The *Journal of Cotton Science 8:99–104 (2004)*
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MOLECULAR BIOLOGY AND PHYSIOLOGY

Foliar Antioxidant Enzyme Responses in Cotton after Aphid Herbivory

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ABSTRACT

Cotton aphid (*Aphis gossypii* G.) populations in the field have fluctuated over the past years, yet it is unknown whether infestation by these insects alters cotton (*Gossypium hirsutum* L.) physiology. Although it is important to determine if aphids contribute to plant stress, there is only limited information on plant stress responses after aphid herbivory. In an attempt to quantify plant stress, the activity of cotton foliar antioxidant enzymes after aphid feeding was examined. Cotton aphids were collected from cotton fields at Lonoke, AR, and reared in the laboratory. Fifty aphids were transferred to cotton (*G. hirsutum* 'Stoneville 474') leaves of the same age and size, and were allowed to increase in numbers without restriction. Leaf samples were collected for protein extraction and antioxidant enzyme assays after 6 d and 9 d of aphid exposure. Enzyme activities of catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX), and glutathione reductase (GR) were measured. The initial population of 50 aphids increased to 137 aphids per leaf after 6 d. Of the antioxidant enzymes tested, only GR activity increased in aphid-infested leaves. After day 9 of infestation, there were 255 aphids per leaf, but the activity of foliar antioxidant enzymes was not different from control plants. In general, antioxidant enzyme activity in cotton plants was not altered by the levels of infestation and feeding duration used in this study.

Life in an oxygen-rich atmosphere has led to the evolution of biochemical adaptations that exploit the reactivity of active oxygen species (AOS). AOS consist of free radicals, such as superoxide, hydroxyl radicals, hydrogen peroxide, and singlet oxygen (Noctor and Foyer, 1998). It is generally assumed that the hydroxyl radical and singlet oxygen are so reactive that their production must be minimized, but plants continuously produce AOS at very high rates even under optimal conditions. These AOS are involved in virtually all major areas of aerobic biochemistry and are produced in large quantities by several enzyme systems. AOS attack lipids, proteins, and nucleic acids, causing lipid peroxidation, protein denaturation, and DNA mutation (Yu and Rengel, 1999; and Noctor and Foyer, 1998). Plants possess a defense system to detoxify these potentially dangerous reactive molecules, and antioxidant enzymes mediate these reactions.

In the natural environment, a complex interaction exists between insects and plants, and both entities have evolved complex biochemical mechanisms to ensure the continuity of species. Arthropod herbivory induces biochemical and physiological changes in host plants that may include the production of AOS. For example, glutathione reductase (GR) activity in barley (*Hordeum vulgare* L.) and wheat (*Triticum aestivum* L.) increased after herbivory by aphids (*S. avenae*) (Argandoña, 1994). *Schizaphis graminum* (R.) herbivory increased hydrogen peroxide content and total soluble peroxidase (POX) activity in barley, with a maximum level of POX activity after 30 min of infestation (Argandoña et al., 2001). Bi and Felton (1995) showed that the oxidative status of soybean [*Glycine max* (L.) Merr.] changed following herbivory by *Helicoverpa zea* (B.). Their results implicated primary compounds (e.g., ascorbic acid, proteins), secondary metabolites (e.g., phenolics), and reactive oxygen species (e.g., hydroxyl radical, hydrogen peroxide) as multiple components of induced resistance.

The nutritional and physiological status of the host plants may play an important role on the success of the insect. Reduced success of aphids on previously infested cotton plants was due to the plant's response to the first aphid infestation (Wool and

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Hales, 1996). It has also been suggested that phloem-feeding insects induce responses similar to pathogen infection and activate the salicylic acid-dependent and jasmonic acid/ethylene-dependent signaling pathways (Walling, 2000). This study is an attempt to understand the induction of antioxidants in cotton as a result of aphid herbivory. It was hypothesized that aphid-infested leaves would exhibit higher foliar antioxidant enzyme activity than non-infested plants. In order to understand the biochemical changes induced by a phloem feeding insect, such as *A. gossypii* on cotton, we determined the activity of four foliar antioxidant enzymes after aphid herbivory.

