the KxS^{RES} plant (P=0.029; Table 5).

Discussion

Significant variation in host response to aphids has been reported in switchgrass (Koch et al. 2014a, 2014b, 2014c). These studies facilitated identification of plants within several cultivars (S, K, and KxS) which were categorized as SUS or RES to aphid herbivory. These specific RES and SUS genotypes were evaluated for biochemical responses to aphids. Both GB and YSA caused damage to RES and SUS plants, and in general, the SUS genotypes within each cultivar experienced greater damage when compared to their respective controls. RES genotypes were also damaged but significantly less than their SUS counterparts, suggesting differences in their underlying physiology. Overall, plants from S were damaged to a greater extent than plants with KxS or K genetic backgrounds. These results are consistent with previously published research (Koch et al. 2014a, 2014b, 2014c).

In the short term, the primary impact of phloem feeders, such as aphids, involves changes in sucrose transport and redirection of leaf metabolism that can lead to an impairment of photosynthesis (Macedo et al. 2003, Franzen et al. 2007, Gutsche et al. 2009b, Pierson et al. 2010a, Singh et al. 2011). This inhibition is common among aphid-infested plants. However, a study of aphid-SUS and RES barley (*Hordeum vulgare* L.) (Gutsche et al. 2009b) concluded that prolonged inhibition to photosynthesis in SUS plants may result in damage by ROS accumulation in the cells (but is not detoxified). On the other hand, RES

plants may counteract harmful effects of ROS by up-regulating detoxification mechanisms when exposed to aphid herbivory.

Multiple studies (Apel and Hirt 2004, Kotchoni and Gachomo 2006, Pitzschke et al. 2006) have shown ROS to be early signals alerting gene expression patterns in cells. However, high concentrations of ROS often lead to cellular toxicity and death (Mittler et al. 2004). CAT, POX, and APX are among the cellular repertoire of oxidative enzymes that ameliorate the harmful effects of ROS, especially in plants with resistance to aphid herbivory (Hildebrand et al. 1986, Bi and Felton 1995, Jespersen et al. 1997, Mittler et al. 1999, Argandoña et al. 2001, Chaman et al. 2001, Hiraga et al. 2001, Heng-Moss et al. 2004, Gulsen et al. 2010, Mhamdi et al. 2010, Ramm et al. 2013).

In our studies, observations revealed increased POX activity in switchgrass, especially S^{RES}, when challenged by GB or YSA. This may contribute to the overall resistance to aphid herbivory found in switchgrass. Differences at day 0 were detected within KxS^{RES} plants (Table 2). This supports studies performed by Ramm et al. (2013) and Prochaska et al. (2015), which documented increased POX activity in RES plants when compared to known SUS plants. Overall, plant POXs have been shown to perform a variety of roles, including a fundamental role in the cell wall building process. However, POX have also been shown to aid in auxin catabolism, wound healing, the production or removal of H_2O_2, and the defense response against insect and pathogen attack (Hiraga et al. 2001, Ni et al. 2001, Kawano 2003, Gutsche et al. 2009a, Gill and Tuteja 2010, Ramm et al. 2013, Prochaska et al. 2015). Increased levels

of POX have also been associated with defensive responses to phloem feeding insects in many plant species (Argandoña et al. 2001, Ni et al. 2001, Park et al. 2005, Smith and Boyko 2007, Gutsche et al. 2009a, Liu et al. 2010, Pierson et al. 2010a, Ramm et al. 2013, Prochaska et al. 2015).

This study found APX activity to be significantly elevated in several switchgrass genotypes when infested with GB. However, this response was only observed for KxS^{RES} plants when infested with YSA, suggesting there were distinct host responses to individual aphids. APX is crucial for detoxifying excess cellular H_2O_2 produced in plants (Ishikawa and Shigeoka 2008, Gill and Tuteja 2010). Differences in gene expression coding for APX have been found in plant-pathogen interactions, specifically tobacco and soybean-soybean aphid interactions where APX was found to be up-regulated (2-fold) in response to aphid herbivory (Mittler et al. 1999, Prochaska et al. 2015).

Earlier research had indicated that S was tolerant to GB and SUS to YSA (Koch et al. 2014b), whereas KxS was SUS to GB but tolerant to YSA. Researchers have also shown there can be a diversity of responses determined both by the host and by the aphid (Ryan et al. 1987, Miller et al. 1994, Riedell and Kieckhefer 1995, Rafi et al. 1997, Wilhelmina et al. 2000, Ni et al. 2001, Heng-Moss et al. 2002, 2004, Zhang et al. 2004, Heng-Moss et al. 2006, Franzen et al. 2007, Yuan et al. 2008, Gutsche et al. 2009b, Pierson et al. 2010a, 2010a, Studham and MacIntosh 2012, Prochaska et al. 2013, 2015, Ramm et al. 2013). This suggests that variations observed in our study could be partially attributable to these factors.

CAT levels did not appear to change in response to aphid herbivory. However, trends indicated increased CAT activity in the response to GB. This response was muted or absent when these plants were challenged with YSA. Ramm et al. (2013) observed CAT transcripts to be higher in unchallenged RES buffalograss, although it is uncertain if similar mechanisms are present in switchgrass.

Previous research has also found differential gene expression among several defense related genes when challenged by phloem-feeding insects (when compared to their respective controls). This information may help elucidate the underlying defensive mechanisms within a given plant (Bi and Felton 1995, Wilhelmina et al. 2000, Torres et al. 2002, Apel and Hirt 2004, Heng-Moss et al. 2004, Ralph et al. 2006, Thompson and Goggin 2006, Couldridge et al. 2007, Franzen et al. 2007, Browse and Howe 2008, Gutsche et al. 2009a, Liu et al. 2010, Pierson et al. 2010a, Suzuki and Mittler 2012, Ramm et al. 2013, Prochaska et al. 2015).

Through transcriptional profiling, Gutsche et al. (2009a) detected nearly 900 differentially expressed genes in wheat challenged by the Russian wheat aphid (RWA), *Diuraphis noxia* (Mordvilko). Of the differentially expressed genes, several were associated with plant defense and ROS scavenging. This included two POX genes that were up-regulated when challenged by RWA and may be significant to our overall understanding of the tolerant response in barley (Gutsche et al. 2009a). Similar responses have been found in other plant-phloem feeding insect systems such as soybean, wheat, and buffalograss (Franzen et al.

2007, Pierson et al. 2010a, Ramm et al. 2013, Prochaska et al. 2015). The previously discussed research demonstrates that POX, APX, and CAT play a role in the plant defense mechanism to aphids.

From genes found to be significant in next generation sequencing (NGS) switchgrass-GB interactions (Donze-Reiner *unpublished*), we selected genes that would potentially expand our knowledge of switchgrass-YSA resistance mechanisms. This includes a terpene synthase (TPS) gene found to be differentially expressed in switchgrass K^{SUS} when the infested plant is compared to it's respective control (Fold change ~55; Table 4). Additionally, day 0 comparisons detected increased TPS expression levels in the RES genotype of S^{SUS} (Fold change ~5; Table 5). Previous studies have found TPS to play various ecological roles in plants including pollinator attraction (Pichersky and Gershenzon 2002, Chen et al. 2011), insect predator attraction to herbivores (Unsicker et al. 2009, Chen et al. 2011), and chemical/physical barriers to oviposition by insects (Paré and Tumlinson 1999, Pichersky and Gershenzon 2002, Keeling and Bohlmann 2006, Cheng et al. 2007, Heiling et al. 2010, Chen et al. 2011, Falara et al. 2011). In some plants, observations have shown that plants can emit volatile terpenoids in response to insect herbivory (Miller et al. 2005, Mumm and Hilker 2006), and can act directly against insect and pathogen attack, even while induced volatile emissions function indirectly to attract natural enemies of attacking insects (Chen et al. 2011). Similar observations, involving TPS in relation to insect herbivory defense activities, have been found in rice (Yuan et al. 2008), sorghum (Shuang and Chen, *unpublished*), tomato (Falara et

al. 2011), and maize (Schnee et al. 2002, 2006, Köllner et al. 2009). In addition, there is some evidence that the switchgrass K is behaving in this way; however, further research is needed to fully understand the role of TPS in K.

Observations also found a WRKY gene to be differentially expressed in both K^{RES} and K^{SUS}. Within the switchgrass K^{RES}, a comparison of infested and control treatments documented a nearly 4-fold change in gene expression. Similar comparisions have shown a nearly 5-fold change in K^{SUS} (Table 4). In general, WRKY genes are involved in plant defense against aphids in wheat, *Arabidopsis*, and soybean (Lapitan et al. 2008, Pandey and Somssich 2009, Botha et al. 2010, van Eck et al. 2010, Prochaska et al. 2015) when challenged by herbivores.

Our study shows a potential role for aminotransferases in switchgrass-YSA interactions in K^{SUS} with a 15-fold change in gene expression occured as a result of YSA herbivory (Table 4). Further evaluations, however, are needed to better understand the role of aminotransferases in switchgrass-YSA systems. Aminotransferases are enzymes that catalyze the transfer of an amino group. Previous work has shown that aminotransferases confer plant resistance to pathogens (Eckardt 2004, Taler et al. 2004). In addition, studies by Smith and Boyko (2007) found differential gene expression for aminotransferases in sorghum (*S. bicolor*)-GB interactions, suggesting these enzymes may play a role in plant resistance to arthropod herbivory.

Observations within our study suggest that pathogenesis-related proteins (PR) appear to be an important factor in the cascade of defensive strategies

occurring in the S^{RES} plant (P=0.03; Table 2). In plants, sources of stress (abiotic or biotic) can result in damage. However, damage occurring from these stresses can remain limited as a result of the plant's defensive response where the plant reaction may be associated with an integrated set of metabolic cascades that may be vital in impeding further stress ingress (van Loon and van Strien 1999). As part of these responses, various genes, such as PR, can be induced. PR, as coded by the specific host plant, can occur systematically with the development of systemic acquired resistance (van Loon and van Strien 1999). PRs have also been found to be an important part of the defensive pathway in tomato, rice, and *Arabidopsis* when confronted by insect herbivores or other stressors (Fidantsef et al. 1999).

In three switchgrass genotypes (S^{SUS}, K^{RES}, and K^{SUS}), observations found significant differences between infested and control plants, suggesting that nucleotide binding site-LRR proteins may play a role in switchgrass defense (Tables 2 and 4). Similar observations were found at day 0 in S^{SUS} (Table 5). In plants, several aphid resistance genes were found to encode nucleotide binding site-LRR proteins (Crute and Dunn 1980, Chen et al. 1997, Milligan et al. 1998, Rossi et al. 1998, van der Biezen and Jones 1998, Nombela et al. 2003, Takken et al. 2006, Wroblewski et al. 2007, Prochaska et al. 2015). Overall, these large and often abundant proteins aid in the detection of a diverse array of pathogens including bacteria, viruses, fungi, insects, and nematodes.

