

# Soybean Nitrogen Relations and Root Characteristics after *Cerotoma trifurcata* (Coleoptera: Chrysomelidae) Larval Feeding Injury<sup>1</sup>

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**Abstract** Bean leaf beetle, *Cerotoma trifurcata* (Förster), is a pest of economic importance to soybean (*Glycine max* [L.] Merr.) production in the U.S. This greenhouse study was conducted to characterize larval feeding damage effects on nitrogen (N) assimilation and root system characteristics in soybean. Pots containing individual plants ( $n = 15$ ) were exposed to 1 of 3 treatment levels that varied in infestation intensity: high intensity (19 larvae per pot), low intensity (5 larvae), and an uninfested control group. After 3 wk, the plants were dissected, and the fresh and dry weights of the roots and shoots were recorded. Also, the number of nodules, number of damaged nodules, and the volume of nodules were compared among the treatments. Leaf and pod chlorophyll, nitrate-N, ureide-N, amino-N, and total N concentrations were measured and compared among the treatments. Nodules were the only organs on the root system that were visibly damaged by larval feeding. External nodule surfaces were scarred, and frequently the internal matrices of the nodules were completely excavated by the larvae. Significantly more nodules per plant were found in the infested treatments, and damaged nodules were significantly more abundant on plants in the high infestation compared with those in the low infestation treatment. Also, plants from the highly infested treatment had significantly smaller nodules than the uninfested control, suggesting that the infested plants responded to larval damage by producing additional nodules. Leaf and pod ureide-N and total N concentrations were significantly lower in infested plants. Thus, feeding by *C. trifurcata* larvae affects the characteristics of nodules as well as the concentrations of N assimilates within soybean plants.

**Key Words** bean leaf beetle, *Cerotoma trifurcata*, *Glycine max*, integrated pest management, nitrogen, nodule, ureide

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*Cerotoma trifurcata* (Förster), the bean leaf beetle, is a pest of current and growing concern in North America. This insect is endemic to the New World, and adults are known to feed on a range of plants, many of which are leguminous (Helm et al. 1983, Kogan et al. 1980). *Cerotoma trifurcata* is a perennial problem in soybeans (*Glycine max* L.), causing multiple forms of damage. Adults defoliate plants from early vegetative stages and feed on developing pods (Smelser and Pedigo 1992a, b). *Cerotoma trifurcata* adults also transmit viruses to soybeans, further reducing yields and profits (Mabry et al. 2003, Walters 1969). This is a multivoltine pest throughout much of its range, and several bouts of damage are often inflicted to a single soybean crop.

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Outbreaks of adult *C. trifurcata* in the Midwest and Great Plains frequently merit insecticide treatment.

The behavior of the subterranean larval stage remains poorly understood. Studies on the larvae of *C. trifurcata* have mostly focused on rearing (Herzog et al. 1974, Zeiss et al. 1996) and the effects of soil structure and moisture levels on larval survival and movement (Marrone and Stinner 1983a, b, 1984). There are few reports on the feeding behavior of larvae outside of rearing regimens, and it is currently unknown how feeding by *C. trifurcata* larvae affects soybean production, although anecdotal reports suggest that this life stage may be significant (Kogan et al. 1980, Newsom et al. 1978).

