

Article

# Light Energy Partitioning under Various Environmental Stresses Combined with Elevated CO<sub>2</sub> in Three Deciduous Broadleaf Tree Species in Japan

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**Abstract:** Understanding plant response to excessive light energy not consumed by photosynthesis under various environmental stresses, would be important for maintaining biosphere sustainability. Based on previous studies regarding nitrogen (N) limitation, drought in Japanese white birch (*Betula platyphylla* var. *japonica*), and elevated O<sub>3</sub> in Japanese oak (*Quercus mongolica* var. *crispula*) and Konara oak (*Q. serrata*) under future-coming elevated CO<sub>2</sub> concentrations, we newly analyze the fate of absorbed light energy by a leaf, partitioning into photochemical processes, including photosynthesis, photorespiration and regulated and non-regulated, non-photochemical quenchings. No significant increases in the rate of non-regulated non-photochemical quenching (J<sub>NO</sub>) were observed in plants grown under N limitation, drought and elevated O<sub>3</sub> in ambient or elevated CO<sub>2</sub>. This suggests that the risk of photodamage caused by excessive light energy was not increased by environmental stresses reducing photosynthesis, irrespective of CO<sub>2</sub> concentrations. The rate of regulated non-photochemical quenching (J<sub>NPQ</sub>), which contributes to regulating photoprotective thermal dissipation, could well compensate decreases in the photosynthetic electron transport rate through photosystem II (J<sub>PSII</sub>) under various environmental stresses, since J<sub>NPQ</sub>+J<sub>PSII</sub> was constant across the treatment combinations. It is noteworthy that even decreases in J<sub>NO</sub> were observed under N limitation and elevated O<sub>3</sub>, irrespective of CO<sub>2</sub> conditions, which may denote a preconditioning-mode *adaptive response* for protection against further stress. Such an *adaptive response* may not fully compensate for the negative effects of lethal stress, but may be critical for coping with non-lethal stress and regulating homeostasis. Regarding the three deciduous broadleaf tree species, elevated CO<sub>2</sub> appears not to influence the plant responses to environmental stresses from the viewpoint of susceptibility to photodamage.

**Keywords:** chlorophyll fluorescence; drought; elevated O<sub>3</sub>; N limitation; non-photochemical quenching; photodamage

## 1. Introduction

Although light is essential for plant growth, plants can suffer from excessive light, especially when combined with other environmental stresses. Light energy absorbed by a leaf is mainly consumed

by photochemical processes such as electron flow to photosynthesis, photorespiration and alternative pathways [1].

Conversely, absorbed light energy is also dissipated by non-photochemical processes divided into two parts: Constitutive, non-regulatory, non-photochemical quenching, and regulatory light-induced, non-photochemical quenching [2–5]. When photosynthetic electron transport is suppressed under environmental stresses, an increase in the fraction of non-regulatory, non-photochemical quenching suggests that plants cannot fully dissipate excess energy through a regulated process [5–9]. Non-regulated, non-photochemical quenching consists of chlorophyll fluorescence internal conversions and intersystem crossing, which leads to the formation of  $^1\text{O}_2$  via the triplet state of chlorophyll ( $^3\text{chl}^*$ ) [10–13].  $^1\text{O}_2$  can lead to PSII photodamage directly, or via inhibiting PSII repair processes [14–16]. Non-regulated, non-photochemical quenching can be a measure of oxidative stress, as the level of lipid peroxidation indicated by malondialdehyde (MDA) accumulation was closely correlated with the quantum yield of non-regulated, non-photochemical quenching ( $Y(\text{NO})$ ) in *Arabidopsis thaliana* under a water deficit imposed by withholding the water supply [17].

Plants can acclimate to various environmental conditions by adjusting their leaf physiological characteristics to prevent photodamage [18,19]. For example, within a canopy, sun leaves grown under higher irradiance have a higher photosynthetic capacity with higher area-based leaf nitrogen ( $N_{\text{area}}$ ) than shade leaves [20], a mechanism contributing to maximize photosynthetic carbon gain at the whole plant level by utilizing limited nitrogen optimally [21]. The net photosynthetic rate is known to be proportional to  $N_{\text{area}}$ , since an increase in  $N_{\text{area}}$  suggests an increase in Rubisco, a major photosynthetic enzyme [22]. As Rubisco is a key enzyme catalyzing both photosynthesis and photorespiration, electron flow through PSII consumed by the processes also increases with increasing  $N_{\text{area}}$  [23]. As energy dissipation through photosynthetic electron transport is closely related to  $N_{\text{area}}$  [23], such  $N_{\text{area}}$ -related photosynthetic acclimation along the light gradient within the canopy, can also contribute to suppress the risk of photodamage in response to the maximum irradiance during sunflecks, in combination with xanthophyll-related photoprotective energy dissipation [24,25].

Environmental stresses such as N limitation, drought and elevated  $\text{O}_3$ , causing a reduction in photosynthesis, would increase excessive light energy via a reduction in photosynthetic electron consumption, since photosynthetic carbon assimilation needs NADPH, generated via electron transport [26]. In the coming future, environmental stresses such as nitrogen limitation, as a relative constraint on plant-growth enhancement under elevated  $\text{CO}_2$  [27], drought [28], and high  $\text{O}_3$  exposure [29–31], are predicted to occur more frequently under global warming and elevated  $\text{CO}_2$  concentrations.

We hypothesized that, even under various environmental stresses such as N limitation, drought and elevated  $\text{O}_3$  under  $\text{CO}_2$  enrichment in the coming future, the non-regulated, non-photochemical quenching should be kept under a certain level, to prevent photosynthetic apparatus from oxidative damage [14–16]. This is achieved by a functional coordination of energy dissipation primarily through N-required electron transport [23], and complementarily through xanthophyll-related thermal energy dissipation, which does not require N investment [8].

To test the hypothesis, we newly analyzed data from previously published works from our research group using three deciduous broadleaf tree species [32–34], where the response of plants to different environmental factors was assessed by chlorophyll fluorescence, so as to assess the fate of absorbed light energy consumed by photochemical processes, and dissipated through constitutively non-regulatory and regulatory light-induced, non-photochemical quenching. We also assessed the light energy not absorbed by a leaf, involved in a bulk loss in chlorophyll pigments, which also has a protective role against photodamage via a reduction in absorbed light energy.

