

Midday depression in photosynthesis: Effect on sucrose-phosphate synthase and ribulose-1,5-bisphosphate carboxylase in leaves of *Prosopis juliflora* (Swartz) DC.

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Abstract

The midday depression in net photosynthetic rate (P_N) and stomatal conductance (g_s) in *Prosopis juliflora* was studied in relation to two key enzymes of carbon metabolism. Diurnal gas exchange measurements carried out in autumn on *P. juliflora* showed a pronounced depression in P_N and g_s along with a decrease in apparent carboxylation efficiency (CE*) during midday. The activities of sucrose-phosphate synthase (SPS) and ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO) also showed large diurnal fluctuations. Initial RuBPCO activity (that present *in vivo*) and total activity (fully carbamylated activity) increased gradually with increase in irradiance, in the morning reaching a maximum by 08:00 h. The RuBPCO activity declined sharply during midday due to decrease in initial activity. The drop in the % activation of RuBPCO indicated that the deactivation of RuBPCO was achieved *via* a decarbamylation mechanism. There was a marked similarity in the diurnal patterns of SPS activity and the P_N . During a diurnal rhythm, SPS activity increased after irradiation, reaching a maximum at 08:00 h and then declined during midday. Diurnal fluctuations in SPS activity could be due to the changes in the amount of protein (changes in V_{max}) as well as to the changes in kinetic properties (changes in V_{lim}). Hence the midday decline in photosynthesis is closely associated with the regulation of RuBPCO and SPS activities.

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Abbreviations: BICINE, N,N-bis(2-hydroxyethyl)glycine; CA1P, carboxy arabinitol 1-phosphate; Chl, chlorophyll; CHX, cycloheximide; C_i , internal CO_2 concentration; CE*, apparent carboxylation efficiency; DTT, dithiothreitol; EDTA, ethylene diamine tetraacetic acid; F6P, fructose-6-phosphate; G6P, glucose-6-phosphate; g_s , stomatal conductance; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid; P_i , inorganic phosphate; PMSF, phenylmethylsulfonylfluoride; P_N , net photosynthetic rate; PPFD, photosynthetic photon flux density; RuBP, ribulose-1,5-bisphosphate; RuBPCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; SPS, sucrose-phosphate synthase; UDPG, uridine-diphosphate-glucose; VPD, vapour pressure deficit.

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Additional key words: diurnal change; net photosynthetic rate; stomatal conductance.

Introduction

Daily carbon gain in trees under field conditions is a complex process influenced by the diurnal and seasonal changes in environmental factors that affect export of photoassimilates and their partitioning. Diurnal measurements indicate that trees often exhibit a midday depression in both P_N and g_s (Küppers *et al.* 1986). The midday depression phenomenon studied to a large extent in Mediterranean trees (Lange *et al.* 1982) is associated with high VPD (Prado *et al.* 1995), high temperature (Singh *et al.* 1996) and/or strong irradiance (Raschke and Resemann 1986, Correia *et al.* 1990, Palanisamy 1996). Though the midday decline offers a well controlled mechanism that the trees seemingly have evolved to overcome radiation and temperature stresses, the cause of midday depression is still not unequivocally elucidated. The decline in P_N may be due to stomatal and non-stomatal limitations of photosynthesis. In several cases, during midday depression the apparent carboxylation efficiency decreased while the internal CO_2 concentration (C_i) remained constant (Gunasckera and Berkowitz 1992). On the other hand, the patchy stomatal closure during midday can decrease local C_i and subsequently inhibit photosynthesis (Beyschlag *et al.* 1992). Though extensive work has been done on environmental effects on midday depression, the status of metabolic reactions during midday depression has not yet been assessed. To understand the physiological adaptation of trees, it is important to know how midday depression would affect the metabolic processes of carbon fixation, partitioning and export.

During gas exchange studies on several trees growing on the marginal land of north India, we have observed that *P. juliflora* shows typical midday depression of P_N , g_s and CE^* , during the period from July to October (Pathre *et al.* 1995). In order to understand the status of metabolic reactions in *P. juliflora* during midday depression in photosynthesis, we studied the behaviour of two key enzymes, *viz.* RubPCO which is involved in carbon fixation and SPS that controls carbon partitioning.

