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Effect of CO_2 Enrichment on Photosynthetic Rates, Enzyme Activitiy and End Products of two Poplar Clones, I-214 (*Populus euramericana*) and Peace (*P. koreana x P. trichocarpa*)

Shin-Young Park and Akio Furukawa

National Institute for Environmental Studies, Onokawa 16-2, Tsukuba, Ibaraki, 305, Japan

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Two comparative poplar clones (I-214: *Populus euramericana, Peace: P koreana x P. trichocarpa*) were exposed to two CO_2 concentrations (350 or 2,000 $\mu L\ L^{-1}$ CO_2) for 21 days. When both poplar clones were compared at growth conditions, the net photosynthetic rate (P_N) in CO_2-enriched (2,000 $\mu L\ L^{-1}$ CO_2 = $C_{2,000}$) plants become about 50-60% higher than that of 350 $\mu L\ L^{-1}$ CO_2 (=C_{350}) plants on 7 days treatment. But the enhancement of P_N by high CO_2 was not maintained throughout all the experimental period. At 21 days, there was no difference of photosynthetic rates between C_{350} and $C_{2,000}$ plants. In contrast with photosynthesis, the response of leaf conductance to the elevated CO_2 concentration was very different between I-214 and Peace. During all experimental period, leaf conductance (g_s) of $C_{2,000}$ plants is 50% lower than that of the C_{350} plants for I-214, while there is no difference of g_s between the plants of C_{350} and $C_{2,000}$ for Peace. The results of g_s in Peace indicate that decreased photosynthetic rate after 21 days in $C_{2,000}$ plants for two poplar clones is possibly due to non-stomatal factors. To investigate the non-stomatal factors, starch accumulation and ribulose-1,6-bisphosphate carboxylase (RuBPCase) were measured. We found significant accumulation of starch in two poplar clones exposed to high CO_2, especially starch of I-214 in $C_{2,000}$ become 3.5 times higher than in C_{350} plants at 21 days. This suggests that high proportion of photosynthates was directed into starch. After 21 days, the activity of ribulose-1, 6-bisphosphate carboxylase of $C_{2,000}$ plants become decreased in 40-50% compared with that of the C_{350} plants. Two poplar clones show the same trend to RuBPCase declines under high CO_2 concentration, although the decline is more significant for I-214. The results reported here suggest that starch accumulation and decreased RuBPCase activity in $C_{2,000}$ plants can be partly ascribed to the loss of photosynthetic efficiency of high CO_2-grown poplar plants.

Key Words : Photosynthetic rate, Leaf Conductance, Starch, High CO_2

1. Introduction

Wide variation exists among C_3 plant species in their photosynthetic and growth responses to elevated CO_2 levels. A sudden increase in atmospheric CO_2 enhances net photosynthesis because CO_2 is the substrate for photosynthesis, and because higher concentration of CO_2 result in more favorable competition with O_2 for ribulose -1, 5-bisphosphate carboxylase/oxygenase, the primary enzyme of the photosynthetic pathway. However, continued exposure to elevated CO_2 leads to decline the initial stimulated photosynthesis. This type of decrease in photosynthetic rate has often been reported in many crop species and herbaceous plants (Stitt, 1991; Mousseau and Saugier, 1992; Poorter 1993), either as partial or occasionally as complete acclimation. Considerable evidence has accumulated from studies with herbaceous plants that photosyn-

thetic acclimation is somehow linked to a lack of adequate sinks for the additional carbon being fixed, and feedbacks *via* starch accumulation and biochemical down-regulation of Rubisco are postulated (Bows, 1991; Arp, 1991; Morcuende *et al.*, 1996). Although, it is important to examine the possibility of photosynthetic acclimation to elevated CO_2 in trees, the evidence in trees supporting the linkage between acclimation and sink strength is much more limited.

