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A. Summary of Research Progress

1. Effects of Loblolly Pine grown under elevated CO₂ on pine sawfly performance

Two separate experiments will be reported: (1) using early (2nd) instar larvae on plants with four levels of CO₂ with no nutrient limitation, and (2) using penultimate instar (5th) larvae fed plants grown under four CO₂ and two nutrient levels in two feeding trials from plants from two different seasons, spring and fall. For each experiment the CO₂ plant growth conditions were the same: pine seedlings were grown in open-topped chambers under three levels of CO₂: (1) ambient (2) 150 :L/L CO₂ above ambient (+150) and (3) 300 :L/L above ambient (+300). A fourth treatment, non-chambered ambient (NC) was used to assess chamber effects. Nutrient treatments were either low nitrogen or nutrient unlimited. Performance of larvae was determined by calculating the relative growth and consumption rates, as well as the digestibility of the larvae. Leaf samples were taken to determine leaf nitrogen, starch, and water contents.

A. Response of Susceptible Young Insects

Although similar to other feeding trials, this experiment used second instar larvae because early instar insects are widely considered to be more sensitive to plant chemical composition. Larvae responded strongly to CO₂ growth conditions in terms of relative consumption, which substantially increased as CO₂ levels rose. Larvae feeding on plants in the +300 treatment consumed approximately 18% more leaf biomass than those feeding on ambient grown plants. This response can be explained, in part, by changes in the leaf nitrogen content and starch:nitrogen ratio. Leaf nitrogen content significantly declined while the starch:nitrogen ratio significantly increased as CO₂ levels increased. This illustrates a clear compensatory feeding response to lower leaf nutritional quality. The starch:nitrogen ratio resulted from both a substantial increase in leaf starch content in the +300 treatment and by a lower quantity of leaf nitrogen in the +150 leaves, which were, surprisingly, similar in starch content to ambient grown plants. Also strongly related to the insect consumption rate was a significant decline in leaf volatile terpenes as CO₂ concentration increased. Total monoterpene content declined by 39% in plants grown at the +300 :L/L CO₂ treatment, and other compounds followed similar trends. Given their presumed role in insect defense, this reduction is an important CO₂ response which has been demonstrated here for the first time. Since the leaf volatile terpene content demonstrated strong chamber effects in most compounds (i.e. NC different than AMB), while leaf nitrogen did not, and relative consumption levels were similar between NC and AMB treatments, it appears leaf nitrogen may be a better predictor of insect feeding behavior.

Although consumption increased on elevated CO₂ grown plants, the relative growth rate of larvae was not different between treatments. This may be partially explained by the nitrogen economy of the larvae. The nitrogen consumption rate of larvae significantly declined in the elevated CO₂ treatments, in spite of higher biomass consumption, due to the severe nitrogen deficit in elevated CO₂ grown plants. In spite of this lower nitrogen intake, the nitrogen accumulation rate of larvae was not different between treatments. This is due to a significant increase in the nitrogen utilization efficiency of the larvae. Therefore, larvae maintained similar growth rates, in spite of lower nitrogen ingestion, by increasing their ability to utilize nitrogen.

The results of this study are currently in press (see Appendix 3).

B. Seasonality of CO₂ Effects

Red headed pine sawfly has a spring and fall generation in the Southeastern U. S. Hence, seasonal changes could lead to different responses to carbon dioxide fertilization. For example, changes in the nutritional and chemical compositions may differentially affect sawfly performance in different seasons. The spring feeding trials were conducted in May and the fall trials in October. Mature needles from the current years growth were used for the feeding trials and chemical analysis.

In the spring, leaf nitrogen content significantly declined with increasing CO₂ concentration, with no main nutrient effect, but a significant CO₂ X nutrient interaction. Both the low nitrogen and nutrient unlimited plants grown at elevated CO₂ had lower leaf nitrogen contents. Interestingly, the overall highest levels were found in the low nitrogen nutrient treatment. A step-wise reduction in leaf nitrogen as CO₂ levels increase, as would be predicted with carbohydrate dilution, was observed in the nutrient unlimited plants. Leaf nitrogen levels stimulated a compensatory increase in consumption by larvae, but only in the low nitrogen plants.

