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Effect of nodulation, nitrogen fixation and CO_2 enrichment on the physiology, growth and dry mass allocation of seedlings of *Alnus rubra* Bong.

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SUMMARY

Inoculated and uninoculated *Alnus rubra* Bong. seedlings were grown for 47 days in atmospheres containing ambient (350 μl CO_2 l^{-1}) and elevated (650 μl CO_2 l^{-1}) levels of CO_2, with and without combined nitrogen (20 mg l^{-1}) supplied as ammonium nitrate. Five plants from each treatment were harvested 15, 30, and 47 days after exposure to CO_2 treatments began. Evidence for the presence of a positive feedback loop between nitrogen fixation and photosynthesis was observed in nodulated plants growing at elevated CO_2. These plants had greater whole-plant photosynthesis and nitrogenase activity, leaf area and nitrogen content, as well as nodule and plant dry mass, relative to nodulated plants grown at ambient CO_2 and non-nodulated plants grown at both CO_2 levels. This feedback may be an important way in which the potential carbon drain of nitrogen fixation on the host plant could be compensated; increased nitrogen availability resulting in stimulated leaf area growth and whole-plant photosynthesis. The relative amount of dry mass allocated below ground decreased for all seedlings over time, and the amount allocated above ground increased. This shift in allocation occurred slowly and at a constant rate in non-nodulated plants and more rapidly and abruptly when plants were nodulated. The proportion of dry mass allocated below ground was consistently greater in non-nodulated plants. Dry mass allocation to the stem was increased when combined nitrogen was applied and was greatest in nodulated plants grown at high CO_2. Dry mass partitioning among other organs was not directly affected by nodulation, CO_2 enrichment, or other treatment interactions.

Key words: *Alnus rubra*, nodulation, nitrogen fixation, CO_2 enrichment, dry mass allocation.

INTRODUCTION

The assimilation of all forms of nitrogen (N) requires metabolic energy and represents a carbon (C) cost to both nitrogen (N_2)-fixing and non-N_2-fixing plants. Assimilation of atmospheric dinitrogen is considered by some to be by far the most costly (Gutschick, 1978; Pate, Atkins & Rainbird, 1981; Schubert, 1982; Dixon & Wheeler, 1983; Tjepkema, Schwintzer & Benson, 1986), though others have reported these costs to be only slightly greater than those required to assimilate ammonium (NH_4^+) (Sellstedt & Huss-Danell, 1986) and nitrate (NO_3^-) (Sellstedt, 1986). When comparing the dry mass yields of N_2-fixing trees with those of non-fixing trees, the impact of the relatively high C costs of symbiotic N_2 fixation to the N_2-fixing host plant, and their effects on dry mass allocation to the stem, must be considered. It is unclear, however, how nodulated and non-nodulated plants respond to photosynthetic enhancement through means other than the provision of additional nitrogen. This is important because genetic enhancement of photosynthetic capability is frequently advocated as a means of yield improvement, and because of the global experiment in CO_2 fertilization currently underway.

The costs of symbiotic N_2 fixation have been estimated for some important agronomic legumes (Minchin & Pate, 1973; Pate, Layzell & Atkins, 1979; Atkins *et al.*, 1980; Sheikholeslam, Fishbeck & Phillips, 1980; Paul & Kucey, 1981) and several actinorhizal plants (Tjepkema & Winship, 1980; Tjepkema, 1985). The effect of the symbiosis on photosynthate partitioning among leaves, stems, roots and nodules has also been reported for actinorhizal plants (Ingestad, 1980; Tjepkema, 1985;

Sellstedt & Huss-Danell, 1986; Sellstedt, 1986). However, no physiological mechanism by which actinorhizal plants may be able to compensate for the high C costs of the N_2-fixing symbiont has been reported. Additionally, the effect of nodulation on photosynthesis and dry mass partitioning under conditions of increased C availability to the plant (from CO_2 enrichment) has not been characterized.

Norby (1987) demonstrated that CO_2 enrichment of inoculated actinorhizal (*Alnus glutinosa* (L.) Gaertn. and *Elaeagnus angustifolia* L.) and legume (*Robinia pseudoacacia* L.) trees resulted in increased whole-plant nitrogenase activity and growth. This effect had been previously demonstrated with several agronomic legumes (Hardy & Havelka, 1976; Phillips *et al.*, 1976; Masterson & Sherwood, 1978; Finn & Brun, 1982). Results from experiments using woody, N_2-fixing trees conform to the established view that symbiotic N_2 fixation is dependent on host plant photosynthesis, and that the two processes are closely linked in both legume (Lindstrom, Newton & Wilson, 1952; Quispel, 1958; Wheeler, 1971; Lawrie & Wheeler, 1973, 1974; Hardy & Havelka, 1976) and actinorhizal symbioses (Quispel, 1958; Wheeler, 1969; Wheeler, 1971; Wheeler & Lawrie, 1976; Gordon & Wheeler, 1978; Dawson & Gordon, 1979; Huss-Danell & Sellstedt, 1983, 1985).

Another important way in which N_2 fixation and the nodules may affect yield is by alteration of C allocation of the host, especially to the stem. N_2-fixing root nodules are strong C sinks (Tjepkema, 1985; Bormann & Gordon, 1984) and potentially large amounts of C may be diverted away from lower priority sinks, such as the stem (Bormann & Gordon, 1984). This would decrease yield. Alternatively, N_2-fixing plants may compensate by fixing more C, or fixing it at a higher rate (Paul & Kucey, 1981), thereby increasing the total amount of C available to fill sinks. This could enable lower priority sinks to be filled sooner than they would be in non-fixing plants.

The objective of this study was to determine whether CO_2-enhanced photosynthesis resulted in greater dry mass accumulation in nodulated versus non-nodulated plants under conditions of moderate nitrogen limitation, similar to those found on many sites in the field, and to describe a mechanism by which this might occur. We did this firstly by testing for the presence of a positive feedback loop between photosynthesis and N_2 fixation (root nodules with active nitrogenase). This was achieved by comparing whole-plant dry mass production of nodulated and non-nodulated (NH_4NO_3-supplied) *Alnus rubra* Bong. seedlings grown in ambient and enriched CO_2 atmospheres (normal and enhanced photosynthesis, respectively). Secondly, the effects, if any, that such a feedback might have on patterns of dry mass allocation were evaluated during seedling ontogeny. Finally, we attempted to determine whether any differences between nodulated and non-nodulated plants were associated with differences in the efficiencies of photosynthesis (per unit leaf area) and N_2 fixation (nitrogenase activity per nodule dry mass) or with differences in the total capacities of these processes (whole-plant rates).

