# Diurnal changes in the stomatal, mesophyll, and biochemical limitations of photosynthesis in well-watered greenhouse-grown strawberries

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## Abstract

The diurnal variations in the factors of photosynthesis reduction under well-watered greenhouse conditions remain poorly understood. We conducted diurnal measurements of gas exchange and chlorophyll fluorescence in strawberries (*Fragaria × ananassa* Duch.) for three sunny days. Quantitative limitation analysis was also conducted to investigate the diurnal variations of photosynthetic limitations [stomatal ($S_L$), mesophyll ($MC_L$), and biochemical limitation ($B_L$)]. Under well-watered greenhouse conditions, a photosynthesis reduction was observed, and the respective limitations exhibited different diurnal changes based on the environmental stress severity. The main limitation was $S_L$, varying between 11.3 and 27.1% around midday, whereas $MC_L$ and $B_L$ were in 4.3–14.2% and 1.7–8.5%, respectively, under relatively moderate conditions. However, both $S_L$ (11.2–34.2%) and $MC_L$ (4.8–26.4%) predominantly limited photosynthesis under relatively severe conditions, suggesting that stomatal closure was the main limitation and that the decline in mesophyll conductance was not negligible under strong environmental stress, even under well-watered greenhouse conditions.

## Introduction

On sunny days, plants actively engage in photosynthesis; however, despite sufficient solar radiation, the photosynthetic rate often decreases around midday. Under strong solar radiation, a decrease in photosynthetic rate around midday, known as midday depression of photosynthesis (hereafter midday depression), is frequently observed (Roessler and Monson 1985, Raschke and Resemann 1986, Ayari *et al.* 2000, Muraoka *et al.* 2000, Pelletier

## Highlights

- Photosynthetic rate was reduced even under well-watered greenhouse conditions
- Stomatal closure predominantly limited photosynthesis under moderate conditions
- Mesophyll conductance also contributed to limitation under severe conditions

*et al.* 2016). Notably, identifying the environmental factors limiting the photosynthetic rate is essential for preventing crop yield loss caused by a decrease in photosynthesis.

Owing to the high solar heat load, environmental conditions in greenhouse agriculture often become un-favorable for photosynthesis, especially on sunny days in warmer climate regions (He *et al.* 2007, López *et al.* 2012). As the photosynthetic process consists of diffusion of $CO_2$ from the atmosphere to the carboxylation site, which was thought to be largely regulated by stomatal functioning and activity of biochemical process at the carboxylation site (Wilson *et al.* 2000), the regulation of photosynthesis by environmental factors has been investigated in greenhouse-grown crops in relation to stomatal and biochemical limitations (Ayari *et al.* 2000, Fan *et al.* 2010, Yuan *et al.* 2016, Rodrigues *et al.* 2018, Sarabi *et al.* 2019). Common environmental stresses in greenhouse agriculture include high light and temperature stresses, which inhibit the activity of photosynthetic enzymes and electron transport chains and induce biochemical limitations (He *et al.* 2007, Hu *et al.* 2009, Fan *et al.* 2010, Lu *et al.* 2017, Szymańska *et al.* 2017). Although high light and temperature conditions are the primary contributors to biochemical limitations, they can indirectly increase stomatal limitation ($S_L$). The transpiration rate is elevated under high light and temperature conditions to suppress an increase in leaf temperature. If water uptake from the roots is insufficient to compensate for water loss *via* transpiration, plants close their stomata to prevent further water loss (Robinson and Bower 1988). In addition, an increase in air temperature results in an elevated vapor pressure deficit (VPD), which increases $S_L$ by inducing stomatal closure (Lu *et al.* 2015, Zhang *et al.* 2015, Yuan *et al.* 2016). Depending on stress severity, water stress increases stomatal and nonstomatal limitations. Under mild water stress conditions, $S_L$ is the predominant factor for the decrease in photosynthetic rate, whereas nonstomatal limitation increases under severe water stress conditions (Yuan *et al.* 2016, Song *et al.* 2020). These studies showed that different photosynthetic limitations (*e.g.*, stomatal or biochemical limitations) regulate photosynthesis depending on the environmental conditions. As the

environmental conditions within the greenhouse change dynamically throughout the day, photosynthetic limiting factors also change throughout the day. Moreover, rather than a single physiological factor, multiple concurrent operating factors can limit the photosynthetic rate. Therefore, to understand how environmental factors limit the photosynthetic rate, diurnal changes in the relative proportions of photosynthetic limitation factors require evaluation. Because only a few studies have investigated dynamic changes in photosynthetic limitations during the day under greenhouse conditions (Chen *et al.* 2014, Zhang *et al.* 2021), diurnal changes in photosynthetic limitations in greenhouse crops remain underexplored.

Quantitative limitation analysis, which was proposed by Jones (1985) and further extended by Wilson *et al.* (2000) and Grassi and Magnani (2005), is one of the common methods for investigating dynamic changes in photosynthetic limitations. Traditionally, the constraints imposed by environmental stress on photosynthesis have been analyzed in terms of $S_L$ and $B_L$ (*i.e.*, maximum carboxylation rate) (Wilson *et al.* 2000), whereas Grassi and Magnani (2005) showed the importance of including mesophyll conductance in the analysis. Since then, quantitative limitation analysis has often been used under field conditions to examine the changes in relative proportions of photosynthetic limitations (*i.e.*, stomatal, mesophyll, and biochemical limitations) under limited soil water conditions over relatively long periods (Grassi and Magnani 2005, Galle *et al.* 2009, Grassi *et al.* 2009, Flexas *et al.* 2009, Egea *et al.* 2011, Zhu *et al.* 2021). However, this quantitative analysis was infrequently used to examine greenhouse-grown horticultural crops (Chen *et al.* 2014, Zhang *et al.* 2021). Because the environmental conditions inside a greenhouse differ from those in the field, changes in photosynthetic limitations and their proportion to reductions in photosynthetic rate would differ from previous field-based studies conducted under water stress. For example, Grassi *et al.* (2009) reported that diffusional limitation (*i.e.*, stomatal and mesophyll limitations) was the dominant limiting factor for photosynthesis under water stress conditions, whereas the proportion of biochemical limitation was higher when water stress was

