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**Effects of Elevated Carbon Dioxide and Nitrogen Fixation on a Legume-Based System: A Test with Soybean and the Soybean Looper, *Pseudoplusia includens***

Angela M. Spickard

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EFFECTS OF ELEVATED CARBON DIOXIDE AND NITROGEN FIXATION  
ON A LEGUME-BASED SYSTEM: A TEST WITH SOYBEAN AND THE  
SOYBEAN LOOPER, *PSEUDOPLUSIA INCLUDENS*

by

Angela M. Spickard

A Thesis  
Submitted to the  
Faculty of The Graduate College  
in partial fulfillment of the  
requirements for the  
Degree of Master of Science  
Department of Biological Sciences

Western Michigan University  
Kalamazoo, Michigan  
June 2000

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2000

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Angela M. Spickard

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Angela M. Spickard, M.S.

Western Michigan University, 2000

Wild-type and non-nodulated soybean were grown at  $367 \pm 10$  ppm [ambient] and  $756 \pm 10$  ppm [elevated] CO<sub>2</sub> to determine whether nitrogen fixation could minimize the effects of elevated CO<sub>2</sub> on plant nutritional and defensive chemistry. Relative to non-nodulated soybean, wild-type soybean had higher concentrations of leaf nitrogen and proteinase inhibitors, as well as lower C:N ratios and lower phenolics. Elevated CO<sub>2</sub> resulted in decreased leaf nitrogen and proteinase inhibitor contents, and increased C:N ratios and phenolics in both soybean strains. Constitutive levels of phenolics and proteinase inhibitors occurred as predicted by the Carbon/Nutrient Balance hypothesis (Bryant et al., 1983). However, induction of phenolics only occurred in plants grown at ambient CO<sub>2</sub>, and induction of trypsin inhibitors only occurred in wild-type plants. Nodulation had the greatest effect on performance of *P. includens*; larvae fed non-nodulated plants displayed lower consumption, digestion, and growth rates. Increased larval consumption of elevated CO<sub>2</sub>-grown plants was sufficient to prevent decreased growth, suggesting that host-plant association with nitrogen-fixing symbionts may buffer this legume-feeding insect against CO<sub>2</sub>-induced changes in host plant chemistry.

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## CHAPTER I

# EFFECTS OF ELEVATED CARBON DIOXIDE AND NITROGEN FIXATION ON THE CARBON/NUTRIENT BALANCE AND CHEMICAL DEFENSES OF SOYBEAN, *GLYCINE MAX* L.

### Introduction

With the predicted doubling of atmospheric carbon dioxide (CO<sub>2</sub>) in the next 50 to 75 years (Tans et al., 1990; Wigley and Raper, 1992), the effects of elevated CO<sub>2</sub> on plant nutritional quality and defensive chemistry have been the focus of much recent study. When grown under elevated CO<sub>2</sub>, plants generally display increased photosynthesis, biomass, and water use efficiency, but decreased leaf nitrogen (Wong, 1979; Rogers et al., 1984; Bazzaz, 1990; Johnson and Lincoln, 1991; Lindroth et al., 1993; Lincoln et al., 1993; Tschaplinski et al., 1995; Reitz et al., 1997; Lawler et al., 1997; Karowe et al., 1997; Karowe and Gray, in preparation). However, the effects of elevated CO<sub>2</sub> on plant chemical defenses are much less well documented, and appear to be less predictable.

Within a plant species, a suite of chemical defenses may exist including both carbon-based compounds such as phenolics, terpenes, and lignin, and nitrogen-based compounds such as alkaloids, non-protein amino acids, and proteinase inhibitors. Presumably, the primary function of these compounds is to defend the plant from insect herbivory and/or pathogenic infection. In addition, each class of compounds

may exist at a constitutive level within a plant and/or may be “induced” as part of a plant’s response to such attacks. Induction of chemical defenses in response to damage appears to be a common strategy, presumably because it allows plants to allocate valuable, but limited, resources (i.e. carbon and nitrogen) to defense when it is most necessary (reviewed by Karban and Myers, 1989; Sagers and Coley, 1995; Hunter and Shultz, 1995; Karban and Baldwin, 1997).

The Carbon/Nutrient Balance (CNB) hypothesis (Bryant et al., 1983) provides a plausible strategy by which plants might allocate limited resources to the production of chemical defenses. The CNB hypothesis proposes that a plant’s investment in defense depends primarily on resource availability in the local environment; essentially, the influence of available resources on plant carbon:nitrogen (C:N) ratio is predicted to dictate the levels of carbon- and nitrogen-based defensive compounds produced. Experimental evidence supporting the predictions of the CNB hypothesis include increased production of tannins and lignin in *Salix aquatica* (Waring et al., 1985), mono- and sesquiterpenes in *Heterothera subaxillaris* (Mihaliak and Lincoln, 1989), papyrific acid and other phenolics in *Betula papyrifera* (Bryant et al., 1987), and furanocoumarins in *Pastinaca sativa* (Zangerl and Berenbaum, 1987) when these species were grown in either a low nitrogen or high light environment. However, nearly as many studies of carbon-based compounds reveal patterns that are inconsistent with the CNB hypothesis. For instance, shading affected neither monoterpenoids in *Satureja douglasii* (Lincoln and Langenheim, 1978), nor the majority of defensive metabolites tested in *Populus balsamifera* (Reichardt et al.,

1991). Moreover, Bryant et al. (1987) observed no effect of nutrient levels on pinosylvin and pinosylvin methyl ester in *Alnus crispa*. The relatively few studies of nitrogen-containing defenses provide even less support. Shading and photosynthetic inhibition did not increase nitrogen-containing norditerpenoid alkaloids in *Delphinium barbeyi* (Ralphs et al., 1998), nor did nitrogen availability affect cyanogenic glycosides in *Lotus corniculatum* (Briggs, 1990). Clearly, the ability of the CNB hypothesis to predict phenotypic changes in carbon-based and, particularly, nitrogen-containing defenses is presently unclear.

It now appears certain that plants will be subjected to increased atmospheric CO<sub>2</sub> throughout at least the next century. Given the nearly universal observation that plant C:N ratios increase under elevated CO<sub>2</sub> (e.g. Bazzaz, 1990; Lindroth et al., 1993; Lawler et al., 1997; Karowe et al., 1997), one consequence of increasing atmospheric CO<sub>2</sub> may be increases in carbon-based chemical defenses; this is a direct prediction of the CNB hypothesis. Supportive evidence for this prediction and for the CNB hypothesis includes a 160% increase in carbon-based cardenolides when *Digitalis lantana* was grown under 1000 ppm CO<sub>2</sub> (Stuhlfauth et al., 1987), and 15% and 28% increases in condensed tannin concentrations in gray birch and in yellow birch, respectively, grown under 700 ppm CO<sub>2</sub> (Traw et al., 1996). In contrast, no change was observed among various carbon-based defenses in sagebrush at 650 ppm CO<sub>2</sub> (Johnson and Lincoln, 1991), in *Plantago lanceolata* at 700 ppm CO<sub>2</sub> (Fajer et al., 1992), or in celery at 718 ppm CO<sub>2</sub> (Reitz et al., 1997), suggesting that the

responses of plant chemical defenses to elevated CO<sub>2</sub> may be chemical- and/or species-specific.

Little research has investigated the effects of elevated CO<sub>2</sub> on nitrogen-containing defenses. With increased plant C:N ratios under elevated CO<sub>2</sub>, the CNB hypothesis also predicts that plants will produce lower levels of chemical defenses that contain nitrogen. One of the few studies that has investigated elevated CO<sub>2</sub>-induced changes in nitrogen-containing defenses found that glucosinolates decreased in wild mustard, but not in radish or turnip at 720 ppm CO<sub>2</sub> (Karowe et al., 1997). In addition, Rufty et al. (1989) reported decreased levels of nitrogen-containing pyridine alkaloids, along with increased levels of carbon-based polyphenols and cuticular diterpenoids, in tobacco grown under elevated CO<sub>2</sub>; this is the only previous study of which we are aware that has investigated the effects of elevated CO<sub>2</sub> on both nitrogen- and carbon-based chemical defenses within the same plant. Therefore, from these limited results it is not yet clear whether nitrogen-containing defenses respond in a predictable manner to elevated CO<sub>2</sub>.

The extent of induction for many chemical defenses can also be influenced by resource availability and C:N ratios (Bryant et al., 1993; Hunter and Shultz, 1995). Therefore, we expect carbon-based compounds to be more readily inducible as a result of the increased carbon availability from higher C:N ratios. In contrast, nitrogen-containing defenses are expected to be less inducible because of the nitrogen limitation in these same plants. To our knowledge, previous studies have only investigated the effects of elevated CO<sub>2</sub> on the induction of carbon-based chemical

defenses. Roth et al. (1998) and Lindroth and Kinney (1998) both found no interaction between CO<sub>2</sub> and defoliation on polyphenolics in aspen and sugar maple, thus lending no support to the prediction that carbon-based defenses will be more inducible under elevated CO<sub>2</sub>. However, the effects of elevated CO<sub>2</sub> on chemical defense induction in herbaceous plants and/or on other carbon-based or nitrogen-containing defenses are not known.

Legumes (Leguminosae) represent one class of plants that may escape many effects of elevated CO<sub>2</sub> due to their mutualistic relationship with nitrogen fixing microbial symbionts. This mutualistic association not only provides legumes with a flexible nitrogen source, but also provides a carbon sink for excess photosynthates; thus, nitrogen fixation may buffer legumes against (i.e. prevent or minimize) increases in C:N ratio under elevated CO<sub>2</sub>. Indeed, legume species grown under elevated CO<sub>2</sub> generally display increased total plant nitrogen fixation (Finn and Brun, 1982; Norby, 1987; Ryle et al., 1992; Tissue et al., 1997) and nitrogenase activity (Tissue et al., 1997). Presumably as a consequence, several legume species display no change or only slight significant decreases in foliar nitrogen content under elevated CO<sub>2</sub> (Ryle et al., 1992; Ayers et al., 1993; Tissue et al., 1997; Karowe and Migliaccio, in preparation).

Soybean (*Glycine max* L.) is an agricultural legume crop that produces a suite of chemical defenses including both carbon-based and nitrogen-containing compounds. A range of carbon-based phenolics, such as tannins and phytoalexins, occur in soybean and have been shown to be effective feeding deterrents (Chiang and

Norris, 1983; Fischer et al., 1990). In addition, soybean contains nitrogen-rich serine proteinase inhibitors, including chymotrypsin and trypsin inhibitors, which can retard insect growth (Steffens et al., 1978; Broadway and Duffey, 1986, Oppert et al., 1993). Moreover, both phenolics (Chaing and Norris, 1983) and proteinase inhibitors (i.e. trypsin inhibitors; Bi et al., 1994) are induced in soybean in response to insect attack. Although the toxicity and biochemical synthesis of soybean defenses are well characterized, the effects of elevated CO<sub>2</sub> and nitrogen fixation on their production have not been investigated. It is plausible that the association with nitrogen-fixing bacteria may buffer this legume against significant changes in C:N ratio in a CO<sub>2</sub> enriched environment, resulting in little or no change in either phenolics or proteinase inhibitor concentrations.

In general, plants are often thought to be nitrogen limited, especially those plants that are unable to fix nitrogen and/or that exist in a high light or low nutrient environment (review by Mattson, 1980). Therefore, metabolic functions requiring nitrogen in plants with low nitrogen or high C:N ratios may compete for this limited resource. Such competition for nitrogen would be manifested as tradeoffs between nitrogen-demanding functions, such as the production of nitrogen-rich defenses including chymotrypsin and trypsin inhibitors. Moreover, it is plausible that increased investment in constitutive levels of proteinase inhibitors may lead to a decrease in nitrogen available for the induction of these defenses. With the possibility that nitrogen may decrease (or C:N increase) under elevated CO<sub>2</sub> or in plants that are incapable of nitrogen fixation, within-plant competition for nitrogen

may increase, therefore leading to more readily visible tradeoffs among constitutive and/or induced levels of these nitrogen-rich defenses.

In the present study, we investigate the role of nitrogen fixation in maintaining the carbon/nutrient balance and chemical defenses of soybean under elevated CO<sub>2</sub>. In addition, we attempt to reveal whether plant tradeoffs exist for nitrogen-containing defenses under situations where nitrogen may be limited. A particularly powerful approach to evaluate the role of nitrogen fixation under elevated CO<sub>2</sub>, and to detect tradeoffs in nitrogen-requiring functions, is to compare two soybean strains that differ only in the ability to harbor the nitrogen fixing bacterium specific to soybean, *Bradyrhizobium japonicum*. The *rj1* non-nodulating mutant and wild-type strains of the soybean variety, Harosoy, are believed to have identical genotypes with the exception of the mutation responsible for ineffective nodulation (James Harper, personal communication). Therefore, a comparison of *rj1* and wild-type strains should reveal the ability of nitrogen fixation to buffer this legume's C:N ratio and, hence, allocation of resources to chemical defense, against the effects of elevated CO<sub>2</sub>. Specifically in this study, we ask:

1. Does association with nitrogen fixing *Bradyrhizobium japonicum* buffer soybean against (i.e. prevent or minimize) altered carbon and nitrogen contents and/or C:N ratio under elevated CO<sub>2</sub>?
2. If so, does association with *B. japonicum* also buffer constitutive concentrations of carbon-based (phenolics) and nitrogen-containing (proteinase inhibitors) chemical defenses against the effects of elevated CO<sub>2</sub>?

3. Does association with *B. japonicum* buffer induction of phenolics and proteinase inhibitors against the effects of elevated CO<sub>2</sub>?

4. Do tradeoffs exist between constitutive and/or induced levels of nitrogen-containing proteinase inhibitors in soybean, and, if so, are tradeoffs more severe under elevated CO<sub>2</sub> and/or in the absence of *B. japonicum*?

## Materials and Methods

### Growth of Plant Material

All plants were grown during the summer of 1998 at the University of Michigan Biological Station (UMBS) in Pellston, Michigan. Seeds of the wild-type (nodulating) soybean variety, Harosoy, and its *rj1* mutant (non-nodulating) isoline (graciously provided by Dr. James Harper, USDA, University of Illinois, Urbana-Champaign) were germinated in six inch pots using a 1:3 mixture of vermiculite and Hyponex top-soil in the greenhouse. Inoculation of both nodulating and non-nodulating plants was performed when the seeds were sown using a 3X application of peat-based inoculum containing *Bradyrhizobium japonicum* (Urbana Laboratories, St. Josephs, MO).

At seven days post germination, both wild-type and mutant strains were placed randomly in 16 open-topped chambers identical to those described by Karowe et al. (1997), half of which were maintained at  $756 \pm 10$  ppm CO<sub>2</sub> (elevated treatment) and the other half at  $367 \pm 10$  ppm CO<sub>2</sub> (ambient treatment). Chambers were monitored every 20 minutes by an infrared gas analyzer (IRGA) and CO<sub>2</sub>

concentrations were recorded to a computer. Plants were watered and checked for insects daily. At two and four weeks post germination, both nodulated and non-nodulated plants received 300 ml of full strength, low-nitrogen fertilizer (10-60-60; Shultz Rapid Bloom Fertilizer supplemented with Espoma<sup>®</sup> Potash). At the conclusion of plant harvesting, the roots of 10 randomly selected plants from both wild-type and mutant strains were extracted and checked for the presence of nodules. All wild-type plants examined displayed an abundance of roots nodules ranging from white to dark red in color, while nodules were completely absent from the roots of non-nodulating plants.

From each of eight treatment groups, 26 plants were harvested when they had developed 7 to 8 fully expanded leaves, approximately six weeks post-germination (i.e. vegetative stage V7 – V8; Fehr et al., 1971). The eight treatment groups consisted of wild-type and non-nodulating plants, each grown at ambient and elevated CO<sub>2</sub> and sampled at 0 hour and 48 hours after mechanical damage. Leaf carbon and nitrogen contents, C:N ratios, total phenolics, and chymotrypsin and trypsin inhibitors levels were determined for each plant (see below).

