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FOLIAR OXIDATIVE STRESS AND INSECT HERBIVORY: PRIMARY COMPOUNDS, SECONDARY METABOLITES, AND REACTIVE OXYGEN SPECIES AS COMPONENTS OF INDUCED RESISTANCE

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Abstract—Oxidative responses of plants to pathogens and other environmental stresses have received considerable recent attention. We propose that an oxidative response also occurs following attack by herbivores. Our data strongly indicate a shift in the oxidative status of soybean following herbivory by the insect *Helicoverpa zea*. Herbivory caused significant increases in lipid peroxidation and ·OH radical formation. The activity of several oxidative enzymes including lipoxygenases, peroxidase, diamine oxidase, ascorbate oxidase, and NADH oxidase I increased after herbivory on soybean. The enhanced production of phenolic compounds is indicated by an increase in the activity of phenylalanine ammonia lyase in wounded tissues. On the other hand, the level of soybean foliar antioxidants such as ascorbic acid, total carotenoids, nonprotein thiols, and catalase decreased significantly following herbivory. These results implicate 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 oxidative changes in the host plant correspond with increased oxidative damage in the midgut of insects feeding on previously wounded plants. Decreases in nonprotein thiols and reduced ascorbic acid occurred in midgut epithelial tissue from insects feeding on wounded plants compared to the insects on control plants. In contrast, midgut hydroperoxides and dehydroascorbic acid concentrations were greater in insects on wounded plants compared to their counterparts on control plants. We conclude that oxidative responses in soybean may have both positive and negative effects upon the host plant: a decrease in herbivory and an increase in oxidative damage to the plant. The salient benefit to the plant, in terms of insect resistance, is the relative balance between these opposing effects.

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Key Words—Oxidative stress; induced resistance, reactive oxygen species, soybean, midgut, ascorbic acid, *Helicoverpa zea*, lipoxygenase, peroxides.

INTRODUCTION

The role of oxidative stress in plant–pathogen interactions has received considerable attention during the last decade (Sutherland, 1991; Mehdy, 1994). It is now recognized that one of the most rapid responses in the plant cell following invasion by pathogens is an oxidative burst of reactive oxygen species (Doke, 1983; Apostol et al., 1989; Paxton and Groth, 1994; Mehdy, 1994). Reactive oxygen species include hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH$), and superoxide radical (O_2^-). Bursts of reactive oxygen species in plant tissues occur in response to viruses (Doke and Ohashi, 1988), bacteria (Adam et al., 1989; Devlin and Gustine, 1992), fungi (Doke, 1983; Montalbini, 1991; Davis et al., 1993), and nematodes (Zacheo and Bleve-Zacheo, 1988). These oxygen intermediates, particularly $\cdot OH$, are highly reactive and detrimental to living cells due to their ability to induce lipid peroxidation and DNA and protein oxidation (Fridovich, 1978; Halliwell and Gutteridge, 1985; Imlay et al., 1988). Furthermore, H_2O_2 may be an important signal for eliciting several plant defensive responses associated with systemic acquired resistance (Apostol et al., 1989; Chen et al., 1993).

The oxidative plant responses to attack by arthropod herbivores are comparatively unknown (Hildebrand et al., 1986a,b; Felton et al., 1994a,b). Spider mite damage on soybean foliage increased lipid peroxidation, lipoxygenase (LOX), and peroxidase (POX), but did not affect the levels of the antioxidant enzymes catalase (CAT) and superoxide dismutase (Hildebrand et al., 1986b). Foliar levels of antioxidant carotenoids declined with increasing mite populations (Hildebrand et al., 1986a). Phloem-feeding by aphids increased the levels of the antioxidant enzyme glutathione reductase in wheat and barley (Argandona, 1994), induced lipid peroxidation in alfalfa foliar tissues (Dillwith et al., 1991), and increased O_2^- formation in excised lucerne foliage (Jiang and Miles, 1993). Feeding damage to soybean by the phloem-feeding, three-cornered alfalfa hopper increased the activities of LOX, POX, polyphenol oxidase (PPO), and ascorbate oxidase (AOX) (Felton et al., 1994a).

Our laboratory has been examining the oxidative plant response to foliar feeding herbivores (Felton et al., 1994a,b). Herbivory by the corn earworm *Helicoverpa zea* (Lepidoptera: Noctuidae) on soybean increased LOX activity and lipid peroxidation (Bi et al., 1994; Felton et al., 1994b). Oxidative damage to foliar protein from wounded plants was indicated by a loss of free thiols (Bi et al., 1994). Felton et al. (1994a) reported that herbivory by the bean leaf beetle *Ceratoma trifurcata* also caused increases in LOX levels but did not affect POX or PPO in soybean.

The effects of herbivory on other reactive oxygen species, antioxidants, and many oxidative enzymes (e.g., NADH oxidase, diamine oxidases, etc.) have not been demonstrated. We initiated this study to gain a more complete understanding of the oxidative status of the host plant following foliar feeding. The system chosen for this study includes soybean and *H. zea*, a major pest in the mid-Atlantic region of the southeastern United States (Smith and Brim, 1979; Fitt, 1989). Our specific objectives were to: (1) assess the impact of herbivory on the oxidative status of soybean, and (2) assess the impact of induced responses in soybean on insect growth and on the oxidative status of the insect midgut.

