

Interactions of Elevated CO₂ and Nitrogen Fertilization: Effects on Production of *Bacillus thuringiensis* Toxins in Transgenic Plants

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ABSTRACT Elevated atmospheric CO₂ concentrations will cause plants to grow faster, lower nitrogen content per unit of plant tissue, and generate higher carbon to nitrogen (C/N) ratios. We hypothesize that production of transgenic proteins will be reduced, thus reducing the efficiency of *Bacillus thuringiensis* (Bt) transgenes against insect populations. Commercially available transgenic cotton plants expressing the *Cry IAc* gene from Bt were compared with a near isogenic non-Bt cotton line in a split-plot design with two levels of atmospheric CO₂ (ambient, 370 ppm and elevated, 900 ppm) incorporating a 2 × 2 factorial design with two nitrogen (N) fertilization regimes (low, 30 mg N/kg soil/wk and high, 130 mg N/kg soil/wk), and two levels of Bt (presence or absence). Bioassays using *Spodoptera exigua* (Hübner) and quantitative enzyme-linked immunosorbent assays for toxin content indicated reduced Bt protein production in elevated CO₂. The tendency for test insects to consume more foliage from plants with lower N, caused by the elevated CO₂, did not compensate for the reduction in toxin production. N fertilization regime interacted with CO₂ concentration, showing that plants growing in N limited systems would produce substantially less toxin. The use of transgenic plants is becoming increasingly important and will continue to be so in the next decades. At the same time, atmospheric CO₂ increase will affect the effectiveness of this strategy. These observations have implications not only for agricultural use of transgenic plants, but also for the ecological consequences of transfer of Bt toxins to closely related wild plant genotypes.

KEY WORDS *Spodoptera exigua*, global climate change, carbon dioxide, CO₂, cotton

ATMOSPHERIC CARBON DIOXIDE levels have increased from ≈270 to 280 μl/liter in preindustrial times to the current 365 μl/liter (Houghton 1990, Berner 1993, Keeling and Whorf 1998). Predictions regarding future equilibrium levels depend on a variety of scenarios. An analysis of these possible scenarios suggests that stabilization of CO₂ at levels below 750 μl/liter may only occur if emissions are reduced well below 1990 level (Schimel et al. 1994). This goal now appears unachievable and some revised models have suggested future equilibrium levels as high as 1,200 μl/liter. However, most models anticipate CO₂ levels in the range of 700–1,100 μl/liter by the second half of the 21st century (Tans and Bakwin 1995, Houghton 1996, Watson et al. 1996, Sarmiento and Le Quere 1996, Mahlman 1997, Sarmiento et al. 1998, Joos et al. 1999). For our experiments, we chose a level of 900 ppm at the middle of the range of predictions in the most recent models.

Probably the most evident and best studied effect of high atmospheric CO₂ on plants is the fertilization effect (LaMarche et al. 1984). Plants grown under elevated CO₂ conditions have higher photosynthetic rates (Lincoln et al. 1993, Bazzaz 1996). This leads to higher carbon to nitrogen ratios (Lawler et al. 1997),

resulting in lower nitrogen levels per unit of plant tissue (Curtis and Wang 1998). We hypothesized that elevated CO₂ leading to a reduction in N content and to a higher C/N in the plants could affect the production of nitrogen-based proteins in transgenic plants. If this reduction is caused by lower N content, high N availability through fertilization should negate this effect. This would be significant because biological control of insect populations by means of transgenic plants is becoming an increasingly important pest management technique (Estruch et al. 1997). Although *Bacillus thuringiensis* (Bt) proteins are the only transgenes commercially used for insect control, several other proteinaceous compounds such as proteinase inhibitors, amylase inhibitors, and lectins are being studied. Because transgenic plants producing nitrogen-based toxins are likely to be of widespread use well beyond the turn of the century, the expected increase in atmospheric CO₂ may impact the effectiveness of this strategy. Therefore, we used Bt transgenic plants as a model to assess the possible effects of future CO₂ atmospheric levels in the increasingly important use of transgenic plants producing proteins toxic to insects.

