

Relationship between photosynthetic pigments and chlorophyll fluorescence in soybean under varying phosphorus nutrition at ambient and elevated CO₂

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Abstract

To assess the relationship between chlorophyll (Chl) fluorescence (CF) and photosynthetic pigments, soybean was grown under varying phosphorus (P) nutrition at ambient and elevated CO₂ (EC). The EC stimulated, but P deficiency decreased plant height, node numbers, and leaf area concomitantly with the rates of stem elongation, node addition, and leaf area expansion. Under P deficiency, CF parameters and pigments declined except that carotenoids (Car) were relatively stable indicating its role in photoprotection. The CF parameters were strongly related with Chl concentration but not with Chl *a/b* or Car. However, total Chl/Car showed the strongest association with CF parameters such as quantum efficiency and yield of photosystem II. This relationship was not affected by CO₂ treatment. The high correlation between CF and total Chl/Car underscores the significance of the quantification of both, Chl and Car concentrations, to understand the photochemistry and underlying processes of photoprotection and mechanisms of excess energy dissipation in a given environment.

Additional keywords: chlorophyll/carotenoids ratio; energy dissipation; photochemical quenching, relationship; response curve.

Introduction

Photosynthetic pigments such as Chl and Car play central roles in the light reaction of the photosynthetic process of plants by capturing solar energy to be used for CO₂ fixation. The proportion of the Chl fluorescence (CF) emission largely depends on the absorbed solar energy by Chl molecules and performance of photosynthetic apparatus. Therefore, photosynthetic pigments and CF are integral parts of photosynthetic processes and widely used to assess the relative impact of environmental stresses on the photosynthetic properties (van Kooten and Snel 1990, Hendry and Price 1993, Samson *et al.* 1999, Maxwell and Johnson 2000, Roháček 2002, Baker and Rosenqvist 2004, Netto *et al.* 2005). In the light reaction phase of photosynthesis, most of the excitation energy from the PSII is converted into the chemical energy [*e.g.*, ATP and NADP(H)] that is largely consumed in CO₂ fixation and photorespiration coupled in the dark reaction (or light

independent) phase. However, the excess energy is dissipated as heat and a small fraction is lost as fluorescence upon de-excitation of the PSII (Maxwell and Johnson 2000). This CF yield can be easily detected and often associated with the changes in the photochemistry which can be related to the overall photochemical efficiency (Seaton and Walker 1990, Maxwell and Johnson 2000, Roháček 2002, Baker and Rosenqvist 2004, Baker 2008). Thus, assessment of the relationship between photosynthetic pigments and CF provides insight into the photosystem functioning.

The photosynthetic pigments such as total chlorophyll (TChl) and Car and their ratios, Chl *a/b* and TChl/Car, and CF parameters, such as minimal (F_0'), maximal (F_m'), variable (F_v') and steady-state (F_s) fluorescence yields, quantum efficiency by oxidized (open) PSII reaction center in light or PSII efficiency (F_v'/F_m'), quantum yield

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Abbreviations: AC – ambient CO₂; Car – carotenoids; Chl – chlorophyll; EC – elevated CO₂; DAP – days after planting; DAT – days after treatment; F_v'/F_m' – quantum efficiency by oxidized (open) PSII reaction center in light or PSII efficiency; MLAER – main-stem leaf area expansion rate; MNAR – main-stem node addition rate; MSER – main-stem elongation rate; MSLA – main-stem leaf area; MSNN – main-stem node number; PS – photosystem; TChl – total chlorophylls.

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of PSII (Φ_{PSII}) and photochemical quenching (q_p), serve as indicators of plant stress and have been used to assess plant nutrient status and health (Hendry and Price 1993, Maxwell and Johnson 2000, Netto *et al.* 2005). Under stress conditions such as nutrient stress, reduction in CO_2 assimilation also decreases the consumption of the chemical energy created in the process of the light reaction of photosynthesis, which leads to the overexcitation of PSII reaction center due to continued photon absorption by Chl molecules. This excess energy must be dissipated for optimum performance of photosystems and to avoid photoinhibition. The plant has devised mechanisms to dissipate the excess excitation energy by heat, re-emittance as CF or nonradiative mechanisms (Genty *et al.* 1990, Maxwell and Johnson 2000, Ivanov *et al.* 2008b). Car also play a major role *via* the xanthophyll cycle in the dissipation of excess excitation energy of PSII to protect photosystems from the photoinhibition (Havaux 1998, Ort 2001, Pascal *et al.* 2005, Lambers *et al.* 2010).

The P deficiency leads to decreased biomass and crop yield, whereas opposite was observed for plants grown under CO_2 enrichment conditions. Therefore, P deficiency and EC often have opposite effects on crop growth and their co-existence are inevitable under natural conditions because natural resources of P are limited and the CO_2 concentration is projected to increase in the future (Cordell *et al.* 2009, IPCC 2013). The plant tissue nutrient

concentration tends to be lower under EC vs. ambient CO_2 (AC) (Cure *et al.* 1988, Singh *et al.* 2013b). Therefore, the interaction between P deficiency and growth CO_2 might alter nutrient allocation among plant parts which, in turn, affects biosynthesis and degradation of photosynthetic pigments. Previous reports indicated that P deficiency affected the metabolism of other mineral nutrients such as N by altering their uptake and utilization (Israel *et al.* 1990, Rufty *et al.* 1991, Singh *et al.* 2013a, Singh *et al.* 2014a). Plants exposed to a CO_2 -enriched environment or stress situation might also reflect lesser Chl concentration, weaker thylakoid membrane and greater Chl *a/b* ratio (Lambers *et al.* 2010, Zai *et al.* 2012). Stability of thylakoid membrane is necessary for biosynthesis and positioning of Chl molecules which might affect the overall CF yield. Netto *et al.* (2002) found a good correlation between photochemical quenching represented by q_p and *SPAD-502* value (signifying TChl) in papaya (*Carica papaya* L.). Although photosynthetic pigments and CF parameters characterize crop health and photosynthetic properties of leaves or plant canopy, studies evaluating their relationships under various growth conditions are limited (Netto *et al.* 2002, Netto *et al.* 2005, Zai *et al.* 2012). The objective of this paper was to assess relationships between photosynthetic pigments and chlorophyll fluorescence parameters in soybean grown at a range of P nutrition under ambient and elevated CO_2 concentrations.

Materials and methods

Plant culture and treatments: Soybean [*Glycine max* (L.) Merr., cv. Spencer] seeds (five seeds per pot) were planted in 20 pots (volume 7.6 L) filled with washed concrete sand in each of six controlled environment growth chambers (EGC Corp., Chagrin Falls, OH, USA) at the USDA-ARS facility in Beltsville, MD, USA. The experiment was repeated once over time using the same six chambers. One plant per pot was maintained and watered with full-strength Hoagland's nutrient solution (Hewitt 1952) from emergence to 12 days after planting (DAP). After that, the treatments were initiated in the factorial combination of two concentrations of CO_2 (ambient, AC, 400 and elevated, EC, $800 \mu\text{mol mol}^{-1}$) and three concentrations of phosphate (0.5, 0.1, and 0.01 mM P) in modified Hoagland's nutrient solution. The details of the experimental condition are given by Singh *et al.* (2014a). In brief, a 28/22°C day/night (12-h/12-h) air temperature and a PAR of $900 \mu\text{mol}(\text{photon}) \text{m}^{-2} \text{s}^{-1}$ at plant canopy were maintained in each chamber.

Plant height, main-stem node numbers (MSNN) and leaf length on each main-stem nodes were measured weekly in seven plants from 14 to 55 DAP. Plant height was also measured at the end of the experiment. Leaf lengths were converted to main-stem leaf area (MSLA) using a sigmoid curve equation with three parameters (*SigmaPlot version 11, Systat Software Inc., San Jose,*

CA, USA) as:

$$y = 91.76 / \{1 + \text{EXP}[-(x-9.44)/2.38]\} \quad (r^2 = 0.97, n = 534,$$

y – area in cm^2 and x – leaf length in cm) developed from relationship between the lengths of several leaves and leaf area obtained at the time of destructive harvests. The relationships of plant height, MSNN and MSLA with time (DAP) were used to estimate stem elongation rate (MSER), node addition rate (MNAR) and leaf area expansion rate (MLAER) of the main stem, respectively. The rates (MSER, MNAR and MLAER) were calculated for each measurement day except first measurement using equation as rate on a specific DAP = $[(V_c - V_p)/(D_c - D_p)]$, where V_c – current measured value and V_p – the previously measured value, D_c – current DAP, D_p – previous DAP. Since the rapid growth of these traits was mainly associated with linear part of the curves residing between first five measurements (between 14 and 42 DAP), the average rate in this period was taken as the representative of MSER, MNAR and MLAER (Singh *et al.* 2014b). Plants were harvested at 30 and 45 DAP to avoid plant competition. After that, seven plants per chamber were maintained until the end of the experiment.

