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RESEARCH PAPERS

Phosphoenolpyruvate Carboxylase during Maturation and Germination Sorghum Seeds: Enzyme Activity and Regulation[1]

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Abstract—Sorghum (*Sorghum bicolor* (L.) Moench) is a species of great socio-economic and ecological importance for countries in arid and semi-arid climate. In C_4 plants like sorghum, phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) plays a key role in seed development and germination. In this work, the PEPC activity shows an increase followed by a decrease at the early and later stages of maturation, respectively. In germinating seeds, the PEPC activity quickly increases after soaking. The L-malate test and the ratio of PEPC activity determined at pH 8.0 and 7.1, indicates, that PEPC is phosphorylated at the early stages of maturation then becomes dephosphorylated at the later stages and during seed germination, PEPC takes back its phosphorylated form. The determination of the affinity constant showed different K_M depending on the seed developmental stage. As there is no PEPC-C4 isoform in developing sorghum seeds, this result indicates that the different K_M observed during seed maturation could be a result of a post-translational regulation such as phosphorylation or ubiquitination of a pre-existing isoform. This regulation enhances the PEPC activity at early stages of seed development.

Keywords: Sorghum bicolor, C_4 plant, seed development, phosphoenolpyruvate carboxylase, enzyme activity, phosphorylation, L-malate, glucose-6-phosphate

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INTRODUCTION

Phosphoenolpyruvate carboxylase (PEPC) is a cytosolic enzyme that is widely present in bacteria, green algae, and in higher plants. PEPC catalyzes the irreversible β-carboxylation of phosphoenolpyruvate (PEP) in the presence of HCO_3^- to yield oxaloacetate and inorganic phosphate (Pi). Oxaloacetate is subsequently converted to malate by malate dehydrogenase (MDH; EC 1.1.1.37). In C_3 plants, malate can either react with a malic enzyme resulting in the production of CO_2 and pyruvate or it can be transported into mitochondria for use in the citric acid cycle (TCA cycle) [1]. In C_4 and crassulacean acid metabolism (CAM) plants, photosynthetic PEPCs have been widely studied due to their central role in atmospheric CO_2 fixation [2]. Besides its role in the C_4 and CAM photosynthesis, PEPC has been implicated in various an array of physiological contexts functions such as the anapleurotic pathway (i.e. replenishment of TCA cycle intermediates when carbon skeletons are removed for other metabolic functions like nitrogen assimilation and amino acid biosynthesis), seed maturation and germination and stomatal opening [3, 4].

Like most enzymes, PEPC activity is subjected to allosteric modulation by metabolic effectors with glucose-6-phosphate (Glc-6-P) and triose phosphates as activators and malate, aspartate and glutamate as inhibitors [4] PEPC can be also regulated through reversible phosphorylation catalyzed by an endogenous PEPC protein kinase (PEPC-K), and dephosphorylation by a protein phosphatase type 2A (PP2A) [5]. PEPC phosphorylation typically reduces the enzyme's sensitivity to malate inhibition while simultaneously enhancing Glc-6-P activation. This feature allows a strong protection against the L-malate, produced by the PEPC itself, which keep the catalytic rate at levels required for physiological context [6].

C_4, CAM and C3 PEPC isoforms in leaves have been widely studied [7–9], but limited information is available concerning the properties of PEPC in seeds. PEPC activity was found in seeds of different plants (castor oil plant, maize, barley, wheat, grape and sor-

[1] The article is published in the original.

[2] These authors contributed equally to this work.

Abbreviations: CAM—crassulacean acid metabolism; DPA—days post-anthesis; DPI—days post-imbibition; EDTA—ethylenedinitrilotetraacetic acid; EGTA—ethylene-bis(oxyethylenenitrilo)tetraacetic acid; HEPES—4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; MDH—malate dehydrogenase; MOPS—4-morpholinepropanesulfonic acid; PEP—phosphoenolpyruvate; PEPC—phosphoenolpyruvate carboxylase; PEPC-K—PEPC protein kinase.

ghum) [5, 10–15]. However, the phosphorylation state of PEPC from these different seeds was variable. In fact, in developing castor oil seeds, PEPC was found in its phosphorylated form. On the contrary, in mature dry seeds, only non-phosphorylated PEPC was present [12, 16]. In developing barley seeds PEPC was represented by its phosphorylated form. However, despite the presence of PEPC kinase in the dry seeds, PEPC was stored in its non-phosphorylated form [11]. PEPC in sorghum seeds has been studied in germinating seeds and in mature dry seeds. To complete the cycle, this study was conducted on sorghum seed PEPC during development stages. Thus, the developmental profiles for PEPC activity, the apparent phosphorylation status (sensitivity to L-malate) and possible interactions with metabolite effectors in developing sorghum seeds have been investigated and compared to germinating seeds.

