Drought stress strengthens the link between chlorophyll fluorescence parameters and photosynthetic traits

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Chlorophyll fluorescence (ChlF) has been used to understand photosynthesis and its response to climate change, particularly with satellite-based data. However, it remains unclear how the ChlF ratio and photosynthesis are linked at the leaf level under drought stress. Here, we examined the link between ChlF ratio and photosynthesis at the leaf level by measuring photosynthetic traits, such as net CO₂ assimilation rate (Aₙ), the maximum carboxylation rate of Rubisco (V_{cmax}), the maximum rate of electron transport (J_{max}), stomatal conductance (gₛ), and total chlorophyll content (Chlₜ). The ChlF ratio of the leaf level such as maximum quantum efficiency of PSII (Fᵥ/Fₘ) based on fluorescence kinetics. ChlF intensity ratio (LD_{685}/LD_{740}) based on spectrum analysis was obtained. We found that a combination of the stomatal limitation, non-stomatal limitation, and Chlₜ regulated leaf photosynthesis under drought stress, while J_{max} and Chlₜ governed the ChlF ratio. A significant link between the ChlF ratio and Aₙ was found under drought stress while no significant correlation in the control, which indicated that drought stress strengthens the link between the ChlF ratio and photosynthetic traits. These results suggest that the ChlF ratio can be a powerful tool to track photosynthetic traits of terrestrial ecosystems under drought stress.
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Abstract
Chlorophyll fluorescence (ChlF) has been used to understand photosynthesis and its response to climate change, particularly with satellite-based data. However, it remains unclear how the ChlF ratio and photosynthesis are linked at the leaf level under drought stress. Here, we examined the link between ChlF ratio and photosynthesis at the leaf level by measuring photosynthetic traits, such as net CO₂ assimilation rate (A_n), the maximum carboxylation rate of Rubisco (V_{cmax}), the maximum rate of electron transport (J_{max}), stomatal conductance (g_s) and total chlorophyll content (Chl_t). The ChlF ratio of the leaf level such as maximum quantum efficiency of PSII (F_v/F_m) based on fluorescence kinetics. ChlF intensity ratio (LD_{685}/LD_{740}) based on spectrum analysis was obtained. We found that a combination of the stomatal limitation, non-stomatal limitation, and Chl_t regulated leaf photosynthesis under drought stress, while J_{max} and Chl_t governed the ChlF ratio. A significant link between the ChlF ratio and A_n was found under drought stress while no significant correlation in the control, which indicated that drought stress strengthens the link between the ChlF ratio and photosynthetic traits. These results suggest that the ChlF ratio can be a powerful tool to track photosynthetic traits of terrestrial ecosystems under drought stress.
The duration and frequency of drought are expected to increase due to global warming (Pachauri et al. 2014). Drought stress increases the frequency of forest fires and the death rate of trees (Anderegg et al. 2013; Phillips et al. 2009), limits leaf photosynthesis and plant productivity (Akhhka et al. 2011; Tezara et al. 2003) and decreases the gross primary production (GPP) (Lee et al. 2013; Li et al. 2019). The terrestrial ecosystem GPP driven by leaf photosynthesis is tightly related to chlorophyll fluorescence (ChlF) (Murchie & Lawson 2013). In the light reaction of leaf photosynthesis, one of the main de-excitation processes for light absorption of the light-harvesting pigments is the emission of ChlF (Aasen et al. 2019). At the regional scale, solar-induced chlorophyll fluorescence (SIF) is observed based on solar irradiance and vegetation irradiance (Smith et al. 2018). ChlF opens a new perspective as a functional proxy of the terrestrial ecosystem GPP (He et al. 2019). However, it remains uncertain whether the link between ChlF and photosynthetic traits will be constrained by drought stress.

