Monthly variability in the photosynthetic capacities, leaf mass per area and leaf nitrogen contents of rice (*Oryza sativa* L.) plants and their correlations

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Abstract

The monthly variability in the photosynthetic capacities, leaf mass per area (LMA) and leaf nitrogen (N) contents of rice (*Oryza sativa* L.) plants is important for determining the growth conditions, estimating the biomass and yield, and developing a rice growth model. Moreover, the correlations among LMA, leaf N content, and photosynthetic capacity parameters, such as the maximum rate of carboxylation ($V_{\text{max}}$) and the maximum rate of electron transport ($J_{\text{max}}$), have not been fully investigated. The objectives of this study were (1) to analyze the monthly variability in $V_{\text{max}}$, $J_{\text{max}}$, LMA, and leaf N content; and (2) to investigate the correlations among $V_{\text{max}}$, $J_{\text{max}}$, LMA, leaf N content, and available N content in the soil. We measured the monthly changes in $V_{\text{max}}$ and $J_{\text{max}}$ in 120 leaves of two rice cultivars, Koshihikari and Akitakomachi, subjected to standard-N and non-N treatments using gas-exchange measurements under open-air field conditions. $V_{\text{max}}$ and $J_{\text{max}}$ values adjusted to a 25°C leaf temperature were significantly greater after the standard-N treatment compared with the non-N treatment in both cultivars, and the values of the photosynthetic capacities tended to decrease over time. In particular, these values significantly decreased at the ripening stage compared with the previous growth stages. The correlations among $V_{\text{max}}$ and $J_{\text{max}}$ at a 25°C leaf temperature, leaf N content based on mass and area, and SPAD readings were significant for both cultivars but were not correlated with LMA. As indicators of photosynthetic performance, the monthly variability in $V_{\text{max}}$ and $J_{\text{max}}$ for the fully expanded top or flag leaves were expressed easily as functions of the SPAD readings, rather than the leaf N contents based on mass and area, which are relatively expensive and labor intensive to measure. This research provides useful information for developing a universal rice growth model.

Key words: Maximum rate of carboxylation, Maximum rate of electron transport, Photosynthesis, Rice leaves

1. Introduction

Global rice (*Oryza sativa* L.) production is close to 500 million tons (USDA, 2018), and more than 90% of the world’s rice is grown and consumed in Asia. Between 1961 and 2014, the rice harvest area and rice production increased by 41% and 243%, respectively, but neither has changed significantly in the past five years (FAO, 2018). Nevertheless, ~35%–60% of the calories consumed by three billion people come from rice (Liu and Li, 2016). As the global population continues to swell, the depletion of available land is becoming a more serious problem; therefore, significant improvements in rice productivity must be made to achieve sustainable agricultural production. Leaf photosynthesis is an essential process for the formation of rice biomass and yield. To achieve the necessary rice yield improvements, it is important to evaluate rice’s photosynthetic capacity and its monthly variability as related to leaf mass per area (LMA) and leaf nitrogen (N) content based on leaf mass ($N_{\text{mass}}$) and leaf area ($N_{\text{area}}$).

The biochemical model based on Farquhar et al. (1980) is widely used to estimate the rate of leaf photosynthesis, and the main parameters in this model are the maximum rate of Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) carboxylation ($V_{\text{max}}$) and the maximum rate of photosynthetic electron transport ($J_{\text{max}}$). These two parameters, which are related to the maximum photosynthetic capacity, have strong correlations with the leaf N content because of the large allocations of leaf N to Rubisco, stomatal enzymes, and photosystem I and II (Makino, 2011; Taylaran et al., 2011). However, if the monthly changes in the photosynthetic model’s parameters and the light extinction coefficient of the crop in the vertical direction are unknown, then the amount of photosynthesis in the entire canopy cannot be properly obtained (Dennis et al., 2001). Therefore, it is necessary to investigate the monthly changes in $V_{\text{max}}$, $J_{\text{max}}$, LMA, and leaf N content, as well as their correlations.

Photosynthetic capacity and both LMA and leaf N content, which is also an important trait that is strongly correlated with leaf structural and biochemical traits, must vary according to the growth stage and available N content in the soil. Moreover, the

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photosynthetic capacity generally decreases from the vegetative to the reproductive stage owing to the leaf senescence (Makino et al., 1984). Some studies have investigated the dependencies of \( V_{\text{max}} \) and \( J_{\text{max}} \) on leaf biochemical (e.g., leaf N content) attributes (Hasegawa et al., 2016; Liu and Li., 2016; Xiong et al., 2016; Cai et al., 2018; Ikawa et al., 2018). Although several physiological factors are associated with variations in LMA and leaf N content, those associated with monthly variations in \( V_{\text{max}} \) and \( J_{\text{max}} \) have not been fully examined in rice. The specific objectives of this study were as follows: (1) to analyze the monthly variability in \( V_{\text{max}}, J_{\text{max}}, \) LMA, and leaf N content; and (2) to research the correlations among \( V_{\text{max}}, J_{\text{max}}, \) LMA, leaf N, and available N contents.