METHODS AND MATERIALS

Plants and Insects. Eggs of *H. zea* were obtained from the University of Arkansas Insect Rearing Facility. Larvae were maintained on a wheat germ-based artificial diet until used in the experiment (Chippendale, 1970).

Soybean seeds (cv. Forrest or Hutcheson) were soaked in water for 6 hr and then incubated at 28°C for 24 hr. Preliminary experiments indicated that both cultivars showed similar oxidative responses to herbivory. Germinated seeds were sown in 430-ml polystyrene foam cups filled with soil mixture (Redi Earth Peat-Lite Mix) in the greenhouse. Plants were used in experiments at the V3 stage unless otherwise noted (Fehr et al., 1971). The cups were arranged in a completely randomized experimental design. Plants were watered every two days. Fertilizer (N/P/K = 20:20:20) was used weekly. Conditions were as follows: (1) 14-hr photophase, using high-pressure sodium light (1000 W) and (2) day temperature, 33 ± 2°C and night temperature, 20 ± 2°C.

Induction of Oxidative Responses. To determine if feeding by *H. zea* larva causes oxidative responses, soybean plants were grown in the greenhouse, treated with insects, and assayed for antioxidants and oxidants. A single fourth-instar *H. zea* was placed on each of five plants, and plants were placed individually in screen cages to prevent larval escape. Five control plants were placed in cages without larvae. After three days, fully expanded leaflets were excised from the uppermost node and assayed for enzymes and antioxidants as described below. Leaflets from the treated plants contained both damaged and undamaged leaflets. Preliminary results indicated that no significant differences existed between these leaflets in the variables assayed. A period of three days was initially chosen to investigate the oxidative changes because maximal systemically induced resistance to insects occurred within this period (Bi et al., 1994; Felton et al., 1994a). Percent defoliation was from ca. 20% by visual estimation using calibrated standards (Kogan and Kuhlman, 1982). The soybean cultivar Forrest was used for LOX, PPO, and nonprotein thiol determinations, whereas Hutcheson was

used for POX, AOX, ascorbate peroxidase (APX), ascorbic acid (ASC), dehydroascorbic acid (DHA), CAT, diamine oxidase (DAO), NADH oxidase I, carotenoids, lipid peroxidation, and ·OH assays. Assays were replicated three times, and the data were analyzed by one-way completely randomized ANOVA and mean differences were determined by LSD at $P = 0.05$.

Time Course for Induction of Oxidative Enzymes. To determine the time course of oxidative changes following feeding by *H. zea*, V4-stage soybean plants (cv. Hutcheson) were grown in the greenhouse, treated with insects, and assayed as described above. The levels of LOX (pH 5.5), POX, and AOX were determined after 24, 48, 72, and 96 hr of feeding by larvae. Six plants per treatment were used for each time period.

Plant Enzyme Assays. To assay for foliar enzymes, 1 g of foliage was homogenized in 10 ml 0.1 M ice-cold K Pi buffer, pH 7.0, containing 1% PVP and 0.5 mM EDTA. The homogenate was centrifuged at −2°C for 20 min at 10,000*g*, and the supernatant was used immediately as the enzyme source for the assays described below. A SLM-AMINCO 3000 diode array spectrophotometer with rate analysis software was used for all assays.

To assay for LOX, 0.25 mM linoleic acid was used as a substrate, and the rate of change in A_{234} was measured (Grayburn et al., 1991). The reaction mixture contained 50 μl of enzyme homogenate and 1 ml of substrate. LOX activity was assayed at pH 5.5, 7.0 (0.1 M K Pi), and 8.5 (0.1 M Na borate) to account for the activities of several isozymes. An extinction coefficient of 27 mM^{-1} cm^{-1} was used.

To assay for POX activity, guaiacol was used as the hydrogen donor according to the procedure of Ridge and Osborne (1970). Fifty microliters of enzyme solution was mixed with 1.0 ml of substrate containing 1 mM H_2O_2 and 2 mM guaiacol in 0.1 M K Pi, pH 7.0. POX activity was estimated from the increase in A_{470}.

To determine PPO activity, the procedure of Kahn and Miller (1987) was followed. Twenty-five microliters of enzyme solution was mixed with 200 μl of 20 mM Tiron (4,5-dihydroxy-1,3-benzenedisulfonic acid disodium salt) in 0.1 M K Pi buffer, pH 7.0, and PPO activity was estimated from A_{420}.

NADH oxidase I activity was measured as a decrease in A_{340} due to the loss of NADH (Brightman et al., 1991). The reaction mixture contained 100 μl enzyme extract in 2.9 ml 20 mM Tris Mes, pH 7.0, containing 1 mM KCN and 300 μM NADH. An extinction coefficient of 6.22 mM^{-1} cm^{-1} was used.

