Limitations of photosynthesis in *Phaseolus vulgaris* under drought stress: gas exchange, chlorophyll fluorescence and Calvin cycle enzymes

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Abstract

In this article, the effects of drought stress (DS) on gas exchange, chlorophyll (Chl) *a* fluorescence and Calvin cycle enzymes in *Phaseolus vulgaris* are evaluated. Three-week-old plants were exposed to DS by receiving only so much water every evening to ensure 30% field capacity water content overnight. After three days under these conditions, we observed that DS induced a decline of the CO_2 assimilation. Gas-exchange data showed that the closure of stomata during DS did not lead to a concomitant decline in calculated intercellular CO_2 concentration. Moreover, DS plants showed a reduction of the photochemical Chl fluorescence quenching, photosystem II quantum yield and electron transport rate and a higher pH gradient and more heat dissipation as compared to controls. The activity of Calvin cycle enzymes, Rubisco, sFBPase, and Ru5PK, decreased strongly in DS plants as compared to controls. Data analysis suggest that the decrease of CO_2 assimilation under drought conditions is not related to a diminished capacity of the use of NADPH and ATP but probably to the decline of enzyme activity involved in RuBP regeneration (Ru5PK).

Additional key words: Calvin cycle enzymes; chlorophyll fluorescence; drought stress; *Phaseolus vulgaris*; photosynthetic rate.

Introduction

Drought stress (DS) is considered to be one of the main environmental factors that strongly limit growth and yield of plants worldwide (Chaves *et al.* 2003). Global change is expected to exacerbate water limitations in semiarid areas (IPCC 2001).

It is well known that inhibition of photosynthesis is one of the primary physiological consequences of DS (Chaves 1991, Cornic 1994, Lawlor 1995). While it is well established that stomatal closure is one of the first responses to soil drying (Chaves 1991, Yordanov *et al.* 2000), there has been some controversy concerning which limitation prevails as drought progresses. Recent studies have shown that the reduced CO_2 diffusion from the atmosphere to the site of carboxylation as a result of stomatal closure and reduced mesophyll conductance is the main cause for decreased photosynthesis under drought stress conditions (Correia *et al*. 1999, Cornic 2000, Chaves and Oliveira 2004, Ennahli and Earl 2005, Grassi and Magnani 2005, Flexas *et al*. 2006). Nevertheless, other studies have shown that drought stress also induces metabolic impairment, particularly decreases in Rubisco activity/ RuBP regeneration (Gunasekera and Berkowitz 1993, Flexas and Medrano 2002, Lawlor and Cornic 2002, Bota *et al.* 2004, Dias and Brüggemann 2007) and reduction of ATP production (Lawlor 1995, Tezara *et al*. 1999). Differences among species and variations of the rates of DS imposition, as well as other environmental stresses, may also play a role in the relative importance of stomatal *vs*. nonstomatal limitations under DS conditions (Chaves 1991).

Photosynthesis and photosynthetic capacity are progressively reduced under drought conditions. As

Received 14 August 2009, *accepted* 29 January 2010.
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Abbreviations: Chl – chlorophyll; DS – drought stress(ed); ETR – electron transport rate; F_m – maximum fluorescence in the dark-adapted state; F_m' – fluorescence during the saturating pulse in the steady state; F_m'' – (hypothetical) maximum fluorescence in the steady state; F_o' – minimum fluorescence after switch off the actinic light; F_v – maximal variable fluorescence; F_t – variable fluorescence in the stady state; PPFD – photosynthetic photon flux density; PS – photosystem; q_P – photochemical quenching; Rubisco – ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBP – ribulose-1,5-bisphosphate; Ru5PK – ribulose-5-phosphate kinase; sFBPase – stromal fructose-1,6-bisphosphatase; q_E – fast relaxing non-photochemical quenching; q_I – slowly relaxing non-photochemical quenching; WUE_i – intrinsic water use efficiency; Φ_{PSII} – quantum yield of PSII.