## 2. Materials and Methods

This study is in part a collective re-analysis of previously published data [32–34]. Regarding “N limitation under elevated  $\text{CO}_2$ ” and “drought under elevated  $\text{CO}_2$ ”, regulated

and non-regulated non-photochemical quenchings were newly calculated based on data from the previous studies [32,33]. Conversely, regarding “elevated O<sub>3</sub> under elevated CO<sub>2</sub>”, all data except for A<sub>n</sub> were not published previously (cf. [34]).

### 2.1. N Limitation under Elevated CO<sub>2</sub>

Data of Japanese white birch (*Betula platyphylla* var. *japonica*) seedlings grown under limited N and elevated CO<sub>2</sub> were obtained from the study by Kitao et al. [32]. Experiments of N limitation under elevated CO<sub>2</sub> were conducted using a natural daylight phytotron (26/16 °C, day/night; ca. 90% of full sunlight) in Hokkaido Research Center, Forestry and Forest Products Research Institute (FFPRI) in Sapporo, Japan (43°N, 141°E; 180 m above sea level). Details are described in Kitao et al. [32]. One-year-old seedlings of Japanese white birch (*Betula platyphylla* var. *japonica*), a pioneer tree species, 15 to 20 cm in height, were transplanted in free-draining plastic pots filled with clay loam soil mixed with Kanuma pumice soil (1:1 in volume). Pots were placed on trays to prevent nutrient drainage. Each of two CO<sub>2</sub> treatments: 360 μmol mol<sup>-1</sup> (ambient CO<sub>2</sub> treatment, A-CO<sub>2</sub>); and 720 μmol mol<sup>-1</sup> (elevated CO<sub>2</sub> treatment, E-CO<sub>2</sub>) were replicated in two chambers. Two nitrogen levels were applied: 700 mg per plant (adequate nitrogen, +N), or 100 mg per plant (limited nitrogen, -N). The former treatment was conducted as 100 mg N pot<sup>-1</sup> week<sup>-1</sup> for 7 weeks during CO<sub>2</sub> treatment, whereas the latter one was conducted as 100 mg N pot<sup>-1</sup> only once at the onset of CO<sub>2</sub> treatment. We supplied relatively high N for +N treatment to provide adequate N to plants, so as to reach their normal state relative to nursery-grown seedlings. Area-based leaf N (N<sub>area</sub>) in the seedlings grown in +N treatment was comparable to those grown in the nursery of FFPRI (data not shown). Conversely, we supplied substantially low N for -N treatment, expecting photosynthetic down-regulation under N limitation [35].

### 2.2. Drought under Elevated CO<sub>2</sub>

Data of Japanese white birch seedlings grown under limited water supply and elevated CO<sub>2</sub> were obtained from the study by Kitao et al. [33]. Experiments of drought under elevated CO<sub>2</sub> were also conducted for 1-year-old seedlings of Japanese white birch in the phytotron in Hokkaido Research Center, FFPRI, as described above. Details are described in Kitao et al. [33]. Each of the two CO<sub>2</sub> treatments i.e., 360 (ambient CO<sub>2</sub> treatment: A-CO<sub>2</sub>) and 720 μmol mol<sup>-1</sup> (elevated CO<sub>2</sub> treatment: E-CO<sub>2</sub>) was replicated in three chambers. Six randomly selected seedlings in each chamber were supplied daily with 100 mL of water or nutrient solution (once per week) (adequate water supply), while the other six seedlings (totally 12 seedlings) received only 100 mL of nutrient solution once weekly (drought). Each plant received a total of 100 mg N during the experiment, which corresponded to limited N treatment, as described above. The lowest predawn leaf water potential (i.e., measured just prior to the scheduled watering), which was in equilibrium with the soil water potential, was A-CO<sub>2</sub> + adequate water supply: -0.13, A-CO<sub>2</sub> + drought: -0.52, E-CO<sub>2</sub> + adequate water supply: -0.12 and E-CO<sub>2</sub> + drought: -0.39 MPa [33]. The values of water potential in the drought treatment were moderate, since no wilting in the seedlings was observed. Leaves flushed and developed during the drought treatment were used for the measurements.

### 2.3. Elevated O<sub>3</sub> under Elevated CO<sub>2</sub>

Data of Japanese oak (*Quercus mongolica* Fisch. ex Ledeb. var. *crispula* (Blume) H. Ohashi) and Konara oak (*Q. serrata* Murray) seedlings grown under elevated O<sub>3</sub> and CO<sub>2</sub> were obtained from the study by Kitao et al. [34]. Experiments of elevated O<sub>3</sub> under elevated CO<sub>2</sub> were conducted in a free-air concentration-enrichment (FACE) exposure system, consisting of 12 plots (3 replicates per treatment), located at the nursery of FFPRI in Tsukuba, Japan (36°00'N, 140°08'E, 20 m a.s.l.).

Details are described in Kitao et al. [34]. One-year-old seedlings of Japanese oak and Konara oak, gap-dependent mid-successional tree species, approximately 5 cm in height under dormancy, were transplanted directly to the ground in the plots. The treatments were as follows: Control (unchanged ambient air), elevated CO<sub>2</sub> (Target set, 550 μmol mol<sup>-1</sup>), elevated O<sub>3</sub> (Target set,

twice-ambient), and elevated CO<sub>2</sub> + O<sub>3</sub> (550 μmol mol<sup>-1</sup> CO<sub>2</sub> and twice-ambient O<sub>3</sub>). Plants were grown under the treatments for two growing seasons. Measurements of gas exchange and chlorophyll fluorescence were conducted in the second growing season.

