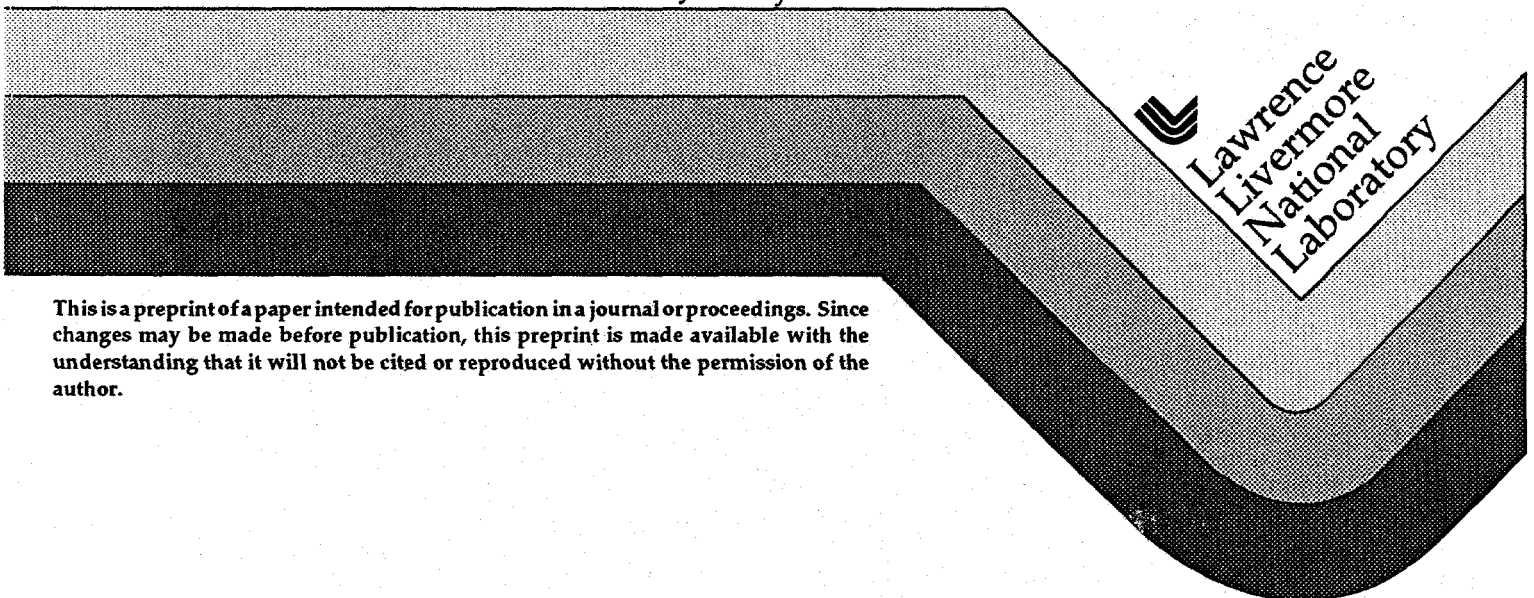


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Sierra-Nevadan Dominant Species:
*Pinus Ponderosa***

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THE EFFECT OF ELEVATED CARBON DIOXIDE ON A SIERRA-NEVADAN DOMINANT SPECIES: *Pinus ponderosa*.

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Abstract

The impact of increasing atmospheric CO₂ has not been fully evaluated on western coniferous forest species. Two year old seedlings of *Pinus ponderosa* were grown in environmentally controlled chambers under increased CO₂ conditions (525 $\mu\text{L}\cdot\text{L}^{-1}$ and 700 $\mu\text{L}\cdot\text{L}^{-1}$) for 6 months. These trees exhibit morphological, physiological, and biochemical alterations when compared to our controls (350 $\mu\text{L}\cdot\text{L}^{-1}$). Analysis of whole plant biomass distribution has shown no significant effect to the root to shoot ratios, however needles subjected to elevated CO₂ exhibited an increased overall specific needle mass and a decreased total needle area. Morphological changes at the needle level included decreased mesophyll to vascular tissue ratio and variations in starch storage in chloroplasts. The elevated CO₂ increased internal CO₂ concentrations and assimilation of carbon. Biochemical assays revealed that ribulose-bisphosphate carboxylase (RuBPCase) specific activities increased on per unit area basis with CO₂ treatment levels. Sucrose phosphate synthase (SPS) activities exhibited an increase of 55% in the 700 $\mu\text{L}\cdot\text{L}^{-1}$ treatment. These results indicate that the sink-source relationships of these trees have shifted carbon allocation toward above ground growth, possibly due to transport limitations. This research is funded by the Western Regional Center of the National Institute for Global Environmental Change (WESTGEC) W/GEC 92-037.

