

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

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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) is 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.

Subjects Ecology, Ecosystem Science, Plant Science, Climate Change Biology

Keywords V_{cmax} , J_{max} , g_s , Chlorophyll fluorescence spectrum, Remote sensing, Chlorophyll content

INTRODUCTION

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, Kane & Anderegg, 2013; Phillips et al., 2009*), limits leaf photosynthesis and plant productivity (*Akhkha, Boutraa & Alhejely, 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,

Submitted 25 February 2020
Accepted 4 September 2020
Published 24 September 2020

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Academic editor

Yann Salmon

Additional Information and
Declarations can be found on
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DOI 10.7717/peerj.10046

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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 CO₂ 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 CO₂ from the stomata to the intercellular spaces, and 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 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 CO₂ from the leaf intercellular spaces to the sites of the dark reactions of photosynthesis in chloroplasts (Flexas *et al.*, 2012; Rancourt, Ethier & Pepin, 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.*, 2014; Chen, Yu & Huang, 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, Marais & Steyn, 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, Humphries & Falkowski, 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, Karavaev & Gunar, 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, Szabo & Lichtenthaler, 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 because it is 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 would be inhibited by stomatal and non-stomatal limitations under drought stress, (ii) the relationship between the ChlF ratio and chlorophyll content might be changed under drought stress, and (iii) the 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 $\mu\text{mol m}^{-2} \text{s}^{-1}$ 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 (θ_g) of the drought was measured about $8 \pm 2\%$ by the weighing method on November 23, 2018, while the soil moisture content of the control was about $15 \pm 1\%$. 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 Chl_t .

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 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The carbon dioxide concentration of the reference chamber was set as 400, 100, 50, 100, 400, 600, 800, 1,000 $\mu\text{mol mol}^{-1}$. A total of 54 A_n/C_i curves were taken (i.e., 3 samples per treatment \times 7 times per sample \times 2 treatments + 6 samples per treatment on the first day \times 2 treatments = 54 curves). Before measuring the A_n/C_i curve, the leaves were adapted for 5 min at a CO₂ concentration of 400 $\mu\text{mol mol}^{-1}$. 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 8,000 $\mu\text{mol m}^{-2} \text{s}^{-1}$, a duration of 1,000 ms, the output rate of 100 Hz, and a margin of 5 points.

The laser-induced chlorophyll fluorescence system was 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 (Fig. 1). The light source output power was 40 mW, and the corresponding light source input voltage was 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 200–1,100 nm. The spectrometer was equipped with a USB port on the side, which was 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, Huang & Zhang, 2009). SMA905 fiber adapter (DingSuo

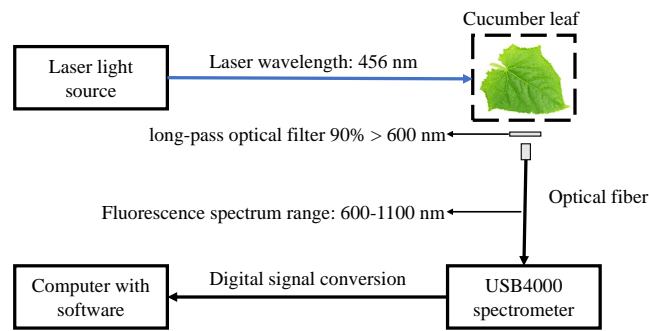


Figure 1 Schematic diagram of laser-induced chlorophyll fluorescence experimental system.

Full-size [DOI: 10.7717/peerj.10046/fig-1](https://doi.org/10.7717/peerj.10046/fig-1)

Technologies, China) was 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 1,000 μm , 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_t . Starting from November 24, 2018, Chl_t 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 \times 3 times per sample \times 2 treatments + 6 samples per treatment on the first day \times 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 0.1 g 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_t in this study was expressed as follows:

$$\text{Chl}_a = 12.72 \times A_{663} - 2.59 \times A_{645} \times 0.1 \quad (1)$$

$$\text{Chl}_b = 22.88 \times A_{663} - 4.67 \times A_{645} \times 0.1 \quad (2)$$

