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Contribution of leaf nitrogen to photosynthetic gas exchange in contrasting rice (*Oryza sativa* L.) cultivars during the grain-filling period

S. SHIMODA

National Agriculture and Food Research Organization, Western Region Agricultural Research Center (NARO/WARC), 6-12-1 Nishifukastu, Fukuyama, Hiroshima 721-8514, Japan

Abstract

Photosynthetic parameters and leaf carbon isotope composition ($\delta^{13}C$) in contrasting rice genotypes in relation to supplemental nitrogen (N) application and water management during the grain-filling period were compared. The changes in stomatal conductance (g_s) and ratio of intercellular to ambient CO_2 mole fraction (C_i/C_a) depended on the leaf nitrogen concentration (leaf N) in both 'Hinohikari' (temperate *japonica* genotype) and 'IR36' (*indica* genotype). In 'Hinohikari', $\delta^{13}C$ reflects photosynthetic gas exchange during the grain-filling period, which is indicated by the significant response of $\delta^{13}C$ to leaf N. In contrast, in 'IR36' $\delta^{13}C$ did not depend on leaf N. This varietal difference in $\delta^{13}C$ to leaf N can be attributed to a difference in the timing of leaf senescence. In 'IR36', leaf N and photosynthetic parameters decreased more rapidly, indicating earlier senescence and a shorter grain-filling period in comparison with 'Hinohikari'. The significant increase in shoot dry mass in 'Hinohikari' resulting from supplemental N application, compared with nonsignificant effect observed in 'IR36', suggests that the timing of senescence in relation to the grain-filling period has a preponderant influence on productivity.

Additional key words: carbon discrimination; nitrogen supply; photosynthetic CO_2 assimilation; stomatal conductance.

Introduction

Application of N fertilizers before and during the grain-filling stage is well known to improve grain yield (Dingkuhn *et al.* 1991a), and the characteristics of leaf photosynthetic assimilates in response to N application have been studied in different rice genotypes, but N-use efficiency and final grain yield have received the most attention (Mae 1997, Nagata *et al.* 2001). The photosynthetic activity response to increased leaf N content has been investigated in different rice varieties (Kuroda and Kumura 1990, Sasaki and Ishii 1992), but photosynthetic parameters during the ripening period have received little attention, even though plant responses have been demonstrated to vary depending on the phenological stage.

During rice cultivation, N fertilizer is commonly applied twice, early application, during field preparation, as a basal dressing, and later application, at the reproductive stage, as supplemental fertilizer. The application of supplemental N fertilizer after the crop generally improves the percentage of ripened grains and increases grain yield in rice (Mae 1997). Supplemental N fertilizer applied shortly before heading increases leaf N content without increasing leaf area, thus enhancing photosynthetic activity during the ripening period. In rice, enhanced photosynthetic activity during the grain-filling period contributes most the final carbon content of the mature grain (Yoshida 1981, Peng *et al.* 2004). Although a number of researchers have compared cultivar differences in photosynthetic capacity in rice, little attention has been paid to the N level and few have focused on the effects of supplemental N application on photosynthetic rate during the grain-filling stage (Hossain *et al.* 2007). Recently, Hirasawa *et al.* (2010) showed that different rates of supplemental N application shortly before heading result in differences in the photosynthetic rate among selected varieties of rice despite identical levels of leaf N. To determine how photosynthetic traits

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Phone: +81-84-923-4100, fax: +81-84-924-7893, e-mail: sss@affrc.go.jp

Abbreviations: C_i/C_a – ratio of intercellular to ambient CO_2 mole fraction; D – dry treatment; DAT – days after transplanting; DM – dry mass; g_s – stomatal conductance; leaf N – leaf nitrogen concentration; P_N – net photosynthetic rate; SWC – volumetric soil water content; W – wet treatment; $\delta^{13}C$ – carbon isotope composition.

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vary among different cultivars, it is necessary to compare the responses to leaf N changes among the cultivars.

Observations of $\delta^{13}C$ are useful as an alternative tool for evaluating the impact of water stress on crop performance and for estimation of water-use efficiency (Pieters and Núñez 2008). Although the use of $\delta^{13}C$ as an indicator of the physiological response to variation in water availability has been proposed, the interaction of this effect with the N regime remains unclear in various crop species. Even though previous studies have predicted that $\delta^{13}C$ would increase with higher leaf N, in fact, no change or a decrease in $\delta^{13}C$ after N application has been demonstrated in wheat (*e.g.*, Cabrera-Bosquet *et al.* 2007) and rice (Kondo *et al.* 2004). In rice, $\delta^{13}C$ increased with increases in basal N, and it decreased when the water deficit intensity increased (Scartazza *et al.* 1998, Kondo *et al.* 2004), indicating a strong influence of N application at the early growth stage. Water status is an important environmental constraint that influences all physiological processes involved in plant growth and development. Early drainage, intended to improve the operating efficiency of large harvesting machines, has been introduced recently, but it often reduces grain quality and yield during hot summers in Japan (Morita 2008, Shimoda 2011). Therefore a better understanding of the effect of water status on photosynthesis during the ripening period in rice is needed. Moreover, the combined effect of water deficits and low N on $\delta^{13}C$ during the ripening period has not been assessed, even though environmental conditions during ripening are known to affect rice production.

