Indirect effects of insect herbivory on leaf gas exchange in soybean

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ABSTRACT

Herbivory can affect plant carbon gain directly by removing photosynthetic leaf tissue and indirectly by inducing the production of costly defensive compounds or disrupting the movement of water and nutrients. The indirect effects of herbivory on carbon and water fluxes of soybean leaves were investigated using gas exchange, chlorophyll fluorescence and thermal imaging. Herbivory by *Popillia japonica* and *Helicoverpa zea* (Boddie) caused a 20–90% increase in transpiration from soybean leaflets without affecting carbon assimilation rates or photosynthetic efficiency (Φ_{PSII}). Mechanical damage to interveinal tissue increased transpiration up to 150%. The spatial pattern of leaf temperature indicated that water loss occurred from injuries to the cuticle as well as from cut edges. A fluorescent tracer (sulforhodamine G) indicated that water evaporated from the apoplast approximately 100 μm away from the cut edges of damaged leaves. The rate of water loss from damaged leaves remained significantly higher than from control leaves for 6 d, during which time they lost 45% more water than control leaves (0.72 mol H_2O per cm of damaged perimeter). Profligate water loss through the perimeter of damaged tissue indicates that herbivory may exacerbate water stress of soybeans under field conditions.

Key-words: *Glycine max*; chlorophyll fluorescence imaging; herbivorous insects; herbivory; photosynthesis; thermal imaging; transpiration.

INTRODUCTION

In addition to directly damaging photosynthetic tissue, herbivores may indirectly affect gas exchange in remaining leaf tissue by diverting resources to defence or disrupting the transport of nutrients and water (Welter 1989; Sack, Cowan & Holbrook 2003; Nykänen & Koricheva 2004), but these effects are highly variable. For example, in wild parsnip (*Pastinaca sativa*) damage by cabbage loopers (*Trichoplusia*

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ni) caused a three-fold greater reduction in photosynthesis than predicted based solely on tissue loss (Zangerl *et al.* 2002). In contrast, an increase in photosynthetic efficiency in cucumber (*Cucumis sativum*) compensated for ~ 80% of the leaf area removed by herbivory (Thomson *et al.* 2003). The mechanisms underlying the indirect effects of foliar damage on photosynthesis and other physiological processes are not well understood and vary for different plant-insect combinations (Hunter 2001; Peterson, Shannon & Lenssen 2004).

Insect damage frequently initiates complex and often interacting processes that affect gas exchange in the remaining leaf tissue. The induction of an arsenal of defence compounds (Karban & Myers 1989; Leon, Rojo & Sanchez-Serrano 2001; Kessler & Baldwin 2002) can divert carbon and nitrogen away from primary metabolism, and many secondary compounds alter the photochemical status of the leaf. For example, changes in pigment content following mechanical damage (Herde *et al.* 1999) and the production of reactive oxygen species (Bi & Felton 1995; Thordal-Christensen *et al.* 1997; Leon *et al.* 2001; Bown, Hall & MacGregor 2002) may reduce photosystem II operating efficiency (Φ_{PSII}) and carbon assimilation rates in damaged leaves. At the molecular level, herbivory may up-regulate defence genes at the expense of genes coding for rate-limiting constituents of photosynthetic metabolism (Schenk *et al.* 2000).

While the indirect effect of herbivory on photosynthesis has received some attention, its potential influence on leaf water status is poorly understood. Acting in a similar way to wind damage (Wilson 1980; Hoad *et al.* 1998), herbivory disrupts the integrity of leaves, creating the possibility for uncontrolled water loss from cut edges or abraded cuticle (Ostlie & Pedigo 1984; Welter 1989). Evaporation may occur from the mesophyll or water may travel directly to the cut edge through the apoplasm or symplasm of epidermal and mesophyll cells (Cany 1990; Barbour & Farquhar 2003).

Technologies that generate spatially resolved measurements can reveal previously undetected effects of herbivory on photosynthesis and transpiration. Using gas-exchange methods and several species of legumes including soybean, Peterson *et al.* (2004) were unable to detect effects of herbivores on photosynthesis and stomatal conductance in tis-

sues adjacent to those that were damaged. However, spatial maps of chlorophyll fluorescence of soybean (*Glycine max* L.) leaves revealed that insect locomotion across leaf surfaces caused localized reductions in photosynthesis and corresponding increases in the production of reactive oxygen species and the signalling molecule 4-aminobutyrate (Bown *et al.* 2002).

The objective of this research was to quantify the spatial pattern of photosynthesis and transpiration across individual soybean leaves following herbivory. Using chlorophyll fluorescence and thermal imaging coupled with gas-exchange measurements, we investigated the effects of foliar damage on carbon and water fluxes through the damaged leaf as well as the spatial pattern of photosystem II operating efficiency (Φ_{PSII}). We also examined the path of water transport through injured leaves using a fluorescent dye tracer and the experimental disruption of major veins.

MATERIALS AND METHODS

Plants and insects

For insect feeding trials, soybeans (*Glycine max* L., cv. Pioneer 93B15) were grown in 5-L pots in a growth chamber with a 14-h photoperiod (400 μmol m^{-2} s^{-1} PFD, 25 °C), 8-h dark period (20 °C), and 1-h simulated dawn and dusk (22 °C, 80–100 μmol m^{-2} s^{-1} PFD). Plants used for mechanical damage experiments were grown in a different growth chamber under similar temperature and humidity but lower irradiance (150 μmol m^{-2} s^{-1} PFD). Plants were watered in excess daily and fertilized (Miracle-Gro; Scotts Miracle-Gro Products, Inc., Marysville, OH, USA) once each week. The soil mix contained 75% (v/v) peat moss and equal volumes of perlite, dolomitic limestone and gypsum (Sun Gro Horticulture Canada Ltd, Seba Beach, Canada). The soil and seeds were sterilized before planting and the pots were rotated within the growth chamber twice each week to minimize the potential effects of heterogeneity in environmental conditions.

