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Estimation of photorespiration rate by simultaneous measurements of CO_2, gas exchange rate, and chlorophyll fluorescence quenching in the C_3 plant *Vigna radiata* (L.) Wilczek and the C_4 plant *Amaranthus mongostanus* L.

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Abstract

So far the photorespiration rate (R_P) in a leaf has been determined as the difference between the net photosynthetic rates (P_N) measured in 21 % O_2 air ($P_{N21\%}$) and 3 % O_2 air ($P_{N3\%}$). In the C_3 plant *Vigna radiata* and the C_4 plant *Amaranthus mongostanus* L., P_N and chlorophyll fluorescence quenching in leaves were monitored simultaneously. R_P of leaves *in situ* was estimated as termed R_{PE} from the electron transport rates through photosystem 2 (PS2), and compared with R_{PO} ($P_{N3\%} - P_{N21\%}$). In *V. radiata* R_{PO} was 11.9 μmol(CO_2) m^{-2} s^{-1} and the ratio of R_{PO} to $P_{N21\%}$ was 42.2 %, whereas the ratio of R_{PE} to $P_{N21\%}$ was 25.7 %. This suggests that R_{PO} may be over-estimated for the real R_P in normal air. In *A. mongostanus*, P_N was almost not changed with a decrease in O_2 concentration from 21 to 3 %, whereas the quantum yield of PS2 was evidently affected by the change in O_2 concentration. This fact shows the presence of photorespiration in this C_4 species, where R_{PE} was equivalent to 3.8 % of $P_{N21\%}$.

Additional key words: electron transport rate; photosynthesis; photosystem 2; mungbean; quantum yield.

Introduction

Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO) has a dual function as carboxylase in carbon assimilation and also as oxygenase in photorespiration. Its functional characteristics in leaf vary with the relative concentrations of CO_2 and O_2 in ambient air. The intercellular CO_2 concentration in leaves growing under field conditions greatly changes with stomata aperture, which may often give a significant effect to both CO_2 assimilation rate and photorespiration rate in various ways depending on other environmental factors such as air temperature, moisture, and photosynthetic photon flux density (PPFD).

Photorespiration has often been regarded as a negative function in biomass production, because a considerable part of CO_2 photosynthetically fixed in a leaf is released to the atmosphere through C_2 cycle. On the other hand, photorespiration protects photosynthetic and other physiological functions in plant by dissipating the chemical energy excessively produced by photochemical systems. This functional importance is more accentuated when plants, particularly C_3 plants, are subjected to stresses such as high irradiance and water deficits (Heber *et al.* 1996, Kozaki and Takeba 1996).

So far the photorespiration rate (R_P) in C_3 plants has been usually determined and evaluated as the value obtained by subtracting $P_{N21\%}$ from $P_{N3\%}$. However, some methodological defects have been pointed out for this method. Under a low O_2 concentration, such as 3 % in air, the activity of oxygenase in RuBPCO is restricted, but the carboxylase activity is enhanced instead. Hence, the subtracted value is an over-estimated value compared to the real R_P in 21 % O_2 air (deVeau and Burris 1989, Tokuda

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Abbreviations: Chl – chlorophyll; F_m' – maximum fluorescence in light; F_t – steady-state fluorescence in light-adapted state; g_s – stomatal conductance; J_T – the rate of electron transport through photosystem 2; k_c – the number of electron equivalents required to reduce 1 molecule CO_2 in 3 % O_2 air; k_N – the number of electron equivalents required to reduce 1 molecule CO_2 in 21 % O_2 air; k_r – the number of electron equivalents required to release 1 molecule CO_2 in photorespiration; NAD-ME – NAD-malic enzyme; P_G – gross photosynthetic rate, $P_G = P_N + R_D$; P_N – net photosynthetic rate; $P_{N21\%}$ – net photosynthetic rate measured in 21 % O_2 air; $P_{N3\%}$ – net photosynthetic rate measured in 3 % O_2 air; R_P – photorespiration rate; R_{PE} – photorespiration rate estimated by energetic calculation; R_{PO} – photorespiration rate estimated by subtracting $P_{N21\%}$ from $P_{N3\%}$; PPFD – photosynthetic photon flux density; PS – photosystem; R_D – dark respiration rate; RuBPCO – ribulose-1,5-bisphosphate carboxylase/oxygenase; ΔCO_2 – the difference of CO_2 concentration between sample air and reference air which flow through the leaf chamber; ΔH_2O – the difference in H_2O vapour pressure between sample air and reference air which flow through the leaf chamber; Φ_e – quantum yield of photosystem 2.

et al. 1999). In order to know R_P that is more accurate or closer to the real value, it is essential to determine it in normal air containing 21 % O_2.

