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Diurnal and seasonal modulation of sucrose phosphate synthase activity in leaves of *Prosopis juliflora*

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Abstract

Diurnal changes of sucrose-phosphate synthase (SPS) activity in different seasons were measured in *Prosopis juliflora* (Swartz) DC. leaves. SPS activity showed large variations with two distinct peaks, one around 09:00 and another at 21:00. The diurnal pattern was apparently not due to circadian rhythm since the activities were directly related to the changes in environmental parameters (irradiance, temperature, and leaf to air vapour pressure deficit) in different seasons. During the day, the enzyme showed changes in kinetic properties, differential sensitivity to allosteric modulators, differential response to ATP concentration, to concentration of endogenous sucrose, and to protein phosphatase inhibitors. These results taken together indicate the modulation of SPS in synchrony with photosynthesis and suggest the existence of multiple levels of modulation, presumably as an adaptive response to changing environmental extremes.

Additional key words: allosteric modulation, photosynthesis, phosphorylation/dephosphorylation, sucrose synthesis.

Introduction

Sucrose and starch are the major products of the carbon assimilation pathway in most plants. Sucrose-phosphate synthase (SPS) plays a key role in the partitioning of carbon into sucrose and starch (Huber and Huber 1996). SPS is itself under a complex regulation mechanism that involves, *1*) a fine control by allosteric effectors like glucose 6-phosphate (G6P, activator) and inorganic phosphate (Pi, inhibitor), and *2*) a coarse control by protein synthesis and covalent modification (Stitt *et al.* 1988, Loewe *et al.* 1996). Further, the enzyme activity is mediated by irradiance, development, senescence, *etc*. The different mechanisms for regulation of SPS activities have been reported in various plant species. In spinach SPS was activated in light by dephosphorylation catalyzed by a SPS protein phosphatase (SPS-PP) (Huber and Huber 1990a, Siegl *et al.* 1990). In dark, on the other hand, spinach SPS was inactivated by phosphorylation catalyzed by a SPS protein kinase (Huber and Huber 1990b). Changes in activation (dephosphorylation) state of SPS could be measured indirectly, by comparing SPS activity at *a*) limiting hexose phosphate concentrations and in presence of Pi (V_{lim} activity), since under these conditions only the dephosphorylated form is active, and *b*) at saturating concentrations of hexose phosphates without Pi (V_{max} activity) which allows the activity of both phosphorylated and dephosphorylated forms to be measured (Krapp and Stitt 1995). Phosphorylation of SPS does not affect apparent maximum activity (V_{max}^{app}) of SPS but enhances the sensitivity of the enzyme to inhibition by Pi and the activator G6P.

Diurnal changes in SPS, have been basically attributed to changes in the irradiance (PPFD), temperature, leaf to air vapour pressure deficit (VPD) and sucrose contents during the day (Stitt *et al.* 1988). However, systematic

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Abbrevations: EDTA - ethylenediaminetetraacetic acid; F6P - fructose-6-phosphate; G6P - glucose-6-phosphate; MOPS - N-morpholinopropanesulfonic acid; PGI - phosphoglucose isomerase; Pi - inorganic phosphate; PMSF - phenylmethylsulfonyl fluoride; P_N - net photosynthetic rate; PPFD - photosynthetic photon flux density; SPS - sucrose-phosphate synthase; SPS-PP - SPS protein phosphatase; S6P - sucrose-6-phosphate; T_l - leaf temperature UDPG - uridine 5'-diphosphoglucose; VPD - leaf to air vapour pressure deficit.

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studies on effects of such parameters on SPS activities are lacking. Most of the earlier work on the diurnal variation in SPS was carried out with the measurement of only V_{max} activity. The measurement of V_{lim} activity is also an important parameter to understand the regulation of the enzyme. Our previous studies on SPS from *Prosopis juliflora,* showed that in autumn the V_{lim} and the V_{max} activity of SPS underwent characteristic changes during midday depression in photosynthesis as an adaptation to high VPD and high PPFD that primarily caused the depression in photosynthesis and stomatal conductance in the leaves (Sinha *et al.* 1997b, Pathre *et al.* 1998). *P. juliflora* is among the trees growing successfully in the northern part of India under seasonal extremes of temperature and humidity and has been one of the major tree species of the Institute′s biomass program at Lucknow. The geographical location of Lucknow is 26° 30' - 27° 10' N, 80° 30' - 81° 13' E, its altitude is 123 m above sea level, with annual rainfall of approximately 500 mm. While studying the ecophy-siology of *P. juliflora* it was observed that the diurnal changes in photosynthetic parameters have a chara-cteristic pattern with respect to the season, as an indicator of the ability of the plant to adjust its photosynthesis to changes in environmental parameters (Pathre *et al.* 1998, Shirke 2001). In the present study, we have determined the diurnal patterns and modulation of SPS activities in leaves of *P. juliflora* during different seasons.

