Contribution of diffusional and non-diffusional limitations to the midday depression of photosynthesis which varies dynamically even under constant environmental conditions**

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Abstract. Both diffusional and non-diffusional limitation factors and their contribution to the phenomenon known as midday depression were studied; a decrease in photosynthesis due to environmental stress. Measurements of leaf gas exchange (transpiration rate, stomatal conductance, photosynthetic rate) and related parameters (intercellular CO_2 concentration, maximum carboxylation, diffusional limitation index, leaf water potential) were determined over time from cucumber plants under constant environmental conditions. The experiment may be divided into three periods. Period 1 occurred when both photosynthetic rate and stomatal conductance increased, period 2 occurred when photosynthetic rate decreased but stomatal conductance remained constant, period 3 occurred with the decrease in both photosynthetic rate and stomatal conductance. Photosynthetic rate increased immediately, post-illumination, and reached its maximum value during period 1, then it decreased to half this value for the remainder of the experiment, indicating that a midday depression had occurred. During period 2, diffusional limitation index was around 50% and then increased while maximum carboxylation sharply decreased, suggesting the existence of both non-diffusional and diffusional limitations. In period 3, stomatal conductance decreased due to decreasing leaf water potential, and diffusional limitation index increased, suggesting that diffusional limitation was dominant at this stage. Thus, it was found that there are multiple dominant factors in midday depression, and that these factors are dynamic even under constant environmental conditions.

Keywords: environmental stress, intercellular CO_2 concentration, photosynthetic rate, stomatal conductance

INTRODUCTION

On sunny days, when there is a sufficient amount of solar radiation, photosynthetic activity often drops around midday. This phenomenon is known as the "midday depression of photosynthesis (hereafter: midday depression)", which is caused by various environmental factors such as water deficit, high temperature, and excessive sunlight (Roessler and Monson, 1985; Raschke and Resemann, 1986; Hirasawa *et al.*, 1989). As photosynthesis is the fundamental physiological reaction for crop production, midday depression should be avoided to reduce yield loss. In greenhouse crop production, environmental control systems are used to improve crop growth and alleviate environmental stresses (He *et al.*, 2007; Hidaka *et al.*, 2016). In order to operate the environmental control systems more effectively and efficiently to avoid midday depression, it is necessary to understand the mechanism of midday depression. However, on a sunny day, when midday depression occurs, multiple environmental stressors such as high temperature, high evaporative demand, and high light levels tend to occur simultane-

ously. In this case, midday depression would be caused by multiple physiological processes such as stomatal closure (Kitano *et al.*, 1993; Hirasawa and Hsiao, 1999), a reduction in photosynthetic enzyme activity (Liu and Huang, 2008), and photoinhibition (He *et al.*, 2007), which makes it more difficult to understand the mechanism of midday depression.

The midday depression of photosynthesis has two limiting factors: diffusional and non-diffusional. Diffusional limitation refers to CO_2 uptake, which is determined in part by the conductivities of stomata, the path through the mesophyll cells inside the leaf, and the leaf surface boundary layer. A low conductivity of stomata (decrease in stomatal conductance) was often observed during the midday depression (Roessler and Monson, 1985; Raschke and Resemann, 1986; Yokoyama *et al.*, 2018), and therefore it should be regarded as one of the main factors behind midday depression. The other contributing limitations (*e.g.*, photoinhibition and sugar accumulation) are collectively referred to as non-diffusional limitations. Photoinhibition, excess light-induced damage to the photosynthetic electron transport system, can reduce the rates of electron transfer and carboxylation as performed by Rubisco (Hu *et al.*, 2009). The accumulation of sugar in the leaves can suppress the exchange of phosphate translocators in the chloroplast envelope membrane, but it is difficult to confirm to what extent this occurs without using methods which are tissue destructive (Araya *et al.*, 2006). There is a persistent debate over the limitations to midday depression (Grassi *et al.*, 2005), which have not been completely elucidated as yet due to the many challenges associated with evaluating the individual impacts of these limitations and their changes over time.

In the present study, a basic experiment was conducted to analyse the midday depression of photosynthesis dynamically with regard to diffusional and non-diffusional limitations in cucumber plants under constant environmental conditions, thereby simplifying plant response and analysis.

