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Effects of Leaf Epidermis Peeling on the CO_2 Exchange Rate and Chlorophyll Fluorescence Quenching, and Estimation of Photorespiration Rate from Electron Transport in Mungbean (*Vigna radiata* (L.) Wilczek) Leaves.

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CO_2 gas exchange rates (CER) in a peeled leaf (PL) and nonpeeled leaf (NL) of mungbean (*Vigna radiata* (L.) Wilczek) were measured in a saturating light intensity to identify their responses to CO_2 concentrations ($[CO_2]$) and leaf temperatures. The CER of PL was regarded as an indicator for the photosynthetic potential in mesophyll. When measured at 35 °C, CER was enhanced by peeling the epidermis, whereas it was less effective at 25 °C and almost not effective at 15 °C. In $[CO_2]$–CER response curves, CER of PL had a highest plateau in 500 to 1,000 μmolmol^{-1} $[CO_2]$ at 35 °C, whereas at both 25 and 15 °C CER showed a peak in 500 μmolmol^{-1} $[CO_2]$. When measured in the air of 20% O_2 concentration ($[O_2]$) in a light intensity of 300 μmolm^{-2}s^{-1} photosynthetically photon flux density (PPFD) at 25 °C, the CO_2 quantum yield (Φ_{CO2}) in PL was higher than that in NL, whereas the photosystem II (PSII) quantum yield (Φ_e) was a little lower in PL. The number (k) of electron equivalents required to fix 1 mol CO_2 was greatly increased in NL due to the increased photorespiration. In 2% $[O_2]$ k of PL was 4.62, which was a little larger than a theoretical minimum (k=4). From the relationships between k, CER and adenosine 5'–triphosphate (ATP) required for photosynthesis, the rates of photorespiration were estimated in different environments.

INTRODUCTION

Mungbean, *Vigna radiata* (L.) Wilczek, is positioned as one of the important protein providing foodstuffs in the tropical and subtropical countries. Many of the studies dealing with mungbean have been conducted mainly on the productivity of field–grown cultivars, but little information is available for physiological aspects in photosynthesis. Accumulating data of the photosynthetic characteristics is necessary as basic information for genetic and physiological improvement of production.

The stomatal resistance is one of the restricting factors for gas exchange, and by removing the resistance the mesophyllic activity in photosynthesis can be determined. Experimentally this activity is known by measuring CO_2 exchange rate (CER) of a leaf the epidermis of which has been peeled (Kubota *et al.*, 1991 and 1992). The investigation of mesophyllic activity controlled by environmental factors is expected to offer many suggestions for understanding the photosynthetic characteristics of this crop. To clarify the responses of the mesophyllic activity to CO_2 concentration ($[CO_2]$) and leaf temperature is one of the subjects in this study.

Photosynthesis consists of two categories in energy balance, energy production by

photosystem and its consumption by CO_2 assimilation, and the situation of electron transport from photosystem to CO_2 assimilation site is diagnosed by the chlorophyll fluorescence quenching analysis (Schreiber *et al.*, 1986, 1998). When peeled leaves are used for the diagnosis, the relationship between CO_2 assimilation and electron transport should be more clearly interpreted.

The relationships between photosynthesis and chlorophyll fluorescence quenching in leaves were discussed by Genty *et al.* (1989), Marco *et al.* (1990), Krall *et al.* (1990, 1991a, 1991b) and Harbinson *et al.* (1990). In our study the effect of peeling the epidermis or removing the stomatal resistance on CER, photorespiration and electron transport in photosystem II (PSII) were investigated in relation to leaf temperature, $[CO_2]$ and O_2 concentration ($[O_2]$). On the basis of CER and electron transport, photorespiration rates were estimated in different environments, and the role of photorespiration and its usefulness as a selection criterion in mungbean breeding were discussed.

MATERIAL AND METHODS

A mungbean cultivar, Chinese, was used as the experimental material. Two plants were grown in a 8-L pot filled with sandy soil in the experimental field of Kyushu University (33° 35' N, 130° 23' E). Seeds were sown on June 26 in 1997. A compound chemical fertilizer (N:P:K=16:16:16 in %) was applied 2 g/pot two weeks after germination of seeds. Full expanded young leaves excised from plants at the pre-flowering stage in the mid summer were used for measurements.