### 2. Materials and Methods

#### 2.1 Experimental site and plant materials

Certified seeds of two rice cultivars, *O. sativa* L. cv. Koshihikari and *O. sativa* L. cv. Akitakomachi, which were selected by treatment with a salt solution, were grown in plastic trays (pot diameter, 16 mm; pot height, 25 mm; pot number, 448/sheet) with three seedlings per pot in May (DOY 122) 2017 at the Field Museum Honmachi at the Tokyo University of Agriculture and Technology (139.47°E, 35.67°N), Japan. Each pot was filled with sterilized nursery soil (0.35:0.52:0.52 g, N:P:K, 5:5:5 kg/0.1 ha). Plants were grown in a plastic greenhouse and irrigated with sufficient water to avoid drought stress. After germination, the young seedlings were transplanted in the field on DOY 146 with 30 cm spacings between rows and 15 cm spacings between plants.

The experiment had a split-plot design, and two replications were conducted because the paddy field was relatively small (0.2 ha), and the soil quality was almost uniform throughout the paddy field. The dominant soil at this site is gray lowland soil, which is the typical soil of paddy fields in Japan, and the site has been in continuous rice cultivation for over 30 years. Each block contained four plots (plot size: 5 m \( \times \) 5 m) of Koshihikari and Akitakomachi under each of the two nitrogen (N) treatments. Two fertilizer treatments were administered, non-N fertilization (NN) and standard-N (SN), with N:P:K 5:5:5 kg/0.1 ha as the base manure and N:K 1.5:1.5 kg/0.1 ha as additional manure supplied 2 weeks before heading. Rice cultivars Koshihikari and Akitakomachi were harvested on 23 September (DOY 266) and 9 September (DOY 252). In all of the experimental plots, irrigation and weed and pest control were conducted according to the normal management practices in this area.

#### 2.2 Gas exchange measurements

Steady-state gas-exchange measurements were taken with a portable photosynthesis system (LI-6800, LI-COR, Lincoln, NE, USA) in 2017 using the youngest fully expanded leaves of rice plants in each plot at between 0900 and 1400 h to avoid afternoon stomatal closure. Prior to measurements, CO\(_2\) and H\(_2\)O concentrations were zeroed using infrared gas analyzer zero calibrations. Then, the leaf in the chamber was placed at the same value as the external photon flux density for several minutes. The photosynthetic photon flux density was then ramped up to 1,800 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) using a red-blue LED artificial light source, which can take 10–60 min. To minimize the difference between the leaf and external temperature and avoid sudden changes in the former, leaves were kept at 25°C, 30°C, and 35°C. The CO\(_2\) concentration in the leaf chamber and the leaf-to-air vapor pressure deficit were set to 400 \( \mu \text{mol mol}^{-1} \) and 1.2 ± 0.2 kPa, respectively. After the standard deviations of differences between the CO\(_2\) and H\(_2\)O concentrations into and out of the leaf chamber reached less than 1.0 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) and 0.1 mmol mol\(^{-1}\), respectively, over a 15 s time period, the CO\(_2\) concentration in the leaf chamber was changed to the following: 400, 300, 150, 50, 400, 500, 800, 1,200, 1,500, and 1,800 \( \mu \text{mol mol}^{-1} \) to measure the net assimilation rate, \( A \), in response to the CO\(_2\) concentration in intercellular spaces, \( C_i \), forming an \( A-C_i \) curve. After equilibration to a steady state, the data in the \( A-C_i \) curve were recorded and matched with infrared gas analyzers at every CO\(_2\) concentration. The \( A-C_i \) curves were measured in 66 leaves from Koshihikari (\( n = 11, 8, 8 \), and 6 in June, July, August, and September for both N treatments, respectively) and in 54 leaves from Akitakomachi (\( n = 11, 8, 8 \), and 8 in June, July, and August for both N treatments, respectively) sampled randomly from each block.

#### 2.3 Measurements of LMA, and leaf N and available N contents

Leaves were extracted from closed leaf chambers after gas-exchange measurements were taken, and then, the leaf relative chlorophyll content was estimated using a SPAD meter (SPAD-502 Plus; Konica Minolta, Inc., Tokyo, Japan). Next, 3–6 samples per leaf were collected using a leaf punch (8 mm; Fujirwara Scientific Company Co., Ltd., Tokyo, Japan), and these samples were oven-dried at 60°C for 1 week to achieve a constant weight. Next, the leaf dry mass was measured, and LMA was calculated using leaf dry mass and leaf area. \( N_{\text{mass}} \) was measured by dry combustion with a CN CORDER MT-700 (Yanaco Technical Science Co., Ltd., Tokyo, Japan), and \( N_{\text{mass}} \) was calculated by multiplying \( N_{\text{mass}} \) by LMA.

To measure the available N content in the soil, which represents the N that is available for plants and is highly correlated with leaf N uptake (Matsumoto, 2002), soil was collected at five points at 5–15 cm soil depths within each plot every 2 weeks, and then the collected soil samples were mixed for each plot. These soil samples were analyzed by Miraizo Co., Ltd., Oita, Japan (http://mirai-zou.co.jp/) to estimate the available N content.