#### Leaf Nitrogen and Carbon Contents and C:N Ratio

Leaf percent carbon and nitrogen and C:N ratio were measured on one lateral leaflet from the third fully expanded leaf (Figure 1). Individual leaflets (Figure 1) were placed in a glassine envelope, flash-frozen in liquid nitrogen, and stored at -

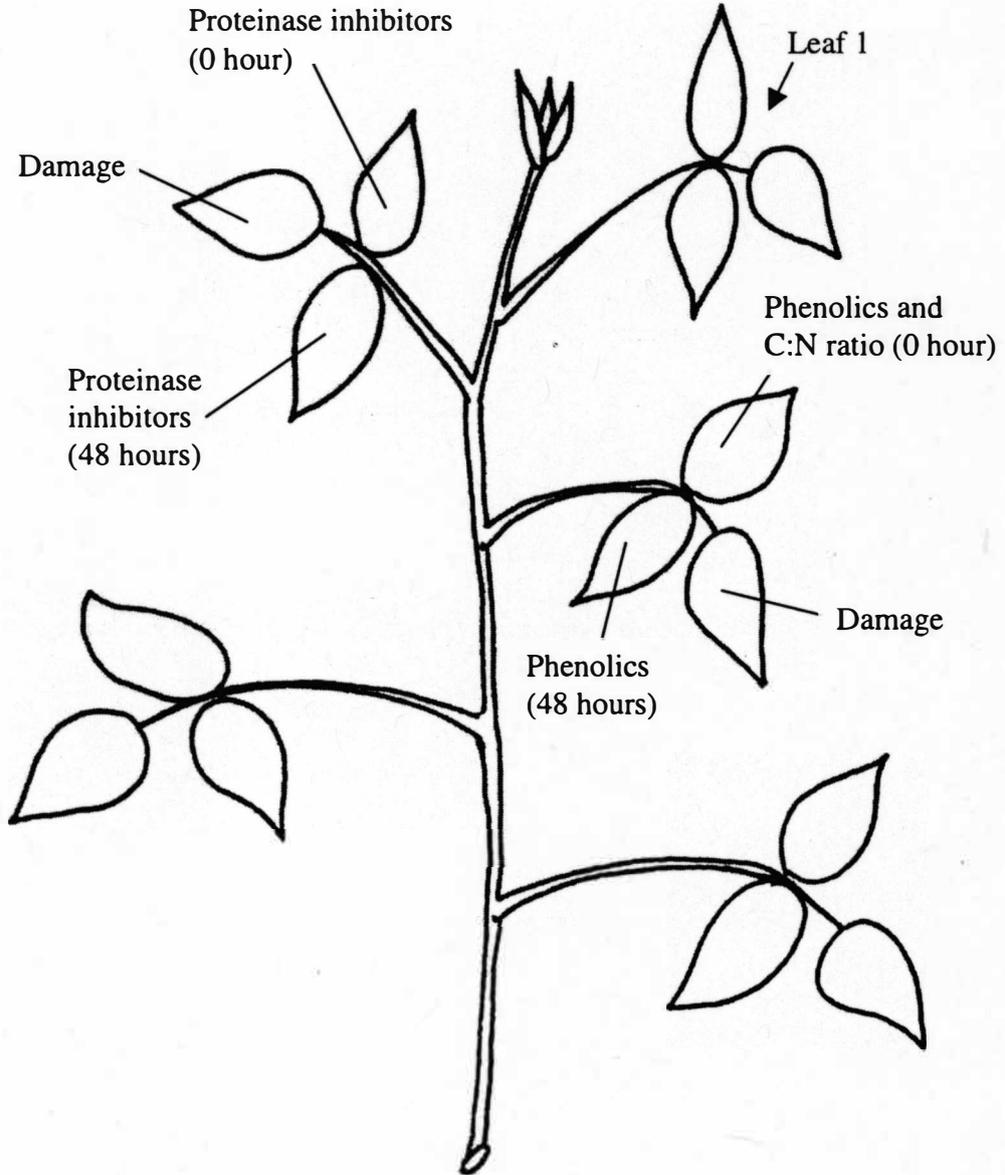


Figure 1. Plant Harvest Regime for Chemical Analyses.

80°C. Samples were freeze dried, ground to a fine powder using a Wiley Mill, and analyzed for carbon and nitrogen content and total phenolic concentrations (see below). For each sample, carbon, hydrogen, and nitrogen contents were determined using a Perkin Elmer CHN Elemental Analyzer at UMBS, and nitrogen and carbon contents were expressed as percentages of dry weight.

### Leaf Total Phenolic Content

Total leaf phenolic content was determined using a modification of methods described in Appel and Shultz (1994) and Appel and Maines (1995). Twenty milligrams of dried leaf tissue was extracted and analyzed for total phenolic content. Extraction of leaf tissue was preceded by three washes using 1 ml ethyl ether. After each wash, samples were pelleted by centrifugation at 4,600g for 5 minutes and the ether was removed. Phenolic extraction was performed by suspending the resulting pellet in 0.4 ml 90:10% methanol:water and sonicating on ice for 30 minutes. Samples were centrifuged again at 4,600g for 5 minutes and the supernatant was collected in a fresh tube. Phenolic extractions were repeated twice more using 0.4 ml 90% methanol until a final volume of 1.2 ml was collected. The final extract was dried using a rotary evaporator and stored at -80°C until samples were analyzed for total phenolics.

Total phenolic content for each leaf sample was determined using the Folin-Denis assay (Swain and Goldstein, 1964). Dried leaf extracts were resuspended in 1 ml deionized H<sub>2</sub>O and diluted 1:20 in 1 ml deionized H<sub>2</sub>O for each reaction. To

each reaction tube, 1 ml Folin-Denis reagent (75 mM sodium tungstate, 1.3 mM phosphomolybdic acid, 2.8% phosphoric acid) was added for three minutes, followed by 1 ml 2N sodium carbonate for a final volume of 3 ml. Samples were incubated for 1 hr at room temperature for color development. Sample absorbency was determined at 725 nm against a blank of deionized water and total phenolics were expressed as  $\mu\text{g}/\text{mg}$  dry leaf material. All samples were run in triplicate.

#### Purification of Phenolic Standard

A total of 16 g dried leaf material (2 g from each treatment group) was extracted, purified, and pooled for use as a semi-pure native standard to determine absolute levels of total phenolics for each treatment group. Extraction of bulk leaf material was performed by washing 2 g (x8) ground leaf powder with 10 ml ethyl ether three times. After each wash, bulk samples were centrifuged at 1,000g for 3 minutes and the ether was discarded. The final pellet was extracted with 40 ml 90% methanol for 30 minutes while sonicating on ice. Samples were centrifuged for 5 minutes at 3,000g and the supernatant was collected in a fresh tube. Phenolic extraction was performed three more times using 20 ml 90% methanol until a total of 100 ml was collected. The bulk extract was taken to dryness using a rotary evaporator and resuspended in 50 ml deionized H<sub>2</sub>O. Purification of the aqueous solution was performed by mixing 25 ml bulk extract with 25 ml ethyl ether for 15 minutes in open topped flasks to remove chlorophyll and lipid contaminants. After each ether purification, bulk mixtures were centrifuged at 500g for 3 minutes and the

aqueous phase was retained. Ether purifications were performed at least 5 more times until all chlorophyll was removed. The final purified extract was taken to complete dryness in a rotary evaporator and the resulting crystallized powder was analyzed for purity and used as a semi-pure native standard for total phenolic analysis.

To determine standard purity, four replicates of 8 mg of crystallized powder was chromatographed on pre-coated silica gel plates (Brinkman) using ethyl acetate:acetone:methanol (50:15:12.5, v/v) with 1% tetra-hydrofluorine. The standard was separated into 7 bands with average Rf values of 0 (band 1, composed of two distinct bands at the origin), 0.10 (band 2), 0.32 (band 3), 0.49 (band 4), 0.56 (band 5), 0.67 (band 6), and 0.91 (band 7). Bands were detected under short-wave UV light, scraped from the plate, and eluted using 4 ml 95% ethanol. The elutants were taken to dryness using a rotary evaporator and a final weight for each band was determined. Mass balance yielded 99% average recovery of the original standard mass. Bands 1 and 2 each represented 25% of the original mass eluted from the silica plate, while band 3 represented 16%, band 4 represented 15%, and bands 5, 6, and 7 represented 4%, 8%, and 7%, respectively. Each band was then dissolved in methanol and analyzed for UV/visible spectrum maxima and phenolic activity using the Folin-Denis assay.

To determine spectrum maxima, each band was analyzed under wavelengths ranging from 200-500 nm. Bands 3, 4, 5, and 6 all yielded spectrum maxima that were analogous with simple phenolics (265-280 nm) and with a variety of phenolic compounds described by Waterman and Mole (1994). Bands 3 and 4 had strong

spectrum maxima at 342 nm and 265 nm, while bands 5 and 6 yielded weaker maxima at 342 nm and 265 nm. Bands 1, 2, and 7 displayed no maxima within the spectrum range indicative of phenolic compounds. The addition of two drops 1N NaOH to each band caused a bathochromic shift of approximately 10 nm for the 265 nm maximum displayed by bands 3-6, 40 nm shift for the 342 nm maximum displayed by bands 3 and 4, and 55 nm for the 325 nm maximum for bands 5 and 6, thus providing additional evidence that the compounds comprising each of these four bands are phenolics. No shifts in maxima were observed for bands 1, 2, and 7.

In general, Folin-Denis assays supported spectrum analyses. Folin-Denis activity was highest for bands 3 and 4 (2.3 and 3.2 Abs/mg, respectively) and intermediate for bands 5 and 6 (1.3 and 1.0 Abs/mg, respectively). Consistent with spectrum analysis, band 7 displayed minimal phenolic activity (0.30 Abs/mg). However, bands 1 and 2 yielded moderate activity (0.8 Abs/mg for bands 1 and 0.6 Abs/mg for band 2). Because spectrum analysis of bands 1 and 2 did not reveal maxima, or shifts in maxima, consistent with phenolic compounds, we believe that these bands are most likely non-phenolic compounds capable of producing false positives by Folin-Denis. We therefore conclude that only bands 3, 4, 5, and 6 are true phenolics; together, these bands comprise 78.6% of the total phenolic activity in our semi-pure standard. Therefore, we reduced by 21.4% our estimates of absolute levels of total phenolics in leaf samples (determined by Folin-Denis only) to correct for false positives.

### Leaf Proteinase Inhibitor Contents

Chymotrypsin and trypsin inhibitor levels were determined using methods based on those of Broadway and Duffey (1986) and Broadway (1989). Leaf tissue from one lateral leaflet from the second fully expanded leaf (Figure 1) was harvested without the midrib, weighed, and homogenized on ice in 3x volume of 1 mM HCl. The homogenate was centrifuged at 14,000g for 5 minutes at 4°C. After centrifugation, the supernatant was transferred to a fresh tube, spun again at 14,000g for 5 minutes at 4°C, and stored on ice until proteinase inhibitor assays were performed.

Chymotrypsin inhibitor activity was determined by mixing leaf extract 1:1 with TLCK-treated bovine chymotrypsin (0.1 mg/ml 1 mM HCl) for 10 minutes at room temperature. After incubation, 100 µl of the sample mixture was added to 2.9 ml substrate buffer (1 mM benzoyl-L-tyrosine ethyl ester in 50% MeOH, mixed 1:1 with 0.05 Tris, pH 8.0), and chymotrypsin activity was monitored at 256 nm every 30 seconds for 3 minutes to generate a reaction curve. The resulting slope of each sample curve was compared to uninhibited chymotrypsin mixed 1:1 with 1 mM HCl, and percent chymotrypsin inhibition was calculated. Each sample was repeated in triplicate.

Trypsin inhibitor activity was quantified by mixing leaf extract 1:1 with TPCK treated bovine trypsin (0.1 mg/ml 1 mM HCl) and incubating at room temperature for 10 minutes. A 100 µl aliquot of the sample mixture was then transferred to 2.9 ml substrate buffer (0.05 M Tris, pH 8.0) containing 1.04 M p-

tolulene-sulfonyl-L-arginine methyl ester (TAME). Trypsin activity was monitored at 247 nm every 30 seconds for 3 minutes. The slope of the linear reaction curve was determined and compared to uninhibited trypsin mixed 1:1 with HCl. Each sample extract was assayed in triplicate.

### Induction of Plant Chemical Defenses

To determine whether induction occurred, concentrations of chemical defenses were compared within individual leaves before and after mechanical damage. Prior to mechanical damage, one opposite leaflet from the second fully expanded leaf was harvested for trypsin and chymotrypsin inhibitor contents, and one opposite leaflet from the third fully expanded leaf was analyzed for total phenolics (Figure 1). Undamaged leaflets sampled just prior to mechanical damage are hereafter referred to as “pre-damage leaflets”. Damage was then performed by placing 25  $\mu$ l of 40  $\mu$ M jasmonic acid (Sigma) on terminal leaflets of the second, third and fourth fully expanded leaves, followed by crushing with a garlic press. The chemical elicitor jasmonic acid has been shown to induce plant chemical defenses when exogenously applied to leaves (Thaler et al., 1996), and therefore was used along with mechanical wounding to mimic insect herbivory. To standardize damage, one press on each side of the midrib in the mid-section of each leaflet was made. In addition, every effort was made to ensure jasmonic acid was evenly distributed where mechanical damage was performed.

Approximately 48 hours post-damage, the opposite leaflet for both leaves 2 and 3 was harvested for induced proteinase inhibitor and phenolic analysis, respectively. Undamaged leaflets sampled at 48 hours after mechanical damage are hereafter referred to as “post-damage leaflets”. The extent of induction was determined separately for each plant by calculating the absolute change in proteinase inhibitor and phenolic concentrations between pre-damage and post-damage leaflets.

It was not possible to obtain both 0 and 48 hour samples from the same leaflet due to minimal leaf material; therefore, we measured constitutive and induced levels in opposite leaflets. To evaluate the implicit assumption that opposite leaflets initially contained comparable constitutive levels of chemical defenses, we harvested a subsample of 15 wild-type plants and compared levels of both phenolics and proteinase inhibitors among opposite leaflets at 0 hour. Linear regression analysis of all chemical defenses for opposite leaflets showed a strong positive correlation, supporting this assumption. Analyses of chymotrypsin and trypsin inhibitors in opposite leaflets of leaf 2 were significantly positively correlated ( $r^2 = 0.50$ ,  $df = 14$ ,  $p = 0.003$ ;  $r^2 = 0.54$ ,  $df = 14$ ,  $p = 0.002$ , respectively; data not shown), as were total phenolic levels in opposite leaflets of leaf 3 ( $r^2 = 0.77$ ,  $df = 13$ ,  $p = <0.0001$ ; data not shown).

#### Determination of Plant Tradeoffs in Chemical Defense

In the present study, regression analysis was used to test for tradeoffs between constitutive and induced levels of both chymotrypsin and trypsin inhibitors at each

combination of soybean strain and CO<sub>2</sub> level. For correlations involving induced levels of chymotrypsin and/or trypsin inhibitors, we calculated the absolute change of each proteinase inhibitor for each plant by subtracting levels within pre-damage leaflets from levels within post-damage leaflets. We interpreted a negative correlation as evidence for a trade-off.

### Statistical Analyses

To determine whether nitrogen fixation affected the response of soybean leaf carbon, nitrogen, and C:N ratio, and/or constitutive levels of chemical defenses within pre-damaged leaflets to elevated CO<sub>2</sub>, two-way analyses of variance (ANOVAs) were performed with CO<sub>2</sub> level and nodulation status as main effects. Of particular interest is the interaction between elevated CO<sub>2</sub> and nitrogen fixation, which indicates whether nitrogen fixation significantly mitigates the effects of elevated CO<sub>2</sub> on soybean nutritional quality and secondary chemistry. Because constitutive trypsin inhibitor levels were not normally distributed, Kruskal-Wallis analyses were performed separately for wild-type and non-nodulated plants and for ambient and elevated CO<sub>2</sub> treatments.

To determine if induction occurred, a paired t-test was used to compare levels of each chemical defense between pre-damage and post-damage leaflets. This analysis was performed separately for each treatment group. In addition, regression analysis was used to determine correlations between leaf carbon, nitrogen, and C:N ratio content, and levels of each chemical defense.

## Results

### Effects of Elevated CO<sub>2</sub> and Nodulation on Leaf Nitrogen and Carbon Contents and C:N Ratio

Leaf nitrogen content differed significantly between soybean strains and to a lesser degree between CO<sub>2</sub> levels (Tables 1 and 2). Overall, nitrogen content was much higher in wild-type plants versus non-nodulated plants at both CO<sub>2</sub> levels (66% higher at ambient CO<sub>2</sub> and 60% higher at elevated CO<sub>2</sub>; Table 1). In addition, growth under elevated CO<sub>2</sub> resulted in a 31% decrease in leaf nitrogen content among wild-type plants and a 20% decrease among non-nodulated plants. Leaf nitrogen content also exhibited a significant CO<sub>2</sub> x nodulation interaction, primarily because the absolute decrease between CO<sub>2</sub> treatments for wild-type plants was much greater than for non-nodulated plants. This significant interaction, however, most likely resulted from the already existing low levels of nitrogen in non-nodulated plants at ambient CO<sub>2</sub>, thus making a decrease in nitrogen comparable to that seen among wild-type plants virtually impossible.

Leaf carbon content also varied significantly between soybean strains, but not between CO<sub>2</sub> levels (Tables 1 and 2). Surprisingly, carbon content was higher in wild-type plants compared to plants that were incapable of harboring nitrogen fixing symbionts. In addition, a significant CO<sub>2</sub> x nodulation interaction was observed, apparently because under elevated CO<sub>2</sub> wild-type plants showed a 1% reduction in carbon content, while non-nodulated plants showed a 1% increase.