In a previous paper (Park *et al.*, 1995) we reported that the initial beneficial effect of CO_2 enrichment on plant growth of I-214 was not maintained during all experimental period. Many studies have quantified this long-term decline, yet there is still no consensus on the causes of this phenomenon. Spencer and Bowes (1986) suggested that reduced stomatal conductance of leaves maintained at high CO_2 could partly explain the acclimation. Yelle *et al.* (1989) reported a significant decrease of stomatal conductance of tomato leaves under high CO_2. However, the constant value of internal CO_2 concentration throughout the experiment suggested that reduced stomatal conductance could not explain lower photosynthetic rates. This indicates the importance of feedback inhibition and starch accumulation damage separately from stomatal effects. Attempts to establish the nonstomatal nature of the feedback inhibition of photosynthesis have been made in many crop species (Goldschmidt and Huber, 1992; Morcuende *et al.*, 1996; Greiner *et al.*, 1996), but few studies of this nature have been done in trees. The first objective of this study was to verify the contribution of stomatal and non-stomatal factors to the photosynthetic rate of two poplar clones to high CO_2, especially using by the mutant poplar of Peace, which have unresponsive stomate to environmental factors (Furukawa *et al.*, 1990). The second objective was to identify whether the decline of photosynthesis to high CO_2 results from starch accumulation and/or change in the activity of RuBPCase in two poplar clones.

2. Materials and Methods

2.1. Plant materials

I-214 (*Populus euramericana*) and Peace (*P. koreana* x *P. trichocarpa*) were propagated by cuttings. Cuttings were cultivated for 3 weeks in a greenhouse by hydroponic culture (Hyponex Japan, N : P_2O_5 : K = 6 : 40 : 5). One week before the treatment of enriched CO_2 , the plants were transferred into the controlled environment room at day/night temperature regime of 25/25°C with a light/dark period of 14/10 hour and 70% RH (relative humidity). Light was provided by metal halide lamps giving PFD (photon flux density) of 400 μmol m^{-2} s^{-1} at the level of pot surface. After one-week pre-conditioning, plants were transferred to small growth chambers (0.9m x 0.6m x 2.2m) in which CO_2 concentrations were kept at 350 and 2,000 μL L^{-1}, respectively. Each chamber was made of transparent acrylite and contained two fans to mix internal air and a cooling device to maintain consistent air temperature in the chamber. Chamber temperatures were maintained at 27-28℃. In each chamber, CO_2 was injected automatically through a mass-flow-controller and continuously monitored by an infrared gas analyzer (Fuji, Model ZAP).

2.2. Gas exchange measurements

On 7, 14, and 21 days after CO_2 treatments (350 or 2,000 μL L^{-1}), gas exchange of single attached leaves was measured with a water-cooled aluminum assimilation chamber (3 × 4cm) as described by Furukawa *et al.* (1983). All mea-

surements were made at 25℃, and a quantum irradiance of 700 μmol m^{-2} s^{-1}. Photosynthesis and transpiration were determined in fully expanded 9th to 11th leaf from the top of plants. Leaf conductance and intercellular CO_2 (C_i) concentrations were calculated as in Farquhar and Sharkey (1982).

2.3. Carbohydrate measurements

Leaf disks (1 cm in diameter) were taken at 05:00, 1 h before light were turned on when the starch level was the lowest, and at 17:00, 10 h after exposure to light when leaf starch level was the highest. The same leaves were used for both samplings. Three leaf discs were extracted with 1 ml boiling HEPES buffer (pH 7.4) for 2 min, homogenated, then centrifuged for 10 min at 14,000 rpm and divided into soluble and insoluble fractions. The insoluble fraction, containing starch, was re-extracted two times with 80% methanol until the insoluble matter was free from soluble fraction. Starch was digested with amyloglucosidase (BM) and released glucose was determined enzymatically (Keppler and Decker 1974). Standard curves were run with amylopectin.

2.4. Enzyme extraction and assays:

For the leaf enzyme assays, leaf disks were taken from leaves grown at 350 or 2,000 μL L^{-1} CO_2 for 21 days and plunged immediately into liquid N_2. The frozen leaf samples were ground on ice in a chilled mortar and pestle with a 1ml grinding medium containing 100 mM Tricine-NaOH (pH8.0), 8 mM $MgCl_2$, 50 mM 2-mercaptoethanol, 1 mM Na_2-EDTA, and 5%(w/v) insoluble polyvinyl-pyroridine-40. The homogenate was centrifuged at 10,000g for 10 min at 4°C and the supernatant was used as the enzyme preparation. Ribulose bisphosphate carboxylase (RuBPCase) was assayed spectrophotometrically at 30°C (Nakamura *et al.*, 1989). The levels of RuBPCase protein from leaves of I-214 grown at 350 or 2,000 μL L^{-1} CO_2 for 21 days were determined by a single radical immunodiffusion method (SRID).

