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Growth, Mineral Nutrients, Photosynthesis and Related Physiological Parameters of *Citrus* in Response to Nitrogen Deficiency

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Abstract: Limited data are available on the physiological responses of *Citrus* to nitrogen (N) deficiency. 'Xuegan' (*Citrus sinensis* (L.) Osbeck) and 'Shantian pummelo' (*Citrus grandis* (L.) Osbeck) seedlings were fertilized with nutrient solution at a N concentration of 0, 5, 10, 15 or 20 mM for 10 weeks. N deficiency decreased N uptake and N concentration in leaves, stems and roots and disturbed nutrient balance and homeostasis in plants, thus inhibiting plant growth, as well as reducing photosynthetic pigment levels and impairing thylakoid structure and photosynthetic electron transport chain (PETC) in leaves, hence lowering CO_2 assimilation. The imbalance of nutrients intensified N deficiency's adverse impacts on biomass, PETC, CO_2 assimilation and biosynthesis of photosynthetic pigments. *Citrus* displayed adaptive responses to N deficiency, including (a) elevating the distributions of N and other elements in roots, as well as root dry weight (DW)/shoot DW ratio and root-surface-per-unit volume and (b) improving photosynthetic N use efficiency (PNUE). In general, N deficiency had less impact on biomass and photosynthetic pigment levels in *C. grandis* than in *C. sinensis* seedlings, demonstrating that the tolerance of *C. grandis* seedlings to N deficiency was slightly higher than that of *C. sinensis* seedlings, which might be related to the higher PNUE of the former.

Keywords: *Citrus grandis*; *Citrus sinensis*; CO_2 assimilation; nitrogen deficiency; photosynthetic nitrogen use efficiency

1. Introduction

After carbon, nitrogen (N) is an essential element required in larger amounts by plants than any other elements. N, as a component of chlorophyll (Chl), nucleic acids, amino acids, proteins, plant hormones, coenzymes and secondary metabolites, accounts for 1–5% of the total plant dry matter. Therefore, N plays a key role in various metabolic processes of plants such as Chl biosynthesis and photosynthesis [1,2]. N is one of the main elements limiting crop productivity, especially in the agricultural system with aerobic soil, in which nitrate is easily leached under the condition of high rainfall [3]. The common N deficiency symptoms of crops include arrested vegetative growth, leaf yellowing, narrow leaves and reduced yields [2,4,5]. To meet the required high crop yields, N fertilizers have been widely applied [3,6]. The amounts of N fertilizers applied in the world from 11.4 Tg fertilizer N year^{-1} in 1961 to 107.7 Tg fertilizer N year^{-1} in 2019 [7]. Therefore, it is becoming increasingly imperative to find a widely applicable method to lower the amount of N fertilizer used without reducing yields [8]. Understanding the physiological responses of crops to N deficiency is key for the improvement of N use efficiency and the reduction in N fertilization.

Leaf CO_2 assimilation (A_{CO2}) relies heavily on the functions of photosynthetic pigments and proteins (enzymes), which occupy the majority of N in leaves [9–12]. Therefore, N deficiency causes a decrease in A_{CO2}, which is often accompanied by decreases in the concentrations of photosynthetic pigments such as Chl and carotenoids (Car) and the activities of photosynthetic enzymes such as ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) [3,9–14]. N is highly related to stomatal conductance (g_s) and/or stomatal movement [9]. In a study, Zhao et al. [15] suggested that the reduction in A_{CO2} in N deficiency sorghum leaves was mainly caused by lower g_s, because its reduction was accompanied by decreases in both g_s and intercellular CO_2 concentration (C_i). However, growing evidence is showing that N-deficiency-induced reduction in leaf A_{CO2} is primarily caused by non-stomatal factors, because the reduction is accompanied by increased C_i and/or the ratio of intercellular to ambient CO_2 concentration (C_i/C_a), despite the reduced g_s [10–12,14,16–19].

Scientific reports on N deficiency's effects on primary photochemistry and electron transport of photosystem II (PSII) are inconsistent. Using Chl *a* fluorescence techniques, a lot of researches have shown that the reduction in leaf A_{CO2} was associated with a reduced electron transport rate and raised thermal dissipation of absorbed light energy [9,14,16,20–24]. However, Cruz et al. [17] indicated that N deficiency induced a decrease in A_{CO2} in cassava leaves, which was mainly caused by a decline in biochemical capacity for carboxylation, rather than electron transport, in which the maximum PSII efficiency of dark-adapted leaves (F_v/F_m) was not significantly altered by N deficiency. Unchanged F_v/F_m also have been obtained in the N-deficient leaves of sorghum [18], maize and wheat [25]. Polyphasic Chl *a* fluorescence (OJIP) transients and related fluorescence (OJIP test) parameters have been widely used to reveal the alterations of PSII photochemistry caused by various nutrients deficiencies, such as phosphorus (P), potassium (K), calcium (Ca), sulfur (S), iron (Fe), boron (B), magnesium (Mg), manganese (Mn), copper (Cu) and zinc (Zn) in leaves [26–34]. However, limited information is available on N-deficiency-induced alterations of OJIP transients and OJIP test parameters in leaves. So far, all of these studies have focused on herbs (i.e., maize, tomato, radish and wheat) and shrubs (i.e., tea) [14,20,25,28,29].

There were several studies investigating N deficiency's effects on the uptake of nutrients and their concentrations in leaves, stems and/or roots, but the results were not unanimous [3,35–41]. For example, the concentrations of P, K, Ca, Mg, Mn, Cu and Fe in roots and shoots of barley decreased significantly in response to N deficiency, with a few exceptions that only displayed a decreased trend; but the decreased extent depended on elements, plant tissues and genotypes [38]. N deficiency increased the concentrations of P and K in *Parthenocissus tricuspidata* (Sieb. & Zucc.) Planch. roots, stems and leaves (mg g^{-1} DW), but decreased their accumulation in roots, stems and leaves (mg plant^{-1}) [39]. In sugar cane, N deficiency lowered the concentration of N in stems and leaves, increased the concentrations of P, K and Mg in leaves and P, Mg and S in roots, decreased the concentration of Ca in roots, but had little influence on the concentrations of Ca and S in leaves and K in roots. In leaves, N deficiency led to an increase in the ratio of P, K, Mg, Ca or S concentration to N concentration, while in stems, N deficiency caused a decrease in the ratio of N-, K-, Ca-, Mg- or S-to-P concentration [41]. The absence of N lowered the accumulation of N, P, K, Mg, S and Ca in snap bean shoots [35]. In *Spathiphyllum* leaves, N deficiency increased the concentrations of P and Ca, but it decreased the concentrations of N and K [40]. Nasr Esfahani et al. [42] found that nitrate or phosphate (Pi) imbalance (deficiency) caused stronger molecular responses than combined deficiencies of nitrate and Pi in chickpea roots and leaves. N deficiency, therefore, may disturb other nutrient balance and homeostasis, and thus intensify its adverse impacts on growth, biosynthesis of photosynthetic pigments, photosynthetic electron transport chain (PETC) and A_{CO2}. To date, such data are very rare.

To deal with N deficiency, plants have evolved various adaptive mechanisms [6,8,43–46]. Many studies have shown that N deficiency can increase the partitioning of photosynthetic

dry matter to roots, thus increasing the root/shoot ratio. The increase may be related to the preferential allocation of limited N-to-N-deficient roots [3,6,14,36,39,47–53]. Wang et al. [39] observed that, in addition to increasing N distribution in *P. tricuspidata* roots, N deficiency led to an increase in the distributions of P and K in roots. In general, N-deficiency-induced decreases in P, K, Mg, Cu, Ca, Fe and Mn concentrations were less in barley roots than in shoots [38]. Under N deficiency, the increased distributions of the other nutrient elements in roots might contribute to the higher root/shoot ratio. Increasing evidence shows that the alteration of root architecture in response to N deficiency plays a key role in enhancing N acquisition of plants [6,8,44,45,54].

N is a major factor limiting yield for many crops including *Citrus* [5,8,44]. The optimum range of N concentration in *Citrus* leaves is 23–28 g kg^{-1} DW [55]. Yellow leaves may show when foliar N concentration ranges from 12.5 to 17.5 g kg^{-1} DW [5]. Chen et al. [56] indicated that *Citrus* plants were ammonium sensitive. In China, N deficiency often occurs in *Citrus* orchards, which is one of the main causes of yield loss and fruit quality decline [57–61]. Limited data are available on the physiological responses of *Citrus* to N deficiency [10,44,45]. Using *Citrus sinensis* (L.) Osbeck and *Citrus grandis* (L.) Osbeck seedlings as materials, we investigated N deficiency impacts on growth, root architecture (i.e., root length, root surface area, root average diameter and root volume), nutrient elements in leaves, stems and roots, and photosynthetic pigments, gas exchange, OJIP transients and related parameters in leaves. The objectives of this study were (a) to test the hypothesis whether nutritional imbalance aggravates the symptoms of N deficiency, (b) to understand the possible physiological mechanisms of *Citrus* adaptation to N deficiency, and (c) the causes of leaf A_{CO2} decline in response to N deficiency.

2. Materials and Methods

2.1. Seedling Culture and N Treatments

Seedling culture and N treatments were carried out according to Yang et al. [32] with some modifications. Six-week-old uniform 'Xuegan' (*Citrus sinensis* (L.) Osbeck) and 'Shantian pummelo' (*Citrus grandis* (L.) Osbeck) seedlings were planted to 6 L pots (two plants pot^{-1}) filled with sand and cultivated in a greenhouse under natural conditions at Fujian Agriculture and Forestry University, Fuzhou (26°5′ N, 119°14′ E) with annual average sunshine hours, temperature and relative humidity of ~1600 h, 20 °C and 76%, respectively [62]. Seven weeks after transporting, each pot was fertilized thrice weekly with nutrient solution at a N concentration of 0, 5 10, 15 or 20 mM (i.e., macronutrients (Table 1) and micronutrients: 20 μM Fe-EDTA, 10 μM H_3BO_3, 2 μM $ZnSO_4$, 2 μM $MnCl_2$, 0.5 μM $CuSO_4$ and 0.065 μM $(NH_4)_6Mo_7O_{24}$) until part of the nutrient solution started to leak out of the hole at the bottom of the pot (~500 mL pot^{-1}). Ten weeks after N treatments, the recent, fully expanded (approximately 7-week-old) leaves were used for all measurements. Firstly, both leaf gas exchange and OJIP transients were measured. Then, 6 mm-diameter leaf discs from the same seedlings used for the above measurements were harvested at a sunny noon and immediately frozen in liquid N_2, then stored at −80 °C until assay of photosynthetic pigments. These plants unused for disc collection were used for the measurements of biomass and elements.

Table 1. Formula of macronutrients.

N Levels (mM)	Macronutrients (mM)								pH	EC (mS cm^{-1})
	K_2SO_4	$CaCl_2$	$MgSO_4$	KH_2PO_4	KNO_3	$Ca(NO_3)_2$	$(NH_4)_2SO_4$	NH_4Cl		
0	2.5	5	2	1	0	0	0	0	6.1	3.6
5	1.25	5	2	1	2.5	0	1.25	0	6.1	3.9
10	2.5	2.5	2	1	0	2.5	0	5	6.1	4.3
15	1.25	2.5	2	1	2.5	2.5	1.25	5	6.1	4.7
20	2.5	0	2	1	0	5	0	10	6.1	5.1

2.2. Biomass, Total Root Length, Root Surface Area, Root Average Diameter, Root Volume, and Leaf Photosynthetic Pigments

Leaf, stem and root DW was weighted after being dried to a constant weight at 70 °C.

The image of clean root was captured using digital scanner (Epson Expression 10000XL, Epson America, San Jose, CA, USA) at 600-dpi resolution. Total root length, root surface area, root average diameter and root volume were analyzed using WinRHIZO software (Version 2009b, Regent Instruments, Montreal, QC, Canada) [56].

Leaf Chl, Chl *a*, Chl *b* and carotenoids (Car) were assayed after being extracted with 80% acetone [63].

2.3. Elements in Leaves, Stems and Roots

Recent fully expanded mature leaves, the middle parts of stems and fibrous roots were collected for element analysis. N, K, P, Mg, Ca, S, Mn, Zn, B, Cu and Fe were extracted and measured as described by Cai et al. [64] and Long et al. [65].

Element uptake per plant (plant total element content) was the sum of element content (element concentration × tissue DW) in leaves, stems and roots. Element uptake per root DW was calculated as plant total element content/root DW. Element distribution in leaves, stems or roots (%) was calculated as element content in leaves, stems or roots/plant total element × 100, respectively [64,65].

