

Effects of nitrogen supply restriction on gas exchange and photosystem 2 function in flag leaves of a traditional low-yield cultivar and a recently improved high-yield cultivar of rice (*Oryza sativa* L.)

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Abstract

The effects of nitrogen (N) supply restriction on the CO₂ assimilation and photosystem 2 (PS2) function of flag leaves were compared between two contrastive Japanese rice cultivars, a low-yield cultivar released one century ago, cv. Shirobeniya (SRB), and a recently improved high-yield cultivar, cv. Akenohoshi (AKN). Both cultivars were solution-cultured at four N supply levels from N4 (control) to N1 (the lowest). With a reduction in N-supply, contents of N (LNC), ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO), and chlorophyll (Chl) in flag leaves decreased in both cultivars. In parallel with this, the net photosynthetic rate (P_N), mesophyll conductance (g_m), and stomatal conductance (g_s) decreased. P_N was more dominantly restricted by g_m than g_s . The values of P_N , g_m , and RuBPCO content were larger in AKN than SRB at the four N supply levels. The content of Chl greatly decreased with N deficiency, but the reduction in the maximum quantum yield of PS2 was relatively small. Quantum yield of PS2 (Φ_{PS2}) decreased with N deficiency, and its significant cultivar difference was observed between the two cultivars at N1: a high value was found in AKN. The content ratio of Chl/RuBPCO was also significantly low in AKN. The low Chl/RuBPCO is one of the reasons why AKN maintained a comparatively high P_N and Φ_{PS2} at N deficiency. The adequate ratio of N distribution between Chl and RuBPCO is the important prerequisite for the efficient and sustainable photosynthesis in a flag leaf of rice plant under low N-input.

Additional key words: chlorophyll; chlorophyll fluorescence; dry matter production; leaf area; mesophyll conductance; net photosynthetic rate; non-photochemical quenching; quantum yield, ribulose-1,5-bisphosphate carboxylase/oxygenase; stomatal conductance.

Introduction

The improvement of leaf photosynthesis is one of the fundamental steps of increasing the yield of rice cultivars in Japan (Jiang *et al.* 1988, Kuroda and Kumura 1990, Sasaki and Ishii 1992, Zhang and Kokubun 2004). Among the leaves of a rice plant, flag leaves play an important role in determining the matter production and grain yield (Murata and Matsushima 1975, Black *et al.* 1995). From these findings, it may be predicted that the photosynthetic improvement in a flag leaf is one of the important steps to sustain the productivity of rice plants under low nitrogen (N) input conditions. So far many

studies have been conducted on the response of growth and photosynthesis to N applications using different rice cultivars (Osada and Murata 1962, Hayami 1982, Sarker *et al.* 2002), but few reports on effects of N-saving are available. In a number of studies, crop photosynthesis has been discussed mainly from the viewpoint of gas exchange rate; however, we consider that in order to deepen the understanding of photosynthetic situation in a leaf, it should be examined and discussed on the basis of the mutual relationship between gas exchange and photosystem functions.

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Abbreviations: AKN – cv. Akenohoshi; C_a – atmospheric CO₂ concentration; C_i – intercellular CO₂ concentration; Chl – chlorophyll; F_0 – initial Chl fluorescence of a dark-adapted leaf; F_m – the maximum Chl fluorescence of a dark-adapted leaf; F_m' – the maximum Chl fluorescence detected in “actinic light”; F_s – steady Chl fluorescence in “actinic light”; F_v – variable Chl fluorescence; g_s – stomatal conductance; g_m – mesophyll conductance; LA – leaf area per plant; LNC – leaf N content; NEP – N utilization efficiency in photosynthesis; NPQ – non-photochemical quenching; P_N – net photosynthetic rate; PPFD – photosynthetic photon flux density; PS2 – photosystem 2; RuBPCO – ribulose-1,5-bisphosphate carboxylase/oxygenase; SNC – N concentration in the solution; SRB – cv. Shirobeniya; TDM – total dry mass per plant; Φ_{PS2} – PS2 quantum yield.

