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HERBIVORE FEEDING AND INDUCTION OF SYSTEMIC RESISTANCE IN COTTON PLANTS

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Abstract

Cotton plants defend themselves against feeding injury from the beet armyworm, *Spodoptera exigua*, by direct and indirect (incurred through natural enemies of the herbivore) resistance, both of which can be systemic and inducible. Numerous studies have demonstrated that plant signals are transmitted upward in the cotton plant, but no attempts have been made to examine the potential that the systemic response is transported downward in cotton, although such movement has been demonstrated in the lima bean. Therefore, we examined the direction of systemically induced resistance in cotton using the beet armyworm as eliciting herbivore. In addition, we evaluated the roles that herbivore density and duration of herbivory play in the process of induction and the systemic response in cotton plants. Plants should be favored to respond differentially to variations in the magnitude and duration of defoliation because resistance induction is assumed to be costly. Results confirmed upward transmission of induced systemic resistance (ISR) in cotton, as bioassay *S. exigua* caterpillars reared on both young, expanding leaves and mature leaves following previous *S. exigua* herbivory on lower leaves performed worse than those on control plants. ISR was not observed in leaves lower than actual damaged leaves, however, as bioassay *S. exigua* caterpillars raised on leaves immediately below the damaged leaves performed equally well in comparison with their counterparts from control plants. The duration of feeding did not affect the magnitude of ISR, provided that the feeding damage was kept at the same level. The feeding damage following 1 d herbivory was not statistically significant than that following 3 d herbivory. Therefore, the levels of ISR of 1 and 3 d herbivory did not differ. The relationship between amount of feeding damage inflicted within the same period of time and magnitude of ISR in young, expanding leaves was best expressed as quadratic. The magnitude of ISR increased as feeding damage mounted before reaching a peak value, then it leveled off or even decreased as feeding damage kept increasing.

Introduction

Gossypium hirsutum L. is one of the four main cotton species planted throughout the world. Both constitutive and induced resistance (IR) have been observed in cotton. 'Built-in' plant traits, such as morphological foliar form (reviewed in Niles, 1980), stored terpenoid aldehydes (Stipanovic et al., 1986), and volatiles such as monoterpenes and sesquiterpenes (Loughrin et al., 1994) stored in lysigenous glands (Elzen et al., 1985), are constitutive. They are present independent of wounding.

IR in cotton is induced in the area surrounding the wound site (induced local resistance, hereafter referred as to ILR) after herbivore feeding. Green leafy volatiles (GLVs) (e.g., (Z)-3-hexenal, (Z)-3-hexenol, and (Z)-3-hexenyl acetate), acyclic monoterpenes, sesquiterpenes, homoterpenes, and indole are typical volatile plant secondary compounds expressed in ILR in cotton (Loughrin et al., 1994; McCall et al., 1994; Turlings et al., 1995; Paré and Tumlinson, 1997, 1998). These compounds are released at the onset of herbivore feeding or after some period (ca. 12-24 h) of continuous feeding (Loughrin et al., 1994). Several monoterpenes, sesquiterpenes, and indole are demonstrated to be *de novo* synthesized (Paré and Tumlinson, 1997).

In addition to ILR, several of the inducible volatile and non-volatile plant compounds are found to be released from intact cotton leaves located above the actual feeding site (hereafter referred as to induced systemic resistance or ISR). ISR traits such as gossypol (Parrott, 1990) and terpenoid aldehydes (McAuslane et al., 1997) are non-volatile and are induced in young developing leaves. They inflict negative effects directly on herbivore performance (direct resistance). Volatile plant compounds such as (Z)-3-hexenyl acetate, acyclic monoterpenoid, sesquiterpenes and homoterpenes are also induced in large quantities (Loughrin et al., 1994; Röse et al., 1996; Paré and Tumlinson,

1997, 1998) following herbivory, and parasitoids have been demonstrated to respond to these herbivore-induced plant volatiles. The parasitoids *Cotesia marginiventris* and *Microplitis croceipes* are attracted to cotton plants damaged by the beet armyworm, *Spodoptera exigua,* and corn earworm, *Helicoverpa zea*, respectively (Röse et al., 1998). *Cardiochiles nigriceps*, a parasitoid of the tobacco budworm, *Heliothis virescens*, flies more frequently to host damaged plants (De Moraes et al., 1998). The resistance realized through the action of natural enemies is termed indirect resistance. Employing natural enemies of herbivores as indirect plant resistance can be so striking that these entomophagous natural enemies are referred to as 'plant bodyguards' (Dicke and Sabelis, 1988; Whitman, 1994; Cortesero et al., 2000).

Studies conducted so far on ISR in cotton have clearly demonstrated the upward transmission of plant signal within the plant (McAuslane et al., 1997; Paré and Tumlinson, 1998). No investigations of the potential downward transmission of ISR in cotton have been conducted, although ISR is indicated to move downward to the rhizosphere in lima bean plants. ISR messenger within lima bean can be collected from the leaf petioles and even roots of lima bean plants damaged by the spider mite *Tetranychus urticae* (Dicke et al., 1993; Chamberlain et al., 2001; Dicke and Dijkman 2001). Predatory mites, *Phytoseiulus persimilis*, are attracted to uninfested lima bean plants that are incubated in elicitor-collecting water compared to uninfested plants incubated in control water.