Measurement of CER

A leaf, the abaxial epidermis of which was peeled, is termed here PL, and an nonpeeled leaf is NL. CERs in PL and NL were measured at leaf temperatures of 15, 25 and 35 °C in the air of different $[CO_2]$ levels from 50 to 2,000 μmolmol^{-1}.

A closed system assimilation chamber (LD-2, Hansatech, UK) was partly remodeled and used here as an open system chamber to measure CER of a detached leaf (Fig. 1). The sampled air was monitored with an infrared CO_2 analyzer (LI-6262, LI-COR, USA). The air flow speed was 0.4 Lmin^{-1} and the reading lag-time of the measurement system was 4 sec.

Water temperature in the water tanks equipped on the chamber (Fig. 1) was adjusted with a water temperature controller (CTER42WS, Komatsu-Yamato, Japan). A light of 2,000 μmolm^{-2}s^{-1} photosynthetically photon flux density (PPFD) was illuminated to a leaf disc (2.4 cm in diameter), and CER of the abaxial side in both PL and NL was measured. As a light source a slide projector (HILUX-HR, RIKAGAKU, Japan) with a 1 kw halogen lamp was used.

To prevent a leaf disk from drying, the air pumped into the chamber was moistened over 85% in relative humidity using a dew point controller (L-610, LI-COR, USA). The air sampled from the chamber was dehumidified by a dry agent (magnesium perchlorate) before it was sent into the CO_2 analyzer.

$[CO_2]$ in the air was adjusted by mixing N_2, O_2 and CO_2 with a gas mixer (GM-3A, KOFLOC, Japan), and a CO_2 absorbent (soda lime) was used for the fine adjustment of $[CO_2]$. After the full openness of stomata was confirmed with NL, the CER was measured

in 50 to 2,000 μmolmol^{-1} [CO_2]. The response of CER in PL was also measured at the same levels of [CO_2].

Simultaneous measurement of chlorophyll fluorescence and CER

The intensity of chlorophyll fluorescence at PSII was monitored with a portable fluorescence measurement system (MFMS-2T, Hansatech, UK). A leaf disc was fixed in the assimilation chamber (Fig. 1) and then placed in the dark for 30 min before fluorescence measurement in order to make the plastoquinone completely oxidized. After the dark treatment, an extremely weak measuring beam (3.2 μmolm^{-2}s^{-1} PPFD, 4.8 kHz) was applied to determine the initial fluorescence (F_o) of the leaf of unphotosynthetic situation. Then a 2 sec pulse illumination of 1,800 μmolm^{-2}s^{-1} PPFD was given to read the maximum fluorescence (F_m).

After this, the leaf was redarkened for 2 min, and then a red actinic light (300 μmolm^{-2}s^{-1} PPFD) was continuously illuminated to let photosynthetic function start. Time courses of fluorescence (F_s) and CER were simultaneously monitored for 10 min, during which fluorescence peaks (F_m') were measured by giving pulses of white saturation light at 20 sec intervals in the first 3 min and then at 30 sec intervals in the following 7 min. These were measured at 25 °C in the air of 2 and 20% [O_2]. The levels of [O_2] were adjusted with the gas mixer described above.

Based on measurements of fluorescence, non photochemical quenching (NPQ), quantum yield (Φ_e) at PSII and CO_2 quantum yield (Φ_{CO2}) were calculated from the