Chl *a* fluorescence measurements: The rate of CO_2 assimilation and CF measurements were made several

times between 26 and 52 days after treatment (DAT) on the uppermost fully expanded leaves between 9:00 and 13:00 h using a portable photosynthetic system (*LI-COR 6400XT*, *LI-COR Inc.*, Lincoln, NE, USA) with an integrated fluorescence chamber head (*LI-COR 6400-40* leaf chamber fluorometer). In the leaf chamber of the *LI-COR 6400XT*, the PAR was set to 1,500 $\mu\text{mol}(\text{photon}) \text{m}^{-2} \text{s}^{-1}$, the leaf temperature was controlled to 28°C using an integrated Peltier cooler, CO₂ was maintained to match the CO₂ treatments, and the relative humidity varied between 45 and 60%.

The rate of CO₂ assimilation and the steady-state CF (F_s) measurements were taken when a steady state (around 4–6 min) was obtained. Photosynthesis was driven by the actinic light sources of the instrument that uses three blue (centered 475 nm) and 27 red light emitting diodes (LEDs) (centered 630 nm). For the CF measurements, the F_s was measured first followed by maximal fluorescence (F_m') by providing a 0.8 s of the saturating light flash of >8,000 $\mu\text{mol}(\text{photon}) \text{m}^{-2} \text{s}^{-1}$ PAR using 27 red LEDs. Immediately, in the following step, the minimal fluorescence of the light-adapted leaf (F_o') was obtained by providing a dark flash by turning off the actinic light briefly while using far-red LEDs (centered wavelength of 740 nm). The far-red radiation drives PSI to help drain PSII electrons. The variable fluorescence yield in the light-adapted leaves (F_v'), efficiency of energy harvesting by oxidized (open) PSII reaction centers in light (F_v'/F_m'), proportion of the PSII unit in open state or photochemical quenching (q_p) and photochemical quantum yield (Φ_{PSII}) were determined following Genty *et al.* (1989) and Maxwell and Johnson (2000) using the equation $F_v' = F_m' - F_o'$; $F_v'/F_m' = (F_m' - F_o')/F_m'$; $q_p = (F_m' - F_s)/(F_m' - F_o')$; $\Phi_{\text{PSII}} = (F_m' - F_s)/F_m'$. These parameters along with quantum yield of CO₂ assimilation (Φ_{CO_2}) were automatically measured and computed from the instrument software (details are available in *LI-6400XT Instruction Manual, version 6*, *Li-Cor Inc.*, Lincoln, NE, USA). The response of CF parameters to PAR (light-response curve) was measured in nine steps initiating from an upper limit of 2,000 $\mu\text{mol}(\text{photon}) \text{m}^{-2} \text{s}^{-1}$ and decreased stepwise to

20 $\mu\text{mol}(\text{photon}) \text{m}^{-2} \text{s}^{-1}$. The response of CF parameters to sub-stomatal CO₂ concentration (C_i or CO₂ response curve) was measured in 11 steps from the treatment ambient CO₂ (C_a , 400 or 800 $\mu\text{mol} \text{mol}^{-1}$), then decreasing stepwise down to 50 $\mu\text{mol} \text{mol}^{-1}$ C_a then increasing stepwise to the upper limit of 1,500 $\mu\text{mol} \text{mol}^{-1}$ C_a after bringing back to the ambient C_a to restore the original rate of CO₂ assimilation. The response to PAR or C_i was measured between 38 and 45 DAT.

Quantification of photosynthetic pigments and tissue phosphorus concentration:

The concentrations of Chl and Car were determined from the leaves that were used for photosynthetic measurements. These pigments were extracted by placing two 0.95 cm² leaf disks for each leaf in a vial containing 5 mL of dimethyl sulfoxide and incubating in the dark for 24 h. Thereafter, the absorbance of the supernatant was measured at 470, 648, and 664 nm by using a *UV-2101-PC* spectrophotometer (*Shimadzu Corp.*, Columbia MD, USA). Then the pigments were estimated by using the equations of Lichtenthaler (1987). The dried leaves were ground using a *Wiley Mill* (*Wiley® Mill, Thomas Scientific*, NJ, USA) to pass through a 1-mm screen. The tissue P concentration was measured in the Agriculture Diagnostic Laboratory, University of Arkansas, AR, USA.

Data analysis: To test for the effect of treatments and their interaction on MSER, MNAR, and MLAER, *PROC MIXED* (*SAS Enterprise Guide, 4.2, SAS Institute Inc.*, NC, USA) was used for analysis of variance (*ANOVA*) using mean data from each repetition of the experiment (thus, two replication). Treatments and their interaction were considered as fixed effects and repetition of the experiment as a random effect. To establish the significance of relationship among CF parameters, photosynthetic pigments and leaf P concentration, *PROC REG* and *PROC GLM* procedures for linear and polynomial second order regressions analysis (significant when $P \leq 0.05$), respectively, were conducted using individual measurements.

Results

Plant growth and development: Plant height and MSNN followed a sigmoidal growth pattern exhibiting a rapid initial growth roughly until 45 DAP across treatments (Fig. 1A,B). Thereafter, plant height and MSNN did not increase substantially. The lowest P treatment at AC had the smallest plant height (131 cm) and the lowest MSNN (15) at maturity. The EC increased plant height and MSNN across P treatments. The MSLA also increased rapidly, exhibiting a curvilinear response as plant aged (Fig. 1C). The MSLA declined under P deficiency but increased at EC across P treatments. The plant growth rates, MSER, MNAR and MLAER were significantly affected by the treatments (P and CO₂) (Fig. 1D–F). On average under P

deficiency, MSER and MNAR declined approximately 8% and MLAER 33% as compared to the full P treatment. The EC increased these growth rates by 5 to 16.5%.

Photosynthetic pigments vs. P concentration: Averaged across CO₂, the mean leaf P concentration for the treatments 0.5, 0.1, and 0.01 mM P were 3.26, 1.15, and 0.77 mg g⁻¹, respectively (Fig. 2). TChl concentration ranged from 54 to 107 $\mu\text{g} \text{cm}^{-2}$ and Car from 16 to 23.5 $\mu\text{g} \text{cm}^{-2}$ across the treatments. Chl pigments (*i.e.*, Chl *a*, Chl *b*, TChl) showed a polynomial second order relationship with leaf P concentration and tended to increase with leaf P (Fig. 2A–C). Car concentration and Chl *a/b* ratio did not

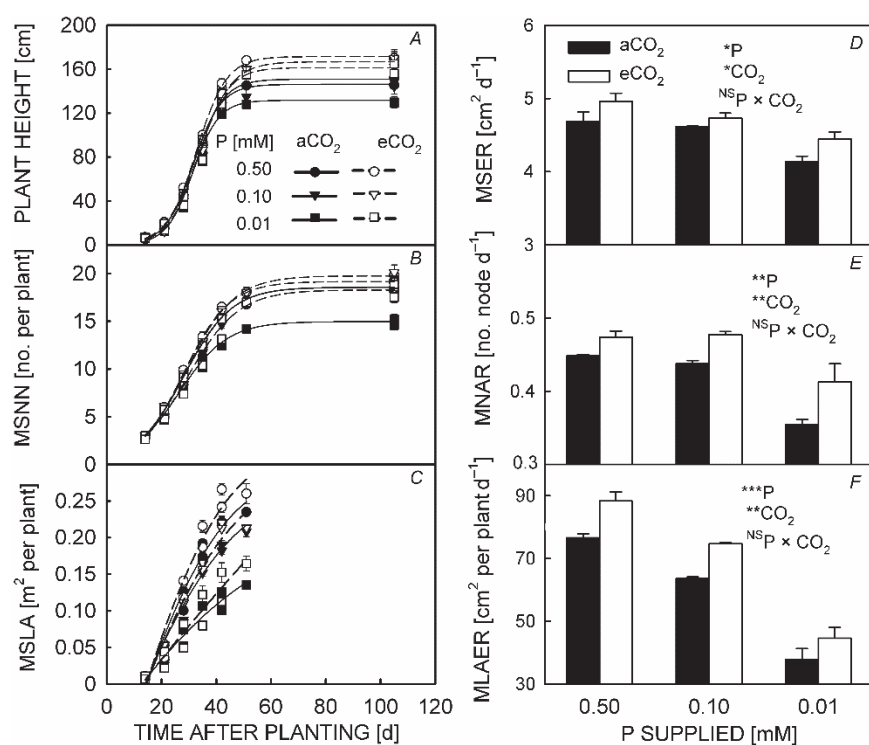


Fig. 1. Response of (A) plant height, (B) main-stem node number (MSNN), and (C) main-stem leaf area (MSLA), (D) main-stem elongation rate (MSER), (E) main-stem node addition rate (MNAR) and (F) main-stem leaf area expansion rate (MLAER) of soybean to three phosphorus (P) treatments grown at either ambient (aCO₂, 400 μmol mol⁻¹) or elevated (eCO₂, 800 μmol mol⁻¹) CO₂ concentration. Error bars represent standard error of 14 individuals (A–C) and two replications from the repeated experiment (D–F). The *, **, *** (D–E) represent significant at $P < 0.05$, $P < 0.01$, $P < 0.001$, respectively; NS – not significant ($P > 0.05$). Error bars smaller than the symbols are not visible.

show a significant relationship with leaf P concentration (Fig. 2D,E). The TChl/Car ratio increased with leaf P concentration exhibiting a curvilinear response (Fig. 2F). The EC tended to decrease the concentration of photosynthetic pigments but did not affect the Chl *a/b*, and TChl/Car ratios (Fig. 2E,F).

