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Light Energy Partitioning under Various Environmental Stresses Combined with Elevated CO₂ in Three Deciduous Broadleaf Tree Species in Japan

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Received: 28 March 2019; Accepted: 30 May 2019; Published: 3 June 2019



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 O₃ in Japanese oak (*Quercus mongolica* var. *crispula*) and Konara oak (*Q. serrata*) under future-coming elevated CO₂ 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_{NO}) were observed in plants grown under N limitation, drought and elevated O₃ in ambient or elevated CO₂. This suggests that the risk of photodamage caused by excessive light energy was not increased by environmental stresses reducing photosynthesis, irrespective of CO₂ concentrations. The rate of regulated non-photochemical quenching (J_{NPQ}), which contributes to regulating photoprotective thermal dissipation, could well compensate decreases in the photosynthetic electron transport rate through photosystem II (J_{PSII}) under various environmental stresses, since J_{NPQ}+J_{PSII} was constant across the treatment combinations. It is noteworthy that even decreases in J_{NO} were observed under N limitation and elevated O₃, irrespective of CO₂ 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 CO₂ appears not to influence the plant responses to environmental stresses from the viewpoint of susceptibility to photodamage.

Keywords: chlorophyll fluorescence; drought; elevated O₃; 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_{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_{area} , since an increase in N_{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_{area} [23]. As energy dissipation through photosynthetic electron transport is closely related to N_{area} [23], such N_{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 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 CO_2 [27], drought [28], and high O_3 exposure [29–31], are predicted to occur more frequently under global warming and elevated CO_2 concentrations.

We hypothesized that, even under various environmental stresses such as N limitation, drought and elevated O_3 under 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 CO_2 ” and “drought under elevated CO_2 ”, regulated

and non-regulated non-photochemical quenchings were newly calculated based on data from the previous studies [32,33]. Conversely, regarding “elevated O₃ under elevated CO₂”, all data except for A_n were not published previously (cf. [34]).

2.1. N Limitation under Elevated CO₂

Data of Japanese white birch (*Betula platyphylla* var. *japonica*) seedlings grown under limited N and elevated CO₂ were obtained from the study by Kitao et al. [32]. Experiments of N limitation under elevated CO₂ 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 CO₂ treatments: 360 μmol mol⁻¹ (ambient CO₂ treatment, A-CO₂); and 720 μmol mol⁻¹ (elevated CO₂ treatment, E-CO₂) 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⁻¹ week⁻¹ for 7 weeks during CO₂ treatment, whereas the latter one was conducted as 100 mg N pot⁻¹ only once at the onset of CO₂ 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_{area}) 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 CO₂

Data of Japanese white birch seedlings grown under limited water supply and elevated CO₂ were obtained from the study by Kitao et al. [33]. Experiments of drought under elevated CO₂ 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 CO₂ treatments i.e., 360 (ambient CO₂ treatment: A-CO₂) and 720 μmol mol⁻¹ (elevated CO₂ treatment: E-CO₂) 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-CO₂ + adequate water supply: -0.13, A-CO₂ + drought: -0.52, E-CO₂ + adequate water supply: -0.12 and E-CO₂ + 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 O₃ under Elevated CO₂

Data of Japanese oak (*Quercus mongolica* Fisch. ex Ledeb. var. *crispula* (Blume) H. Ohashi) and Konara oak (*Q. serrata* Murray) seedlings grown under elevated O₃ and CO₂ were obtained from the study by Kitao et al. [34]. Experiments of elevated O₃ under elevated CO₂ 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 CO₂ (Target set, 550 μmol mol⁻¹), elevated O₃ (Target set,

twice-ambient), and elevated CO₂ + O₃ (550 μmol mol⁻¹ CO₂ and twice-ambient O₃). 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 CO₂ enrichment”, or a leaf chamber fluorometer (Li-6400-40, Li-Cor) for plants grown under “drought, and elevated O₃ under elevated CO₂”. Details are described in Kitao et al. [32–34]. The net photosynthetic rate (A_n), 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_m, and Y(NPQ) = F/F_m′ – F/F_m, where F, F_m and F_m′ 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 O₃ under elevated CO₂, we measured F, F_m′ and F_o′ (the minimum fluorescence yield during illumination) during the gas exchange measurements, but did not measure F_m. We measured F_v/F_m 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 O₃-treated plants [34]. Since F_o′ is estimated as F_o′ = F_o/(F_v/F_m + F_o/F_m′) [36], F_m can be estimated as F_m = (F_m′ × F_o′ × F_v/F_m)/((F_m′ – F_o′) × (1 – F_v/F_m)). This would be a practical approach to determine F_m 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_{PSII}) was calculated as J_{PSII} = Y(II) × ABS × light intensity × 0.5 [6]. Analogous to J_{PSII}, the rate of regulatory thermal dissipation (J_{NPQ}) and the rate of non-regulatory energy dissipation via heat or fluorescence (J_{NO}) 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_{chl}) was estimated as J_{chl} = (1 – ABS) × light intensity × 0.5.