Introduction:

Atmospheric CO₂ concentrations are expected to double during the next century. These projected increases and associated alterations in climate will potentially have substantial impacts upon forest ecosystems. The importance of forests and their interactions with climate are considerable. Forests occupy 22 percent of the earth's land area (excluding polar regions), while accounting for two-thirds of terrestrial photosynthesis (Waring and Schlesinger 1985). The extent of the impact associated with elevated CO₂ concentrations on forest biomass accumulation (carbon sinks) remains uncertain, due to differential species and genotypic responses and the level of habitat optimization (irradiance, temperature, moisture, and nutrient availability).

It is well documented that increasing the ambient level of CO₂ concentration enhances growth and increases dry matter of many plants experiencing otherwise ideal conditions (Kramer, 1981) and is a widely employed practice in the horticultural industries. The growth enhancement results from adaptations which optimize photosynthetic carbon acquisition and allocation (Acock and Allen, 1985; Eamus and Jarvis, 1989). These adaptive responses can occur at several levels of

biological organization: biochemical (Van Oosten *et al.*, 1992), physiological (Tissue *et al.*, 1993), anatomical (Thomas and Harvey, 1983) and morphological (Radoglou and Jarvis, 1990). Coordinate changes at these self-integrating organizational levels might serve to increase the fitness of organisms acclimating to elevated CO₂. Perennial plants, particularly longer-lived species such as trees, have the opportunity to adjust to the altered environment by amassing adaptive responses through long-term exposures.

The potential for increased carbon assimilation associated with elevation of CO₂ in the atmosphere could, however, be restricted by limitations imposed in biochemical and physiological processes. Several investigations have demonstrated that following an initial enhancement of carbon assimilation, a reduction in growth rate and assimilation occurs, referred to as acclimation (Brown, 1991, Yelle *et al.*, 1991, Spenser and Bowes, 1986; Surano *et al.*, 1986). The principle physiological and biochemical mechanisms individually suggested as feedback regulatory points for carbon assimilation are: 1) stomatal limitations, decrease in stomatal conductance associated with increases in leaf internal CO₂ concentrations (C_i); 2) alterations in the activity level or content of ribulose-1,5-bisphosphate carboxylase (RuBPCase); and 3) the accumulation of high levels of primary photosynthate within the chloroplast as starch resulting in the disruption of organelle integrity.

Additionally, changes in leaf anatomy might also contribute to either up or down regulation of photosynthetic carbon assimilation. Several anatomical responses to elevated CO₂ have been previously reported (Conroy *et al.*, 1986, Mousseau and Enoch, 1989) which include increases in leaf area, mesophyll areas, larger cells and increased internal leaf airspaces. These structural changes would all serve to enhance photosynthetic processes. Changes in the opposite direction might be viewed as contributing to down-regulation or to balancing processes which might serve to maintain normal growth patterns. These growth responses observable at the whole plant and leaf structural level would be dependent upon the genetic background.

None of these mechanisms have individually been shown to account for the observed data, suggesting that they should not be viewed as independent regulatory mechanisms. Since these physiological processes are biochemically linked through metabolism, each component serving in turn as a sink then as a source for a subsequent processes, it is logical then to examine the relative activities of selected carbon sources as they correlate with intermediate (carbon allocation patterns) and long-term carbon sinks (biomass accumulation and carbon reserves).

The following conceptual control model emerges as a controlling process for carbon accumulation which is regulated but not limited overall by a series of self-integrating source/sink feedback loops (Fig. 1). The salient points in this coordinating control model are: 1) stomatal and mesophyll limitations to carbon assimilation; 2) biochemical limitations, changes in the levels and activities of carboxylation, starch synthesis and sucrose synthesis; 3) carbon allocation to biomass and carbohydrate storage.