MATERIALS AND METHODS

Chemicals. All chemical and biochemical reagents were purchased from Sigma (United States) except phosphoenolpyruvate (PEP) and β-nicotinamide adenine dinucleotide reduced disodium salt (NADH) which were purchased from Roche Diagnostics (Switzerland), b-mercaptoethanol was obtained from Amresco (United States) and Bradford reagent was obtained from Bio-Rad (United States).

Plant material. *Sorghum bicolor* (L.) Moench plants were cultivated in a growth chamber. The environmental conditions in the growth chamber were 16-hour day (30°C)/8-hour night (22°C). Seeds were harvested at 5, 10, 15, 20, 25, 30 and 35 days post-anthesis (DPA), and immediately stored at –80°C until use.

For seedlings, seeds were sterilized in 5% (v/v) NaOCl for 15 min and thoroughly washed with sterile water. Seeds were germinated either on filter paper soaked in sterile distilled water, in Petri dishes. The dishes were placed in darkness in a sterile chamber at 24°C. Seeds were harvested at 0, 3, 6, 8, 10, 12 and 15 days post-imbibition (DPI), and immediately stored at –80°C until use.

Enzyme extraction. Seeds were extracted in four volumes of a 100 mM Tris-HCl, pH 8.0, buffer containing 10 mM $MgCl_2$, 10% (v/v) glycerol, 1% (w/v) polyvinylpyrrolidone, 50 mM NaF, 1 mM Na_2MoO_4, 14 mM β-mercaptoethanol, 1 mM phenylmethanesulfonyl fluoride, 1 mM EDTA (ethylenedinitrilotetraacetic acid), 1 mM EGTA (ethylene-bis(oxyethylenenitrilo)tetraacetic acid), 4 μg/mL leupeptin, 4 μg/mL chymostatin. The homogenates were centrifuged at 12000 *g* for 15 min at 4°C. The supernatant was precipitated with ammonium sulfate (60% saturation), the saturated supernatant was centrifuged again in the same conditions and the resulting pellet was suspended in the extraction buffer and used for enzyme assays.

Enzyme assay and electrophoresis. Phosphoenolpyruvate carboxylase activity was assayed at 30°C by following NADH oxidation at 340 nm using a BioTek Epoch microplate reader and the following optimized assay conditions: 100 mM HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid) pH 7.3, 5 mM $MgCl_2$, 5 mM $NaHCO_3$, 2.5 mM PEP, 0.2 mM NADH and 3 U/mL of desalted porcine muscle malate dehydrogenase. PEPC activity was expressed as the amount of enzyme extract which catalyzes the transformation of 1 μmol of substrate per minute and per milligram of protein at 30°C. Activity values presented are the average of at least six independent measurements.

The samples were subjected to non-denaturing polyacrylamide gels at 4°C (80 V for 3 h) in an electrophoresis cell (Bio-Rad). After protein migration, the gels were treated and revealed as described by Rivoal et al. [17].

Kinetic studies. A BioTek Epoch microplate reader spectrophotometer was used for all kinetic studies. To determine the effect of pH, enzyme activity was measured as described above except that the buffer was 100 mM MOPS (4-morpholinepropanesulfonic acid) for pH 6.5, 7.1, and 7.3, and 100 mM Tris-HCl for pH 7.5 and 8.0. Since the inhibitory effect of malate and the activation effect of Glc-6-P are exerted at suboptimal pH, malate and Glc-6-P sensitivity of PEPC was measured at pH 7.3. The malate and Glc-6-P concentrations used were chosen to encompass the concentration causing 50% inhibition (IC_{50}) or 50% activation (K_a) of initial PEPC activity. Apparent K_M values were evaluated from Lineweaver-Burk plots over the range of 0.1 to 2.5 mM PEP. The IC_{50} and K_a values were determined using Microsoft Excel Office 2010, using the range of 0 to 2 mM L-malate and Glc-6-P. The phosphorylation state of PEPC was determined by the malate test (malate inhibition at suboptimal pH of 7.3 and expressed as the IC_{50}) and/or by the velocity test (ratio of PEPC activity determined at pH 8 and 7.1, which decreases when the enzyme becomes phosphorylated due to an increase in velocity at pH 7.1), as proposed by Giglioli-Guivarch et al. [18]. All kinetic parameters represent means of at least six separate determinations.

Estimation of protein. Soluble protein content was determined by the method of Bradford [19], using the Bio-Rad dye reagent and bovine serum albumin as a standard. To remove pipetting error, each sample was run in triplicate and with two volumes.