*Abbreviations*: $B_L$ – contribution of maximum carboxylation rate to relative change in photosynthetic rate; $C_a$ – $CO_2$ concentration inside the chamber; $C_c$ – $CO_2$ concentration at the carboxylation site; $C_i$ – intercellular $CO_2$ concentration; $E_a$ – activation energy; ETR – electron transport rate; $F_m'$ – maximal fluorescence yield of the light-adapted state; $F_s$ – steady-state fluorescence yield; $g_m$ – mesophyll conductance; $g_{m,ref}$ – reference value of mesophyll conductance; $g_{sc}$ – stomatal conductance to $CO_2$; $g_{sc,ref}$ – reference value of stomatal conductance to $CO_2$; $g_{sw}$ – stomatal conductance to water vapor; $g_{tot}$ – total conductance to $CO_2$ between the leaf surface and carboxylation site; $I$ – solar radiation outside the greenhouse; $K_c$ – Michaelis constants for carboxylation; $K_m$ – Michaelis–Menten constant; $K_o$ – Michaelis constants for oxygenation; $l_b$ – relative limitation of maximum carboxylation rate to relative change in photosynthetic rate; $l_{mc}$ – relative limitation of mesophyll to relative change in photosynthetic rate; $l_s$ – relative limitation of stomatal conductance to relative change in photosynthetic rate; $MC_L$ – contribution of mesophyll conductance to relative change in photosynthetic rate; $O$ – $O_2$ concentration; $P_{Nmax}$ – light-saturated net photosynthetic rate; $P_{Nmax,ref}$ – reference photosynthetic rate assuming the reference values of stomatal conductance, mesophyll conductance, and maximum carboxylation rate are reached at the same time; $R$ – universal gas constant; $R_d$ – mitochondrial respiration rate in the light; RH – relative humidity inside the greenhouse; RuBP – ribulose-1,5-bisphosphate; $S_L$ – contribution of stomatal conductance to relative change in photosynthetic rate; $T_A$ – air temperature inside the greenhouse; $V_{cmax}(C_c)$ – maximum carboxylation rate based on $CO_2$ concentration at the carboxylation site at 25°C; $V_{cmax}(C_c,T_{leaf})$ – maximum carboxylation rate based on $CO_2$ concentration at the carboxylation site at the respective $T_{leaf}$; $V_{cmax,ref}$ – reference value of maximum carboxylation rate; VPD – vapor pressure deficit inside the greenhouse; α – the leaf absorptance; β – portioning of absorbed quanta between PSI and PSII; $\Gamma^*$ – $CO_2$ compensation point in the absence of mitochondrial respiration; $\Phi_{PSII}$ – effective quantum yield of PSII photochemistry; $\Psi_w$ – leaf water potential.

less severe. Because greenhouse-grown horticultural crops are generally well-watered, the biochemical limitation would play a greater role in the photosynthetic regulation of greenhouse-grown crops than it would in studies conducted under water stress.

This study examines how different photosynthetic limitations (*i.e.*, stomatal, mesophyll, and biochemical limitations) regulate photosynthesis in response to diurnal changes under greenhouse microclimate conditions. We hypothesize that diffusional limitations (*i.e.*, stomatal and mesophyll) have a relatively smaller proportion than biochemical limitations in well-watered greenhouse-grown strawberries. To achieve this, the gas exchange and chlorophyll fluorescence of greenhouse-grown strawberries were measured on a diurnal scale for three days under varying environmental conditions, followed by quantitative limitation analysis to evaluate the diurnal changes in photosynthetic limitations.

## Materials and methods

**Plant materials and growth conditions**: Strawberry nurseries (*Fragaria × ananassa* Duch. cv. Fukuoka S6) were grown in a greenhouse (37 m long × 9 m wide × 4.5 m high) located at the National Agricultural and Food Research Organization (Kyushu Okinawa Agricultural Research Center, Japan; 33°18.4'N, 130°32.8'E). The nurseries, which were selected from mother stocks, were planted in plastic pots filled with a 3:5:2 (v:v:v) mixture of peat moss, coconut shell, and charcoal in early June 2020. A nutrient solution (*OK F-1, OAT Agrio Co., Ltd.*, Tokyo, Japan; electrical conductivity = 0.6 dS m$^{-1}$) was supplied at a rate of 300 mL per day per plant at 9:00, 11:00, 13:00, 15:00, and 17:00 h each day. To induce anthesis, nutrient supplementation was stopped between mid-August and mid-September; thus, only water was supplied during this period. Flower buds differentiated on the first inflorescence until mid-September, and other growth conditions were set, as described by Hidaka *et al.* (2019). On 1 October 2020, 288 plants were transplanted on cultivation beds (15 m long × 30 cm wide × 80 cm high) and filled with the culture soil (*Good Soil, Kaneya Co., Ltd.*, Aichi, Japan) in a greenhouse (20 m long × 8 m wide × 4 m high) located at Ito Plant Experiment Fields & Facilities, Faculty of Agriculture, Kyushu University, Fukuoka, Japan (33°35.5'N, 130°12.9'E), with 20-cm intervals between plants and inter-row spacing of 15 cm (Fig. 1). The plants were supplied with a nutrient solution (same as that described above) at a rate of 200–300 mL per day per plant depending on the air temperature inside the greenhouse, at 1-h intervals during daytime (6:00–18:00 h). The greenhouse was equipped with an oil heater (*HK2027TEV, NEPON Inc.*, Tokyo, Japan) and a ventilation system comprising roof and side-wall windows to keep the air temperature between 8 and 22°C. In addition, to prevent excessive high temperature and drying inside the greenhouse, the fine mist cooling system (the mist nozzle diameter, 0.15 mm; dynamic injection pump flow rate, 1.5 L min$^{-1}$; *Green.com Inc.*, Tokyo, Japan) was operated for 2 min at 15-min intervals from 10:00 to
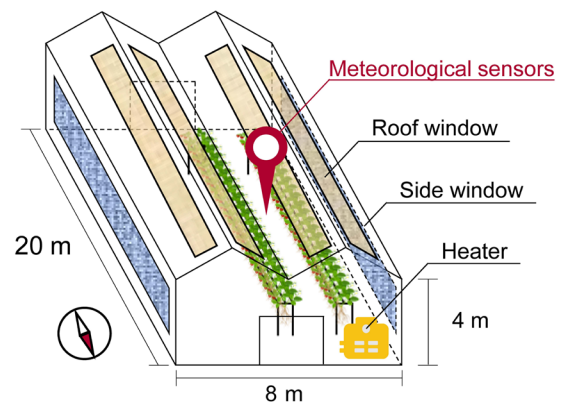


Fig. 1. Schematic diagram of the greenhouse in which the strawberry plants used in the experiment were cultivated. To regulate the environment inside the greenhouse, a heater for heating, a fine mist-cooling system for cooling, and skylight and side windows for ventilation were installed. The red mark in the figure indicates the sensor group installed to measure the microclimates (PPFD, $T_A$, and RH) inside the greenhouse.

16:00 h only on sunny days. Flowers were pollinated by bees.

Inside the greenhouse, the microclimates, such as PPFD, air temperature ($T_A$), and relative humidity (RH) were measured using a quantum sensor (*PAR-02D, Prede Co., Ltd.*, Tokyo, Japan) and a temperature/humidity sensor (*HMP60, Vaisala*, Helsinki, Finland), respectively. A single set of meteorological sensors was placed around the center of the greenhouse between plant rows at a height of 1.2 m (near the canopy top; Fig. 1). Data were measured at 5-s intervals, and 10-min means were logged in a programmable data logger (*CR-1000, Campbell Scientific Inc.*, Logan, USA). The vapor pressure deficit (VPD) inside the greenhouse was calculated from $T_A$ and RH. In addition, the outside solar radiation ($I$) at the same interval was obtained from a meteorological station (pyranometer: *CHF-SE20-JM, Climatec Inc.*, Tokyo, Japan) located about 120 m away from the greenhouse used in the experiment.

