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# Temperature modulation of the activity and malate inhibition of the phosphoenolpyruvate carboxylase from leaves of *Alternanthera pungens*, compared to that of *Lycopersicom esculentum*

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**Abstract:** Temperature caused marked modulation of phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) in leaf discs of *Alternanthera pungens* (C<sub>4</sub> plant) as well as *Lycopersicon esculentum* (C<sub>3</sub> species). The optimal incubation temperature for PEPC activity in *A. pungens* was 45 °C compared to 30 °C in *L. esculentum*. *A. pungens* lost nearly 61% of PEPC activity on exposure to a low temperature of 15 °C, compared to only about a 33% loss in the case of *L. esculentum*. The C<sub>4</sub> enzyme was less sensitive to supra-optimal temperature and more sensitive to sub-optimal temperature than that of the C<sub>3</sub> species. Further as the temperature was raised from 15 °C to 50 °C, there was a sharp decrease in malate sensitivity of PEPC. The extent of such a decrease in C<sub>4</sub> plants was more than that in C<sub>3</sub> species. Arrhenius plots that were constructed by plotting the activity of PEPC against the reciprocal of temperature in the absence or presence of malate exhibited abrupt changes or “break-points” at only one point of 17°C in *A. pungens* while at two points corresponding 17°C and 27°C in case of *L. esculentum*. The activation energy of PEPC from *A. pungens* was less compared to that of *L. esculentum* in the temperature range of 10 to 27°C. However, the activation energy of PEPC from *A. pungens* was less than that of *L. esculentum* above the temperature of 27°C. The activation energy increased by 2 to 4 fold at temperatures below 17°C, in case of both *A. pungens* and *L. esculentum*. Thus, our results show the activity and malate sensitivity of PEPC can be influenced in relation to high temperature tolerance of C<sub>4</sub> plants, which can be physiologically significant.

**Keywords:** Temperature, Malate Sensitivity, Cold Sensitivity, PEPC, Arrhenius Plots, Activation Energy

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## 1. Introduction

Phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) which catalyses the primary step of phosphoenolpyruvate (PEP) carboxylation in C<sub>4</sub> and CAM plants, is regulated by internal metabolites, its common inhibitor being malate and its activator glucose-6-phosphate (Glu-6-P). Stupendous progress has been made in our knowledge of biochemistry and molecular biology of PEPC in not only C<sub>4</sub> plants, but also C<sub>3</sub> species and legume root nodules. Several authors have periodically reviewed the literature on the properties, regulation and functions of C<sub>4</sub> PEPC particularly in the past decade [1-10].

C<sub>4</sub> plants differ from C<sub>3</sub> plants in several features, including their light and temperature responses [11, 12]. The temperature optima for photosynthesis and growth in C<sub>4</sub> plants are usually higher than those for C<sub>3</sub> plants [13]. However, the C<sub>4</sub> plants are quite sensitive to cold temperatures. The cold sensitivity C<sub>4</sub> pathway has been suggested to be related to the cold sensitivity of key C<sub>4</sub> enzymes, such as pyruvate phosphate dikinase (PPDK) or PEPC [14-16].

The cold sensitivity of PPDK in C<sub>4</sub> plants is well established and the mechanism of cold inactivation of PPDK is studied in detail [15, 17-18]. In contrast, the reports on cold sensitivity of PEPC have been quite conflicting. There

are reports which suggest PEPC is sensitive to cold temperature [19, 20], while others could not detect any significant change in the properties of PEPC at cold temperature [18, 21]. Further, some of these experiments involved either long term exposures of plants or short term exposure of purified enzymes and thus involved diverse experimental material.

On illumination, the activity of PEPC is enhanced by 2-3 fold along with a marked decrease in the malate sensitivity of the enzyme. These changes during the light activation are due to mainly the phosphorylation of the enzyme [3, 22-23]. Despite being a cytosolic enzyme, the C<sub>4</sub> PEPC is modulated markedly by light as well as temperature. The individual effects of either temperature or light on the activity and regulatory properties of C<sub>4</sub> PEPC have been studied extensively [24-26]. However, there are only a few reports on the interactive influence of light and temperature on C<sub>4</sub> PEPC [20, 27]. The interaction between light and temperature while modulating both the activity and regulatory properties of PEPC in leaf discs and leaves of *Amaranthus hypochondriacus* has recently been reported [28]. Earlier studies were carried out *in vitro*. Compared to the available literature on the properties and mechanism of light activation of PEPC, in C<sub>4</sub> plants, the literature on the regulation by temperature of PEPC is quite limited [2]. The present study is an attempt to characterize the temperature responses of PEPC from a typical C<sub>4</sub> plant, *Alternanthera pungens* and compare with that of a C<sub>3</sub> plant, *Lycopersicon esculentum*. Experiments were conducted on leaf discs so as to simulate physiological situation *in vivo*. The results indicate that the changes in kinetic and regulatory properties of PEPC are examined critically with temperature changes encountered by C<sub>4</sub> and C<sub>3</sub> species.

