Elevated CO_2 Increases Root Mass and Leaf Nitrogen Resorption in Red Maple (*Acer rubrum* L.)

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Abstract: To understand whether the process of seasonal nitrogen resorption and biomass allocation are different in CO_2-enriched plants, seedlings of red maple (*Acer rubrum* L.) were exposed to three CO_2 concentrations (800 μL L^{-1} CO_2 treatments—A800, 600 μL L^{-1} CO_2 treatments—A600, and 400 μL L^{-1} CO_2 treatments—A400) in nine continuous stirred tank reactor (CSTR) chambers. Leaf mass per area, leaf area, chlorophyll index, carbon (C), nitrogen (N) contents, nitrogen resorption efficiency (NRE), and biomass allocation response were investigated. The results indicated that: (1) Significant leaf N decline was found in senescent leaves of two CO_2 treatments, which led to an increase of 43.4% and 39.7% of the C/N ratio in A800 and A600, respectively. (2) Elevated CO_2 induced higher NRE, with A800 and A600 showing significant increments of 50.3% and 46.2%, respectively. (3) Root biomass increased 33.1% in A800 and thus the ratio of root to shoot ratio was increased by 25.8%. In conclusion, these results showed that to support greater nutrient and water uptake and the continued response of biomass under elevated CO_2, *Acer rubrum* partitioned more biomass to root and increased leaf N resorption efficiency.

Keywords: elevated CO_2; *Acer rubrum*; senescent leaf N; nitrogen resorption efficiency; biomass response

1. Introduction

The initial effect of elevated CO_2 increases net primary production (NPP) by enhancing leaf photosynthesis in most plant communities. To sustain the high rates of forest NPP observed under elevated CO_2, the requirement for nitrogen would also increase the close relationship of photosynthesis [1] and the synthesis of proteins required for the construction and maintenance of living tissue. However, studies confirm that growth at elevated CO_2 results in significant reductions in N content in leaves (mass basis) and a relatively higher C/N [2] ratio. Elevated CO_2 which may cause nitrogen (N) concentrations to decline in green leaves has been well investigated [3]. The possible reasons for N decline in leaf include dilution effects [4], increase of less N demand (increased use of photosynthetic nitrogen efficiency and downregulation of photosynthetic enzymes) [5–7], less N available (carbon enrichment of the rhizosphere leads to progressively greater limitations of the nitrogen available to plants) [7–9], and CO_2 inhibition of nitrate assimilation [10]. Apart from this direct effect on photosynthesis, sugar sensing and signaling pathways are reported as important pathways to indirectly regulate the photosynthesis process [11].

It is well known that elevated CO_2 increases plant growth and leads to greater biomass production. However, which plant organ is allocated the greater production of carbohydrates varies within and between species. There is a wide range of root responses from large increases [12], to no response [13,14],

and decreased responses [15]. The functional balance of shoot root biomass partitioning models predict that as plant tissue C/N ratios rise, root biomass allocation will increase to ensure greater uptake of N to balance the extra C input due to elevated CO_2 [16]. It is also reported that greater root growth is related to the sugar signaling pathway which acts on regulating nutrient uptake and transport [11]. In addition, there has been considerable debate concerning whether elevated CO_2 results in shifts in the root to shoot ratio (R/S). Thus, identifying the ecological attributes that predict root responses remains challenging.

Nutrient resorption is defined as the process by which nutrients are mobilized from senescent leaves and transported to other plant parts [17]. The differences between nitrogen (N) in green leaves and N of abscised leaves are dependent on the process of N resorption [18,19]. Nitrogen resorption efficiency is calculated as N resorption divided by the initial N content (N in green leaves). However, the reports of the effects of elevated CO_2 on litter N are less than on green leaves [3,20]. Litter N inherently is more difficult to detect because factors that affect senescence and resorption are increasingly variable. Although significant declines in green leaf N content in elevated CO_2 have been reported [18], few significant effects have been reported on N resorption independently of warming, lacking light, soil nitrogen, and water conditions. Nitrogen resorption may become an increasingly important source of N as a higher net primary production induced by elevated CO_2. Biomass allocation will be a critical feature to address the environmental challenges, and its relationship to foliar N resorption is poorly understood.