2.5. Electron microscopy

Transmission electron microscopy was used to determine the location and size of starch grains in plants grown at elevated CO_2 concentration. Leaf samples, collected at 15:00 from fully expanded leaves grown under 350 and 2,000 μL L^{-1} CO_2 for 21 days, were cut into 1 mm squares and fixed in 4% glutaraldehyde, post-fixed in 2% osmium tetroxide, then dehydrated and infiltrated with Spurr's resin. Thin sections made with a LKB Nova ultramicrotome were post-stained with uranyl acetate and lead citrate. Micrographs were made with a JEOL JEM 2,000FX transmission electron microscope.

3. Results

A typical irradiance response curves of P_N at external CO_2 of 350 μL L^{-1} was shown in Fig. 1. In both poplar clones, photosynthetic response to irradiance was similar and almost saturated at 700 μmol m^{-2} s^{-1}. All other experiments on P_N and g_s were conducted at 700 μmol m^{-2} s^{-1}. The effect of elevated CO_2 on P_N and g_s in two poplar clones were measured at 7, 14 and 21 days after CO_2 treatment (Fig. 2). When two poplar clones were measured at their growth CO_2 condition, P_N was significantly higher in $C_{2,000}$ plants at 7d of treatment. However, the increase in P_N of I-214 and Peace by CO_2 enrichment declined with following prolonged treatment and

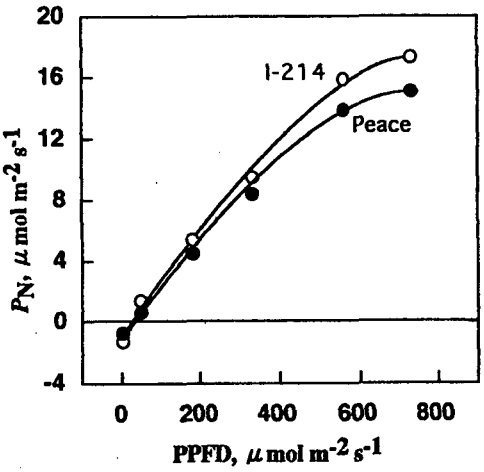

Fig. 1. Photosynthetic response to irradiance of two poplar clones grown under 350μL L^{-1} CO_2. Measurements were made at 350μL L^{-1} CO_2.

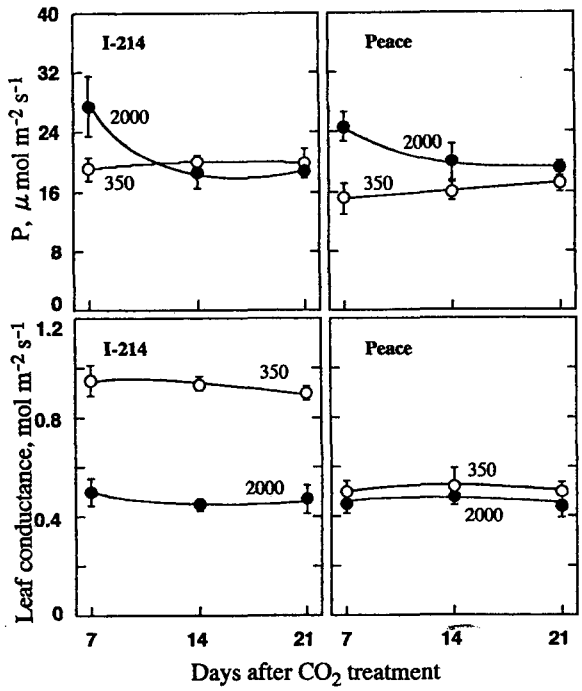

Fig. 2. Photosynthetic rates and leaf conductance for plants grown at 350 or 2,000 μL L^{-1} CO_2. They were measured at the level under which they were grown. Vertical bars indicated ±SD of the means of three independent measurements on different plants.

there was no difference between C_{350} and $C_{2,000}$ plants at 21 days. Under the same measurement condition with P_N, g_s of I-214 in $C_{2,000}$ plants was 50% lower than that of C_{350} plants at 7 days. Thereafter it had little changed with following prolonged treatment. On the other hand, there was little difference in g_s between C_{350} and $C_{2,000}$ plants of Peace throughout all the treatment period. Photosynthetic rates increased with increasing C_i and saturated at approximately 700 μL L^{-1} CO_2 in C_{350} and $C_{2,000}$ plants. However, at any given C_i the photosynthetic rate of $C_{2,000}$ plants was substantially lower than those in C_{350} plants(Fig. 3).