## 2. Materials and Methods

### 2.1. Plant Material and Growth Conditions

Plants of *Alternanthera pungens* H.B.K (C<sub>4</sub> plant) was propagated by transplantation of cuttings and *Lycopersicon esculentum* Mill (C<sub>3</sub> species) were raised from seeds. The plants were grown in earthen pots filled with soil supplemented with farmyard manure. They were grown outdoors in the field, in the campus of the University of Hyderabad under a natural photoperiod of approximately 12 h and temperatures of 30-40 °C day/25-30 °C night. The upper fully expanded leaves were harvested, about 2-3 h after sunrise. Leaf discs (each of *ca.* 0.2 cm<sup>2</sup>) were prepared from 4- to 6- week-old plants of *Alternanthera pungens* and 2- to 4-week-old plants of *Lycopersicon esculentum*.

### 2.2. Extraction and Assay of PEPC

The extraction and assay of PEPC were conducted following the standard method previously described [24, 23]). The temperature range tested in *A. pungens* (C<sub>4</sub> plant) was 15°C to 55°C and for *L. esculentum* (C<sub>3</sub> species) was 15°C – 50°C. Thirty leaf discs (each of *ca.* 0.2 cm<sup>2</sup> and a total weight

of 125 mg) were extracted in a chilled mortar and pestle with 500 ml of extraction medium containing 100 mM TRIS-HCl (pH 7.3), 10 mM MgCl<sub>2</sub>, 2 mM K<sub>2</sub>HPO<sub>4</sub>, 1 mM EDTA, 10% (v/v) glycerol, 10 mM β-mercaptoethanol, 10 mM NaF, 2 mM phenylmethylsulphonyl fluoride (PMSF), 10 mg ml<sup>-1</sup> chymostatin was used, instead of PMSF. However, PMSF was used routinely, as it was found to be quite effective in avoiding proteolysis in the case of *A. pungens*. The homogenate was centrifuged at 15 000 g for 5 min and the supernatant was used as 'crude extract'. A small aliquot was kept aside, prior to centrifugation, for chlorophyll estimation.

Maximum PEPC activity was assayed by coupling to NAD-malate dehydrogenase (NAD-MDH) and monitoring NADH oxidation at 340 nm in a Shimadzu 1601 UV-Visible spectrophotometer at a temperature of 30 °C. The assay mixture (1 ml) contained 50 mM TRIS-HCl (pH 7.3), 5 mM MgCl<sub>2</sub>, 0.2 mM NADH, 2 U of NAD-MDH, 2.5 mM PEP, 0.05 mM NaHCO<sub>3</sub>, and leaf extract equivalent to 1 µg of chlorophyll. The sensitivity of PEPC to malate was checked by adding malate to make a final concentration of 0.5 mM in the assay mixture. Each assay was done in triplicate for each sample.

### 2.3. Incubation of Leaf Discs at Different Temperature

Thirty leaf discs were floated on distilled water in a 5 cm diameter Petri dishes and were left in darkness for 2 h. After predarkening, the leaf discs were incubated 30 min at required temperature in the range of 15°C to 50°C in a thermo-statically controlled water bath. At the end of 30 min in each temperature, the leaf discs were extracted (as described above) and the extract was examined for PEPC activity.

### 2.4. Estimation of Chlorophyll

Chlorophyll was estimated by extraction with 80% (v/v) acetone [29].