In general, photorespiration in C_4 plants is almost completely depressed because malate and aspartate produced in mesophyll cells at the initial reaction of photosynthesis in C_4 plants provide highly concentrated CO_2 to RuBPCO in the bundle sheath cells (Edwards and Walker 1983, Pearcy and Ehleringer 1984, Edwards *et al.* 1985). However, the existence of photorespiration is known in C_4 species by observing the incorporation of $^{18}O_2$ into metabolites formed as a consequence of photorespiration (Berry *et al.* 1978, deVeau and Burris 1989). Maroco *et al.* (1997, 2000) showed that the CO_2 assimilation rate in three subtypes of C_4 plants increased at an optimal O_2 concentration of 5 to 10 %. This may indicate that C_4 plants conduct a considerable photorespiration depending on the environment. But gas exchange measurements are inadequate as a method to detect slight differences between the P_N in C_4 plant observed at 3 and 21 % O_2.

Recently many studies have been performed on the chemical energy sharing between CO_2 assimilation and other metabolisms (photorespiration and Mehler reaction), using the chlorophyll (Chl) fluorescence quenching diagnosis (Di Marco *et al.* 1994, Di Martino *et al.* 1999, Tsuyama and Kobayashi 1999, Miyake and Yokota 2000, Muraoka *et al.* 2000, Ruuska *et al.* 2000). Nevertheless, not much information is available on R_P and the relation of R_P to $P_{N21\%}$ in crop leaves evaluated by Chl fluorescence diagnosis and their specific and cultivar characteristics. To know real values of R_P in crop leaves, it is essential to deepen the understanding of the role of photorespiration as a function necessary for growth and productivity maintenance of the plant.

In this study we examined how to estimate a more correct R_P of leaves *in situ* based on the changing electron transport rates by a simultaneous monitoring of CO_2 exchange rate and Chl fluorescence quenching. The P_N and R_P were calculated and compared between the C_4 plant, *Amaranthus mongostanus*, and the C_3 plant, *Vigna radiata*.

Materials and methods

The C_3 plant mungbean, *V. radiata* (L.) Wilczek cv. Chinese and the C_4 plant *A. mongostanus* L. were grown in 8 500 cm^3 pots (two plants per pot for *V. radiata*, one plant per pot for *A. mongostanus*) filled with sandy soil containing a chemical compound fertiliser (N, P, and K each 0.8 g per pot) during summer season in the experimental field of Kyushu University (33°35'N, 130°23'E).

Gas exchange and Chl fluorescence were simultaneously measured with full expanded, attached leaves of both species. P_N, R_D, and transpiration rate were measured with a sandwich-type assimilation chamber (*PLD-B, ADC*, Hoddesdon, UK) in an open gas exchange system. Sampled air was monitored with an infrared CO_2 analyser (*LI6262, Li-Cor*, Lincoln, USA). The O_2 concentration in air pumped to the leaf chamber was alternatively shifted 3 times between 21 and 3 % O_2 for about 100 min. The air containing 350 μmol(CO_2) mol^{-1} and 3 or 21 % O_2 was prepared by mixing N_2, O_2, and CO_2 with a gas mixer (*GM-3A, KOFLOC*, Kyoto, Japan). CO_2 absorbent (soda lime) was used for the final adjustment of CO_2 concentration. The air had been moisture-saturated at 18.3 °C by a dew point generator (*LI610, Li-Cor*, Lincoln, USA) before it was sent to the assimilation chamber, and the air flow rate through the chamber was 16.67 cm^3 s^{-1}. Leaf temperature was adjusted to 30.2 ±0.6 °C by circulating temperature-controlled water to the radiator attached to the chamber. A metal halogen lamp (*HILUX-HR, Rikagaku Co.*, Japan) provided actinic irradiation of 1 600 μmol m^{-2} s^{-1} PPFD.

