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An *in vitro* Coupled Assay for PEPC with Control of Bicarbonate Concentration

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[Abstract] Phosphoenolpyruvate carboxylase (PEPC) catalyzes a critical step in carbon metabolism in plants and bacteria, the irreversible reaction between bicarbonate and phosphoenolpyruvate to produce the C_4 compound oxaloacetate. This enzyme is particularly important in the context of C_4 photosynthesis, where it is the initial carbon-fixing enzyme. Many studies have used kinetic approaches to characterize the properties of PEPCs from different species, different post-translational states, and after mutagenesis. Most of these studies have worked at a fixed saturating concentration of bicarbonate. Controlling the concentration of bicarbonate is difficult at low concentrations because of equilibration with atmospheric CO_2. We describe here a simple, repeatable, and gas-tight assay system for PEPC that allows bicarbonate concentrations to be controlled above *ca.* 50 µM.

Keywords: PEPC, C_4, Bicarbonate assay, Gas-controlled assay, Malate dehydrogenase coupled assay

[Background] The enzyme phosphoenolpyruvate carboxylase (PEPC; E.C. 4.1.1.31) catalyzes the essentially irreversible reaction between bicarbonate and phosphoenolpyruvate (PEP) to form oxaloacetate and inorganic phosphate. This reaction is a critical step in carbon metabolism in plants and bacteria, but the enzyme is most widely studied in the context of its critical role in C_4 photosynthesis, where it is responsible for primary carbon fixation. In typical PEPC assays, the enzyme is coupled to malate dehydrogenase (MDH), which converts oxaloacetate to malate, consuming NADH, and leading to a decrease in absorbance at 340 nm. This reliable coupled assay has been used extensively to compare PEPCs from different species and to understand the consequences of post-translational modification on inhibitor binding and affinity for PEP (Janc *et al.*, 1992; Duff *et al.*, 1995; Blasing *et al.*, 2000; Jacobs *et al.*, 2008; Paulus *et al.*, 2013). Studies where the concentration of bicarbonate is varied are more challenging, as background bicarbonate, arising from equilibration with atmospheric CO_2, is found at concentrations above the K_m for bicarbonate (often less than 100 µM), preventing accurate kinetic measurements. Measuring the rate of the PEPC reaction at low bicarbonate concentrations requires that background bicarbonate be removed as much as possible, and careful gas-tight assay procedures. Accurate kinetic measurements also require determining the concentration of residual bicarbonate, allowing calculation of the correct concentration of substrate.

We describe here a set of methods to remove most background bicarbonate, quantify the remaining amount, and reliably assay the enzyme without significant contamination from atmospheric CO_2. Using these methods, we can reliably reduce background bicarbonate to less than 50 µM.

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Overview: The methods described here are simple and repeatable. The major assay components, buffer and water, are sparged with nitrogen gas to reduce the background bicarbonate concentration. Assays are constructed and sealed under nitrogen to minimize contamination from atmospheric carbon dioxide. Assays are then initiated by the addition of PEPC, delivered with a gastight syringe. Background bicarbonate is determined using an endpoint assay.

Limitations: While we have found these methods suitable for measuring the properties of a wide range of PEPC enzymes (Bauwe, 1986; Phansopa *et al.*, 2020), they are not universally applicable. For PEPC enzymes with $K_m^{bicarbonate}$ much below the residual background bicarbonate, the experimental design and data analysis described here are not suitable, so an alternative approach using the integrated Michaelis-Menten equation is recommended. This alternative approach requires additional controls to overcome problems associated with product inhibition, enzyme instability, and product instability leading to regeneration of CO_2 in solution; these are not described here and the interested reader should consult the careful work of Bauwe (1986), and DiMario and Cousins (2019).

Also, as the product oxaloacetate is converted into malate in the assay described here, it is not possible to carry out product inhibition studies using oxaloacetate. This limitation could be resolved by detecting a substrate or the other product. An elegant alternative detection system for gaseous CO_2, coupled to the substrate concentration through carbonic anhydrase, has been described using membrane-inlet mass spectrometry (DiMario and Cousins, 2019). Our attempts to develop an alternative assay to monitor the production of inorganic phosphate using the purine nucleotide phosphorylase assay (Webb, 1992) were unsuccessful, due to inhibition of PEPC by components of the coupling system (unpublished work).