Table 1

Leaf Nitrogen and Carbon Contents, C:N Ratios, and Levels of Chemical Defenses for Soybean Grown at Ambient and Elevated CO<sub>2</sub>

Measure of Plant Quality	Wild-type/ Ambient CO <sub>2</sub>	Wild-type/ Elevated CO <sub>2</sub>	Non-Nodulated/ Ambient CO <sub>2</sub>	Non-Nodulated/ Elevated CO <sub>2</sub>
Leaf Nitrogen <sup>a</sup> (%)	2.9 ± 0.4	2.0 ± 0.5	1.0 ± 0.2	0.8 ± 0.1
Leaf Carbon <sup>a</sup> (%)	44.0 ± 0.7	43.5 ± 0.6	40.3 ± 0.7	40.6 ± 0.7
Leaf C:N Ratio <sup>a</sup>	15.3 ± 2.0	22.4 ± 5.0	39.5 ± 6.3	51.7 ± 7.1
Constitutive Phenolics (µg/mg <sup>b</sup> )	85.5 ± 11.7	91.3 ± 11.0	115.9 ± 13.4	122.7 ± 18.8
Δ <sup>c</sup> Total Phenolics	9.4 ± 10.6*	<0.0 ± 12.2	9.6 ± 19.9*	4.9 ± 19.1
Constitutive CI (%)	56.3 ± 8.6	50.9 ± 10.0	16.8 ± 13.7	7.7 ± 9.8
Δ <sup>c</sup> CI (%)	0.5 ± 8.8	3.9 ± 16.8	0.8 ± 11.9	2.5 ± 10.6
Constitutive TI (%)	12.9 ± 7.2	6.6 ± 4.9	0.8 ± 1.8	0.3 ± 1.4
Δ <sup>c</sup> TI (%)	4.8 ± 10.0*	3.4 ± 5.7*	1.2 ± 4.7	<0.0 ± 2.0

\*Indicates 48 hour values are significantly higher than 0 hour values by a paired t-test; <sup>a</sup>Values for leaf nitrogen and carbon contents, and C:N ratios obtained from pre-damage leaflets; <sup>b</sup>Total phenolics expressed as µg phenolic per mg dry leaf weight; <sup>c</sup>Δ represents absolute change in chemical defense during 48 hours; calculated by subtracting constitutive levels of chemical defenses in pre-damage leaflets from induced levels in post-damage leaflets for each plant.

Table 2

Two-way Analysis of Variance<sup>a</sup> for Total Leaf Nitrogen and Carbon Contents and C:N Ratios of Soybean

Source of Variation	SS	df	MS	F	p
<b>Leaf Nitrogen Content</b>					
Main effects					
CO <sub>2</sub> Level	8.2	1	8.2	68.3	<0.0001
Nodulation	62.6	1	62.6	520.0	<0.0001
Interaction					
CO <sub>2</sub> x Nodulation	2.6	1	2.6	21.4	<0.0001
Error	11.8	98	0.1		
Total	85.0	101			
<b>Leaf Carbon Content</b>					
Main effects					
CO <sub>2</sub> Level	0.3	1	0.3	0.7	n.s.
Nodulation	274.1	1	274.1	587.3	<0.0001
Interaction					
CO <sub>2</sub> x Nodulation	3.6	1	3.6	7.7	0.0068
Error	45.7	98	0.5		
Total	323.7	101			
<b>Leaf C:N Ratio</b>					
Main effects					
CO <sub>2</sub> Level	2382.6	1	2382.6	80.1	<0.0001
Nodulation	18231.8	1	18231.8	613.2	<0.0001
Interaction					
CO <sub>2</sub> x Nodulation	164.2	1	164.2	5.5	0.0208
Error	2913.8	98	29.7		
Total	23717.9	101			

<sup>a</sup>Two-way analysis of variance for leaf nitrogen and carbon contents, and C:N ratios obtained from pre-damage leaflets. Corresponding means are reported in Table 1.

These changes in carbon and nitrogen contents resulted in significant differences in leaf C:N ratios (Tables 1 and 2). As expected, C:N ratio was lowest for

wild-type plants grown under ambient CO<sub>2</sub> and highest for non-nodulated plants grown under elevated CO<sub>2</sub>. Changes in C:N ratio were driven by changes in percent nitrogen (i.e. plants with higher nitrogen contents had lower C:N ratios), and occurred despite higher carbon content among wild-type plants. In addition, a significant CO<sub>2</sub> x nodulation interaction existed; the absolute increase in C:N ratio under elevated CO<sub>2</sub> was much greater for non-nodulated plants than for wild-type plants.

### Effects of Elevated CO<sub>2</sub> and Nodulation on Constitutive Levels of Total Phenolics and Proteinase Inhibitors

#### Total Phenolics

Constitutive levels of total phenolics within pre-damage leaflets differed among treatment groups (Tables 1 and 3); both CO<sub>2</sub> and nodulation influenced the production of these carbon-based defenses. Relative to wild-type plants, non-nodulated plants contained 26% more phenolics. In addition, growth under elevated CO<sub>2</sub> resulted in similar proportional increases in phenolic content of wild-type and non-nodulated plants (6.4% and 5.5%, respectively); hence, there was no significant CO<sub>2</sub> x nodulation interaction.

#### Proteinase Inhibitors

Both chymotrypsin and trypsin inhibitor levels also differed significantly among treatment groups (Tables 1 and 3). Chymotrypsin inhibitor content was much lower in non-nodulated plants. To a lesser extent, chymotrypsin inhibitor levels were

Table 3

## Two-way Analysis of Variance for Constitutive Levels of Total Phenolics and Chymotrypsin Inhibitors in Soybean

Source of Variation	SS	df	MS	F	p
<b>Total Phenolic Content</b>					
Main effects					
CO <sub>2</sub> Level	1037.4	1	1037.4	5.3	0.0241
Nodulation	24859.7	1	24859.7	125.8	<0.0001
Interaction					
CO <sub>2</sub> x Nodulation	6.3	1	6.3	0.03	n.s.
Error	19767.6	100	197.7		
Total	45671.0	103			
<b>Chymotrypsin Inhibitors</b>					
Main effects					
CO <sub>2</sub> Level	1132.2	1	1132.2	8.4	0.0047
Nodulation	43705.5	1	43705.5	322.7	<0.0001
Interaction					
CO <sub>2</sub> x Nodulation	158.7	1	158.7	1.2	n.s.
Error	13004.0	96	135.5		
Total	58135.3	99			

also lower in plants grown under elevated CO<sub>2</sub> (Table 3). No significant CO<sub>2</sub> x nodulation interaction was detected, despite a 54.2% proportional decrease in chymotrypsin inhibitors for non-nodulated plants at elevated CO<sub>2</sub> compared to a 9.6% decrease for wild-type plants.

Due to non-normality, constitutive trypsin inhibitor content was analyzed by Kruskal-Wallis tests. When plants grown at ambient and elevated CO<sub>2</sub> were considered together, trypsin inhibitors were significantly lower among non-nodulated plants (KW = 52.45, n = 86, p < 0.001). In addition, trypsin inhibitors were also

nearly significantly lower at elevated CO<sub>2</sub> (KW = 3.52, n = 86, p = 0.061). The effect of CO<sub>2</sub> appeared to depend on whether plants were associated with nitrogen fixing symbionts; among wild-type plants trypsin inhibitors decreased significantly under elevated CO<sub>2</sub> (KW = 8.48, n = 44, p = 0.004), but not among non-nodulated plants (KW = 0.83, n = 42, p = 0.36). However, the ability to detect significant decreases in trypsin inhibitors among non-nodulated plants under elevated CO<sub>2</sub> was most likely constrained due to the already existing low levels of trypsin inhibitors under ambient conditions.

#### Effects of Elevated CO<sub>2</sub> and Nodulation on the Induction of Phenolics and Proteinase Inhibitors

Induction of total leaf phenolics occurred only under ambient CO<sub>2</sub>, and did so for both wild-type and non-nodulated plants. Among plants grown at ambient CO<sub>2</sub>, total phenolic levels within post-damage leaflets were on average 9.9% higher for wild-type plants (paired t = 4.52, df = 25, p = <0.001) and 7.6% higher for non-nodulated plants (paired t = 2.45, df = 25, p = 0.022). No induction of phenolics occurred for either soybean strain under elevated CO<sub>2</sub>.

Induction of chymotrypsin inhibitors did not occur for either soybean strain at ambient or elevated CO<sub>2</sub>. However, trypsin inhibitors were induced in wild-type plants, and did so at both ambient (paired t = 2.25, df = 21, p = 0.035) and elevated (paired t = 2.79, df = 21, p = 0.011) CO<sub>2</sub>. Induction of trypsin inhibitors did not occur in non-nodulated plants at either CO<sub>2</sub> level.

### Are Levels of Chemical Defenses Consistent With the Carbon/Nutrient Balance Hypothesis?

Overall, constitutive levels of total phenolics and chymotrypsin and trypsin inhibitors were strongly correlated with leaf C:N ratios and carbon and nitrogen contents (Table 4). The phenolic content of leaves was positively correlated with C:N ratio, indicating that those plants having higher carbon content relative to nitrogen (i.e. non-nodulated plants grown under elevated CO<sub>2</sub>) produce greater amounts of constitutive phenolics. Conversely, constitutive levels of total phenolics were negatively correlated with both leaf carbon and nitrogen contents, largely because those plants that contained the highest levels of carbon also contained the highest levels of nitrogen (i.e. wild-type plants grown under ambient CO<sub>2</sub>).

Levels of leaf chymotrypsin and trypsin inhibitors were negatively correlated with leaf C:N ratio, and positively correlated with leaf carbon and nitrogen contents. Unlike phenolics, nitrogen-rich proteinase inhibitors negatively correlated with plant C:N ratios, thus being more concentrated in leaves that contained less carbon relative to nitrogen. In addition, levels of both chymotrypsin and trypsin inhibitors occurred at higher levels in leaves that contained more carbon and, probably more causally, in leaves that had more nitrogen.

No significant correlation was detected between leaf C:N ratios and/or carbon or nitrogen levels, and the absolute change of phenolics or chymotrypsin inhibitors between pre-damage and post-damage leaflets. However, the absolute change of trypsin inhibitors after plant damage negatively correlated with leaf C:N ratios and

Table 4

Correlations Between Soybean Leaf Nitrogen and Carbon Contents and C:N Ratios, and Constitutive and Post-damage Levels of Chemical Defenses

Plant Chemical Defense	Leaf C:N Ratio	% Leaf Nitrogen	% Leaf Carbon
Constitutive Phenolics	m = 0.485 R = 0.665 p = <0.001	m = -0.028 R = -0.651 p = <0.001	m = -0.061 R = -0.721 p = <0.001
Constitutive Chymotrypsin Inhibitors	m = -1.35 R = -0.879 p = <0.001	m = 22.05 R = 0.856 p = <0.001	m = 10.63 R = 0.813 p = <0.001
Constitutive Trypsin Inhibitors	m = -0.299 R = -0.652 p = <0.001	m = 5.14 R = 0.703 p = <0.001	m = 2.35 R = 0.596 p = <0.001
$\Delta^a$ Phenolics	m = 0.057 R = 0.042 p = 0.673	m = -0.528 R = -0.023 p = 0.815	m = -0.829 R = -0.072 p = 0.474
$\Delta^a$ Chymotrypsin Inhibitors	m = 0.024 R = 0.030 p = 0.769	m = -0.885 R = -0.065 p = 0.515	m = 0.347 R = 0.051 p = 0.614
$\Delta^a$ Trypsin Inhibitors	m = -0.108 R = -0.249 p = 0.022	m = 1.49 R = 0.214 p = 0.050	m = 0.947 R = 0.253 p = 0.020

<sup>a</sup> $\Delta$  represents absolute change in chemical defense over a 48 hour period. Sample sizes ranged from 84-102 for each analysis.

positively correlated with nitrogen and carbon contents (Table 4). These results indicate that plants having lower C:N ratios, as well as higher levels of nitrogen and carbon, were capable of inducing more trypsin inhibitors when damaged.

#### Tradeoffs Between Constitutive and Induced Proteinase Inhibitor Levels

When all treatment groups were considered together, constitutive levels of chymotrypsin and trypsin inhibitors were significantly positively correlated, indicating that individual plants with high levels of one type of proteinase inhibitor had high levels of the other (Table 5). However, this relationship was not observed within any of the four treatment groups, suggesting that the overall positive correlation was driven by differences in proteinase inhibitor levels between treatment groups (levels of both inhibitors were much higher in both wild-type groups than in both non-nodulated groups). Nonetheless, the absence of a negative correlation within any treatment group suggests that soybean does not encounter a trade-off between investment in constitutive chymotrypsin inhibitor and investment in constitutive trypsin inhibitor, even when nitrogen is extremely limiting (i.e. for non-nodulated plants under elevated CO<sub>2</sub>).

Similarly, we observed no tradeoff between constitutive levels of either proteinase inhibitor and post-damage change in the other. We observed a significant positive correlation between constitutive levels of chymotrypsin inhibitors and post-damage change in trypsin inhibitors (i.e. constitutive CI vs.  $\Delta$ TI, Table 5) when all treatment groups were pooled, but not when treatment groups were analyzed

Table 5

## Correlations Between Constitutive and Induced Levels of Chymotrypsin and Trypsin Inhibitors in Soybean

Variable 1:	Constitutive CI	Constitutive CI	Constitutive TI	Constitutive CI	Constitutive TI	$\Delta^*$ %CI	Constitutive CI	Constitutive TI
Variable 2:	Constitutive TI	$\Delta^*$ TI	$\Delta^*$ %CI	$\Delta^*$ %CI	$\Delta^*$ %TI	$\Delta^*$ %TI	Post-Damage CI	Constitutive TI
All Treatment Groups	m = 0.180 R = 0.607 p = <0.001	m = 0.065 R = 0.224 p = 0.043	m = 0.253 R = 0.131 p = 0.234	m = -0.116 R = 0.267 p = 0.007	m = -0.069 R = 0.072 p = 0.510	m = -0.008 R = -0.015 p = 0.891	m = 0.884 R = 0.903 p = <0.001	m = 0.931 R = 0.698 p = <0.001
Wild-type / Ambient CO <sub>2</sub>	m = -0.351 R = -0.298 p = 0.202	m = -0.227 R = -0.298 p = 0.190	m = 0.379 R = 0.320 p = 0.169	m = -0.644 R = -0.629 p = 0.001	m = -0.619 R = -0.443 p = 0.039	m = 0.060 R = 0.071 p = 0.766	m = 0.356 R = 0.407 p = 0.048	m = 0.381 R = 0.291 p = 0.189
Wild-type / Elevated CO <sub>2</sub>	m = 0.653 R = 0.397 p = 0.075	m = 0.101 R = 0.273 p = 0.245	m = 0.939 R = 0.260 p = 0.243	m = -0.781 R = -0.642 p = 0.001	m = -0.523 R = -0.449 p = 0.036	m = -0.175 R = -0.056 p = 0.803	m = 0.219 R = 0.228 p = 0.273	m = 0.477 R = 0.417 p = 0.054
Non-nodulated / Ambient CO <sub>2</sub>	m = -0.736 R = -0.106 p = 0.656	m = 101 R = 0.273 p = 0.245	m = -0.028 R = -0.004 p = 0.986	m = -0.319 R = -0.462 p = 0.020	m = -1.23 R = -0.482 p = 0.027	m = -0.254 R = -0.092 p = 0.693	m = 0.681 R = 0.744 p = <0.001	m = -0.228 R = -0.102 p = 0.661
Non-nodulated / Elevated CO <sub>2</sub>	m = 2.56 m = 0.364 p = 0.104	m = -0.041 R = -0.211 p = 0.358	m = 0.534 R = 0.066 p = 0.776	m = -0.677 R = -0.628 p = 0.001	m = -1.06 R = -0.782 p = <0.001	m = -0.222 R = -0.037 p = 0.873	m = 0.323 R = 0.360 p = 0.071	m = -0.063 R = -0.074 p = 0.751

\* $\Delta$  represents absolute change in chemical defense over a 48 hour period. Sample sizes ranged from 82-100 for pooled treatment group analyses and from 23-25 for individual treatment group analyses.

separately. No significant correlation was observed between constitutive levels of trypsin inhibitors and post-damage change in chymotrypsin inhibitors, either overall or within any treatment group.

Interestingly, our data suggest a possible tradeoff between constitutive levels and post-damage change of the same proteinase inhibitor. Whether all four treatment groups were considered together or individually, constitutive chymotrypsin levels were negatively correlated with the change in chymotrypsin inhibitors 48 hours after damage (i.e. constitutive CI vs.  $\Delta$ CI, Table 5). Moreover, growth under elevated CO<sub>2</sub> resulted in a much stronger negative correlation for non-nodulated plants ( $p = 0.020$  vs.  $p = 0.001$ ), and a slightly stronger negative correlation for wild-type plants. Similarly, constitutive trypsin inhibitor levels were negatively correlated with the change in trypsin inhibitors 48 hours after damage within each treatment group (though no significant correlation was observed when all treatment groups were pooled, because both constitutive levels and post-damage change were higher in wild-type than in non-nodulated plants). For non-nodulated plants, but not for wild-type plants, this apparent tradeoff was much stronger under elevated CO<sub>2</sub>.