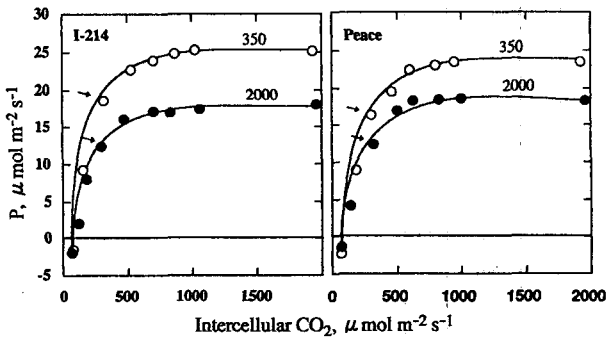

Fig. 3. The relationship between photosynthesis and internal CO_2 concentration for the I-214 and Peace, when grown in either 350 (○) or 2,000 (●) μL L^{-1} CO_2. Measurements were made at 700 μmol m^{-2} s^{-1}. The arrows (→) indicate the points obtained at an external CO_2 of 350 μL L^{-1}. Mean values are shown : n=3.

CO_2 treatment had a profound effect on the leaf starch accumulation. In both poplar clones, starch contents in $C_{2,000}$ plants were higher than those of C_{350} plants, except that sampled at 05:00 on 7 days treatment of I-214, and higher in the leaves sampled at 17:00 than 05:00 (Fig. 4 A and B). These responses of starch to high CO_2 were more significant for I-214. The period of CO_2

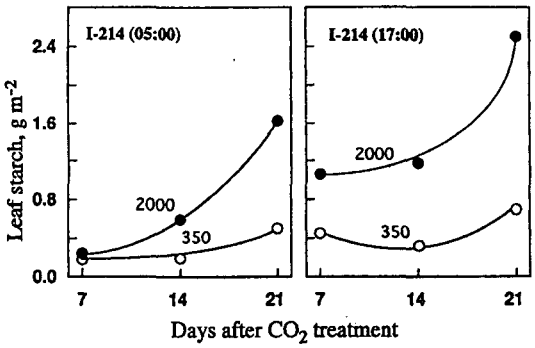

Fig. 4a.

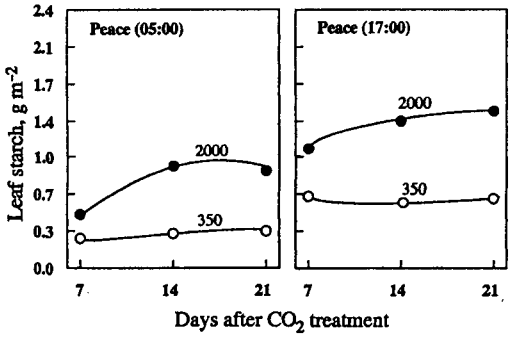

Fig. 4b.

Fig. 4. Effects of CO_2 treatments on leaf starch contents in I-214 (A) and Peace (B) at 05:00 and 17:00.

treatment also affected on the starch accumulation. More prolonged treatment resulted in more significant accumulation of starch For CO_2-enriched I-214, starch contents sampled at 05:00 and 17:00 after 21 days were more than 6 and 2 times greater than those of 7days, respectively. At 21 days, starch contents in $C_{2,000}$ plants of I-214 sampled at 05:00 and 17:00 were more than 3 times greater than in the C_{350} plants. Compared with plants of Peace grown at 2,000 for 21 days, starch contents sampled at 05:00 and 17:00 were 44% and 32% higher in plants of I-214, respectively. These results became more apparent on electron micrographs (Fig. 5 and 6). Electron

Fig. 5a.

Fig. 5b.

Fig. 5. Electron micrographs of chloroplast in leaf tissue of I-214 grown at 350 (A) or 2,000 (B) $\mu L\ L^{-1}$ CO_2 for 21 days. Leaf samples were obtained at 15:00.

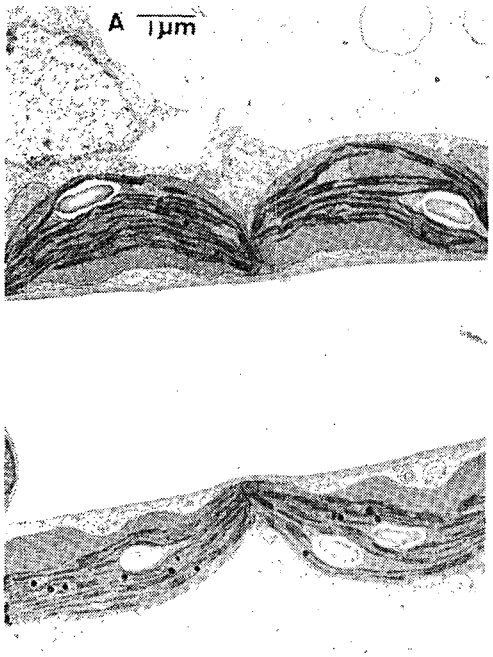

Fig. 6a.