In our studies, we first examine the direction of ISR using *S. exigua* as an eliciting herbivore. We evaluated ISR through its direct effect on the performance of *S. exigua* caterpillars placed on intact leaves of induced plants. Bioassays of herbivores on detached leaves can be problematic, particularly if the bioassay is conducted for more than one day. Detached corn and lima bean leaves release volatile plant secondary compounds in higher quantities than intact leaves (Arimura et al., 2001; Schmelz et al., 2001). It also is difficult to maintain the turgidity of excised leaves for a prolonged period even if the petioles are covered with wet cotton ball (personal experience).

The release of some volatile inducible terpenes induced by *S. exigua* follows a diurnal pattern in cotton (Loughrin et al., 1994). But the amount seems not to increase day by day as herbivore feeding continues. The quantities of some other terpenes that do not follow diurnal patterns even decline after about 24 h of continual herbivore feeding. This may suggest that either cotton plants actively avoid over-investment of resources in defense or their ability to defend themselves against herbivory is limited. ISR may have the same fate. Hence, we then separately investigated the magnitude of ISR in response to various lengths of feeding time while keeping the amount of feeding damage about the same, and the magnitude of ISR in response to differential levels of feeding damage that was inflicted within a fixed period of time.

Materials and methods

Cotton plants and herbivores

Cotton plants, *Gossypium hirsutum* (variety FiberMax 989), were grown in a greenhouse in plastic flower pots (15 cm in diameter) filled with peat moss and potting soil. Sta-Green all purpose plant fertilizer ca. 1 tea spoon per pot was evenly mixed with peat moss and potting soil before potting. Day/night cycle was about 14L:10D. Plants were watered as needed. Plants with 5 or 6 fully expanded leaves were used in all the experiments. Cotton plants for different treatments were generally matched for height and size of leaves. If difference in these two traits was noticed, plants with different traits were then arranged into different blocks before being randomly assigned to treatments and control within a block. All experimental cotton plants were so spaced to avoid direct leaf contact with each other. We also assumed no plant-plant communications through airborne messengers.

Beet armyworm (BAW), *S. exigua* caterpillars originated from a laboratory colony in the Department of Entomology, UGA, on the Tifton campus. Newly-emerged caterpillars were reared on semi-artificial diet until they were early second instars (ca 3 d old). Early second-instar caterpillars were used throughout the experiments unless otherwise noted.

Direction of ISR transmission evaluated through direct resistance

The experiment was a 3×2 factorial design with leaf positions and induction condition as two factors. Leaf positions tested were the third fully expanded leaf (L3), the fifth fully expanded leaf (L5), and the seventh young expanding leaf (L7) (cotyledons numbered as node 0). Induction condition entails induced and control. Fifteen early second-instar BAW caterpillars (induction BAW) were caged on the fourth fully-expanded true leaf (L4) for 2 d to elicit ISR. Cages were made according to Cortesero et al. (1997), but modified. Instead of perforating the plastic soft-

drink lids, we cut a disk (3 cm in diameter) out of the center of the lids and glued with fine mesh gauze. Another 10 early second-instar BAW caterpillars (bioassay BAW) were separately caged on L3, L5, and L7 for bioassay 2 d after removing the induction caterpillars. Only one leaf position was used for bioassay on each cotton plant to avoid potential interactions due to feeding on more than 1 leaf position in the same plant. All 6 treatments were replicated 4 times except 1 treatment—induced and L5, where one replicate in the treatment was lost. ISR induction feeding damage was quantified daily over a 3-d period using images from a digital camera (Canon D-30 camera, Japan) and digital imaging software -- Image Processing and Analysis in Java (ImageJ, version 1.34s, available in public domain). Leaf area eaten per caterpillar (cm^2 per caterpillar) on Days 1, 2, and 3 was calculated. Numbers of bioassay BAW caterpillars recovered were recorded and and caterpillars were weighed daily with a Mettler Analytical Balance (AE 100, Mettler Instrument Corp., Switzerland). Average weight gain of bioassay BAW (g per caterpillar) was calculated by subtraction of initial weight from weight measured daily and then divided by numbers of bioassay BAW caterpillars collected. Bioassay BAW weight was measured daily for 5 d.

Bioassay BAWs were reared in cups with semi-artificial diet in an environmental chamber after the greenhouse bioassay. They were checked daily for pupation and emergence.

Does feeding time play a role in ISR?