#### 2.4 Biochemical modelling to estimate \( V_{\text{max}} \) and \( J_{\text{max}} \)

The biochemical photosynthetic model of Farquhar et al. (1980), modified by Sharkey et al. (2007) and Sharkey (2015) was used to estimate \( V_{\text{max}} \) and \( J_{\text{max}} \). \( A \) is calculated as the minimum of the Rubisco-limited rate of CO\(_2\) assimilation (\( A_r \)), ribulose 1,5-bisphosphate (RuBP) regeneration-limited rate of CO\(_2\) assimilation (\( A_j \)) or triose phosphate utilization (TPU)-limited rate of CO\(_2\) assimilation (\( A_d \)).

\[
A = V_{\text{max}} \left[ \frac{C_r - F_s}{C_r + K_c (1 + O/K_c)} \right] = R_d, \tag{1}
\]
\[ A_i = J - \frac{C_i - F*)}{4C_i + SF*} - R_d \]  
\[ A_i = 3TPU - R_d \]  
where \( V_{\text{max}} \) represents the maximum rate of carboxylation, \( C_c \) represents the chloroplast partial pressures of \( \text{CO}_2 \), \( F* \) represents the \( \text{CO}_2 \) compensation point in the absence of day respiration (\( R_d \)), and \( K_c \) and \( K_o \) are the Michaelis–Menten coefficients of Rubisco for the carboxylation and oxygenation reactions, respectively. \( O \) is the partial pressure of oxygen at Rubisco. \( J \) represents the photosystem II electron transport rate.

\( V_{\text{max}} \) was estimated by fitting the model to the measured \( A-C_i \) data for \( C_i < 200 \mu\text{mol mol}^{-1} \) as Rubisco-limited, and \( J_{\text{max}} \) was estimated using the \( A-C_i \) data for \( C_i > 300 \mu\text{mol mol}^{-1} \) as RuBP-regeneration limited, with the last \( C_i \) point as \( TPU \)-limited in accordance with Sharkey et al. (2007). In the present study, we assumed that the value of mesophyll conductance (\( g_m \)) is constant across the entire \( C_i \). Although this assumption is somewhat speculative, it is acceptable for estimating the monthly variability in \( V_{\text{max}} \) and \( J_{\text{max}} \) under two fertilizer treatments with the average \( g_m \), \( C_c \) is estimated by using Eqs. 1, 2, and 3 where \( C_c = (C_i - A/g_m) \). Using non-linear curve fitting to minimize the sum of squared model deviations from the \( A-C_i \) data, \( g_m \) was determined. Therefore, \( V_{\text{max}} \) and \( J_{\text{max}} \) are sensitive to the \( g_m \) value.

The temperature response for the Michaelis constants for carboxylation and oxygenation (\( K_c = 27.24 \text{ Pa} \) and \( K_o = 16.58 \text{ Pa} \) at 25°C, respectively), and the photorespiratory compensation point \( (F*) = 3.74 \text{ Pa} \) at 25°C), were corrected as described in Harley et al. (1992). In the present study, to analyze the monthly variability of the photosynthetic parameters and facilitate comparisons without considering temperature responses, \( V_{\text{max}} \) and \( J_{\text{max}} \) at a 25°C leaf temperature were estimated using the dependence of reaction rates on temperature. The temperature dependences of \( K_c, K_o, \), and \( F* \) were as follows:

\[ f(T_i) = f(298) \exp \left[ \left(1 - 298/T_i \right) H_i / 298 R \right], \]  
where \( f(T_i) \) represents a given parameter value at a \( T_i \) leaf temperature, \( f(298) \) represents the given parameter value at a 25°C (298 K) leaf temperature, \( H_i \) represents the enthalpy of activation, and \( R \) represents the gas constant.

In the present study, the temperature responses of \( V_{\text{max}} \) and \( J_{\text{max}} \) assumed to be unaffected by organic/inorganic environmental factors. \( V_{\text{max}} \) and \( J_{\text{max}} \) at a 25°C leaf temperature, \( V_{\text{max,25}} \) and \( J_{\text{max,25}} \) respectively, were estimated using the following equation (Harley et al., 1992):

\[ f(V_{\text{max,25}}, J_{\text{max,25}}) = \frac{f(V_{\text{max}}, J_{\text{max}})}{(1 + \exp \left[ \Delta S T_i - H_i / RT_i \right])} \exp \left( - H_i / RT_i \right), \]  
where \( V_{\text{max}}, J_{\text{max}}, T_i \), and \( \Delta S \) represent the values of \( V_{\text{max}} \) and \( J_{\text{max}} \) at leaf temperature \( T_i \), respectively. \( H_i \) represents the enthalpy of deactivation, \( c \) represents a scaling constant and \( \Delta S \) \( (0.65 \text{ K} \text{ mol}^{-1}) \) represents entropy. \( H_o, H_p, \) and \( c \) were obtained by fitting Eqs. 4 and 5 using a nonlinear least-squares regression (Table S1). The temperature responses of \( V_{\text{max}} \) and \( J_{\text{max}} \) fitted using Eqs. 4 and 5 are shown in Fig. S1.