DAO activity was measured with 10 mM putrescine (diaminobutane) in 0.1 M K Pi, pH 7.0 (Federico et al., 1985). The reaction was initiated with 1 ml enzyme extract in 3 ml of substrate. After incubation at 37°C for 10 min, the reaction was stopped by adding 0.5 ml of 10% (w/v) TCA followed by 0.05 ml (10 mg/ml) *o*-aminobenzaldehyde in 95% ethanol. The absorbance of the Δ^1-pyrroline complex was measured at A_{430} after removal of protein by centrif-

ugation at 3000*g* for 20 min. An extinction coefficient of 1.86 mM^{-1} cm^{-1} was used (Federico et al., 1985).

AOX activity was measured according to the procedures of Felton and Summers (1993). The reaction mixture contained 100 μl enzyme homogenate, 1 ml of 0.1 M K Pi buffer (pH 7.0) with 0.15 mM ASC, 0.5 mM EDTA, and 0.002% metaphosphoric acid. The decrease in A_{265} of ASC was monitored, and an extinction coefficient of 14.7 mM^{-1} cm^{-1} was used.

APX activity was measured following Mittler and Zilinskas (1991) with the addition of 1 mM ASC to the extraction buffer. The reaction mixture contained 0.50 mM ASC, 50 mM K Pi buffer, pH 7.0, 0.1 mM H_2O_2, and 50 μl enzyme homogenate in a 1.5-ml total volume. Corrections were made for ASC disappearance due to nonenzymatic oxidation and H_2O_2-independent oxidation. An extinction coefficient of 2.8 mM^{-1} cm^{-1} for A_{290} was used.

CAT activity was monitored by following the loss of H_2O_2 at 240 nm (Aebi, 1984). The substrate was 0.059 M H_2O_2 in 0.05 M K Pi buffer, pH 7.0, containing 1 mM EDTA. The reaction was initiated by adding 100 μl enzyme homogenate to 2 ml of substrate, and enzyme activity was determined from the linear portion of the rate curve (A_{450} to A_{400}).

To assay for phenylalanine ammonia lyase (PAL) activity, the enzyme extract was prepared following Degousee et al. (1994). Briefly, 1 g fresh weight of soybean foliage was homogenized in 10 ml Na borate buffer, pH 8.8, containing 2.5% PVP and 15 mM 2-mercaptoethanol and then centrifuged at 20,000*g* for 15 min. The reaction mixture contained 100 μl supernatant from the enzyme extract and 1 ml 20 mM L-Phe. Reaction mixture was incubated for 1 hr at 40°C, and the change in A_{290} was measured against a blank without substrate (Degousee et al., 1994). An extinction coefficient of 10 mM^{-1} cm^{-1} was used.

Chemical Antioxidants. Carotenoid analysis followed the procedure of Hildebrand et al. (1986a). One gram of fresh foliage from each plant was lyophilized and then pulverized in a mortar and pestle. The pulverized sample was extracted with 8 ml of petroleum ether containing 0.45 mM butylated hydroxytoluene. The resulting mixture was incubated for 15 min at 30°C in a water bath, vortexed, and centrifuged at 2000*g* for 30 min. The A_{450} of the supernatant was measured, and an $E^{1\%}$ (Extinction coefficient of 1% concentration) value of 25,000 was used for carotenoid content.

The concentrations of foliar ASC and DHA were determined by a spectrophotometric assay (Law et al., 1983). Total ASC (ASC + DHA) was measured after reduction of DHA by DTT. Reduced ASC was estimated in the absence of DTT. DHA was estimated from the difference of total ASC and reduced ASC. Five hundred milligrams of foliage was homogenized in 5 ml of 5% metaphosphoric acid and centrifuged at 10,000*g* for 20 min. The reaction mixture for total ASC contained 0.02 ml of tissue aliquot, 0.05 ml of 150 mM Na Pi (pH 7.4) containing 5 mM EDTA, and 0.01 ml of 10 mM DTT. After 10

min at room temperature, 0.01 ml of 5 mM *N*-ethylmaleimide was added to remove DTT. Color was developed with 0.04 ml of 10% TCA, 0.04 ml of 44% orthophosphoric acid, 0.04 ml of 4% α,α'-dipyridyl in 70% ethanol, and 0.02 ml of 3% $FeCl_3$. The color development was processed at 25°C for 3 hr, and the A_{525} was read. The assay for reduced ASC omitted the DTT and *N*-ethylmaleimide, using 0.02 ml water as a replacement. L-ASC was used as a standard.

To assay for nonprotein thiols, 500 mg foliage was homogenized in 5 ml 5% metaphosphoric acid and centrifuged at 10,000*g* for 20 min. The color was developed by adding 0.1 ml of supernatant to 2.5 ml of 2.5 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) in 0.2 M K Pi buffer, pH 8.0 (Anderson and Wetlaufer, 1975). The A_{412} was recorded and concentrations determined from a standard curve for glutathione.