Statistical analysis. SPSS 10.0.5 package for Windows, version 10.0.1 was used for all statistical analyses. One-way ANOVA model and Tukey's post-hoc tests were performed to determine significant differences among means ($P < 0.05$). Different letters indicate significant differences at 5% level.

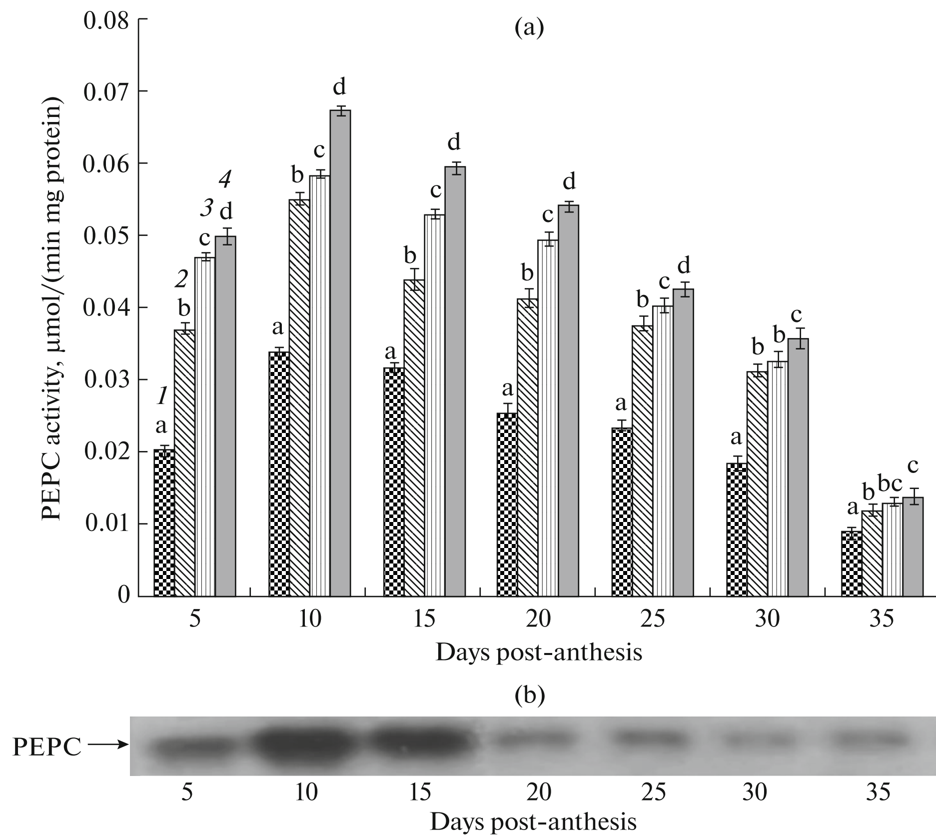

Fig. 1. (a) PEPC activity during maturation of sorghum seeds and effect of the reaction-medium pH. *1*—pH 6.5, *2*—pH 7.1, *3*—pH 7.5, *4*—pH 8.0. Each value represents the mean of six replicates. Each value represents the mean ± SE obtained from six independent measurements. Means were compared by Tukey's test. Different letters indicate significant differences between groups at the 5% level; (b) in gel PEPC activity, 30 µg of seeds protein were used for each sample.

RESULTS

PEPC Activity during Maturation and Germination of Sorghum Seeds and Effect of pH

The PEPC activity is detected in all stages of seed development; however, the PEPC activity is higher during the early stages (5 to 15 DPA) and gradually decreases until reaching its lowest level at the maturation stage (Fig. 1a). This result was confirmed by the in gel PEPC activity (Fig. 1b). Fig. 1a showed also that the effect of pH on the activity of PEPC during seed maturation depends on the developmental stage. In fact, this activity increases by 2—3-fold between pH 6.5 and 8.0 for stages 5 to 20 DPA. However, the PEPC activity becomes less sensitive to pH changes between 20 DPA to mature seeds.

Concerning the PEPC activity in germinating seeds, Fig. 2a showed that at the early germination stages, the PEPC activity gradually increased and attained its maximum at the 6 DPI for which, the activity is 9—10-fold higher than at the first stage. Afterwards, PEPC activity decreased significantly until the last stage (15 days after imbibition). In-gel PEPC activity experiments (Fig. 2b) confirm these results. In fact, the intensity of the PEPC band increases between non-imbibed seeds to 8 DPI and decreased thereafter (Fig. 2b). Concerning the effect of pH on the PEPC activity of sorghum germinating seeds, Fig. 2a showed that in dry seeds there was no significant effect. However, when the seeds are imbibed, increasing pH had a positive effect on this activity. In fact, the PEPC activity increased by 5—7-folds between pH 6.5 and 8.0 from the 3rd to the 10th DPI. Beyond the 10th DPI, the influence of the increase in pH has decreased until 15 DPI where no effect has been detected.