This research suggests cysteine-rich secretory proteins (CRSP) may play a role in the plant's defensive response to YSA herbivory with differential gene

expression in two switchgrass genotypes, K^{SUS} and S^{SUS} (Tables 2 and 4). CRSP have been found in host plant-pathogen and host plant-antifungal activity in relation to defense (Blein et al. 2002, Rivas and Thomas 2005, Chisholm et al. 2006, Parker 2009, Miyakawa et al. 2014).

Finally, a gene of unknown function (*Pavir.J10977*) will require further study as it appears to be a significant player in the defensive response of switchgrass to cereal aphid herbivory (Tables 2 and 4). This gene is differentially regulated in K^{SUS} and S^{SUS}, by as much as 42 fold (S^{SUS}) in infested treatments when compared to the aphid controls. At day 0, significant differences were detected ($P = 0.02$) and differed by a near 40 fold difference in S^{SUS}.

Conclusions

This research identified a range of host responses to two different aphid species. There was a stronger plant response to GB feeding than to feeding by YSA. This was consistent with observed damage ratings. Selection for switchgrass resistance to one or both aphids appears feasible as all identified RES genotypes outperformed the comparable SUS genotypes. This study also indicated substantial variation in host response within the switchgrasses to the two aphid species. Overall, this project provides a better understanding of the mechanisms contributing to the defensive response to GB and YSA in the switchgrass cultivars K, KxS, and S.

Table 1. Gene ID, gene description, and gene primers (FWD and REV) used for RT-qPCR in switchgrass plants challenged by YSA.

GeneID	Gene Description	Gene Primer (FWD)	Gene Primer (REV)
Pavir.Ba00166	Peroxidase	5'-GGCCTTCA TGGAGGGTTCTC-3'	5'-GGTTCACG TTGGTGTCGTTG-3'
Pavir.Ba00167	Peroxidase	5'-CATGACTG CTTTGTCCAGGC-3'	5'-ATGTTGGC GATGACGTCGAA-3'
Pavir.Ba00168	Peroxidase	5'-GGAACAAG AACCTCGACCCA-3'	5'-AGCAGGTT GGTGTAGTAGGC-3'
Pavir.Ca00085	WRKY112	5'-GGTGCCAT CTAGCCTAGGAG-3'	5'-GTGGTGGT CTCGAGGATGAT-3'
Pavir.Cb00592	Pathogenesis-related protein	5'-GTGAAGTC GGAGATGGTGGT-3'	5'-TCTTGATG AGGCCGAGGTAG-3'
Pavir.Ib00618	Aminotransferase	5'-TCGGCTAT GGCTGAGTATGC-3'	5'-CTCCGTCC GAGATGAACACC-3'
Pavir.J10977	Unknown	5'-CTCTCCTC CTCGTCTCATCG-3'	5'-GTGTTGTG CCGTATGTTGGT-3'
Pavir.J11635	Terpene Synthesis	5'-AGAGCACT CGACTACCTGGA-3'	5'-CCCCATCT TCTCCAACGTGT-3'
Pavir.J28677	NB-ARC domain (LRR)	5'-ACAAAGCT GGCTCAGATGGT-3'	5'-CAACAGCA GAAACCAGCAAA-3'
Pavir.J37785	Cysteine-rich secretory protein	5'-GTACGACC ACGACAGCAACT-3'	5'-TAGTACGG GCTCTGTCCCTC-3'

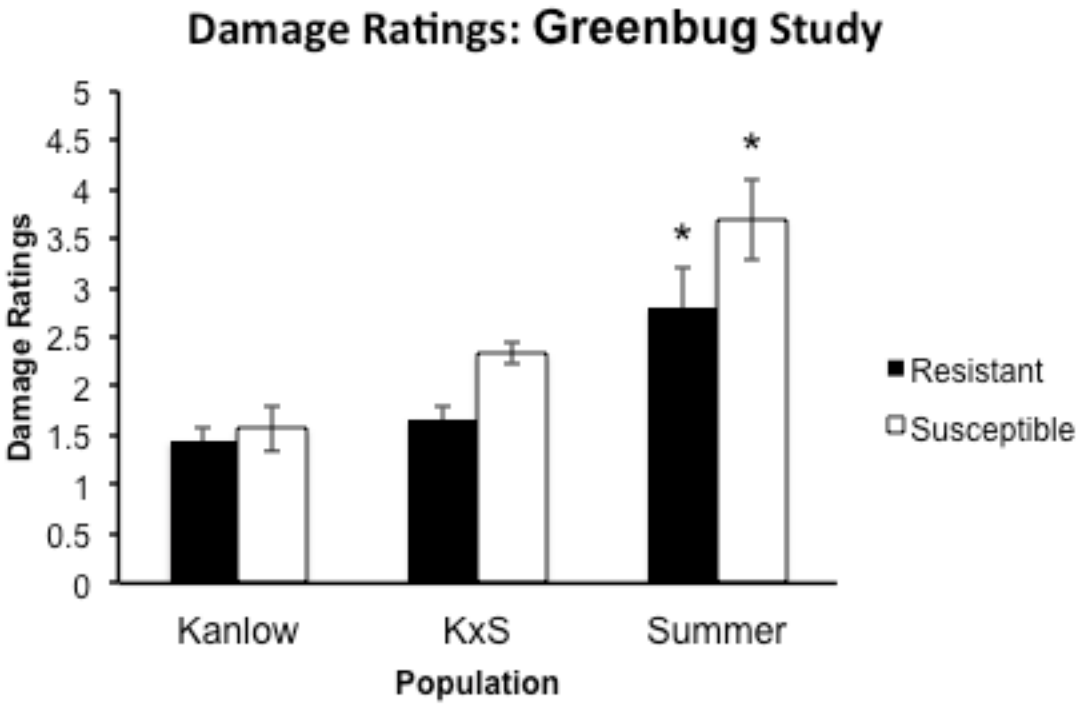

Figure 1. Damage ratings for RES and SUS switchgrass genotypes from (A) Summer, (B) KxS, and (C) Kanlow plants when challenged with greenbugs. (*) indicates significant differences between infested and control treatments within a given genotype ($P<0.05$). Damage ratings for all controls were scored at 1.0 ± 0.0. Values are the means ± SE (n = 5).

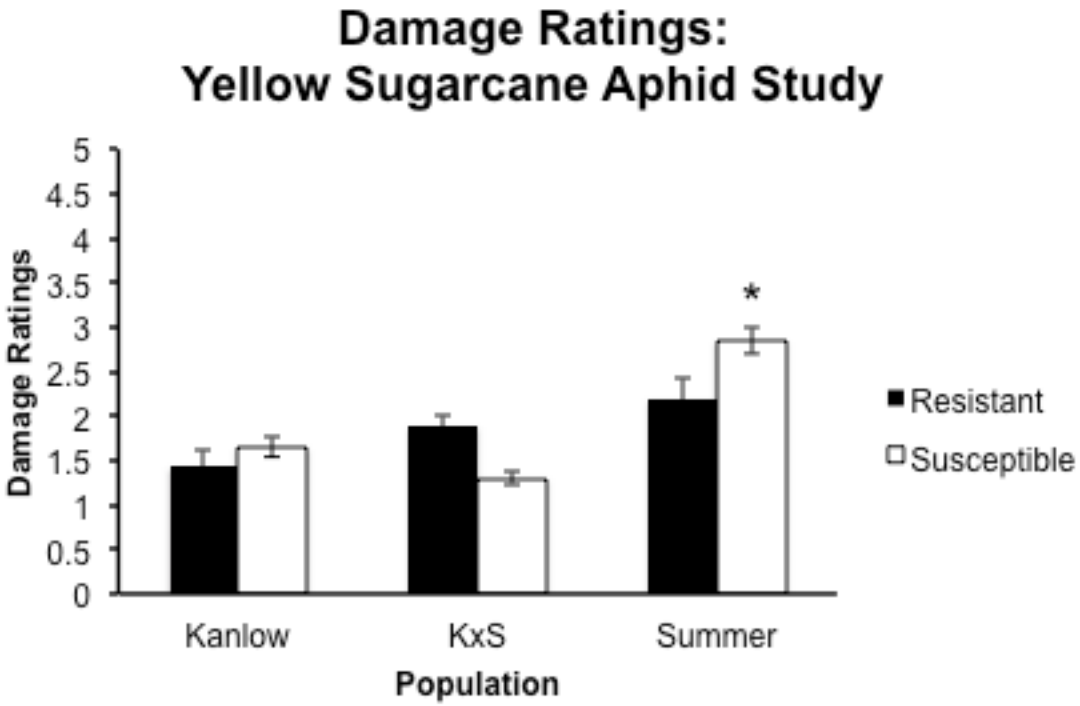

Figure 2. Damage ratings for RES and SUS switchgrass genotypes from (A) Summer, (B) KxS, (C) Kanlow plants when challenged with yellow sugarcane aphids. (*) indicates significant differences between infested and control treatments within a given genotype ($P<0.05$). Damage ratings for all controls were scored at 1.0 ± 0.0. Values are the means ± SE (n = 5).

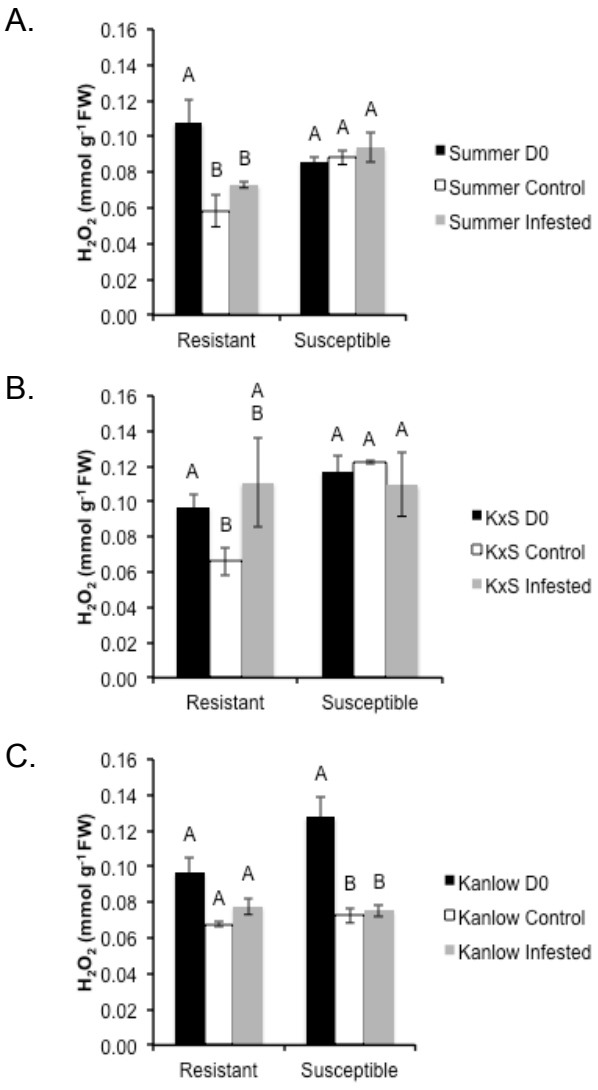

Figure 3. H_2O_2 content among RES and SUS switchgrass genotypes from (A) Summer, (B) KxS, and (C) Kanlow plants when challenged with greenbug. Different letters indicate significant differences ($P<0.05$). Values are the means ± SE ($n = 5$).