Red maple (*Acer rubrum* L.) was selected for this experiment because it is one of the most common and widespread deciduous trees of eastern and central North America, growing on a wide range of soil types in both moist and dry biomass. The main objective of the experiment is to study the leaf N content, N resorption efficiency, and biomass allocation responses to elevated CO_2, independently of warming, lacking light, soil nitrogen, and water conditions. We want to know whether the process of seasonal leaf N resorption and biomass allocation are different in CO_2-enriched plants. The hypothesis tested is that elevated CO_2 would increase biomass allocation to the roots and increase leaf N resorption for more N demand in a condition of adequate nutrients and water.

2. Materials and Methods

2.1. Experimental System and Design

The experiment was investigated in nine continuously stirred tank reactor (CSTR) chambers (Figure 1), which were located inside a glass greenhouse at the University of Massachusetts, Amherst. More details of the operation of our CSTR chambers and CO_2 control system have been described previously [21–23] and the CO_2 concentration fluctuation range was ±10 μL L^{-1} around the target CO_2 concentrations. The time course of irradiance inside the greenhouse was adjusted the day before depending on the weather forecast, so that the irradiance inside the greenhouse was similar to the natural environment. The photoperiod varied with the season outside the greenhouse, mostly from 6 a.m. to 7:30 p.m. All seedlings were exposed to natural light since the chambers were located in a separate single glass greenhouse, but a supplementary irradiance was also supplied with nine 400 W metal Halide lights (Metal Arc, Sylvania, Worcester, MA, USA) in order to better simulate outdoors irradiance.

The irradiance inside was 75%–85% of that outside. The maximum radio flux was 630 lx. The temperature inside the greenhouse (only analyzed the day we collected data) varied from 18.16 °C to 35.8 °C and the average value was 35.8 °C. The relative humidity varied from 33.99% to 81.96%, and the average value was 58%. The temperature and relative humidity were on average 4.8 °C higher and 15% lower than outside, respectively. The average temperature and relative humidity in the 800 and 600 μL L^{-1} chambers were 0.34 °C higher and 2% lower, respectively, than in the control chamber [24]. CO_2 concentration enrichment was started from June 26, 2014 to November 28, 2014 (156 days) with pure CO_2 supplied continuously for 24 hours. One LI–7000 CO_2/H_2O analyzer monitor

was used to measure and record the CO_2 concentration (Li–Cor Inc., Lincoln, NE, USA). The CO_2 concentration inside every chamber was checked and adjusted every two days to make sure the target CO_2 concentration settings could be reached. Three CO_2 concentration treatments (800 μL L^{-1}—A800, 600 μL L^{-1}—A600, and 400 μL L^{-1}—A400) were set up randomly among nine chambers with three replications each. Twenty-seven seedlings, uniform in basal diameter and height, were carefully picked up and divided into nine groups, so there were three seedlings grown inside each chamber. All seedlings in chambers were watered every other day with the same volume of water as needed.

Figure 1. The nine continuously stirred tank reactor (CSTR) chambers in the experiment.

2.2. Plant Material and Management

Two-year-old nursery-grown red maple (*Acer rubrum* L.) seedlings grew from seeds (natural settings) were obtained from the Forrest Keeling nursery (fknursery.com) in Elsberry. Red maple, one of the most common and widespread deciduous trees of eastern and central North America, is adaptable to a very wide range of site conditions. All seedlings were transplanted immediately in 1-gallon pots to the greenhouse on June 3. The growing medium used in pots was an inorganic potting mixture (MM 300 growing medium) (SunGro, Washington, WA, USA). All seedlings were acclimated to the greenhouse environment from June 3 to June 25 (23 days) until all seedlings grew well, then all seedlings with pots were moved into the CSTR chambers on June 26. All seedlings were well-watered every other day. All seedlings were fertilized with a soluble fertilizer (16–17–18; Peters Professional; Scotts, OH, USA) (3.9 g L^{-1}) every week and a micronutrient soluble trace element mix (36.9 mg L^{-1}) [22] was applied once on August 8.