In this context, we first tested whether cotton plants grown under elevated CO₂ would show differences in leaf carbon (C), leaf nitrogen (N), or in carbon to nitrogen (C/N) ratios. We then tested for differences in Bt toxin production and did insect bioassays to test for differences on insect performance. We are aware

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that potential negative effects from reduced toxin production might be ameliorated. Previous research with high C/N ratios indicates that herbivorous insects consume more foliage to ingest the required nitrogen or, in some cases, simply grow more slowly (Lincoln et al. 1986, Osbrink et al. 1987). Some results (Fajer et al. 1989, Slansky and Wheeler 1989) show that insect weight gain is positively correlated with nitrogen concentration in the plant material offered, and amount of foliage consumption is negatively correlated with foliar nitrogen concentration (Marks and Lincoln 1996). Thus, we also examined whether previously reported increases in food consumption by herbivores might balance any potential reduction in Bt toxin production.

Currently, Bt transgenic plants are effective in controlling target insect pests (Hilder and Boulter 1999). Unfortunately, nontarget insects have also been affected (Hilbeck et al. 1998). Consequently, the impact of Bt transgenic plants on the fate of nontarget insects is of concern. Additionally, Bt resistance has been observed in target insect species and may become increasingly common in the future (Roush 1998). Not only can studying the effect of elevated CO₂ on Bt toxin production in plants be useful in evaluating ecological consequences on nontarget organisms, but also the information can be used to model the impact that the elevated CO₂ levels we expect may have on resistant and partially resistant populations of target insects pests.

Spodoptera exigua (Hübner) was chosen as the nontarget lepidopteran because this insect occurs on many plants that have been engineered with the Cry IAc toxin from Bt. This polyphagous pest is often found on commercially planted cotton (Adamczyk et al. 1998, Akey and Henneberry 1998).

Recent evidence suggests that gene flow via pollination of a transgene from a genetically modified crop to a closely related wild species can occur (Ellstrand and Prentice 1995, Timmons et al. 1995, Arriola and Ellstrand 1996, Darmency et al. 1998). The risks of dominant transgenetic traits that confer fitness advantages relative to the wild parental lines are potentially serious (Adam et al. 1993, Ellstrand 1996, Arriola and Ellstrand 1997). Concerns have escalated with the commercial release and increasing use of transgenic crops such as potatoes, corn, and cotton. To test whether elevated CO₂ levels and the possible subsequent lower leaf nitrogen might affect production of proteinaceous toxins in plants growing in typically low nitrogen natural areas, we examined nitrogen availability effects on C/N ratios, Bt toxin production, and insect development under both low and elevated CO₂ environments.

Materials and Methods

Test Chambers and CO₂ Control. Twelve chambers consisting of 5.08-cm PVC pipe frame and transparent Teflon film (modified after Osbrink et al. 1987) were used for the plant growth experiments. The chambers were 127 cm wide by 122 cm high by 102 cm diameter

with a volume of 1.58 m³. Blowers attached to each chamber (Dayton Shaded Pole Blowers, Dayton Electric, Dayton, IL) allowed for 0.49–0.51 air exchanges per minute. The chambers were placed inside a temperature-controlled greenhouse. Six chambers were maintained at 360–380 μl/liter CO₂ (ambient CO₂ treatment) and six were maintained at 900 μl/liter CO₂ (elevated CO₂ treatment) at the midrange of the most current predictions for future equilibrium levels. Each chamber was randomly assigned to a CO₂ treatment. CO₂ levels were monitored in all the chambers and controlled in the elevated CO₂ treatments. CO₂ was monitored with a nondispersive Infrared Gas Analyzer (IRGA, Type GMP111, Vaisala OY, Helsinki, Finland). Two rotatory pumps (Q-Com, Irvine, CA) each collected air samples for 30 s from six of the chambers in sequence. Thus, each chamber was individually sampled every three minutes.

Each of the six high CO₂ chambers has two separate lines for CO₂ gas injection. One line was set to inject gas continuously at a rate that raised the CO₂ level inside the elevated CO₂ chambers to ≈870 μl/liter. A computer-controlled solenoid valve in the second line was opened when the CO₂ level in that particular chamber was 870 μl/liter or below, and it was closed when the CO₂ level was 900 μl/liter or above. Four metal halide 1,000-W lamps provided additional light to maintain a photoperiod of 14:10 (L:D) h.

Plants. Twenty cotton plants (*Gossypium hirsutum* L.) were sown individually in 3-liter pots in each of the 12 environmental chambers. Ten plants were of a transgenic cotton line containing the Bt gene for the production of the CryIAC protein (Deltapine Nuctn 33B, Delta and Pine Land, Casa Grande, AZ). The remaining 10 plants were of a near isogenic line without the Bt gene (Deltapine DP5415). Five of the 10 plants of per CO₂/N treatment received a nitrogen fertilization level of 130 mg N/kg soil per week (the "high" nitrogen level) and received a nitrogen fertilization level of 30 mg N/kg soil per week (the "low" nitrogen level) (after Rogers et al. 1996). In all the experiments, plants were between 40 and 45 d of age at use. Each plant was used only once and leaf tissue not used within 24 h of removal from the plant was discarded.