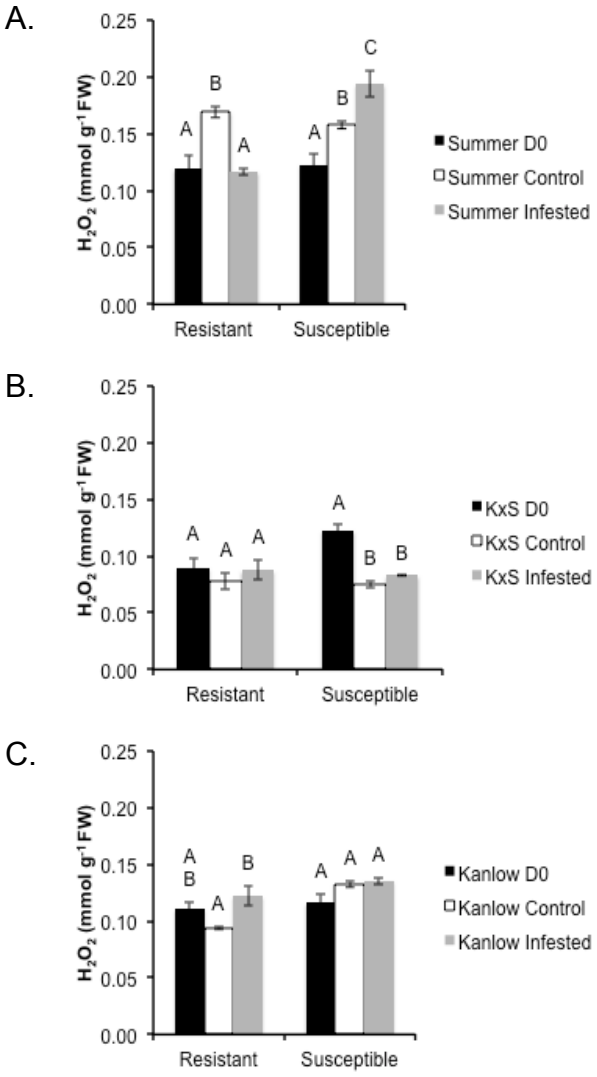

Figure 4. H_2O_2 content among RES and SUS switchgrass genotypes from (A) Summer, (B) KxS, and (C) Kanlow plants when challenged with yellow sugarcane aphid. Different letters indicate significant differences ($P<0.05$). Values are the means ± SE (n = 5).

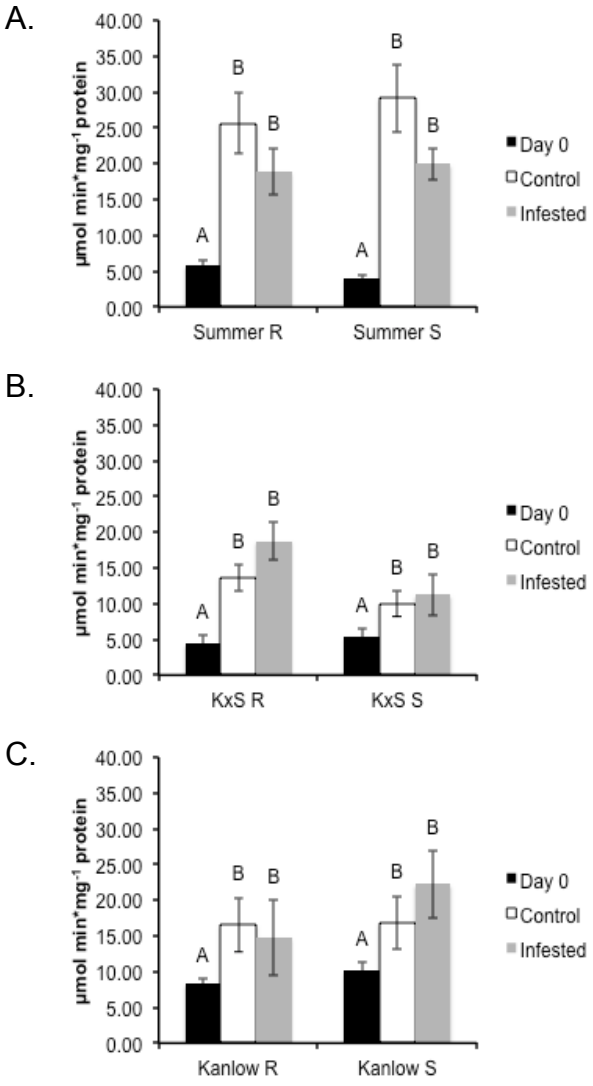

Figure 5. Peroxidase specific activity among RES and SUS switchgrass genotypes from (A) Summer, (B) KxS, and (C) Kanlow plants when challenged with greenbug. Different letters indicate significant differences ($P<0.05$). Values are the means ± SE ($n = 5$).

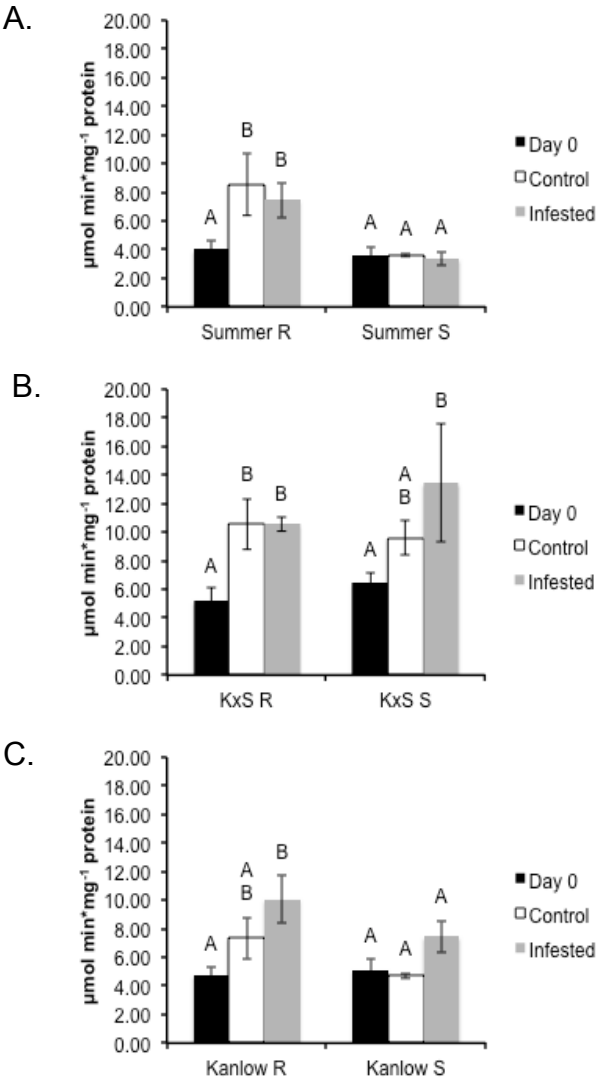

Figure 6. Peroxidase specific activity among RES and SUS switchgrass genotypes from (A) Summer, (B) KxS, and (C) Kanlow plants when challenged with yellow sugarcane aphid. Different letters indicate significant differences ($P<0.05$). Values are the means ± SE (n = 5).

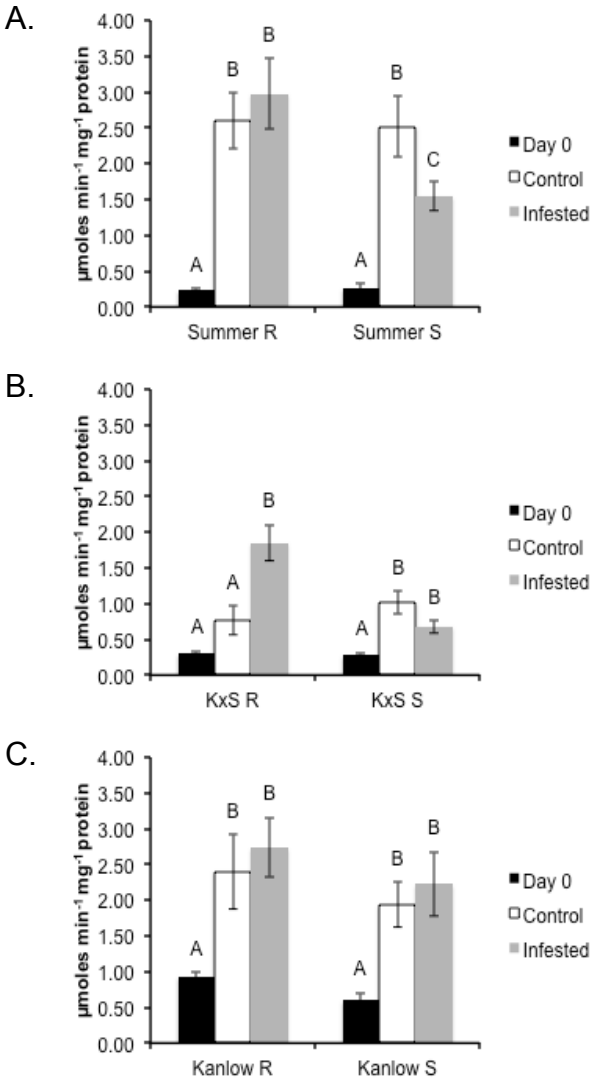

Figure 7. Ascorbate peroxidase specific activity among RES and SUS switchgrass genotypes from (A) Summer, (B) KxS, and (C) Kanlow plants when challenged with greenbug. Different letters indicate significant differences ($P<0.05$). Values are the means ± SE (n = 5).

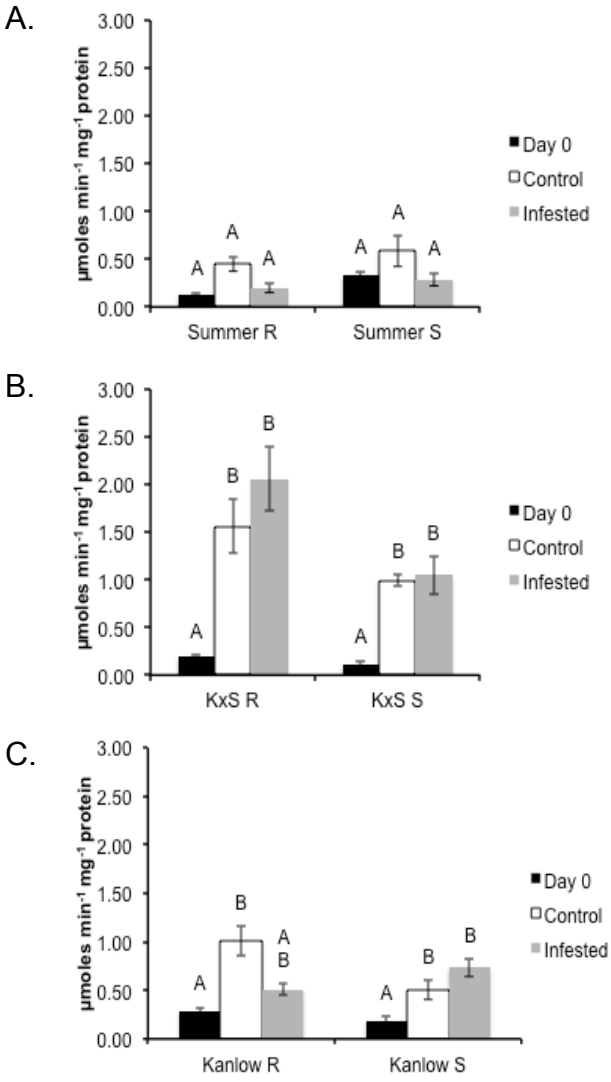

Figure 8. Ascorbate peroxidase specific activity among RES and SUS switchgrass genotypes from (A) Summer, (B) KxS, and (C) Kanlow plants when challenged with yellow sugarcane aphid. Different letters indicate significant differences ($P<0.05$). Values are the means ± SE ($n = 5$).