Materials and methods

The experiments were conducted on one year-old potted plants of *P. juliflora* in a warm-weather period after the last monsoon rains in autumn of 1995. Diurnal exchange of CO_2 and water vapour was measured from dawn to dusk on attached fully expanded leaves, using a *Li-Cor* model 6200 portable photosynthesis system, with 1000 cm^3 chamber (*Li-Cor*). Each leaf was fully exposed and oriented normal to irradiation during measurements to ensure the highest possible PPFD. The equations used for calculation were those reported by Caemmerer and Farquhar (1981) as detailed in the *Li-Cor* technical reference manual (1987). The IRGA was calibrated using authentic gases from *Li-Cor* (U.S.A.). Diurnal changes in P_N in response to change in CO_2 concentration were measured according to McDermitt *et al.* (1989).

RuBPCO was extracted from leaf tissue samples frozen in liquid nitrogen by grinding leaflets at 0-4 °C in an extraction buffer (pH 8.2) containing 100 mM Bicine, 20 mM MgCl₂, 1 mM EDTA, 5 mM DTT, and 5 mM ascorbate. The extract was then centrifuged at 13 000×g for 30 s and supernatant was analysed for RuBPCO activity. For each sample, the RuBPCO activity was determined twice: immediately after leaf extraction, yielding the "initial" activity. The total activity was measured after fully carbamylating the enzyme by adding 100 µmol of 100 mM NaH¹⁴CO₃ to 900 mm³ of the initial extract and incubating at 25 °C for 15 min. Activities were determined at 25 °C from the rate of ¹⁴CO₂ incorporation into acid-stable compounds (Pierce *et al.* 1982). The % activation of the enzyme was calculated as (initial activity/total activity × 100) according to Butz and Sharkey (1989).

For SPS assay, frozen leaflets were ground in a chilled mortar in an extraction buffer containing 0.1 M HEPES-KOH buffer, pH 7.4, 5 mM MgCl₂, 1 mM EDTA, 25 mM β-mercaptoethanol, 1 mM PMSF, and 0.2 % Triton X-100. The homogenates were centrifuged at 13 000×g for 5 min and the supernatant immediately desalting on Sephadex G-25 equilibrated with the extraction buffer without Triton X-100. The maximum activity (V_{max}) of the enzyme was assayed in a reaction mixture of 100 mm³ final volume consisting of 50 mM HEPES-KOH (pH 7.4), 15 mM MgCl₂, 5 mM UDPG, 4 mM F6P, and 20 mM G6P. The limiting assay (V_{lim}) was carried out as described above except that 10 mM P_i was included and the concentrations of F6P and G6P were lowered to 2 and 10 mM, respectively. The reactions were initiated by the addition of enzyme extract, the samples were incubated at 25 °C for 20 min. The assay was terminated with 30 % KOH, and unreacted F6P was destroyed by placing the tubes in boiling water for 10 min. Appropriate controls were run for comparison. After cooling, 1.0 cm³ of 0.14 % (m/v) anthrone in 13.8 M H₂SO₄ was added and the tubes were incubated at 40 °C for 20 min prior to measuring absorbance at 620 nm. The ratio of two activities (V_{lim}/V_{max} × 100) was considered as the activation state and was expressed as % (Huber and Huber 1991).

Chlorophyll (Chl) content in the samples was determined according to the method described by Coombs *et al.* (1985).

Results and discussion

Under sub-tropical Indian climatic condition in autumn *P. juliflora* experiences a typical midday depression in *P_N* and *g_s* like the Mediterranean plant species. Fig. 1B shows typical daily patterns of *P_N* and *g_s* for *P. juliflora* in November. Midday depression in both *P_N* and *g_s* was evident with a sharp 50 % decline in *P_N* and 60 % in *g_s* as compared to the maximum morning values. The *C_i* did not dramatically change between 08:00 and 16:00 h (results not shown) but vapour pressure deficit (VPD) increased continuously until it reached a maximum of 4.5 kPa at noon (Fig. 1A). The PPFD was above 1000 µmol m⁻² s⁻¹ through most of the day and was well above the *P_N* saturation irradiance for *P. juliflora* (Fig. 1A). The depression in *P_N* occurred primarily due to high VPD and/or high irradiance. The CE* as calculated

from the P_N/C_i curve was also lowered during the midday depression. Also the diurnal measurements of RuBPCO showed a decrease in the initial activity and activity ratio during the depression (Figs. 1C and 2).