Recently, the functional situation in the photosystem 2 (PS2) in a leaf has come to be readily diagnosed by the introduction of the PAM-instrumentation. Some researchers reported that N deficiency reduced the maximum quantum yield of PS2 (F_v/F_m) and the quantum yield of PS2 (Φ_{PS2}) (Verhoeven *et al.* 1997, Lima *et al.* 1999, Huang *et al.* 2004), however, others suggested that N deficiency has no significant effects on PS2 functions (Ciompi *et al.* 1996, Lu and Zhang 2000). Therefore, a unified view has not ever been formed concerning effects of N deficiency on PS2 functions.

As for rice cultivars, studies conducted from both viewpoints of gas exchange and PS2 functions are few; particularly, little information is available for effects of N deficiency on these functions. To date, we have continued studies on the N-saving rice cultivation, in which the effects of N supply restriction on the gas exchange and

PS2 function were investigated using different rice cultivars (Araki *et al.* 2005, Kumagai *et al.* 2006).

In the present study, photosynthetic features of a flag leaf of two contrastive Japanese rice cultivars, cv. Shirobeniya (SRB) and cv. Akenohoshi (AKN) were investigated. SRB had been cultivated widely in the western region of Japan approximately one century ago, while AKN is an improved *japonica-indica* cross characterized by having a high leaf photosynthetic rate and high grain yield (Jiang *et al.* 1988, Kubota *et al.* 1988). The responses of gas exchange rate and PS2 functions to the restriction of N supply were compared between the two cultivars, and by clarifying their cultivar difference, we obtained the fundamental information of improving photosynthetic sustainability in a flag leaf under low N-input growth conditions.

Materials and methods

Rice cultivars used and their cultivation: The water-soaked seeds of two Japanese rice cultivars, cv. Shirobeniya (SRB) and cv. Akenohoshi (AKN), were sown in nursery boxes at the beginning of July, 2005. Three weeks after sowing, young shoots were transplanted and grown for two weeks in the solution-culture bath (0.5 m³) containing the standard solution of Yoshida *et al.* (1971), which consists of 1.43 mM NH₄NO₃, 0.51 mM K₂SO₄, 1.00 mM CaCl₂, 1.67 mM MgSO₄, 0.32 mM NaH₂PO₄, 0.04 mM FeCl₂, 9.09 μM MnCl₂, 0.08 μM (NH₄)₆Mo₇O₂₄, 18.2 μM H₃BO₃, 0.15 μM ZnSO₄, 0.16 μM CuSO₄, and 3.57 mM Na₂SiO₃. Then, the plants were separated into four groups and grown in the four baths each containing the solution of different N concentration of four levels until eight weeks after transplanting. Four plants of each cultivar were grown in each solution bath. N concentrations in the solution (SNC) at the treatment onset were 2.86 (N4 = control), 2.14 (N3), 1.43 (N2), and 0.71 mM (N1). SNC was monitored at one-week intervals with an ion meter (*IM-22P*, *Toa-DKK*, Japan), and pH in the solutions was adjusted every day within the range of 5.0 to 5.5. SNC decreased with plant growth as shown in Fig. 1. Each solution was renewed at two-week intervals. The N concentration in each renewed solution was adjusted to the same SNC level that was measured in each bath at the renewal time. The concentrations of nutritional components other than N were adjusted to the standard solution level.

The plant growth was periodically surveyed, and when plants reached the heading stage in eight weeks after transplanting, gas exchange and Chl fluorescence emission were simultaneously measured on a flag leaf of a main culm by the methods described below. After the measurements, four plants were sampled and divided into four parts: the leaf, culm, root, and dead part, and they were dried at 80 °C for 7 d in an oven to weigh their dry mass (DM). Leaf area (LA) was measured with an

automatic area meter (*AAM-8*, *Hayashi-denko*, Japan).

Leaf N content (LNC): A dried flag leaf was powdered, and the content of N was determined according to the semi-micro Kjeldahl procedure.