Metabolite Effects on PEPC Activity during Maturation and Germination of Sorghum Seeds

It is at present well studied that PEPC is controlled by a metabolic regulation which is translated by activation or inhibition of enzyme activity through a variety of photosynthetic compounds. In this study, the PEPC activity of maturing and germinating seeds was monitored in the presence of different concentrations ranging from 0.1 to 2 mM Glc-6-P or L-malate at pH 7.3 and suboptimal concentration of PEP (0.2 mM).

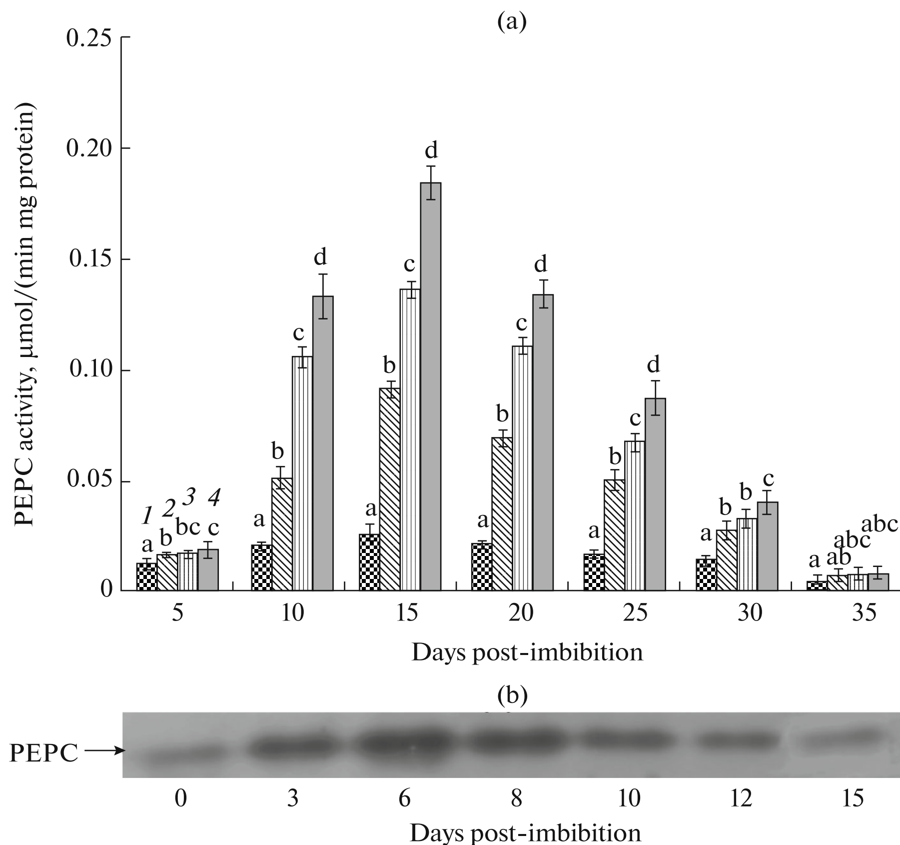

Fig. 2. (a) PEPC activity during germination of sorghum seeds and effect of the reaction-medium pH. *1*—pH 6.5, *2*—pH 7.1, *3*—pH 7.5, *4*—pH 8.0. Each value represents the mean of six replicates. Each value represents the mean ± SE obtained from six independent measurements. Means were compared by Tukey's test. Different letters indicate significant differences between groups at the 5% level; (b) in gel PEPC activity, 30 μg of seeds protein were used for each sample.

Activator. We studied the effect of Glc-6-P on the PEPC activity during maturation and germination (Table 1) of sorghum seeds. It seems that this effector is weakly active on the PEPC of developing seeds compared to germinating seeds. In fact, in the maturing seeds, PEPC activity increases slightly with increasing concentration of Glc-6-P. The activation caused by 2 mM of this effector does not exceed 19% at the initial stages of maturing seeds and reaches 26% at the last stage. On the contrary, in the germinating seeds, the PEPC activity increased significantly with increasing concentration of Glc-6-P. Consequently, 2 mM concentration increases the initial activity of the enzyme, 44% at the first four stages and 78% at the latter stages.