Light-response curves of Chl fluorescence parameters:

The Φ_{CO_2} increased with PAR between 20 and 200 μmol(photon) m⁻² s⁻¹, thereafter declined as PAR was increased to 2000 μmol(photon) m⁻² s⁻¹ (Fig. 3A). The Φ_{CO_2} also declined markedly in P-deficient leaves. The EC increased Φ_{CO_2} especially in the 0.50 mM P-treated plants. The F_o' , F_m' , and F_v' decreased as PAR increased and were substantially lower under P deficiency (Fig. 3B–D). The F_s increased hyperbolically with PAR at 0.50 mM and showed a small increase at 0.10 mM P-treated plants (Fig. 3E). However, F_s decreased with PAR in the lowest P (0.01 mM)-treated plants. The F_v'/F_m' , Φ_{PSII} , and q_p also exhibited a decreasing pattern as PAR increased and the effect of CO₂ appeared to be small (Fig. 3F–H). The Φ_{PSII}/Φ_{CO_2} values at 20 μmol m⁻² s⁻¹ were not realistic and were either negative or very high (Fig. 3I). However, Φ_{PSII}/Φ_{CO_2} tended to decrease between 50 and 200 μmol(photon) m⁻² s⁻¹ and stayed fairly consistent when PAR was increased thereafter. The Φ_{PSII}/Φ_{CO_2} was greater under P deficiency but smaller at EC vs. AC.

The CO₂ response curves of Chl fluorescence: The Φ_{CO_2} increased hyperbolically as C_i increased and reached saturation over 1,000 μmol mol⁻¹ (Fig. 4A). The Φ_{CO_2}

declined several folds under P deficiency. The EC also decreased Φ_{CO_2} , particularly in the two higher P treatments. The F_o' remained fairly stable in response to changes in C_i but severely decreased under P deficiency (Fig. 4B). The F_m' and F_v' showed a similar increasing trend with C_i and decreased under P deficiency (Fig. 4C,D). The F_s remained relatively stable in response to changes in C_i and severely decreased under P deficiency while exhibiting a noticeable increase at EC (Fig. 4E). The F_v'/F_m' , Φ_{PSII} , and q_p increased with C_i and showed lower values under P deficiency and EC (Fig. 4F–H). The Φ_{PSII}/Φ_{CO_2} sharply decreased as C_i increased up to approximately 400 μmol mol⁻¹ and did not differ between treatments (Fig. 4I).

Photosynthetic pigments vs. Chl fluorescence: The relationship of TChl, Car, Chl *a/b*, and TChl/Car with various CF parameters were established using regression analysis (Figs. 5–8). The F_o' , F_m' , F_v' , and F_s tended to increase with Chl *a*, Chl *b*, TChl or Car concentration (Fig. 5). However, the coefficient of determination (r^2) of the regression analysis of F_o' , F_m' , F_v' , and F_s was greater and mainly significant ($P < 0.05$) with Chl concentration (Fig. 5A–L). These parameters showed an insignificant ($P > 0.05$) relationship with carotenoids (Fig. 5M–P). The F_o' , F_m' , F_v' , and F_s often exhibited a slightly greater response to photosynthetic pigments at EC (Fig. 5).

The Φ_{CO_2} , F_v'/F_m' , q_p , and Φ_{PSII} increased linearly with increase in Chl concentration (*i.e.*, Chl *a*, Chl *b*, TChl) (Fig. 6A–D,F–I,K–N). The linear correlation of Φ_{CO_2} , F_v'/F_m' , q_p , and Φ_{PSII} with Car concentration tended to be

positive but were often insignificant (Fig. 6P–S). The response of Φ_{CO_2} to TChl was markedly greater at EC vs. AC (Fig. 6K). The $\Phi_{\text{PSII}}/\Phi_{\text{CO}_2}$ decreased as TChl or Car concentration increased and was always smaller at EC vs. AC (Fig. 6E,J,O,T).

The Chl *a/b* values ranged from 3.55 to 4.7 and TChl/Car values from 3.2 to 5.1 (Fig. 7). Relationships between TChl/Car and CF parameter showed the greatest r^2 values (Fig. 7–8). The F_m' , F_v' , linearly increased with Chl *a/b* especially at EC (Fig. 7B,C). The F_o' , F_m' , F_v' , and F_s increased with TChl/Car exhibiting strong linear

Discussion

Plant growth and development: The sensitivity of soybean to treatments was confirmed by the significant effect of P nutrition and CO_2 on the growth and developmental rates. The reduction in the various soybean growth parameters under P deficiency was in accordance with the other C_3 crops (Reddy and Zhao 2005, Fleisher *et al.* 2012, Singh *et al.* 2013b). The growth reduction resulted from slower developmental rates of plant stem, leaf addition, and smaller leaf area. Decreased plant height was associated with the lower number of main-stem nodes under P deficiency. Plants grown at low P supply also had smaller main-stem leaf area. The growth rates, MSER, MNAR and MLAER, were significantly reduced by P deficiency. However, EC increased these growth rates across P treatments (Fig. 1). A marked reduction of MSER and MNAR was mainly found in the lowest P treatment. The decreases in plant growth due to nutrient stress including P has been previously reported in other crops and can be attributed to the limited availability of the plant resources such as phosphorus nutrient and decreased overall photosynthesis (Zhao *et al.* 2005, Fleisher *et al.* 2012, Singh *et al.* 2013a).

Photosynthetic pigments and Chl fluorescence: The observed leaf tissue P concentration in the 0.50 mM P treatment was comparable to the observations made in other field studies (Cassman *et al.* 1981, Walker *et al.* 1985). Chl concentration (*i.e.*, TChl, Chl *a* or Chl *b*) in P-deficient plants declined, but Car concentration was not affected significantly indicating its importance in the photoprotection of PSII (Pascal *et al.* 2005). The observed reduction in the photosynthetic pigments under P deficiency was consistent with the other studies (Crafts-Brandner 1992, Singh *et al.* 2013a). Singh and Reddy (2016) suggested the decrease in Chl concentration as a mechanism to avoid excess light harvesting to protect PSII from photodamage under P deficiency. The Chl *a/b* and the TChl/Car ratios remained fairly stable across leaf P concentration except for a few values at the lower leaf P concentrations. Since nitrogen was not limiting in this study, Chl *a/b* ratio might have been maintained without a substantial impact of P deficiency. The observed Chl *a/b* was approximately between 3 and 5 which was in accord-

relationships (Fig. 7E–H). The EC did not show a marked effect on the relationship of F_o' , F_m' , F_v' , and F_s with either Chl *a/b* or TChl/Car ratio. The Φ_{CO_2} , F_v'/F_m' , q_p , and Φ_{CO_2} increased with Chl *a/b* particularly at EC concentration (Fig. 8A–D). However, these parameters showed a strong linear correlation with TChl/Car ratio and increased with it across CO_2 concentration (Fig. 8F–I). The relationship of $\Phi_{\text{PSII}}/\Phi_{\text{CO}_2}$ with Chl *a/b* was not significant but decreased as TChl/Car ratio increased (Fig. 8E,J). The $\Phi_{\text{PSII}}/\Phi_{\text{CO}_2}$ showed greater values at EC across Chl *a/b* or TChl/Car (Fig. 8E,J).

ance with reported values under the standard light environment (Lichtenthaler and Wellburn 1983, Netto *et al.* 2005).

Light and CO_2 dependence of photosynthetic processes are often described by studying their responses to varying light intensity or C_i concentration. Most of the measured parameters tended to be smaller at high PAR or greater at high C_i concentration across P treatments. The decline in CF parameters such as F_v'/F_m' , Φ_{PSII} , and q_p at high PAR indicated that excess energy absorbed by photosynthetic pigments was not used for CO_2 fixation, thus decreasing the efficiency of PSII and photochemical quenching (Genty *et al.* 1990, Maxwell and Johnson 2000). The decline in CF parameters, such as F_v'/F_m' , Φ_{PSII} , and q_p , at low C_i concentration might have been caused by a severe reduction in the carboxylation rate, thus inhibiting utilization of absorbed energy of the photosystem in the carbon assimilation.