Japanese beetles (*Popillia japonica*) were collected in a soybean field at the University of Illinois at Urbana-Champaign (UIUC) South Farms and used the same day they were collected. Corn earworm caterpillars (*Helicoverpa zea* Bodie) from a colony maintained by the Department of Entomology at UIUC were reared individually in cups on an artificial diet as described by Waldbauer, Cohen & Friedman (1984). Adult Japanese beetles and fourth or fifth instar corn earworms were used for all experiments. These two species represent different feeding guilds; Japanese beetles are skeletonizers, consuming interveinal tissue and carefully avoiding major and minor veins, whereas earworms are indiscriminate leaf chewers consuming the leaf lamella as well as minor and major veins.

Experimental trials

Three Japanese beetles were placed in a fine mesh bag over one lateral leaflet per plant of the topmost (third) fully expanded trifoliate on 10- to 14-day-old soybean plants (V4 developmental stage; $n = 9$). The beetles were allowed to feed for 24 h. On a different set of plants ($n = 8$), one earworm was placed in a clip cage (5 cm^2) on one lateral leaflet per plant of the fully expanded third trifoliate. Insects were removed the following morning; the leaf area consumed was quantified by digital image analysis (Scion Image; Scion Corp., Frederick, MD, USA) and varied between 50 and 80% of the total leaflet area (12–14 cm^2 average) for Japanese beetles and 40–100% of the area in the clip cages for earworms. Rates of carbon dioxide and water vapour exchange and spatial patterns of chlorophyll fluorescence (described below) of damaged and control leaflets were measured 30 min after removal of the insects (this was designated day 1). A gas-exchange chamber (5.0 cm × 7.5 cm) was placed over the damaged tissue and included undamaged tissue and the holes created by the insects. In most cases, all of the insect-damaged tissue fit inside the cuvette. Identical measurements were performed on corresponding regions of undamaged lateral leaflets on the same plants. Measurements were made mid-morning under steady-state conditions and continued for 4 d after the beginning of the experiment.

The potential effect of herbivory on stomatal conductance (g_{st}) immediately adjacent to the tissue removed was examined in another experiment where a Japanese beetle was placed on one lateral leaflet of the fully expanded third trifoliate per plant ($n = 10$). The area removed did not exceed 20% of the total area at the end of the feeding period. Measurements were initiated within 30 min after removal of the beetles (designated day 1) and continued for 8 d. Gas exchange and chlorophyll fluorescence were measured by placing a small cuvette (LI-COR LI-6400-40; total area of 2 cm^2; LI-COR Inc., Lincoln, NE, USA) on intact tissue immediately adjacent and distal to the damaged areas on leaflets (no damaged tissue was placed in the chamber). Measurements made on the corresponding portion of the undamaged sister leaflet served as the control.

To determine if mechanical injury simulates insect damage, for each of eight plants approximately 18% of the interveinal tissue on a lateral leaflet was removed by making 12 evenly spaced holes (5.5 mm diameter). The holes were made on both sides of the midrib and were clustered between second-order veins in the middle of the leaflet. The gas exchange cuvette was placed directly over the damaged area, and the rates of carbon dioxide and water exchange as well as chlorophyll fluorescence were measured mid-morning approximately 1 h after the induction of damage and at the same time on each of the following 14 d. Two hours after the end of the light period on the first day, the rate of dark respiration was measured on each damaged and control leaflet.

In a separate experiment designed to simulate damage to the main vein by a chewing insect, a single punch (1-cm diameter) was applied to the midrib of a lateral leaflet either near the petiole (1 cm from the base of the leaf; $n = 5$), distal (1 cm from the tip of the leaf; $n = 5$) or medial (in the centre of the leaf; $n = 5$). The effects of damaging

the midvein were quantified by measuring gas-exchange and chlorophyll fluorescence with the leaf chamber fluorometer of the LI-COR gas-exchange system (LI-COR 6400-40; total area of 2 cm²; Li-COR Inc.). Measurements were made at a higher irradiance (250 μmol m^{-2} s^{-1}, PFD) than the other experiments yielding higher rates of gas exchange. The cuvette was placed close to the centre of the leaflets, between the positions of the medial and distal midvein punches, and on the corresponding areas of the undamaged sister leaflets.

In all experiments the lateral leaflet opposite to the one subjected to herbivory or mechanical damage was used as a control. To confirm that the lateral leaflet of the same trifoliate was an appropriate control and did not exhibit a systemic response to herbivory, lateral soybean leaflets were mechanically damaged and compared with their undamaged sister leaflet and with comparable lateral leaflets on completely undamaged plants ($n = 7$). Mechanical damage similar in magnitude to tissue removed by herbivory had no effect on the rate of CO_2 exchange, stomatal conductance or chlorophyll fluorescence of the sister leaflet (data not shown).

Water movement was tracked in damaged and intact soybean leaflets by infiltrating with the fluorescent dye sulforhodamine G (Biotium, Inc., Hayward, CA, USA) as in Canny (1990) and Wright *et al.* (2000). This dye is polar and does not traverse cell membranes (Gaff & O-Ogola 1971). Following mechanical damage ($n = 5$) or insect damage ($n = 5$), the petioles of soybean leaves were cut under water and placed in a 1% (w/v) aqueous solution of the dye. Undamaged leaves from a separate set of plants ($n = 5$) served as controls. The path of water movement through pigmented leaves or those cleared of chlorophyll and other pigments by boiling in 90% ethanol for 10–20 min was visualized with epifluorescence ($\lambda_{ex} = 568$ nm; emission filter 605 DF 32 nm) and differential interference contrast (DIC) microscopy at 2–40 × magnification (Nikon E600FN Physiostation; Fryer Co Inc, Huntley, IL, USA).

