Differential inhibition of photosynthesis under drought stress in *Flaveria* species with different degrees of development of the C_4 syndrome

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Abstract

The effect of drought stress (DS) on photosynthesis and photosynthesis-related enzyme activities was investigated in *F. pringlei* (C_3), *F. floridana* (C_3-C_4), *F. brownii* (C_4-like), and *F. trinervia* (C_4) species. Stomatal closure was observed in all species, probably being the main cause for the decline in photosynthesis in the C_3 species under ambient conditions. *In vitro* ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO) and stromal fructose 1,6-bisphosphatase (sFBP) activities were sufficient to interpret the net photosynthetic rates (P_N), but, from the decreases in P_N values under high CO_2 (C_a = 700 μmol mol^{-1}) it is concluded that a decrease in the *in vivo* rate of the RuBPCO reaction may be an additional limiting factor under DS in the C_3 species. The observed decline in the photosynthesis capacity of the C_3-C_4 species is suggested to be associated both to *in vivo* decreases of RuBPCO activity and of the RuBP regeneration rate. The decline of the maximum P_N observed in the C_4-like species under DS was probably attributed to a decrease in maximum RuBPCO activity and/or to decrease of enzyme substrate (RuBP or PEP) regeneration rates. In the C_4 species, the decline of both *in vivo* photosynthesis and photosynthetic capacity could be due to *in vivo* inhibition of the phospho*enol*-pyruvate carboxylase (PEPC) by a twofold increase of the malate concentration observed in mesophyll cell extracts from DS plants.

Additional key words: bundle sheath cells; C_3/C_4 metabolism; CO_2 compensation concentration; malic enzyme; NADP-malate dehydrogenase; phospho*enol*pyruvate carboxylase; pyruvate-orthophosphate dikinase; RuBP; RuBPCO; stromal fructose 1,6-bisphosphatase; water potential.

Introduction

The evolution of the C_4-metabolic pathway probably occurred initially about 70 million years ago as a consequence of the decrease of atmospheric CO_2 concentrations at the end of the Cretaceous period and beginning of the Paleocene, and C_4 species spread around 8–10 million years ago, when atmospheric CO_2 concentration reached a minimum of 200 μmol mol^{-1} (Ehleringer *et al.* 1991, Edwards *et al*. 2001). However, due to fossil fuel and the massive clearing of forests the concentration of atmospheric CO_2 is expected to increase up to 700 μmol mol^{-1} in the next century (Sage and Coleman 2001). Also, average atmosphere temperature is increasing and climate warming is expected to increase the frequency and severity of drought (Gregory *et al.* 1997). Drought stress (DS) is one main environmental factor limiting photosynthesis, growth, and yield of plants. Observed decreases of the photosynthetic capacity under drought stress (DS) have been attributed to lower mesophyll CO_2 availability, as a consequence of stomatal closure (*e.g.* Chaves 1991, Cornic 2000) and/or to non-stomatal effects (*e.g.* Berkowitz 1998, Flexas and Medrano 2002, Lawlor 2002). While the majority of DS experiments have been conducted in C_3 plants, fewer have been reported from C_4 plants showing the involvement of both stomatal and non-stomatal reasons for the inhibition of photosynthesis in the latter (Du *et al.* 1996).

Environmental stresses such as DS or limited nutrient availability generally reduce the response of C_3 plants, but not of C_4 plants to increasing CO_2 concentration; this suggests that C_4 plants will continue to maintain their competitive advantages over C_3 plants under DS despite the present increase of the atmospheric CO_2 concentration

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Abbreviations: BS − bundle sheath cells; C − control; C_a − external CO_2 concentration; C_i − intercellular CO_2 concentration; DS − drought stress; FM − fresh mass; g_s − stomatal conductance; HB − homogenization buffer; MDH − malate dehydrogenase; ME − malic enzyme; P_N − net photosynthetic rate; PEPC − phospho*enol*pyruvate carboxylase; PPDK − pyruvate-orthophosphate dikinase; RuBP − ribulose-1,5-bisphosphate; RuBPCO − ribulose-1,5-bisphosphate carboxylase/oxygenase; sFBP − stromal fructose 1,6-bisphosphatase.

(Wand *et al.* 1999, Ward *et al.* 1999). The genus *Flaveria* (Asteraceae) contains not only C_3 and C_4 species but also various intermediates (C_3-C_4 and C_4-like species) which represent stages in the evolutionary transition from C_3 to C_4 photosynthesis (Monson and Moore 1989). We tested the hypothesis that—given comparable developmental stages, leaf properties, and a similar genetic background—the gradual development of the C_4 syndrome within a genus is beneficial for drought tolerance of photosynthesis even under nowadays or increasing CO_2 concentrations. Additionally, we compared various possible enzymatic steps for their possible role in the non-stomatal limitation of photosynthesis in the different photosynthetic pathways.