The net CO$_2$ assimilation rate ($A_n$) of leaves decreases during drought stress due to stomatal limitation and non-stomatal limitation (Ashraf & Harris 2013; Chaves et al. 2002; Flexas & Medrano 2002). On the one hand, stomatal limitation means that drought stress affects the diffusion process of CO$_2$ from the stomata to the intercellular spaces then reduces $A_n$ (Flexas et al. 2014; Lawlor & Tezara 2009). A study of pine seedlings found that the limitation of stomatal conductance ($g_s$) to $A_n$ increased during drought stress (Anev et al. 2016). On the other hand, non-stomata limitations include biochemical limitation and mesophyll conductance ($g_m$) limitation (Salmon et al. 2020). Drought stress decreases the maximum carboxylation rate of Rubisco ($V_{cmax}$) and the maximum rate of electron transport ($J_{max}$) then reduces $A_n$ (Flexas et al. 2004; Niinemets & Keenan 2014; Rho et al. 2012). A study of Eucalyptus and Quercus found that the $V_{cmax}$ and $J_{max}$ in the drought were significantly lower than those in the control (Zhou et al. 2014). Also, drought stress leads to a reduction of $g_m$, which limits the diffusion of CO$_2$ from the leaf intercellular spaces to the sites of the dark reactions of photosynthesis in chloroplasts (Flexas et al. 2012; Rancourt et al. 2015). Recent studies have incorporated the effects of stomatal and non-stomatal limitations for predicting the response of photosynthesis to drought stress (Drake et al. 2017; Salmon et al. 2020). Thus, understanding the relative contributions of stomatal limitation versus non-stomatal limitation to the decline of $A_n$ is fundamental to project the effect of drought stress (Campos et al. 2014a; Chen et al. 2015; Gimeno et al. 2019).

ChlF is a fast, accurate, and non-destructive probe, which can be utilized to obtain information about the metabolism of photosystem II (PSII) (Baker 2008). Photosynthetically active radiation is absorbed by chlorophyll and accessory pigments of chlorophyll-protein complexes and migrated to the reaction centers of photosystems I (PSI) and II (PSII), where the conversion of the quantum photosynthetic process takes place, and is then consumed by photochemistry, heat dissipation, or re-emitted as ChlF (Porcar-Castell et al. 2014). Due to the competition between these three processes, ChlF can be used to obtain photosynthesis information (Maxwell & Johnson 2000; Murchie & Lawson 2013). In recent years, researchers have used the changes of...
ChlF to explore photosynthetic apparatus under different environmental situations (Badr & Brueggemann 2020; Hajihashemi et al. 2020; Iqbal et al. 2019; Xu et al. 2020).

Most studies on ChlF are based on polyphasic fluorescence transient (OJIP) to obtain fluorescence kinetic parameters, such as the maximum quantum efficiency of PSII reaction centers ($F_v/F_m$), the photochemical ($qP$) and non-photochemical (NPQ) quenching (Mathobo et al. 2017). By analyzing the fluorescence kinetic curves, we can obtain abundant information about the structure and the function of PSII during stress conditions (Krause & Weis 1991; Stirbet et al. 2018). $F_v/F_m$ is the maximal quantum efficiency of PSII reaction centers which positively correlated with the activity of primary PSII photochemistry (Butler 1978; Stirbet et al. 2018). Low $F_v/F_m$ represents that light energy absorbed by PSII reaction centers may be underutilized (Fracheboud & Leipner 2003). PSII is considered to be a susceptible component of the photosynthetic machinery and will often bear the brunt of stress conditions, which leads to a decrease in $F_v/F_m$ (Demmig-Adams & Adams 2018; Long 1994). For example, a study of *Viburnum* found that the $F_v/F_m$ significantly decreased during a severe drought (Tribulato et al. 2019). Likewise, Li et al. (2008) analyzed the effect of drought stress on the photochemical efficiency of leaves and found that the $F_v/F_m$ was decreased while the NPQ increased during severe drought stress. The decrease in $F_v/F_m$ indicates the down-regulation of photosynthesis or photoinhibition under stress (Lichtenthaler & Rinderle 1988; Van Kooten 1990). Therefore, fluorescence kinetic parameters have been used to determine photosynthetic traits successfully.

Laser-induced fluorescence spectrum analysis is a specific technique that provides a new approach to monitor vegetation physiology remotely (Gouveia-Neto et al. 2011; Utkin et al. 2014). Leaves have two fluorescence emission peaks located in the 685 nm of the red region ($LD_{685}$) and the 740 nm of the far-red region ($LD_{740}$) (Buschmann 2007), which are closely related to the chlorophyll content (Chl$_t$) (Kalmatskaya et al. 2016; Nyachiro et al. 2001). $LD_{685}$ and $LD_{740}$ both increase with the increases of Chl$_t$ at low Chl$_t$, while in the case of higher Chl$_t$, $LD_{685}$ will decrease due to re-absorption of the emitted red band fluorescence by the chlorophyll absorption bands (Baker 2008; Buschmann 2007). It has been demonstrated that $LD_{685}/LD_{740}$ is a good inverse indicator of the Chl$_t$ and reflects the active degree of photosynthesis (Baker 2008; D'Ambrosio et al. 1992). However, there has been a lack of synchronous observation for fluorescence kinetic parameters and fluorescence spectrum, which can be used to evaluate the response of leaf to drought stress (Magney et al. 2017).