**Measurements of photosynthetic parameters and leaf water potential**: Diurnal changes in photosynthetic parameters [light-saturated photosynthetic rate ($P_{Nmax}$), stomatal conductance to water vapor ($g_{sw}$), and chlorophyll (Chl) fluorescence] under light-saturated conditions were measured using a portable gas-exchange system (*LI-6400, LI-COR*, Lincoln, NE, USA) equipped with a leaf chamber fluorometer (*LI-6400-40, LI-COR*, Lincoln, NE, USA) on 22, 23, and 30 May 2021. This measurement was conducted to evaluate the diurnal changes in mesophyll conductance ($g_m$) using the variable $J$ method (Harley *et al.* 1992) and the maximum carboxylation rate using the one-point method (De Kauwe *et al.* 2016). The measurements were conducted on randomly selected fully expanded sun leaves from 6:30 to 18:00 h at an interval of about 30 min under light-saturating PPFD of 1,500 µmol m$^{-2}$ s$^{-1}$

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with a $CO_2$ concentration of 400 µmol mol$^{-1}$. In addition, the air temperature and VPD inside the chamber were allowed to follow ambient conditions. Moreover, while the diurnal changes in photosynthetic parameters were measured, gas exchange under natural light was also measured (Fig. 1S, *supplement*).

From the fluorescence measurements, the effective quantum yield of PSII photochemistry ($\Phi_{PSII}$) was determined as follows:

$$\Phi_{PSII} = F_m' - F_s/F_m' \qquad (1)$$

where $F_s$ is the steady-state fluorescence yield at a PPFD of 1,500 µmol m$^{-2}$ s$^{-1}$ and $F_m'$ is the maximum fluorescence measured with a light-saturating pulse. Then, the electron transport rate (ETR) was calculated as follows:

$$ETR = \alpha \times \beta \times \Phi_{PSII} \times PPFD \qquad (2)$$

where $\alpha$ is the leaf absorptance, which was assumed to be 0.85 (Evans and Loreto 2000), and $\beta$ is the portioning of absorbed quanta between PSI and PSII, which was assumed to be 0.5 (Laisk and Loreto 1996).

Diurnal changes in leaf water potential ($\Psi_w$) were also measured along with the gas-exchange measurements every two hours from 6:00 to 18:00 h ($n = 3$ for each measurement point). $\Psi_w$ was measured using a pressure chamber (*Pump-up Chamber*, *PMS Instruments*, Albany, OR, USA). The measurements of $\Psi_w$ were made on different plants used for the gas-exchange measurements to avoid any effects by sampling leaves for the $\Psi_w$ measurement.

**Assessing the risk of intercellular $CO_2$ concentration miscalculations**: To derive a reliable value of $V_{cmax}$ ($C_c$) and $g_m$, an accurate measurement of intercellular $CO_2$ concentration ($C_i$; *i.e.*, accurate measurement of $P_{Nmax}$ and $g_{sw}$) is important (Pons *et al.* 2009). The effect of cuticular transpiration on $g_{sw}$ calculation is negligible when transpiration through the stomata is sufficiently large relative to cuticular transpiration. However, when $g_{sw}$ becomes lower under high evaporative demand conditions, $g_{sw}$ could be overestimated due to a relative increase in cuticular transpiration, resulting in an error in $C_i$ value (*e.g.*, Boyer *et al.* 1997, Márquez *et al.* 2021). Thus, we recalculated $C_i$ to account for the effect of cuticular transpiration. Cuticular conductance was determined using the method of Sack *et al.* (2003). Briefly, strawberry leaves were dried on a laboratory bench for 2−4 h, and the leaves were weighed at intervals of 1−5 min. Leaf temperature ($T_{leaf}$), air temperature, and relative humidity were measured by the infrared thermography camera (*FLIR i5*, *IRIE SHOKAI Co., Ltd.*, Tokyo, Japan) and the temperature–humidity recorder (*TR-72Ui*, *T&D Co.*, Nagano, Japan), respectively, and used to calculate leaf-to-air VPD. Cuticular transpiration was estimated from the slope of the decline in leaf weight and then normalized by the leaf area. The leaf area was estimated from the measured leaf's picture using *ImageJ v. 1.53* software. Finally, cuticular conductance was evaluated by dividing cuticular transpiration by leaf-to-air VPD. Moreover, to avoid errors, $CO_2$ leakage in the

gas-exchange measurement was corrected, as described by Flexas *et al.* (2007a).

Because patchy stomatal closure could cause an error in $C_i$ when plants are subjected to stress conditions, we checked the reliability of the measured $C_i$ value based on the $C_i$−$g_{sw}$ relationship, as described by Grassi *et al.* (2009). Owing to the sharp increase in $C_i$ for $g_{sw}$ values less than 0.05 mol m$^{-2}$ s$^{-1}$ (Fig. 2S, *supplement*), data below this threshold were omitted from the estimation of photosynthetic parameters.

**Calculation of mesophyll conductance and biochemical capacity parameters**: From the method proposed by Harley *et al.* (1992), $g_m$ was calculated as follows:

$$g_m = P_{Nmax} \times \frac{ETR - 4(P_{Nmax} + R_d)}{C_i - \Gamma^* \left[ ETR + 8\left( P_{Nmax} + R_d \right) \right]} \qquad (3)$$

where $P_{Nmax}$ and $C_i$ are taken from gas-exchange measurements at saturating light, and ETR is calculated by Eq. 2. $R_d$ is the mitochondrial respiration rate in the light, estimated by multiplying the maximum carboxylation rate on intercellular $CO_2$ concentration by 0.015, and $\Gamma^*$ is the $CO_2$-compensation point in the absence of mitochondrial respiration defined by Bernacchi *et al.* (2001).

From the transport equation for $CO_2$ diffusion into the leaf, the $CO_2$ concentration at the carboxylation site ($C_c$) was calculated as follows:

$$C_c = C_i - P_{Nmax}/g_m \qquad (4)$$

From the measurement of gas exchange under light-saturated conditions, we calculated the maximum carboxylation rate based on $CO_2$ concentration at the carboxylation site at the respective $T_{leaf}$ [$V_{cmax}(C_c, T_{leaf})$] by the one-point method (De Kauwe *et al.* 2016), as follows:

$$V_{cmax}\left( C_c, T_{leaf} \right) = \frac{P_{Nmax}}{\left( \dfrac{C_c - \Gamma^*}{C_c + K_m} - 0.015 \right)} \qquad (5)$$

where $K_m$ is the Michaelis−Menten constant defined by Bernacchi *et al.* (2001). Other Rubisco kinetics and their temperature dependencies were also taken from Bernacchi *et al.* (2001). Moreover, we used the following Arrhenius function to standardize $V_{cmax}(C_c, T_{leaf})$ to 25°C:

$$V_{cmax}\left( C_c, T_{leaf} \right) = V_{cmax}\left( C_c \right) \exp \left[ \frac{E_a \left( T_{leaf,K} - 298.15 \right)}{298.15 \, R \, T_{leaf,K}} \right] \qquad (6)$$

where $V_{cmax}(C_c)$ is the maximum carboxylation rate based on $CO_2$ concentration at the carboxylation site at 25°C; $R$ is the universal gas constant (8.314 J mol$^{-1}$ K$^{-1}$); and $E_a$ is the activation energy obtained by fitting Eq. 6 to the relationship between $V_{cmax}(C_c, T_{leaf})$ and $T_{leaf}$ (61.00 kJ mol$^{-1}$; Fig. 3S, *supplement*).