Gas exchange and Chl fluorescence: The gas exchange rate of a flag leaf was measured with a sandwich-type assimilation chamber (*PLC-4B*, *Shimadzu*, Japan) of open system. The measurement was carried out under the conditions of 30±1 °C in leaf temperature, 380±10 cm³ m⁻³ in CO₂ concentration, 60 % in relative humidity, and 500 μmol m⁻² s⁻¹ in photosynthetic photon flux density (PPFD). The leaf area used in the measurements was 5.9 cm² and the air flow rate into the assimilation chamber was 0.706 mmol s⁻¹. CO₂ concentration and water vapour pressure in the reference and sample air were monitored with an infrared CO₂ and H₂O gas analyzer (*Li-6262*, *LI-COR*, USA). Based on the measured values, the values of net photosynthetic rate (P_N), stomatal conductance (g_s), mesophyll conductance (g_m), and intercellular CO₂ concentration (C_i) were calculated according to the method of Long and Hallgren (1985). The parameter g_m was obtained on the assumption that CO₂ concentration in mesophyll was zero.

The Chl fluorescence of PS2 was monitored simultaneously with the measurement of gas exchange rate using a fluorescence probe (*PAM-2000*, *Walz*, Germany) equipped on the assimilation chamber. With a leaf dark-adapted for 1 h, the initial fluorescence (F_0) in the non-photosynthetic situation was determined under a measuring beam (3.2 μmol m⁻² s⁻¹ PPFD); thereafter, the maximum fluorescence (F_m) was measured by imposing 0.8-s saturation pulse of 8 000 μmol m⁻² s⁻¹ PPFD onto the leaf. Then, the leaf was continuously irradiated by an "actinic light" of 500 μmol m⁻² s⁻¹ PPFD. After the steady-state fluorescence (F_s) was recorded, a 0.8-s saturation pulse

was given at 30-s intervals to determine the maximum fluorescence in the light-adapted state (F_m').

Based on the data obtained, the following parameters were calculated: the maximum quantum yield, $F_v/F_m = (F_m - F_0)/F_m$ (according to van Kooten and Snel 1990); PS2 quantum yield, $\Phi_{PS2} = (F_m' - F_s)/F_m'$ (Genty *et al.* 1989); non-photochemical quenching, $NPQ = (F_m - F_m')/F_m'$ (Bilger and Björkman 1990).

Contents of soluble protein, Chl, and RuBPCO (EC 4.1.1.39): After the measurement of gas exchange and Chl fluorescence, leaf discs of 5 mm diameter were sampled and frozen in liquid N, and stocked at -80°C . For the measurement of Chl content, three discs of them were soaked in 96 % ethyl-alcohol in the dark for two days until the leaf colour was bleached. After this extraction procedure, Chl content in the alcohol solution was estimated from the absorbances at 649 and 665 nm using a spectrophotometer (*UV-1200*, Shimadzu, Japan),

Results and discussion

As shown in Fig. 1, SNCs in all treatments had gradually declined with the plant growth and, except at N4 (control), were eventually depleted in seven, six, and five weeks after transplanting at N3, N2, and N1, respectively. Thus both cultivars grown at N1 suffered from the most severe N deficiency at the heading stage.

Table 1 shows the total dry mass (TDM), LA, and LNC of a flag leaf at the heading stage in both cultivars.

Table 1. Total dry mass, TDM [g per plant], leaf area, LA [m^2 per plant], and leaf nitrogen content, LNC of a flag leaf, [g m^{-2}] at the heading stage in two rice cultivars (SRB = Shirobeniya, AKN = Akenohoshi) grown in different N concentrations. Values in parentheses indicate the ratio compared to control. Same letters in the column represent not significant by the Tukey's test at 5 % level.

| | | TDM | LA | LNC | |
|-----|--------------|----------|-------|--------|-------|
| SRB | N4 (control) | 37.2 ab | (100) | 1.37 b | (100) |
| | N3 | 35.6 ab | (96) | 1.13 c | (82) |
| | N2 | 27.2 bc | (73) | 0.94 d | (68) |
| | N1 | 22.8 c | (61) | 0.73 e | (53) |
| AKN | N4 (control) | 41.2 a | (100) | 1.79 a | (100) |
| | N3 | 35.2 ab | (85) | 1.28 b | (72) |
| | N2 | 31.7 abc | (77) | 1.14 c | (64) |
| | N1 | 32.3 abc | (78) | 0.79 e | (44) |

There is a significant positive relationship between P_N and LNC in rice cultivars (Ishihara *et al.* 1979, Makino *et al.* 1985, Kuroda and Kumura 1990). Fig. 2 shows the photosynthetic gas exchange parameters of both cultivars observed at the heading stage. P_N decreased with a reduction in N supply, and a cultivar difference was observed in all the N treatments: the value of P_N in AKN was higher than that in SRB (Fig. 2A). Many of the studies described that the genetically improved rice cultivars had higher leaf photosynthetic rates than the traditional

according to the method described by Wintermans and de Mots (1965).