Research on the larval feeding of *Cerotoma* spp. on various host plants compels the idea that *C. trifurcata* larvae may damage soybean plants. Studies of *Cerotoma facialis* Erickson showed that larval infestations on the roots of *Phaseolus vulgaris* L. seedlings were lethal to the crop (Cardona et al. 1982). Larvae of another congener, *Cerotoma arcuata* Olivier, are known to remove the nodules from infested plants and reduce the root mass (Teixeira et al. 1996). Feeding by this species resulted in a reduction in shoot nitrogen (N) and yield. Defoliation by *C. arcuata* adults did not reduce yields on its own, leading the authors to conclude that subterranean larvae of *C. arcuata* were the most injurious life stage to the plant, which is also true with some other insect pests of agronomic importance such as the corn rootworm complex. Ninety years ago, *C. trifurcata* was observed to feed on the nodules of cowpea (*Vigna unguiculata* [L.] Walp.) and other legumes (Leonard and Turner 1918, McConnell 1915), and larvae of *C. trifurcata* are found aggregated near the base of soybean plants, where nodules are most abundant (Anderson and Waldbauer 1977, Riedell et al. 2005). In a cursory state report, Newsom et al. (1978) found that applying soil insecticides to eliminate *C. trifurcata* larvae from soybean plots resulted in higher numbers of nodules, larger nodules, greater leaf area, and higher yields compared with unsprayed plots. Their conclusion was that larval feeding is important to soybean production, and that the system merits further research.

Nodules contain symbiotic N-fixing bacteria (*Rhizobium* spp.) and are a major source of N in soybeans. The N fixed in soybean nodules is translocated to the aboveground organs and tissues as ureides (allantoin and allantoate), and to a lesser extent as the amino acid asparagine (McClure and Israel 1979, Schubert 1986, Todd et al. 2006). The amount of available soil N often restricts crop productivity in agricultural systems (Schubert 1986). Thus N-fixation in nodules provides an important source of N needed for photosynthesis, growth, and seed production in soybeans. Destruction of these organs by nodule-feeding insects may have important implications for crop yield and seed composition.

With this research, we describe the feeding behavior of *C. trifurcata* larvae on soybeans and document its effects on crop N relationships. Specifically, we describe the physical damage caused by *C. trifurcata* larvae to soybean root systems. Moreover, we measured and compared the concentration of several N-containing compounds in plants infested with high or low densities of *C. trifurcata* larvae and compared these levels with uninfested plants.

## Materials and Methods

**Insects.** *Cerotoma trifurcata* adults were collected from 2 fields of alfalfa (*Medicago sativa* L.) near Vermilion, SD, USA, on 12 May 2006. Beetles ( $n = 200$ ) were

caged in aggregate and kept in an environmental chamber at 16:8 h (L:D), 27°C, and 40-60% relative humidity. Beetles of mixed sex ratio were divided into groups of 10 per 250-ml plastic cup with a fine mesh covering over the lid. Sprigs of cowpea foliage and green bean (*P. vulgaris*) pods were fed to the beetles over 3-4 wk, and water was offered from a saturated cotton wick. Females began laying eggs within 8 d of being brought into the laboratory. Oviposition substrates included soybean and cowpea foliage and water-soaked (for more than 24 h) cowpea cotyledons with their seed coats removed. Eggs were isolated from the adults daily and placed on dampened paper toweling in 30-ml plastic cups with tight-fitting plastic lids. Newly-laid eggs were refrigerated at 6°C until 16 d before the infestation to synchronize hatching (they were refrigerated for fewer than 7 d). Bean leaf beetle larvae hatch in 6-10 d when eggs are held at 27°C (JGL, unpubl. data). Thus, all larvae for the infestation were younger than 10 d old.

Larvae were reared for 1-10 d preinfestation to improve survivorship in the soybean pots (neonate larvae are sensitive to manipulation). When hatch was imminent (the dark larval head capsule was visible through the chorion), half a cowpea cotyledon was placed in the rearing container. The cowpea cotyledon was soaked for at least 24 h on damp paper toweling and its seed coat was removed. It was then soaked in 0.5% cupric sulfate solution for 5 min before placing it into the larval container. These rearing protocols were adapted from Herzog et al. (1974). Larvae were reared at densities of fewer than 50 larvae per 0.7-l (10.5 × 10.5 cm basal area) plastic container with tight fitting plastic lids and a 4-cm<sup>2</sup> diam screened hole for aeration. The paper toweling was dampened with water as needed (initially 2 ml), and the cowpeas were checked daily for fungal contaminants. Larvae frequently were transferred using fine paintbrushes to clean containers with fresh cowpeas and paper toweling. Using this time- and labor-intensive rearing procedure, preimaginal development requires approximately 30 d (JGL, unpubl. data).