**Quantitative limitation analysis**: To evaluate the contributions of stomatal, mesophyll, and biochemical limitations on the midday depression of photosynthesis

in strawberry plants, quantitative limitation analysis proposed by Jones (1985) and further extended by Wilson *et al.* (2000) and Grassi and Magnani (2005) was performed. According to Grassi and Magnani (2005), relative changes in light-saturated photosynthetic rates can be expressed in terms of parallel relative changes in stomatal conductance, mesophyll conductance, and biochemical capacity [*i.e.*, $V_{cmax}(C_c)$]:

$$\frac{dP_{Nmax}}{P_{Nmax}} = S_L + MC_L + B_L = l_s\frac{dg_{sc}}{g_{sc}} + l_{mc}\frac{dg_m}{g_m} + l_b\frac{dV_{cmax}(C_c)}{V_{cmax}(C_c)} \quad (7)$$

$$l_s = \frac{g_{tot}/g_{sc} \times \partial P_{Nmax}/\partial C_c}{g_{tot} + \partial P_{Nmax}/\partial C_c} \quad (8)$$

$$l_{mc} = \frac{g_{tot}/g_m \times \partial P_{Nmax}/\partial C_c}{g_{tot} + \partial P_{Nmax}/\partial C_c} \quad (9)$$

$$l_b = \frac{g_{tot}}{g_{tot} + \partial P_{Nmax}/\partial C_c} \quad (10)$$

where $g_{sc}$ is stomatal conductance to $CO_2$ (*i.e.*, $g_{sw}/1.6$), $g_{tot}$ is total conductance to $CO_2$ between the leaf surface and carboxylation site (*i.e.*, $1/g_{tot} = 1/g_{sc} + 1/g_m$); $S_L$, $MC_L$, and $B_L$ are the contributions of stomatal conductance, mesophyll conductance, and maximum carboxylation rate based on the $CO_2$ concentration at the carboxylation site at 25°C to relative change in photosynthetic rate (*i.e.*, $dP_{Nmax}/P_{Nmax}$), respectively; $l_s$, $l_{mc}$, and $l_b$ are the corresponding relative limitations ($0 < l_i < 1$, i = s, mc, b). Then, $dP_{Nmax}/P_{Nmax}$ was defined as follows:

$$\frac{dP_{Nmax}}{P_{Nmax}} \approx \frac{P_{Nmax,ref} - P_{Nmax}}{P_{Nmax,ref}} \quad (11)$$

$$P_{Nmax,ref} = \frac{V_{cmax,ref}\left[C_a - P_{Nmax,ref}\left(1/g_{sc,ref} + 1/g_{m,ref}\right) - \Gamma^*\right]}{\left[C_a - P_{Nmax,ref}\left(1/g_{sc,ref} + 1/g_{m,ref}\right) + K_c\left(1 + O/K_o\right)\right]} - R_d \quad (12)$$

where $g_{sc,ref}$, $g_{m,ref}$, and $V_{cmax,ref}$ are the reference values of $g_{sc}$, $g_m$, and $V_{cmax}(C_c)$ defined as the maximum measured values; $P_{Nmax,ref}$ is the reference photosynthetic rate assuming $g_{sc,ref}$, $g_{m,ref}$, and $V_{cmax,ref}$ are reached simultaneously; $O$ is the $O_2$ concentration; $K_c$ and $K_o$ are the Michaelis constants for carboxylation and oxygenation defined by Chen *et al.* (2014), respectively; $R_d$ is the day respiration rate. In addition, $\partial P_{Nmax}/\partial C_c$ in Eqs. 7–10 was calculated as follows:

$$\partial P_{Nmax}/\partial C_c = V_{cmax}\left[\Gamma^* + K_c\left(1 + O/K_o\right)\right]/\left[C_c + K_c\left(1 + O/K_o\right)\right]^2 \quad (13)$$

**Statistical analysis**: Descriptive statistics [determination of means and standard error of all parameters, *i.e.*, $\Psi_w$, $P_{Nmax}$, $g_{sc}$, $g_m$, $V_{cmax}(C_c)$, ETR, and respective limitations] was done using the *R* software (*version 4.0.2*, *R Development Core Team*, Vienna Austria). Linear regression was used to analyze the relationship between $P_{Nmax}$ and respective parameters [$g_{sc}$, $g_m$, $V_{cmax}(C_c)$, ETR, $C_i$, $C_c$]. The significance of the correlations between $P_{Nmax}$ and respective parameters was checked with *Pearson*'s correlation test using *R* software.

## Results

**Diurnal changes in microclimate conditions in the greenhouse**: Although all three days were relatively sunny, with a maximum PPFD around 1,800 μmol m⁻² s⁻¹, differences in microclimate conditions were observed each day (Fig. 2). The lowest average PPFD of 631.1 μmol m⁻² s⁻¹, $T_A$ of 20.8°C, and VPD of 0.79 kPa were observed on 22 May, whereas the highest average PPFD of 717.7 μmol m⁻² s⁻¹, $T_A$ of 25.5°C, and VPD of 1.72 kPa were observed on 30 May. On 23 May, the average PPFD, $T_A$, and VPD were 698.3 μmol m⁻² s⁻¹, 24.6°C, and 1.31 kPa, respectively, which were intermediate between 22 and 30 May.

**Diurnal changes in leaf water potential and photosynthetic parameters**: The diurnal changes in $\Psi_w$ were similar among the measurement days, which decreased during the morning periods and then increased in the afternoon periods (Fig. 3). $\Psi_w$ at 6:00 h was –0.08, –0.09, and –0.07 on 22, 23, and 30 May, respectively, showing that strawberry plants had an appropriate water status in the early morning. During the morning periods, $\Psi_w$ on 30 May was relatively lower than those on 22 and 23 May. The lowest $\Psi_w$ value was observed between 12:00 and 14:00 h, which was –0.65, –0.90, and –0.91 on 22, 23, and 30 May, respectively. $\Psi_w$ tended to increase from 14:00 to 18:00 h.

Midday depression was observed depending on the environmental stress on each measurement day (Figs. 4; 4S, *supplement*). Owing to the severe environmental stress around midday (10:00 to 14:00 h) on 30 May, $g_{sw}$ was lower than 0.05 mol m⁻² s⁻¹, which resulted in an unreliable $C_i$ value (Fig. 2S). Therefore, most data between 10:00 and 14:00 h were discarded, and fewer data points were shown (Fig. 4C,F,I,L).

