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Interactions between light and temperature during the *in vitro* as well as *in situ* modulation of phosphoenolpyruvate carboxylase in leaves of a C_3 plant *Pisum sativum L.*

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ABSTRACT

The C_4 phosphoenolpyruvate carboxylase (PEPC) is well known for its modulation by light and temperature. We have attempted to address such coordinated effects of light and temperature on C_3 PEPC in leaves of *Pisum sativum*. Experiments were done with dark-adapted leaf discs as well as with leaves collected at different times during the day and in each month during the year. The modulation of C_3 PEPC by temperature was much stronger than that by light. The optimum temperature for *in vitro* activity of C_3 PEPC was 25°C while 20°C was sub-optimal and 30°C to 45°C were supra-optimal. The photo-activation of PEPC was more at 25°C than at 45°C. The activation of C_3 PEPC by temperature was more in the dark than in the light. In *in situ* conditions, a diurnal rhythm in the activity and regulatory properties of C_3 PEPC was more prominent at colder than at warm temperatures. However, no consistent trends in the seasonal changes in PEPC activity/ regulatory properties were observed. Scatter plots suggested that light had a greater influence on PEPC activity while temperature exerted a much greater effect on its regulatory properties. Thus, C_3 PEPC was also modulated by a natural variation in light and temperature under *in situ* conditions. These observations correlated well with the light and temperature requirements of C_3 plants.

KEYWORDS

C_3 PEPC; Light; Temperature; Diurnal rhythm; Glucose-6-phosphate; Malate

ARTICLE HISTORY

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Introduction

Phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) is a key enzyme involved in primary carbon fixation in the leaves of C_4 and Crassulacean Acid Metabolism (CAM) plants. In C_3 plants, the enzyme is responsible for housekeeping, by providing carbon skeletons for amino acid and protein synthesis. Unlike C_4 plants, C_3 plants do not have a CO_2 concentrating mechanism (the C_4 cycle), which enables them to function optimally under conditions of high light intensities and warm temperatures, which promote photorespiration. Hence, C_3 plants differ from C_4 plants in their optimal light and temperature requirements [1,2]. Parallelly the responses of PEPC from C_3 plants to light and/or temperature may also vary from those of C_4 PEPC.

There have been only a few studies made on the effects of light on the activity and regulatory properties of C_3 PEPC. Unlike C_4 PEPC, C_3 isoform is not activated by light [3,4]. However, Rajagopalan et al. have reported that PEPC from C_3 species is activated by up to 1.6-fold upon exposure to light but with no significant changes in either sensitivity to malate or activation by Glu-6-P [5]. In contrast, PEPC from wheat leaves showed up to a 3.8-fold increase in activity due to protein phosphorylation [6] and also showed a decrease in malate sensitivity upon illumination [7]. Although to a lesser extent when compared to C_4 plants, light enhances PEPC activity in various C_3 plants with a concomitant decrease in malate sensitivity and an enhancement in activation by Glu-6-P [8,9].

Illumination of protoplasts obtained from barley leaves led to an increase in PEPC activity [10] along with a reduction in malate sensitivity [11] and a reduction in $K_{0.5}$ (PEP) but with no accompanying changes in V_{max} [12].

In comparison to the reports on the responses of C_3 PEPC to light, studies on the effects of varying temperatures are very limited. Chinthapalli et al. have reported C_3 PEPC to be more active at 30°C than at lower or higher temperatures and that the sensitivity of the enzyme to malate decreases with an increase in temperature [13]. Thus, there is a need for a comprehensive study on the effects of light as well as temperature on the activity and regulatory properties of C_3 PEPC. Further, there are no studies on the interactive influence of light and temperature on C_3 PEPC, either under in vitro or in situ conditions. It is not clear if the activity/regulatory properties of C_3 PEPC follow any diurnal rhythm. There have been two reports regarding the effect of N nutrition on diurnal rhythms of C_3 PEPC. The PEP carboxylation rates in nitrate grown, pea plants showed a diurnal rhythm but without any concomitant diurnal changes in PEPC activity/regulatory properties/protein levels, which may be due to direct allosteric effects but not due to changes in protein phosphorylation levels [14]. In leaves of N-sufficient tobacco plants, PEPC transcript levels were higher at night than during the day, and the PEPC activity was highest during the morning, whereas in N-limited plants, both the mRNA and activity levels remained low during the day [15]. Thus, further studies on the possible occurrence of a diurnal and seasonal rhythm in C_3 PEPC are also required.

