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# Photosynthetic nitrogen- and water-use efficiencies in C<sub>3</sub> and C<sub>4</sub> subtype grasses grown under two nitrogen supply levels

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## ABSTRACT

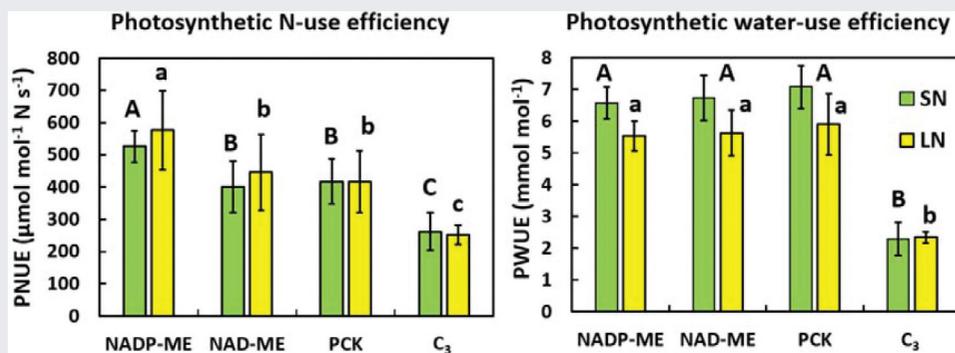
C<sub>4</sub> plants have higher photosynthetic nitrogen- and water-use efficiencies (PNUE and PWUE) than C<sub>3</sub> plants. Some studies on C<sub>4</sub> grasses have reported that NADP-malic enzyme (NADP-ME) grasses have higher PNUE than NAD-malic enzyme (NAD-ME) grasses, whereas other studies have suggested that this difference is caused by the traits of phylogenetic lineage. Further extensive studies are required to properly understand the PNUE and PWUE of C<sub>4</sub> grasses. We investigated the PNUE and PWUE for 22 grass species of four photosynthetic types [4 NADP-ME, 6 NAD-ME, 6 phosphoenolpyruvate carboxykinase (PCK), and 6 C<sub>3</sub> species] grown under two N supply levels. Under the same N level, the three C<sub>4</sub> types showed higher photosynthetic rate and lower stomatal conductance and transpiration rate than the C<sub>3</sub> type, but these parameters were similar in the C<sub>4</sub> types. Although there were no significant differences in leaf mass per area and N content per leaf area among the four types, these traits tended to be somewhat lower in the NADP-ME type than in other types. As a result, PNUE was highest in the NADP-ME type, lowest in the C<sub>3</sub> type, and intermediate in the NAD-ME and PCK types. PWUE was much higher in the C<sub>4</sub> types than in the C<sub>3</sub> type but similar in the C<sub>4</sub> types. The N supply level did not greatly affect the trends of PNUE and PWUE. This study suggests that the NAD-ME and PCK grasses have the N-related physiological traits similar to each other but differing from the NADP-ME grasses.

## ARTICLE HISTORY

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C<sub>3</sub> grasses; C<sub>4</sub> grasses; C<sub>4</sub> biochemical subtype; nitrogen supply; photosynthetic nitrogen-use efficiency; photosynthetic water-use efficiency



## Introduction

Photosynthesis is one of the most important physiological processes that are involved in plant growth and crop yield. Currently, the photosynthetic carbon metabolism of plants is classified into three types: C<sub>3</sub>, C<sub>4</sub> and crassulacean acid metabolism (CAM). C<sub>4</sub> plants, plants performing C<sub>4</sub> type photosynthesis, attract a great deal of attention since the discovery, because they have higher photosynthetic capacity and productivity than C<sub>3</sub> and CAM plants (Brown, 1999; Ehleringer & Monson, 1993; Ermakova et al., 2020).

The higher photosynthetic capacity of C<sub>4</sub> plants is achieved by a CO<sub>2</sub> concentrating mechanism that functions in concert with two types of cells, mesophyll and bundle sheath (BS) cells. Atmospheric CO<sub>2</sub> is primarily fixed as C<sub>4</sub> acids (malate and aspartate) in the mesophyll cells. The formed C<sub>4</sub> acids are transported to adjacent BS cells to be decarboxylated. Released CO<sub>2</sub> is re-fixed by ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco). This biochemical process called the C<sub>4</sub> pathway raises the concentration of CO<sub>2</sub> around Rubisco of the BS cells. As a result, oxygenase activity of Rubisco

(photorespiration) is suppressed (Kanai & Edwards, 1999; Schlüter & Weber, 2020). The  $C_4$  pathway is divided into three biochemical subtypes following the major enzyme involved in the decarboxylation of  $C_4$  acids in the BS cells: NADP-malic enzyme (NADP-ME), NAD-malic enzyme (NAD-ME), and phosphoenolpyruvate carboxykinase (PCK) types (Gutierrez et al., 1974; Kanai & Edwards, 1999). In general,  $C_4$  plants exhibit higher photosynthetic rate in environments with high light, high temperature, and water deficiency than  $C_3$  plants, in which photorespiration is accelerated. Reflecting their photosynthetic traits,  $C_4$  plants are notably prevalent in grasslands and semi-arid lands of tropical and subtropical regions (Christin & Osborne, 2014; Ehleringer & Monson, 1993; Sage, 2004).

$C_4$  plants occur in 19 families of the angiosperm. In these  $C_4$  families, the grass family (Poaceae) includes a large number of  $C_4$  species (Sage et al., 2012). This family contains more than 11,000 species, of which 42% is composed of  $C_4$  species (Grass Phylogeny Working Group II, 2012; Osborne et al., 2014). They have evolved within a branch of the family termed the PACMAD (Panicoideae, Arundinoideae, Chloridoideae, Micrairoideae, Aristidoideae, and Danthonioideae) clade. The subfamilies Panicoideae and Chloridoideae include abundant  $C_4$  species (Grass Phylogeny Working Group II, 2012).  $C_4$  vegetation (grasses) account for 23% of annual terrestrial primary production (Still et al., 2003) and include many valuable grain, forage, and bioenergy crops and serious weeds (Brown, 1999; Jones, 2011; Tubeileh et al., 2016). Thus, it is important to understand the physiological and ecological traits of  $C_4$  grasses. The photosynthetic biochemistry of  $C_4$  grasses is more complex than those of other  $C_4$  families, because all three biochemical subtypes occur in  $C_4$  grasses (Gutierrez et al., 1974). To date, PCK type  $C_4$  species have been identified only in the grass family (Christin et al., 2008; Voznesenskaya et al., 2006). It is unknown the reason why these biochemical variants have evolved in  $C_4$  grasses. It remains unclear whether the differences in  $C_4$  biochemistry result in differences in physiological and ecological behavior of  $C_4$  grasses.