On 22 May, $P_{Nmax}$ was maintained at around 15 μmol m⁻² s⁻¹ until around 13:30 h and then sharply decreased to 12.2 μmol m⁻² s⁻¹ by 14:30 h. $g_m$ exceeded $g_{sc}$ throughout the day. $g_m$ peaked (0.27 mol m⁻² s⁻¹) at around 8:30 h and decreased to 0.17 mol m⁻² s⁻¹ by 13:30 h, whereas $g_{sc}$ showed a relatively constant value of 0.09 mol m⁻² s⁻¹ from 8:30 to 13:30 h and decreased to 0.06 mol m⁻² s⁻¹ by 14:30 h. $V_{cmax}(C_c)$ gradually increased during the morning period and showed a relatively constant value of around 130 μmol m⁻² s⁻¹ and then decreased after 14:45 h. On 23 May, $P_{Nmax}$ peaked (13.7 μmol m⁻² s⁻¹) at around 7:30 h and then decreased to about half of the maximum value by 12:00 h. In addition, $g_m$ was almost constant around 0.20 mol m⁻² s⁻¹ during the morning periods and then decreased to 0.10 mol m⁻² s⁻¹ in the afternoon, whereas $g_{sc}$ peaked (0.07 mol m⁻² s⁻¹) at 7:30 h and then decreased to 0.04 by 14:00 h. $g_m$ and $g_{sc}$ were almost the same value in
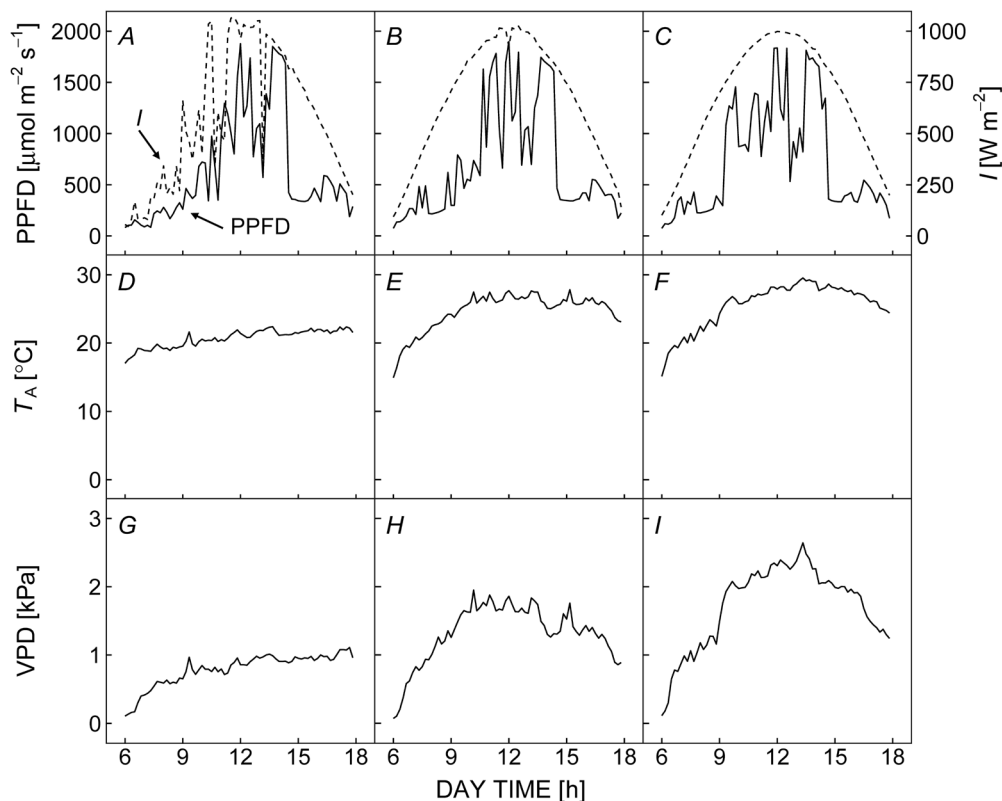
Fig. 2. Diurnal changes in outside solar radiation (*I*; *dashed line*) and PPFD (*solid line*) (*A–C*), air temperature ($T_A$) (*D–F*), and vapor pressure deficit (VPD) (*G–I*) inside the greenhouse. The respective rows indicate the day when gas-exchange measurements were conducted (*i.e.*, *A, D, G*: 22 May; *B, E, H*: 23 May; *C, F, I*: 30 May).
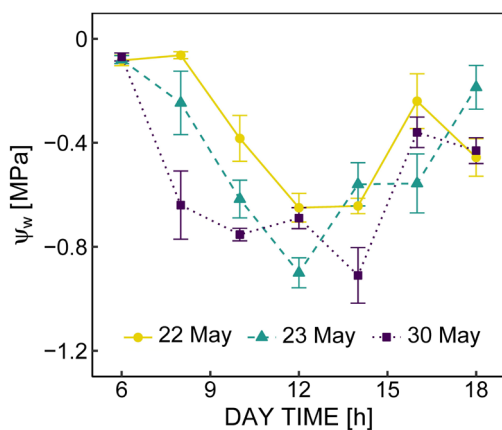


Fig. 3. Diurnal changes in leaf water potential ($\Psi_w$) on the day when gas-exchange measurements were conducted (22, 23, and 30 May). Means ± SE (*n* = 3) are shown.

the afternoon period compared with those in the morning period. $V_{cmax}(C_c)$ tended to increase from 6:30 h, peaking (136.4 μmol m$^{-2}$ s$^{-1}$) at around 10:50 h and then decreasing thereafter. On 30 May, $P_{Nmax}$ peaked (13.4 μmol m$^{-2}$ s$^{-1}$) at 8:30 h and then constantly decreased to 7.8 μmol

m$^{-2}$ s$^{-1}$ by 15:00 h. $g_m$ peaked (0.33 mol m$^{-2}$ s$^{-1}$) at 8:30 h and then sharply decreased to 0.16 mol m$^{-2}$ s$^{-1}$ by 10:00 h. $g_m$ gradually decreased to 0.10 mol m$^{-2}$ s$^{-1}$ by 15:00 h. $g_{sc}$ peaked (0.07 mol m$^{-2}$ s$^{-1}$) at 8:30 h and decreased to 0.05 mol m$^{-2}$ s$^{-1}$ by 13:30 h. $V_{cmax}(C_c)$ increased in the early morning and varied between 146.1 and 92.7 μmol m$^{-2}$ s$^{-1}$.

**Relationship between photosynthetic rate and parameters**: $P_{Nmax}$ and diffusion conductance (*i.e.*, $g_{sc}$ and $g_m$) were significantly positively correlated on all three measurement days (Fig. 5A,D,G). No significant correlation was found between $P_{Nmax}$ and $V_{cmax}(C_c)$ (Fig. 5B,E,H). A significant positive correlation existed between $P_{Nmax}$ and $C_i$ on 22 May but was negligible on 23 and 30 May (Fig. 5C,F,I). However, $P_{Nmax}$ and $C_c$ were significantly positively correlated on all three measurement days (Fig. 5C,F,I).