The experiment was arranged as a randomized complete block design with feeding time as treatments. Treatments and control were each replicated 4 times. Ten, 15, and 30 early second-instar BAW caterpillars were allowed to feed on the third fully expanded leaf of different cotton plants for 1 (T1), 2 (T2), and 3 (T3) d, respectively. Caterpillars were replaced daily with the same density designated for treatment in order to exclude the possible effect of herbivore age on ISR. Feeding area caused by each induction BAW caterpillar was quantified and calculated as described above. Another 10 caterpillars of the same species and age (bioassay BAW) were caged on the sixth leaf (still expanding) right after removal of induction BAW for bioassay. The average weight of each bioassay BAW caterpillar was determined each day throughout the experiment. BAW caterpillars used in the experiments were very small, we assume the individual weight was negligible. Because the sixth leaf was not sufficient to support caterpillar feeding for 6 d, caterpillars were reared on the fifth leaf on Days 5 and 6.

Does feeding damage play a role in ISR?

Five, 10, 20, and 30 early second-instar BAW caterpillars were allowed to feed on the third fully expanded leaf for 24 h to inflict differential levels of feeding damage. Total feeding damage was measured using the method described before. Another 5 BAW caterpillars of the same age were caged on the sixth leaf (a young expanding leaf) right after removal of induction BAW. Numbers of bioassay BAW caterpillars were recorded and total weight determined daily for 5 d as described above. Average bioassay BAW weight was calculated as total weight of bioassay BAW caterpillars divided by numbers of caterpillar recovered. Because the sixth leaf was too small for caterpillars to feed for 5 d, caterpillars were moved to the fifth leaf on Days 4 and 5. The experiment was arranged as a randomized complete block design with four treatments and control each blocked 5 times.

Statistical analyses

Leaf area consumed, average weight gain, average weight of bioassay BAW, pupal weight, days from onset of bioassay to pupation, and days from pupation to adult emergence per caterpillar in all experiments were analyzed with PROC GLM (version 8, SAS Institute Inc., Cary, NC). Percent of BAW pupae yielding adults was analyzed with non-parametric method (PROC NPAR1WAY, WILCOXON). Data were checked for model assumptions before analysis. Data were untransformed unless otherwise noted.

Results

Direction of ISR transmission evaluated through direct resistance

ISR in the young leaf (L7) reduced herbivore mass from day 1 of bioassay (Fig. 1A). Average weight gains of each bioassay BAW reared on L7 of the damaged cotton plants were 86% less within 1 d, 88% within 2 d, 86% within 3 d, 84% within 4 d, and 86% within 5 d less than corresponding weight gains of BAW reared on L7 of control plants. The weight gain differences of bioassay BAW between ISR and control were all statistically significant ($p = 0.0295$, 0.0297, 0.0323, 0.0366, respectively for 1, 2, 3, 4 d), except for those on D5, which were nearly significant ($p = 0.0543$). Leaf areas eaten on L7 of damaged cotton plants were reduced from the onset of the experiment compared to corresponding areas of control plant (Fig. 1B). Although the differences in leaf area consumed between L7 of damaged and control plants were not statistically significant all 3 d, each bioassay BAW on L7 of damaged plant

consumed half the area by D1, one third the area by D2 and D3 of BAW counterparts on L7 of control plants. Time from onset of the bioassay to pupation of bioassay BAW on damaged plants was 18.2±0.63 d, which was significantly longer than the time to pupation of bioassay BAW on control plants (15.2±0.63 d) (Table 1). No significant difference was observed in time from pupation to adult emergence, percent adult emergence or pupal weight between treatment and control (Table 1).

