Corn Defense Responses to Nitrogen Availability and Subsequent Performance and Feeding Preferences of Beet Armyworm (Lepidoptera: Noctuidae)

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ABSTRACT Many studies have reported the effect of nitrogen (N) fertilization on plant constitutive defense responses; however, little is known about their effects on plant induced defense patterns and its consequence for insect herbivores. In our experiments, the effects of N availability on growth, nutritional quality (N content, protein/carbohydrate [P:C] ratio, modified gross energy [MGE]), and constitutive phenolics of corn, *Zea mays* L. were quantified. Moreover, the indirect effects of N fertilization on the beet armyworm, *Spodoptera exigua* Hübner through larval performance and feeding preference were examined. N fertilization increased plant growth, and depressed defense traits by increasing N content and the P:C ratio, as well as decreasing the constitutive concentration of phenolics. Subsequently, beet armyworm showed higher performance and preferentially fed on high-N corn because of its low defense traits. After beet armyworm feeding, high-N corn significantly deterred larval feeding, and had negative effects on the performance of beet armyworm through decreasing P:C ratio and increasing induced phenolics. On the contrary, there were no significant changes in P:C ratio and phenolics in low-N corn after feeding damage. Larval performance and preference were also not affected by induced compounds in low-N corn, which suggested that the expression of induced defense was dependent on N availability. The result indicates that N availability can exert a variety of bottom-up effect on plant defense patterns to influence insect population dynamics, and thereby may represent a source of variation in plant-insect interactions.

KEY WORDS *Spodoptera exigua*, *Zea mays*, N availability, insect-plant interactions

Worldwide, corn is the most widely grown crop for human consumption, animal feed, and industrial materials and annually revenues are worth many billions of dollars. Consequently, because of its agronomic importance corn has been a keystone model organism for basic research for nearly a century.

Nitrogen (N) is probably the most critical resource limiting plant and insect growth in terrestrial environments, and has the potential to alter plant-insect interactions (Mattson 1980, Cease et al. 2012). N can exert a variety of bottom-up effect on plant defense patterns through nutrition and secondary metabolites, which often affects the performance and preference of insect herbivores. Insects that feed on plants containing low-N generally have lower growth, feeding preference, and survival rate than on plants with high-N fertilization (Chen et al. 2008).

In plants, nutrient deficiency and secondary metabolites constitute the first line of plant defense to avoid insect attack under different N environments (Feeny 1976, Ohnmeiss and Baldwin 1994, Novriyanti et al. 2012). Limited N fertilization can decrease plant N and protein production, and increase carbohydrates, resulting in changes of nutrient balance such as protein/carbohydrate ratios (P:C ratio) and energy (modified gross energy, MGE) (Bryant et al. 1983, Kytö et al. 1996). In response, insects have evolved effective feeding strategies to depress the negative effects from plant defense (Bede et al. 2007, Walling 2008). For example, larvae of fall armyworm, *Spodoptera frugiperda* Smith, preferentially selected balanced nutrient intake or high P:C ratio diets (Waldbauer et al. 1984). Moreover, limited N fertilization can depress insect performance because of high constitutive phenolic levels in plants, which is one of the most clearly demonstrated carbon-based secondary metabolites (Koricheva 2002, Underwood and Rausher 2002). Compared with low fertilization plant, *Populus tremuloides* Michx with low-N fertilization had significantly higher phenolics. Increasing phenolic extracts from aspen leaves decreased the growth of aspen tortrix, *Choristoneura conflictana* Walker (Bryant et al. 1987). Therefore, herbivorous insects prefer

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plants with low levels of constitutive secondary metabolites (Underwood and Rausher 2002, Piubelli et al. 2003, Pöykkö et al. 2005).

If nutrient and secondary metabolite barriers are breached, inducible responses in plants are important in reducing further insect attack (Baldwin and Schmelz 1994, Glynn et al. 2003, Olson et al. 2009). Changes in growth patterns, nutrients, and secondary metabolites induced by insect herbivory are likely to both have positive or negative effects on the performance and feeding preference of insects (Martinsen et al. 1998, Stout et al. 2009, Hamm et al. 2010). For example, herbivory from the stem-boring moth, *Endoclita excrescens* Butler, changed the nutritional status and biomass of willow trees, *Salix* spp., and increased the growth and survival of the leaf beetle, *Plagiodera versicolora* Laicharting by inducing compensatory regrowth (Utsumi and Ohgushi 2008). In contrast, feeding damage of *Helicoverpa zea* Boddie in soybean raised the level of induced plant defense by reducing in the foliar protein levels, resulting in a 52% decrease in larval weight (Bi et al. 1994). Similarly, insect feeding can also change feeding preference as a result of elevated phenolic levels in plants (Hartley and Lawton 1987, Bi and Felton 1995).

The expression of induced responses in plants can also be influenced by N availability (Coley et al. 1985, Glynn et al. 2003, Olson et al. 2009). Lou and Baldwin (2004) reported that high-N inputs increased inducible carbon-based rutin, chlorogenic acid, and diterpene glycosides as well as nicotine and trypsin. However, few studies have explicitly addressed the issues relating to the variation of inducible responses and constitutive defenses through plant chemistry and insect feeding preference and performance under different N fertilization regimes (Koricheva 2002, Craine et al. 2003, Nykänen and Koricheva 2004). Therefore, there is still a significant gap in our understanding of N effects on plant-insect interactions. This report will evaluate the effects of N fertilization on corn defense patterns and its consequences on beet armyworm larvae, *Spodoptera exigua* Hübner feeding preference and performance. These results will contribute to a broader understanding of the indirect effects of N fertilization on responses of plant in metabolic processes and insect herbivory.