2.4. Gas Exchange, OJIP Transients and Related Fluorescence Parameters in Leaves

Gas exchange was made with a CIRAS-2 portable photosynthesis system (PP Systems, Herts, UK) between 9:00 and 11:00 a.m. on a sunny day [26]. Water use efficiency (WUE) and photosynthetic N use efficiency (PNUE) were calculated as A_{CO2}/transpiration rate (Tr) [62] and A_{CO2}/leaf N concentration [66], respectively. Leaf OJIP transients were measured after plants had been stored in the dark for 3 h at room temperature using the Handy PEA (Hansatech Instruments Limited, Norfolk, UK). Fluorescence parameters were calculated according to Kalaji et al. [29] and Jiang et al. [67].

2.5. Statistical Analysis

The results were the means ± SE of 3–12 replicates. Except for the mean OJIP transients (only means), a plant in different pots was a repeat. Data were analyzed by two-way ANOVA (five (N levels) × two (species)) followed by the least significant difference (LSD) at $p < 0.05$ level. Calculation of Pearson correlation coefficients (PCCs) were performed with the SPSS statistical software (version 17.0, IBM Corp., Armonk, NY, USA) [26,68].

3. Results

3.1. Effects of N Supply on Seedling Growth

As shown in Figure 1, 0 mM N treatment significantly reduced root length, root surface area, root average diameter, root volume, root DW, stem DW, leaf DW, shoot DW and whole plant DW by 30%, 44%, 20%, 55%, 23%, 46%, 49%, 48% and 41%, respectively, in *C. grandis* seedlings and 29%, 36%, 25%, 53%, 41%, 67%, 65%, 66% and 58%, respectively, in *C. sinensis* seedlings, and significantly increased the ratio of root/shoot DW by 48% and 74% in *C. grandis* and *C. sinensis* seedlings, respectively, relative to 20 mM N treatment, while other N treatments had no significant effect on these ten parameters, with only a few exceptions. The root DW/shoot DW ratio was higher in *C. sinensis* seedlings than in *C. grandis* seedlings or similar between the two depending on N supply, while the reverse was the case for the other nine parameters. As shown in Figure S1, N deficiency symptoms such as vein chlorosis or yellow vein in older leaves and weak, yellowish and small new leaves were observed in 0 mM N-treated seedlings, but not in 5–20 mM N-treated seedlings [4,5]. Therefore, seedlings treated with 0 mM N were considered N deficient, and those treated with 5–20 mM N were considered N sufficient.
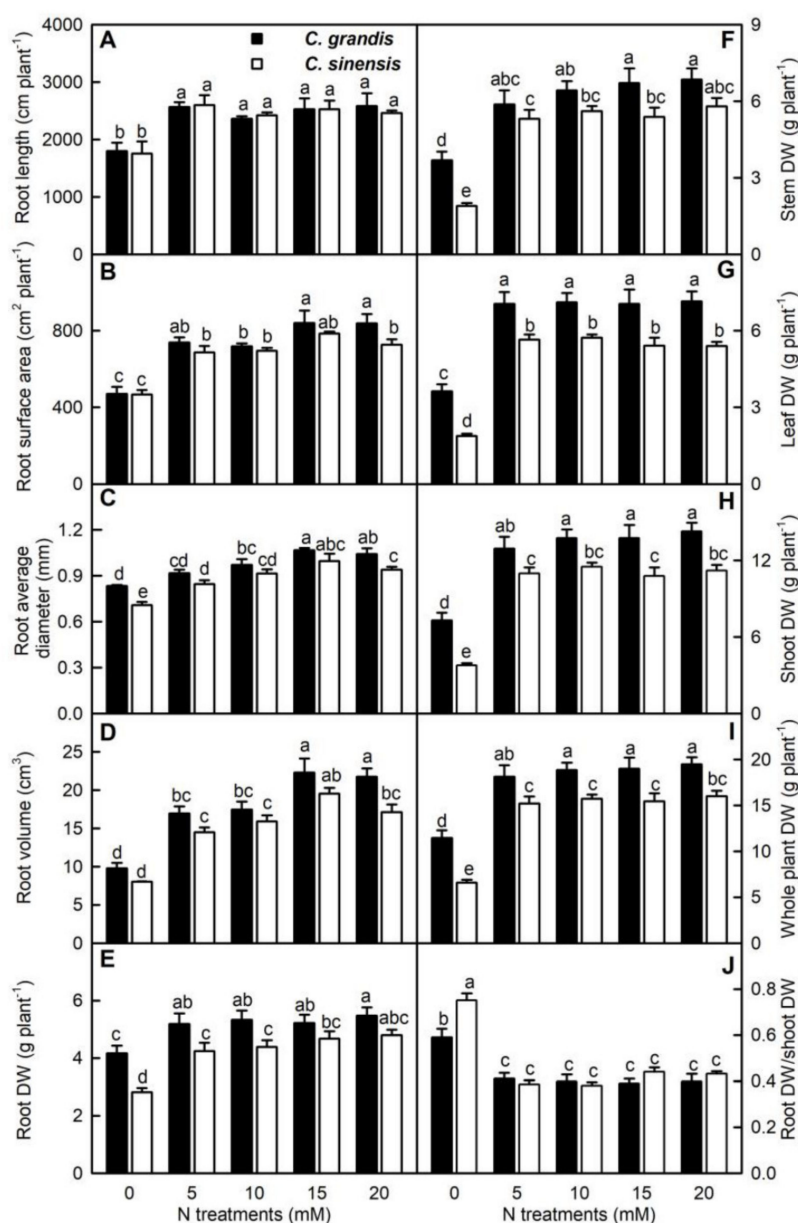
Figure 1. Effects of nitrogen (N) supply on mean (±SE, *n* = 3–4 for (**A**–**D**) or 12 for (**E**–**J**)) root length (**A**), root surface (**B**), root average diameter (**C**), root volume (**D**), root dry weight (DW, (**E**)), stem DW (**F**), leaf DW (**G**), shoot DW (**H**), whole plant DW (**I**) and root DW/shoot DW ratio (**J**) in *Citrus grandis* and *Citrus sinensis* seedlings. Different letters above the bars indicate a significant difference at *p* < 0.05. The same notation will be used in Figures 2–7 and 9.

3.2. Effects of N Supply on Element Concentrations in Roots, Stems and Leaves

N concentration in leaves and roots increased with increasing N supply, and N concentration in stems increased as N supply increased from 0 to 5 mM, after which it remained relatively stable with the elevating N supply. P concentration in *C. grandis* and *C. sinensis* leaves, *C. sinensis* stems and *C. grandis* roots decreased as N supply increased from 0 to 5 mM, but it then remained unchanged with increasing N supply; P concentration in *C. grandis* stems decreased as N supply increased from 0 to 5 mM, but it then rose with increasing N supply; P concentration in *C. sinensis* roots decreased or remained stable as N supply increased from 0 to 15 mM, and then increased at 20 mM N. The concentrations of K, Ca, Mg and S in leaves, stems and roots decreased or remained stable with increasing N supply. The concentrations of N, K, Ca and Mg in leaves, and P, K, Ca, Mg and S in roots,

were higher in *C. sinensis* seedlings than those in *C. grandis* seedlings or similar between the two at each given N supply, while the concentrations of N, P, K, Ca, Mg and S in roots, N in stems, and P and S in leaves were higher in *C. grandis* seedlings than those in *C. sinensis* seedlings or similar between the two at each given N supply, with the exceptions that the concentrations of P and S in 0 mM N-treated leaves and P in 0 and 20 mM N-treated roots were higher in *C. sinensis* seedlings than those in *C. grandis* seedlings (Figure 2).
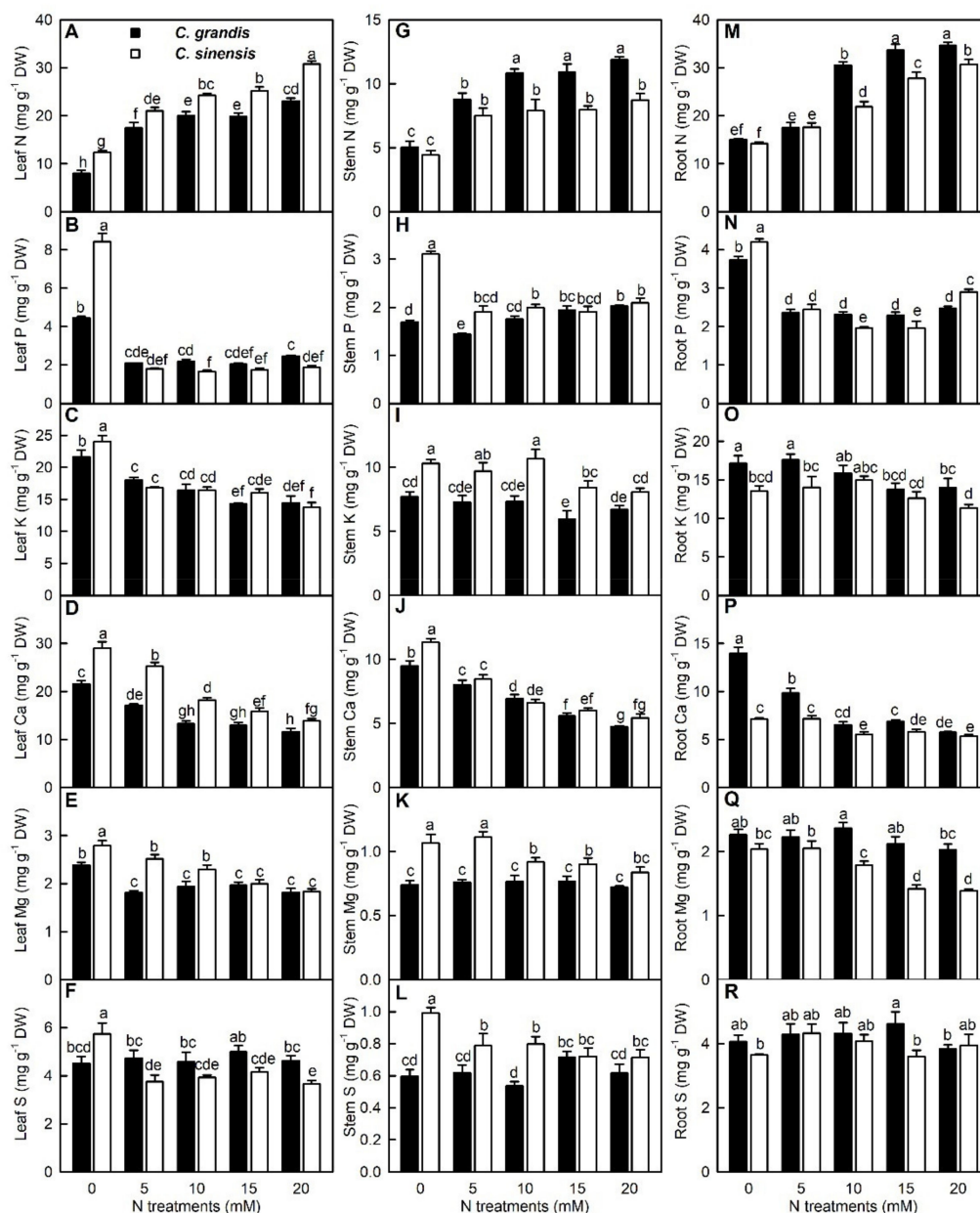

Figure 2. Effects of N supply on mean (±SE, *n* = 4) concentrations of N, P, K, Ca, Mg and S in leaves (**A**–**F**), stems (**G**–**L**) and roots (**M**–**R**).