2.5 Statistical analyses

All statistical analyses were performed using the SPSS software package [Statistical Package for the Social Sciences for Windows 12 (2014), Chicago, IL, USA]. Two-way ANOVA were used to test the main effects and factor interactions of the N treatment and month on photosynthesis capacity, LMA, and leaf N content. Results were assumed to be statistically significant when \( P < 0.05 \). A linear regression analysis was used to test for correlations between parameters and were significant at \( P < 0.05 \). In the present study, a statistical analysis of varietal differences in the correlation between photosynthetic capacity and leaf N content was not performed. Moreover, the ANCOVA with an \( N_{\text{max}} \) covariate was used in examining the differences in the mean values of \( V_{\text{max}} \) and \( J_{\text{max}} \) that are related to the effect of month while taking into account the influence of an \( N_{\text{max}} \).

3. Results

3.1 Correlations among LMA, and leaf N and available N contents

Table 1 shows the monthly values of available N content, LMA, \( N_{\text{max}} \), and \( N_{\text{max}} \) as well as SPAD readings for both cultivars. There was no significant correlation between LMA and available N content in either cultivar (\( R = 0.25, P = 0.35 > 0.05 \) for Koshihikari; \( R = 0.20, P = 0.53 > 0.05 \) for Akitakomachi). When data from June, July, and August were used, the correlation between \( N_{\text{max}} \) and available N content was statistically significant for both cultivars (\( R = 0.87, P < 0.05 \) for Koshihikari; \( R = 0.90, P < 0.05 \) for Akitakomachi). However, there was no significant correlation when all of the data, including September, were used (\( R = 0.17, P = 0.51 > 0.05 \) for Koshihikari). Similarly, \( N_{\text{max}} \) and available N content were statistically significant (\( R = 0.85, P < 0.05 \) for Koshihikari; \( R = 0.90, P < 0.05 \) for Akitakomachi), except in data from September. SPAD readings and available N content were also statistically significant (\( R = 0.87, P < 0.05 \) for Koshihikari; \( R = 0.82, P < 0.05 \) for Akitakomachi), except in data from September. Moreover, the correlation between LMA and SPAD readings when all of the data used was statistically significant (\( R = 0.59, P < 0.05 \) for Koshihikari; \( R = 0.54, P < 0.05 \) for Akitakomachi). The correlation between \( N_{\text{max}} \) and SPAD readings was statistically significant (\( R = 0.92, P < 0.05 \) for Koshihikari; \( R = 0.79, P < 0.05 \) for Akitakomachi), and the correlation between \( N_{\text{max}} \) and SPAD readings was also statistically significant (\( R = 0.92, P < 0.05 \) for Koshihikari; \( R = 0.91, P < 0.05 \) for Akitakomachi).

3.2 Effect of N treatment and month on LMA and leaf N content

The two-way ANOVA showed that there was non-N treatment-by-month interaction for LMA, but there were significant month-dependent LMA differences for both cultivars (Table 1). For Akitakomachi, LMA was significantly greater under NN conditions than under SN conditions. Furthermore, LMA under NN conditions for Koshihikari tended to be greater than under SN conditions but the difference was not significant (\( P = 0.20 > 0.05 \)). The LMA for Koshihikari in September was significantly greater compared with that in other months, and the LMA for Akitakomachi in August was significantly greater than that in July. Regardless of the N treatment, LMA values during the ripening stage, which was roughly equivalent to September for Koshihikari and August for Akitakomachi, were greater than
Table 1. Monthly variability of the available nitrogen content in the soil (available N), leaf mass area (LMA), nitrogen content based on leaf area (N\text{area}), SPAD readings, and mesophyll conductance (g\text{m}) under two N conditions. Data are presented as means ± SDs of total leaves for each plot with two replications. Data followed by the same letters in the same row are not significantly different among months. ** and *** indicate significance at the 0.05 and 0.01 levels, respectively. n.s. indicates no significance at the 0.05 level. Multiple comparisons were conducted using Tukey’s HSD test.