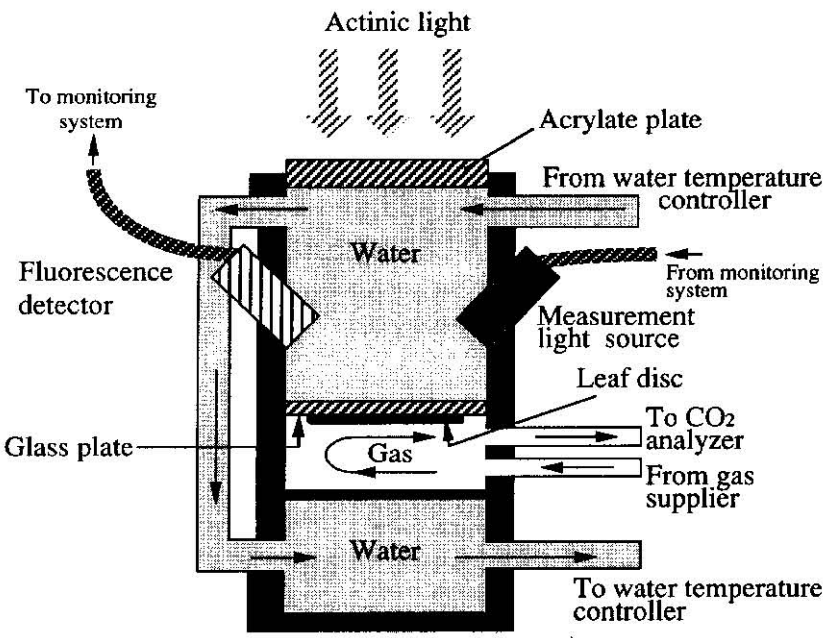

Fig. 1. Diagram of the measurement system of CO_2 assimilation and chlorophyll fluorescence.

equations (1), (2) and (3), respectively.

$$NPQ = (F_m - F_m') / F_m' \quad \cdots\cdots\cdots\cdots (1)$$

$$\Phi_e = (F_m' - F_s) / F_m' \quad \cdots\cdots\cdots\cdots (2)$$

$$\Phi_{CO2} = CER / (L \times i) \quad \cdots\cdots\cdots\cdots (3)$$

where L is a light intensity supplied to a leaf disk, L=300μmolm^{-2}s^{-1} PPFD is used here; i is the ratio of the photon absorbed by chlorophyll to the incident photon, i=0.8 is used.

The equation (4) represents the relationship between CER and Φ_e (Genty *et al.*).

$$CER = \Phi_e \times 0.5 \times L \times i / k \quad \cdots\cdots\cdots\cdots (4)$$

Assuming the photon is evenly distributed to the two photosystems, 0.5 is used in the equation (4). The parameter k is the number of electron equivalents required to reduce 1mol CO_2 and calculated from the equation (5).

$$k = \Phi_e \times 0.5 \times L \times i / CER \quad \cdots\cdots\cdots\cdots (5)$$

RESULTS AND DISCUSSION

[CO_2]–CER response curves in PL and NL detached from plants grown in the mid summer were determined at 15, 25 and 35 °C, and shown in Fig. 2. CERs of both leaves at 35 °C linearly increased with [CO_2] from 50 to 350μmolmol^{-1}; however, at a higher level of [CO_2] than 350μmolmol^{-1} CER of PL was higher than that of NL, and had a highest plateau of 26.0 to 26.8μmolm^{-2}s^{-1} and 21.0 to 21.5μmolm^{-2}s^{-1} in 500 to 1,000μmolmol^{-1} [CO_2] in PL and NL, respectively. Islam *et al.* (1994) suggested that maintaining stomatal aperture was the primary step toward the improvement of CER of mungbean cultivars. Also our results demonstrate that if the stomatal resistance is removed, the CER significantly increases with an increase in [CO_2]. But at a higher level above 1,000μmolmol^{-1} [CO_2], CER slightly decreased. As a reason for this, it is considered that the reproduction rate of substrate ribulose bisphosphate (RuBP) becomes a limiting factor to photosynthesis, and also the excessive supply of substrate CO_2 is likely one of the inhibitors to the mesophyllic activity.

CER decreased with decreasing leaf temperature from 35 to 15 °C (Fig. 2). The peaks of CER in NL appeared at a [CO_2] of 500μmolmol^{-1}, showing 18.2 and 13.4μmolm^{-2}s^{-1} at 25 and 15 °C, respectively, and those of PL were 22.0 and 13.7μmolm^{-2}s^{-1}, respectively. The results in Fig. 2 indicate that the epidermis peeling was less effective on CER at 25 °C and not effective at 15 °C. At low temperatures the leaves had a peak of CER at a lower level of [CO_2], the fact of which suggests that enzymes, such as Rubisco, related to CO_2 assimilation are inactivated at low temperatures so that the mesophyll photosynthetic activity may be saturated at a comparatively low [CO_2] level.