The CF parameters highly declined under P deficiency across the measured PAR or C_i concentrations signifying optimum tissue P concentration is critical to attaining the maximum fluorescence activity and photosynthesis. The differences between the P treatments were more obvious above 500 $\mu\text{mol}(\text{photon})\text{m}^{-2}\text{s}^{-1}$ PAR and above 200 $\mu\text{mol}\text{mol}^{-1}$ of C_i concentration. The P deficiency reduced Φ_{CO_2} concomitantly with CF parameters, such as F_o' , F_m' , F_v'/F_m' , q_p , and Φ_{PSII} , which was in accordance with earlier reports (Lauer *et al.* 1989, Singh *et al.* 2013a, Singh and Reddy 2014, Singh and Reddy 2015). The F_o' is the indicative of open PSII reaction center if the primary electron acceptor (Q_A) is maximally oxidized (*i.e.*, ready to accept electrons). Whereas, the F_m' is indicative of closed PSII reaction centers as if Q_A is fully reduced and cannot accept electrons. The decrease in F_v'/F_m' was caused more by the reduction of F_m' than that of F_o' suggesting the accumulation of reduced Q_A in PSII reaction center and decreased photochemical efficiency in P-deficient leaves (Singh and Reddy 2015). It was supported by a concomitant decrease in F_v' under P deficiency. The decreased F_s in P-deficient leaves also suggested inhibited electron transport (Singh and Reddy 2015). The observed increase of $\Phi_{\text{PSII}}/\Phi_{\text{CO}_2}$ ratio in P-deficient leaves especially in response to PAR might have been caused by the consumption of electrons in the processes other than CO_2

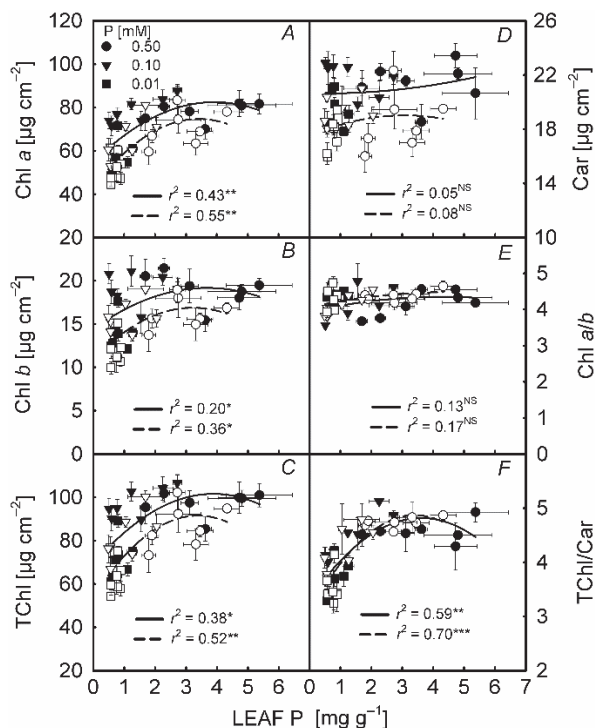


Fig. 2. Response of (A) chlorophyll (Chl) *a*, (B) Chl *b*, (C) total Chl (TChl), and (D) carotenoids (Car) concentration, (E) Chl *a/b* and (F) TChl/Car to leaf tissue phosphorus (P) concentration of soybean uppermost fully expanded leaves grown at either ambient (filled symbols and solid lines, 400 $\mu\text{mol mol}^{-1}$) or elevated (open symbols and dashed lines, 800 $\mu\text{mol mol}^{-1}$) CO_2 under different P treatments. Error bars represent the standard error of 3–5 individuals measured between 26 and 53 d after treatment. The lines are fit of “polynomial second order equation”. The asterisk(s) *, **, *** and ^{NS} – significance levels of a given regression fit as $P \leq 0.05$, $P \leq 0.01$, $P \leq 0.001$ and $P > 0.05$, respectively. Error bars smaller than the symbols are not visible.

fixation, such as photorespiration, nitrogen metabolisms or nonradiative dissipation mechanisms (Edwards and Baker 1993, Maxwell and Johnson 2000, Ivanov *et al.* 2008b).

Relationships between photosynthetic pigments and Chl fluorescence parameters:

Most of the CF parameters showed good correlation with photosynthetic pigments. Stronger associations of CF parameters were mainly observed with Chls or TChl/Car ratio. Chl *a* showed greater coefficient of determination ($r^2 = 0.21$ – 0.68) than that of Chl *b* concentration ($r^2 = 0.01$ – 0.58) when regressed with CF parameters. The correlation of CF parameters was greater with TChl ($r^2 = 0.22$ – 0.64) than with Car ($r^2 = 0.03$ – 0.23) concentration. This might be attributed to the dependence of the light absorption and fluorescence emission on the concentration of Chl molecules in the chloroplast (Miranda *et al.* 1981). This was also supported by the strong association of Chl concentration with maximal and variable CF yields (*i.e.*,

F_v' and F_m') and their ratio (F_v'/F_m') representing the efficiency of energy harvesting by PSII reaction centers. Similarly, the quantum yields of CO_2 fixation (*i.e.*, Φ_{CO_2}) and PSII (Φ_{PSII}) were also strongly correlated with the Chl *a*, Chl *b* or TChl concentration. The poor correlation of CF parameters with Chl *a/b* ratio might be due to insignificant change of Chl *a/b* across leaf P concentration.

The fluorescence yield from green leaves depends on the Chl concentration while Car serves in the dissipation of excess excitation as heat. In the current study, TChl concentration declined, but the Car concentration did not vary significantly across leaf P concentrations demonstrating its importance in the protection of the PSII from photodamage (Pascal *et al.* 2005, Singh *et al.* 2013c). As one of the necessary mechanisms to dissipate the excess excitation energy, Car participate in the xanthophyll cycle to minimize photoinhibition (Demmig-Adams 1990, Hendry and Price 1993). In this cycle, violaxanthin is converted to antheraxanthin through de-epoxidation and finally into zeaxanthin which participates in the regulation of heat dissipation from PSII (Demmig-Adams 1990, Havaux 1998, Ort 2001). Relatively poor correlation of Car concentration with CF parameters might be partly caused by the involvement of Car in the radiationless (or as heat) quenching of the excess energy (Demmig-Adams 1990). However, the TChl/Car ratio showed the strongest relationship ($r^2 = 0.30$ – 0.76) with the CF parameters. The TChl/Car was strongly associated ($r^2 > 0.60$) with F_v'/F_m' and Φ_{PSII} representing the efficiency of energy harvesting by PSII and quantum yield, respectively.

The decline in TChl/Car was similar to the responses observed for the CF parameters as shown by their strong linear relationships. This suggests the biosynthesis and degradation of Chls and Car in the chloroplast were coordinated to optimize photosynthetic processes while protecting PSII from the photoinhibition. In fact, the reduction in TChl/Car at lower leaf P concentration resulted from a greater decrease in Chl than Car concentration. Therefore, stability in the Car concentration appeared to be a major mechanism of PSII protection from photodamage in P-deficient soybean. This was supported by the highest r^2 observed when the TChl/Car ratio was regressed with Φ_{CO_2} , F_m' , F_v' , F_v'/F_m' ratio and Φ_{PSII} .

Nonetheless, a significant decrease in Φ_{CO_2} and CF parameters, such as F_v'/F_m' , Φ_{PSII} , and q_p , under P deficiency clearly indicated reduced CO_2 assimilation and impairment of photosystems. The cumulative effect of decreased Φ_{CO_2} , F_v'/F_m' , and Φ_{PSII} translated into increased number of quantum per CO_2 fixation (Edwards and Baker 1993). In fact, this was shown by the increase in $\Phi_{\text{PSII}}/\Phi_{\text{CO}_2}$ under P deficiency. This $\Phi_{\text{PSII}}/\Phi_{\text{CO}_2}$ represents an alternative electron sink, and its high value is often associated with reduced photochemical efficiency of PSII as also reported in P-deficient cotton (Edwards and Baker 1993, Singh *et al.* 2013a).