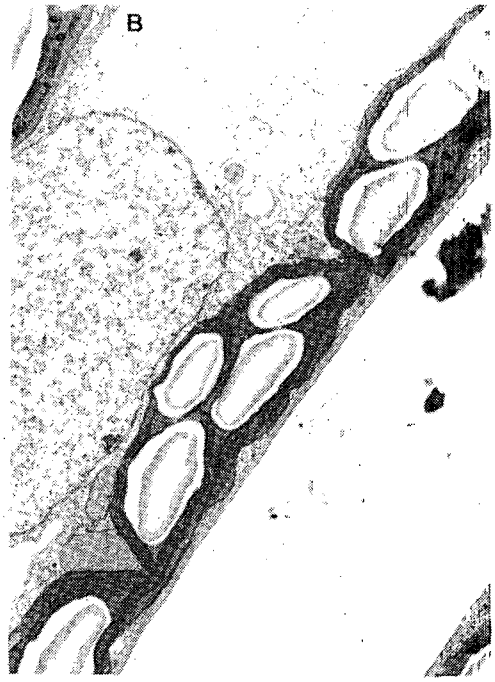

Fig. 6b.

Fig. 6. Electron micrographs of chloroplast in leaf tissue of Peace grown at 350 (A) or 2,000 (B) μL L^{-1} CO_2 for 21 days. Leaf samples were obtained at 15:00.

micrographs of chloroplasts in leaves of $C_{2,000}$ showed the development of excessively large aberrant starch granules. This symptom became more pronounced in I-214 (Fig. 5B). For I-214, the configuration of grana stacks in $C_{2,000}$ plants appear to have been disturbed and distorted by these large irregularly shaped, starch grains (Fig 5 and 6).

Table 1. A comparison of RuBPCase activity and RuBPCase concentration for I-214 and Peace when grown at either 350 or 2,000 μL L^{-1} CO_2 for 21 days.

poplar	CO_2	Activity	Concentration
	μL L^{-1}	mmol m^{-2} min^{-1}	μg cm^{-2}
I-214	350	1.57 ± 0.06	598 ± 10.4
	2,000	0.82 ± 0.05	452 ± 62.0
Peace	350	1.24 ± 0.03	n.a.
	2,000	0.79 ± 0.02	n.a.

* Mean values ± SD are shown: n=4 (n.a. = not assayed).

At 21 days after treatment, RuBPCase activity and RuBPcase concent- ration were decreased 47.8 and 24.5% in leaves of $C_{2,000}$ plants as compared to in C_{350}. This decrease was significant for I-214, although the two poplar clones showed the same trend to the activity of RuBPCase decline under high CO_2 concentration (Table 1).

4. Discussion

As is commonly found with C_3 plants (Fisher *et al.* 1981, Rogers *et al.* 1986), short term CO_2 enrichment resulted in a 40-70% increase of P_N in two poplar clones. However, the initial enhancement of photosynthetic rates of both poplar clones were not maintained during the

whole experimental period. At 21 days, P_N in $C_{2,000}$ plants for two poplar clones were similar to or less than those of C_{350} plants even when measured at their growth conditions, $C_{2,000}$ plants were measured at 2,000 $\mu L\ L^{-1}$ CO_2. These results, therefore, indicated that the growth enhancement in $C_{2,000}$ plants during the first two harvest periods (Park *et al.*, 1995) was mainly due to the increased photosynthetic rates at that time, and the inhibition of growth enhancement during the last harvest period (21 days), particularly in I-214, resulted from the declined photosynthetic rate by CO_2 enrichment. Inhibition of photosynthetic enhancement in $C_{2,000}$ plants in this study was not associated with decreased g_s. This is because that g_s in $C_{2,000}$ plants at 7days was 50% lower than in C_{350}, however P_N in $C_{2,000}$ plants was higher than in C_{350} plant. Our results of g_s in mutant poplar of Peace supported that reduced g_s could not explain lower photosynthetic rates, because photosynthetic rate in $C_{2,000}$ plants was declined although g_s was not changed by CO_2 treatment.