To avoid any potential effects of plant responses to damage during sampling on Bt production or acceptability to herbivores, a separate set of 240 plants (20 in each chamber with the same design as above) had C and N analyses conducted on one of the first two true leaves and one of the two uppermost leaves. To test for possible differences in Bt production throughout the plant and because of leaf age, Bt was quantified from the first two leaves and the two uppermost leaves. The carbon and nitrogen analyses were conducted by the University of California's Division of Agriculture and Natural Resources Analytical Laboratory, Davis, CA. The Bt protein analyses were conducted in our laboratory using a commercially available Btk enzyme-linked immunosorbent assay (ELISA) test (Btk ELISA PathoScreen kit, Agdia, Elkhart, IN) (Sundaram et al. 1995).

Insect Bioassays. The insect bioassays were conducted with cohorts of neonate *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) standardized for age by eclosion within the previous 12 h. This insect was selected because preliminary trials indicated that the *Cry IAc* Bt toxin was mildly toxic for this species; mortality on the Bt transgenic plants was 31.7% compared with the mortality on the controls on nontransgenic plants of 10.0% or less (C.E.C., unpublished data). This relatively mild effect allowed comparisons to determine if environmental changes would increase or decrease the effects of transgenic plants on insect performance. This determination would not be possible using insects targeted by the toxin. All insects used were from a laboratory colony maintained at the Department of Entomology, University of California at Riverside. For each replicate within a treatment, five neonates were reared individually in 28.4-ml plastic cups lined with agar to keep the plant material turgid and suitable for ingestion (Diawara et al. 1996). The larvae were fed foliage ad libitum from the cotton plants described previously. The plant material was changed every other day throughout the test. At no time were the insects allowed to consume all of the available food. Results from a previous experiment indicated that the insects are not directly affected by the elevated CO₂ levels used in this study (C.E.C., unpublished data). The cups with individual insects were placed on trays and maintained inside an incubator at 28 ± 2°C, ambient CO₂, and a photoperiod of 14:10 (L:D) h. Trays were randomized inside the incubator and treatments in each tray were rotated on a daily basis to eliminate any potential positional effects.

Individual larval weights were measured at days 5, 7, and 10. From these data we calculated relative growth rates (RGR = mg biomass gained/mg of larval biomass/d; Waldbauer 1964) for each treatment for the periods day 5 to day 7, day 7 to day 10, and day 5 to day 10. We also measured days to pupation, pupal weight, days to adult eclosion, and mortality occurring in the larval and larval plus pupal stages.

Statistical Analysis. The experimental design was a split-plot arrangement with CO₂ level (ambient or elevated) as the whole plots, including a 2 × 2 factorial for two levels of Bt (presence or absence) and two levels of nitrogen fertilization (high and low). We had a total of eight treatments replicated six times. Treatments were assigned at random to each chamber. For the insect bioassays we had five insects for each replicate. Analysis of variance (ANOVA) (Super ANOVA, Abacus Concepts 1993) was used to separate treatments at the $P < 0.05$ level. Dependent variables were tested for normality and transformed where appropriate. Because weights of individual insects were measured over time, a repeated measures analysis was performed for this variable. When significant, two-way interactions were analyzed using contrast analyses to determine the amount of variation in the interaction explained by each of the variables.