The quantum yield of PS2 (Φ_e) was measured with a Chl fluorometer (*PAM-2000, Walz*, Effeltrich, Germany), and the head of the fluorescence probe guide was fixed on the assimilation chamber at the position where it did not shade the leaf. Φ_e was calculated from the Eq. (1) proposed by Genty *et al.* (1989):

$$\Phi_e = (F'_m - F_t)/F'_m \quad (1)$$

where F_t is Chl fluorescence emitted by leaf, measured at the steady state, and F'_m is the fluorescence spike shown by giving 1.2 s pulse of photosynthesis saturating irradiation of 8 000 μmol m^{-2} s^{-1} PPFD.

The rate of electron transport through PS2 (J_T) was calculated from Eq. (2):

$$J_T = \Phi_e L \times 0.5 \times a \quad (2)$$

where L is the PPFD at leaf surface (1 600 μmol m^{-2} s^{-1}). Assuming that the photons are evenly distributed to the two photosystems, 0.5 is used in Eq. (2). a is the ratio of Chl-absorbed photons to the incident photons. Values of a, 0.935 and 0.921, were determined with *V. radiata* and *A. mongostanus*, respectively.

The parameter k_c is the number of electron equivalents required to reduce 1 molecule of CO_2 in the Calvin cycle. The theoretical minimum value of k_c is 4, but in our study its value was calculated from Eq. (3) using the parameters measured without photorespiration in 3 % O_2 air. k_c determined in 21 % O_2 air is termed k_N.

$$k_c = J_T/P_G \quad (3)$$

where P_G is a sum of $P_N + R_D$. The Eq. (3) is rewritten as Eq. (4) using parameters of RuBP carboxylation rate (V_c) and RuBP oxygenation rate (V_o), and then Eq. (5) is deduced:

$$P_G = V_c - 0.5\ V_0 \quad (4)$$

$$J_T = k_c\ V_c + 0.5\ k_r\ V_0 \quad (5)$$

where k_r is the number of electron equivalents required to release 1 molecule of CO_2 in photorespiration. The equation $J_T = 4\ V_c + 4\ V_0$ proposed by Farquhar *et al.* (1980) has been frequently used in recent studies (Di Martino *et al.* 1999, Miyake and Yokota 2000, Muraoka *et al.* 2000). The number of electrons required to fixing 1 molecule O_2 in the C_2 cycle is between 4 and 6. For instance, Di Marco *et al.* (1994) used the equation of $J_T = 4\ V_c + 6\ V_0$. We used the experimentally determined value as coefficients in Eq. (5).

k_r is estimated from the Eq. (6) that is based on the ratio of 18.5 ATP and 9 ATP, both of which are chemically equivalent energies consumed for release of 1 molecule CO_2 in C_2 cycle and fixation of 1 molecule CO_2 in C_3 cycle, respectively:

$$k_r = k_c\ 18.5/9.0 \quad (6)$$

In C_4 plants, k_r is calculated from Eq. (7) because the CO_2 concentrating mechanism requires additional 2 ATP to convert pyruvate to phosphoenolpyruvate in each turn of the C_4 cycle in the NAD-malic enzyme (NAD-ME) subtype such as *A. mongostanus* (Table 1):

$$k_r = k_c\ 18.5/11.0 \quad (7)$$

When the values of J_T, k_c, V_c, or CO_2 assimilation rate measured in 3 % O_2 air and k_r are substituted in the Eq. (5), R_P can be obtained from Eq. (8):

$$R_{PE} = 0.5\ V_0 \quad (8)$$

The photorespiration ratio based on chemical energy is written as $R_{PE}/P_{N21\%}$, where $P_{N21\%}$ is a value measured in 21 % O_2 air. The R_P determined by subtracting $P_{N21\%}$ from $P_{N3\%}$ is termed R_{PO} here, and in this case the photorespiration ratio is $R_{PO}/P_{N21\%}$.