**Plants.** Forty-five soybean (cv. '91B91'; Pioneer Hi-Bred International Inc., Des Moines, IA, USA) plants were established in the greenhouse on 4 May 2006. Individual plants were contained in fiber pots filled with 9 L of a 60:40 mixture of sifted field soil:perlite. The soil was collected from a field site to which pesticides had never been applied. Mean ( $\pm$  SEM) minimum and maximum daily greenhouse temperatures were 24.4  $\pm$  0.2 and 38.5  $\pm$  1.3°C and natural daylight was supplemented with a 16:8 h (L:D) photophase provided by a mixture of 50% sodium vapor and 50% metal halide lamps (200  $\mu$ Mol s<sup>-1</sup> m<sup>-2</sup> photosynthetically active radiation at canopy height). Plants received 300 ml of tap water twice daily from a drip irrigation system. A 10-ml glass pipette (1 cm diam) was inserted into each of the pots just beneath the germinating seedling. To establish a favorable soil environment for *C. trifurcata* larval movement in the soil, two earthworms (*Lumbricus terrestris* L.; DMF Bait Co., Waterford, MI, USA) approx. 13 cm in length, were placed in each pot. Dead worms were replaced within the first 24 h of their establishment. Insecticidal soap (2% v/v; AllPro Insecticidal Soap 40%, Value Garden Supply, St. Joseph, MO, USA) was applied to the plants weekly to curtail low populations of thrips and soybean aphids (*Aphis glycines* Matsumura). Plants were infested with bean leaf beetle larvae at the full bloom growth stage (R2; Ritchie et al. 1997), which is approximately when ureide-N concentrations in soybean shoot organs begin to increase (Riedell et al. 2005).

**Infestation of plants.** The 45 plants were randomly and evenly assigned to 3 larval infestation levels—low (5 larvae per pot), high (19 larvae per pot), and an uninfested control treatment. Pots were arranged in the greenhouse in a completely

random design with 15 replications per treatment. Before infestation, a small amount of water (<5 ml) was poured over the pipette to dampen the soil. The pipette was twisted out of the soil, and larvae were placed directly onto the root system that had grown to surround the pipette. Larvae were transferred into the hole using paintbrushes, and the hole was gently filled with field soil that had been sifted through an 80-mesh screen and contained 150 g kg<sup>-1</sup>. The sifted soil was gently tamped down. This larval infestation protocol was adopted from Riedell (1989). Larvae burrowed into the soil surrounding the hole within 5 min of their placement. Soil moisture at 3-4 cm deep and within 2.5 cm of the plant stem was maintained at 128-186 g kg<sup>-1</sup> (LIC Soil Moisture Meter, Lincoln, NE). A single application of insecticidal soap was applied after infestation to minimize foliar pest damage.

Larvae were allowed to feed on soybean plants for 14-16 d, after which the plants were at the beginning seed stage (R5; Ritchie et al. 1997) when shoot ureide-N concentrations are at their peak (Riedell et al. 2005). Each shoot was severed at the soil surface, and leaves, pods, and stems were separated and examined. The fiber pots were cut away from the root systems. Soil from 5 of the 15 high level infestation pots was mechanically removed from the root system and examined for the presence of living larvae. We opted to examine soil from only 5 pots with this qualitative measure of larval survival because searching the soil and root mass for larvae was found to be damaging to the plants, and could only be conducted at the expense of the root system structural measurements. For these and the remaining pots, soil and perlite were gently removed from the root system with a stream of tap water. Soil-free root systems were placed in sealed plastic bags that had been partially filled with 50% ethanol and were refrigerated at 11°C until further examination.