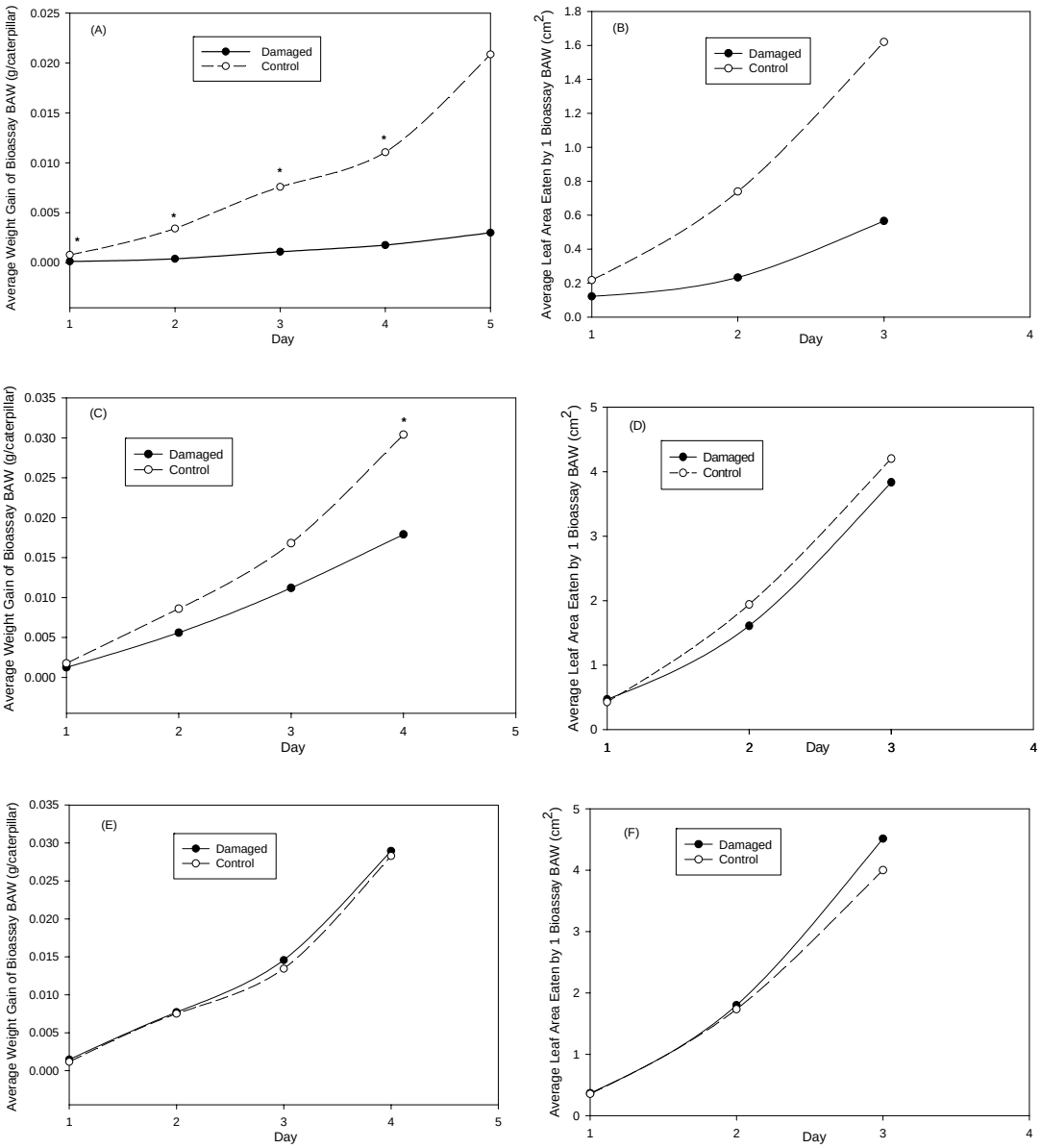

Fig. 1. Average weight gain and leaf area eaten of bioassay BAW caterpillars reared on leaves with ISR and on control plants over the course of several days. (A) and (B), on still expanding leaves (L7); (C) and (D), on leaves immediately above damaged leaves (L5); (E) and (F), on leaves immediately below damages leaves (L3).

ISR was observed in the leaf immediately above the induction leaf (L5) in form of weight gain of bioassay BAW. The weight gains of bioassay BAW raised on L5 of damaged plants were consistently less than those of BAW reared on L5 of control plants (Fig. 1C). Each bioassay BAW caterpillar gained ca. 30% within 1 d, 35% within 2 d, 33% within 3 d, and 41% within 4 d, less mass on L5 of damaged plants in comparison to its counterpart on L5 of control plants, though weight gain differences between bioassay BAW reared on L5 of damaged and control plant were not

significant until Day 4 (p = 0.0403). Leaf areas eaten by bioassay BAW on L5 of damaged plant within 1, 2, and 3 d were not different from those eaten on L5 of control plants (Fig. 1D). No significant differences between bioassay BAW reared on damaged and control plants were observed in terms of time from onset of bioassay to pupation, time from pupation to adult emergence, and percent adults emerged (Table 1). However, mean pupal weight of each bioassay caterpillar on damaged plant was found to be ca. 16% higher than that on control plant (Table 1).

Table 1 Effects of ISR direction expression by leaf position on bioassay BAW life history variables[1].

	Time from bioassay to pupation (Mean±SEM) (d)	Time from pupation to adult emergence (Mean±SEM) (d)	Percent of adults emerged (Mean±SEM) (%)	Pupal weight (Mean±SEM) (g)
L7				
Damaged	18.18±0.63*	10.91±0.22	96.43±0.07	0.13±0.01
Control	15.17±0.63	10.41±0.22	100.00±0.0	0.13±0.00
L5				
Damaged	14.07±0.03	10.50±0.23	96.67±0.06	0.13±0.00**
Control	14.05±0.03	9.79±0.20	100.00±0.00	0.11±0.00
L3				
Damaged	14.06±0.07	9.89±0.23	100.00±0.0	0.12±0.01
Control	14.10±0.07	9.53±0.23	97.22±0.06	0.12±0.01

[1] L7, young expanding leaf; L5, mature leaf immediately above damaged leaf; L3, mature leaf immediately below damaged leaf. * and ** significant difference between damaged and control plants at 0.05 and 0.01 level, respectively.

BAW rearing on the leaf immediately below (L3) the induced leaf had no measurable effect on BAW development or leaf consumption. The differences between those variables on damaged and control plants measured over a period of several days were small and not significant (Fig. 1E and 1F). No significant differences between bioassay BAW reared on damaged and control plants were observed in terms of time from onset of bioassay to pupation, time from pupation to adult emergence, percent adults emerged, and pupal weight (Table 1).