MATERIALS AND METHODS

Plant Material

A mixture of *A. rubra* seed collected from two trees in Olympia, Washington (USA), was soaked for 24 h in distilled water, sown on moist, sterile Redi-earth® potting mix (Grace-Sierra Horticultural Products Co., 62 Whittemore Ave., Cambridge, MA 02140, USA) and placed on a greenhouse mist bench to germinate. After 4 weeks, 200 seedlings with two to three true leaves were removed from the germination medium and transplanted into 130 ml plastic tree tubes (one seedling per tube) containing a sterilized mix of two parts perlite to one part Redi-earth® (v/v). Five days after transplanting, 100 plants were inoculated with *Frankia* strain ArI3 (Berry & Torrey, 1979) by pipetting 1.0 ml of a suspension of *Frankia* cells to the potting medium at the base of the stem [1.0 ml packed cell volume (Nittayajarn & Baker, 1989) of ArI3 diluted with 100 ml distilled water]. Tubes were placed in racks on a shaded greenhouse bench and watered to the drip point twice daily with distilled water. Two weeks later, all plants were transported from Yale to the Duke University Phytotron in Durham, North Carolina (USA). Upon arrival, each seedling, along with the plug of potting medium surrounding its roots, was transplanted to a 500 ml plastic cup with drainage holes at the bottom. Cups were filled with a sterilized mix of equal parts of quartz gravel, vermiculite and Turface® (montmorillonite, fritted clay: Applied Industrial Materials Corp., 1 Parkway North, Suite 400, Deerfield, IL, USA) to insure adequate drainage, aeration, and moisture holding capacity.

Experimental Design and Growth Conditions

Plants in both inoculated and uninoculated groups were sorted into three size classes to minimize initial variability among treatments, and five plants from each size class were assigned to treatments varying in atmospheric CO_2 concentration (350 or 650 μl CO_2 l^{-1}) and nutrient solution concentration of NH_4NO_3-N. Previous experience with red alder seedlings demonstrated that uninoculated plants receiving nutrient solution without N do not grow. Therefore, no plants were assigned to this treatment. Plants were randomly located in each growth chamber.

Growth conditions in both chambers were the same except for CO_2 concentration. Cool-white, 120 W fluorescent and 100 W incandescent lights

provided a photosynthetic photon flux density of 400 μmol m^{-2} s^{-1}. Relative humidity was maintained at 70%, and the photoperiod was 16 h at 26 °C and the dark period 8 h at 20 °C. Racks, each holding nine plants, were systematically rotated every third day within a chamber, and plants from one chamber were switched with those from the other chamber every week to minimize the potential effects of varied growth conditions within and between chambers, respectively.

Seedlings were watered to the drip point at the beginning of the photoperiod with either 1/4-strength N-free Hoagland's nutrient solution at pH 6.0 or the same solution with a growth-limiting amount (20 mg l^{-1}) of N added as NH_4NO_3. Uninoculated plants were supplied with growth-limiting amounts of combined N in order to compare their performance to inoculated (nodulated) plants and to test effectively the feedback hypothesis. Preliminary experimentation showed this level of N to simulate levels commonly found at field sites, to be only marginally growth limiting (after 4 weeks) and not to inhibit nodulation in inoculated plants. In the current study 20 mg l^{-1} N was rather more growth limiting. Pots were watered to the drip point with deionized water in the evening (9–10 h into the photoperiod) to displace residual nutrient solution and to prevent salt damage to plants.

Atmospheric CO_2 concentrations of 350 and 650 μl CO_2 l^{-1} were chosen to simulate approximate present and projected future global levels (Baes *et al.*, 1977).

Measurements

Growth and N Analysis: Five plants from each treatment were harvested 15, 30, and 47 d after final transplanting. Seedlings were dissected into leaves, stems, roots and nodules, dried at 70 °C for 2 d, and weighed. Leaf area was measured on all three sampling days using a Licor LI 3100/1 automatic leaf area meter. Seedling height was measured weekly.

Dried tissue from plants harvested on day 47 was ground in a Wiley mill to pass through a number 20 sieve and stored in scintillation vials. Total N in a 50–200 mg subsample of ground material was determined using standard Kjeldahl digestion procedures and spectrophotometric analysis on a Technicon Autoanalyzer Model II (Crooke & Simpson, 1971; Nelson & Sommers, 1973). Total and % N content were calculated for each plant and all plant parts.

N_2 Fixation: Nitrogenase activity of intact, inoculated plants was measured on day 47 using the acetylene (C_2H_2) reduction assay. Each pot was enclosed in a gas-tight 4 l polyethylene tub fitted with a rubber septum such that only the below ground portion of the plant was exposed to 0·10 atm C_2H_2. Acetylene was introduced through the septum using a 1500 ml gas-tight syringe and hypodermic needle. After introduction of acetylene, the tub was vented with a hypodermic needle to restore atmospheric pressure inside. The assay commenced 3 h into the photoperiod. Plants remained in their own growth chamber and were incubated for 2·5 h. Gas samples (5·0 ml) were removed from the tub with a 5·0 ml disposable hypodermic syringe 0, 15, 30, 60, 90 and 150 min after the incubation began. Samples were stored in 15-ml gas-tight serum vials for subsequent gas chromatographic analysis. The volume of gas removed each time from the tub was immediately replaced with an equal volume of room air. Ethylene (C_2H_4) gas standards (5·0 ml) were also injected into serum vials and analyzed along with the other samples. C_2H_4 production was measured on a Hewlett Packard Model 5890A gas chromatograph fitted with a flame ionization detector. The stainless steel column was 1·22 m × 1·7 mm i.d., filled with 80/100 mesh Poropak T, and the oven temperature was at 100 °C. N_2 carrier gas flow rate was 50 ml min^{-1}. Since all plants were watered 2 h prior to the beginning of the assay, all free water had drained from the pots. This permitted rapid gas diffusion. Constant rates of C_2H_2 reduction were observed after 15 min and were maintained for the entire assay period for all plants. No C_2H_4 accumulation was detected when nodulated plants were incubated in the absence of C_2H_2, nor when pots containing only mix were incubated with and without C_2H_2. Although the incubation method did not permit evaluation of possible C_2H_2-induced decline of nitrogenase activity (Minchin *et al.*, 1983), comparative treatment effects could be reasonably estimated. Subsequent evaluation of red alder from the same seed source, inoculated with the same *Frankia* strain grown under similar conditions, but using an open, constant flow C_2H_2 reduction assay system (Silvester *et al.*, 1989) showed no C_2H_2-induced decline.

Photosynthesis: Leaf gas exchange measurements began immediately after the C_2H_2 reduction assay was completed. Pots were removed from their tubs and watered to the drip point with deionized water at 26 °C. The most recently fully expanded leaf (Leaf Plastochron Index 3, or LPI 3) was enclosed in a temperature-controlled leaf cuvette in an open infrared gas analysis system. Photosynthetic performance of this leaf was found to be representative of that particular plant, and variability between plants in a treatment was minimized by using fully expanded leaves at the same stage of development. Photosynthesis was measured at 26 °C and a photosynthetic photon flux density of 1500 μmol m^{-2} s^{-1} (assumed to be saturating). Rates were calculated using equations of von Caemmerer & Farquhar (1981). Measurements were made on all plants

within a 5-h period near the end of the photoperiod on two consecutive days; day 46 and 47 at 350 and 650 μl CO_2 l^{-1}, respectively. Rates reported in this paper are from plants measured at the same CO_2 concentration at which they were grown. Each plant was removed from its chamber immediately before its photosynthesis was measured and was immediately returned afterwards. Sampling order was random. Whole-plant photosynthesis (capacity) was calculated by multiplying single leaf rates by plant leaf area. This was justified because self- and mutual shading was not significant, and because rates measured on leaves at LPI 3 accurately represented rates measured on leaves at greater LPI in a preliminary experiment.