MATERIALS AND METHODS

Experimental conditions. The experiment was repeated three times in a growth chamber programmed for 14 (light)/10 (dark) hours, with a temperature of 16º C during the dark hours that increased to 28º C at noon, and 75% relative humidity (i.e. to simulate field conditions in May at Clarkedale, AR). Three seeds of the cotton cultivar Stoneville 474 (Stoneville Pedigreed Seed; Scott, MS) were sown in each 2-L pot filled with Sunshine mix #2 (Sun Gro Horticulture, Pine Bluff, Arkansas). One week later, plants were thinned to one plant per pot. Twelve potted plants for each experiment were watered with half-strength Hoagland's nutrient solution to maintain a well-watered and fertilized status. Each pot was placed in a 20-cm diameter tray that was placed inside a 25-cm diameter tray. The latter tray was filled with water and a small amount of detergent to restrict the movement of wingless aphids. Twelve plants were divided into two groups, one group receiving aphids and the other without aphids. Both groups were separated by a mosquito net to restrict the movement of winged aphids. Pots within each group were moved around the growth chamber every other day to allow more uniform conditions among plants. The first unfurled true leaf from the apex was tagged with a jeweler's tag at 14 days after planting (DAP). Six days later, aphids were transferred with a moist camel hair paintbrush to the tagged leaf. Fifty aphids (wingless adults plus nymphs) were individually transferred to one leaf of each plant. In addition, the remaining leaves were infested with 5 aphids per leaf. Aphids were allowed to increase without restriction.

Cotton aphids used were collected from infested cotton fields at Lonoke, Arkansas, and were reared in the laboratory.

Aphid counting. Aphids on the adaxial and abaxial surfaces of the leaves were counted daily, using a magnifying glass and a hand counter. Aphids were counted at approximately 1600 h. In order to achieve uniform aphid infestation on the first 2 d following the aphid transfer, additional aphids were added to the leaves that had lower numbers of aphids. While counting aphids, the leaves were handled with extreme care to avoid possible tissue damage and disturbance of the aphids. Non-infested leaves were also inspected. A graphic representation of the increase in aphid population was obtained by plotting the mean number of aphids on six leaf samples for each experiment against the number of days that plants were exposed to aphids. The values are expressed as the average number of aphids per leaf per day.

Leaf sampling. After the plants had been exposed to 6 d of aphid feeding (26 DAP), three plants per treatment were sprayed with 1% (v/v) sodium dodecyl sulfate to cause the removal of the stylets (Moran and Thompson, 2001). Plants were rinsed with deionized water one hour prior to collecting the leaves. Leaf samples were collected at 1700 h. The tagged leaves were sealed in plastic bags, dipped in liquid nitrogen and kept at -70º C for protein extraction. The same procedure was repeated on the second leaf sampling in which leaves were exposed to 9 d of aphid feeding (29 DAP).

Protein extraction. For protein extraction, the protocol of Anderson et al. (1992) with slight modifications was used. All chemicals used in these experiments were obtained from Sigma (St. Louis, Missouri), unless otherwise indicated. The extraction buffer consisted of 50 mM PIPES-NaOH, pH 6.8, 6 mM DL-cysteine hydrochloride, 10 mM D-isoascorbic acid, 1 mM EDTA, 1% polyvinylpyrrolidone-10, and 0.3% Triton X-100. One gram of frozen tissue was homogenized in a 15-mL centrifuge tube containing 0.5 g of insoluble polyvinylpolypyrrolidone, one drop of Antifoam A, and 4 mL of ice-cold extraction buffer. During all steps, the tissue samples were held on ice, and the contents in the centrifuge tube were mixed thoroughly using a Polytron homogenizer (Brinkmann Instruments; Westbury, New York). The contents of the tubes were centrifuged at 13,000 x g at 4º C for 20 min in a Beckman Allegra 64 R centrifuge (Beckman Instruments Inc.; Palo Alto, California). Soluble plant extracts were desalted using G-25 Sephadex PD-10 columns (Amersham Pharmacia Biotech; Uppsala, Sweden). Prior to use,

the columns were rinsed three times and equilibrated with 100 mM Tris-HCl, pH 7.0. One milliliter of plant extract was added to the top of the Sephadex column, and eluted with 3 mL of 100 mM Tris-HCl, pH 7.0. Catalase (CAT) activity was measured immediately because it is very unstable, and the remaining eluate was stored at -70º C.

Antioxidant enzyme assays. All enzyme activities are expressed as units per gram fresh weight of tissue. For CAT, POX and ascorbate peroxidase (APX), 1 unit of enzyme is defined as the amount necessary to decompose 1 µmol substrate/min at 25º C. One unit of GR is defined as the amount of enzyme required to reduce 1 nmol substrate/min at 25º C. All spectrophotometric analyses were performed on a BioSpec 1601 UV/VIS spectrophotometer (Shimadzu; Columbia, Maryland). All enzyme assays were repeated in triplicate. For each assay 100 mM Tris-HCl at pH 7.0 was used as a blank control.