MATERIALS AND METHODS

Cucumber (*Cucumis sativus* L. cv. 'Natusuzumi') seeds were sown in plastic pots (9.5 cm in diameter and 10 cm in height) at a rate of one seed per pot, and filled with vermiculite at the beginning of September 2017. After germination, the pots were kept in an experimental greenhouse located at the Faculty of Agriculture, Kyushu University (N 33°37', E 130°25') and maintained with an air temperature of 20-27°C during the day and 14-18°C at night, the environment was controlled by a heat pump in addition to ventilation built into the roof and side windows. The plants were kept moist through the pots being placed in a container where water was always stored at a water level of <1 cm, with tap water until the cotyledons developed, after which they were grown with a standard nutrient solution

(Otsuka AgriTechno Co. Ltd., Japan) with an electrical conductivity of 2.0 dS m^{-1}. The nutrient solution contained 17.1 mmol (NO_3^-) L^{-1}, 1.1 mmol (PO_4^{3-}) L^{-1}, 1.6 mmol (SO_4^{2-}) L^{-1}, 8.4 mmol (K^+) L^{-1}, 1.5 mmol (Mg^{2+}) L^{-1}, and 3.9 mmol (Ca^{2+}) L^{-1}.

All of the measurements were conducted on the 14th and 15th October, 2017 in the lab at the Faculty of Agriculture, Kyushu University. Upon reaching the 4th leaf stage, the transpiration rate (Tr), stomatal conductance (g_s) and photosynthetic rate (*A*) were measured under constant conditions for the 3rd fully expanded leaf of each plant (n = 3) using a portable leaf chamber system (LI-6400, LI-Cor Biosciences, USA). Lighting was provided by 10 LEDs (LLM031, Stanley, Japan) with a photosynthetic photon flux density (PPFD) of 1806 ± 133.2 μmol m^{-2} s^{-1} at the leaf surfaces. The LED light was white, and the peaks of the spectral distributions appeared at wavelengths of 450 and 550 nm (Yoneda *et al.*, 2020). The air temperature and vapour pressure deficit were maintained at 25°C and 2.5 kPa, respectively, which was identical to the ambient conditions in the laboratory. Moreover, the reference CO_2 concentration of the chamber was maintained at 400 μmol mol^{-1}. The rate of gas exchange was measured under dark conditions for 30 min and under a PPFD of 1806 ± 133.2 μmol m^{-2} s^{-1} for 8 h and every 30 min afterwards.

Chlorophyll autofluorescence was also assessed in the 3rd expanded leaf from each plant, using a portable fluorometer (MINI-PAM, Walz, Effeltrich, Germany). The photochemical efficiency of the photosystem II (F'_V/F'_M) was evaluated by measuring the steady-state fluorescence (*F*) and maximum fluorescence (F'_M) during a light-saturating pulse under experimental conditions using the following formula (Genty *et al.*, 1989):

$$F'_V/F'_M = \frac{F'_M - F}{F'_M}. \tag{1}$$

Thirty minutes prior to and 8.5 h after illumination, the basic (F_O) and maximum (F_M) fluorescence was measured, and the difference between them (F_V) was calculated. The maximum fluorescence of the dark-adapted leaves for 2 h was then calculated in terms of $F_V/F_M = (F_M - F_O)/F_M$.

The leaf water potential (Ψ_W) was measured using a pressure chamber (Model670, PMS, USA) at the start and end of the lighting period (0 and 8 h from the start of actinic light application). Note that the plants for the Ψ_W measurements were prepared separately from those for gas exchange measurement .

The one-point-method, described by Kauwe *et al.* (2016), was used to determine the maximum carboxylation rate (V_{CMAX}) of Rubisco (n = 3), which was calculated using the following equation:

$$V_{CMAX} = A \left(\frac{c_i - K_m}{c_i - \Gamma^*} - 0.015 \right), \tag{2}$$

where: C_i is the intercellular CO_2 concentration, K_m is the Michaelis-Menten constant, Γ^* is the CO_2 compensation point. K_m and Γ^* characterize the gas exchange rate of the leaf and were estimated as in Bernacchi *et al.* (2001). In order to satisfy the assumption of the one-point method that the photosynthetic rate is below Rubisco limitation, experimental leaves were exposed to light-saturated conditions with a PPFD of 1806 ± 133.2 µmol m^{-2} s^{-1}.