The characteristics of C_3 PEPC were assessed in extracts (*in vitro*) prepared either from leaf discs exposed to temperature and/or light treatments or from leaves collected from the field (*in situ*) at the required time of the day. Experiments were performed in different months of the year

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2009 to examine critically whether there are any significant diurnal and seasonal variations in the properties of PEPC in the leaves of *P. sativum*, a C_3 plant. Our university campus, being located in a typical, subtropical environment, offers a good choice, with significant fluctuations in the intensity of incident light as well as temperature during the day and during the year. The physiological/biochemical properties of C_3 PEPC were studied, followed by the molecular analysis of protein levels, phosphorylation status, and mRNA levels.

Materials and Methods

Plant material and growth conditions

Plants of *Pisum sativum* L. (cv. Arkel) were raised from seeds purchased from Pocha Seeds Co. Ltd., Pune, or Maharashtra Hybrid Seed Co., Mumbai. The seeds were soaked overnight in water, surface sterilized with 0.2% (v/v) sodium hypochlorite solution for 15 min, and then washed for 1 hr under running tap water. The seeds were kept covered in a moist black cloth at 25°C until they germinated (usually 3 days). The germinating seeds were then sown in plastic trays filled with soil and farmyard manure (3:1, v/v) and were watered twice daily. The plants were grown in a greenhouse at average temperatures of 33°C day/25°C night and a natural photoperiod of approximately 12 hr for *in vitro* or outdoors for *in situ* studies as required.

Harvest of leaf tissue

The second and third fully expanded leaves were picked from 8-15-day old plants. The leaves were harvested about 2 to 3 hr after sunrise for *in vitro* studies, and leaf discs were punched as described below. For *in situ* studies, the leaf samples were harvested at the time needed, immediately frozen, and stored in liquid nitrogen until used for the preparation of total protein extract for PEPC enzyme assays.

Temperature and/or light treatments

Discs of *ca*. 0.2 cm^2 were punched from leaves underwater with the help of a sharp paper punch. Leaf discs (each of ca. 0.2 cm^2; total weight approximately 125mg) were kept in 25mL beakers containing distilled water and left in darkness for 2 hr. The dark-adapted leaf discs were incubated for the required time period and temperature in thermostatically controlled circulatory water baths. When required, illumination as white light at an intensity of 1000 $\mu molm^{-2}s^{-1}$ was supplied simultaneously by arranging four Philips Comptalux flood bulbs (150W each). The light was allowed to pass through a 10cm thick water filter, which helped to dissipate the heat and to maintain an optimal temperature during illumination. At the end of each incubation period, the leaf discs were quickly extracted, and the extract was assayed for PEPC activity as described next.

Extraction and assay of PEPC

The extraction and assay of PEPC were as already described [13,16]. The leaf discs or frozen leaf material was quickly extracted using a chilled mortar and pestle in ice, i.e., at 4°C with 1 mL of extraction medium containing 100mM Tris-HCl (pH ± 7.3), 10mM $MgCl_2$, 2mM K_2HPO_4, 1mM EDTA, 10% (v/v) glycerol, 10mM β-mercaptoethanol, 10mM NaF, 2mM PMSF, $10\mu gmL^{-1}$ chymostatin and 2% (w/v) insoluble PVP. The homogenate was centrifuged at 15,000g for 5min, and the supernatant was used as a "crude extract". A small aliquot was kept aside for chlorophyll estimation prior to centrifugation.

Maximum PEPC activity was assayed by coupling to NAD-MDH and monitoring NADH oxidation at 340nm in a Shimadzu 1601 UV-visible spectrophotometer at 30°C (irrespective of the preincubation temperature of the leaf discs or leaves). The assay mixture (1mL) contained 50mM Tris-HCl (pH 7.3), 5mM $MgCl_2$, 0.2mM NADH, 2 U MDH, 2.5mM PEP, 10mM $NaHCO_3$ and leaf extract equivalent to 1µg of chlorophyll. The sensitivity of PEPC to malate was checked by adding malate to a final concentration 2mM/mL of assay mixture. Activation by Glu-6-P was similarly checked by adding Glu-6-P to a final concentration of 2mM/mL of assay mixture; 0.5mM PEP and 0.05mM $NaHCO_3$ were used.

Estimation of chlorophyll and protein

Chlorophyll was estimated by extraction with 80% (v/v) acetone [17]. Total protein content was estimated by the method of Lowry et al. by using bovine serum albumin (BSA) as a standard [18].

Replications and statistical analysis

All *in vitro* experiments were repeated 3 to 5 times on different days. All *in situ* assays were performed three times for each sample. The average values ± SE are presented. Statistical analysis of the data was done using the software Sigma plot (version 11.0).