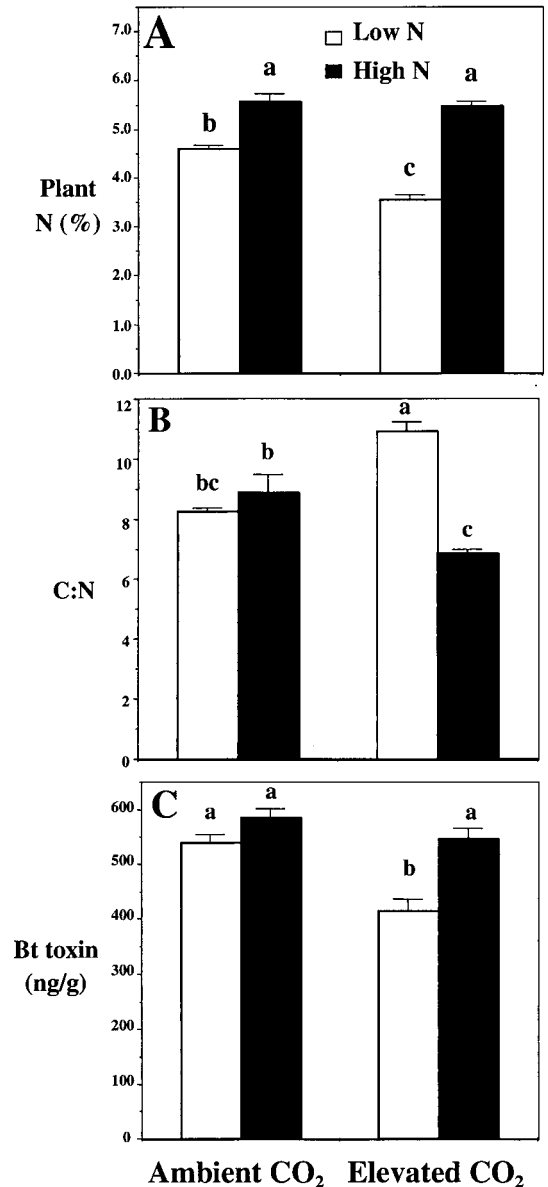


Fig. 1. Significant CO₂ × N two-way interactions on plants. (A) Significant CO₂ × N two-way interaction for foliar N. (B) Significant CO₂ × N two-way interaction for C/N. (C) Significant CO₂ × N two-way interaction for plant Bt toxin production. Bars with the same lowercase letter are not significantly different at $P = 0.05$.

Results and Discussion

Effects on the Plants. No significant CO₂ × N × Bt three-way interaction effects were found for leaf nitrogen, leaf C, or C/N ratios. There were significant two-way CO₂ × N interaction effects on total leaf nitrogen (Fig. 1A) and C/N ratios (Fig. 1B). Plants grown in elevated CO₂ atmospheres had significantly lower leaf nitrogen content and significantly higher C/N ratios. This effect was reduced by increasing the

Table 1. ANOVAs comparing the effects of CO₂, nitrogen fertilization, and Bt presence or absence on plant parameters

	Plant nitrogen			Plant C/N			Bt toxin production		
	F	df	P	F	df	P	F	df	P
CO ₂	18.055	1	0.013	10.017	1	0.034	3.363	1	0.141
N	134.706	1	<0.001	77.437	1	<0.001	30.951	1	<0.001
Bt	0.325	1	0.579	0.064	1	0.805	—	—	—
CO ₂ × N	6.983	1	0.022	7.036	1	0.021	7.738	1	0.007
CO ₂ × Bt	0.665	1	0.431	0.255	1	0.623	—	—	—
N × Bt	0.149	1	0.706	0.300	1	0.594	—	—	—
CO ₂ × N × Bt	1.287	1	0.279	1.292	1	0.278	—	—	—
Leaf	—	—	—	—	—	—	0.541	1	0.464
Residual	—	16	—	—	16	—	—	91	—

nitrogen fertilization level. Plants grown in elevated CO₂ had 12.46% less foliar nitrogen than plants grown under current ambient CO₂ levels, and plants receiving the high fertilization level had 27.86% more foliar nitrogen than plants in the low fertilization treatment. There was no significant CO₂ × N interaction effect on total leaf C. There were no significant two-way interactions between Bt presence/absence and CO₂, or between Bt presence/absence and N for any of the plant variables measured. Thus, total foliar nitrogen content of the plants was not dependent on whether the plant was transgenic or nontransgenic (Table 1).

Contrast analyses of the significant CO₂ × N two-way interactions on plant N content showed that the main effect of CO₂, explained 14.6% of the interaction variance, while the main effect of N explained 81.2% of the interaction variance. On plant C/N ratios, contrast analysis showed that the main effect of CO₂ explained 16.7% of the interaction variance, and the main effect of N explained 76.3%, of that interaction variance.

Effects on Bt Protein Production. We tested two leaves from the top and two from the bottom of each plant. A significant CO₂ × N interaction was observed for Bt toxin production (Fig. 1C, Table 1). In elevated CO₂, plants grown with low N had lower N contents, and this negatively impacted the production of Bt toxin. Contrast analysis of this interaction on Bt toxin content showed that the main effect of CO₂ accounted for 39.7% of the interaction variance, and 48.1% was caused by the main effect of N. Thus, transgenic plants grown in elevated CO₂ produced lower levels of Bt toxin than those plants grown in ambient CO₂. High levels of nitrogen fertilization partially compensated for the CO₂ effect and allowed the plants to produce

Bt toxin levels closer to those of plants grown in ambient CO₂ (Fig. 1C).