The aim of this study was to clarify differences in leaf gas exchange in contrasting rice varieties in response to supplemental N application and water status. The goal of N application in this study was to improve photosynthetic activity during the ripening period, which may lead to different results in comparison with previous basal N control experiments.

Materials and methods

An irrigated paddy field with Gray Lowland soil at the National Agricultural Research Center for the Western Region in Fukuyama, Hiroshima, Japan (34°30′N, 133°23′E; 2 m a. s. l.) was used as the experimental site. The mean annual temperature in 2009 was 15.5°C at this site. Water-soaked seeds of 'Hinohikari' (temperate *japonica*) and 'IR36' (*indica*) were sown on 11 May 2009. 'Hinohikari' is currently the leading cultivar in western Japan. 'IR36' is a high-yield rice variety that is representative of the *indica* subspecies. Three weeks after the sowing, on 4 June, the young shoots were transplanted, three to a hill. Hills were spaced 0.2 m apart and rows 0.3 m apart in plots of four rows of nine hills each. Basal fertilizer was applied to the field at 4 g(N) m^{-2}, 6 g(P_2O_5) m^{-2}, and 4 g(K_2O) m^{-2} before the shoots were transplanted. Rice plants (36 hills) of each cultivar were grown either without (treatment 0) or with supplemental N fertilization [4 g(N) m^{-2}; treatment 4] in each of two concrete block frames (1.8 m by 22.4 m; two cultivars by two N top dressing treatments per frame) maintained under different water management treatments. The dry treatment (D) frame was drained on 14 August, whereas the soil in the wet treatment (W) frame was kept submerged until plant maturity. Thus, each cultivar was grown under four treatment regimes (W0, W4, D0, and D4), and a total of 144 hills of each cultivar were used for each experiment. Four replicates of each experiment were performed in eight adjacent frames (one pair of frames per experiment). The usual practice in western Japan is to apply supplemental N fertilization twice, at about 25 and 10 d before heading. The earlier supplemental N fertilization affects primarily the number of panicles and the canopy structure. In this study, supplemental N fertilization was applied only once, on 10 August, about 10 d before heading, so that these morphological traits would not be changed by the supplemental N application. 'Hinohikari' and 'IR36' headed on 22 and 18 August 2009, at 79 and 75 d after transplanting (DAT), respectively. To determine yield and shoot dry mass, 144 hills of each experiment were harvested at maturity (2 October). The harvested plants were separated into panicles and non-panicle parts, which were then dried at 70°C to a constant mass. The recorded yield was adjusted to a grain water content of 15%.

Fig. 1 shows rainfall and soil water content during the rice-growing season in 2009. Volumetric soil water content was measured with time domain reflectometers (*TDR CS615, Campbell Scientific*, Logan, UT, USA). Two sensors were inserted at 0–0.10 m depth, and measurements were recorded at 1-h intervals by a data logger (*CR23X, Campbell Scientific*). Precipitation was measured by a rain gauge (*B-011–00, Yokogawa*, Tokyo, Japan) near the paddy field.

Leaf gas-exchange measurements were performed on fully expanded topmost and second leaves using a portable photosynthesis system (*LI-6400, Li-COR Inc.*, Lincoln, NE, USA). Gathered data included net photosynthetic rate (P_N), g_s and C_i/C_a. Two leaves from each of four different plants (three plants on 26 August) of the four treatment regimes W0, W4, D0, and D4 (only W0 and W4 on 14 August) of each cultivar were measured between 9:00 h and 13:00 h of the local time under a constant saturated light level of 1,000 μmol m^{-2} s^{-1} provided by red/blue light-emitting diodes on clear days. The leaf chamber temperature was 30.7 ± 0.2°C, the reference CO_2 concentration was maintained at 380 μmol mol^{-1}, and the relative humidity was 60 ± 10%. Gas-exchange parameters were recorded as soon as the topmost expanded leaf was enclosed in the chamber and the system software indicated that CO_2, H_2O, and air flow in the chamber had stabilized. After the measurements, the leaves were harvested and their δ^{13}C and N content were determined as described below.