Materials and methods

Plants: All genotypes originally were kindly provided by Prof. Westhoff, University of Düsseldorf, Germany and propagated and cultivated in glasshouses of the Botanical Garden of the University of Frankfurt, Germany. Plants of *F. trinervia* (NADP-ME C_4) were grown from seeds and *F. pringlei* (C_3), *F. floridana* (C_3-C_4), and *F. brownii* (C_4-like) from cuttings in a mixture of 50 % sand and 50 % peat. After 2–3 weeks, when the cuttings presented roots, the plants were transferred to a climate chamber into plastic trays (11×11×12 cm^3) containing a mixture of 1 kg(dry mass) soil (25 % sand, 25 % organic matter, 50 % peat), of which sufficient material was prepared in advance for all the experiments to ensure constant drought conditions throughout the study. The plants were grown in a climate chamber at 23 °C, 40–60 % relative humidity, 400 μmol(CO_2) mol^{-1}, 14/10 h day/night rhythm with 400 μmol(photon) m^{-2} s^{-1}, provided by *Osram* 1 000 W lamps, and received water daily. 3–4-week-old plants were exposed to DS by receiving only so much water every evening to ensure 30 % field capacity water content overnight. After 3–4 d, this resulted in estimated soil water potentials between –0.25 and –1.80 MPa during the DS treatment, which persisted for three further days.

Determination of plant water potentials: Water potentials on abscised stems was measured with a *SKPM 1400* pressure chamber (*SKYE Instruments*, Powys, Wales, UK) according to Scholander (1965).

Gas exchange measurements: *In situ* net photosynthetic rate (P_N), stomatal conductance (g_s), and intercellular CO_2 concentration (C_i) under growth CO_2 conditions and under experimentally varied CO_2 concentrations were determined with a *LI-6200* infrared gas analyzer (*LiCor*, Lincoln, NE, USA) at 1 000 (*F. pringlei*) or 2 000 μmol(photon) m^{-2} s^{-1} (*F. floridana, F. brownii*, and *F. trinervia*) "white light" either under growth chamber conditions [400 μmol(CO_2) mol^{-1}] or under experimentally varied CO_2 concentrations (Fig. 4).

Enzyme activities: Immediately after measuring the individual P_N of the youngest fully developed leaf during 15 min of irradiation [1 000 (*F. pringlei*) or 2 000 μmol (photon) m^{-2} s^{-1} (other species) for full activation of all light-regulated enzymes], enzyme activities were determined according to Du *et al.* (1996) in the same leaf section. Two leaf discs of 11 mm diameter for *F. trinervia, F. pringlei,* and *F. floridana* and four leaf discs of 6 mm diameter for *F. brownii* were punched from the measured leaf section and homogenized at 0 °C with 2 cm^3 homogenization buffer (HB), 20 mg *Polyclar AT* (*SERVA*, Heidelberg, Germany) and some sand with mortar and pestle. HB consisted of 50 mM Tris/HCl, pH 7.9, 8 mM $MgCl_2$, 5 mM Na-pyruvate, 1 mM EDTA, 2 mM K_2HPO_4, 20 mM dithiothreitol, and 0.3 % (m/v) bovine serum albumin. The homogenate was centrifuged in Eppendorf cups at 9 000×*g*. The supernatant was immediately used for the enzyme assays, which were performed subsequently on each homogenization before a new sample was produced from another leaf. Samples for the measurements of malate dehydrogenase (MDH), malic enzyme (ME), and phospho*enol*pyruvate carboxylase (PEPC) were kept on ice until measurement. Samples for ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO) determination were incubated in 20 mM $MgCl_2$, 10 mM $NaHCO_3$ for 20 min on ice prior to measurement for full activation (Lilley and Walker 1974). In the C_4 species and in both intermediate species, fully activated RuBPCO activities in the extracts usually did not match P_N. However, the same RuBPCO assay produced sufficient activities in the C_3 species. All attempts to increase the measured RuBPCO activities by variation of the homogenization procedure (grinding under liquid N_2) or increasing antioxidant or phenol-binding component concentrations failed. Since ME activities were in excess of P_N and a microscopic analysis revealed no intact bundle sheath strands in the preparations, insufficient homogenization of bundle sheath cells was excluded as a likely reason for the low RuBPCO rates. Samples for pyruvate-orthophosphate dikinase (PPDK) determination were immediately used at room temperature, since PPDK became inactivated on ice. In the case of stromal fructose 1,6-bisphosphatase (sFBP), new extracts were prepared from one leaf disc of 11 mm diameter for *F. trinervia, F. pringlei,* and *F. floridana* and two leaf discs of 6 mm diameter for *F. brownii* and enzyme activities were measured according to Brüggemann *et al.* (1994).

Electrophoresis: For electropheretic and Western blot analyses of leaf extracts according to standard procedures (Laemmli 1970, Beyel and Brüggemann 2005), preparations in HB without bovine serum albumin were

performed and aliquots equivalent to defined leaf areas were run on 15 % PAA gels. Proteins were identified both by Coomassie Brilliant Blue staining and by electroblotting and incubation with anti-RuBPCO large subunit (*Secale cereale*) antiserum (Dr. Schmidt, University of Frankfurt, Germany, 1 : 5 000 dilution) or anti-PEPC (*Sorghum bicolor*) antiserum (Prof. Westhoff, University of Düsseldorf, Germany, 1 : 5 000 dilution).