**Observations of plant injury.** The effects of larval feeding were assessed on both the above- and below-ground portions of the soybean plants. The shoot fresh weights were recorded to the nearest 0.001 g using an electronic balance. Pod fresh weight also was examined separately as a function of yield. Shoot organs were dried at 60°C for 72 h in a forced-air oven, and dry weights were recorded.

The preserved root systems were examined microscopically for signs of larval feeding injury, and nodules were removed. The total numbers of nodules were enumerated for each plant, as were the number of nodules that displayed signs of insect feeding. Nodule volume from each root system was calculated with fluid displacement in graduated cylinders, and the total displacement was divided by the number of nodules to determine mean volume per nodule per plant. Fresh weight of the root system (nodules had been removed) was measured to the nearest 0.001 g using an electronic balance. Root systems were dried at 60°C for 72 h and dry weights were recorded.

**Plant nutrient analysis.** Dried pods and leaves were ground to pass through a 2-mm screen in a Wiley mill (Arthur Thomas Co., Philadelphia, PA) and were weighed. Samples from each plant were then subjected to total N (Pikul et al. 2001), ureide-N (Patterson et al. 1981), amino-N (Yemm and Cocking 1955), and NO<sub>3</sub>-N (Cataldo et al. 1975) analyses.

**Data analysis.** The mean fresh and dry weights of the above- and below-ground portions of the plants were compared among the treatments using ANOVA (SYSTAT Software 2004). The mean numbers and volumes of nodules per plant were compared among the treatments using ANOVA. The mean numbers of damaged nodules per plant were compared among treatments using the nonparametric Kruskal-Wallis Analysis of Variance (SYSTAT Software 2004). Total N, ureide-N, amino-N, and

NO<sub>3</sub>-N were compared among the treatments using ANOVA, and statistical outliers were excluded from analyses (never more than 8% of samples, depending on the analyses). When means were found to be significantly different ( $\alpha = 0.05$ ), they were separated using the least significant difference (LSD) means comparisons (SYSTAT Software 2004).

## Results

**Feeding damage to plants.** Mean fresh and dry weights of shoots (aboveground organs) and roots (belowground organs) per plant were statistically similar among the treatments (shoots [fresh]:  $F = 0.33$ ;  $df = 2, 42$ ;  $P = 0.72$ ; shoots [dry]:  $F = 1.22$ ;  $df = 2, 42$ ;  $P = 0.31$ ; root [fresh]:  $F = 0.44$ ;  $df = 2, 42$ ;  $P = 0.65$ ; root [dry]:  $F = 1.31$ ;  $df = 2, 42$ ;  $P = 0.28$ ). Leaf numbers per plant were statistically similar among the treatments ( $F = 0.64$ ;  $df = 2, 42$ ;  $P = 0.53$ ), as were the mean fresh weights of pods per plant ( $F = 0.43$ ;  $df = 2, 42$ ;  $P = 0.66$ ).

Larvae and pupae were recovered from 3 of the 5 infested pots examined, indicating that the insects were able to grow and develop during the 2-wk exposure period in our greenhouse experiment (data not shown). Larval feeding injury was observed on the root nodules. Damaged nodules were easily identified because they were relatively darker brown compared with healthy nodules. Frequently, the larvae created a hole in the external tissues of nodules and completely removed the contents of the nodules. Other symptoms of feeding damage were small feeding craters or scars on the surface of the nodules (Fig. 1). There was no visible feeding damage observed on the tap or lateral roots. Larval feeding on the root hairs, as reported by McConnell (1915) on cowpeas, would have been difficult to observe using the methods used during this experiment.