The assay of Beers and Sizer (1952) was used to measure CAT. Substrate solution for CAT was 59 mM H_2O_2 in 50 mM KPO_4 buffer at pH 7.0. Assays were initiated by the addition of 0.1 mL of protein extract to 1.9 mL of deionized water and 1 mL of substrate solution. The disappearance of H_2O_2 was measured as the decrease in absorbance at 240 nm.

The technique of Nickel and Cunningham (1969) for measurement of POX activity was followed. The POX reaction solution consisted of 30 mM $NaPO_4$ buffer at pH 6.0, 120 mM H_2O_2, 17 mM $Na_2S_2O_3$, and 0.3 mM 2,3′,6-trichloroindophenol (Acros, New Jersey). The assay mixture consisted of 950 µl POX reaction solution, and 50 µl of plant extract. The hydrogen peroxide-dependent oxidation of reduced 2,3',6-trichloroindophenol was monitored at 675 nm.

For the APX assay, the protocol of Anderson et al. (1992) was followed using 0.1 mL protein extract in a final reaction volume of 0.9 mL. The ascorbic acid-dependent reduction of H_2O_2 at 265 nm was recorded. The reaction cocktail contained 92 mM HEPES-KOH at pH 7.0, 0.11 mM EDTA (disodium salt), pH 7.0, 0.16 mM Na ascorbate, and 0.11 mM H_2O_2,

GR was measured using the assay of Schaedle and Bassham (1977). The GR reaction solution consisted of 50 mM Tris-HCl, pH 7.5, 0.15 mM NADPH+H, 0.5 mM oxidized glutathione, 3 mM $MgCl_2$. Assays were initiated by the addition of 50 µL of plant extract in a final reaction volume of 1 mL. The glutathione-dependent oxidation of NADPH+H was measured at 340 nm.

Statistical analysis. All statistical analyses were conducted using SAS, version 8.1 (SAS Institute Inc.; Cary, NC). The experiments were arranged in a completely randomized design with three replications. Three cotton leaves were collected per sampling time per treatment. *T*-tests at $P = 0.05$ using the pooled error mean square from the ANOVA were used. A total of thirty-six tissue samples were assayed for each antioxidant enzyme, and each data point represents the mean of 27 values per treatment per sampling time.

RESULTS AND DISCUSSION

Growth of aphid populations. The number of aphids per leaf per day was averaged over the three experiments (Fig. 1), and was not statistically analyzed. Fifty aphids per leaf were initially used to quantify changes in antioxidant enzyme activity in response to aphid herbivory. The average number of aphids per leaf was 137 and 255 on days 6 and 9, respectively. The University of Arkansas Extension Service classifies infestation levels as light when 1 to 10 aphids per leaf are present on an occasional plant, medium when 11 to 25 aphids per leaf are present on numerous plants and some leaves curling on edges, and heavy when more than 26 aphids per leaf are present on numerous plants and leaves are crinkling and curling (Johnson, 1994).

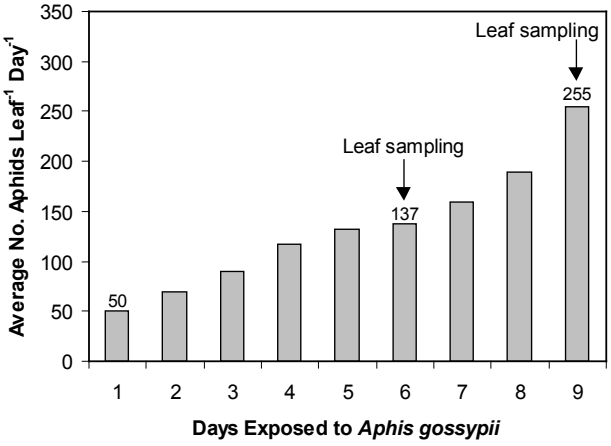

Figure 1. *Aphis gossypii* infestation on cotton leaves. Values are the means of three experiments with three replicates each. Arrows indicate when the leaf samples were collected. The number of aphids at the beginning (50), first sampling (137) and second sampling (255) are indicated.