Does feeding time play a role in ISR?

The ANOVA results and multiple comparisons of initial feeding damage are summarized in Table 2. The damaged areas of treatments T1, T2, and T3 were all significantly different from 0. The damaged areas of T1 (11.8±0.36 cm^2) and T3 (12.3±0.36 cm^2) were not statistically different from one another (p = 0.3223). However, the damaged area of T2 (13.1±0.36) was 1.32 cm^2 greater than that of T1, and T1 and T2 were significantly different (p = 0.0403).

Table 2 Summary of ANOVA table and multiple comparisons of initial feeding damage inflicted by same number of BAW caterpillars but with various feeding time.

ANOVA table

Source	DF	Type III SS	F Value	Pr > F
Block	3	1.2822	0.83	0.5227
Treatment	2	3.5193	3.43	0.1016
Error	6	3.0785		

Multiple comparisons among treatments

Treatment*	Mean±SEM** (cm^2)
T1	11.79±0.36 a
T2	13.11±0.36 b
T3	12.34±0.36 ab

*T1, 1 d feeding; T2, 2 d feeding; T3, 3 d feeding; * *different letters following mean±SEM implies that they are different from each other at α=0.05 level. Pairwise t-test was used to perform multiple comparisons across treatments.

One day's feeding by bioassay BAWs on induced leaves of T2 and T3 reduced the weights of BAW significantly compared to those feeding on control leaves (p = 0.0103 and 0.0249, respectively) (Table 3). The bioassay BAW weight from T1 was not significantly different from BAW reared on control plants (p = 0.3649). The patterns were consistent for 3 d (Table 3), but on Day 3, bioassay BAW of T1 plants weighed ca. 30% less in comparison with corresponding BAW on control plants. From Day 4 to Day 6, bioassay BAW weights of all T1, T2, and T3 BAW were consistently and significantly lower than those of BAW on control plants (Table 3).

Table 3 Average weight of bioassay BAW and leaf area eaten over a period of several days.

Weight of bioassay BAW (Mean±SEM) (mg/caterpillar)*						
Treatment**	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Control	2.4±0.00a	5.3±0.00a	11.8±0.00a	28.5±0.00a	57.0±0.00a	116.0±0.01a
T1	2.2±0.00ab	4.0±0.00ab	8.5±0.00ab	15.3±0.00b	30.2±0.00b	56.7±0.01b
T2	1.7±0.00b	2.5±0.00b	4.6±0.00b	9.8±0.00b	16.1±0.00b	35.2±0.01b
T3	1.8±0.00b	2.9±0.00b	6.0±0.00b	11.5±0.00b	20.5±0.00b	38.0±0.01b

Leaf area eaten (Mean±SEM) (cm^2/caterpillar)***		
Control	0.268±0.03a	0.814±0.12a
T1	0.254±0.03a	0.567±0.12ab
T2	0.121±0.03b	0.339±0.12b
T3	0.144±0.03b	0.419±0.12b

*T1, 1 d feeding; T2, 2 d feeding; T3, 3 d feeding; * * and *** different letters following mean±SEM implies that they are different from each other at α=0.05 level. Pairwise t-test was used to perform multiple comparisons across treatments.

Each bioassay BAW of T1, T2, and T3 consumed less leaf mass within 24 h than BAW feeding on control leaves did within the same period of time (Table 3). Within the first 24 h, the leaf area consumed by each bioassay BAW on T1 was 2.20±0.00 mg, which was not significantly different from that consumed on control leaf (2.4±0.00 mg) (p = 0.7639). The leaf mass eaten by each bioassay BAW on T2 and T3 in the first 24 h were 1.7±0.00 and 1.8±0.00 mg, respectively, which were both significantly lower than corresponding leaf mass consumed in the controls (p = 0.0102 and 0.0228, respectively). Each bioassay BAW on T2 and T3 consumed significantly less leaf area than its counterpart on T1 plants. Within the second 24 h, bioassay BAW on T2 and T3 plants also consumed significantly less leaf that that BAW on controls. Though the leaf mass consumed by bioassay BAW on T1 was not significantly different from that on control (p = 0.1887), it was 34% less.

Does feeding damage play a role in ISR?