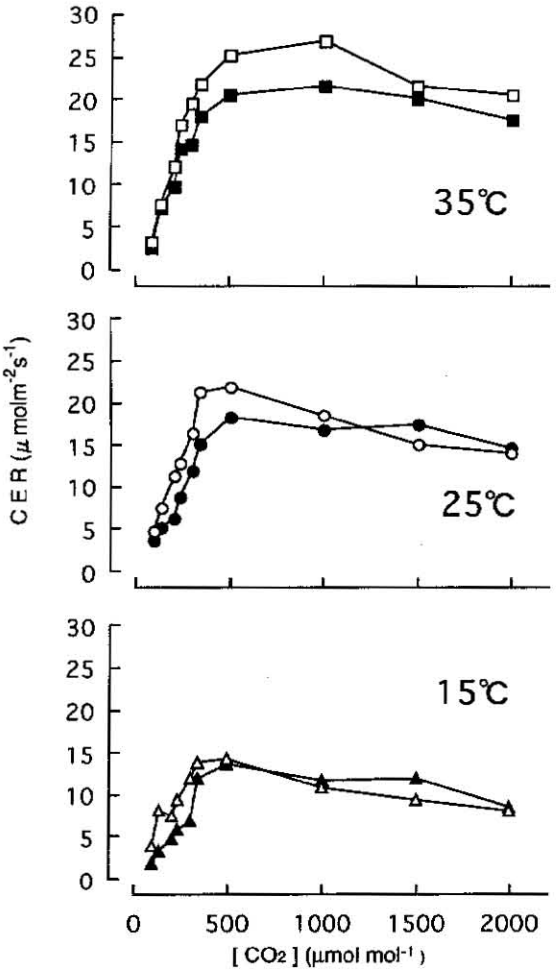

Fig. 2. Responses of CER to $[CO_2]$ in PL (open) and NL (closed) measured at different leaf temperatures in a light intensity of 2,000 μmol $m^{-2}s^{-1}$ PPFD.

CER in C_3 plants is obtained as a balanced value between CO_2 assimilation by RuBPCarboxylase (RuBPCase) and CO_2 release (photorespiration) by RuBPOxygenase (RuBPOase). The intercellular CO_2 concentration (Ci) of leaf adjusts the relative activity of carboxylase and oxygenase, by which CER and electron transport situation are changed. Ci varies with stomatal resistance.

In Table 1, CER, NPQ, Φ_e, Φ_{CO2} and k at 35 °C are compared between PL and NL. All these parameters were shown as averages during the photosynthetically steady state. CER of NL, 4.39 μmolm^{-2}s^{-1}, was 45.5% of that (9.65 μmolm^{-2}s^{-1}) of PL, but Φ_e was 16.3% higher in NL (0.487) than in PL (0.419). The calculated value of k=13.31 in NL was about

Table 1. Effects of the leaf epidermis peeling on CER, NPQ, Φ_{CO_2}, Φ_e, and k. The measurement condition; 350 μmolmol^{-1} [CO_2], 20% [O_2], 300 μmolm^{-2}s^{-1} PPFD and 35 °C.

	CER (μmolm^{-2}s^{-1})	NPQ	Φ_{CO_2}	Φ_e	k
NL	4.39	0.470	0.018	0.487	13.31
	(45.5)	(136.2)	(45.0)	(116.3)	(255.5)
PL	9.65	0.345	0.040	0.419	5.21

PL, peeled leaf; NL, nonpeeled leaf; ( ), % of NL/PL

2.6-fold higher than that (5.21) of PL. This may be caused by the additional energy requirement by the increase in photorespiration rate in NL. NPQ (0.470) in NL was 36.2% higher than that of PL, the fact of which may suggest that a relatively large portion of the total energy was dispersed as heat or nonphotochemical quenching in NL because the CO_2 assimilation was restricted by stomatal resistance.