Statistical Analysis

Whole-plant growth, % dry mass allocated to plant parts, whole-plant net photosynthesis, specific nitrogenase activity (SNA), and tissue N content were calculated. Sample means and standard errors were calculated for all parameters for each sampling day. Analysis of variance using SAS General Linear Model (GLM) procedures (SAS Institute Inc., Box 8000, Cary, NC, 27511–8000, USA) was performed to evaluate treatment effects. Each parameter analyzed was treated as the dependent variable in an SAS linear regression model with treatment variables: combined N (concentration of N in nutrient solution), nodulation (inoculated or uninoculated), CO_2 (atmospheric CO_2 concentration), and all possible treatment variable interaction terms as independent variables in the model (Equation 1).

$$\text{Dependent variable } (Y) = a + b_1 \text{ (combined N)} + b_2 \text{ (nodulation)} + b_3([CO_2]) + b_4(\text{combined N} \times [CO_2]) + b_5 \text{ (nodulation} \times [CO_2]) \quad (1)$$

SAS GLM procedures also generated an analysis of variance table. Probability of a significant difference, when not specifically stated, was: $P < 0{\cdot}05$.

RESULTS

Dry mass production and height growth

Dry mass of all plants increased over the course of the experiment (Table 1). Dry mass of each organ generally increased in proportion to its plant dry mass; organs of larger plants grew more rapidly than organs of smaller plants. This pattern was consequently also true for above and below ground growth. Whole-plant, leaf, stem, and root growth was enhanced by nodulation, application of combined N, elevated CO_2, and by interactions between nodulation and elevated CO_2, and applied N and elevated CO_2. The presence or absence of nodules was the strongest determinant of both whole-plant and organ dry mass production, followed by combined N treatment. CO_2 concentration and treatment interactions were weaker determinants. The positive effect of CO_2 enrichment on whole-plant dry mass was greatest when plants were supplied with combined N. Nodulated plants grown at high CO_2 and supplied with combined N grew most rapidly.

Height growth closely followed plant dry mass production and was in general positively affected by the same treatments and interactions. The exception was that CO_2 concentration had no independent, direct effect on height growth (Fig. 1).

Dry mass allocation and seedling development

Dry mass allocation within a plant, expressed as a percentage of the ratio between organ dry mass and plant dry mass, was primarily affected by the presence or absence of nodules. Nodulated plants allocated relatively more dry mass above ground, to leaves and stem, and less below ground, to roots and nodules, than did non-nodulated plants (Fig. 2). Percent dry mass allocated below ground decreased for all plants over time. This shift in allocation occurred relatively early and abruptly in nodulated plants and more gradually and evenly in non-nodulated seedlings. Application of combined N increased dry mass allocation to the stem. Allocation to the stem was consistently greatest in nodulated plants grown at high CO_2.

Nodule characteristics

All inoculated plants sampled on days 15, 30, and 47 had nodules, and the number per plant increased significantly by day 47 (Table 2). Nodules appeared to be well distributed throughout each of these root systems on days 30 and 47. Overall, neither CO_2 nor combined N had a significant effect on the number of nodules per plant, although plants growth at high CO_2 and those supplied with combined N tended to have slightly more. Inoculated plants supplied with combined N had the greatest total nodule dry mass (Table 1). CO_2 enrichment had no apparent affect on nodule dry mass per plant. Among inoculated plants, neither CO_2 nor combined N had a significant effect on nodule dry mass as a percentage of whole-plant dry mass. Nodulated plants supplied with combined N and grown at high CO_2 tended to allocate slightly less dry mass to nodules, but allocated significantly less (2·1 %) than other plants on day 47 (Table 2).

Frankia contamination of uninoculated plants was detected on day 47. One or two relatively large nodules were observed on each uninoculated plant. We do not know if these nodules had nitrogenase activity since these plants were not assayed.

Leaf characteristics

Leaf area per plant, number of leaves per plant,

Table 1. *Whole-plant dry mass (mg) of leaves, stem, roots, nodules, and above and below ground organs of seedlings of* Alnus rubra [*mean* ± *SE; n = 5, except where n = 3* (*) *and where n = 4*(†)]

Treatment				Sampling day		
Nodulation	[Nitrogen] (mg l^{-1})	[CO_2] ($\mu l\ l^{-1}$)	Plant part(s)	15	30	47‡
−	20	350	Leaves	28·7 (3·4)†	138·5 (21·5)	395·6 (114·5)
			Stem	10·3 (0·4)†	52·9 (6·2)	202·6 (34·3)
			Roots	67·7 (10·8)†	201·6 (28·0)	384·9 (26·1)
			Nodules	0·0 (0·0)†	0·0 (0·0)	20·2 (7·9)
			Above ground	36·0 (2·7)†	191·4 (27·6)	598·2 (147·8)
			Below ground	67·7 (9·0)†	201·6 (28·0)	405·1 (29·3)
			All	106·7 (8·6)†	393·0 (53·9)	1003·3 (160·6)
−	20	650	Leaves	40·6 (8·3)	149·5 (12·6)	556·7 (129·7)*
			Stem	12·2 (1·8)	47·5 (4·1)	264·2 (62·6)*
			Roots	100·1 (15·6)	238·4 (36·2)	558·5 (126·4)*
			Nodules	0·0 (0·0)	0·0 (0·0)	43·3 (15·7)*
			Above ground	52·8 (10·0)	197·0 (15·9)	820·9 (189·8)*
			Below ground	100·1 (15·6)	238·4 (36·2)	601·8 (141·8)*
			All	152·9 (25·4)	435·4 (46·1)	1422·7 (331·6)*
+	0	350	Leaves	34·6 (2·6)	95·9 (12·3)	994·3 (53·5)
			Stem	13·1 (1·2)	33·6 (3·9)	466·2 (28·4)
			Roots	62·3 (8·9)	54·0 (7·5)	720·6 (70·2)
			Nodules	2·3 (0·4)	9·6 (0·9)	70·2 (9·2)
			Above ground	47·7 (3·4)	129·5 (15·4)	1460·5 (76·6)
			Below ground	64·6 (9·0)	63·6 (7·1)	790·8 (75·7)
			All	112·3 (10·8)	193·1 (22·1)	2251·2 (146·5)
+	0	650	Leaves	34·3 (5·2)	144·4 (21·4)	1237·4 (151·2)
			Stem	13·6 (1·9)	36·7 (3·4)	567·9 (72·8)
			Roots	56·5 (7·5)	84·6 (10·2)	711·8 (71·3)
			Nodules	0·8 (0·3)	13·9 (4·5)	79·0 (7·6)
			Above ground	47·9 (7·1)	181·1 (24·5)	1805·3 (221·1)
			Below ground	57·3 (7·4)	98·5 (12·4)	790·8 (78·5)
			All	105·2 (14·4)	279·6 (35·6)	2596·1 (298·5)
+	20	350	Leaves	45·1 (2·4)	359·7 (44·1)	1608·2 (189·7)
			Stem	16·9 (1·1)	127·8 (11·2)	892·3 (151·1)
			Roots	72·0 (8·1)	242·0 (27·2)	1326·2 (198·4)
			Nodules	1·9 (0·6)	32·1 (2·7)	118·3 (22·6)
			Above ground	62·0 (3·3)	487·5 (55·0)	2500·5 (324·3)
			Below ground	73·9 (8·5)	274·1 (29·3)	1444·5 (219·6)
			All	135·9 (8·6)	761·6 (80·6)	3945·0 (499·0)
+	20	650	Leaves	45·3 (2·9)	500·7 (49·4)	2488·9 (370·2)*
			Stem	16·4 (1·1)	174·1 (15·2)	1587·5 (235·5)*
			Roots	67·5 (8·3)	211·6 (11·5)	2595·7 (500·4)*
			Nodules	1·8 (0·3)	33·5 (1·5)	144·9 (24·9)*
			Above ground	61·7 (4·0)	674·8 (62·7)	4076·4 (601·7)*
			Below ground	69·3 (8·4)	245·1 (11·8)	2740·6 (525·2)*
			All	131·0 (10·2)	919·9 (74·4)	6816·9 (1021·9)*