No treatment group, together or separately, exhibited a significant correlation between post-damage changes in trypsin and chymotrypsin inhibitors (Table 5).

## Discussion

In the present study, we found that both CO<sub>2</sub> concentration and the ability to harbor nitrogen fixing *B. japonicum* strongly affected soybean leaf carbon and

nitrogen content, C:N ratio, and constitutive and induced levels of both carbon- and nitrogen- based chemical defenses. Overall, wild-type plants had substantially higher nitrogen and proteinase inhibitor levels, and much lower C:N ratios and total phenolic levels than non-nodulated plants. In addition, when both wild-type and non-nodulated plants were grown under elevated CO<sub>2</sub>, C:N ratios and constitutive amounts of total phenolics increased, and nitrogen and constitutive proteinase inhibitor contents decreased.

The CNB hypothesis predicts that the production of chemical defenses depends on plant C:N ratios (Bryant et al., 1983). Therefore, environmental factors such as low nutrient availability and/or elevated CO<sub>2</sub>, which may decrease leaf nitrogen contents and increase C:N ratios, should affect levels of plant chemical defenses. We interpret the CNB hypothesis to predict an increase in constitutive levels and inducibility of carbon-based chemical defenses and a decrease in constitutive levels and inducibility of nitrogen-based chemical defenses among plants with high C:N ratios, i.e. those exposed to elevated CO<sub>2</sub> and/or lacking nitrogen-fixing mutualists.

Consistent with these predictions, constitutive levels of carbon-based phenolics increased under elevated CO<sub>2</sub> and in non-nodulated plants. In addition, constitutive phenolic levels were positively correlated with C:N ratios, suggesting that carbon in excess of that required for growth was invested in foliar phenolic compounds. Constitutive levels of proteinase inhibitors also provided some support for the CNB hypothesis. Overall, both chymotrypsin and trypsin inhibitor levels

decreased under elevated CO<sub>2</sub> (the decrease in trypsin inhibitors was nearly significant) and in non-nodulated plants. Moreover, constitutive levels of both inhibitors were negatively correlated with C:N ratios.

However, we also obtained results that were clearly not consistent with the predictions of the CNB hypothesis. Although the CNB hypothesis attributes the production of chemical defenses primarily to plant C:N ratio, constitutive phenolics were no more strongly correlated with plant C:N ratio than with nitrogen or carbon content alone. Moreover, constitutive levels of trypsin inhibitors were more strongly correlated with nitrogen content than with C:N ratio. This result suggests that foliar nitrogen content, rather than C:N ratio, may be the most important determinant of soybean proteinase inhibitor levels. We also did not observe a CO<sub>2</sub> effect on constitutive trypsin inhibitors among non-nodulated plants despite much higher C:N ratios among non-nodulated plants grown under elevated CO<sub>2</sub>. However, our power to detect a CO<sub>2</sub> effect in this case may have been quite low, due to the extremely low trypsin inhibitor levels among non-nodulated plants at ambient CO<sub>2</sub>.

The induction of chemical defenses can also be influenced by resource availability and C:N ratios (Bryant et al., 1993; Hunter and Shultz, 1995), as predicted by the CNB hypothesis. However, very few studies have investigated the effect of elevated CO<sub>2</sub> on the inducibility of carbon-based chemical defenses and, to our knowledge, no study has investigated the effect of elevated CO<sub>2</sub> on the inducibility of nitrogen-based defenses. We interpreted the CNB hypothesis to predict an increase in inducibility of carbon-based phenolics and a decrease in

inducibility of nitrogen-based proteinase inhibitors in plants grown under elevated CO<sub>2</sub> and/or lacking nitrogen fixing symbionts. However, our results generally were inconsistent with these predictions. Induction of phenolics occurred only among soybean grown at ambient CO<sub>2</sub>, despite their lower C:N ratios, and was no stronger for wild-type than for non-nodulating plants, again despite large differences in C:N ratios. Post-damage change in phenolic levels also was not correlated with C:N ratio. These results are broadly consistent with those of Roth et al. (1998) and Lindroth and Kinney (1998), who found that induction of several carbon-based polyphenols in aspen occurred equally under ambient and elevated CO<sub>2</sub>.

We also did not observe an effect of elevated CO<sub>2</sub> on inducibility of nitrogen-rich proteinase inhibitors. Contrary to the prediction of the CNB hypothesis, wild-type plants grown under elevated CO<sub>2</sub> were capable of inducing trypsin inhibitors to the same extent as wild-type plants grown under ambient CO<sub>2</sub>, despite higher C:N ratios among elevated grown plants. Moreover, post-damage change in trypsin inhibitors was no more strongly correlated with C:N ratio than with foliar carbon content, suggesting that C:N ratio is not the primary determinant of inducibility of trypsin inhibitors. Finally, post-damage change in chymotrypsin inhibitors was not correlated with C:N ratio (or with leaf nitrogen or carbon content). However, this provides weak evidence against the CNB hypothesis because chymotrypsin inhibitors were not inducible in this study.

We observed two patterns of induction that are consistent with the predictions of the CNB hypothesis. Induction of trypsin inhibitors occurred only among wild-

type plants, which had much lower C:N ratios than non-nodulated plants. In addition, damage-induced change in trypsin inhibitors was negatively correlated with C:N ratios.

Prior studies of the effects of elevated CO<sub>2</sub> on plant chemical defenses have also provided mixed support for the CNB hypothesis. Although several studies have reported increases in carbon-based chemical defenses under elevated CO<sub>2</sub> (Stuhlfauth et al., 1987; Traw et al., 1996; Penuelas et al., 1996), many have found no significant change in carbon-based defenses (Lincoln and Couvet, 1989; Johnson and Lincoln, 1990; Johnson and Lincoln, 1991; Fajer et al., 1992; Penuelas et al., 1996; Reitz et al., 1997). Moreover, Fajer et al. (1992) found that the carbon-based iridoid glycosides, catalpol and aucubin, actually decreased in *P. lanceolata* exposed to elevated CO<sub>2</sub>, and suggest that other factors, such as hormonal changes and defense-related enzyme activity, may be stronger determinants than C:N ratio of plant chemical defense levels. The much smaller number of studies of CO<sub>2</sub>-induced changes in nitrogen-based defenses also provide limited support for the CNB hypothesis (Rufy et al., 1989; Karowe et al., 1997).

It can be argued that the failure of the CNB hypothesis to accurately predict patterns of induction should not be surprising. Because the CNB hypothesis is primarily an evolutionary hypothesis that attributes the production of chemical defenses to the environment in which a plant has evolved, it may be inappropriate to expect this hypothesis to accurately predict phenotypically plastic responses of plants to new environmental conditions, e.g. to increases in atmospheric CO<sub>2</sub>.

Another primary goal of this study was to determine whether association with nitrogen fixing mutualists will buffer legumes against changes in nitrogen content, C:N ratio, and constitutive and induced levels of chemical defenses under elevated CO<sub>2</sub>. When possible, we considered the presence of a CO<sub>2</sub> x nodulation interaction as the primary indicator of whether “buffering” by nitrogen fixation occurred.

Consistent with our hypothesis, C:N ratios exhibited a significant CO<sub>2</sub> x nodulation interaction; the effect of elevated CO<sub>2</sub> on C:N ratio was substantially less for wild-type plants than for non-nodulated plants. This suggests that nitrogen fixation may buffer the C:N ratio of soybean under elevated CO<sub>2</sub>. Induction of trypsin inhibitors also appeared to require the presence of nitrogen-fixing symbionts, since it occurred only in wild-type plants. That induction was approximately equal for wild-type plants under both ambient and elevated CO<sub>2</sub> suggests association with nitrogen-fixing symbionts may maintain the inducibility of this defense under elevated CO<sub>2</sub>.

However, because non-nodulated plants grown under ambient CO<sub>2</sub> did not exhibit induction of trypsin inhibitors, we cannot determine whether nitrogen fixation truly “buffers” this defensive response.

The significant CO<sub>2</sub> x nodulation interaction detected for leaf nitrogen content argues for a different conclusion. Growth under elevated CO<sub>2</sub> resulted in a greater absolute decrease in nitrogen content among wild-type plants than among non-nodulated plants. However, it is likely that the extremely low nitrogen content of non-nodulated plants grown under ambient CO<sub>2</sub> limited the maximum possible decrease in nitrogen we could have observed among non-nodulated plants grown

under elevated CO<sub>2</sub>. Had they displayed an absolute decrease equal to that of wild-type plants, foliar nitrogen content of non-nodulated plants under elevated CO<sub>2</sub> would have been 0.1%; it is not likely that soybean can survive with much less than the observed level of 0.8% foliar nitrogen. Therefore, we believe that the severe nitrogen deficiency experienced by non-nodulated plants precluded us from determining whether nitrogen fixation buffers plant nitrogen content against a CO<sub>2</sub>-induced decrease. Similarly, though the significant CO<sub>2</sub> effect on constitutive trypsin inhibitor levels in wild-type plants, but not in non-nodulated plants, might suggest that nitrogen fixation will not buffer soybean against decreases in these nitrogen-containing defenses, the very low trypsin inhibitor levels in non-nodulated plants at ambient CO<sub>2</sub> again may have precluded us from determining whether nitrogen fixation buffers trypsin inhibitor levels against a CO<sub>2</sub>-induced decrease.

Our results suggest that nitrogen fixation will not buffer soybean against CO<sub>2</sub>-induced changes in constitutive levels of phenolics and chymotrypsin inhibitors. The absence of a CO<sub>2</sub> × nodulation interaction indicates that constitutive levels of these defenses will be similarly influenced by elevated CO<sub>2</sub> regardless of a plant's association with nitrogen-fixing symbionts.

Decreased foliar nitrogen content may also explain why non-nodulated plants contained slightly but significantly less carbon than wild-type plants. Given that nitrogen deficiency has been shown to cause decreases in chlorophyll, total protein, and ribulose biphosphate carboxylate activity, and therefore in photosynthetic and carbon fixation rates (Rufty et al., 1984), the reduced carbon content of non-

nodulated plants may have resulted from reduced plant nitrogen content. The strong positive correlation between leaf nitrogen and carbon contents in all treatments also suggests that carbon acquisition is nitrogen-dependent.

When nitrogen is limiting, a primary tradeoff in nitrogen allocation may exist between growth and defense (Herms and Mattson, 1992). Since we did not measure plant biomass, we cannot determine whether soybean encounters a tradeoff between growth and defense. However, we can determine whether tradeoffs exist between nitrogen-containing defense traits, and whether such tradeoffs are more severe when foliar nitrogen is more limiting, i.e. for plants grown under elevated CO<sub>2</sub> and/or that cannot nodulate.

We investigated the existence of nitrogen-containing tradeoffs between constitutive and induced levels of nitrogen-rich chymotrypsin and trypsin inhibitors. When plants within each of the four treatment groups were analyzed separately, we did not detect any tradeoff (identified as a significant negative correlation) between constitutive levels of chymotrypsin and trypsin inhibitors, between constitutive levels of either proteinase inhibitor and post-damage change in the other proteinase inhibitor, or between post-damage changes in the two proteinase inhibitors.

However, we did observe a negative correlations between constitutive levels and post-damage change of the same proteinase inhibitor, suggesting a tradeoff between investment in constitutive defense by each proteinase inhibitor and ability to respond to damage by producing additional amounts of the same proteinase inhibitor. For instance, plants containing high constitutive levels of chymotrypsin inhibitors

produced lower additional amounts of chymotrypsin inhibitors within post-damage leaflets (assuming equal turnover rates for all treatment groups). This tradeoff existed within each treatment group and, particularly for non-nodulating plants, was stronger under elevated CO<sub>2</sub>. The change in trypsin inhibitors 48 hours after damage was also negatively correlated with constitutive levels of trypsin inhibitors for all treatment groups; for non-nodulated plants, this apparent tradeoff was also much stronger under elevated CO<sub>2</sub>. These results suggest that soybean encounters a tradeoff between pre-attack and post-attack defense by proteinase inhibitors, and that the tradeoff is most severe particularly for the most nitrogen-limited plants. Moreover, because growth under elevated CO<sub>2</sub> greatly increases the severity of these tradeoffs for non-nodulated plants but not for nodulated plants, these results suggest that association with nitrogen-fixing bacteria buffers soybean against CO<sub>2</sub>-induced increases in the severity of these tradeoffs.

It should be noted that negative correlations between constitutive levels and post-damage change could arise as a statistical artifact, since post-damage change is defined as post-damage level (within post-damage leaflets) minus constitutive level (within pre-damage leaflets). For instance, if constitutive and post-damage levels of chymotrypsin inhibitors are not correlated, high constitutive levels would tend to produce low values of post-damage change. A spurious negative correlation would be less likely if constitutive and post-damage levels are positively correlated. Because we did not observe a positive correlation between constitutive and post-damage levels of chymotrypsin inhibitors within wild-type or non-nodulated plants at

elevated CO<sub>2</sub>, or between constitutive and post-damage levels of trypsin inhibitors within any treatment group (with the possible exception of wild-type plants at elevated CO<sub>2</sub>), we must be particularly cautious in interpreting negative correlations between constitutive and post-damage change within these groups as evidence of nitrogen-based tradeoffs. Nonetheless, this argument does not compromise the observation that, for non-nodulated plants, growth under elevated CO<sub>2</sub> resulted in a much stronger negative correlation between constitutive and post-damage change in trypsin inhibitor levels.

Over the last two decades, many studies have used the CNB hypothesis to predict changes in plant chemical defenses under elevated CO<sub>2</sub>. The equivocal support for the predictions of the CNB hypothesis in this and previous studies suggest that CO<sub>2</sub>-induced changes in both carbon-based and nitrogen-containing plant defenses generally are not driven by CO<sub>2</sub>-induced changes in foliar C:N ratios. While the CNB hypothesis often accurately predicts evolved strategies of plant chemical defense, phenotypically plastic responses apparently are determined by factors other than the relative levels of carbon and nitrogen within leaves. The species- and/or chemical-specific responses of plant defenses to elevated CO<sub>2</sub> suggest that species- and/or chemical-specific mechanisms control the phenotypic response of plants to this particular form of environmental variation.

To date, most research on the response of plant chemical defenses to elevated CO<sub>2</sub> has concentrated on constitutive levels of carbon-based defenses. Consequently, we know very little about the influence of elevated CO<sub>2</sub> on constitutive levels of

nitrogen-containing defenses, or on the inducibility of carbon-based or nitrogen-containing defenses. The current study is unusual in its attempt to address the effects of elevated CO<sub>2</sub> on a nitrogen-containing defense and on the inducibility of both carbon- and nitrogen-containing defenses, and in its attempt to determine whether association with nitrogen-fixing symbionts can buffer plants against CO<sub>2</sub>-induced changes in chemical defenses. Clearly, additional studies are necessary before we can fully understand the ecological consequences of elevated CO<sub>2</sub> levels that are almost certain to occur during this century.

## CHAPTER II

### EFFECTS OF ELEVATED CARBON DIOXIDE, NITROGEN FIXATION, AND MECHANICAL DAMAGE ON LARVAL PERFORMANCE OF THE SOYBEAN LOOPER, *PSEUDOPLUSIA INCLUDENS*

#### Introduction

It is well established that elevated CO<sub>2</sub> lowers the nutritional quality of many plant species by diluting leaf nitrogen, increasing leaf carbon to nitrogen (C:N) ratio, and/or decreasing leaf water content (Wong, 1979; Rogers et al., 1984; Bazzaz, 1990; Johnson and Lincoln, 1991; Lindroth et al., 1993; Lincoln et al., 1993; Tschaplinski et al., 1995; Reitz et al., 1997; Lawler et al., 1997; Karowe et al., 1997). Because the growth and fitness of insect herbivores is highly dependent on the nitrogen content of their host plants, it is very likely that increases in atmospheric CO<sub>2</sub> will have direct adverse effects on herbivorous insects (review by Coviella and Trumble, 1999). In addition, levels of some plant chemical defenses, such as carbon-based phenolics, may also increase under elevated CO<sub>2</sub> (Stuhlfauth et al., 1987; Traw et al., 1996; Penuelas et al., 1996; Lawler et al., 1997); such increases may also negatively influence the performance of foliage-feeding insects.