2.3. Leaf Chlorophyll Index

The chlorophyll index was measured weekly on the same marked leaves used for leaf area, measuring N content weekly from September 5 until complete senescence using a nondestructive SPAD-502 chlorophyll meter (Konica Minolta, Tokyo, Japan). SPAD, a surrogate expressed for chlorophyll index, estimated the amount of chlorophyll present by measuring the amount of light transmitting through a leaf. The Minolta SPAD has two LEDs that emit red (650 nm, chlorophyll absorbs light and is unaffected by carotene) and infrared (940 nm, no absorption occurs) wavelengths through an intact leaf sample. Minolta SPAD data was found to be quite well correlated with leaf N [25] and leaf chlorophyll contents [26], and therefore it has been used to estimate the leaf chlorophyll index nondestructively in other atmospheric CO_2 enrichment studies [27]. The SPAD value in this paper was represented as the average value of the first, second, and third bud-break leaves.

2.4. Elemental Carbon (C), Nitrogen (N) Contents of Senescent Leaves and Nitrogen Resorption Efficiency (NRE)

In the plants, natural leaf abscission was preceded by the autumnal leaf senescence, a process involving changes in the color of the leaves and a decline in their photosynthetic capacity [28]. In our experiment, the onset of senescence was established on October 7. Senescent leaves were easily identified in the red maple by displaying yellow colors and by becoming detached from the plants with very gentle shaking. The greenhouse did not have wind or extremely low temperatures. Some leaves remained attached to the stem without photosynthetic activity, these were tested by very gentle shaking to determine whether they could be considered to be falling leaves. The senescent leaves were collected, and the elemental contents of carbon and nitrogen were measured using an NC2500 elemental analyzer (CE Instruments, Milan, Italy). The N content ($mg\ g^{-1}$) in green leaves was estimated by the regression equation between SPAD and N in senescent leaves ($Y_N = 0.0639X_{SPAD} + 0.0113$, $R^2 = 0.51$, $p < 0.05$). Nitrogen resorption efficiency (NRE) was calculated as the percentage reduction of nitrogen between green and senescent leaves using the following formula [19,27]:

$$NRE = 100\% \times [(\text{green leaf N} - \text{senescent leaf N})/\text{green leaf N}] \tag{1}$$

2.5. Leaf Mass per Area (LMA) and Whole Leaf Area

Leaf mass per area determinations (LMA, $g\ m^{-2}$) were collected on September 15t. LMA was calculated by weighting the dry mass of leaf disks of a known area. The leaf area of red maple was calculated using the formula [29]:

$$\text{Leaf area} = 3.676 + 0.49 \times L \times W \tag{2}$$

where L and W are the longest length and the width of red maple leaves, respectively. Whole plant leaf area was calculated by the sum of the products of leaf number and leaf area in upper, middle, and latter leaves, respectively.

2.6. Biomass Measurements

Root, stem, and leaves were separated and collected at the end of the experiment. The dry mass was determined after drying at 85 °C for 48 hours to constant mass.

2.7. Data Analysis

There are three chambers at each CO_2 concentration and three CO_2 levels.

The effects of elevated CO_2 on all parameters except SPAD and whole leaf area were analyzed by one-way ANOVA with CO_2 concentration treatments as the treatment factor. For SPAD and whole leaf area, split-plot RM-ANOVA was applied as CO_2 levels, time and the interaction of CO_2 and time as the treatment factor. When the CO_2 effect was significant, post-hoc comparisons were taken using the LSD test. Normality (Kolmogorov–Smirnov test) and homogeneity of variance (Levene's test) of the data were checked prior to analysis. Results were considered significant when $p < 0.05$. All the analyses were performed by the SPSS statistics software (Version 18.0, SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Leaf Mass per Area (LMA), Leaf SPAD, and Whole Plant Area