Materials and Methods

Insects. Eggs of beet armyworm were obtained from Henan Jiyuan Baiyun Industry Co. Ltd. (Jiyuan, Henan, China). New hatched larvae were reared on a tomato-based artificial diet, and kept in glass tubes (one larva per tube) in climate chambers (Safe PRX-450030, Ningbo, Zhejiang, China) at 26 ± 1°C, a photoperiod of 14:10 (L:D) h until pupation. The main ingredients in the artificial diet included wheat germ 94.0 g, canned tomato paste 45.0 g, yeast 35.0 g, methyl parahydrobenzoate 1.6 g, sorbic acid 0.8 g, ascorbic acid 2.6 g, agar 11.0 g, linoleic acid 1.0 ml, and distilled 750 ml water (Wu and Gong 1997). Emerged adults were mated in a cage (length: width: height = 60 cm: 60 cm: 60 cm), and

allowed to oviposit on wax paper for 7 d. Cotton soaked with 10% honey water was placed in a plastic petri dish as a food source for adults.

Corn Plants. Seeds of corn, *Zea mays* L. ('Nongda 4967') were obtained from the Chinese Academy of Agricultural Sciences, Beijing, China. The seeds were germinated in vermiculite and watered daily. After 4 d, germinated seeds were transplanted into 0.5 liter free-draining pots (one plant per pot). Corn plants were grown in a 1:1 soil mixture of vermiculite and sand, and kept in climate chambers (26°C day, 23°C night, 60% relative humidity [RH], a photoperiod of 14:10 (L:D) h).

Experimental Design. Five tests were separately conducted to determine individual or interaction effects of N availability and damage status on plants and insects. They were relative growth, nutritional analysis and phenolics for corn, and feeding preference and performance for beet armyworm larvae.

All tests were designed as two-way factorial arrangements to investigate plant and insect responses. The two factors were N availability and damage status. The N availability factor included as high-N and low-N levels. The damage status factor had three levels, including nondamage, mechanical, and feeding damage. Therefore, there were totally six treatments of corn in each experiment. Plants treatments included: 1) nondamage high-N corn, 2) nondamage low-N corn, 3) mechanical damage high-N corn, 4) mechanical damage low-N corn, 5) feeding damage high-N corn, and 6) feeding damage low-N corn.

For N factor, corn plants were supplied with high-N or low-N fertilization after seedling emergence. Two concentrations of N were separately supply to corn plants. The high-N treatment with a N concentration of 9 mmol/L (2:1 ratio of KNO_3: $Ca(NO_3)_2$-$4H_2O$) and low-N treatment with a N concentration of 0.3 mmol/L (2:1 ratio KNO_3:$Ca(NO_3)_2$-$4H_2O$). KCl and $CaCl_2$ were, respectively, added 5.8 and 2.9 mmol/L to replace the missing K and Ca. Besides N, mixture saltes were added into the nutrient solution, which consisted of 0.75 mmol/L $MgSO_4$-$7H_2O$, 0.5 mmol/L KH_2PO_4, 0.25 mmol/L NaCl, 0.25 mmol/L K_2SO_4, 60 μmol/L Fe-Na EDTA, 50 μmol/L H_3BO_3, 15 μmol/L $MnCl_2$-$4H_2O$, 2 μmol/L $ZnSO_4$-$7H_2O$, 0.25 μmol/L $CuSO_4$-$5H_2O$, and 0.2 μmol/L Na_2MoO_4-$2H_2O$ (Schmelz et al. 2003). All plants were supplied with 50 ml of nutrient solution and ≈100 ml water per plant every 2 d.

For damage factor, plants were exposed to three levels until four-leaf stage. Nondamage treatment was designed to place five clip cages (40 ml; 5 cm in height, 4 cm in diameter) in the second and third uppermost leaves without *S. exigua* larva. Mechanical damage treatment was artificially damage to mimic the damage from insect feeding. Approximately 40% leaf area of the second and third uppermost leaves was excised with sterilized scissors. Feeding damage treatment was established by placing five fifth instar larvae feeding in a clip cage under light, until ≈40% of leaf surface was removed (Noble 1958). To extension the contact time between larval oral secretion and corn damage leaves, clip cages were removed every 3 and 4 h; all treatments lasted ≈24 h.

We used 750 plants for five tests and six treatments. There were 124 plants for relative growth, 258 plants for nutritional analysis, 108 plants for phenolics analysis, 260 plants for feeding preference, and newly hatched larval and fifth instar larval feeding in insect performance experiments. All germinated seeds were randomly separated into six treatments after transplanting in each test.

Plant Relative Growth and Nutritional Quality. Relative plant relative growth was determined for all plants by measuring corn height from the shoot tip to the stem collar on the 14th and 18th day after transplanting, and was calculated as equation 1.

$$G = (H_2 - H_1) / H_1 \quad\quad [1]$$

Where G is plant relative growth; H_1 is plant height at day 14 and H_2 is plant height at day 18 after transplanting.