| Variable          | Cultivar | Treatment | June     | July     | August   | September |
|-------------------|----------|-----------|----------|----------|----------|-----------|
| Available N       | Koshihikari | NN        | 6.42 ± 1.1 | 5.66 ± 0.1 | 4.43 ± 0.3 | 6.76 ± 0.5 |
|                   |          | SN        | 6.75 ± 1.1 | 5.68 ± 0.5 | 4.64 ± 0.4 | 6.92 ± 0.1 |
|                   | Akitakomachi | NN        | 6.50 ± 0.7 | 6.03 ± 0.5 | 5.24 ± 1.0 | —         |
|                   |          | SN        | 7.07 ± 1.0 | 6.46 ± 0.8 | 5.34 ± 0.6 | —         |
| LMA (g m\text{−2}) | Koshihikari | NN        | 51.0 ± 7.0 | 47.5 ± 3.5 | 51.7 ± 5.8 | 65.2 ± 3.9 |
|                   |          | SN        | 50.8 ± 6.3 | 45.8 ± 1.5 | 51.5 ± 6.3 | 62.9 ± 2.1 |
|                   |          | Mean      | 50.9 ± 6.5 a | 46.7 ± 2.7 a | 51.6 ± 5.4 a | 64.5 ± 3.1 b |
|                   | Akitakomachi | NN        | 50.2 ± 6.8 | 48.0 ± 4.3 | 56.7 ± 8.6 | —         |
|                   |          | SN        | 46.8 ± 6.2 | 44.3 ± 4.4 | 53.0 ± 4.9 | —         |
|                   |          | Mean      | 48.5 ± 6.3 ab | 46.1 ± 4.5 b | 54.7 ± 6.7 a | —         |
| N\text{area} (%)  | Koshihikari | NN        | 3.95 ± 0.41 | 3.21 ± 0.49 | 2.55 ± 0.36 | 1.36 ± 0.10 |
|                   |          | SN        | 4.47 ± 0.74 | 3.40 ± 0.60 | 2.94 ± 0.35 | 1.44 ± 0.19 |
|                   |          | Mean      | 4.23 ± 0.64 a | 3.31 ± 0.52 b | 2.75 ± 0.36 c | 1.40 ± 0.14 d |
|                   | Akitakomachi | NN        | 3.51 ± 0.41 | 3.05 ± 0.33 | 2.25 ± 0.33 | —         |
|                   |          | SN        | 4.17 ± 0.41 | 3.47 ± 0.52 | 2.54 ± 0.27 | —         |
|                   |          | Mean      | 3.81 ± 0.41 a | 3.26 ± 0.47 b | 2.40 ± 0.31 c | —         |
| SPAD (−)          | Koshihikari | NN        | 2.01 ± 0.26 | 1.52 ± 0.23 | 1.32 ± 0.54 | 0.89 ± 0.14 |
|                   |          | SN        | 2.27 ± 0.39 | 1.56 ± 0.35 | 1.51 ± 0.15 | 0.91 ± 0.11 |
|                   |          | Mean      | 2.15 ± 0.36 a | 1.54 ± 0.28 b | 1.42 ± 0.34 b | 0.90 ± 0.12 c |
| g\text{m} (mol m\text{−2}s\text{−1}) | Koshihikari | NN        | 0.158 ± 0.021 | 0.126 ± 0.028 | 0.128 ± 0.016 | 0.073 ± 0.008 |
|                   |          | SN        | 0.162 ± 0.014 | 0.129 ± 0.020 | 0.133 ± 0.025 | 0.086 ± 0.010 |
|                   |          | Mean      | 0.160 ± 0.018 a | 0.127 ± 0.025 b | 0.130 ± 0.020 b | 0.079 ± 0.009 c |
|                   | Akitakomachi | NN        | 0.164 ± 0.016 | 0.132 ± 0.023 | 0.134 ± 0.019 | —         |
|                   |          | SN        | 0.171 ± 0.009 | 0.137 ± 0.015 | 0.135 ± 0.021 | —         |
|                   |          | Mean      | 0.168 ± 0.013 a | 0.135 ± 0.019 b | 0.135 ± 0.020 b | —         |
thereof of the previous growth stage (Table 1).

There was non-N treatment-by-month interaction for N\textsubscript{mass} for either cultivar, but the N\textsubscript{mass} was significantly increased under SN conditions for both cultivars. The N\textsubscript{mass} values for both cultivars were significantly different among months, and they substantially decreased as the growth stages advanced. Furthermore, for the N\textsubscript{mass} values of both cultivars, non-N treatment-by-month interaction was detected. There were significant monthly differences in N\textsubscript{mass} for both cultivars, and N\textsubscript{mass} values decreased as the growth stages progressed. N\textsubscript{mass} and N\textsubscript{area} were significantly greater under SN conditions compared with under NN conditions for both cultivars (Table 1).

Non-N treatment-by-month interaction was detected on the SPAD readings of both cultivars. SPAD readings were significantly greater under SN conditions than under NN conditions. Moreover, SPAD readings were significantly different among months, and they substantially decreased as the growth stages proceeded (Table 1).

3.3 Monthly variability of V\textsubscript{cmax} and J\textsubscript{max}

The correlation between A and C\textsubscript{i} was used to assess the biochemical limitations of photosynthesis in each month and under two N treatments. A two-way ANOVA was conducted to determine whether the N treatment and month independently affected rice photosynthetic capacity parameters and whether they are mutually influential.