The LMA showed no responses to elevated CO_2 (Figure 2). For SPAD measurements, a decreasing trend was observed during the experiment in all treatments (Figure 3). SPAD values in A800 and A600 were 22.3% and 17.3% lower, respectively, on August 20, when the largest difference between treatments was observed (Figure 3). The whole plant leaf area was not significantly different among treatments (Figure 3).
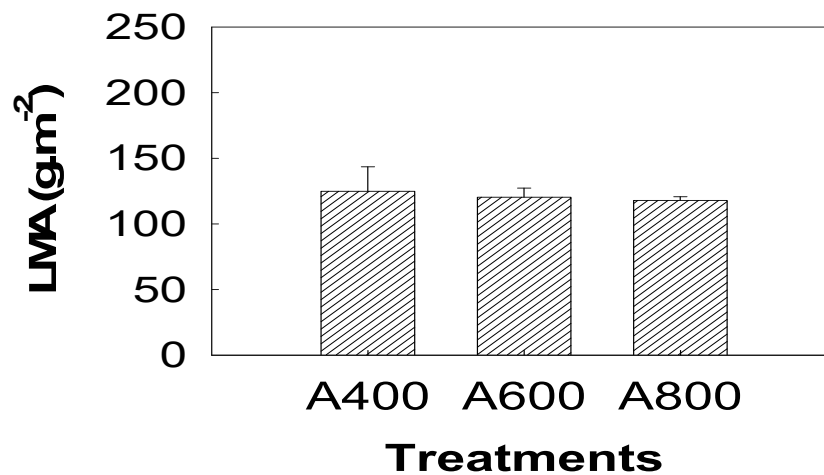
Figure 2. Effects of elevated CO_2 (A400—400, A600—600, and A800—800 µL L^{-1}) on leaf mass per area (LMA) of red maple (*Acer rubrum* L.). The values shown are mean ± SD. There were no significant differences among (CO_2) treatments when $p < 0.05$, $n = 3$.
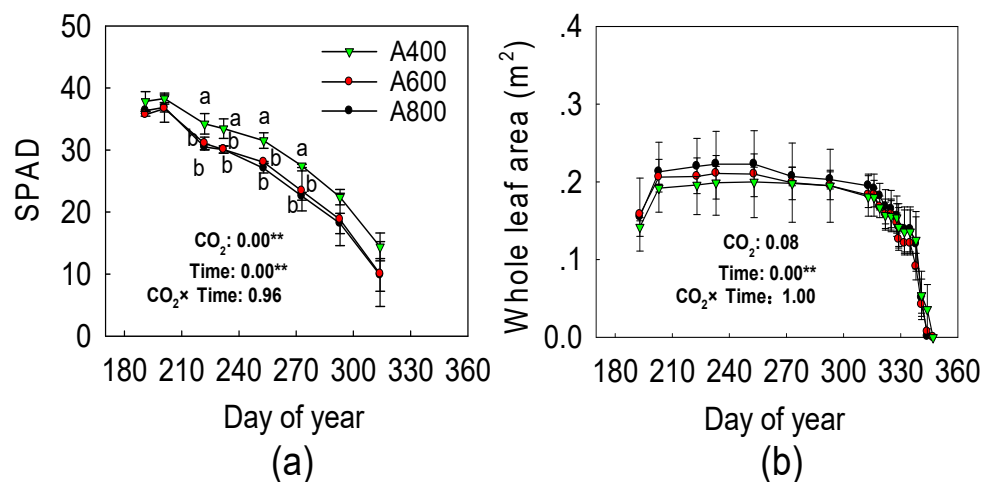

Figure 3. Effects of elevated CO_2 (A400—400, A600—600, and A800—800 µL L^{-1}) on mean SPAD value (**a**) and whole plant leaf area (**b**) of red maple (*Acer rubrum* L.). The values shown are mean ± SD. Different lowercase letters above bars mean significant multiple comparison results among [CO_2] treatments when $p < 0.05$, $n = 3$. The ANOVA results of (CO_2) treatments (CO_2), time, and the interaction ($CO_2 \times$ time) are shown in the figure, ** means $p < 0.01$.