These studies address the proposed underlying biochemical and structural feedback loops which may be involved in the regulation of the observed variable carbon assimilation responses of forest species to elevated CO₂. The results are being employed to evaluate the proposed mechanistic control model, which might be of generalized value in predicting future plant performance.

Methods and Materials:

Plant Material and Growth Conditions

The plant material used in this study was open pollinated, 2-year old *Pinus ponderosa* seedlings. The parent populations for the species are located at mid-elevation (ca. 1500 m) in the central Sierra Nevada of California. The seedlings were grown in the standard growth medium of the U.S. Forest Service Chico Tree Improvement Center (45% peat, 45% vermiculite, 10% perlite by volume). The pot volume, 18 L was large enough that pot restrictions on carbon sink size (Thomas and Strain 1991, Arp 1991) did not occur during the study period.

The seedlings were acclimated to growth chamber conditions for six months prior to the initiation of elevated CO₂ treatments. The potted seedlings were grown in eight controlled environment chambers (Environator Corp.), each equipped with eighteen high output fluorescent tubes and ten incandescent bulbs providing 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (photosynthetically active radiation) at canopy level. The light regime was stepped to prevent sudden irradiance induced water stress. A 16 hour photoperiod was broken into 30 min. per dawn/dusk with incandescent bulbs only, 2 hours per dawn/dusk at one-half maximum irradiance and 11 hours full irradiance. Chamber air mixing was accomplished by internal fans providing air flow of at least 0.5 m s^{-1} . All plants received a complete "time-release fertilizer" treatment and were maintained at approximately 20% soil moisture.

CO₂ Treatments

The three CO₂ treatments were 350 $\mu\text{L}\cdot\text{L}^{-1}$ CO₂, 525 $\mu\text{L}\cdot\text{L}^{-1}$ CO₂ and 700 $\mu\text{L}\cdot\text{L}^{-1}$ CO₂. Constant CO₂ concentrations within the growth chambers were maintained using a data acquisition system and controller (DAS) to control a set point solenoid which was used to automatically inject bottled CO₂ (to increase the atmospheric CO₂ concentration within the chamber) or circulate the atmosphere of the growth chamber through a soda lime CO₂ scrubbing column (to decrease the CO₂ concentration within the chamber).

The concentrations of CO₂ in the growth chambers were monitored using a dedicated CO₂ analyzer (Horiba Model PIR-2000). Chamber atmospheres were sampled 12 times per hour at canopy height, at the center of the chamber, for approximately 1 minute, and the measures of CO₂ concentration were averaged over the 1 minute sampling period. The DAS used data from the ambient CO₂ analyzers to open or close the set-point solenoids to maintain CO₂ concentrations within $\pm 5\%$ of the treatment concentration. Fumigation was continuous throughout the exposure period.

All analyzers were zeroed and span checked daily and had a complete multipoint calibration every month. Carbon dioxide analyzers were calibrated with Scripts-traceable bottled standard gases.

Growth Measurements

Growth measurements were made on a weekly basis and included assessments of stem length measured from the cotyledon scar to the shoot apex, stem caliper measured at the cotyledon scar in two separate orientations. Root to shoot ratios, total biomass and specific leaf masses were measured at periodic harvests.

Physiological Measurements

Gas exchange measurements were made using a system comprised of a temperature controlled cuvette (DDG-9920, Data Design Group, La Jolla, CA) and an infra-red gas analyzer (Li-6250, Licor, Inc., Lincoln, NB). Temperature of the cuvette was maintained at a setpoint value of 22°C using a temperature controller (CN9111, Omega Engineering, Inc., Stamford, CT). Vapor pressure deficit within the chamber was constantly maintained using a dew-point generator (Licor Inc., Lincoln, NB). Photosynthetically active radiation was supplied at 700 $\mu\text{mol m}^{-2} \text{s}^{-1}$ using a Q-Beam light source.

Gas exchange analyses were repeated three times during the study in conjunction with periodic sampling for enzyme and starch assays.