There were significant differences among all the treatments in the number of nodules per plant ( $F = 5.25$ ;  $df = 2, 42$ ;  $P = 0.009$ ) (Table 1), as well as the number of damaged nodules per plant ( $\chi^2 = 36.79$ ;  $df = 2$ ;  $P < 0.0001$ ) (Table 1). Although greater numbers of nodules were found on plants from the high infestation treatment than from the uninfested treatment, average nodule volumes were significantly smaller on plants from the high infestation treatment than from the uninfested treatment ( $F = 4.13$ ;  $df = 2, 42$ ;  $P = 0.023$ ) (Table 1). The result of these interactions is that total nodule volume per plant was similar among the treatments ( $F = 0.23$ ;  $df = 2, 42$ ;  $P = 0.79$ ).

**Soybean N assimilates.** Leaf N assimilate concentrations produced in response to the three treatments are presented in Table 2. Ureide-N concentration in the leaves decreased significantly as the number of larvae per pot was increased ( $F = 5.74$ ;  $df = 2, 40$ ;  $P = 0.006$ ). Although there were no significant differences in chlorophyll, nitrate-N, or amino-N in the leaves across the different treatments (chlorophyll:  $F = 2.63$ ;  $df = 2, 42$ ;  $P = 0.08$ ; nitrate-N:  $F = 1.73$ ;  $df = 2, 42$ ;  $P = 0.19$ ; amino-N:  $F = 2.13$ ;  $df = 2, 42$ ;  $P = 0.13$ ), the total N concentration in the high infestation treatment was significantly less than the two other treatments ( $F = 5.71$ ;  $df = 2, 42$ ;  $P = 0.006$ ).

The pod N assimilate concentrations produced in response to the 3 treatments are presented in Table 3. Ureide-N concentrations were significantly lower in treatments that received larvae compared with the untreated plants ( $F = 10.19$ ;  $df = 2, 42$ ;  $P < 0.001$ ). Total N was also significantly lower in the damaged treatments versus the untreated control ( $F = 10.29$ ;  $df = 2, 37$ ;  $P < 0.001$ ). Nitrate-N and amino-N