‡ Presented graphically in Figure 2.

and average area per leaf were greatest in nodulated plants (Table 3). This effect became more pronounced over time. Application of combined N amplified the primary effect of nodulation. Effect of combined N also became more noticeable over time with respect to leaf area per plant and average area per leaf. Nodulated plants grown at high CO_2 had the largest area per leaf. These plants also tended to have the greatest leaf area per plant (no statistically significant effect; $P = 0{\cdot}0807$). Over time, positive effects became apparent of interactions between CO_2 enrichment and application of combined N, and particularly between CO_2 enrichment and nodulation, on average area per leaf. CO_2 enrichment had no direct effects (independent of other treatments) on the leaf characteristics evaluated.

Photosynthesis

Photosynthesis of all plants was enhanced by CO_2 enrichment (Fig. 3). Of all the treatments tested, only an increase in atmospheric CO_2 affected photosynthetic efficiency, or CO_2 exchange rate per unit leaf area, and this enhancement of photosynthetic

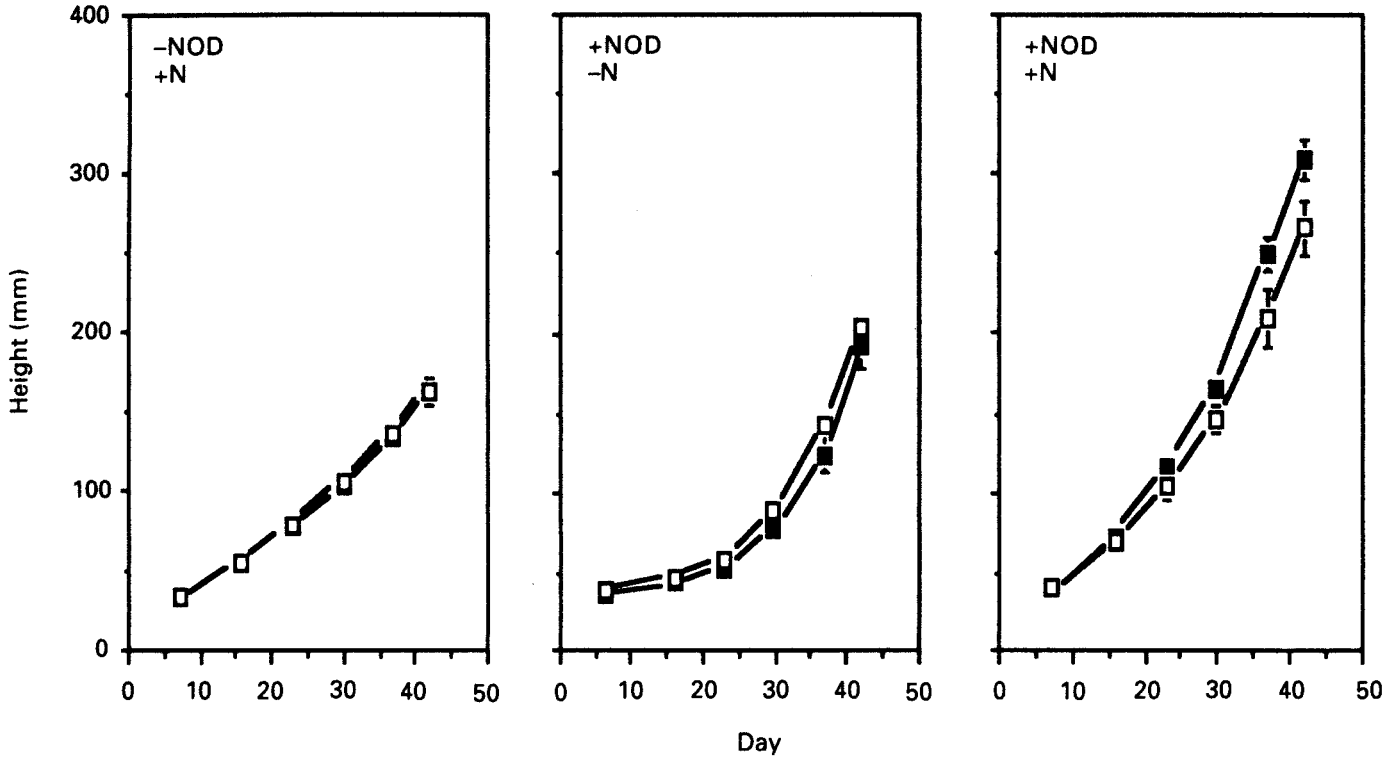

Figure 1. Increase in height of seedlings of *Alnus rubra* grown in atmospheres containing 350 (□) and 650 (■) μl CO_2 l^{-1}. Key: (−NOD, +N) non-nodulated, 20 mg N l^{-1}; (+NOD, −N) nodulated, 0 mg N l^{-1}; and (+NOD, +N) nodulated, 20 mg N l^{-1}; (means ± SEs).