Fe concentration in *C. grandis* and *C. sinensis* roots and leaves, and *C. grandis* stems decreased or remained stable with increasing N supply with the only exception that Fe concentration in *C. grandis* leaves was higher at 15 mM N than at 10 and 20 mM N, while its concentration in *C. sinensis* stems increased or remained unchanged with increasing N supply. The concentrations of Mn and Zn in *C. sinensis* roots, stems and leaves, Zn in *C. grandis* leaves, and Cu in *C. grandis* and *C. sinensis* leaves increased as N supply increased

from 0 to 10 mM, but they then decreased or remained unchanged with further increasing N supply. The concentrations of Mn in *C. grandis* leaves, stems and roots, Zn in *C. grandis* stems and roots, and Cu in *C. grandis* roots increased as N supply increased from 0 to 5 mM, and then decreased or remained relatively stable with further increasing N supply with the only exception that Mn concentration in stems was higher at 20 mM N than that at 0–15 mM N. B concentration in leaves was higher at 0 mM N than that at 5–20 mM N, while its concentration in stems and roots did not significantly alter in response to N supply. Cu concentration in stems increased or remained stable with increasing N supply. The concentrations of Fe in leaves and stems, Mn and Zn in leaves, stems and roots, and Cu in stems were higher in *C. sinensis* seedlings than those in *C. grandis* seedlings or similar between the two at each given N supply with the only exception that Fe concentration in 0 mM N-treated stems was higher in *C. grandis* than in *C. sinensis* seedlings, while the concentrations of Fe in roots, and Cu in leaves and roots were higher in *C. grandis* than those in *C. sinensis* seedlings or similar between the two at each given N supply with the exception that Cu concentration in 20 mM N-treated leaves and roots was higher in *C. sinensis* seedlings than that in *C. grandis* seedlings (Figure 3).
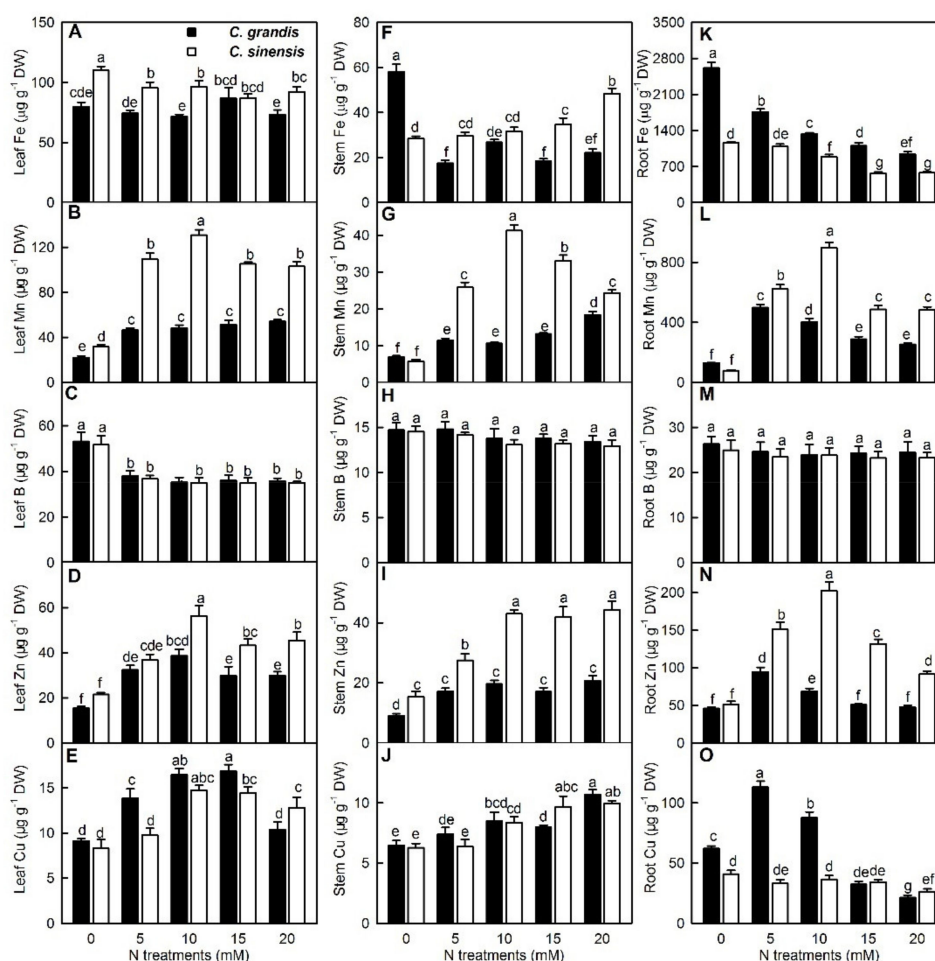

Figure 3. Effects of N supply on mean (±SE, *n* = 4) concentrations of Fe, Mn, B, Zn and Cu in leaves (**A–E**), stems (**F–J**) and roots (**K–O**).

Compared with 20 mM N treatment, N deficiency significantly increased the ratios of P/N, K/N, Ca/N, Mg/N, S/N, Fe/N, B/N and Cu/N by 423%, 333%, 436%, 280%, 182%, 214%, 331% and 154%, respectively, in *C. grandis* leaves and by 1022%, 336%, 420%, 279%, 290%, 199%, 270% and 62%, respectively, in *C. sinensis* leaves, by 97%, 171%, 371%, 142%, 128%, 520%, 158% and 42%, respectively, in *C. grandis* stems and by 192%, 151%, 310%, 151%, 1713%, 15% (the only exception without significant difference), 121% and 24%, respectively, in *C. sinensis* stems, and by 250%, 184%, 463%, 159%, 146%, 427%, 150% and 573%, respectively, in *C. grandis* roots and 212%, 157%, 187%, 216%, 99%, 334%, 131% and 275%, respectively, in *C. sinensis* roots. The absence of N, therefore, led to an increase in all the eight elements in roots, stems and leaves relative to N. N deficiency lowered Mn/N ratio in *C. sinensis* leaves, stems and roots by 23%, 53% and 65%, respectively, relative to 20 mM N treatment, but it was similar in *C. grandis* roots, stems and leaves between 0 and 20 mM treatments. Zn/N ratio in *C. sinensis* leaves and *C. grandis* roots and leaves was higher at 0 mM N than that at 20 mM N, but the reverse was the case for Zn/N ratio in *C. sinensis* stems. Zn/N ratio in *C. grandis* stems and *C. sinensis* roots was similar between 0 and 20 mM N treatments (Figures S2 and S3).

In leaves, the ratios of K/N, Ca/N, Mg/N, S/N, Fe/N, B/N and Cu/N were not significantly lower at *C. grandis* than those at *C. sinensis* at each given N supply with the exceptions that the ratios of Ca/N and Mg/N were higher in the latter at 5 mM N; the ratio of P/N was higher (lower) in *C. sinensis* than in *C. grandis* at 0 mM (5–20 mM) N; the ratios of Mn/N and Zn/N were not significantly lower in *C. sinensis* than in *C. grandis* (Figures S2 and S3).

In stems, the ratios of all the ten elements to N were higher in *C. sinensis* than those in *C. grandis* at given N supply with a few of exceptions (Figures S2 and S3).

In roots, the ratios of P/K, S/N, Mn/N, B/N and Zn/B were not significantly lower in *C. sinensis* than those in *C. grandis* at each given N supply with the only exception that the ratio of Mn/N was significantly lower in the former at 0 mM N, but the ratios of K/N, Ca/N, Mg/N, Fe/N and Cu/N were not significantly lower in *C. grandis* than those in *C. sinensis* with the only exception that the ratio of K/N was significantly lower in the former at 10 mM N (Figures S2 and S3).

3.3. Effects of N Supply on Nutrient Uptake

For *C. grandis* seedlings, N uptake per plant (root DW) increased with increasing N supply, and Fe uptake per plant (root DW) decreased with increasing N supply. P uptake per plant (root DW) decreased as N supply increased from 0 to 5 mM, but it then increased or remained relatively stable with further increasing N supply. K, Ca, Mg, S, Cu, B, Mn and Zn uptake per plant (root DW) increased significantly as N supply increased from 0 to 5 mM, after which they decreased or remained relatively stable with further increasing N supply with the exceptions that Ca and B uptake per root DW did not significantly alter at the range of 0–5 mM N and that S uptake per root DW was higher at 15 mM than at 10 mM. For *C. sinensis* seedlings, N uptake per plant (root DW) increased with the increase in N supply. P uptake per plant (root DW) decreased or remained stable as N supply rose from 0 to 15 mM, and then reduced at 20 mM N. K, Ca, Mg, S and B uptake per plant (root DW) significantly increased as N supply rose from 0 to 5 mM, but they then decreased or remained relatively stable with the increase in N supply with the only exception that Fe uptake per root DW did not significantly differ between 0 and 5 mM N treatments. Cu, Mn and Zn uptake per plant (root DW) significantly rose as N supply rose from 0 to 10 mM, and then decreased or did not significantly alter with further increasing N supply (Figure 4).
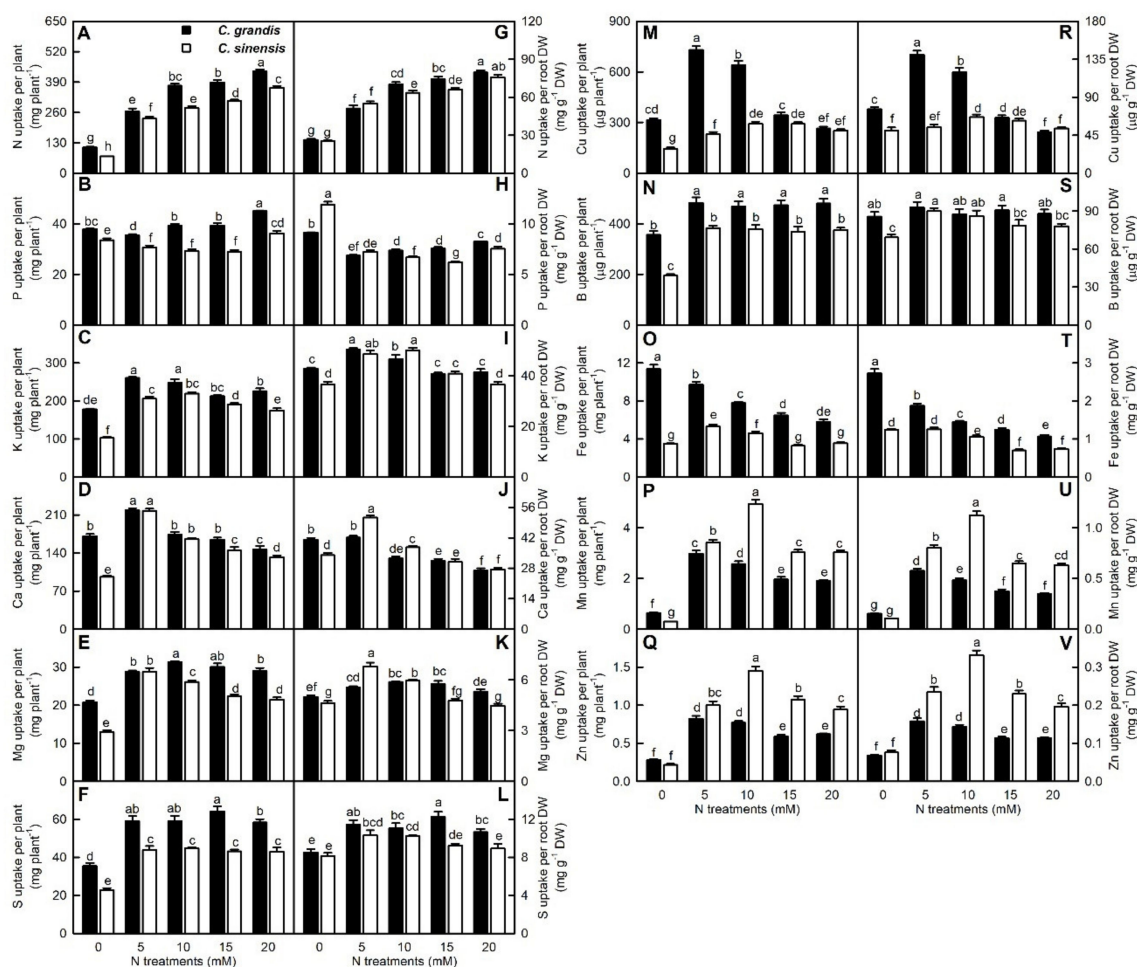
Figure 4. Effects of N supply on mean (±SE, *n* = 4) element uptake per plant (**A–F**,**M–Q**) and per root DW (**G–L**,**R–V**).

As shown in Figure 4, N, P, K, Ca, Mg, Cu, B and Fe uptake per plant (root DW) were higher in *C. grandis* seedlings than those in *C. sinensis* seedlings or similar between the two at each given N supply with the exceptions that P uptake per root DW at 0 mM N, K uptake per root DW at 10 mM N, Ca uptake per root DW at 5 and 10 mM N, and Mg uptake per root DW at 5 mM N were higher in *C. sinensis* than those in *C. grandis* seedlings, while Mn and Zn uptake per plant (root DW) were higher in *C. sinensis* than those in *C. grandis* seedlings or similar between the two at each given N supply with the exception that Mn uptake per plant was higher in *C. grandis* than in *C. sinensis* seedlings at 0 mM N.