#### 2.4. Measurements of Gas Exchange and Chlorophyll Fluorescence

Measurements of gas exchange and chlorophyll fluorescence were conducted with a portable photosynthesis measuring system (Li-6400, Li-Cor, Lincoln, NE, USA), combined with a portable fluorometer (PAM-2000, Walz, Effeltrich, Germany) for plants grown under “N limitation with CO<sub>2</sub> enrichment”, or a leaf chamber fluorometer (Li-6400-40, Li-Cor) for plants grown under “drought, and elevated O<sub>3</sub> under elevated CO<sub>2</sub>”. Details are described in Kitao et al. [32–34]. The net photosynthetic rate (A<sub>n</sub>), quantum yield of PSII electron transport (Y(II)), quantum yield of non-regulate, non-photochemical quenching in PSII (Y(NO)), and finally the quantum yield of regulated, non-photochemical quenching in PSII (Y(NPQ)) [2–5] were measured at a photosynthetic steady state under saturating light intensities provided by a red/blue LED array (Li-6400-40, Li-Cor), with blue light comprising 10% of the total PPFD. We measured Y(NO) and Y(NPQ), based on the simple approach: Y(NO) = F/F<sub>m</sub>, and Y(NPQ) = F/F<sub>m</sub>′ – F/F<sub>m</sub>, where F, F<sub>m</sub> and F<sub>m</sub>′ is the relative fluorescence yield at steady state illumination, the relative maximum fluorescence yield in dark-adapted conditions, or that during illumination, respectively [4,5]. Regarding the data sets of drought and elevated O<sub>3</sub> under elevated CO<sub>2</sub>, we measured F, F<sub>m</sub>′ and F<sub>o</sub>′ (the minimum fluorescence yield during illumination) during the gas exchange measurements, but did not measure F<sub>m</sub>. We measured F<sub>v</sub>/F<sub>m</sub> on the following day, after an overnight dark-adaptation in the same leaves for the gas exchange, and chlorophyll fluorescence measurements with the photosynthesis system (Li-6400, Li-Cor) for drought-treated plants [33], and with a portable fluorometer (Mini-PAM, Walz) for O<sub>3</sub>-treated plants [34]. Since F<sub>o</sub>′ is estimated as F<sub>o</sub>′ = F<sub>o</sub>/(F<sub>v</sub>/F<sub>m</sub> + F<sub>o</sub>/F<sub>m</sub>′) [36], F<sub>m</sub> can be estimated as F<sub>m</sub> = (F<sub>m</sub>′ × F<sub>o</sub>′ × F<sub>v</sub>/F<sub>m</sub>)/((F<sub>m</sub>′ – F<sub>o</sub>′) × (1 – F<sub>v</sub>/F<sub>m</sub>)). This would be a practical approach to determine F<sub>m</sub> for many samples in the field after the fluorescence measurements during daytime. Leaf absorptance (ABS) was calculated from a calibration curve between SPAD readings (measured with a SPAD chlorophyll meter, SPAD 502, Minolta, Osaka, Japan) and leaf absorptance [32–34]. Based on the chlorophyll fluorescence parameters, the electron transport rate (J<sub>PSII</sub>) was calculated as J<sub>PSII</sub> = Y(II) × ABS × light intensity × 0.5 [6]. Analogous to J<sub>PSII</sub>, the rate of regulatory thermal dissipation (J<sub>NPQ</sub>) and the rate of non-regulatory energy dissipation via heat or fluorescence (J<sub>NO</sub>) were estimated from Y(NPQ) × ABS × light intensity × 0.5 and Y(NO) × ABS × light intensity × 0.5, respectively [4]. Light energy not absorbed by chlorophyll in a leaf (J<sub>chl</sub>) was estimated as J<sub>chl</sub> = (1 – ABS) × light intensity × 0.5.

#### 2.5. Leaf N Content

Regarding ‘elevated O<sub>3</sub> under elevated CO<sub>2</sub>’, the leaves were sampled after the measurements and used for a determination of N<sub>area</sub> by the combustion method, using an analysis system composed of an N/C determination unit (SUMIGRAPH, NC 800, Sumika Chem. Anal. Service, Osaka, Japan), a gas chromatograph (GC 8A, Shimadzu, Kyoto, Japan), and a data processor (Chromatopac, C R6A, Shimadzu).

#### 2.6. Statistical Analysis

In the study on N limitation under elevated CO<sub>2</sub>, individual seedlings across the two chambers were used as the sample unit (n = 4–6). Two-way Analysis of Variance (ANOVA) (N × CO<sub>2</sub>) was used to test the differences in the treatment means of A<sub>n</sub>, J<sub>PSII</sub>, J<sub>NPQ</sub>, J<sub>PSII</sub>+J<sub>NPQ</sub>, J<sub>NO</sub> and J<sub>chl</sub>. In the study on drought under elevated CO<sub>2</sub>, statistics are based on the individual plot (CO<sub>2</sub> × water regime) in each chamber as the sample unit (n = 3). Three to six plants were measured in each plot.

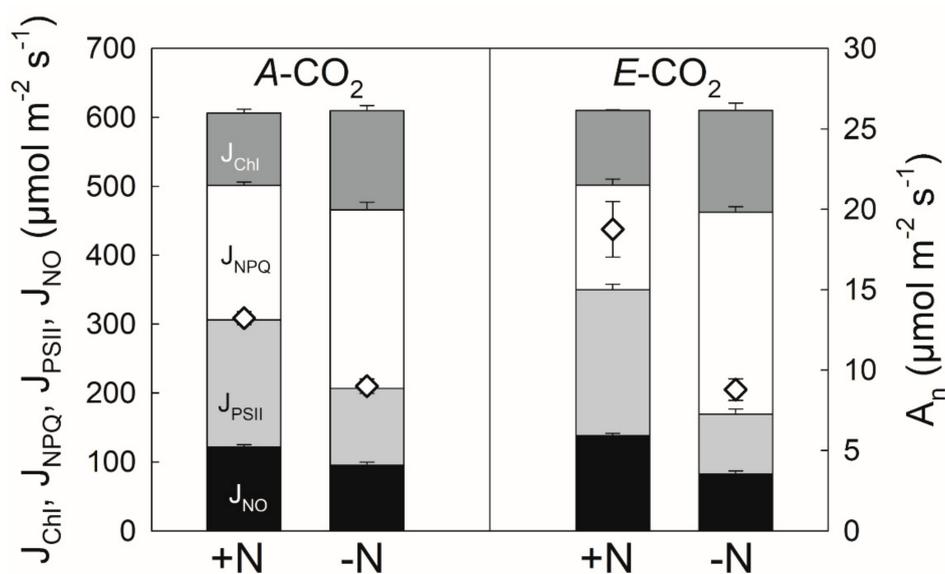
A mean value from these plants was used as the estimate for that sample unit. Two-way ANOVA, with one between-subjects factor (CO<sub>2</sub>) and one within-subject factor (water regime), was used to test treatment differences in A<sub>n</sub>, J<sub>PSII</sub>, J<sub>NPQ</sub>, J<sub>PSII</sub> + J<sub>NPQ</sub>, J<sub>NO</sub> and J<sub>chl</sub>. In the study on elevated O<sub>3</sub> under