Mesophyll and bundle sheath cells' separation and malate determinations in *F. trinervia*: Half of a leaf was excised and irradiated for 15 min with 2 000 μmol (photon) m^{-2} s^{-1} "white light" in a moist chamber and immediately ground carefully at 0 °C with 2 cm^3 of 7 % $HClO_4$ and sand in a mortar and pestle to avoid bundle sheath disruption. The homogenate was then filtered through 80 μm aperture nylon net. The filtrate was neutralized with 5 M K_2CO_3 on ice and centrifuged for 5 min at 9 000×*g* in an Eppendorf cup. The supernatant was termed mesophyll extract. The material remaining on the net was further ground under liquid nitrogen to ensure complete disruption of the bundle sheath cells and then extracted with 7 % $HClO_4$, neutralized with 5 M K_2CO_3, and insoluble material pelleted by centrifugation (see above) to yield bundle sheath extract. Bundle sheath and mesophyll extracts were kept on ice until being used for the determination of malate according to Lowry and Passoneau (1972).

Microscopy analysis showed that the tissue remaining in the net contained intact bundle sheath cells and the filtrate was absent of them. Characteristic enzymes from the two cell-types (PEPC for the mesophyll cells and ME for bundle sheath cells) were determined from parallel preparations in HB instead of 7 % $HClO_4$ (Table 3). Enzyme activities showed a good separation by this procedure. Assuming that the filtrate mainly contained mesophyll cell fragments and the remainder in the net contained mainly intact bundle sheath cells, the fresh mass of the leaf sample used for the separation procedure was multiplied by the percentage of mesophyll (49.6 %) or bundle sheath cells (14.3 %) in a leaf of *F. trinervia*, determined from microscopy analysis, for the estimates of malate concentrations or enzyme activities for the two types of tissue.

Results

Gas exchange rates under ambient conditions: Of the four *Flaveria* species, the C_4 and the C_3-C_4 intermediate species revealed the highest P_N under growth conditions [control (C) plants: 30–40 μmol m^{-2} s^{-1}]. Upon exposure to DS, P_N decreased with decreasing leaf water potential in all species. Drought-stressed *F. trinervia* plants (C_4) achieved the lowest plant water potentials (down to –1.9 MPa), but P_N values were above 6.5 μmol m^{-2} s^{-1} in all cases (Fig. 1*D*). During DS, the lowest average P_N values were obtained in the C_3 species *F. pringlei* (4.9±2.8 μmol m^{-2} s^{-1}) (Fig. 1*A*).

g_s decreased in all four *Flaveria* species under DS (Fig. 2). In *F. trinervia* and *F. brownii,* the relative decrease was small due to low initial g_s. In DS plants, *F. pringlei* and *F. trinervia* presented, on average, the lowest g_s (0.030±0.015 and 0.052±0.018 mol m^{-2} s^{-1}, respectively) (Fig. 2*A*,*D*).

The *LiCor 6200* provides an internal algorithm programme for the calculation of C_i values according to the equations developed by Caemmerer and Farquhar (1981). While these equations yield reliable values under conditions, when cuticular resistance is negligible (*i.e.* stomata are open), they tend to overestimate C_i at closed stomata, since they assume the same diffusion resistances for H_2O and CO_2 (Boyer *et al.* 1997). Nevertheless, the calculated data are summarized in Table 1.

P_N/C_i response curves: The photosynthetic response to an experimentally induced decrease of C_a was measured in C plants in the four species. While *F. pringlei* (Fig. 3*A*) revealed a typical RuBPCO-controlled pattern with a CO_2 compensation concentration around 50–100 μmol mol^{-1}, *F. trinervia* clearly showed PEPC-controlled kinetics (Fig. 3*D*). In *F. pringlei* (Fig. 3*B*) and *F. brownii* (Fig. 3*C*), lower CO_2 compensation concentrations (<50 μmol mol^{-1}) were obtained with shapes of the P_N/C_i curves resembling *F. pringlei*, except for a steeper initial slope in *F. brownii*.

At high C_a (700 μmol mol^{-1}), average P_N declined under DS from 27–30 to 5–10 (*F. pringlei*), 40 to 10–20 (*F. floridana*), 25–30 to 15–20 (*F. brownii*), and 35 to 12–18 (*F. trinervia*) μmol(CO_2) m^{-2} s^{-1}, respectively.

Table 1. C_i values [μmol(CO_2) mol^{-1}] calculated from gas exchange data in leaves of control and drought stressed plants of *Flaveria.* Means ± SD.

	Control	Stressed
F. pringlei	295 ± 13	145 ± 18
F. floridana	294 ± 20	229 ± 42
F. brownii	252 ± 22	219 ± 19
F. trinervia	256 ± 39	224 ± 41

***In vitro* activities of key enzymes of the Calvin cycle and electrophoretic studies:** Two putative bottleneck enzymes from the Calvin cycle, RuBPCO and sFBP, were measured in all the *Flaveria* species. In the C_3 species *F. pringlei*, a decrease of about 64 % of the RuBPCO activity was observed in DS plants, but the values obtained were always above the P_N. In *F. brownii*, RuBPCO activity in DS plants was lower than in C plants, and the activities were always below the P_N. RuBPCO activities in the other two species showed