Results and discussion

Table 1 shows the values of k_c given theoretically and k_r determined experimentally. The theoretical minimum value of k_c is 4.00, and that of NAD-ME subtype is 4.89. It is a little larger than 4.00, because the chemical energy required for fixing 1 molecule of CO_2 in this subtype is equivalent to 11 molecules of ATP compared to 9 ATP molecules in C_3 plants. The theoretical value of k_r, 8.22, is more than two-fold the k_c value because the energy used for releasing 1 molecule CO_2 in photorespiration is equivalent to 18.5 ATP.

The experimentally determined k_c value in *V. radiata* obtained by Eq. (3) was 4.63±0.12 as shown in Table 1, which was higher than the theoretical minimum value of 4.00. According to Björkman and Demmig (1987) the mean value of maximum quantum yields for 37 C_3 species was 0.106 (O_2 intake/photon absorbed), which corresponds to k_c = 4.72 that is calculated on the basis of 1 CO_2 fixation in C_3 cycle. Using a peeled leaf of *V. radiata* without gas exchange restriction, Tokuda *et al.* (1999) obtained 4.62 for k_c, which is close to the value determined here.

In the C_3 plant *V. radiata,* the difference of CO_2 concentration (ΔCO_2) between sample air and reference air showed clear oscillations according to the oxygen concentration change in the air flown to the leaf chamber (Fig. 1). These oscillations mean that P_G increased with a change in O_2 concentration from 21 to 3 %. In contrast to this, Φ_e showed a counter directional change with change in P_G (Fig. 1). The difference of H_2O vapour pressure (ΔH_2O) between sample air and reference air was almost constant. This means that stomatal conductances (g_s) were not significantly different between the measurements in 21 and 3 % O_2. g_s was about 0.3 mmol m^{-2} s^{-1} (Table 2). This g_s is sufficient to perform gas exchange between the ambient air and the leaf of *V. radiata*.

Table 1. The experimental values of electron equivalent to reduce or release in photorespiration 1 CO_2 molecule in *V. radiata* and *A. mongostanus* and the theoretical minimum values in the Calvin cycle and C_4 cycle of NAD-ME type. Means ± SE of three replications. k_c = the number of electron equivalents to reduce 1 CO_2 molecule in the Calvin cycle. k_r = the number of electron equivalents to release 1 CO_2 molecule in photorespiration.

	Theoretical minimum value		Experimental value	
	C_3	C_4	*V. radiata* (C_3)	*A. mongostanus* (C_4)
k_c	4.00	4.89	4.63±0.12	5.05±0.22
k_r	8.22	8.22	9.52	8.49

Zelitch (1971) reported that photosynthetic rate increased by 33 to 50 % in C_3 plants when O_2 concentration of the ambient air decreased from 21 % to 3 or 1 %. Akita (1980) also reported that such an increase in P_N of 40 rice cultivars was 32.5 % and that in 6 dicotyledonous plants it was 40.8 % at a leaf temperature of 30 °C and irradiance of 1 450 μmol m^{-2} s^{-1}. As shown in Fig. 1 and Table 2, the increase in P_N of *V. radiata* by restricting photorespiration in 3 % O_2 air was about 42 %, which was almost similar to the results of Zelitch and Akita.

R_{PE} in *V. radiata* was 7.3±0.4 μmol(CO_2) m^{-2} s^{-1} (Table 2). It was lower than R_{PO} [11.9±0.2 μmol(CO_2) m^{-2} s^{-1}]. $R_{PE}/P_{N21\%}$ was 25.7±0.9 %, lower than $R_{PO}/P_{N21\%}$ (42.2±0.9 %). In wheat, DeVeau and Burris (1989) estimated R_P using the rate of incorporation of $^{18}O_2$ into glycollate. They estimated a photorespiration ratio of 26.9 %, which is similar to the value of 25.7±0.9 % obtained here in *V. radiata*. R_{PO} is inevitably an overestimate of the real R_P performed in the normal air. Under the non-photorespiratory condition such as in air with

3 % O_2, RuBP is not consumed in photorespiration and accumulated in leaf, and this may result in a significant promotion of the carboxylase activity and CO_2 assimilation.

The value of k_N was 8.03 (Table 2), which was about twice the theoretical minimum value ($k_c = 4$) due to the additional requirement of chemical energy by photorespiration. J_T/P_N was 8.6, which was close to the range 8.7 to 10.8 obtained by Krall and Edwards (1992) in wheat under ambient air.