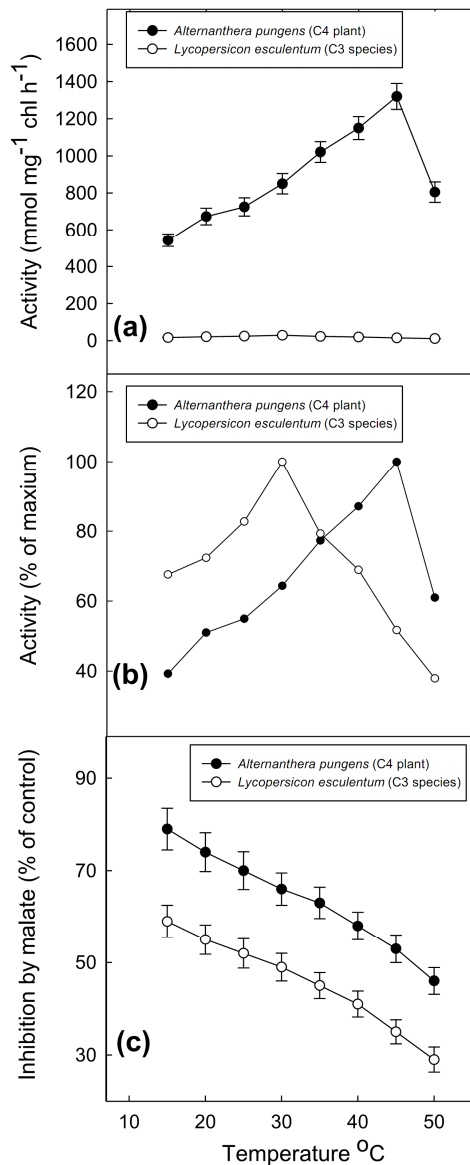
### 2.5. Replications and Statistical Analysis

All experiments were repeated 3 to 5 times on different days. The average values ±SE are presented. Statistical analysis of the data was done using the software Sigmaplot (version 10.0).

## 3. Results

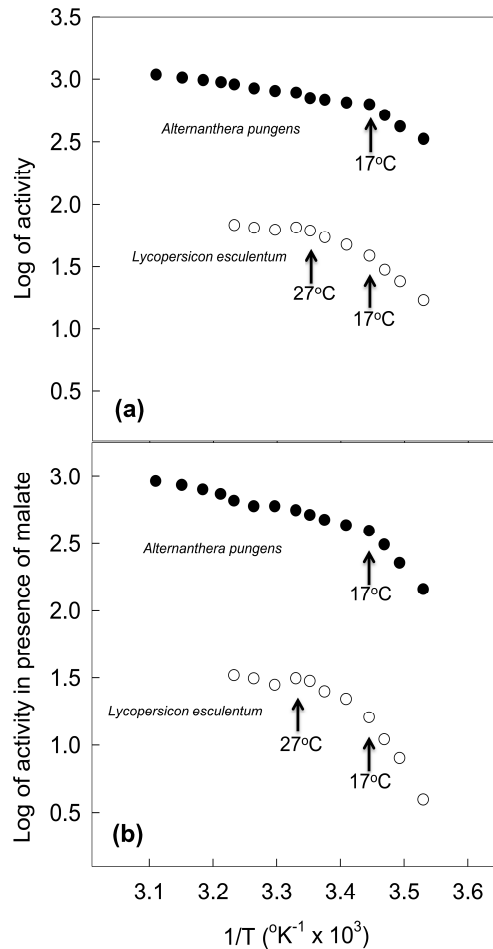
The optimal temperature for PEPC activity in leaf discs of *A. pungens* was 45°C compared to 30°C in *L. esculentum* (Figure 1 (a)). The response of enzyme to temperature was quite dramatic when plotted as the % of maximum activity (Figure 1 (b)). The decrease at 40°C was much higher in case of *L. esculentum* than that of *A. pungens*. Similarly the decrease in activity of PEPC at temperatures above 15°C was much greater in the case of *A. pungens* than that in *L. esculentum*. Thus, the C<sub>4</sub>PEPC was less sensitive to supra-optimal temperatures and more sensitive to sub-optimal temperatures than that of C<sub>3</sub> species. As the temperature was

raised from 15 °C to 50 °C, there was a sharp decrease in malate sensitivity of PEPC. The extent of such a decrease in C4 plants was more than that in C3 species when the enzyme was assayed at 0.05 mM bicarbonate (Figure 1 (c)). The extent of malate inhibition was always higher in case of *L. esculentum* than that of *A. pungens*.