a consequence, lower light intensity is required to saturate photosynthesis. Under these conditions, plants cannot use all the excess of light energy for photosynthesis, increasing the susceptibility of plants to photoinhibition (Flexas and Medrano 2002). Chl fluorescence measurements have become a widely used method to study the functioning of the photosynthetic apparatus and are a powerful tool to study the plants' response to environmental stress (Pankovic *et al.* 1999, Maroco *et al.* 2002, Ennahli and Earl 2005, Grassi and Magnani 2005, Massacci *et al.* 2008). In C_3 plants, both maximum quantum yield of PSII photochemistry (measured by the ratio of F_v/F_m, *i.e.* the ratio of variable to maximum Chl fluorescence emission) (Genty *et al.* 1989, Cornic *et al.* 1992, Pankovic *et al.* 1999) and Φ_{PSII}, the quantum yield of photosystem (PS) II under a given light intensity (Pankovic *et al.* 1999, Maroco *et al.* 2002, Ennahli and Earl 2005) are usually not changed by mild DS. However, they are significantly reduced by nonphysiologically low relative water contents, when photoinhibition occurs (Cornic 1994, Pankovic *et al.* 1999, Flexas and Medrano 2002). Electron transport is very resistant to drought (Kaiser *et al.* 1981, Sharkey and Badger 1982, Ennahli and Earl 2005) and can remain unchanged during mild DS (Sharkey and Badger 1982).

The present work was undertaken to contribute to a better understanding of the effects of DS on plant performance in C_3 plants. With that purpose, the effects of DS on water status, gas exchange rates, Chl fluorescence, and Calvin cycle enzyme activities were determined and compared to well watered plants (control) in a typical mesophytic C_3 plant (*Phaseolus vulgaris*), which is grown throughout the temperate and subtropic regions and is, depending on natural precipitation conditions, frequently exposed from moderate to severe DS in the field.

Materials and methods

Plant growth and experimental conditions: Plants of *Phaseolus vulgaris* L. cv Saxa were grown in 1 kg pots ($11\times11\times12$ cm^3) containing a soil mix of 25% sand, 25% organic matter and 50% peat. Plants were grown in a growth chamber at 23°C, 40–60% relative humidity, 14/10 h day/night rhythm with a photosynthetic photon flux density (PPFD) of 150–200 μmol m^{-2} s^{-1} provided by *Osram* lamps. Three-week-old plants were exposed to DS by receiving only so much water every evening to ensure a water content corresponding to 30% field capacity overnight. After 3 days, plants had reached a water potential between –0.82 and –1.18 MPa during the DS treatment, which persisted for two further days.

Plant water status: Whole-plant water potentials were measured with a *SKPM 1400* pressure chamber (*SKYE Instruments*, Powys, Wales, UK) on abscised stems just above the soil surface, according to Scholander *et al.* (1965).

Gas exchange and Chl fluorescence measurements: Oxygen evolution was measured with a leaf-disc oxygen electrode (*Hansatech*, Kings Lynn, UK) at 2% O_2, 4.5% CO_2 at different light intensities (50, 120, 400, and 1,000 μmol m^{-2} s^{-1} PPFD) in control and DS plants. CO_2 concentration was kept constant by a 1 M Na_2CO_2/$NaHCO_3$ (1:19, v/v) buffer included in a sponge in the measuring chamber. Temperature during measurements was 23ºC. This gas composition will overcome any stomatal effect, especially in drought-treated plants, and will allow to measure the photosynthetic potential under optimal conditions without photorespiration.