**Diurnal changes in photosynthetic limitations**: On 22 May, the photosynthetic rate was limited by 44.8% in total (*i.e.*, $S_L + MC_L + B_L$), largely due to $S_L$ (26.5%) and $B_L$ (11.8%) at around 7:30 h (Fig. 6A). From 8:30 to 13:30 h, the limitation sum varied between 21.1 and 28.9%. During this period, $S_L$ and $MC_L$ were the main limiting factors for photosynthesis, whereas $B_L$ was relatively lower, varying between 1.6 and 4.4%. The limitation sum
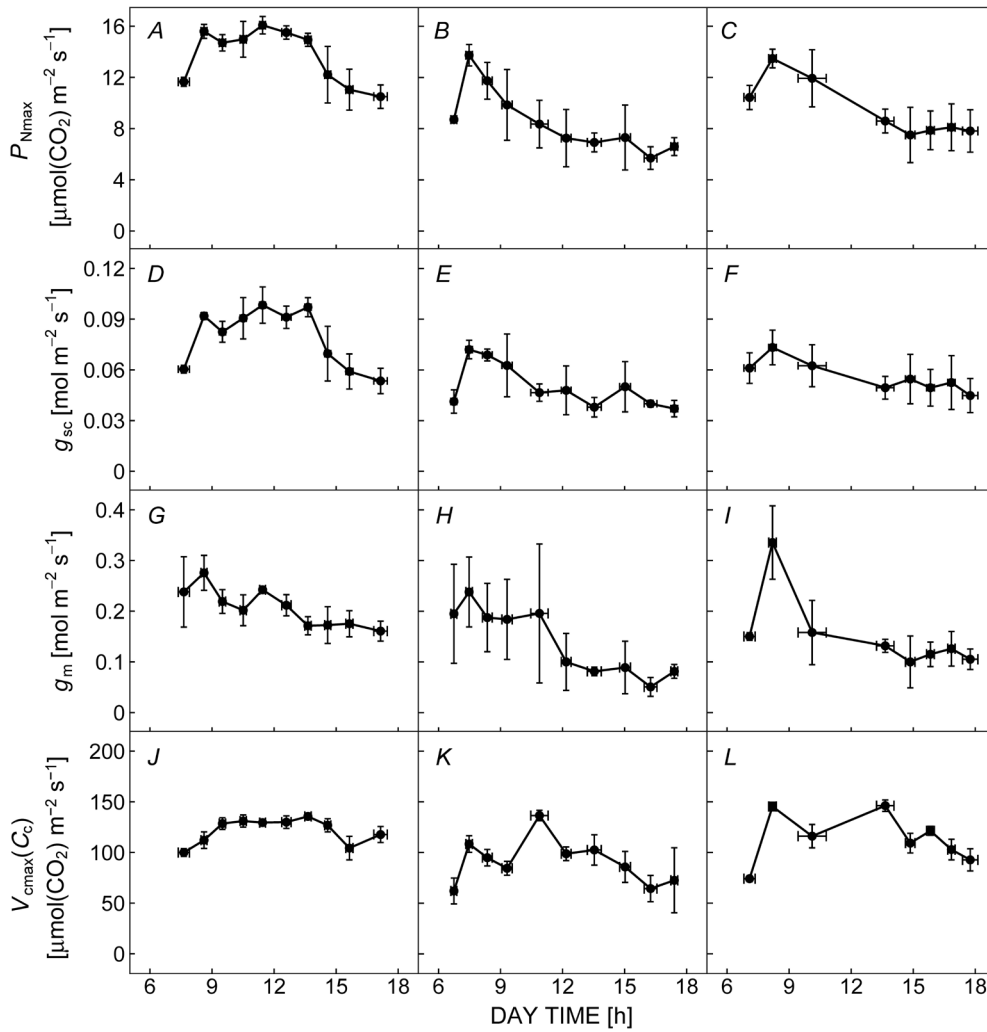
Fig. 4. Diurnal changes in light-saturated photosynthetic rate ($P_{Nmax}$) (*A–C*), stomatal conductance to $CO_2$ ($g_{sc}$) (*D–F*), mesophyll conductance ($g_m$) (*G–I*), and maximum carboxylation rate based on the $CO_2$ concentration at the carboxylation site [$V_{cmax}(C_c)$] (*J–L*). The respective rows indicate the day when gas-exchange measurements were conducted (*i.e.*, *A, D, G, J*: 22 May; *B, E, H, K*: 23 May; *C, F, I, L*: 30 May). The measurements were conducted from 6:30 to 18:00 h (Japan standard time). Means ± SE (*n* = about 3) are shown.

sharply increased at around 14:30 h mainly due to an increase in $S_L$. On 23 May, the limitation sum was 59% at around 6:45 h and then sharply decreased to 31% at around 7:30 h mainly due to $S_L$ (Fig. 6*B*). From 7:30 to 9:20 h, $B_L$ increased from 9.7 to 18.9%, thereby increasing the limitation sum from 31.5 to 47.2%. While $B_L$ decreased and had a relatively lower value than $S_L$ and $MC_L$ after 9:20 h, $S_L$ and $MC_L$ tended to increase, thereby increasing the total limitation sum. On 30 May, the limitation sum decreased from 43.2 to 23% at around 8:10 h and then increased thereafter (Fig. 6*C*). $S_L$ and $MC_L$ were relatively higher than $B_L$ throughout most of the day.