Materials and Reagents

1. Pipette tips
2. 0.2 µm pore filter
3. 1.5 ml mini-centrifuge tubes (Eppendorf, catalog number: 0030120159)
4. 0.5 ml sealable UV-Cuvette (Fisher Scientific, catalog number: 10386712)
5. Whatman membrane filters, nylon pore size 0.2 µm, diameter 47 mm (Millipore Sigma, catalog number: WHA7402004)
6. Parafilm (Bemis, catalog number: 11772644)
7. Two rubber septa for ST/NS 24/40 joint (Millipore Sigma, catalog number: Z553980)
8. Two glass stoppers for ST/NS 24/40 joint (Millipore Sigma, catalog number: Z229571)
9. 12.5 mm rubber septa for sealing cuvettes (Millipore Sigma, catalog number: Z167274)
10. Three pieces of 1 m long 4 mm bore rubber tubing (Fisher Scientific, catalog number: 11876293)
11. A glass funnel
12. Two 5 ml syringes (Terumo, catalog number: Z116866)
13. Two 120 mm, 21-gauge needles (Sterican, catalog number: 466 5643)
14. Two 40 mm, 21-gauge small needles (BD Microlance 3, catalog number: 304432)

15. PEPC Enzyme: Wild type or mutant PEPC purified from *E. coli*, stored at -80°C
16. Phosphoenolpyruvate trisodium salt (PEP, Millipore Sigma, catalog number: P7002), store at -20°C
17. Malate dehydrogenase enzyme (MDH, Millipore Sigma, catalog number: M2634), store at 4°C
18. β-Nicotinamide adenine dinucleotide (NADH, Millipore Sigma, catalog number: 10107735001), store at -20°C
19. Magnesium chloride ($MgCl_2$, Millipore Sigma, catalog number: 63069)
20. Potassium bicarbonate ($KHCO_3$, Millipore Sigma, catalog number: 60339)
21. Tricine (Millipore Sigma, catalog number: T0377)
22. Tris(hydroxymethyl)aminomethane (Tris base) (Millipore Sigma, catalog number: 252859)
23. Potassium chloride (KCl, Millipore Sigma, catalog number: T0377)
24. Potassium hydroxide (KOH, Millipore Sigma, catalog number: 757551)
25. Ultrapure deionised water
26. Tris-HCl (see Recipes)
27. Tricine-KOH (see Recipes)

Equipment

1. Pipettes
2. 1700 series gastight syringe with a cemented needle (Hamilton, catalog number: 80200)
3. Nitrogen gas supply
4. Nitrogen gas canister (99.998% minimum N_2, BOC, catalog number: 44-W)
5. Two 250 ml two-neck round bottom flasks (Millipore Sigma, catalog number: Z516872)
6. Glass single bank manifold with three positions (Millipore Sigma, catalog number: Z532169)
7. Magnetic stirrers
8. Magnetic stirrer plate
9. A split-beam spectrophotometer with temperature control. For example, a Cary 300 UV-Vis spectrophotometer (Agilent). A cell changer is not essential but it is convenient.

Software

1. Cary WinUV software (Agilent, www.agilent.com)
2. Igor Pro (Version 7.0.8.1; Wavemetrics Inc., Lake Oswego, Oregon, www.wavemetrics.com) or equivalent package capable of non-linear regression analysis.

Procedure

A. Preparation of CO_2-free assay components

This procedure takes more than 12 h, so should be begun the day before assays are planned. The

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aim is to remove as much dissolved HCO_3^- as practically possible, by sparging the major assay components with nitrogen gas. Water and buffer make up *ca.* 75% of the assay solution, and are sufficiently stable to be sparged with nitrogen for extended periods. A typical degassing set-up will involve one solution of buffer and one solution of deionised water (Figure 1).