The relationship between internal CO_2 concentration and photosynthetic rate in $C_{2,000}$ plants demonstrated a strong non-stomatal limitation to photosynthesis. At 21 days, the photosynthetic rates of $C_{2,000}$ plants in both poplar clones were lower than those of C_{350} plants when both were measured at the same external CO_2 concentration. This indicates that the inhibition of photosynthesis in $C_{2,000}$ plants is mainly due to the decrease in internal CO_2 utilization, but not by the inhibition of CO_2 uptake. Delucia *et al.* (1985) also found no consistent differences in stomatal limitation of P_N between Gossypium hirsutum grown under 350, 675 and 1,000 $\mu L\ L^{-1}$ CO_2. Pammenter *et al.* (1993) and Greiner de Mothes *et al.* (1996) also reported that P_N and g_s were not linearly related at saturated CO_2 concentration, probably due to end product feedback inhibition of photosynthesis.

The literature of CO_2 enrichment contains a substantial debate as to whether the massive production and build-up of carbohydrates, particularly starch, cause a decline in photosynthesis (Azcon-Bieto, 1983; Vu *et al.*, 1989; Allen, 1990; Rowland-Bamford *et al.*, 1990). DeLucia *et al.* (1985) demonstrated that starch concentration increased gradually throughout the light period and declined to the previous morning's level by the end of the dark period. And also indicated degradation and/or translocation of carbohydrates was insufficient in the high CO_2 grown plants to reduce the starch pool to the previous morning's level by the end of the dark period. Our results for starch suggests that overnight translocation of photosynthates is very limited in $C_{2,000}$ plants, because starch contents in $C_{2,000}$ plants of sampled at 05:00 were more than 3 times higher compared with C_{350} plants on 14 and 21 days. However there was little difference between C_{350} and $C_{2,000}$ plants at 7days treatment. This suggests that the decline of photosynthesis in $C_{2,000}$ plants measured on 14 and 21 days can be attributed to the accumulation of starch. Electron micrographs of chloroplasts from leaves of C_{350} and $C_{2,000}$ at 21 days support it and offer additional evidence that excessively large aberrant starch granules in plants grown at elevated CO_2 concentration affect chloroplast structure. In contrast to a normal chloroplast, the configuration of grana stacks appears to be disturbed and distorted by these large irregularly shaped, starch grains. The distortion was usually more pronounced in I-214 than in Peace. This can be attributed to lower levels of starch in peace than in I-214 leaves. Because the photosynthetic rates after 21 days on CO_2 treatment are similar between ambient- and enhanced-CO_2 grown plants, higher starch concentration in the enhanced plants indicate that

photosynthetically fixed carbon is partitioned into starch synthesis. The excess starch accumulation in $C_{2,000}$ plants for I-214 could inhibit the plant growth, especially in conditions where the amount of photosynthates exceeds the capacity of consumes. The most often cited mechanisms to account for a negative effect of carbohydrate on photosynthesis include feedback inhibition and chloroplast disruption.

Irrespective of the mechanism linking starch production and photosynthetic CO_2 assimilation, a decline in the latter appears to be mediated through changes in RuBPCase activity. In both poplar clones, reduced photosynthetic efficiency in $C_{2,000}$ plants was associated with decreased RuBPCase activity (table 1). Lower RuBPCase activities in CO_2-enhanced plants have been reported in many herbaceous plants including bean (Porter and Grodzinski, 1984), rice (Rowland-Bamford *et al.*, 1991) and soybean (Vu *et al.*, 1983). The differing slopes of photosynthetic rate to internal CO_2 concentration in both clones can also be explained by the fact that lower carboxylase activities in the enhanced plants. The quantitative determination of RuBPCase suggests that the decrease in activity of RuBPCase in $C_{2,000}$ plants is some due to the decreased RuBPCase protein content but not to the inactivation of the enzyme.

In conclusion, plants after 21 days CO_2 treatment produce a high level of starch accumulation, show decreased RuBPCase activity and a decline in potential net photosynthesis in two poplar clones. The decline in photosynthesis in the high CO_2 plants seems to be a result of non-stomatal limitations that contributed to higher internal resistances. It is suggested that carbohydrate induced feedback inhibition and possibly physical damage at the chloroplast level are responsible for limiting photosynthesis in the high CO_2 grown plants. Strong sinks or rapid translocation may avoid such acclimation responses.

Further researches to study the effects of high CO_2 on the mesophyll resistance need to uncover the biochemical and physiological reasons underlying the efficiency loss.

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