Materials and methods

Plants and sampling: Two-year old *Prosopis juliflora* (Sw.) DC. plants, growing in 10 dm^3 plastic pots on the terrace garden of the Institute under natural conditions throughout the year, were used for all experiments. The plants were watered everyday and were supplied with Hoagland's solution fortnightly. The experiments were conducted from 1997 through 1999. In *P. juliflora* new leaves are produced during February (1st flush) and July (2nd flush) and it takes about one month for the complete development of the leaves. Therefore, taking into account the seasonal pattern for leaf growth, diurnal cycles were studied in four different periods representing typical different seasons in India, March (spring), May (summer), September (monsoon) and December (winter) (Table 1). All measurements were made with fully developed leaves that were 1 to 5 months old.

Table 1. Leaf age and the environmental parameters during sampling for diurnal studies of SPS activities in *P. juliflora*. The values of the environmental parameters are monthly average values determined from 4 to 6 years of periodic observations obtained by weather station (saturating PPFD is considered as > 0.7 mmol $m^{-2}s^{-1}$).

Leaf sampling	Leaf age 1st flush	2nd flush	Temperature [°C]	PPFD [mmol $m^{-2}s^{-1}$]	$PPFD_{sat}$ duration [h]	VPD [kPa]
March	1 month		29.6	1.55	9	2.7
May	3 months		37.0	1.44	10	4.8
September		2 months	34.5	1.66	10	3.1
December		5 months	23.5	1.31	7.5	2.0

Measurement of photosynthesis parameters: Gas exchange measurements were made at every 2 h from dawn to dusk, on clear days using *LI-6200* portable photosynthesis system fitted with a 1 dm^3 chamber (*LI-COR*, Lincoln, USA). To measure the rate of photosynthesis, the leaf was fully exposed and oriented to normal light to ensure the measurements of gas exchange at the highest possible PPFD. The parameters T_l, VPD and PPFD were recorded simultaneously.

Extraction and determination of enzyme activity: Leaves were quickly frozen and stored in liquid nitrogen. For extraction, the frozen leaves were ground with a chilled mortar and pestle in 0.1 M N-morpholino-propanesulfonic acid (MOPS) buffer, pH 7.5 containing 10 mM $MgCl_2$, 1 mM ethylene diamine tetra acetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 25 mM β-mercaptoethanol and 0.02 % (v/v) Triton X-100 in the ratio of 1:3 (m/v). The homogenate was quickly centrifuged at 13 000 *g* for 2 min and the supernatant was immediately desalted on a *Sephadex G-25* column. The column was pre-equilibrated and eluted with the extraction buffer without Triton-X-100. The desalted extract was assayed immediately for SPS activity, determined by measuring the sucrose-6-phosphate (S6P) formed (Huber *et al*. 1989). Unless stated otherwise the V_{max} activity of the enzyme was assayed in a reaction mixture of 0.1 cm^3 final volume, consisting of 50 mM MOPS (pH 7.5), 15 mM $MgCl_2$, 10 mM uridine 5'-diphosphoglucose (UDPG), 4 mM fructose-6-phosphate (F6P) and 20 mM glucose-6-phosphate (G6P) while the V_{lim} activity was also assayed as above except that 10 mM Pi was additionally included

and the concentrations of UDPG, F6P and G6P were lowered to 5, 2 and 10 mM, respectively. It is known that the V_{max} activity is profoundly influenced by the UDPG concentrations (Lunn and Hatch 1997). The effects of varying UDPG concentrations were first determined in pilot experiment and it was observed that 10 mM UDPG is saturating concentration under our assay conditions (data not shown). Hence for all V_{max} assays, this concentration was used. The reactions were initiated by the addition of 0.05 cm^3 enzyme extract and incubated at 25 °C. The assays were terminated at 0 and 20 min with 30 % KOH and unreacted hexose phosphates were destroyed by placing the tubes in boiling water for 10 min. After cooling, 1.0 cm^3 of 0.14 % (m/v) anthrone in 13.8 M H_2SO_4 was added and the tubes were incubated at 40 °C for 20 min prior to measuring the absorbance at 620 nm. For kinetic analysis the reactions were carried out in an assay containing 50 mM MOPS (pH 7.5), 15 mM $MgCl_2$, and the other additions as described in the figure legend.

Determination of *in vivo* effects of metabolites and inhibitors: To investigate the *in vivo* effect of inhibitors or metabolites, the leaf at a given time was excised under water and the petiole of the leaf was immersed into the inhibitor or metabolite solution in a Petri dish. For control, the leaf was fed with distilled water under similar conditions. The leaf was left in dark at room temperature for 3 h to allow uptake of the solution. After 3 h, the leaf was analysed for the enzyme activity either immediately or after 30 min exposure to sunlight [≈1 mmol m^{-2} s^{-1}].