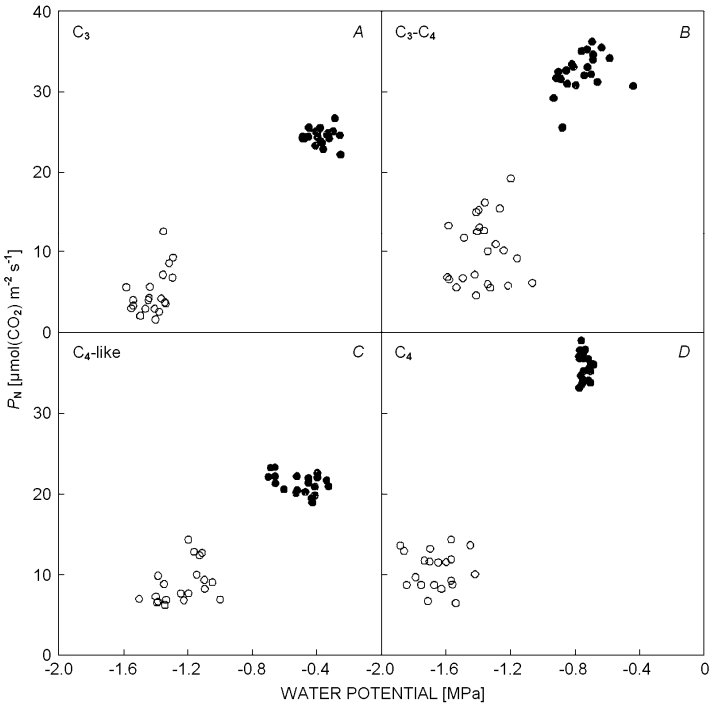

Fig. 1. Response of net photosynthetic rate (P_N) to decreasing plant water potential in control (●) and drought stressed (○) plants of *F. pringlei* (*A*), *F. floridana* (*B*), *F. brownii* (*C*), and *F. trinervia* (*D*).

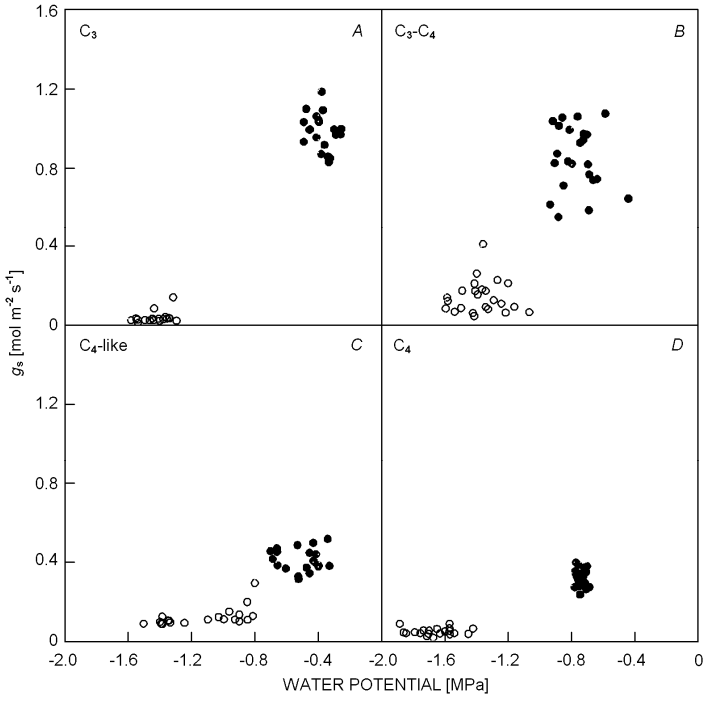

Fig. 2. Response of stomatal conductance (g_s) to decreasing plant water potential in control (●) and drought stressed (○) plants of *F. pringlei* (*A*), *F. floridana* (*B*), *F. brownii* (*C*), and *F. trinervia* (*D*).

similar values under both C and DS conditions. However, these activities were insufficient to explain P_N in C plants, but exceeded P_N of DS plants (Table 2).

In accordance with the change in *in-vitro* enzyme activity, Coomassie-stained gels and Western blots of leaf extracts revealed visible decreases of the amount of RuBPCO large subunit protein only in *F. pringlei* and *F. brownii*, but not in the other two species (data not shown).

Stromal FBP activities in both intermediates were only slightly affected by water stress. However, in *F. pringlei* and *F. trinervia* decreases of almost 50 % in the activities under DS were observed. According to the stoichiometry of the Calvin cycle (1 FBP to 3 CO_2), *in vitro* sFBP activities always exceeded the P_N in all four species (Table 2).

Table 2. RuBPCO and sFBP activities and net photosynthetic rates, P_N [μmol m^{-2} s^{-1}] in *F. pringlei*, *F. floridana*, *F. brownii,* and *F. trinervia* under control (C) and drought stress (DS) conditions. Means ± SD.

	P_N		RuBPCO		3 × sFBP	
	C	DS	C	DS	C	DS
F. pringlei	24.4 ± 1.0	5.0 ± 2.8	38.1 ± 4.6	13.5 ± 3.8	69.5 ± 8.0	38.8 ± 10.1
F. floridana	32.2 ± 2.7	10.3 ± 4.2	22.8 ± 6.3	19.1 ± 6.0	54.8 ± 10.1	45.1 ± 11.3
F. brownii	21.4 ± 1.5	8.0 ± 2.4	13.5 ± 1.9	5.7 ± 1.7	79.8 ± 14.6	70.3 ± 11.2
F. trinervia	36.0 ± 1.6	10.5 ± 2.4	19.4 ± 4.6	19.9 ± 4.5	81.9 ± 11.0	43.3 ± 6.1

Table 3. C_4-enzyme activities and photosynthetic rates [μmol m^{-2} s^{-1}] in *F. floridana* and *F. brownii* under control (C) and drought stress (DS) conditions. Means ± SD.