elevated CO<sub>2</sub>, all statistics were based on the mean value of the individual plot (CO<sub>2</sub> × O<sub>3</sub> regime) as the sample unit ( $n = 3$ ). These values were then averaged to provide the sample estimate for that replicate. Three-way ANOVA, with two between-subjects factors (CO<sub>2</sub> and O<sub>3</sub>) and one within-subject factor (species), was used to test the differences in  $A_n$ ,  $J_{PSII}$ ,  $J_{NPQ}$ ,  $J_{PSII} + J_{NPQ}$ ,  $J_{NO}$  and  $J_{Chl}$ , and leaf N.

### 3. Results

#### 3.1. Nitrogen Limitation under Elevated CO<sub>2</sub>

When compared at the growth CO<sub>2</sub>, i.e., 360  $\mu\text{mol mol}^{-1}$  for the ambient-CO<sub>2</sub>-grown plants, and 720  $\mu\text{mol mol}^{-1}$  for the elevated-CO<sub>2</sub>-grown plants, higher  $A_n$  was observed in plants grown under elevated CO<sub>2</sub> than in ambient-CO<sub>2</sub> plants with adequate N supply, whereas no enhancement in  $A_n$  under elevated CO<sub>2</sub> was observed with a limited N supply (Figure 1, Table 1). Conversely, no enhancement in  $J_{PSII}$  was observed in plants grown under elevated CO<sub>2</sub> with an adequate N supply, whereas the limited N supply resulted in lower  $J_{PSII}$  irrespective of CO<sub>2</sub> treatments.  $J_{NPQ}$  was significantly higher in plants grown under limited N supply than those under adequate N supply. The sum of  $J_{NPQ} + J_{PSII}$  was not significantly different among the treatment combinations. As ABS was lower in the plants grown with limited N supply, higher  $J_{Chl}$  was observed in those plants. As a consequence of the increased  $J_{Chl}$  in addition to  $J_{NPQ}$ , lower  $J_{NO}$  was observed in the plants grown with limited N supply, in spite of significantly lower  $J_{PSII}$ , irrespective of CO<sub>2</sub> treatment.



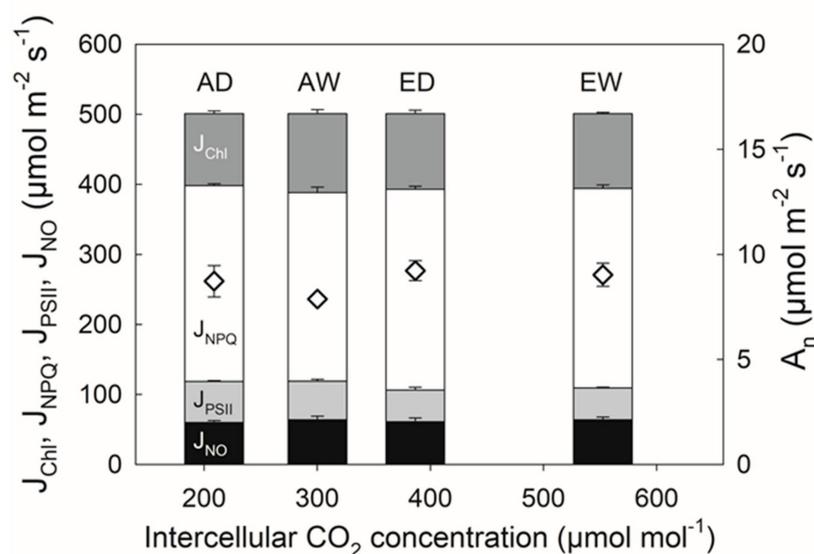
**Figure 1.** Fate of light energy partitioning in the seedlings of Japanese white birch grown with N limitation under elevated CO<sub>2</sub>.  $J_{Chl}$ ,  $J_{NPQ}$ ,  $J_{PSII}$  and  $J_{NO}$  were measured in the seedlings of Japanese white birch grown under ambient (A-CO<sub>2</sub>) and elevated CO<sub>2</sub> (E-CO<sub>2</sub>) with adequate (+N) and limited N supply (-N). Open diamonds indicate net photosynthetic rate ( $A_n$ ). Measurements were conducted for fully-developed mature leaves (leaf age was approx. 1 month) under respective growth CO<sub>2</sub> concentrations (i.e., 360  $\mu\text{mol mol}^{-1}$  for ambient-CO<sub>2</sub>-grown plants, and 720  $\mu\text{mol mol}^{-1}$  for elevated-CO<sub>2</sub>-grown plants) at saturating light (1200  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ). Values are means  $\pm$  se ( $n = 4-6$ ). Data were obtained from Kitao et al. [32].  $J_{Chl}$ ,  $J_{NPQ}$  and  $J_{NO}$  were newly calculated based on data from the previous study.

**Table 1.** *F* values of Analysis of Variance (ANOVA) to test the effects of various environmental stresses (N limitation, drought and elevated O<sub>3</sub>) under ambient or elevated CO<sub>2</sub> on J<sub>NO</sub>, J<sub>PSII</sub>, J<sub>NPQ</sub>, J<sub>PSII</sub>+J<sub>NPQ</sub>, J<sub>Chl</sub>, and A<sub>n</sub> measured at respective growth CO<sub>2</sub> concentrations. Significant effects are indicated in the table by \*\*\*: *p* ≤ 0.001, \*\*: *p* ≤ 0.01, \*: *p* ≤ 0.05, and ns: non-significant. Data were obtained from Kitao et al. [32–34].