Foliar oxidative response to aphid herbivory. Aphids did not affect CAT activity in cotton leaves (Fig. 2). Furthermore, aphid-infested and non-infested leaves showed similar CAT activity on day 6 (P = 0.9987) and on day 9 (P = 0.0949). CAT levels in soybean leaves decreased significantly following herbivory by *H. zea* (Bi and Felton; 1995). Similar studies on two-spotted spider mite (*Tetranychus urticae* K.) damage on soybean foliage [*Glycine max* (L.) M.] showed no affect in the levels of CAT (Hildebrand et al., 1986), but alfalfa (*Medicago sativa* L.) plants resistant to the spotted alfalfa aphid [*Therioaphis maculata* (B.)] contained higher activities of CAT than susceptible plants (Dillwith et al., 1991). *Helicoverpa zea* larvae feeding on cotton foliage induced increased levels of endogenous H_2O_2, salicylic acid, CAT, and APX (Bi et al., 1997). Inhibition of CAT activity has been suggested to be caused by the induction of salicylic acid, a potent CAT inhibitor (Chen et al., 1993), but this inhibition of CAT does not occur in cotton leaves, even at relatively high concentrations (1 mM) of salicylic acid (Bi et al., 1997).

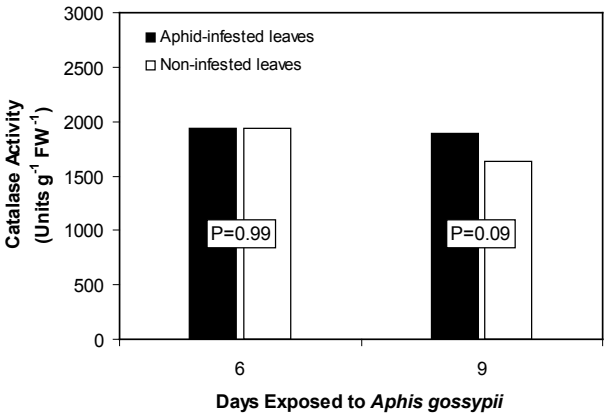

Figure 2. Catalase activity in non-infested (□) and aphid-infested leaves (■), after 6 and 9 d of exposure to *Aphis gossypii*. FW = fresh weight. Values are the means of twenty-seven observations.

The levels of POX were similar in aphid-infested and non-infested leaves both on day 6 (P = 0.3269) and on day 9 (P = 0.2352) (Fig. 3). Phytophagous Homoptera and Heteroptera secrete catechol oxidase and POX in their saliva (Peng and Miles, 1988; Miles and Peng, 1989), which enters the plant during feeding. The biological significance of POX is debatable, since similar oxidases occur in both plants and insects. Aphid feeding has been observed to induce localized and plant-wide increases in mRNA transcription and enzyme activity of several pathogenesis-related proteins, including POXs and chitinases (Stout et al., 1998; Fidantsef et al., 1999). On soybean, POX increased in foliage damaged by two-spotted spider mite (Hildebrand et al., 1986), and larval *H. zea* feeding increased the foliar POX activity 1.6 times (Bi and Felton, 1995).

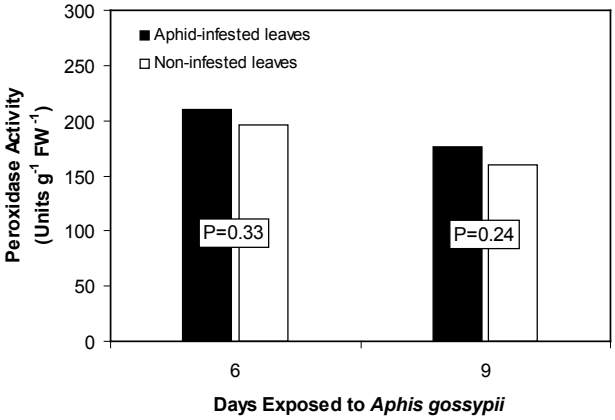

Figure 3. Peroxidase activity in non-infested (□) and aphid-infested leaves (■), after 6 and 9 d of exposure to *Aphis gossypii*. FW = fresh weight. Values are the means of twenty-seven observations.

Foliar APX activity was not affected by aphids after 6 d infestation (Fig. 4). After 9 d of aphid infestation, the APX activity increased, but not significantly (P = 0.09). APX increased in soybean and cotton foliage after herbivory by *H. zea* (Bi and Felton, 1995; Bi et al., 1997).

