Influence of leaf herbivory, root herbivory, and pollination on plant performance in *Cucurbita moschata*

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Abstract. 1. Plants experience herbivory on many different tissues that can affect reproduction directly by damaging tissues and decreasing resource availability, or indirectly via interactions with other species such as pollinators.

2. This study investigated the combined effects of leaf herbivory, root herbivory, and pollination on subsequent damage, pollinator preference, and plant performance in a field experiment using butternut squash (*Cucurbita moschata*). Leaf and root herbivory were manipulated using adult and larval striped cucumber beetles (*Acalymma vittatum* F.), a cucurbit specialist.

3. Leaf herbivory reduced subsequent pistillate floral damage and powdery mildew (*Sphaerotheca fuliginea*) infection. In spite of these induced defences, the overall effect of leaf herbivory on plant reproduction was negative. Leaf herbivory reduced staminate flower production, fruit number, and seed weight. In contrast, root herbivory had a minimal impact on plant reproduction.

4. Neither leaf nor root herbivory altered pollinator visitation or floral traits, suggesting that reductions in plant performance from herbivory were as a result of direct rather than indirect effects. In addition, no measured aspect of reproduction was pollen limited.

5. Our study reveals that although leaf herbivory by the striped cucumber beetle can protect against subsequent damage, this protection was not enough to prevent the negative impacts on plant performance.

Key words. *Acalymma vittatum*, butternut squash, *Cucurbita moschata*, direct effects, herbivory, indirect effects, *Peponapis pruinosa*, pollen limitation.

Introduction

In both agricultural and natural systems, plants exist in a complex environment where they interact with antagonists and mutualists on several different tissues. Although leaf herbivory, root herbivory, and pollination have traditionally been examined separately, studies linking above- and belowground processes are increasingly becoming the focus of research between insects and plants. Plant traits involved in defence, attraction, and reproduction can be affected by both leaf and root herbivory separately. However, several recent studies have examined the combined effects of damage to both above- and belowground plant tissues and have found both positive and negative effects

on plant traits and fitness (Van der Putten *et al.*, 2001; Bardgett & Wardle, 2003; Blossey & Hunt-Joshi, 2003; Bezemer & Van Dam, 2005), indicating the importance of considering multiple interactions simultaneously.

Leaf herbivory can negatively affect plant performance directly and indirectly by changing the plant's interaction with other organisms. Leaf herbivory can reduce pollinator attraction by altering floral traits (Strauss, 1997; Lehtila & Strauss, 1999; Mothershead & Marquis, 2000), which can be especially detrimental to species that rely exclusively on pollinators for reproduction. The negative indirect effects of herbivory have been well studied in natural systems, but much less so in agricultural systems where pollination may be critical for crop yield. For example, cantaloupe plants with leaf damage had reduced pollinator visitation and yield compared with undamaged controls. Enhanced pollination eliminated the effects of herbivory on cantaloupe reproduction, allowing damaged plants to produce as much fruit as undamaged plants (Strauss & Murch, 2004).

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In contrast, supplemental pollination did not alter the cucumber's ability to compensate for herbivory in the greenhouse (Thomson *et al.*, 2003). In order to determine whether effects of leaf and root herbivory on plant performance are direct or indirect, pollen limitation should be quantified. This may be particularly important in agroecosystems, where recent pollinator declines (Steffan-Dewenter *et al.*, 2005; Klein *et al.*, 2007) may leave the plant more susceptible to pollen limitation.

Root herbivory often has direct negative effects on plants, but can indirectly benefit the plant aboveground by defending against herbivores or attracting mutualists. Root damage can induce plant defences aboveground, which may reduce the fitness of shoot herbivores (Bezemer *et al.*, 2004; Hol *et al.*, 2004; Bezemer & Van Dam 2005; Soler *et al.*, 2005). Root herbivores can also influence above ground organisms such as predators (Masters *et al.*, 2001), parasitoids (Masters *et al.*, 2001; Wackers & Bezemer, 2003; Poveda *et al.*, 2005) and even hyperparasitoids (Soler *et al.*, 2005) via plant-mediated mechanisms. In addition, a few studies have linked root herbivory with aboveground mutalists. Root herbivory increased pollinator visitation to wild mustard (Poveda *et al.*, 2005), and mechanical root damage increased extrafloral nectar production in cotton (Wackers & Bezemer, 2003), which may increase the recruitment of natural enemies. Root damage has been hypothesised to induce a stress response that reduces water content and increases the movement of soluble nitrogen and carbohydrates to plant foliage, improving the performance of aboveground herbivores (Masters *et al.*, 1993). A similar mechanism could potentially explain pollinator or natural enemy attraction to plants with root damage, although too few studies have been conducted at this point to make generalisations. These examples suggest that root herbivory can influence aboveground trophic levels, but the direct or indirect effects of these interactions on plant reproduction are still to be investigated in many cases.