Plant nutritional quality were analyzed by measuring N content, P:C ratio, and MGE. Samples were taken from the of corn plant after 18 d transplant. Foliar N content for each treatment group was measured with a Kjeltec N analysis system (Foss automated Kjeltec TM instruments, model 2100, Hillerød, Denmark) coupled to a 1002 distilling unit and a 2006 digestion unit (Foss Tecator AB, Hogamas, Sweden) (Bradstreet 1954). Three corn plants were mixed as a replicate. There were 18 plants and six replicates in each treatment.

Protein and carbohydrate were measured to calculate P:C ratio and MGE. All samples were cut from third and fourth corn leaves in each treatment, ground and stored in liquid N. Five corn plants in each sample were mixed and divided into two lots to quantify protein and carbohydrate, respectively. Five replicates and 25 corn seedlings were used per treatment.

Levels of protein were determined in total protein quantitative kit (Nanjing Jiancheng Science and Technology Co., Ltd., Nanjing, Jingsu, China) using bovine serum albumin (BSA) as standards. The samples were ground with 3 ml pH 7.8 phosphate buffer in an ice bath and then centrifuged at 4°C, 7,378 × g for 10 min (Eppendorf centrifuge 5417R, Hamburg, Germany). The supernatants were used to analyze the protein following the Coomassie brilliant blue G-250 method using a spectrophotometer (Hitachi U-3310, Tokyo, Japan) (Martin and Martin 1982). Five 0.1–1 mg/ml BSA protein standards were prepared. Both protein standards and samples were added to 5 ml Coomassie brilliant blue G-250 dye reagent, incubated at room temperature for 5 min, and then the absorbance was measured at 595 nm.

Carbohydrate levels were measured by the phenol-sulfuric acid method (Dubois et al. 1956, Zhao et al. 2002). Samples were homogenized with 10 ml of 80% ethanol and centrifuged at 295 × g for 20 min. The supernatants were collected and then mixed in 1 ml of 80% ethanol extract with 1 ml of 5% phenol solution and 5 ml of 96% sulfuric acid and held in a water bath at 30°C for 20 min. Levels of carbohydrate were determined against standards, prepared by using a known concentration of glucose.

The balance of nutrition was evaluated by measuring the P:C ratio, and energy shortage quantified by determining MGE levels. P:C ratio (Behmer 2009) and MGE levels (Shimada and Saitoh 2006) were estimated using equations (2) and (3), respectively.

$$\text{P:C ratio} = P/C \quad\quad [2]$$

$$MGE = (23.7 \times P) + (17.2 \times C) \quad\quad [3]$$

Where: P is protein (%), C is carbohydrate (%), 23.7 is the chemical energy content coefficient of proteins, 17.2 is the chemical energy content coefficient of carbohydrates (Robbins 1983, Shimada and Saitoh 2006).

Plant Phenolics. For determination of plant secondary metabolites in the corn plants after beet armyworm damage, levels of vanillic acid, caffeic acid, and tannic acid in leaf tissue were analyzed with the method described by Häkkinen et al. (1998) and Peng et al. (1999). Briefly, the leave samples were weighed, ground in liquid N and ultrasonicated in a solution of 1% HCl and 99% methanol (pH 3) for 30 min. Samples were homogenized for two hours to allow the release of phenolics. The extracts were centrifuged at 903 × g for 10 min and filtered through a 0.45 μm syringe filter (Whatman, Maidstone, England, United Kingdom). Liquid samples (20 μl) were injected into an high-performance liquid chromatography (HPLC) (Agilent 1100 series HPLC, Agilent Technologies, Waldbronn, Germany) system fitted with diode-array detector (G1315B) and equipped with a Sepax HP-C18 column (Sepax Technologies, Changzhou, China) and operated by HP ChemStation software. A constant solvent flow was used with solvents A (acetonitrile) and B (0.5% acetic acid in millipore ultrapure water) at the ratio of 0% A from 0 to 5 min, 5% A from 5 to 10 min, 10% A from 10 to 40 min, 30% A from 40 to 50 min, and 0% A from 50 to 60 min. A quaternary pump was used for mixing the mobile phase to avoid pressure fluctuations because of the mixing of methanol in water. The flow rate was 1 ml/min. Chromatograms could be tested between 270 and 300 nm, while the peak responses were analyzed at 278 nm.

Phenolic acids were identified and quantified by comparing retention time and product ion spectrum with those in the user library created from the standard compounds (Häkkinen et al. 1998). The relative responses for each pure compound were calculated from five dose–response curves of the phenolics. Total phenolic concentration was calculated from the sum of phenolic concentrations in each sample. Three corn plants were mixed as a replicate. There were six replicates in each treatment.

Larval Feeding Preference. Dual choice experiments were assessed between nondamage high-N and nondamage low-N treatments. While for the triple choice experiments assessed: 1) feeding preference among nondamage high-N, mechanical damage high-N, and feeding damage high-N treatments, and 2) feeding preference among nondamage low-N, mechanical damage low-N, and feeding damage low-N.