For the measurement of soluble protein content, six leaf discs were used. These discs were powdered in liquid N in a mortar; then a chilled extraction buffer that contains 100 mM $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ (pH 7.0), 1 mM phenylmethanesulfonyl fluoride, 1 % (m/v) insoluble polyvinyl polypyrrolidone, and 1 % (c/v) 2-mercaptoethanol was added to it, and the powder was further ground. The obtained solution sample was transferred into Eppen tubes and applied to a centrifugation ($12\,000\times g$, 5 min, 4°C). Then, Bradford reagent (*Bio-Rad*, USA) was added to the supernatant according to the method of Bradford (1976), and the amount of soluble protein in the sample was determined by the absorbance at 595 nm with the spectrophotometer. The amount of RuBPCO in the soluble protein obtained above was quantified by SDS-polyacrylamide gel electrophoresis, according to the method described by Makino *et al.* (1985).

With a reduction in N supply from N4 to N1, LNC decreased: the value of LNC at N1 decreased to 44 and 53 % of N4 in AKN and SRB, respectively. On the other hand, TDM and LA observed at N1 were 78 and 59 % in AKN, and 61 and 37 % in SRB, respectively. This suggests that AKN is likely to have a higher sustainability in TDM and LA in N-deficient growth condition than SRB.

cultivars (Kuroda and Kumura 1990, Sasaki and Ishii 1992, Zhang and Kokubun 2004). As for AKN, Jiang *et al.* (1988) reported that this cultivar showed a particularly vigorous photosynthesis at the heading stage.

The values of g_s and g_m decreased with reduction of N supply, and a cultivar difference was found in these two parameters at all the N treatments: AKN had larger values than SRB (Fig. 2B,C). On the other hand, the ratio of C_i to reference air CO_2 concentration (C_a) (C_i/C_a) was almost constant in both cultivars (Fig. 2D). These facts may

indicate that the reduction of photosynthetic capacity with N supply restriction was due to depressed mesophyll function rather than stomatal limitation. This finding is coincident with the reports by Ciompi *et al.* (1996) and Huang *et al.* (2004). However, several other reports described that a reduction in LNC induced stomatal closure and g_s depression, by which P_N of a rice leaf was

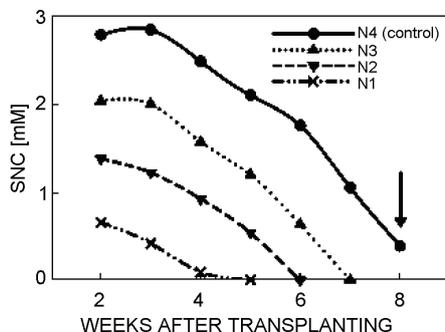


Fig. 1. Changes in N concentration in the solutions (SNC) with growth period. SNCs at the treatment onset were 2.86, 1.43, 1.14, and 0.71 mM in N4 (control), N3, N2, and N1, respectively. Arrow indicates the times of measurement and sampling at the heading stage.

