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Induction of Soybean Resistance to the Mexican Bean Beetle (Coleoptera: Coccinellidae)

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ABSTRACT: We tested chemical and insect feeding-induced insect resistance on soybean plants. The chemical induction effects of jasmonic acid (JA) and salicylic acid (SA) were investigated. We also evaluated the effects of plants stressed with previous insect herbivory. A larval antibiosis screening technique (LAST) and a preference test were performed in petri dishes using Mexican bean beetles, *Epilachna varivestis* Mulsant, to monitor the effects of resistance in soybeans. Results from the LAST assessment showed evidence of both chemical and insect feeding induction of resistance. JA applied to the plant resulted in greater insect mortality and less larval damage than did the controls, indicating an increased resistance to herbivorous stress. In contrast, plants treated with SA had slightly less resistance. In the previously insect-damaged study, plants that had portions of lower leaves consumed by the beetle developed a higher level of resistance to subsequent feeding of the upper leaves. Preference studies were inconclusive because of a general lack of insect feeding. Induced resistance could provide a means to enhance the natural resistance of soybeans to pests, benefiting both consumers and producers.

Soybean lines have natural defense mechanisms to prevent insect injury (Lin and Kogan, 1990). These defenses can be triggered as a result of a stress, i.e., induced resistance, or can be constantly present and stress-independent, i.e., constitutive resistance. Studies have confirmed both types of resistances in soybeans. Induced resistance has resulted from mechanical injury, insect herbivory, UV radiation, and pathogen infection (Lin and Kogan, 1990; Lin et al., 1990; Conconi et al., 1996). Constitutive resistance, also recognized in the soybean (Chaing et al., 1986, 1987), has been used in cultivar development. Although a number of resistant cultivars have been released, none have been popular on the market due to the effects of reduced yields (Boethel, 1999).

Much work is continuing in the area of induced resistance in soybeans. Systemic acquired resistance is the resistance induced in response to a challenger and spread systemically throughout the plant (Ryals et al., 1996; Weiler, 1997; Willits and Ryals, 1998). One or more signals can travel from the local wound to unwounded areas and systemic acquired resistance is established. Once this resistance is established it may function for up to several weeks (Thaler, 1999).

Recent studies indicate that various secondary metabolites that act as plant growth regulators may play a role in induced resistance and have an effect on insect development, including jasmonates, salicylic acid, brassinosteroids, peptides, and lipochitooligosaccarides (Raskin, 1992; Tizio, 1996; Creelman and Mullet, 1997). Jasmonic acid (JA) is an example of a compound that is exerted systemically throughout the plant following injury, enhancing plant defense (Weiler, 1997). Contrasted with this, research has shown that salicylic acid (SA) can shut down a plant's response to injury (Pan et al., 1998). Salicylates, when taken in by a plant, inhibit allene oxide synthase activity, a necessary component in

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the octadecanoid pathway (Creelman and Mullet, 1997). When the production of allene oxide synthase is inhibited, JA production is reduced, resulting in a reduced defense against the challenger (Doares et al., 1995). This can occur whether it is a pathogen or an insect (Creelman and Mullet, 1997). We hypothesize that a plant sprayed with SA will become less able to defend itself.

The Mexican bean beetle (MBB) *Epilachna varivestis* Mulsant (Coleoptera: Coccinellidae) is a significant insect pest in certain soybean growing areas of the U.S. (Edwards et al., 1994). It feeds only in plants of the Leguminosae family, therefore having a considerable host specificity (Lin and Kogan, 1990). MBB has also been a targeted insect in a breeding program in Ohio to derive resistant soybeans (Cooper and Hammond, 1988, 1995, 1999).

Studies were conducted to test induced resistance in soybeans to the MBB, through chemical stresses where plants were sprayed with varying concentrations of JA and SA, and physical stresses where plants were previously defoliated. We hypothesized that (1) surface-applied JA will make the soybeans less susceptible to MBB larval damage and SA would make soybeans more susceptible, (2) increasing the concentrations of JA and SA would enhance their respective effects, and (3) previously insect-defoliated plants would induce resistance to subsequent insect feeding.

Materials and Methods

Glysophate-tolerant soybean seeds, locally grown in central OH (a mixture of cultivars Asgrow 2701 and 3001) were germinated under fluorescent lights, 17 hr days, at 21 ± 2°C in seed starters, transplanted to 10 cm pots, and then placed in a greenhouse equipped with sodium lights at 17 hr, 21–24°C days and 15–18°C nights. Plants used in the experiment were randomized and assigned to a specific treatment. Rows of plants were sorted by treatment and separated from the adjacent row. Pots within rows and rows themselves were reordered every other day to account for any unequal illumination of light or other factors that could possibly affect the plant's growth and development.