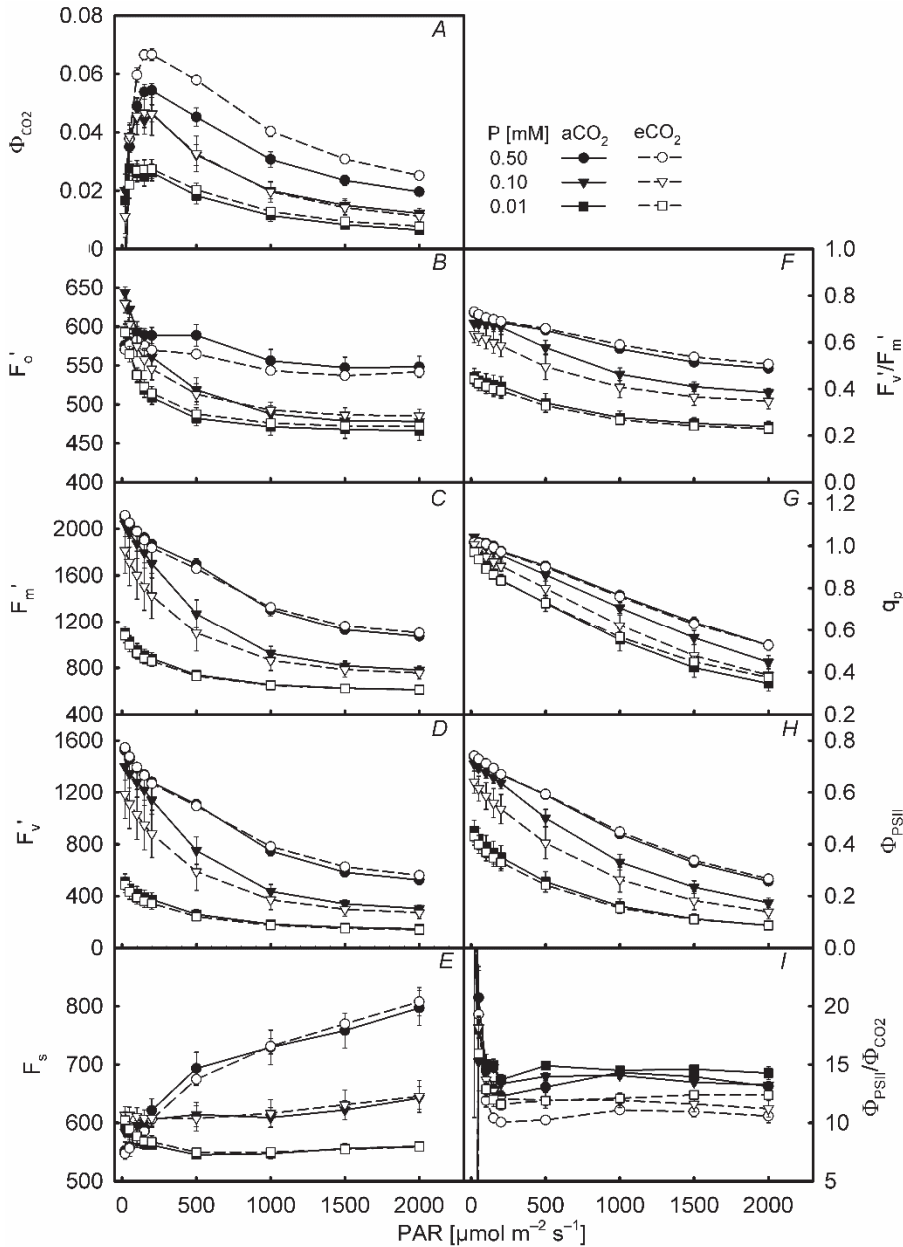


Fig. 3. Response of (A) quantum yield of CO₂ assimilation (Φ_{CO_2}), (B) minimal (F_0'), (C) maximal (F_m'), (D) variable (F_v') and (E) steady-state (F_s) chlorophyll fluorescence yields, (F) efficiency of energy harvesting by oxidized (open) PSII reaction centers in light (F_v'/F_m'), (G) proportion of the PSII unit in open state or photochemical quenching (q_p), (H) photochemical quantum yield (Φ_{PSII}), and (I) $\Phi_{\text{CO}_2}/\Phi_{\text{PSII}}$ to photosynthetically active radiation (PAR) measured between 38 and 45 d after treatment in uppermost fully expanded leaves of soybean grown at either ambient (aCO₂, 400 $\mu\text{mol mol}^{-1}$) or elevated (eCO₂, 800 $\mu\text{mol mol}^{-1}$) CO₂ under different phosphorus (P) treatments. Error bars represent the standard error of 6–8 individuals. Error bars smaller than the symbols are not visible.

The effect of EC on the CF parameters, such as F_v'/F_m' , Φ_{PSII} , and q_p , was minor across the range of Chl and Car concentrations. In fact, EC did not alter the relationship of CF parameters with the TChl/Car ratio. The lower sensitivity of many CF parameters to EC condition across P treatments or photosynthetic pigments might be explained due to adjustment in the partitioning of electron transport products (ATP, NADPH) between carbon assimilation and photorespiration (Genty *et al.* 1990). The excess energy

caused by suppressed photorespiration under EC, might lead to greater excitation pressure in PSII and favoring energy dissipation through non-fluorescence quenching or nonradiative mechanisms (Genty *et al.* 1990, Maxwell and Johnson 2000, Ivanov *et al.* 2008b). Evidence suggested that the nonradiative dissipation involves reaction center quenching which might cause lower Chl fluorescence yield without the decrease in the photochemical efficiency (Olaiola and Yamamoto 1994, Ivanov *et al.* 2008a)

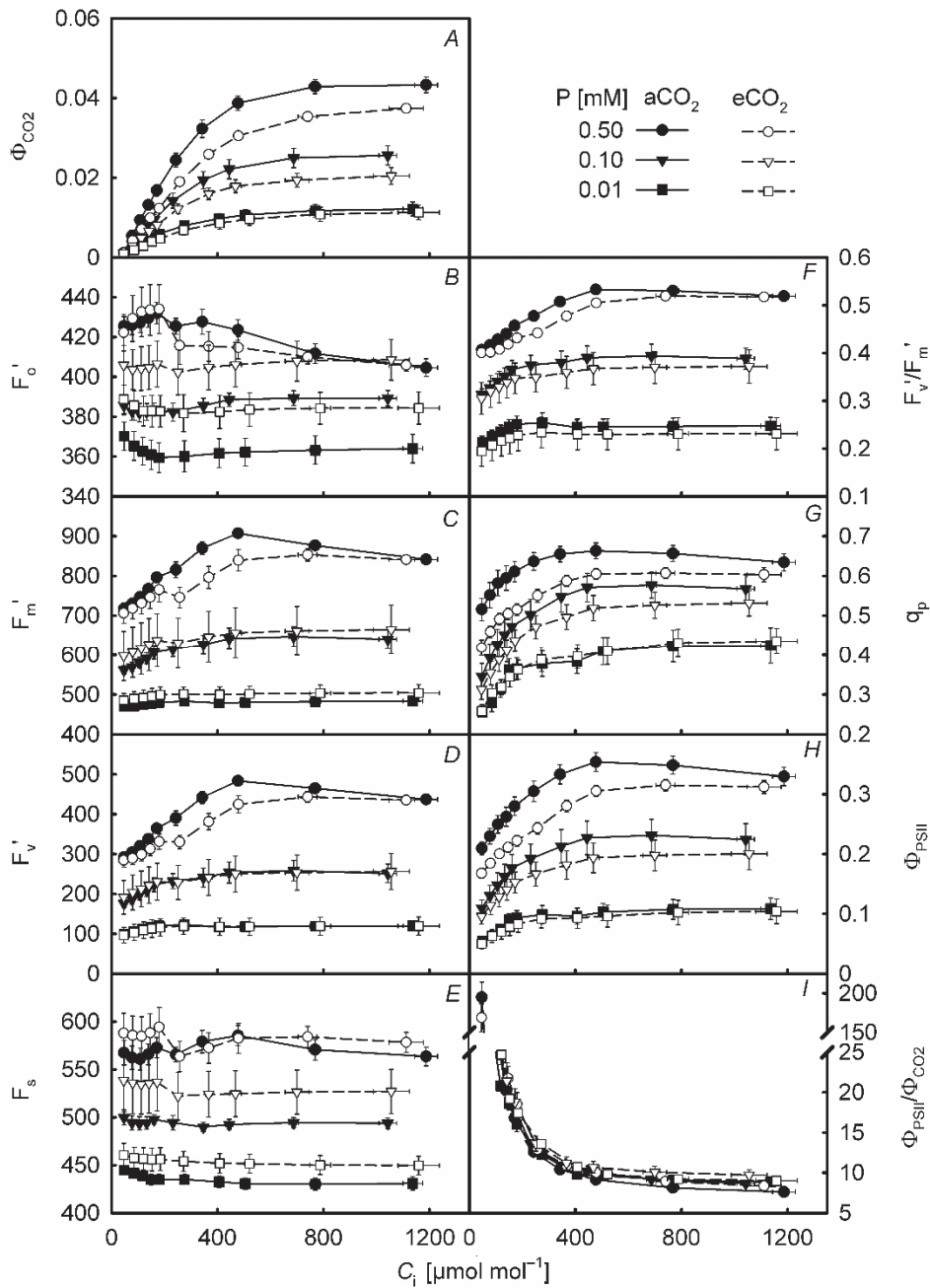


Fig. 4. Response of (A) quantum yield of CO₂ assimilation (Φ_{CO_2}), (B) minimal (F'_0), (C) maximal (F'_m), (D) variable (F'_v) and (E) steady-state (F_s) chlorophyll fluorescence yields, (F) efficiency of energy harvesting by oxidized (open) PSII reaction centers in light (F'_v/F'_m), (G) proportion of the PSII unit in open state or photochemical quenching (q_p), (H) photochemical quantum yield (Φ_{PSII}), and (I) $\Phi_{\text{CO}_2}/\Phi_{\text{PSII}}$ ratio to sub-stomatal CO₂ concentration (C_i) measured between 38 and 45 d after treatment in uppermost fully expanded leaves of soybean grown at either ambient (aCO₂, 400 $\mu\text{mol mol}^{-1}$) or elevated (eCO₂, 800 $\mu\text{mol mol}^{-1}$) CO₂ under different phosphorus (P) treatments. Error bars represent the standard error of 6–8 individuals. Error bars smaller than the symbols are not visible.