The N- and water-use efficiencies (NUE and WUE) are critical concepts to understand the N and water economy of plants. There are a variety of ways to express the NUE and WUE. The photosynthetic N- and water-use efficiencies (PNUE and PWUE) are frequently used to understand the NUE and WUE at a leaf level (Brown, 1978; Ghannoum et al., 2011). The PNUE is defined as net photosynthetic rate ( $P_N$ ) per unit of leaf N content, whereas the PWUE is expressed as the ratio of  $P_N$  to transpiration rate ( $T_s$ ). As these values represent instantaneous efficiency, biomass production per unit of N in

the plant and per unit of water transpired are also used. The NUE and WUE in both photosynthesis and biomass production are about twice as high in  $C_4$  plants compared to  $C_3$  plants (Brown, 1978; Ghannoum et al., 2011; Jones, 2011; Vogan & Sage, 2011).  $C_4$  plants can attain higher  $P_N$  under less leaf N than  $C_3$  plants due to the  $CO_2$  concentrating mechanism (Brown, 1978; Ghannoum et al., 2011). In  $C_4$  leaves, less N is allocated to Rubisco and more to thylakoid components than in  $C_3$  leaves (Makino et al., 2003). The specific activity and catalytic turnover rate ( $k_{cat}$ ) of Rubisco are higher in  $C_4$  plants than in  $C_3$  plants (Seemann et al., 1984). Higher PWUE in  $C_4$  plants is due to that higher  $P_N$  is achieved under lower stomatal conductance ( $g_s$ ) (Ghannoum et al., 2011).

There are some conflicting reports about the PNUE of  $C_4$  subtype grasses; NADP-ME grasses have higher PNUE than NAD-ME grasses (Ghannoum et al., 2005), whereas there is no consistent difference in PNUE between them (Taub & Ler dau, 2000). With regard to the PNUE of PCK grasses, only few species have been investigated (Pinto et al., 2016, 2014). Meanwhile, it has been reported that the pattern of large-scale distribution of  $C_4$  grasses for annual precipitation differs among the  $C_4$  subtypes (Ellis et al., 1980; Schulze et al., 1996). However, it is uncertain whether the distribution patterns of  $C_4$  subtypes are accounted for by their physiological traits such as PWUE. Further extensive studies are required to properly understand the PNUE and PWUE of  $C_4$  subtype grasses.

In this study, we investigated the PNUE and PWUE of  $C_3$  and  $C_4$  subtype grasses grown under two N supply levels, together with some gas exchange and physiological traits of leaves. In particular, it is interesting to evaluate the PNUE and PWUE of PCK grasses, because the available data are limited.

## Materials and methods

### Plant materials

Six  $C_3$  species and 16  $C_4$  species (4 NADP-ME, 6 NAD-ME, and 6 PCK species) of grasses were examined in this study (Table 1). According to recent classification of subfamilies in the Poaceae (Grass Phylogeny Working Group II, 2012), the  $C_3$  species examined belonged to three subfamilies, Ehrartoideae, Panicoideae, and Arundinoideae. The NADP-ME and PCK species belonged to Panicoideae, and the NAD-ME species did to two subfamilies, Chloridoideae and Panicoideae (Table 1). The  $C_4$ -acid decarboxylation type of each  $C_4$  species was determined on the basis of data from previous studies and leaf anatomical observation by the authors (Table 1). Seeds of *Brachiaria* species were provided by Prof. Y. Kawamoto, Faculty of Agriculture,

**Table 1.** The grass species examined in this study.

Photosynthetic type	Species	Use	Subfamily	Tribe		
NADP-ME	<i>Digitaria ciliaris</i>		Panicoideae	Paniceae	Leaf anatomy by authors	
	<i>Echinochloa crusgalli</i>		Panicoideae	Paniceae	Gutierrez et al. (1974)	
	<i>Zea mays</i>	G, F	Panicoideae	Andropogoneae	Gutierrez et al. (1974)	
	<i>Zea nicaragensis</i>	GR	Panicoideae	Andropogoneae	Leaf anatomy by authors	
NAD-ME	<i>Eleusine coracana</i>	G	Chloridoideae	Cynodonteae	Leaf anatomy by authors	
	<i>Eleusine indica</i>		Chloridoideae	Cynodonteae	Gutierrez et al. (1974)	
	<i>Leptochloa chinensis</i>		Chloridoideae	Cynodonteae	Leaf anatomy by authors	
	<i>Panicum coloratum</i> cv. Solai	F	Panicoideae	Paniceae	Ohsugi et al. (1982)	
	<i>Panicum dichotomiflorum</i>	F	Panicoideae	Paniceae	Ohsugi et al. (1982)	
	<i>Panicum miliaceum</i>	G	Panicoideae	Paniceae	Gutierrez et al. (1974)	
	<i>Brachiaria brizantha</i>	F	Panicoideae	Paniceae	Ueno et al. (2005)	
	<i>Brachiaria decumbens</i>	F	Panicoideae	Paniceae	Ueno et al. (2005)	
PCK	<i>Brachiaria humidicola</i>	F	Panicoideae	Paniceae	Ueno et al. (2005)	
	<i>Panicum maximum</i>	F	Panicoideae	Paniceae	Gutierrez et al. (1974)	
	<i>Urochloa mollis</i>		Panicoideae	Paniceae	Leaf anatomy by authors	
	<i>Urochloa texana</i>		Panicoideae	Paniceae	Ueno et al. (2005)	
	C <sub>3</sub>	<i>Oryza minuta</i>	GR	Ehrhartoideae	Oryzeae	
		<i>Oryza punctata</i>	GR	Ehrhartoideae	Oryzeae	
<i>Oryza rufipogon</i>		GR	Ehrhartoideae	Oryzeae		
<i>Oryza sativa</i> cv. Nipponbare		G	Ehrhartoideae	Oryzeae		
<i>Panicum bisulcatum</i>			Panicoideae	Paniceae		
<i>Phragmites communis</i>			Arundinoideae	Arundineae		

Notes: Use: F, forage; G, grain; GR, genetic resource.