Chemically-induced resistance procedure: Treatments for the chemically-induced resistance experiment were derived from methyl jasmonate, (95+%, IUPAC name 3-oxo-2-(2-pentenyl)cyclopentaneacetic acid, methyl ester, $C_{13}H_{20}O_3$, MW 224.3, obtained as a gift from R. A. Creelman) and methyl salicylate (99%, IUPAC name 2-hydroxybenzoic acid methyl ester, $C_8H_8O_3$, MW 152.1, obtained from Sigma Chemical Co., St. Louis, MO). First, stock solutions of SA (5%) and JA (1%) were prepared with 5 and 1 ml concentrated (~1M or nearly 100%) SA and JA added to 95 and 99 ml ethanol, respectively, as was a control with 5 ml water in 95 ml ethanol. Two levels each of JA and SA concentrations were then prepared: 0.015% and 0.03% JA, referred to as JA1 and JA2, respectively, and 0.05% and 0.1% SA, referred to as SA1 and SA2, respectively. Concentration levels were chosen based on previous work by Browse (Browse, pers. comm.). Final concentrations were then made by diluting 15 (0.015%) or 30 ml (0.03%) of JA stock with 985 or 970 ml deionized water, by diluting 10 (0.05%) or 20 (0.1 %) ml of SA stock with 990 or 980 ml deionized water, and by diluting the control stock of 20 ml with 980 ml deionized water. In each of the five final solutions, six drops of Tween™ 20 liquid detergent per liter of solution were added to allow equal distribution of the treatment solution on the plant leaf surface and to aid in the absorption of the solution.

The solutions were poured into hand spray bottles and the appropriate plants were sprayed every-other day, beginning 12 days prior to the start of the petri-dish larval assessment (collectively termed as the larval antibiosis screening technique (LAST) (Rufener et al., 1987)), and continued until the end of the assessment when adult MBB eclosed.

Each plant was sprayed with 4 ml of the treatment solution when plants were small, progressing to 6 ml near the end of the experiment. All plants were sprayed with the same dosage and volume. A total of seven treatments were used and were referred to as control, JAN, JA2, SA1, SA2, Both1 (JA1 + SA1), and Both2 (JA2 + SA2) (see above paragraph for concentrations). There were five replications of the treatments. A replicate set of plants were grown with the same treatments (however, no leaves were removed) to determine whether the chemical treatments altered plant growth patterns.

Insect feeding-induced resistance procedure: MBB adults and eggs were obtained from a colony reared on green bean, *Phaseolus vulgaris* L., at the Ohio Agricultural Research and Development Center in Wooster, OH. Prior to emergence three egg masses with 75–100 eggs were placed on each of the plants undergoing the physically-induced treatment. Eclosed larvae were allowed to feed on the bottom tier of leaves (first three trifoliates) of each plant for 7 days, severely damaging the lower foliage. After three days plants were used in the preference test and the larval antibiosis screening technique. There were 10 replications of these treatments.

Larval antibiosis screening technique: For both chemically-induced resistance and insect feeding-induced resistance, the LAST bioassay was performed using larvae in petri dishes and recording their mortality rate, developmental periods, weights, and plant consumption. One trifoliate leaf from the upper-half of the plant was taken from each plant and placed in a petri dish (95 × 15 mm) with filter paper moistened with tap water. Five randomly chosen neonate larvae were placed in each petri dish. Larvae were allowed to feed for 3 wk, after which the remaining live larvae were weighed. Mortality of larvae in each petri dish was monitored every 2–4 days. Leaves were exchanged with another leaf from the same plant every 2–4 days, depending on the level of leaf tissue consumption. The removed leaves were pressed and from the only first collection a 6.7 cm^2 sample was excised from each leaf and scanned for area with a LICOR™ leaf-area meter (Model LI-3100, LICOR, Lincoln, NE). The 6.7 cm^2 sample was used, rather than the entire leaf, to control for original area; it was a square block that occupied most of each leaf so that the sample was highly representative of the entire, but variously sized leaves. Visual inspection of leaves from the other collections indicated the results from other collections would be similar until mortality increased so that less beetles were feeding on certain treatments. The leaf-area meter thus measured the remaining (non-consumed) portion of each leaf block, such that the lower the number, the more consumed.

Data collected included percentage larval mortality, larval weight (average weight per larva per petri dish), total developmental period, and remaining leaf area. For chemical treatments, data were analyzed in S-plus (Statistical Sciences, 1993), using analysis of variance for treatment effects, with Tukey's multicomparison test to detect differences (P = 0.05) among treatments for those variables with a significant ANOVA. For the insect-feeding experiment, results were analyzed with t-test since there were only two treatments.