Leaf location on the test plants did not affect Bt toxin levels; there was no difference in Bt toxin production between leaves from the top and the bottom of the plants. Flower buds produced only 20% of the Bt toxin level found in leaves. These results suggest that in the elevated CO₂ conditions expected in the 21st century, Bt toxin production will be affected by a significant interaction between CO₂ level and nitrogen availability.

Effects on Insects. There were no significant three-way CO₂ × N × Bt interactions for any of the insect variables measured. This allowed investigation of the two-way interactions (Table 2).

Insect growth parameters relevant for the dynamics of insect populations were strongly affected by significant two-way CO₂ × Bt and CO₂ × N interactions (Table 3). The number of days required for larval development (days to pupation) was affected by a significant CO₂ × Bt interaction. In ambient CO₂, there was a significant increase in days to pupation for insects fed transgenic plants. This difference was not evident in elevated CO₂. Pupal weight was also affected differentially (Tables 2 and 3). In ambient CO₂, pupal weight was significantly higher in nontransgenic plants. This difference was not observed in elevated CO₂. Similarly, the number of days to adult eclosion (larval plus pupal stages) was affected by a significant CO₂ × Bt interaction. In ambient CO₂, insects on nontransgenic plants developed faster than insects on transgenic plants (Table 2). Developmental times to adult eclosion were not significantly different between treatments in elevated CO₂.

Table 2. ANOVAs comparing the effects of CO₂, nitrogen fertilization, and Bt presence or absence on insect parameters

	Days to pupation			Pupal weight			Days to adult			Mortality		
	F	df	P	F	df	P	F	df	P	F	df	P
CO ₂	0.051	1	0.821	0.792	1	0.852	0.792	1	0.375	0.678	1	0.562
N	2.582	1	0.110	2.264	1	0.631	2.264	1	0.135	0.553	1	0.462
Bt	47.494	1	<0.001	38.092	1	<0.001	38.092	1	<0.001	11.523	1	0.002
CO ₂ × N	4.122	1	0.044	6.312	1	0.290	6.312	1	0.013	0.084	1	0.773
CO ₂ × Bt	5.078	1	0.025	4.690	1	0.032	7.553	1	0.007	0.026	1	0.873
N × Bt	1.223	1	0.270	1.001	1	0.478	1.001	1	0.319	0.015	1	0.903
CO ₂ × N × Bt	0.376	1	0.540	0.384	1	0.522	0.384	1	0.536	0.015	1	0.903
Residual	—	182	—	—	181	—	—	148	—	—	38	—

Table 3. Mean ± SE for the CO₂ × Bt and CO₂ × N interactions on days to pupation, pupal weight, and days to adult eclosion

	Ambient CO ₂ ± SE	Elevated CO ₂ ± SE
CO ₂ × Bt interaction effect on days to pupation (days)		
Bt Plants	21.071 ± 0.568a	19.625 ± 0.614ab
Non-Bt Plants	16.278 ± 0.518c	17.222 ± 0.533bc
CO ₂ × Bt interaction effect on pupal weight (g)		
Bt Plants	0.082 ± 0.003c	0.089 ± 0.002b
Non-Bt Plants	0.097 ± 0.002a	0.094 ± 0.002ab
CO ₂ × Bt interaction effect on days to adult eclosion (days)		
Bt Plants	28.600 ± 0.471a	27.414 ± 0.715ab
Non-Bt plants	23.277 ± 0.569c	25.400 ± 0.677b
CO ₂ × N interaction effect on days to pupation (days)		
Low N	19.300 ± 0.580a	18.128 ± 0.569ab
High N	17.370 ± 0.678b	18.362 ± 0.621ab
CO ₂ × N interaction effect on days to adult eclosion (days)		
Low N	26.773 ± 0.637a	25.882 ± 0.717a
High N	24.132 ± 0.665b	26.450 ± 0.721a

Means followed by the same letter within each interaction do not differ significantly at *P* = 0.05.

Significant two-way CO₂ * N interactions also affected insect development. Consistent with the CO₂ * Bt interactions, both days to pupation and days to adult were less in the high N treatment in ambient CO₂ (Table 3). There were no significant differences in elevated CO₂.