Mean initial feeding damage of 4 treatments caused by induction BAWs with the densities of 5, 10, 20, and 30 early 2nd-instar caterpillars for 24 h was statistically significant from 0 (table 4). The differences among the 4 treatments were all significant as well. The weights of bioassay BAW raised on the treatment and the control plants over 5 d are shown in Fig. 2. Average weight of 1-d-old bioassay BAW reared on 5 BAWs treatment (2.10±0.10mg) was not significantly different from corresponding bioassay BAW of control (2.20±0.10 mg) (p = 0.2846). Weight of 1-d-old bioassay BAW from the 10, 20, and 30 BAWs treatments were significantly lower than that of BAW on control plants (p = 0.0285, 0.0038, and 0.0204, respectively). Compared to bioassay BAW of the 5 BAWs treatment, the weights of bioassay BAWs of the 20 BAWs treatment were statistically lower on Day 1 (p = 0.0372). No other significant difference was observed on Day 1. On Day 2, weights of bioassay BAW in all treatments (5, 10, 20 and 30 BAWs) were significantly lower than those of BAWs in the control (p = 0.0251, 0.0041, 0.0006, and 0.0016, respectively). The differences between treatments were not significant. A similar pattern was observed on Day 3. The weight of 4-d-old bioassay BAW of the 10, 20 and 30 BAW treatments were significantly lower than corresponding BAW in the control. In comparison to the weight of bioassay BAW in the control (16.5±1.40 mg), the weight of bioassay BAW of 5 BAW treatment (12.8±1.40 mg) was ca. 23% less, though statistically they were not different (p = 0.0919). On Day 5, the weights of BAW in the 5, 10, 20, and 30 BAW treatments were statistically lower than corresponding BAW weight in the control treatment (p = 0.0408, 0.0027, 0.0013, and 0.0016, respectively).

The relationship between ISR (expressed as weight of bioassay BAW caterpillar, the lighter the weight of caterpillar, the stronger the ISR) and feeding damage area was best represented by quadratic expressions. The pattern was consistent over the experimental course of 5 d (Fig. 3).

Discussion

Direction of ISR transmission evaluated through direct resistance

Induced systemic resistance (ISR) in young, expanding leaves above the feeding site has been observed in cotton (Alborn et al., 1996; McAuslane et al., 1997; Paré and Tumlinson, 1998). Our experiment confirmed the occurrence of ISR in cotton plants, and of upward movement of the response. The average weight gains of bioassay BAW reared on undamaged young expanding leaves (L7) of cotton plants with one mature leaf (L4) damaged by BAW

Table 4 Summary of ANOVA table and multiple comparisons of initial feeding damage inflicted by various densities of induction BAWs.

ANOVA table

Source	DF	Type III SS	F Value	Pr > F
Block	4	1.4825	1.09	0.4029
Treatment	3	139.3252	137.12	<0.0001
Error	12	4.0643		

Multiple comparisons among treatments

Treatment	Mean±SEM* (cm^2)
5 BAWs	1.2880±0.2603 a
10 BAWs	2.6466±0.2603 b
20 BAWs	5.0770±0.2603 c
30 BAWs	8.2314±0.2603 d

* different letters following mean±SEM implies that they are different from each other at α=0.01 level. Pairwise *t*-test was used to perform multiple comparisons across treatments.

before bioassay were consistently and significantly lower over a period of 5 d than weight gains of BAW on undamaged L7 of cotton plant (Fig. 1A). The pattern was mirrored in the leaf area eaten by each bioassay BAW (Fig. 1B). Bioassay BAW caterpillars (3-d-old) fed on L7 of induced plants needed 3 d more time to complete larval development to pupation in comparison to those fed on L7 of control plants (Table 1). Bioassay BAW caterpillars were fed with identical artificial diet after 5 d bioassay on leaf still attached to plant. It's likely that the difference might be greater provided that bioassay BAWs were restricted to feed on live plant throughout the larval period.

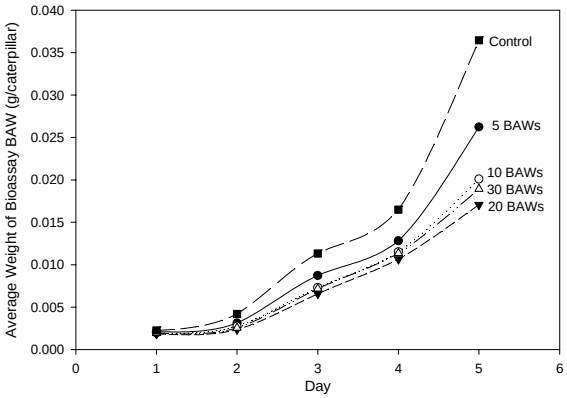

Fig. 2 Average weight of bioassay BAW caterpillars reared on leaves from plants with different levels of feeding damage and control plant over 6 days.

ISR on mature leaves immediately above the damaged leaf was suggested from our results, but it was not so strong as that in young leaves. Bioassay BAW reared on undamaged mature leaves (L5) from plants with L4 damaged before bioassay consistently gained less biomass than those reared on L5 from control plants (Fig. 1C). Within 4 d, weight gain of bioassay BAW on L5 from damaged plant was ca. 41% lower than that of bioassay BAW on L5 of