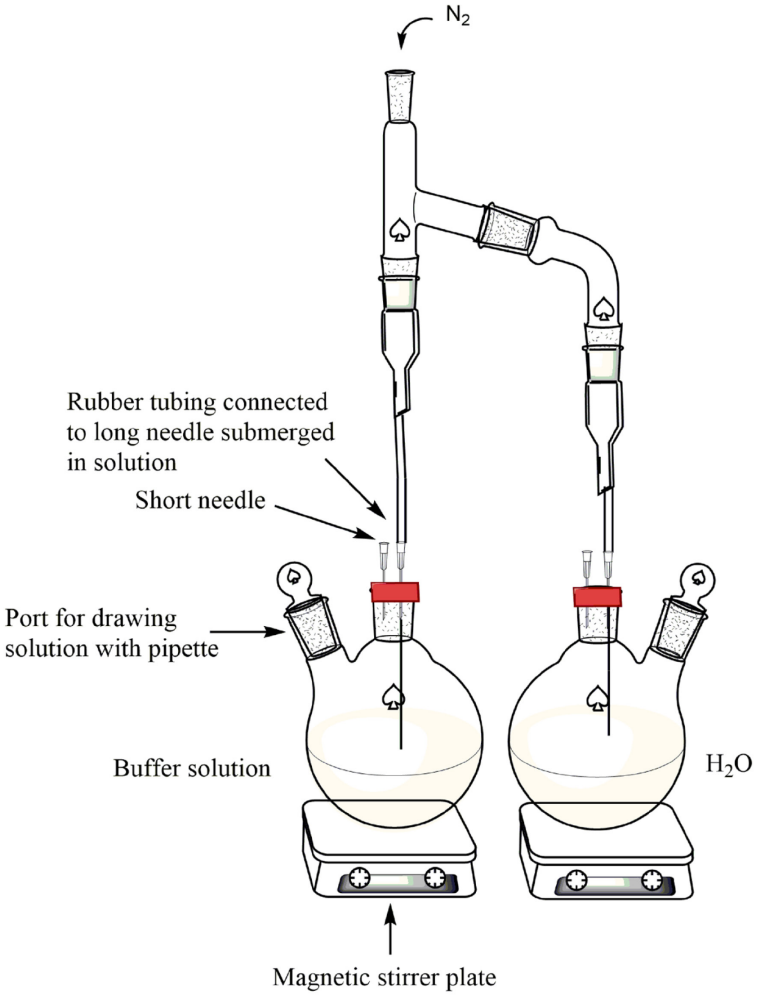

Figure 1. A typical degassing set-up to reduce the concentration of dissolved CO_2 in the assay components.

1. Prepare buffer stock solutions (200 mM Tris-HCl pH 7.4, or Tricine-KOH pH 8.0, as described below) and obtain ultrapure water. Decant the solutions (*ca.* 50 ml) into separate double-neck round bottom flasks, and add a magnetic stirrer. Seal one of the necks of the round bottom flasks with a rubber septum, and seal the other with a glass stopper. Septa may be reinforced with parafilm to prevent gas leaks.
2. Through one septum, pass a long needle, ensuring the tip of the needle is submerged in the assay component. Pass a short needle through the same septum. Create a manifold for the needle by inserting the end of the tubing into the syringe body. Use parafilm to seal the join between the syringe body and the tubing. Connect the long needle to the nitrogen supply.

3. To begin the sparging process, turn on the gas supply indicated by bubbles emanating from the long needle. Ensure the bubbles are produced at no more than *ca.* one bubble per second. A faster supply will result in the loss of assay solution. Turn on the magnetic stirrer. Check all joins for any nitrogen leaks, and patch with parafilm if necessary.
4. Extensively sparge with nitrogen in the tightly sealed container for 12 h prior to use.
5. After a sparging period, turn off the nitrogen supply. Connect the set-up to a high-purity nitrogen cylinder. Bubble the gas for 1 h prior to the first assay at a similar rate, *ca.* one bubble per second.
6. Remove the needles and seal the flask until use. As some background bicarbonate will remain after this procedure, the background concentration will need to be determined as described below in Step B4 and Procedure C. The reduced bicarbonate concentration will be stable for eight hours.

B. Instrument set-up, reagent preparation, and quality control

The concentration determinations described here must be carried out every day that kinetic measurements will be performed, and not just when stock solutions are prepared, defrosted, or enzymes purified. It is convenient to measure the concentrations of enzyme and substrates during the final sparge of buffer with high-purity nitrogen (1 h, as described in Step A5).

1. Instrument set-up

   This procedure requires access to a high-quality split-beam spectrophotometer with temperature control. We have not found plate-readers suitable, due to the difficulties in preventing assays equilibrating with atmospheric CO_2; if this could be solved, this procedure would be much less laborious. The instructions are given for Cary spectrophotometers, but could easily be adapted to equipment from any comparable supplier. The spectrophotometer equipment will require room for the cuvette to be seated with a gastight seal.