Ribulose-1,5-bisphosphate Carboxylase

Ribulose-1,5-bisphosphate carboxylase (E.C. 4.1.1.39) was extracted by the procedures of Tissue *et al.* 1993. The needles were frozen in liquid nitrogen then homogenized in a buffer, containing 100 mM HEPES (pH 7.5), 10 mM MgCl_2 , 5 mM EDTA, 0.7% polyethylene glycol, 14 mM β -mercaptoethanol and 2% polyvinylpyrrolidone. The homogenate was centrifuged in a microcentrifuge 9000g for 30s, then the supernatant was rapidly frozen with liquid nitrogen until spectrophotometrically analyzed by the procedures of Van Oosten *et al.* 1992 employing a Hewlett Packard Diode Array spectrophotometer equipped with a thermo-regulated cuvette holder at 22°C.

Starch Measurements

Starch content of needles was quantitatively determined by enzymatic hydrolysis according to the procedures of Haissig and Dickson (1979). Needles were frozen and ground into a fine powder then triple extracted with methanol:chloroform:water (12:5:3 by volume). The pellets were enzymatically digested with α -amylase to completely hydrolyze the starch to glucose. The liberated glucose was subsequently quantified by the activity of glucose oxidase (E.C. 1.1.3.4) coupled with the reduction of o-dianisidine dihydrochloride chromophore which exhibits an absorbance maximum at 450 nm following an incubation of 30 min at 30°C. Glucose concentrations were determined by comparison with a standard curve.

Sucrose Phosphate Synthase

Sucrose phosphate synthase (SPS, E.C. 2.3.1.14) was extracted by the procedures of Kalt-Torres, *et al.* (1987) with the following modifications. Frozen needle tissue was homogenated with 50 mM HEPES-NaOH (pH 7.5), 10 mM MgCl_2 , 1 mM EDTA, and 2.5 mM dithiothreitol. The extract was filtered through cheesecloth and then centrifuged at 25,000 g for 10 min. The supernatant was subsequently passed through a G-25 gel exclusion column. Collected fractions were analyzed for SPS activity. SPS activity was measured spectrophotometrically using the continuous assay of Kerr *et al.* (1987). This assay is based on a coupled enzyme reaction which can be followed continuously at 340 nm. The reaction mixture contained 10 mM

fructose-6-phosphate, 28 mM UDP-glucose, 10mM glucose-6-phosphate (as an activator), 11 mM MgCl₂, 0.4 mM phosphoenolpyruvic acid, 0.15 mM NADH, 1.0 mM ATP, 10 Units of lactate dehydrogenase, 4 Units nucleotide diphosphate kinase, and enzyme extract. Prior to initiation of the reaction samples were thermally equilibrated at 22°C.

Morphological Measurements

Microscopy

Transmission electron microscopy (TEM) was utilized in the evaluation of starch accumulation and the associated disruption of chloroplast structure and function. Light microscopy (LM) examinations were coupled with digital analysis for quantification of alterations in leaf anatomy. These studies focused on the extent to which leaf structural sinks (percent leaf represented by mesophyll, parenchyma and vascular tissues) are influenced by elevated CO₂.

In conjunction with periodic physiological and biochemical sampling, needles were harvested, immediately diced into small pieces and immersed into cold (4°C) primary fixative, 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2). Following primary fixation, secondary fixation in 2% OsO₄, and ethanol dehydration, the tissues were processed for TEM, and LM.

LM and TEM tissues were embedded in a Spurr's/Epon resin mixture, and viewed using Reichert Diapon light microscopes or Hitachi S-300 transmission electron microscope.

Morphometry

Morphometry was used to quantify changes in leaf structure and starch content coinciding with changes in physiological and biochemical performance. One μm cross-sections of leaves/needles were studied by microscopy to quantify the cross-sectional area and cellular structure of the photosynthetic tissues. Resulting micrographs were digitally encoded for quantification and statistical analysis of ultrastructure using a computer-based morphometry package (Global Lab Image).

Results:

Ponderosa pine seedlings grown at elevated CO₂ under otherwise identical conditions exhibited distinct changes in growth patterns. Increases in stem elongation of 20 and 25% at 525 $\mu\text{L}\cdot\text{L}^{-1}$ and 700 $\mu\text{L}\cdot\text{L}^{-1}$ CO₂, respectively, were observed when compared with ambient CO₂ growth. No CO₂ effect was seen on changes in stem caliper (Table 1). Growth at elevated levels of CO₂ exhibited a trend toward lower root/shoot ratios but were not statistically significant (Table 1).