In the C_4 plant *A. mongostanus*, the change in O_2 concentration in the leaf chamber from 21 to 3 % gave almost no increase in ΔCO_2, whereas it significantly affected Φ_e. Increased values of Φ_e were observed in air with 21 % O_2, though the variation range was considerably small compared to that in *V. radiata* (Fig. 1). Nevertheless, this fact may suggest that in this C_4 species a small amount of chemical energy is consumed through photorespiration.

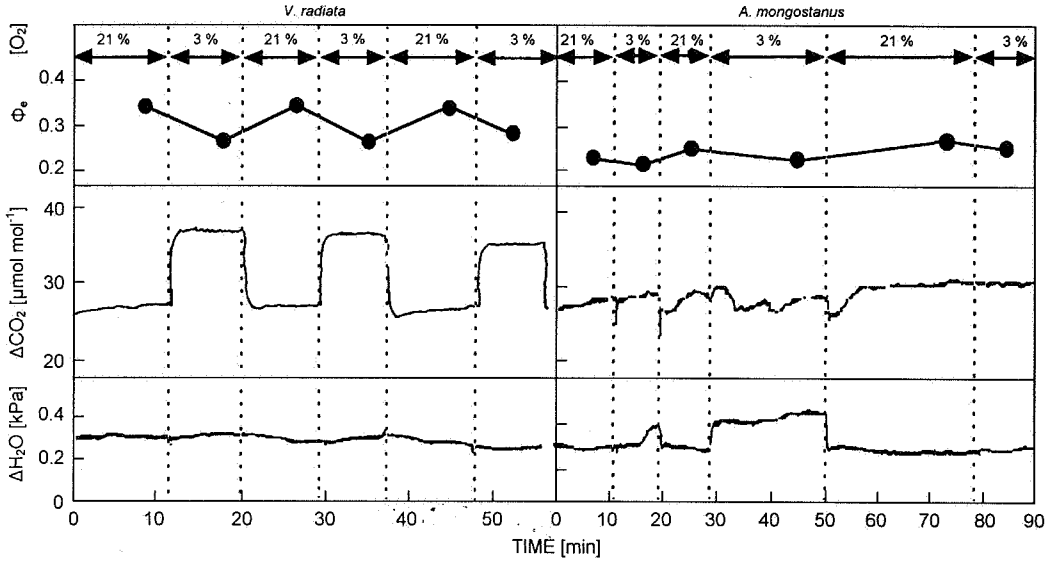

Fig. 1. Time courses of quantum yield of photosystem 2 (Φ_e), the difference of CO_2 concentration (ΔCO_2), and the difference of H_2O vapour pressure (ΔH_2O) between sample air and reference air in *V. radiata* and *A. mongostanus* leaves with alternate changes between 21 and 3 % O_2 in the ambient air at 1 600 μmol m^{-2} s^{-1} PPFD and leaf temperature of 30.2±0.6 °C.

Table 2. Values of parameters related to electron transport, photosynthesis, and photorespiration in *V. radiata* and *A. mongostanus* measured in air containing 3 and 21 % O_2. For symbols see the list of abbreviations.

	V. radiata (C_3)		*A. mongostanus* (C_4)	
	21 % O_2	3 % O_2	21 % O_2	3 % O_2
Φ_e	0.326±0.004	0.265±0.004	0.249±0.011	0.229±0.011
k_c	-	4.63±0.12	-	5.05±0.22
k_N	8.03±0.15	-	5.56±0.20	-
J_T [μmol m^{-2} s^{-1}]	243.8±3.1	198.2±2.7	181.5±7.9	167.5±8.8
P_N [μmol(CO_2) m^{-2} s^{-1}]	28.3±0.6	40.2±0.7	31.6±0.3	32.0±0.9
J_T/P_N	8.61±0.19	4.93±0.15	5.74±0.21	5.23±0.23
P_G [μmol(CO_2) m^{-2} s^{-1}]	30.3±0.5	42.8±0.6	32.7±0.3	33.1±0.5
g_s [mol(CO_2) m^{-2} s^{-1}]	0.303±0.009	0.300±0.012	0.298±0.006	0.353±0.054
V_c [μmol(CO_2) m^{-2} s^{-1}]	37.6±0.8	42.8±0.6	33.9±0.1	33.1±0.9
V_0 [μmol(CO_2) m^{-2} s^{-1}]	14.6±0.8	-	2.41±0.47	-
R_{PE} [μmol(CO_2) m^{-2} s^{-1}]	7.3±0.4	-	1.2±0.2	-
R_{PE}/P_N [%]	25.7±0.9	-	3.8±0.8	-
R_{PO} [μmol(CO_2) m^{-2} s^{-1}]	11.9±0.2	-	0.43±1.00	-
R_{PO}/P_N [%]	42.2±0.9	-	1.4±3.3	-