Results

Changes in activity levels of C_3 PEPC in leaf discs of *P. sativum* upon incubation at different temperature

A remarkable dependence of PEPC activity on temperature was observed when leaf discs of *P. sativum* were incubated at varying temperatures for 45min (Figure 1A and 1B). The optimum temperature for *in vitro* activity of C_3 PEPC was 25°C. The activity decreased slightly upon incubation at 20°C (sub-optimal) but was much less when compared to the reduction upon exposure to supra-optimal temperatures (30°C to 45°C). Thus, C_3 PEPC was most active at moderate temperatures, less sensitive to cold temperatures, and most sensitive to warm temperatures. For further studies about the interactive effects of light and temperature on C_3 PEPC, the temperatures of 30°C were taken as the pre-incubation control, 25°C as the optimal, 20°C as the sub-optimal, and 40°C as the supra-optimal for incubation of leaf discs. The results are described in the next section.

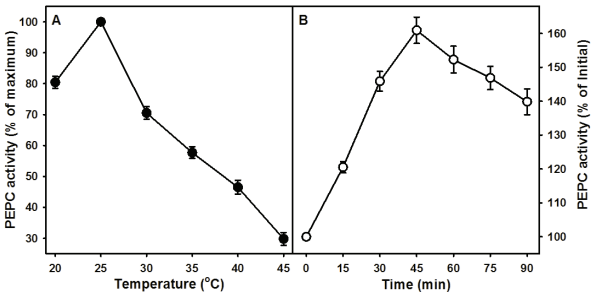

Figure 1. The activity of C_3 PEPC in extracts from leaf discs of *P. sativum* upon incubation at different temperatures (A) or under illumination (B). The changes caused by temperature or light are all statistically significant ($P<0.01$). Standard errors if not seen are within the symbols.

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Effect of light and temperature on the activity of C_3 PEPC in leaf discs of *P. sativum*

Incubation of leaf discs at 25°C led to a dramatic increase in C_3 PEPC activity with time. A 1.6-fold increase in C_3 PEPC activity over initial was observed at 25°C at an optimal incubation time of 45 min. This activation was however only as high as 1.4-fold at 20°C, but decreased by 0.6-fold at 40°C (Figure 2). Thus, the C_3 PEPC from *P. sativum* is optimally active at the moderate temperature of 25°C than at either 20°C or at 40°C.

The PEPC activity was observed to increase by up to 2.0-fold at 25°C upon 45 min of illumination. This activation by light was however comparatively lower, i.e., 1.6-fold at 20°C and 1.1-fold at 40°C (Figure 2). The L/D ratio was higher (1.5) at 25°C than at 20°C (1.1), suggesting that light activation is more at 25°C than at 20°C (Table 1). However, the higher L/D ratio at 40°C (1.9) could be attributed to very low PEPC activity in the dark at this temperature. Thus, the light activation of C_3 PEPC was being modulated as a factor of temperature. The 25°C/40°C and 20°C/40°C ratios were higher in the dark than in light, suggesting that the activation of C_3 PEPC by temperature was more in the dark than in light. The 25°C /40°C and 20°C /40°C ratios were higher when compared to the L/D ratios, suggesting a greater effect of temperature than light in modulating C_3 PEPC.

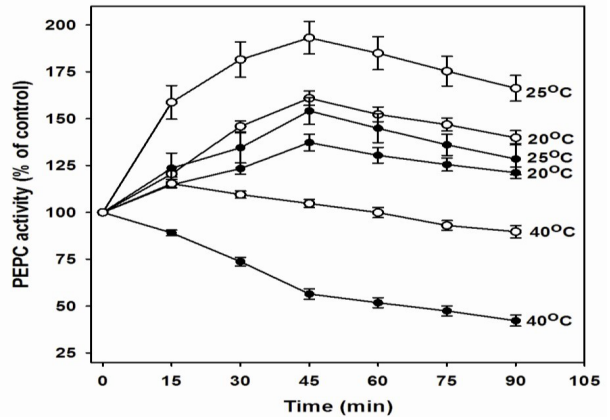

Figure 2. Effect of temperature on the activity of C_3 PEPC in extracts from dark-adapted (●) and light adapted (○) leaf discs of *P. sativum*. The changes caused by temperature are all statistically significant (P<0.01). Standard errors if not visible are within the symbols.

Table 1. Modulation of C_3 PEPC by effectors in relation to light and/or temperature treatments in leaf discs of *P. sativum in vitro.*

Modulator	Activation by Light (L/D Ratio)			Stimulation by temperature			
				(25/40 °C)		(20/40 °C)	
	25 °C	20 °C	40 °C	Dark	Light	Dark	Light
Control* (No Effector)	1.5	1.1	1.9	2.8	1.8	2.4	1.5
Malate* (2mM)	1.6	1.5	2.3	4.9	3.4	3.6	2.3
Glu-6-P** (2mM)	1.5	1.3	2.1	4.3	3.1	3.3	2

The average values of 3-5 replications are shown. The values obtained were all statistically significant (P<0.01).