The Carbon-Nutrient Balance (CNB) hypothesis (Bryant et al., 1983) predicts that carbon-based defenses of plants will increase with increasing C:N ratio, e.g. in plants grown under elevated CO<sub>2</sub>. In support of the CNB hypothesis, under elevated CO<sub>2</sub>, carbon-based cardenolides increased in *Digitalis lantana* (Stuhlfauth et al.,

1987), condensed tannins increased in both gray and yellow birch (Traw et al., 1996), and total phenolics and condensed tannins increased in *Eucalyptus tereticornis* (Lawler et al., 1997). However, increases in carbon-based defenses are by no means universal. Various carbon-based defenses did not increase under elevated CO<sub>2</sub> in sagebrush (Johnson and Lincoln, 1991), plantain (Fajer et al., 1992), or celery (Reitz et al., 1997). Thus, the effect of elevated CO<sub>2</sub> on carbon-based chemical defenses apparently is chemical- and/or species-specific.

With few exceptions, insects increase consumption rates by 15-80% when fed plants grown under elevated CO<sub>2</sub> (reviewed by Lincoln et al., 1993). For example, *Spodoptera* caterpillars increased consumption of elevated CO<sub>2</sub>-grown peppermint on average by 22% (Johnson and Couvet, 1989). Larvae of *Junonia coenia* also increased consumption rates by 20-40% when fed plantain grown at 770 ppm CO<sub>2</sub> (Fajer, 1989), and *Melanoplus* grasshoppers consumed more sagebrush grown at 650 ppm CO<sub>2</sub> (Johnson and Lincoln, 1990).

Presumably, increased consumption by insect herbivores is a compensatory response to decreased nitrogen content of plants grown under elevated CO<sub>2</sub> (Scriber and Feeny, 1979; Mattson, 1980; Mattson and Scriber, 1986). However, compensatory feeding under elevated CO<sub>2</sub> often is not sufficient to prevent decreased growth rate (Lincoln et al., 1986; Akey and Kimball, 1989; Fajer, 1989; Lindroth et al., 1993), or increased developmental time (Fajer, 1989; Akey and Kimball, 1989; Lindroth and Kinney, 1998). For instance, the relative growth rate (RGR) of the grasshopper *Melanoplus differentialis* (but not of *M. sanguinipes*) decreased by 40% when fed sagebrush grown at 650 ppm CO<sub>2</sub> (Johnson and Lincoln, 1991). Gypsy

moth larvae experienced a 63% reduction in RGR, and forest tent caterpillars displayed a 25% reduction in RGR when fed aspen grown at 642 ppm CO<sub>2</sub> (Lindroth et al., 1993). In addition, Lawler et al. (1997) reported reduced pupal weight and increased mortality for *Chrysophtharta flaveola* larvae fed eucalyptus grown at elevated CO<sub>2</sub>. Reduced pupal weight and prolonged developmental time were also reported for beet armyworm larvae fed elevated-grown cotton seedlings (Akey and Kimball, 1989).

Legumes (Leguminosae) represent one class of plants that may escape many effects of elevated CO<sub>2</sub> due to their mutualistic relationship with nitrogen-fixing bacteria. This mutualistic association provides legumes not only with a flexible nitrogen source, but also with a carbon sink for excess photosynthate. Therefore, nitrogen fixation may prevent the C:N ratio of legumes from increasing under elevated CO<sub>2</sub> (i.e. nitrogen fixation may “buffer” legumes against increases in C:N ratio under elevated CO<sub>2</sub>; Chapter I). Indeed, legume species grown under elevated CO<sub>2</sub> generally display increased total plant nitrogen fixation (Finn and Brun, 1982; Norby, 1987; Ryle et al., 1992; Tissue et al., 1997) and nitrogenase activity (Tissue et al., 1997). Presumably as a consequence, several legume species display no change or only slight significant decreases in foliar nitrogen content under elevated CO<sub>2</sub> (Tissue et al., 1997; Ryle et al., 1992; Ayers et al., 1993; Karowe et al., in preparation).

In addition, it is possible that legume-feeding insect herbivores may escape the typical adverse effects of elevated CO<sub>2</sub> due to the association of their host plants with nitrogen fixing bacteria. If consumption rates of insects are determined

primarily by foliar nitrogen content (Scriber and Feeney, 1979; Mattson, 1980; Mattson and Scriber, 1986), and levels of plant chemical defenses depend primarily on plant C:N ratio, as predicted by the CNB hypothesis, then legume-feeding insects would not consume greater amounts of leaf material or chemical defenses when feeding on host plants grown under elevated CO<sub>2</sub>. Studies on legume feeders under elevated CO<sub>2</sub>, however, have yielded equivocal results. No difference in the relative growth rate or instar duration was found for the soybean looper, *Pseudoplusia includens*, fed elevated CO<sub>2</sub>-grown soybean (Lincoln et al., 1984; 1986). In addition, Karowe et al. (in preparation) reported that larvae of *Colias philodice* fed their host plant, *Trifolium pratense*, also showed virtually no difference in consumption, digestibility, or growth between CO<sub>2</sub> treatments. However, *C. philodice* larvae fed *Melilotus alba* grown under elevated CO<sub>2</sub> displayed decreased digestion efficiency and pupal weight (Karowe et al., in preparation). Osbrink et al. (1987) also found a reduction in pupal weight for *Trichoplusia ni* when fed elevated-grown lima beans. These results suggest that some, but not all, legume-feeding insect herbivores may be buffered against the adverse effects of elevated CO<sub>2</sub>.

Soybean (*Glycine max* L.) is an agriculturally important legume that is susceptible to attack by the soybean looper, *Pseudoplusia includens* (Lepidoptera: Noctuidae). Presumably to aid in defense against such insect herbivores, soybean produces a suite of chemical defenses including both carbon- and nitrogen-containing compounds. Several soybean carbon-based phenolics, such as tannins and phytoalexins, have been shown to be effective feeding deterrents (Chiang and Norris, 1983; Fischer et al., 1990). In addition, soybean contains nitrogen-rich serine

proteinase inhibitors, including chymotrypsin and trypsin inhibitors, that can retard insect growth (Steffens et al., 1978; Broadway and Duffey, 1986; Oppert et al., 1993). If association with nitrogen-fixing symbionts buffers this legume against significant changes in C:N ratio in a CO<sub>2</sub> enriched environment, it is plausible that little or no change in either phenolics or proteinase inhibitor concentrations would occur under elevated CO<sub>2</sub>. Consequently, herbivores of soybean may not experience the adverse effects on growth typically associated with feeding on host plants grown under elevated CO<sub>2</sub>.

In the present study we investigate the effects of elevated CO<sub>2</sub> on *P. includens* by comparing performance of larvae fed wild-type and non-nodulated soybean strains grown under ambient and elevated CO<sub>2</sub>. We hypothesize that association of soybean with nitrogen-fixing bacteria will prevent leaf carbon, C:N ratio, and levels of carbon-based defenses (phenolics) from increasing, and leaf nitrogen and levels of nitrogen-containing defenses (chymotrypsin and trypsin inhibitors) from decreasing under elevated CO<sub>2</sub>. As a result, we anticipate that growth of host plants under elevated CO<sub>2</sub> will not affect the performance of soybean loopers fed wild-type soybean, but will result in decreased performance of soybean loopers fed non-nodulated soybean. In addition, we evaluate whether performance of *P. includens* is correlated with levels of leaf nitrogen, carbon, C:N ratio, water, total phenolics and/or proteinase inhibitors. Specifically, in this research we ask:

1. Does host plant association with the nitrogen-fixing bacteria, *Bradyrhizobium japonicum*, buffer larval growth and performance of the soybean

looper, *Pseudoplusia includens*, against the adverse effects typically associated with growth of host plants under elevated CO<sub>2</sub>?

2. Is performance of the soybean looper correlated with foliar levels of nitrogen, carbon, C:N ratio, water, total phenolics and/or proteinase inhibitors?

## Materials and Methods

### Growth of Plant Material

All plants were grown during the summer of 1998 at the University of Michigan Biological Station (UMBS) in Pellston, Michigan. Seeds of the wild-type (nodulated) soybean variety, Harosoy, and its *rj1* mutant (non-nodulated) isolate (generously provided by Dr. James Harper, USDA, University of Illinois, Urbana-Champaign) were germinated in 6-inch pots using a 1:3 mixture of vermiculite and Hyponex top soil in the greenhouse. Inoculation of both wild-type and non-nodulated plants was performed when the seeds were sown using a 3X application of peat-based inoculum containing *Bradyrhizobium japonicum* (Urbana Laboratories, St. Josephs, MO).

Seven days after germination, both wild-type and non-nodulated strains were placed randomly in 16 open-topped chambers identical to those described by Karowe et al. (1997), half of which were maintained at  $756 \pm 10$  ppm CO<sub>2</sub> (elevated treatment) and the other half at  $367 \pm 10$  ppm CO<sub>2</sub> (ambient treatment). Chambers were monitored every 20 minutes by an infrared gas analyzer (IRGA) and CO<sub>2</sub> concentrations were recorded to a computer. Plants were watered and checked for

insects daily. At two and four weeks after germination, both wild-type and non-nodulated plants received 300 ml of full strength, low-nitrogen fertilizer (10-60-60; Shultz Rapid Bloom Fertilizer supplemented with Espoma<sup>®</sup> Potash). At the conclusion of plant harvesting, the roots of 10 randomly selected plants from both wild-type and non-nodulated strains were examined for the presence of nodules. All wild-type plants displayed an abundance of root nodules ranging from white to dark red, while nodules were completely absent from the roots of non-nodulated plants.

From each of eight treatment groups, 8-12 plants were harvested when they had developed 7 to 8 fully expanded leaves, approximately six weeks after germination (i.e. vegetative stage V-7 to V-8; Fehr et al., 1971). The eight treatment groups consisted of wild-type and non-nodulated plants, each grown at ambient and elevated CO<sub>2</sub> and sampled at 0 and 48 hours after mechanical damage (see below). Leaf carbon and nitrogen contents, C:N ratio, water content, total phenolics, and chymotrypsin and trypsin inhibitor levels were determined, and fourth instar *P. includens* feeding trials were performed for each plant (see below).

#### Leaf Nitrogen, Carbon, and Water Contents, and C:N Ratio

Leaf percent nitrogen and carbon, C:N ratio, and leaf water content were measured on one half of a lateral leaflet from the fourth fully expanded leaf (Figure 2; the other half of the same leaflet was used for a feeding trial as described below). The half leaflet was harvested without the midrib, weighed, placed in a glassine envelope, and stored at -80°C for several weeks. Samples were then dried at 60°C for 72 hours, weighed again to obtain a wet:dry conversion for determining plant water

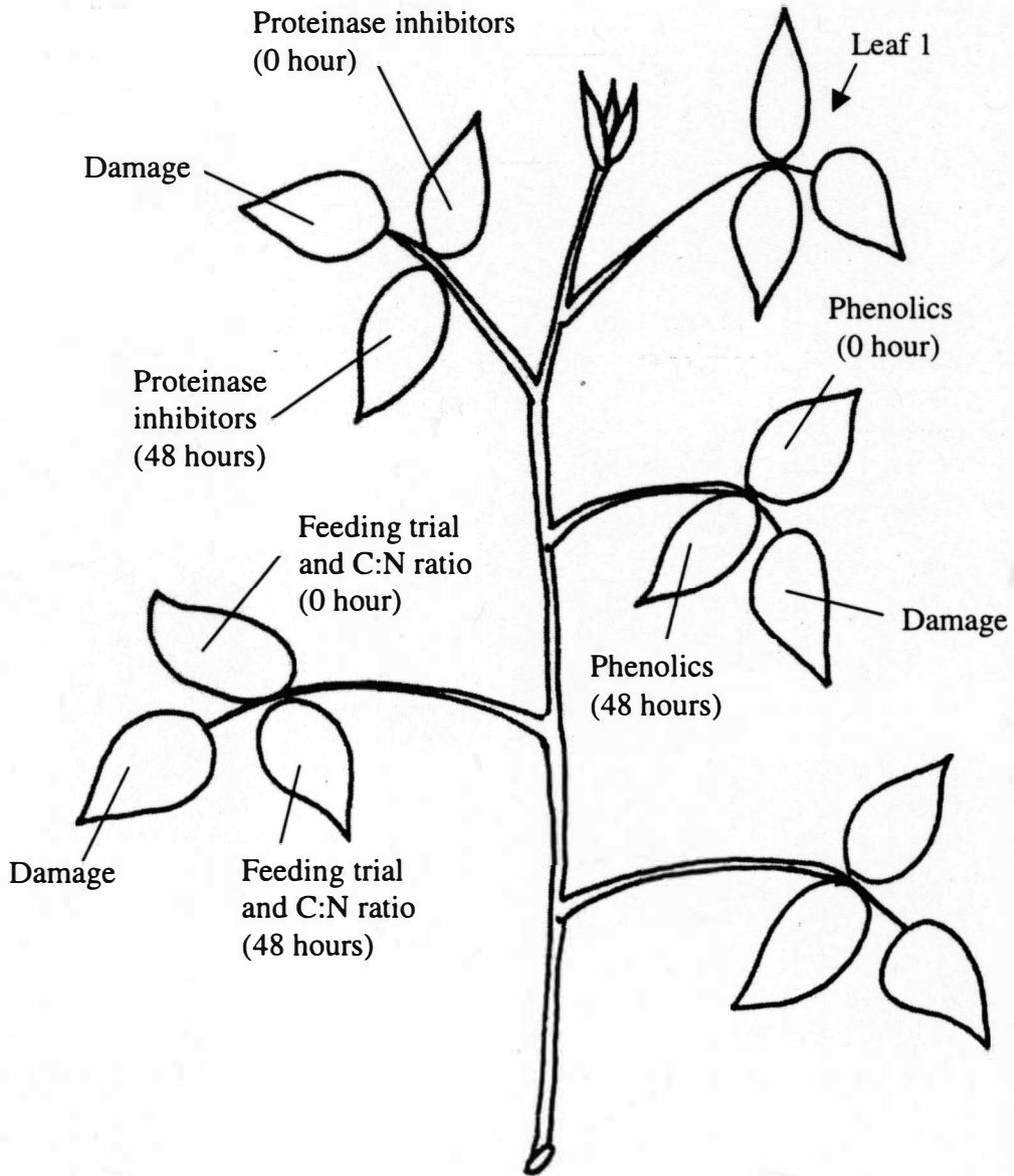


Figure 2. Plant Harvest Regime for Chemical Analyses and Larval *Pseudoplusia includens* Feeding Trials.

content, and ground to a fine powder using a Wiley Mill. For each sample, carbon, hydrogen, and nitrogen contents were determined using a Perkin Elmer CHN Elemental Analyzer at UMBS, and nitrogen and carbon contents were expressed as percentages of leaf dry weight.

### Leaf Total Phenolic Content

Total leaf phenolic content was determined on the third fully expanded leaf (Figure 2), using methods described in Spickard and Karowe (in preparation). Briefly, 20mg of dried leaf tissue was washed three times using 1 ml ethyl ether. After each wash, samples were pelleted by centrifugation at 4,600g for 5 minutes and the ether was removed. Phenolic extraction was performed by suspending the resulting pellet in 0.4 ml 90:10% methanol:water and sonicating on ice for 30 minutes. Samples were centrifuged again at 4,600g for 5 minutes and the supernatant was collected in a fresh tube. Phenolic extractions were repeated twice more using 0.4 ml 90% methanol until a final volume of 1.2 ml was collected. The final extract was dried using a rotary evaporator and stored at -80°C until samples were analyzed for total phenolics.

Total phenolic content for each leaf sample was determined using the Folin-Denis assay (Swain and Goldstein, 1964). Dried leaf extracts were resuspended in 1 ml deionized H<sub>2</sub>O and diluted 1:20 in 1 ml deionized H<sub>2</sub>O for each reaction. To each reaction tube, 1 ml Folin-Denis reagent (75 mM sodium tungstate, 1.3 mM phosphomolybdic acid, 2.8% phosphoric acid) was added for three minutes, followed by 1 ml 2N sodium carbonate for a final volume of 3 ml. Samples were incubated for

1 hr at room temperature for color development. Sample absorbency was determined at 725 nm against a blank of deionized water. Total phenolic concentration was estimated using a semi-pure native standard and expressed as  $\mu\text{g}/\text{mg}$  dry leaf material. Standard purification and purity evaluations were performed using methods described in Spickard and Karowe (in preparation). All samples were run in triplicate.

### Leaf Proteinase Inhibitor Contents

Chymotrypsin and trypsin inhibitor levels were determined using methods based on those of Broadway and Duffey (1986) and Broadway (1989). Leaf tissue from one lateral leaflet from the second fully expanded leaf (Figure 2) was harvested without the midrib, weighed, and homogenized on ice in 3x volume of 1 mM HCl. The homogenate was centrifuged at 14,000g for 5 minutes at 4°C. After centrifugation, the supernatant was transferred to a fresh tube, spun again at 14,000g for 5 minutes at 4°C, and stored on ice until proteinase inhibitor assays were performed.