| Treatment  | Effect   | <i>F</i> -statistics |                     |                     |                                      |                     |                     |
|--|--|----------------------|---------------------|---------------------|--------------------------------------|---------------------|---------------------|
|  |  | J <sub>NO</sub>      | J <sub>PSII</sub>   | J <sub>NPQ</sub>    | J <sub>PSII</sub> + J <sub>NPQ</sub> | J <sub>Chl</sub>    | A <sub>n</sub>      |
| N limitation   | CO <sub>2</sub> (F <sub>1,13</sub> )                 | 0.69 <sup>ns</sup>   | 0.47 <sup>ns</sup>  | 0.81 <sup>ns</sup>  | 0.11 <sup>ns</sup>                   | 0.03 <sup>ns</sup>  | 51.8 <sup>***</sup> |
|  | N (F <sub>1,13</sub> )                               | 106 <sup>***</sup>   | 133 <sup>***</sup>  | 124 <sup>***</sup>  | 0.19 <sup>ns</sup>                   | 28.6 <sup>***</sup> | 322 <sup>***</sup>  |
|  | CO <sub>2</sub> × N (F <sub>1,13</sub> )             | 13.3 <sup>**</sup>   | 9.63 <sup>**</sup>  | 18.4 <sup>***</sup> | 3.08 <sup>ns</sup>                   | 0.00 <sup>ns</sup>  | 71.4 <sup>***</sup> |
| Drought  | CO <sub>2</sub> (F <sub>1,4</sub> )                  | 0.01 <sup>ns</sup>   | 25.1 <sup>**</sup>  | 2.68 <sup>ns</sup>  | 0.00 <sup>ns</sup>                   | 0.01 <sup>ns</sup>  | 3.75 <sup>ns</sup>  |
|  | Drought (F <sub>1,4</sub> )                          | 0.71 <sup>ns</sup>   | 0.40 <sup>ns</sup>  | 3.90 <sup>ns</sup>  | 2.23 <sup>ns</sup>                   | 1.39 <sup>ns</sup>  | 0.74 <sup>ns</sup>  |
|  | CO <sub>2</sub> × Drought (F <sub>1,4</sub> )        | 0.02 <sup>ns</sup>   | 0.40 <sup>ns</sup>  | 1.86 <sup>ns</sup>  | 1.28 <sup>ns</sup>                   | 2.04 <sup>ns</sup>  | 0.29 <sup>ns</sup>  |
| Elevated O <sub>3</sub>  | CO <sub>2</sub> (F <sub>1,8</sub> )                  | 5.00 <sup>ns</sup>   | 5.46 <sup>*</sup>   | 16.4 <sup>**</sup>  | 0.50 <sup>ns</sup>                   | 0.05 <sup>ns</sup>  | 35.6 <sup>***</sup> |
|  | O <sub>3</sub> (F <sub>1,8</sub> )                   | 5.54 <sup>*</sup>    | 6.60 <sup>*</sup>   | 14.9 <sup>**</sup>  | 0.07 <sup>ns</sup>                   | 0.72 <sup>ns</sup>  | 25.5 <sup>***</sup> |
|  | CO <sub>2</sub> × O <sub>3</sub> (F <sub>1,8</sub> ) | 0.91 <sup>ns</sup>   | 1.35 <sup>ns</sup>  | 0.08 <sup>ns</sup>  | 2.54 <sup>ns</sup>                   | 2.46 <sup>ns</sup>  | 0.94 <sup>ns</sup>  |
|  | Species (F <sub>1,8</sub> )                          | 1.36 <sup>ns</sup>   | 12.7 <sup>***</sup> | 0.42 <sup>ns</sup>  | 4.36 <sup>ns</sup>                   | 2.13 <sup>ns</sup>  | 39.3 <sup>***</sup> |
|  | CO <sub>2</sub> × Species (F <sub>1,8</sub> )        | 0.08 <sup>ns</sup>   | 2.35 <sup>ns</sup>  | 0.22 <sup>ns</sup>  | 0.50 <sup>ns</sup>                   | 0.35 <sup>ns</sup>  | 6.64 <sup>*</sup>   |
|  | O <sub>3</sub> × Species (F <sub>1,8</sub> )         | 0.10 <sup>ns</sup>   | 18.0 <sup>**</sup>  | 1.15 <sup>ns</sup>  | 4.79 <sup>ns</sup>                   | 4.32 <sup>ns</sup>  | 8.59 <sup>*</sup>   |
| CO <sub>2</sub> × O <sub>3</sub> × Species (F <sub>1,8</sub> ) | 4.43 <sup>ns</sup>                                   | 23.9 <sup>**</sup>   | 7.58 <sup>*</sup>   | 0.97 <sup>ns</sup>  | 6.32 <sup>*</sup>                    | 2.30 <sup>ns</sup>  |                     |

### 3.2. Drought under Elevated CO<sub>2</sub>

Measurements of gas exchange and chlorophyll fluorescence were conducted at the growth CO<sub>2</sub> (i.e., 360 μmol mol<sup>-1</sup> for the ambient-CO<sub>2</sub>-grown plants and 720 μmol mol<sup>-1</sup> for the elevated-CO<sub>2</sub>-grown plants) when soils were most dried on the previous day of irrigation (i.e., just prior to the scheduled watering). Intercellular CO<sub>2</sub> concentration (C<sub>i</sub>) was higher under elevated CO<sub>2</sub>, but lower under drought (Figure 2). Irrespective of the large variation of C<sub>i</sub> itself, J<sub>Chl</sub>, J<sub>NPQ</sub>, J<sub>NO</sub> and J<sub>PSII</sub>+J<sub>NPQ</sub> were not significantly different among the treatment combinations (Figure 2, Table 1). Only J<sub>PSII</sub> was significantly lower in the plants grown under elevated CO<sub>2</sub>, whereas no significant difference in A<sub>n</sub> was observed among the treatment combinations.