Butternut squash plants (*Cucurbita moschata* Duch. ex Poir) provide an ideal system to demonstrate the effects of both leaf and root herbivory on pollination and reproduction, because striped cucumber beetles (*Acalymma vittatum* F.) are specialist herbivores on aboveground cucurbit tissues as adults, and on roots as larvae (Bellows & Divers, 2002). *Cucurbita* species also rely exclusively on pollinators for reproduction (Hurd *et al.*, 1971). We investigated how leaf herbivory, root herbivory, and pollination jointly affect: (1) subsequent leaf damage, (2) pollinator preference, and (3) plant performance. We predict that leaf and root herbivory will have direct negative effects on plant performance by reducing leaf and root area, as well as resources for fruit and seed production. We also predict that leaf herbivory may have a negative indirect effect on plant fitness by altering floral traits and making plants less attractive to pollinators, as has been demonstrated in several natural systems (Strauss, 1997; Lehtila & Strauss, 1999; Mothershead & Marquis, 2000). Root herbivory may have some positive indirect effects on plant performance by increasing pollinator preference, as has been seen in other studies (Poveda *et al.*, 2005). This study will reveal the individual and combined impacts of leaf herbivory, root herbivory, and pollination on several plant responses in butternut squash.

Materials and methods

Study system

Cucurbita moschata (Cucurbitaceae), or butternut squash, is an annual that originated in South America (Whitaker, 1956). Plants are self-compatible but monoecious, requiring a pollinator to vector pollen from staminate to pistillate flowers. Flowers are five lobed, bright yellow-orange, actinomorphic, occur within the axils of the leaves, and last a single day (Whitaker & Davis, 1962; Robinson & Decker-Walters, 1997). Staminate flowers typically outnumber pistillate flowers in *Cucurbita* species (Tepedino, 1981). Butternut squash is a popular crop grown worldwide (Robinson & Decker-Walters, 1997). In Massachusetts, up to 40% of vegetable crop acreage is invested in cucurbits, including butternut squash (USDA, 2002).

The herbivore *Acalymma vittatum* (Coleoptera: Chrysomelidae), the striped cucumber beetle, is a major cucurbit crop pest in the northeastern United States (Boucher & Durgy, 2004). Striped cucumber beetles and related Diabroticite beetles have evolved as specialist herbivores on cucurbits, compulsively feeding in response to cucurbitacins (Metcalf *et al.*, 1980). Striped cucumber beetles feed exclusively on cucurbit shoots and roots as well as flowers and pollen (Andersen & Metcalf, 1987). Adult striped cucumber beetles overwinter as adults, then emerge in the spring and feed on foliar parts. Females lay eggs that hatch into root-feeding larvae, which then emerge during late summer as new adults (Bellows & Divers, 2002). Diabroticite beetles feed on native as well as domesticated cucurbits (Tallamy & Krischik, 1989), such that interactions studied on domesticated species may be relevant to native wild congeners.

Butternut squash, like many cucurbits, depend on pollinators for pollen transfer (Whitaker & Davis, 1962; Robinson & Decker-Walters, 1997). In an agricultural system where resources are plentiful, pollen limitation can become more significant than resource limitation (as in Strauss & Murch, 2004). Typical pollinators of butternut squash in Virginia, West Virginia, and Maryland (Schuler *et al.*, 2005) as well as in western Massachusetts (Cavanagh, Adler and Hazzard, in prep) include bumble bees (*Bombus sp.*), squash bees (*Peponapis pruinosa* Say), and honey bees (*Apis mellifera* L.). Squash bees are specialist pollinators that forage exclusively on both wild and domestic *Cucurbita* (Hurd *et al.*, 1971). These solitary bees are ground nesters that collect both nectar and pollen (Willis & Kevan, 1995). *Bombus* sp. and *Apis mellifera* are both generalist pollinators.

Propagation and study site

We germinated butternut squash seeds (cv. Waltham Butternut, Johnny's Selected Seeds, Winslow, ME, U.S.A.) in Metromix 360 soil (The Scotts Co., Marysville, OH) in the greenhouse (University of Massachusetts, Amherst, U.S.A.) under natural light on 20 May 2005. On 17 June 2005 at the fourth leaf stage, we transplanted 160 butternut squash plants to a 1672 m^2 plot at the University of Massachusetts South Deerfield Research

Farm. Plants were spaced 3 m apart on all sides. Prior to planting, 19-19-19 NPK fertilizer was applied once at a rate of 1 kg per 17.8 m^2.

Experimental design and treatments

We manipulated leaf herbivory (enhanced and natural), root herbivory (enhanced and natural), and pollination (enhanced and natural) after transplanting in a factorial design, for eight total combinations. We assigned plants to treatments in a randomized block design, with one replicate per treatment combination per block and 20 blocks, for a total of 160 plants.

We conducted a pre-treatment herbivory census to estimate the average amount of natural herbivory at the site on 23 June 2005. This census was used to determine treatment herbivory levels. We estimated foliar tissue on a scale of 0 to 4 (0 = no damage, 1 = 1–25% damage, 2 = 26–50% damage, 3 = 51–75% damage, 4 = 76–100% damage). The average damage score per plant was 1.24 ± 0.04 (mean $\pm$ SE, $n = 158$) with a minimum score of 0 and a maximum score of 2.33.