Determination of soluble sugars, starch and chlorophyll contents: The frozen leaf samples were ground in 1 M $HClO_4$ (usually 1.0 cm^3 for 10cm^2 leaf area) and centrifuged. The supernatant was used to determine sucrose content by the method of Jones *et al.* (1977). The pellet, which contained starch, was washed and suspended in 0.2 cm^3 of 0.2 M MES buffer (pH 4.5). The suspension was treated with 14 units of amylo-glucosidase and 0.4 units amylase at 55 °C for 2 h. The reaction mixture was centrifuged and glucose in the supernatant was determined as above. Chlorophyll and phaeophytin were extracted with acetone and content in the samples was determined according to the method described by Coombs *et al.* (1985) and Vernon (1960), respectively.

Results

The diurnal changes in SPS activity and photosynthesis parameters were followed throughout the period of study, beginning from March (spring) with the newly developed leaves and ending in December (winter) (Fig. 1) with the leaves produced in July (also see Table 1). In spring photosynthesis rate in *P. juliflora* leaves showed parallel response to PPFD, irrespective to the changes in VPD and temperature (Fig. 1*A*,*B*). The rate was more or less constant throughout the photoperiod. The V_{max} and V_{lim} activity also increased gradually with increase in PPFD and peaked around 10:00 and then decreased continuously and reached to minimum around 18:00. The second peak of SPS activities, though not prominent appeared at 21:00 (Fig. 1*B*). Summer in Lucknow is usually very hot and dry with the leaf temperatures greater than 40 °C around midday and VPD greater than 7.5 KPa (Fig. 1*C*). The maximum PPFD attained in May was 1.7 mmol m^{-2} s^{-1} and was lower than that in March. The typical pattern of P_N during May showed an early peak around 08:00 and then declined sharply till midday and remained constant to the new low value till 16:00 (Fig. 1*D*). In May the V_{max} activities of SPS (Fig. 1*D*) were low, almost 50 % of that of March (Fig. 1*B*) with two distinct peaks, one at 08:00 parallel to the P_N peak and the second peak at 21:00.

In monsoon, which begins in July, the plants were relieved from temperature and VPD stress (Fig. 1*E*) of the summer season and also produce a second flush of new leaves. These new leaves showed almost constant rates of P_N during most of the photoperiod (Fig. 1*F*), a response similar to that observed in spring season but the overall rate of photosynthesis was 50 % higher than that in spring. SPS activity showed response parallel to P_N showed peak at 8:00 during the photoperiod (Fig. 1*F*). The second peak of V_{max} activity appeared at 23:00 showed slight shift as compared to other seasons.

In winter the diurnal changes in P_N were more or less parallel to the PPFD with the maximum rate at around 10:00 (Fig. 1*H*). The temperatures ranged from 10 to 30 °C with low VPD (Fig. 1*G*). During diurnal cycle the V_{max} and the V_{lim} activity of enzyme increased sharply with increase in PPFD during morning hours. Both the activities reached a maximum around 10:00 and remained fairly constant throughout the photoperiod, though photosynthesis rate decreased gradually after 10:00. After the sunset both the activities started rising again giving a second peak (Fig. 1*H*). The rise in the V_{max} activity in the second peak was more than three fold with respect to the predawn value, while V_{lim} activity was same as that observed in the morning peak.

The variation in SPS activity observed may be attributed to circadian rhythms too. In order to establish that the diurnal changes in SPS activity observed during photoperiod are not due to any endogenous rhythm, the

Prosopis plants were subjected to a darkness period extended by 3 to 16 h. At different times plants were taken from darkness, exposed to saturating light [≈ 1 mmol m^{-2} s^{-1}] for 30 min to attain the steady state P_N. It was observed that all the plants, irrespective to the period of extended darkness, showed almost same P_N [≈ 10 μmol m^{-2} s^{-1}] (data not shown).