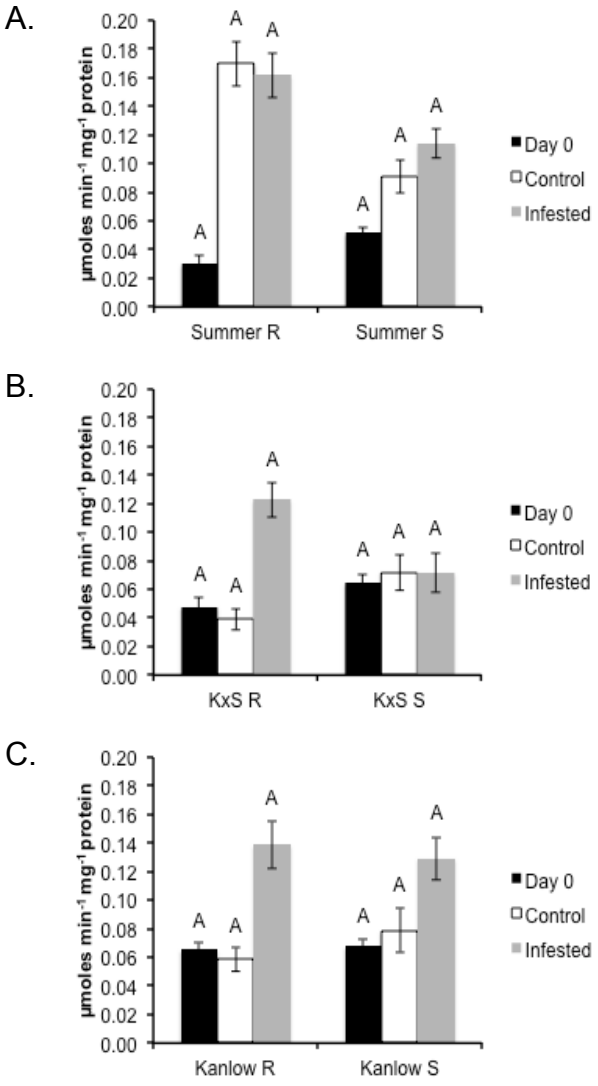

Figure 9. Catalase specific activity among RES and SUS switchgrass genotypes from (A) Summer, (B) KxS, and (C) Kanlow plants when challenged with greenbug. Different letters indicate significant differences ($P<0.05$). Values are the means ± SE ($n = 5$).

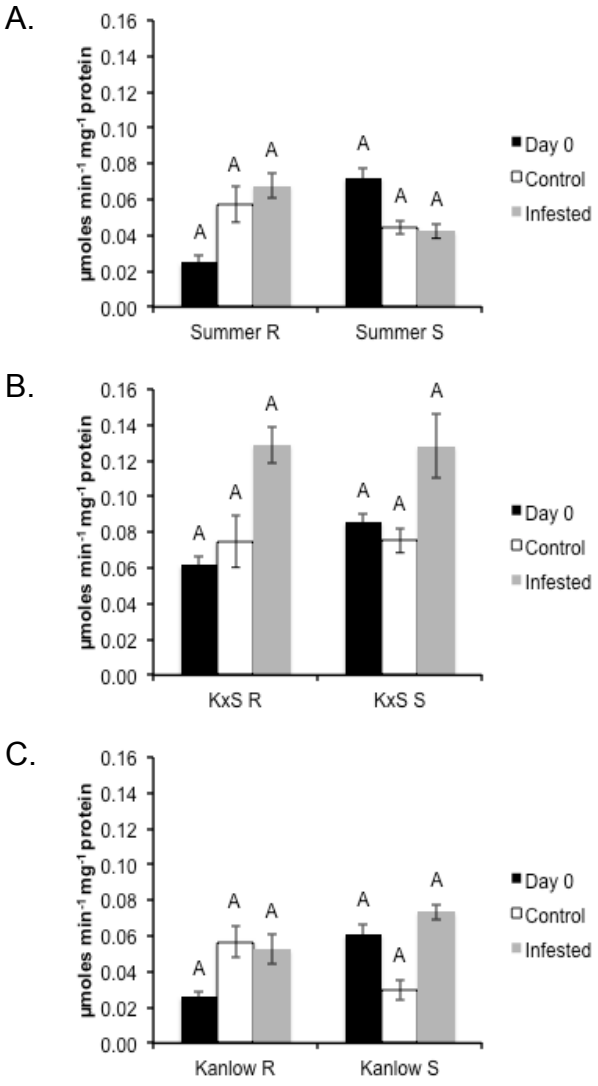

Figure 10. Catalase specific activity among RES and SUS switchgrass genotypes from (A) Summer, (B) KxS, and (C) Kanlow plants when challenged with yellow sugarcane aphid. Different letters indicate significant differences ($P<0.05$). Values are the means ± SE (n = 5).

Table 2. Gene description, gene ID, ΔΔCt, gene fold change, and T-Test value for YSA infested and uninfested switchgrass plants of Summer 10 DAI. Significant differences in bold (P<0.05). Values are the means ± SE (n = 3).

Summer		Resistant Day 10 Infested vs. Control			Susceptible Day 10 Infested vs. Control		
		ΔΔCt	Fold change	T-Test	ΔΔCt	Fold change	T-Test
Peroxidase	Pavir.Ba00166	3.11	0.12	0.14	0.16	0.89	0.86
Peroxidase	Pavir.Ba00167	**4.72**	**0.04**	**0.03**	-1.14	2.21	0.43
Peroxidase	Pavir.Ba00168	**4.68**	**0.04**	**0.002**	0.58	0.67	0.63
WRKY112	Pavir.Ca00085	1.61	0.33	0.20	0.64	0.64	0.49
Pathogenesis-related protein	Pavir.Cb00592	**8.13**	**0.00**	**0.03**	-1.69	3.22	0.23
Aminotransferase	Pavir.Ib00618	0.06	0.96	0.96	-2.06	4.16	0.26
Unknown	Pavir.J10977	-0.67	1.59	0.68	**-5.42**	**42.70**	**0.05**
Terpene Synthesis	Pavir.J11635	2.10	0.23	0.16	-0.08	1.06	0.77
NB-ARC domain (LRR)	Pavir.J28677	1.65	0.32	0.17	**-3.48**	**11.19**	**0.05**
Cysteine-rich secretory protein	Pavir.J37785	0.78	0.58	0.57	**-6.65**	**100.62**	**0.01**

Table 3. Gene description, gene ID, ΔΔCt, gene fold change, and T-Test value for YSA infested and uninfested switchgrass plants of KxS 10 DAI. Significant differences in bold (P<0.05). Values are the means ± SE (n = 3).

KxS		Resistant Day 10 Infested vs. Control			Susceptible Day 10 Infested vs. Control		
		ΔΔCt	Fold change	T-Test	ΔΔCt	Fold change	T-Test
Peroxidase	Pavir.Ba00166	**-6.76**	**108.69**	**0.00**	-2.07	4.18	0.52
Peroxidase	Pavir.Ba00167	2.63	0.16	0.09	0.63	0.65	0.68
Peroxidase	Pavir.Ba00168	-1.99	3.97	0.25	2.75	0.15	0.46
WRKY112	Pavir.Ca00085	**-3.43**	**10.79**	**0.06**	-3.58	11.96	0.32
Pathogenesis-related protein	Pavir.Cb00592	-1.03	2.04	0.32	-2.05	4.13	0.23
Aminotransferase	Pavir.Ib00618	2.50	0.18	0.71	-3.12	8.70	0.07
Unknown	Pavir.J10977	-0.03	1.02	0.99	1.02	0.49	0.35
Terpene Synthesis	Pavir.J11635	-2.42	5.36	0.40	1.06	0.48	0.54
NB-ARC domain (LRR)	Pavir.J28677	-3.24	9.47	0.25	-0.85	1.81	0.59
Cysteine-rich secretory protein	Pavir.J37785	-1.40	2.63	0.54	0.47	0.72	0.78

Table 4. Gene description, gene ID, ΔΔCt, gene fold change, and T-Test value for YSA infested and unifested switchgrass plants of Kanlow 10 DAI. Significant differences in bold (P<0.05). Values are the means ± SE (n = 3).

Kanlow		Resistant Day 10 Infested vs. Control			Susceptible Day 10 Infested vs. Control		
		ΔΔCt	Fold change	T test	ΔΔCt	Fold change	T test
Peroxidase	Pavir.Ba00166	0.10	0.93	0.90	-5.40	42.28	0.22
Peroxidase	Pavir.Ba00167	0.73	0.60	0.68	-0.38	1.30	0.76
Peroxidase	Pavir.Ba00168	1.70	0.31	0.61	-2.43	5.38	0.39
WRKY112	Pavir.Ca00085	**-1.93**	**3.80**	**0.02**	**-2.19**	**4.57**	**0.05**
Pathogenesis-related protein	Pavir.Cb00592	-0.67	1.59	0.52	-4.37	20.67	0.07
Aminotransferase	Pavir.Ib00618	2.56	0.17	0.40	**-3.92**	**15.16**	**0.01**
Unknown	Pavir.J10977	-2.87	7.33	0.20	**-3.09**	**8.50**	**0.04**
Terpene Synthesis	Pavir.J11635	-1.95	3.87	0.07	**-5.77**	**54.70**	**0.03**
NB-ARC domain (LRR)	Pavir.J28677	**-5.61**	**48.74**	**0.05**	**-4.53**	**23.03**	**0.03**
Cysteine-rich secretory protein	Pavir.J37785	-2.33	5.04	0.33	**-3.06**	**8.34**	**0.01**

Table 5. Gene description, ΔΔCt, gene fold change, and T-Test value for day 0 RES and day 0 SUS switchgrass plants of Summer (S), KxS, and Kanlow (K). Significant differences in bold (P<0.05). Values are the means ± SE (n = 3).