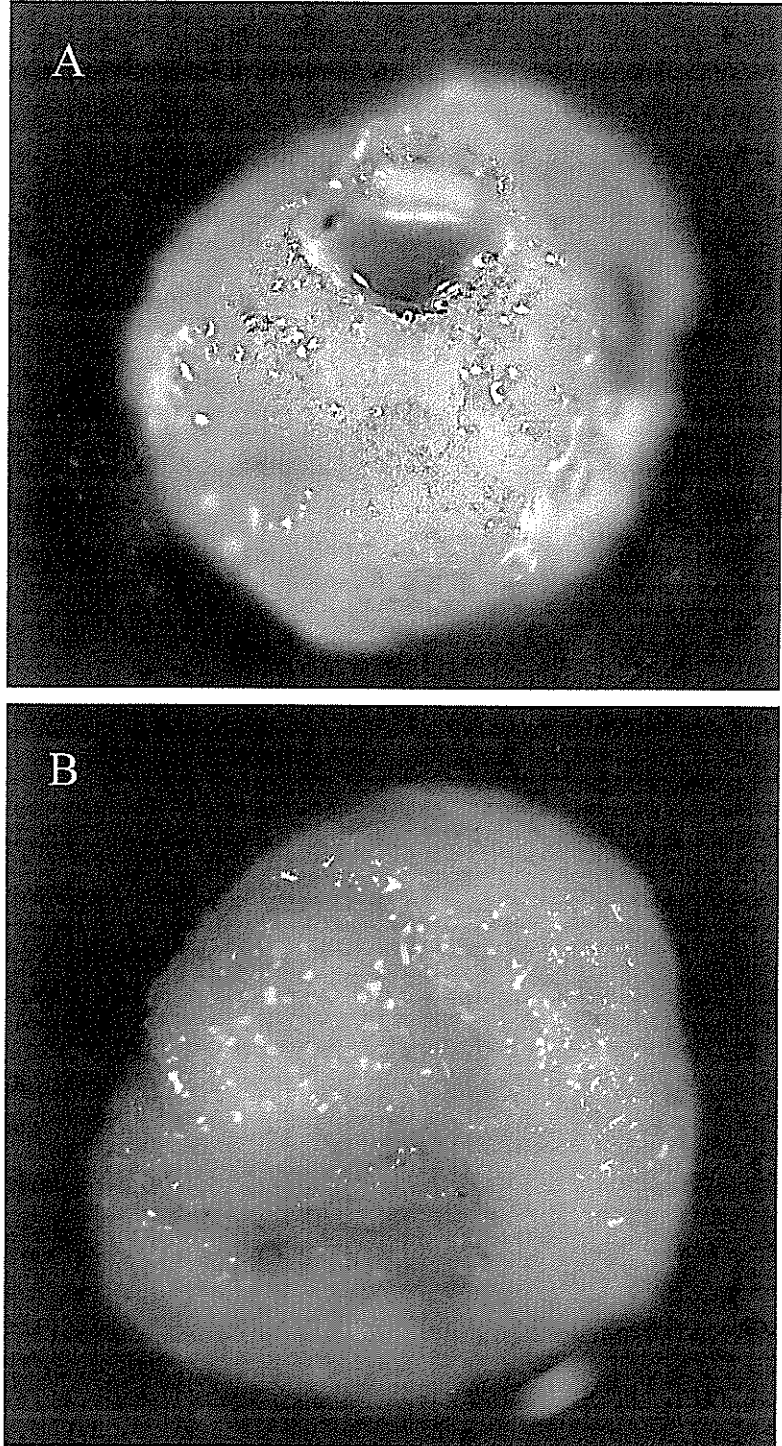


Fig. 1. Soybean nodules damaged (A) or undamaged (B) by bean leaf beetle larvae.

**Table 1. Mean ( $\pm$  SEM) nodule characteristics on soybean plants infested with different levels of *C. trifurcata* larvae**

	Nodules per plant	Damaged nodules per plant	Total nodule volume per plant (cm <sup>3</sup> )	Volume per nodule (cm <sup>3</sup> )
None	142 $\pm$ 15a	0a	3.58 $\pm$ 0.01	0.028 $\pm$ 0.003a
Low	160 $\pm$ 13b	13 $\pm$ 2b	3.27 $\pm$ 0.01	0.022 $\pm$ 0.002ab
High	215 $\pm$ 21c	30 $\pm$ 3c	3.43 $\pm$ 0.01	0.019 $\pm$ 0.002b

Means within a column flowed by the same lower case letter are not significantly different (LSD,  $\alpha = 0.05$ ).

were similar among the treatments (nitrate-N:  $F = 0.50$ ;  $df = 2, 42$ ;  $P = 0.61$ ; amino-N:  $F = 0.92$ ;  $df = 2, 42$ ;  $P = 0.41$ ). Chlorophyll was significantly different among the treatments ( $F = 7.01$ ;  $df = 2, 40$ ;  $P = 0.002$ ), with undamaged plants having pods with significantly higher chlorophyll content than the two treatments that received larvae.

### Discussion

These results demonstrate that larvae of *C. trifurcata* consume soybean root nodules and that this feeding affects nodule morphological characteristics and reduces their contribution of N fixation assimilates (ureides) to the overall N nutrition of the plant. A previous report found that larvae fed on the roots, root hairs, and nodules of cowpeas (McConnell 1915). In contrast, the only damage to soybean root systems observed in our study was inflicted on the nodules. Larvae fed externally on the outer tissues of the nodules, often creating craters in the external surface. When the internal matrix of the nodule was reached, then the larvae would completely excavate the contents. McConnell (1915), Leonard and Turner (1918), and Kogan et al. (1980) observed similar nodule injury caused by *C. trifurcata* larvae to cowpeas and soybeans and documented the larval injury photographically.