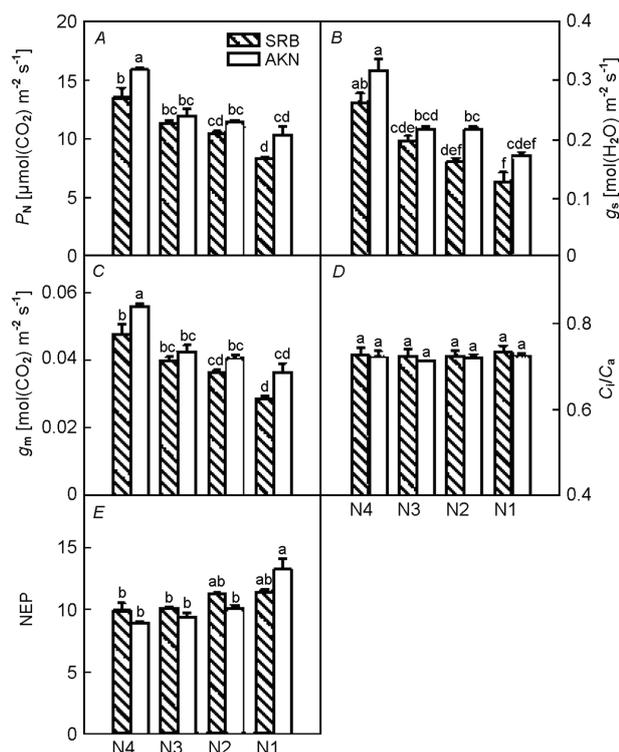


Fig. 2. Effects of N supply restriction on gas exchange parameters (A: net photosynthetic rate, P_N ; B: stomatal conductance, g_s ; C: mesophyll conductance, g_m ; D: the ratio of intercellular to atmospheric CO_2 concentration, C_i/C_a ; E: N utilization efficiency in photosynthesis, NEP) of a flag leaf at the heading stage in two rice cultivars. Means \pm SE of three replications. Bars followed by the same letter represent non significant difference by the Tukey's test at 5 % level.

strongly restricted (Ishihara *et al.* 1979, Kuroda and Kumura 1990). In our study, g_m is recognized as the major determinant of P_N . The parameter g_m is regarded as an indicator of the photosynthetic potential in mesophyll (Islam *et al.* 1994), which is closely related to the energetic economics between CO_2 assimilation system and energy production system. The analysis based on the functional balance between CO_2 assimilation and PS2 is necessary to obtain a more fundamental understanding of N responses in photosynthesis and their cultivar difference.

N utilization efficiency in photosynthesis (NEP) increased with a reduction in N supply in both cultivars (Fig. 2E). The value of NEP in AKN was lower at N2 to N4, but higher at N1 than that in SRB. There are a number of studies on N utilization efficiency in the growth and photosynthesis of rice cultivars (Makino *et al.* 1994, Mae 1997, Ishimaru *et al.* 2001), of which the report by Mae (1997) suggested that there were two main determinants of NEP: the kinetics of RuBPCO and the ratio of RuBPCO content to LNC. This hypothesis also emphasized the importance of the mesophyll function, as we mentioned above. At the next step, the mesophyll function was discussed in relation to the photosynthetic energy balance between CO_2 assimilation and PS2 functions based on the RuBPCO and Chl contents in a leaf.

Fig. 3 shows the Chl fluorescence parameters of both cultivars observed at the heading stage. F_v/F_m showed a slight decrease with a reduction in N supply (Fig. 3A). This fact indicates that the energy-producing function in PS2 was not so strongly deteriorated by N deficiency. The cultivar difference in F_v/F_m was small, although slightly higher values were shown in AKN. Some researchers also reported that F_v/F_m and Φ_{PS2} were negatively influenced by N deficiency (Verhoeven *et al.* 1997, Lima *et al.* 1999, Huang *et al.* 2004). The cultivar difference in Φ_{PS2} was evident in N1: a significantly large value was found in AKN (Fig. 3B). This parameter represents the fraction of energy utilized for photosynthetic electron transport in thylakoid membranes of chloroplast (Genty *et al.* 1989). The result in our study may imply that AKN was able to maintain a higher efficiency in the electron transport of PS2 in N1.

The value of NPQ was increased by the restriction of N supply in both cultivars (Fig. 3C). A substantial increase was observed in SRB that showed a greater depression in P_N as shown in Fig. 2A. NPQ is frequently adopted as a parameter indicating the activity of xanthophyll-cycle that is closely related with heat dissipation for the energy excessively accumulated in a leaf. This activity is enhanced under the N-deficient growth condition, and plays a crucial role in preventing the photo-damage (Verhoeven *et al.* 1997, Lu and Zhang 2000, Chen *et al.* 2003). The leaf showing a lower NPQ may have a higher efficiency and sustainability in photosynthesis.