In the fall, the nitrogen dynamics in the plants at this late season period were much different than from the spring experiment. Overall nitrogen contents were much higher for the nutrient unlimited plants, which showed a significant step-wise reduction as CO₂ levels rose. Contrary to the spring needles, the low nitrogen plants responded most strongly to nutrient levels, not CO₂ conditions. That is, even though leaf nitrogen contents were lower on the elevated CO₂ grown plants, there were no significant differences between any treatment. Relative consumption followed a similar response to nutrient treatments as did leaf nitrogen. On plants grown without nutrient limitation there was a strong, compensatory increase in consumption, presumably due to lower leaf nitrogen content in these needles, while on the low nitrogen plants consumption rates were similar regardless of CO₂ regime. Thus, significant differences were observed between seasons in the response to carbon dioxide enrichment.

C. Fecundity and Mortality of Red Headed Pine Sawfly

At the Duke University site, we have extended our study to a longer term experiment examining elevated CO₂ and soil nutrient levels on the loblolly pine. The objective of this study was to assess possible CO₂ effects on herbivore population size, as well as consumption and growth. We conducted a field experiment to examine the effects of the loblolly pine growth conditions on the developmental rate and fecundity of an important insect pest of this tree species, the red-headed pine sawfly. Very young insects were bagged directly on plants within the chambers and allowed to go through their larval life cycle. Using the final weight of insect pupae and numbers of eggs per females we will be able to assess potential population level effects on the insects. Leaf samples collected during the experiment will allow us to quantify key components of the leaf to evaluate their role in effects on the insects. These include leaf nitrogen, starch, soluble sugars, and defensive monoterpenes.

2. Response of Two Lepidopterans to White Oak under Elevated CO₂

At Oak Ridge National Laboratory, we conducted a series of experiments in collaboration with Dr. R. Norby. Feeding experiments were conducted to examine how white oak, in its fourth growing season of elevated CO₂ growth conditions, may affect two widespread and important insect pest of this species; gypsy moth and forest tent caterpillar. Several feeding experiments

examining the responses of both insects at different ages, as well as an examination of long term effects on gypsy moth fecundity and developmental rates, were conducted. As above, leaf samples were collected for chemical analysis. In addition, detailed analysis on the ability of the insects to utilize available nitrogen will be carried out to examine how insects may adjust to alterations in leaf chemistry due to growth conditions. These chemical analysis are also presently ongoing. The field portions of these studies have been completed and much of the data analysis of the consumption and growth information has been completed.

3. Source/Sink Control of Allelochemical Production

The primary experimental objective of this portion of the research is to measure the regulation and activity of a key enzyme in polyphenol production to evaluate sink strength in a defined biosynthetic pathway. Our goal is to measure several components of sink strength, including gene regulation and catalysis. In addition, these results will allow us to draw strong mechanistically based inferences about partitioning of carbon under elevated CO₂ conditions.

Our measure of gene regulation under elevated carbon dioxide is to assess the level of messenger RNA coding for the key enzyme chalcone synthase (CHS). The isolation of chalcone synthase mRNA from pine needles is interfered with, perversely, by the very polyphenol products of the pathway. These compounds are well known to bind with proteins, but also bind onto other polymers such as nucleic acids. We have used the traditional methods of isolation of RNA with little success. We have recently been successful using a procedure established for another conifer which adopts techniques used to allow enzyme isolation from polyphenol-containing leaves. We are currently isolating a homologous probe directly from Loblolly Pine. The sequence of the CHS mRNA from the European pine, *P. sylvestris*, has recently been published. Using this sequence, we have had constructed oligonucleotide primers which will allow us to produce a homologous probe from a total RNA extract of Loblolly Pine using the polymerase chain reaction. Needles from ambient and elevated grown plants have been harvested in conjunction with the insect feeding trials and are stored at -80E.

In addition to loblolly pine we will assess chalcone synthase activity in french bean. We have obtained from another laboratory a probe for the chalcone synthase mRNA derived from french bean and transformed the insert-containing plasmid into a bacterium. The probe was amplified by growth of the bacteria on Amp containing media, separation of plasmid DNA, and isolating the plasmid at 7.4kb on the gel. The plasmid DNA was cut with appropriate restriction enzymes and the 1.4kb insert eluted from a gel. The probe was radioactively labeled with ³²P by nick translation. The probe was successful in detecting CHS mRNA from bean.