	Day 0 S Resistant vs. Susceptible		Day 0 KxS Resistant vs. Susceptible		Day 0 K Resistant vs. Susceptible	
	Fold change	**T-Test**	**Fold change**	**T-Test**	**Fold change**	**T-Test**
Peroxidase	**-28.46**	**0.01**	**10.86**	**0.006**	-10.87	0.27
Peroxidase	-5.95	0.11	-1.07	0.949	-3.38	0.28
Peroxidase	-2.16	0.49	**0.26**	**0.029**	-2.70	0.65
WRKY112	1.16	0.91	-1.66	0.686	-42.32	0.14
Pathogenesis-related protein	-28.82	0.16	4.23	0.345	**47.62**	**0.03**
Aminotransferase	-2.94	0.32	16.59	0.239	-1.87	0.62
Unknown	**-40.07**	**0.02**	7.83	0.481	1.46	0.71
Terpene Synthesis	**-5.39**	**0.01**	-1.28	0.228	2.28	0.73
NB-ARC domain (LRR)	**-4.27**	**0.003**	7.98	0.398	-1.41	0.70
Cysteine-rich secretory protein	-7.07	0.08	0.18	0.243	1.02	0.99

Supplement Table 1A. Statistical analysis of H_2O_2 content comparisons among RES and SUS Summer (S), KxS, and Kanlow (K) switchgrasses among greenbug infested comparisons (P<0.05). Three treatments were included: infested plants (I), control plants (C), and day 0 (D0) plants.

A) Greenbugs

Differences of population*genotype*treatment Least Squares Means

Pop	Gen	Trt	_Pop	_Gen	_Trt	Est	SE	DF	t Value	Pr > |t|
K	R	C	K	R	D0	-0.029	0.014	40	-2.02	0.050
K	R	C	K	R	I	-0.010	0.015	40	-0.66	0.513
K	R	D0	K	R	I	0.019	0.014	40	1.32	0.195
K	S	C	K	S	D0	-0.055	0.014	40	-3.86	0.000
K	S	C	K	S	I	-0.003	0.015	40	-0.16	0.871
K	S	D0	K	S	I	0.053	0.014	40	3.68	0.001
KxS	R	C	KxS	R	D0	-0.031	0.014	40	-2.25	0.030
KxS	R	C	KxS	R	I	-0.045	0.015	40	-2.93	0.006
KxS	R	D0	KxS	R	I	-0.014	0.014	40	-1.03	0.309
KxS	S	C	KxS	S	D0	0.005	0.015	40	0.34	0.733
KxS	S	C	KxS	S	I	0.013	0.015	40	0.83	0.410
KxS	S	D0	KxS	S	I	0.007	0.015	40	0.49	0.628
S	R	C	S	R	D0	-0.049	0.015	40	-3.24	0.002
S	R	C	S	R	I	-0.015	0.015	40	-0.95	0.347
S	R	D0	S	R	I	0.035	0.015	40	2.29	0.028
S	S	C	S	S	D0	0.002	0.015	40	0.15	0.883
S	S	C	S	S	I	-0.006	0.015	40	-0.38	-0.707
S	S	D0	S	S	I	-0.008	0.015	40	-0.53	0.601

Supplement Table 1B. Statistical analysis of H_2O_2 content comparisons among RES and SUS Summer (S), KxS, and Kanlow (K) switchgrasses among yellow sugarcane aphid infested comparisons. Three treatments were included: infested plants (I), control plants (C), and day 0 (D0) plants.

B) Yellow Sugarcane Aphid

Differences of population*genotype*treatment Least Squares Means

Pop	Gen	Trt	_Pop	_Gen	_Trt	Est	SE	DF	t Value	Pr > |t|
K	R	C	K	R	D0	-0.017	0.012	41	-1.42	0.164
K	R	C	K	R	I	-0.028	0.012	41	-2.35	0.024
K	R	D0	K	R	I	-0.011	0.012	41	-0.93	0.357
K	S	C	K	S	D0	0.016	0.012	41	1.37	0.178
K	S	C	K	S	I	-0.003	0.012	41	-0.22	0.828
K	S	D0	K	S	I	-0.019	0.012	41	-1.59	0.120
KxS	R	C	KxS	R	D0	-0.011	0.011	41	-1.08	0.288
KxS	R	C	KxS	R	I	-0.010	0.012	41	-0.81	0.424
KxS	R	D0	KxS	R	I	0.002	0.011	41	0.17	0.863
KxS	S	C	KxS	S	D0	-0.048	0.011	41	-4.30	0.000
KxS	S	C	KxS	S	I	-0.008	0.012	41	-0.69	0.492
KxS	S	D0	KxS	S	I	0.040	0.011	41	3.56	0.001
S	R	C	S	R	D0	0.050	0.011	41	4.67	<.0001
S	R	C	S	R	I	0.053	0.012	41	4.42	<.0001
S	R	D0	S	R	I	0.003	0.011	41	0.27	0.790
S	S	C	S	S	D0	0.035	0.012	41	2.96	0.005
S	S	C	S	S	I	-0.036	0.012	41	-3.03	0.004
S	S	D0	S	S	I	-0.071	0.012	41	-5.98	<.0001

Supplement Table 2A. Statistical analysis of peroxidase specific activity among RES and SUS Summer (S), KxS, and Kanlow (K) switchgrasses among greenbug infested comparisons (P<0.05). Three treatments were included: infested plants (I), control plants (C), and day 0 (D0) plants.

A) Greenbugs

Differences of population*genotype*treatment Least Squares Means

Pop	Gen	Trt	_Pop	_Gen	_Trt	Est	SE	DF	t Value	Pr > |t|
K	R	C	K	R	D0	0.685	0.258	72	2.65	0.010
K	R	C	K	R	I	0.115	0.237	72	0.48	0.631
K	R	D0	K	R	I	-0.570	0.261	72	-2.18	0.032
K	S	C	K	S	D0	0.511	0.250	72	2.04	0.045
K	S	C	K	S	I	-0.278	0.227	72	-1.22	0.225
K	S	D0	K	S	I	-0.789	0.244	72	-3.23	0.002
KxS	R	C	KxS	R	D0	1.127	0.301	72	3.74	0.000
KxS	R	C	KxS	R	I	-0.318	0.237	72	-1.34	0.183
KxS	R	D0	KxS	R	I	-1.445	0.294	72	-4.91	<.0001
KxS	S	C	KxS	S	D0	0.603	0.296	72	2.04	0.045
KxS	S	C	KxS	S	I	-0.122	0.262	72	-0.47	0.643
KxS	S	D0	KxS	S	I	-0.725	0.292	72	-2.48	0.015
S	R	C	S	R	D0	1.481	0.270	72	5.49	<.0001
S	R	C	S	R	I	0.308	0.222	72	1.39	0.169
S	R	D0	S	R	I	-1.231	0.274	72	-4.49	<.0001
S	S	C	S	S	D0	1.970	0.295	72	6.69	<.0001
S	S	C	S	S	I	0.378	0.218	72	1.73	0.087
S	S	D0	S	S	I	-1.593	0.300	72	-5.31	<.0001

Supplement Table 2B. Statistical analysis of peroxidase specific activity among RES and SUS Summer (S), KxS, and Kanlow (K) switchgrasses among yellow sugarcane aphid infested comparisons (P<0.05). Three treatments were included: infested plants (I), control plants (C), and day 0 (D0) plants.

B) Yellow Sugarcane Aphid

Differences of population*genotype*treatment Least Squares Means

Pop	Gen	Trt	_Pop	_Gen	_Trt	Est	SE	DF	t Value	Pr > |t|
K	R	C	K	R	D0	0.430	0.271	72	1.59	0.117
K	R	C	K	R	I	-0.314	0.227	72	-1.38	0.172
K	R	D0	K	R	I	-0.744	0.258	72	-2.89	0.005
K	S	C	K	S	D0	-0.083	0.294	72	-0.28	0.777
K	S	C	K	S	I	-0.462	0.272	72	-1.70	0.094
K	S	D0	K	S	I	-0.378	0.266	72	-1.42	0.159
KxS	R	C	KxS	R	D0	0.704	0.249	72	2.83	0.006
KxS	R	C	KxS	R	I	0.000	0.206	72	0.00	0.998
KxS	R	D0	KxS	R	I	-0.704	0.249	72	-2.83	0.006
KxS	S	C	KxS	S	D0	0.393	0.237	72	1.65	0.103
KxS	S	C	KxS	S	I	-0.339	0.201	72	-1.69	0.096
KxS	S	D0	KxS	S	I	-0.731	0.224	72	-3.26	0.002
S	R	C	S	R	D0	0.761	0.279	72	2.73	0.008
S	R	C	S	R	I	0.140	0.234	72	0.60	0.551
S	R	D0	S	R	I	-0.621	0.285	72	-2.17	0.033
S	S	C	S	S	D0	0.000	0.339	72	0.00	1.000
S	S	C	S	S	I	0.069	0.345	72	0.20	0.842
S	S	D0	S	S	I	0.069	0.345	72	0.20	0.842

Supplement Table 3A. Statistical analysis of ascorbate peroxidase specific activity among RES and SUS Summer (S), KxS, and Kanlow (K) switchgrasses among greenbug infested comparisons (P<0.05). Three treatments were included: infested plants (I), control plants (C), and day 0 (D0) plants.

A) Greenbugs

Differences of population*genotype*treatment Least Squares Means

Pop	Gen	Trt	_Pop	_Gen	_Trt	Est	SE	DF	t Value	Pr > |t|
K	R	C	K	R	D0	1.472	0.412	252	3.57	0.000
K	R	C	K	R	I	-0.328	0.412	252	-0.80	0.426
K	R	D0	K	R	I	-1.800	0.412	252	-4.37	<.0001
K	S	C	K	S	D0	1.342	0.412	252	3.25	0.001
K	S	C	K	S	I	-0.287	0.412	252	-0.70	0.486
K	S	D0	K	S	I	-1.629	0.412	252	-3.95	0.000
KxS	R	C	KxS	R	D0	0.463	0.412	252	1.12	0.263
KxS	R	C	KxS	R	I	-1.085	0.412	252	-2.63	0.009
KxS	R	D0	KxS	R	I	-1.548	0.412	252	-3.76	0.000
KxS	S	C	KxS	S	D0	0.744	0.412	252	1.81	0.072
KxS	S	C	KxS	S	I	0.339	0.412	252	0.82	0.412
KxS	S	D0	KxS	S	I	-0.406	0.412	252	-0.98	0.326
S	R	C	S	R	D0	2.365	9.412	252	5.74	<.0001
S	R	C	S	R	I	-0.376	0.412	252	-0.91	0.363
S	R	D0	S	R	I	-2.741	0.412	252	-6.65	<.0001
S	S	C	S	S	D0	2.260	0.412	252	5.48	<.0001
S	S	C	S	S	I	0.971	0.412	252	2.35	0.019
S	S	D0	S	S	I	-1.289	0.412	252	-3.13	0.002

Supplement Table 3B. Statistical analysis of ascorbate peroxidase specific activity among RES and SUS Summer (S), KxS, and Kanlow (K) switchgrasses among yellow sugarcane aphid infested comparisons (P<0.05). Three treatments were included: infested plants (I), control plants (C), and day 0 (D0) plants.