*Assayed at 2.5mM PEP; **Assayed at 0.5mM PEP.

Effect of light and temperature on the regulatory properties of C_3 PEPC in leaf discs of *P. sativum*

Experiments were done to study the responses of C_3 PEPC to malate and Glu-6-P under the influence of light and temperature. The results are summarized in Table 2. The sensitivity of PEPC to 2 mM malate decreased from 55% to 40% when incubated at 25°C. However, this relief in inhibition by malate was much less, from 55% to 48% at 20°C, than at 25°C. In contrast, at 40°C, the malate sensitivity increased from 55% to 65%. Thus, malate inhibition was less at 25°C than at either 20°C or 40°C. Upon illumination, the malate sensitivity decreased further to a low of 20% at 25°C but only up to 33% at 20°C. However, the malate sensitivity still increased to 57% at 40°C.

Maximum activation of C_3 PEPC with 2 mM Glu-6-P was observed at 25°C (2.1-fold). However, this activation was only 1.9-fold at 20°C and 1.4-fold at 40°C . Upon illumination of leaf discs, the activation of C_3 PEPC by Glu-6-P further increased to 2.6-fold at 25°C and 2.2-fold at 20°C but up to only 1.6-fold at 40°C. Thus, 25°C was observed to be the optimum temperature with regards to reduction in malate sensitivity and increase in Glu-6-P activation when compared to either 20°C or 40°C under both dark and light conditions. Hence, temperature modulated the regulatory properties of PEPC not only in the dark but also under the light.

Table 2. Modulation of the regulatory properties of C_3 PEPC in relation to light and/or temperature treatments in leaf discs of *P. sativum in vitro*.

Temperature	2mM Malate*		2mM Glu-6-P**	
	Dark	Light	Dark	Light
30 °C (Control)	55% ± 1.5	-	160% ± 4.1	-
25 °C	40% ± 1.1	20% ± 0.04	210% ± 4.1	260% ± 4.1
20 °C	48% ± 1.3	33% ± 1.2	193% ± 8.6	215% ± 4.1
40 °C	65% ± 2.0	57% ± 1.4	140% ± 4.1	156% ± 4.1

All values are averages of 3-5 replications ± SE and are statistically significant (P<0.01).

*Assayed at 2.5mM PEP; **Assayed at 0.5mM PEP.

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Pattern of modulation of C_3 PEPC activity during a daily natural cycle of light and temperature in leaves of *P. sativum*

PEPC activity in *P. sativum* was determined at regular intervals of 3 hr from 6.00 hr to 24.00 hr during the course of a particular day during different months of the year 2009, along with the light intensities and temperatures (data for August is not available). No consistent, clear trends in the diurnal changes in PEPC activity were observed. For e.g., in May, the PEPC activity remained high throughout the day (Figure 3A), but in December, PEPC activity started to rise from 6.00 hr to reach a peak at 15.00 hr and then decreased to reach a minimum at 24.00 hr (Figure 3A). When the maximum PEPC activities during a typical day for all the months of the year were compared, there appeared to be no seasonal differences in PEPC activity in *P. sativum* (Figure 4A). The data for two months- May and December is also shown in a tabular form (Table 2).

As the day progressed, the light intensity reached its maximum by 12 PM and the temperature by 15.00 h and then decreased by 24.00 hr. This trend was also consistent for all months of the entire year. The temperatures and light intensities were significantly higher during the summer [e.g., May] and least during the winter [e.g., December] (Figures 3 D and 3 E).