Inhibitor. The effect of L-malate on PEPC during the maturation and germination of sorghum seeds is shown in Table 2. The percentage inhibition of L-malate at 0.1 mM does not exceed 11% during the early stages of the maturation whereas at the last two stages, this concentration causes more than 50% inhibition. Concerning the effect of L-malate on PEPC during the germination of sorghum seeds (Table 2), we showed that the enzyme activity decreases significantly with increasing concentration of L-malate. We showed also that the PEPC activity at early stages of germination is less sensitive to L-malate unlike the last two stages.

PEPC Phosphorylation Status during Maturation and Germination of Sorghum Seeds

The kinetic parameters of PEPC during seed maturation are resumed in Table 3. Results showed that the IC_{50} in the green seeds was high and reached a maximum of 1.1678 mM at the third stage. Thereafter, the IC_{50} decreases to 0.073 mM in mature seeds. The ratio of the specific activity at pH 8 and 7.1 is also high at the early stages and decreases with the progress of seed development and stabilizes at the last stages of development (25–35 DPA). Table 3 shows also that the K_a in maturing seeds is high at the first stage and then decreases to its lowest value at the fifth stage and subsequently increases at the mature stage.

Regarding K_M, it showed that the affinity of the PEPC of maturing sorghum seeds for its substrate varies depending on the development stage. Thus, we noticed that the K_M increased at the early stages to

Table 1. Effect of Glc-6-P on the activity of PEPC from sorghum seeds at different maturation and germination stages

		PEPC activity, μmol/(min mg protein)					
Glc-6-P		0 mM	0.1 mM	0.5 mM	1 mM	2 mM	K_a
Days post-anthesis	5	0.019 ± 0.000[a]	0.021 ± 0.001[ab]	0.022 ± 0.003[bc]	0.023 ± 0.003[bc]	0.024 ± 0.002[d]	5.876 ± 0.006
	10	0.033 ± 0.001[a]	0.034 ± 0.005[ab]	0.035 ± 0.003[abc]	0.036 ± 0.002[bc]	0.039 ± 0.002[d]	5.670 ± 0.003
	15	0.030 ± 0.003[a]	0.031 ± 0.002[ab]	0.032 ± 0.001[ab]	0.033 ± 0.003[c]	0.035 ± 0.001[d]	5.498 ± 0.002
	20	0.030 ± 0.003[a]	0.031 ± 0.001[ab]	0.032 ± 0.001[c]	0.033 ± 0.002[bcd]	0.035 ± 0.002[cd]	5.292 ± 0.004
	25	0.022 ± 0.001[a]	0.024 ± 0.008[ab]	0.025 ± 0.006[bc]	0.026 ± 0.004[bc]	0.028 ± 0.002[d]	5.063 ± 0.009
	30	0.022 ± 0.002[a]	0.023 ± 0.001[ab]	0.024 ± 0.004[abc]	0.025 ± 0.004[abc]	0.026 ± 0.002[d]	5.196 ± 0.002
	35	0.011 ± 0.001[a]	0.012 ± 0.001[b]	0.013 ± 0.002[bc]	0.015 ± 0.001[bc]	0.016 ± 0.001[d]	5.776 ± 0.001
Days post-imbibtion	0	0.017 ± 0.003[a]	0.019 ± 0.003[ab]	0.021 ± 0.005[abc]	0.022 ± 0.003[abcd]	0.024 ± 0.006[bcd]	5.776 ± 0.004
	3	0.060 ± 0.002[a]	0.062 ± 0.002[ab]	0.067 ± 0.003[bc]	0.077 ± 0.005[bcd]	0.088 ± 0.006[cd]	2.356 ± 0.011
	6	0.092 ± 0.001[a]	0.096 ± 0.002[ab]	0.101 ± 0.002[bc]	0.111 ± 0.007[c]	0.140 ± 0.007[d]	2.263 ± 0.013
	8	0.076 ± 0.008[a]	0.078 ± 0.009[ab]	0.084 ± 0.003[abc]	0.090 ± 0.007[c]	0.109 ± 0.008[d]	2.304 ± 0.002
	10	0.074 ± 0.002[a]	0.076 ± 0.009[ab]	0.085 ± 0.001[abc]	0.100 ± 0.008[cd]	0.114 ± 0.006[d]	1.743 ± 0.008
	12	0.035 ± 0.009[a]	0.042 ± 0.002[b]	0.047 ± 0.001[bc]	0.056 ± 0.003[c]	0.065 ± 0.002[d]	1.126 ± 0.006
	15	0.005 ± 0.002[a]	0.005 ± 0.002[a]	0.007 ± 0.003[b]	0.007 ± 0.001[bc]	0.009 ± 0.003[c]	1.225 ± 0.003

K_a values: concentrations of activator producing 50% activation; Glc-6-P: glucose-6-phosphate. Each value represents the mean ± SE obtained from six independent measurements. Means were compared by Tukey's test. Different letters indicate significant differences between groups at the 5% level.

reach a maximum of 0.194 mM at 15 DPA then declined to 0.067 mM at the mature stage (35 DPA).