2.5. Leaf N Content

Regarding ‘elevated O₃ under elevated CO₂’, the leaves were sampled after the measurements and used for a determination of N_{area} 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 CO₂, individual seedlings across the two chambers were used as the sample unit (n = 4–6). Two-way Analysis of Variance (ANOVA) (N × CO₂) was used to test the differences in the treatment means of A_n, J_{PSII}, J_{NPQ}, J_{PSII}+J_{NPQ}, J_{NO} and J_{chl}. In the study on drought under elevated CO₂, statistics are based on the individual plot (CO₂ × 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 (CO₂) and one within-subject factor (water regime), was used to test treatment differences in A_n, J_{PSII}, J_{NPQ}, J_{PSII} + J_{NPQ}, J_{NO} and J_{chl}. In the study on elevated O₃ under

elevated CO₂, all statistics were based on the mean value of the individual plot (CO₂ × O₃ 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 (CO₂ and O₃) 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 CO₂

When compared at the growth CO₂, i.e., 360 $\mu\text{mol mol}^{-1}$ for the ambient-CO₂-grown plants, and 720 $\mu\text{mol mol}^{-1}$ for the elevated-CO₂-grown plants, higher A_n was observed in plants grown under elevated CO₂ than in ambient-CO₂ plants with adequate N supply, whereas no enhancement in A_n under elevated CO₂ was observed with a limited N supply (Figure 1, Table 1). Conversely, no enhancement in J_{PSII} was observed in plants grown under elevated CO₂ with an adequate N supply, whereas the limited N supply resulted in lower J_{PSII} irrespective of CO₂ 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 CO₂ treatment.

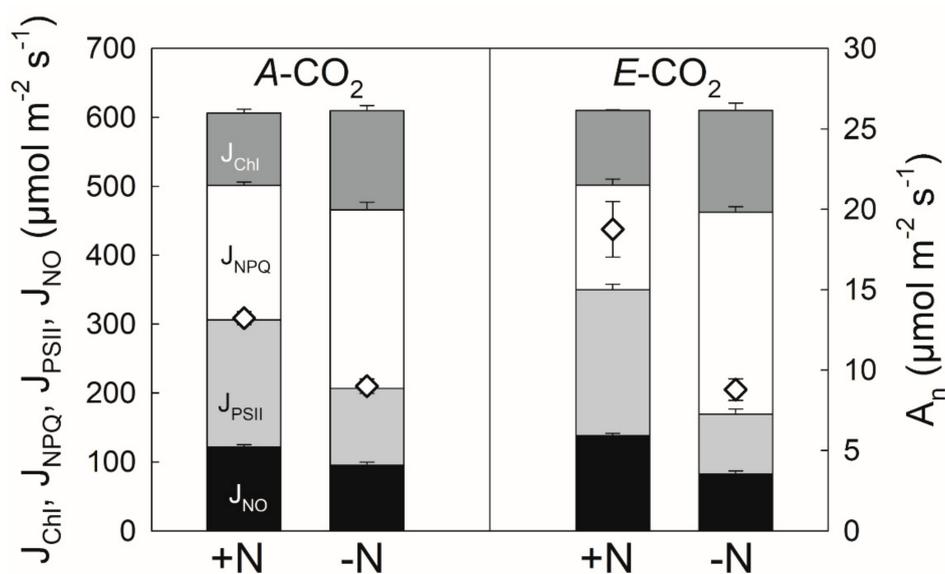


Figure 1. Fate of light energy partitioning in the seedlings of Japanese white birch grown with N limitation under elevated CO₂. J_{Chl} , J_{NPQ} , J_{PSII} and J_{NO} were measured in the seedlings of Japanese white birch grown under ambient (A-CO₂) and elevated CO₂ (E-CO₂) with adequate (+N) and limited 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 CO₂ concentrations (i.e., 360 $\mu\text{mol mol}^{-1}$ for ambient-CO₂-grown plants, and 720 $\mu\text{mol mol}^{-1}$ for elevated-CO₂-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 O₃) under ambient or elevated CO₂ on J_{NO}, J_{PSII}, J_{NPQ}, J_{PSII}+J_{NPQ}, J_{Chl}, and A_n measured at respective growth CO₂ concentrations. Significant effects are indicated in the table by ***: $p \leq 0.001$, **: $p \leq 0.01$, *: $p \leq 0.05$, and ns: non-significant. Data were obtained from Kitao et al. [32–34].