Gas exchange and chlorophyll fluorescence measurements

Gas exchange and the spatial pattern of chlorophyll fluorescence of intact and damaged leaflets were measured simultaneously with an open path gas-exchange system (LI-COR 6400, LI-COR Inc.) and an imaging chlorophyll fluorometer (Walz Imaging PAM; Walz GmbH, Effeltrich, Germany). The top half of the cylindrical gas-exchange chamber (LI-COR 6400-05) was removed and replaced with a flat glass panel with an antireflective coating (NT46-103; Edmund Industrial Optics, Barrington, NJ, USA), through which chlorophyll fluorescence was imaged (< 5% signal loss).

The portion of the leaflet inside the gas-exchange cuvette was maintained at 23–24 °C (50% RH) and 400 μL L^{-1} CO_2 in the reference stream. Actinic illumination at the leaf surface was provided by the blue LED light source of the imaging fluorometer. This illumination was not, however,

uniform over the entire leaf surface. The area viewed by the fluorometer (2 cm × 3 cm) received 250 μmol m^{-2} s^{-1} PFD. The total leaf area inside of the gas exchange cuvette was approximately twice as large as the area viewed by the fluorometer and the average irradiance over the entire leaf area was 160 μmol m^{-2} s^{-1} PFD. Because of this difference, measurements of gas exchange and chlorophyll fluorescence parameters were not strictly comparable. During gas-exchange measurements, the rest of the plant was illuminated with a halogen lamp (200–300 μmol m^{-2} s^{-1} PFD). To correct the gas-exchange measurements for the actual leaf area in the cuvette, leaves were photographed and their area measured using digital image analysis (Scion Image; Scion Corp). All measurements were performed on attached leaves.

Chlorophyll fluorescence parameters were measured under light-adapted conditions after the leaf had reached constant rates of carbon dioxide and water exchange. The minimum fluorescence in the light-adapted state (F') was measured with the measuring pulse from the fluorometer (Baker *et al.* 2001) using an intensity of 5 and camera gain of 7 (instrument settings). An image of the maximum fluorescence (F_m') was collected following a one-second saturating pulse (approximately 2500 μmol m^{-2} s^{-1}). Photosystem II efficiency (Φ_{PSII}) was calculated as the quotient $(F_m' - F')/F_m'$ (Oxborough *et al.* 2000; Baker *et al.* 2001). At a given incident irradiance and leaf absorptance, Φ_{PSII} is directly proportional to the rate of electron transport though the photosystem II reaction centre (Genty, Briantais & Baker 1989; Rolfe & Scholes 1995).

Thermal imaging

The source of water loss from insect-damaged soybean leaflets was investigated by measuring the spatial pattern of leaf temperature with an infrared thermal camera (ThermaCAM SC1000 Infrared Camera; FLIR Systems, Portland, OR, USA; wavelengths: 3–5 μm). We assumed that variation in latent heat loss associated with transpiration was the dominant process controlling variations in leaf surface temperature (Jones 1999). Earworms were placed in mesh bags over soybean leaflets ($n = 6$) for 2 h; during this period they removed from 1 to 10% of the leaflet area. The spatial pattern of chlorophyll fluorescence and gas-exchange was measured as described previously within 1 h of removing the insects. Following the gas exchange measurements, plants were moved to a darkened growth chamber at 35 °C to allow the stomata to close ($g_{st} \leq 0.03$ mol m^{-2} s^{-1}). To confirm that the stomata had closed, the conductance of undamaged portions of damaged leaflets was estimated with the gas exchange system. Measurements were made as close to the damaged areas as possible without including cut edges in the cuvette. Similar measurements were also performed on the control leaflets. With the stomata closed, most of the water loss from leaves came from injuries. During thermal imaging, the leaf was kept horizontal and flat by suspending it in the centre of a large circular frame as in Walter, Feil & Schurr (2002). Temper-
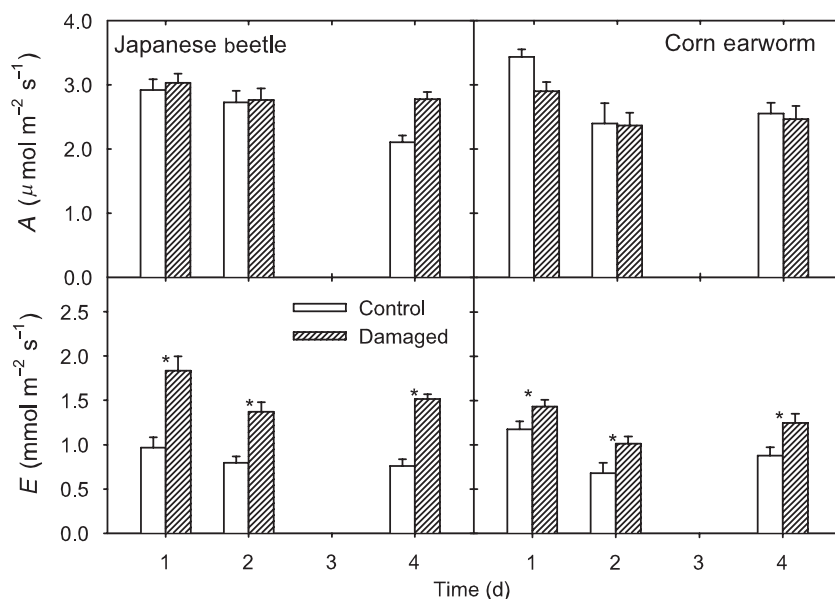
Figure 1. The effects of herbivory by Japanese beetle (left) and corn earworm (right) on net photosynthesis (A) and transpiration (E). The sister leaflets on the same leaf served as the controls. Mid-morning measurements were taken directly over the damaged areas of leaflets and the corresponding region of the control leaflets. The first measurements were made 1–2 h after the insects were removed. Overall, there were no significant differences in carbon assimilation rates between damaged and control leaves for either Japanese beetle ($P = 0.81$) or corn earworm ($P = 0.14$). Each bar represents the average of nine independent measurements for Japanese beetle and eight for corn earworm damage, and the error bars represent $\pm 1 SE$ (time was the repeated factor). Asterisks indicate statistically significant differences; $P \leq 0.05$.