Feeding preference apparatus were made from 120 mm diameter petri dish (Fig. 1; Showler 2001). One
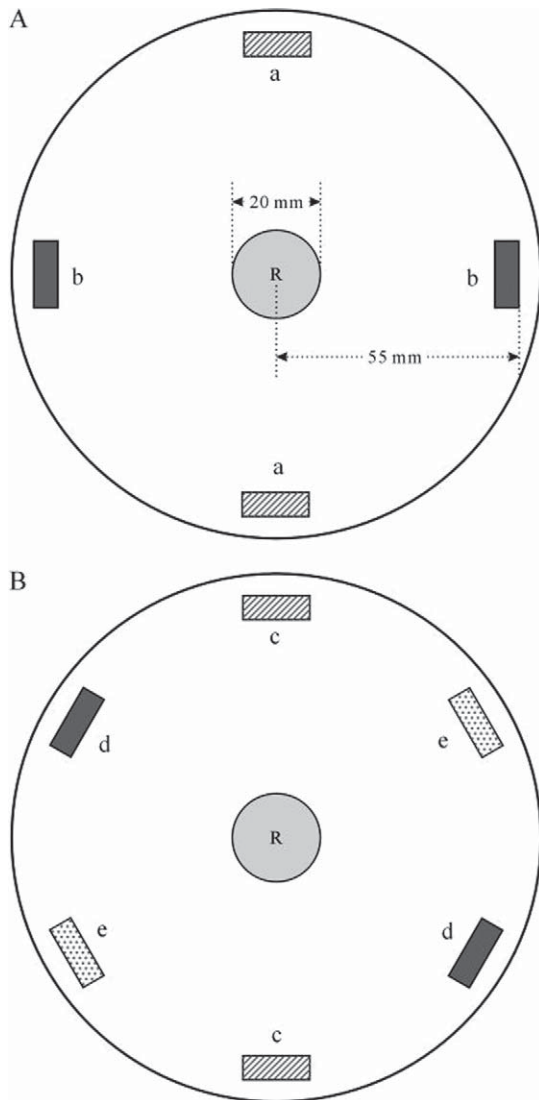
Fig. 1. Schematic drawing of the dual choice and triple choice test apparatus. (A) Dual choice feeding preference: selection between nondamaged high-N and nondamaged low-N treatments. (B) Triple choice feeding preference: feeding preference between nondamaged, mechanical damaged, and feeding damaged treatments. Lower case letters (from a to e) indicate different treatment corn leaves; R indicates where larvae were released.

fourth instar larva was starved for 6 h and was then placed into the center area of the apparatus that was lined with a water-moist filter paper to maintain the humidity using a fine brush. For dual choice experiments, four pieces of leaves (12 by 17 mm) cut from the tips of the third leaf (from the apex) were placed around the edge of the feeding apparatus equidistant from the larva and the leaf pieces (Fig. 1a). For triple feeding preference, six leaf pieces (two each of nondamaged, mechanical damaged, and feeding damaged treatments in high-N or low-N plants) were placed in

the apparatus. The two leaves from each feeding damaged treatment were placed diagonally from each other (Fig. 1b). The position of the larvae relative to the leaves then was recorded at 30, 60, 120, and 180 min after initial placement in the center of the arena. Preference were recorded as the percentage of choice of 10 larvae, and there were six replicates (six replicates $\times$ 10 larvae = 60 larvae) used for each treatment.

Larval Performance. Newly hatched and fifth instar larvae were used to test beet armyworm performance. Ten newly hatched larvae fed with different treatment corn and kept at 26°C in a glass tube (7 cm in height, 2 cm in internal diameter) for 5 d. Five days after feeding, larval survival and growth in each tube were recorded. Variable insect performance was calculated as Behmer (2009) equation (4). Here the growth rate equals total growth (e.g., milligrams) per day per insect, and survival means the average number of surviving insects. Therefore, "growth rate $\times$ survival" is average growth per day per insect (milligrams per day). Every 50 newly hatched larvae in five tubes (5 tubes $\times$ 10 larvae = 50 larvae) was reckoned as a replicate and six replicates were used for each treatment (50 larvae $\times$ 6 replicates = total 300 larvae in per treatment). In each glass tube, three pieces of plant leaves (1.5 $\times$ 2 cm) and moist filter paper were placed at the bottom and renewed every 24 h. Tubes were capped with a cotton plug to maintain humidity.

Variable insect performance

$$= \text{growth rate} \times \text{survival} \quad [4]$$

For fifth instar larval performance, a newly ecdysed larva was placed in a corn plant and fed for 24 h. To prevent insect escape, the aerial parts of corn and larva were covered by a mesh bag (9 $\times$ 12 cm) and tied with a plastic rope and prarmfilm warp. Initial and final weight of larva was recorded during the 24 h. The relative growth was calculated as equation (5).

$$\text{Relative growth} = (W_2 - W_1)/W_1 \quad [5]$$

Where: W_1 is weight of newly ecdysed fifth instar larva and W_2 is larva weight after 24 h of feeding. Pearson correlation coefficients were calculated to examine the correlation relationships between relative growth of larvae and plant chemicals.