Chymotrypsin inhibitor activity was determined by mixing leaf extract 1:1 with TLCK-treated bovine chymotrypsin (0.1 mg/ml 1 mM HCl) for 10 minutes at room temperature. After incubation, 100  $\mu\text{l}$  of the sample mixture was added to 2.9 ml substrate buffer (1 mM benzoyl-L-tyrosine ethyl ester in 50% MeOH, mixed 1:1 with 0.05 Tris, pH 8.0), and chymotrypsin activity was monitored at 256 nm every 30 seconds for 3 minutes to generate a reaction curve. The resulting slope of each sample curve was compared to uninhibited chymotrypsin mixed 1:1 with 1 mM HCl, and

percent chymotrypsin inhibition was calculated. Each sample was repeated in triplicate.

Trypsin inhibitor activity was quantified by mixing leaf extract 1:1 with TPCK treated bovine trypsin (0.1 mg/ml 1 mM HCl) and incubating at room temperature for 10 minutes. A 100  $\mu$ l aliquot of the sample mixture was then transferred to 2.9 ml substrate buffer (0.05 M Tris, pH 8.0) containing 1.04 M p-tolulene-sulfonyl-L-arginine methyl ester (TAME). Trypsin activity was monitored at 247 nm every 30 seconds for 3 minutes. The slope of the linear reaction curve was determined and compared to uninhibited trypsin mixed 1:1 with HCl. Each sample extract was assayed in triplicate.

### Plant Damage Regime

To determine whether mechanical damage affects the performance of fourth instar larvae, for instance via induction of chemical defenses, feeding trials were conducted using lateral leaflets of the same leaf before and after mechanical damage. Immediately prior to damage, one lateral leaflet from the second fully expanded leaf was harvested for trypsin and chymotrypsin inhibitor contents, one lateral leaflet from the third fully expanded leaf was harvested for total phenolics, and one lateral leaflet from the fourth fully expanded leaf was harvested for leaf nitrogen, carbon, C:N ratio, and water contents, and for insect feeding trials (Figure 2). Undamaged leaflets sampled just prior to mechanical damage are hereafter referred to as “pre-damage leaflets.” Damage was then performed by placing 25  $\mu$ l of 40  $\mu$ M jasmonic acid (Sigma) on terminal leaflets of the second, third, and fourth fully expanded leaves,

followed by crushing with a garlic press. The chemical elicitor jasmonic acid has been shown to induce plant chemical defenses when exogenously applied to leaves (Thaler et al., 1996), and therefore was used along with mechanical wounding to mimic insect herbivory. To standardize damage, one press on each side of the midrib in the mid-section of each leaflet was made. In addition, every effort was made to ensure jasmonic acid was evenly distributed where mechanical damage was performed. Forty-eight hours after plants were damaged, post-damage levels of proteinase inhibitors and total phenolics were determined for each plant using the remaining (undamaged) lateral leaflet from leaves 2 and 3, respectively, and post-damage feeding trials and levels of nitrogen, carbon, C:N ratio, and water content were determined for the remaining (undamaged) lateral leaflet of leaf 4 (Figure 2). Undamaged leaflets sampled at 48 hours after mechanical damage are hereafter referred to as “post-damage leaflets.”

#### *Pseudoplusia includens* Larval Performance

The effects of elevated CO<sub>2</sub>, nitrogen fixation, and mechanical damage on larval growth and performance were determined for fourth instar *P. includens* larvae. Two feeding trials were performed using the two opposite leaflets from leaf 4 of each plant. Immediately prior to mechanical damage, one larva received one half of one lateral leaflet of leaf 4 (the pre-damage leaflet). Forty-eight hours after mechanical damage, a different larva received one half of the opposite lateral leaflet of leaf 4 (the post-damage leaflet; Figure 2). Eggs of *P. includens* were generously provided from a colony maintained by Dr. Seth Young, University of Arkansas. Larvae were reared

on BioServ<sup>®</sup> soybean looper diet in 25 ml polystyrene cups in an environmental chamber under a 16:8 - light:dark cycle with a corresponding 25:20 °C temperature cycle. Fresh diet was given every 2-3 days.

Upon molting into the fourth instar, each test larva was placed in an 11-cm petri dish with moistened filter paper and starved for 1 hour. Frass produced during this starvation period was discarded before the feeding trial began. Each larva was then weighed and given one half of a weighed pre-damage or post-damage lateral leaflet from leaf 4. After feeding for 48 hours, the larva was dried to a constant weight at 60 °C and weighed. To provide a conversion factor for estimating the initial dry weight of test larvae, 10 freshly molted fourth instar larvae were starved for 1 hour, weighed, dried to a constant weight at 60°C, and reweighed. The dry weight of leaf material provided in each trial was estimated using a conversion factor generated from the other half of the same leaflet, which was harvested for CHN analyses as described above.

All frass produced during the feeding trial and all uneaten food were dried to constant weight at 60°C and weighed. For all larvae, relative consumption rate (RCR), approximate digestibility (AD), efficiency of conversion of ingested food (ECI), efficiency of conversion of digested food (ECD), and relative growth rate (RGR) were calculated based on dry weights. Formulas for these standard nutritional indices, as described by Waldbauer (1968), are presented in Table 6. Sample sizes were 8-12 larvae from each of eight treatment groups (2 CO<sub>2</sub> x 2 soybean strains x 2 harvest times) yielding a total of 81 feeding trials.

Table 6

Formulas for Standard Gravimetric Nutritional Indices as in Waldbauer (1968)

Nutritional Index	Formula
Relative consumption rate (RCR)	= (food ingested)/ (average larval weight)(days)
Approximate digestibility (AD)	= 100 x (food ingested - frass)/ (food ingested)
Efficiency of conversion of digested food (ECD)	= 100 x (larval weight gained)/ (food ingested - frass)
Efficiency of conversion of ingested food (ECI)	= 100 x (larval weight gained)/ (food ingested)
Relative growth rate (RGR)	= (weight gained)/ (average larval weight)(days)

#### Effects of Plant Chemical Defenses on Larval Performance

Leaflet size precluded using a single leaflet to measure levels of leaf nitrogen, carbon, water, and chemical defenses, and to conduct insect feeding trials. Therefore, we measured levels of proteinase inhibitors (chymotrypsin and trypsin inhibitors) within leaf 2, total phenolics within leaf 3, and leaf nitrogen, carbon, water, and C:N ratio within leaf 4. However, only leaf 4 was used in feeding trials. Therefore, inferences about the effects of chemical defenses on insect performance in this study are based on the assumption that levels of proteinase inhibitors in leaf 4 are positively correlated with levels in leaf 2, and that levels of phenolics in leaf 4 are positively correlated with levels in leaf 3. To evaluate these assumptions, we determined inter-

leaf correlations for pre-damage (constitutive) and post-damage levels of all three chemical defenses using 9-14 additional wild-type plants grown in the greenhouse at Western Michigan University.

Pre-damage levels of phenolics were significantly positively correlated between leaves 3 and 4 ( $r^2 = 0.39$ ,  $df = 13$ ,  $p = 0.017$ ; data not shown). However, contrary to expectation, pre-damage levels of chymotrypsin and trypsin inhibitors were not significantly correlated between leaves 2 and 4 ( $r^2 = 0.22$ ,  $df = 8$ ,  $p = 0.20$ ;  $r^2 = 0.01$ ,  $df = 8$ ,  $p = 0.99$ , respectively; data not shown).

In part because chymotrypsin inhibitors were not inducible in either leaf 2 or leaf 4, a nearly significant positive correlation existed between post-damage levels of chymotrypsin inhibitors in leaves 2 and 4 ( $r^2 = 0.41$ ,  $df = 8$ ,  $p = 0.06$ ; data not shown). However, due to differential induction among leaves, no correlation between leaves 2, 3, and 4 was found for post-damage levels of either trypsin inhibitors in leaves 2 and 4 ( $r^2 = 0.04$ ,  $df = 8$ ,  $p = 0.62$ ; data not shown) or total phenolics in leaves 3 and 4 ( $r^2 = 0.05$ ,  $df = 13$ ,  $p = 0.46$ ; data not shown). In fact, comparison of pre- and post-damage opposite leaflets indicated that no induction of any chemical defense occurred in leaf 4 (chymotrypsin inhibitors: paired  $t = 1.0$ ,  $df = 8$ ,  $p = 0.33$ ; trypsin inhibitors: paired  $t = 0.5$ ,  $df = 8$ ,  $p = 0.63$ ; phenolics: paired  $t = 1.5$ ,  $df = 13$ ,  $p = 0.15$ ; data not shown), despite the induction of both trypsin inhibitors and phenolics in leaves 2 and 3, respectively (chymotrypsin inhibitors: paired  $t = 2.0$ ,  $df = 8$ ,  $p = 0.087$ ; trypsin inhibitors: paired  $t = 3.2$ ,  $df = 8$ ,  $p = 0.013$ ; phenolics: paired  $t = 2.4$ ,  $df = 13$ ,  $p = 0.032$ ; see Chapter I).

These results support the assumption that pre-damage levels of total phenolics in leaf 3 and post-damage levels of chymotrypsin inhibitors in leaf 2 are good predictors of corresponding levels of these defenses in leaf 4. However, we could not confirm the assumptions that constitutive levels of proteinase inhibitors, induced levels of trypsin inhibitors, or phenolics measured in leaves 2 and 3 are positively correlated with corresponding levels of these defenses in leaf 4. Therefore, inferences about the effect of soybean chemical defenses on performance of *P. includens* larvae fed leaf 4 will be restricted to constitutive levels of total phenolics and post-damage levels of chymotrypsin inhibitors.

Interestingly, constitutive levels of both chymotrypsin and trypsin inhibitors were significantly lower in leaf 4 than in leaf 2. Chymotrypsin inhibitors were on average 13% lower in leaf 4 (paired  $t = 3.4$ ,  $df = 16$ ,  $p = 0.004$ ), and trypsin inhibitors were approximately 48% lower (paired  $t = 6.6$ ,  $df = 16$ ,  $p = <0.001$ ). In contrast, levels of total phenolics were 11% higher in leaf 4 than in leaf 3 (paired  $t = 4.52$ ,  $df = 13$ ,  $p = 0.001$ ).

### Statistical Analyses

To determine whether nitrogen fixation affected the response to elevated  $CO_2$  of soybean, pre-damage levels of leaf nitrogen, carbon, water, C:N ratio, total phenolics and/or post-damage levels of chymotrypsin inhibitors, two-way analyses of variance (ANOVAs) were performed with  $CO_2$  level and nodulation status as main effects. We interpreted a significant  $CO_2$  x nodulation interaction as possible evidence that nitrogen fixation buffers soybean nutritional quality and/or chemical

defenses against the effects of elevated CO<sub>2</sub>. To determine the effects of mechanical damage on leaf quality, levels of leaf nitrogen, carbon, C:N ratio, and water were compared between opposite pre-damage and post-damage leaflets by paired t-tests. This analysis was performed separately for each treatment group.

Three-way ANOVAs were used to determine the effects of CO<sub>2</sub> level, nodulation status, and mechanical damage on RCR, AD, ECD, ECI, and RGR of *P. includens* larvae. Of particular interest are the CO<sub>2</sub> x nodulation interactions, which indicate whether nitrogen fixation is capable of buffering *P. includens* against reduced performance typically associated with host plant growth under elevated CO<sub>2</sub>.

Correlation analyses were used to determine the relationship between leaf nutritional quality (C:N ratio, nitrogen, carbon, and water contents) and levels of each chemical defense. In addition, correlations were determined between leaf C:N ratio, nitrogen, carbon, and water contents and each measure of larval performance. Finally, correlations were determined between each measure of larval performance and pre-damage levels of total phenolics and post-damage levels of chymotrypsin inhibitors. Because leaf nitrogen was strongly correlated with pre-damage levels of total phenolics and post-damage levels of chymotrypsin inhibitors, the effects of these chemical defenses on larval performance was determined using residual variation remaining after each measure of performance had been regressed against leaf nitrogen.

## Results

### Effects of Elevated CO<sub>2</sub> and Nodulation on Leaf Nitrogen, Carbon, and Water Contents, and C:N Ratio

Leaf nitrogen content of pre-damage leaflets was significantly higher in wild-type plants, and in plants grown under ambient CO<sub>2</sub> (Tables 7 and 8). Overall, the nitrogen content of leaf 4 was much higher in wild-type plants than in non-nodulated plants at both CO<sub>2</sub> levels (71% higher at ambient CO<sub>2</sub> and 67% higher at elevated CO<sub>2</sub>; Table 7). In addition, growth under elevated CO<sub>2</sub> resulted in a 32% decrease in leaf nitrogen among wild-type plants and a 22% decrease among non-nodulated plants. Leaf nitrogen content also exhibited a significant CO<sub>2</sub> x nodulation interaction, primarily because the absolute decrease between CO<sub>2</sub> treatments for wild-type plants was much greater than for non-nodulated plants (Tables 7 and 8).

Carbon content of pre-damage leaflets of leaf 4 also differed significantly between soybean strains and CO<sub>2</sub> levels (Tables 7 and 8). Surprisingly, carbon content was highest in wild-type plants at ambient CO<sub>2</sub>, followed by wild-type plants at elevated CO<sub>2</sub>, and was lowest in plants that were incapable of harboring nitrogen-fixing symbionts. In addition, a significant CO<sub>2</sub> x nodulation interaction was observed, apparently because wild-type plants showed a 3% reduction in carbon content at elevated CO<sub>2</sub>, while carbon content of non-nodulated plants increased slightly (0.5%) at elevated CO<sub>2</sub>.

These changes in carbon and nitrogen contents of pre-damage leaflets resulted in significant differences in leaf C:N ratio (Tables 7 and 8). As expected, C:N ratio

Table 7

Mean Values ( $\pm$  SD)<sup>a</sup> for Leaf Nitrogen, Carbon, C:N Ratio, Water Content, Total Phenolics and Proteinase Inhibitors of Soybean Grown at Ambient and Elevated CO<sub>2</sub>

Nodulation	CO <sub>2</sub>	Leaf Nitrogen (%)	Leaf Carbon (%)	Leaf C:N Ratio	Leaf Water (%)	Pre-Damage Phenolics ( $\mu$ g/mg)	Post-Damage Chymotrypsin Inhibitors (%)
Wild-type (nodulating)	Ambient	3.1 $\pm$ 0.5	44.2 $\pm$ 0.5	14.7 $\pm$ 2.3	69.7 $\pm$ 4.0	87.1 $\pm$ 13.2	58.2 $\pm$ 7.8
	Elevated	2.1 $\pm$ 0.4	42.9 $\pm$ 0.5	20.7 $\pm$ 3.4	64.5 $\pm$ 5.1	97.3 $\pm$ 9.3	50.6 $\pm$ 9.9
Non-Nodulating	Ambient	0.9 $\pm$ 0.1	39.7 $\pm$ 0.8	43.4 $\pm$ 6.6	72.2 $\pm$ 4.6	119.4 $\pm$ 12.5	8.5 $\pm$ 13.8
	Elevated	0.7 $\pm$ 0.1	39.9 $\pm$ 0.4	61.8 $\pm$ 7.3	68.3 $\pm$ 3.1	134.2 $\pm$ 18.3	6.2 $\pm$ 10.8

<sup>a</sup> Leaf nitrogen, carbon, C:N ratio, and water contents were all measured in pre-damage leaflets of leaf 4, total phenolics were measured in pre-damage leaflets of leaf 3, and chymotrypsin inhibitors were measured in post-damage leaflets of leaf 2. Sample sizes range from 8-12 individuals for each treatment group.

Table 8

Two-way Analysis of Variance for Total Leaf Nitrogen, Carbon, C:N Ratio,  
and Water Content of Pre-damage Leaves<sup>a</sup>

Source of Variation	SS	df	MS	F	p
<b>Leaf Nitrogen Content</b>					
Main effects					
CO <sub>2</sub> Level	3.7	1	3.7	35.7	<0.0001
Nodulation	32.3	1	32.3	311.7	<0.0001
Interaction					
CO <sub>2</sub> x Nodulation	1.1	1	1.1	10.6	0.0024
Error	3.8	37	0.1		
Total	40.7	40			
<b>Leaf Carbon Content</b>					
Main effects					
CO <sub>2</sub> Level	2.9	1	2.9	10.4	0.0026
Nodulation	140.1	1	140.1	498.2	<0.0001
Interaction					
CO <sub>2</sub> x Nodulation	5.4	1	5.4	19.3	<0.0001
Error	10.4	37	0.3		
Total	155.1	40			
<b>Leaf C:N Ratio</b>					
Main effects					
CO <sub>2</sub> Level	1469.3	1	1469.3	51.5	<0.0001
Nodulation	12085.2	1	12085.2	423.4	<0.0001
Interaction					
CO <sub>2</sub> x Nodulation	378.2	1	378.2	13.3	0.0008
Error	1056.1	37	28.5		
Total	16344.8	40			
<b>Leaf Water Content</b>					
Main effects					
CO <sub>2</sub> Level	202.5	1	202.5	11.3	0.0018
Nodulation	97.1	1	97.1	5.4	0.0256
Interaction					
CO <sub>2</sub> x Nodulation	3.7	1	3.7	0.2	n.s.
Error	663.6	37	17.9		
Total	970.5	40			

<sup>a</sup>Two-way analysis of variance for leaf nitrogen, carbon, C:N ratio, and water content are for pre-damage leaflets. Corresponding means are reported in Table 7.

was lowest for wild-type plants grown under ambient CO<sub>2</sub> and highest for non-nodulated plants grown under elevated CO<sub>2</sub>. Changes in C:N ratio were driven by changes in percent nitrogen (i.e. plants with higher nitrogen contents had lower C:N ratio), and occurred despite higher carbon content among wild-type plants. In addition, a significant CO<sub>2</sub> x nodulation interaction existed; the absolute increase in C:N ratio under elevated CO<sub>2</sub> was much greater for non-nodulated plants than for wild-type plants.