In this study, cucumber was used as an ideal test plant due to its short growth period, easy survival, and widely used in ecophysiology research (Li et al. 2008). A drought experiment was conducted over an 8-day from November 24 to December 1, 2018. Gas exchange parameters, fluorescence kinetic parameters, fluorescence spectrum, and chlorophyll content were measured in cucumber leaf. Here, our overall objective was to assess the response of photosynthesis traits and ChlF ratio to drought stress based on synchronous observation of gas exchange and fluorescence under drought stress. We hypothesize that (i) photosynthesis will be inhibited by stomatal and non-stomatal limitations under drought stress, (ii) the relationship between ChlF ratio and
chlorophyll content may be changed under drought stress, and (iii) ChlF ratio can be used to reflect photosynthesis.

### Materials & Methods

#### Plant material and experimental design

Cucumbers (*Cucumis sativus L.*) were used as plant material, which was cultured in plastic seedling pots (12×8×10 cm) and cultivated in a growth chamber. Growth chamber temperature was 20-25°C at day and 15-18 °C at night then light intensity was 1200 μmol m⁻² s⁻¹ with relative humidity (RH) at 75%. The potting soil was a composite culture substrate composed of wood chips, peat, pine bark, and sand. Six mature cucumbers were divided randomly into two treatments: drought and control, with three replicates per treatment. The drought treatment started on November 21, 2018. The soil moisture content (θᵥ) of the drought was measured about 8 ± 2% by the weighing method on November 23, 2018, while the soil moisture content of the control was about 15 ± 1%. During the experiment, the plants of the drought treatment were not irrigated, while the plants of the control group were irrigated daily. The upmost, sunlit, dark green, fully unfolded and mainstem leaves were used to measure gas exchange and $F_{v}/F_{m}$, and adjacent leaves were used to measure laser-induced chlorophyll fluorescence and Chlᵣ.

#### Measurement of the CO₂ response curve and chlorophyll fluorescence

Typical $A_{n}/C_{i}$ curves (light-saturated net CO₂ assimilation rate versus intercellular CO₂ concentrations) were measured using the Li-6800 portable photosynthesis system (LI-COR Inc., USA) from 8:00 to 11:30 after two days of drought treatment. The upmost fully unfolded, mainstem leaves were measured at leaf temperature of 25°C, RH of 50-60%, and photosynthetic photon flux density (PPFD) of 1500 μmol m⁻² s⁻¹. The carbon dioxide concentration of the reference chamber was set as 400, 100, 50, 100, 400, 600, 800, 1000 μmol mol⁻¹. A total of 54 $A_{n}/C_{i}$ curves were taken (i.e., 3 samples per treatment ×7 times per sample × 2 treatments + 6 samples per treatment on the first day × 2 treatments = 54 curves ). Before measuring the $A_{n}/C_{i}$ curve, the leaves were adapted for 5 minutes at a CO₂ concentration of 400 μmol mol⁻¹.

Measurements of the $A_{n}/C_{i}$ curve were taken when gas exchange had equilibrated (taken to be when the coefficient of variation for the CO₂ partial pressure differential was below 1% between the sample and reference analyzers). This condition was typically achieved within 1-2 min after a stable CO₂ concentration had been reached.

$F_{v}/F_{m}$ was measured using the Li-6800 fluorescence leaf chamber (LI-COR Inc., USA) connected to an LI-6800 portable photosynthesis system after dark treatment for one night. In the evening before the measurement, the upmost fully unfolded, mainstem leaves used to measure the dark-adapted fluorescence parameters were wrapped with tin foil. The rectangular flash was configured with a red target of 8000 μmol m⁻² s⁻¹, a duration of 1000 ms, the output rate of 100 Hz, and a margin of 5 points.

The laser-induced chlorophyll fluorescence system is composed of blue laser light source with a peak emission of 456 nm (Dslaser, China), USB4000 grating spectrometer, VIS-NIR band optical fiber (Ocean Optics, USA), Long-pass optical filter (AT600lp, Chroma Technology Corp,
USA), and computer with software (Figure 1). The light source output power is 40 mW, and the corresponding light source input voltage is 5.7 V. The spectrometer used in the experiment has a resolution of 1.5 nm, an integral time of 3.8 ms-10 s, and detector covers of 200-1100 nm. The spectrometer is equipped with a USB port on the side, which is connected to the computer and directly powered by the computer. The linear array CCD detector (Toshiba, Japan) of the USB4000 spectrometer has a pixel count of 3648 (Li et al. 2009). SMA905 fiber adapter (DingSuo Technologies, China) is used as a connector to match the VIS-NIR band optical fiber and USB4000 spectrometer. VIS-NIR band optical fiber has an optical fiber core diameter of 1000 um, numerical aperture of 0.22, and divergent Angle of 25.4°. The included angle between light source and leaves is 45°, the fiber is perpendicular to the leaves with a distance of 4.5 cm. The long-pass optical filter with a transmission wavelength range greater than 600 nm, and transmittance greater than 90% to prevent the influence of reflected light on the fluorescence spectrum. The chlorophyll fluorescence of 650-850 nm was received by optical fiber then collected by the spectrometer. SpectraWiz software (StellarNet Inc., Tampa FL, USA) was used to set up to collect three spectra and take the average, the integrating time with 600 ms.