$$\text{Chl}_t = \text{Chl}_a + \text{Chl}_b. \quad (3)$$

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_{cmax} and J_{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 (Haiye, Haoyu & Lei, 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_t 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_t compared with control ($P < 0.05$). The averages of A_n were $1.0 \pm 0.1 \mu\text{mol m}^{-2} \text{s}^{-1}$ and $2.0 \pm 0.2 \mu\text{mol m}^{-2} \text{s}^{-1}$ in drought and control (Fig. 2A). The averages of V_{cmax} and J_{max} were $92.0 \pm 5.0 \mu\text{mol m}^{-2} \text{s}^{-1}$ and $117.0 \pm 5.0 \mu\text{mol m}^{-2} \text{s}^{-1}$ in drought, respectively (Figs. 3A and 3C). For control, the averages of V_{cmax} and J_{max} were $105.9 \pm 4.3 \mu\text{mol m}^{-2} \text{s}^{-1}$ and $141.0 \pm 5.2 \mu\text{mol m}^{-2} \text{s}^{-1}$, respectively (Figs. 3A and 3C). Compared to control, the averages of V_{cmax} and J_{max} decreased 13.1% and 17.1%, while the g_s and Chl_t in drought ($0.1 \pm 0.01 \text{ mol m}^{-2} \text{s}^{-1}$ and $1.6 \pm 0.1 \text{ mg g}^{-1}$) were reduced by 27.1% and 21.5% compared with control ($0.1 \pm 0.01 \text{ mol m}^{-2} \text{s}^{-1}$ and $2.1 \pm 0.1 \text{ mg g}^{-1}$) (Figs. 3E and 3G). Compared to the control plants, the averages of F_v/F_m decreased 6.8% (0.74 ± 0.01 to 0.69 ± 0.13), while $\text{LD}_{685}/\text{LD}_{740}$ 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_t was observed in the control, whereas the correlation was positive ($R^2 = 0.56$, $P < 0.001$) in the drought stress (Figs. 4A–4D).

A significant positive correlation between F_v/F_m and V_{cmax} was found in the drought ($R^2 = 0.13$, $P = 0.03$). Meanwhile, a marginally positive correlation between F_v/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