Statistical Analysis. Two-way ANOVAs were used to analyze N availability, damaged status and their interaction effects on plant responses (relative growth, N concentration, P:C ratio, MGE levels) and larval performance (Eubanks and Denno 2001, Denno et al. 2002). Data of plant relative growth were normalized with an arc-sine transformation before analyses. t test and Tukey test were separately adopted to analyze the differences of the means between N treatments at each damage level and among the damage levels at each N-level (Dhileepan 2001).

Data on preference of beet armyworm were not normally distributed and transformations did not adequately correct this anomaly. Moreover, parametric test cannot be used to analyze the preference results as the data violate the assumption of independence (Peterson and

Table 1. Two-way ANOVA analyses for N levels and damaged treatments with and without beet armyworm on relative growth and nutritional quality of corn

Source of variation	Relative growth[a] (cm/d)				N content (%)				P:C ratio (g/g)				MGE (KJ/g)			
	df	MS	F	P	df	MS	F	P	df	MS	F	P	df	MS	F	P
N level	1	0.67	39.50	<0.01	1	9.24	303.37	<0.01	1	1.81	32.88	<0.01	1	0.54	0.16	0.11
Damage	2	0.04	2.18	0.11	2	0.03	1.05	0.36	2	0.32	5.87	<0.01	2	6.06	1.76	0.48
N level × damage	2	0.03	1.68	0.20	2	0.04	1.14	0.33	2	0.21	3.81	0.03	2	2.07	0.60	0.44
Error	118	0.02			31	0.03			24	0.06			24	3.44		

P:C ratio, protein/carbohydrate ratio; MGE, modified gross energy.
[a] Proportion data were arc-sine transformed before analysis to meet the assumption of GLM.

Renaud 1989, Roa 1992). Thus, these data were analyzed using Friedman test by Nonparametric analyses (Lockwood 1998, Hägele and Rowell–Rahier 1999). Pearson correlations were used to analyze the relationship between chemical concentration of corn and larval growth. All statistical procedures, including tests for normality and homoscedasticity, were carried out using SPSS 11.5 (IBM Corp., NY). Untransformed means and SEs are reported in the figures and tables.

Results

Plant Growth and Nutritional Quality. The interaction of N and damage significantly affected the P:C ratio of corn (Table 1; $P = 0.03$). There were no significant interactions between N availability and damaged status on relative growth ($P = 0.20$) and MGE levels ($P = 0.44$) of corn.

The average relative growth of nondamaged high-N corn was 0.17 cm/d and 1.54 times higher than nondamaged low-N corn (Table 2; $t = 2.44$; $P = 0.02$). N content was the highest in nondamage high-N corn (Table 2; V_{max} mean = 3.30 ± 0.04%; $t = -10.61$; $P < 0.01$). N availability significantly increased the P:C ratio of corn ($t = -5.19$; $P < 0.01$). The P:C ratio in nondamage high-N corn was 1.17 g/g and the highest of all treatments, while MGE levels were not affected by N availability ($t = -0.01$; $P = 0.99$).

Feeding damage in high-N corn also affected foliar P:C ratios, which decreased by 19.66 and 52.99% in the

mechanical and feeding damage plants, respectively, compared with the nondamage corn (Table 2; $F = 5.36$; df = 2, 15; $P < 0.05$), while there were no differences in P:C ratio for low-N corn ($F = 2.09$; df = 2, 15; $P = 0.17$). Damage status had no significant effects on plant relative growth and MGE levels in both high- and low-N corn (Table 1; growth high-N: $F = 2.05$; df = 2, 62; $P = 0.14$; low-N: $F = 1.84$; df = 2, 56; $P = 0.17$; MGE high-N: $F = 1.78$; df = 2, 15; $P = 0.21$; low-N: $F = 0.67$; df = 2, 15; $P = 0.53$).

Plant Phenolics. Vanillic, caffeic, and tannic acid extracted from corn were analyzed by HPLC. The interaction of N availability and damaged status had a significant effect on tannic acid (Table 3; $P < 0.01$). However, vanillic, caffeic acid, and total phenolic concentration in corn were not affected by the combined effects of N availability and damaged status (vanillic acid, $P = 0.29$; caffeic acid $P = 0.88$; total phenolics $P = 0.63$).

Total phenolic acid concentration was significantly ($t = 2.48$; $P = 0.03$) decreased by N availability in nondamaged corn (Table 4). However, individual phenolic concentrations of corn were not affected by N availability ($P > 0.05$). The average levels of vanillic acid and tannic acid in the feeding damage corn were 0.20 and 0.27 μg/g, respectively, which were significantly higher than in the nondamage corn in the high-N treatment (Table 4; vanillic acid $F = 5.06$; df = 2, 15; $P = 0.02$; tannic acid $F = 5.31$; df = 2, 15; $P = 0.02$). In addition, the concentration of vanillic acid and tannic

Table 2. Effect of N levels and damaged treatments with and without beet armyworm on relative growth and nutritional quality of corn (mean ± SE)