3.2. Leaf Carbon (C), Nitrogen (C) Contents, and Nitrogen Resorption Efficiency (NRE)

The results indicated that N content of senescent leaves in A800 and A600 significantly decreased by 28.1% and 27.5%, respectively (Figure 4). The C content experienced no response to elevated CO_2 (Figure 4). The C/N ratio in A800 and A600 was 43.4% and 39.7% higher, respectively, than in A400. The N resorption efficiency in A800 and A600 increased 50.3% and 46.2%, respectively (Figure 5).
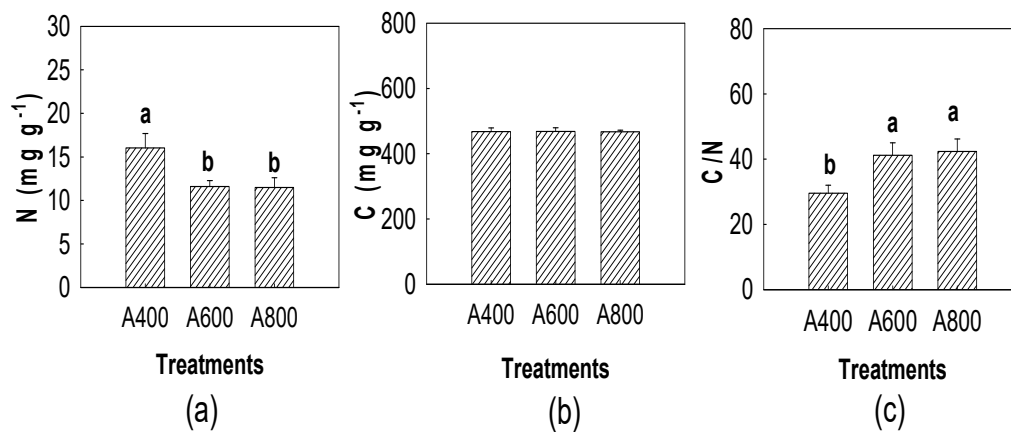
Figure 4. Effects of elevated CO_2 (A400—400, A600—600, and A800—800 μL L^{-1}) on leaf mass-based N contents (**a**) leaf mass-based C contents (**b**) and C/N ratio (**c**) in senescent leaves of red maple (*Acer rubrum* L.). The values shown are mean ± SD. Different lowercase letters above bars mean significant multiple comparison results among CO_2 treatments when $p < 0.05$, $n = 3$.
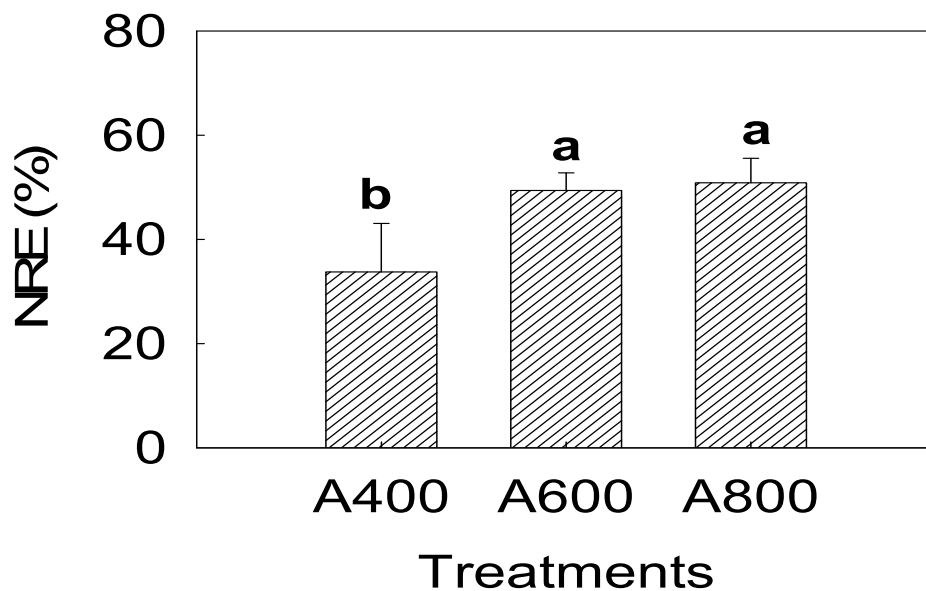

Figure 5. Effects of elevated CO_2 (A400—400, A600—600, and A800—800 μL L^{-1}) on nitrogen resorption efficiency (NRE) of red maple (*Acer rubrum* L.) under three CO_2 concentration treatments (A400—400, A600—600, and A800—800 μL L^{-1}). The values shown are mean ± SD. Different lowercase letters above bars mean significant multiple comparison results among CO_2 treatments when $p < 0.05$, $n = 3$.