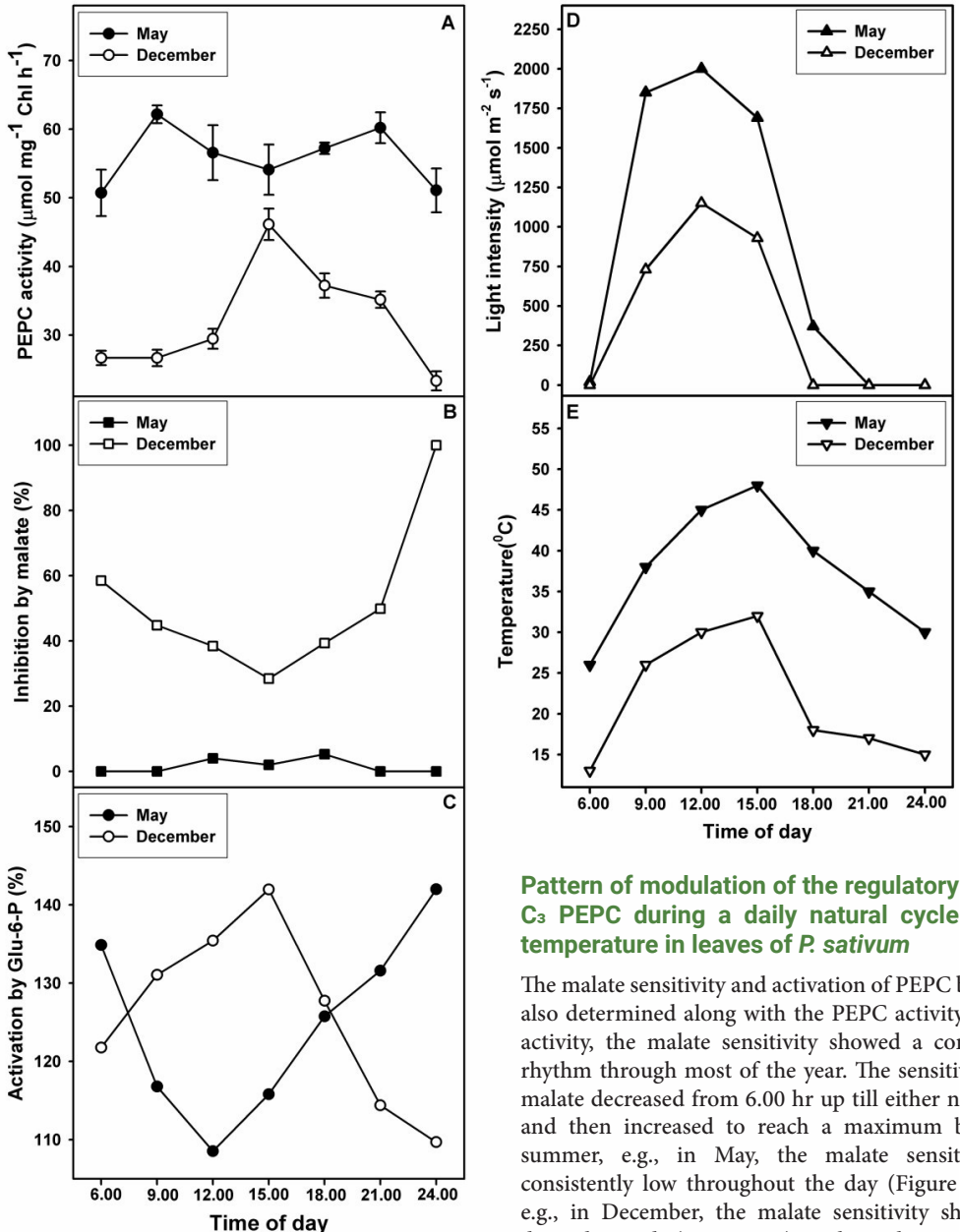

Figure 3. Patterns of change in PEPC activity (A), inhibition by malate (B), activation by Glu-6-P (C), light intensity (D) and temperature (E) during the course of a typical day in May and December, 2009. Standard errors if not visible fall within the symbols.

Pattern of modulation of the regulatory properties of C_3 PEPC during a daily natural cycle of light and temperature in leaves of *P. sativum*

The malate sensitivity and activation of PEPC by Glu-6-P were also determined along with the PEPC activity. Unlike PEPC activity, the malate sensitivity showed a consistent diurnal rhythm through most of the year. The sensitivity of PEPC to malate decreased from 6.00 hr up till either noon or 15.00 hr and then increased to reach a maximum by 24.00 hr. In summer, e.g., in May, the malate sensitivity remained consistently low throughout the day (Figure 3B). In winter, e.g., in December, the malate sensitivity showed a clearer diurnal trend (Figure 3B). When the minimum malate sensitivity recorded during a typical day for all the months of the year was compared, there appears to be no seasonal differences in the sensitivity of PEPC to malate in *P. sativum* (Figure 4B).