control plants. The weight gain difference was statistically significant (p = 0.0403). Our finding was consistent with Alborn et al. (1996), that mature leaves cut from cotton plants whose oldest two true leaves had been fed on by two third-instar *Spodoptera littoralis* for 16 h were avoided by bioassay conspecifics in the feeding choice tests. However, McAuslane et al. (1997) observed no significant effects of ISR (gland density, total glands, quality and quantity of terpenoid aldehydes produced) on upper mature leaves on plants with the two oldest leaves being on fed by *S. exigua* for 24 h. One possible explanation is that McAuslane et al. (1997) used 2 third-instar caterpillars while we used 15 early second-instars to induce ISR. The age difference of inducing caterpillars might partly account for the inconsistent results, since herbivore age has been suggested to affect the production of parasitoid-attracting volatile synomones (Takabayashi et al., 1995; Gouinguené et al., 2003). Second, feeding time and damage amount might also account for some of the difference. In our experiment, leaves were fed by BAW for 2 d, with 15.6 cm^2 leaf consumed. Third, the potential effects caused by different bioassay methods couldn't be excluded. Bioassays of herbivores on detached plant leaves could be problematic, as noted above.

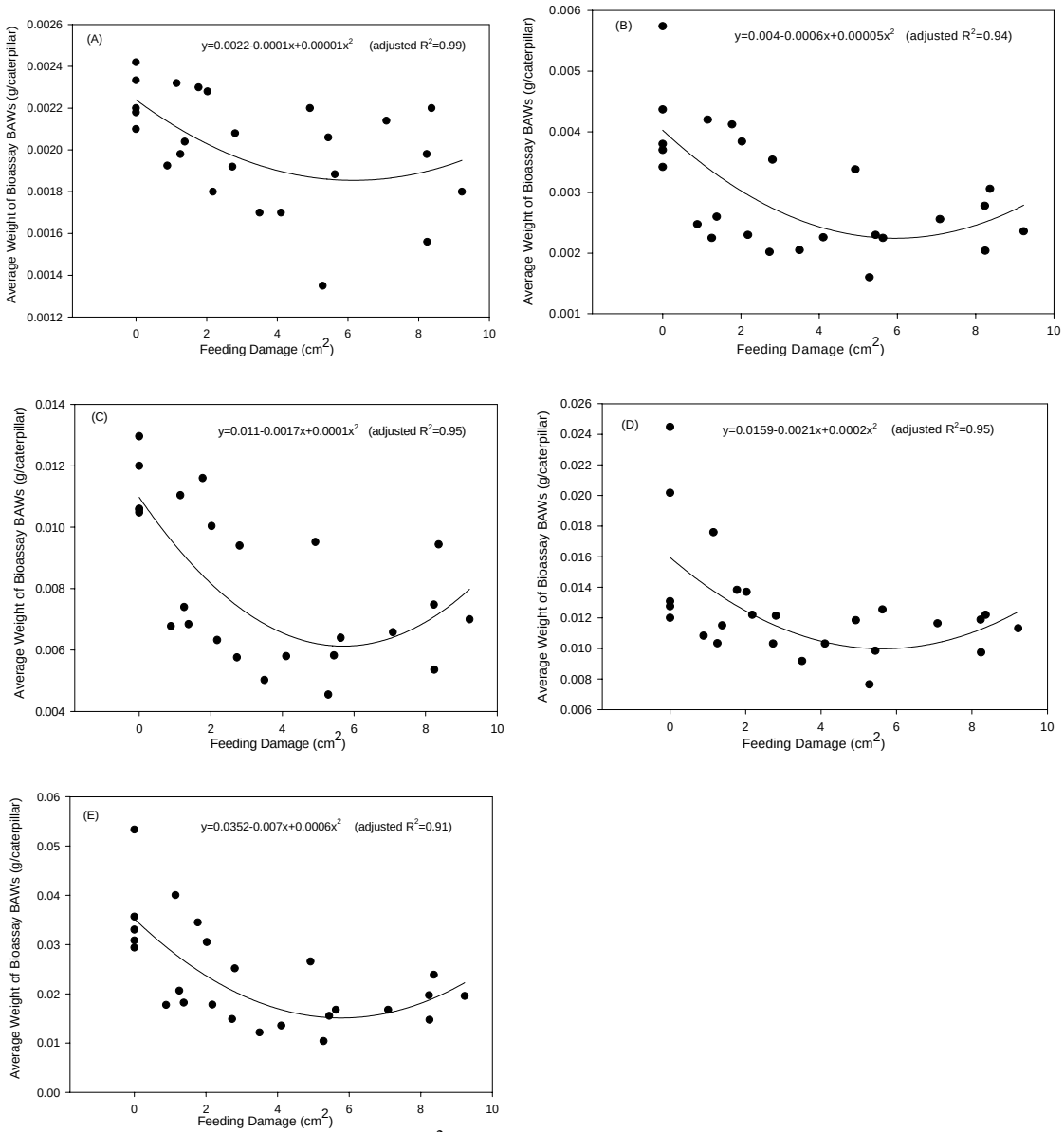

Fig. 3 Quadratic regression ($y = y_0+ax+bx^2$) of average weight of bioassay BAWs against feeding damage over a period of 5 d. (A), 1-d-old BAWs, $p(y_0)$<0.0001, $p(a)$=0.0335, and $p(b)$=0.1187; (B), 2-d-old BAWs, $p(y_0)$<0.0001, $p(a)$=0.0036, and $p(b)$=0.0244; (C), 3-d-old BAWs, $p(y_0)$<0.0001, $p(a)$=0.0008, and

$p(b)$=0.0067; (D), 4-d-old BAWs, $p(y_0)$<0.0001, $p(a)$=0.0069, and $p(b)$=0.0292; and (E), 5-d-old BAWs, $p(y_0)$<0.0001, $p(a)$=0.0009, and $p(b)$=0.0077.