3.3. Biomass

With respect to A400, the total dry biomass significantly increased in A800 (by 19.2%) but effects were not significant for A600. The root biomass of A800 also increased by 33.1% and thus the mass ratio of roots to shoots was increased by 25.8% (Figure 6). However, leaf, stem, and leaves did not allocate more biomass under elevated CO_2.
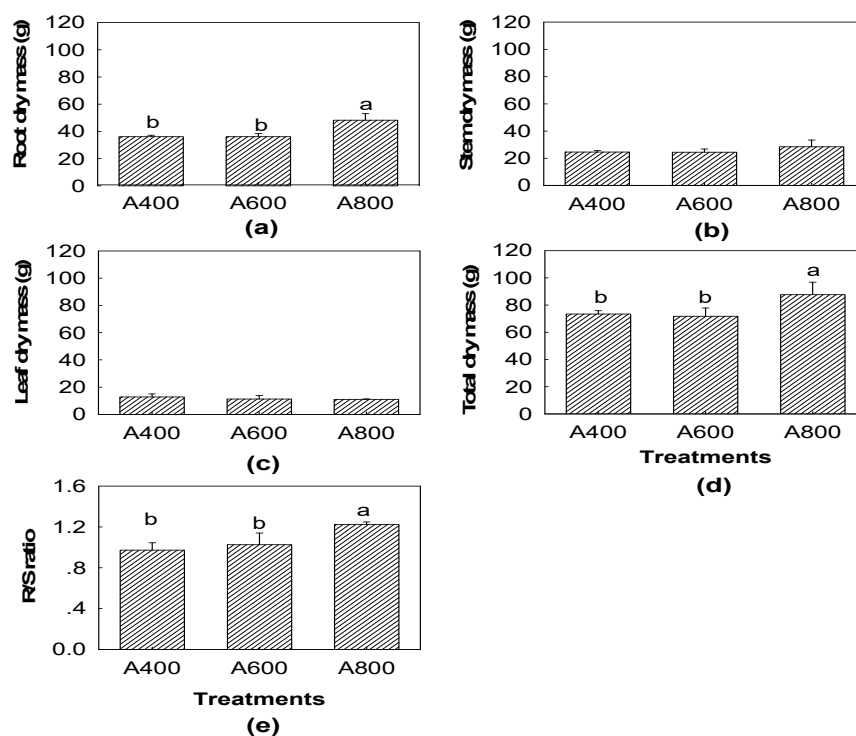
Figure 6. Dry mass of roots (**a**) stem, (**b**) leaves, (**c**) total, (**d**) mass ratio of roots to shoots (R/S ratio, and (**e**) of red maple (*Acer rubrum* L.) under three CO_2 concentration treatments (A400—400, A600—600, and A800—800 $\mu L\ L^{-1}$). The values shown are mean ± SD. Different lowercase letters above bars mean significant multiple comparison results among CO_2 treatments when $p < 0.05$, $n = 3$.

4. Discussion

A long-lived leaf usually acts as a storage organ for a while, especially for Rubisco (Ribulose-1,5-bisphosphate) enzyme which may play a significant role as an instantaneous storage protein [30]. Decreased leaf photosynthesis and nitrogen in the autumn is the first step of leaf senescence (decreased N and increased C/N ratio) for deciduous trees. In our study, N in A800 and A600 all significantly decreased (28.1% and 27.5%, respectively, Figure 4). These results are comparable with Norby et al (2000) [18], who found that CO_2 enrichment reduced leaf N content by 25% in red maple (*Acer rubrum* L.) under elevated (+300 $\mu L\ L^{-1}$) atmospheric CO_2 content. Unlike Curtis (1998) [2], who found reduced N content only when using a leaf mass basis N, but not when using a leaf area basis (due to increased leaf area and leaf weight rather than N reallocation), we found the N mass-based contents decreased without changes in the leaf mass per area (LMA) and the leaf area (Figures 2 and 3). This result suggested that in *Acer rubrum* the significant decrease in leaf N contents under CO_2 enrichment is not caused by changes in leaf density. Meanwhile, the increased C/N ratio of senescent leaf litter is a determinant of long-term allocational carbon and nitrogen and growth responses to CO_2 enrichment. It was reported that although less nitrogen is invested in Rubisco total nitrogen demand of green tissues would reduce, which comprised up to 60% of soluble leaf protein with nitrogen [31,32]. However, although the Rubisco content decreased because more nitrogen was allocated to RuBP (Ribulose-1,5-bisphosphate) regeneration and to Pi (phosphate) regeneration [11,33], the specific activity of plants increased and thus maintained sufficient photosynthetic capacity during the growing season [31].