In the present study, there were non-N treatment-by-month interaction effects on V\textsubscript{cmax,25} and J\textsubscript{max,25} according to the ANOVA (Fig. 1a and 1b). However, individual N treatment and month effects on V\textsubscript{cmax,25} and J\textsubscript{max,25} were statistically significant for both cultivars. For Koshihikari, V\textsubscript{cmax,25} and J\textsubscript{max,25} were significantly different among the months, except for between July and August, and for Akitakomachi, there were significant differences among months, except for V\textsubscript{cmax,25} and J\textsubscript{max,25} between July and August. V\textsubscript{cmax,25} and J\textsubscript{max,25} were significantly greater under SN conditions compared with under NN conditions for both cultivars, and the values of these parameters generally tended to decrease over time. For Koshihikari, the V\textsubscript{cmax,25} decreased 70% under SN conditions and 75% under NN conditions, and the J\textsubscript{max,25} decreased 70% under SN conditions and 68% under NN conditions from June to September. For Akitakomachi, the V\textsubscript{cmax,25} decreased 41% under SN conditions and 39% under NN conditions, and the J\textsubscript{max,25} decreased 44% under SN conditions and 34% under NN conditions from June to August.

Fig. 1. Monthly variability in (a) maximum rate of carboxylation (V\textsubscript{cmax,25}) and (b) maximum rate of electron transport (J\textsubscript{max,25}) adjusted to a 25°C leaf temperature under non-nitrogen (NN) and standard nitrogen (SN) conditions for the two rice cultivars Koshihikari and Akitakomachi. The data are presented as means ± SDs of the total leaves from each plot with two replications. Data followed by the same lowercase and capital letters indicate no significant differences among months for cultivars Koshihikari and Akitakomachi, respectively, at the 0.05 level. ** and *** indicate significant differences at the 0.05 and 0.01 levels, respectively. n.s. indicates not significant at the 0.05 level. Multiple comparisons were conducted using Tukey’s HSD test.
An ANCOVA with an N<sub>area</sub> covariate (P < 0.05), revealed that <i>V</i><sub>max,25</sub> values in June for both cultivars were significantly greater than in other months. Similarly, <i>J</i><sub>max</sub> values for both cultivars were significantly greater in June than in other months (Table 2).

### 3.4 Correlations among <i>V</i><sub>max</sub>, <i>J</i><sub>max</sub>, LMA, and leaf N content

The correlation coefficients between <i>V</i><sub>max,25</sub> and LMA, N<sub>area</sub>, N<sub>mass</sub>, and SPAD readings were −0.32 (P < 0.05), 0.84 (P < 0.05), 0.83 (P < 0.05), and 0.84 (P < 0.05), respectively, for Koshihikari and −0.41 (P < 0.05), 0.81 (P < 0.05), 0.72 (P < 0.05), and 0.77 (P < 0.05), respectively, for Akitakomachi (Fig. 2a, 2b, 2c and 2d). Furthermore, the correlation coefficients between <i>J</i><sub>max,25</sub> and LMA, N<sub>area</sub>, N<sub>mass</sub>, and SPAD were −0.30 (P < 0.05), 0.81 (P < 0.05), 0.85 (P < 0.05), and 0.80 (P < 0.05), respectively, for Koshihikari and −0.20 (P = 0.149 > 0.05), 0.81 (P < 0.05), 0.80 (P < 0.05), and 0.85 (P < 0.05), respectively, for Akitakomachi (Fig. 3a, 3b, 3c and 3d).

The <i>J</i><sub>max,25</sub>/<i>V</i><sub>max,25</sub> ratios in September for Koshihikari and in August for Akitakomachi were significantly greater than in other months, except between June and August for Akitakomachi (Table 3).

### 4. Discussion

#### 4.1 Monthly variability in LMA and leaf N content

Leaf N content in rice plants can substantially affect the leaf’s anatomical structures and functions (Lee et al., 2011; Xiong et al., 2015, 2016). When there is a deficiency in the leaf N content, rice growth is generally severely inhibited, which can lead to the accumulation of non-structural carbohydrates in leaves and a subsequent increase in LMA (Pan et al., 2011). LMA values in July, which was roughly equivalent to the tillering stage, were lower than in other months for both cultivars. Generally, the size of chloroplast within mesophyll cells potentially increases LMA during N supplementation because chloroplasts are much denser than vacuoles (Pooter et al., 2009; Xiong et al., 2015). However, an increase in cell size during leaf expansion is usually accompanied by increases in the size/area of the vacuole and intercellular air space, which potentially decrease leaf density and LMA (Volkenburgh, 1999). Therefore, LMA values were lower in expanding leaves during both N treatments (Table 1), and the effect of an increased LMA, caused by the

### Table 2. Analysis of covariance with a leaf N content based on an N<sub>area</sub> covariate. Data are presented as means ± SDs of total leaves for each plot, with two replications. Data in the same column followed by the same letters were not significantly different among months (P < 0.05).