Striped cucumber beetles were vacuum collected for both leaf and root herbivory treatments at Riverland Organic Farm (Sunderland, MA). To determine the effects of leaf herbivory, we manipulated leaf damage by applying striped cucumber beetles to butternut squash leaves. Three herbivory bouts were applied over 3 weeks (24 June, 30 June, and 6 July) to mirror the timing of naturally occurring beetle damage. Enhanced leaf herbivory was applied by covering two emerging leaves (on separate vines) per plant per herbivory bout with a mesh bag containing three to six striped cucumber beetles. Natural leaf herbivory plants had leaves bagged without beetles as a control. Beetles fed for 4–5 days before bags were removed. Enhanced leaf herbivory treatments resulted in ~50% area removal per leaf on a total of six leaves per plant. Based on the pre-treatment herbivory census, this damage was well within the range of naturally occurring damage per leaf. Based on leaf counts conducted before and after damage was imposed, we estimate plants produced an average of 12 leaves by the end of the damage treatments. The removal of 50% of 6 leaves is therefore an overall area removal of 25%, imposed over a 3-week period.

To determine the effects of root herbivory, we manipulated root damage by applying striped cucumber beetle eggs to butternut squash roots. Root herbivory was applied after leaf herbivory to mimic the beetle life cycle (Bellows & Divers, 2002; Smyth *et al.*, 2002). We treated plants once when naturally occurring adult beetle activity had subsided (during 11 and 13 July 2005), to co-occur with the timing of natural larval herbivory. Adult striped cucumber beetles were mated in the lab using existing protocols (Cuthbert *et al.*, 1968; Branson *et al.*, 1988; Smyth *et al.*, 2002), and eggs were collected and stored in a 12 °C incubator for up to 2 weeks before use. Seven- to 12-day-old eggs were pooled into a single application. Eggs were suspended in a 0.5% agar solution (Fisher Scientific, Pittsburg, PA) at a density of 110.0 ± 2.39 (mean $\pm$ SE, $n = 20$) per plant and applied to the roots using a plastic 20-ml syringe. Natural root herbivory plants received 20 ml of agar without eggs as a control. The congeners *A. bivittula* and *A. albidovittata* laid 156.80 ± 41.78 and 43.36 ± 12.12 eggs, respectively, daily per squash host (Walsh, 2003). In addition, a single female *A. vittatum* can lay up to 50 eggs in one night (R. R. Smyth, pers. comm.). Thus, a one-time application of 110 eggs was well within the realm of naturally occurring oviposition rates. Subsamples from the egg/agar solution were allowed to hatch in 8.5-cm Petri plates to determine egg viability (number of hatched eggs/total eggs), which was $80.35 \pm 3.62\%$ (mean $\pm$ SE, $n = 10$).

In addition to the herbivory treatments, we manipulated pollination to determine if plants were pollen limited and if herbivory altered pollen limitation. We collected pollen from greenhouse-grown plants every day at around 05.00 hours from at least 20 different staminate flowers. We hand-pollinated all pistillate flowers on enhanced pollination plants between 05.00 and 08.00 hours 5 days per week, from 18 July until 2 September 2005. We applied saturating amounts of pollen evenly to all three stigma lobes with a paintbrush. Plants in the natural pollination treatment were not manipulated, and all plants received natural insect pollination from bees in the field.

Response measurements

Subsequent damage. We determined how treatments influenced subsequent leaf damage, floral damage, and powdery mildew infection. Leaf damage was measured three times (2, 12 and 19 August 2005) and data were averaged across dates to create one score per plant. The eight youngest leaves were scored on a randomly chosen runner on a scale of 1 to 7 (1 = 0–1%, 2 = 2–5%, 3 = 6–10%, 4 = 11–20%, 5 = 21–50%, 6 = 51–75%, 7 = 76–100% damage as a result of herbivory). Individual leaf scores were averaged for one score per plant. We estimated floral damage using flowers collected for floral traits (see below) on a scale of 0 to 5 (0 = no damage, 1 = 0 to 1%, 2 = 2 to 5%, 3 = 6 to 10%, 4 = 11 to 20%, 5 = >20% damage). We recorded the amount of powdery mildew (*Sphaerotheca fuliginea*) infection on 19 August 2005. Damage was estimated as the percentage of leaves per plant (including the leaves scored in the herbivory censuses) showing symptoms of powdery mildew infection on a scale of 1 to 4 (1 = 0–25% of leaves, 2 = 26–50%, 3 = 51–75%, 4 = 76–100%).