The basis of determination of C<sub>4</sub> biochemical subtype is also shown for each C<sub>4</sub> species.

University of the Ryukyus, Okinawa, Japan, and the Agricultural Research Service, Plant Introduction Station, USDA, USA. Seeds of wild species of *Oryza* were provided from the National Institute of Genetics, Mishima, Shizuoka, Japan. Young shoots of *Phragmites communis* were collected at the riverside of Muromi River, Fukuoka, Japan. Seeds of other species were either those from the seed stock of our laboratory, which were collected from wild plants naturally growing in Fukuoka and Tsukuba, Japan and stored in our laboratory, or seeds purchased from market (*Zea mays*, *Eleusine coracana*, *Panicum miliaceum*, and *P. maximum*).

### Growth condition

Seeds were germinated on perforated multi-well nursery boxes filled with loam soil granules and grown for about 3 to 5 weeks in a greenhouse at the experimental field of Kyushu University. Seeds of wild species of *Oryza* were kept under a temperature of 45°C for 5 days to break dormancy. Healthy seedlings were transplanted to 5 L pots (one plant per pot) with sandy loam soil containing standard N (SN, 0.6 g N per pot) and low N (LN, 0.2 g N per pot). Each pot also contained 0.66 g of phosphorus and 0.83 g of potassium. The plants were grown in the greenhouse [natural sunlight, wherein the maximum photosynthetic photon flux density was about 1500  $\mu\text{mol m}^{-2} \text{s}^{-1}$  at plant height; the mean minimum and maximum temperatures were 25.1°C and 37.8°C, respectively] for 4–7 weeks from July to September. The uppermost fully expanded leaves from each of three plants grown under each N level were used for measurements. In general, plants were

watered daily at the early growth stage and subsequently twice a day. *Oryza* species and *Phragmites communis* were grown under waterlogged conditions by setting the pots in a pool (about 15 cm in water depth) in the same greenhouse.

### Gas exchange traits and PWUE

The leaves from each of three plants at each N supply level were used for measurements of gas exchange traits. The measurement was conducted between 800 and 1400 with an infrared CO<sub>2</sub>/H<sub>2</sub>O gas analyzer (Li-6262, LI-COR, Inc., Nebraska, USA) installed in an open gas-exchange system, as described in Kumagai et al. (2014). Gas exchange traits were measured at leaf temperature (30°C), relative humidity (60%), and ambient CO<sub>2</sub> (403 ± 4  $\mu\text{L L}^{-1}$ ) and O<sub>2</sub> (21%, v/v) concentrations. Light was provided by a metal halide lamp (LS-M180; Sumita Optical Glass Inc., Saitama, Japan) at a photosynthetic photon flux density of 1500  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . Prior to determination of photosynthetic rate, leaves were acclimated to the environment in a chamber for about 30 min.  $P_N$ ,  $g_{sr}$  and  $T_r$  were calculated as described in Long and Hallgren (1985). PWUE was calculated by  $P_N$  divided by  $T_r$ .

### Leaf mass per area, chlorophyll and N contents, and PNUE

The same leaves used for the gas exchange measurement were used for determination of leaf mass per area (LMA) and chlorophyll (Chl) content. Leaf samples (2.4–2.5 cm<sup>2</sup>)

were air dried at 80°C for 1 day and weighted. LMA was calculated by dividing dry weight by leaf area. Chl was extracted from leaf samples (2.4–2.5 cm<sup>2</sup>) in 80% acetone, and Chl content was measured spectrophotometrically according to Arnon (1949). The leaf samples for N content were collected from other fully expanded leaves, air dried at 80°C for 1–2 days, and milled to a fine powder. The N content of leaf sample (0.3 g of powder) was determined using a micro-Kjeldahl procedure (Tsutsumi et al., 2017). The PNUE was calculated by  $P_N$  divided by leaf N content.

### Statistical analysis

The data were presented as means of three plants  $\pm$  SD for the gas exchange and other physiological parameters of leaves. The data were analyzed in Bell Curve for Excel (Social Survey Research Information Co., Ltd., Shinjuku, Tokyo, Japan). The significance of differences in the parameters between the N supply levels in each species was tested by *t*-test. The significance of differences in the parameters among the four photosynthetic types in each N supply level was tested by ANOVA, following by Tukey–Kramer post hoc tests. Pearson's correlation coefficients between the parameters were also calculated. *P* values less than 0.05 or 0.01 were considered to be statistically significant.