## Discussion

**Diurnal variations in photosynthetic limiting factors**: In the present study, diurnal changes in photosynthetic limitations were investigated in well-watered greenhouse-grown strawberries. A diurnal variation in the proportion of photosynthetic limitation was observed, varying between measurement days under different environmental conditions (Fig. 6). While $S_L$ accounted for a large proportion of the photosynthetic limitation on all three measurement days, $MC_L$ also accounted for a large proportion of the photosynthetic limitation under severe environmental conditions (*i.e.*, 23 and 30 May). Contrary to our hypothesis, $B_L$ was relatively lower than $S_L$ and $MC_L$ on all three measurement days. This finding was unexpected because previous studies have reported that diffusional limitation is larger under water stress conditions, while the relative importance of $B_L$ increases when water stress is less pronounced (Grassi *et al.* 2009). Strawberry plants' stomatal sensitivity to plant water status may account for the higher proportion of $S_L$. Jensen *et al.*
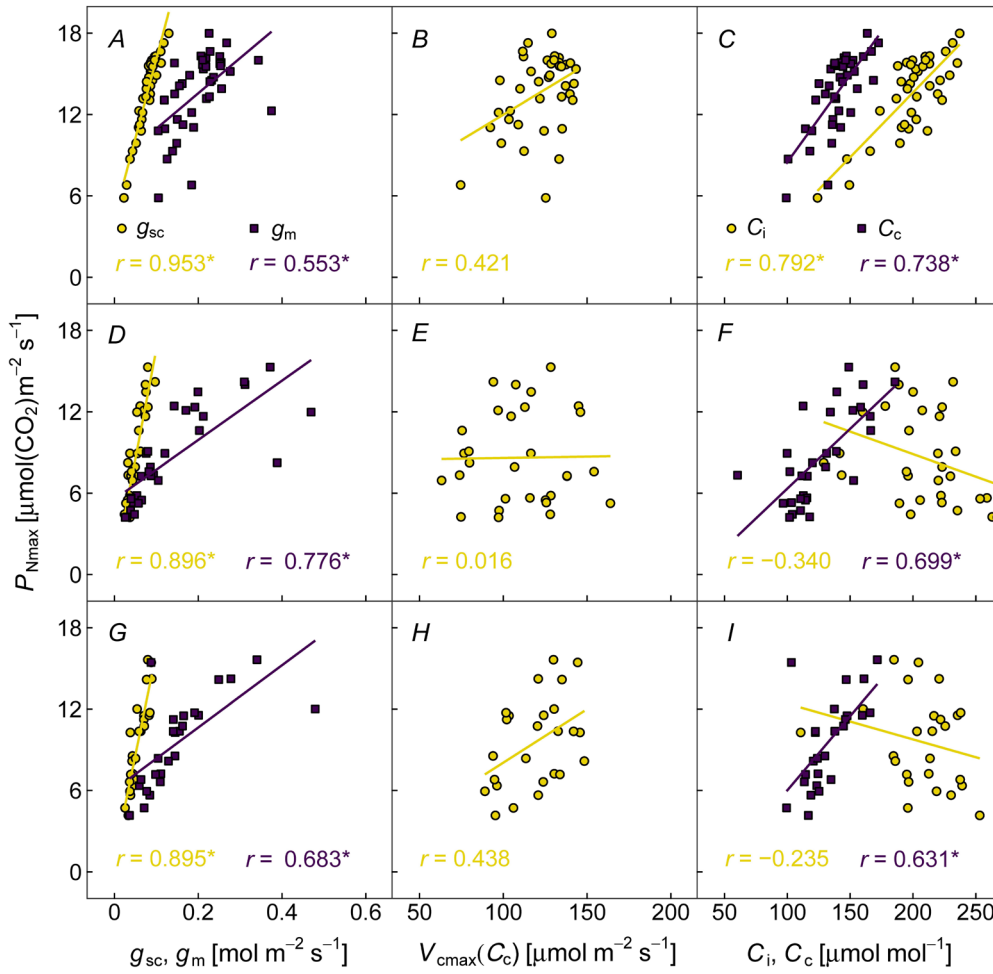
Fig. 5. Relationships among light-saturated photosynthetic rate ($P_{Nmax}$) and stomatal conductance to $CO_2$ ($g_{sc}$; *yellow circle*) and mesophyll conductance ($g_m$; *purple circle*) (*A,D,G*), maximum carboxylation rate based on $CO_2$ concentration at the carboxylation site [$V_{cmax}(C_c)$; *yellow circle*; *purple circle*] (*B,E,H*), and intercellular $CO_2$ concentration ($C_i$; *yellow circle*) and the leaf $CO_2$ concentration at the carboxylation site ($C_c$; *purple circle*) (*C,F,I*). The respective columns indicate the day when gas-exchange measurements were conducted (*i.e.*, *A, B, C*: 22 May; *D, E, F*: 23 May; *G, H, I*: 30 May). *The solid lines* indicate respective linear regression. The *Pearson*'s correlation coefficients (*r*) and statistical significance are also shown (*: $p<0.001$).

(2009) reported that the soil moisture at which a decrease in $g_{sw}$ was observed surpassed that at which a decrease in leaf water potential was observed, suggesting conservative stomatal behavior in strawberry plants. In our study, the observed $\Psi_w$ when $g_{sc}$ decreased was between –0.6 and –0.7 MPa on all measurement days, which was insufficient to induce stomatal closure for most crops (West and Gaff 1976, Syvertsen 1982, Wankmüller and Carminati 2022). This result suggests that strawberry plants conserve plant water status by closing their stomata at an early stage of water stress, thereby preventing a decrease in biochemical activity caused by severe water stress. In addition, Klamkowski and Treder (2006) reported that $g_{sw}$ of 0.05 mol m$^{-2}$ s$^{-1}$ was the threshold of stomatal and nonstomatal limitations in strawberry plants. Because $g_{sw}$ below 0.05 mol m$^{-2}$ s$^{-1}$ was not used in our analysis to ensure photosynthetic parameter accuracy, $S_L$ was

expected to be one of the dominant limiting factors for photosynthesis.

In addition to $S_L$, $MC_L$ accounted for a large proportion of photosynthetic limitations under severe environmental conditions (*i.e.*, 23 and 30 May). While $P_{Nmax}$ showed significant positive correlations with both $C_i$ and $C_c$ when the environmental condition was less severe, it showed a significant positive correlation with only $C_c$ (Fig. 5*C,F,I*) under severe environmental conditions. This result suggests that $P_{Nmax}$ was limited by the supply of $CO_2$ from intercellular air spaces to chloroplasts, which in turn caused $g_m$ to be the main limiting factor of $P_{Nmax}$. Although recent studies have shown the effect of $g_m$ on photosynthetic limitation (*e.g.*, Flexas *et al.* 2009), studies on photosynthetic limitations in horticultural crops have been investigated in terms of stomatal and nonstomatal limitations (Ayari *et al.* 2000, Fan *et al.* 2010, Yuan *et al.*
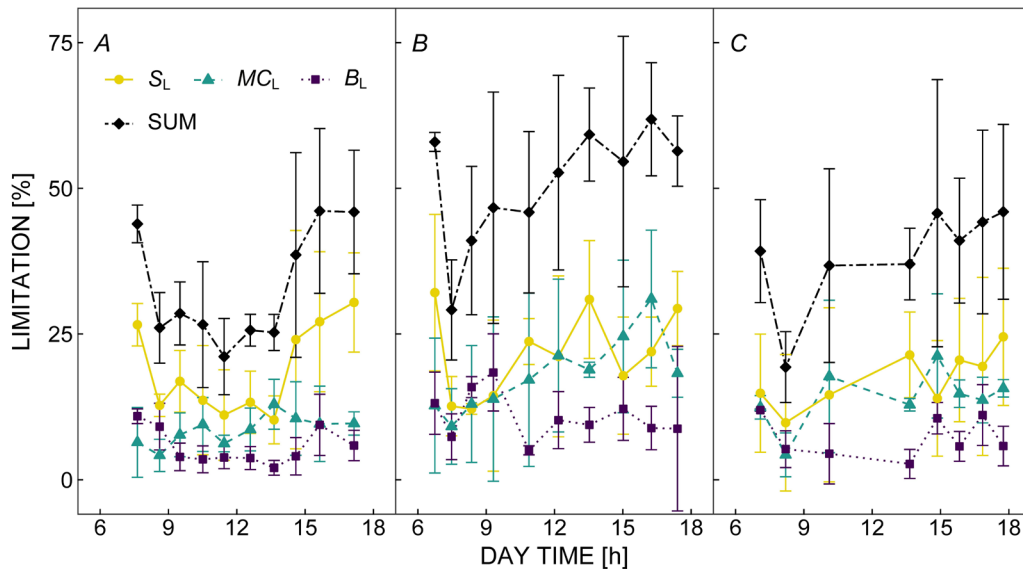
Fig. 6. Diurnal changes in the limitations to light-saturated photosynthetic rate on the day when gas-exchange measurements were conducted (*i.e.*, *A*: 22 May; *B*: 23 May; *C*: 30 May). $S_L$, $MC_L$, $B_L$, and SUM indicate stomatal limitation (*yellow circle*), mesophyll conductance limitation (*blue triangle*), biochemical limitation (*purple square*), and the sum of each limitation (*black rhombus*), respectively. The measurements were conducted from 6:30 to 18:00 h (Japan standard time). Means ± SE (*n* = 3–5) are shown.