Floral traits and pollinator preference. We measured floral traits for up to two staminate and two pistillate flowers per plant from 2 August until 1 September between 05.00 and 11.00 hours, using digital calipers and a plastic ruler. On all flowers, we measured display width (distance across flower from the tip of one petal to the other), petal area (estimated as length × width), nectary diameter, and flower dry weight. Flower dry weights were obtained from tissue dried in a convection oven at 45 °C for at least a week. Nectar production (volume and sugar content) from bagged flowers was measured using micropipette tubes and a portable refractometer. Staminate floral trait measurements included anther area (estimated as length × width) and nectary opening size (average nectary opening area × number of nectary holes). Pistillate floral trait measurements included ovary area (estimated as length × width) and stigma surface area (measured on one stigma lobe, estimated as length × width).

We observed pollinators from 17 July, at the onset of flowering, until 2 September 2005. Staminate and pistillate flowers were observed for 5 min each, at least once per plant between 05.00 and 12.00 hours. We recorded bee species, number of visits, visit duration, and number of rejections (when a pollinator approaches a flower and departs without landing). The response variables were: number of visits, time per visit, and number of rejections by bees, as well as the number of striped cucumber beetles inside flowers. In this study, 77% of pollinator visitors to butternut squash flowers were squash bees (*P. pruinosa*) and 20% were bumble bees (*Bombus sp.*), so analyses focused on these taxa. The remaining 3% were honey bees (*A. mellifera*) and other species. Nectar collecting was the major pollinator behaviour observed for both staminate and pistillate flowers (>93% of all behaviour observations).

Plant performance. We examined the effects of herbivory and pollination on plant performance by measuring flower number, fruit, and seed yield. We counted all staminate and pistillate flowers 5 days a week from flowering onset on 18 July until 2 September 2005, when production had almost entirely ceased. Fruits were harvested on 8 September 2005. For each plant, we recorded total fruits produced and total fruit fresh weight. Weight per fruit was calculated as total fruit weight/total fruit number. For two randomly chosen fruits per plant, we dried, counted, and weighed all seeds. Seeds were categorised as developed or undeveloped; undeveloped seeds were hollow, indicating no embryo within the seed coat. Weight per seed (total seed weight/number of developed seeds) was calculated. In addition, from 14 September until 21 October 2005 we destructively harvested plants to measure dry root biomass.

Statistical analysis

We examined the effects of leaf herbivory, root herbivory, pollination, and their interactions on subsequent damage, pollinator preference, and plant performance. All data were averaged within plant to use plant as the unit of replication. Data were analysed with SAS 9.1 statistical software (SAS Institute Inc., Cary, NC) using the General Linear Models (GLM) procedure with type III sums of squares. The basic model analysed the effects of leaf herbivory, root herbivory, pollination, their interactions, and block (a fixed factor) on several response variables. Date and time were included as covariates when appropriate, and removed if non-significant. MANOVA and subsequent ANOVAS were conducted unless otherwise noted. The response variables analysed were: (1) subsequent damage, measured as subsequent leaf damage, pistillate and staminate floral damage, striped cucumber beetle number and presence/absence, and powdery mildew infection. Each of these was considered a separate response and analysed with ANOVA, except for cucumber beetle presence/absence, which was analysed with logistic regression. (2) Floral traits and pollinator preference, measured as pistillate and staminate floral traits in separate MANOVAS (using ANOVA for nectar traits since nectar was not available on some plants), pollinator preference (using ANOVA as some plants had no pollinator visitation data), and plant performance (one MANOVA including all measures of flower, fruit, and seed production). Assumptions of

normality were examined using normal probability plots and the Shapiro-Wilks test. Staminate floral damage and floral traits were normal without transformation; all other data were log $(x + 1)$ transformed to meet assumptions of normality. Two plants died, leaving 158 plants for analysis.

Results

Subsequent damage

Subsequent leaf damage was not affected by leaf herbivory, root herbivory, pollination, or any treatment interactions (ANOVA, $F_{1,445} < 2.31$, $P > 0.13$ for all), although damage varied with block ($F_{19,445} = 2.94$, $P < 0.0001$) and date ($F_{2,445} = 93.31$, $P < 0.0001$).

Enhanced leaf herbivory reduced pistillate flower damage scores by 18% (ANOVA, $F_{1,68} = 6.19$, $P < 0.02$, Fig. 1) but had no effect on staminate flower damage ($F_{1,114} = 1.59$, $P = 0.21$). Root herbivory, pollination, and interaction terms had no significant effects on pistillate or staminate flower damage ($F < 1.68$, $P > 0.20$ for all). Block had a significant effect on staminate ($F_{19,114} = 2.23$, $P < 0.005$) but not pistillate floral damage ($F_{19,68} = 1.27$, $P = 0.24$).

The presence of striped cucumber beetles in flowers was not affected by treatments, their interactions, or block (logistic regression, Wald $\chi^2 < 2.48$, $P > 0.11$ for all). In the subset of plants whose flowers contained beetles, the treatments and treatment interactions had no effect on the number of beetles per flower (ANOVA, $F_{1,102} < 3.13$, $P > 0.08$ for all). Only block had a significant effect ($F_{18,102} = 1.84$, $P = 0.03$).