## Results

### Gas exchange traits

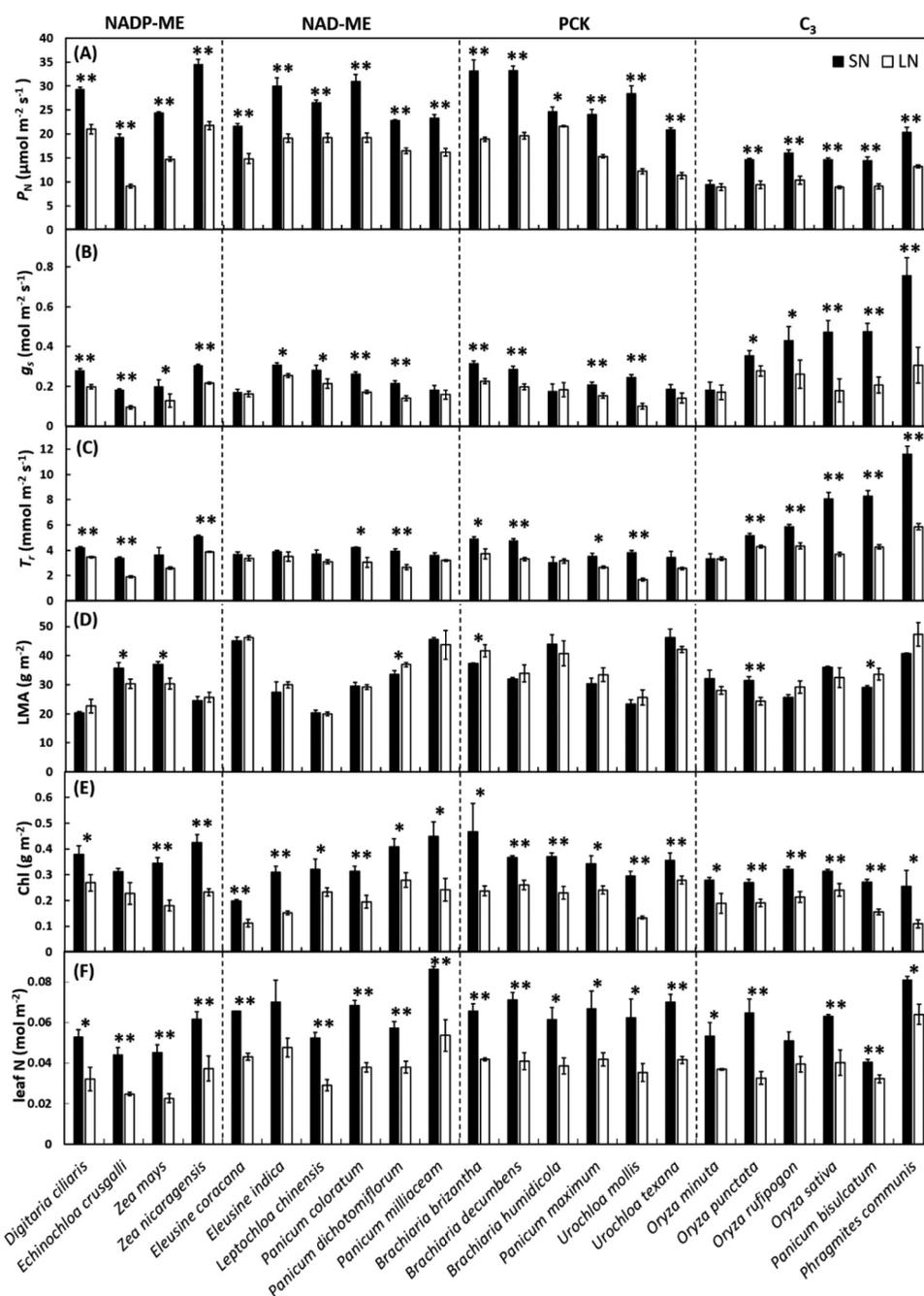
There was a considerable variation in  $P_N$  among species of each photosynthetic type (Figure 1(a)). The mean of  $P_N$  was higher in the three C<sub>4</sub> types than in the C<sub>3</sub> type. However, there was no significant difference in the mean value of  $P_N$  among the three C<sub>4</sub> types (Figure 2(a)). Significant differences in the mean of  $P_N$  were found between SN and LN plants of all grasses except *Oryza minuta* (C<sub>3</sub>) (Figure 1(a)). Although a large species variation was found in  $g_s$  of the C<sub>3</sub> type, there were only small variations in  $g_s$  of the C<sub>4</sub> types (Figure 1(b)). *Oryza minuta* had low  $g_s$ , as in C<sub>4</sub> grasses (Figure 1(b)). In SN plants, the mean of  $g_s$  was higher in the C<sub>3</sub> type than in the three C<sub>4</sub> types, but there was no difference in mean values of  $g_s$  among the four photosynthetic types in LN plants (Figure 2(b)).  $P_N$  was positively correlated with  $g_s$  in each photosynthetic type, but the slope of regression lines markedly differed between the C<sub>3</sub> and C<sub>4</sub> grasses (Figure 3(a); Table 2). In SN plants,  $T_r$  also showed a trend similar to  $g_s$  (Figures 1(c), 2(c)). In LN plants, however, the mean of  $T_r$  was highest in the C<sub>3</sub> type, lowest in the NADP-ME and PCK types, and intermediate in the NAD-ME type (Figure 1(c), 2(c)).

### LMA, Chl content, and leaf N content

There was a large variation in LMA among species of each photosynthetic type (Figure 1(d)). Although there was a trend that the mean of LMA was somewhat lower in the NADP-ME type than in other types, it did not significantly differ among the four types (Figure 2(d)). In *Echinochloa crusgalli* (NADP-ME), *Zea mays* (NADP-ME), and *Oryza punctata* (C<sub>3</sub>), LMA was higher in SN plants than in LN plants, whereas in *Panicum dichotomiflorum* (NAD-ME), *Brachiaria brizantha* (PCK), and *P. bisulcatum* (C<sub>3</sub>), it was higher in LN plants than in SN plants (Figure 1(d)). There were no significant correlations between  $P_N$  and LMA in all photosynthetic types (Figure 3(b); Table 2). The Chl content per leaf area was higher in the SN plants than in the LN plants in all species except *E. crusgalli* (Figure 1(e)). Although there was a trend that the C<sub>3</sub> type had somewhat lower Chl content per leaf area than the C<sub>4</sub> types, there was no significant difference among them (Figure 2(e)). The Chl content per leaf area was positively correlated with  $P_N$  in the NADP-ME and PCK types but not in the NAD-ME and C<sub>3</sub> types (Figure 3(c); Table 2). The leaf N content per leaf area was significantly higher in SN plants than in LN plants in all species except *Eleusine indica* (NAD-ME) and *Oryza rufipogon* (C<sub>3</sub>) (Figure 1(f)). Although the NADP-ME type had somewhat lower N content per leaf area than other types, there was no significant difference among the four types (Figure 2(f)). There were strong positive correlations between  $P_N$  and N content per leaf area in each photosynthetic type (Figure 3(d); Table 2). The NADP-ME grasses had higher  $P_N$  than the NAD-ME and PCK grasses at leaf N content of more than about 0.04 mol m<sup>-2</sup> (Figure 3(d)).