Examination of the above ground biomass revealed that a decrease in total leaf area of 33% and 47% at 525 $\mu\text{L}\cdot\text{L}^{-1}$ and 700 $\mu\text{L}\cdot\text{L}^{-1}$ CO₂ growth levels, respectively. Analysis of the foliar tissues showed an increase in specific leaf mass (gDW/cm² needle) of 14% at both 525 $\mu\text{L}\cdot\text{L}^{-1}$ and 700 $\mu\text{L}\cdot\text{L}^{-1}$ CO₂ (Table 1). A portion of this increase could be attributed to starch accumulation, the remainder to structural changes. Detailed examination of the needle anatomy and cellular distributions of immature expanding (sink) needles at all CO₂ growth levels did not display changes in needle cross-sectional area, percentage of cross-section area occupied by mesophyll or vascular tissues. However, similar examinations of the mature (source) needles revealed an increase in cross-sectional area, an 8% reduction in mesophyll tissues and a 4% increase in total vascular area at both 525 $\mu\text{L}\cdot\text{L}^{-1}$ and 700 $\mu\text{L}\cdot\text{L}^{-1}$ CO₂ (Table 2). These results

taken collectively suggest that acclimation of mature photosynthetic tissues and changes in internal allocation of fixed carbon are occurring with prolonged exposure to increased CO₂.

Physiologically, the plants grown at elevated CO₂ exhibited substantial increases in instantaneous levels of CO₂ fixation when measured at their respective CO₂ growth levels (Table 3). Gas exchange analysis showed that the trees grown at elevated CO₂, 525 μL·L⁻¹ and 700 μL·L⁻¹ had a 2 and 3 fold increase in assimilation rates. There was no significant change in stomatal conductances but elevated CO₂ exposure increased internal CO₂ concentrations (C_i). Internal CO₂ concentrations (C_i) remained at approximately 63% of external (C_e) CO₂ concentrations.

Biochemical measurements of two key regulatory enzymes in the photosynthetic pathway revealed adjustments in the metabolic processes. Both ribulose-bis-phosphate carboxylase (RuBPCase) and sucrose phosphate synthase (SPS) showed up-regulated specific activity measured on a unit area basis in response to increased external CO₂ concentrations. RuBPCase showed a response at 525 μL·L⁻¹ and 700 μL·L⁻¹ CO₂ of 17 and 30% respectively. SPS responded only with growth at 700 μL·L⁻¹ CO₂. After 6 months there was a 55% increase in specific activity per unit area of mature source leaves (Table 4).

Discussion and Conclusions:

Pinus ponderosa seedlings responded to growth at elevated CO₂ at several levels of biological organization after 6 months of exposure. At the whole plant level the trees exhibited increases in stem length but did not increase in caliper when compared to growth ambient levels of CO₂ (350 μL·L⁻¹). Other changes that were observed included decreases in total plant needle area, and a trend toward reduction in root/shoot ratio at the highest level of CO₂ exposure (700 μL·L⁻¹). Changes in the root /shoot ratio have not been measured in many studies, however, of those reporting root/shoot ratios in coniferous tree species, most have indicated no effect (summarized in Rodgers and Krupa, 1994). These data suggest alterations in the internal carbon allocation patterns are being affected by elevated CO₂, shifting toward above ground biomass.

Microscopic techniques were collectively employed to assess structural changes associated with alterations in the strengths of source/sink relationships. Foliar tissue measurements revealed that specific needle mass increased by 15%. Increases in specific leaf mass with growth at elevated CO₂ are commonly reported (Gunderson and Wullschleger, 1994). This increase in needle mass was partly accounted for by increased starch deposition, however, shifts in the internal needle anatomy also occurred. Mature needles grown at elevated CO₂ showed a decrease in the amount of mesophyll tissue and an increase in total vascular tissue measured as a percentage of mid-needle cross-sectional area. Similar results were obtained for *Pinus taeda* (Thomas and Harvey, 1983). While the rearrangements of cellular proportions were not statistically significant (resulting partly from the wide genetic background of the experimental materials) they consistently exhibited a trend toward increasing the vascular/mesophyll ratios.