Elevated CO<sub>2</sub> had the greatest effect on the water content of pre-damage leaflets. Growth of soybean under elevated CO<sub>2</sub> resulted in 7.5% and 5.4% decreases in water content among wild-type and non-nodulated plants, respectively (Tables 7 and 8). Nodulation had a marginal, yet significant, effect on water content. Non-nodulated plants contained 3% more water than wild-type plants grown at ambient CO<sub>2</sub>, and 6% more water than wild-type plants grown at elevated CO<sub>2</sub>. No CO<sub>2</sub> x nodulation interaction was detected for leaf water content.

#### Effects of Mechanical Damage on Leaf Nitrogen, Carbon, and Water Contents, and C:N ratio

Mechanical damage had little effect on leaf nutritional quality. Leaf nitrogen content did not differ significantly between pre-damage and post-damage opposite leaflets for any treatment group except possibly non-nodulated plants grown at elevated CO<sub>2</sub>. Plants within this treatment group displayed a nearly significant 5% decrease in nitrogen content in response to damage (paired  $t = 2.24$ ,  $df = 7$ ,  $p = 0.060$ ; data not shown). Damage did not, however, affect the carbon content or C:N ratio of

plants within any treatment group. In addition, damage had very little effect on leaf water content. Only non-nodulated plants at elevated CO<sub>2</sub> displayed a significant 4% increase in water content in post-damage leaflets (paired  $t = 3.28$ ,  $df = 6$ ,  $p = 0.017$ ; data not shown).

### Effects of Elevated CO<sub>2</sub> and Nodulation on Leaf Phenolics and Chymotrypsin Inhibitors

#### Pre-Damage Total Phenolics

Both CO<sub>2</sub> and nodulation significantly influenced pre-damage (constitutive) levels of total phenolics in soybean leaves (Tables 7 and 9). Relative to wild-type plants, non-nodulated plants contained 26% more phenolics. Growth under elevated CO<sub>2</sub> resulted in similar increases in phenolic content of wild-type and non-nodulated plants (10.2 and 14.8 µg/mg, respectively); hence, there was no significant CO<sub>2</sub> x nodulation interaction.

#### Post-Damage Chymotrypsin Inhibitors

Post-damage levels of chymotrypsin inhibitors were significantly lower in non-nodulated than in wild-type plants (Tables 7 and 9). However, chymotrypsin inhibitor levels were not significantly affected in plants grown under elevated CO<sub>2</sub>. No significant CO<sub>2</sub> x nodulation interaction was detected for chymotrypsin inhibitors.

Table 9

Two-way Analysis of Variance for Pre-damage Total Phenolic Content and Post-damage Chymotrypsin Inhibitor Content<sup>a</sup>

Source of Variation	SS	df	MS	F	p
<b>Pre-Damage Total Phenolic</b>					
Main effects					
CO <sub>2</sub> Level	1547.3	1	1547.3	8.0	0.0074
Nodulation	11849.6	1	11849.6	61.5	<0.0001
Interaction					
CO <sub>2</sub> x Nodulation	53.5	1	53.5	0.3	n.s.
Error	7127.5	37	192.6		
Total	21473.0	40			
<b>Post-Damage Chymotrypsin Inhibitors</b>					
Main effects					
CO <sub>2</sub> Level	245.2	1	245.2	2.2	n.s.
Nodulation	22365.7	1	22365.7	200.4	<0.0001
Interaction					
CO <sub>2</sub> x Nodulation	71.0	1	71.0	0.6	n.s.
Error	4128.6	37	111.6		
Total	26709.8	40			

<sup>a</sup>Phenolic content was measured in pre-damage leaflets of leaf 3 and chymotrypsin inhibitor content was measured in post-damage leaflets of leaf 2. Corresponding means are presented in Table 7.

### Correlations Between Soybean Nutritional Quality and Chemical Defenses

Among pre-damage leaflets, levels of total phenolics were strongly positively correlated with C:N ratio and negatively correlated with nitrogen and carbon contents (Table 10). Among post-damage leaflets, chymotrypsin inhibitors were negatively

correlated with C:N ratio and positively correlated with nitrogen and carbon contents.

Neither chemical defense was significantly correlated with leaf water content.

Table 10

Correlations Between Soybean Leaf Nutritional Quality and Chemical Defenses<sup>a</sup>

Plant Chemical Defense	Leaf Nitrogen (%)	Leaf Carbon (%)	Leaf C:N Ratio	Water Content (%)
Pre-Damage Total Phenolics	m = -17.63 R = -0.768 p = <0.001	m = -9.10 R = -0.773 p = <0.001	m = 0.897 R = 0.783 p = <0.001	m = 0.691 R = 0.147 p = 0.359
Post-Damage Chymotrypsin Inhibitors	m = 22.17 R = 0.870 p = <0.001	m = 11.29 R = 0.881 p = <0.001	m = -1.05 R = -0.862 p = <0.001	m = -0.953 R = -0.181 p = 0.263

<sup>a</sup>Leaf nitrogen, carbon, C:N ratio, and water content were measured in leaf 4, pre-damage levels of total phenolics were measured in leaf 3, and post-damage levels of chymotrypsin inhibitors were measured in leaf 2. Sample size was 39-41 for each correlation.

Effects of Elevated CO<sub>2</sub>, Nodulation, and Mechanical Damage on Growth and Performance of *Pseudoplusia includens*

Overall, performance of *P. includens* larvae was most affected by the nodulation status of soybean, and to a lesser degree by CO<sub>2</sub> level and mechanical damage (Tables 11 and 12). Compared to larvae fed wild-type plants, larvae fed non-nodulated plants displayed significantly lower relative consumption rate (RCR) and nearly significantly lower approximate digestibility (AD). In addition, these larvae showed significantly lower conversion efficiencies for digested (ECD) and ingested

Table 11

Mean Values ( $\pm$  SD) for Measures of Performance for Fourth Instar *Pseudoplusia includens* Larvae Fed Soybean Grown at Ambient and Elevated CO<sub>2</sub>

Soybean Strain and CO <sub>2</sub> Level	Damage	<i>n</i>	RCR (g/g/d)	AD (%)	ECD (%)	ECI (%)	RGR (g/g/d)
Wild-type/ Ambient	pre-damage	9	3.41 $\pm$ 0.40	44.88 $\pm$ 14.45	15.63 $\pm$ 6.06	6.53 $\pm$ 1.65	0.222 $\pm$ 0.057
	post-damage	11	3.57 $\pm$ 0.88	32.00 $\pm$ 8.61	24.85 $\pm$ 12.75	7.16 $\pm$ 2.58	0.248 $\pm$ 0.062
Wild-type/ Elevated	pre-damage	12	4.34 $\pm$ 0.60	41.80 $\pm$ 16.50	17.38 $\pm$ 8.11	6.45 $\pm$ 2.22	0.278 $\pm$ 0.076
	post-damage	12	3.67 $\pm$ 1.18	39.11 $\pm$ 8.54	16.72 $\pm$ 5.53	6.71 $\pm$ 3.18	0.222 $\pm$ 0.069
Non-nodulated/ Ambient	pre-damage	8	2.95 $\pm$ 0.72	36.24 $\pm$ 15.10	0.24 $\pm$ 12.43	-0.76 $\pm$ 4.34	-0.006 $\pm$ 0.109
	post-damage	9	2.84 $\pm$ 1.05	31.66 $\pm$ 12.35	-3.87 $\pm$ 17.31	-0.40 $\pm$ 4.43	0.019 $\pm$ 0.133
Non-nodulated/ Elevated	pre-damage	12	3.79 $\pm$ 1.62	34.82 $\pm$ 12.60	-3.36 $\pm$ 11.24	-0.54 $\pm$ 2.90	0.005 $\pm$ 0.101
	post-damage	8	3.44 $\pm$ 1.35	31.97 $\pm$ 15.73	4.14 $\pm$ 14.00	-0.11 $\pm$ 5.60	0.049 $\pm$ 0.131

Table 12

Three-way Analysis of Variance for Measures of Larval Performance  
for *Pseudoplenia includens*<sup>a</sup>

Source of Variation	SS	df	MS	F	p
<b>RCR</b>					
Main Effects					
CO <sub>2</sub>	7.37	1	7.37	6.47	0.0131
Nodulation	4.72	1	4.72	4.15	0.0452
Damage	1.15	1	1.15	1.01	n.s.
Interactions					
CO <sub>2</sub> x Nodulation	0.21	1	0.21	0.19	n.s.
CO <sub>2</sub> x Damage	1.43	1	1.43	1.26	n.s.
Nodulation x Damage	0.003	1	0.003	0.00	n.s.
CO <sub>2</sub> x Nodulation x Damage	0.41	1	0.41	0.36	n.s.
Error	81.98	72	1.14		
<b>AD</b>					
Main Effects					
CO <sub>2</sub>	0.001	1	0.001	0.06	n.s.
Nodulation	0.06	1	0.06	3.76	0.0563
Damage	0.06	1	0.06	3.72	0.0576
Interactions					
CO <sub>2</sub> x Nodulation	0.003	1	0.003	0.19	n.s.
CO <sub>2</sub> x Damage	0.02	1	0.02	1.00	n.s.
Nodulation x Damage	0.01	1	0.01	0.47	n.s.
CO <sub>2</sub> x Nodulation x Damage	0.01	1	0.01	0.50	n.s.
Error	1.24	72	0.02		
<b>ECD</b>					
Main Effects					
CO <sub>2</sub>	0.0005	1	0.0005	0.04	n.s.
Nodulation	0.73	1	0.73	57.94	<0.0001
Damage	0.02	1	0.02	1.38	n.s.
Interactions					
CO <sub>2</sub> x Nodulation	0.01	1	0.01	1.13	n.s.
CO <sub>2</sub> x Damage	0.0004	1	0.0004	0.03	n.s.
Nodulation x Damage	0.003	1	0.003	0.26	n.s.
CO <sub>2</sub> x Nodulation x Damage	0.06	1	0.06	4.45	0.0383
Error	0.91	72	0.01		

Table 12--Continued

Source of Variation	SS	df	MS	F	p
<b>ECI</b>					
Main Effects					
CO <sub>2</sub>	<0.0001	1	<0.0001	0.00	n.s.
Nodulation	0.10	1	0.10	84.76	<0.0001
Damage	0.0003	1	0.0003	0.29	n.s.
Interactions					
CO <sub>2</sub> x Nodulation	0.0001	1	0.0001	0.11	n.s.
CO <sub>2</sub> x Damage	<0.0001	1	<0.0001	0.01	n.s.
Nodulation x Damage	<0.0001	1	<0.0001	0.00	n.s.
CO <sub>2</sub> x Nodulation x Damage	<0.0001	1	<0.0001	0.02	n.s.
Error	0.08	72	0.001		
<b>RGR</b>					
Main Effects					
CO <sub>2</sub>	0.006	1	0.006	0.68	n.s.
Nodulation	1.02	1	1.02	114.8	<0.0001
Damage	0.002	1	0.002	0.21	n.s.
Interactions					
CO <sub>2</sub> x Nodulation	0.0001	1	0.0001	0.02	n.s.
CO <sub>2</sub> x Damage	0.005	1	0.005	0.54	n.s.
Nodulation x Damage	0.01	1	0.01	1.41	n.s.
CO <sub>2</sub> x Nodulation x Damage	0.01	1	0.01	1.42	n.s.
Error	0.66	74	0.66		

<sup>a</sup>Corresponding means are given in Table 11.

(ECI) food. Consequently, larvae fed non-nodulated plants displayed a 94% decrease in relative growth rate (RGR) compared to larvae fed wild-type plants. CO<sub>2</sub> level had a significant effect only on RGR, which was 19% greater for larvae fed plants grown under elevated CO<sub>2</sub>. Mechanical damage had a nearly significant effect on AD, which was 16% lower for larvae fed post-damage leaflets.

No CO<sub>2</sub> x nodulation interaction was detected for any measure of *P. includens* performance (Table 12). However, it should be noted that the extremely low values of ECD, ECI, and RGR for larvae fed non-nodulated plants at both ambient and

elevated CO<sub>2</sub> may have made it impossible to detect a CO<sub>2</sub> x nodulation interaction effect for these measures of larval performance. A three-way interaction among CO<sub>2</sub>, nodulation, and mechanical damage was detected for larval ECD.

#### Effects of Leaf Nutritional Quality on Larval Performance

Surprisingly, RCR of *P. includens* was not significantly correlated with leaf nitrogen, carbon, or C:N ratio (Table 13). However, RCR was significantly negatively correlated with leaf water content. AD was nearly significantly positively correlated with leaf nitrogen content ( $p = 0.064$ ) and negatively correlated with C:N ratio ( $p = 0.057$ ), but was not correlated with leaf carbon or water content.

As expected, ECD, ECI, and RGR were significantly positively correlated with leaf nitrogen content and negatively correlated with leaf C:N ratio (Table 13). In addition, ECD, ECI, and RGR were significantly positively correlated with carbon content; however, we believe this simply reflects the fact that plants that contained more nitrogen also contained more carbon. ECD and ECI were not correlated with leaf water content. However, RGR was negatively correlated with leaf water content.

#### Effects of Soybean Chemical Defenses on Larval Performance

As described above, inferences about the effect of soybean chemical defenses on performance of *P. includens* larvae will be restricted to pre-damage levels of total phenolics and post-damage levels of chymotrypsin inhibitors. No measure of performance of larvae fed leaf 4 was significantly correlated with either pre-damage levels of total phenolics or post-damage levels of chymotrypsin inhibitors (Table 13).

Table 13

Correlations Between *Pseudoplusia includens* Larval Performance and Soybean Nutritional Quality and Chemical Defenses

	Leaf Nitrogen (%)	Leaf Carbon (%)	Leaf C:N Ratio	Water (%)	Pre-Damage Total Phenolics <sup>a</sup>	Post-Damage Chymotrypsin Inhibitors (%) <sup>a</sup>
n	81	81	81	81	41	40
RCR	m = 0.039 R = 0.035 p = 0.755	m = 0.108 R = 0.192 p = 0.088	m = -0.005 R = -0.083 p = 0.462	m = -0.088 R = -0.388 p = <0.001	m = -0.001 R = -0.025 p = 0.878	m = 0.003 R = 0.076 p = 0.646
AD	m = 0.028 R = 0.208 p = 0.064	m = 0.013 R = 0.188 p = 0.095	m = -0.001 R = -0.214 p = 0.057	m = 0.001 R = 0.036 p = 0.751	m = <0.001 R = 0.025 p = 0.877	m = 0.001 R = 0.119 p = 0.472
ECD	m = 0.096 R = 0.642 p = <0.001	m = 0.048 R = 0.641 p = <0.001	m = -0.005 R = -0.619 p = <0.001	m = -0.004 R = -0.123 p = 0.277	m = <-0.001 R = -0.067 p = 0.678	m = <-0.001 R = -0.001 p = 0.975
ECI	m = 0.034 R = 0.704 p = <0.001	m = 0.017 R = 0.684 p = <0.001	m = -0.002 R = -0.704 p = <0.001	m = -0.001 R = -0.121 p = 0.285	m = <0.001 R = 0.003 p = 0.986	m = <0.001 R = 0.018 p = 0.911
RGR	m = 0.102 R = 0.694 p = <0.001	m = 0.054 R = 0.719 p = <0.001	m = -0.005 R = -0.723 p = <0.001	m = -0.007 R = -0.240 p = 0.031	m = <-0.001 R = -0.065 p = 0.686	m = <0.001 R = 0.091 p = 0.575

<sup>a</sup>Correlations between measures of larval performance and pre-damage levels of phenolics and post-damage levels of chymotrypsin inhibitors were determined using residual values obtained from larval measure vs. % leaf nitrogen correlations.

These results indicate that neither pre-damage levels of phenolics or post-damage levels of chymotrypsin inhibitors could explain the remaining residual variation in insect performance after correlations with leaf nitrogen were performed.