There were no three-way or two-way interactions for the mortality or weight variables, allowing comparisons based on main effects. There was a significant Bt main effect on insect mortality. Larval mortality was significantly higher on Bt transgenic plants, and overall mortality (to adult eclosion) was also higher in Bt plants (Table 3). For insect weights, the repeated measures analysis showed a significant CO₂ main effect (Table 4). Insects fed on plants grown in elevated CO₂ grew significantly slower than insects fed on plants grown in ambient CO₂. Not surprisingly, the repeated measures analysis showed also a significant Bt main effect on weight. Insects fed transgenic Bt plants grew significantly slower than insects fed non-Bt plants (Table 4).

In conclusion, these results support the hypothesis that the lower N content per unit of plant tissue caused

by the elevated CO₂ will result in lower toxin production by transgenic plants when nitrogen supply to the plants is a limiting factor. Because there were no effects on C concentration in the plants, changes in C/N ratios were the result of modification of N rather than C. These results are consistent with previous reports where lower N content per unit of plant tissue led to higher C/N ratios in plants grown in elevated CO₂ conditions (Lincoln et al. 1986, Trumble et al. 1993, Rogers et al. 1996). Atmospheric CO₂ levels at the concentrations expected in the next few decades will affect strategies for use of transgenic plants producing protein toxins. Under the reduced N fertilization levels desired for sustainable agriculture, and in many agricultural regions in the world where N input is strongly limited by fertilizer price, transgenic plants grown in elevated CO₂ will have lower total nitrogen leaf content and possibly lower toxin concentrations.

Our data suggests that, as currently designed, transgenic plants will require additional N inputs to maintain effectiveness in elevated atmospheric CO₂. Unfortunately, nitrogen fertilizers, which were credited for much of the 'green revolution' of the 1950s and 1960s, also have been responsible for significant increases in petrochemical use and for present concerns with environmental damage caused by nitrification associated with runoff from agricultural lands (Johnson and Parker 1993, Larocque and Banton 1995, Lindau et al. 1997).

As a result of reduced toxin production in elevated atmospheric CO₂, transgenic plants producing N-based toxins are likely to engender substantial concerns among agriculturists and naturalists. Even current transgenic plants that produce sublethal levels of toxins for nontarget pest insects risk speeding the development of resistance to the toxin. The expected elevated atmospheric CO₂ levels will interact with N availability, differentially affecting population parameters that are important in the dynamics of natural populations of insects. Elevated CO₂ appears to eliminate differences between transgenic and nontrans-

Table 4. Means ± SE for CO₂ and Bt main effects on insect larval weights (g)

Day	Main effect	
	CO ₂ ^a	
	Ambient CO ₂ ± SE	Elevated CO ₂ ± SE
5	0.003 ± 0.001	0.003 ± 0.001
7	0.016 ± 0.002	0.014 ± 0.002
10	0.073 ± 0.008	0.062 ± 0.007
	Bt ^b	
	Bt Plants	Non-Bt Plants
5	0.002 ± 0.001	0.005 ± 0.001
7	0.006 ± 0.001	0.024 ± 0.002
10	0.026 ± 0.003	0.109 ± 0.009

^a Repeated measures showed a significant difference between ambient and elevated CO₂ at *P* = 0.05.

^b Repeated measures showed a significant difference for Bt presence/absence at *P* = 0.05.

genic plants for some key insect developmental/fitness variables including length of the larval stage and pupal weight. Thus, because of the reduced levels of toxin production and altered insect development and fitness variables, nontarget pest population growth rates may not be affected to the same extent in future. Extrapolating our results to other systems should be attempted with caution, but we anticipate that elevated CO₂ atmospheric levels could have similar effects for other proteins under study for eventual use in transgenic plants, such as proteinase inhibitors, amylase inhibitors, and lectins.

Finally, recent reports have highlighted the danger of Bt gene transference into wild relatives of transgenic crop lines (Ellstrand and Prentice 1995; Arriola and Ellstrand 1996, 1992). We were initially concerned that this transfer would confer an evolutionary advantage to those species most capable of outcrossing. Unlike outcrossing with crops bred for horticultural traits (synchronized ripening or larger fruit, for instance) that may not enhance survival, toxin production would allow some species or lines to outcompete others. Through this mechanism, genotypic variation would be reduced. Our results indicate that plants growing in the wild on low fertility soils are likely to have low levels of toxin production. Therefore, the postulated advantage conferred by the eventual transference of Bt genes into the wild relatives may not be as substantial as anticipated, at least in low fertility areas. Nonetheless, in and near agricultural sites with higher N availability, higher protein production could exacerbate the relative advantages for those wild relatives most capable of incorporating genes from transformed crops.

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