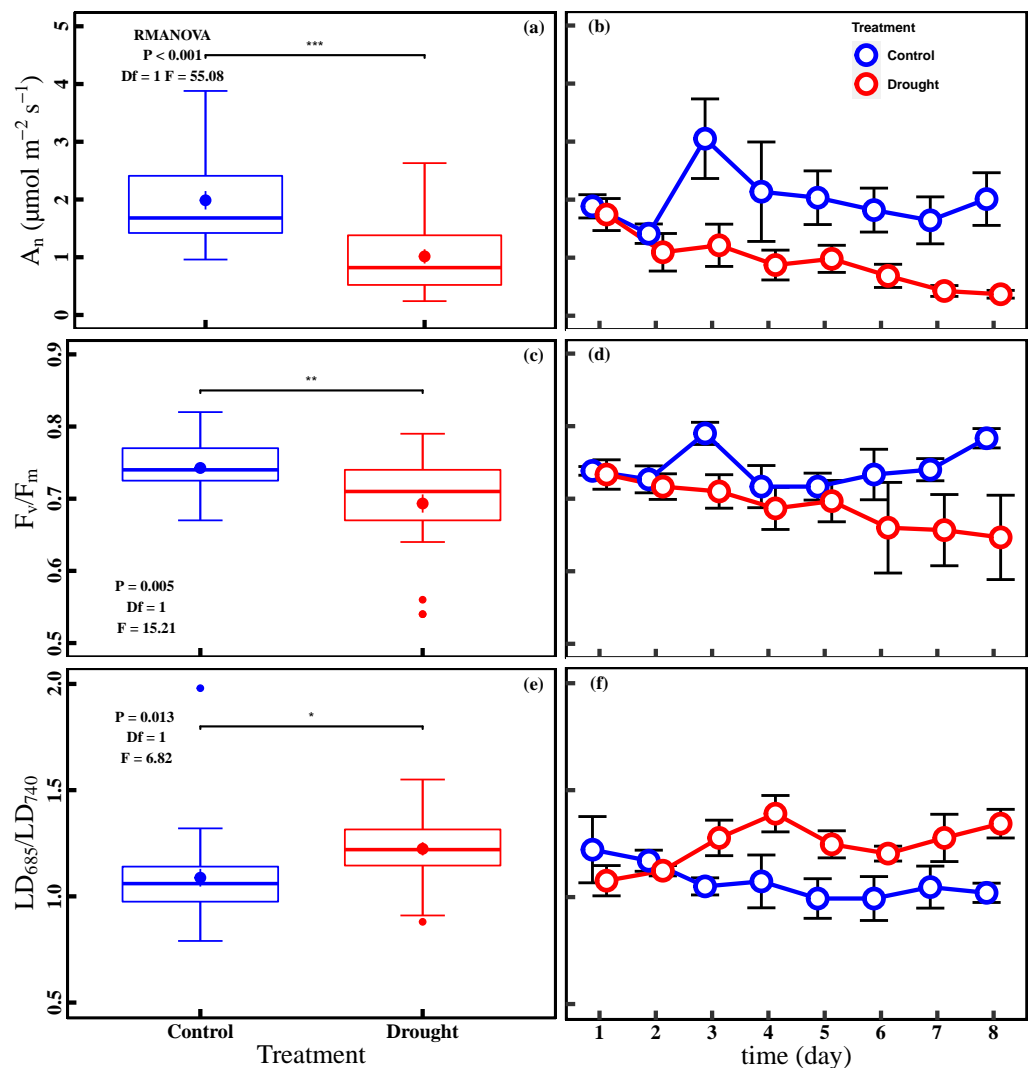


Figure 2 Variations of net CO₂ assimilation rate and chlorophyll fluorescence parameters in control and drought stress. (A, B) A_n (net CO₂ assimilation rate $\mu\text{mol m}^{-2} \text{s}^{-1}$), (C, D) F_v/F_m (maximum quantum efficiency of PSII), (E, F) LD_{685}/LD_{740} (Laser induced chlorophyll 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 three replicates per treatment per date (six replicates 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.

Full-size DOI: [10.7717/peerj.10046/fig-2](https://doi.org/10.7717/peerj.10046/fig-2)

correlation was found between F_v/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_v/F_m and Chl_t in the control group, whereas a significant positive correlation was found between F_v/F_m and Chl_t under drought stress ($R^2 = 0.29$, $P = 0.02$) (Figs. 4E–4H).