**Figure 1.** The activity of PEPC in extracts from leaf discs of *Alternanthera pungens* (C<sub>4</sub> plant) and *Lycopersicon esculentum* (C<sub>3</sub> species) after exposure to varying temperatures. (a) The activity of PEPC is represented as either enzyme units of  $\mu\text{mol mg}^{-1} \text{Chl h}^{-1}$  (b) or % of its maximum. The activity of PEPC in extracts from leaf discs of *Alternanthera pungens* (C<sub>4</sub> plant) and *Lycopersicon esculentum* (C<sub>3</sub> species) after exposure to varying temperatures. The preincubation time for leaf discs was 30 min for each temperature. The experiments were done on at least three different days and the average values  $\pm$ SE are represented. The maximal activity of PEPC in *A. pungens* and *L. esculentum* were  $1273 \pm 98$  and  $29.4 \pm 2.4 \mu\text{mol mg}^{-1} \text{Chl h}^{-1}$ , respectively. (c) Effect of temperature on the malate sensitivity of PEPC in extracts prepared from the leaf discs of *Alternanthera pungens* (C<sub>4</sub> plant) or *L. esculentum* (C<sub>3</sub> species), exposed to different temperatures. The activity was measured at 30 °C and assayed in the absence or presence of either 0.5 mM malate (*A. pungens*) or 2 mM malate (*L. esculentum*). Further details were as described in figure 1b.

Arrhenius plots were constructed by plotting the activity of PEPC against the reciprocal of temperature in the absence (Figure 2 (a)) or presence of malate (Figure 2 (b)). These enzyme activities were measured after exposing the leaf discs to a range of 10°C to 45°C in case of *A. pungens* and 10°C to 35°C in case of *L. esculentum*. Arrhenius plots exhibited abrupt changes or “break-points” at only one point of 17°C in *A. pungens*(C<sub>4</sub>) while at two points corresponding 17°C and 27°C in case of *L. esculentum* (C<sub>3</sub>). The patterns of Arrhenius curves in presence of malate were quite similar to those in the absence, with similar breaks in the slope.



**Figure 2.** Arrhenius plots of PEPC activity in the absence of malate or in the presence of malate during the assay. The points are averages of three to five separate experiments. The activity of PEPC was determined in the extracts prepared from leaf discs of *Alternanthera pungens* (C<sub>4</sub> plant) or *Lycopersicon esculentum* (C<sub>3</sub> species), exposed to varying temperatures. Further details were as in Figure 1. The ‘break-points’ are indicated by arrows.

The activation energy of PEPC from *A. pungens* was less than that from *L. esculentum* in the temperature range of 10 to 27°C (Table 1). However, the activation energy of PEPC from *A. pungens* was less compared to *L. esculentum* above the temperature of 27°C. The activation energy increased by 2 to 4 fold at temperatures below 17°C, in case of both *A. pungens* (C<sub>4</sub>) and *L. esculentum* (C<sub>3</sub>).

## 4. Discussion

The temperature can cause quite striking changes in not only the activity but also the regulatory properties of PEPC in both C<sub>3</sub> and C<sub>4</sub> plants. The optimal temperature for PEPC in *A. pungens* (C<sub>4</sub>) was 45°C compared to 30°C in *L. esculentum* (C<sub>3</sub> species) is not surprising (Figure 1). The activities of PEPC and NADP-ME in desalted extracts from different species of sugarcane showed no large changes after incubating the enzyme at various temperatures from 30°C to 0°C [16]. In contrast, PEPC in extracts of *P. maximum* lost up to 50% of its activity after incubation for 60 min at 0°C while the enzyme from *P. miliaceum* was stable [21]. Our results confirm that the C<sub>4</sub> PEPC is quite sensitive to sub-optimal temperatures compared to the PEPC of C<sub>3</sub> species.

The sharp increase in the activity of PEPC with temperature, particularly above 15°C, could be physiologically significant, as the temperature is expected to rise from about 10 to 15°C in the morning to 35 to 40°C at midday, on a typically clear and sunny day. The maintenance of high enzyme activity at warm temperatures, together with a sharp decrease in the malate sensitivity of PEPC was also noticed in other C<sub>4</sub> plants [24]. A combination of light and warm temperature could amplify the photoactivation of the PEPC, as observed in case of *Egeria densa* [30] and *Amaranthus paniculatus* [20]. The marked reversibility of the effect of temperature on PEPC in case of both C<sub>4</sub> and C<sub>3</sub> plants [24] is an additional indication of the possible physiological relevance of temperature effects on PEPC.

These results demonstrate clearly the marked changes induced by temperature in the sensitivity of PEPC to malate. As the temperature was raised from 15 °C to 50 °C, there was a marked decrease in malate sensitivity of PEPC. The extent of such a decrease in C<sub>4</sub> plants (79% to 46%) was more than that in C<sub>3</sub> species (59% to 29%) (Figure 2). Thus, PEPC appeared to be highly sensitive to malate at cold temperatures, while becoming relatively insensitive to malate at warm temperatures. The extent of malate inhibition is quite high in *L. esculentum* compared with *A. pungens*. Again the limited reports in the literature had conflicting

observations. At low temperature, the sensitivity of PEPC to malate was very high in maize [31], but was quite low in *Bryophyllum fedtschenkoi* [32]. Lowering the temperature from 25 °C to 3 °C not only decreased the catalytic capacity of PEPC, but also caused a considerable reduction (about 10-fold) in the sensitivity of PEPC to malate [32].