Enhanced leaf herbivory significantly reduced powdery mildew infection (ANOVA, $F_{1,129} = 5.97$, $P < 0.02$; Fig. 1). Root herbivory, pollination and all treatment interactions did not affect powdery mildew infection ($F_{1,129} < 0.98$, $P > 0.32$ for all), although block had a significant effect ($F_{19,129} = 4.80$, $P < 0.0001$).
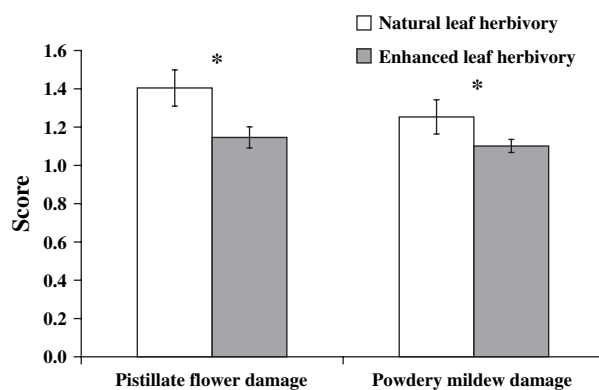

Fig. 1. Effects of leaf herbivory on subsequent pistillate flower damage and powdery mildew infection. Pistillate flower damage scale: 0 = no damage, 1 = 0 to 1% damage, 2 = 2 to 5%, 3 = 6 to 10%, 4 = 11 to 20%, 5 = >20% damage. Powdery mildew infection scale: 1 = 0–25% of leaves infected, 2 = 26–50%, 3 = 51–75%, 4 = 76–100%.

Floral traits and pollinator preference

Pistillate and staminate floral traits were not significantly affected by leaf herbivory, root herbivory, pollination, or their interactions (MANCOVA, Wilks' $\lambda > 0.89$, $P > 0.14$ for all). Measurement date and block affected both pistillate and staminate floral traits (Wilks' $\lambda < 0.68$, $P < 0.0001$ for both).

Pistillate flowers produced on average four times more nectar than staminate flowers, and staminate flowers produced nectar that was 19% more concentrated than pistillate flowers (Table 1). The treatment interaction of root herbivory × pollination significantly affected staminate nectar traits (MANCOVA, Wilks' $\lambda = 0.87$, $P < 0.007$), which was driven by an effect on sugar concentration (ANCOVA, $F_{1,75} = 8.70$, $P < 0.004$). Plants with enhanced root herbivory and enhanced pollination or with natural root herbivory and natural pollination produced nectar that was slightly more concentrated than plants in the natural root herbivory and enhanced pollination treatment or the enhanced root herbivory and natural pollination treatments (Table 1). Date, time, and block also affected staminate nectar production (Wilks' $\lambda < 0.36$, $P < 0.0001$ for all). Staminate nectar production was not affected by any other treatments or treatment interactions (MANCOVA, Wilks' $\lambda > 0.93$, $P > 0.07$ for all). Pistillate nectar production was not affected by any treatments or interactions (MANCOVA, Wilks' $\lambda > 0.88$, $P > 0.10$ for all), but was affected by block and time (Wilks' $\lambda < 0.59$, $P < 0.0001$ for both).

Leaf herbivory, root herbivory, pollination, their interactions, and block had no significant effect on any measure of bee visitation or rejections to staminate or pistillate flowers (ANOVA, $F < 1.63$, $P > 0.06$ for all).

Plant performance

Leaf herbivory had an overall significant effect on plant performance (MANOVA, Wilks' $\lambda = 0.81$, $P < 0.0005$). Leaf herbivory reduced staminate flower production by 10% but had no effect on pistillate flower production (Table 2, Fig. 2A). Plants with enhanced leaf herbivory also produced 21% fewer fruits (Table 2, Fig. 2B) and 8% lighter seeds (Table 2, Fig. 2C) compared with plants with natural leaf herbivory. Leaf herbivory had no effect on root weight (Table 2).

Root herbivory had no significant effect on plant performance (MANOVA, Wilks' $\lambda > 0.92$, $P > 0.20$ for all). However, it

is interesting to note that plants with enhanced root herbivory had heavier roots (10.81 ± 0.44 g compared with 9.93 ± 0.47 g; Table 2) and produced 8% heavier fruits (1.23 ± 0.03 kg compared with 1.13 ± 0.03 kg; Table 2) than plants with natural root herbivory.

Enhanced pollination did not significantly affect plant performance (MANOVA, Wilks' $\lambda = 0.93$, $P > 0.27$), although plants with enhanced pollination produced 8% heavier fruits than naturally pollinated plants (1.23 ± 0.03 kg compared with 1.13 ± 0.03 kg; Table 2).

The treatment interactions of leaf herbivory, root herbivory, and pollination had no significant effects on plant performance (MANOVA, Wilks' $\lambda > 0.90$, $P > 0.07$ for all). Block significantly affected plant performance (MANOVA, Wilks' $\lambda = 0.22$, $P < 0.0001$), particularly total staminate flowers, total fruit number, and weight per fruit ($F_{19,128} > 1.97$, $P < 0.05$ for all, Table 2).