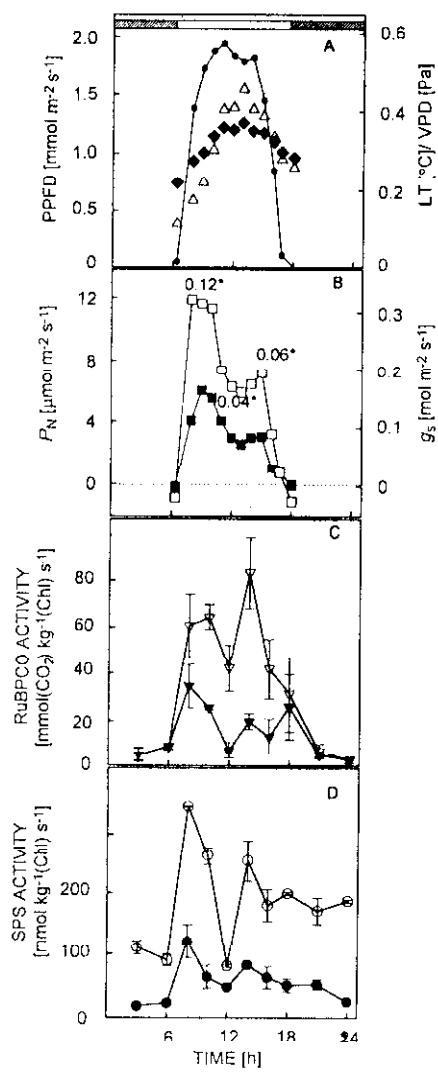


Fig. 1. Daily time course of: (A) irradiance (PPFD, ●), vapour pressure deficit (VPD, Δ) and leaf temperature (LT, ♦), the shaded bars at the top of the figure correspond to the dark period; (B) net photosynthetic rate (P_N , □) and stomatal conductance (g_s , ■), * represents the values for apparent carboxylation efficiency; (C) initial activity (▼) and total activity (▽) of RuBPCO; (D) V_{lim} activity (●) and V_{max} activity (○) of SPS measured in leaves of *P. juliflora*. Each point (bar) represents the mean (\pm SE) of three to four replicates at each sampling time.

At very low PPFD values prior to sunrise, the activation of RuBPCO in *P. juliflora* was nearly 100 %, though the initial and total activities of RuBPCO were extremely low (Figs. 1C and 2). With increasing PPFD both the activities increased up to 10:00 h but the activation levels were decreased to 40 %, because the increase in total activity was larger (>20 fold) than that of initial activity (<10 fold). The PPFD reached a maximum at 2000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at 10:00 h and remained high (>1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$) for most of the day. At midday the % RuBPCO activation was further decreased to 20 % with decline in the initial activity but the total activity remained

high. In the afternoon the total activity increased sharply to a maximum of 83 mmol kg⁻¹(Chl) s⁻¹ at 13:00 h while the initial activity remained low thereby maintaining activation levels around 20-25 %. Both the activities then decreased along with the decrease in PPFD. The activation levels of RuBPCO thereafter increased continuously after 16:00 h and reached 100 % by midnight.

The % activation reflects the carbamylation state of RuBPCO (Butz and Sharkey 1989). Therefore, the decrease in % activation during midday can be interpreted as arising due to a decarbamylated state of the enzyme. The carbamylation state increased only after 16:00 h, when P_N had recovered from the midday decline. The decarbamylation of RuBPCO indicates that the diurnal regulation of RuBPCO in *P. juliflora* is markedly different from that in crop species. In spinach or other crop species the carbamylation of RuBPCO increases with increase in PPFD and the enzyme remains in carbamylated form as long as the PPFD is high, the carbamylation decreasing only when PPFD decreases in late afternoon (Kobza and Seemann 1989). For the regulation of RuBPCO two major mechanisms have been proposed: (a) carbamylation of a lysine residue in the active site of RuBPCO, (b) binding of the inhibitor CA1P to the active site (Salvucci 1989, Seemann *et al.* 1990). While both regulatory mechanisms can be utilized following changes in irradiance, only modulation of the carbamylation state may regulate RuBPCO in response to changes of $p(\text{CO}_2)$ or $p(\text{O}_2)$ (Sharkey *et al.* 1986, Sage *et al.* 1988). These mechanisms have been deduced from controlled and steady state gas exchange experiments. In the field, however, plants do not experience such conditions, thus the variation observed in RuBPCO of *P. juliflora* in autumn may be due to a combination of different mechanisms. In early morning at low PPFD the enzyme shows high activation and very low activity suggesting the involvement of tight binding of the inhibitor (Kobza and Seemann 1989). But as the PPFD increased most of the regulation was accomplished by changes in the carbamylation state of the enzyme. The decarbamylation observed could not be due to decrease in PPFD, as the irradiance was saturating for most of the day. Therefore, the decarbamylation might have occurred due to changes in partial pressures of CO₂ as mentioned above. The C_i dependent deactivation of RuBPCO by decarbamylation has been reported in C₃ annuals *Chenopodium album* and *Phaseolus vulgaris* (Sage *et al.* 1990). In these plants under steady state conditions, at PPFD of 1750 $\mu\text{mol m}^{-2} \text{s}^{-1}$, the activation state of RuBPCO was increased when C_i was decreased from 45 to 10 Pa. However, below a C_i of 10 Pa, the activation state decreased as C_i was reduced to the compensation concentration. Under natural environment, low C_i may occur as a result of patchy stomatal closure (Sharkey and Seemann 1989). During midday P_N depression in *P. juliflora*, the apparent C_i values were calculated on the basis of gas exchange parameters. If non-uniform stomatal closure (patchy) had occurred, then the actual C_i might have been much lower than the calculated value (Beyschlag *et al.* 1992). It was not possible to measure the patchiness in the leaflets of *P. juliflora* leaves (as leaf closes all its leaflets once plucked) using available techniques like water infiltration or ¹⁴CO₂ incorporation (Mott *et al.* 1993), and hence the actual C_i could not be estimated. However, in view of the wide occurrence of the patchy stomatal closure during midday under high VPD (Cardon *et al.* 1994) and