The decrease in malate sensitivity of PEPC can also occur due to the proteolysis of the enzyme. However, the authors are confident that this is not the reason during these observations. There was no detectable change in the protein levels as indicated by the western blots [24]. The changes in activity of PEPC due to temperature were reversible to a marked extent [24].

Arrhenius plots revealed differences between not only the C<sub>3</sub> and C<sub>4</sub> plants, but also the pattern in presence or absence of malate (Figure 3 (a and b)). As the temperature was raised, the activation energy was lowered in both *A. pungens* (C<sub>4</sub> plant) and in *L. esculentum* (C<sub>3</sub> species). The changes in activation energy as indicated by discontinuities (“breakpoints”) in Arrhenius plots at a critical temperature can be an indication of the cold lability of PEPC from different species [23, 33-34]. The break at 27°C in case of C<sub>3</sub> plants suggests that the C<sub>3</sub> enzyme does not respond much to temperatures, above 27°C. In contrast, the absence of such break and the continuation of slope indicate that the activation energy continues to decrease for C<sub>4</sub>-PEPC as the temperature rises from 27°C to 45°C. The presence of malate increased significantly the activation energy in both C<sub>3</sub> and C<sub>4</sub> species (Table 1). Such increase in activation energy of PEPC in presence of malate, an inhibitor, is logical as malate being an effective inhibitor may slow down the thermodynamic responses of PEPC. But in presence of malate the activation energy increased nearly two-fold over that in the absence of malate, during the temperature range of 10°C to 27°C in both *A. pungens* and *L. esculentum*. The limited studies made earlier on the activation energy of PEPC again were conflicting. Some of the reports indicate discontinuity in the Arrhenius plots of PEPC [19] while others did not observe such break points [18].

**Table 1.** Activation energy (kcal mol<sup>-1</sup>) of PEPC in extracts from leaf discs of *Alternanthera pungens* (C<sub>4</sub> plant) or *Lycopersicon esculentum* (C<sub>3</sub> species) exposed to different temperatures.

Temperature range (°C)	<i>Alternanthera pungens</i> (C <sub>4</sub> plant)	<i>Lycopersicon esculentum</i> (C <sub>3</sub> species)
<b>Activation energy (kcal mol<sup>-1</sup>)</b>		
Enzyme activity in the absence of malate		
10-17	13.8	18.3
17-27	3.8	9.1
27-35 or 27-45 <sup>a</sup>	2.9	1.23
Enzyme activity in the presence of malate		
10-17	23.0	31.0
17-27	6.1	12.5
27-35 or 27-45 <sup>a</sup>	4.5	1.03

<sup>a</sup> The range was 27-35°C for *Lycopersicon esculentum* and 27-45°C for *Alternanthera pungens*

For *Amaranthus cruentus* the activation energy rapidly increased below 20°C, but it is not clear whether it extrapolates to infinity at the same temperature as in *Sorghum bicolor*, or at a slightly lower temperature [35]. The

present data indicates that C<sub>4</sub> plants have special adaptation mechanism, which modifies to survive and maintain high rate of photosynthesis under conditions of high temperature. This is to conclude that high temperature tolerance of C<sub>4</sub>

plants is not only the presence of heat shock proteins, but may be a greater part of thermo stability of the carboxylating enzymes. Further, studies are essential to characterize molecular basis of modulation by temperature of PEPC in C4 plants.

## 5. Conclusions

Temperature caused a dramatic modulation of PEPC in leaf discs of *Alternanthera pungens* (C<sub>4</sub>) compared to that of *Lycopersicon esculentum* (C<sub>3</sub>). There was a strong contrast in the temperature optima for PEPC activity in leaf discs of *Alternanthera pungens* and *Lycopersicon esculentum*. The steep increase in activity of PEPC with rise in temperature could be physiologically significant, as the temperature is expected to rise from about 10 to 15°C in the morning to 35 to 40°C at midday, on a clear and sunny day. As the temperature was raised, the activation energy was lowered in both *Alternanthera pungens* and *Lycopersicon esculentum*. The changes in activation energy as indicated by discontinuities ("breakpoints") in Arrhenius plots at a critical temperature can be an indication of the cold lability of PEPC from different species.

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