The value of k_c in *A. mongostanus* was 5.05±0.22 (Table 2), a little higher than a theoretically determined minimum value of 4.89 in C_4 plants (Table 1). The value of 5.05±0.22 was slightly lower than the value of 5.20 determined by Krall and Edwards (1992) in maize in air at 80 Pa CO_2 and 3 % O_2 under a high irradiance of 1 800

μmol m^{-2} s^{-1} PPFD. The value of k_N was 5.56±0.20, which was higher than k_c (5.05±0.22) in *A. mongostanus*. This is the evidence that chemical energy consumption increases with the increase in O_2 concentration in air, and this may suggest the functioning of photorespiration in this species.

The photorespiration ratio, $R_{PE}/P_{N21\%}$, was 3.8 % in *A. mongostanus* (Table 2). Based on rates of incorporation of $^{18}O_2$ into glycollate, the photorespiration ratio in maize was 3 % in mature leaves and 11 % in young seedlings (deVeau and Burris 1989). Furthermore, the photorespiration ratio estimated from the model of C_4 photosynthesis by Jenkins *et al.* (1989) was also similar to that shown here for *A. mongostanus*. According to Ueno (2001), RuBPCO functions not only in bundle sheath cells but also in ordinary epidermal cells, guard cells, and companion cells, whereas phosphoenolpyruvate carboxylase was not detected in ordinary epidermal cells and parts of guard cells in *Amaranthus edulis*. Hence photorespiration may be, though slightly, constantly functioning in this species.

In the C_4 species *A. mongostanus*, Chl fluorescence is emitted in mix from chloroplasts included both in mesophyll cells and in bundle sheath cells. Chl fluorescence emission in C_4 plants was already determined by Mayne *et al.* (1975) using isolated chloroplasts from different C_4 subtypes. They found a functional Chl activity ratio of 27 : 73 for mesophyll : bundle sheath in NAD-ME subtypes such as *A. mongostanus*. More detailed studies were conducted on Hill reaction and PS2 potential in C_4 species by Ku *et al.* (1974) and Edwards *et al.* (1976).

In C_4 species, as mentioned above, Φ_e calculation is based on the total value of Chl fluorescence emitted from the two different tissues. If C_4 and C_3 cycles work independently consuming chemical energies produced by mesophyll cells and bundle sheath cells, respectively, it may be difficult to determine the value of k_c in *A. mongostanus*. However, the k_c value may be determined from such a mixed value of Chl fluorescence in C_4 plants, because the chemical energy produced in mesophyll cells and bundle sheath cells is available in common for the use in C_3 cycles in bundle sheath cells through a chemical energy shuttle. Hatch (1987) and Leegood (1997) demonstrated the shuttle for chemical energy transport between mesophyll and bundle sheath. In this shuttle a part of phosphoglyceric acid transported from the bundle sheath to mesophyll was reduced by NADPH in mesophyll, and triose phosphate formed from phosphoglyceric acid was returned to the bundle sheath to maintain pools of C_3 cycle intermediates in all three subtypes of C_4 species.

We conclude that not only photorespiration rate in C_3 species can be more correctly evaluated but also a low rate of photorespiration in C_4 species can be detected based on the chemical energy production and consumption balance using the Chl fluorescence quenching diagnosis. The values obtained by this method are useful to deepen understanding of the functional role of photorespiration *in situ*, and the accumulation of data becomes valuable in elucidating the specific and variety features in growth sustainability and stress tolerance in relation to the dissipation of excessive energy in crops.

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