Measurement of chlorophyll content

The leaves used to measure the ChlF spectrum were cut out and used to measure Chl. Starting from November 24, 2018, Chl was measured every other day. The control and drought were repeated three times (repeated six times on the first day), for a total of four measurements. A total of 30 chlorophyll content were collected (i.e., 3 samples per treatment ×3 times per sample × 2 treatments + 6 samples per treatment on the first day × 2 treatments =30 ). Acetone and anhydrous ethanol were mixed into the extract at a volume of 1:1. The leaves were cut into filaments and weighed at 0.1g in the test tube containing the mixture, which was placed in the dark place. After the material was completely white, the optical density at 663 nm and 645 nm was measured by spectrophotometer (MAPADA, China). The Chl in this study was expressed as follows:

\[ \text{Chl}_a = 12.72 \times A_{663} - 2.59 \times A_{645} \times 0.1 \]  
\[ \text{Chl}_b = 22.88 \times A_{663} - 4.67 \times A_{645} \times 0.1 \]  
\[ \text{Chl}_t = \text{Chl}_a + \text{Chl}_b \]  

Where Chl\(_a\) is the chlorophyll A content (mg g\(^{-1}\)), and Chl\(_b\) is the chlorophyll B content (mg g\(^{-1}\)), and Chl\(_t\) is the total chlorophyll content (mg g\(^{-1}\)). The A\(_{645}\) and A\(_{663}\) are absorbances at wavelengths 645 and 663, respectively.

Statistical Analysis

\( V_{\text{max}} \) and \( J_{\text{max}} \) were estimated by fitting the \( A_n/C_i \) curves using a spreadsheet-based software developed by Sharkey (Sharkey 2016). The chlorophyll fluorescence collection program for the spectrometer was written based on the underlying program of the spectrometer with MATLAB software (Yu Haiye 2009). Dark current noise is removed from the chlorophyll fluorescence spectrum curve and the curve is smoothed by Savitzky-Golay filtering (Gorry 1990).

We repeated measurements of the same six individuals. Repeated Measures ANOVA (RMANOVA) was used to test the effects of drought stress on photosynthetic traits and
chlorophyll fluorescence parameters. The effects were considered to be significantly different if
P < 0.05. Besides, a mixed-effect linear model was used to evaluate the effect of V_{cmax}, J_{max}, g_s,
and Chl_i on A_n and chlorophyll fluorescence parameters. The individual plant was used as a
random term. Similar method was used to test the relation between A_n and chlorophyll
fluorescence parameters. All statistical analyses were performed using SPSS 25.0 (SPSS Inc.,
USA).

Results

The response of leaf photosynthetic traits and ChlF ratio to drought

The drought stress caused a significant reduction in A_n, V_{cmax}, J_{max}, g_s, and Chl_i compared with
control (P < 0.05). The averages of A_n were 1.0 ± 0.1 μmol m^{-2} s^{-1} and 2.0 ± 0.2 μmol m^{-2} s^{-1} in
drought and control (Figure 2a). The averages of V_{cmax} and J_{max} were 92.0 ± 5.0 μmol m^{-2} s^{-1} and
117.0 ± 5.0 μmol m^{-2} s^{-1} in drought, respectively (Figure 3a, c). For control, the averages of V_{cmax}
and J_{max} were 105.9 ± 4.3 μmol m^{-2} s^{-1} and 141.0 ± 5.2 μmol m^{-2} s^{-1}, respectively (Figure 3a, c).

Compared to control, the averages of V_{cmax} and J_{max} decreased 13.1% and 17.1%, while the g_s
and Chl_i in drought (0.1 ± 0.01 mol m^{-2} s^{-1} and 1.6 ± 0.1 mg g^{-1}) were reduced by 27.1% and
21.5% compared with control (0.1 ± 0.01 mol m^{-2} s^{-1} and 2.1 ± 0.1 mg g^{-1}) (Figure 3e, g).