deactivation of RuBPCO under low C_i as shown in C_3 annuals (Sage *et al.* 1990), the decarbamylation of RuBPCO observed in *P. juliflora* could be a result of low C_i . If RuBPCO would be regulated so that the capacity to consume RuBP would balance the RuBP regeneration capacity (Sage *et al.* 1990), then under saturating irradiance and low C_i , the RuBP consumption would be poor and the RuBP pool could be maintained by deactivation of RuBPCO through its decarbamylation. It is also speculated that below 10 Pa the carbamylation of RuBPCO by RuBPCO activase is CO_2 limited (Sage *et al.* 1990). Thus the deactivation of RuBPCO may be a secondary effect resulting from low C_i and not a direct effect of stress on the g_s .

In addition to the change in RuBPCO activity at midday, a drastic decrease in SPS activity was also observed. *In vivo*, SPS exists in kinetically active (dephosphorylated) and inactive (phosphorylated) forms. The V_{max} assay measured at saturating concentrations of hexose phosphates allows the study of the activity of both forms. The activity of the dephosphorylated enzyme or kinetically active form (V_{lim}) could be measured at limiting hexose phosphate concentrations and in presence of P_i (Siegl and Stitt 1988, Huber and Huber 1992). Diurnal changes observed in V_{max} activity of SPS in spinach by Stitt *et al.* (1988) may be due to uncharacterized mechanisms which alter the V_{max} or the amount of SPS protein present in the leaf, while variations in V_{lim} activity in the presence of P_i are attributed to the kinetically active (dephosphorylated) form (Stitt *et al.* 1988). Fig 1D shows diurnal fluctuations in V_{max} and V_{lim} activities of SPS from *P. juliflora*. Before sunrise the activities were low (the activity ratio of V_{max} to V_{lim} was only 0.25). After sunrise both the activities increased along with increase in PPFD and P_N , and reached a maximum at 08:00 h. Since the relative increase in V_{lim} was larger than that of V_{max} , the % activation was also increased. As the day progressed, both the activities of SPS decreased with a decrease in P_N and g_s (at midday depression), while the PPFD remained high ($= 2000 \mu\text{mol m}^{-2} \text{s}^{-1}$). The V_{max} activity dropped to its lowest value at 12:00 h (to the same value as the predawn one), but V_{lim} at the same time was still twice that of its predawn value. This unequal inhibition was reflected in the % activation curve showing that at 12:00 h there was 65 % activation of SPS (Fig. 2). In the afternoon the SPS activities again increased along with the

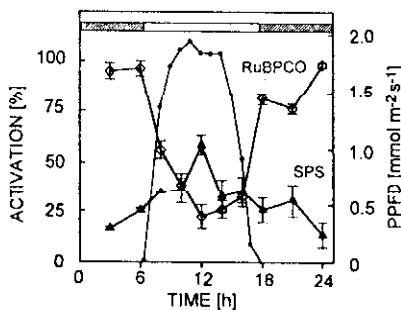


Fig. 2. Diurnal changes in PPFD (●), % activation of RuBPCO (◊) and SPS (▲); the shaded bars at the top of the figure correspond to the dark period.

recovery of photosynthesis. The increase in the V_{max} activity was larger (2.5 fold) than that of V_{lim} (<2 fold). After 14:00 h both the activities decreased along with the

decrease in PPFD and P_N . In the evening the % activation remained in the range of 25-30 % reaching the lowest value at 24:00 h due to the substantial decrease in the V_{lim} activity while the V_{max} activity remained constant [167 mmol kg⁻¹(Chl) s⁻¹]. Both activities reached their lowest value after midnight.