We have established the procedure for assaying the activity of the chalcone synthase enzyme. The activity is determined by measuring the production of naringenin by HPLC. Our instrumentation is sensitive enough to measure approximately 2-5 pmol or about 2 ng. We are currently purifying one of the substrates of chalcone synthase for the assay, coumaroyl-CoA. We have also established the procedure for assaying the quantity of an inducible chalcone synthase-catalyzed antimicrobial compound, phaseollin, of bean by HPLC. This assay is also sensitive enough to complement the assays on gene activity and enzyme activity, i.e. all can be accomplished from a single leaf. Thus, we are able to measure gene activity, enzymatic catalysis and product accumulation under elevated carbon dioxide conditions. This will allow us to make strong inferences about sink intensity, carbon partitioning, and plant defenses under elevated carbon dioxide.

Two plant growth chambers with CO₂ control are now available at the University of

South Carolina and we are currently growing bean plants under ambient and elevated CO₂ to be used for this experiment.

This study has been complete and a manuscript prepared (see Appendix 4).

4. Growth and reproduction of grasshoppers feeding on a C₄ grass under elevated carbon dioxide

Previous examinations have concentrated on the consumptive responses of insect to the changed characteristics of plants growing under elevated CO₂. However, the influence of CO₂ on insect herbivores and herbivory, may be more profound through effects on reproduction than solely on consumption. In addition, experiments examining CO₂ effects throughout life cycles are needed.

This experiment determined the effect of elevated CO₂ on the reproduction, food consumption, growth, and mortality of two orthopterans: Melanoplus sanguinipes and M. differentialis when maintained throughout their lifecycle at 350 ml/l CO₂ and 650 ml/l CO₂ and fed the C₄ grass Andropogon gerardii grown at these two CO₂ levels. The carbon: nitrogen ratio of A. gerardii was significantly higher under elevated CO₂. The size at adulthood of both species was lower under elevated CO₂. The fecundity of M. sanguinipes was reduced significantly at elevated CO₂, but M. differentialis failed to reproduce in either treatment. Biomass consumption was higher for M. sanguinipes at elevated CO₂, but relative nitrogen consumption rate was lower. There was no detectable effect of CO₂ treatment on the final survivorship of M. sanguinipes. These results suggest that leaf feeding insects may have reduced reproduction as well as increased consumptive responses under expected elevated atmospheric CO₂ conditions.

This study has been completed and a manuscript prepared (see appendix 5)

5. Elevated carbon dioxide and temperature effects on growth and defense of Big Sagebrush

The interactive effects of elevated CO₂ with jointly varying physical factors is important for our understanding of how CO₂ effects may occur. Projected changes in CO₂ are presumed to be accompanied by altered climates, particularly elevated temperatures and perhaps altered moisture regimes.

This experiment was conducted to examine the joint effects of elevated carbon dioxide and temperature, as well as moisture, treatments on the growth and phytochemical production of Big Sagebrush, Artemisia tridentata. Increasing the atmospheric carbon dioxide concentration from a current ambient 350 ml/l up to 650 ml/l led to significantly increased root, shoot and total biomass. On the other hand, shoot and root biomass, as well as total plant biomass, plant height and average area of leaves, decreased in the 5° elevated temperature treatment (37°/30° versus 32°/25° day/night). Further, root, shoot and total plant biomass showed significant interactions between CO₂ and temperature treatments. The water supply treatments (once daily versus twice daily) did not affect biomass growth and significantly decreased only leaf water content. Leaf nitrogen content was significantly reduced in elevated CO₂ and there was a significant interaction between CO₂ and temperature effects. The temperature and water treatments also resulted in a significant interaction effect for the leaf nitrogen concentration. Surprisingly, the total elemental carbon content also decreases as the carbon dioxide concentration increased.