	F. floridana		*F. brownii*	
	C	DS	C	DS
P_N	32.2 ± 2.7	10.3 ± 4.2	21.4 ± 1.5	8.0 ± 2.4
PEPC	15.6 ± 3.3	15.3 ± 2.1	37.3 ± 6.7	24.4 ± 6.2
NADP-MDH	22.3 ± 4.0	17.0 ± 4.3	24.7 ± 2.8	21.1 ± 0.3
NADP-ME	7.4 ± 1.5	6.1 ± 1.2	49.7 ± 11.7	36.5 ± 11.4
PPDK	6.2 ± 1.0	5.0 ± 1.3	15.0 ± 3.3	12.0 ± 2.6

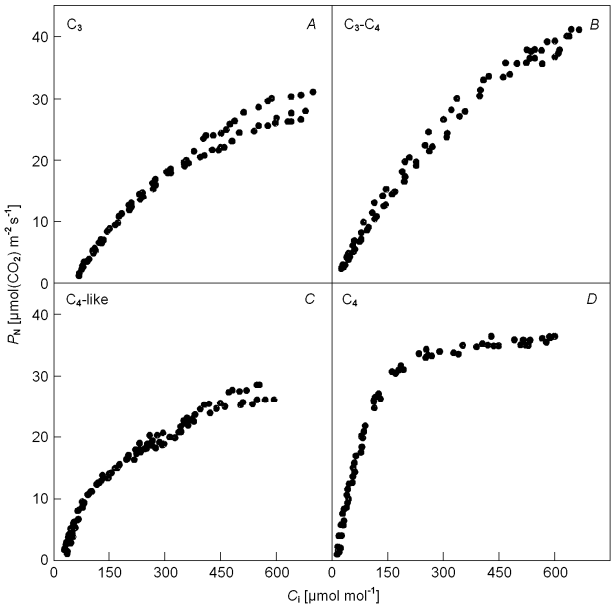

Fig. 3. Response of net photosynthetic rate (P_N) to intercellular CO_2 concentration (C_i) in control plants of *F. pringlei* (*A*), *F. floridana* (*B*), *F. brownii* (*C*), and *F. trinervia* (*D*).

C_4-enzyme activities: In the C_4 plant *F. trinervia*, *in vitro* PEPC, MDH, and ME activities in both C and DS plants and PPDK activities in DS plants were always higher than the P_N (Fig. 4). However, PPDK activities in C plants were very similar to P_N. All four enzymes on average showed lower activities in DS plants than in C plants. Electrophoretic analysis of PEPC protein content revealed a slight decrease in one out of two independent preparations (data not shown).

Since both intermediate species are able to fix CO_2 at least partially through the C_4 pathway (Monson *et al.* 1986, Chastain and Chollet 1989), C_4 enzyme activities were also measured in these species. *F. brownii* always presented higher enzyme activities than the intermediate *F. floridana*, but lower ones than the C_4 species, *F. trinervia* (Table 3, Fig. 4). The average enzyme activities measured in C and DS plants of *F. brownii* were higher than 65 % of the P_N. In *F. floridana*, the activities of the C_4 cycle enzymes measured in DS plants were at least 30 % of the P_N while in C plants only PEPC and MDH were above this percentage. In both species, only a small decrease in the C_4 enzyme activities was observed in plants under DS (Table 3). In Western blots stained with anti-PEPC antiserum, a slight decrease of PEPC protein content in leaf extracts from DS *F. brownii* was visible (data not shown).

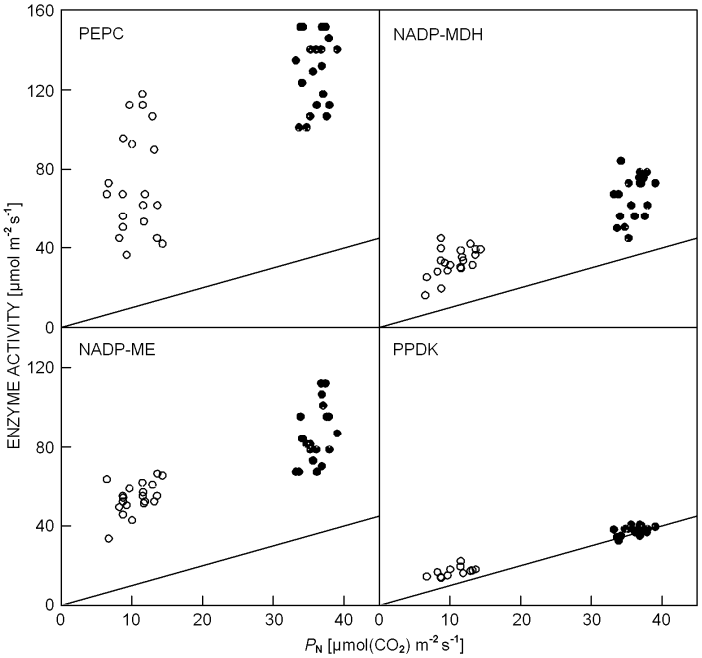

Fig. 4. C_4-enzyme activities compared to net photosynthetic rate (P_N) in control (●) and drought stressed (○) plants of *F. trinervia*. The solid line represents a 1 : 1 relationship between enzyme activity and photon-saturated P_N, both on leaf area basis.