Compared to the control plants, the averages of F_s/F_m decreased 6.8% (0.74 ± 0.01 to 0.69 ±
0.13), while LD_{685}/LD_{740} increased 10.7% (1.1 ± 0.4 to 1.2 ± 0.3).

Control factors for A_n and ChlF ratio

Significant positive correlation was found between A_n and V_{cmax} in the control (R^2 = 0.15, P =
0.03) and drought stress (R^2 = 0.45, P < 0.001). There was a significant positive correlation
between J_{max} and A_n in the control (R^2 = 0.12, P = 0.04) and under drought stress (R^2 = 0.60, P <
0.001). Besides the significant positive correlation (R^2 = 0.11, P = 0.05) between g_s and A_n in the
control, a significant positive correlation (R^2 = 0.48, P < 0.001) in the drought stress was
observed. A poor correlation (R^2 = 0.07, P = 0.84) between A_n and Chl_i was observed in the
control, whereas the correlation was positive (R^2 = 0.56, P < 0.001) in the drought stress (Figures
4a-d).

A significant positive correlation between F_s/F_m and V_{cmax} was found in the drought (R^2 = 0.13, P
= 0.03). Meanwhile, a marginally positive correlation between F_s/F_m and J_{max} was observed in
the control (R^2 = 0.09, P = 0.09), and a significant positive correlation was observed in the
drought stress (R^2 = 0.28, P = 0.03). In addition, a significant positive correlation has been found
between F_s/F_m and g_s in the control (R^2 = 0.28, P = 0.003) while the correlation was poor under
drought stress (R^2 = 0.01, P = 0.22). No significant correlation (R^2 = 0.03, P = 0.27) was
observed between F_s/F_m and Chl_i in the control group, whereas a significant positive correlation
was found between F_s/F_m and Chl_i under drought stress (R^2 = 0.29, P = 0.02) (Figures 4e-h).

A significant negative correlation was found between LD_{685}/LD_{740} and V_{cmax} in drought stress
(R^2 = 0.18, P = 0.009), while there was no significant correlation between LD_{685}/LD_{740} and V_{cmax}
in control (R^2 = 0.03, P = 0.50). There was no significant correlation between LD_{685}/LD_{740} and
J_{max} in control (R^2 = 0.03, P = 0.69), while a significant negative correlation was observed
between LD$_{685}$/LD$_{740}$ and J$_{\text{max}}$ in drought stress ($R^2 = 0.13$, $P = 0.04$). No significant correlation was observed between LD$_{685}$/LD$_{740}$ and $g_s$ in control ($P = 0.13$) and drought stress ($P = 0.09$).

There was no significant correlation between the LD$_{685}$/LD$_{740}$ and Chl$_t$ in the control ($R^2 = 0.06$, $P = 0.68$), while a significant negative correlation between LD$_{685}$/LD$_{740}$ and Chl$_t$ in drought stress ($R^2 = 0.17$, $P = 0.02$) (Figures 4i-l).

**Correlation between $A_n$ and ChlF ratio**

There was a marginally positive correlation between $A_n$ and $F_v/F_m$ in the control ($R^2 = 0.08$, $P = 0.09$). However, the correlation between $A_n$ and $F_v/F_m$ was significant positive in drought stress ($R^2 = 0.28$, $P = 0.003$). Similarly, there was no significant correlation between $A_n$ and LD$_{685}$/LD$_{740}$ in the control ($R^2 = 0.02$, $P = 0.45$), while a significant negative correlation was found between $A_n$ and LD$_{685}$/LD$_{740}$ in drought stress ($R^2 = 0.17$, $P = 0.02$) (Figures 5a-b).

**Discussion**

**Combination of the stomatal limitation, non-stomatal limitations, and chlorophyll content regulated leaf photosynthesis under drought stress**

As expected, drought stress significantly decreased the leaf photosynthesis of cucumber (Figure 2a). Although drought stress is known to reduce leaf photosynthesis, the processes responsible for the key limitations are still a matter of debate (Chaves et al. 2002; Flexas & Medrano 2002; Pinheiro & Chaves 2011). Here, our study found that the combination of the stomatal limitation, non-stomatal limitations, and chlorophyll content regulated the decrease of leaf photosynthesis under drought stress (Figures 4a, b, c, d). Increasing evidence shows that leaf photosynthesis under drought stress is not limited by a single process (Zhou et al. 2015). It has been demonstrated that stomatal closure reduces photosynthesis and transpiration while improving water use efficiency due to acclimation under drought stress (Feller 2016; Lamaoui et al. 2018).