Increased temperature led to a significantly decrease in total leaf volatile mono- and sesquiterpene content. As temperature increased, the concentration of leaf sesquiterpene lactones also increased. Carbon dioxide and temperature treatments also resulted in a significant interaction effect on leaf sesquiterpene lactones concentration. In addition, the combination of carbon dioxide and water treatments statistically had a significant effect on leaf coumarin content.

From this experiment, it is apparent that increasing atmospheric carbon dioxide concentration can lead to an increase in plant yield or biomass, at least when other stresses do not limit plant growth. The growth reduction from elevated temperature may be, at least partly, ameliorated by elevated CO₂ conditions. On the other hand there is also a decline in the quality of leaf nutrients or nitrogen content under elevated CO₂. The changes in production of leaf secondary metabolite may ultimately contribute to change dynamics of other organism and the energy flow among organism among current and potential future environments.

This study has been completed and a manuscript prepared (see appendix 6).

6. Biomass Allocation Patterns of Defoliated Sagebrush Grown Under Two Levels of Carbon Dioxide

We grew sagebrush (*Artemisia tridentata*) plants under projected (650 uL/L) and ambient (350 uL/L) levels of CO₂. One half of the plants in each CO₂ treatment were subjected to three episodes of 40% defoliation. Carbon dioxide enrichment did not significantly affect leaf volatile terpene yield, although defoliated plants from both CO₂ regimes exhibited lower mean yields ($P < 0.06$). Defoliation and carbon dioxide enrichment reduced leaf nitrogen concentration, with the lowest mean concentration being found in the defoliated-high CO₂ grown plants. Defoliation decreased shoot biomass by 33% under ambient conditions, but shoot biomass was reduced by only 16% under the projected CO₂ conditions. The projected rise in atmospheric carbon dioxide may facilitate greater plant tolerance of chronic herbivory primarily thru enhanced regrowth and poorer leaf nutritional quality to subsequent herbivores.

This study has been completed and a manuscript submitted (see Appendix 7).

7. Response of a Grass-Fungal Endophyte Mutualism to Elevated Carbon Dioxide

Previous studies have shown that insects commonly consume more when fed leaf tissue grown under CO₂ enrichment, but with few negative effects on growth. On the other hand, lepidopteran larvae fed tissue infected with *Balansiae* fungal endophytes (which produce toxic alkaloids) typically eat less but also suffer negative effects on growth and survival. This study was carried out to understand how these 2 factors may interact to affect larval consumption and growth in fall armyworm, *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae). Infected and uninfected ramets of a single genotype of tall fescue (*Festuca arundinacea* Schreb.) were grown under CO₂ concentrations of 400 and 700 mliter CO₂/liter of air. Relative consumption of leaf tissue by larvae was 32% greater in the high CO₂ treatment compared with leaves grown under low CO₂ concentrations, but was not influenced by infection.

As expected, larvae had significantly decreased relative growth rates when fed infected tissue, with their growth rates somewhat increased under high CO₂ levels. Increased CO₂ level and infection both led to significantly reduced efficiency of conversion of ingested food. These 2 factors also interacted so that the lowest efficiency of conversion of ingested food was seen when

both infection and an enriched atmospheric CO₂ environment were present. As global atmospheric CO₂ levels continue to increase, it appears that fungal endophytes will continue to be important in turfgrasses as protection against insect herbivores and may lead to increased fitness for infected plant genotypes.

This study has been completed and a manuscript has been submitted (see Appendix 8).

B. Human Resources

1. Graduate students Associated with Project

Ying Liu, M.S. Degree, December 1994

The Response of Plant Secondary Metabolism Under Enriched Carbon Dioxide and Wounding

Ray S. Williams, Ph.D. Degree, December 1995

Responses of Leaf Feeding Insects and Their Host Trees to Enriched Carbon Dioxide Atmospheres

Sandra L. Johnson, Ph.D. expected December 1996

The Impact of Herbivory on Allocation Responses to Elevated Carbon and Nitrogen Availability by *Heterotheca subaxillaris*

2. Post-doctoral Research Associate

Kaiping Han. (June-August) 1994. Response of Loblolly Pine Allelochemicals to Elevated Carbon Dioxide.

3. Summer Research Associate

Dr. Susan Marks, Assistant Professor, Winthrop College, Summer 1994