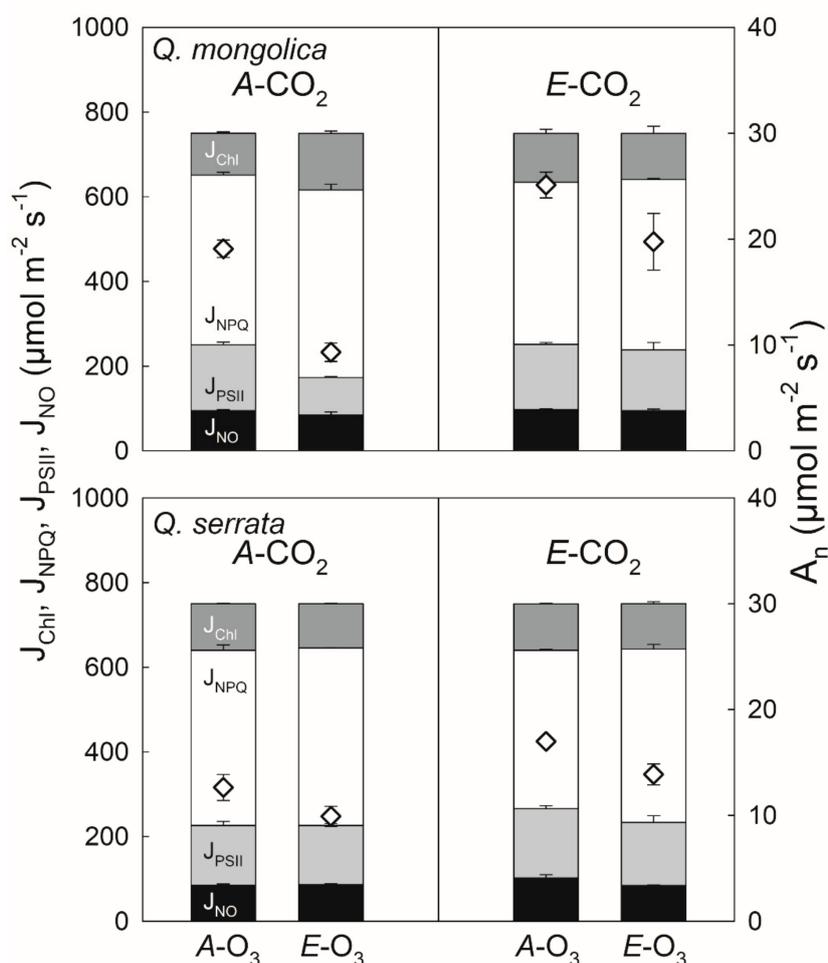


**Figure 2.** Fate of light energy partitioning with drought under elevated CO<sub>2</sub>. Data are plotted as a function of intercellular CO<sub>2</sub> concentration. J<sub>Chl</sub>, J<sub>NPQ</sub>, J<sub>PSII</sub> and J<sub>NO</sub> were measured in the seedlings

of Japanese white birch grown under ambient and elevated  $\text{CO}_2$  with adequate (daily) and limited (once-weekly) water supply. AD: Ambient  $\text{CO}_2$  + once-weekly irrigation; AW: Ambient  $\text{CO}_2$  + daily-irrigation; ED: Elevated  $\text{CO}_2$  + once-weekly irrigation; EW: Elevated  $\text{CO}_2$  + daily-irrigation. Open diamonds indicate net photosynthetic rate ( $A_n$ ). Measurements were conducted for fully-developed mature leaves (leaf age was approx. 1 month) under the most dried conditions under respective growth  $\text{CO}_2$  concentrations (i.e.,  $360 \mu\text{mol mol}^{-1}$  for ambient- $\text{CO}_2$ -grown plants, and  $720 \mu\text{mol mol}^{-1}$  for elevated- $\text{CO}_2$ -grown plants) at saturating light ( $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$ ). Values are means  $\pm$  se ( $n = 3$ ). Data were obtained from Kitao et al. [33].  $J_{\text{Chl}}$ ,  $J_{\text{NPQ}}$  and  $J_{\text{NO}}$  were newly calculated based on data from the previous study.

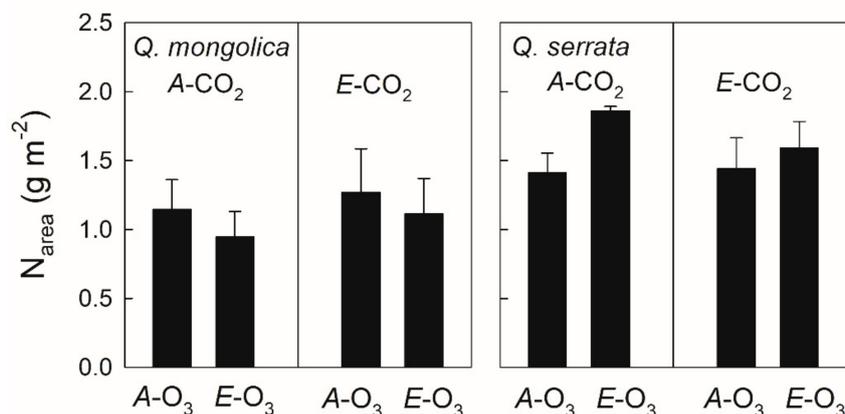
### 3.3. Elevated $\text{O}_3$ under Elevated $\text{CO}_2$

$A_n$  measured at the respective growth  $\text{CO}_2$  (i.e.,  $380 \mu\text{mol mol}^{-1}$  for the ambient- $\text{CO}_2$ -grown plants and  $550 \mu\text{mol mol}^{-1}$  for the elevated- $\text{CO}_2$ -grown plants) increased under elevated  $\text{CO}_2$ , but decreased under elevated  $\text{O}_3$  (Figure 3, Table 1).  $A_n$  was significantly different between *Q. mongolica* and *Q. serrata*, and the effects of  $\text{CO}_2$  and  $\text{O}_3$  were also different between species (Table 1).  $J_{\text{PSII}}$  increased under elevated  $\text{CO}_2$ , but decreased under elevated  $\text{O}_3$ , whereas  $J_{\text{NPQ}}$  decreased under elevated  $\text{CO}_2$  but increased under elevated  $\text{O}_3$ . As a result, no significant differences were observed in  $J_{\text{PSII}} + J_{\text{NPQ}}$  among the treatment combinations or across species.  $J_{\text{Chl}}$  was neither affected by  $\text{CO}_2$ ,  $\text{O}_3$  nor species. Significantly lower  $J_{\text{NO}}$  was observed in the plants grown under elevated  $\text{O}_3$ . Area-based leaf N content ( $N_{\text{area}}$ ) was not significantly different among the treatment combinations, whereas significantly higher  $N_{\text{area}}$  was observed in *Q. serrata* (Figure 4, Table 2).