B) Yellow Sugarcane Aphid

Differences of population*genotype*treatment Least Squares Means

Pop	Gen	Trt	_Pop	_Gen	_Trt	Est	SE	DF	t Value	Pr > |t|
K	R	C	K	R	D0	1.206	0.545	252	2.21	0.028
K	R	C	K	R	I	0.634	0.445	252	1.43	0.155
K	R	D0	K	R	I	-0.572	0.598	252	-0.96	0.340
K	S	C	K	S	D0	0.733	0.632	252	1.16	0.247
K	S	C	K	S	I	-0.274	0.478	252	-0.57	0.567
K	S	D0	K	S	I	-1.007	0.607	252	-1.66	0.098
KxS	R	C	KxS	R	D0	1.840	0.559	252	3.29	0.001
KxS	R	C	KxS	R	I	-0.169	0.281	252	-0.60	0.548
KxS	R	D0	KxS	R	I	-2.009	0.553	252	-3.64	0.000
KxS	S	C	KxS	S	D0	1.571	0.625	252	2.51	0.013
KxS	S	C	KxS	S	I	0.063	0.373	252	0.17	0.866
KxS	S	D0	KxS	S	I	-1.508	0.629	252	-2.40	0.017
S	R	C	S	R	D0	0.850	0.679	252	1.25	0.211
S	R	C	S	R	I	0.528	0.610	252	0.87	0.387
S	R	D0	S	R	I	-0.323	0.746	252	-0.43	0.666
S	S	C	S	S	D0	0.432	0.561	252	0.77	0.442
S	S	C	S	S	I	0.455	0.565	252	0.81	0.421
S	S	D0	S	S	I	0.023	0.622	252	0.04	0.970

Supplement Table 4A. Statistical analysis of catalase specific activity among RES and SUS Summer (S), KxS, and Kanlow (K) switchgrasses among greenbug infested comparisons (P<0.05). Three treatments were included: infested plants (I), control plants (C), and day 0 (D0) plants.

A) Greenbugs

Differences of population*genotype*treatment Least Squares Means

Pop	Gen	Trt	_Pop	_Gen	_Trt	Est	SE	DF	t Value	Pr > |t|
K	R	C	K	R	D0	0.825	1.904	252	0.43	0.665
K	R	C	K	R	I	0.128	1.536	252	0.08	0.934
K	R	D0	K	R	I	-0.697	1.943	252	-0.36	0.720
K	S	C	K	S	D0	-0.788	1.817	252	-0.43	0.665
K	S	C	K	S	I	-0.970	1.770	252	-0.55	0.584
K	S	D0	K	S	I	-0.182	1.376	252	-0.13	0.895
KxS	R	C	KxS	R	D0	0.187	1.398	252	0.13	0.894
KxS	R	C	KxS	R	I	-0.595	1.173	252	-0.51	0.613
KxS	R	D0	KxS	R	I	-0.781	1.248	252	-0.63	0.532
KxS	S	C	KxS	S	D0	-0.136	1.279	252	-0.11	0.916
KxS	S	C	KxS	S	I	-0.610	1.161	252	-0.53	0.600
KxS	S	D0	KxS	S	I	-0.474	1.112	252	-0.43	0.670
S	R	C	S	R	D0	0.859	1.958	252	0.44	0.661
S	R	C	S	R	I	-0.178	1.448	252	-0.12	0.902
S	R	D0	S	R	I	-1.036	1.910	252	-0.54	0.588
S	S	C	S	S	D0	-0.533	1.542	252	-0.35	0.730
S	S	C	S	S	I	0.051	1.754	252	0.03	0.977
S	S	D0	S	S	I	0.584	1.568	252	0.37	0.710

Supplement Table 4B. Statistical analysis of catalase specific activity among RES and SUS Summer (S), KxS, and Kanlow (K) switchgrasses among yellow sugarcane aphid infested comparisons (P<0.05). Three treatments were included: infested plants (I), control plants (C), and day 0 (D0) plants.

B) Yellow Sugarcane Aphid

Differences of population*genotype*treatment Least Squares Means

Pop	Gen	Trt	_Pop	_Gen	_Trt	Est	SE	DF	t Value	Pr > |t|
K	R	C	K	R	D0	0.825	1.904	252	0.43	0.665
K	R	C	K	R	I	0.128	1.536	252	0.08	0.934
K	R	D0	K	R	I	-0.697	1.943	252	-0.36	0.720
K	S	C	K	S	D0	-0.788	1.817	252	-0.43	0.665
K	S	C	K	S	I	-0.970	1.770	252	-0.55	0.584
K	S	D0	K	S	I	-0.182	1.376	252	-0.13	0.895
KxS	R	C	KxS	R	D0	0.187	1.398	252	0.13	0.894
KxS	R	C	KxS	R	I	-0.595	1.173	252	-0.51	0.613
KxS	R	D0	KxS	R	I	-0.781	1.248	252	-0.63	0.532
KxS	S	C	KxS	S	D0	-0.136	1.279	252	-0.11	0.916
KxS	S	C	KxS	S	I	-0.610	1.161	252	-0.53	0.600
KxS	S	D0	KxS	S	I	-0.474	1.112	252	-0.43	0.670
S	R	C	S	R	D0	0.859	1.958	252	0.44	0.661
S	R	C	S	R	I	-0.178	1.448	252	-0.12	0.902
S	R	D0	S	R	I	-1.036	1.910	252	-0.54	0.588
S	S	C	S	S	D0	-0.533	1.542	252	-0.35	0.730
S	S	C	S	S	I	0.051	1.754	252	0.03	0.977
S	S	D0	S	S	I	0.584	1.568	252	0.37	0.710

CHAPTER 4:

EVALUATION OF SECOND-GENERATION TETRAPLOID SWITCHGRASS, *PANICUM VIRGATUM* L., TO GREENBUG, *SCHIZAPHIS GRAMINUM* (RONDANI), FOR RESISTANCE SELECTION

Introduction

Switchgrass, *Panicum* virgatum L., is a leading candidate for biomass energy production in the US (Vogel et al. 2011). When grown on a commercial scale, switchgrass will likely become a suitable host to a wide range of insect pests (Heng-Moss et al. 2014). Such scenarios have occurred in the past when other native grasses with relatively low pest densities have been commercialized and grown in large monoculture settings (Heng-Moss et al. 2002, 2003).

Switchgrass has been demonstrated to be a suitable host for different feeding guilds of insects (Prasifka et al. 2009a, 2009b, Dowd and Johnson 2009, Schaeffer et al. 2011, Koch et al. 2014a, 2014b, 2014c). Work with two aphids, greenbugs (GB), *Schizaphis graminum* (Rondani), and the yellow sugarcane aphid (YSA), *Sipha flava* (Forbes), have confirmed that different categories of resistance are present in tetraploid switchgrasses (Koch et al. 2014b, 2014c).

Among the tetraploid switchgrasses evaluated, cultivar Summer (S), an upland forage type plant, was categorized as tolerant to GB and susceptible to the YSA. The lowland cultivar Kanlow (K), on the other hand, was categorized as having both antibiosis and antixenosis to both aphid species (Koch et al. 2014b, 2014c) although both susceptible (SUS) and resistant (RES) genotypes were

found within both cultivars. Koch et al. (2014b, 2014c) suggested the presence of significant underlying genetic diversity for resistance to these aphid species in the different switchgrass cultivars.

As of late, the use of insect-resistant plants has become a popular and effective approach for managing insect pests. This is an attractive pest management strategy because it offers reduced insecticide use resulting in a reduction in both input costs and environmental hazards. Smith (1999, 2005) has documented hundreds of insect-resistant cultivars are grown throughout the U.S., and suggests that plant resistance may increase the overall efficiency of insect biological control agents by reducing the vigor of insect pests through the interactions of insect-resistant plants and natural enemies.

Breeding or selecting for plant tolerance (Smith 2005) appears to be a reasonable approach for minimizing the development of new insect biotypes although the genetics underlying the tolerance response in plants has not been fully elucidated. Based on a number of studies that have evaluated tolerance to aphids and other piercing-sucking insects (especially in grasses), certain themes have emerged for both short- and long-term plant responses to herbivory. Among these responses are an ability to overcome (1) inhibition to photosynthesis, (2) increased cellular reactive oxygen species (ROS) and (3) inhibition to growth and development (Mittler et al. 1999, Kawano 2003, Apel and Hirt 2004, Heng-Moss et al. 2004, Kotchoni and Gachomo 2006, Franzen et al. 2007, Dowd and Johnson 2009, Gutsche et al. 2009a, Prasifka et al. 2009a, Gill and Tuteja 2010, Liu et al. 2010, Donze-Reiner *unpublished*, Prochaska

unpublished). Research also suggested that tolerant plants may have a higher basal level of detoxifying enzymes and proteins compared to SUS plants, thus reducing the plant's ability to protect itself from high ROS accumulations (Vleeshouwers et al. 2000, Ramm et al. 2013). Potentially, this may allow a tolerant plant to utilize a greater portion of available resources for growth and development, rather than needing to mount the defensive response required by SUS plants.

Plant defense responses can arise from multiple sources (Smith 2005, Kim et al. 2008). It appears that modulations in photosynthesis, cellular redox control, and maintaining growth appear to be important traits found within RES (tolerant) plant systems (Gawrońska and Kiełkiewicz 1999, Strauss and Agrawal 1999, Heng-Moss et al. 2004, Smith 2005, Franzen et al. 2007). The goal of this research was to develop switchgrass lines that could be used to probe the genetics underlying host responses to aphids using molecular methods.

Materials & Methods

Plant material. Switchgrass plants from two cultivars, S and K, were screened for resistance to GBs and YSAs as described by Koch et al. (2014a). Plants were grown to two host developmental stages, the 2nd and 5th leaf stage, to test for host suitability. Five apterous, adult aphids were placed on each plant and caged. Plants were evaluated weekly performing a visual damage rating and counting total aphid number.

Following screenings, plants from the two cultivars determined to be highly SUS (rating of 3.5+) and highly resistant (rating of 2.0 or less) to aphid herbivory were transplanted into isolation crossing blocks at the University of Nebraska-Lincoln ARDC research field site (near Ithaca, NE). Four isolation plots were established (1 isolation site per category: susceptible (SUS) and resistant (RES) among two cultivars (K and S): Kanlow susceptible (K^{SUS}) (n=7), Kanlow resistant (K^{RES}) (n=4), Summer susceptible (S^{SUS}) (n=4), and Summer resistant (S^{RES}) (n=3)) with at least a quarter mile (0.40 km) separation between sites to improve cross-fertilization within each isolation. Following plant maturity, seeds were harvested from individual plants to screen the various half-sib families.

Screening of half-sib families. Screenings were performed with seedlings obtained from the various half-sib families. In total, 20 seedlings from each half-sib family were randomly assigned to a control (uninfested) or infested group (10 each). Infested treatments were challenged with GBs at the 2nd host developmental leaf stage using a completely randomized design. All plants were enclosed using a tubular plastic cage (4 cm diameter by 46 cm in height) with organdy fabric-covered vents to confine aphids to their respective plants. Ten apterous, adult aphids were transferred to treatment plants using a fine bristle paintbrush, then caged. Experimental replicates were maintained in the greenhouse at 25 ± 7°C with a photoperiod of 16:8 hour (L:D). Replicates were evaluated weekly for total aphids and visual damage based on a 1-5 rating scale (Heng-Moss et al. 2002, Koch et al. 2014a). Experiments were concluded when

the 10 plants from at least one of the half-sib families reached a mean damage rating of 3.5.