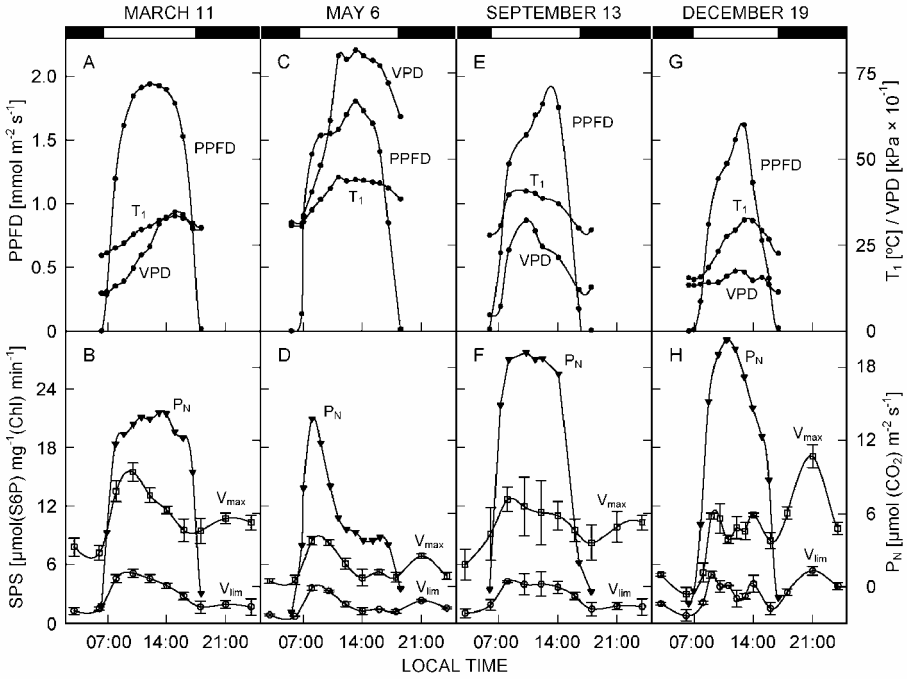

Fig. 1. Daily patterns of PPFD, T_l ,VPD; P_N (*closed triagles*) and SPS activities, V_{lim} (*open circles*) and V_{max} (*open squares*) in leaves of *P. juliflora*, measured during, March (*A*,*B*), May (*C*,*D*), September (*E*,*F*), and December (*G*,*H*). The data for photosynthesis rate and environmental parameters are the representative of at least four measurements and generally did not vary by more than 3 %, while the data for the enzyme activity are means ± SD of four different leaves.

Earlier an increase in V_{lim} activity during light activation of *Prosopis* SPS has been shown to be due to the dephosphorylation of enzyme on the basis of studies carried out with okadaic acid, an inhibitor of type 1 or 2A protein phosphatase (Pathre *et al.* 2000). During diurnal cycle, the activation of V_{lim} activity was observed in both morning and night leaf samples in different seasons. To investigate whether the appearance of two peaks of SPS were due to dephosphorylation of the enzyme, the leaves of *P. juliflora* were fed at different times with 10 nM okadaic acid. The leaves were excised under water at 05:30 and at 17:30 and fed with 10 nM okadaic acid and water (as control) in dark for 3 hours. The leaves excised at 05:30 and treated with okadaic acid were exposed to light [≈ 1 mmol m^{-2} s^{-1}] for 30 min and both V_{max} and V_{lim} activities were determined. The data in Table 2 show that the V_{lim} activity in day leaf samples was inhibited while that from the night samples was not affected. In controls (samples fed with water) the V_{lim} activity increased in a manner same as that observed in intact leaves during diurnal measurements. The inhibition of the V_{lim} activity in morning samples indicates that the light activation of SPS observed after sunrise may be due to the dephosphorylation of the enzyme, but the activation of V_{lim} activity observed in the night is apparently not due to the dephosphorylation of the enzyme. The V_{max} activity in all the samples remained unaffected by the treatment of okadaic acid (Table 2).

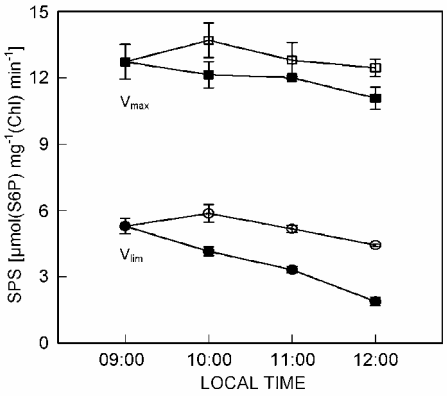

Fig. 2. Reversal of light activation of V_{lim} activity by okadaic acid. The leaves of *P. juliflora* were cut under water and fed with 10 nm okadaic acid (*closed points*) or water (*open points*) at 09:00 in saturating light and the samples were harvested in liquid nitrogen at every hour till 12:00 and assayed for V_{lim} (*circles*) and V_{max} (*squares*) activities. The results are the means ± SE of four extracts from different plants.

Table 2. Effect of okadaic acid on day and night peak activities of SPS [μmol(S6P) mg^{-1}(Chl) min^{-1}]. Excised leaves of *P. juliflora*, were fed with either 10 nM okadaic acid or water (as control) for 3 h in dark. The SPS activities were determined after 30 min exposure to saturating light in the 09:00 samples and after quenching in liquid nitrogen directly after feeding treatment for the 21:00 samples.