### PNUE and PWUE

There was a considerable variation in PNUE among species of each photosynthetic type (Figure 4(a)). In *Leptochloa chinensis* (NAD-ME) and *B. humidicola* (PCK), PNUE was higher in LN plants than in SN plants, whereas in *B. brizantha*, *O. rufipogon*, and *P. bisulcatum*, it was higher in SN plants than in LN plants (Figure 4(a)). The mean of PNUE was highest in the NADP-ME type and lowest in the C<sub>3</sub> type, and intermediate in both the NAD-ME and PCK types irrespective of N supply level (Figure 5(a)). There was no large variation in PWUE among species of each photosynthetic type (Figure 4(b)). In the C<sub>4</sub> grasses, PWUE was higher in SN plants than in LN plants or did not significantly differ between them (Figure 4(b)). In the C<sub>3</sub> grasses, the response of PWUE to N supply level considerably varied among the species (Figure 4(b)). The



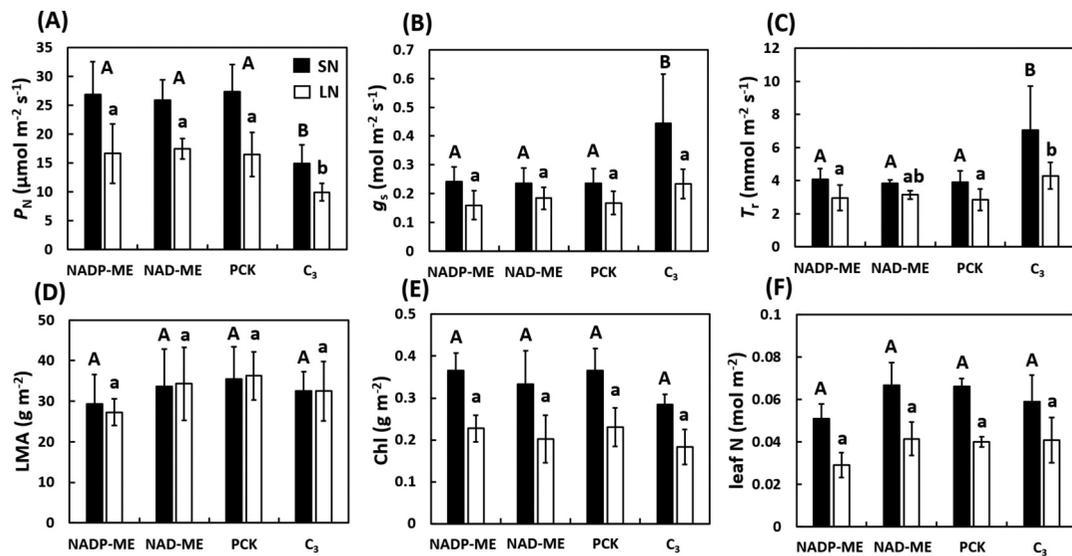
**Figure 1.** Comparison of (a) net photosynthetic rate ( $P_N$ ), (b) stomatal conductance ( $g_s$ ), (c) transpiration rate ( $T_r$ ), (d) leaf mass per area (LMA), (e) chlorophyll (Chl) content, and (f) N content per leaf area in NADP-malic enzyme (NADP-ME), NAD-malic enzyme (NAD-ME), phosphoenolpyruvate carboxykinase (PCK), and C<sub>3</sub> grasses grown under two N supply levels. Notes: Mean  $\pm$  SD ( $n = 3$  plants). Significant at  $P$ : \*  $< 0.05$ ; \*\*  $< 0.01$ . SN, standard N; LN, low N.

mean of PWUE was much higher in the three C<sub>4</sub> types than in the C<sub>3</sub> type, and there was no difference among the three C<sub>4</sub> types (Figure 5(b)).

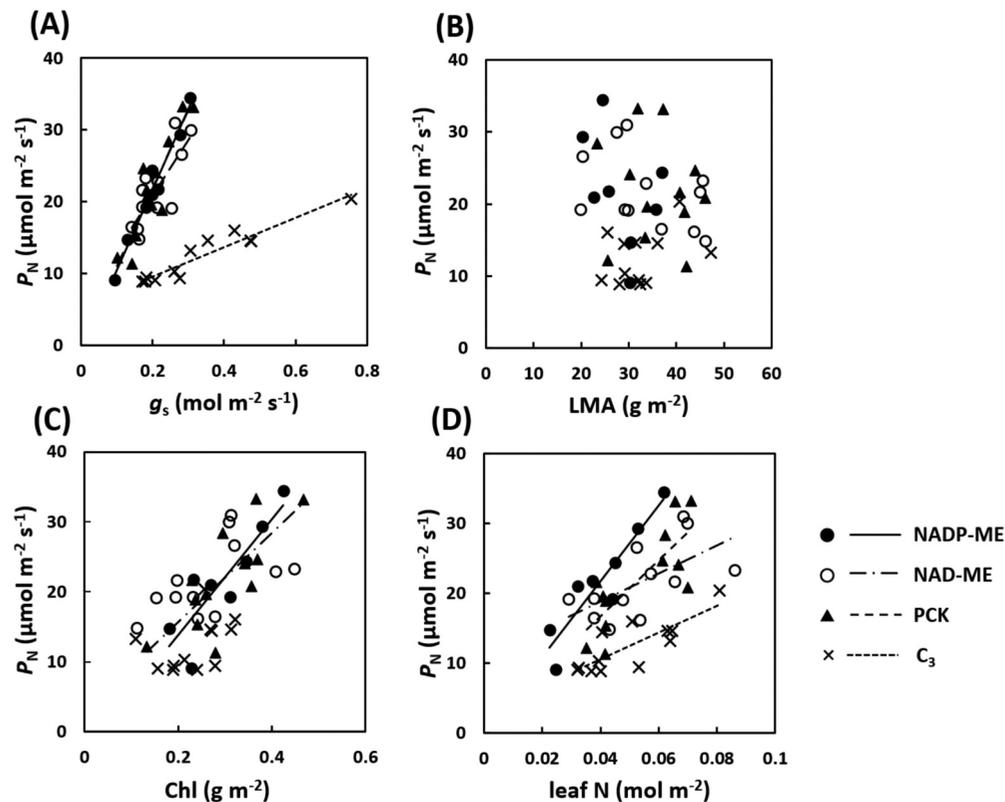
## Discussion

The resource use efficiency provides an important clue to explicate the ecological and adaptive aspects of C<sub>4</sub> subtype plants. Although several studies on

PNUE and PWUE have been undertaken for C<sub>4</sub> grasses (reviewed in Ghannoum et al., 2011), it is important for our understanding to integrate data from diverse plants grown under various environmental conditions. Our study provided the data obtained from C<sub>4</sub> subtype grasses including the PCK type grown under high irradiance and high temperature in midsummer. These light and temperature conditions are relatively higher than those



**Figure 2.** Comparison of (a) net photosynthetic rate ( $P_N$ ), (b) stomatal conductance ( $g_s$ ), (c) transpiration rate ( $T_r$ ), (d) leaf mass per area (LMA), (e) chlorophyll (Chl) content, and (f) N content per leaf area among NADP-ME, NAD-ME, PCK, and C<sub>3</sub> grasses grown under two N supply levels. Notes: Mean  $\pm$  SD ( $n = 4-6$  species). Bars with the same letter are not significantly different ( $P < 0.05$ ). SN, standard N; LN, low N.