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

3.2. Drought under Elevated CO₂

Measurements of gas exchange and chlorophyll fluorescence were conducted at the growth CO₂ (i.e., 360 μmol mol⁻¹ for the ambient-CO₂-grown plants and 720 μmol mol⁻¹ for the elevated-CO₂-grown plants) when soils were most dried on the previous day of irrigation (i.e., just prior to the scheduled watering). Intercellular CO₂ concentration (C_i) was higher under elevated CO₂, but lower under drought (Figure 2). Irrespective of the large variation of C_i itself, J_{Chl}, J_{NPQ}, J_{NO} and J_{PSII}+J_{NPQ} were not significantly different among the treatment combinations (Figure 2, Table 1). Only J_{PSII} was significantly lower in the plants grown under elevated CO₂, whereas no significant difference in A_n was observed among the treatment combinations.

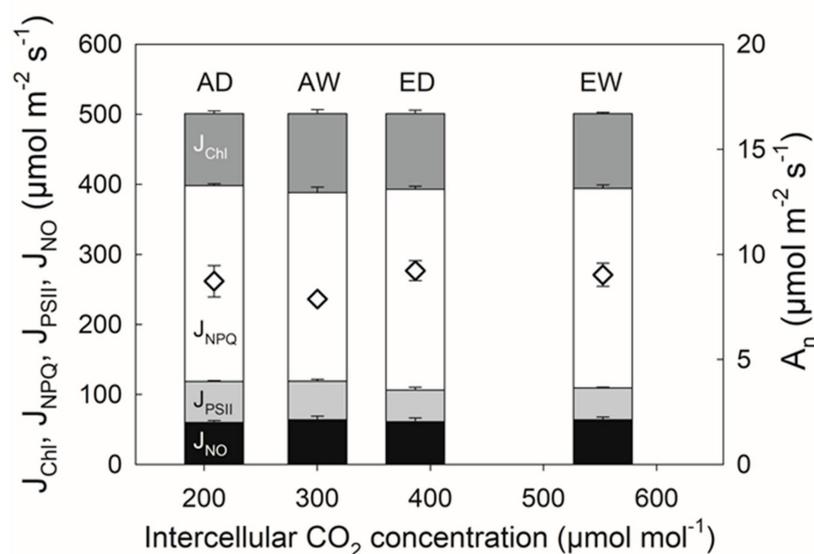


Figure 2. Fate of light energy partitioning with drought under elevated CO₂. Data are plotted as a function of intercellular CO₂ concentration. J_{Chl}, J_{NPQ}, J_{PSII} and J_{NO} were measured in the seedlings

of Japanese white birch grown under ambient and elevated CO_2 with adequate (daily) and limited (once-weekly) water supply. AD: Ambient CO_2 + once-weekly irrigation; AW: Ambient CO_2 + daily-irrigation; ED: Elevated CO_2 + once-weekly irrigation; EW: Elevated 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 CO_2 concentrations (i.e., $360 \mu\text{mol mol}^{-1}$ for ambient- CO_2 -grown plants, and $720 \mu\text{mol mol}^{-1}$ for elevated- 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_{Chl} , J_{NPQ} and J_{NO} were newly calculated based on data from the previous study.

3.3. Elevated O_3 under Elevated CO_2

A_n measured at the respective growth CO_2 (i.e., $380 \mu\text{mol mol}^{-1}$ for the ambient- CO_2 -grown plants and $550 \mu\text{mol mol}^{-1}$ for the elevated- CO_2 -grown plants) increased under elevated CO_2 , but decreased under elevated O_3 (Figure 3, Table 1). A_n was significantly different between *Q. mongolica* and *Q. serrata*, and the effects of CO_2 and O_3 were also different between species (Table 1). J_{PSII} increased under elevated CO_2 , but decreased under elevated O_3 , whereas J_{NPQ} decreased under elevated CO_2 but increased under elevated 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_{Chl} was neither affected by CO_2 , O_3 nor species. Significantly lower J_{NO} was observed in the plants grown under elevated O_3 . Area-based leaf N content (N_{area}) was not significantly different among the treatment combinations, whereas significantly higher N_{area} was observed in *Q. serrata* (Figure 4, Table 2).