Plant Hydroxyl Radical Formation, Lipid Peroxidation, and Total Foliar Peroxides. To assay for ·OH formation in soybean, DMSO was used as a specific detector for ·OH formation in vivo (Babbs et al., 1989; Popham and Novacky, 1991). DMSO may also react with the potent oxidant peroxynitrite (Crow et al., 1994). The wounded DMSO treatment contained five greenhouse-grown plants treated with one larva per plant and watered with 0.5% DMSO. The control DMSO treatment contained five plants that were treated identically except that no larvae were placed on the plants. The remaining 10 control plants contained five plants with larvae and five without. Twelve hours prior to infesting plants with larvae, the DMSO-treated plants were watered to saturation with 0.5% DMSO and control plants were watered with tap water. Larvae were placed on treatment plants for 24 hr. The watering procedure was repeated three times during the experiment. Foliage was then excised from the two uppermost nodes (2 g/plant), quickly frozen with dry ice, and then ground with a mortar and pestle. The resulting slurry was extracted with distilled water and centrifuged to remove solid materials (Babbs et al., 1989). Removal of interfering chemicals and the color reaction with fast blue BB dye was as described (Babbs et al., 1989). The A_{420} of the methane sulfinic acid product was read, and concentrations were calculated based upon a standard curve. The A_{420} of the appropriate water-treated controls was subtracted from the DMSO treatments. Nonspecific absorbance in the water treatments was 28% of the DMSO treatments.

Lipid peroxidation was assayed using the thiobarbituric acid assay (Dhindsa and Matowe, 1981). One gram of fresh foliage was homogenized in 5 ml H_2O, and 5 ml of 5 mM thiobarbituric acid in 20% TCA was added. The mixture was heated to 95°C for 30 min and then quickly cooled in an ice-bath. The cold mixture was centrifuged at 10,000*g* for 20 min, and the A_{532} and A_{600} were recorded. The nonspecific A_{600} was subtracted from the A_{532}. Thiobarbituric-reactive substances were expressed as malondialdehyde equivalents using the extinction coefficient of 155 mM^{-1} cm^{-1}.

Total hydroperoxides (ROOH) were measured following Jiang et al. (1992). The method is based on the hydroperoxide-mediated oxidation of Fe^{2+} to Fe^{3+} under acidic condition. The Fe^{3+} forms a Fe^{3+}–xylenol orange complex measured at 560 nm. The extinction coefficients for lipid hydroperoxides and H_2O_2 are very similar in this assay (Jiang et al., 1991). Foliar samples were homogenized in the same buffer used for preparation of enzyme extract, but with the addition of 2.5% TCA and 1 mM BHT. After centrifugation of the homogenate, 100 μl of the sample was incubated with a 900-μl reaction mixture containing 100 μM xylenol orange, 250 μM Fe^{2+}, 25 mM H_2SO_4, and 4 mM BHT in 90% (v/v) methanol. After a 30-min incubation, the A_{560} was recorded and concentrations of hydroperoxides were calculated based on a standard curve with H_2O_2.

Effect of Induced Responses on Oxidative Status of Insect Midgut. To determine the effect of induced responses in soybean plants on larval growth and the oxidative status of the midgut epithelium, V6-stage soybean plants (cv. Hutcheson) were grown in cages in the field (Felton et al., 1994a). Three third-instar *H. zea* were placed on each treatment plant for three days. After this time period, the third instars were removed from plants and discarded. Five plants from each treatment were assayed for POX, LOX (pH 5.5), and LOX (pH 7.0). Newly molted fifth instars were then placed on each remaining control and treatment plant. Initial weights of these larvae were not significantly different ($P = 0.514$). Mean weight of larvae placed on the treatment plants was 125.7 $\pm$ 6.5 mg, and mean weight of larvae on control plants was 121.5 $\pm$ 5.0 mg. A total of 30 larvae per treatment was tested.

After three days, larvae were removed from each plant and weighted. The midgut epithelium was removed, weighed, and homogenized (Summers and Felton, 1994). Midgut tissue was pooled with three larvae per replicate providing a total of 10 replicates per treatment. ASC and ROOH were assayed using the assays described above and modified following Summers and Felton (1994). Nonprotein thiols were determined with DTNB in absolute methanol (Hu, 1994). Because of the movement of larvae between plants in their respective cages, the relative growth rate (milligrams per day/per milligram of larva) was calculated using the mean initial weights rather than individual larval weights (Felton et al., 1994a).

RESULTS

Foliar Oxidative Response to Herbivory. Larval *H. zea* feeding on soybean increased the activities of several foliar oxidative enzymes. LOX activities increased by 2.5-fold when measured at pH 5.5 ($P < 0.001$), 2.81-fold at pH 7.0 ($P < 0.001$), and 3.34-fold at pH 8.5 ($P < 0.001$) compared with the control foliage (Table 1). The feeding also significantly increased the activities of several other foliar oxidases (Table 1) such as POX by 1.6-fold ($P = 0.006$),