In situ determinations of net photosynthetic rate (P_N), stomatal conductance (g_s), and calculated intercellular CO_2 concentration (C_i) at saturating PPFD (400 μmol m^{-2} s^{-1}) were determined with a *LI-6200* infrared gas analyzer (*LiCor*, Lincoln, NE, USA) under growth chamber conditions [370 μmol(CO_2) mol^{-1}]. Gas exchange measurements were performed in the growth chamber. The relative humidity of air entering the cuvette was set at 60% and the cuvette temperature was 23ºC. Flow rate of air through the cuvette was set at 250 μmol s^{-1}. Plants were illuminated during 15–20 min before the photosynthesis measurements. C_i was calculated according to von Caemmerer and Farquhar (1981) and the intrinsic water use efficiency was calculated as the ratio of P_N/g_s. Measurements were performed in the youngest fully developed leaf. The gas analyzer was calibrated every day according to the manufacturer's recommendations.

Chl *a* fluorescence was measured in leaf discs with a *PAM-101* Chl fluorometer (*Walz*, Effeltrich, Germany) under the same conditions as the O_2 evolution measurements. Photochemical quenching (q_P) and quantum yield of PSII were calculated according to Brüggemann *et al.* (1992) and Genty *et al.* (1989). Non-photochemical quenching (NPQ) of Chl fluorescence parameters, *i.e.* fast relaxing NPQ (q_E) and slowly relaxing NPQ (q_I) were determined according to Schreiber *et al.* (1986) and Brüggemann *et al.* (1992). With F_m being maximum fluorescence in the dark-adapted state, F_t the variable fluorescence in the stady state (*i.e.* after 5 min illumination), F_m' fluorescence during the saturating pulse in the steady state, F_o' the minimum fluorescence immediately after switching off the actinic light and F_m'' the (hypothetical) maximum fluorescence in the steady state, if no photoinhibition had occurred during the measurements (obtained by linear extrapolation to the moment of switching off the actinic light of the F_m' values obtained during saturating flashes every 3 min in the dark for 15 min, following the actinic lights), the calculated parameters are as follows: $q_P = (F_m' - F_t)/(F_m' - F_o')$, $q_I = (F_m - F_m'')/(F_m - F_o')$ and $q_E = (F_m'' - F_m')/(F_m'' - F_o')$.

As described by Krall and Edwards (1992), the electron transport rate (ETR) was estimated as ETR = ($\Delta F/F_m'$) × PPFD × 0.5 × 0.84. PPFD is the photosynthetic photon flux density incident on the leaf, 0.5 is the factor that assumes equal distribution of energy between the two photosystems, and the leaf absorbance used was 0.84 because is the most common value for C_3 plants (Björkman and Demmig 1987).

Calvin cycle enzymes: Immediately after gas exchange measurements, leaf samples were collected from the youngest fully developed leaf, and used for enzyme activities determinations. Rubisco (EC 4.1.1.39) was extracted and the activity assayed as described by Lilley and Walker (1974) and Dias and Brüggemann (2007). This assay follows NADPH oxidation measured spectrophotometrically at 340 nm. Total activity was achieved after incubation in 20 mM $MgCl_2$ and 10 mM $NaHCO_3$ for 20 min. Ribulose-5-phosphate kinase (Ru5PK; EC 2.7.1.19) was measured using the same protocol for Rubisco, however the reaction was started with ribulose-5-phosphate (0.5 mM). Stromal fructose-1,6-bisphosphatase (sFBPase; EC 3.1.3.11) was extracted and assayed according to Brüggemann *et al.* (1994). This assay is coupled to NADP reduction measured spectrophotometrically at 340 nm.

Statistics: Gas exchange and leaf water potential data are the averages of three determinations per plant from 6 plants per experiment (control and DS); oxygen production data are the average of individual measurements in 10 plants per experiment; Chl fluorescence, Rubisco and Ru5PK data are the averages of individual measurements of 5 plants and 9 plants for sFBPase per experiment. Comparisons between means (control and DS) were evaluated by *t*-test at a significant level set to 0.05. The data were analysed applying the program *Sigma Stat for Windows 3.1*. (*Aspire Software International,* Ashburn, VA, USA)

Results

Gas exchange: Fig. 1 shows the light-response curves of O_2 evolution under nonphotorespiratory conditions for control and DS bean plants. Control plants always showed a significantly higher rate of O_2 evolution than DS plants under all light intensities. The rate of O_2 evolution increased up to PPFD of 400 µmol m^{-2} s^{-1} in control and stressed plants, and decreased at PPFD of 1,000 µmol m^{-2} s^{-1} in both treatments.