Time	Feeding	V_{lim}	V_{max}
05:30	before	1.50 ± 0.18	7.45 ± 0.34
09:00	water	4.82 ± 0.16	12.51 ± 0.44
09:00	okadaic acid	2.20 ± 0.27	12.12 ± 0.44
17:30	before	1.33 ± 0.11	6.13 ± 0.29
21:00	water	3.44 ± 0.07	10.77 ± 0.47
21:00	okadaic acid	3.03 ± 0.18	10.22 ± 0.50

To investigate further whether continuous dephosphorylation is required to maintain the high V_{lim} activity observed in the morning, the leaves were excised under water at 09:00 and fed with water (control) and 10 nM okadaic acid for next 3 h in light [≈ 1 mmol m^{-1} s^{-1}]. After three hours an almost 50 % decrease in the V_{lim} activity was observed in the samples fed with okadaic acid relative to that in control samples while the V_{max} activity remained relatively constant in both control and okadaic acid treated samples (Fig. 2).

The changes in phosphorylation status of SPS in *P. juliflora* during the day was determined indirectly by incubating the enzyme isolated at 09:00 and at 21:00 with increasing concentrations of ATP and monitoring the changes in V_{lim} activity (Fig. 3). ATP inhibited the enzyme extracted at 09:00 to a greater extent (50 %) than that of enzyme extracted at 21:00 (25 %). The difference in inhibition clearly indicates that the enzyme in the leaves harvested at 21:00 appears to be more phosphorylated than the day enzyme.

Diurnal profiles of SPS activity in *P. juliflora* showed that both V_{max} and V_{lim} activities were low in predawn samples. Both the activities increased several folds after sunrise in all the seasons. To determine the changes in kinetic properties of SPS on light activation the enzyme was isolated at 03:00 and 09:00. The study of saturation kinetics of SPS in crude extracts, though complicated due to the presence of phosphoglucose isomerase (PGI) can still be carried out, if the concentration of F6P and G6P used was in a 1:5 ratio (Stitt *et al.* 1988). In our study also, we used F6P and G6P in a ratio of 1:5. The desalted preparation of the enzyme showed hyperbolic saturation curves for UDPG and F6P (Fig. 4*A*,*B*). The higher activity at 09:00 appeared to be due to decrease in K_m from 12.98 mM (predawn) to 6.7 mM and increase in V_{max}^{app} for UDPG. The K_m for F6P was not affected by light activation. It has been observed that SPS in *P. juliflora* is activated by G6P and inhibited by Pi in both desalted as well as partially purified extracts (Sinha *et al*. 1997a). It is possible that these metabolites may be involved in the activation/de-activation of the enzyme observed predawn or at 09:00. To investigate the effects of these metabolites, SPS isolated predawn and at 09:00, was treated with increasing concentration of Pi. Differential inhibition was shown indicating that the predawn enzyme was more susceptible to Pi as compared to that at 09:00 (Fig. 4*C*). The activation by G6P was, however, found to be similar in both the types of enzyme (data not shown). At 10 mM Pi, the K_m for UDPG of the enzyme isolated predawn and at 09:00 increased to 26 mM and 8.69 mM from 12.96 mM and 6.7 mM, respectively. The V_{max} of the enzyme was, however, not affected (inset of Fig. 4*C*). It was also observed that Pi did not have any effect on the K_m for F6P.

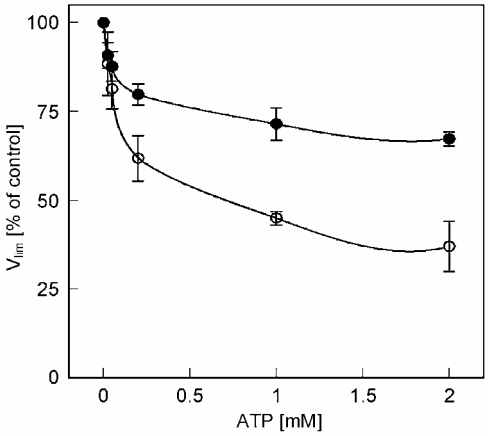

Fig. 3. ATP dependent inactivation of SPS in *P. juliflora* leaves harvested at 09:00 (*open circles*) and at 21:00 (*closed circles*) and incubated with increasing concentrations of ATP at 25 °C prior to the assay of V_{lim} activity. The results are the mean ± SE of four leaves.