Generally viewed, the ratios of P, K, Ca, Mg, S, Fe, B and Cu uptake per plant to N uptake per plant increased with the decrease in N supply, while plant total Mn/N and Zn/N ratios for *C. grandis* (*C. sinensis*) increased significantly as N supply increased from 0 to 5 (10) mM, then decreased with further increasing N supply. It is noteworthy that both plant total Mn/N and Zn/N ratios in *C. grandis* seedlings were 33% and 80% higher at 0 mM N than those at 20 mM, respectively. The absence of N, thus, caused an increase in the uptake of the 10 elements per plant in *C. sinensis* and *C. grandis* seedlings relative to N uptake per plant with the exceptions of B and Zn uptake per plant in *C. sinensis* seedlings. Plant total P/N, Ca/N, Mn/N and Zn/N ratios were not significantly lower in *C. sinensis* than those in *C. grandis* seedlings with the exceptions that plant total Ca/N and Mn/N ratios were significantly lower in the former at 0 mM N, while plant total S/N, Fe/N, B/N and Cu/N ratios were not significantly lower in *C. grandis* than those in *C. sinensis* seedlings at each given N supply. The plants' total K/N ratio at 0–5 mM N and Mg/N ratio at 0 and 20 mM N were significantly higher in *C. grandis* than those in *C. sinensis*

seedlings, while plant total K/N ratio at 10–15 mM N and Mg/N ratio at 5–10 mM N were significantly higher in the latter (Figure 5).
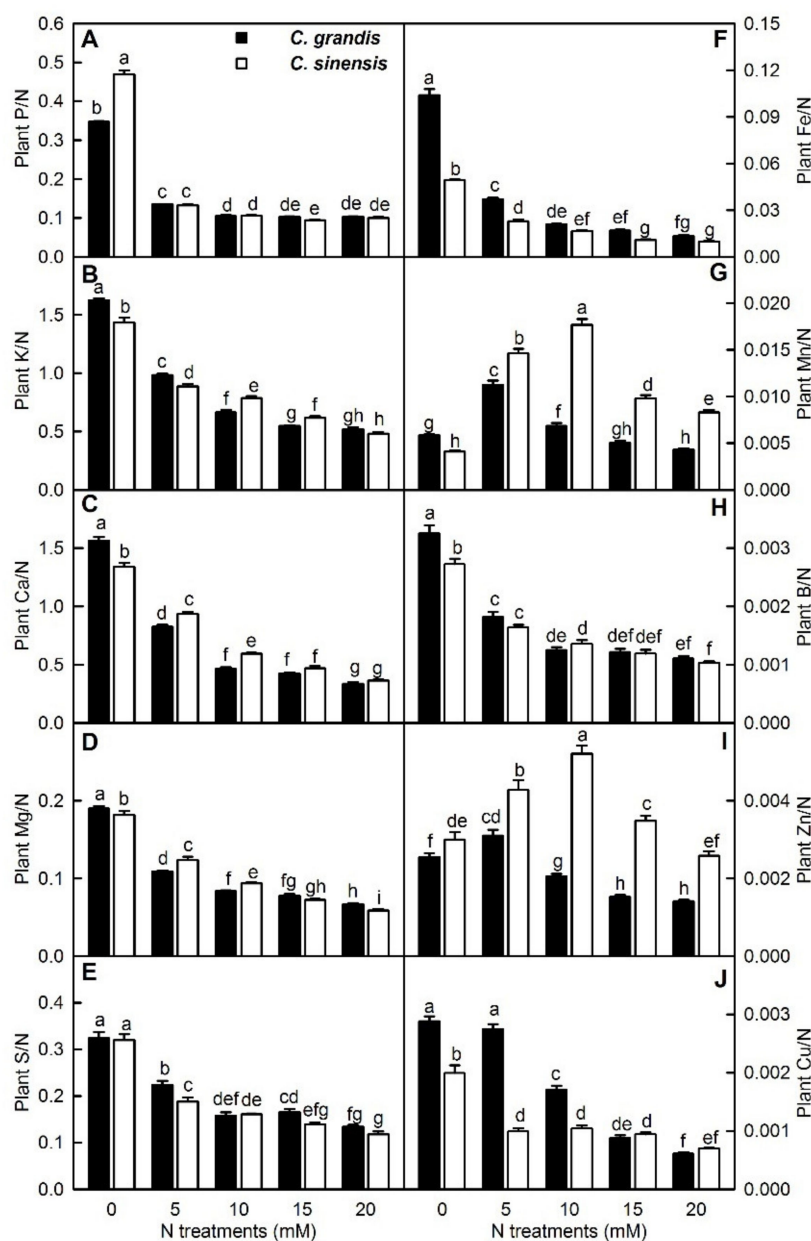

Figure 5. The effects of N supply on mean (±SE, *n* = 4) ratios of P (**A**), K (**B**), Ca (**C**), Mg (**D**), S (**E**), Fe (**F**), Mn (**G**), B (**H**), Zn (**I**) and Cu (**J**) uptake per plant to N uptake per plant.

PCCs were calculated for whole plant DW, plant total element content (element uptake per plant) and the ratios of plant total element content to total N content (Figure S4). Whole-plant DW was significantly and positively related to plant total N, B, S, Mg or K content (*r* > 0.8866), and displayed an upward trend with increasing plant total Cu, Mn, Ca, Zn or P content; but it was significantly and negatively related to plant total P/N, Mg/N, Ca/N, K/N, S/N or B/N ratio (*r* < −0.7809), and displayed a downward trend with increasing plant total Fe/N, Cu/N or Zn/N ratio. A positive and significant relationship existed between any two parameters of plant total N, B, S, Mg and K contents (*r* > 0.6366) or plant total P/N, Mg/N, Ca/N, K/N, S/N and B/N ratios (*r* > 0.8728).

3.4. Effects of N Supply on Element Distributions in Roots, Stems and Leaves

As shown in Figure S5, N distribution in roots significantly decreased as N supply increased from 0 to 5 mM, and then increased or remained relatively stable with increasing N supply, while N distribution in stems and leaves significantly rose as N supply increased from 0 to 5 mM, and then decreased or remained unchanged with further increase in N supply. P distribution in roots and leaves decreased or remained stable with the increase in N supply with the only exception that P distribution in *C. sinensis* roots was higher at 20 mM N than at 5–15 mM N, while P distribution in stems increased with the increase in N supply with the only exception that P distribution in *C. sinensis* stems was lower at 20 mM N than at 10 mM N. K distribution in roots and leaves did not significantly alter in response to N supply except for an increase in K distribution in roots at 0 mM N, while K distribution in stems increased or remained stable with the increase in N supply. Ca distribution in roots decreased or remained unchanged as N supply decreased from 20 to 5 mM N, and then increased at 0 mM N; Ca distribution in leaves significantly increased as N supply increased from 0 to 5 mM, and then decreased or remained stable with further increase in N supply; Ca distribution in stems did not significantly alter in response to N supply except for an increase in 10 mM N-treated *C. grandis* and 20 mM N-treated *C. sinensis* seedlings. Mg distribution in roots decreased or remained unchanged with the increase in N supply, while the reverse was the case for Mg distribution in stems and leaves with the only exception that Mg distribution in leaves was lower in 20 mM N-treated *C. sinensis* than in 10 mM N-treated *C. sinensis* seedlings. N supply had little influence on S distribution in roots, stems and leaves except for an increase in S distribution in *C. grandis* and *C. sinensis* roots and a decrease in S distribution in *C. grandis* leaves. As shown in Figure S6, Cu, Fe, Mn and Zn distributions in roots decreased or remained unchanged with the increase in N supply with the exceptions that Mn distribution in *C. grandis* roots was significantly lower at 0 mM N than at 5 mM N, and that Mn distribution in *C. sinensis* roots was significantly lower at 0 mM N than at 10 mM N, while their distributions in stems and leaves increased or remained unchanged with the increase in N supply except that Fe distribution in *C. grandis* stems was significantly higher at 0 mM N than at 5 mM N, and that Cu distribution in *C. grandis* leaves was significantly higher at 15 mM N than at 20 mM N. N supply had little influence on B distribution in roots, stems and leaves except for an increase in B distribution in 0 mM N-treated *C. sinensis* roots and a decrease in B distribution in 0 mM N-treated *C. sinensis* stems. Compared with 20 mM treatment, 0 mM N-treated *C. grandis* seedlings displayed increased distributions of N, P, K, Ca, Mg, S, Cu, Fe, B and Zn in roots and P in leaves, decreased distributions of P, K, Mg, Cu, B, Mn and Zn in stems, N, Ca, S, Cu, Fe, Mn and Zn in leaves, and unaltered distributions of B in roots, N, Ca, S and Fe in stems and K, Mg and B in leaves, while 0 mM N-treated *C. sinensis* seedlings had elevated distributions of N, K, Mg, Cu, B, Fe and Zn in roots and P in leaves, reduced distributions of P, K, Mg, Cu, B, Fe, Mn and Zn in stems and N, Mg, Cu, Fe and Zn in leaves, and unchanged distributions of P, Ca, S and Mn in roots, N, Ca and S in stems and K, Ca, S, B and Mn in leaves. To conclude, N supply altered element distributions in roots, stems and leaves.

As shown in Figures S5 and S6, the distributions of N, P, K, Ca, Mg, Cu, Fe and Mn in roots, N and Ca in stems, and P, S and Zn in leaves were higher in *C. grandis* than those in *C. sinensis* seedlings or similar between the two at each given N supply except that *C. sinensis* seedlings had higher distributions of P and Mn in 20 mM N-treated roots and P in 0 mM N-treated leaves relative to *C. grandis* seedlings, while the reverse was the case for the distributions of S and Zn in roots, P, K, Mg, S, Cu, Fe, Mn and Zn in stems and N, Ca, Mg, Cu, Fe and Mn in leaves except that *C. grandis* seedlings had higher distributions of Cu and Mn in 20 mM N-treated stems and Cu in 15 mM N-treated leaves relative to *C. sinensis* seedlings.

3.5. Effects of N Supply on Gas Exchange and Photosynthetic Pigments in Leaves

N deficiency decreased A_{CO2}, g_s, Tr, WUE by 48%, 21%, 18% and 36%, respectively, in *C. grandis* leaves and by 46%, 28%, 30% and 24%, respectively, in *C. sinensis* leaves, but increased C_i and C_i/C_a by 20% and 21%, respectively, in *C. grandis* leaves and by 21% and 20%, respectively, in *C. sinensis* leaves. The six parameters did not significantly alter over the range of 5–20 mM N supply. All the six parameters were similar between *C. grandis* and *C. sinensis* leaves at each given N supply with the only exception that Tr was significantly lower in *C. sinensis* than in *C. grandis* leaves at 0 mM N. PNUE, which decreased with the increase in N supply, was significantly lower in *C. sinensis* than that in *C. grandis* leaves at each given N supply. N deficiency increased A_{CO2}/Chl by 22% in *C. sinensis* leaves relative to 20 mM N, but not in *C. grandis* leaves. A_{CO2}/Chl was lower in *C. grandis* leaves than that in *C. sinensis* leaves at 0 mM N, but it was lower in the latter at 5 and 15 mM N (Figure 6).
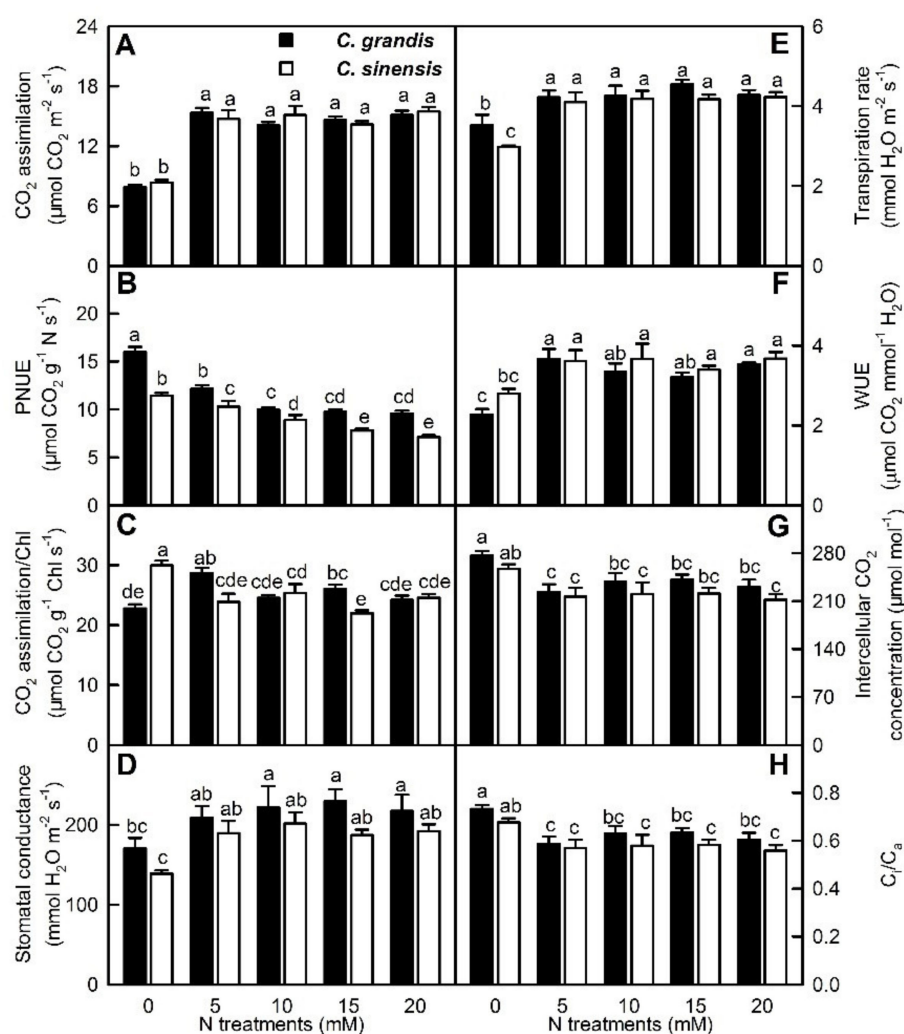

Figure 6. Effects of N supply on mean (±SE, *n* = 3–4) CO_2 assimilation (A_{CO2}, (**A**)), photosynthetic N use efficiency (PNUE, (**B**)), A_{CO2}/chlorophyll (Chl, (**C**)), stomatal conductance (g_s, (**D**)), transpiration rate (Tr, (**E**)), water use efficiency (WUE, (**F**)), intercellular CO_2 concentration (C_i, (**G**)) and ratio of intercellular to ambient CO_2 concentration (C_i/C_a, (**H**)) in leaves.