The value, k=5.21 shown in PL, is not so different from the theoretical minimum of k=4. This may suggest that the epidermis peeling is effective in promoting gas exchange and reducing photorespiration. If Ci comes close to the ambient [CO_2] level of 350 μmolmol^{-1} by removing the stomatal resistance, the CO_2 fixation is presumed to reach a nearly saturating level as predicted from Fig. 2.

Fig. 3 (A, B and C) represents the time courses of CER, NPQ and Φ_e measured with PL at 25 °C in 20 and 2% [O_2]. CER quickly increased with time at the initial phase (Fig. 3A). Kobza and Edwards (1987) reported that RuBPCarboxylase-Oxygenase (Rubisco) in *Triticum aestivum* L. was light-activated within 1 min but the time required for reaching the steady state of photosynthesis was more than several minutes. It was also confirmed that the initial slope of CER in PL had a close relation with the light activation of Rubisco (Kubota *et al.*, 1994). In Fig. 3A the initial slope in 2% [O_2] indicates the light activation of RuBPCase, whereas that in 20% [O_2] is shown as a joined value of RuBPCase and RuBPOase activations. The time required to reach the full activation was different in 2 and 20% [O_2]; it was less than 200 sec in the former and about 300 sec in the latter. Saturating values of CER in 2 and 20% [O_2] were 12.50 and 7.88 molm^{-2}s^{-1}, respectively (Fig. 3A), and both NPQ and Φ_e were a little higher in 20% [O_2] (Fig. 3B, C).

CER, NPQ, Φ_e, Φ_{CO_2} and k in PL measured in 2 and 20% [O_2] are shown in Table 2. CERs in 20 and 2% [O_2] are termed $CER_{20\%}$ and $CER_{2\%}$ here, respectively. $CER_{20\%}$ of 7.88 μmolm^{-2}s^{-1} shown in Table 1 was 63.0% of 12.50 μmolm^{-2}s^{-1} of $CER_{2\%}$. Contrary to this, the value of k, 7.75, in 20% [O_2] was significantly higher than that (4.62) in 2% [O_2]. The photorespiration in 20% [O_2] is considered as one of the causes of increasing the value of k, and if photorespiration is completely restricted, the theoretical minimum of k is 4. The value k=4.62 obtained here in 2% [O_2] is 16% higher than the theoretical one.

Nine mol adenosine triphosphate (ATP) is consumed in the Calvin cycle in which 1 mol CO_2 is fixed by RuBPCase to produce 2 mol 3-phosphoglycerate (PGA) at the first step and 1 mol RuBP is reproduced at the final step. On the other hand, 1 mol CO_2 is