Although the total nodule volume per plant was similar among the treatments, nodule characteristics were altered in injured plants (Table 1). In addition to having more nodules, the most heavily infested plants had a significantly smaller average nodule volume than the uninfested plants (Table 1), suggesting that heavily-infested

**Table 2. Mean ( $\pm$  SEM) concentrations (mg/g of dry weight) of chlorophyll, N assimilates, and total N in the leaves of uninfested soybeans, and plants infested with low and high levels of *C. trifurcata* larvae (5 and 19 larvae per plant, respectively)**

	Chlorophyll	Ureide-N	Nitrate-N	Amino-N	Total N
None	6.11 $\pm$ 0.24	0.73 $\pm$ 0.10a	0.53 $\pm$ 0.02	0.98 $\pm$ 0.04	39.6 $\pm$ 0.8a
Low	5.42 $\pm$ 0.28	0.78 $\pm$ 0.06b	0.49 $\pm$ 0.01	1.05 $\pm$ 0.04	40.3 $\pm$ 0.4a
High	5.25 $\pm$ 0.32	0.54 $\pm$ 0.04c	0.51 $\pm$ 0.02	0.93 $\pm$ 0.04	37.6 $\pm$ 0.5b

Means within a column flowed by the same lower case letter are not significantly different (LSD,  $\alpha = 0.05$ ).

**Table 3. Mean ( $\pm$  SEM) concentrations (mg/g of dry weight) of chlorophyll, N assimilates, and total N in the pods of uninfested soybeans, and plants infested with low and high levels of *C. trifurcata* larvae (5 and 19 larvae per plant, respectively)**

	Chlorophyll	Ureide-N	Nitrate-N	Amino-N	Total N
None	0.84 $\pm$ 0.04a	7.73 $\pm$ 0.29a	0.28 $\pm$ 0.02	2.08 $\pm$ 0.10	32.8 $\pm$ 0.5a
Low	0.65 $\pm$ 0.02b	6.26 $\pm$ 0.33b	0.29 $\pm$ 0.01	1.97 $\pm$ 0.07	30.5 $\pm$ 0.4b
High	0.73 $\pm$ 0.04b	5.82 $\pm$ 0.32b	0.27 $\pm$ 0.01	1.93 $\pm$ 0.07	30.5 $\pm$ 0.3b

Means within a column followed by the same lower case letter are not significantly different (LSD,  $\alpha = 0.05$ ).

plants responded to larval feeding by producing additional nodules. Field observations by Anderson and Waldbauer (1977) indicated that soybean plants found in association with *C. trifurcata* larvae had about 50% more nodules than uninfested plants. These results are similar to ours in that we found a 52% increase in the number of nodules on plants given the high infestation larval treatment when compared with uninfested controls (Table 1). In red clover, Nutman (1952) found that nodule excision led to an increase in the number of nodules subsequently formed. Thus, our observation that damage to nodules by *C. trifurcata* larvae resulted in a proliferation of smaller nodules on the root system supports and extends that of Nutman (1952). At this point, it is not clear how alterations in the size distribution of nodules will affect the larval populations of *C. trifurcata*.

In this study, nodule damage caused by larval feeding decreased the concentration of N fixation assimilates (ureides) and total N in the shoot organs of soybean plants. Thus, feeding by *C. trifurcata* larvae potentially has serious implications for the production of soybeans. Leonard and Turner (1918) found that nodule destruction by *C. trifurcata* larvae was associated with lower total N levels in cowpea plants. Our research showed that feeding by *C. trifurcata* larvae reduced the ureide-N and total N concentrations of soybean leaves and pods, particularly in the highly infested pots (Tables 2, 3). No differences were observed in the amino-N contents, as would be expected as soybeans do not translocate substantial quantities of N-fixation products as amino acids. In conclusion, although soybeans compensate for nodule destruction caused by *C. trifurcata* larvae by producing more small nodules, feeding still disrupts fixed N accumulation in affected plants. How larval feeding affects soybean yields, and whether this life stage of the pest merits management action by producers should be addressed by future research.

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