Non-stomatal limitations were defined as the sum of the contributions of mesophyll conductance and leaf biochemistry, which directly reflect the biochemical process of photosynthesis (Grassi & Magnani 2005). The decrease in $V_{\text{cmax}}$ and J$_{\text{max}}$ may result from a decrease in the amount of active Rubisco and an inadequate supply of ATP or NADPH or to a low enzymatic activity during the photosynthetic carbon reduction cycle (Campos et al. 2014b; Flexas et al. 2004; Peña-Rojas et al. 2004). Recent studies found that stomatal and non-stomatal limitations to photosynthesis are coordinated on similar timescales, and suggested that non-stomatal limitations should be included in predict the model of photosynthesis response to drought (Drake et al. 2017; Salmon et al. 2020). A study of three Mediterranean species has found that the decrease of $A_n$ was simultaneous regulated by stomatal and biochemical limitations during drought stress (Varone et al. 2012). In addition, Chl$_t$ is the main pigment that absorbs photosynthetically active radiation and can indirectly reflect the integrity of the photosynthetic device (Streit et al. 2005). Chl$_t$ can be used as a functional trait to evaluate drought stress (Pilon et al. 2018; Pilon et al. 2014). Therefore, our observations suggest that the combination of the stomatal limitation, non-stomatal limitation, and chlorophyll content should be taken into consideration in process-based models for simulating photosynthesis in terrestrial ecosystems under drought stress.
**J\textsubscript{max} and Chl\textsubscript{t} governed ChlF ratio under drought stress**

Leaf \( F_v/F_m \) is a crucial chlorophyll fluorescence parameter for evaluating the health or integrity of the internal apparatus under drought stress (Krause & Weis 1991; Urban et al. 2017). Here, we found that \( F_v/F_m \) was significantly decreased under drought stress (Figure 2b), which revealed that the PSII may be damaged under drought stress, and the primary reaction of photosynthesis may be inhibited (Lichtenthaler & Rinderle 1988). The fluorescence parameters of the leaves are changed in two ways under stress conditions. The minimal fluorescence (\( F_o \)) increases due to obstruction of the electron flow through PSII, and plastoquinone acceptor (QA\textsuperscript{+}) cannot be completely oxidized during stress. Simultaneously, the reduction of \( F_m \) during stress may be affected by decreased activity of the water-splitting enzyme complex and perhaps a concomitant cyclic electron transport within or around PSII (Porcar-Castell et al. 2014). Therefore, \( F_v/F_m \) will decrease under drought stress. Our finding was consistent with a previous studying, in which drought stress inhibited the photochemical activity of PSII and decreased leaf \( F_v/F_m \) (Meng et al. 2016). Meanwhile, our study showed that \( F_v/F_m \) was largely related to \( J_{\text{max}} \) and Chl\textsubscript{t} under drought stress (Figures 4f, h). It has been proposed that \( J_{\text{max}} \) is decreased by drought stress, which prevents the electron from rapidly transferring back, and hinders the whole photochemical process (Baker & Rosenqvist 2004; Khatri & Rathore 2019). Similarly, the decrease in Chl\textsubscript{t} will weaken the photochemical process, which demonstrates the dependence of the light absorption and fluorescence emission on the concentration of chlorophyll molecules in the chloroplast (Nyachiro et al. 2001). Thus, the significant linear relationship between \( F_v/F_m \) and \( J_{\text{max}} \) and Chl\textsubscript{t} observed in drought stress jointly indicates the importance of \( J_{\text{max}} \) and Chl\textsubscript{t} in governing chlorophyll fluorescence.

In our study, the \( \text{LD}_{685}/\text{LD}_{740} \) based on spectral analysis was significantly increased under drought stress (Figure 2c). This finding was similar to the study of Meng et al. (2016), in which the fluorescence intensity ratio increased when the PSII was damaged. Moreover, \( \text{LD}_{685}/\text{LD}_{740} \) was largely regulated by \( J_{\text{max}} \) and Chl\textsubscript{t} under drought stress (Figure 4j, l). The previous study found that changes in the chlorophyll content resulted in changes of more than 90% for the \( F_{690}/F_{735} \) ratio (Csintalan et al. 1998). There was a significant negative correlation between the \( \text{LD}_{685}/\text{LD}_{740} \) and Chl\textsubscript{t} under drought stress (Figure 4l). The chlorophyll absorption spectrum overlaps with the chlorophyll fluorescence emission spectrum in the red band, which results in the \( \text{LD}_{685} \) being decreased by re-absorption in the case of higher chlorophyll content. The effect of re-absorption on the red band is stronger than that of the far-red band, and therefore the \( \text{LD}_{685}/\text{LD}_{740} \) will decrease (Buschmann 2007). The \( \text{LD}_{685}/\text{LD}_{740} \) represents an ideal tool for evaluating the change of Chl\textsubscript{t} and reflects the photochemical activity of PSII indirectly under drought stress (Figure 4j, l). Spectral analysis has been used to directly assess ecosystem functioning under climate change. For instance, Gameiro et al. (2016) found a significant linear relationship between the fluorescence intensity ratio and leaf water content of *Arabidopsis*. Norikane et al. (2003) successfully monitor the growth of tomato under drought stress based on spectral analysis. Here, synchronous observation of \( \text{LD}_{685}/\text{LD}_{740} \) and \( F_v/F_m \) based on spectral...
analysis and fluorescence kinetics suggest that LD_{685}/LD_{740} can be used as an indicator for detection of plant stress.