In situ leaf P_N, g_s, calculated C_i and intrinsic water use efficiency (WUE_i) as a function of leaf water status are shown in Fig. 2. All these parameters were measured at saturating light (400 µmol m^{-2} s^{-1}) as observed in Fig. 1. The imposition of DS decreased water potential from a range of –0.29 and –0.55 (control) to –0.82 and –1.18 MPa (DS). P_N and g_s decreased with the drop of the plant water potential. Control plants showed always higher P_N and g_s than DS plants (Fig. 2*A,C*). The closure of stomata during DS did not lead to a concomitant decline in calculated C_i (Fig. 2*B*). WUE_i increased with decreasing plant water potential (Fig. 2*D*).

Responses of Chl fluorescence parameters to DS: The response of q_P, q_E, and q_I to changes in light intensity for control and DS plants is shown in Fig. 3. q_P decreased with increasing light intensities and control plants presented significantly higher means of q_P than DS plants under all PPFD (Fig. 3*A*). q_E and q_I increased with PPFD and DS plants had always a significantly higher level of q_E and q_I for each light intensity. Between 50–120 µmol m^{-2} s^{-1}, q_E showed a strong increase in both treatments, and then increased slowly (Fig. 3*B*). q_I increased strongly from 50 to 400 µmol m^{-2} s^{-1} under DS conditions, and stabilized after that (Fig. 3*C*). In control plants, q_I increased slowly with the increase of PPFD.

Fig. 4*A* shows the quantum yield of PSII (Φ_{PSII}) of control and DS bean plants. The values of Φ_{PSII} decreased with the increasing PPFD. Control plants always showed a significantly higher Φ_{PSII} for each light treatment, compared to plants exposed to drought conditions, except for 1,000 µmol m^{-2} s^{-1}.

Estimated ETR in bean plants under control and drought conditions for different light intensities are presented in Fig. 4*B*. In control plants ETR increased strongly up to 400 µmol m^{-2} s^{-1} achieving a mean value of 47.8 ± 1.2 µmol m^{-2} s^{-1}, but a further increase of PPFD to 1,000 µmol m^{-2} s^{-1} decreased ETR to a mean of 34.0 ± 6.6 µmol m^{-2} s^{-1}. DS bean plants showed an increase of

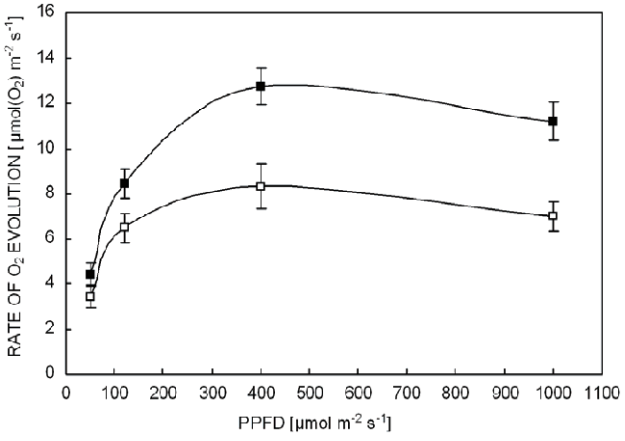

Fig. 1. Light-dependency curves of O_2 evolution in control (*closed squares*) and drought stress *P. vulgaris* plants (*open squares*). Values are means ± SD (*n* = 10).