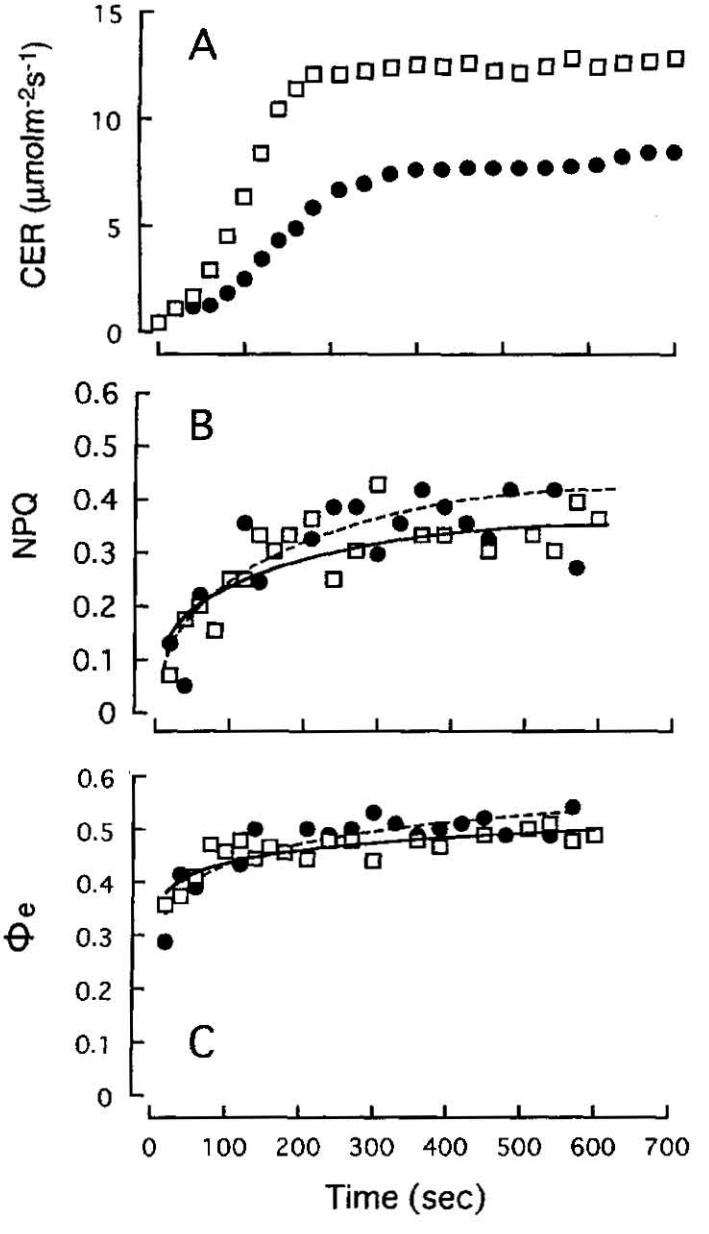

Fig. 3. Time courses of CER (A), NPQ (B) and Φ_e (C) in PL determined in the air of 2% [O_2] (open) and 20% [O_2] (closed). The other measurement conditions; 25 °C, 350 μmol mol^{-1} [CO_2] and 300 μmolm^{-2}s^{-1} PPFD.

Table 2. CER, NPQ, Φ_{CO_2}, Φ_e and k of PL determined in 2% [O_2] and 20% [O_2]. The measurement condition; 350 μmolmol^{-1} [CO_2], 300 μmolm^{-2}s^{-1} PPFD and 25 °C.

	CER (μmolm^{-2}s^{-1})	NPQ	Φ_{CO_2}	Φ_e	k
20% [O_2]	7.88 (63.0)	0.361 (103.4)	0.33 (63.5)	0.509 (105.9)	7.75 (167.8)
2% [O_2]	12.50	0.349	0.052	0.481	4.62

( ); % of 20% [O_2] / 2% [O_2]

released by photorespiration after 3 mol PGA has been produced by RuBPOase, and reproduction of 1.5 mol RuBP from this 3 mol PGA requires 13.5 mol ATP in chloroplasts (Oliver, 1998). In the biochemical reaction of fixing 1 mol CO_2, k=4 is required theoretically, and 9 mol ATP is consumed. On the other hand, the photorespiration (1 mol CO_2 release) consumes 13.5 mol ATP which is 1.5 times that required for 1 mol CO_2 fixation. If k has a parallel relation with the amount of ATP, k=6 (=4×1.5) is given for photorespiration.

The photorespirationless CER determined in 2% [O_2] is expressed as the equation (6).

$$CER_{2\%} = \Phi_{e2\%} \times L_0 / 4 \quad \cdots\cdots\cdots\cdots (6)$$

where $\Phi_{e2\%}$ is a value of Φ_e determined in 2% [O_2], and L_0=0.5×L×i.

In the natural air condition where the photorespiration is performed, CER is expressed as the equation (7).

$$CER = \Phi_e \times L_1 / 4 - \Phi_e \times L_2 / 6 \quad \cdots\cdots\cdots\cdots (7)$$

$$L_0 = L_1 + L_2 \quad \cdots\cdots\cdots\cdots (8)$$

where L_0 is shared between L_1 and L_2 for CO_2 fixation and photorespiration, respectively.