ature differences on the same image were precise to $\pm 0.07\ ^\circ C$.

Statistical analysis

The gas exchange and chlorophyll fluorescence data were analysed for each experiment separately using repeated measures multivariate analyses of variance (RM ANOVA; SigmaStat 3.0; SPSS Inc., Chicago, IL, USA and Systat 8.6; Systat Software Inc., Point Richmond, CA, USA). Time was the repeated factor; damage was the independent variable, and assimilation, transpiration, stomatal conductance, temperature and Φ_{PSII} were the dependent variables. Statistical significance was $P \leq 0.05$. When the overall P was significant, post-hoc multiple comparisons were conducted with the Holm-Sidak test (versus control).

RESULTS

Herbivory by Japanese beetle and corn earworm caused substantial increases (20–90%) in water loss from soybean leaflets for several days after insects were removed (Fig. 1), but did not affect the rate of net photosynthesis measured as carbon exchange (Japanese beetle, $P = 0.81$; corn earworm, $P = 0.14$) or stomatal conductance of nearby undamaged tissue ($P = 0.62$; Fig. 2).

Rates of dark respiration were unaffected by herbivory (insect-damaged leaf: $-2.9 \pm 0.1\ \mu mol\ CO_2\ m^{-2}\ s^{-1}$; undamaged sister leaflet: $= -2.9 \pm 0.2\ \mu mol\ CO_2\ m^{-2}\ s^{-1}$; $n = 8$, $P = 0.97$) or mechanical damage to interveinal tissue (data not shown). Herbivory by Japanese beetle had no effect on the spatially averaged values of Φ_{PSII} (control: 0.42 ± 0.01; damaged: 0.42 ± 0.02, relative units), and neither did damage by corn earworm (control: 0.39 ± 0.02; damaged: 0.38 ± 0.01)

In the areas immediately adjacent to caterpillar or beetle damage, typically within 100–500 μm of the cut edges, Φ_{PSII}

was reduced by more than 50% relative to areas away from the damage or relative to control leaves (Fig. 3a). However, when the average value was calculated for the entire leaf surface, there was no significant effect of damage on Φ_{PSII}. Herbivory caused a reproducible but small (3–4%) and transient (30–120 min) increase in Φ_{PSII} on undamaged areas of the leaf (compare values for the undamaged areas in Fig. 3a with the values in the control leaf in Fig. 3b). This increase in Φ_{PSII} was within the magnitude of variation in control leaves and therefore could not be resolved at $P \leq 0.05$.
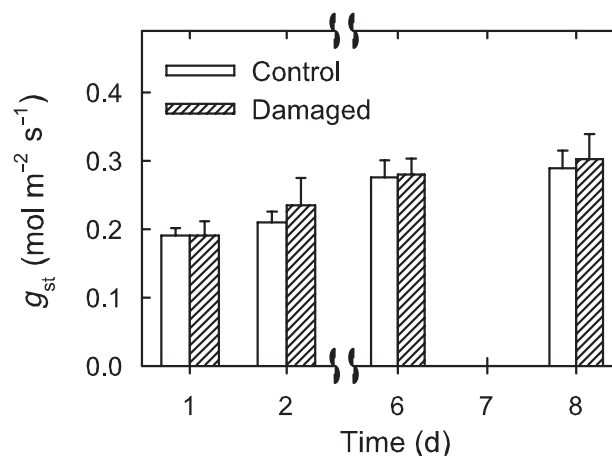