Preference test: The preference test was performed once for 6–8 hr to monitor adult insect preference or non-preference (Kogan, 1972). A cork borer was used to excise a 1-cm diam disk of leaf tissue. Disks were weighed and placed in a petri dish with moistened filter paper. From each plant leaf that was used for the preference test, a corresponding check disk was also taken. This check disk was weighed fresh and dry in order to get a percent water estimate.

Six separate tests were conducted as follows: (A) previously eaten (insect feeding-induced) vs. not previously eaten (control); (B) SA, JA, Both, and control; (C) JA1, JA2, previously eaten, and control; (D) SA1, SA2, and control; (E) JA1, JA2, and control; and

Table 1. Means (±SEM) of the LAST experiment for chemically- and insect feeding-induced resistance.

Treatment	No. of dishes[a]	Total larvae number	Percentage mortality	Mean larval weight, mg	Developmental time, d	Remaining leaf area cm²
			Chemically-induced resistance[b]			
Control	4	13	48.0 ± 33.5 A	18.6 ± 2.5	47.1 ± 0.3	6.1 ± 0.24 AB
JA1	4	4	84.0 ± 8.9 AB	19.0 ± 3.9	50.8 ± 5.7	6.0 ± 0.16 AB
JA2	1	1	96.0 ± 8.9 B	18.0 ± 0.0	54.0 ± 0.0	6.4 ± 0.10 B
SA1	5	9	64.0 ± 26.1 AB	19.2 ± 3.4	49.6 ± 2.3	6.1 ± 0.21 AB
SA2	5	14	44.0 ± 26.1 A	16.7 ± 4.2	46.9 ± 4.1	5.7 ± 0.87 A
Both1	1	1	96.0 ± 8.9 B	15.2 ± 0.0	50.0 ± 0.0	6.4 ± 0.20 B
Both2	1	1	96.0 ± 8.9 B	16.9 ± 0.0	50.0 ± 0.0	6.4 ± 0.23 B
			Insect feeding-induced resistance[c]			
Control	9	22	56.0 ± 28.0 A	16.4 ± 2.7	48.1 ± 6.2	6.1 ± 0.62 A
Previously eaten	1	2	96.0 ± 12.6 B	13.5 ± 0.0	51.0 ± 0.0	6.6 ± 0.12 B

[a] Number of dishes with live larvae remaining out of 5 and 10 replicates for the chemically-induced resistance experiment and the insect feeding-induced resistance experiment, respectively.

[b] Means with differing letters contained within a column are significantly different (ANOVA, using Tukey's multicomparison test).

[c] Means with differing letters contained within a column are significantly different using a *t*-test.

(F) Both1, Both2, and control. Ten replications were used in Tests A–C, while the number of replicates were reduced to three in tests D–F.

Each plant disc was pinned to a sheet of filter paper (Whatman #1, size 7) with a map tack. Adult MBB were starved for 20 hr prior to the experiment. Two adult MBBs were placed in each dish for treatments A–C and allowed to feed for 7 hr. For treatments E–F, three adults were placed in each dish and allowed to feed for 8 hr. After the adults had been removed, the leaf disks were allowed to dry at 55°C and then weighed. Corresponding check disks were similarly dried and weighed, and then used to determine the dry leaf material that had been consumed by calculating an expected weight for each disk based on the percent water of each individual leaf. The weight of dry matter actually consumed in the preference test was calculated by subtracting the actual dry weight (test disk) from the theoretical dry weight (control disk).

Results

Chemically-induced resistance: MBB mortality was greatest, being nearly 100%, with JA2, Both1 and Both2 ($F = 6.7$; d.f. = 6, 28; Pr > $F = 0.00018$) (Table 1). SA2 had significantly less mortality compared with JA2 or either Both1 and Both2 treatments, and was equal to the control. Because mean larval weight and mean developmental time were based on averages of the larvae within a petri dish and JA2, Both1, and Both2 only had one surviving larvae in one dish each at the end of the experiment, statistics could not be properly run on these two variables. However treatments SA2, Both1, and Both2 had numerically lower mean larval weights. With developmental period, the SA2 treatment had the shortest time to pupate, while the JA treatments, especially JA2, had the longest development time (Table 1). A statistically significant difference was obtained for remaining leaf area, with SA2 having the lowest value ($F = 3.3$; d.f. = 6, 28; Pr > $F = 0.014$), indicating that larvae growing in the presence of SA2 consumed a significantly higher proportion of the leaves, while the JA2- and Both-treated plants had less consumption (Table 1).

The plants grown as controls with all seven treatments but with no harvesting of leaves

for insect feeding were not significantly different in dry weight (data not presented). There were no apparent differences in plant growth based on dry weight.