N deficiency significantly reduced Chl *a*, Chl *b*, Chl *a* + *b* and Car concentrations and Chl *a/b* ratio by 45%, 42%, 44%, 35% and 5%, respectively, in *C. grandis* leaves and by 57%, 55%, 56%, 48% and 4% (the only exception without significant difference), respectively, in *C. sinensis* leaves, but significantly increased Car/Chl, Chl/N and Car/N ratios by 14%, 78% and 102%, respectively, in *C. grandis* leaves and by 17%, 31% and 54%, respectively, in

C. sinensis leaves relative to 20 mM N. The concentrations of Chl *a*, Chl *b*, Chl *a* + *b* and Car were higher in *C. grandis* than in *C. sinensis* leaves at 0 mM N, but were not lower in the latter at 5–20 mM N. However, the ratios of Chl *a*/*b*, Car/Chl, Chl/N and Car/N were not lower in *C. grandis* leaves than those in *C. sinensis* leaves at each given N supply (Figure 7).
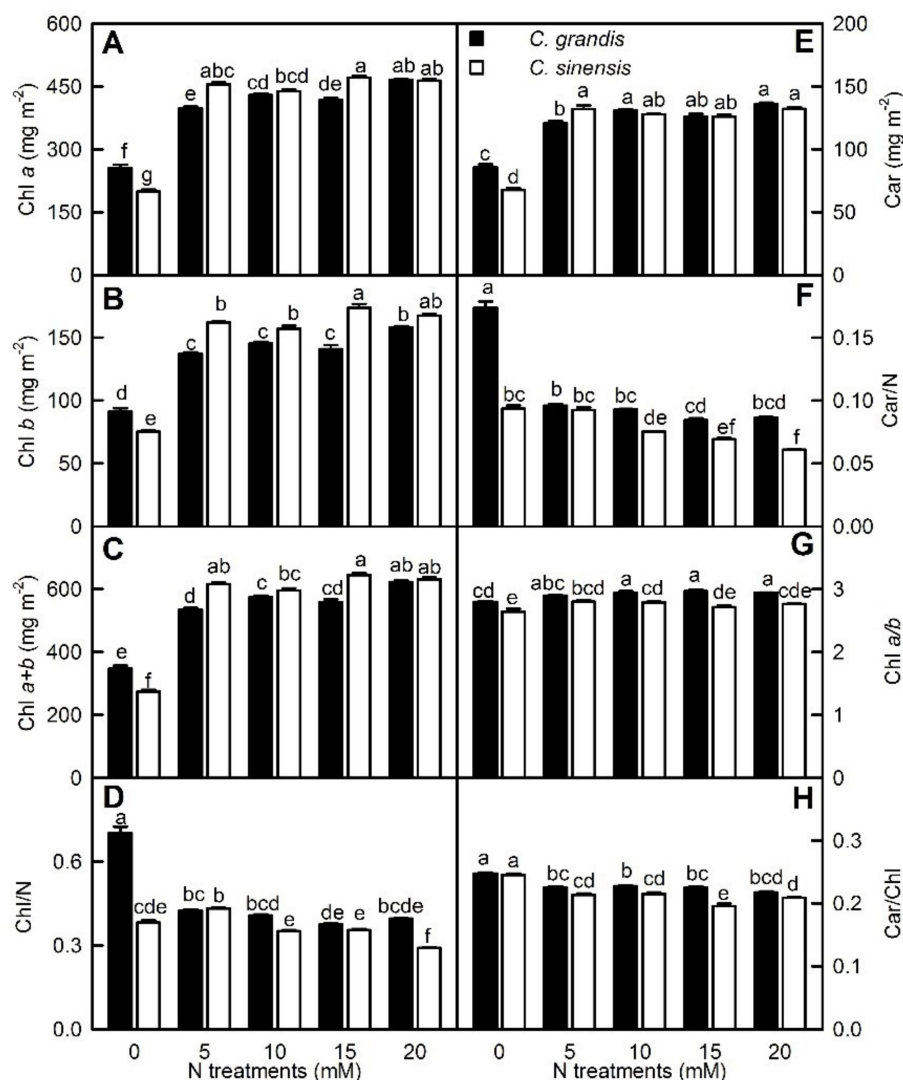

Figure 7. Effects of N supply on mean (±SE, *n* = 4) Chl *a* (**A**), Chl *b* (**B**), Chl *a* + *b* (**C**), Chl/N (**D**), carotenoids (Car, **E**), Car/N (**F**), Chl *a*/*b* (**G**) and Car/Chl (**H**) in leaves.

3.6. Effects of N Supply on OJIP Transients and Related Parameters in Dark-Adapted Leaves

We observed that the heterogeneity of samples (leaves) was greater at 0 mM N than at 5–20 mM N, and that OJIP transients from N deficiency *C. grandis* and *C. sinensis* leaves had greatly increased O-step, slightly increased (*C. sinensis*) or unaltered (*C. grandis*) P-step, positive ΔL-, ΔK-, ΔJ-, and ΔI-bands, positive Δ_{O-K}, Δ_{O-J} and Δ_{J-I} bands, but negative Δ_{I-P} bands compared with OJIP transients from 20 mM N-treated leaves, with greater alterations of ΔL-bands, ΔK-bands and Δ_{I-P} bands and less alterations of Δ_{J-I} bands in N deficiency *C. sinensis* than in N deficiency *C. grandis* leaves. Few, if any, differences existed among the OJIP transients from the 5, 10, 15 and 20 N-treated leaves (Figures 8 and S7).
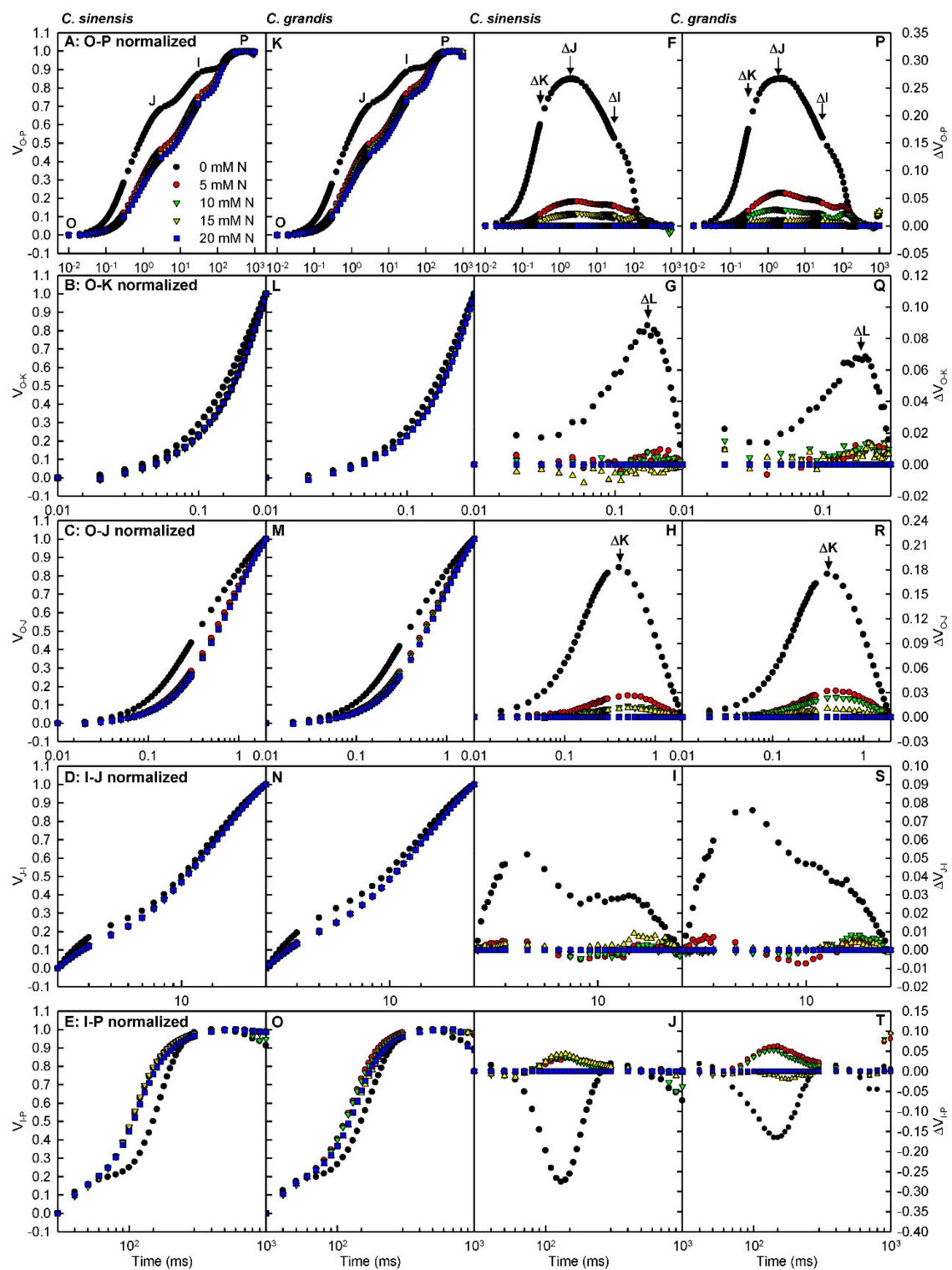
Figure 8. Effects of N supply on the mean OJIP transients of 10 measured samples expressed as the kinetics of relative variable fluorescence: between F_o and F_m (O-P normalized): $V_{O-P} = (F_t - F_o)/(F_m - F_o)$ (**A,K**) and the differences of the five samples to the reference sample treated with 20 mM N (ΔV_{O-P}; **F,P**); between F_o and $F_{300\mu s}$ (O-K normalized): $V_{O-K} = (F_t - F_o)/(F_{300\mu} - F_o)$ (**B,L**) and the differences of the five samples to the reference sample (ΔV_{O-K}; (**G,Q**)); between F_o and F_J (O-J normalized): $W_{O-J} = (F_t - F_o)/(F_J - F_o)$ (**C,M**) and the differences of the five samples to the reference sample (ΔV_{O-J}; (**H,R**)); between F_J and F_I (J-I normalized): $W_{J-I} = (F_t - F_J)/(F_I - F_J)$ (**D,N**) and the differences of the five samples to the reference sample (ΔV_{J-I}; (**I,S**)); and between F_I and F_m (I-P normalized): $V_{I-P} = (F_t - F_I)/(F_m - F_I)$ (**E,O**) and the differences of the five samples to the reference sample (ΔV_{I-P}; (**J,T**)).