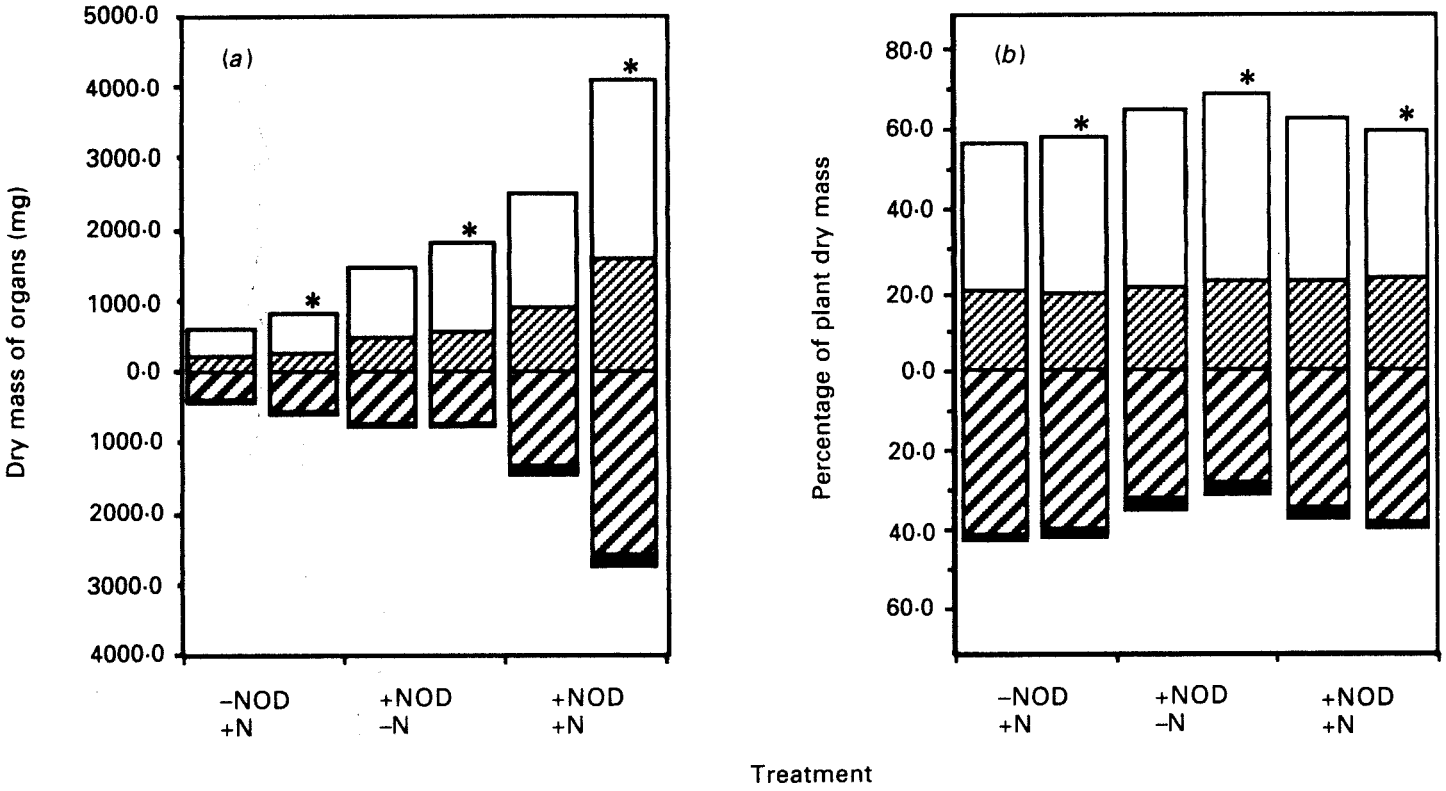

Figure 2. Dry mass (*a*) and % of whole-plant dry mass (*b*) allocated at final harvest (day 47) to leaves (□), stems (▨), roots (▨), and nodules (■), of seedlings of *Alnus rubra* grown in atmospheres containing 350 (bars without *) and 650 (bars with *) μl CO_2 l^{-1}. Key: (−NOD, +N) non-nodulated, 20 mg N l^{-1}; (+NOD, −N) nodulated, 0 mg N l^{-1}; and (+NOD, +N) nodulated, 20 mg N l^{-1}. Each segment represents the mean of five plants.

efficiency occurred independently of N supply. Photosynthetic capacity, or whole-plant CO_2 exchange rate, was stimulated by the same treatments as those affecting dry mass production and leaf area growth. Interaction between nodulation and CO_2 also resulted in plants with the greatest photosynthetic capacity. This was further promoted by the addition of combined N.

Nitrogenase activity

CO_2 enrichment had a slight positive effect (P =

Table 2. *Nodule characteristics of seedlings of* Alnus rubra: *number per plant, nodule weight ratio [(nodule dry mass/whole-plant dry mass) × 100], average dry mass per nodule (mean ± SE), and fraction of plants per treatment with nodules; n = 5, except where n = 3(*), or where n = 4(†)*

Treatment				Sampling day		
Nodulation	[Nitrogen] (mg l^{-1})	[CO_2] ($\mu l\ l^{-1}$)	Nodule parameters	15	30	47
−	20	350	Number plant^{-1}	0·0†	0·0	1·6 (0·4)
			Nodule weight ratio (%)	·	·	1·8 (0·5)
			Dry mass nodule^{-1} (mg)	·	·	11·3 (2·4)
			Fraction of plants with nodules	0/4†	0/5	5/5
−	20	650	Number plant^{-1}	0·0	0·0	1·7 (0·7)*
			Nodule weight ratio (%)	·	·	2·9 (0·4)*
			Dry mass nodule^{-1} (mg)	·	·	27·2 (5·7)*
			Fraction of plants with nodules	0/5	0/5	3/3*
+	0	350	Number plant^{-1}	3·6 (0·9)	14·6 (2·9)	21·2 (3·7)
			Nodule weight ratio (%)	2·1 (0·4)	5·4 (1·0)	3·1 (0·3)
			Dry mass nodule^{-1} (mg)	1·0 (0·5)	0·8 (0·1)	3·6 (0·6)
			Fraction of plants with nodules	5/5	5/5	5/5
+	0	650	Number plant^{-1}	3·6 (1·4)	17·8 (1·8)	32·2 (5·4)
			Nodule weight ratio (%)	0·8 (0·3)	5·0 (1·2)	3·1 (0·1)
			Dry mass nodule^{-1} (mg)	0·2 (0·0)	0·8 (0·2)	2·6 (0·3)
			Fraction of plants with nodules	5/5	5/5	5/5
+	20	350	Number plant^{-1}	5·0 (1·4)	20·2 (1·6)	33·0 (3·4)
			Nodule weight ratio (%)	1·4 (0·4)	4·3 (0·3)	2·9 (0·2)
			Dry mass nodule^{-1} (mg)	0·4 (0·1)	1·6 (0·1)	3·6 (0·5)
			Fraction of plants with nodules	5/5	5/5	5/5
+	20	650	Number plant^{-1}	5·6 (0·2)	30·8 (5·2)	37·0 (2·5)*
			Nodule weight ratio (%)	1·3 (0·2)	3·7 (0·3)	2·1 (0·2)*
			Dry mass nodule^{-1} (mg)	0·3 (0·1)	1·2 (0·2)	4·0 (0·7)*
			Fraction of plants with nodules	5/5	5/5	3/3*

Table 3. *Leaf area per plant, number of leaves per plant, and average area per leaf of seedlings of* Alnus rubra *[mean ± SE; n = 5, except where n = 3(*) or where n = 4(†)]*