Statistical analysis. Generalized mixed model analysis (PROC GLIMMIX, SAS Institute 2011) was conducted on damage ratings and aphid counts to determine any significant differences. Where appropriate, means were separated using Fisher's least significant differences (LSD) procedure ($\alpha = 0.05$).

Results

Half-sib family screening. Damage: Several interesting trends occurred in the specific half-sib families analyzed. Within K^{RES}, 4 half-sib families were evaluated (Kanlow resistant (KR) and family number; KR-1, KR-2, KR-3, and KR-4) (Figure 1A). No significant differences were detected between aphid-infested and aphid-control plants in half-sib families KR-1 (damage: 1.1 ± 0.1) and KR-2 (1.4 ± 0.1) when compared to their respective controls (damage: 1.0 ± 0.0; $P<0.05$). However, significant differences were detected in half-sib families KR-3 (damage: 1.7 ± 0.2) and KR-4 (damage: 2.5 ± 0.0) when compared to their respective controls (damage: 1.0 ± 0.0; $P=0.0023$ and $P<0.0001$) (Figure 1A). Seven half-sib families were evaluated in the genotypes K^{SUS} (Kanlow susceptible (KS); KS-1, KS-2, KS-3, KS-4, KS-5, KS-6, and KS-7). Significant differences were detected in 6 of the 7 treatments (aphid-infested and aphid-control) examined (Figure 1B). Again, controls were rated as 1.0 ± 0.0 among all half-sib families within the genotype K^{SUS}. Mean damage ratings were observed as: 2.3 ± 0.2 (KS-1; $P<0.0001$), 2.2 ± 0.3 (KS-2; $P<0.0001$), 1.6 ± 0.2 (KS-3;

P=0.005), 2.5 ± 0.3 (KS-5; P<0.0001), 3.1 ± 0.3 (KS-6; P<0.0001), and 2.2 ± 0.2 (KS-7; P<0.0001). No significant differences were found in the remaining half-sib family (KS-4; 1.2 ± 0.1; P<0.05) (Figure 1B).

Within S^{RES} selections, significant differences were found between treatments (aphid-infested and aphid-control) in three of the screened half-sib families (Summer resistant (SR); SR-1; damage: 1.6 ± 0.2 (P=0.017), SR-2; damage: 2.3 ± 0.2 (P<0.0001), and SR-3; damage: 1.6 ± 0.1 (P=0.013)). All controls had a mean damage rating of 1.0 ± 0.0 (Figure 2A).

Significant differences in damage ratings were also detected in the four S^{SUS} half-sib families (Summer susceptible (SS); SS-1; damage: 1.6 ± 0.2 (P=0.002), SS-2; damage: 2.3 ± 0.2 (P<0.0001), SS-3; damage: 2.7 ± 0.3 (P<0.0001), and SS-4; damage: 3.0 ± 0.2 (P<0.0001)) when compared to their respective controls (1.0 ± 0.0) (Figure 2B). An overall check of randomly selected plants from the S base population had a damage rating of 2.0 ± 0.0 which was significantly greater (P<0.0001) than those observed in the respective uninfested control plants (Figure 2B).

Overall, aphid infested K^{RES} plants had a mean damage rating of 1.6 ± 0.1, whereas K^{SUS} plants had a mean damage rating of 2.1 ± 0.1. Within S, an average mean damage rating of 1.8 ± 0.1 was observed for S^{RES} plants, and a mean damage rating of 2.2 ± 0.1 in S^{SUS} plants. Overall, these findings (on a population level) are in agreement with previous studies performed by Koch et al. (2014a). Statistical analysis for damage among half-sib families within a

switchgrass genotype (K^{RES}, K^{SUS}, S^{RES}, and S^{SUS}) are shown in Supplement Table 1.

Significant differences were found in cumulative aphid days (CAD) among the switchgrass families (K^{RES}, K^{SUS}, S^{RES}, and S^{SUS}) when challenge by GBs (compared to their respective controls). K^{SUS} had a mean of 274.2 ± 28.3 CAD (KS control: 0.0 ± 0.0 CAD; P<0.0001), while K^{RES} had 191.8 ± 23.0 CAD (K^{RES} control: 0.0 ± 0.0 CAD; P<0.0001) when challenged with GBs. S^{RES} had 273.9 ± 28.8 CAD (S^{RES} control: 0.0 ± 0.0 CAD; P<0.0001) over S^{SUS}, which had 154.4 ± 17.5 CAD (S^{SUS} control: 0.0 ± 0.0 CAD; P<0.0001) when infested by GBs. Statistical differences were also found between half-sib families within a given genotype. Statistical analysis for CAD among half-sib families within a switchgrass genotype (K^{RES}, K^{SUS}, S^{RES}, and S^{SUS}) is shown in Supplement Table 2.

Discussion

Previous research has established that significant variation exists in the RES response of lowland (K) and upland (S) switchgrass to aphid herbivory by GBs and the YSAs (Koch et al. 2014b, 2014c). These studies demonstrated that K expresses high levels of antibiosis which negatively impact the biology or life history of an insect (Smith 2005, Dogramaci et al. 2007), with lower CAD and damage ratings, relative to other switchgrasses screened. In the current study, damage ratings were always greater within S plants (average rating of 2.1) when

compared to K plants (average rating of 1.8), which is consistent with earlier studies.

Overall, RES genotypes appear better able to withstand GB feeding pressure, as evidenced by the lower damage ratings of the K^{RES}-K^{SUS} half-sibs (Figure 1) relative to the S^{RES}-S^{SUS} half-sib families (Figure 2). Visual plant damage included chlorosis and tissue necrosis. In some replicates, especially in S^{SUS} half-sib plants, GB populations may have waned due to a lack of healthy tissue for feeding. These observations may not be evident in our data as CAD integrates aphid density over time.

Screening of half-sib families established two important details: (1) the original selection of the parent plants as SUS or RES to GB was validated through screening of the half-sib progeny, and (2) freely intermating only plants within each category (RES or SUS) and within each cultivar (K or S) resulted in half-sib families with a continued inherited SUS or RES response. These data suggest molecular (RNA-Seq and/or biochemical analyses) methods can be used to probe these half-sib families to ascertain traits that may be associated with resistance. However, continued screening and identification of new half-sib and full-sib families will be needed to continue genetic refinement of the resistance mechanisms acting on phloem-feeding insects within these switchgrasses.

This research provides valuable baseline information needed for the continued selection of switchgrass genotypes with resistance to insect pests. Knowledge provided by the study furthers our understanding of the

compensatory mechanisms resulting from aphid herbivory. Understanding these defensive mechanisms at a molecular level will help facilitate the identification of genetic markers that can be used to phenotype elite switchgrass germplasms and can be used to develop new cultivars with improved resistance to phloem-feeding insects.

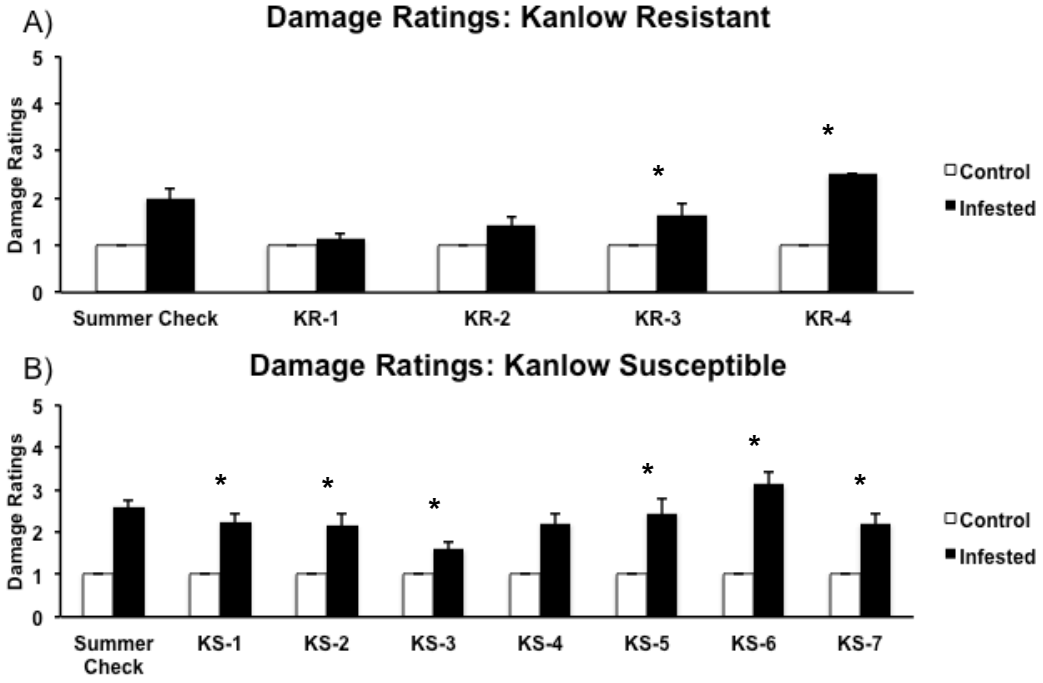

Figure 1. Damage ratings among Kanlow switchgrass half-sib families from (A) RES and (B) SUS genotypes. (*) indicates significant differences between infested and control treatments within a given genotype ($P<0.05$). Damage ratings for all controls were scored at 1.0 ± 0.0. Values are the means ± SE (n = 10).

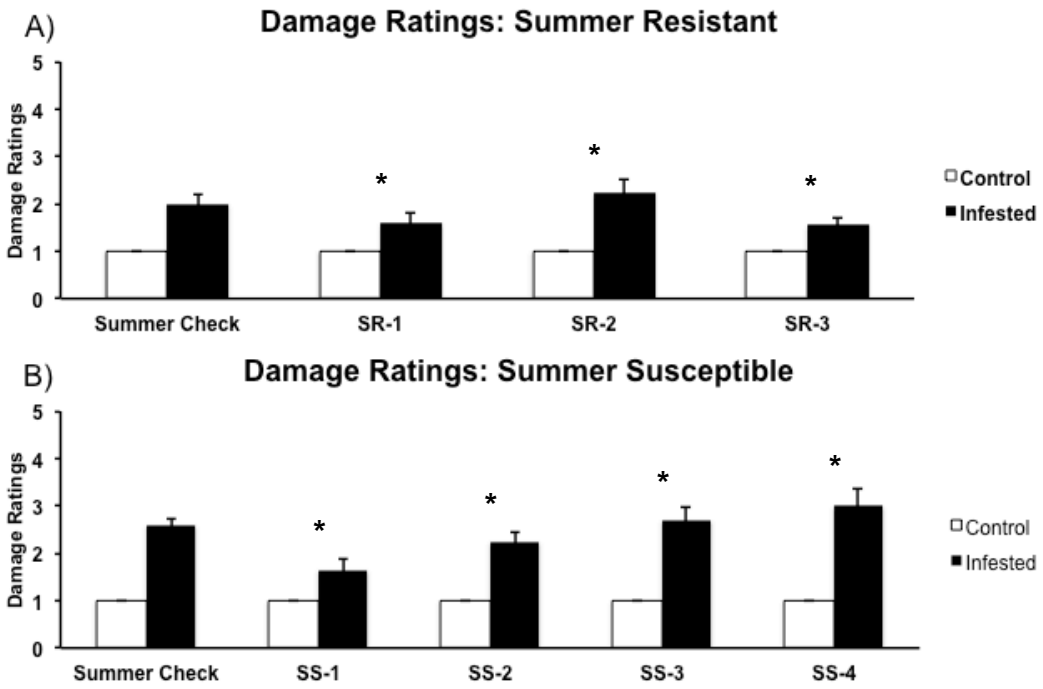

Figure 2. Damage ratings among Summer switchgrass half-sib families from (A) RES and (B) SUS genotypes. (*) indicates significant differences between infested and control treatments within a given genotype ($P<0.05$). Damage ratings for all controls were scored at 1.0 ± 0.0. Values are the means ± SE (n = 3).