**Figure 3.** Fate of light energy partitioning under elevated  $\text{O}_3$  and  $\text{CO}_2$ . Here,  $J_{\text{Chl}}$ ,  $J_{\text{NPQ}}$ ,  $J_{\text{PSII}}$  and  $J_{\text{NO}}$  were measured in the seedlings of Japanese oak (*Q. mongolica*) and Konara oak (*Q. serrata*) grown

under ambient ( $A-O_3$ ) and elevated  $O_3$  ( $E-O_3$ ), combined with ambient ( $A-CO_2$ ) and elevated  $CO_2$  ( $E-CO_2$ ). Open diamonds indicate the net photosynthetic rate ( $A_n$ ). Measurements were conducted for fully-developed mature leaves (leaf age was approx. 2 months) under respective growth  $CO_2$  concentrations (i.e.,  $380 \mu\text{mol mol}^{-1}$  for ambient- $CO_2$ -grown plants, and  $550 \mu\text{mol mol}^{-1}$  for elevated- $CO_2$ -grown plants) at saturating light ( $1500 \mu\text{mol m}^{-2} \text{s}^{-1}$ ). Values are means  $\pm$  se ( $n = 3$ ).  $A_n$  was obtained from Kitao et al. [34].



**Figure 4.** Area-based leaf N content ( $N_{\text{area}}$ ) in the seedlings of Japanese oak (*Q. mongolica*) and Konara oak (*Q. serrata*) grown under ambient ( $A-O_3$ ) and elevated  $O_3$  ( $E-O_3$ ), combined with ambient ( $A-CO_2$ ) and elevated  $CO_2$  ( $E-CO_2$ ). Values are means  $\pm$  se ( $n = 3$ ).

**Table 2.**  $F$  values of three-way ANOVA with two between-subjects factors ( $CO_2$  and  $O_3$ ) and one within-subject factor (species), to test the effects of  $CO_2$  ( $F_{1,8}$ ),  $O_3$  ( $F_{1,8}$ ), species ( $F_{1,8}$ ),  $CO_2 \times O_3$  ( $F_{1,8}$ ),  $CO_2 \times$  Species ( $F_{1,8}$ ),  $O_3 \times$  Species ( $F_{1,8}$ ), and  $CO_2 \times O_3 \times$  Species ( $F_{1,8}$ ) on area-based leaf nitrogen content ( $N_{\text{area}}$ ). The symbols ns and \* denote non-significant ( $p > 0.05$ ) and significant ( $p \leq 0.05$ ) effects, respectively.

| F-Statistics                     |                    |
|----------------------------------|--------------------|
| Effect                           | $N_{\text{area}}$  |
| $CO_2$                           | 0.01 <sup>ns</sup> |
| $O_3$                            | 0.25 <sup>ns</sup> |
| $CO_2 \times O_3$                | 0.27 <sup>ns</sup> |
| Species                          | 7.03*              |
| $CO_2 \times$ Species            | 0.58 <sup>ns</sup> |
| $O_3 \times$ Species             | 1.94 <sup>ns</sup> |
| $CO_2 \times O_3 \times$ Species | 0.25 <sup>ns</sup> |

## 4. Discussion

### 4.1. Nitrogen Limitation under Elevated $CO_2$

Nitrogen plays a key role in photosynthesis, since Rubisco, a key-enzyme of photosynthesis, is the largest sink of N in a leaf [22], and also a considerable amount of N is involved in proteins related to linear electron transport [23,37]. Plants grown under elevated  $CO_2$  often show photosynthetic acclimation typically accompanied with a decrease in the maximum capacity of Rubisco carboxylation, known as photosynthetic down-regulation, particularly under limited nitrogen availability [27,35]. When Japanese white birch seedlings were grown under the combinations of  $CO_2$  and N treatments, leaves showed higher  $N_{\text{area}}$  with higher N supply, but lower  $N_{\text{area}}$  under elevated  $CO_2$  treatment [32]. In the present study, elevated  $CO_2$  had no effect on  $J_{NO}$ , whereas limited N decreased  $J_{NO}$ , suggesting a lower risk of photodamage under N limitation, irrespective of lower  $A_n$  [5]. The decreases in electron

transport rate ( $J_{PSII}$ ) by N limitation and photosynthetic down-regulation under elevated  $CO_2$  were fully-compensated by regulated thermal energy dissipation ( $J_{NPQ}$ ), since the sum of  $J_{PSII}$  and  $J_{NPQ}$  was not significantly different across the treatment combinations. Conversely, the decrease in  $J_{NO}$  under limited N resulted mainly from the increased loss of absorbed light energy, indicated by the increase in  $J_{Chl}$ .

#### 4.2. Drought under Elevated $CO_2$

Drought-induced stomatal closure leads to low intercellular  $CO_2$  ( $C_i$ ) [1]. Leaves developed under long-term drought display higher photosynthetic capacity, accompanied with higher  $N_{area}$ , thus compensating the reduced photosynthetic performance under low  $C_i$  [38–40]. In the present study, seedlings of Japanese white birch were grown under elevated  $CO_2$  and long-term drought with limited N supply. Photosynthetic capacity, indicated by the maximum rate of Rubisco carboxylation ( $V_{c,max}$ ), was previously shown to increase by long-term drought accompanied with higher  $N_{area}$ , whereas elevated  $CO_2$  decreased  $V_{c,max}$  with lower  $N_{area}$  [33]. In combination of changes in  $V_{c,max}$  with different  $C_i$ ,  $A_n$  was not significantly different among the treatment combinations. In spite of similar  $A_n$ ,  $J_{PSII}$  decreased under elevated  $CO_2$ , maybe because of a suppression of photorespiration under elevated  $CO_2$  ( $720 \mu mol mol^{-1}$ ) [41]. The decrease in  $J_{PSII}$  under elevated  $CO_2$  was well compensated by a regulated photoprotective reaction ( $J_{NPQ}$ ) [2,5], leading to unchanged  $J_{NO}$  under the combinations of  $CO_2$  and water treatments. An increase in  $Y(NO)$  was reported in mature leaves of *A. thaliana* under water deficit by withholding water, whereas a less extent of increase in  $Y(NO)$  was observed in young leaves, suggesting higher acclimating capacity, preventing oxidative damage in younger leaves [17]. In the present study, as the leaves of Japanese birch seedlings had flushed and developed during the relatively moderate drought treatment, they might fully acclimate to long-term drought, preventing photodamage [38–40].