Photosynthetic carbon assimilation (A) measured by gas exchange analysis showed increased relative photosynthetic activities (A elevated/ A ambient) of 1.66 and 2.13 for 525 μL·L⁻¹ and 700 μL·L⁻¹ CO₂ respectively. Similar enhancements of assimilation have been observed in other coniferous species (Grulke *et al.*, 1993; Conroy *et al.*, 1990a,b;1986). Stomatal

conductances in the present study were not significantly affected by increases in CO₂ growth levels. This observation accounts for the near constant ratio of C_i/C_a of 0.63 at all growth levels. The increased C_i correlated with increased assimilation rates. Biochemical assessment of RuBCase specific activity per unit needle area revealed increases in activity at 525 μL·L⁻¹ and 700 μL·L⁻¹ CO₂. These results suggest that either an increase in the enzyme concentration was affected or there was an up-regulation of the enzyme activity. We did not observe any significant change in the total needle soluble protein content associated with CO₂ treatments. Increases in RuBCase activity in mature exporting needles (sources) correlated with increased chloroplast starch deposition.

Increase starch deposition in the exporting needles also correlated with increased SPS specific activity. Sucrose phosphate synthase is a key enzyme in the sucrose synthesis pathway and its activity might be used to measure mobilization of photoassimilate prior to translocation. In those mature needles which exhibited enhanced photosynthesis, starch accumulation, decreased mesophyll area, and increased vascular tissue, the specific activity of SPS per unit area was increased by 55% above levels at ambient CO₂ growth.

These data collectively suggest that Ponderosa pine seedlings are responding to increased CO₂ by optimization of carbon acquisition mechanisms and changing carbon and possible nitrogen allocation patterns. Shifts in the sink/source relationships are possibly due to transient transport limitations.

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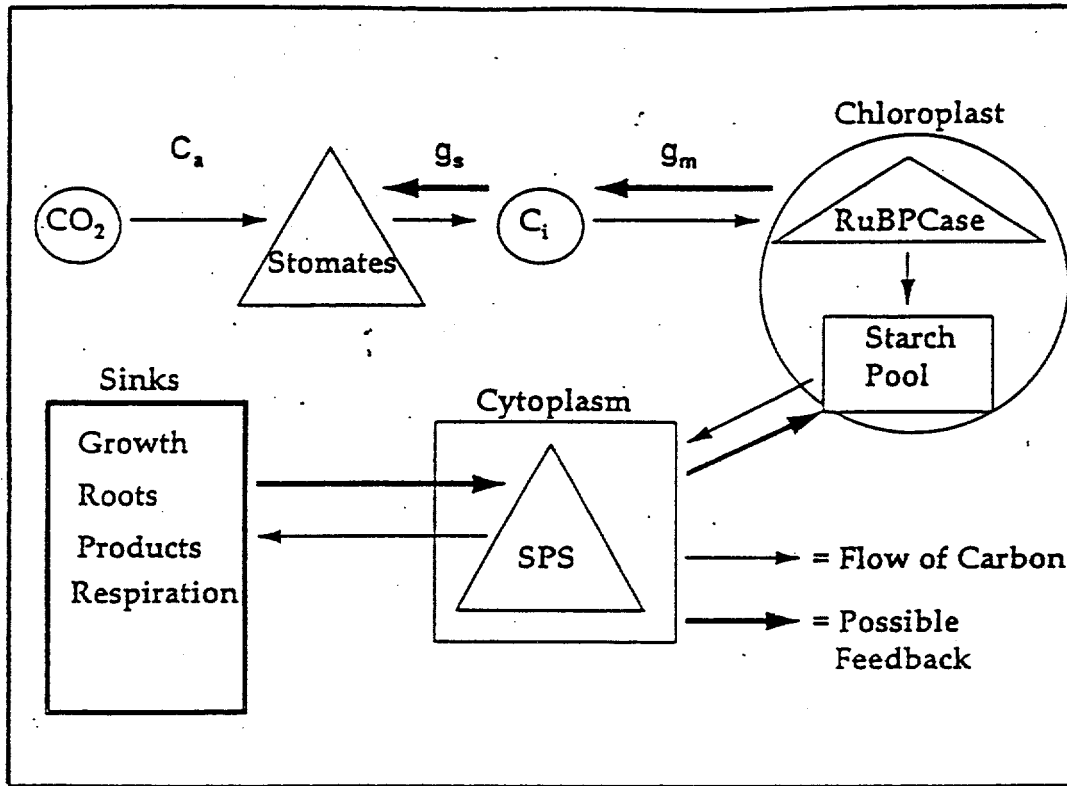


Figure 1. Conceptual model of carbon allocation patterns with potential feedback regulation.