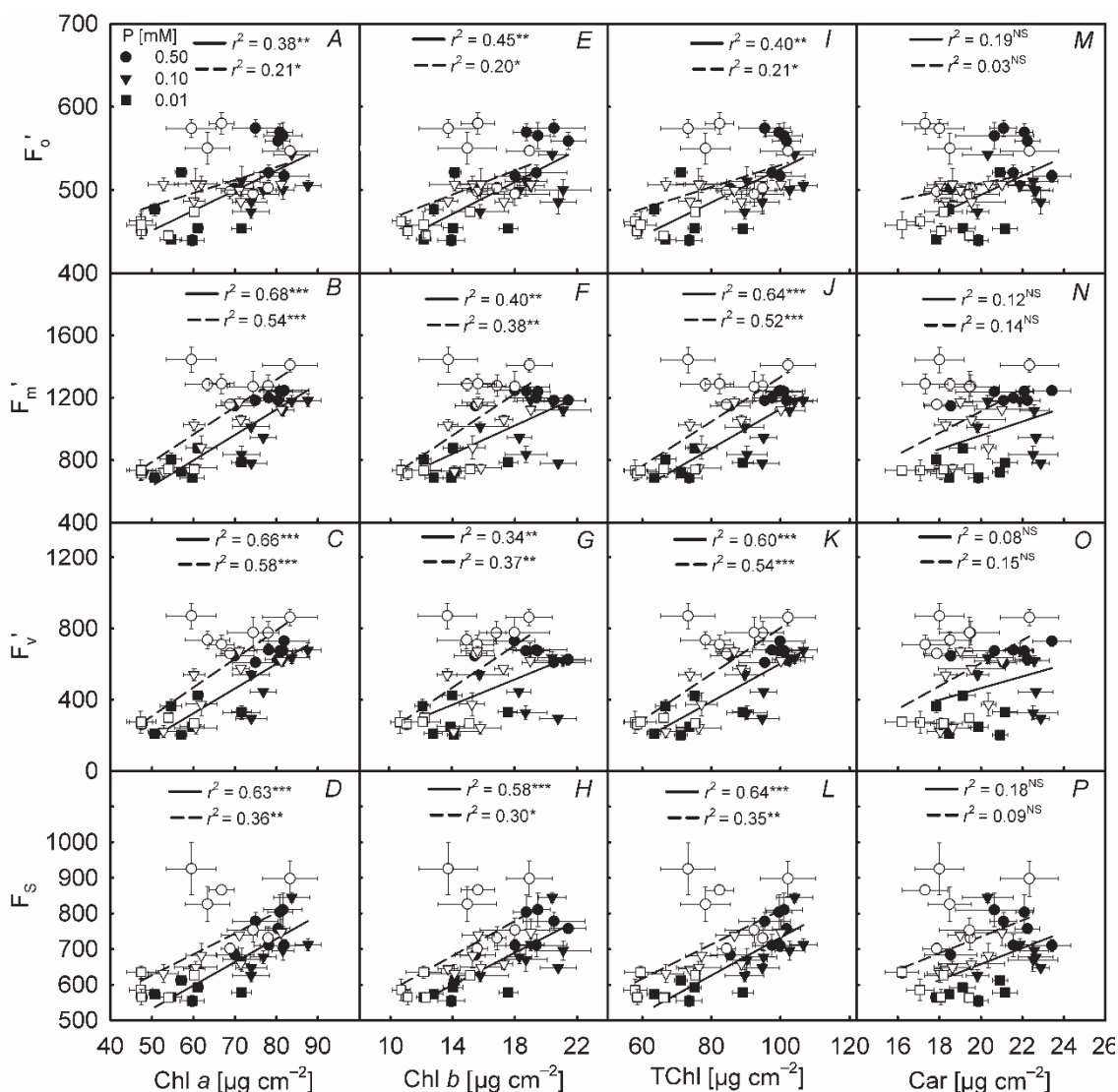


Fig. 5. The relationship of minimal (F_o'), maximal (F_m'), variable (F_v') and steady-state (F_s) chlorophyll fluorescence yield with (A–D) chlorophyll (Chl) a, (E–H) Chl b, (I–L) total Chl (TChl) and (M–P) carotenoids (Car) concentration of soybean uppermost fully expanded leaves grown at either ambient (filled symbols and solid lines, 400 $\mu\text{mol mol}^{-1}$) or elevated (open symbols and dashed lines, 800 $\mu\text{mol mol}^{-1}$) CO₂ concentration under three phosphorus (P) treatments. Error bars represent the standard error of 3–5 individuals measured between 26 and 53 d after treatment. Lines are the linear regression fit. The asterisk(s) *, **, *** and ^{NS} – significance levels of a given regression fit as $P \leq 0.05$, $P \leq 0.01$, $P \leq 0.001$, and $P > 0.05$, respectively. Error bars smaller than the symbols are not visible.

In fact, Φ_{CO_2} as an indicator of CO₂-fixation capacity exhibited a pronounced increase at EC across P nutrition or photosynthetic pigments, despite the lesser sensitivity of other CF parameters.

In summary, P deficiency significantly decreased soybean vegetative growth and developmental rates of main-stem elongation, node addition and leaf area expansion, whereas elevated CO₂ stimulated. The photosynthetic pigments and CF parameters were more sensitive to P deficiency than to the elevated CO₂ concentration. The CF parameters showed good correlation with chlorophyll and its ratio with carotenoids concentration but a relatively poor correlation with Chl a/b ratio and carotenoids. Under

P deficiency, the decreases in chlorophyll concentration (TChl, Chl a, Chl b) were accompanied by the decline in CF parameters. However, carotenoids concentration ratio was fairly stable across leaf P concentration signifying its importance in the mechanism to minimize photoinhibition of photosystems by dissipating excess excitation energy of PSII under P deficiency. The correlation between CF and chlorophylls was attributed to the dependence of energy absorption and fluorescence emission on chlorophyll molecules. The strongest relationship of CF parameters including F_v'/F_m' and Φ_{PSII} was observed with TChl/Car ratio. The elevated CO₂ did not modify the relationship of CF parameters with the TChl/Car ratio. Results suggested