Although the experimental value of k=4.62 (Table 2) is a little larger than the theoretical k=4, assuming here that k=4.62 is the fundamental value to fix 1 mol CO_2, a value of k required for photorespiration is predicted as 6.93 (4.62×1.5) or 6.62 (4×1.5+0.62). When k=6.93 is used, the equation (8) is rewritten as the equation (9).

$$CER = \Phi_e \times L_1 / 4.62 - \Phi_e \times L_2 / 6.93 \quad \cdots\cdots\cdots\cdots (9)$$

By substituting CER and Φ_e shown in Table 1 and 2 into the equation (9), and L_0=120 (=300×0.5×0.8) μmolm^{-2}s^{-1} into the equation (8), the total CO_2 fixation rate ($\Phi_e \times L_1$/4.62), photorespiration rate ($\Phi_e \times L_2$/6.93) and the photorespiration ratio (Photorespiration rate/Total CO_2 fixation rate) are calculated and listed up in Table 3. The highest photorespiration rate and ratio, 3.30 μmolm^{-2}s^{-1} and 42.9%, are estimated in NL in 20%

Table 3. Estimated rates of photorespiration and total fixed CO_2 of PL based on the data listed in Table 1 and 2.

	$[CO_2]$ (μmolmol^{-1})	$[O_2]$ (%)	°C	Φ_{e}	k	CER	TC	PR	PR/TC (%)
						(μmolm^{-2}s^{-1})			
NL	350	20	35	0.487	13.31	4.39	7.70	3.30	42.9
PL	350	20	35	0.419	5.21	9.65	10.14	0.49	4.9
PL	350	20	25	0.509	7.75	7.88	10.02	2.14	21.3
PL	350	2	25	0.481	4.62	12.50	12.50	0	0

TC, Total CO_2 fixation rate; PR, Photorespiration rate, PR/TC; Photorespiration ratio.

$[O_2]$. Reduction in these values of PL in 20% $[O_2]$ is considered to depend on the increase in Ci by epidermis peeling. The photorespiration ratio in PL measured at 35 °C in 20% $[O_2]$ is 4.9%, which is a considerably low compared to that (21.3%) in PL at 25 °C. The epidermis peeling promotes gas exchange and hence the photorespiration is restricted as mentioned above. However, as shown in table 3, the strength of the restriction is considerably different among the individual measurements of PL. As a reason for this it may be considered that the relative humidity in the assimilation chamber, though being kept over 85% here, was not controlled so rigorously, so that change in air moisture could affect the evapotranspiration and Ci.

The photorespiration in leaf is estimated from the electron transport according to the method described above, but a further examination is necessary to determine more adequate values of k and also to make more rigorous the environmental control of the measurement system. The cyclic electron transport from PSI and Mehler reaction are not taken into account here for the sake of simplicity. Also because it may be considered that these functions are less operative in our experimental condition where the light intensity used is not so high (300μmolm^{-2}s^{-1} PPFD).

In general, photorespiration rate is estimated by subtracting $CER_{20\%}$ from $CER_{2\%}$. However the calculated in this manner is not thought to represent the real rate of photorespiration in 20% $[O_2]$. The calculation, as tried here, based on electron transport may be considered to have a better consistency to the real photorespiration rate than that obtained by the subtraction ($CER_{2\%}$–$CER_{20\%}$). The obtained values are expected to be a useful indicator for evaluating the photorespiratory traits among genotypes.

To maintain stomatal aperture and raise mesophyllic activity are essential for improving photosynthesis of leaves grown in adequate growth environments. But such a C_3 plant as mungbean grown in the tropics frequently closes stomata by being subjected to water deficits in strong solar radiation and high temperature. If CO_2 assimilation is restricted in high light intensities, the chemical energy produced by photosystem is accumulated in chloroplasts, which becomes a cause of damaging the photosynthetic apparatus. Under the condition like this, dispersing the accumulated energy is an indispensable role of photorespiration.

Cultivars having a high photorespiratory ability is considered to disperse effectively excessive energy in leaf, the function of which allows leaves to retard senescence and maintain a photosynthetic activity for a longer time. However, having a high photores-

piration rate concurrently means a large production loss by CO_2 release. In order to utilize the genetic variation in photorespiratory characteristics as an effective selection criterion in breeding, it is necessary to deepen the study on the relative strength of its positive and negative effects on mungbean production in relation to the growth conditions.

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