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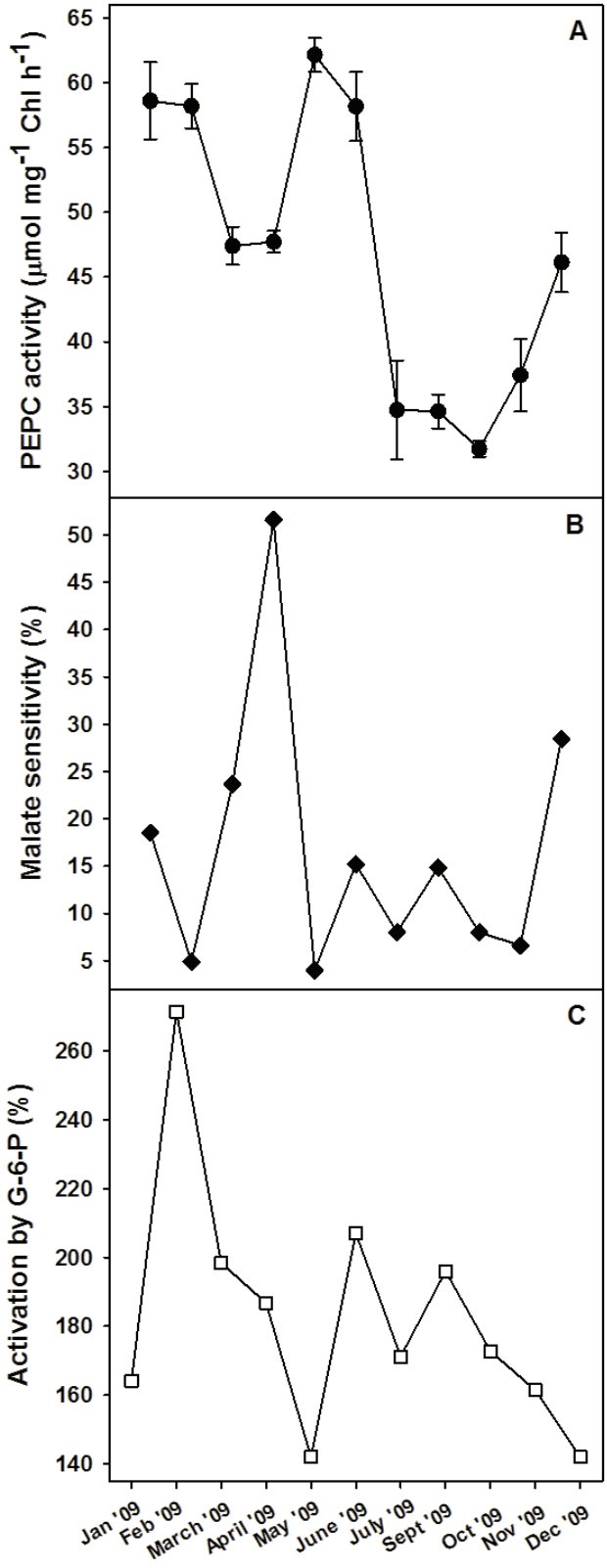

Figure 4. Maximum PEPC activity (A), least of PEPC sensitivity to malate (B) in and maximum sensitivity to Glu-6-P of PEPC (C) in leaves of *P. sativum* observed during a typical day of each month of the year, 2009. All values are averages of three replications ± SE.

The activation by Glu-6-P, however showed inconsistent trends in the diurnal changes over the year. In summer, e.g., in May, the levels of PEPC activation by Glu-6-P remained low during the day but high during the night (Figure 3C). On the contrary, in winter, e.g., in December, the activation by Glu-6-P showed an opposite diurnal trend- increasing from the morning, reaching a peak by 15.00 hr, and then decreasing (Figure 3C). On comparison of the maximum levels of activation by Glu-6-P during a typical day for all the months of the year, no seasonal differences in these levels in *P. sativum* could be observed (Figure 4C).

Thus, the activity, malate sensitivity, and activation by Glu-6-P of C_3 PEPC did not show a consistent diurnal variation over different months. There was also no marked seasonal variation that could be observed in these properties of C_3 PEPC. The ranges of these variations are summarized in Table 2. Scatter plots (Figure 5) were used to calculate the correlation coefficients between light or temperature and PEPC activity, malate sensitivity, and activation by Glu-6-P data obtained at 15.00 hr over the year (Table 3). These suggested that light has a greater influence on PEPC activity while temperature exerted a much greater effect on the regulatory properties of C_3 PEPC. Thus, C_3 PEPC was also modulated by a natural variation in light and temperature under *in situ* conditions.

Table 3. The range of changes in C_3 PEPC activity, malate sensitivity, activation by Glu-6-P, light intensities, and temperatures during the course of a typical day in May and December, 2009 in leaves of *P. sativum*.

Property/parameter	Ranges during the day	
	May-2009	Dec-2009
PEPC activity (µmol mg^{-1} Chl h^{-1})	51-62	23-46
Inhibition by malate (%)*	5-0	100-28
Activation by Glu-6-P (%)**	109-135	110-142
Light intensity (µmol m^{-2} s^{-1})	0-2000	0-1150
Temperature (°C)	26-48	13-32

*Assayed at 2.5mM PEP; **Assayed at 0.5mM PEP.