**Drought stress strengthens the relationship between net CO\(_2\) assimilation rate (A\(_n\)) and ChlF ratio**

Our study reported a significant relationship between the A\(_n\) and ChlF ratio under drought stress, while no significant correlation was found in the control (Figure 5a, b). The strengthening relationship between A\(_n\) and ChlF ratio may be ascribed to variations of J\(_{\text{max}}\) and Chl\(_t\) under drought stress. On the one hand, the reduction of F\(_v\)/F\(_m\) and J\(_{\text{max}}\) under drought indicated that the photosynthetic electron transport was damaged. Drought stress damages the reaction center of PSII and inhibits the electron transfer process of photosynthesis, which reduces the light energy conversion efficiency of PSII (Brestic et al. 1995; Cornic & Fresneau 2002; Longenberger et al. 2009). On the other hand, drought stress deforms the leaf chloroplast layer structure and reduces chlorophyll content (Batra et al. 2014). So, J\(_{\text{max}}\) and Chl\(_t\) became limiting factors for the A\(_n\) and ChlF ratio under drought stress (Figures 4b, d, f, h, j, l). The strengthening relationship between A\(_n\) and ChlF ratio has been observed in previous studies (Murchie & Lawson 2013; Su et al. 2015). For example, Wang et al. (2018) found a significant linear relationship between A\(_n\) and F\(_v\)/F\(_m\) in the soybean experiment under drought conditions. Batra et al. (2014) found similar results by studying the ChlF ratio characteristics of mung beans under drought conditions. Therefore, ChlF ratio based on spectral analysis and fluorescence kinetics was a better indicator of the photosynthetic capacity under drought stress.

**Conclusions**

Our results demonstrate that the decrease in cucumber leaf photosynthesis is regulated by stomatal limitation, non-stomatal limitation, and chlorophyll content under drought stress. We recommend that incorporated the effects of stomatal, non-stomatal limitations and chlorophyll content, and applied it to the prediction of plant photosynthesis response to drought stress. The J\(_{\text{max}}\) and Chl\(_t\) are key limiting factors for the ChlF ratio under drought stress, and the ChlF can characterize plant photosynthetic capacity as new technology under drought stress.

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**Figure 1**

Schematic diagram of laser-induced chlorophyll fluorescence experimental system
Figure 2

Variations of net CO$_2$ assimilation rate and chlorophyll fluorescence parameters in control and drought stress

(a,b) $A_n$ (net CO$_2$ assimilation rate $\mu$mol m$^{-2}$ s$^{-1}$), (c,d) $F_v/F_m$ (maximum quantum efficiency of PSII), (e,f) LD$_{685}$/LD$_{740}$ (Laser induced chlorophyll fluorescence intensity ratio). In the box plot, the points and short error bars represent the mean ($\pm$SE) of $n = 27$ per treatment, and the line and long error bars represent the median line and 95% CI, respectively. In the line chart, the points and error bars reflect the mean ($\pm$SE) of 3 replicate per treatment per date (replicate 6 per treatment on the first day). The blue and red indicates the control and drought treatment, respectively. RMANOVA was used estimate the effect of treatment: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; n.s. not significant.
Figure 3

Variations of photosynthetic traits and total chlorophyll concentration in control and drought stress