	Plant damaged type		
	Nondamaged	Mechanical	Larval feeding
Relative growth (cm/d)			
High-N	0.17 ± 0.02Aa	0.19 ± 0.02Aa	0.15 ± 0.02Aa
Low-N	0.11 ± 0.02Ba	0.07 ± 0.02Ba	0.08 ± 0.02Ba
N content (%)			
High-N	3.30 ± 0.04Aa	3.40 ± 0.05Aa	3.19 ± 0.13Aa
Low-N	2.29 ± 0.09Ba	2.30 ± 0.04Ba	2.30 ± 0.19Ba
P:C ratio (g/g)			
High-N	1.17 ± 0.10Aa	0.94 ± 0.11Aa	0.55 ± 0.09Ab
Low-N	0.48 ± 0.09Ba	0.31 ± 0.03Ba	0.39 ± 0.03Aa
MGE (KJ/g)			
High-N	10.43 ± 1.11Aa	9.50 ± 0.73Aa	8.31 ± 0.36Aa
Low-N	10.43 ± 1.11Aa	9.02 ± 0.78Aa	9.60 ± 0.49Aa

P:C ratio, protein/carbohydrate ratio; MGE, modified gross energy.
Upper case letters (A and B) indicate significantly different means within a column (*t* test; $P < 0.05$). Lower case letters (a and b) indicate significantly different means within a row (Tukey test; $P < 0.05$).

Table 3.　Two-way ANOVA analyses for N levels and damaged treatments with and without beet armyworm on phenolic production in corn

Source of variation	Vanillic acid (μg/g)				Caffeic acid (μg/g)				Tannic acid (μg/g)				Total phenolics (μg/g)			
	df	MS	F	P	df	MS	F	P	df	MS	F	P	df	MS	F	P
N level	1	0.15	7.72	<0.01	1	0.13	14.82	<0.01	1	0.001	0.15	0.71	1	0.61	20.18	<0.01
Damage	2	0.11	5.59	<0.01	2	0.03	0.35	0.71	2	0.01	1.69	0.20	2	0.23	7.65	<0.01
N level × damage	2	0.03	1.30	0.29	2	0.01	−0.13	0.88	2	0.05	5.70	<0.01	2	0.01	0.48	0.63
Error	30	0.02			30	0.01			30	0.01			30	3.44		

acid in feeding damage corn was 1.69 times higher than in the nondamage high-N corn ($F = 9.10$; df = 2, 15; $P < 0.01$). However, feeding damage did not ($P > 0.05$) influence phenolic levels in low-N treatments.

Larval Feeding Preference. Dual choice experiments showed that 43.30–71.67% of beet armyworm larvae preferred high-N corn at 60, 120, and 180 min after their introduction to the food source (Fig. 2a; 30 min: $P = 0.41$; 60, 120, and 180 min: $P = 0.01$). The triple-choice experiments showed that 36.67% of larvae had a positive response to nondamage high-N corn, which was two times higher than the preference for mechanically damage or feeding-damage corn (Fig. 2b; 30, 60, and 180 min $P > 0.05$; 120 min $P = 0.04$). However, larvae did not show differences in feeding preference among the three damage treatments in the low-N corn (Fig. 2c; $P > 0.05$).

Larval Performance. The performance of newly hatched and fifth instar larvae of beet armyworm showed that variable insect performance and larval growth rate were significantly lower in low-N corn (Fig. 3a; $P < 0.05$). Average relative growth of fifth instar larvae fed on nondamage low-N corn was 46.68% lower than those fed on nondamage high-N corn. Variable performance of larvae fed on feeding damaged corn was 0.25 mg/d/insect which was 22.06% lower than those fed on nondamage corn in the high-N treatment (Fig. 3b; high-N: $F = 4.47$; df = 2, 15; $P < 0.01$), whereas larvae fed on low-N corn did not show significant differences (newly hatched $F = 0.30$; df = 2, 15; $P = 0.75$; fifth instar $F = 1.97$; df = 2, 70; $P = 0.15$). There were no significant interaction effects of N and damage on

beet armyworm larvae (newly hatched $F = 1.52$; $P = 0.24$; fifth instar $F = 0.75$, $P = 0.48$).

Relationship Between Chemical Levels in Corn and Larval Relative Growth. Correlative relationships between chemical levels of corn and larval relative growth of beet armyworm were examined using Pearson's correlation. The relative growth of larvae were positively correlated with N content and P:C ratio (Table 5) and negatively correlated with vanillic acid and total phenolics.

Discussion

This study showed that N supply can lead to an increase of relative growth and nutritional quality (foliar N content, P:C ratio) of corn plant, and lower levels of total phenolic acids. This result was consistent with the predication of carbon-nutrient balance hypothesis (Bryant et al. 1983). Plants grow in N-rich environments invest more resource to growth rather than to defense herbivores. N fertilization increases plant expense of carbon-based secondary metabolite production, and decreases in phenolic levels. A similar example was found in a cotton study, which plants grown with low-N had higher amounts of phenolics compared with plants grown with high-N levels (Coviella et al. 2002).