Our data on diurnal measurements show that, after sunrise SPS activity increased with an increase in P_N. The assimilated carbon by P_N leads to an accumulation of sucrose and starch. The typical pattern in September showed that both sucrose and starch levels increased in the morning and reached a maximum around 08:00 and then sucrose content decreased gradually, while starch levels continued to increase till 12:00 and remained high till sunset. After sunset the content of starch decreased continuously while that of sucrose either increased or remained constant depending on the season. The accumulation and decline in sucrose content, was observed to follow closely the activation state (V_{lim}/V_{max}) of SPS (Fig. 5). To elucidate the role of sucrose, *Prosopis* leaves were cut predawn under water and fed with 25 mM sucrose solution in dark for 3 h and then harvested in liquid nitrogen in the dark or after subjecting for 30 min to saturating light. The V_{lim} activity in control leaves (fed

Table 3. Alteration of SPS activity [µmol(S6P) mg^{-1}(Chl) min^{-1}] by feeding metabolites to leaves *of P. juliflora*. The leaves of *P. juliflora* were cut under water at 05:00 (predawn) and fed with 25 mM sucrose, 10mM Pi or 25 mM mannose for 3.5 h in dark or 3 h in dark and exposed to saturating light for 30 min. The leaves cut and fed with distilled water under similar conditions of dark and light served as control. After 3.5 h the enzyme was extracted and assayed under V_{max} and V_{lim} conditions as mentioned in material and methods after desalting. The results are the mean ± SE obtained from four different leaves.

		Control	Sucrose	Pi	Mannose
V_{lim}	dark	1.23 ± 0.13	1.15 ± 0.23	1.14 ± 0.14	3.58 ± 0.30
	light	5.94 ± 0.51	2.07 ± 0.28	2.59 ± 0.08	8.76 ± 0.43
V_{max}	dark	6.57 ± 0.44	7.77 ± 0.36	8.46 ± 0.98	9.01 ± 0.57
	light	12.89 ± 1.01	9.14 ± 0.80	10.27 ±0.56	15.87 ± 0.29

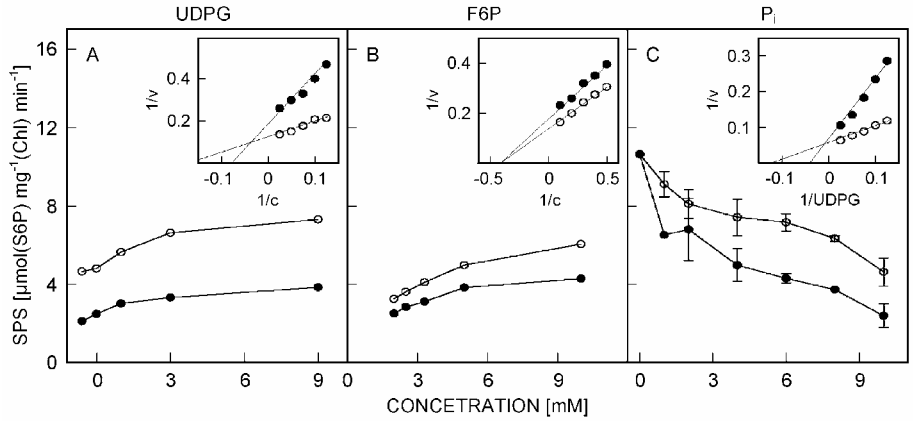

Fig. 4. Substrate saturation kinetics for UDPG and F6P of SPS in *P. juliflora*. The leaves were harvested at 03:00 (*closed circles*) and at 09:00 (*open circles*) and SPS activity was measured in desalted extracts. *A* - the activity of SPS was measured with 4 mM F6P, 20 mM G6P and 5 - 40 mM UDPG in the reaction mixture; *B* - with 10 mM UDPG and 1 - 10 mM F6P added with G6P in the ratio of 1:5 in the reaction mixture, the insets show the respective Lineweaver-Burk plots of the substrate saturation curves; *C* - effect of Pi inhibition on SPS activity (SPS assayed in absence of G6P was considered as 100 % activity) and the data plotted are the representative of four different sets of experiments; the inset shows the Lineweaver-Burk plot for UDPG in presence of Pi.

with water) increased almost 5 fold while that in sucrose fed leaves showed only a marginal increase (68 %). The V_{max} activity appears to be less affected by sucrose but still had much less activity as compared to that in the control leaves (Table 3). The increase in P_N after sunrise also reportedly changes the concentration of Pi at sub-cellular level as Pi is taken up in chloroplast and triose phosphates are exported to the cytosol (Gerhardt *et al.* 1987). To investigate the effect of changing cytosolic concentration of Pi on SPS activity, the predawn leaves were fed with 10 mM Pi or 25 mM mannose solution. When Pi was supplied to the leaves, it substantially decreased both the activities in light though the inhibition in V_{lim} activity was higher than that in V_{max} activity indicating that the increase in Pi concentration may have affected the phosphorylation status of the enzyme (Table 3). Mannose (used for sequestering of Pi), when fed to the predawn leaves increased the V_{lim} activity drastically even in dark (3 fold) and more than seven fold in light. The V_{max} activities also increased considerably in mannose fed leaves as compared to that in the control leaves.