#### 4.3. Elevated $O_3$ under Elevated $CO_2$

Tropospheric ozone ( $O_3$ ) levels continue to increase globally [42,43], concurrently occurring with an increase in atmospheric  $CO_2$  concentration [44]. Contrary to elevated  $CO_2$ , which may enhance plant growth in the short term [45,46], elevated  $O_3$  generally reduces plant growth via a reduction in photosynthetic rate and increased respiration rate [30,47]. Deciduous broadleaf trees native to Japan, Japanese oak (*Quercus mongolica*) and Konara oak (*Q. serrata*), were exposed to free air enriched with elevated  $O_3$  (twice ambient  $O_3$ ) and/or  $CO_2$  ( $550 \mu mol mol^{-1}$  as target).  $A_n$  in the fully-expanded second-flushed leaves, measured at each growth  $CO_2$ , reduced by elevated  $O_3$  but enhanced by elevated  $CO_2$ , irrespective of species. As  $A_n$  was enhanced under elevated  $CO_2$  with no difference in  $N_{area}$  among the treatment combinations, photosynthetic down-regulation, which is often induced by elevated  $CO_2$  under limited N availability [32,35], was not apparent in the present study of a free-air  $CO_2$  and  $O_3$  exposure without limitations of root growth [34]. Furthermore, reduced leaf N, accompanied with a reduction in  $A_n$  under elevated  $O_3$  [48], was not observed in the present study, suggesting that causes other than leaf N reduction might be predominant to decrease  $A_n$ , such as an oxidative stress in the chloroplast [49].  $J_{PSII}$  was also reduced by elevated  $O_3$ , but increased by elevated  $CO_2$ , as well as  $A_n$ . In contrast,  $J_{NPQ}$  was increased by elevated  $O_3$ , but decreased by elevated  $CO_2$ , which might fully compensate the changes in  $J_{PSII}$ , as indicated by the constant  $J_{PSII}+J_{NPQ}$ . It is noteworthy that  $J_{NO}$  decreased under elevated  $O_3$ , which means that elevated  $O_3$  would not necessarily increase the risk of photodamage in these species.

#### 4.4. Regulated and Non-regulated Non-photochemical Quenching under Elevated $CO_2$

In the present study, we investigated the fate of light energy absorbed by a leaf under various environmental stresses combined with elevated  $CO_2$ . We particularly focused on  $J_{NO}$ , a measure of constitutive, non-regulated, non-photochemical energy dissipation, because an increase in  $J_{NO}$  suggests an increase in the risk of photodamage [2,5]. As a whole, photoprotective thermal energy

dissipation indicated by  $J_{NPQ}$  may well compensate for the decreases in  $J_{PSII}$  under environmental stresses, since  $J_{PSII} + J_{NPQ}$  was rather constant throughout the various stresses, even under elevated  $CO_2$ . If plants can keep  $J_{PSII}$  constant, there is a high potential for preventing the accumulation of excess energy [25,38–40]. However, if  $J_{PSII}$  is restricted under limited N supply or by other environmental stress such as elevated  $O_3$ , xanthophyll-related regulated thermal energy dissipation ( $J_{NPQ}$ ) would act as an efficient safety valve, which does not need N investment [8].

Furthermore, although drought and elevated  $CO_2$  had no effects on  $J_{NO}$ , N limitation and elevated  $O_3$  resulted in decreases in  $J_{NO}$ , in contrast to expected stress responses (i.e., increases in  $J_{NO}$ ), which can be considered as an *adaptive response* in the framework of pre-conditioning to cope with further environmental stresses [50,51]. By doing so,  $J_{NO}$  may be decreased to such an extent that will offset high increases that would occur under further stress. This novel mechanism builds upon an extended body of literature showing the biological capacity of a variety of organisms to display hormetic *adaptive responses* which eventually act as biological shields against following health threats [50–52]. Such *adaptive responses* for coping with stress are activated by low/mild severity of stress, at levels that are (often far) lower than the level beyond which toxicological, adverse responses occur [50–52]. This suggests that NPQ can compensate for the effects of following more severe environmental stress, but if the stress is too severe (e.g., acute exposure), increased NPQ may not be enough to fully compensate for the negative effects of stress.

Whereas it was difficult to explicitly determine the factor inducing lower  $J_{NO}$  under elevated  $O_3$  (maybe the integrated effects of  $J_{NPQ} + J_{Chl}$ ), an increase in  $J_{Chl}$  apparently contributed to reducing  $J_{NO}$  under limited N. Thus, in addition to the fractions of absorbed light energy partitioning, based on chlorophyll fluorescence ( $Y(II)$ ,  $Y(NPQ)$  and  $Y(NO)$ ), reduced chlorophyll pigments should be taken into account as a photoprotective reaction for assessing environmental stresses by using chlorophyll fluorescence measurements [53].

Similar to the present study, a stable or even lower  $Y(NO)$  due to the decline in  $Y(II)$ , accompanied with the increase in  $Y(NPQ)$ , was also reported in paraquat-exposed *Arabidopsis thaliana* [11,18] and in Al-exposed *A. thaliana* [12]. The decrease in  $J_{NO}$  may denote also decreased ROS production [17]. Non-regulated, non-photochemical quenching consists of chlorophyll fluorescence internal conversions and intersystem crossing, which leads to the formation of singlet oxygen ( $^1O_2$ ) via the triplet state of chlorophyll ( $^3chl^*$ ) [10,11,13]. Since  $J_{NO}$  declined, it seems that  $J_{NPQ}$  was sufficient enough to protect plants from ROS, by exhibiting lower  $^1O_2$  production, and preventing the photosynthetic apparatus from oxidative damage [12].

## 5. Conclusions

Based on the results from three deciduous broadleaf tree species in the present study, even when photosynthesis and  $J_{PSII}$  were reduced by environmental stresses, photoprotective mechanisms including  $J_{NPQ}$  and  $J_{Chl}$  could suppress the rise of  $J_{NO}$  in the leaves developed under the stresses, consequently preventing photodamage even under future-coming elevated  $CO_2$  conditions.

**Author Contributions:** M.K., and H.T. designed the study. M.K., H.T., S.K., H.H., K.Y. and M.K. collected the photosynthetic data, performed the analysis, and hence equally contributed to this study. M.K. led the writing with input from E.A. and T.K. All authors also discussed the results and commented on the manuscript.

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