Our findings showed that N deficiency decreased maximum primary yield of photochemistry of PSII (F_v/F_o), total electron carriers per reaction center (RC; S_m or EC_o/RC), F_v/F_m (TR_o/ABS), quantum yield for electron transport (φ_{Eo} or ET_o/ABS), efficiency with which an electron can move from the reduced intersystem electron acceptors to the photosystem I (PSI) end electron acceptors (δ_{Ro} or RE_o/ET_o), quantum yield for the reduction in end acceptors of PSI per photon absorbed (φ_{Ro} or RE_o/ABS), probability that a trapped exciton moves an electron into the electron transport chain beyond Q_A^- (ψ_{Eo} or ET_o/TR_o), efficiency with which a trapped exciton can move an electron into the electron transport chain from Q_A^- to the PSI end electron acceptors (ρ_{Ro} or RE_o/TR_o), electron transport flux per RC (ET_o/RC), reduction in end acceptors at PSI electron acceptor side per RC (RE_o/RC), reduction in end acceptors at PSI electron acceptor side per cross section (CS; RE_o/CS_o), maximum amplitude of IP phase, and total performance index ($PI_{abs,total}$) by 26%, 49%, 6%, 48%, 23%, 60%, 45%, 58%, 14%, 34%, 48%, 65% and 90%, respectively, in *C. grandis* leaves and by 26%, 45%, 5%, 47%, 22%, 58%, 44%, 56%, 9%, 29%, 44%, 64% and 90%, respectively, in *C. sinensis* leaves, and increased minimum fluorescence (F_o), approximated initial slope (in ms^{-1}) of the fluorescence transient V = f(t) (M_o), relative variable fluorescence at the J- (2 ms, V_J) and I- (30 ms, V_I) steps, absorption flux per RC (ABS/RC), quantum yield for energy dissipation (DI_o/ABS), dissipated energy flux per RC (DI_o/RC), trapped energy flux per RC (TR_o/RC), and dissipated energy flux per CS (DI_o/CS_o) by 31%, 167%, 68%, 21%, 68%, 28%, 114%, 58% and 67%, respectively, in *C. grandis* leaves and by 35%, 179%, 70%, 22%, 73%, 28%, 123%, 64% and 74%, respectively, in *C. sinensis* leaves relative to 20 mM N. Interestingly, N deficiency increased maximum fluorescence (F_m) by 6% in *C. sinensis* leaves and decreased maximum variable fluorescence (F_v) by 4% in *C. grandis* leaves relative to 20 mM N, but had no significant impact on both F_m in *C. grandis* leaves and F_v in *C. sinensis* leaves. There were relatively little differences in all 24 parameters among 5, 10, 15 and 20 mM N-treated leaves. F_o, M_o, V_J, V_I, S_m, ABS/RC, ET_o/ABS, ET_o/TR_o, DI_o/RC, TR_o/RC, RE_o/CS_o and maximum amplitude of IP phase did not significantly differ between *C. grandis* and *C. sinensis* leaves except that F_o and V_I at 20 mM N and S_m at 15 mM N were slightly higher in *C. grandis* leaves than in *C. sinensis* leaves, and that ET_o/ABS at 5 mM N was slightly higher in *C. sinensis* than in *C. grandis* leaves. F_m, F_v, F_v/F_o, F_v/F_m, RE_o/ET_o, RE_o/ABS, RE_o/TR_o, ET_o/RC, RE_o/RC and $PI_{abs,total}$ were higher in *C. sinensis* than in *C. grandis* leaves or similar between the two at each given N supply. DI_o/ABS and DI_o/CS_o were higher in *C. grandis* than in *C. sinensis* leaves or similar between the two at each given N supply (Figure 9).

3.7. Correlation Coefficient Matrices of Gas Exchange, Photosynthetic Pigments, Element Concentrations and Fluorescence Parameters in Leaves

PCCs were calculated using the means of gas exchange, photosynthetic pigments, element levels and fluorescence parameters in leaves in order to understand the relationships between them (Figure 10). There was a significant negative or positive relationship ($r > 0.6349$ or < -0.6447) between any two parameters of the 11 parameters for gas exchange and photosynthetic pigments (C_i/C_a, Tr, WUE, A_{CO2}, PNUE, g_s, C_i, Chl *a*, Chl *b*, Chl and Car) with a few exceptions including the relationships between C_i/C_a and g_s ($r = -0.4153$), WUE and g_s ($r = 0.5439$), C_i and g_s ($r = -0.4096$), PNUE and g_s ($r = -0.3309$), and Tr and PNUE ($r = -0.5329$). Leaf A_{CO2}, Chl or Car was significantly and positively related to leaf N, Zn, Cu, Mn, ET_o/ABS, ET_o/TR_o, RE_o/CS_o, F_v/F_m, RE_o/ABS, RE_o/TR_o, F_v/F_o, maximum amplitude of IP phase, RE_o/ET_o, RE_o/RC, $PI_{abs,total}$, S_m or ET_o/RC ($r > 0.6129$) with a few exceptions including the relationships between Car and Mn ($r = 0.5871$), Car and Cu ($r = 0.5909$), and Chl and Cu ($r = 0.5513$), and significantly and negatively related to leaf S, Ca, Mg, P, K, B, Cu/N, B/N, S/N, K/N, Ca/N, Mg/N, Fe/N, P/N, F_o, M_o, V_J, V_I, ABS/RC, DI_o/ABS, TR_o/RC, DI_o/RC or DI_o/CS_o ($r < -0.6565$) with a few exceptions including the relationships between S and A_{CO2} ($r = -0.5549$), Cu/N and A_{CO2} ($r = -0.5882$), and Cu/N and Car ($r = -0.5039$), while the reverse was the case for PNUE with a few exceptions including PNUE in relation to Cu ($r = -0.4670$), S ($r = 0.4122$), Ca ($r = 0.4477$),

Mg ($r = 0.3908$) and P ($r = 0.4718$). Leaf A_{CO2}, Chl, Car or PNUE was not significantly related to leaf F_v, Mn/N, Zn/N, Fe or F_m.
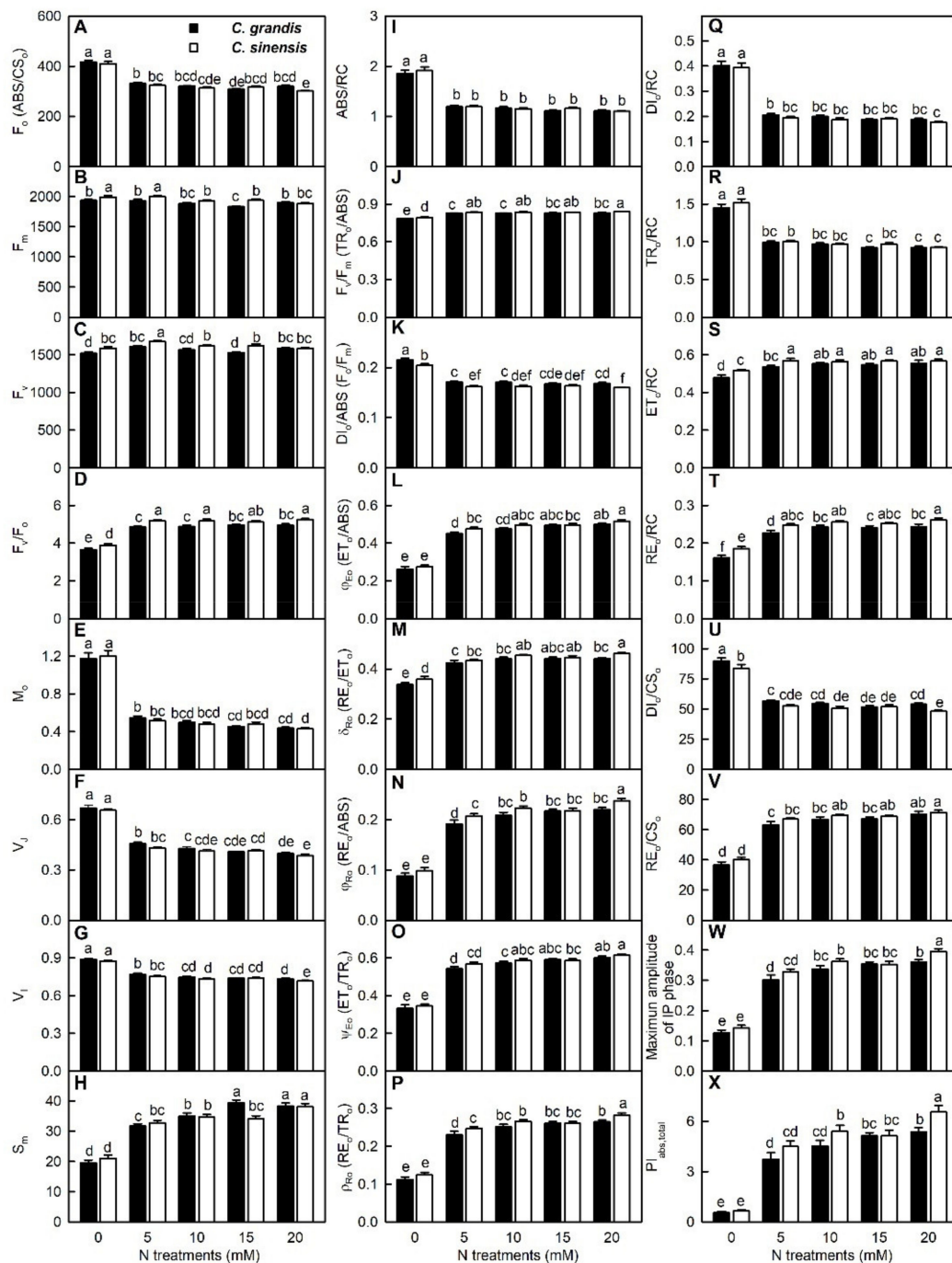

Figure 9. Effects of N supply on mean ($\pm$SE, $n = 10$) F_o (ABS/CS$_o$, (**A**)), F_m (**B**), F_v (**C**), F_v/F_o (**D**), M_o (**E**), V_J (**F**), V_I (**G**), S_m (EC$_o$/RC, (**H**)), ABS/RC (**I**), F_v/F_m (TR$_o$/ABS, (**J**)), DI$_o$/ABS (F_o/F_m, (**K**)), φ_{Eo} (ET$_o$/ABS, (**L**)), δ_{Ro} (RE$_o$/ET$_o$, (**M**)), φ_{Ro} (RE$_o$/ABS, (**N**)), ψ_{Eo} (ET$_o$/TR$_o$, (**O**)), ρ_{Ro} (RE$_o$/TR$_o$, (**P**)), DI$_o$/RC (**Q**), TR$_o$/RC (**R**), ET$_o$/RC (**S**), RE$_o$/RC (**T**), DI$_o$/CS$_o$ (**U**), RE$_o$/CS$_o$ (**V**), maximum amplitude of IP phase (**W**), and PI$_{abs,total}$ (**X**) in dark-adapted *C. grandis* and *C. sinensis* leaves.
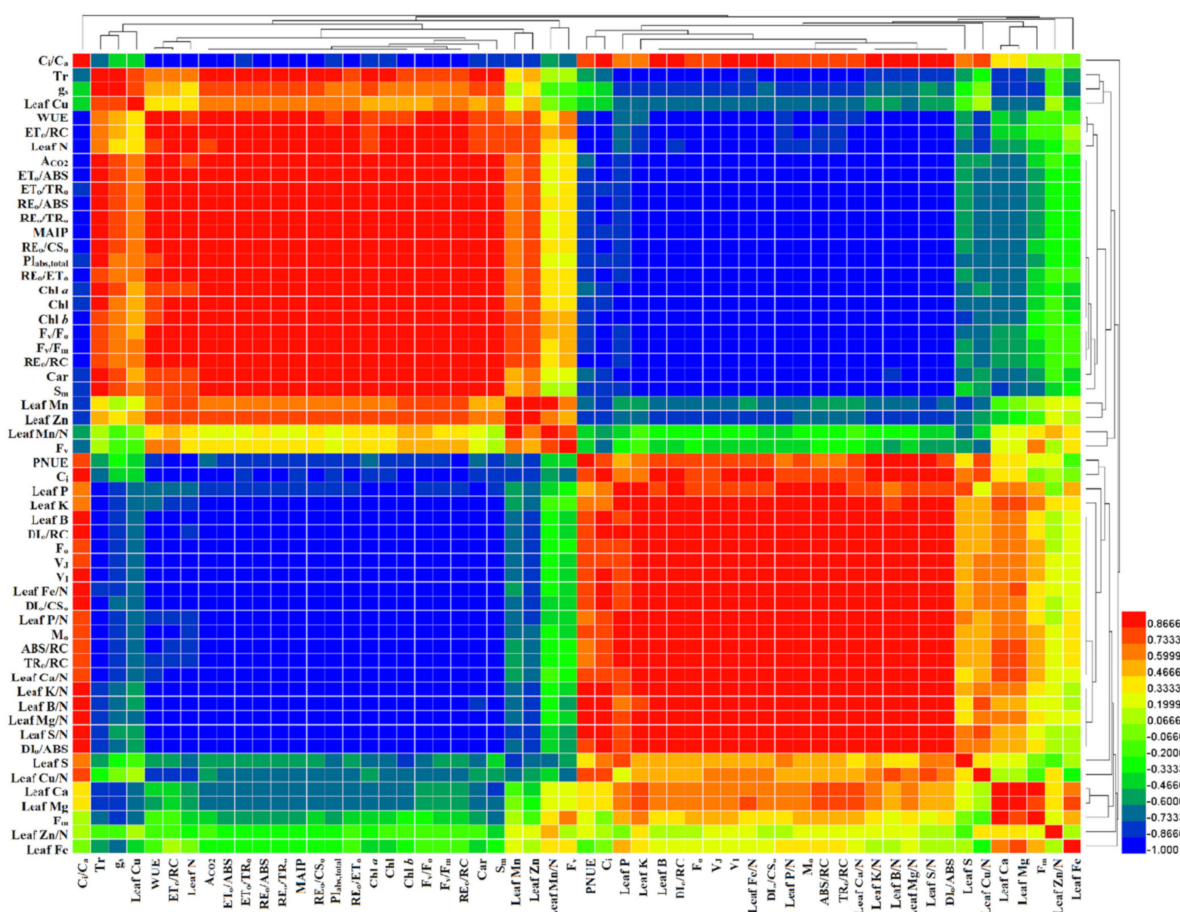
Figure 10. Matrices of Pearson correlation coefficients (PCCs) for the mean gas exchange ($n = 3$–4), photosynthetic pigments ($n = 4$), element concentrations and ratios ($n = 4$) and fluorescence parameters ($n = 10$) in *C. grandis* and *C. sinensis* leaves. A_{CO2}, CO_2 assimilation; MAIP, maximum amplitude of IP phase; Tr, transpiration rate. Data for element concentrations and ratios came from Figures 2, 3, S2 and S3. Data for gas exchange and pigments came from Figures 6 and 7. Data for fluorescence parameters came from Figure 9.