2016, Rodrigues *et al*. 2018, Sarabi *et al*. 2019), with only a few studies considering $g_m$ (Chen *et al*. 2014, Zhang *et al*. 2021). In our study, $g_m$ was almost constant or increased during the early morning and then decreased toward the afternoon (Fig. 4*D–F*). $g_m$ can respond to several environmental and physiological factors, such as light intensity (Douthe *et al*. 2011, Xiong *et al*. 2015), ambient $CO_2$ concentration (Düring, 2003, Rho *et al*. 2011), leaf temperature (von Caemmerer and Evans 2015, Xiong *et al*. 2015), and water stress (Grassi and Magnani 2005, Flexas *et al*. 2009, Galle *et al*. 2009). In our study, a decrease in $g_m$ was observed when PPFD exceeded 1,000 μmol m$^{-2}$ s$^{-1}$. Because $g_m$ increases with increasing light intensity (Flexas *et al*. 2007b) and ambient $CO_2$ concentration inside the studied greenhouse was relatively constant due to ventilation, changes in leaf temperature and plant water status might have affected the changes in $g_m$ in our study. von Caemmerer and Evans (2015) reported that $g_m$ increased when the leaf temperature was 15–40°C in *Oryza sativa*, *Nicotiana tabacum*, *Gossypium hirsutum*, *Glycine max*, and *Eucalyptus pauciflora*, whereas the $g_m$ values of *Triticum aestivum*, *Lophostemon confertus*, and *Quercus engelmannii* were relatively constant or decreased for leaf temperature exceeding 30°C. In our study, the leaf temperature was between 30 and 36°C when a decrease in $g_m$ was observed, suggesting that high leaf temperature might decrease $g_m$ in strawberry plants. In addition to high leaf temperatures, a decrease in $g_m$ has been extensively reported under water stress conditions (Grassi and Magnani 2005, Flexas *et al*. 2009, Galle *et al*. 2009). The overall trends of $g_m$ and $\Psi_w$ were relatively similar in our study. For example, sharp decreases in $g_m$

and $\Psi_w$ were observed in the morning periods on 30 May, whereas decreases in $g_m$ and $\Psi_w$ were observed around noon on 22 and 23 May. Therefore, a decrease in $\Psi_w$ might be involved in $g_m$ reduction.

**Implications for future research**: Although many studies have applied quantitative limitation analysis to investigate the relative proportion of photosynthetic limitations (*e.g.*, Grassi and Magnani 2005, Flexas *et al*. 2009, Galle *et al*. 2009, Varone *et al*. 2012, Chen *et al*. 2014, Xiong *et al*. 2017, Zhang *et al*. 2021), to the best of our knowledge, none of these studies, including this study, have considered limitation by boundary layer conductance, which is an important factor for heat and mass exchange processes of leaves. The limitation of photosynthesis by boundary layer conductance might be negligible if the wind speed is high. However, the influence of boundary layer conductance is important under calm wind conditions, which are often observed inside greenhouses (Boulard *et al*. 2002). Kitaya *et al*. (2003) reported that gas exchange in sweet potato leaves was strongly limited by boundary layer conductance when the wind speed was less than 0.2 m s$^{-1}$. In addition, as lower boundary layer conductance limits heat exchange of the leaf surface, leaf-to-air temperature difference would be larger when boundary layer conductance is small (Kimura *et al*. 2017). These findings show that if the boundary layer conductance is low during the daytime, it would affect photosynthesis, both directly and indirectly, by acting as resistance to gas exchange and by increasing leaf temperature. Therefore, incorporating boundary layer conductance into quantitative limitation analysis will provide more insights into how diffusional and nondiffusional limitations affect photosynthetic rates.

Quantitative limitation analysis stems from the biochemical model of $C_3$ photosynthesis, which is defined as the photosynthetic rate limited by the carboxylation of RuBP (ribulose-1,5-bisphosphate) or by RuBP regeneration (Farquhar *et al.* 1980). In our study, RuBP carboxylation was assumed to always limit the photosynthetic rate. However, light intensity inside greenhouses frequently fluctuates mainly due to a greenhouse structure (Cabrera-Bosquet *et al.* 2016; Fig. 2*A–C*), thereby fluctuating the limiting step of photosynthetic rate. Kimura *et al.* (2022) reported that PPFD inside the greenhouse varied spatiotemporally due to changes in sun position and greenhouse structures (*i.e.*, the spatial variability of the daily integral of PPFD was 40%). In other words, because the light-receiving conditions of leaves change frequently, the limiting factor of the photosynthetic rate would also often change (*i.e.*, when leaves receive adequate light, the photosynthetic rate is limited by RuBP carboxylation; when the light is interrupted by a greenhouse structure, the photosynthetic rate is limited by RuBP regeneration). Therefore, to more accurately assess the limiting factors of leaf photosynthetic rate in greenhouse-grown plants, the limitation by ETR and maximal electron transport rate should also be evaluated based on the light-receiving conditions of leaf surfaces (Chen *et al.* 2014).

**Conclusion**: Quantitative limitation analysis, which distinguishes the factors of photosynthesis reduction into the three photosynthetic limitations ($S_L$, $MC_L$, and $B_L$), revealed a decrease in $P_{Nmax}$ even under well-watered greenhouse conditions. The respective limitations also showed different diurnal changes depending on the environmental stress on each measurement day. Under relatively moderate environmental stress, $S_L$ was the primary limitation of $P_{Nmax}$, whereas $S_L$ and $MC_L$ were the primary limitations under relatively severe stress, suggesting that stomatal closure was the main photosynthetic limitation and that the decline in mesophyll conductance was not negligible, especially under strong environmental stress and even under well-watered greenhouse conditions. This study provides a foundation for enhancing the greenhouse-grown crop's yield loss. To more accurately elucidate the limiting factors of leaf photosynthetic rate in greenhouse-grown plants in the future, quantitative limitation analysis must be improved by incorporating (*1*) the limitation by boundary layer conductance and (*2*) evaluating $B_L$ with a suitable method according to limitation of leaf photosynthetic rate [*i.e.*, limited by the RuBP carboxylation rate when the light is received well (Grassi and Magnani 2005) and limited by the RuBP regeneration rate when the light is interrupted by a greenhouse structure (Chen *et al.* 2014)].

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