The harvested leaves were oven-dried at 80°C for at least 48 h and then ground into fine powder in a vibration mill (*Heiko Co. Ltd*., Tokyo, Japan) for the analysis of their leaf N and δ^{13}C. Approximately 2 mg of leaf sample were weighed in a tin cup and combusted in an elemental analyzer (*Flash EA 1112, ThermoFinnigan Co*., Bremen, Germany) coupled to an isotope ratio mass spectrometer (*Delta Plus Advantage, ThermoFinnigan Co*., Bremen, Germany) by a *Conflo III* interface.

One-way analysis of variance (*ANOVA*) was used to evaluate the effects of N application and drainage on δ^{13}C, gas exchange, and the yield component of each cultivar. *Pearson*'s correlation test was used to assess the statistical significance of the correlation coefficients. Statistical analyses were conducted with R statistical software (*R version 2.10.1, R Foundation for Statistical Computing*, Vienna, Austria).

Results

After the dry treatment frames were drained, soil volumetric water content within them decreased markedly from 0.36 to 0.20 (Fig. 1), but it recovered to above 0.30 m^3 m^{-3} after precipitation events on 12 and 30 September. Leaf N was similar in both 'Hinohikari' and 'IR36' before supplemental N (Fig. 2). In the regimes without supplemental N (D0 and W0), leaf N from 73 to 91 DAT was constant at about 1.7% in 'Hinohikari' and decreased to 1.3% in 'IR36'. In the supplemental N regimes (D4 and W4), leaf N increased after N application. Drainage was performed 4 d after supplemental N application; leaf N in the early drainage regimes (D0 and D4) rapidly decreased as ripening progressed and approached the D4 values.

P_N decreased more rapidly in 'IR36' than in 'Hinohikari' with ripening (Fig. 3*A*,*B*). In 'IR36' in the D0 and D4 regimes, P_N decreased from 18.0 to 5.2 µmol(CO_2) m^{-2} s^{-1} and from 17.8 to 6.2 µmol(CO_2) m^{-2} s^{-1}, respectively, from 73 to 109 DAT. g_s was higher in 'IR36' than in 'Hinohikari' immediately after supplemental N application (Fig. 3*C*,*D*). g_s was lower in 'IR36' than in 'Hinohikari' from 99 DAT. In 'IR36', g_s was larger in wet regimes than in dry regimes at 91 and 109 DAT, and showed no clear response to N application at 83 DAT. Most values of g_s at 99 DAT were lower than 0.15 mol(H_2O) m^{-2} s^{-1}, but g_s was higher than 0.20 mol(H_2O) m^{-2} s^{-1} in the W4 regime in both cultivars. C_i/C_a ranged from 0.64 to 0.79 in 'Hinohikari' and from 0.61 to 0.83 in

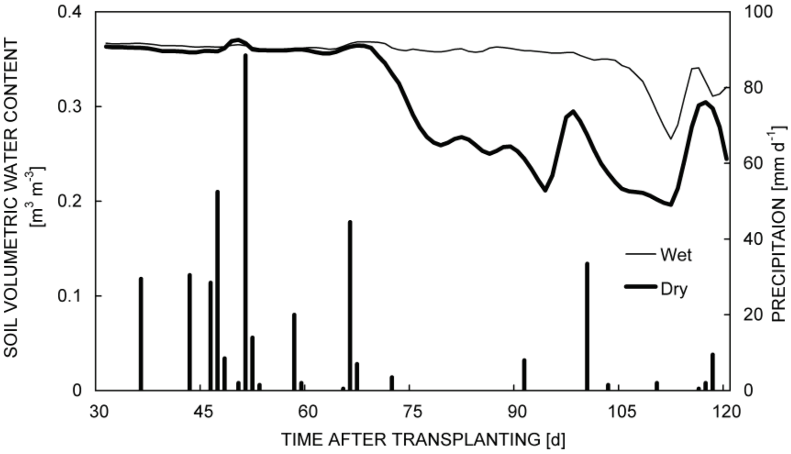

Fig. 1. Changes in soil volumetric water content (*thick line*, W treatment; *thin line*, D treatment) and precipitation (*vertical bars*) during the rice growing season.