Low nutritional quality of plants that results from nutritional deficiency or the presence of secondary metabolites can offer "quality starvation" and act as sublethal defenses against herbivores (Moran and Hamilton 1980, Clancy and Price 1987). Our bioassay experiments revealed that beet armyworm larvae pre-

Table 4.　Effects of N levels and damaged treatments with and without beet armyworm on phenolic concentrations in corn (mean ± SE)

	Plant damaged type		
	Nondamaged	Mechanical	Larval feeding
Vanillic acid (μg/g)			
High-N	0.11 ± 0.02Ab	0.12 ± 0.01Ab	0.20 ± 0.03Aa
Low-N	0.16 ± 0.05Aa	0.23 ± 0.05Aa	0.43 ± 0.12Aa
Caffeic acid (μg/g)			
High-N	0.18 ± 0.03Aa	0.17 ± 0.01Ba	0.20 ± 0.03Ba
Low-N	0.28 ± 0.05Aa	0.30 ± 0.04Aa	0.32 ± 0.03Aa
Tannic acid (μg/g)			
High-N	0.11 ± 0.02Ab	0.15 ± 0.03Aab	0.27 ± 0.05Aa
Low-N	0.18 ± 0.05Aa	0.25 ± 0.03Aa	0.27 ± 0.015Aa
Total phenolics (μg/g)			
High-N	0.40 ± 0.05Bb	0.45 ± 0.04Bb	0.67 ± 0.06Aa
Low-N	0.62 ± 0.11Aa	0.79 ± 0.07Aa	0.89 ± 0.11Aa

Upper case letters (A and B) indicate significantly different means within a column (t test; $P < 0.05$). Lower case letters (a and b) indicate significantly different means within a row (Tukey test; $P < 0.05$).
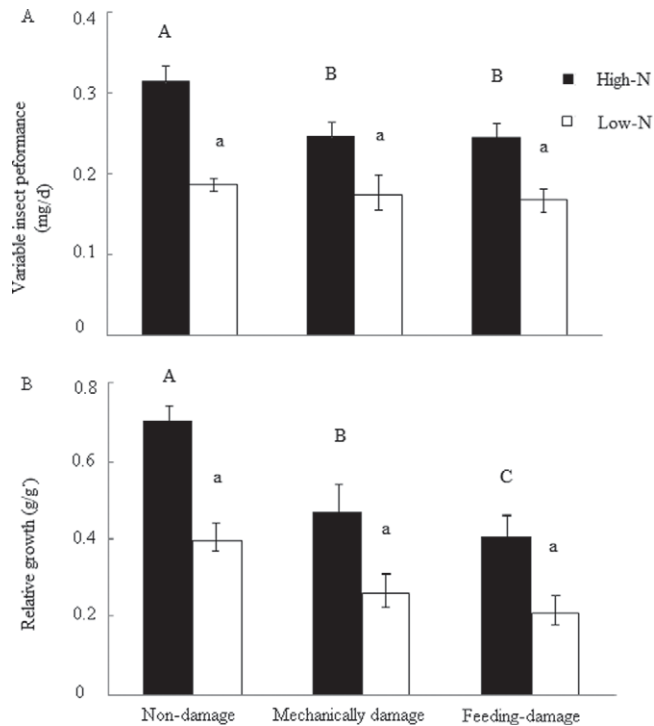
Fig. 2. Effect of N levels and damaged treatments on the relative growth and performance of beet armyworm larvae. (A) Variable performance after 5 d of feeding, $n = 10$ per treatment (i.e., column). Newly hatched larvae were used to calculate variable performance. (B) Fifth instar larvae were used to calculate relative growth (g/g). Upper case letters (A and B) indicate significantly different means between N treatments (t test; $P < 0.05$). Lower case letters (a and b) indicate significantly different means within damage treatments (Tukey test; $P < 0.05$).

ferred to high-N corn, and the performance of fifth instar larvae and newly hatched larvae were higher on high-N corn than those on low-N corn. High P:C ratio in corn was positively correlated with larval performance (Table 5) and may play a key role in feeding attraction. A similar result was found in an artificial diet study by Merkx–Jacques et al. (2008). Beet armyworm larvae preferred protein-rich (P:C = 1.1) than carbohydrate-rich diet (P:C = 0.5 and 0.2) and generated preingestive nutritional regulation. Excess carbohydrate also exhibited deleterious effects on larval development and growth. MGE also can influence insect performance by altering larvae digestion and ingestion (Bowen et al. 1995, Dickman et al. 2008). However, our results showed that there were no differences in MGE between high- and low-N plants. The MGE in corn under different N availability did not play a key role in larval performance of beet armyworm.

In our study, increased performance of beet armyworm in the high-N treatment also resulted from a decreased carbon-based secondary metabolite. Levels of total phenolics were lower in high-N corn than in low-N corn and then had a negative effect on larval relative growth. This is probably because the phenolics inhibit herbivore digestion by binding to the consumed plant proteins making them more difficult to digest, by interfering with protein absorption and digestive enzymes (Summers and Felton 1994, Barbehenn et al. 2005). Corn

phenolics were also found to have a negative effect on maize weevil (Serratos et al. 1987, Classen et al. 1990).

However, nutritional quality can be induced to generate a new change by feeding damage. Feeding damage corn in the high-N treatment decreased the P:C ratio and in turn decreased the fifth instar larval growth. Brody and Karban (1989) reported that reduced nutritional quality of cotton may be one of the primary mechanisms underlying induced defense to mites. Recent studies reported that feeding-regulated protein by insects could target and interfere with digestive and absorptive process of insect digestive canal, thus playing a critical role in postingestive plant defense (Chen and Ni 2011). Moreover, insect feeding could also lead to increased phenolic levels of plants and/or could have negative effects on insects (Bi et al. 1994, Karban and Baldwin 1997). The high-N corn increased vanillic, tannic acid, and total phenolic concentrations after feeding induction, while only vanillic acid and total phenolics were negatively correlated with beet armyworm growth, which is consistent with the work of Ren (2010). This implies that vanillic acid can decrease larval survival and growth rate and is correlated with insect defense.