| Month   | V<sub>max,25</sub> | J<sub>max,25</sub> |
|---------|------------------|------------------|
|         | Koshihikari      | Akitakomachi     | Koshihikari    | Akitakomachi   |
| June    | 170.1 ± 36.4a    | 169.3 ± 39.0a    | 181.1 ± 33.4a  | 166.8 ± 25.1a  |
| July    | 91.4 ± 23.5b     | 115.2 ± 30.8b    | 90.0 ± 18.7b   | 102.3 ± 14.5b  |
| August  | 102.5 ± 17.8b    | 100.7 ± 17.1b    | 103.6 ± 3.7b   | 101.0 ± 6.7b   |
| September | 47.0 ± 20.4c  | –                | 56.3 ± 13.1c   | –              |

**Fig. 2.** Correlations between (a) leaf mass per area (LMA), (b) nitrogen content based on leaf mass (N<sub>mass</sub>), (c) nitrogen content based on leaf area (N<sub>area</sub>), and (d) SPAD readings and the maximum rate of carboxylation (<i>V</i><sub>max,25</sub>) adjusted to a 25°C leaf temperature for the two rice cultivars Koshihikari and Akitakomachi. Cross points and the solid line represent Koshihikari, and filled squares and the dotted line represent Akitakomachi. ** indicates significance at the 0.05 levels. n.s. indicates no significance at the 0.05 level. The lines represent the linear regressions for all data. For Koshihikari, <i>V</i><sub>max,25</sub> = −1.95LMA + 211.90; <i>V</i><sub>max,25</sub> = 42.66N<sub>mass</sub> + 20.67; <i>V</i><sub>max,25</sub> = 91.19N<sub>area</sub> − 32.64; <i>V</i><sub>max,25</sub> = −5.48SPAD − 77.13. For Akitakomachi, <i>V</i><sub>max,25</sub> = −2.70LMA + 265.52; <i>V</i><sub>max,25</sub> = 45.57N<sub>mass</sub> − 16.36; <i>V</i><sub>max,25</sub> = 81.38N<sub>area</sub> − 4.43; <i>V</i><sub>max,25</sub> = 6.64SPAD − 110.80.
accumulation of non-structural carbohydrates in leaves, might be offset by increases in the size/area of the vacuole and intercellular air space. Therefore, LMA values of expanding leaves were relatively lower than those of expanded leaves, which resulted in a relatively greater decrease in the percentage of N\textsubscript{mass} from June to July. Thus, determining the variations in LMA responses includes assessing the leaf’s developmental stage, chloroplast size and width, cell wall components, non-structural carbohydrates in leaves, and plant cultivar.

N\textsubscript{mass} decreased as the growth stage progressed (Table 1). A similar trend was observed for N\textsubscript{area}. These results corroborate those of Sheehy et al. (1998). The declines of N\textsubscript{mass} and N\textsubscript{area} with increasing biomass can probably be attributed to a decrease in the fraction of total plant N content associated with photosynthesis in relation to the concomitant increase in the N fraction used for structural and storage constituents, as shown in Bélanger and Gastal (2000). The leaf N content was significantly correlated with the available N content, except for data in September for Koshihikari and August for Akitakomachi, which suggested that the leaf N content is determined mainly by the available N content, except during the ripening stage. The leaf N content can be estimated accurately using SPAD readings.

![Graphs showing correlations between LMA, N\textsubscript{mass}, N\textsubscript{area}, and SPAD readings.](image)

**Fig. 3.** Correlations between (a) leaf mass per area (LMA), (b) nitrogen content based on leaf mass (N\textsubscript{mass}), (c) nitrogen content based on leaf area (N\textsubscript{area}), and (d) SPAD readings and the maximum rate of electron transport (J\textsubscript{max}) adjusted to a 25°C leaf temperature for the two rice cultivars Koshihikari and Akitakomachi. Cross points and the solid line represent Koshihikari, and filled squares and the dotted line represent Akitakomachi. ** indicates significance at the 0.05 level. n.s. indicates no significance at the 0.05 level. The lines represent linear regressions for all data. For Koshihikari, J\textsubscript{max,25} = -1.80LMA + 213.56; J\textsubscript{max,25} = 41.57N\textsubscript{mass} - 9.71; J\textsubscript{max,25} = 95.57N\textsubscript{area} - 31.93; J\textsubscript{max,25} = -5.30SPAD - 63.46. For Akitakomachi, J\textsubscript{max,25} = -1.24LMA + 188.84; J\textsubscript{max,25} = 42.66N\textsubscript{mass} - 10.69; J\textsubscript{max,25} = 83.89N\textsubscript{area} - 3.23; J\textsubscript{max,25} = 6.89SPAD - 123.66.