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

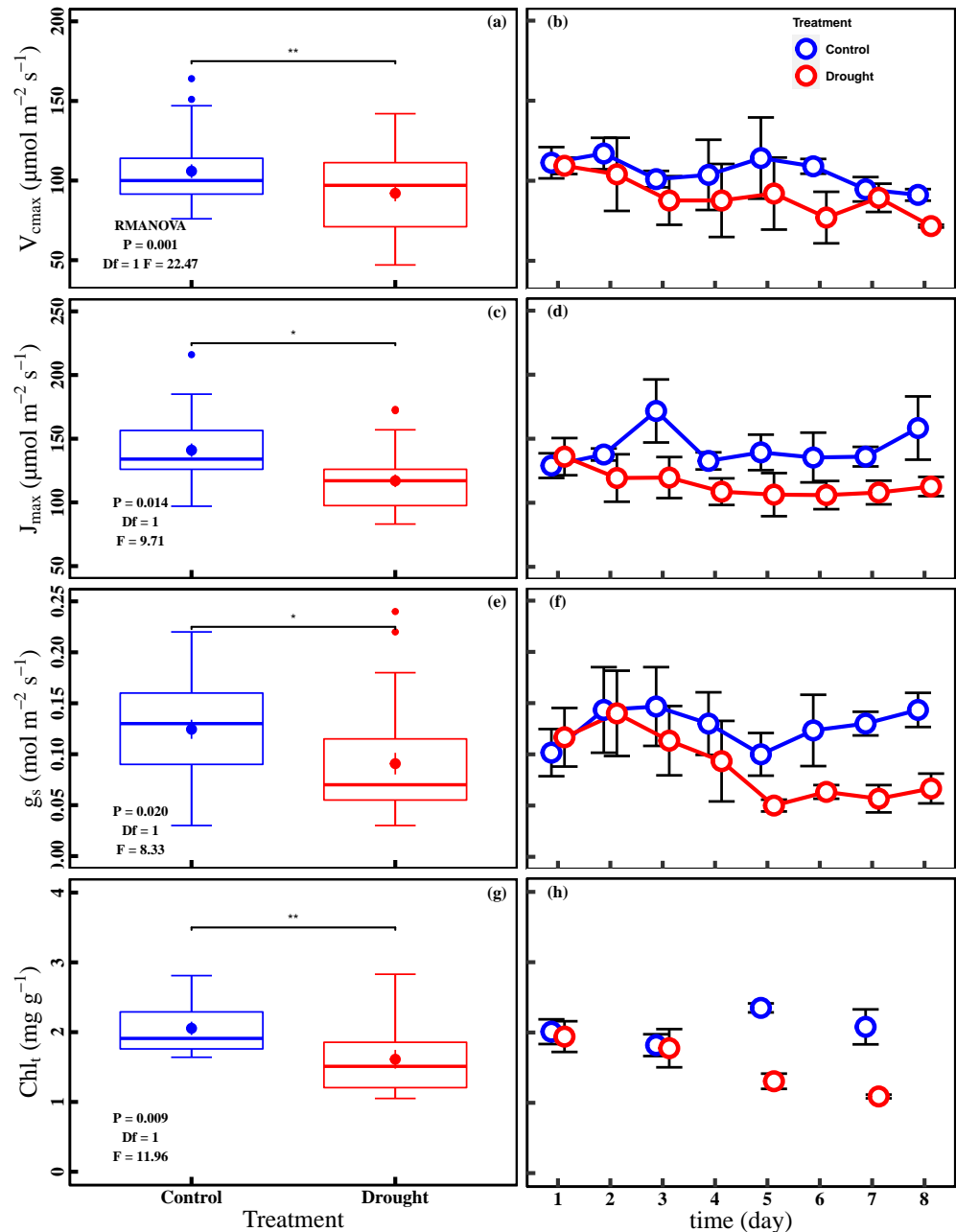


Figure 3 Variations of photosynthetic traits and total chlorophyll concentration in control and drought stress. (A, B) V_{cmax} (Maximum carboxylation rate, $\mu\text{mol m}^{-2} \text{s}^{-1}$), (C, D) J_{max} (Maximum photoelectron transfer rate, $\mu\text{mol m}^{-2} \text{s}^{-1}$), (E, F) g_s (stomatal conductance, $\text{mol m}^{-2} \text{s}^{-1}$), (G, H) Chl_t (Total chlorophyll concentration, mg g^{-1}) in control and drought stress. In the box plot, the points and short error bars represent the mean ($\pm\text{SE}$) of $n = 27$ per treatment ($n = 15$ of Chl_t 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 ($\pm\text{SE}$) of three replicates per treatment per date (six replicates 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.

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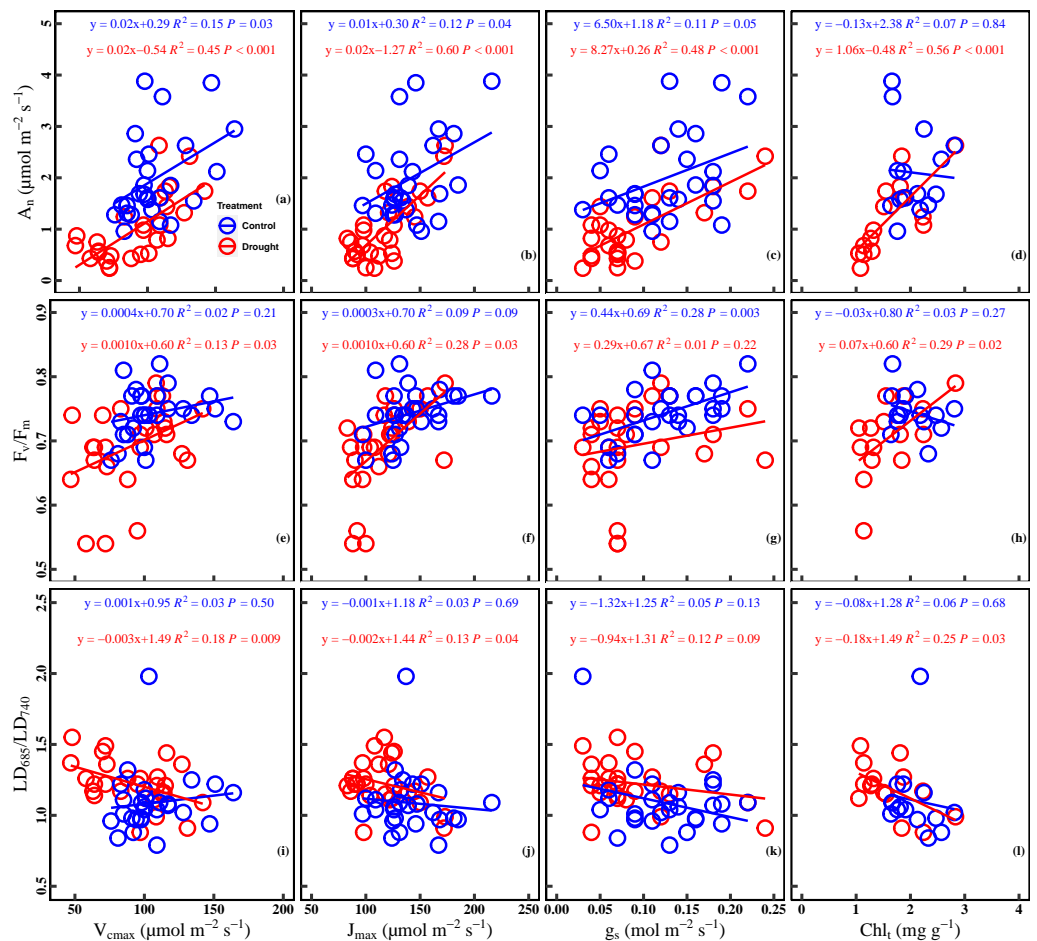