The results in Table 4 showed that the IC_{50} for L-malate of PEPC during seed germination has four phases. In fact, in dry seeds, the IC_{50} was very low (0.73 mM), it greatly increased after the third day of imbibition to reach 0.602 mM, and then, gradually increased to a value of 2.655 mM at the last stage. Contrary to the IC_{50}, the K_a decreased during imbibition and stabilized at the last two stages. Concerning the report activity pH 8.0/7.1 it's nearly doubled between the dry seeds and seeds imbibing for 72 h. This ratio improved by following a gradual way until the last stage where it is at its minimum. Concerning the K_M, it increased by 72% from the first to the third stage and subsequently decreased in a progressive manner to achieve its minimum at the last stage.

DISCUSSION

During maturation and germination, sorghum grains undergo several metabolic changes. The products resulting from the assimilation of the photosynthetic carbon are exported and accumulated in the storage organs such as the seed. These storage products are remobilized to support germination and seedling establishment after germination or to satisfy high local demand of carbon for specific processes [20]. PEPC is a key enzyme that plays a very important role in carbon metabolism. This enzyme has been the subject of several studies during the maturation and germination of grains of barley, maize and grains of some oil seeds [5, 12, 21], in tomato fruits during their development [22] and in rice seeds [3, 23]. In this work we have focused on the PEPC of sorghum grains during their development and germination.

In order to study the evolution of the enzymatic activity of the PEPC in the sorghum grains, we measured the enzymatic activity during the maturation and the germination of the sorghum grains. Our results show the presence of PEPC activity at all stages of maturation and germination of sorghum seeds. However, this activity varies according to the stage of development. Indeed, at the early stages of maturation, where the PEPC activity was higher, this enzyme plays a key role in the carbon assimilation, necessary for carbohydrate and protein reserves synthesis and, which would explain the increase in PEPC activity at these stages. As the seed becomes mature, the production of the reserves decreases and as a result, the PEPC activity also decreases [11, 22, 24, 25]. During germination, PEPC activity increased during the early stages and decreased thereafter. These variations in PEPC activity during germination may be explained by the fact that during germination the embryo requires carbon and nitrogen skeletons, consequently, it uses the anapleurotic function of the PEPC in order to supply the embryo and vegetative parts in carbon skeletons [11, 26].

Table 2. Effect of L-malate on the activity of PEPC from sorghum seeds at different maturation and germination stages