**Figure 3.** The relationships between net photosynthetic rate ( $P_N$ ) and (a) stomatal conductance ( $g_s$ ), (b) leaf mass per area (LMA), (c) chlorophyll (Chl) content, and (d) N content per leaf area in NADP-ME, NAD-ME, PCK, and C<sub>3</sub> grasses grown under two N supply levels.

**Table 2.** Correlation coefficients ( $r$ ) from linear regression analysis and statistical significance of the relationships between net photosynthetic rate and other physiological traits in NADP-ME, NAD-ME, PCK, and  $C_3$  grasses.

Trait	NADP-ME	NAD-ME	PCK	$C_3$
$g_s$	0.983**	0.803**	0.906**	0.952**
LMA	-0.418 <sup>NS</sup>	-0.435 <sup>NS</sup>	-0.132 <sup>NS</sup>	0.366 <sup>NS</sup>
Chl content	0.871**	0.554 <sup>NS</sup>	0.771**	0.444 <sup>NS</sup>
Leaf N content	0.926**	0.623*	0.783**	0.809**

Significant at  $P$ : \* < 0.05; \*\* < 0.01; NS, not significant.

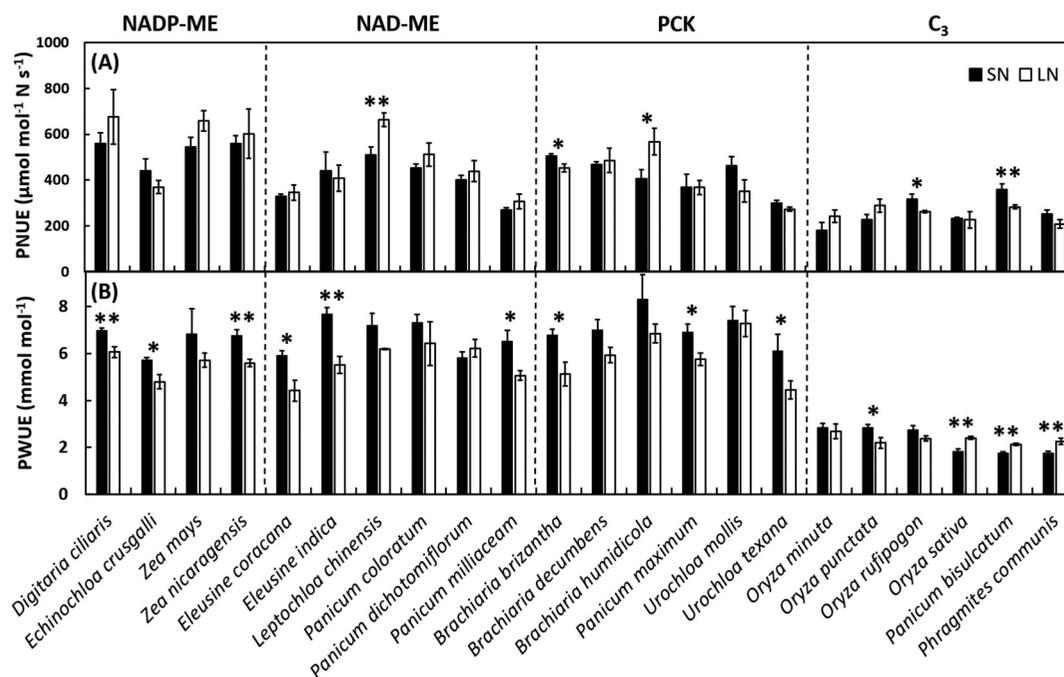
in previous studies (Ghannoum et al., 2005; Taub & Lerda, 2000; Taylor et al., 2010). However, many  $C_4$  grasses may experience these environmental conditions in natural habitats.

### The relationships between gas exchange and other physiological traits

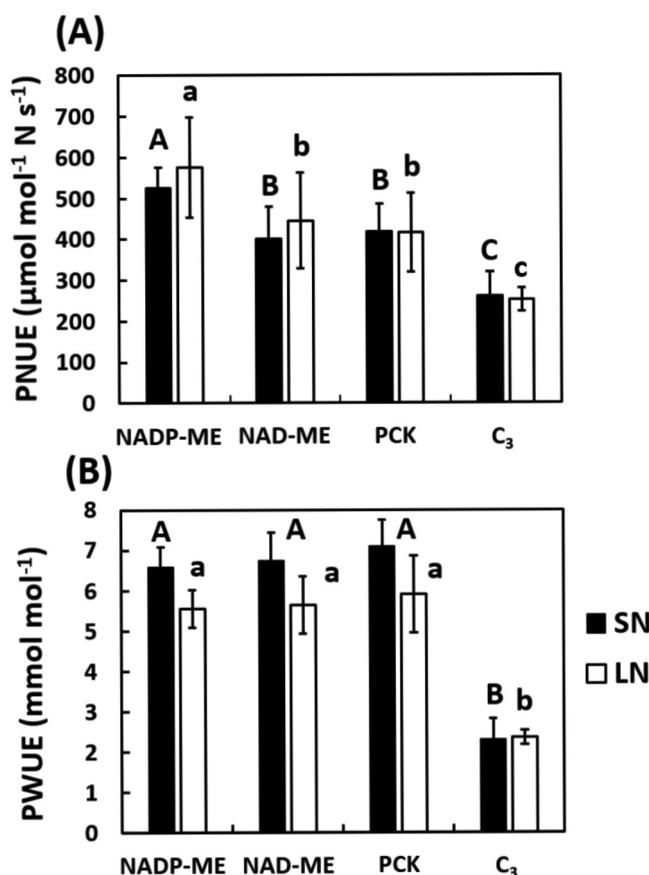
Our study showed that the  $C_4$  grasses have higher  $P_N$  than the  $C_3$  grasses under both N supply levels irrespective of the  $C_4$  subtypes (Figure 2(a)). There was a trend that  $P_N$  values in our study were lower than those reported by previous studies (e.g. Hirasawa et al., 2010; Yabiku & Ueno, 2017). This is probably due to relatively lower N condition applied in this study.  $P_N$  of the  $C_3$  grass *Phragmites communis* was similar to or rather higher than those of some  $C_4$  grasses (*Echinochloa crusgalli*, *Urochloa mollis*, and *U. texana*) under SN and/or LN supply level (Figure 1(a)). This  $C_3$  grass may be worthy