Nitrogen (N) resorption is defined as the process where nitrogen and other nutrients are mobilized from senescent leaves and then transported to other plant organs [17]. The N resorption efficiency (mass-based) was 50.8% at A800 and 49.4% at A600 in leaves of red maple (Figure 5), which are comparable to a mean resorption efficiency of 49%–59% of some plants obtained by other researchers [34–36] but lower than previous measurements of resorption (around 70%) in *Acer*

rubrum [37]. The N resorption efficiency in our experiment should be underestimated because N reduction in green leaves was more pronounced than senescent leaves [33] while we estimated N in green leaves through the relationship between SPAD and N in senescent leaves.

More N resorbed and stored in permanent tissues or organs, more nitrogen can be used for early growth in the next year, especially in the case when N availability is insufficient to meet the demands of developing foliage [38]. However, senescence is an aging process of losing functions and structures, whose ultimate goal is to remove nutrients from senescent leaves and transport them to other plant organs [17,39].

Elevated CO_2 increases carbohydrate production by stimulating photosynthesis. The proportion (amounts) of the extra carbohydrates accumulated under elevated CO_2 in leaves partitioning among plant organs is not well known. It is also affected by species, plant growth method [40], availability of carbon sinks [41], duration of the experiment [42], or nonstructural carbohydrate accumulation by its role as a signaling molecule (sugar sensing and signaling pathways) [11], etc. Our results indicated that the root to shoot ratio increased and more biomass was allocated to root than to shoot and leaf (Figure 6). There are many similar research results on a general increase in root biomass and root to shoot ratio [43]. Growth in roots would be stimulated when carbohydrates are transported more to roots, and therefore the balance of nutrient/water uptake and transport would also be improved [11]. Root biomass in A800 treatments increased 33.1%, which is comparable with a meta-analysis by Nie et al. (2013), who found the biomass of total root was increased by 28.8% [43]. Under elevated CO_2, biomass, distribution among root, stems, and foliage should be due to the proportion of new C allocation to different tissue types, different turnover rates of the tissue types, or by the two processes in combination [20]. Therefore, increased root biomass is the more typical mechanism supporting greater N uptake and the continued response of NPP under elevated CO_2 [44].

It is noted that the impact of our results also has limitations. First, seedlings and saplings in a natural environment must deal with the far greater seasonal amplitude of environmental variability than adult trees. Two-year seedlings grown from seeds in ambient air are possibly more characteristic [45,46] to elevated CO_2 than mature, well-established trees for accelerating growth instead of acclimation. Second, the seedlings may experience a sort of priming effect and not suffer from acclimation of photosynthesis in a relatively short CO_2 fumigation period. Thus, our results only occurred when soil nutrition and soil water were not limited, and complementary long-term experiments would be needed.

5. Conclusions

In summary, we found that red maple (*Acer rubrum* L.) allocated more biomass to roots, leaf C/N ratio decreased, and N resorption from senescent leaves increased under 800 μL L^{-1} CO_2 concentration treatment after one growing season. More growth in root may be helpful to improving nutrient and water uptake, and therefore would help to maintain the balance of nutrients within the plant as a sink. The nitrogen resorption is an increasingly important N source especially when facing high CO_2 concentration future. Higher N resorption efficiency of senescent leaves makes possible more nitrogen transfer to other plant organs for early growth next year. Biomass allocation and proportion will be a critical feature to address the environmental challenges. Nitrogen partitioning in plants and leaf senescence dynamics in forests grown under elevated CO_2 is paramount and complementary long-term experiments will also be needed.

Author Contributions: Conceptualization, W.M.; methodology, W.M. and L.L.; Formal Analysis, L.L.; investigation, L.L.; data curation, L.L.; writing—original draft preparation, L.L.; writing—Review and editing, X.W.; supervision, W.M. and X.W.; project administration, W.M.; funding acquisition, W.M. and L.L.

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