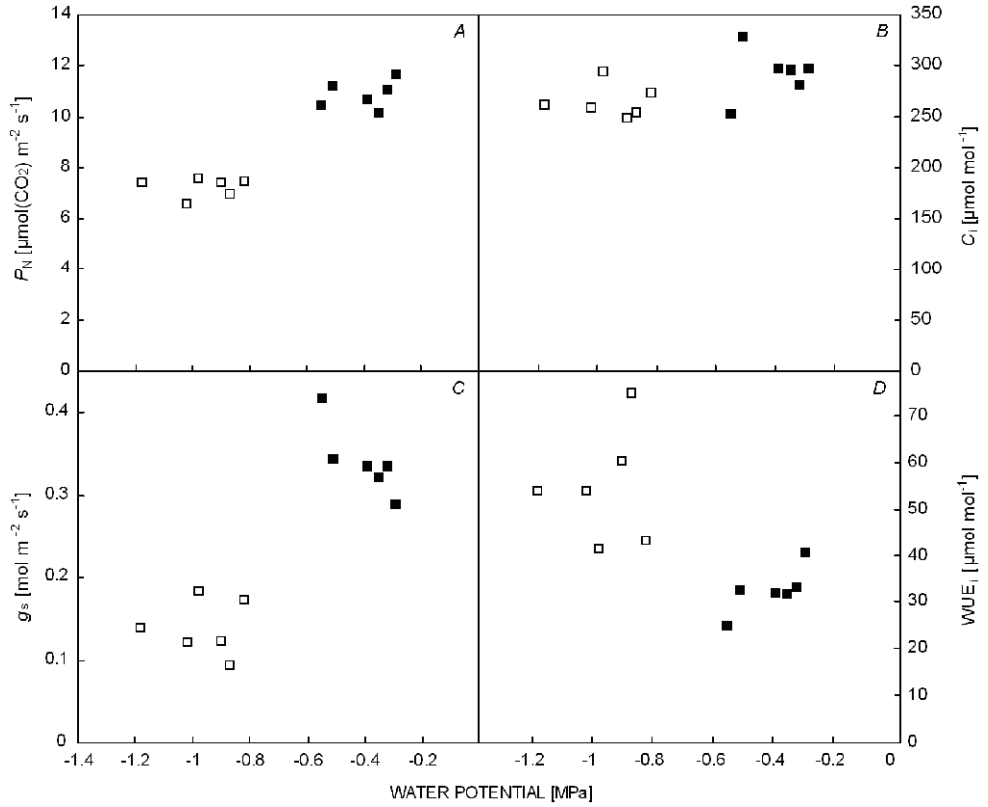

Fig. 2. *A*: In situ photosynthesis (P_N), *B*: calculated intercellular CO_2 concentration (C_i), *C*: stomatal conductance (g_s) and *D*: intrinsic water use efficiency (WUE_i) as affected by decreasing plant water pressure in individual control (*closed squares*) and drought stress *P. vulgaris* plants (*open squares*) ($n = 6$).

ETR also until 400 µmol m^{-2} s^{-1} (ETR mean 25.6 ± 1.74 µmol m^{-2} s^{-1}) and then decreased to 23.9 ± 3.5 µmol m^{-2} s^{-1} under higher PPFD (1,000 µmol m^{-2} s^{-1}). Control plants always showed a significantly higher ETR for each light intensity, as compared to drought plants, except at 1,000 µmol m^{-2} s^{-1} (Fig. 4*B*).

Calvin cycle enzymes: Three of the putative bottleneck enzymes of the Calvin cycle were measured in control and DS plants of *P. vulgaris*. Fig. 5 shows the maximum activities of Rubisco, Ru5PK and sFPBase in control and DS plants of *P. vulgaris*. A significant decrease in enzyme activity was observed under DS conditions. Rubisco, the key enzyme of the carboxylation phase of the Calvin cycle, and Ru5PK, the key enzyme in the regenerative phase of the Calvin cycle, were reduced by 49% relative to control plants (Fig. 5*A,B*). The activity of sFBPase was decreased by 35% in DS plants as compared to the controls (Fig. 5*C*).