L-Malate		PEPC activity, μmol/(min mg protein) 0 mM	0.1 mM	0.2 mM	0.5 mM	1 mM	2 mM	IC_{50}
Days post-anthesis	5	0.019 ± 0.000^{a}	0.017 ± 0.000^{b}	0.016 ± 0.001^{b}	0.013 ± 0.001^{c}	0.011 ± 0.001^{d}	0.008 ± 0.000^{e}	1.107 ± 0.013
	10	0.033 ± 0.002^{a}	0.029 ± 0.001^{b}	0.026 ± 0.001^{c}	0.024 ± 0.001^{d}	0.018 ± 0.001^{e}	0.012 ± 0.001^{f}	1.405 ± 0.011
	15	0.029 ± 0.002^{a}	0.025 ± 0.001^{b}	0.025 ± 0.002^{b}	0.020 ± 0.001^{c}	0.016 ± 0.001^{d}	0.012 ± 0.001^{e}	1.678 ± 0.009
	20	0.033 ± 0.002^{a}	0.024 ± 0.001^{b}	$0.016 \pm .001^{c}$	0.010 ± 0.000^{d}	0.008 ± 0.000^{e}	0.007 ± 0.000^{e}	0.924 ± 0.002
	25	0.026 ± 0.003^{a}	0.020 ± 0.003^{b}	0.019 ± 0.002^{b}	0.017 ± 0.002^{c}	0.014 ± 0.002^{d}	0.013 ± 0.002^{d}	0.812 ± 0.001
	30	0.022 ± 0.002^{a}	0.011 ± 0.001^{b}	0.010 ± 0.001^{bc}	0.009 ± 0.001^{c}	0.007 ± 0.000^{d}	0.004 ± 0.000^{e}	0.090 ± 0.004
	35	0.020 ± 0.002^{a}	0.009 ± 0.000^{b}	0.009 ± 0.001^{bc}	0.008 ± 0.001^{bc}	0.006 ± 0.001^{d}	0.005 ± 0.000^{d}	0.073 ± 0.001
Days post-imbibition	0	0.026 ± 0.002^{a}	0.012 ± 0.001^{b}	0.011 ± 0.001^{bc}	0.010 ± 0.001^{bc}	0.008 ± 0.001^{d}	0.007 ± 0.001^{d}	0.073 ± 0.002
	3	0.060 ± 0.007^{a}	0.055 ± 0.005^{a}	0.042 ± 0.006^{b}	0.034 ± 0.007^{b}	0.019 ± 0.007^{c}	0.016 ± 0.005^{c}	0.602 ± 0.001
	6	0.092 ± 0.015^{a}	0.067 ± 0.019^{b}	0.037 ± 0.008^{c}	0.031 ± 0.003^{cd}	0.023 ± 0.003^{de}	0.021 ± 0.006^{de}	0.631 ± 0.003
	8	0.076 ± 0.008^{a}	0.048 ± 0.007^{b}	0.038 ± 0.005^{c}	0.029 ± 0.002^{cd}	0.023 ± 0.005^{de}	0.018 ± 0.005^{e}	0.761 ± 0.008
	10	0.074 ± 0.012^{a}	0.055 ± 0.011^{b}	0.045 ± 0.007^{bc}	0.037 ± 0.008^{cd}	0.029 ± 0.006^{de}	0.026 ± 0.008^{de}	1.146 ± 0.013
	12	0.042 ± 0.010^{a}	0.037 ± 0.008^{ab}	0.028 ± 0.008^{bc}	0.025 ± 0.004^{bcd}	0.024 ± 0.009^{bcde}	0.022 ± 0.006^{cde}	1.383 ± 0.023
	15	0.007 ± 0.004^{a}	0.006 ± 0.001^{a}	0.006 ± 0.002^{a}	0.005 ± 0.002^{b}	0.005 ± 0.004^{b}	0.004 ± 0.001^{b}	2.655 ± 0.003

IC_{50} values = concentrations of inhibitor producing 50% inhibition. Each value represents the mean ± SE obtained from six independent measurements. Means were compared by Tukey's test. Different letters indicate significant differences between groups at the 5% level.

Table 3. Kinetic parameters of PEPC from sorghum seeds at different days post-anthesis

Kinetic parameter	Days post-anthesis						
	5	10	15	20	25	30	35
K_M (PEP mM)	0.128 ± 0.001	0.192 ± 0.003	0.194 ± 0.000	0.150 ± 0.002	0.141 ± 0.001	0.098 ± 0.002	0.067 ± 0.001
IC_{50} (L-malate mM)	1.107 ± 0.013	1.405 ± 0.011	1.678 ± 0.009	0.924 ± 0.002	0.812 ± 0.001	0.090 ± 0.004	0.073 ± 0.001
K_a (Glc-6-P mM)	5.876 ± 0.006	5.670 ± 0.003	5.498 ± 0.002	5.292 ± 0.004	5.063 ± 0.009	5.196 ± 0.002	5.776 ± 0.001
Ratio PEPC activity at pH 8.0/7.1	1.464 ± 0.001	1.442 ± 0.001	1.311 ± 0.002	1.350 ± 0.000	1.116 ± 0.001	1.151 ± 0.013	1.143 ± 0.003

K_M = Michaelis constant; IC_{50} and K_a values = concentrations of inhibitors and activators producing 50% inhibition and activation, respectively. Each value represents the mean ± SE obtained from six independent measurements.

Table 4. Kinetic parameters of PEPC from sorghum seeds at different days post-imbibition

Kinetic parameter	Days post-imbibition						
	0	3	6	8	10	12	15
K_M (PEP mM)	0.068 ± 0.001	0.087 ± 0.002	0.093 ± 0.001	0.069 ± 0.000	0.062 ± 0.002	0.048 ± 0.001	0.042 ± 0.002
IC_{50} (L-malate mM)	0.073 ± 0.002	0.602 ± 0.001	0.631 ± 0.003	0.761 ± 0.008	1.146 ± 0.013	1.383 ± 0.023	2.655 ± 0.003
K_a(Glc-6-P mM)	5.776 ± 0.004	2.356 ± 0.011	2.263 ± 0.013	2.304 ± 0.002	1.743 ± 0.008	1.126 ± 0.006	1.225 ± 0.003
Ratio PEPC activity at pH 8.0/7.1	1.143 ± 0.008	2.582 ± 0.003	2.353 ± 0.002	2.247 ± 0.002	1.730 ± 0.001	1.443 ± 0.001	1.163 ± 0.002

K_M = Michaelis constant; IC_{50} and Ka values = concentrations of inhibitors and activators producing 50% inhibition and activation, respectively. Each value represents the mean ± SE obtained from six independent measurements.