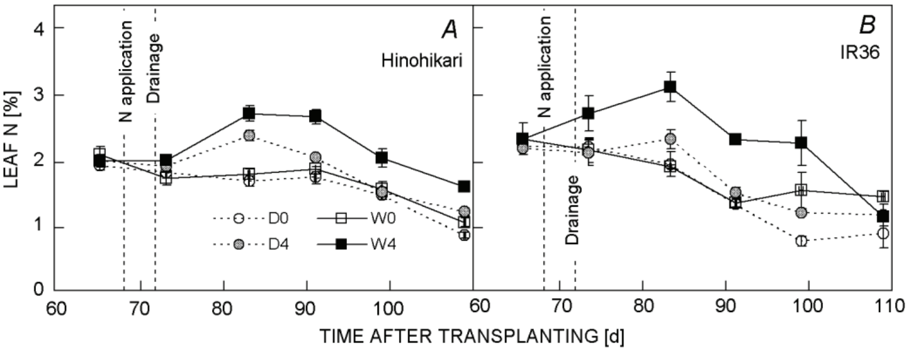

Fig. 2. Changes in leaf nitrogen (N) concentration in 'Hinohikari' (*A*) and 'IR36' (*B*). Treatment regimes (D0, W0, D4, and W4) denote either dry (D) or wet (W) water management practices, either with [4 g(N) m^{-2}] or without [0 g(N) m^{-2}] supplemental N application. The bars indicate the standard error of the mean (SE, $n = 4$).