ISR was not observed on the leaf below the damaged leaf which had been continuously fed on by BAW for 2 d. None of the measured bioassay parameters for bioassay BAW were significant between those from treatment and control plants (Figs. 1E, 1F; Table 1). The parasitoid *Microplitis croceipes*, in no-choice wind tunnel tests, responded the same way to the lower half of plants whose upper half had been fed on by its host the tobacco budworm, *Heliothis virescens*, for 24 h and to the lower half of plants with no feeding damage (unpubl. data). It's likely that the benefits of resource investment in protecting old leaves are lower than costs. So, ISR in old leaves does not occur. These data, however, did not exclude the possibility of ISR being transmitted down below the real feeding site, possibly into the rhizosphere. From an evolutionary and population perspective, it would be advantageous for cotton plants to warn neighboring conspecifics. More studies are needed before making conclusions.

The roles feeding time and feeding damage play in ISR

Feeding on mature leaves by 10 early second instar *S. exigua* caterpillars for 24 h induced ISR in young undamaged cotton leaves. Continuous feeding longer than 24 h increased the induction of ISR in young leaves, but the magnitude of ISR was not significantly different from the magnitude of ISR induced following 24 h feeding, provided the feeding damage of the different treatments was kept at same level. The initial feeding damage of 1 d feeding (T1) was 11.8±0.36 cm^2, which was not significantly different from that of 3 d feeding (T3) (12.3±0.36 cm^2) (Table 2). Therefore, the magnitude of ISR of T1 and T3 did not differ significantly from one other (Table 3). The same pattern was detected between T2 and T3 (Table 3). Loughrin et al. (1994) also found that the release of some volatile inducible terpenes induced by *S. exigua* did not increase day by day as herbivore feeding continues. The quantities of some other terpenes that do not follow diurnal patterns even decline after about 24 h of continual herbivore feeding. Furthermore, after feeding damage reached a certain level, any additional feeding damage might alter the magnitude of ISR a little, but not significantly (Table 3, between T1 and T2). This finding was further confirmed by the relationship between feeding damage and magnitude of ISR (the lighter the bioassay caterpillar, the stronger the ISR) (Fig. 3). Cotton leaves of the same leaf position from different treatments were fed on by different densities of *S. exigua* to inflict various levels of feeding damage but for the same duration of feeding. The relationship was best expressed as quadratic (Fig. 3). The ISR increased as feeding damage mounted before reaching a peak value, then it leveled off or even attenuated as feeding damage kept increasing. This may suggest that either cotton plants actively avoid over-investment of resources in defense since further investment (costs) will exceed benefits, or their ability to protect themselves from herbivory is limited after feeding damage reaches a certain level. From *S. exigua* perspective, aggregative feeding seems to be a strategy adapted to break down cotton plant defense. Typically, *S. exigua* eggs are laid in clusters of from 50 to over 100 eggs on the lower surface of lower leaves of host plants. After egg emergence, 1st-instar caterpillars feed together around the oviposition site until the 3rd-instar, when they start to disperse (personal observation).

Plants act quickly in response to herbivory. ISR in young leaves was found a few hours after onset of herbivory on lower mature leaves. *S. exigua* caterpillars ate significantly less young leaf mass of plants whose 2 oldest mature leaves had been previously fed on by 2 of their conspecifics for as short as 6 hr, in comparison with *S. exigua* caterpillars on control plants with no previous herbivore damage (Alborn, 1996). Phytochemicals (e.g.,jasmonic acid) upregulating defense genes or production of volatile plant secondary metabolites in maize (*Zea mays* cv. Delprim) were increased over 10-fold minutes after mechanical wounding or a combination of mechanical wounding and volicitin (elicitor isolated from oral secretion of *S. exigua*) application, compared to intact maize plants (Schmelz et al., 2003). Nevertheless, the strength of the response is mediated by the amount of feeding damage. The production of headspace volatile (ILR) from spider mite, *Tetranychus urticae*-infested kidney bean plants mainly correlated with the spider mite densities (Meada and Takabayashi, 2001; Horiuchi et al., 2003). Volatile emission peaked as spider mite density peaked. In maize, ILR (expressed as volatile sesquiterpene and indole production) positively correlated with *S. exigua* herbivory levels (Schmelz et al., 2003). No limitation of volatile production was detected in these studies, however. To authors' knowledge, this paper is the first to suggest a limitation of ISR, and the first to elucidate the interacting role of feeding damage and duration of feeding in ISR.

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