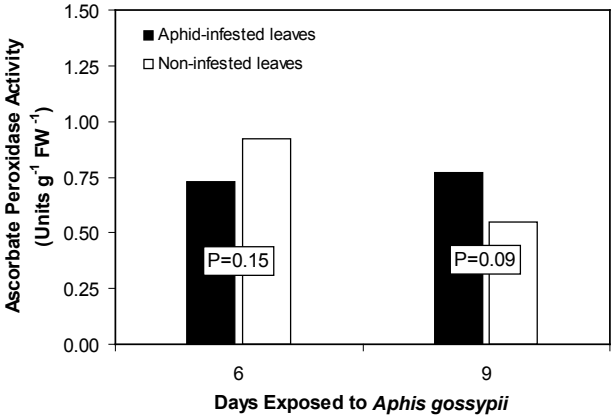

Figure 4. Ascorbate peroxidase activity in non-infested (□) and aphid-infested leaves (■), after 6 and 9 d of exposure to *Aphis gossypii*. FW = fresh weight. Values are the means of twenty-seven observations.

The GR activity was significantly higher (P = 0.0008) in aphid-infested leaves than in non-infested leaves after 6 d of infestation (Fig. 5). The main change of GR activity could be associated with a plant defensive mechanism against physiological damage produced by the aphids (Argandoña, 1994). On day 9, aphid-infested leaves had numerically higher enzyme activity than non-infested leaves, but it was not statistically significant (P = 0.1556). Probably, the highest activities of this enzyme occurred within 6 d after the damage with the activation of other antioxidant enzymes. GR activity increased 75% in roots and 31% in leaves of barley infested with aphids (*S. avenae*) for 3 d, and 125 and 32% for 9 d (Argandoña, 1994) . The same author found a similar response in wheat plants infested with aphids, in which GR activity increased 30 and 58% in the roots and leaves, respectively. A similar transient increase in GR activity was observed on cotton plants tolerant to NaCl stress (Gossett et al., 1994a; 1994b). The expression levels of all *Arabidopsis thaliana* (L.) genes involved with oxidative stress responses, except glutathione-S-transferase, were influenced by 72 h of *M. persicae* feeding, but not 96 h (Moran et al., 2002).

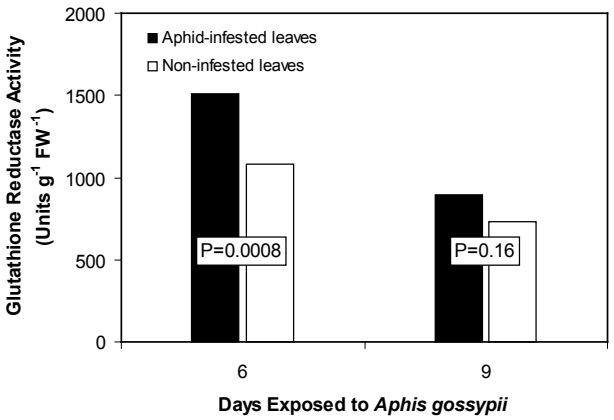

Figure 5. Glutathione reductase activity in non-infested (□) and aphid-infested leaves (■), after 6 and 9 d of exposure to *Aphis gossypii*. FW = fresh weight. Values are the means of twenty-seven observations.

In the cotton-aphid system, the activity of most foliar antioxidant enzymes was not increased, unlike the response in barley and wheat (Argandoña 1994, Argandoña et al., 2001), alfalfa (Dillwith et al., 1991), and *Arabidopsis* (Moran et al., 2002). Our data showed that foliar antioxidant responses vary depending on the aphid and plant combination. Some plants may be more susceptible to aphid herbivory.

CONCLUSIONS

Aphid herbivory for 6 and 9 d on cotton did not alter the foliar CAT, POX, and APX activities compared with non-infested leaves. The activity of GR was significantly higher in aphid-infested leaves than in non-infested leaves on day 6. In wheat and barley, GR activity was also highest at 3 and 9d, (Argandoña, 1994). This is the first report of antioxidant enzyme responses in cotton after *A. gossypii* herbivory. It would be worthwhile evaluating plant responses within 3 d of aphid herbivory to examine the enzyme activities at the beginning of the damage. The constitutive levels of CAT, POX and APX seemed relatively high in cotton leaves, which is in agreement with the results reported by Gossett et al. (1996). A separate study showed no alterations of photosynthesis or respiration rates after 9 d of aphid feeding (unpublished data). In general, antioxidant enzyme activity in cotton plants was not altered by the levels of infestation and feeding duration used in this study.

ACKNOWLEDGEMENT

We thank Dr. Nilda Burgos, Dept. Crop, Soil, and Environmental Sciences, University of Arkansas for allowing us to use her laboratory equipment, and Dr. Ronald McNew, Agricultural Statistics Lab, University of Arkansas for assistance with the statistical analysis. Dr. Kenneth Korth, Dept. Plant Pathology, University of Arkansas provided critical review of the manuscript. We appreciate the suggestions provided by anonymous reviewers.

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