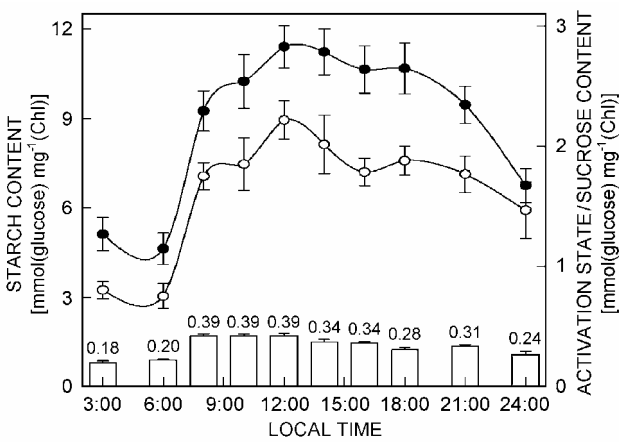

Fig. 5. Diurnal changes in starch (*closed circles*), and sucrose (*open circles*) content and activation state (V_{lim}/V_{max}) of SPS (represented by *bars*) of *P. juliflora* leaves in September. The results are the means ± SE of four leaves.

Discussion

The results of the present investigation show that *P. juliflora* exhibits prominent diurnal changes in SPS activity in different seasons. The diurnal variations in the activity of SPS were, however, not due to any intrinsic or circadian rhythms but due to the transient changes in environmental conditions. Further, the changes observed are complementary to the changes in the rate of P_N. Although in Fig. 1 we have presented the diurnal observations of a single day in a given season, the repeatability of the pattern was checked several times during the study period. Similarly, *in vivo* experiments described in the paper were also repeated in different months randomly, however, the data is shown for only a particular set of experiments. In general, the activity of *Prosopis* SPS was highest in winter and lowest in summer. Further, SPS activity was low predawn and high in the morning and night in all the seasons. The morning peak activity was observed around 09:00 when PPFD and P_N reached maximum, while the night peak occurred around 21:00. Though peak SPS activity initially paralleled the P_N maxima, the activity did not show a similar parallel response in the subsequent hours. For example, in spring and in monsoon, the P_N remained constant from 08:00 to 15:00 but SPS activity declined gradually after 09:00. This shows that the activity pattern is not solely governed by photosynthesis.

The diurnal changes observed in *Prosopis* SPS can be attributed to extrinsic factors like changes in irradiance, temperature and VPD as well as intrinsic factors like the concentration of metabolites and complex regulatory properties of the enzyme. The measurement of diurnal changes in SPS activities reported earlier have been made with the view that irradiance may be the most important factor for the activation of the enzyme, and thus most of the data on diurnal changes of SPS activities was generated under greenhouse or growth chamber conditions and not under natural conditions as has been done in the present study. Nevertheless our data showed some comparisons with the available information in case of the other plants. In plants such as spinach (Stitt *et al.* 1988, Huber *et al.* 1992), tobacco (Huber *et al.* 1984), cotton (Hendrix and Huber 1986), eelgrass (Zimmerman *et al.* 1995), rice (Seneweera *et al.* 1995) and tomato (Jones and Ort 1997), there are reports of only V_{lim} activity undergoing diurnal changes while V_{max} remained constant throughout the day. In certain other plants like barley and maize diurnal fluctuations in V_{max} activity were also observed along with that in V_{lim} activity (Sicher and Kremer 1984, Bruneau *et al.* 1991, Lunn and Hatch 1997). The activation of both V_{max} and V_{lim} activity and sensitivity to Pi suggest that regulation of *Prosopis* SPS may be similar to group I plants like barley, in which SPS activation is sensitive to irradiance and Pi. Huber *et al*. (1985) has observed that the diurnal changes in SPS activity in pea resulted in two peaks, one in the morning and another at night as observed in *P. juliflora*, while in soybean SPS, two peaks of activity, one broad peak early in the dark period and the other at the end of the dark period were observed. Further, it was also shown that soybean SPS has weak allosteric regulation (Nielsen and Huber 1989). The data available for seasonal changes in SPS activity are only for spruce needles, where high SPS activity was reported before the bud break due to changes in the amounts of SPS protein (Loewe *et al.* 1996).