TABLE 1. EFFECT OF HERBIVORY ON FOLIAR ENZYME ACTIVITIES IN SOYBEAN

Enzyme	Control	Wounded	LSD (0.05)
AOX			
(nmol/min/g fresh weight)	219.0 (±33.3)[a]	468.1 (±89.3)	95.2
APX			
(μmol/min/g fresh weight)	2.95 (±0.49)	4.442 (±0.51)	0.76
CAT			
(μmol/min/g fresh weight)	143 (±30)	92 (±25)	45.1
DAO			
(μmol/min/g fresh weight)	447 (±77)	704 (±104)	92.0
LOX (pH 5.5)			
(nmol/min/g fresh weight)	420 (±63)	1050 (±148)	160
LOX (pH 7.0)			
(nmol/min/g fresh weight)	309 (±89)	867 (±165)	177
LOX (pH 8.5)			
(nmol/min/g fresh weight)	210 (±68)	701 (±171)	204
NADH oxidase I			
(nmol/min/g fresh weight)	72.6 (±33.4)	112.9 (±17.7)	34.3
PAL			
(nmol/min/g fresh weight)	9.9 (±5.1)	23.3 (±11.5)	9.03
POX			
(nmol/min/g fresh weight)	23.0 (±5.5)	36.8 (±6.1)	8.5
PPO			
(Δ OD/min/g fresh weight)	6.5 (±0.7)	8.5 (±1.2)	NS

[a]Numbers in parentheses are standard deviations.

DAO by 1.57-fold ($P < 0.001$), AOX by 2.14-fold ($P = 0.006$), and NADH oxidase I by 1.56-fold ($P = 0.026$) (Table 1). PAL activity was increased 2.35-fold ($P = 0.001$) by herbivory, but PPO was not significantly affected ($P = 0.06$) (Table 1). The level of the antioxidant enzyme CAT decreased 36% ($P = 0.03$) in damaged foliage compared to control foliage; however, another antioxidant enzyme, APX, increased 1.5-fold ($P = 0.002$) following herbivory (Table 1).

Feeding by *H. zea* in soybean also resulted in significant changes in foliar antioxidants as indicated by a 26% loss of ASC ($P = 0.003$) and 104% higher DHA ($P = 0.02$) level compared to controls (Table 2). The ratio of ASC to DHA was reduced from 11.68 to 3.62 reflecting the significant change in redox status. Herbivory resulted in a greater than 33% decrease in carotenoid concentration ($P < 0.001$) and 8% loss of nonprotein thiols ($P < 0.001$) in comparison with those in control foliage (Table 2).

TABLE 2. EFFECT OF HERBIVORY ON FOLIAR ANTIOXIDANTS IN SOYBEAN

Antioxidant	Control	Wounded	LSD (0.05)
Total ASC (μg/g fresh weight)	621.5 (±50.1)[a]	462.8 (±40.5)	70.6
Reduced ASC (μg/g fresh weight)	572.5 (±50.2)	362.6 (±41.0)	71.6
DHA (μ/g fresh weight)	49.0 (±11.6)	100.2 (±39.5)	42.4
ASC:DHA	11.68	3.62	
Total carotenoids (μ/g fresh weight)	126 (±20)	84 (±12)	21.0
Nonprotein thiols (ng/g fresh weight)	279 (±5.8)	257 (±4.8)	7.7

[a] Numbers in parentheses are standard deviations.

Folivory by *H. zea* caused an increase in ·OH flux by 3.87-fold ($P = 0.038$) in wounded soybean foliage compared to the undamaged control treatment (Table 3). Lipid peroxidation increased in wounded tissue as indicated by a 17% increase in malondialdehyde equivalents ($P = 0.009$) (Table 3). Total hydroperoxide levels were increased by 2.26-fold ($P = 0.002$) in the wounded tissue compared to the control treatment (Table 3).

Time Course for Induction of Oxidative Enzymes. Herbivory caused significant increases in LOX after 24 hr of feeding, which continued throughout the sampling period ($P < 0.05$; Figure 1). Maximal increase occurred at 72 hr for LOX and for POX, but the POX increase was significant ($P < 0.05$) only

TABLE 3. EFFECT OF HERBIVORY ON FOLIAR LIPID PEROXIDATION, TOTAL HYDROPEROXIDES, AND HYDROXYL RADICAL (·OH) LEVELS IN SOYBEAN

Oxidant	Control	Wounded	LSD (0.05)
Malondialdehyde equivalents (μmol/g fresh weight)	1.35 (±0.07)	1.58 (±0.19)	0.17
Total hydroperoxides (nmol/g fresh weight)	71.9 (±47.1)	162.4 (±26.3)	50.6
Hydroxyl radical (nmol/g fresh weight)	1.75 (±0.90)	6.78 (±1.36)	2.45

[a] Numbers in parentheses are standard deviations.