Table 4. Enzyme activities [µmol kg^{-1}(FM) s^{-1}] and malate concentrations [mmol kg^{-1}(FM)] in mesophyll and bundle sheath extracts from *F. trinervia* under control (C) and drought stress (DS) conditions. Means ± SD.

	C		DS	
	Mesophyll	Bundle sheath	Mesophyll	Bundle sheath
PEPC	8.5 ± 1.4	4.7 ± 0.9	5.2 ± 0.9	2.6 ± 0.2
NADP-ME	1.2 ± 0.2	5.0 ± 0.3	2.3 ± 0.5	5.2 ± 0.4
Malate	35.9 ± 9.2	32.9 ± 7.6	72.3 ± 15.8	35.9 ± 9.2

Malate contents in mesophyll and bundle sheath cells from *F. trinervia*: Malate is a known inhibitor of PEPC (Wedding *et al*. 1990). In order to study whether a putative malate feedback inhibition may occur under DS, as postulated for *Sorghum bicolor* (Beyel and Brüggemann 2005), mesophyll and bundle sheath cell extracts were prepared and malate contents were measured. Malate contents increased twofold in mesophyll extracts of DS plants. However, in bundle sheath extracts, malate contents in C and DS plants were similar (Table 4).

Discussion

Stomatal *vs.* non-stomatal effects: A decrease in P_N during water deficit can be attributed to both stomatal and non-stomatal effects. Stomatal closure is considered the first line of defence against water loss (Chaves 1991), and a parallel decrease of P_N and g_s under DS has generally been reported (Medrano *et al.* 1997, Correia *et al.* 1999, Castrillo *et al*. 2001, Maroco *et al.* 2002). Also in the four *Flaveria* species of this study, DS lead to stomatal closure (Fig. 2). As in other C_3 species (Sharkey and Seemann 1989, Vassey and Sharkey 1989, Lal *et al.* 1996, Escalona *et al.* 1999), stomatal closure strongly reduced the intercellular CO_2 availability in *F. pringlei* (Table 1). In the other three species, the *LiCor* calculation revealed no significant decrease of C_i, in accordance with previous studies on other C_4 plants (Premachandra *et al.* 1994, Prakash and Rao 1996, Yu *et al.* 2004, Beyel and Brüggemann 2005). However, the reliance of C_i calculations from CO_2 and H_2O exchange rates in DS plants is strongly under debate, not only because of the masked effects of stomatal patchiness (Terashima *et al.* 1988, Mansfield *et al.* 1990, Terashima 1992), but also because of the different diffusion resistances of the cuticle for CO_2 and H_2O (Boyer *et al.* 1997). Therefore, no unequivocal conclusions on possible stomatal limitations of photosynthesis could be drawn for the other three species, and measurements of photosynthetic enzymes were performed to identify possible non-stomatal limiting factors.

In *F. pringlei*, fully activated *in vitro* RuBPCO activity and RuBPCO protein content decreased in DS plants, although the activities still exceeded the P_N. Since the calculated decline of C_i alone would still allow for P_N higher than those observed, provided the calculations were reliable and the P_N/C_i curve for C plants (Fig. 3*A*)

would still be applicable to DS plants, a decline of the *in vivo* RuBPCO activity may well contribute to the limitation of photosynthesis under DS. Similarly, Kicheva *et al.* (1994) in wheat, Kanechi *et al.* (1996) in coffee, and Panković *et al.* (1999) in sunflower observed decreases of *in vitro* RuBPCO activity under DS. In principle, several factors may contribute to even further declines of RuBPCO activity *in vivo* through side effects of DS: stromal acidification (Berkowitz *et al.* 1983), increase of stromal ionic strength (Kaiser 1982), or accumulating RuBPCO oxidation through over-excitation, which can play a role under long-term chilling stress (Brüggemann *et al.* 1995). Limitation of RuBP regeneration by the decreased sFBP activity (Table 2) appears unlikely, since the latter enzyme activity still vastly exceeded the P_N.

Positive P_N in the C_3-C_4 intermediate and the C_4-like species were already observed at C_i below 50 μmol mol^{-1} (Fig. 4*B*,*C*). These results are attributed to reduced photorespiratory loss of CO_2 by (partially expressed) C_4-metabolism (*F. brownii*) and by recycling the photorespired CO_2 in the bundle sheath cells in the case of C_3-C_4 intermediate species (*F. floridana*), respectively (Monson 1989). C_3-C_4 intermediate species are able to re-assimilate up to 70 % of the photorespired CO_2 (Hunt *et al.* 1987). In the C_4-like intermediate species this mechanism is not so important since the reduced photorespiration rates are mainly due to the high level of development of the C_4 pathway (Cheng *et al.* 1988, Ku *et al.* 1991).