Figure 2. The effect of herbivory by Japanese beetle on stomatal conductance to water vapour (g_{st}) for tissue immediately adjacent and distal to the damage on soybean leaves. Measurements on the equivalent region of an adjacent undamaged sister leaflet served as the control. The first mid-morning measurements were taken 1–2 h after the insects were removed. Each bar represents the average of 10 independent measurements and the error bars represent $\pm 1 SE$. The differences between treatments were not statistically significant ($P = 0.62$). A different group of plants from those used for Fig. 1 was used for this experiment.
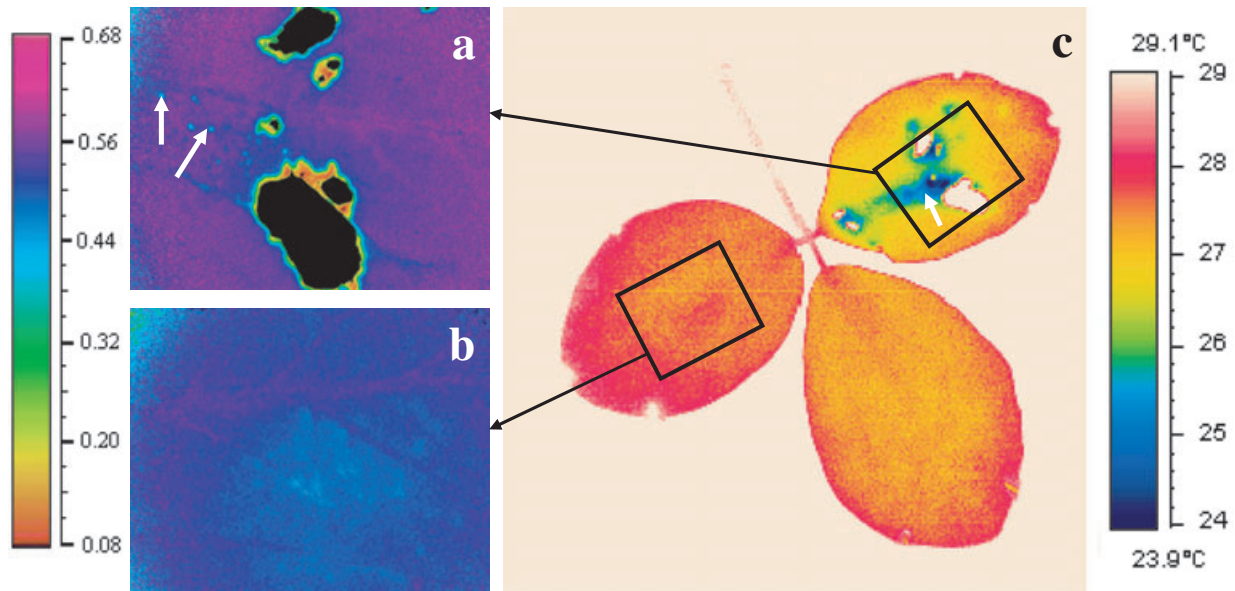
Figure 3. The effects of herbivory by corn earworm on the spatial pattern of Φ_{PSII} (damaged leaflet (a), undamaged leaflet (b)) and leaf temperature (c) on soybean. All images were taken within 1 h after the removal of the insects. The white arrows in the false colour image of chlorophyll fluorescence (a) point to minute mandible bites by the caterpillars (lower Φ_{PSII}) otherwise invisible to the naked eye, but deep enough to penetrate the cuticle. The corresponding white arrow in the false colour thermal image (c) indicates the large effect these bites had on water loss (blue area extending towards the petiole). Yellow, green and blue areas on the thermal image indicate zones affected by evaporative cooling. Areas with lower Φ_{PSII} correspond to high temperatures surrounding the holes. The transient increase in Φ_{PSII} (approximately 3%) can be observed on the damaged leaflet (purple areas; a).

Regions of the leaf immediately adjacent (50–300 μm) to caterpillar damage were 1–2 °C cooler than regions of the same leaf away from the damage or comparable regions on the undamaged sister leaflet (Fig. 3c). As the thermal images were obtained in the dark the patterns of leaf temperature were not influenced by stomatal conductance. Other cool areas not adjacent to holes corresponded to small mandible scrapes on the adaxial leaf surface (white arrows in Fig. 3a). Insofar as lower temperatures represent greater energy loss as latent heat and thus greater rates of evapotranspiration, the thermal images suggest that water evaporated rapidly from cut edges and other areas of direct caterpillar damage.

The water supply to areas of leaves damaged by insects or by mechanical damage was maintained through minor veins (apparently unrestricted), even veins severed by herbivores and open to the atmosphere, as shown by the transport of the fluorescent dye sulforhodamine G (Fig. 4a). At the point of evaporation the dye precipitated as a dark-red residue, indicating that the transition from liquid to vapour phase occurred in continuous bands around the entire circumference of holes, approximately 100–250 μm from the damaged edges, and was not associated with severed veins (Fig. 4b & c). In leaves cleared of pigments (Fig. 4b & c), this pattern indicated that water exited the vascular tissue, moved apoplastically and evaporated from the leaf through damaged surfaces.

The mesophyll of soybean leaves is compartmentalized by bundle sheath extensions. Glycerin infiltrated cut edges only up to fourth-order minor veins (Fig. 4d), indicating that bundle sheath extensions associated with these minor veins were effective barriers to the diffusion of water vapour in the mesophyll. Thus, water exited vascular tissue no further than 300 μm from the edge of damage, travelled apoplastically in the liquid state for 100–200 μm and evaporated at its equilibrium point with the vapour state, approximately 100 μm away from the edge.

Since the plants used for one of the mechanical damage experiments (lamellar damage) were grown at lower light intensities than those used to examine the effects of mechanical severing of the mid vein or the insect feeding trials, their overall rates of gas exchange were lower. Nonetheless, as with herbivory, mechanical damage to interveinal tissue caused significant increases in transpiration (Fig. 5). The rate of water efflux was up to 150% greater from damaged leaves on the first day of measurement ($P < 0.01$) and remained higher than control leaves for 6 d (Fig. 5). Assuming stomatal conductance was constant and remained at the level of the control leaves for the 16-h photoperiod, and only non-stomatal water loss occurred in the dark from damaged tissue, under the conditions of the experiment, control leaflets would transpire 36 mol of water over the first week following damage, whereas damaged leaflets would lose 51 mol. The difference of 15 mol (approximately 270 mL), attributed solely to uncontrolled water loss through damaged surfaces, amounted to a cumulative loss of 0.72 mol H_2O per cm of damaged perimeter; namely an average of 1.2 μmol H_2O (cm damaged edge)$^{-1}$ s^{-1}, for the first week following damage.