RuBPCO is the main component of CO_2 assimilation

apparatus in a leaf, which strongly influences P_N . In a mature leaf of rice, approximately 80 % of the total N accumulated in the leaf is located in chloroplasts (Morita 1980), and approximately 30 % of the total N in the leaf is allocated for the construction of RuBPCO (Makino *et al.* 1985). Likewise, Chl also plays a vital role in determining leaf photosynthetic rate by regulating the production of biochemical energy. Then in Fig. 4, RuBPCO and Chl contents and their ratio are represented for both cultivars. The values of RuBPCO and Chl contents decreased similarly in both cultivars with a reduction in N supply (Fig. 4A,B). The cultivar difference was very large in RuBPCO content, however, it was small in Chl content. The large RuBPCO content in AKN means a large energy sink for CO_2 assimilation and this is one of the reasons why AKN was able to achieve a higher P_N under the N deficiency.

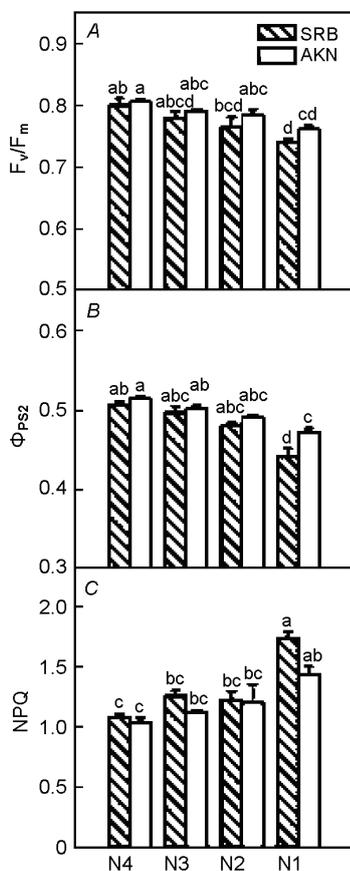


Fig. 3. Effects of N supply restriction on Chl fluorescence parameters (A: the maximal quantum yield, F_v/F_m ; B: PS2 quantum yield, Φ_{PS2} ; C: non-photochemical quenching, NPQ) of a flag leaf at the heading stage in two rice cultivars. Means \pm SE of three replications. Bars followed by the same letter represent non significant difference by the Tukey's test at 5 % level.

Chl/RuBPCO ratio is a parameter reflecting the mutual relationship between energy production site and CO_2 assimilation site in a leaf, and is closely related to the photosynthetic efficiency. Evans and Terashima (1987) and Terashima and Evans (1988) reported that the contents of RuBPCO and Chl in a leaf were decreased by restricting N supply. Similar phenomenon was also observed in our study (Fig. 4A,B), but Chl/RuBPCO ratio showed a significant difference between the two cultivars: the lower values were observed in AKN

through all the N treatments (Fig. 4C). This is the most interesting finding in this study, suggesting that AKN has a more efficient energy flow in the photosynthetic system of a leaf. The leaf with a high Chl/RuBPCO ratio is considered to be beneficial in light harvesting; particularly in the case leaves are receiving low irradiance. However, flag leaves of a rice plant are positioned at the top of a canopy; therefore, they are most frequently exposed to excessively high irradiance. In such circumstances, flag leaves showing a high Chl/RuBPCO ratio are easily plunged into the physiologically dangerous state in which the production of photochemical energy greatly exceeds the consumption, and excessive energy is accumulated in a leaf. Chen *et al.* (2003) reported that rice plants grown under N deficiency were more susceptible to photo-damage when exposed to strong irradiance. Flag leaves play a vital role as the main source organ at the maturing stage (Murata and Matsushima 1975, Black *et al.* 1995); therefore, the photosynthetic damage in flag leaves may result in serious depression of yield. It may be desirable for a flag leaf to have a large content of RuBPCO and relatively low Chl/RuBPCO ratio in order to perform an efficient and sustainable photosynthetic activity. To maintain the Chl/RuBPCO ratio at an appropriate level is one of the fundamental steps to improve the photosynthetic