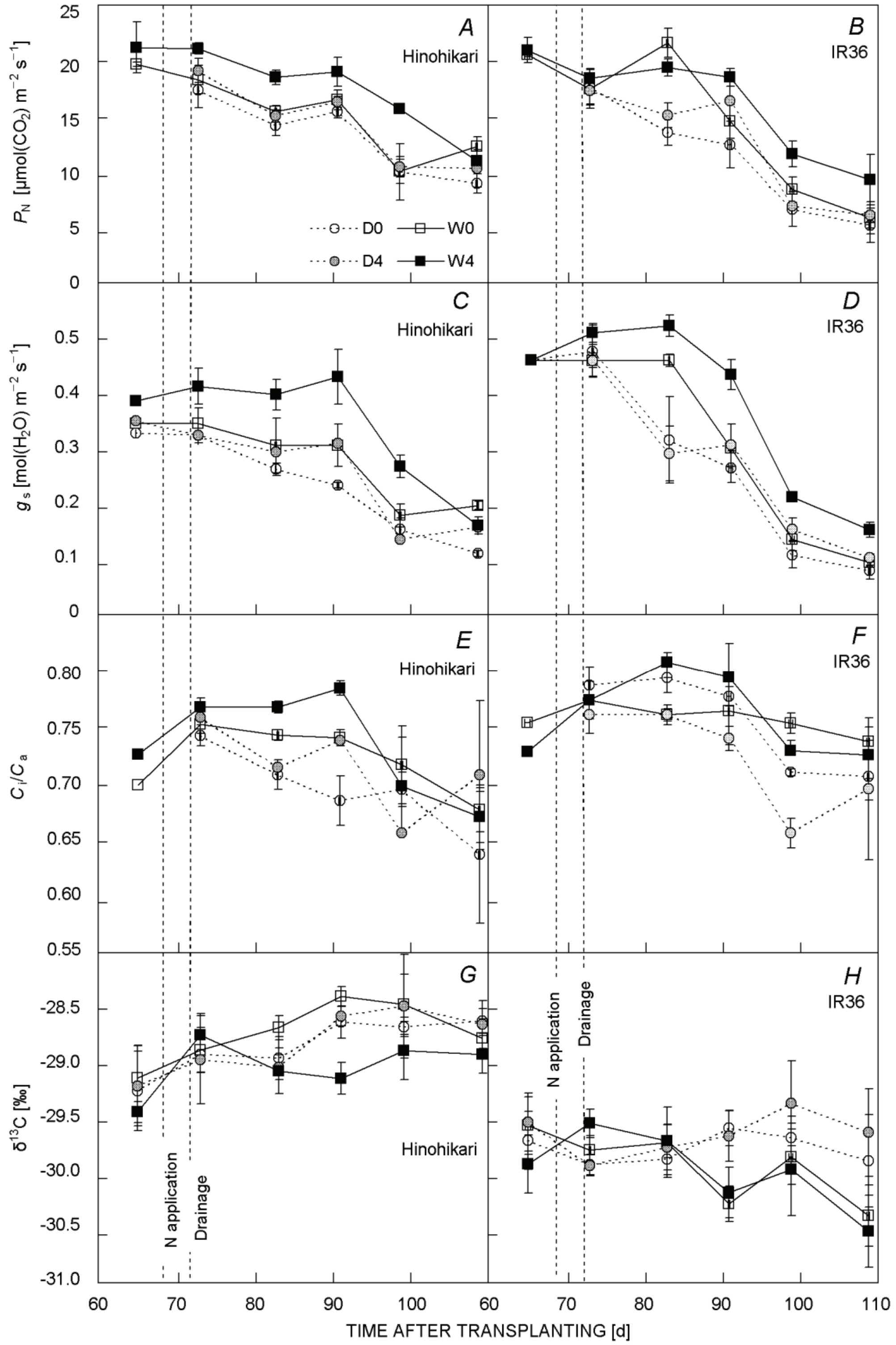

Fig. 3. Changes in the net photosynthetic rate (P_N) in 'Hinohikari' (*A*) and 'IR36' (*B*); changes in stomatal conductance (g_s) in 'Hinohikari' (*C*) and 'IR36' (*D*); changes in the ratio of the intercellular to the ambient CO$_2$ mole fraction (C_i/C_a) during the grain-filling period in 'Hinohikari' (*E*) and 'IR36' (*F*); and leaf carbon isotope composition (δ^{13}C) in 'Hinohikari' (*G*) and 'IR36' (*H*). The bars indicate the standard error of the mean (SE, $n = 4$).

'IR36' (Fig. 3*E*,*F*). C_i/C_a showed different responses to water and N treatments in both cultivars. In 'Hinohikari', although at 73 DAT, C_i/C_a showed an initial rise in the supplemental N regime, at 83 DAT it decreased in response to the dry treatments. 'IR36' showed lower values in D4 after 83 DAT, but no clear trend among the treatment regimes.

Before supplemental N application, δ^{13}C was –30.0‰ to –29.6‰ in 'IR36' and –29.5‰ to –29.2‰ in 'Hinohikari' (Fig. 3*G*,*H*). In 'Hinohikari', δ^{13}C was higher than in 'IR36', and it tended to increase with ripening, whereas in 'IR36', it increased or was constant in dry regimes and decreased in wet regimes with ripening. Ten days before harvest, in the wet treatment without top dressing (W0), δ^{13}C was –30.6‰ to –29.7‰ in 'IR36' and –28.9‰ to –28.7‰ in 'Hinohikari'. N application in the wet regime showed clear and significant effects on isotopic discrimination in 'Hinohikari'. δ^{13}C in 'Hinohikari' at 91 DAT was higher in the wet treatment with N top dressing (W4, –29.2‰) than in the wet treatment without supplemental N (W0, –28.4‰; Fig. 3*G*). Although variation in δ^{13}C in 'IR36' was independent of supplemental N, δ^{13}C was slightly higher in the wet treatments (D0 and D4) than in the dry ones (W0 and W4; Fig. 3*H*).

g_s and C_i/C_a are respectively shown as a function of leaf N. g_s was strongly dependent on leaf N in 'Hinohikari' (Fig. 4*A*; $R^2 = 0.67$, $p<0.001$) and 'IR36' (Fig. 4*B*; $R^2 = 0.68$, $p<0.001$). The slope and intercept of the g_s-N relationship in 'Hinohikari' ($g_s = 0.17 \times$ leaf N – 0.03) were similar to those in 'IR36' ($g_s = 0.20 \times$ leaf N – 0.06).