The index of the diffusional limitation (*IDL*) to photosynthesis (the inhibition of CO_2 uptake) was evaluated using the following equation (Muraoka *et al.*, 2000) (n = 3):

$$IDL = 100 \left(1 - \frac{A}{A_{400}} \right). \qquad (3)$$

A_{400} is the photosynthetic rate calculated by applying the assumption that the CO_2 concentration in the chloroplast is 400 µmol mol^{-1} and was estimated by incorporating measurements of T_L and F'_V/F'_M at 30 min intervals in Eq. (3), according to Farquhar *et al.* (1980).

The measurement under lighting experiment was divided into 3 periods according to the transition of A and g_s (this is elaborated below), which could become indices of the midday depression. The difference in leaf gas exchange characteristics (A, Tr, g_s, C_i, F'_V/F'_M, F_V/F_M, V_{CMAX}, *IDL*, and Ψ_W) between the different measurement periods were analysed using the Student's *t*-test. Slopes were also calculated for a given parameter change over time and assessed using the *t*-test.

RESULTS AND DISCUSSION

Figure 1 shows the time course changes in A, Tr, g_s, and C_i before and after illumination with a PPFD of 1806 ± 133.2 µmol m^{-2} s^{-1} at the leaf surface. A increased immediately following illumination and reached its peak at 15.7 µmol m^{-2} s^{-1} after 1.5 h, then it gradually decreased. At 8 h post-illumination, A had dropped to half of its maximum value, at 7.7 µmol m^{-2} s^{-1}. Simultaneously, Tr and g_s increased during the first hour, after which they remained constant at around 8 mmol m^{-2} s^{-1} and 0.35 mol m^{-2} s^{-1}, respectively. After 5.0 h, Tr and g_s began to decrease, eventually reaching approximately 4.5 mmol m^{-2} s^{-1} and 0.2 mol m^{-2} s^{-1}, respectively, 8 h post-illumination.

Temporal trends in A and g_s are often regarded as indices of midday depression (Yabuki *et al.*, 1963), therefore the experiment was divided into 3 periods: period 1 included the first 1.5 h where both A and g_s increased, for period 2 the time range was 1.5-5.0 h where A decreased but g_s held constant, and finally, period 3 consisted of hours 5.0-8.0 h where both of A and g_s decreased. A change in the leaf gas-exchange parameters (A, Tr, g_s, C_i, F'_V/F'_M, V_{CMAX}, and *IDL*) from period 2-3, as well as the rate (slope) of change over time for each parameter were assessed statistically (Table 1).
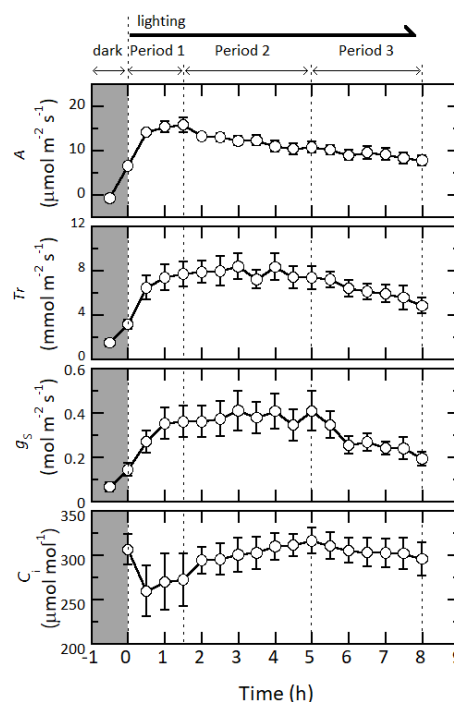

Fig. 1. Time change in the photosynthetic rate (A), transpiration rate (Tr), stomatal conductance (g_S), intercellular CO_2 concentration (C_i) of a cucumber leaf under the conditions with an air temperature of 25°C, a vapour pressure deficit of 2.5 kPa, a CO_2 concentration of 400 µmol mol^{-1} before and after the application of light with a photosynthetic photon flux density (PPFD) of 1806 $\pm$ 133.2 µmol m^{-2} s^{-1} on the leaf surfaces. The mean and standard error bars are shown (n = 3).