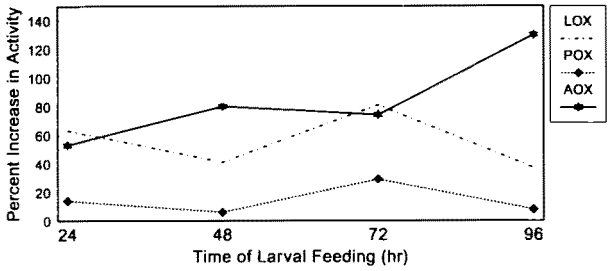

FIG. 1. Effect of length of herbivory on oxidative enzymes in soybean foliage. AOX = ascorbate oxidase; LOX = lipoxygenase measured at pH 5.5; and POX = peroxidase.

at 72 hr of herbivory. AOX was significantly increased throughout all time periods ($P < 0.05$) and was maximal after 96 hr of larval feeding.

Effect of Induced Responses on Oxidative Status of Insect Midgut. The oxidative shift in soybean plants caused by insect feeding was indicated by a 2.09-fold increase in LOX (pH 5.5), a 1.44-fold increase in LOX (pH 7.0), and a 1.42-fold increase in POX (Table 4). The ingestion of previously wounded plant tissue by larvae affected relative growth rate and the oxidative status of the midgut. The relative growth rate of larvae feeding on the wounded plants was reduced by more than 62% compared to larvae on control plants ($P < 0.001$; Table 4). Evidence that oxidative damage occurred in larvae ingesting wounded tissue was indicated by the fact that total ASC was decreased by ca. 15% ($P = 0.002$), reduced ASC was decreased by more than 20% ($P < 0.001$), and the oxidized form of ASC, DHA, was increased by 2.25-fold ($P = 0.005$). Accordingly, the ratio of midgut ASC to DHA decreased from 26.50 in larvae on control plants to 9.33 in larvae feeding on wounded plants. The levels of nonprotein thiols were significantly decreased in the wounded treatment by 48% ($P < 0.001$). Total hydroperoxides, an indicator of oxidative stress, were more than 66% higher in larvae feeding on the wounded plants compared to larvae on control plants ($P = 0.035$).

DISCUSSION

Plant chemical defense against insects is largely ascribed to plant secondary metabolites (Rosenthal and Berenbaum, 1991) and to a lesser extent primary metabolites such as amino acids, water, vitamins, etc. (Slansky, 1990). Our data indicate that the induced responses to herbivory in soybean entail a three-fold, integrated response (Figure 2) that includes: (1) enhanced secondary metabolite production (e.g., phenylalanine ammonia lyase; Liu et al., 1992;

TABLE 4. EFFECT OF HERBIVORY IN SOYBEAN ON FOLIAR OXIDATIVE ENZYMES, LARVAL GROWTH, AND OXIDATIVE STATUS OF INSECT MIDGUT EPITHELIUM

Chemical	Control	Wounded	LSD (0.05)
Plant enzymes			
LOX (pH 5.5)			
(nmol/min/g fresh weight)	189 (±31)[a]	395 (±79)	88
LOX (pH 7.0)			
(nmol/min/g fresh weight)	138 (±16)	199 (±35)	46
POX			
(Δ OD/min/g fresh weight)	29.6 (±3.9)	41.9 (±4.3)	6.6
Insect factors			
Relative growth rate			
(mg/da/mg larva)	0.204 (±0.07)	0.077 (±0.05)	0.02
Midgut total ASC			
(mg/g fresh weight)	1.10 (±0.08)	0.93 (±0.11)	0.09
Midgut reduced ASC			
(mg/g fresh weight)	1.06 (±0.09)	0.84 (±0.08)	0.08
Midgut DHA			
(mg/g fresh weight)	0.04 (±0.01)	0.09 (±0.03)	0.02
Midgut ASC:DHA	26.50	9.33	
Midgut nonprotein thiols			
(μmol/g fresh weight)	5.03 (±1.37)	2.61 (±1.04)	1.34
Midgut hydroperoxides			
(μmol/g fresh weight)	0.36 (±0.07)	0.60 (±0.17)	0.20

[a] Numbers in parentheses are standard deviations.

Table 1); (2) a loss in the quantity and/or quality of essential nutrients and antioxidants (e.g., protein, Bi et al., 1994; ascorbic acid, Table 2); and (3) the production of reactive oxygen species (e.g., hydroxyl radical, Table 3; hydrogen peroxide, superoxide radical). However, at present it is not known if these oxidative responses are the direct result of herbivory or the result of microbes entering open wounds caused by insect feeding. Nonetheless, we suggest that the net impact upon the herbivore involves both nutritional and oxidative stress (Figure 2).

The role of reactive oxygen species has been largely overlooked as a component of plant defense (Norris and Liu, 1992; Ahmad, 1992; Appel, 1993; Norris, 1994) for several reasons. First, there are no clearly defined pathologies or diseases associated with oxidative stress in insects as have been defined for mammals and humans (e.g., amytrophic lateral sclerosis, atherosclerosis, etc.; Felton, 1995). Second, because many consider oxidative stress to be a chronic phenomenon in animals, some scientists have assumed that insects are too short-