Treatment				Sampling day		
Nodulation	[Nitrogen] (mg l^{-1})	[CO_2] ($\mu l\ l^{-1}$)	Leaf parameter (area in cm^2)	15	30	47
−	20	350	Area plant^{-1}	8·9 (0·7)†	33·9 (4·9)	102·4 (31·4)
			Leaves plant^{-1}	·	11·4 (0·9)	23·0 (3·6)
			Area leaf^{-1}	·	3·0 (0·3)	4·1 (0·6)
−	20	650	Area plant^{-1}	9·8 (2·1)	29·1 (1·8)	112·8 (28·6)*
			Leaves plant^{-1}	·	14·2 (1·2)	30·3 (3·8)*
			Area leaf^{-1}	·	2·1 (0·2)	3·6 (0·7)*
+	0	350	Area plant^{-1}	6·9 (0·6)	27·1 (3·4)	261·4 (12·0)
			Leaves plant^{-1}	·	11·0 (0·4)	36·6 (1·8)
			Area leaf^{-1}	·	2·5 (0·2)	7·1 (1·0)
+	0	650	Area plant^{-1}	6·3 (0·8)	29·7 (4·3)	287·8 (40·2)
			Leaves plant^{-1}	·	12·8 (1·6)	38·4 (4·4)
			Area leaf^{-1}	·	2·3 (0·3)	7·4 (0·4)*
+	20	350	Area plant^{-1}	13·4 (0·9)	100·7 (13·3)	348·0 (47·3)
			Leaves plant^{-1}	·	20·0 (2·0)	55·0 (5·9)
			Area leaf^{-1}	·	5·0 (0·4)	6·3 (0·5)
+	20	650	Area plant^{-1}	11·7 (0·7)	105·0 (10·3)	484·2 (73·8)*
			Leaves plant^{-1}	·	27·0 (2·9)	53·3 (3·8)*
			Area leaf^{-1}	·	4·0 (0·4)	9·4 (2·2)*

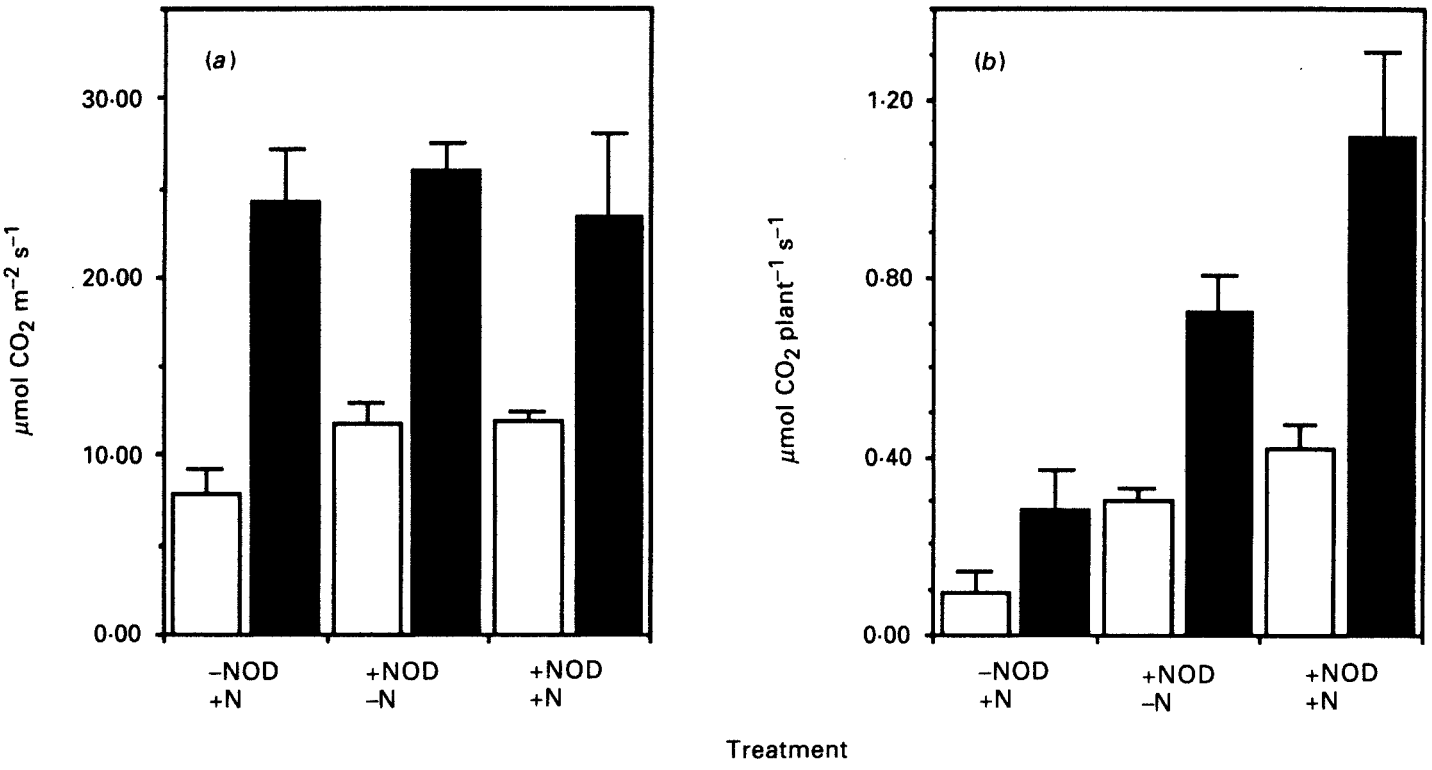

Figure 3. Photosynthetic rate per unit leaf area (efficiency) (*a*) and rate per plant (capacity) (*b*) at final harvest (days 46 and 47) of seedlings of *Alnus rubra* grown in atmospheres containing 350 (open bars) and 650 (solid bars) μl CO_2 l^{-1}. Key: (−NOD, +N) non-nodulated, 20 mg N l^{-1}; (+NOD, −N) nodulated, 0 mg N l^{-1}; and (+NOD, +N) nodulated, 20 mg N l^{-1}. Each bar represents the mean (+SE) of five plants.