Except for F_m and F_v, a significant negative or positive relationship existed between any two fluorescence parameters ($r > 0.8345$ or < -0.8392).

Leaf N was significantly and positively related to leaf A_{CO2}, Tr, WUE, Chl *a*, Chl *b*, Chl, Car, Mn, Zn, F_v/F_o, S_m, F_v/F_m, ET_o/ABS, RE_o/ET_o, RE_o/ABS, ET_o/TR_o, RE_o/TR_o, ET_o/RC, RE_o/RC, RE_o/CS_o, maximum amplitude of IP phase or $PI_{abs,total}$ ($r > 0.6722$); and significantly and negatively related to leaf PNUE, C_i, C_i/C_a, S, P, K, B, P/N, K/N, Ca/N, Mg/N, S/N, Cu/N, Fe/N, B/N, F_o, M_o, V_J, V_I, TR_o/RC, ABS/RC, DI_o/RC, DI_o/CS_o or DI_o/ABS ($r < -0.6485$), but not significantly related to g_s, Ca, Mg, Fe, Cu, Mn/N, Zn, F_m or F_v.

There was a significant and positive relationship between any two parameters of leaf P, K, Ca, Mg and B ($r > 0.6825$) or leaf P/N, K/N, Ca/N, Mg/N, S/N, Fe/N, B/N and Cu/N ($r > 0.6372$) with a few exceptions including Cu/N in relation to P/N ($r = 0.5031$) and Ca/N ($r = 0.5870$).

4. Discussion

4.1. The Tolerance of C. grandis Seedlings to N Deficiency Was Slightly Higher than That of C. sinensis Seedlings

Plants need adequate amount of N for normal growth, development and reproduction. The availability of N to roots plays a key role in plant growth and photosynthetic pigment biosynthesis [2,13,35]. Regressive analysis showed that whole plant DW was positively

related to N uptake per plant, but negatively related to the ratio of P, Mg, Ca, K, S or B uptake per plant to N uptake per plant (Figure S4), and that leaf Chl *a*, Chl *b*, Chl *a* + *b* or Car concentration was positively related to leaf N, but negatively related to leaf P/N, K/N, Ca/N. Mg/N, S/N, Fe/N or B/N ratio (Figure 10), suggesting that the imbalance of plant (leaf) nutrients might aggravate the N-deficiency-induced decrease in plant growth (leaf photosynthetic pigment biosynthesis). Here, N deficiency affected root, stem, leaf, shoot and whole plant DW (Figure 1), and leaf concentrations of Chl *a*, Chl *b*, Chl *a* + *b* and Car (Figure 7) more in *C. sinensis* than those in *C. grandis* seedlings. In addition, N-deficiency-induced alterations of OJIP transients were slightly greater in *C. sinensis* than those in *C. grandis* leaves, as indicated by the more pronounced positive ΔV_{O-K} and ΔV_{O-J} bands, and negative ΔV_{I-P} band with the exception that the positive ΔV_{J-I} band was more pronounced in *C. grandis* leaves than that in *C. sinensis* leaves (Figure 8). These results indicated that *C. grandis* seedlings had slightly higher tolerance to N deficiency than that of *C. sinensis* seedlings. PNUE increases in response to N deficiency. A higher PNUE can make plants use N effectively for biomass production and improve N use efficiency. There were genotypic differences in PNUE of maize and olives, and PNUE in N-efficient genotypes was higher than that of N-inefficient genotype. A genotypic difference in PNUE has in fact been observed in maize, and PNUE of N-efficient genotypes was higher than that of N-inefficient genotype [69,70]. Boussadia et al. [66] found that PNUE increased in N deficiency 'Meski' olive leaves, but remained unchanged in 'Koroneiki' olive leaves, concluding that 'Meski' had an improvement of N budget in its leaves than 'Koroneiki', and hence might be better adapted to N deficiency. The higher tolerance of *C. grandis* to N deficiency could be explained in this way, because N-deficiency-induced increase in PNUE was greater in *C. grandis* than that in *C. sinensis* leaves and PNUE was higher in the former than in the latter at each given N supply (Figure 6).

4.2. N Deficiency Disturbed Nutrient Balance and Homeostasis, and Altered Nutrient Distributions in Roots, Stems and Leaves

N has both antagonism and synergism on other elements, affecting the uptake and distributions of other elements, and ultimately determining the contents of other elements in different organs of plants [3,37]. Here, N deficiency increased or did not affect P, K, Ca, Mg, S, Fe and B concentrations in roots, stems and leaves and Cu concentration in roots with the exceptions that N deficiency lowered the concentrations of P in *C. grandis* stems and Fe in *C. sinensis* stems (Figures 2 and 3). N deficiency led to an increase in the ratios of P, K, Ca, Mg, S, Fe, B and Cu concentrations to N concentration in roots, stems and leaves (Figures S2 and S3), and the ratios of P, K, Ca, Mg, S, Fe, B and Cu uptake per plant to N uptake per plant (Figure 5) in *C. sinensis* and *C. grandis* seedlings relative to 20 mM N treatments with the exception that the ratio of stem Fe/N in *C. sinensis* seedlings did not significantly differ between 0 mM and 20 mM N treatments. Therefore, N had antagonistic effect on the uptake of these elements. In maize, Schlüter et al. [71] observed that N-starvation led to a strong accumulation of Pi in leaves accompanied by the downregulation of genes involved in Pi-starvation response, demonstrating the importance of Pi homeostasis in stress adaption. Excessive N supply was considered as a cause of *Citrus* Cu deficiency in Florida [5]. There was report showing that N absorption could lower the uptake of P and K in plants [37]. Additionally, N deficiency affected Mn and Zn concentrations and the ratios of Mn/N and Zn/N in roots, stems and leaves, as well as the ratios of Mn (Zn) uptake per plant to N uptake per plant in *C. grandis* and *C. sinensis* seedlings with the exceptions that Zn concentration and Mn/N in *C. grandis* roots, Zn/N in *C. sinensis* roots and Zn uptake per plant/N uptake per plant in *C. sinensis* seedlings did not differ significantly between 0 and 20 mM N treatments (Figures 3, 5 and S3). Lack of N, therefore, altered nutrient uptake per plant to N uptake per plant, thus distributed nutrient balance and homeostasis in leaves, stems and roots. N-deficiency-induced imbalance of nutrients has been obtained in peace lily leaves [40], sugar cane leaves and stems [41], barley [49], grapevine [72], pear [3], *Cyclocarya paliurus* (Batal.) Iljinskaja [37], snap bean [35] and sorghum [36].

The distributions of 11 elements in roots were not significantly lower at 0 mM N than those at 20 mM, while the reverse was the case for their distributions in leaves and stems with the exceptions that P distributions in leaves was significantly higher at 0 mM N than that at 20 mM N (Figures S4 and S5). On the whole, lack of N, therefore, increased the distributions of nutrients in roots, but decreased their distributions in shoots. N-deficiency-induced increase in N, P, K distributions in roots has been obtained in *P. tricuspidata* [39] and maize [6] plants.

4.3. Citrus Displayed Adaptive Responses to N Deficiency

In addition to suppressing plant growth, N deficiency also affects dry matter allocation [15,50]. According to the classical theory of 'functional equilibrium' between shoots and roots, under N deficiency, both the relative deficiency of N and the relative surplus of carbohydrates in plants will have less influence on root development than on shoot development [52]. Here, root DW decreased to a less extent than shoot DW in response to N deficiency, and the ratio of root DW/shoot DW increased (Figure 1). Similar reports for increased partitioning of photosynthetic dry matter to the roots and the increase in the ratio of root DW/shoot DW are well recorded for other N deficiency plants including *Betula pendula* Roth. [51], maize [6,52], lettuce [73], peace lily [40], wheat [48,74,75], sorghum [15], swithgrass [76], pear [3], *P. tricuspidata* [39], soybean [53], snap bean [35], rice [47], sweet potato [77], chickpea [42] and pumpkin [50]. Our results demonstrated that among the four parameters (root length, root surface area, root average diameter and root volume) for root architecture, root volume had the greatest decrease under N deficiency, thus leading to increased root-surface-per-unit volume (Figure 1). Plasticity of root architecture plays a role in the acclimation to N deficiency [6]. Yang et al. [48] observed that low N-induced increase in the ratio of root DW/shoot DW was greater in a newly formed hexaploid wheat with stronger tolerance to N deficiency than its parents, which might be an adaptive growth strategy for the hexaploid because relative more roots fed relative less shoots with N, thereby causing higher N accumulation in the shoots. Active elongation of thin roots to search for N has also been considered as the survival strategy for plants [50]. Thus, N-deficiency-induced increases in the root-surface-per-unit volume due to reduced root diameter and the ratio of root DW/shoot DW might facilitate N uptake and acquisition, thus improving *Citrus* tolerance to N deficiency. However, the difference in N deficiency tolerance between *C. sinensis* and *C. grandis* could not be explained in this way, because N-deficiency-induced increase in the ratio was greater in the former than in the latter (Figure 1). Under low-N stress, N rather than carbon should be the most limited resource for root growth [46]. Here, we observed that N concentration reduced to a less extent in N deficiency roots than that in N deficiency stems and leaves, and N deficiency increased N distribution in roots (Figures 2 and S2), indicating that the limited N in N deficiency seedlings was preferentially allocated to the roots to maintain their growth. To conclude, N deficiency *Citrus* seedlings enhanced their ability to acquire N by increasing N distribution in roots and altering their dry matter partitioning to favor root growth and by optimizing root architecture. In addition, the preferential allocation of the other elements (K, Ca, Mg, Cu, B, Mn and Zn in N deficiency roots (Figures S5 and S6) might contribute to higher ratio of root DW/shoot DW (Figure 1).

4.4. Possible Causes for N-Deficiency-Induced Decrease in A_{CO2} in Leaves

Regressive analysis showed that leaf A_{CO2} (PNUE) was positively (negatively) related to leaf N concentration, but negatively (positively) related to leaf P/N, K/N, Ca/N, Mg/N, S/N, Fe/N, B/N or Cu (Figure 10), implying that the imbalance of leaf nutrients might intensify N-deficiency-induced decrease in A_{CO2}. The higher C_i and C_i/C_a in N-deficient leaves (Figure 6) and negative relationships between A_{CO2} and C_i as well as A_{CO2} and C_i/C_a (Figure 10) indicated that N-deficiency-induced decrease in A_{CO2} was mainly caused by non-stomatal factors, as obtained in 'Cleopatra mandarin' (*Citrus reticulata* L.) [10], apple [12], grape [78], rice [47] and soybean [79]. However, Zhao et al. [15] suggested

that N-deficiency-induced decrease in sorghum leaf A_{CO2} was firstly caused by stomatal closure, because both C_i and g_s decreased with the decrease in A_{CO2}.