## Discussion

In a wide variety of plant species, exposure to elevated atmospheric CO<sub>2</sub> decreases leaf nitrogen, increases C:N ratio, and/or lowers water content (Wong, 1979; Rogers et al., 1984; Bazzaz, 1990; Johnson and Lincoln, 1991; Lindroth et al., 1993; Lincoln et al., 1993; Tschaplinski et al., 1995; Reitz et al., 1997; Lawler et al., 1997; Karowe et al., 1997). Presumably in response to decreased plant nutritional quality, insect herbivores typically increase consumption of plants grown at elevated CO<sub>2</sub>, but nevertheless often display reduced growth (Akey and Kimball, 1989; Fajer, 1989; Johnson and Lincoln, 1991; Lindroth et al., 1993; Lawler et al., 1997; also reviews by Lincoln et al., 1993; Coviella and Trumble, 1999). In this study, we used soybean and the soybean looper to test the hypothesis that association with nitrogen-fixing bacteria buffers legumes against (i.e. minimizes or prevents) elevated CO<sub>2</sub>-induced increases in leaf C:N ratio and carbon-based chemical defenses, and decreases in leaf nitrogen and nitrogen-containing defenses, and thereby buffers legume-feeding insect herbivores against adverse effects of CO<sub>2</sub>-induced changes in host plant quality. To test these hypotheses, we calculated quantitative nutritional indices for fourth instar *P. includens* larvae fed nodulated and non-nodulated soybean grown under ambient and twice ambient CO<sub>2</sub>.

The strongest adverse effects on *P. includens* larval performance in this study were associated with lack of nodulation. Relative growth rate declined 94% when larvae were fed non-nodulated plants, due to significantly lower RCR, ECD, and ECI, and nearly significantly lower AD, relative to larvae fed wild-type plants. By itself,

elevated CO<sub>2</sub> had a much less pronounced adverse effect on *P. includens*. Relative growth rate did not differ between larvae fed soybean grown at ambient and elevated CO<sub>2</sub>, in part because larvae fed elevated-grown plants increased their consumption rates.

We did not detect any evidence that host plant association with nitrogen-fixing bacteria buffered *P. includens* against the adverse effects of plant growth under elevated CO<sub>2</sub>; i.e., we observed no significant CO<sub>2</sub> x nodulation interaction for any measure of insect performance. However, we do not believe we can confidently exclude this possibility. We believe the extremely low RGR of larvae fed non-nodulated soybean at ambient CO<sub>2</sub> (0.013) may have severely diminished the possibility of detecting a further effect of elevated CO<sub>2</sub>.

In general, though not without exception, herbivores of non-legumes are adversely affected when their host plants are grown under elevated CO<sub>2</sub> (Akey and Kimball, 1989; Fajer, 1989; Johnson and Lincoln, 1991; Lindroth et al., 1993; Lawler et al., 1997; but see, Lincoln and Couvet, 1989; Johnson and Lincoln, 1991). In contrast, among *P. includens* larvae fed wild-type (nodulated) soybean, RGR was slightly, though not significantly, higher for larvae fed plants grown at elevated CO<sub>2</sub> than for larvae fed plants grown at ambient CO<sub>2</sub>. Moreover, *P. includens* required only a modest (ca. 15%) increase in RCR to achieve equal growth on elevated-grown soybean. In contrast, non-legume feeders often display reduced growth despite increases of RCR of up to 80% (reviewed by Lincoln et al., 1993). We interpret the lack of effect of elevated CO<sub>2</sub> on growth of *P. includens* fed nodulated soybean as

modest support for the hypothesis that nodulation buffers higher trophic levels against the adverse effects of host plant growth under elevated CO<sub>2</sub>.

Typically, insect herbivores increase their consumption of plants grown under elevated CO<sub>2</sub> (Johnson and Couvet, 1989; Fajer, 1989; Johnson and Lincoln, 1990; Karowe and Gray, in preparation; also see reviews by Lincoln et al., 1993; Coviella and Trumble, 1999), presumably as a compensatory response to decreased leaf nitrogen content. Our results support these earlier findings; overall, fourth instar *P. includens* displayed an overall 18% increase in consumption of elevated-grown soybean. However, our results indicate that consumption rate of *P. includens* is not determined solely by plant nitrogen content. For instance, the observation that soybean loopers consumed significantly less of the plants that contained the least nitrogen (non-nodulated plants) contradicts the general observation that insects compensate for decreased plant nitrogen by consuming more (Scriber and Feeney, 1979; Mattson, 1980; Mattson and Scriber, 1986). Moreover, the lack of correlation between RCR and leaf nitrogen or C:N ratio, suggests that larvae fed plants low in nitrogen did not always increased consumption.

One plausible explanation for the lack of compensatory feeding on non-nodulated plants is that their extremely low nitrogen levels may have caused non-nodulated plants to be less acceptable to larvae; nitrogen levels below 1% may actually inhibit, rather than stimulate, consumption. In a study investigating the effects of leaf nitrogen content on *P. includens*, Wier and Boethel (1995) determined that non-nodulated soybean with less than 1.2% nitrogen caused 100% larval mortality. In addition, this concentration was determined to be the minimum

concentration where survival mechanisms, such as increased number of stadia, could be used by soybean looper larvae (consistent with our observation that larvae fed non-nodulated soybean essentially did not grow). Therefore, it is possible that the decrease in RCR of *P. includens* fed non-nodulated soybean in the present study reflects the response of larvae to extremely low levels of nitrogen (0.7-0.9%) in non-nodulated plants.

Alternatively, the observed decrease in RCR may have been a response to the 37% increase in total phenolics in non-nodulated plants at both ambient and elevated CO<sub>2</sub>. Plant phenolics, especially many flavonoids (e.g. catechin, rutin, glyceollin) can function as significant feeding deterrents to a number of insect herbivores (Chiang and Norris 1983; Bernays et al., 1991; Liu et al., 1993; Schoonhoven et al., 1998). Therefore, within non-nodulated soybean the level of feeding deterrents (e.g. flavonoids) was likely higher, and therefore might explain why insects fed less on these plants, despite their low nitrogen contents. However, this hypothesis does not explain the observed lack of correlation between RCR and pre-damage phenolic levels.

Finally, leaf toughness may also explain why larvae fed less on leaves from non-nodulated plants. Although we did not determine lignin concentration or overall toughness of leaves, other studies have suggested that plants with low nitrogen content (less than 2%) contain more carbon-based structural components that make leaves tougher to eat (Wheeler and Center, 1996; Lawler et al., 1997; Murakami and Wada, 1997). Therefore, the ability of *P. includens* to compensate for reduced plant

nitrogen by increasing consumption of non-nodulated plants may have been hindered by increased leaf toughness.

When compensatory feeding of low nitrogen plants under elevated CO<sub>2</sub> is not adequate, one detrimental consequence for insects is decreased RGR. For example, the grasshopper, *Melanoplus differentialis* (Johnson and Lincoln, 1991), early instar *Junonia coenia* (Fajer, 1989), gypsy moth larvae, *Lymantria dispar*, and forest tent caterpillars, *Malacosoma disstria* (Lindroth et al., 1993), all displayed reduced growth rates when fed host plants grown under elevated CO<sub>2</sub>. In addition, pupal weight was reduced and developmental time was prolonged for both *Chrysophtharta flaveola* fed eucalyptus grown under elevated CO<sub>2</sub> (Lawler et al., 1997) and armyworm larvae fed cotton seedlings grown under elevated CO<sub>2</sub> (Akey and Kimball, 1989). The grasshopper, *Melanoplus sanguinipes*, (Johnson and Lincoln, 1991) and *Spodoptera eridania* larvae (Lincoln and Couvet, 1989), however, displayed no difference in RGR when fed their host-plants grown under elevated CO<sub>2</sub>.

In contrast to non-legume feeding insects, the few existing studies on the effects of elevated CO<sub>2</sub> on legume-feeding insect herbivores have produced a greater disparity among results. For instance, consistent with the results of this study, *P. includens* larvae displayed no difference in RGR when fed nodulated soybean grown under elevated CO<sub>2</sub> (Lincoln et al., 1984; 1986). Similarly, *Colias philodice* displayed no change in pupal weight when fed elevated-grown *Trifolium pratense*, but displayed reduced digestion and lower pupal weight when fed elevated-grown *Melilotus alba* (Karowe et al., in preparation). Pupal weight was also lower for *Trichoplusia ni* fed elevated-grown lima bean (Osbrink et al., 1987). It is therefore

not yet clear how legume feeders generally will be affected by host plant exposure to elevated CO<sub>2</sub>.

We found that host plant growth under elevated CO<sub>2</sub> had no adverse effect on RGR of *P. includens*. Moreover, our results do not provide strong evidence that nitrogen fixation is capable of buffering insect herbivores against reduced growth due to elevated CO<sub>2</sub>-induced changes in plant quality. However, as previously mentioned, the extremely strong adverse effects on larval performance of removing nitrogen fixation may have seriously compromised our ability to detect a buffering CO<sub>2</sub> x nodulation interaction. Larvae fed non-nodulated plants displayed much lower ECD, ECI, and RGR compared to larvae fed nodulated plants, most likely due to the extremely low nitrogen levels (often <1%) of non-nodulated plants at both CO<sub>2</sub> levels. Therefore, we believe it would be premature to exclude the possibility that host plant association with nitrogen-fixing bacteria buffers higher trophic levels against adverse effects of plant growth under elevated CO<sub>2</sub>. In addition, we believe that our method of comparing nodulated and non-nodulated strains of the same plant species is a powerful approach for determining the ecological significance of nitrogen fixation in the face of rapidly rising atmospheric CO<sub>2</sub>. If the extreme nitrogen limitation of non-nodulated plants had been eased with higher fertilizer application, this method may have yielded more conclusive results.

Few studies have addressed the influence of elevated CO<sub>2</sub>-induced changes in leaf allelochemicals on the behavior and growth of insect herbivores. The Carbon-Nutrient Balance hypothesis (Bryant et al., 1983) predicts that carbon-based chemical defenses, such as phenolics and terpenes, will increase in plants having higher C:N

ratios (however, nitrogen-containing defenses may decrease as observed in the present study). Therefore, it is possible that, under elevated CO<sub>2</sub>, many insects may encounter additional burdens of increased carbon-based chemical defenses, along with lower nitrogen concentrations within their host plants.

Studies investigating the effects of CO<sub>2</sub>-induced changes of plant chemical defenses on insects have, however, provided equivocal results. For example, Johnson and Lincoln (1990) reported that consumption of sagebrush by two species of *Melanoplus* grasshoppers was negatively correlated with concentrations of leaf volatiles. Although concentrations of these carbon-based chemical defenses were not affected by elevated CO<sub>2</sub>, at least one *Melanoplus* species was not able to compensate for elevated CO<sub>2</sub>-induced decreases in leaf nitrogen, apparently due to its sensitivity to these allelochemicals. In contrast, Lincoln and Couvet (1989) found that *Spodoptera* caterpillars increased consumption of plant tissue grown under elevated CO<sub>2</sub>, despite increased amounts of volatile monoterpenes. Furthermore, Lincoln and Kinney (1998) found no difference in growth rates or final mass of gypsy moth larvae fed aspen or sugar maple, despite varying levels of phenolics and condensed tannins under elevated CO<sub>2</sub> and/or after defoliation. The impact of altered chemical defenses under elevated CO<sub>2</sub> on insect growth and performance therefore remains unclear.

We did not detect any evidence that soybean phenolics or chymotrypsin inhibitors influence any measure of *P. includens* performance. It is possible that, although it is a generalist, *P. includens* has adapted to these compounds. While a variety of plant phenolics have been shown to negatively affect insect herbivores, several generalist lepidopterans appear to be unaffected by these chemical defenses.

For instance, performance of the silkworm, *Callosamia promethea* (Manuwoto and Scriber, 1986) and the tree-feeding lymantriid, *Orgyia leucostigma* (Karowe, 1989), was not diminished on diets containing high levels of phenolics. Similarly, while proteinase inhibitors adversely affect many insect herbivores by stimulating the hyper-production of proteolytic enzymes within the insect gut (Broadway and Duffey, 1986), several lepidoterans, including *Pieris rapae* and *Helicoverpa zea*, were not adversely affected by the proteinase inhibitors of their host plants (Broadway, 1996). These insects secrete “inhibitor-resistant” proteases that are not inhibited by the proteinase inhibitors of their host plants (Broadway, 1996). It should not be too surprising, therefore, that performance of *P. includens* is not clearly influenced by the phenolic or proteinase inhibitor content of soybean.

Measuring plant chemistry within the same leaf (or leaves) that is used for an individual feeding trial is a powerful approach to evaluate correlations between plant nutritional quality, chemical defenses, and insect performance. For example, Fajer (1989) determined leaf nitrogen, water, and iridoid glycoside contents within the same leaves that were used for individual *Junonia coenia* feeding trials. Therefore, Fajer (1989) was able to conclude with greater than usual confidence that leaf nitrogen content, but not leaf water or iridoid glycoside contents, was most likely responsible for modifications in feeding behavior and differences in growth rates for larvae fed elevated CO<sub>2</sub>-grown plants. However, despite its obvious advantages, this approach has rarely been used, most likely due to limitations imposed by leaf size.

Unfortunately, limited leaf material precluded us from conducting all chemical analyses and feeding trials using a single leaf and/or leaflet. Instead, we

estimated pre-damage and post-damage concentrations of chemical defenses in leaf 4, which was fed to larvae, by measuring levels of defenses in leaves 2 and 3. We also performed inter-leaf correlations to check the assumption that phenolic and proteinase inhibitor levels measured in leaves 2 and 3 accurately reflect levels in leaf 4.

Surprisingly, constitutive levels of both proteinase inhibitors were not correlated between leaves, and levels of all chemical defenses displayed significant differences in concentrations among leaves. In addition, we also found differential induction of both trypsin inhibitors and total phenolics among leaves 2, 3, and 4. These results raise questions concerning conclusions about plant-insect interactions that are based on plant chemistry measured in leaves other than those used for feeding trials. In particular, leaves among many herbaceous plants can differ greatly in chemical composition and morphology due to their smaller size and shorter developmental time.

Mechanical damage had very little effect on *P. includens* larval performance. We observed only one consequence of damage: larvae digested damaged plants significantly less efficiently. However, this decrease in AD did not appear to affect insect growth. In addition, a significant three-way interaction among CO<sub>2</sub>, nodulation, and mechanical damage was also detected for ECD, suggesting that the effects of mechanical damage depend on CO<sub>2</sub> level and nodulation status. To our knowledge, only two other studies have addressed the combined effects of elevated CO<sub>2</sub> and mechanical damage on insect performance. Lindroth and Kinney (1998) and Roth et al. (1998) evaluated the effects of elevated CO<sub>2</sub> and defoliation on the larval performance of gypsy moths, *Lymantria dispar* (Lindroth and Kinney, 1998) and

forest tent caterpillars, *Malacosoma disstria* (Roth et al., 1998) fed aspen and maple. Exposure of aspen to elevated CO<sub>2</sub>, but not defoliation, resulted in lower larval growth rates and final larval weights for both insect species. However, Roth et al. (1998) found that both elevated CO<sub>2</sub> and defoliation affected the growth of forest tent caterpillars fed maple; larvae fed defoliated maple displayed decreased consumption and digestive efficiency. In contrast to Roth et al. (1998), neither CO<sub>2</sub> level nor defoliation affected growth of gypsy moth larvae fed maple (Lindroth and Kinney, 1998).

One final point worth mentioning is that, under elevated CO<sub>2</sub>, increased larval developmental time, particularly coupled with increased consumption, may dramatically increase the probability that larvae will be detected and killed by natural enemies (Karowe, unpublished data). Volatiles from damaged leaves serve as cues for gravid females of many parasitoid species (Pivnick 1993; Steinberg et al., 1993). Therefore, increased leaf damage by foliage-feeding insects, coupled with increased exposure time, may make many insect herbivores more susceptible to natural enemies in a CO<sub>2</sub>-enriched environment (reviewed by Coviella and Trumble, 1999).

We are only beginning to understand the possible effects of global atmospheric changes, including elevated CO<sub>2</sub>, on ecosystems and ecological processes. Over the past two decades, it has become clear that a number of changes in plant chemistry and physiology occur when plants are exposed to elevated CO<sub>2</sub>, and that CO<sub>2</sub>-induced changes in plant quality often result in reduced performance of higher trophic levels, including insect herbivores. However, these effects of elevated CO<sub>2</sub> are by no means universal among plant-herbivore systems. In particular, it is

plausible that the unusual association of legumes with nitrogen-fixing bacteria may cause them, and therefore their herbivores as well, to be less affected by rising atmospheric CO<sub>2</sub>. Although we found only weak evidence that association with nitrogen-fixing symbionts “buffers” legume-based systems against the typical effects of elevated CO<sub>2</sub>, we believe it would be premature to reject this hypothesis, particularly given the tremendous ecological and economic importance of legumes.

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