The magnitude of induced defenses is dependent on resource availability (Lou and Baldwin 2004, Chen and Ni 2011, Sampedro et al. 2011). Hunter and Schultz (1995) found that the expression of
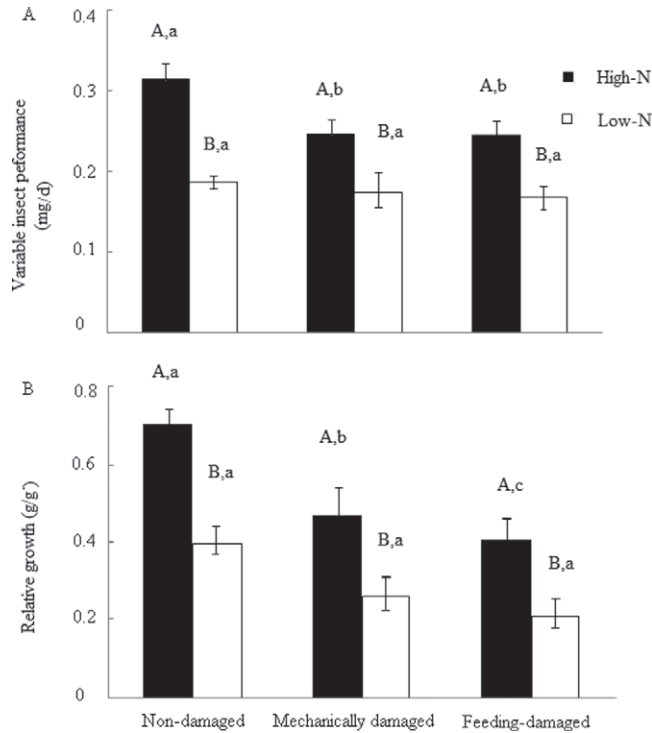
Fig. 3. Effect of N levels and damaged treatments on beet armyworm larval feeding preferences (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$).

induced defenses to sap-sucking insects may be suppressed by fertilization in red and chestnut oak. In contrast, Mutikainen et al. (2000) found that induced defense in silver birch to autumnal moth, *Epirrita autumnata* Borkhausen, were expressed only in fertilized plants. In our study, there was no significant evidence to show strong induced defense in low-N corn. Moreover, larval growth, survival, and feeding preference of beet armyworm did not show significant differences between the different damaged treatments in the low-N corn, which strongly indicates that defense patterns were significantly different between the low and high-N plants.

Plant defense strategies have been hypothesized to vary in response to N availability and these responses are associated with the relative costs and benefits between constitutive and induced defenses in many hypotheses, such as the "carbon-nutrient balance hypothesis" (Bryant et al. 1983) and the "resource availability hypothesis" (Coley et al. 1985, Craine et al. 2003). Plants in resource-rich environments allocate resources into growth and subsequent induced defense against herbivores are more advantageous than constitutive defense. This plant defense pattern is associated with the relative increased opportunity cost in potential growth as the result of less competitive status, and the allocation costs of secondary metabolites (Coley et al. 1985). However, based on the current study, plants grown in resource-rich environments had lower constitutive defense which enhanced an increased insect feeding selection pressure by beet armyworm on corn. Therefore, plants in resource-rich environments have higher plasticity of induced defense to depress further insect attack than in resource-poor environments. Similar results were found in 68 woody plant studies and nine species of herbaceous plants that illustrated that induced defense, especially the production of phenolics involves protein-precipitation, had higher levels in resource-rich plants after insect damage more than in resource-poor plants (Nykänen and Koricheva 2004, Van Zandt 2007). In contrast to high-N plants, plants growing in low-N environments have a surplus of carbon, and it is adaptive for plants to allocate some of their excess carbon to constitutive defense that makes them less susceptible to herbivores (Bryant et al. 1983).

Table 5. Pearson correlation analysis between the performance of beet armyworm larvae and chemical levels of corn

	Larval relative growth (g/g/d)	
	Pearson correlations coefficient	P
N concn (%)	0.35	<0.01
P:C ratio (g/g)	0.54	<0.01
MGE (KJ/g)	0.12	0.29
Vanillic acid (μg/g)	−0.49	<0.01
Caffeic acid (μg/g)	−0.17	0.16
Tannic acid (μg/g)	0.17	0.15
Total phenolic (μg/g)	−0.35	<0.01

P:C ratio, protein/carbohydrate ratio; MGE, modified gross energy.

In summary, this study highlights the complexities of N regulation mechanisms underlying plant-insect interactions. Plants grown under different N environments trade-off constitutive and induce defense to modify insect population dynamics. If this scenario is extrapolated to a population level or large crop scale, it can give insights to better understand insect-plant interactions to develop optimized management practices for increasing N fertilizer use efficiency in crops, which could benefit from improved plant defense ability and to help develop agricultural and ecological management strategies.

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