Discussion

The main goal of this study was to examine the combined effects of leaf herbivory, root herbivory, and pollination on subsequent damage, pollinator preference, and plant performance in butternut squash. Enhanced leaf herbivory by the striped cucumber beetle had a stronger negative effect on butternut squash plant performance than root herbivory. Leaf herbivory also had stronger negative effects than root herbivory on plant performance in several natural systems including *Salix planifolia* (Houle & Simard, 1996), *Sonchus oleraceus* (Masters & Brown, 1992), *Sinapis arvensis* (Poveda *et al.*, 2005), and *Lythrum salicaria* (Hunt-Joshi & Blossey, 2005). Leaf herbivory decreased subsequent floral damage and pathogen infection, but this protection did not ameliorate the negative effects of leaf herbivory on reproduction. In addition, butternut plants were not pollen limited in this study, and herbivory (leaf or root) did not affect floral traits or pollinator preference. Overall, there were very few interactions between leaf herbivory, root herbivory, and pollination, indicating that treatment effects were largely additive. In an agroecosystem, plants are supplied with supplemental fertiliser, water and space, and butternut squash may be better able to compensate for low levels of leaf or root damage than natural plant systems. In addition, our herbivore treatments were enhancements of the naturally-occuring herbivory, and control plants were exposed to natural

Table 1. Combined effects of enhanced pollination and root herbivory on nectar volume and sugar concentrations in *Cucurbita moschata*. Numbers represent mean ± SE for each response. Nectar volume is in μl and nectar concentration is in % sucrose equivalents.

Treatment combination	Staminate flowers			Pistillate flowers		
	N	Nectar volume	Nectar concentration	N	Nectar volume	Nectar concentration
Enhanced root herbivory × Enhanced pollination	25	23.10 ± 3.15	33.14 ± 0.74	19	130.51 ± 34.65	24.26 ± 2.15
Enhanced root herbivory × Natural pollination	31	18.65 ± 2.34	32.10 ± 0.93	11	65.32 ± 12.26	28.18 ± 1.40
Natural root herbivory × Enhanced pollination	14	21.76 ± 13.34	32.36 ± 1.34	9	104.98 ± 24.20	22.39 ± 3.25
Natural root herbivory × Natural pollination	13	17.25 ± 2.45	33.58 ± 1.18	6	107.14 ± 53.23	22.67 ± 3.28
Average across treatments	104	19.94 ± 1.25	32.68 ± 0.45	45	88.43 ± 12.35	26.42 ± 0.99

Table 2. ANOVA showing the effects of leaf herbivory, root herbivory, pollination, their interactions, and block on plant performance.

Source	d.f.	Staminate flowers		Pistillate flowers		Weight per fruit		Fruit number		Seed number		Weight per seed		Root weight	
		MS	F	MS	F	MS	F	MS	F	MS	F	MS	F	MS	F
Leaf herbivory	1	0.61	**13.15***	0.06	0.49	0.02	0.34	1.55	**14.57***	0.04	1.56	0.00	**7.75**	0.00	0.02
Root herbivory	1	0.15	3.15	0.02	0.16	0.27	**5.90***	0.01	0.11	0.06	2.49	0.00	0.37	0.27	1.47
Pollination	1	0.00	0.00	0.07	0.65	0.30	**6.56***	0.01	0.11	0.01	0.40	0.00	0.15	0.04	0.24
Leaf × Root	1	0.08	1.77	0.10	0.86	0.00	0.01	0.07	0.64	0.00	0.02	0.00	0.52	0.27	1.48
Leaf × Pollination	1	0.11	2.41	0.00	0.03	0.01	0.12	0.11	0.99	0.00	0.01	0.00	0.96	0.03	0.17
Root × Pollination	1	0.05	1.02	0.00	0.00	0.07	1.62	0.01	0.12	0.02	0.90	0.00	**10.41**	0.05	0.25
Leaf × Root × Poll	1	0.05	1.09	0.06	0.55	0.17	3.86	0.21	1.94	0.00	0.13	0.00	**4.00***	0.13	0.73
Block	19	0.14	**2.92***	0.18	1.55	0.09	**2.04***	0.21	**1.97***	0.03	1.32	0.00	1.20	0.20	1.09
Error	128	0.05		0.11		0.05		0.11		0.24		0.00		0.18	

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

herbivory both above and belowground. We chose to enhance rather than eliminate herbivory, because pesticides can have unintended effects on pollinators or the plants themselves. As we did not include treatments in which herbivory was eliminated, our results can be interpreted as a conservative test of the impacts of leaf and root damage on other interactions, and on plant performance in this system.