Discussion

In this study, the effects of the interaction between light and temperature were examined while modulating C_3 PEPC from *P. sativum* under *in vitro* as well as *in situ* conditions. Another objective was to find out if these two factors affected C_3 PEPC in a manner similar to C_4 PEPC. Hence, these experiments were done with leaf discs as well as leaves using the same methodology as was used with C_4 PEPC. In our present study, in contrast to the observations made by Chastain and Chollet and Wang et al., we observed that the PEPC from *P. sativum* was activated by 1.5-2.0 fold by light (Figure 2) [3,4]. The ranges of light activation obtained were in accordance with

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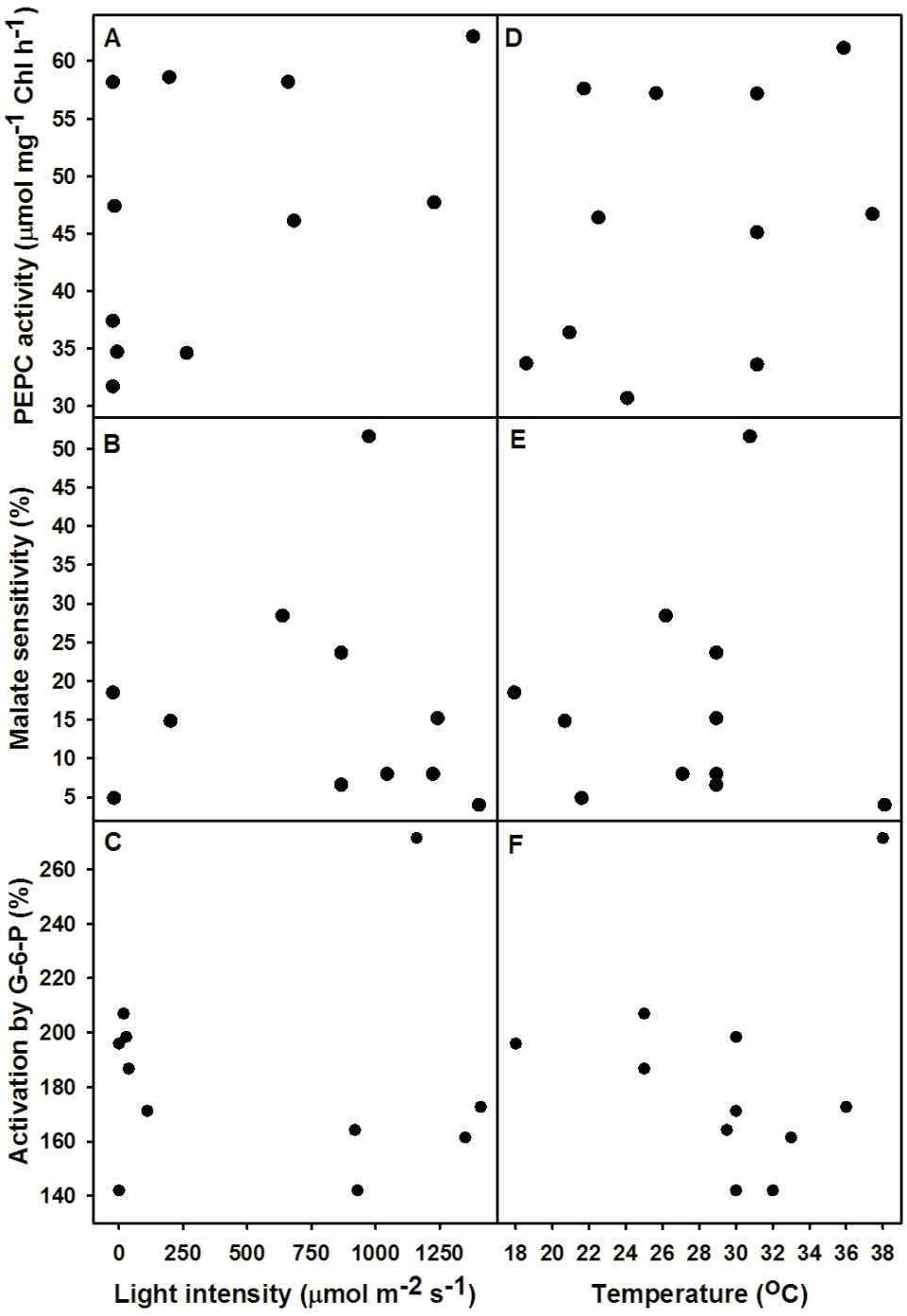

Figure 5. Scatter plots of PEPC activity (A), sensitivity to malate (B) and activation by Glu-6-P (C) versus the maximum light intensity during a typical day of the different months of the year. Scatter plots of PEPC activity (D), sensitivity to malate (E) and activation by Glu-6-P (F) versus the maximum temperature during a typical day of the different months of the year.

those reported by Rajagopalan et al. and Gupta et al. but not as high as those reported by Van Quy et al. [5,6,8]. However, in a further extension to these studies, such a stimulation of PEPC activity by light was found to be better at cold temperatures than at warm temperatures (Figure 2).