Insect feeding-induced resistance: Significantly greater mortality ($t = -4.12$, d.f. = 18, $Pr > t = 0.0006$) was observed when insects were fed leaves from the previously defoliated soybeans (Table 1). In this experiment, a total of 22 larvae pupated while only two larvae on previously-injured plants survived. Although insufficient samples were obtained for statistics (only 1 petri dish with surviving larvae on previously defoliated soybeans), these two surviving larvae on previously-defoliated soybeans also had corresponding numerically lower average weights and longer development periods compared to controls. Leaves from the previously-eaten plants also had a statistically greater leaf area remaining after feeding than the control ($t = -2.59$, d.f. = 18, $Pr > t = 0.0185$).

Preference test: No significant trends were observed in any of the experiments (data not presented). During the course of the preference test, i.e., the 6–8 hr feeding period, there was a general lack of insect feeding. This might have been a result of the adult beetles being reared on green beans, which is a preferred species of plant relative to soybeans, resulting in a strong non-preference for feeding on soybeans. A longer starvation period, or a conditioning period of soybean feeding, was perhaps required.

Discussion

Results from the LAST assessment for chemically-induced resistance generally supported the hypothesis that JA would influence soybean plants to become more resistant. The SA2-treated plants also had slightly lower mortality, faster development time and more leaf consumption than controls, and less resistance compared to JA-treated plants. Contrary to our hypothesis, the treatments that included JA and SA together (Both1 and Both2) did not show intermediate effects. Plants treated with both JA and SA at both concentrations were similar to JA-treated plants where high mortality was observed.

In the separate control test, we found that neither the JA nor SA treatments caused any difference in growth or final biomass to plants that were not subjected to insect attack or harvesting of leaves (data not presented). The exogenously applied compounds did not affect overall plant growth, it only affected the resistance of the plant to insect attack.

Plants treated with JA became more resistant to the MBB. Jasmonic acid is the end product of the octadecanoid defense pathway in a plant, and it leads to a plant's production of defensive proteins (Creelman and Mullet, 1997). Although we did not biochemically analyze the plants for confirmation, we believe that the exogenously applied JA stimulates a greater production of these defensive compounds and makes the plant more resistant. Larvae feeding on JA-treated plants will, therefore, tend to have higher mortality and slower development than larvae feeding on control plants. The overall effect also results in less total leaf consumption on plants treated with JA.

Though not statistically convincing, overall trends show that plants treated with the higher level of SA were slightly more susceptible to MBB compared with controls. SA2-treated plants had the most surviving larvae (14 out of 25 initial larvae), the fastest development time (46.9 days), and the most leaf tissue consumption of all treatments (1 cm^2 consumed out of 6.7 cm^2 of leaf material) (Table 1). These SA2 plants appeared more susceptible to MBB as compared to JA-treated plants. Salicylic acid tends to block the production of allene oxide synthase, which is a necessary enzyme to produce JA through the octadecanoid pathway (Pan et al., 1998). Jasmonic acid acts as a signal for the plant to produce a defensive reaction when attacked. With its defense impaired, a SA-influenced plant is unable to produce the necessary defensive compounds such as proteinase inhibitors

(herbivore anti-feedants) and becomes "defenseless" to the challenger. As a result, the plant is consumed at a faster rate and the feeding larvae have a lower mortality and faster development than otherwise might be expected. In an experiment on tomatoes with tobacco hornworm, JA was also found to protect the plant while SA decreased resistance to hornworm attack (Iverson et al., 2001).

With insect feeding-induced resistance, previously defoliated plants did show a large increase in resistance, with only two MBB out of 50 being alive at the end of the study, along with markedly lower mean larval weight, longer development time, and larger remaining leaf area. When a challenger attacks a plant, a local and systemic wound response is created which results in the accumulation of proteinase inhibitors. At the site of wounding, many reactions occur, including the production of systemin and oligogalacturonides which are inducers of jasmonic acid biosynthesis, causing a rapid increase in the accumulation of octadecanoids, including JA. These octadecanoids activate proteinase inhibitor synthesis. Then, one or more signals travel from the local wound to the unwounded areas and systemic acquired resistance is established. Once established, it can last for a few days to several weeks. In this experiment, the effects of acquired resistance were apparent and lasted for several weeks.

Induced resistance holds promise for the future of the agricultural industry. A plant not spending energy in constantly producing constitutive resistance could direct that much more energy into producing its seed. Upon attack, a quick buildup of proteinase inhibitors within the affected plant, along with the emission of volatile exudate signals to neighboring plants, could prevent severe loss of yield due to insect damage, while at the same time, maximize production in times of little or no insect outbreaks. Meanwhile, applications of JA-like compounds could supplement the arsenal against insects, without the harmful byproducts of many synthetic pesticides. Enhancing the natural immunity systems in agricultural crops is an area with potentially huge dividends towards enhancing the world food supply.

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