Carbon assimilation rates remained relatively constant
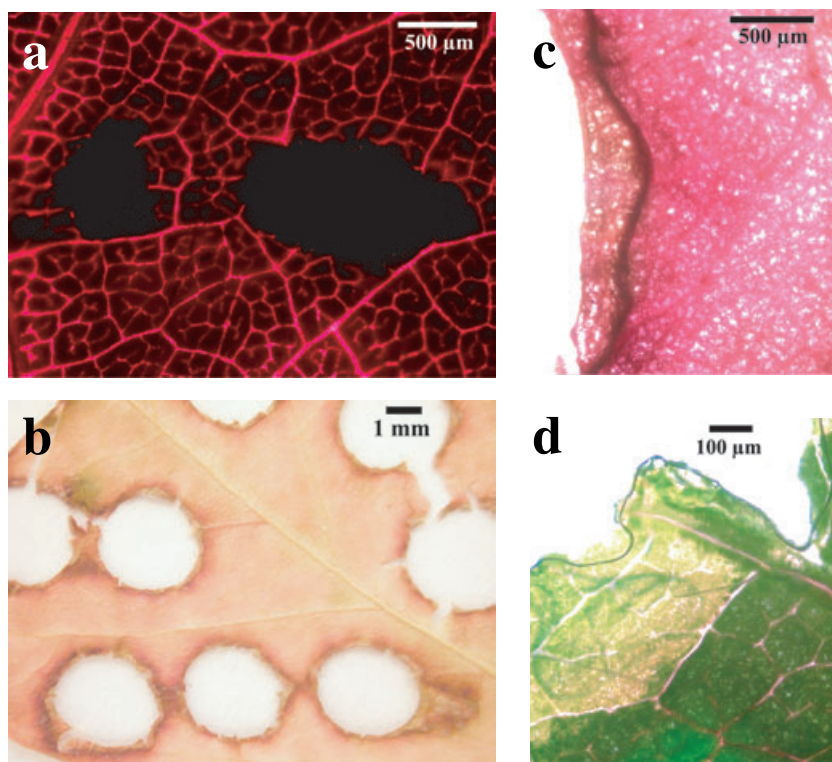
Figure 4. The effect of mechanical damage or herbivory by corn earworm on the movement of a fluorescent dye (sulforhodamine G) through a soybean leaf. (a) Fluorescence micrograph of a leaf following herbivory and subsequently fed the dye, illustrating the water moving to the ends of severed veins. (b) Reflected light image of a portion of a cleared soybean leaf illustrating the accumulation of the dye at the liquid water/vapour interface around each hole. (c) Transmitted light micrograph of a mechanically damaged leaf (cleared of biological pigments). The dark vertical band represents the accumulation of dye at the liquid water/vapour interface at approximately 200 μm from the cut edge. (d) Diffusion and capillary uptake of glycerin at the cut edge of a leaf subjected to herbivory. The more transparent area infiltrated by glycerin illustrates that the bundle sheath extensions in soybean leaves block movement in the apoplast past fourth-order veins.

throughout the experiment for both control and damaged leaflets, with no significant differences between damaged and undamaged leaflets overall ($P = 0.34$). There were also no significant differences in the spatially averaged Φ_{PSII} between control (0.44 ± 0.03, relative units) and damaged leaflets (0.43 ± 0.02) for the duration of the experiment ($P = 0.60$). The spatial pattern of Φ_{PSII} on the mechanically damaged leaflets was similar to insect-damaged leaves, with highly localized reductions in Φ_{PSII} of $\geq 50\%$ immediately adjacent to the cut edges (within 500 μm of the edge; data not shown).

Damage to the midvein at the base of the leaflet, near the petiole, caused significant reductions in carbon assimilation rates and stomatal conductance of the remaining tissue ($P < 0.01$) for up to 6 d after the damage was inflicted (Fig. 6). However, spatially averaged Φ_{PSII} was significantly (average 8%; $P = 0.02$) higher in damaged leaflets. Damage near the tip of the leaflet had no effect on the rate of carbon assimilation rates ($P = 0.60$), stomatal conductance ($P = 0.58$), or Φ_{PSII} ($P = 0.63$).

DISCUSSION

In addition to the direct loss of photosynthetic tissue, insect and mechanical damage to soybean leaves caused profligate water loss from the perimeter of the injuries for an extended period (Figs 1 & 5). Under the conditions of these experiments, damaged leaves experienced approximately 40% greater water loss over 1 week than undamaged leaves. With the exception of localized reductions in photosystem II operating efficiency (Φ_{PSII}), typically confined to regions immediately adjacent to the cut edges, damage to interveinal tissue had no effect on the rates of photosynthetic or respiratory carbon exchange or stomatal conductance of the remaining undamaged tissues. The disruption of the midvein, however, caused substantial reductions in net photosynthesis and stomatal conductance 'downstream' from the damage (Fig. 6). For field conditions, where soybeans experience higher incident irradiance and thus greater stomatal conductance, the contribution of the indirect water loss may be less than that calculated for the conditions of these experiments. Conversely, this value would increase with the higher vapour pressure deficit typical of field conditions. The indirect effects of herbivory on soybeans may be considerable, depending not only on the environmental conditions but also on the type of damage and thus vary with insect feeding guild.

The contrasting responses of soybeans to interveinal and midvein damage suggest that different feeding habits among insect taxa (Labandeira *et al.* 1994) are likely to have different effects on the physiology of remaining leaf tissues. Although the herbivores used in this study represented two different feeding guilds, their effects on leaf physiology were similar because neither insect cut large veins. However, many mandibulate foliage feeders (Orthoptera, and most Coleoptera, Hymenoptera, and Lepidoptera) indiscriminately sever large veins on soybean, and probably cause substantial reductions in photosynthesis downstream from the point of damage.