Various methods have been used to study the PEPC phosphorylation: incorporation of ^{32}P into PEPC, immunochemical detection of phospho-serine in the N-terminal domain of PEPC, lower activity after treatment with phosphatases, change in sensitivity of PEPC with L-malate or change in activity ratio at pH 8.0 and 7.1 [5, 11]. The difference in the kinetic parameters of the PEPC is not universal for all vegetable PEPCs. Therefore, this study aimed to determine the kinetic parameters of the PEPC of the sorghum grain during maturation and germination.

Our results showed that PEPC is sensitive to the effect of L-malate and pH in developing grains. Thus, the IC_{50} and the activity ratio at pH 8.0/7.1 increased in the early days and then decreased from 20 DPA to the mature seeds (35 DPA). After the imbibition of the grains, the IC_{50} and PEPC activity ratio at pH 8.0/7.1 increased during germination. This means that the state of phosphorylation evolves with the time of these two life cycle processes. During maturation, PEPC phosphorylated at the early days of maturation and dephosphorylated at the later days of maturation. The phosphorylation of the enzyme occurred again during the first days of germination. These results are consistent with the work done on sorghum leaves. They also correspond to those obtained on wheat and barley for which the PEPC is stored in its non-phosphorylated form in mature grains and it is only after the imbibition that the enzyme becomes phosphorylated [11]. A recent study by Cerný et al. [21] suggested that a homotetrametric class-1 PEPC type exists in a phosphorylated form in mature corn kernels [21]. However, previous work on sorghum grains [16, 18] showed that despite the presence of PEPC kinase in dry seeds, the PEPC enzyme is dephosphorylated. Indeed, during the maturation of the grains, the environment of the seed is very enriched by the L-malate which prevented the phosphorylation of the enzyme and it is only after imbibition of the grains that this acid is used. This would cause phosphorylation of PEPC by a PEPC-K already present in the dry seed.

It is now well established that PEPC is regulated metabolically by Glc-6-P allosteric activation and it is inhibited by L-malate [4, 10]. Our study showed a significant increase in PEPC activity at the early days of maturation and seed germination by Glc-6-P. In contrast, L-malate inhibits PEPC activity, this inhibition is important at the later days of maturation and early days of germination. These changes in inhibition and activation by L-malate and Glc-6-P at suboptimal pH during maturation and germination of sorghum grains reflect changes in the apparent phosphorylation state of PEPC. Indeed, phosphorylation modulates the metabolic regulation of PEPC by weakening the inhibitory effect of L-malate and by increasing the activity at alkaline pH [27]. This post-translational modification is seen as a mechanism of protection of PEPC against this retro-inhibition caused by L-malate in physiologi-

cal condition [3]. The results of a previous work [28] showed that the PEPC activity in the grapes is more important in the stages where the concentration of malic acid reaches its maximum. It has recently been shown that the phosphorylated form of PEPC extracted from maize grain is less inhibited by L-malate [21]. Photosynthetic metabolites are not only effectors of PEPC activity but also their level of phosphorylation by PEPC-K, which is also metabolically regulated in the same sense as PEPC [29].

The determination of the affinity constant showed different K_M depending on the seed developmental stage. This result may indicate the presence of two forms of PEPC as it may indicate differential regulation of a preexisting isoform. The study conducted by Ruiz-Ballesta et al. [30] on the post-translational modification of multiple phosphoenolpyruvate carboxylase isoenzymes in developing sorghum seeds, showed no expression of the photosynthetic isoform (C_4-isoform) during seed development. This result indicates that the different K_M observed during seed maturation could be a result of a post-translational regulation such as phosphorylation or ubiquitination of a pre-existing isoform. In fact, Ruiz-Ballesta et al. [30] showed differential expression of three gene isoforms of the PEPC-C_3 during seed maturation, however, no studies on the regulation of these isoforms have been conducted. Therefore, the present study initiates the work on the regulation of PEPC in developing seeds and argued an enhancement of the PEPC activity during the early stages of seed maturation with differential affinity to PEP compared to mature seeds.

In conclusion, we have shown that PEPC activity was detected at various stages of developing and germinating sorghum seed with different extent between stages. We showed that the apparent phosphorylation status changes with the process of developing and germinating seed. Moreover, PEPC from sorghum seed was subjected to metabolic control (G-6-P and L-malate). The sensitivity of PEPC to these effectors reflects its phosphorylation status. In regard with the results presented in this study, we speculate that PEPC might play a key role in seed maturation and germination.

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