Our observations that PEPC activity in *P. sativum* was higher at 25°C than at warmer temperatures (30°C to 45°C) (Figure 1) correlated well with those made by Chinthapalli et al., who have reported 30°C to be optimal for the activity of C_3 PEPC [13]. The activation of C_3 PEPC upon exposure to optimum temperature was more in the dark than in light (Table 1). The higher 25°C /40°C and 20°C /40°C ratios, when compared to the L/D ratios (Table 1), suggested that activation due to incubation at optimal temperature was more efficient than light in enhancing the activity of C_3 PEPC *in vitro*. In the case of C_4 PEPC, photoactivation was higher at warm temperatures, and the effect of light in modulating PEPC activity was higher than that of temperature [19,20].

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Along with the activation of C_3 PEPC, we also observed a concomitant decrease in malate sensitivity and an increase in activation by Glu-6-P by light (Table 1). Such a response was contrary to that reported by Rajagopalan et al. but in accordance with some of the other previous reports, such as those by Gupta et al., Smith et al., and Dupont et al. [5,8,9,11]. In addition to this, we observe further that such modulation of the regulatory properties of C_3 PEPC by light was more prominent at lower than at warm temperatures (Table 1). However, the modulation of the activity and regulatory properties of C_3 PEPC by temperature was further enhanced in light, just as in the case with C_4 PEPC (Figure 2). Thus, we study and report for the very first time an interaction between light and temperature while modulating not only the activity but also the regulatory properties of C_3 PEPC under *in vitro* conditions. With C_4 PEPC, the modulation of the regulatory properties by light was more prominent at warm temperatures [20].

Both the activity and regulatory properties of C_3 PEPC under *in situ* conditions usually showed a clear diurnal rhythm only when the light intensities and temperatures are lower [e.g., in December] (Figures 3 and 4). The sensitivity of C_3 PEPC to malate showed ab consistent diurnal rhythm throughout the year, being lower during the day. This would be necessary and important for the enzyme to function optimally during the day. This observation of an occurrence of a diurnal rhythm in the activity and regulatory properties of C_3 PEPC was in contrast to that made by Leport et al. but similar to that by Scheible et al. A seasonal rhythm in the activity and properties of C_3 PEPC over the course of a typical year was however not observed to be consistent or prominent (Figure 4) [14]. The correlation coefficients obtained suggested that light better modulated C_3 PEPC activity, whereas temperature had a greater effect on its regulatory properties (Table 4). The diurnal rhythm in the activity and regulatory properties of C_4 PEPC was more prominent in warm months with a clear seasonal rhythm [20].

Table 4. Correlation coefficient relations between light or temperature with the modulation of the activity and regulatory properties of C_3 PEPC in leaves of *P. sativum*.

Parameter	PEPC activity (μmol mg^{-1} Chl h^{-1})	Inhibition by Malate (%)*	Activation by Glu-6-P (%)**
Light (μmol m^{-2} s^{-1})	0.48	-0.01	0.02
Temperature (°C)	0.37	-0.02	0.11

*Assayed at 2.5mM PEP; **Assayed at 0.5mM PEP.

This is the first study on the interactions between light and temperature during the regulation of activity and regulatory properties of C_3 PEPC under *in vitro* or *in situ* conditions. C_3 plants differ from C_4 plants in their light and temperature requirements [1,2]. The optimum temperature for photosynthesis and growth is lower in C_3 plants when compared to C_4 plants [21]. The photosynthetic rates were higher in the C_3 than the C_4 subspecies of *Alloteropsis semialata* under low winter temperatures [22]. The observations made under *in vitro* and *in situ* conditions correlated well with the light and temperature requirements of C_3 plants. The higher activity, lower malate sensitivity and increased response to Glu-6-P at temperatures around 25°C than at warm temperatures suggested that the optimal conditions for C_3 PEPC relate well to the optimal growth temperature of C_3 plants. Under field conditions, a diurnal rhythm in the activity and regulatory properties of C_3 PEPC was more prominent in colder than warm temperatures. The ambient light intensities are usually subdued on cold days, indicating that the C_3 plants prefer temperate regions and can tolerate low light conditions.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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