**Table 3.** J\textsubscript{max,25}/V\textsubscript{cmax,25} ratios of the rice leaves for each month under two N conditions. Data are presented as means ± SDs of total leaves for each plot with two replications. Data followed by the same letters in the same row are not significantly different among months. ** indicates significance at the 0.05. n.s. indicates no significance at the 0.05 level. Multiple comparisons were conducted using Tukey’s HSD test.

| Cultivar       | Treatment | June   | July   | August  | September |
|----------------|-----------|--------|--------|---------|-----------|
| Koshihikari    | NN        | 1.03 ± 0.18 | 1.00 ± 0.16 | 1.07 ± 0.21 | 1.30 ± 0.23 |
|                | SN        | 1.09 ± 0.33 | 0.97 ± 0.25 | 0.96 ± 0.29 | 1.12 ± 0.18 |
|                | Mean      | 1.06 ± 0.26 | 0.99 ± 0.21 | 1.02 ± 0.25 | 1.22 ± 0.21 |
|                | Treatment | n.s.   |        |         |           |
|                | Month     |        | **     |         |           |
|                | Treatment × Month | n.s.   |        |         |           |
| Akitakomachi   | NN        | 0.99 ± 0.22 | 1.00 ± 0.09 | 1.07 ± 0.09 | –         |
|                | SN        | 0.98 ± 0.17 | 0.80 ± 0.22 | 0.94 ± 0.05 | –         |
|                | Mean      | 0.99 ± 0.20 | 0.90 ± 0.17 | 1.01 ± 0.07 | –         |
|                | Treatment |        | **     |         |           |
|                | Month     |        | **     |         |           |
|                | Treatment × Month | n.s.   |        |         |           |
Moreover, the monthly variability of the leaf N content, owing to leaf N content accumulation, recycling, and translocation, reflected the rice growth phases and leaf age. In the present study, a comprehensive analysis that considered variables such as leaf N content, available N content, photosynthetically active radiation, air temperature, and water temperature was not performed. A future work will focus mainly on the effects of climate, water, fertilization and leaf age on the monthly changes in the leaf N content.

4.2 Monthly changes in $V_{\text{cmax}}$ and $J_{\text{max}}$

The $V_{\text{cmax}}$ and $J_{\text{max}}$ values indicated that there were monthly variations in both cultivars. The greatest $V_{\text{cmax}}$25 and $J_{\text{max}}$25 values occurred in June, which is the initial vegetative stage, and declined progressively during the growing season, and these values were lowest during the ripening stage (Fig. 1). The available N content was significantly decreased from July to August; therefore, the leaf N content and SPAD readings were also significantly decreased. However, the suppression of the inactive Rubisco was not performed. A future work will focus mainly on the effects of climate, water, fertilization and leaf age on the monthly changes in the leaf N content.

Thus, the suppression of the inactive Rubisco was significantly decreased compared with during other stages, but its value for Akitakomachi was a bit unclear (Table 1). Therefore, relatively great decrease in $V_{\text{cmax}}$25 attributed to $g_{\text{m}}$ and flag leaves of rice have lower $V_{\text{cmax}}$ per unit Rubisco values than younger leaves (Seneweera et al., 2002). In the present study, $g_{\text{m}}$ for Koshihikari during the ripening stage was significantly decreased compared with during other stages, but its value for Akitakomachi was a bit unclear (Table 1). Therefore, relatively great decrease in $V_{\text{cmax}}$25 attributed to $g_{\text{m}}$ and flag leaves caused the greater $J_{\text{max}}$25/$V_{\text{cmax}}$25 ratio during the ripening stage. Moreover, the slope of the regression of $J_{\text{max}}$ to $V_{\text{cmax}}$ must be attributable to the variations in N reallocation (Borjigidai et al., 2006), the internal conductance to CO$_2$ diffusion (von Caemmerer, 2000), and the sensitivity of stomata to vapor pressure deficits (Medlyn et al., 2002). However, in the present study, it was not possible to determine these complex effects under open-air field conditions.

LMA strongly affects leaf photosynthesis, and variations in LMA are mainly caused by changes in leaf density (Pooter et al., 2009; Xiong et al., 2016). In the present study, we observed that the correlations between LMA and both $V_{\text{cmax}}$25 and $J_{\text{max}}$25 were significant except for $J_{\text{max}}$25 of Akitakomachi (Figs. 2a and 3a). This is likely related to chemical composition and leaf anatomical traits. Understanding the response of LMA to the growth environment is very important for improving the photosynthetic capabilities of leaves. Further research should be conducted on the influences of structural components such as the amount of intercellular air space, and chemical components such as non-structural carbohydrates, on LMA in rice plants. The effects of leaf age, the causes of LMA variation, and the influence on leaf N content must also be taken into account.

The monthly variability in LMA, leaf N, $V_{\text{cmax}}$ and $J_{\text{max}}$, and their correlations at the leaf level need to be determined to accurately understand the growth conditions and estimate rice yields, and also to develop a universal rice growth model. However, to collect the photosynthetic capacity parameters, such as $V_{\text{cmax}}$ and $J_{\text{max}}$, is expensive and labor intensive; therefore, it is desirable to accurately estimate these parameters cheaply and easily. In this study, SPAD readings, $V_{\text{cmax}}$, and $J_{\text{max}}$ were shown to be strongly correlated. Therefore, the monthly changes in $V_{\text{cmax}}$ and $J_{\text{max}}$ at the top or flag leaf must be expressed as a function of the SPAD readings during the growing season. Furthermore, determining the detailed correlation between SPAD readings and the photosynthetic capability at the canopy level, which would decay vertically downward, is important for evaluating yield potential over a wide area.

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