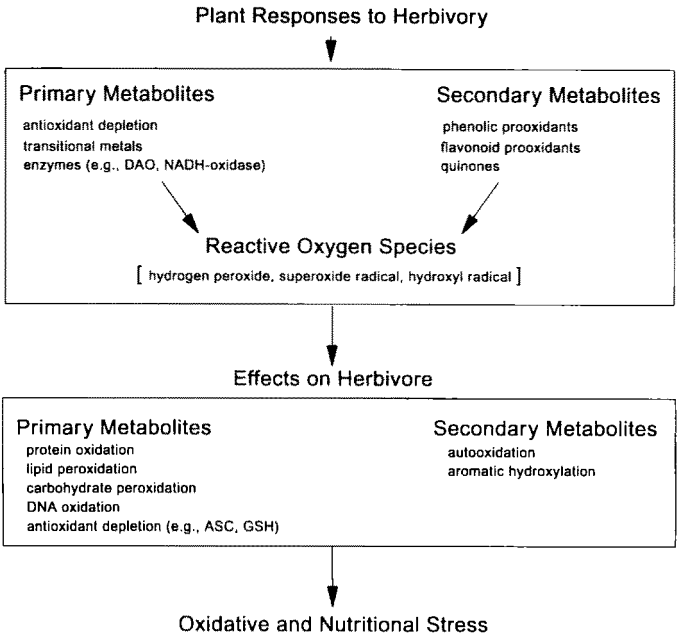

FIG. 2. Threefold response of soybean foliage to insect herbivory. Numerous interactions may take place among the metabolites produced in response to herbivory. For example, phenolic prooxidants may produce reactive oxygen species or may bind to proteins. Alternatively, phenolics may be reduced by primary metabolites (e.g., ascorbate, cysteine) or be oxidized by enzymes requiring reactive oxygen species such as hydrogen peroxide.

lived to be affected by oxidative stress. However, it has been recently shown that even short-lived insects such as *Drosophila melanogaster* can greatly benefit by additional antioxidant protection (Orr and Sohal, 1994). Third, the methodologies for measuring reactive oxygen species (e.g., ESR spectroscopy and spin traps) have been relatively difficult and expensive. Furthermore, these methodologies have been difficult or impossible to deploy for many *in situ* or *in planta* determinations. Fourth, because of the highly transitory behavior of reactive oxygen species, it is essentially impossible to use artificial diets to bioassay their effect directly. Thus, chemical fractionation of plant tissues to isolate and identify potential sources of chemical resistance may fail to account for not only the activities of plant enzymes (e.g., polyphenol oxidase; Felton et al., 1989; peroxidase, Liu et al., 1993), but also for potential contribution of reactive oxygen species. The problems in causally linking insect herbivore performance with oxidative stress are difficult but not insurmountable. Decades of intensive

research in the biomedical field have encountered similar difficulties, but the use of selective inhibitors of antioxidant systems and the ability to genetically modify the expression of antioxidant systems has contributed to rapid progress in recent years.

Several lines of evidence suggest that oxidative stress may be an important factor in insect biology and a potential mechanism of plant resistance to insects. First, insect life-span and fitness can be increased by elevated levels of chemical antioxidants, as well as the overexpression of antioxidant enzymes in transgenic insects (Orr and Sohal, 1994; Felton and Summers, 1995).

Second, selective modification of the antioxidant status of insects has demonstrated the detrimental effects of dietary prooxidants. For instance, specific inhibition of antioxidant enzymes (e.g., superoxide dismutase) decreases insect performance on certain host plants or artificial diets rich in prooxidants (Pritsos et al., 1991; Ahmad, 1992). Elevated levels of dietary chemical antioxidants such as vitamin E, carotenoids, and ascorbate decrease the toxicity of prooxidant compounds (Aucoin et al., 1990; Summers and Felton, 1994).

Third, insects feeding on previously wounded foliage show evidence of oxidative damage in the midgut (Table 4). The toxicological effect of the oxidative stress in the midgut is unknown, but disruptions in absorption, transport, and membrane-bound enzymes may be likely (Yu, 1994). The oxidative change in foliage may alter the nutritional quality of foliage to herbivores as well. The loss of nutrients such as ASC, free fatty acids (e.g., linoleic acid), and carotenoids that occur in damaged foliage may disrupt normal growth and development of insects (Dadd, 1973). Bi et al. (1994) also showed that prior herbivory reduced the nutritional quality of foliar protein to *H. zea*.

Fourth, herbivory causes increases in reactive oxygen species, namely, ·OH (Table 3) in soybean foliage. The ·OH radical is the most reactive known and can attack and damage virtually any molecule found in living cells (Halliwell, 1991). ·OH reacting with lipids, proteins, or DNA can initiate chain reactions that involve lipid peroxidation, protein oxidation and fragmentation, and substantial alteration of DNA (Halliwell, 1991). Several enzymatic systems may contribute to elevated ·OH levels. The ·OH may be formed from H_2O_2 during metal-catalyzed reactions (Halliwell, 1991). Increases in H_2O_2 may arise from several biochemical pathways in different cellular compartments. Our results (Tables 1 and 4) indicate that H_2O_2 may arise from several enzymes that produce reactive oxygen species as primary or secondary products including NADH oxidase, DAO, POX, and LOX (Kanofsky and Axelrod, 1986; Chamulitrat et al., 1991; Sutherland, 1991; Vianello and Macri, 1991; Liu et al., 1993). Alternatively, H_2O_2 accumulation could result from the decreased levels of several scavenging agents such as CAT, ASC, or thiols (Tables 1, 2, and 4). However, the H_2O_2-scavenging enzyme APX increased following herbivory (Table 1). We have not differentiated the chloroplastic vs. cytosolic APX isozymes, and thus