The Figs. 1D and 2 show that the diurnal regulation of SPS of *P. juliflora* is different from that in crop species such as spinach. In spinach, the steady state concentration of SPS protein does not change significantly on a diurnal basis or in response to light/dark transitions. This conclusion was based on immunochemical measurements (Walker and Huber 1989) and verification that the maximum SPS activity remained constant (Stitt *et al.* 1988, Walker and Huber 1989). Also in the short term (*i.e.*, over a period of 4 to 6 h) there appeared a relatively little turn-over of the SPS protein from spinach because the inhibition of cytoplasmic protein synthesis with CHX did not reduce maximum SPS activity (Weiner *et al.* 1992). In *P. juliflora* the diurnal measurements of SPS showed a large increase in V_{max} activity with the increase in PPFD and P_N during morning hours and a subsequent decrease at midday suggesting that the amount of SPS protein underwent changes or that some activation/deactivation (inhibition) processes took place. Secondly, the % activation which increased from 25 % at predawn to a maximum of 65 % at midday indicated that the mechanism of covalent modification was also active (Fig. 2). The involvement of protein synthesis during light activation of V_{max} activity in *P. juliflora* was further confirmed using CHX. Pretreatment of leaves of *P. juliflora* with 10 μ M CHX had no effect on both the SPS activities of leaves maintained in darkness (Table 1) but it substantially inhibited the light dependent increase in V_{max}

Table 1. Effect of preincubation of leaves with cycloheximide on the activation of sucrose-phosphate synthase (SPS) in light. The leaf of *P. juliflora* was cut under water before sunrise. The leaf was fed with 20 μ M CHX through transpiration stream. After 3 h in dark, the leaf was exposed to sunlight (=1000 μ mol m⁻² s⁻¹). After 30 min irradiation, the leaf was excised and assayed for SPS activity under two different conditions (see text for details). The values represent data (\pm SE) obtained from four separate experiments.

Treatment	SPS activity [mmol kg ⁻¹ (Chl) s ⁻¹]		Activation [%]
	V_{lim}	V_{max}	
Intact leaf in dark for 0 min	24.33 \pm 1.17	145.17 \pm 0.33	16.86 \pm 0.93
Intact leaf in dark for 4 h	22.50 \pm 4.33	131.33 \pm 19.00	17.07 \pm 0.98
Intact leaf in light for 30 min	49.67 \pm 2.50	237.67 \pm 11.00	21.02 \pm 2.01
Intact leaf in light for 4 h	66.17 \pm 4.00	203.33 \pm 10.67	32.64 \pm 3.18
Leaf fed with water in dark for 3 h and exposed to light for 30 min	56.50 \pm 4.00	241.67 \pm 13.67	23.39 \pm 0.35
Leaf fed with CHX in dark for 3 h and exposed to light for 30 min	24.17 \pm 1.17	68.33 \pm 4.67	35.74 \pm 4.26

and V_{lim} activities. The values of Table 1 also show that a protein synthesis step is essential for the activation of V_{lim} activity. Changes in the activation (dephosphorylation) state of SPS may play a central role at least in spinach, in adjusting the rate of sucrose synthesis to the availability of photosynthates, and the

demand for sucrose. This could be a general mechanism when diurnal photosynthesis changes in parallel with changes in PPF. However, during the midday P_N depression, as observed in *P. juliflora*, the rate of sucrose synthesis apparently seems to be controlled by decreasing the amount of SPS protein rather than by the change in activation state. This proposal is based on the observation that the SPS is highly active during the midday P_N depression. Therefore, protein turnover step superimposed on the covalent modification may be an additional mechanism in tree species like *P. juliflora* regulating the sucrose synthesis under natural stress (diurnal and also seasonal).

These results show how a complex diurnal rhythm can be resolved into a balance between two interacting regulatory mechanisms, one of which is related to the CO_2 fixation and the other to the utilization of the photosynthates. Our observations indicate that in *P. juliflora*, the midday decline in P_N and g_s may begin at stomatal level but also has systematic downward regulation by decarboxylation of RuRPO and turnover of the SPS protein. These additional modes of regulation may be required in tree species like *P. juliflora* to survive under extreme environments in different seasons.

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