Both Chl and Car, the important photosynthetic pigments, were lowered in N deficiency *Citrus* leaves (Figure 7). Chl is a key component of photosynthesis, responsible for the absorption, transportation and transformation of light energy. Many studies have shown that leaf A_{CO2} decrease with the decrease in Chl level [9,14,22]. Apart from general non-photochemical quenching (NPQ), Car can effectively quench the excited triplet state of Chl and $^1O_2$ and protect thylakoid membranes, PSI and PSII from reactive oxygen species (ROS) damage [79,80]. We found that leaf A_{CO2} significantly decreased with the decrease in Chl, Chl *a*, Chl *b* or Car level (Figure 10), that under N deficiency stress, the decrease in Chl in *C. sinensis* leaves was greater than that of A_{CO2}, while the decrease in Chl and A_{CO2} in *C. grandis* leaves was not significantly different, and that Car declined less in *C. grandis* leaves relative to A_{CO2} in response to N deficiency, while the decrease in Car and A_{CO2} in *C. sinensis* leaves did not significantly differ (Figures 6 and 7). N-deficiency-induced decrease in photosynthetic pigments might play a role in A_{CO2} decline, at least in *C. sinensis* leaves. Zhao et al. [15] observed increased A_{CO2}/Chl ratio in N-limited sorghum leaves, concluding that the higher A_{CO2}/Chl ratio might partially alleviate negative effects of N deficiency on A_{CO2}. N-deficiency-induced an increase in A_{CO2}/Chl ratio has been obtained in maize [81]. The ratio of Chl *a*/*b* was significantly lowered in N deficiency *C. grandis* leaves due to the more reduction in Chl *a* relative to Chl *b*, but not in N deficiency *C. sinensis* leaves (Figure 7). There were mixed reports on N deficiency's effects on Chl *a*/*b* ratio; N deficiency decreased the ratio in apple [12] and *Citrus medica* L. [22], increased it in 'Cleopatra mandarin' [10] and maize [23,69], but had no influence on it in soybean, *Populus* and rice [9]. Under N deficiency, Car decreased less relative to Chl in leaves, and the ratio of Car/Chl increased (Figure 6). Similar results have been obtained on *B. pendula* and *B. pubescens* [82], *C. medica* [22], and sweetpotato [77]. A higher ratio of Car/Chl (Figure 7) might be an adaptation mechanism of photosynthetic function to N deficiency by protecting thylakoid membranes, PSI and PSII from photo-oxidative damage [78,82].

Nutrient deficiencies, including N deficiency, can cause photoinhibition of PSII, thereby lowering leaf photosynthetic rate [14,20,26,29,33,34]. As expected, photoinhibition of PSII was observed in N-deficient leaves [83], as indicated by lower F_v/F_m and ET_o/ABS, and higher DI_o/RC, DI_o/CS_o and DI_o/ABS, as well as markedly altered OJIP transients relative to 20 mM N-treated leaves (Figures 8 and 9). The decline of F_v/F_m in response to N deficiency was caused by an increase in F_o, because F_m increased in N deficiency *C. sinensis* leaves and did not significantly alter in N deficiency *C. grandis* leaves relative to 20 mM N-treated leaves. A higher F_o usually occurs in the damaged antennal complex, which cannot effectively transfer energy to the RC [29]. The increase in F_o has been suggested to results from the decoupling of PSII light-harvesting complex (LHCII) from PSII rather than the degradation of LHCII [20].

The decrease in F_v/F_o indicated that the thylakoid structure of N-deficient leaves was damaged [84]. N-deficiency-induced damage of chloroplast (thylakoid) ultrastructure has been observed by confocal laser scanning and/or transmission electron microscopy in radish [28] and 'Cleopatra mandarin' [10] leaves. There was report suggesting that the reduction in *Citrus* leaf A_{CO2} in response to N deficiency was the direct consequence of the destruction of thylakoid assembly [10]. Regressive analysis showed that F_v/F_o was significantly and positively to leaf A_{CO2}, Chl, Chl *a*, Chl *b* or Car ($r > 0.9251$) (Figure 10). Lack of N, therefore, damaged the structure of thylakoids, thus lowering leaf photosynthetic pigment levels and A_{CO2}.

The positive O-K (ΔL-) band in N-deficient leaves corresponded to less connected antennae complexes with lower energy transfer and absorption efficiency due to improper membrane organization [29] and more fragile PSII units with less stability [85], as indicated by the increase in leaf heterogeneity (Figure S7). The positive O-J (ΔK-) band in N-deficient leaves resulted from the inhibition of electron donation from the oxygen-evolving complex (OEC) to P680$^+$ due to the inactivation of OEC [86]. The positive O-K (ΔL-) and O-J (ΔK-)

bands have been obtained in N-deficient tea [14], maize, tomato [28] and radish [20] and various higher plants submitted to other nutrient deficiencies [26,28–30,33,34].

Imbalance in the energetic connectivity between PSII units led to an increase in the inactivation of RCs, as confirmed by the increase in ABS/RC (Figure 8). The inactivation of RCs might protect N-deficient leaves from photooxidative damage by dissipating excessive absorbed light energy [28,29]. This agreed with our findings that N deficiency had higher Car/Chl ratio (Figure 7) and energy dissipation, as confirmed by increased DI_o/RC, DI_o/CS_o and DI_o/ABS (Figure 9).

The positive ΔJ-band (increased V_J), ΔI-band (increased V_I) and negative I-P band (decreased maximum amplitude of IP phase) indicated that N deficiency had more reductive PSII acceptor side, but more oxidative PSI acceptor side. In other word, PSII acceptor side was more sensitive site to N deficiency than PSII donor side; and PSI was less impaired by N deficiency than that of PSII [26,87]. This was also supported by our findings that N-deficient leaves had higher F_o and lower F_v relative to 20 mM N-treated leaves, which were the characteristic of photoinhibitory damage at PSII acceptor side [88]. Similar results have been reported in N-deficient tea [14] and rapeseed [29]. Unfortunately, IP phase and I-P normalized curves were not investigated in the reports of Kalaji et al. [29] and Lin et al. [14], respectively.

The decreases in RE_o/ET_o, RE_o/ABS, RE_o/TR_o, RE_o/RC, RE_o/CS_o and $PI_{abs,total}$ indicated that N deficiency lowered both the efficiency of electron transport to the PSI end electron acceptors and the reduction in PSI end-electron acceptors. This was confirmed by the more oxidize PSI acceptor side, as confirmed by the decrease in maximum amplitude of IP phase (Figure 9). Similar results have been obtained in N-deficient tea [14], radish [28] and rapeseed [29].

Except for F_v and F_m, each other fluorescence parameter had a significant positive or negative correlation with leaf N concentration, leaf P/N, K/N, Ca/N, Mg/N, S/N, Fe/N, or B/N ratio, but the relationships between fluorescence parameters and leaf N were opposite to those between fluorescence parameters and these ratios (Figure 10). Imbalance of leaf nutrients, therefore, intensified N-deficiency-induced impairment of PETC from the donor side of PSII to the reduction in PSI end-electron acceptors (i.e., photon absorption in LHCII, structural damage of thylakoids, electron supply from OEC, stability of PSII units, Q_A and plastoquinone reduction at PSII acceptor site, and reductions of PSI end-electron acceptors).

Regressive analysis showed that leaf A_{CO2} increased with the increase in F_v/F_o, S_m, F_v/F_m, ET_o/ABS, RE_o/ET_o, RE_o/ABS, ET_o/TR_o, RE_o/TR_o, ET_o/RC, RE_o/RC, RE_o/CS_o, maximum amplitude of IP phase or $PI_{abs,total}$, but it decreased with the increase in F_o, M_o, V_J, V_I, TR_o/RC, ABS/RC, DI_o/RC, DI_o/CS_o or DI_o/ABS. The exceptions were that leaf A_{CO2} was not significantly related to F_v or F_m (Figure 10). These results of this study indicated that the impairment of PETC in N-deficient leaves might contribute to the decrease in A_{CO2}.

5. Conclusions

The results of this study clearly demonstrated that N deficiency decreased N uptake and N concentration in roots, stems and leaves and disturbed nutrient balance and homeostasis in plants, thus reducing plant growth, as well as lowering photosynthetic pigment levels, impairing thylakoid structure and whole PETC in leaves, and thereby decreasing A_{CO2} (Figure 11). The imbalance of nutrients intensified N deficiency adverse impacts on biomass, photosynthetic pigment biosynthesis, PETC and A_{CO2}. *Citrus* seedlings displayed adaptive responses to N deficiency, including (a) increasing the distributions of N and other elements in roots, as well as root DW/shoot DW ratio and root-surface-per-unit volume and (b) enhancing PNUE (Figure 11). The tolerance of *C. grandis* seedlings to N deficiency was slightly higher than that of *C. sinensis* seedlings, which might be related to the higher PNUE of the former. This study provided some novel information on the physiological adaption of *Citrus* to N-deficiency.
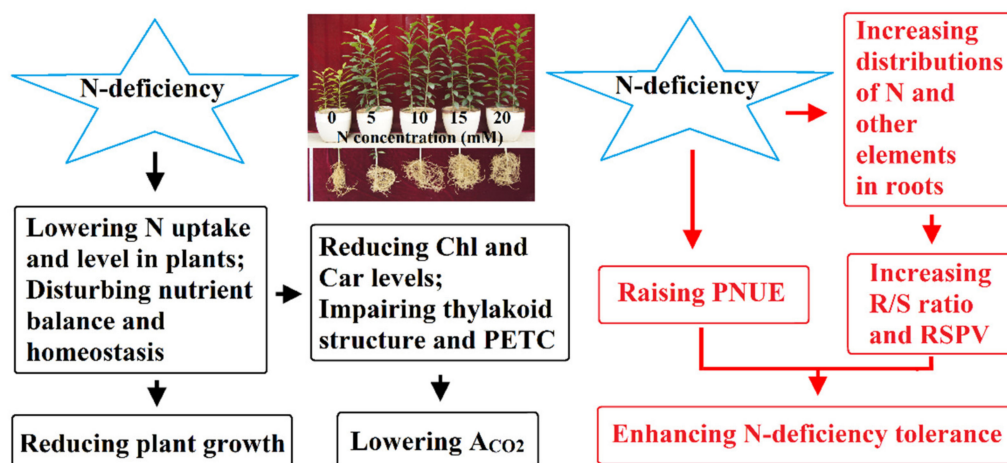
Figure 11. A diagram for the physiological response of *C. grandis* and *C. sinensis* seedlings to N deficiency. A_{CO2}, CO_2 assimilation; Car, Carotenoids; Chl, chlorophyll; N, nitrogen; PETC, photosynthetic electron transport chain; PNUE, photosynthetic N use efficiency; R/S, root DW/shoot DW; RSPV, root-surface-per-unit volume.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy11091859/s1, Figure S1: Effects of nitrogen (N) supply on growth of *Citrus sinensis* (A,B) and *Citrus grandis* (C,D) seedlings, Figure S2: Effects of N supply on mean ($\pm$SE, $n = 4$) ratios of P, K, Ca, Mg or S concentration to N concentration in leaves (A–E), stems (F–J) and roots (K–O) of *Citrus grandis* and *Citrus sinensis* seedlings, Figure S3: Effects of N supply on mean ($\pm$SE, $n = 4$) ratios of Fe, Mn, B, Zn or Cu concentration to N concentration in leaves (A–E), stems (F–J) and roots (K–O) of *Citrus grandis* and *Citrus sinensis* seedlings, Figure S4: Matrices of Pearson correlation coefficients (PCCs) for the mean whole plant DW ($n = 12$), element uptake per plant (plant total element content) and the ratio of element uptake per plant to N uptake per plant ($n = 4$) in *C. grandis* and *C. sinensis* seedlings, Figure S5: Effects of N supply on mean ($\pm$SE, $n = 4$) N, P, K, Ca, Mg and S distribution in roots (A–F), stems (G–L) and leaves (M–R) of *Citrus grandis* and *Citrus sinensis* seedlings, Figure S6: Effects of N supply on mean ($\pm$SE, $n = 4$) Cu, B, Fe Mn and Zn distribution in roots (A–E), stems (F–J) and leaves (K–O) of *Citrus grandis* and *Citrus sinensis* seedlings, Figure S7: Effects of N supply on the high-light-induced OJIP transients of dark-adapted *C. grandis* (A–E) and *C. sinensis* (F–J) leaves plotted on a logarithmic time scale (0.01 to 1000 ms).

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