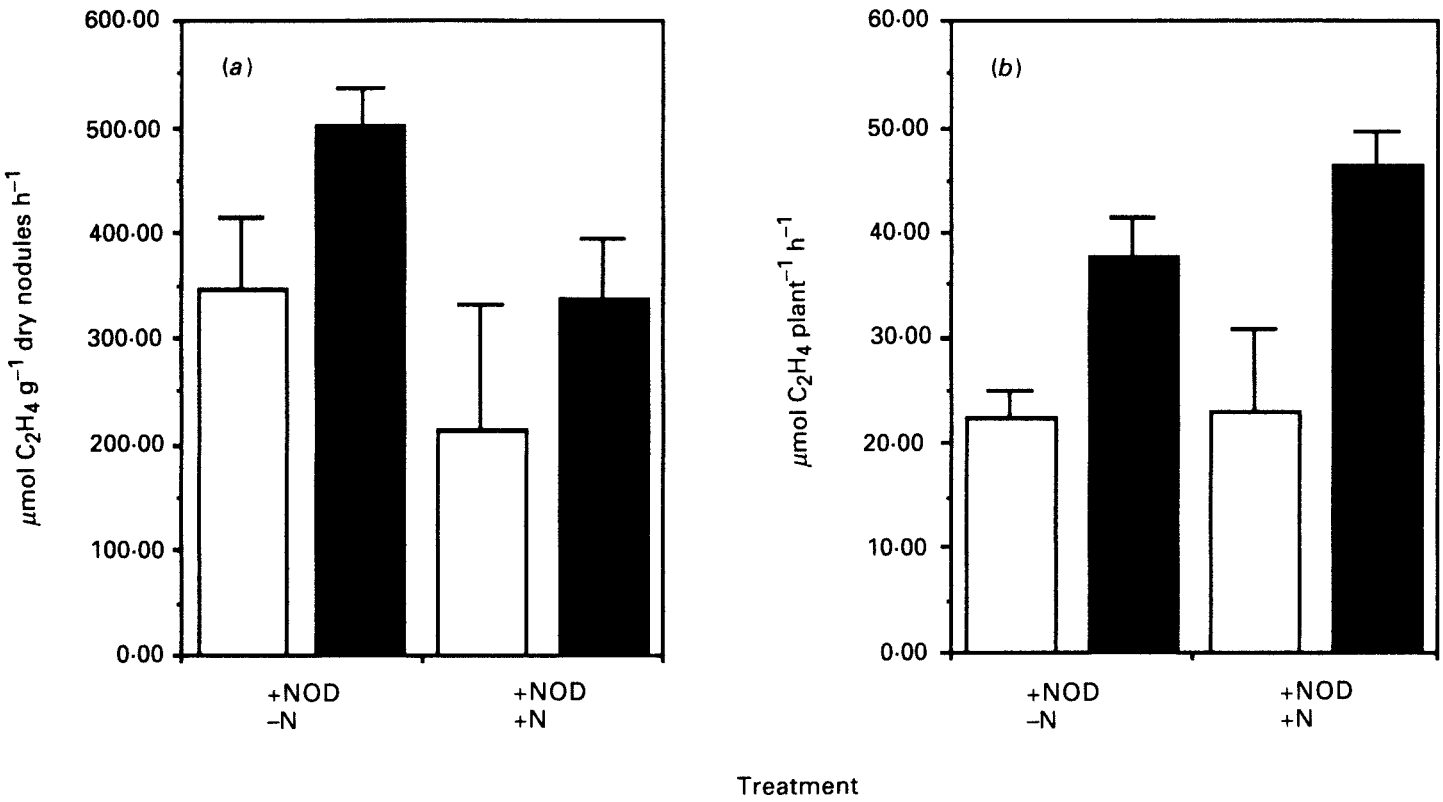

Figure 4. Acetylene reduction rate per gram nodule dry weight (efficiency) (*a*) and rate per plant (capacity) (*b*) at final harvest (days 46 and 47) of seedlings of *Alnus rubra* grown in atmospheres containing 350 (open bars) and 650 (solid bars) μl CO_2 l^{-1}. Key: (−NOD, +N) non-nodulated, 20 mg N l^{-1}; (+NOD, −N) nodulated, 0 mg N l^{-1}; and (+NOD, +N) nodulated, 20 mg N l^{-1}. Each bar represents the mean (+SE) of five plants.

0·0833), and combined N a slight negative effect (P = 0·0702), on SNA (or efficiency) (Fig. 4). CO_2 enrichment had a significant positive effect on whole-plant nitrogenase activity (capacity). The slight negative effect of combined N on SNA was compensated by its strong positive effect on nodule dry mass per plant (Table 1).

Tissue N

The N content of a plant or organ was directly related to its dry mass (Table 4), and was consequently generally affected by the same treatments. The N content of nodulated plants was greater than that of non-nodulated plants, and was further

Table 4. *Nitrogen concentration and content of leaf, stem, root, nodule and whole-plant tissue of seedlings of* Alnus rubra *at final harvest (day 47) [mean ± SE; n = 5, except where n = 3(*)]*

Treatment				Nitrogen	
Nodulation	[Nitrogen] (mg l^{-1})	[CO_2] ($\mu l\ l^{-1}$)	Plant part(s)	Concentration [% (w/w)]	Content (mg)
−	20	350	Leaves	2·38 (0·23)	10·3 (4·0)
			Stem	1·33 (0·16)	2·9 (0·8)
			Roots	1·16 (0·13)	4·4 (0·5)
			Nodules	4·39 (0·29)	0·9 (0·3)
			Above ground	2·01 (0·22)	13·2 (4·7)
			Below ground	1·30 (0·15)	5·3 (0·8)
			All	1·72 (0·21)	18·5 (5·4)
−	20	650	Leaves	2·83 (0·18)*	15·4 (2·9)*
			Stem	1·73 (0·12)*	4·6 (1·0)*
			Roots	1·08 (0·06)*	6·1 (1·7)*
			Nodules	3·79 (0·44)*	1·5 (0·4)*
			Above ground	2·47 (0·22)*	19·9 (3·9)*
			Below ground	1·25 (0·05)*	7·7 (2·1)*
			All	1·96 (0·07)*	27·6 (5·9)*
+	0	350	Leaves	3·28 (0·08)	32·6 (1·5)
			Stem	1·69 (0·06)	7·8 (0·3)
			Roots	1·14 (0·09)	8·0 (0·5)
			Nodules	3·68 (0·54)	2·5 (0·3)
			Above ground	2·77 (0·06)	40·4 (1·5)
			Below ground	1·36 (0·11)	10·5 (0·5)
			All	2·28 (0·07)	50·9 (1·8)
+	0	650	Leaves	2·92 (0·05)	36·1 (4·4)
			Stem	1·61 (0·02)	9·1 (1·1)
			Roots	1·12 (0·11)	8·5 (1·1)
			Nodules	2·30 (0·55)	1·9 (0·5)
			Above ground	2·47 (0·15)	45·2 (5·4)
			Below ground	1·25 (0·05)	10·4 (1·5)
			All	2·14 (0·07)	55·6 (6·5)
+	20	350	Leaves	2·87 (0·11)	45·6 (4·5)
			Stem	1·42 (0·03)	12·5 (1·9)
			Roots	1·18 (0·12)	15·0 (1·6)
			Nodules	2·38 (0·14)	2·8 (0·4)
			Above ground	2·36 (0·08)	58·1 (6·3)
			Below ground	1·27 (0·11)	17·8 (1·9)
			All	1·95 (0·10)	75·9 (8·0)
+	20	650	Leaves	2·48 (0·07)*	62·1 (10·9)*
			Stem	1·36 (0·09)*	21·7 (3·9)*
			Roots	1·24 (0·04)*	32·1 (6·2)*
			Nodules	2·37 (0·09)*	3·4 (0·6)*
			Above ground	2·04 (0·07)*	83·8 (14·8)*
			Below ground	1·30 (0·03)*	35·5 (6·7)*
			All	1·74 (0·04)*	119·3 (20·7)*

increased when nodulated plants were supplied with combined N. Thus, plants with a greater N content also had a higher leaf N content, a greater leaf area, higher rates of whole-plant photosynthesis, and a greater whole-plant dry mass. Nodulated seedlings grown at high CO_2 supplied with combined N had the greatest stem, root, and whole-plant N content.