Enhanced leaf herbivory decreased subsequent pistillate floral damage, but not leaf damage, suggesting that leaf herbivory may have induced resistance in flowers but not leaves. Floral defensive compounds can protect against florivores (Baldwin & Karb, 1995; Adler *et al.*, 2001), although some defences may also deter pollinators, especially if constitutively expressed (Euler & Baldwin, 1996; Strauss *et al.*, 2002; Adler & Irwin, 2005). Expensive reproductive organs, such as flowers are predicted to have constitutive defences (Rhoades, 1979), but recent studies have shown that floral resistance can be induced in some systems (McCall, 2006; McCall & Karban, 2006). For example, leaf herbivory induced higher alkaloids in the nectar of *Nicotiana tabacum* (Adler *et al.*, 2006) and flowers of *N. sylvestris* (Ohnmeiss & Baldwin, 2000) than in the leaves. In *Cucurbita*, striped cucumber beetles feed on cotyledons early in the season, then on true leaves, roots, and flowers later in the season (Andersen & Metcalf, 1987; Bellows & Divers, 2002; Smyth *et al.*, 2002), suggesting that early leaf herbivory could be a reliable cue for impending floral damage (Karban *et al.*, 1999). The mechanism of induced floral resistance in *C. moschata* is unknown and merits further study, particularly since recent work shows that greater numbers of beetles in flowers of the congener *C. pepo* subsp. *texana* are associated with a higher incidence of bacterial wilt (Ferrari *et al.*, 2006, 2007). Cucurbitacins were not found in *C. moschata* blossoms (Andersen & Metcalf, 1987*)*, although other resistance compounds could potentially be induced upon damage. Our study suggests that leaf herbivory may induce defences in flowers but not leaves, a finding consistent with the hypothesis of protecting reproductive organs which are tightly linked with fitness.

Enhanced leaf herbivory protected plants from subsequent infection by the biotrophic fungus, powdery mildew (*S. fuliginea*). Herbivores can indirectly affect subsequent pathogen infection via plant-mediated defence pathways (Stout *et al.*, 2006). Broad-spectrum defences may be needed to resist herbivory as well as pathogen infection. Herbivore and pathogen defence pathways were initially thought to be independent of each other, although cross-talk between the two pathways can cause signalling conflicts (Bostock *et al.*, 2001). Leaf-chewing herbivores trigger the jasmonate defence pathway, which in some cases can protect against pathogens (Penninckx *et al.*, 1996; Thaler *et al.*, 2004; Stout *et al.*, 2006) including a few biotrophic fungi (Glazebrook, 2005). Cucurbitacins may be induced by leaf herbivory (Carroll & Hoffman, 1980; Tallamy, 1985; Agrawal *et al.*, 1999), but they do not protect against microbial pathogens (Tallamy & Krischik, 1989; Apriyanto & Potter, 1990; Moran & Schultz, 1998) and so are unlikely to be the mechanism responsible for the patterns we found. Other compounds such as peroxidase enzymes (Moran & Schultz, 1998) may be employed for pathogen defence. The mechanism behind induced pathogen defence in this system is unknown and merits further study as powdery mildew diseases are a major problem for crop production worldwide (McGrath & Thomas, 1996).

Leaf herbivory significantly reduced subsequent flower damage as well as fungal infection, but this was not enough protection to prevent the negative impacts of herbivory on plant performance. In this study, enhanced leaf herbivory reduced both male and female reproduction. Male reproduction is typically sacrificed over female reproduction in other herbivore-stressed plant systems (Quesada *et al.*, 1995; Lehtila & Strauss, 1999), which can lead to a reduction in pollen export and outcrossing rate (Thomson *et al.*, 2004). In this study, foliar herbivore damage to butternut squash plants significantly reduced staminate flower production by 10%, but reduced pistillate flower production by only 5%. However, female reproduction, in terms of fruit yield and seed weight, was also significantly reduced by leaf herbivory. Measuring male reproduction in terms of siring success was beyond the scope of this study, but merits further research to determine whether damage has stronger effects on male or female function in this species.