of future study, since a related species of *Phragmites* has been reported to possess remarkable traits of photosynthesis (Srivastava et al., 2014). Both  $g_s$  and  $T_r$  are generally higher in the  $C_3$  grasses than in the  $C_4$  grasses (Figure 1(b,c)). The slopes of regression line between  $g_s$  and  $P_N$  clearly differed between the  $C_3$  and  $C_4$  grasses (Figure 3(a)).  $C_4$  plants can exhibit higher  $P_N$  for a given  $g_s$  than  $C_3$  plants due to the possession of  $CO_2$  concentrating mechanism (Ghannoum et al., 2011; Wong et al., 1985). It is interesting to note that in the three  $C_4$  types the regression lines lay one upon another (Figure 3(a)).

Although there was no significant difference in LMA among the three  $C_4$  types, there was a trend that LMA in the NADP-ME type was somewhat lower than those in the NAD-ME and PCK types (Figure 2(d)). Similar trend has been also reported in the NADP-ME and NAD-ME grasses (Ghannoum et al., 2005, 2001). This trend was also recognized in leaf N content (Figure 2(f)) but not in Chl content (Figure 2(e)). These data show that the NADP-ME type has lower LMA, probably thinner leaves, than the NAD-ME and PCK types, resulting in lower N content per leaf area, but has Chl content per leaf area similar to those of the two types. This suggests that the NADP-ME type may allocate less leaf N to soluble proteins including Rubisco than the NAD-ME and PCK types. In fact, Ghannoum et al. (2005) have reported that the allocation ratio of N to soluble proteins in leaves is lower in NADP-ME grasses (36–45%) than in NAD-ME grasses (51–55%). Meanwhile, Pinto et al. (2016) have



**Figure 4.** Comparison of (a) photosynthetic N-use efficiency (PNUE) and (b) photosynthetic water-use efficiency (PWUE) in leaves of NADP-ME, NAD-ME, PCK, and  $C_3$  grasses grown under two N supply levels. Notes: Mean  $\pm$  SD ( $n = 3$  plants). Significant at  $P$ : \* < 0.05; \*\* < 0.01. SN, standard N; LN, low N.



**Figure 5.** Comparison of (a) photosynthetic N-use efficiency (PNUE) and (b) photosynthetic water-use efficiency (PWUE) among NADP-ME, NAD-ME, PCK, and  $C_3$  grasses grown under two N supply levels. Notes: Mean  $\pm$  SD ( $n = 4\text{--}6$  species). Bars with the same letter are not significantly different ( $P < 0.05$ ). SN, standard N; LN, low N.

reported that NADP-ME and PCK grasses have lower LMA and leaf N content than NAD-ME grasses. Thus, further studies may be required for PCK grasses, especially for those of the subfamily Chloridoideae (Table 1).

### PNUE

We confirmed that the three  $C_4$  types have higher PNUE than the  $C_3$  type (Figure 5(a)). Since we carried out this study in midsummer, all the  $C_3$  species examined were chosen from grasses originating in warm environments. Pooideae  $C_3$  grasses originating in cool environments, such as *Poa* and *Triticum*, have been also reported to have PNUE values similar to these summer-active  $C_3$  grasses (Ghannoum et al., 2011; Westbeek et al., 1999). Our previous study on 12 species of *Amaranthus* (NAD-ME type  $C_4$  dicots) found that PNUE values ranged from 260 to 458  $\mu\text{mol mol}^{-1} \text{N s}^{-1}$  (mean  $\pm$  SD = 344  $\pm$  56  $\mu\text{mol mol}^{-1} \text{N s}^{-1}$ ; Tsutsumi et al., 2017). These values were similar to or somewhat lower than those of the NAD-ME and PCK grasses examined here. Another study on 22 maize and 4 teosinte lines (NADP-ME type grasses)

reported that PNUE values ranged from 510 to 862  $\mu\text{mol mol}^{-1} \text{N s}^{-1}$  (mean  $\pm$  SD = 700  $\pm$  84  $\mu\text{mol mol}^{-1} \text{N s}^{-1}$ ; Yabiku & Ueno, 2017). These values were in the highest class of PNUE values found in the NADP-ME grasses examined here. Ghannoum et al. (2005) and Makino and Ueno (2018) showed that PNUE was higher at lower N supply level. In our study, there were no great differences in PNUE between SN and LN plants of each photosynthetic type (Figure 5(A)), although there were some exceptional species (Figure 4(a)).

To date, there are several studies on the PNUE of  $C_4$  subtypes in grasses. Taub and Lerdau (2000) reported that species-level variation in PNUE is large enough to mask any differences that may be due to the  $C_4$  subtypes. However, it should be considered that the number of species examined was small in their study. In a more extensive comparative study, Ghannoum et al. (2005) showed that NADP-ME grasses had higher PNUE than NAD-ME grasses, consistent with the results of our study (Figure 5(a)). It appears that higher PNUE in the NADP-ME type is brought partially by lower leaf N content compared to the NAD-ME type (Figure 2(f))