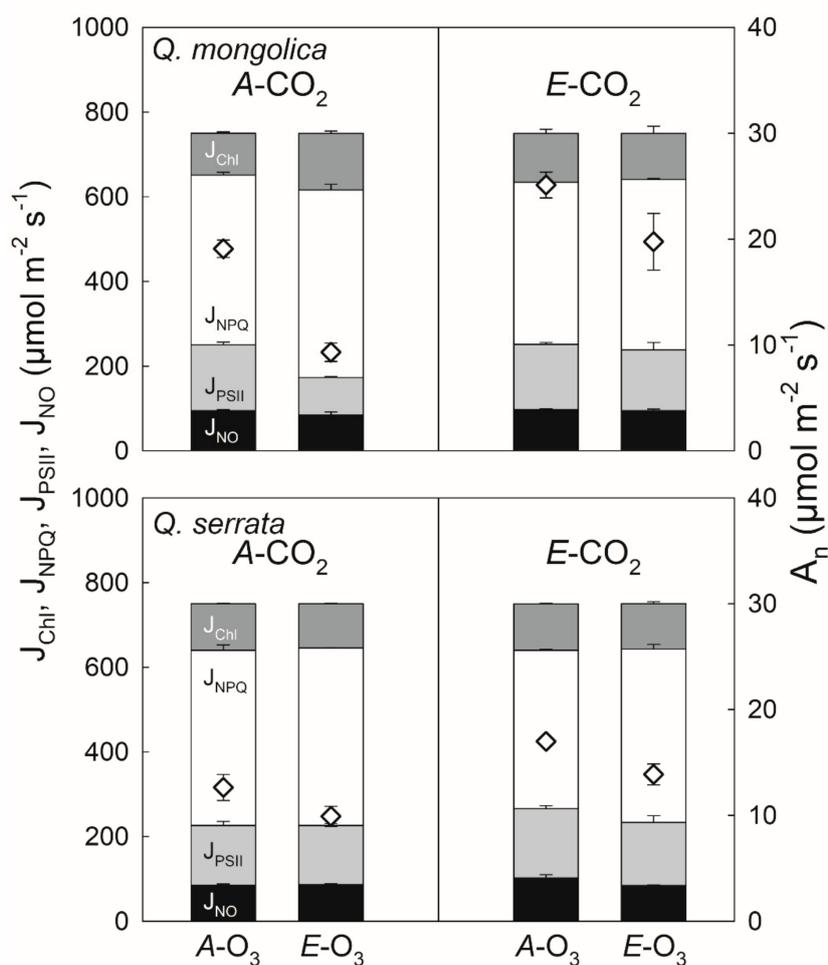


Figure 3. Fate of light energy partitioning under elevated O_3 and CO_2 . Here, J_{Chl} , J_{NPQ} , J_{PSII} and J_{NO} were measured in the seedlings of Japanese oak (*Q. mongolica*) and Konara oak (*Q. serrata*) grown

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].

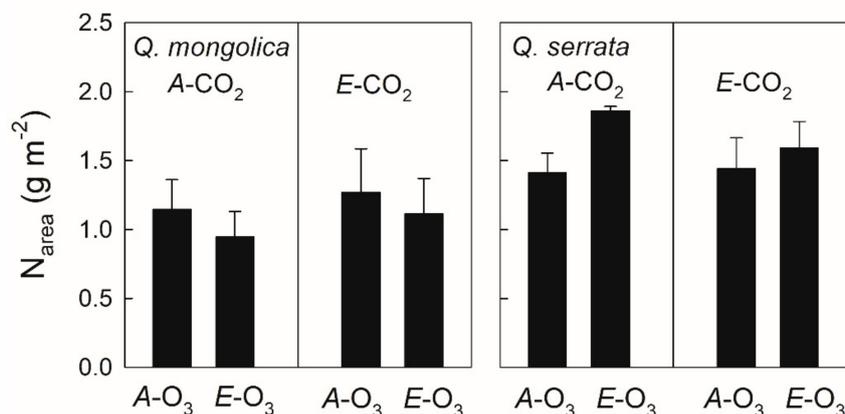


Figure 4. Area-based leaf N content (N_{area}) in the seedlings of Japanese oak (*Q. mongolica*) and Konara oak (*Q. serrata*) grown under ambient ($A-O_3$) and elevated O_3 ($E-O_3$), combined with ambient ($A-CO_2$) and elevated CO_2 ($E-CO_2$). Values are means \pm se ($n = 3$).

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_{area}). The symbols ns and * denote non-significant ($p > 0.05$) and significant ($p \leq 0.05$) effects, respectively.

Effect	F-Statistics	
	N_{area}	
CO_2	0.01	ns
O_3	0.25	ns
$CO_2 \times O_3$	0.27	ns
Species	7.03	*
$CO_2 \times$ Species	0.58	ns
$O_3 \times$ Species	1.94	ns
$CO_2 \times O_3 \times$ Species	0.25	ns

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_{area} with higher N supply, but lower N_{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.

Funding: This study was supported in part by JSPS KAKENHI Grant Number JP17K19301, JP17F17102 and JP17H03839. Evgenios Agathokleous was a JSPS International Research Fellow (ID No: P17102).

Acknowledgments: We thank K Mima and K Sakai for leaf N analyses, and express our sincere appreciation to V. Hurry for his helpful suggestions on our article. E.A. acknowledges multi-year support from The Startup Foundation for Introducing Talent of Nanjing University of Information Science & Technology (NUIST), Nanjing, China.

Conflicts of Interest: The authors declare no conflict of interest.

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