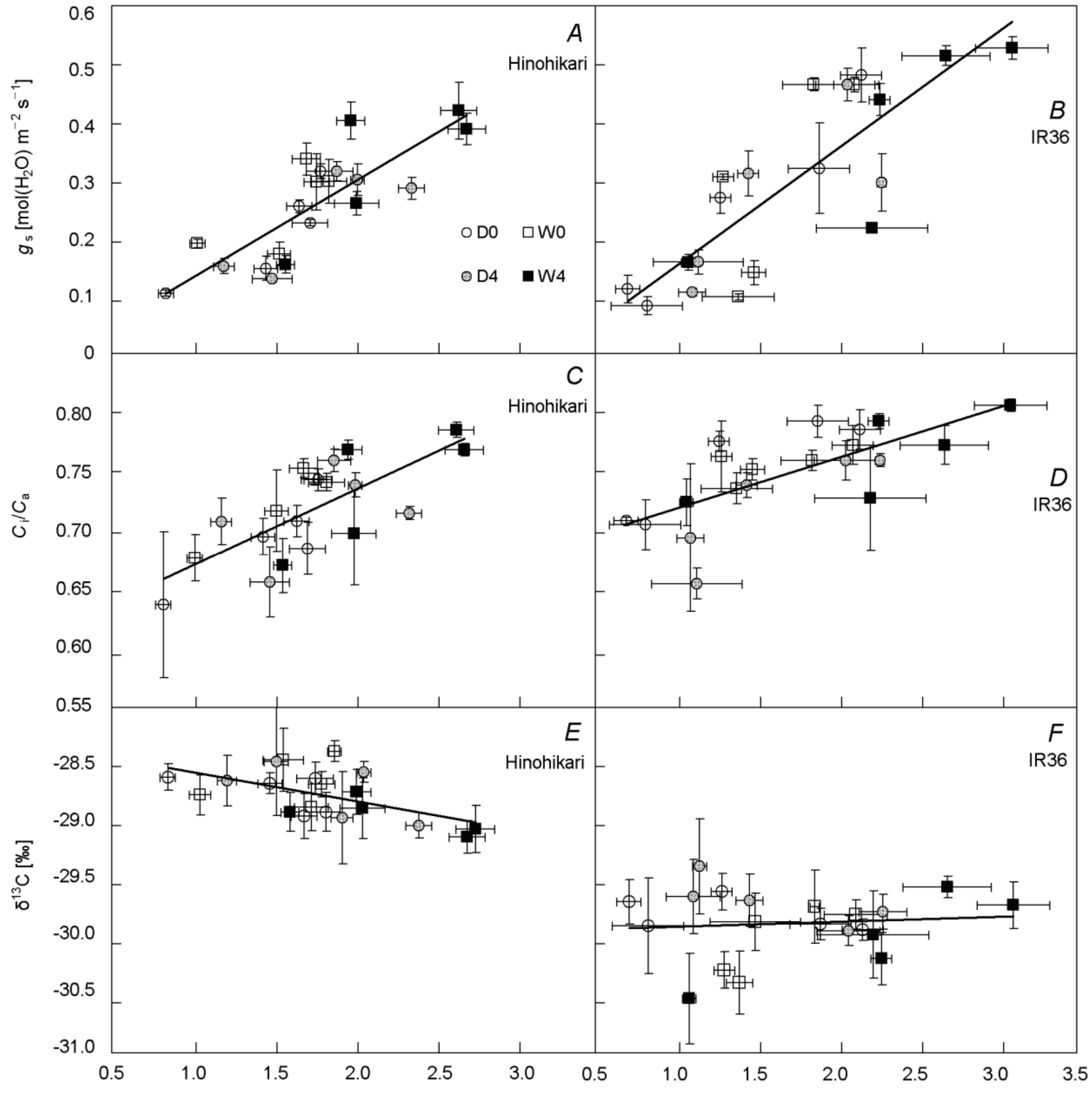

Fig. 4. Relationship between leaf N and g_s during growth in 'Hinohikari' (*A*; $g_s = 0.17 \times$ leaf N – 0.03, $R^2 = 0.67$, $p<0.001$) and 'IR36' (*B*; $g_s = 0.20 \times$ leaf N – 0.04, $R^2 = 0.68$, $p<0.001$); C_i/C_a during growth in 'Hinohikari' (*C*; $C_i/C_a = 0.06 \times$ leaf N + 0.60, $R^2 = 0.53$, $p<0.001$) and 'IR36' (*D*; $R^2 = 0.44$, $C_i/C_a = 0.04 \times$ leaf N + 0.68, $p<0.01$); and δ^{13}C and leaf N during growth in 'Hinohikari' (*E*; $\delta^{13}\mathrm{C} = -0.26 \times$ leaf N – 28.3, $R^2 = 0.31$, $p<0.05$) and in 'IR36' (*F*; $\delta^{13}\mathrm{C} = -0.04 \times$ leaf N – 30.0, $R^2 = 0.01$, ns) with and without supplemental N application. The bars indicate the standard error of the mean (SE, $n = 4$).