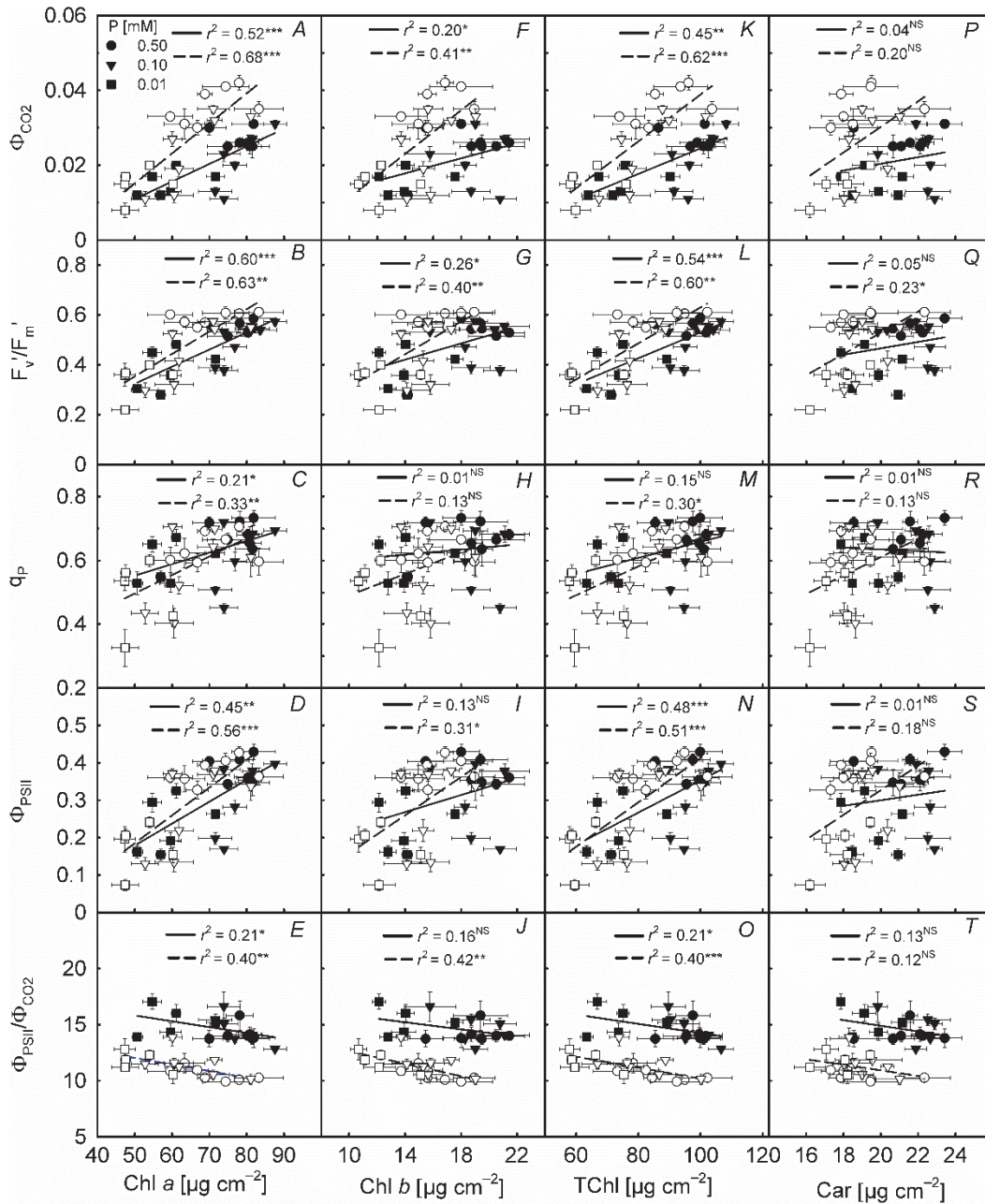


Fig. 6. The relationship of quantum yield of CO₂ assimilation (Φ_{CO_2}), efficiency of energy harvesting by oxidized (open) PSII reaction centers in light (F_v/F_m), proportion of the PSII unit in open state or photochemical quenching (q_p), photochemical quantum yield (Φ_{PSII}) and $\Phi_{\text{CO}_2}/\Phi_{\text{PSII}}$ with (A–E) chlorophyll (Chl) *a*, (F–J) Chl *b*, (K–O) total Chl (TChl), and (P–T) carotenoids (Car) concentration of soybean uppermost fully expanded leaves grown at either ambient (filled symbols and solid lines, 400 $\mu\text{mol mol}^{-1}$) or elevated (open symbols and dashed lines, 800 $\mu\text{mol mol}^{-1}$) CO₂ concentration under three phosphorus (P) treatments. Other details are as in Fig. 5.

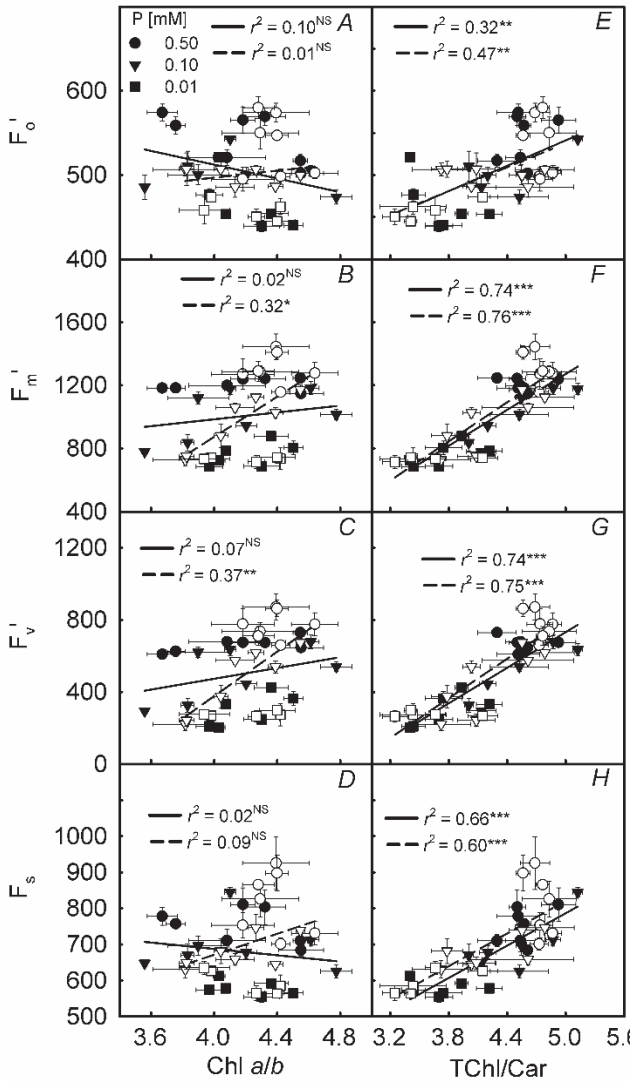


Fig. 7. The relationship of minimal (F_o'), maximal (F_m'), variable (F_v'), and steady-state (F_s) chlorophyll fluorescence with (A–D) chlorophyll *a/b* (Chl *a/b*) and (E–H) total Chl/carotenoids (TChl/Car) ratios of soybean uppermost fully expanded leaves grown at either ambient (filled symbols and solid lines, $400 \mu\text{mol mol}^{-1}$) or elevated (open symbols and dashed lines, $800 \mu\text{mol mol}^{-1}$) CO_2 concentration under three phosphorus (P) treatments. Other details are as in Fig. 5.

that chlorophyll concentration and CF parameters such as F_v'/F_m' and Φ_{PSII} are useful to assess the photochemical processes. However, the high linear association between CF parameters and TChl/Car ratio also emphasized the quantification of both chlorophyll and Car concentrations to understand the photochemistry and underlying processes that minimize the photoinhibition damage to photosystem.

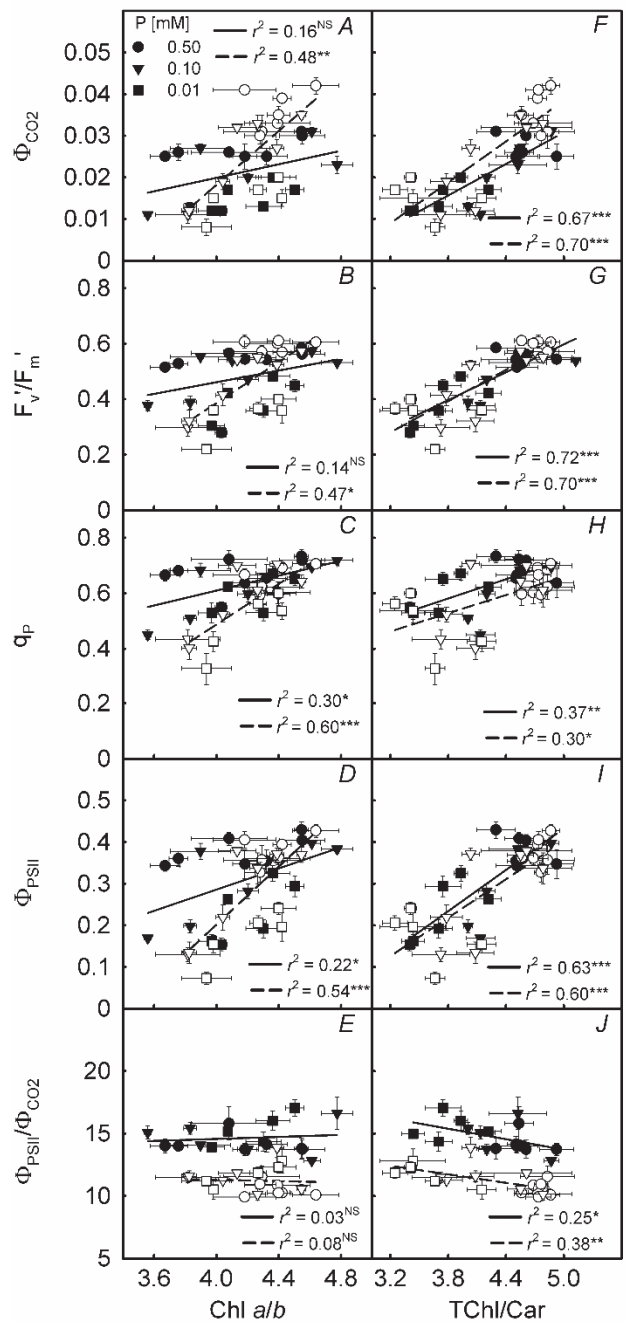


Fig. 8. The relationship of quantum yield of CO_2 assimilation (Φ_{CO_2}), efficiency of energy harvesting by oxidized (open) PSII reaction centers in light (F_v'/F_m'), proportion of the PSII unit in open state or photochemical quenching (q_p), photochemical quantum yield (Φ_{PSII}) and $\Phi_{\text{CO}_2}/\Phi_{\text{PSII}}$ with (A–E) chlorophyll *a/b* (Chl *a/b*) and (F–J) total Chl/carotenoids (TChl/Car) ratios of soybean uppermost fully expanded leaves grown at either ambient (filled symbols and solid lines, $400 \mu\text{mol mol}^{-1}$) or elevated (open symbols and dashed lines, $800 \mu\text{mol mol}^{-1}$) CO_2 concentration under three phosphorus (P) treatments. Other details are as in Fig. 5.