During period 1, A and g_s increased, while C_i decreased. McAusland *et al.* (2016) reported that the stomatal response to changing irradiance from 100 to 1000 µmol m^{-2} s^{-1} stepwise was an order of magnitude slower than photosynthesis. F'_V/F'_M and V_{CMAX}, which are related to photosynthetic activity, sharply increased soon after light was applied (Fig. 2). *IDL* also increased during this period, which indicates that stomatal limitation is a dominant limiting factor of the photosynthetic rate. Thus, the reduction in C_i may be due to slower stomatal opening relative to the induction of photosynthetic activity soon after the application of light. In this period, the gas exchange parameters (A, Tr and g_s) tended to increase, and thus it may be concluded that midday depression had not occurred in period 1.

During period 2, A showed a tendency to decrease, from 15.7 µmol m^{-2} s^{-1} to 10.3 µmol m^{-2} s^{-1} at a significant rate (slope) of -0.43 nmol m^{-2} s^{-2} (Table 1) while g_s held constant. As a result, C_i increased at the significant rate of 3.1 nmol mol^{-1} s^{-1}. F'_V/F'_M and V_{CMAX} decreased sharply at the beginning of the period 2. F'_V/F'_M was constant after a sharp decrease, while V_{CMAX} gradually decreased afterwards. *IDL* decreased to around 50% at the beginning of period 2. These results indicate that a non-diffusional limitation was the dominant factor for the midday depression

Table 1. Values of the average and slope for the photosynthetic rate (A), transpiration rate (Tr), stomatal conductance (g_S), intercellular CO_2 concentration (C_i), photochemical efficiency of photosystem II (F'_V/F'_M), maximum carboxylation rate (V_{CMAX}), and index of diffusional limitation (IDL) during period 2 (1.5-5 h from the start of actinic light application) and period 3 (5-8 h from the start of actinic light application) in the experiment (n = 3)

Parameters	Average		Slope			
	Period 2	Period 3	Period 2	*t*-test	Period 3	*t*-test
A	12.2[a]	8.92[b]	-1.32	***	-0.85	*
Tr	7.75[a]	5.98[b]	-0.11	n.s.	-0.83	***
g_S	0.38[a]	0.26[b]	0.01	n.s.	-0.05	*
C_i	300	303	10.5	***	-4.8	**
F'_V/F'_M	0.17	0.16	-0.01	n.s.	0.003	n.s.
V_{CMAX}	49.4[a]	35.4[b]	-7.7	**	-2.7	*
IDL	59.6[a]	68.2[b]	1.2	n.s.	3.00	n.s.

Different letters for the average value between periods 2 and 3 indicate a significant difference at p < 0.05. *, **, and *** and also indicate the significance of the slope at p < 0.05, 0.01, and 0.001, respectively, and n.s. indicates that the value was not significant.
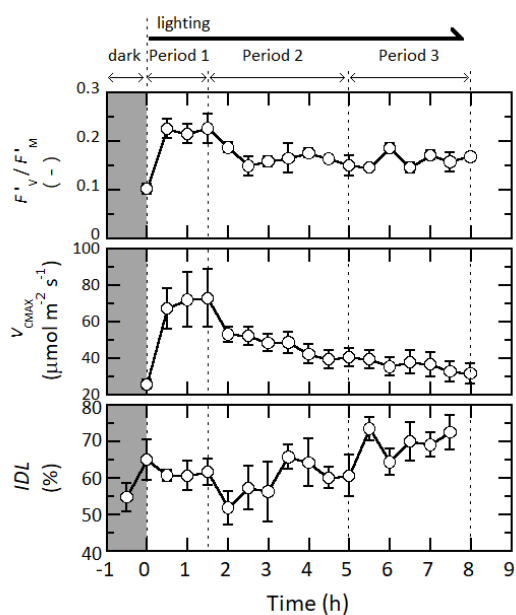