   Turn on the instrument at least 30 min before beginning measurements to allow the lamp to stabilize. Turn on the water bath or Peltier temperature control and set to 25°C.
2. Preparation of coupled assay materials (malate dehydrogenase and NADH)

   Remove NADH from the freezer (-20°C) and allow it to come to room temperature before opening to prevent condensation. Prepare a NADH solution [355 mg in 25 ml for a *ca.* 20 mM stock in water (MiliQ)]. Determine the concentration spectrophotometrically ($\varepsilon_{340} = 6220 M^{-1} cm^{-1}$). These solutions can be frozen (-20°C) in aliquots and remain stable for approximately 1 month. Do not refreeze. Malate dehydrogenase is stored at 4°C until use.
3. Preparation of phosphoenolpyruvate (PEP) stock solution.

   Remove from the freezer (-20°C) and allow to come to room temperature before opening to prevent condensation. Prepare a stock solution (15 ml of total solution with 4.3 g PEP should result in *ca*. 400 mM PEP solution). As PEP is supplied with an unknown amount of water of hydration, the concentration needs to be determined spectrophotometrically by an endpoint assay. Prepare assays in triplicate as described below (Procedure C) with 10 mM bicarbonate

and 100 µM PEP (estimated). A ΔA_{340} = 0.622 is expected under these conditions; determine your stock PEP concentration using the extinction coefficient above. In this assay, it is essential that bicarbonate is in excess of PEP. This stock solution can be frozen (-20°C) and remains stable for a month.

4. Determination of background bicarbonate (total dissolved CO_2).
   The effectiveness of removing background CO_2 needs to be determined with an endpoint assay. If the background bicarbonate concentration is above 50 µM, the major components will need to be sparged for a further hour with high purity nitrogen. Prepare assays in triplicate as described below (Procedure C) with no added bicarbonate and 20 mM PEP. It is essential that PEP is in excess of the background bicarbonate.

C. Endpoint assay of PEP and background bicarbonate

These assays for PEP and background bicarbonate are required to establish accurate substrate concentrations. The method is essentially the same as the kinetic assays, except we are interested in the total change in signal at 340 nm, not the initial rate. Concentrations of PEPC used can be adjusted to ensure that essentially full conversion to products is seen in a reasonable time frame. If you have access to a cell changer, these can be run in parallel.

Troubleshooting tip: If your assays are not gas tight, you will see a slow linear phase in the bicarbonate assay, indicative of gas exchange with the atmosphere.

1. Connect a glass funnel to the normal nitrogen supply to create a field of nitrogen under which to assemble the assay. Add PEP, $MgCl_2$, NADH, KCl, MDH, and bicarbonate (if required) to the empty cuvette (Table 1). Run the nitrogen through the funnel to create a field of nitrogen, and place the cuvette with the components under the nitrogen. After removing the glass stopper, take the sparged water and buffer from the round-bottomed flasks. It is not recommended to stop the nitrogen flow through the buffer and water until all assays are assembled. Replace the glass stopper as quickly as possible to ensure the round bottom flasks are sealed after use. Pipette mix the assay components under nitrogen and seal with a septum.

Table 1. PEPC Assay construction

Component	Final Concentration	Typical volume	Stock Concentration
Buffer (Tris-HCl pH 7.4 or Tricine-KOH pH 8.0)	50 mM	150 µl	200 mM
H_2O (MilliQ, degassed)		Adjust to give 0.6 ml total volume.	
$MgCl_2$	20 mM	60 µl	200 mM
NADH	150 µM	6 µl	15 mM
MDH [a]	0.01 U/µl	0.6 µl	10 U/µl
PEP [b]	Varies		400 mM
$KHCO_3$ [c]	Varies	x µl	6 mM
KCl [d]		y µl = x_{max} - x µl	6 mM
PEPC [e]	50 nM	10 µl	3 µM

Notes:

a. *MDH concentration varies by batch, adjust volumes accordingly.*
b. *Use analytical concentrations of PEP.*
c. *Adjust final concentration of bicarbonate to take into account the background. Note that this is total dissolved CO_2, the true bicarbonate concentration varies with pH and I.*
d. *KCl is added to prevent variation in I resulting from varying the $KHCO_3$ and to keep a constant [K+]; x+y (µl) should be constant.*
e. *Concentration of PEPC stocks vary. As 10 µl is a convenient volume to pipette accurately, it is often useful to produce a 3 µM working stock; check this concentration spectrophotometrically.*

2. Place the assembled assay in the spectrophotometer. Measure the absorbance at 340 nm for approximately 30 seconds, to determine the starting absorbance. Pause data acquisition and deliver 50 nM PEPC (final concentration) with a gas-tight Hamilton syringe (generally *ca.* 100 µl). Gently tip the cuvette from side to side to mix (do not invert) and return the cuvette to the spectrophotometer. Measure the absorbance for 30 min. In this time a stable endpoint will be reached. Record the absorbance of the endpoint. Determine the concentration of background bicarbonate or PEP stock from the difference in absorbance measurements.
3. Repeat the endpoint assay three times to ensure consistent concentrations are determined. Report concentrations as mean and standard deviation.