References

- Baker N.R., Rosenqvist E.: Applications of chlorophyll fluorescence can improve crop production strategies: An examination of future possibilities. – *J. Exp. Bot.* **55**: 1607-1621, 2004.
- Baker N.R.: Chlorophyll fluorescence: a probe of photosynthesis *in vivo*. – *Annu. Rev. Plant Biol.* **59**: 89-113, 2008.
- Cassman K.G., Whitney A.S., Fox R.L.: Phosphorus requirements of soybean and cowpea as affected by mode of N nutrition. – *Agron. J.* **73**: 17-22, 1981.
- Cordell D., Drangert J.-O., White S.: The story of phosphorus: Global food security and food for thought. – *Global Environ. Chang.* **19**: 292-305, 2009.
- Crafts-Brandner S.J.: Phosphorus nutrition influence on leaf senescence in soybean. – *Plant Physiol.* **98**: 1128-1132, 1992.
- Cure J.D., Ruffy T.W., Israel D.W.: Phosphorus stress effects on growth and seed yield responses of nonnodulated soybean to elevated carbon dioxide. – *Agron. J.* **80**: 897-902, 1988.
- Demmig-Adams B.: Carotenoids and photoprotection in plants: A role for the xanthophyll zeaxanthin. – *BBA-Bioenergetics* **1020**: 1-24, 1990.
- Edwards G.E., Baker N.R.: Can CO₂ assimilation in maize leaves be predicted accurately from chlorophyll fluorescence analysis? – *Photosynth. Res.* **37**: 89-102, 1993.
- Fleisher D.H., Wang Q., Timlin D.J. *et al.*: Response of potato gas exchange and productivity to phosphorus deficiency and carbon dioxide enrichment. – *Crop Sci.* **52**: 1803-1815, 2012.
- Genty B., Briantais J.M., Baker N.R.: The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. – *Biochim. Biophys. Acta* **990**: 87-92, 1989.
- Genty B., Harbinson J., Baker N.R.: Relative quantum efficiencies of the two photosystems of leaves in photorespiratory and not photorespiratory conditions. – *Plant Physiol. Bioch.* **28**: 1-10, 1990.
- Havaux M.: Carotenoids as membrane stabilizers in chloroplasts. – *Trends Plant Sci.* **3**: 147-151, 1998.
- Hendry G.A.F., Price A.H.: Stress indicators: chlorophylls and carotenoids. – In: Hendry G.A.F., Grime J.P. (ed.): *Methods in Comparative Plant Ecology* Pp. 148-152. Chapman & Hall, London 1993.
- Hewitt E.J.: Sand and water culture. Methods used in the study of plant nutrition. – In: Technical Communication No. 22. Commonwealth Bureau of Horticulture and Plantation, Commonwealth Agricultural Bureaux Farnham Royal. Pp. 187-190. Maidstone, Kent. Bucks 1952.
- IPCC. Summary for policymakers. – In: Stocker T.F., Qin D., Plattner G.-K. *et al.* (ed.): *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change.* Pp. 29. Cambridge University Press, Cambridge and New York 2013.
- Israel D.W., Ruffy T.W., Cure J.D.: Nitrogen and phosphorus nutritional interactions in a CO₂ enriched environment. – *J. Plant Nutr.* **13**: 1419-1433, 1990.
- Ivanov A., Sane P., Hurry V. *et al.*: Photosystem II reaction centre quenching: Mechanisms and physiological role. – *Photosynth. Res.* **98**: 565-574, 2008a.
- Ivanov A., Hurry V., Sane P. *et al.*: Reaction centre quenching of excess light energy and photoprotection of photosystem II. – *J. Plant Biol.* **51**: 85-96, 2008b.
- Lambers H., Chapin F.S., Pons T.L.: *Plant Physiological Ecology. Chapter 2: Photosynthesis, Respiration, and Long-Distance Transport.* 2nd ed. Pp. 11-162. Springer, New York 2010.
- Lauer M.J., Pallardy S.G., Blevins D.G., Randall D.D.: Whole leaf carbon exchange characteristics of phosphate deficient soybeans (*Glycine max* L.). – *Plant Physiol.* **91**: 848-854, 1989.
- Lichtenthaler H.K., Wellburn A.R.: Determinations of total carotenoids and chlorophylls *a* and *b* of leaf extracts in different solvents. – *Biochem. Soc. T.* **11**: 591-592, 1983.
- Lichtenthaler H.K.: Chlorophylls and carotenoids: Pigments of photosynthesis. – *Methods Enzymol.* **148**: 350-382, 1987.
- Maxwell K., Johnson G.N.: Chlorophyll fluorescence: A practical guide. – *J. Exp. Bot.* **51**: 659-668, 2000.
- Miranda V., Baker N.R., Long S.P.: Limitations of photosynthesis in different regions of the *Zea mays* leaf. – *New Phytol.* **89**: 179-190, 1981.
- Netto A.T., Campostrini E., de Oliveira J.G., Yamanishi O.K.: Portable chlorophyll meter for the quantification of photosynthetic pigments, nitrogen and the possible use for assessment of the photochemical process in *Carica papaya* L. – *Braz. J. Plant Physiol.* **14**: 203-210, 2002.
- Netto A.T., Campostrini E., de Oliveira J.G., Bressan-Smith R.E.: Photosynthetic pigments, nitrogen, chlorophyll *a* fluorescence and SPAD-502 readings in coffee leaves. – *Sci. Hortic.-Amsterdam* **104**: 199-209, 2005.
- Olaizola M., Yamamoto H.Y.: Short-term response of the diadinoxanthin cycle and fluorescence yield to high irradiance in *Chaetoceros muelleri* (Bacillariophyceae). – *J. Phycol.* **30**: 606-612, 1994.
- Ort D.R.: When there is too much light. – *Plant Physiol.* **125**: 29-32, 2001.
- Pascal A.A., Liu Z., Broess K. *et al.*: Molecular basis of photoprotection and control of photosynthetic light-harvesting. – *Nature* **436**: 134-137, 2005.
- Reddy K.R., Zhao D.L.: Interactive effects of elevated CO₂ and potassium deficiency on photosynthesis, growth, and biomass partitioning of cotton. – *Field Crop. Res.* **94**: 201-213, 2005.
- Roháček K.: Chlorophyll fluorescence parameters: The definitions, photosynthetic meaning, and mutual relationships. – *Photosynthetica* **40**: 13-29, 2002.
- Ruffy T.W., Siddiqi M.Y., Glass A.D.M., Ruth T.J.: Altered ¹³NO₃⁻ influx in phosphorus limited plants. – *Plant Sci.* **76**: 43-48, 1991.
- Samson G., Prášil O., Yaakoub B.: Photochemical and thermal phases of chlorophyll *a* fluorescence. – *Photosynthetica* **37**: 163-182, 1999.
- Seaton G.G.R., Walker D.A.: Chlorophyll fluorescence as a measure of photosynthetic carbon assimilation. – *P. R. Soc. B* **242**: 29-35, 1990.
- Singh S.K., Badgujar G., Reddy V.R. *et al.*: Carbon dioxide diffusion across stomata and mesophyll and photo-biochemical processes as affected by growth CO₂ and phosphorus nutrition in cotton. – *J. Plant Physiol.* **170**: 801-813, 2013a.
- Singh S.K., Badgujar G.B., Reddy V.R. *et al.*: Effect of phosphorus nutrition on growth and physiology of cotton under ambient and elevated carbon dioxide. – *J. Agron. Crop Sci.* **199**: 436-448, 2013b.
- Singh S.K., Hoyos-Villegas V., Ray J.D. *et al.*: Quantification of leaf pigments in soybean (*Glycine max* (L.) Merr.) based on wavelet decomposition of hyperspectral features. – *Field Crops Res.* **149**: 20-32, 2013c.
- Singh S.K., Reddy V.R., Fleisher H.D., Timlin J.D.: Growth,

- nutrient dynamics, and efficiency responses to carbon dioxide and phosphorus nutrition in soybean. – J. Plant Int. **9**: 838-849, 2014a.
- Singh S.K., Reddy K.R., Reddy V.R., Gao W.: Maize growth and developmental responses to temperature and ultraviolet-B radiation interaction. – Photosynthetica **52**: 262-271, 2014b.
- Singh S.K., Reddy V.R.: Combined effects of phosphorus nutrition and elevated carbon dioxide concentration on chlorophyll fluorescence, photosynthesis and nutrient efficiency of cotton. – J. Plant Nutr. Soil Sci. **177**: 892-902, 2014.
- Singh S.K., Reddy V.R.: Response of carbon assimilation and chlorophyll fluorescence to soybean leaf phosphorus across CO₂: Alternative electron sink, nutrient efficiency and critical concentration. – J. Photoch. Photobio. B **151**: 276-284, 2015.
- Singh S.K., Reddy V.R.: Methods of mesophyll conductance estimation: its impact on key biochemical parameters and photosynthetic limitations in phosphorus stressed soybean across CO₂. – Physiol. Plantarum **157**: 234-254, 2016.
- van Kooten O., Snel J.F.: The use of chlorophyll fluorescence nomenclature in plant stress physiology. – Photosynth. Res. **25**: 147-150, 1990.
- Walker W.M., Raines G.A., Peck T.R.: Effect of soybean cultivar, phosphorus and potassium upon yield and chemical composition. – J. Plant Nutr. **8**: 73-87, 1985.
- Zai X.M., Zhu S.N., Qin P. *et al.*: Effect of *Glomus mosseae* on chlorophyll content, chlorophyll fluorescence parameters, and chloroplast ultrastructure of beach plum (*Prunus maritima*) under NaCl stress. – Photosynthetica **50**: 323-328, 2012.
- Zhao D., Reddy K.R., Kakani V.G., Reddy V.R.: Nitrogen deficiency effects on plant growth, leaf photosynthesis, and hyperspectral reflectance properties of sorghum. – Eur. J. Agron. **22**: 391-403, 2005.