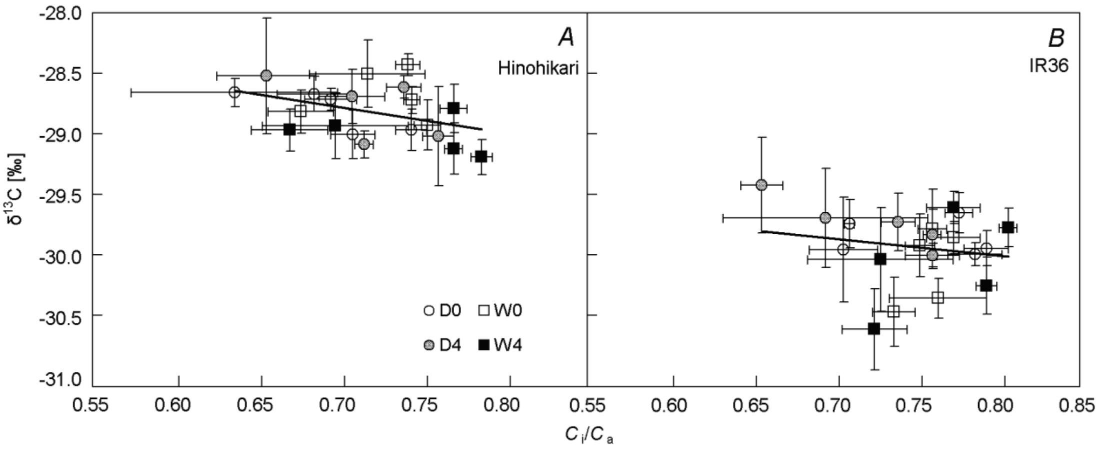

Fig. 5. Relationship between $\delta^{13}C$ and C_i/C_a during growth in 'Hinohikari' (*A*; $\delta^{13}C = -2.1 \times C_i/C_a - 27.3$, $R^2 = 0.18$, ns) and 'IR36' (*B*; $\delta^{13}C = -1.4 \times C_i/C_a - 28.9$, $R^2 = 0.01$, ns). The bars indicate the standard error of the mean (SE, $n = 4$).

Table 1. Shoot dry mass and brown rice yield in 'Hinohikari' and 'IR36'. The figure in parenthesis indicates the standard error of the mean (SE, $n = 4$). n.s. – not significant. * – 5% level of significance. ** – 1% level of significance.

Cultivar	Treatment regime				Significance level		
	D0	W0	D4	W4	Water	N	W × N
Shoot DM [kg m^{-2}]							
Hinohikari	1.72 (0.07)	1.72 (0.17)	1.91 (0.03)	2.08 (0.04)	n.s.	**	n.s.
IR36	1.31 (0.03)	1.49 (0.16)	1.43 (0.04)	1.55 (0.10)	*	n.s.	n.s.
Brown rice yield [kg m^{-2}]							
Hinohikari	0.59 (0.01)	0.57 (0.02)	0.70 (0.01)	0.69 (0.00)	n.s.	**	n.s.
IR36	0.63 (0.01)	0.68 (0.09)	0.66 (0.02)	0.67 (0.08)	n.s.	n.s.	n.s.

C_i/C_a in 'IR36' was slightly lower in the supplemental N treatments at similar leaf N than in the treatments not supplemented with N. There was a significant response of C_i/C_a to leaf N in both 'Hinohikari' (Fig. 4*C*; $C_i/C_a = 0.06 \times$ leaf N $+ 0.60$, $R^2 = 0.53$, $p<0.001$) and 'IR36' (Fig. 4*D*; $C_i/C_a = 0.04 \times$ leaf N $+ 0.68$, $R^2 = 0.44$, $p<0.01$). $\delta^{13}C$ is shown as a function of leaf N and C_i/C_a. $\delta^{13}C$ showed a significant dependence on leaf N in 'Hinohikari' (Fig. 4*E*; $R^2 = 0.31$, $p<0.05$), but not in 'IR36' (Fig. 4*F*; $R^2 = 0.01$, ns). $\delta^{13}C$ was not significantly dependent on C_i/C_a in both 'Hinohikari' (Fig. 5*A*; $R^2 = 0.18$, ns) and 'IR36' (Fig. 5*B*; $R^2 = 0.01$, ns).

In 'Hinohikari', the N application treatment had a significant effect on shoot dry mass and yield but the drainage treatment did not (Table 1). N application resulted in a 9% to 20% increase in shoot dry mass and a 19% increase in yield. In 'IR36', the N top dressing effect was not significant; shoot dry mass showed a 2% to 10% increase, and brown rice yield showed a change of –1% to 5% (Table 1). Drainage was significantly correlated with yield in 'Hinohikari', and with shoot dry mass in 'IR36'.

Discussion

The higher values of P_N and g_s in 'IR36' before heading suggest a higher potential photosynthetic ability of its leaves compared with those of 'Hinohikari'. The maximum g_s value was lower in 'Hinohikari' than in 'IR36'. Furthermore, $\delta^{13}C$ was higher in 'Hinohikari' than in 'IR36' (Fig. 3*G*,*H*). The different $\delta^{13}C$ among cultivars are consistent with previous findings showing that *indica* varieties generally have higher $\delta^{13}C$ than *japonica* varieties (Samejima 1985, Dingkuhn *et al*. 1991b, Kondo *et al*. 2004). Cultivars with higher P_N increase the supply of CO_2 to the plant and relatively lower leaf N decreases the demand for CO_2, so together these may increase C_i/C_a and thus decrease $\delta^{13}C$ (Kanemura *et al*. 2007). The relatively higher P_N in 'IR36' thus caused the CO_2 demand to increase, which in turn decreased $\delta^{13}C$.