Despite the negative impacts of root herbivory seen in several natural and managed plant systems (reviewed in Blossey & Hunt-Joshi, 2003), root herbivory did not significantly affect plant performance. We could not assess the effectiveness of our enhanced root herbivory treatment in terms of increased larvae
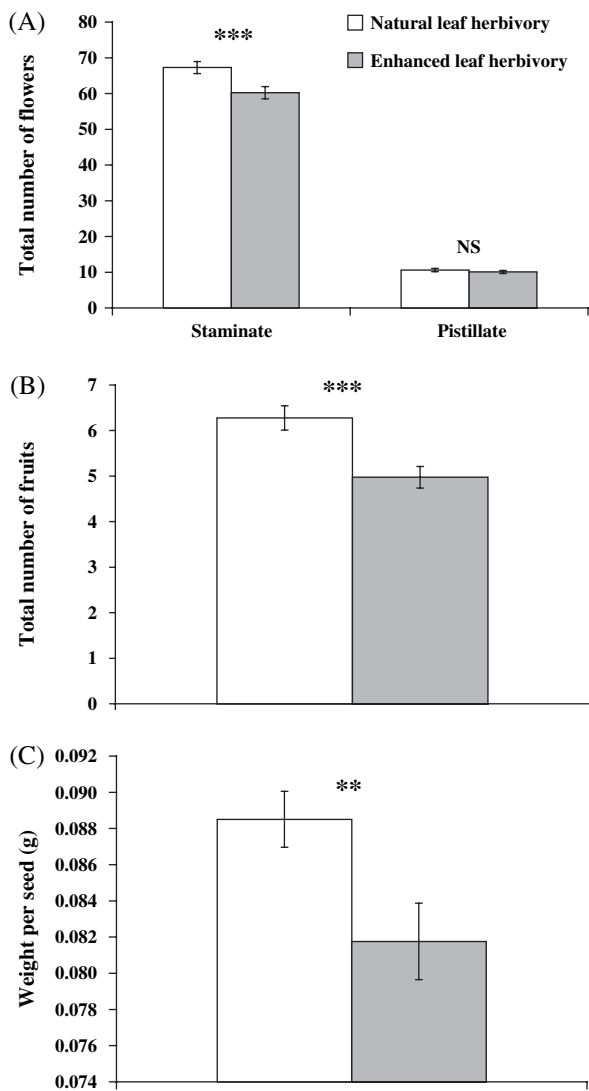
Fig. 2. Effects of leaf herbivory on (A) the total number of staminate and pistillate flowers, (B) the total number of fruits per plant, and (C) weight per seed. Error bars represent ±1 SE of the mean. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

per plant, because it would require destructive sampling. We did examine roots at the end of the season, but at that point larvae had long since emerged and fine root damage was not evident. Thus, the extent to which our treatments enhanced root damage is unknown. While there were no significant effects, root-damaged plants produced slightly heavier roots and fruits, suggesting that butternut squash plants may tolerate or even overcompensate for low levels of root herbivory. Compensation for root damage can occur in other plant systems, but root regrowth is also dependent on soil nutrient availability (Steinger & Mullerscharer, 1992; Houle & Simard, 1996). High amounts of soil nitrogen provided as fertilizer in agroecosystems, may help plants recover from low levels of root herbivory. Root herbivory can have beneficial effects on plants, ranging from increasing

pollinator visitation (Poveda *et al.*, 2005) to inducing aboveground defences (Bezemer *et al.*, 2003, 2004; Hol *et al.*, 2004, Bezemer & Van Dam, 2005). However, a plant's response to root herbivory may be non-linear. Low levels of root herbivory may allow the plant to maintain vigour and devote more resources to defence, whereas high levels of root herbivory may cause extensive stress and be detrimental to plant performance (Soler *et al.*, 2005). Our study suggests that butternut squash plants may have compensated for low levels of root damage, leaving floral traits and pollinator preference largely unchanged. The effect of more severe root herbivory on pollination and plant performance should be examined in future studies, as cucurbit crops can suffer high levels of cucumber beetle infestation.

Plants in this study were not pollen limited for fruit or seed production. There was also no interaction between herbivory (leaf or root) and pollination on fruit or seed yield. In addition, neither leaf nor root herbivory affected pollinator preference or floral traits, indicating that the reduction in plant reproduction from leaf herbivory was not a result of indirect effects on pollination. Leaf herbivory did not make plants less attractive to pollinators, in contrast with other studies that damaged plants continuously or after bud formation (Strauss, 1997; Lehtila & Strauss, 1999; Mothershead & Marquis, 2000). Our results are contrary to another study using a cucurbit crop, which found that cantaloupe plants with damaged leaves were more pollen limited than undamaged plants (Strauss & Murch, 2004). In that study, 20% of the leaf area was removed once on the entire plant, whereas our study removed 50% of the leaf area on six leaves over several weeks. Plants may be better able to compensate for gradual compared with sudden bouts of herbivore damage, resulting in little effect on floral traits, pollinator visitation or pollen limitation.

The lack of pollen limitation in this study may have been due to the high proportion of native squash bee pollinators, which are more efficient pollinators of cucurbits than non-native honey bees (Tepedino, 1981). The addition of managed honey bee colonies for crop pollination may be unnecessary if native squash bee populations are strong. The contribution of native bee communities can provide a critical ecosystem service of crop pollination, as the populations of honey bee colonies continue to decline (Steffan-Dewenter *et al.*, 2005; Klein *et al.*, 2007; Winfree *et al.*, 2007). Species interactions in our butternut squash agroecosystem may also shed light on similar interactions in wild cucurbit congeners that have evolved with comparable specialist herbivores and pollinators, including Diabroticite beetles (Tallamy & Krischik, 1989) and squash bees (Hurd *et al.*, 1971; Meléndez-Ramirez *et al.*, 2002). Our study contributes to the growing body of knowledge linking above- and belowground processes through plant traits and interactions with other organisms. Herbivore, pollinator, and plant interactions should be simultaneously investigated in order to understand the ecological challenges a plant faces in both natural and managed ecosystems.

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