D. Kinetic Assays

The goal of these assays is to determine an accurate set of initial rates, either at fixed (saturating) concentration of PEP, or varying the concentration of both PEP and bicarbonate. Reaction times and enzyme concentrations should be adjusted as needed, to ensure that data are collected in the initial rate phase of the reaction; progress to completion is not required. Assays are essentially constructed as described above. It is often useful to plot initial rates as you are collecting them; this allows 'on-the-fly' adjustment of the experimental design to ensure that an adequate concentration range is covered. In particular, if the lowest concentration of bicarbonate used is above the apparent K_m, then modify your design to include additional points at lower concentrations.

1. Construct the assays as before (Table 1), combining all the non-degassed components on a normal benchtop, then filling the assay with nitrogen gas, adding the sparged components, and sealing the cuvette under the field of nitrogen gas.
2. Initiate the assay by the addition of 50 nM PEPC with a gas-tight Hamilton syringe (as before). Mix by gently shaking the cuvette from side to side (do not invert).
3. Introduce the cuvette to the spectrophotometer and start recording. Generally, absorbance at 340 nm is measured for 15 min.
4. Calculate the initial rate using the instrument software (*e.g.*, kinetic ruler in the Cary WinUV software). For each point, record the concentration of both substrates, enzyme (if varied at any

point during the day), initial rate, units (*e.g.*, μMs^{-1}, $\Delta A_{340}\, min^{-1}$), and note if the trace looks in any way suspicious (*e.g.*, non-linear, substantially lower or higher rate, or noise than expected). Rates will need to be converted into units of change in concentration with time; this can be done either immediately after the end of the assay or batchwise at the end of the day.

5. Each concentration point should be run in triplicate. Assays should not be run in order of concentration; vary concentration of substrates between assays to prevent systematic error arising from any time-dependent decay of reagents.
6. Repeat an endpoint assay halfway through data collection and at the end, to make sure the background bicarbonate has not increased. If it has increased, sparge the buffer solution and water for an hour with high purity nitrogen, and reperform the endpoint assay.
7. Plot the calculated reaction velocities against bicarbonate concentration in Igor Pro (or other suitable software package), then fit using the appropriate kinetic model. Illustrative curves can be found in the literature (*e.g.*, Figure 1 in Moody *et al.*, 2020). As a guide, we find that a v_i/[E] of *ca.* 40 s^{-1} are reasonable at saturating PEP and bicarbonate.

Data analysis

At fixed single concentrations of PEP, the data can be analyzed using the Michaelis-Menten equation (Equation 1) where $v_i/[E_T]$ is the steady-state rate divided by the total enzyme concentration, k_{cat} is the turnover number, K_m is the Michaelis constant, and [S] is the substrate concentration. Estimated standard errors of parameter values are provided directly by the software and standard errors in k_{cat}/K_M can be estimated using propagation of errors; if following this procedure, it is essential to include the covariance term (Equation 3).

$$\frac{v_i}{[\mathrm{E}]_\mathrm{T}} = \frac{k_{cat}[S]}{K_m + [S]} \qquad \text{Equation 1}$$

Alternatively, when both substrates are varied, the data can be described by Equation 2, where A and B are the two substrates.

$$\frac{v_i}{[\mathrm{E}]_\mathrm{T}} = \frac{k_{cat}[A][B]}{K_I^A K_M^B + K_M^A[B] + K_M^B[A] + [A][B]} \qquad \text{Equation 2}$$

In both cases, choose a range of substrate concentrations that span a 0.1 K_M-10 K_M range. Generally, 8 to 12 different substrate concentrations should be used when collecting data at a fixed concentration of PEP (*i.e.*, analyzing using Equation 1). When both substrate concentrations are varied, then at least five concentrations of each (*i.e.*, 25 assays in total) should be used.