Fig. 2. Time change in the photochemical efficiency of photosystem II (F'_V/F'_M), maximum carboxylation rate (V_{CMAX}), and the index of diffusional limitation (IDL) of a cucumber leaf under the conditions of an air temperature of 25°C, and a vapour pressure deficit of 2.5 kPa, a CO_2 concentration of 400 µmol mol^{-1} before and after the application of light with a photosynthetic photon flux density (PPFD) of 1806 ± 133.2 µmol m^{-2} s^{-1} on the leaf surfaces. The mean and standard error bars are shown (n = 3).

in the first half of period 2. In the latter half of period 2, although g_s held constant and the V_{CMAX} value related to non-diffusional limitation decreased, IDL reduced to around 60-65%. The boundary layer conductance is negligible as a component of the diffusional limitation factor because boundary layer conductance in the open chamber is greater than stomatal conductance and also constant as the air inside the chamber is mixed by a fan (Jones, 2014).

Therefore, the decrease in mesophyll conductance might be a reason for the production of an IDL value of around 60-65% during the latter half of the period. Mesophyll conductance has recently been recognized as an important component of the CO_2 diffusion pathway (von Caemmerer and Evans, 2015; Loucos *et al.*, 2017). However, the environmental response of mesophyll conductance differs among species and genotypes, and thus a mechanistic understanding of the environmental response of mesophyll conductance remains elusive (Flexas *et al.*, 2008). Thus, in period 2, although A decreased at a constant rate, the reason for the decrease in A changed dynamically from the non-diffusional to the diffusional limitation.

In period 3, A showed a further decreasing tendency at a significant rate of -0.25 nmol m^{-2} s^{-2}. In addition, Tr decreased at the significant rate of -0.23 µmol m^{-2} s^{-2} and g_s at the significant rate of -17 µmol m^{-2} s^{-2}. Some inhibition of CO_2 uptake likely occurred, due to stomatal closure which was indicated by a decrease in C_i. The decline in g_s cannot be due to an immediate response to environmental change, as environmental conditions were kept constant during the experiment. Rather, the decline in g_s may relate to leaf water status, which is derived from the excess accumulation of transpirational water loss, supported by a significant reduction in water potential throughout the experiment (Fig. 3). In period 3 therefore, the reason for the constant decrease may be attributed to diffusional limitation due to stomatal closure.

CONCLUSIONS

1. Photosynthesis and leaf gas-exchange related parameters decreased throughout the experimental period (2-8 h from the start of the application of actinic light), thereby allowing us to observe the phenomenon of midday depression.
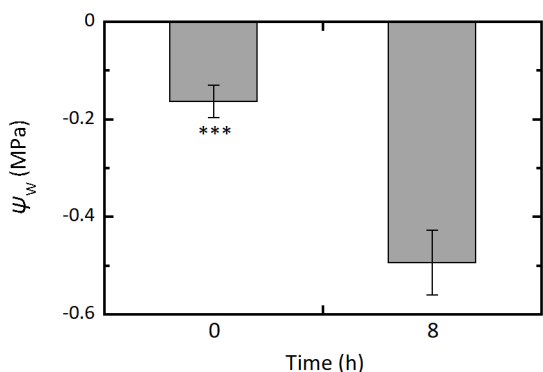
Fig. 3. Leaf water potential (Ψ_W) of a cucumber leaf at the start and end of the application of light with a photosynthetic photon flux density (PPFD) of 1806 ± 133.2 μmol m^{-2} s^{-1} on the leaf surfaces. The mean and standard error bars are shown (n = 3). *** indicates the significance between values before and after the application of light at p < 0.001.

2. It was found that the midday depression of photosynthesis was initially caused by non-diffusional limitation (during the first half of the experiment) but was subsequently caused by diffusional limitation (during the second half). That is, the main factor behind midday depression can change dynamically, even under constant environmental conditions.

3. The work presented here is useful with regard to our understanding of the physiological responses in crops. However, further quantitative assessment of midday depression, and its dynamics in natural conditions, is required.

Conflict of interest: The authors declare no conflict of interest.

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