Diurnal measurements of sucrose accumulation indicate that the gradual decline of SPS during photoperiod and the recovery during night closely followed the accumulation and decline of sucrose. It is thus possible that the SPS activity was balanced during photosynthesis to maintain the concentration of sucrose at threshold level and whatever excess carbon was fixed; it was diverted for the synthesis of starch. If so, the higher activities of SPS may be required during night for the synthesis of sucrose from starch. The accumulation of sucrose increased Pi sensitivity of SPS (Stitt *et al.* 1988) and since the phosphorylated form of SPS is more sensitive to Pi, it could be inferred that the accumulation of sucrose somehow maintained the enzyme in the phosphorylated form. Further the light activation of SPS is caused due to the dephosphorylation of the enzyme by protein phosphatase, the mechanism primarily prevalent in several species (Huber and Huber 1996), also indicates that the accumulation of sucrose may also have a role in the regulation of dephosphorylation of SPS. The feeding of sucrose also decreased the V_{max} activity in *Prosopis* leaves (Table 3). The V_{max} activity of SPS was shown to represent the amount of SPS protein, indicating that the turnover of the enzyme was perturbed by the changing cytosolic concentration of sucrose. In sugar beet leaves, exogenously supplied sucrose represses the expression of SPS gene (Hesse *et al*. 1995).

The feeding of Pi to the leaf can affect SPS enzyme at different levels. First, the increased concentration of Pi may inhibit the enzyme directly by an allosteric inhibition effect. Secondly, Pi could increase the phosphorylation of SPS that makes the enzyme sensitive to Pi inhibition. Third, Pi is known to inhibit the protein phosphatases (Weiner *et al*. 1993) and thus prevent the activation of the enzyme by dephosphorylation in light. Therefore, when leaf was fed with mannose (Pi sequestering reagent), the reduced concentration of Pi resulted in the activation of SPS (Table 3). However, the reasons for increase in V_{max} in mannose fed leaves are not clear.

The mechanism of covalent modification appeared to play a key role during diurnal and seasonal changes of the enzyme. At a time prior to dawn, most of the enzyme remained in the phosphorylated form (low V_{lim} activity) and onset of light, activated the enzyme as observed from

an increase in V_{lim} activity. This increased activation was shown to be due to dephosphorylation of the enzyme. The V_{lim} also increased around 21:00 though the extent of increase varied in different seasons. V_{lim} activity peak at night was much less inhibited as compared to the morning peak, when treated with ATP, indicating different phosphorylation status of the enzyme. The *in vivo* treatment of okadaic acid at 09:00 in light resulted in a rapid decrease of the V_{lim} activity indicating, continuous dephosphorylation is required for the maintenance of high activation state of the enzyme. Further feeding of okadaic acid at different times showed that the V_{lim} activity when measured during morning was inhibited substantially while that measured at night remained unaffected. This differential response indicates that the activation of the V_{lim} activity observed in the morning may be due to the dephosphorylation by type 1 or 2A protein phosphatase. However, the activation of V_{lim} in the night could not be attributed to okadaic acid sensitive dephosphorylation, as okadaic acid had no effect on the activation of the enzyme. Hence it may be predicted that either protein phosphatases other than PP1 or PP2A types and which are not affected be ruled out and needs further attention. Jones and Ort (1997) have envisaged that the circadian regulation of protein phosphatase gene transcription controls the timing of SPS phosphatase activity, which in turn determines the activation of SPS, in case of tomato.

SPS in *P. juliflora* is unstable and is present in very low quantities (Sinha *et al.* 1997a). Therefore, the kinetic properties were studied using desalted extract. In fact the low intra-cellular concentrations and labile nature of SPS enzyme has been the major hurdle in the study of this enzyme in highly purified state. Other workers have also used partially purified or even crude extracts for the study of SPS (Loewe *et al.* 1996, Lunn and Hatch 1997). Like spinach and maize, SPS in *P. juliflora* also showed changes in kinetic properties during diurnal cycle. The kinetic analysis also showed that the increase in V_{max} value during light activation of *Prosopis* SPS (Fig. 4). In addition to changes in V_{max} light activation also reduces the K_m for only one of the substrate, UDPG, unlike in spinach where the light affects the K_m for F6P but not for UDPG. The kinetics of allosteric regulation in SPS is different among various species (Huber *et al.* 1989). In maize and spruce needles the inhibition of SPS by Pi was observed only after G6P activation, while SPS in spinach (Doehlert and Huber 1983) and potato (Reimholz *et al.* 1994) is inhibited by Pi even in absence of G6P. The regulation by Pi appears to play a prominent role during diurnal changes in SPS activity in *P. juliflora* as light activated SPS showed substantial changes in the allosteric properties. The enzyme isolated after sunrise showed less sensitivity to Pi inhibition than that of the enzyme isolated from predawn leaves.

The *Prosopis* SPS have multiple levels of regulation for its temporal activities. In the present paper we show that some of the intrinsic metabolites, possible phosphorylation and dephosphorylation events and environmental factors all contribute to regulation of SPS activities. Diurnal SPS activity variations in different plants also reflect the differences in the regulatory properties of the enzyme. Further, since in most of the species studied so far, SPS appears to be primarily regulated via the mechanism of protein phosphorylation/ dephosphorylation, effected through protein kinases and protein phosphatases, it would also be interesting to study the regulation of these enzymes (kinases and phosphatases) under changing environmental conditions.

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