The importance of vein cutting has often been mentioned but seldom been studied (Morrison & Reekie 1995; Peterson, Higley & Spomer 1996; Meyer 1998; Peterson *et al.*
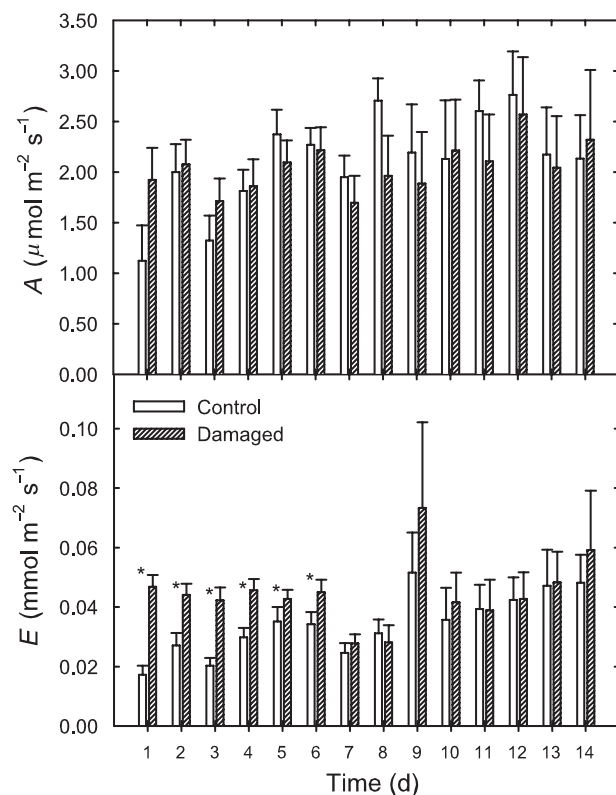
Figure 5. The effects of mechanical damage to interveinal tissue in soybean leaflets on net photosynthesis (A) and transpiration (E). Damage was caused by removing 12 leaf discs (0.24 cm² each) from a lateral leaflet (see Methods). The gas-exchange cuvette was placed directly over the damaged tissue and rates were adjusted for the actual leaf area in the chamber. The first measurements were made 30 min after damage induction. Each bar represents the average of eight independent measurements and the error bars represent ±1SE. Asterisks indicate statistically significant differences; $P \leq 0.05$.

1998). One exception is a study by Oleksyn *et al.* (1998) that demonstrated that punctures to the midveins of *Betula pendula* leaves have a far greater negative impact on photosynthesis than punctures avoiding leaf veins. Damage to soybean by skeletonizers such as the Japanese beetle is likely to have little effect on photosynthesis of remaining tissues but can substantially increase rates of water loss.

Peterson & Higley (1996) and Ostlie & Pedigo (1984) also reported increased transpiration from damaged soybean leaves with little or no change in carbon assimilation rates. There are aspects of leaf physiology in soybean that are responsive to damage. For example, insect damage induces large increases in the production of reactive oxygen species (ROS) and the signalling molecule 4-aminobutyrate (Bown *et al.* 2002) without a corresponding effect on photosynthesis or respiration. Nonetheless, studies with other plant species found significant changes in the photosynthesis near damaged areas, either depressions (Zangerl *et al.* 2002) or compensatory increases (Thomson *et al.* 2003). It remains unclear how the damage–response mechanisms of soybean operate without causing changes in photosynthesis.

The spatial pattern of leaf surface temperature suggests that water evaporated through the edges of holes (exiting the leaf laterally) as well as through small injuries to the cuticle (Fig. 3a & c). Furthermore, deposition of the non-permeating dye sulforhodamine G at the water liquid/vapour interface around the perimeter of cut edges (Fig. 4b & c) indicated that water was lost from cell wall spaces approximately 150 μm from the edge of holes.

Because soybean leaves are heterobaric, bundle sheath extensions obstructed lateral diffusion of water vapour through the mesophyll, which could not therefore contribute to the high rates of evaporation from cut edges. Bundle sheath extensions project from the vascular bundles to the upper and lower epidermis, effectively compartmentalizing the leaf mesophyll. The inability of glycerin to infiltrate cut edges past fourth- or fifth-order veins (Fig. 4d) indicates that these veins are associated with bundle sheath extensions in soybean. In the absence of effective lateral diffusion of water vapour or evaporation from xylem elements, it appears that water moves to cut edges through minor veins and laterally through unsuberized cell wall spaces in the apoplast.

Lateral water transport outside vascular tissue can occur simultaneously through unsuberized cell wall spaces (apoplast) and through the cytosol of nearby cells (symplast; Barbour & Farquhar 2003). As the dye (sulforhodamine G) used to follow water movement cannot enter the symplasm, its deposition at the sites of evaporation around holes suggests that at least some of the water escaping from damaged areas moved to the cut edges through the apoplastic spaces below the epidermis, where bundle sheath extensions do not restrict movement. This, however, does not exclude the possibility of symplastic movement and water probably followed both pathways (Barbour & Farquhar 2003).

Taken together, our results suggest that water moves to damaged edges first by exiting minor veins and moving into the cell wall spaces below the epidermis (Karabourniotis, Bornman & Nikolopoulos 2000). After moving as a liquid through the apoplast, it evaporated into the mesophyll approximately 100 μm away from the edge and diffused to the atmosphere. The region 100–200 μm around the edges of damaged areas coincided with the bands of lower Φ_{PSII} (Fig. 3a & b) and higher temperature (Fig. 3c), suggesting that these regions immediately adjacent to damage had become severely desiccated.