Figure 4 Relationships between chlorophyll fluorescence parameters and photosynthetic traits in the control and drought stress. (A) A_n (net CO_2 assimilation rate $\mu\text{mol m}^{-2} \text{s}^{-1}$) and V_{cmax} (Maximum carboxylation rate, $\mu\text{mol m}^{-2} \text{s}^{-1}$), (B) A_n and J_{max} (Maximum photoelectron transfer rate, $\mu\text{mol m}^{-2} \text{s}^{-1}$), (C) A_n and g_s (stomatal conductance, $\text{mol m}^{-2} \text{s}^{-1}$), (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) $\text{LD}_{685}/\text{LD}_{740}$ (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.

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$\text{LD}_{685}/\text{LD}_{740}$ and V_{cmax} in control ($R^2 = 0.03$, $P = 0.50$). There was no significant correlation between $\text{LD}_{685}/\text{LD}_{740}$ and J_{max} in control ($R^2 = 0.03$, $P = 0.69$), while a significant negative correlation was observed between $\text{LD}_{685}/\text{LD}_{740}$ and J_{max} in drought stress ($R^2 = 0.13$, $P = 0.04$). No significant correlation was observed between $\text{LD}_{685}/\text{LD}_{740}$ and g_s in control ($P = 0.13$) and drought stress ($P = 0.09$). There was no significant correlation between the $\text{LD}_{685}/\text{LD}_{740}$ and Chl_t in the control ($R^2 = 0.06$, $P = 0.68$), while a significant negative correlation between $\text{LD}_{685}/\text{LD}_{740}$ and Chl_t in drought stress ($R^2 = 0.25$, $P = 0.03$) (Figs. 4I–4L).

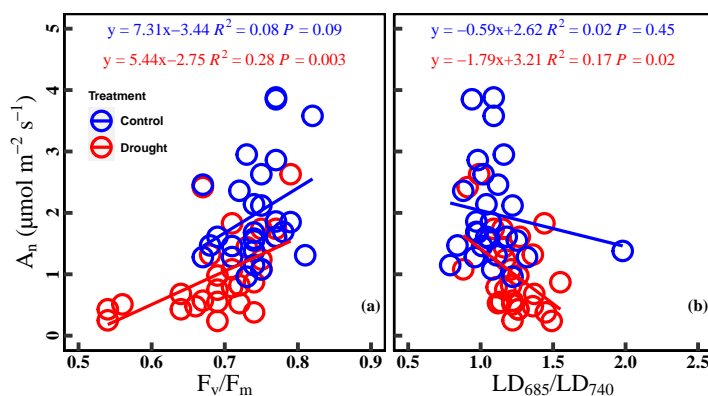


Figure 5 Relationships between net CO₂ assimilation rate and chlorophyll fluorescence parameters in control and drought stress. (A) A_n (net CO₂ assimilation rate μmol m⁻² s⁻¹) and F_v/F_m (maximum quantum efficiency of PSII), (B) or A_n and LD₆₈₅/LD₇₄₀ (Laser-induced chlorophyll fluorescence intensity 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.