(a,b) \( V_{\text{cmax}} \) (Maximum carboxylation rate, \( \mu \text{mol m}^{-2} \text{s}^{-1} \)), (c,d) \( J_{\text{max}} \) (Maximum photoelectron transfer rate, \( \mu \text{mol m}^{-2} \text{s}^{-1} \)), (e,f) \( g_{s} \) (stomatal conductance, \( \text{mol m}^{-2} \text{s}^{-1} \)), (g,h) \( \text{Chlt} \) (Total chlorophyll concentration, \( \text{mg g}^{-1} \)) in control and drought stress. In the box plot, the points and short error bars represent the mean (±SE) of \( n = 27 \) per treatment (\( n = 15 \) of Chl, per treatment), and the line and long error bars represent the median line and 95% CI, respectively. In the line chart, the points and error bars reflect the mean (±SE) of 3 replicate per treatment per date ( replicate 6 per treatment on the first day). The blue and red indicate the control and drought stress, respectively. RMANOVA was used estimate the effect of treatment: \* \( P < 0.05 \); ** \( P < 0.01 \); *** \( P < 0.001 \); n.s. not significant.
Figure 4

Relationships between chlorophyll fluorescence parameters and photosynthetic traits in the control and drought stress

(a) $A_n$ (net CO$_2$ assimilation rate $\mu$mol m$^{-2}$ s$^{-1}$) and $V_{cmax}$ (Maximum carboxylation rate, $\mu$mol m$^{-2}$ s$^{-1}$), (b) $A_n$ and $J_{max}$ (Maximum photoelectron transfer rate, $\mu$mol m$^{-2}$ s$^{-1}$), (c) $A_n$ and $g_s$ (stomatal conductance, mol m$^{-2}$ s$^{-1}$), (d) $A_n$ and Chl$_t$ (Total chlorophyll concentration, mg g$^{-1}$), (e) $F_{v}/F_{m}$ (maximum quantum efficiency of PSII) and $V_{cmax}$, (f) $F_{v}/F_{m}$ and $J_{max}$, (g) $F_{v}/F_{m}$ and $g_{s}$, (h) $F_{v}/F_{m}$ and Chl$_t$, (i) LD$_{685}$/LD$_{740}$ (Laser-induced chlorophyll fluorescence intensity ratio) and $V_{cmax}$, (j) $F_{v}/F_{m}$ and $J_{max}$, (k) $F_{v}/F_{m}$ and $g_{s}$, (l) $F_{v}/F_{m}$ and Chl$_t$. Linear fitting was used for correlation analysis (n = 27 for per treatment and n = 15 of Chl$_t$ for per treatment). The blue line and red line indicate the linear regression for the control and drought stress, respectively.
Manuscript to be reviewed

(a) $y = 0.02x + 0.29 \quad R^2 = 0.15 \quad P = 0.03$

(b) $y = 0.01x + 0.50 \quad R^2 = 0.12 \quad P = 0.04$

(c) $y = 6.59x + 1.18 \quad R^2 = 0.11 \quad P = 0.05$

(d) $y = 8.27x + 0.26 \quad R^2 = 0.48 \quad P < 0.001$

(e) $y = 0.13x + 2.38 \quad R^2 = 0.07 \quad P = 0.84$

(f) $y = 1.06x - 1.86 \quad R^2 = 0.56 \quad P < 0.001$

(g) $y = 0.0004x + 0.70 \quad R^2 = 0.02 \quad P = 0.21$

(h) $y = 0.0010x + 0.60 \quad R^2 = 0.13 \quad P = 0.03$

(i) $y = 0.44x + 0.69 \quad R^2 = 0.28 \quad P = 0.03$

(j) $y = 0.29x + 0.67 \quad R^2 = 0.01 \quad P = 0.22$

(k) $y = 0.07x + 0.60 \quad R^2 = 0.29 \quad P = 0.02$

(l) $y = 0.001x + 0.95 \quad R^2 = 0.03 \quad P = 0.50$

(m) $y = -0.03x + 1.49 \quad R^2 = 0.18 \quad P = 0.99$

(n) $y = -0.92x + 1.18 \quad R^2 = 0.03 \quad P = 0.69$

(o) $y = -1.32x + 1.25 \quad R^2 = 0.05 \quad P = 0.13$

(p) $y = -0.94x + 1.31 \quad R^2 = 0.12 \quad P = 0.09$

(q) $y = -0.18x + 1.49 \quad R^2 = 0.25 \quad P = 0.03$
Figure 5

Relationships between net CO$_2$ assimilation rate and chlorophyll fluorescence parameters in control and drought stress

(a) $A_n$ (net CO$_2$ assimilation rate μmol m$^{-2}$ s$^{-1}$) and $F_v/F_m$ (maximum quantum efficiency of PSII), (b) or $A_n$ and $LD_{685}/LD_{740}$ (Laser-induced chlorophyll fluorescence intensity ratio). Linear fitting was used for correlation analysis ($n = 27$ for per treatment). The blue line and red line indicate the linear regression for the control and drought stress, respectively.