Discussion

Similar to earlier reports on mildly (Sharkey and Seemann 1989, Cornic and Briantais 1991, Cornic and Ghashgaie 1991, Bota *et al.* 2004) and severely DS bean plants (Castrillo *et al*. 2001), the severe DS imposed on bean plants in our study significantly decreased *in situ* photosynthesis rates (Fig. 2*A*). This finding was corroborated under CO_2-saturating conditions (Fig. 1), indicating that despite strong stomatal closure as a first line of defense (Fig. 2*C*), the photosynthetic apparatus itself was inhibited by the stress treatment. The similar calculated C_i values in control and DS plants (Fig. 2*B*) together with the very similar photosynthetic rates obtained under ambient (Fig. 2*A*) and saturating CO_2 (Fig. 1) suggested that intercellular CO_2 might not be the limiting factor for photosynthesis in the stressed plants. However, the calculations of C_i performed by the *LiCor* apparatus according to von Caemmerer and Farquhar (1981) are only reliable under open stomata conditions, since they rely on the assumption that the conductance of the leaf surface for water and CO_2 are similar, which may not be the case under closed stomata (Boyer *et al.* 1997, Flexas *et al*. 2002).

The Chl fluorescence data obtained under non-limiting CO_2 concentrations suggest that the DS plants have a lower capacity for the use of transported electrons - their electron transport chain is more reduced at any

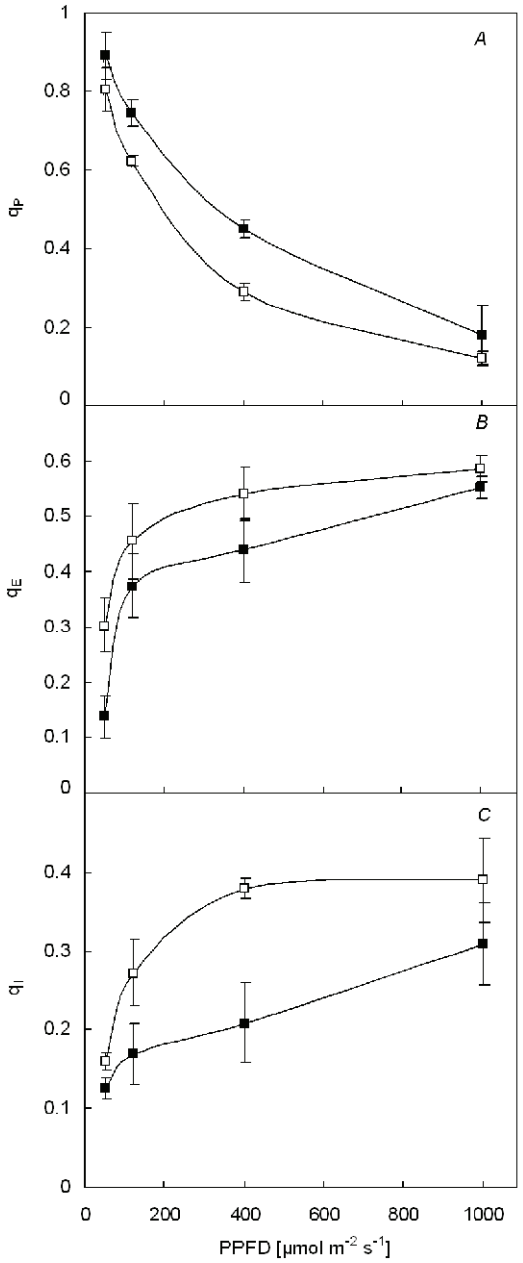

Fig. 3. Light-response curves of the Chl fluorescence quenching parameters q_P (A), q_E (B) and q_I (C) for control (*closed squares*) and drought stress (*open squares*) *P. vulgaris* plants. Values are means ± SD (n = 5).