Table 1. *Pinus ponderosa* growth measurements after six months of CO₂ exposure.

CO ₂ Exposure Level	% Stem Diameter Growth	% Stem Length Growth	Root/Shoot Ratio	Total Needle Area (cm ²)	Specific Needle Mass (g FW/cm ²)
350 uL·L ⁻¹	1.6	6.7	0.840	477.1	0.740
	(0.8)	(1.0)	(0.15)	(135.2)	(0.25)
525 uL·L ⁻¹	2.2	7.9	0.927	315.4	0.890
	(1.2)	(1.9)	(0.17)	(129.9)	(0.52)
700 uL·L ⁻¹	1.9	8.6	0.738	252.0	0.885
	(1.0)	(2.0)	(0.12)	(41.0)	(0.61)

Growth measurements represent the mean values of 9 different plants at each CO₂ exposure level. Values in parentheses are standard deviations. Percent growth represents change in parameter from onset of experiment.

Table 2. *Pinus ponderosa* 1 μ m mid-needle cross-sections after six months of exposure to carbon dioxide.

<u>Measurement</u>	<u>Initial*</u>	<u>CO₂ Exposure Level</u>		
		<u>350 μL \cdot L⁻¹</u>	<u>525 μL \cdot L⁻¹</u>	<u>700 μL \cdot L⁻¹</u>
Total Cross-sectional Area (mm ²)	0.58 (0.15)	0.61 (0.17)	0.76 (0.24)	0.78 (0.19)
	0.86 (0.28)	0.80 (0.14)	0.88 (0.16)	0.87 (0.35)
Mesophyll/Total Cross-sectional Tissue Area (%)	56.33 (3.93)	56.47 (2.16)	51.82 (6.00)	52.20 (1.94)
	54.81 (2.30)	55.88 (2.98)	52.96 (4.30)	53.58 (3.18)
Vascular/Total Cross-sectional Tissue Area (%)	26.24 (2.58)	26.94 (2.13)	30.53 (5.06)	30.25 (1.65)
	27.96 (2.75)	27.35 (3.04)	28.00 (3.68)	27.81 (6.32)

Initial column was derived from tissue collected prior to exposure. Data on the first row was derived from mature needles on the terminal bud. Data on the indented second row was derived from immature tissue on the terminal bud. Vascular tissue includes endodermis, transfusion tissue, xylem and phloem. Thirty-two needles were sampled for initial and treatments. All numbers are means, standard deviations in parentheses.

Table 3. *Pinus ponderosa* photosynthetic data after six months of CO₂ exposure.

CO ₂ Exposure Level	A ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	Cs (cm s^{-1})	Ci ($\mu\text{L L}^{-1}$)	Ci/Ca
350 $\mu\text{L L}^{-1}$	1.0870 (0.2069)	0.0331 (0.0008)	223.0 (23.0)	0.64
525 $\mu\text{L L}^{-1}$	1.8084 (0.4945)	0.0383 (0.006)	347.0 (46.0)	0.66
700 $\mu\text{L L}^{-1}$	2.3213 (0.3526)	0.0333 (0.011)	439.0 (48.0)	0.63

Gas exchange measurements represent mean values of 18 separate plants at each CO₂ exposure level. Values in parentheses are standard deviations.

Table 4. *Pinus ponderosa* biochemical data after six months of CO₂ exposure level.

CO ₂ Exposure Level	SPS Specific Activity (mmol/mg/min/cm ²)	RuBPCase Specific Activity (nmol/mg/min/cm ²)
350 uL.L ⁻¹	0.0107 (0.07)	0.6160 (0.11)
525 uL.L ⁻¹	0.0835 (0.06)	0.7881 (0.15)
700 uL.L ⁻¹	0.3445 (0.09)	0.9599 (0.14)

Enzyme activities represent mean values of 15 isolations at each CO₂ exposure level. Values in parentheses are standard deviations.