do not know the relative importance of differences in H_2O_2 synthesis and degradation between different cellular compartments. Herbivory caused a decline in CAT levels, which have also been implicated in plant–pathogen interactions (Chen et al., 1993). The inhibition of CAT was due to induction of salicylic acid, a potent CAT inhibitor (Chen et al., 1993). Presumably levels of H_2O_2 increase following CAT inhibition and then act as an elicitor of plant defenses (Chen et al., 1993). It is unknown if the decrease in CAT following herbivory is due to enhanced salicylic acid biosynthesis.

Fifth, increases in oxidative enzymes and other oxidative changes in soybean may serve in several manners to enhance insect resistance (Liu et al., 1993; Felton et al., 1994a,b). Increased production of H_2O_2 by DAO (Federico and Angelini, 1986, 1988; Angelini and Federico, 1989; Angelini et al., 1990) may provide substrate for POXs. NADH oxidase occurs in the plasma membrane of plant cells and oxidizes NADH or NADPH to form O_2^- that then dismutates to H_2O_2 (Doke et al., 1991; Brightman et al., 1991). POXs are important in cell wall strengthening, including lignification (Goldberg et al., 1985; Lagrimini et al., 1987), suberization (Espelie and Kolattukudy, 1985; Espelie et al., 1986), and cross-linking of hydroxyproline-rich cell wall protein (Fry, 1986). These cross-linking processes may ultimately decrease the digestibility of plant tissues for herbivores. PAL provides phenolic precursors for these reactions (Goldberg et al., 1985). Moreover, oxidative products of enzymes (e.g., phenolic prooxidants, quinones, lipid hydroperoxides, aldehydes) can impose considerable toxicological assault upon herbivores (Duffey and Felton, 1991; Appel, 1993; Summers and Felton, 1994; Barbehenn and Martin, 1994). Increases in PAL activity (Table 1) may lead to increased levels of phenolic prooxidants such as caffeic acids that are toxic to larvae (Summers and Felton, 1994). The volatile aldehyde products of LOX are repellent to certain insect herbivores including bean bugs *Riptortus clavatus*, strawberry leafy beetles *Galerucella vittaticollis*, and false melon beetles *Atrachya menetriesi* (Mohri et al., 1990). Moreover, the induction of LOXs may be important in defense signal transduction because LOXs are involved in the initial phases of jasmonic acid biosynthesis (Ueda and Kato, 1980; Vick and Zimmerman, 1987; Enyedi et al., 1992). The jasmonates induce the synthesis of several plant defensive proteins or chemicals, including proteinase inhibitors, chalcone synthase, proline-rich cell wall proteins, PAL, and alkaloids (Creelman et al., 1992; Enyedi et al., 1992; Hamberg and Gardner, 1992).

Oxidative responses in plants may have both positive and negative effects upon the host plant: a decrease in herbivory but an increase in oxidative damage to the plant. Oxidative stress is an important factor in plant responses to several environmental stresses including chilling temperatures (Prasad et al., 1994; Gupta et al., 1993), phytopathogens (Devlin and Gustine, 1992; Aver'yanov et al., 1993), NaCl injury (Gossett et al., 1994), and environmental pollutants (Badiani

et al., 1993; Guzy and Heath, 1993). Our data provide more substantive evidence that plant responses to herbivory also include changes in the oxidative status of foliage. Consequently, herbivory may predispose the plant to damage by other environmental stresses that impose oxidative stress to the plant. Conversely, the oxidative changes may be a component of plant defense against herbivores and other pests. The salient benefit to the plant, in terms of insect resistance, may represent the relative balance between these opposing effects.

In summary, we propose that at least five potential functions for oxidative stress in plant–herbivore interactions exist including: (1) direct oxidative injury to the herbivore (Appel, 1993; Felton et al., 1994b; Summers and Felton, 1994); (2) indirect injury to the herbivore through oxidative damage to dietary nutrients and antioxidants (Felton et al., 1989, 1992; Bi et al., 1994; Barbehenn and Martin, 1994); (3) obstruction of access to plant protein and nutrients via rapidly induced oxidative cross-linkage of cell wall proteins (Bradley et al., 1992); (4) formation of volatile compounds that are repellent to herbivores (Mohri, 1990); and (5) signal transduction for elicitation of plant defenses (Apostol et al., 1989; Norris and Liu, 1992; Creelman et al., 1992; Farmer et al., 1992; Chen et al., 1993; Norris, 1994). Thus, induced resistance in certain soybean cultivars may be composed of a threefold, integrated response involving primary metabolites, secondary metabolites, and reactive oxygen species (Figure 2). Further research is needed to determine if these results are generalizable to other plant cultivars and species.

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