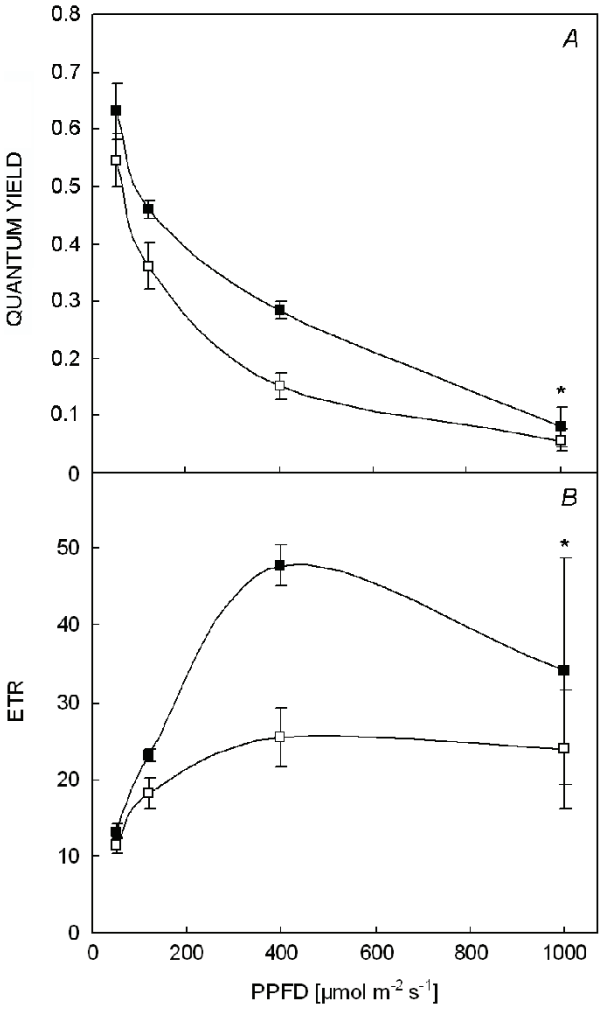

Fig. 4. Light-response curves of the quantum yield of PSII (Φ_{PSII}) (*A*) and estimated ETR (*B*) of control (*closed squares*) and drought stress (*open squares*) *P. vulgaris* plants. Values are means ± SE (n = 5). The DS plants showed significantly different values than the controls (P<0.05), except for PPFD 1,000 µmol m^{-2} s^{-1} (*). ETR is presented in µmol(electron) m^{-2} s^{-1}.

given light intensity and shows a higher pH gradient and more heat dissipation than control plants - their q_E values are higher than in controls under most light intensities. The latter protection mechanism, however, was insufficient to prevent them from a stronger photoinhibition at light intensities at the upper limit of their growth conditions or beyond. Since q_P is lower in DS plants, it could have been expected that their Φ_{PSII} and their calculated ETRs are lower, too, since all three parameters include the value of ΔF as a factor in the calculations. All these results can easily be explained, when a limitation of the dark reactions is taken into account, *i.e*. a nonstomatal limitation of photosynthesis by diminished capacity of the use of NADPH and ATP. While an inhibition of the ATPase has been discussed under certain drought conditions (Lawlor 1995, Tezara *et al.* 1999, Cornic 2000), in our case a limitation of the Calvin cycle key enzyme Ru5PK down to activities found for the overall photosynthesis rates at light- and CO_2-saturation (*i.e*. *ca*. 6 µmol m^{-2} s^{-1}) was observed (Fig. 2*A*). The other two enzymes analyzed, Rubisco and sFBPase also showed decreased rates under DS, but their in vitro activities were higher than maximum photosynthetic rates. Although we cannot exclude the possibility that the measured Ru5PK activities may be higher *in vivo* since our control plants revealed maximum P_N of *ca*. 12 µmol CO_2 m^{-2} s^{-1} and in vitro Ru5PK activities were slightly lower (*ca*. 11 µmol m^{-2} s^{-1}) (Figs. 2*A*, 5*B*), this enzyme appears to be a good