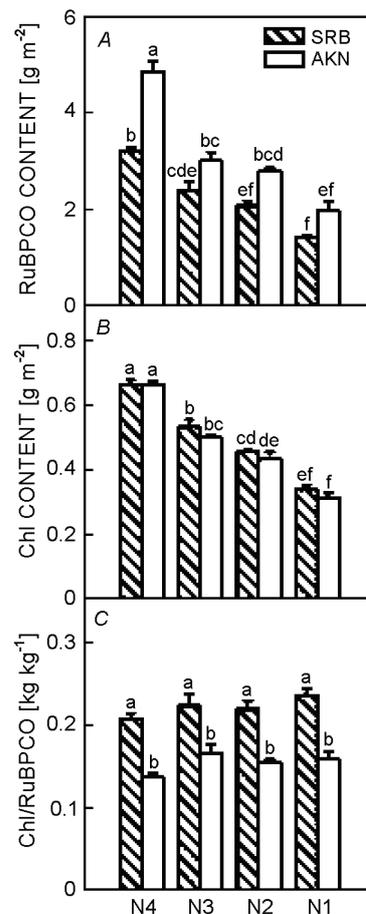


Fig. 4. Effects of N supply restriction on RuBPCO content (A), Chl content (B), and Chl/RuBPCO ratio (C) of a flag leaf at the heading stage in two rice cultivars. Means \pm SE of three replications. Bars followed by the same letter represent non significant difference by the Tukey's test at 5 % level.

sustainability under low N-input growth conditions. We conclude that the ratio of Chl/RuBPCO is one of the

criteria for screening rice genotypes with high photosynthetic sustainability in flag leaves.

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Kitchin, R., Fuller, D.: **The Academic's Guide to Publishing**. – Sage Publications, London – Thousand Oaks – New Delhi 2005. ISBN 1 4129 0083 2 (paperback). 180 pp.

Completion of a research project requires publishing the results in an appropriate way. The reviewed textbook teaches the beginners in science how to fulfil the task. It explains different forms of publication of results: as articles in journals, chapters in books, research reports, papers and presentations at science conferences and in popular media, and as an information on the Internet. A strategy for publishing during different phases of researcher's career (postgraduate, research and teaching posts, tenure and after-tenure) is explained as well as principles of team work. General writing advice is the next step: the necessity of keeping in mind correct grammar and punctuation, ensuring good readability by looking at proper narrative, text structure, clarity, and style, balancing presented information and length of text, giving relevant accessories, and avoiding general mistakes are stressed. Plagiarism, duplication of publications, falsification of results, respecting copyright, *etc.* are the further discussed topics.

The following chapters deal with individual forms of publishing. Selecting of proper journal, good preparation of an article, submission, respecting referees' comments prior to resubmission, as well as special tips (writing a book review or an editorial) are shown next. A special chapter deals with preparing research reports (for example for grant agencies), another one with publishing on the Internet (mailing lists, bulletin boards, websites, issues to consider such as ownership, copyright, contracts, security, design, access, or quality and worth). The last topic is rather new and not all textbooks deal with it. In the chapter on cooperation with popular media there is a very useful box summarizing how to prepare a press release. Relatively much space (six chapters) is dedicated to book publishing (maybe this form of publication is often used in geography, from which research field are many examples presented). The chapters describe book

types, selection of publisher, possibility to publish one's PhD thesis, contacts with selected publisher (writing and submitting a proposal, negotiating a book contract, writing and submitting a manuscript), editing a book, and self-publishing a book.

The following two chapters are certainly not written for beginners in science: how to propose a new journal or book series, and how to edit a journal. Chapter on the production process includes important points such as reading the proofs, preparing indexes; according to my opinion the explanation here should bring more detail.

Very important is the next topic, dealing with oral presentations and scientific posters. An important, but often neglected means of scientific communication is to make and keep contacts. Appropriate recommendations are given here (see overview in box 20.1 Secrets of successful networking). The following chapter (Organizing events) is certainly not prepared for beginners, even if the Conference checklist (box 21.1) will be used by many conference organizers.

Appendix 1 presents useful references and resources. Appendices 2 "Questions journal editors are often asked" and 3 "Refereeing for journals, publishers and conferences" explain facts not discussed in the respective chapters. A good index facilitates use of this textbook.

I think that the difficulty with this textbook lies in the unbalance of chapters—some of them are written for beginners in science and therefore would require more information and details, some of them can be used by more advanced researchers only. If a beginner will try to publish a book or launch a journal, his effort will probably end without an expected success. On the other hand, every researcher can find some useful information in this textbook.

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