The concentration of N in tissues was, in general, inversely related to N content. Therefore, application of combined N had a larger positive effect on growth than on N content. Therefore, leaf, stem, and whole-plant N concentration was lower. Leaf and stem concentrations were lowest in nodulated plants grown at high CO_2. Other treatments and interactions, which generally increased growth, had no effect on tissue N concentration.

DISCUSSION

The possibility of feedback

The first step in testing for the possibility of a feedback was to demonstrate a positive growth response which was associated with an interaction between the N_2-fixation apparatus (presence of root nodules with active nitrogenase) and CO_2 enrichment. The second step was to show that net photosynthesis and nitrogenase activity (either specific and/or whole-plant) was enhanced by CO_2

enrichment. We hypothesized that greater C availability from environmentally enhanced photosynthesis would provide a greater boost in growth in nodulated plants than in non-nodulated plants as a result of a positive interaction (feedback) between CO_2 enrichment (photosynthesis) and active root nodules (N_2 fixation, which was associated with greater N availability for host plant growth). Such an interaction was observed, both with respect to growth and physiology. However, our results do not exclude the possibility (or probability) that other feedbacks normally associated with plant growth were also operating. Our hypothesized feedback would have also been regulated by the availability of water and other nutrients, and by other growth feedbacks.

Photosynthesis and nitrogenase activity, nodule characteristics, and N

Whole-plant photosynthesis was increased by CO_2 enrichment, both because of its large positive effect on photosynthetic efficiency and slight positive effect on leaf area per plant. The level of combined N supplied to non-nodulated plants was growth-limiting, especially to leaves. Therefore, photosynthetic capacity in these plants was limited relative to nodulated plants. Photosynthetic efficiency of non-nodulated plants, however, was not significantly below that of nodulated plants growing at the same CO_2 concentration. This indicated that the growth-limiting level of combined N supplied to non-nodulated plants mainly affected photosynthetic capacity by restricting leaf area growth.

Our results of nitrogenase activity are similar to those reported for soybean grown under CO_2 enrichment (from 800 to 1200 μl CO_2 l^{-1}) (Hardy & Havelka, 1976), however they differ from results of other studies on annual legumes (Masterson & Sherwood, 1978; Finn & Brun, 1982) and N_2-fixing trees (Norby, 1987), which have shown that only whole-plant nitrogenase activity was enhanced by CO_2 enrichment. Phillips et al. (1976) found that short-term exposure of pea plants to elevated CO_2 (from 320 to 1200 μl CO_2 l^{-1}) doubled SNA, while long-term exposure stimulated whole-plant nitrogenase activity by increasing nodule biomass. In our study, whole-plant activity increased because both SNA and nodule dry mass per plant tended to be positively affected by CO_2 enrichment. We cannot absolutely exclude the possibility, however, that contamination may have affected the results in some unobvious way.

Dry mass production and leaf growth

Dry mass production of symbiotic plants grown at ambient CO_2 in our study was comparable to that reported for seedlings of similar age in four other studies using other species of *Alnus* (Tjepkema, 1985; Sellstedt, 1986; Sellstedt & Huss-Danell, 1986; Norby, 1987). Likewise, growth of symbiotic seedlings at elevated CO_2 was similar to that reported for young *A. glutinosa* seedlings under CO_2 enrichment (700 μl CO_2 l^{-1}) (Norby, 1987). Increased plant growth was directly related to improvement of plant N status. Therefore, dry mass production was improved as N availability, from CO_2-enhanced N_2 fixation, was increased. Application of combined N accounted for the larger feedback effect observed on day 47 in nodulated plants growing at high CO_2. The delayed growth response of nodulated plants grown without N to the feedback was likewise attributed to a lack of available substrate N while nodules were forming and beginning to fix N_2. MacConnell & Bond (1957) established that nodule initiation and development was facilitated by supplying newly-inoculated *Myrica gale* L. and *A. glutinosa* with some combined N. Ingestad (1980) also reported that nodule biomass and N_2 fixation in *Alnus incana* (L.) Moench was enhanced by frequently supplying low concentrations of combined N.

Dry mass allocation

For plants grown at both levels of CO_2, changes in dry mass allocation to above and below ground organs conformed well to the resource partitioning model described by Davidson (1969). In his model, partitioning of resources to shoots and roots was expressed as a balance between shoot and root activity, with shoots providing C and roots providing nutrients and water. Therefore, in non-nodulated plants growing under conditions of relative N-deficiency (as indicated by lower plant N content), a greater percentage of their dry mass was allocated to roots, and less to above ground organs (also described by Loomis, 1953; Nátr, 1975; Dawson & Gordon, 1979). Since N can be derived from N_2 fixation, a reduction in dry mass allocation to roots (and a greater allocation to shoots) of nodulated plants (especially those growing in relatively N-poor substrates) is to be expected and has been observed (Ingestad, 1980; Sellstedt, 1986; Sellstedt & Huss-Danell, 1986). The amount of N made available to nodulated plants from N_2 fixation was apparently greater than the N supplied to non-nodulated plants.

Changes in C source and sink activities resulting from the hypothesized feedback were apparent in a small, but statistically significant, increase in % dry mass allocated to the stem. Since stems are not normally primary assimilatory organs for C or nutrients (N), they are also likely to be relatively low priority C sinks (Bormann & Gordon, 1984). When additional C and N were present (in nodulated plants grown at high CO_2 and supplied combined N), some was apparently still available after higher priority assimilatory sinks (leaves, roots, and nodules) had

been filled, and this relative surplus of these resources appears to have been allocated to the stem. If this effect were (or could be) maintained or further enhanced throughout the life of a tree, significant yield improvement of wood fiber could be realized.

Conclusions

This study indicates that a feedback loop between photosynthesis and N_2 fixation may be an important mechanism by which *A. rubra* is able to compensate for the C drain of N_2-fixation. Therefore, dry mass yields of N_2-fixing trees grown under CO_2 enrichment appear not to have been decreased by these C drains and may be comparable to yields of non-fixing trees growing on similar sites. On relatively N-poor sites, growth of fixing trees is likely to be greater than that of non-fixing trees. Longer-term experiments should be conducted using *A. rubra* and other N_2-fixing trees in order to confirm the effect of a feedback on dry mass allocation to the stem. More research is needed to evaluate how important a feedback would be under field conditions, as well as what other potentially important effects this phenomenon might have on plant yield, competition among species, and ecosystem function. The existence of a feedback system would also point to the potential gains in host plant growth that could be realized by choosing optimum (efficient) host/endophyte combinations and to the potential for significant increases in yield of woody biomass of N_2-fixing trees under conditions of CO_2 enrichment. To test our feedback hypothesis definitively and more quantitatively than we have, a double-labelling experiment using $^{14}CO_2$ and $^{15}N_2$ should be performed.

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