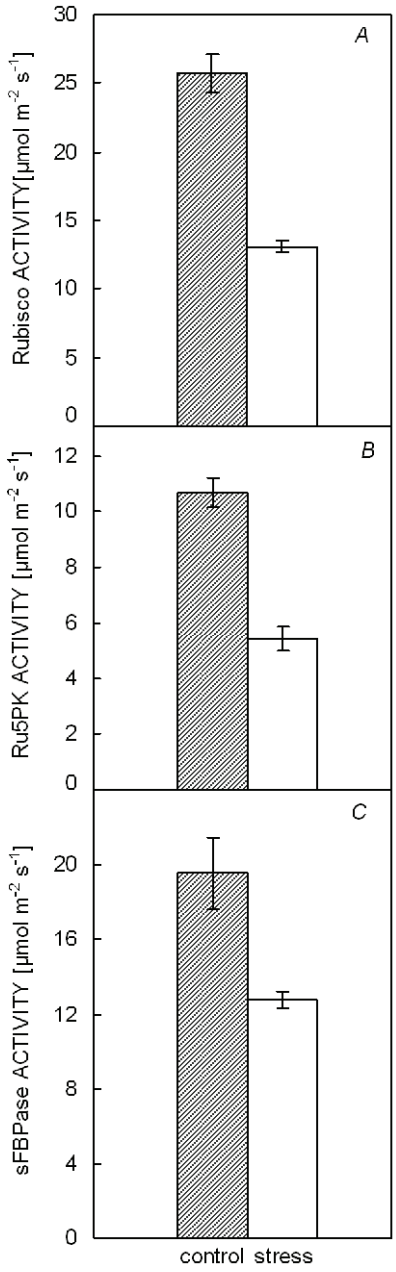

Fig. 5. In vitro activities of key enzymes of the Calvin cycle (Rubisco, Ru5PK, and sFBPase) in control and drought stress *P. vulgaris* plants. Values are means ± SD (Rubisco and Ru5PK $n = 5$, sFBPase $n = 9$).

candidate as the limiting factor for overall photosynthesis in DS bean plants, also explaining all concomitant Chl fluorescence data.

Decreased Rubisco activity (Castrillo *et al*. 2001, Parry *et al*. 2002, Tezara *et al*. 2002) or impaired capacity for RuBP regeneration (Bota *et al.* 2004, Flexas *et al.* 2004, Dias and Brüggemann 2007) have been suggested as the main limitations to photosynthesis under drought conditions. Our results match with the conclusions of the data analysis reported in the literature in *P. vulgaris* and also for other species. A careful look at the studies of Sharkey and Seemann (1989) showed that in mildly drought-stressed bean plants, RuBP and ATP levels did not decline, concluding that reduced photosynthetic rate in intact leaves was primarily caused by stomatal closure. However, when drought stress becomes severe, leaves had negligible rates of photosynthesis and a strong decline in RuBP and ATP occurred. The authors concluded that only by severe drought stress a RuBP regeneration impairment occurs. Similar conclusions were also reported by Bota *et al.* (2004). These authors demonstrated that for several species, including *P. vulgaris*, a decline of the availability of RuBP and Rubisco activity occurred when drought stress was severe, strongly decreasing photosynthesis. Gunasekera and Berkowitz (1993) identified RuBP synthesis (inhibition of the enzyme activity) as a limiting factor during drought stress in tobacco plants. In olive, rosemary, and lavender, Chl *a* fluorescence data also suggest that the decreases in the ability to regenerate RuBP cannot be attributed to a reduction of the ability to produce ATP during the early stages of DS development (Nogués and Baker 2000).

In summary, our results suggest, that while stomatal closure was observed during severe DS imposition, CO_2 availability was not affected and that limitations of CO_2 assimilation in DS plants of *P. vulgaris* could be related to the decline of the enzyme activity involved immediately in RuBP regeneration (Ru5PK).

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