The leaf N effect on $\delta^{13}C$ in 'Hinohikari' (Fig. 4*E*) may result from the increasing demand for CO_2 generated within the leaves. In fact, C_i/C_a tended to increase in response to high leaf N in both cultivars (Fig. 4*C*,*D*). The retranslocation of N to younger leaves was more effective in 'Hinohikari' under the wet treatment, as shown by the higher $\delta^{13}C$ in that treatment. In normally developing leaves, $\delta^{13}C$ reflects the time-integrated photosynthetic ability of the plant during leaf expansion. If new leaves are not produced because of senescence, changes in $\delta^{13}C$ may be caused by respiration or translocation, instead of by carbon assimilation. After the supplemental N treatment, $\delta^{13}C$ was significantly related to leaf N in 'Hinohikari', but not in 'IR36' (Fig. 4*F*), suggesting that in 'Hinohikari', leaf $\delta^{13}C$ reflects photosynthetic gas exchange during the grain-filling period, as indicated by the significant response of $\delta^{13}C$ to leaf N during that period.

Although the slopes of the regression lines of g_s or C_i/C_a against leaf N were similar between 'Hinohikari' and 'IR36' (Fig. 4*A*,*B*,*C*,*D*), the relationship of $\delta^{13}C$ against leaf N was different between the two cultivars (Fig. 4*E*,*F*). The photosynthetic parameters P_N, g_s, and C_i/C_a are sensitive to the plant's physiological condition at the time of measurement, whereas $\delta^{13}C$ values reflect physiological conditions integrated over a longer period (Scartazza *et al.* 1998). The nonsignificant correlations between $\delta^{13}C$ and leaf physiological traits in 'IR36' might be explained by more rapid senescence in that variety. Earlier senescence and a shorter grain-filling period in 'IR36' is suggested by the more rapid decreases in leaf N and the photosynthetic parameters P_N and g_s compared with 'Hinohikari'. Moreover, both photosynthetic parameters were lower in 'IR36' than in 'Hinohikari' from 91 DAT, except in the wet regimes (W0 and W4). Drastic reductions in photosynthetic parameter values are more likely to be related to aging than to other factors such as lower photosynthetic activity or lower levels of Rubisco (Monti *et al.* 2007). The varietal differences in the $\delta^{13}C$-leaf N relationship, attributed to leaf senescence in 'IR36', are a key to estimating photosynthetic ability during the grain-filling period. Recently, Hirasawa *et al.* (2010) showed that the high-yielding *indica* variety 'Takanari' has a higher stomatal aperture even at the same level of leaf N than *japonica* varieties, due not only to its high capacity for accumulating a larger amount of leaf N but also to its higher g_s values at the same level of leaf N. This result for 'Takanari' differs from that for the *indica* variety, 'IR36', in this study. The response of photosynthetic parameters to leaf N is thus not dependent on the genotype, be it *japonica* or *indica*.

Our results imply that N limitation during the grain-filling period constrains gas exchange less severely in 'IR36' than in 'Hinohikari'. The different responses to supplemental N application of P_N, g_s, and $\delta^{13}C$ between cultivars should affect shoot dry mass and yield. In this study, shoot dry mass was significantly increased by supplemental N application in 'Hinohikari', but not significantly increased in 'IR36' (Table 1), thus suggesting that senescence during the grain-filling period plays a preponderant role in productivity. Positive correlations between carbon isotopic discrimination of leaves and both shoot dry mass and yield have been reported previously (*e.g.* Merah *et al.* 2001).

In 'Hinohikari', the N effect on yield was significant but not as large as that found in other studies because N was applied only at the late growth stage. Although in this study an increase of less than 20% was observed in shoot dry mass in the N application regimes (Table 1), an increase about three times as large with basal N or multiple N applications has been observed in rice (Kondo *et al.* 2004) and wheat (Cabrera-Bosquet *et al.* 2007, Serret *et al.* 2008). Previous studies predicted that $\delta^{13}C$ would decrease with higher leaf N, whereas in fact $\delta^{13}C$ shows an increase or no change after N application in crop plants (Kondo *et al.* 2004, Cabrera-Bosquet *et al.* 2007). These results indicate a strong effect of N application at the early growth stage. These studies also demonstrated that large biomass production in a high N regime exacerbates water stress, which accounts for lower P_N and higher $\delta^{13}C$ under aerobic and extreme N conditions. The previously reported positive effect of N on $\delta^{13}C$ suggests that a high N supply increases shoot dry mass, and the leaves then compete for the available water, with a corresponding negative effect on carbon assimilation (Kondo *et al.* 2004, Cabrera-Bosquet *et al.* 2007). Here, the aim of the supplemental N application was to improve photosynthetic activity, and its application was not related to the change in any morphological trait. If no extensive increase in shoot dry mass occurs during the grain-filling period, then $\delta^{13}C$ can be used to infer differences in physiological responses between cultivars.

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