The standard error in k_{cat}/K_m can be calculated from the variances in k_{cat} and K_m, and the covariance (Equation 3 [Bevington and Robinson, 1992]). Here σ_{Km}, σ_{kcat}, $\sigma_{kcat/Km}$, cov(k_{cat}, Km) are the standard error of K_m, k_{cat}, k_{cat}/K_m, and the covariance, respectively. Note that Igor Pro reports the variances

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(*i.e.*, σ^2) in the variance-covariance matrix.

$$\sigma_{kcat/Km} = \frac{k_{cat}}{K_m}\sqrt{\left(\frac{\sigma_{kcat}}{k_{cat}}\right)^2 + \left(\frac{\sigma_{Km}}{K_m}\right)^2 - 2\frac{\mathrm{cov}(k_{cat}, K_m)}{k_{cat}\times K_m}} \qquad \text{Equation 3}$$

Recipes

1. Tris-HCl, pH 7.4
   a. Dissolve 24.23 g of Tris base in *ca.* 900 ml of water, titrate to pH 7.53 (assuming a lab temperature of 20°C).
   b. Adjust for actual lab temperature using $\Delta pK_a/\Delta T$ = -0.028 and bring to a final volume of 1 L in a volumetric flask. The buffer is then passed through a 0.2 µm pore filter.
2. Tricine-KOH, pH 8.0
   a. Dissolve 35.84 g of Tricine free acid in *ca.* 900 ml of water, titrate to pH 8.10 (assuming a lab temperature of 20°C.
   b. Adjust for actual lab temperature using $\Delta pK_a/\Delta T$ = -0.021) and bring to a final volume of 1 L in a volumetric flask. The buffer is then passed through a 0.2 µm pore filter.

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Competing interests

The authors declare no conflict of interests.

References

1. Bauwe, H. (1986). An efficient method for the determination of K_m values for $HCO3^-$ of phosphoenolpyruvate carboxylase. *Planta* 169: 356-360.
2. Bevington, P. R., and Robinson, D. K. (1992). Data reduction and error analysis for the physical sciences. McGraw-Hill, New York.
3. Blasing, O. E., Westhoff, P. and Svensson, P. (2000). Evolution of C_4 phosphoenolpyruvate carboxylase in *Flaveria*, a conserved serine residue in the carboxyl-terminal part of the enzyme is a major determinant for C_4-specific characteristics. *J Biol Chem* 275(36): 27917-27923.
4. DiMario, R. J. and Cousins, A. B. (2019). A single serine to alanine substitution decreases

bicarbonate affinity of phosphoenolpyruvate carboxylase in C4*Flaveria* trinervia. *J Exp Bot* 70(3): 995-1004.

5. Duff, S. M., Andreo, C. S., Pacquit, V., Lepiniec, L., Sarath, G., Condon, S. A., Vidal, J., Gadal, P. and Chollet, R. (1995). Kinetic analysis of the non-phosphorylated, *in vitro* phosphorylated, and phosphorylation-site-mutant (Asp8) forms of intact recombinant C4 phosphoenolpyruvate carboxylase from sorghum. *Eur J Biochem* 228(1): 92-95.
6. Jacobs, B., Engelmann, S., Westhoff, P. and Gowik, U. (2008). Evolution of C4 phosphoenolpyruvate carboxylase in *Flaveria*: determinants for high tolerance towards the inhibitor L-malate. *Plant Cell Environ* 31(6): 793-803.
7. Janc, J. W., O'Leary, M. H. and Cleland, W. W. (1992). A kinetic investigation of phosphoenolpyruvate carboxylase from Zea mays. *Biochemistry* 31(28): 6421-6426.
8. Moody, N. R., Christin, P. A., and Reid, J. D. (2020). Kinetic Modifications of C4 PEPC Are Qualitatively Convergent, but Larger in *Panicum* Than in *Flaveria*. *Front Plant Sci* 11: 1014.
9. Paulus, J. K., Schlieper, D. and Groth, G. (2013). Greater efficiency of photosynthetic carbon fixation due to single amino-acid substitution. *Nat Commun* 4(1): 1518.
10. Phansopa, C., Dunning, L. T., Reid, J. D. and Christin, P. A. (2020). Lateral gene transfer acts as an evolutionary shortcut to efficient C4 biochemistry. *Mol Biol Evol* 37(11): 3094-3104.
11. Webb, M. R. (1992). A continuous spectrophotometric assay for inorganic phosphate and for measuring phosphate release kinetics in biological systems. *Proc Natl Acad Sci U S A* 89(11): 4884-4887.