Supplement Table 1. Statistical analysis of damage ratings among Kanlow R, Kanlow S, Summer R, and Summer S half-sib family comparisons (infested; P<0.05). All controls were scored with a damage rating of 1.0 ± 0.0. Legend: Population (Pop), Genotype (Gen), Treatment (TRT), Estimate (Est), Standard error (SE).

Differences of population*genotype*treatment: Least Squares Means

Pop	Gen	Trt	_Pop	_Gen	_Trt	EST	SE	DF	t Value	Pr > |t|
K1	R	I	K2	R	I	-0.313	0.289	302	-1.08	0.281
K1	R	I	K3	R	I	-0.525	0.280	302	-1.88	0.061
K1	R	I	K4	R	I	-1.375	0.409	302	-3.36	0.001
K1	R	I	S	Check	I	-0.875	0.280	302	-3.13	0.002
K1	S	I	K2	S	I	0.100	0.211	302	0.47	0.636
K1	S	I	K3	S	I	0.650	0.211	302	3.08	0.002
K1	S	I	K4	S	I	1.100	0.211	302	5.21	<.0001
K1	S	I	K5	S	I	-0.200	0.211	302	-0.95	0.345
K1	S	I	K6	S	I	-0.875	0.224	302	-3.91	0.000
K1	S	I	K7	S	I	0.050	0.211	302	0.24	0.813
K1	S	I	S	Check	I	0.250	0.211	302	1.18	0.238
K2	R	I	K3	R	I	-0.213	0.224	302	-0.95	0.344
K2	R	I	K4	R	I	-1.063	0.373	302	-2.85	0.005
K2	R	I	S	Check	I	-0.563	0.224	302	-2.51	0.013
K2	S	I	K3	S	I	0.550	0.211	302	2.60	0.010
K2	S	I	K4	S	I	1.000	0.211	302	4.73	<.0001
K2	S	I	K5	S	I	-0.300	0.211	302	-1.42	0.157
K2	S	I	K6	S	I	-0.975	0.224	302	-4.35	<.0001
K2	S	I	K7	S	I	-0.050	0.211	302	-0.24	0.813

K2	S	I	S	Check	I	0.150	0.211	302	0.71	0.478
K3	R	I	K4	R	I	-0.850	0.366	302	-2.32	0.021
K3	R	I	S	Check	I	-0.350	0.211	302	-1.66	0.099
K3	S	I	K4	S	I	0.450	0.211	302	2.13	0.034
K3	S	I	K5	S	I	-0.850	0.211	302	-4.02	<.0001
K3	S	I	K6	S	I	-1.525	0.224	302	-6.81	<.0001
K3	S	I	K7	S	I	-0.600	0.211	302	-2.84	0.005
K3	S	I	S	Check	I	-0.400	0.211	302	-1.89	0.059
K4	R	I	S	Check	I	0.500	0.366	302	1.37	0.173
K4	S	I	K5	S	I	-1.300	0.211	302	-6.15	<.0001
K4	S	I	K6	S	I	-1.975	0.224	302	-8.81	<.0001
K4	S	I	K7	S	I	-1.050	0.211	302	-4.97	<.0001
K4	S	I	S	Check	I	-0.850	0.211	302	-4.02	<.0001
K5	S	I	K6	S	I	-0.675	0.224	302	-3.01	0.003
K5	S	I	K7	S	I	0.250	0.211	302	1.18	0.238
K5	S	I	S	Check	I	0.450	0.211	302	2.13	0.034
K6	S	I	K7	S	I	0.925	0.224	302	4.13	<.0001
K6	S	I	S	Check	I	1.125	0.224	302	5.02	<.0001
K7	S	I	S	Check	I	0.200	0.211	302	0.95	0.345
S	Check	I	S1	R	I	0.417	0.244	302	1.71	0.089
S	Check	I	S1	S	I	-1.000	0.280	302	-3.58	0.000
S	Check	I	S2	R	I	-0.250	0.224	302	-1.12	0.265
S	Check	I	S2	S	I	-0.700	0.211	302	-3.31	0.001
S	Check	I	S3	R	I	0.438	0.224	302	1.95	0.052
S	Check	I	S3	S	I	-0.250	0.211	302	-1.18	0.238
S	Check	I	S4	S	I	0.350	0.211	302	1.66	0.099
S1	R	I	S2	R	I	-0.667	0.255	302	-2.61	0.009
S1	R	I	S3	R	I	0.021	0.255	302	0.08	0.935

S1	S	I	S2	S	I	0.300	0.280	302	1.07	0.284
S1	S	I	S3	S	I	0.750	0.280	302	2.68	0.008
S1	S	I	S4	S	I	1.350	0.280	302	4.83	<.0001
S2	R	I	S3	R	I	0.688	0.236	302	2.91	0.004
S2	S	I	S3	S	I	0.450	0.211	302	2.13	0.034
S2	S	I	S4	S	I	1.050	0.211	302	4.97	<.0001
S3	S	I	S4	S	I	0.600	0.211	302	2.84	0.005

Supplement Table 2. Statistical analysis of CAD among Kanlow R, Kanlow S, Summer R, and Summer S half-sib family comparisons (P<0.05). All controls were observed with a CAD value of 0.0 ± 0.0. Legend: Population (Pop), Genotype (Gen), Treatment (TRT), Estimate (Est), Standard error (SE).

Differences of population*genotype*treatment: Least Squares Means

Pop	Gen	Trt	_Pop	_Gen	_Trt	EST	SE	DF	t Value	Pr > |t|
K1	R	I	K2	R	I	-0.526	0.497	342	-1.06	0.290
K1	R	I	K3	R	I	-0.761	0.497	342	-1.53	0.127
K1	R	I	K4	R	I	-0.713	0.496	342	-1.44	0.152
K1	R	I	S	Check	I	-0.636	0.249	342	-2.56	0.011
K1	S	I	K2	S	I	0.195	0.000	342	Infty	<.0001
K1	S	I	K3	S	I	0.602	0.000	342	Infty	<.0001
K1	S	I	K4	S	I	-0.507	0.000	342	Infty	<.0001
K1	S	I	K5	S	I	0.123	0.000	342	Infty	<.0001
K1	S	I	K6	S	I	-0.714	0.000	342	Infty	<.0001
K1	S	I	K7	S	I	-0.444	0.000	342	Infty	<.0001
K1	S	I	S	Check	I	0.060	0.000	342	Infty	<.0001
K2	R	I	K3	R	I	-0.235	0.497	342	-0.47	0.637
K2	R	I	K4	R	I	-0.187	0.496	342	-0.38	0.707
K2	R	I	S	Check	I	-0.110	0.248	342	-0.44	0.657
K2	S	I	K3	S	I	0.406	0.000	342	Infty	<.0001
K2	S	I	K4	S	I	-0.702	0.000	342	-Infty	<.0001
K2	S	I	K5	S	I	-0.072	0.000	342	-Infty	<.0001
K2	S	I	K6	S	I	-0.909	0.000	342	-Infty	<.0001
K2	S	I	K7	S	I	-0.639	0.000	342	-Infty	<.0001

K2	S	I	S	Check	I	-0.135	0.000	342	-Infty	<.0001
K3	R	I	K4	R	I	0.048	0.496	342	0.10	0.923
K3	R	I	S	Check	I	0.124	0.248	342	0.50	0.617
K3	S	I	K4	S	I	-1.109	0.000	342	-Infty	<.0001
K3	S	I	K5	S	I	-0.478	0.000	342	-Infty	<.0001
K3	S	I	K6	S	I	-1.316	0.000	342	-Infty	<.0001
K3	S	I	K7	S	I	-1.045	0.000	342	-Infty	<.0001
K3	S	I	S	Check	I	-0.541	0.000	342	-Infty	<.0001
K4	R	I	S	Check	I	0.076	0.247	342	0.31	0.757
K4	S	I	K5	S	I	0.630	0.000	342	Infty	<.0001
K4	S	I	K6	S	I	-0.207	0.000	342	-Infty	<.0001
K4	S	I	K7	S	I	0.063	0.000	342	Infty	<.0001
K4	S	I	S	Check	I	0.568	0.000	342	Infty	<.0001
K5	S	I	K6	S	I	-0.838	0.000	342	-Infty	<.0001
K5	S	I	K7	S	I	-0.567	0.000	342	-Infty	<.0001
K5	S	I	S	Check	I	-0.063	0.000	342	-Infty	<.0001
K6	S	I	K7	S	I	0.271	0.000	342	Infty	<.0001
K6	S	I	S	Check	I	0.775	0.000	342	Infty	<.0001
K7	S	I	S	Check	I	0.504	0.000	342	Infty	<.0001
S	Check	I	S1	R	I	0.363	0.248	342	1.46	0.145
S	Check	I	S1	S	I	0.135	0.000	342	Infty	<.0001
S	Check	I	S2	R	I	-0.302	0.248	342	-1.22	0.223
S	Check	I	S2	S	I	-0.030	0.000	342	-Infty	<.0001
S	Check	I	S3	R	I	-0.484	0.248	342	-1.95	0.052
S	Check	I	S3	S	I	0.609	0.000	342	Infty	<.0001
S	Check	I	S4	S	I	0.344	0.000	342	Infty	<.0001
S1	R	I	S2	R	I	-0.665	0.496	342	-1.34	0.181
S1	R	I	S3	R	I	-0.847	0.351	342	-2.41	0.016

S1	S	I	S2	S	I	-0.165	0.000	342	-1.22	<.0001
S1	S	I	S3	S	I	0.473	0.000	342	Infty	<.0001
S1	S	I	S4	S	I	0.209	0.000	342	Infty	<.0001
S2	R	I	S3	R	I	-0.182	0.351	342	-0.52	0.604
S2	S	I	S3	S	I	0.639	0.000	342	Infty	<.0001
S2	S	I	S4	S	I	0.374	0.000	342	Infty	<.0001
S3	S	I	S4	S	I	-0.265	0.000	342	-Infty	<.0001

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