but similar values of  $P_N$  in the two types (Figure 2(a)). On the other hand, Taylor et al. (2010) has suggested that phylogenetic effects are involved in the difference in PNUE values of  $C_4$  grasses; NADP-ME species of the tribe Andropogoneae in the subfamily Panicoideae have generally higher PNUE values than  $C_3$ , other NADP-ME, and NAD-ME grasses, whereas NADP-ME species of *Aristida* in the subfamily Aristidoideae have PNUE values similar to  $C_3$  grasses. The *Aristida* species have higher leaf N content than the Andropogonoid NADP-ME species (Taylor et al., 2010), and are well known to have unusual Kranz leaf anatomy consisting of a double chlorenchymatous BS and complex cellular accumulation of  $C_3$  and  $C_4$  enzymes (Ueno, 1992). Thus, *Aristida* species may represent an exceptional group in the NADP-ME grasses. Although a limited number of NADP-ME species belonging to the tribes Andropogoneae and Paniceae were examined in this study (Table 1), there was no significant difference in PNUE values between these tribes (Supplemental data 1). Recently, Pinto et al. (2016) examined PNUE for 4 PCK species (3 Panicoideae and 1 Chloridoideae species), 9 NADP-ME, and 11 NAD-ME grass species grown in growth chambers (day/night temperature, 24/19°C), and showed that the NADP-ME grasses had higher PNUE than the NAD-ME grasses, whereas the PCK grasses had intermediate PNUE between these two types. The results of NADP-ME and NAD-ME grasses corresponded to but those of PCK grasses somewhat differed from those of our study (Figure 5(a)). The difference in the trend of PCK grasses may be due to the differences in species examined and/or growth conditions. Taken altogether, it seems that NADP-ME grasses have generally higher PNUE than NAD-ME grasses with some exceptional NADP-ME grasses, whereas PCK grasses have PNUE similar to that of NAD-ME rather than NADP-ME grasses.

Ghannoum et al. (2005) have suggested that higher PNUE of NADP-ME than NAD-ME grasses is achieved with less leaf N, soluble protein, and Rubisco with a faster  $k_{cat}$ . Seemann et al. (1984) found that the specific activity and  $k_{cat}$  of Rubisco of PCK species were similar to those of NAD-ME species but lower than those of NADP-ME species. These data are consistent with the trend of PNUE in the  $C_4$  subtypes found in our study (Figure 5(a)). The major  $C_4$  acid that is produced in the mesophyll cells is malate in the NADP-ME type and aspartate in the NAD-ME and PCK types (Kanai & Edwards, 1999). The  $C_4$  biochemical mechanism is more complex in the NAD-ME and PCK types than in the NADP-ME type. The NAD-ME and PCK types require some enzymes such as alanine aminotransferase and aspartate aminotransferase that are not needed in the NADP-ME type. In particular, that

of PCK type is complicated, because PCK and NAD-ME function together in the decarboxylation step (Kanai & Edwards, 1999; Voznesenskaya et al., 2006). Although photorespiration of  $C_4$  plants is reduced to low level (Kanai & Edwards, 1999; Schlüter & Weber, 2020), the BS cells contain considerable amounts of photorespiratory enzymes such as glycine decarboxylase and glycolate oxidase, especially in the NAD-ME and PCK types (Ueno et al., 2005; Yoshimura et al., 2004). As a result, more enzyme protein, namely more leaf N, may be required for the performance of photosynthesis in the PCK and NAD-ME types. It is uncertain whether the difference in PNUE values among the  $C_4$  subtypes of grasses is associated with their ecological performance. However, it seems that NAD-ME and PCK grasses have higher N requirement than NADP-ME grasses. This characteristic may be related to the abundance of these subtypes (Knapp & Medina, 1999). It is interesting to note that the most successful  $C_4$  crops such as maize, sorghum, and sugarcane are the NADP-ME type.

### PWUE

We found that irrespective of the  $C_4$  types, the  $C_4$  grasses had higher PWUE than the  $C_3$  grasses (Figure 5(b)). Our previous study on NAD-ME type  $C_4$  species of *Amaranthus* reported that PWUE values ranged from 5.6 to 10.4 mmol mol<sup>-1</sup> (mean  $\pm$  SD = 8.5  $\pm$  1.3 mmol mol<sup>-1</sup>; Tsutsumi et al., 2017). These values were higher than those of the  $C_4$  grasses examined here. Another study on maize and teosinte lines (NADP-ME type) reported that PNUE values ranged from 5.3 to 7.0 mmol mol<sup>-1</sup> N s<sup>-1</sup> (mean  $\pm$  SD = 6.1  $\pm$  0.5 mmol mol<sup>-1</sup> N s<sup>-1</sup>; Yabiku & Ueno, 2017). These values were similar to those of the  $C_4$  grasses in our present study. Although the  $C_4$  pathway enables plants to exhibit higher  $P_N$  under lower  $g_s$ , there is no evidence that the difference in  $C_4$  biochemical subtype may be reflected on water physiology of  $C_4$  plants. Ghannoum et al. (2001) have also showed that PWUE and WUE in biomass production are similar in NAD-ME and NADP-ME grasses under well-watered conditions.

There are some reports on the geographical distribution of  $C_4$  subtype grasses for precipitation. In grasses occurring in South West Africa, NADP-ME grasses increase in abundance and NAD-ME grasses become less abundant with increasing mean annual precipitation, whereas PCK grasses show an intermediate pattern between them (Ellis et al., 1980; Schulze et al., 1996). On the other hand, Taub (2000) pointed out from a study on grasses in the United States that the correlations between the

relative species abundance of C<sub>4</sub> subtype grasses and annual precipitation gradients are solely due to the association of the C<sub>4</sub> subtypes with particular grass subfamilies. In our study, there were no significant difference in PWUE between different tribes of C<sub>4</sub> grasses (Supplemental data 1). Liu and Osborne (2015) have suggested that phylogenetic lineage, photosynthetic pathway, and adaptation to habitat water availability act together to influence the water-related physiological traits of C<sub>4</sub> grasses. It should be also considered that PWUE is an instantaneous indicator in leaf water physiology and may not represent water economy over the growth period of plants. Meanwhile, NAD-ME grasses increase their WUE under drought to a greater extent than NADP-ME grasses (Ghannoum et al., 2002). Our study investigated PWUE in plants grown under well-water conditions. The responses of the C<sub>4</sub> subtype grasses to drought stress still remain to be explored.

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No potential conflict of interest was reported by the author(s).

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## Author contributions

OU conceived, and YT and OU designed the study. YT conducted the experiments. OU and YT wrote and approved the manuscript.

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