RuBPCO activities in C plants of *F. floridana* and *F. brownii* were lower than the respective P_N. In *Sorghum*, where a similar finding was observed, Western blots gave no indications of a decrease of RuBPCO protein content in DS plants (Beyel and Brüggemann 2005). According to Rogers *et al.* (2001), who studied pine needle tissue, insufficient extraction of RuBPCO before the analysis may often be the cause for low RuBPCO activities *in vitro* in leaves with strong cell walls. However, any attempt to increase *in vitro* RuBPCO activities of C plants to or beyond their photosynthetic capacity by modifying the extraction procedure (liquid N_2 and excessive grinding) failed both in *Sorghum* (Beyel and Brüggemann 2005) and in *Flaveria*.

F. floridana exhibits characteristics between the C_3 and C_4 pathways (Holaday *et al.* 1984). RuBPCO is present in mesophyll and bundle sheath cells while PEPC and NADP-ME are partially compartmentalized as in a C_4 plant (Casati *et al.* 1999). Since the intermediate species contains both C_3 and C_4 pathway activities, both enzymes, RuBPCO and PEPC, are involved in the first step of the CO_2 assimilation. However, in *F. floridana,* PEPC is only responsible for a portion of the total CO_2 fixed (15 % according to Holaday *et al.* 1984, 30–50 % according to Chastain and Chollet 1989 and to Monson *et al.* 1986). Our results exclude the C_4 enzymes as possible limiting factors, since the activities measured in C and DS plants were similar and were enough to explain above 30 % of P_N. In *F. floridana*, the P_N/C_i curve strongly points to a RuBPCO-reaction limited situation of P_N in C plants under most CO_2 concentrations, be it by CO_2 or by RuBP regeneration (Fig. 3*B*). *In vitro* RuBPCO activities as well as RuBPCO protein contents were similar in C and DS plants (Table 2). While under C_a, stomatal limitation alone may well account for the observed decline in P_N, the decrease of P_N even under higher CO_2 in DS plants of *F. floridana* could be associated to an *in vivo* decrease in the RuBP regeneration cycle. Again, however, measurements of sFBP in *F. floridana* showed only a small decrease in the *in vitro* enzyme activity under DS, probably ruling this enzyme out as a limiting factor as discussed for *F. pringlei* (Table 2).

F. brownii is the most advanced C_3-C_4 intermediate in the genus *Flaveria* in terms of development of the C_4 syndrome (Monson *et al.* 1987). However, the principal photosynthetic carboxylation enzymes are not yet completely compartmentalized between mesophyll and bundle sheath cells as in a C_4 plant. PEPC and NADP-ME enzyme are present in both mesophyll and bundle sheath cells, but RuBPCO is mainly present in the bundle sheath cells chloroplasts (Reed and Chollet 1985). In *F. brownii*, about 65–80 % of the CO_2 is fixed into C_4 acids, whereas about 20 % of CO_2 enters the C_3 cycle directly through RuBPCO (Monson *et al.* 1986, Cheng *et al.* 1988, Chastain and Chollet 1989). The analysis of the P_N/C_i curve and the activity of PEPC suggest that the kinetic of CO_2 uptake at low CO_2 (initial slope) is related to the activity of PEPC as in a C_4 plant, while for high CO_2 it represents either the *in vivo* RuBPCO activity or the *in vivo* PEP regeneration rate (Caemmerer and Furbank 1999). The decline of the maximum P_N in DS plants under elevated CO_2 was in parallel to the decrease in the maximum RuBPCO activity observed in the *in vitro* measurements. Under DS, both PEPC activity and PEPC protein content declined slightly. However, DS plants of *F. brownii* contained sufficient *in vitro* PEPC, MDH, and ME activities to exceed the P_N under elevated CO_2 concentration (Table 3), while PPDK might become limiting in addition to the effect of DS on RuBPCO. Thus, with stomata closed in DS plants, the plants may still perform the P_N allowed by (less) PEPC, but will loose the additional beneficial effect of RuBPCO at higher C_i (cf. P_N/C_i curve, Fig. 3*C*).

In *F. trinervia*, both P_N under ambient CO_2 and under elevated CO_2 decreased to similar values [10–15 μmol(CO_2) m^{-2} s^{-1}] when plants were exposed to DS. The decrease in maximum P_N may reflect decreases in RuBPCO reaction rate and/or in the *in vivo* PEP regeneration rate (cf. Figs. 5 and 6 in Caemmerer and Furbank 1999). However, measurements of the *in vitro* activities of RuBPCO and of PEPC and other enzymes of the C_4-metabolism showed that all major enzymes were sufficient to explain photosynthetic activities of the DS plants (Fig. 4). Only in the case of PPDK in C plants, this enzyme may be a limiting factor for photosynthetic rates. Du *et al.* (1996) also observed a decrease of the PPDK

activity in DS sugarcane plants. In DS plants the two Calvin cycle enzymes, sFBP and RuBPCO, also revealed activities in excess of P_N. However, as in *F. floridana* and *F. brownii*, in C plants the values for RuBPCO activity were below the P_N.