Severing the main vein reduced photosynthesis and stomatal conductance distal to the damage, but the absence of downstream effects of interveinal damage, either mechanical or by insects, suggests that there is considerable redundancy in the hydraulic system of soybean leaves. Highly reticulate minor venation provides considerable buffer against damage (Wagner 1979; Roth-Nebelsick *et al.* 2001), where leaves without this reticulate venation may be more vulnerable to interveinal damage (Shull 1934). As observed by Sack *et al.* (2003) for oak and maple, damage to the main vein of soybean substantially altered gas-exchange and chlorophyll fluorescence downstream on soybean leaves. Thus, the main vein provides a primary path for water
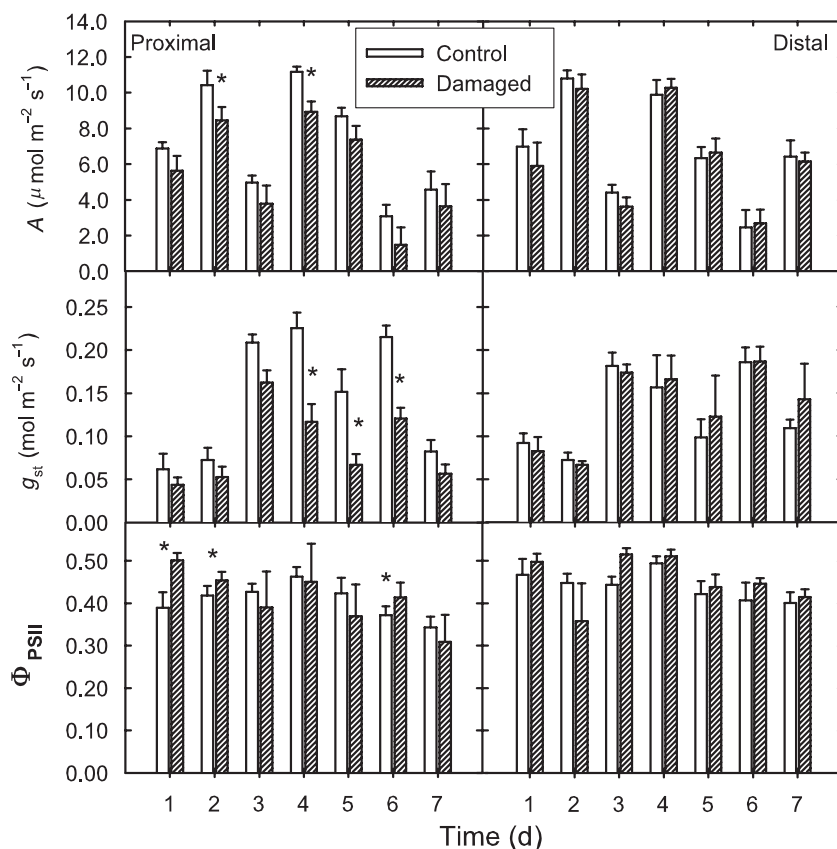
Figure 6. The effects of severing the midvein of a soybean leaflet on net photosynthesis (*A*), stomatal conductance to water vapour (g_{st}) and the photochemical efficiency of photosystem II (Φ_{PSII}). Gas exchange and chlorophyll fluorescence were measured in the centre of a lateral leaflet and the midvein was severed either near the petiole (1 cm from the leaf base; left panels) or near the tip of the leaflet (1 cm from the leaf tip; right panels). The first measurements were made 30–60 min after damage induction. Each bar represents the average of five independent measurements and the error bars represent ±1SE. Asterisks indicate statistically significant differences for treatment effect; $P \leq 0.05$.

transport in soybean leaves and represents a point of vulnerability to large chewing insects, including grasshoppers and large lepidopteran larvae that commonly feed on this species (Kogan & Kuhlman 1982; McPherson & Moss 1989).

Uncontrolled water loss from tissue damaged by chewing insects was sustained for at least 4 d (Fig. 1) and in the case of mechanical damage continued for 6 d (Fig. 5). The cessation of water loss may have resulted from lignification at the edges of wounded tissue (Lagrimini *et al.* 1993; Okey *et al.* 1995). In flax plants, an increase in ligno-suberin around wounds was detected approximately 4 d after the induction of damage (McDougall 1993). Given the similar duration of the profligate water loss from damaged soybean leaves, lignification may have similarly contributed to healing in this species.

Following herbivory there consistently was a small and transient increase in Φ_{PSII} (Figs 3a, b & 6) with no detectable effect on carbon assimilation rates. It is possible that the transient activity of an alternative electron sink may have uncoupled electron transport from primary carbon assimilation. Plants, including soybean, often respond initially to injury by producing reactive oxygen species, particularly superoxides (Leon *et al.* 2001; Bown *et al.* 2002). The superoxide radical is detoxified in chloroplasts by thylakoid-bound superoxide dismutase to produce hydrogen peroxide. Through the Mehler ascorbate peroxidase pathway, hydrogen peroxide can withdraw electrons from the photosynthetic electron transport chain in a reaction medi-

ated by monodehydroascorbate reductase, which uses NADPH (Ort & Baker 2002). The activity of this pathway may explain the transient increase in Φ_{PSII} in damaged leaves, while carbon gain either decreased or remained constant.

Although soybeans are considered tolerant of herbivory, particularly during vegetative growth (Haile, Higley & Specht 1998), profligate water loss from damaged leaf tissue represents a previously hidden cost with potentially large ecological and agronomic implications. Drought stress causes substantial yield losses in soybean (Liu, Andersen & Jensen 2003), and greater rates of water loss and lower water-use efficiency from damaged leaves may exacerbate the effects of drought on productivity. Because of the high perimeter-to-area ratio of skeletonized tissue, enhanced rates of water loss may be particularly severe for herbivores that produce this type of damage, such as the Japanese beetle, which is becoming an increasingly important soybean pest (Kogan & Kuhlman 1982).

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