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Correlation between A_n and ChIF 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 LD₆₈₅/LD₇₄₀ in the control ($R^2 = 0.02$, $P = 0.45$), while a significant negative correlation was found between A_n and LD₆₈₅/LD₇₄₀ in drought stress ($R^2 = 0.17$, $P = 0.02$) (Figs. 5A–5B).

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 (Fig. 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 (Figs. 4A–4D). 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_{cmax} and J_{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.*, 2014; Flexas *et al.*, 2004; Peña Rojas, Aranda & Fleck, 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 simultaneously 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_{max} and Chl_t governed ChF 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, Arrouf & Bidel, 2017). Here, we found that F_v/F_m was significantly decreased under drought stress (Fig. 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^-) 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_{max} and Chl_t under drought stress (Figs. 4F and 4H). It has been proposed that J_{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_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_{max} and Chl_t observed in drought stress jointly indicates the importance of J_{max} and Chl_t in governing chlorophyll fluorescence.

In our study, the LD_{685}/LD_{740} based on spectral analysis was significantly increased under drought stress (Fig. 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, LD_{685}/LD_{740} was largely regulated by J_{max} and Chl_t under drought stress (Figs. 4J and 4L). 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, Tuba & Lichtenthaler, 1998). There was

a significant negative correlation between the LD_{685}/LD_{740} and Chl_t under drought stress (Fig. 4L). The chlorophyll absorption spectrum overlaps with the chlorophyll fluorescence emission spectrum in the red band, which results in the 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 LD_{685}/LD_{740} will decrease (Buschmann, 2007). The LD_{685}/LD_{740} represents an ideal tool for evaluating the change of Chl_t and reflects the photochemical activity of PSII indirectly under drought stress (Figs. 4J and 4L). 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 LD_{685}/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 (Figs. 5A and 5B). The strengthening relationship between A_n and ChlF ratio may be ascribed to variations of J_{max} and Chl_t under drought stress. On the one hand, the reduction of F_v/F_m and J_{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, Sharma & Kumari, 2014). So, J_{max} and Chl_t became limiting factors for the A_n and ChlF ratio under drought stress (Figs. 4B, 4D, 4F, 4H, 4J and 4L). 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, Sharma & Kumari (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 incorporating the effects of stomatal, non-stomatal limitations and chlorophyll content, and applying them to the prediction of plant photosynthesis response to drought stress. The J_{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.

ACKNOWLEDGEMENTS

We gratefully acknowledge Yunhui Tan, Meijuan Fu, and Yao Si for their help with the measurement of the photosynthesis and chlorophyll fluorescence. We thank two anonymous reviewers and editor for their constructive comments.

ADDITIONAL INFORMATION AND DECLARATIONS

Funding

This study was supported financially by the Zhejiang Provincial Natural Science Foundation of China (LY19C030004), the National Key Research and Development Program of China (2017YFB0504000 and 2016YFB0501501), the National Natural Science Foundation of China (41871084 and 31400393), and a grant from State Key Laboratory of Resources and Environmental Information System. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Grant Disclosures

The following grant information was disclosed by the authors:

Zhejiang Provincial Natural Science Foundation of China: LY19C030004.

National Key Research and Development Program of China: 2017YFB0504000, 2016YFB0501501.

National Natural Science Foundation of China: 41871084, 31400393.

State Key Laboratory of Resources and Environmental Information System.

Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Jie Zhuang and Yonglin Wang conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Yonggang Chi and Lei Zhou conceived and designed the experiments, performed the experiments, analyzed the data, authored or reviewed drafts of the paper, and approved the final draft.
- Jijing Chen, Wen Zhou, Jun Song, Ning Zhao and Jianxi Ding analyzed the data, prepared figures and/or tables, and approved the final draft.

Data Availability

The following information was supplied regarding data availability:

The raw measurements are available in [Supplemental File](#).

Supplemental Information

Supplemental information for this article can be found online at <http://dx.doi.org/10.7717/peerj.10046#supplemental-information>.

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