Malate concentrations in *F. trinervia*: The results of the gas exchange measurements had indicated that the decline of the photosynthesis capacity in *F. trinervia* could be due to an inhibition of the CO_2-fixing enzyme PEPC *in vivo* under DS, despite sufficient PEPC activity *in vitro*. PEPC is the key enzyme of carbon assimilation in the C_4 pathway and is located in the cytoplasm of mesophyll cells. The activity of this enzyme is regulated by metabolites, changes in the pH of the cytoplasm, and also by reversible phosphorylation of a serine residue, which alters the sensitivity of the enzyme to these metabolites (Nimmo *et al.* 1986, Chollet *et al.* 1996, Vidal and Chollet 1997). While malate, the major product of the primary CO_2 fixation, is a competitive inhibitor of the PEPC activity, glucose-6-phosphate increases the affinity of PEPC for PEP by reducing the K_m, this positive effector enhances the ability of PEP to compete with malate (Vidal and Chollet 1997). Phosphorylation of PEPC in irradiated leaves markedly increases PEPC activity, reduces the inhibitory effect of malate, and increases the effect of glucose-6-phosphate (Leegood and Walker 1999). Since the observed *in vitro* PEPC activities in DS plants of *F. trinervia* vastly exceeded P_N without protein kinase and ATP in the extraction medium (*i.e.* a technique used by Sawada *et al.* 2002 to assess the phosphorylation state *in vivo*), we concluded that a putative decrease in the *in vivo* phosphorylation state of the enzyme was not responsible for the decline in P_N. Enzyme phosphorylation seems to allow C_4 PEPC to continue to fix CO_2 in the presence of high cytosolic malate concentrations (10–20 mM) in mesophyll cells in order to maintain malate diffusion to bundle sheath cells (Vidal and Chollet 1997). As observed here in *F. trinervia* and also by other authors in *Zea mays* (Leegood 1985, Stitt and Heldt 1985), malate is mainly located in mesophyll cells. However, not all malate is photosynthetically active in the cytoplasm (Hatch 1971), but a considerable amount is probably stored in the vacuole (Leegood 1985). In the literature, malate contents measured in C_4 leaves (*Zea mays*) range from 5–17 (bundle sheath cells) to 35–92 mM (mesophyll cells) (Leegood 1985, Stitt and Heldt 1985). Beyel and Brüggemann (2005) observed *ca.* 80 % increase in total leaf malate content in *Sorghum bicolor* under DS. Different results were observed by Du *et al.* (1998) in sugarcane, where malate contents dropped in leaves of DS plants. In *F. trinervia,* malate contents in the mesophyll extracts doubled from 36 in C up to 72 mmol kg^{-1}(FM) in DS plants. In bundle sheath extracts, these values were similar in C [32 mmol kg^{-1}(FM)] and DS plants [36 mmol kg^{-1}(FM)]. However, we did not observe a significant gradient of overall malate contents between BS and mesophyll cells of C plants, nor did we observe a significant increase in overall malate contents in the BS upon DS. While, therefore, conclusions from the overall contents upon the (critical) cytoplasm contents should be made with care, the data clearly indicate a significant increase of whole cell malate content in the mesophyll as a consequence of feedback accumulation through limited sink activity under DS.

C_4-species of the genus *Flaveria* contain both C_3 and C_4 isoforms of the enzyme PEPC; the C_3 form of PEPC showing a 15-fold higher sensitivity to malate than the C_4-enzyme (Bläsing *et al.* 2002). However, when the pH decreases to 7.3, also the C_4 isoform becomes inactivated at a malate content of 9.4 mM (Bläsing *et al.* 2002). Under photosynthetic conditions, the cytosolic pH undergoes a moderate acidification by dicarbonic acid formation (Yin *et al.* 1993), and DS decreases cytosolic pH further (Berkowitz *et al.* 1983). Even if the malate content in the cytoplasm would only be 10 % of the overall concentration found in mesophyll cells of DS plants (*i.e.* 7 mM, Table 4), at pH 7.3 or lower it would be strongly inhibiting.

Concluding remarks: The results indicate differential sensitivity of photosynthesis towards DS in the four species, depending on different physiological factors. An increase of malate concentration in the mesophyll cells may be the cause for the inhibition of PEPC activity *in vivo* and photosynthesis in the C_4 species. The decline of photosynthesis in the C_4-like intermediate was most probably due to a combination of stomatal closure and partial PEPC and RuBPCO losses in DS plants, while in the C_3-C_4 intermediate species it was probably associated with a reduction of g_s and, possibly, the regenerative phase of the Calvin cycle at elevated CO_2 concentration. In the C_3 species, the decrease in photosynthesis under DS was probably related to both (complete) stomatal closure and to decreases in the rate of the RuBPCO reaction.

The positive P_N observed at very low C_i (<50 μmol mol^{-1}) in both intermediate species represents an advantage under low atmospheric CO_2 when compared to the C_3 species.

Although non-stomatal effects severely decreased P_N in DS plants of the other three species, their P_N under C_a was higher than in *F. pringlei*. As shown in Fig. 2, the dependence of CO_2 fixation on the enzymatic properties of RuBPCO forces the C_3 and C_3-C_4 species to operate at higher g_s under C conditions, leading to lower water use efficiency than in the C_4 and C_4-like species. Under DS, the evolutionary change from a RuBPCO-based to a PEPC-based CO_2 fixing system *via* the C_3-C_4 intermediate and the C_4-like type, results in a secondary advantage, *i.e.* to produce sufficient saccharides to maintain the reduced need of the sinks even with stomata closed.

Since the C_3-C_4 and C_4-like species represent evolutionary intermediates that have evolved from C_3 plants

(Monson and Moore 1989), our results indicate that even under today's high atmospheric CO_2 concentration, the further development of the C_4 pathway in these intermediates will still be beneficial under water-limiting growth conditions.

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