

Original article

Carbon dioxide concentrations are very high in developing oilseeds

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Abstract

A new method has been developed to rapidly determine the total inorganic carbon concentration (gaseous $[\text{CO}_2]$ + aqueous $[\text{CO}_2]$ + $[\text{HCO}_3^-]$ + $[\text{CO}_3^{2-}]$) in developing seeds. Seeds are rapidly dissected and homogenized in 1 N HCl in gas-tight vials. The headspace gas is then analyzed by infrared gas analysis. Developing rapeseed (*Brassica napus* L.) and soybean [*Glycine max* (L.) Merr.] seeds were analyzed and found to have up to 40 and 12 mM total inorganic carbon, respectively. These concentrations are ca. 600–2000-fold higher than in ambient air or values reported for leaves. Carbon dioxide concentrations in rapeseed peaked during the stage of maximum oil synthesis and declined as seeds matured. The consequences for seed metabolism, physiology and carbon economy are discussed.

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1. Introduction

In many seeds large amounts of triacylglycerols are synthesized during seed filling, accounting, for example, for more than 45% of the total seed dry weight of oilseed rape (*Brassica napus* L.) [18]. Because fatty acids contain more carbon and chemical potential energy than either protein or carbohydrates, even seeds such as soybeans with lower oil content (20%), store as much energy in oil as in protein. The primary substrate for oil biosynthesis is the sucrose transported from the photosynthetic tissues to the developing seeds. Sucrose is initially broken down by invertase into hexose units, which are further metabolized via glycolysis or the oxidative pentose phosphate pathway to triose that is used to produce acetyl-CoA units, which are the building blocks of fatty acid synthesis (FAS). For each two carbon unit added to the growing acyl chain of fatty acids, one carbon dioxide is produced by the pyruvate dehydrogenase (PDH) reaction,

and therefore, one third of the carbon supplied as sugars that is devoted to FAS is released as CO_2 . Considering the high rates of FAS during oilseed development, the production of CO_2 is high. For example, a *B. napus* embryo at the midstage of development synthesizes $\sim 5 \mu\text{g}$ of fatty acids per h [19], which will require $\sim 100 \text{ nmol}$ of acetyl-CoA and will be accompanied by $\sim 100 \text{ nmol}$ CO_2 . The gaseous CO_2 concentration in the silique locus has been reported to range between 0.8% and 2.5% [13,14], and it is assumed that seed respiration is the major contributor to this high level of CO_2 . Furthermore, because the seed coat surrounding the developing embryo is a major barrier for gas diffusion [14], it is expected that developing seeds have either an elevated CO_2 concentration within the tissue or have developed mechanisms to deal with such high CO_2 levels, for example, by exporting CO_2 after incorporation into malate. The substantial production of CO_2 during oil synthesis will clearly impact the overall carbon economy of seeds and may also influence several aspects of physiology and metabolism of seeds. However, to our knowledge the CO_2 content of developing seeds has not previously been determined. In order to address this question, we describe a new method for measuring seed CO_2 concentration. This procedure has been used to determine CO_2 levels in developing seeds of rapeseed and soybean and to monitor CO_2 concentrations at different stages of seed filling in developing rapeseeds.

Abbreviations: CO_2 (aq.), aqueous carbon dioxide; CO_2 (g), gaseous carbon dioxide; DAF, days after flowering; FAS, fatty acid synthesis; IRGA, infra red gas analyzer; $p\text{CO}_2$, partial pressure of CO_2 ; PDH, pyruvate dehydrogenase; Rubisco, ribulose-1,5-biphosphate carboxylase/oxygenase; S.E., standard error.

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2. Results and discussion

2.1. Testing the seed CO₂ determination method

In order to determine the CO₂ concentration within seeds we needed a method that could sample a heterogeneous tissue containing seed coat, endosperm liquid and embryo. Because dissection of the seed can allow loss of CO₂ during handling we chose a method in which seeds were sealed in vials (Fig. 1) immediately after removal from the silique. HCl was added to convert all forms of inorganic carbon to CO₂ and the seeds were homogenized with glass beads. Gaseous CO₂ was then determined with an infra red gas analyzer (IRGA). Developing rapeseed (*B. napus* L.) and soybean [*Glycine max* (L.) Merr.] seeds were analyzed and found to have very high total CO₂, up to 40 and 12 mM, respectively. These concentrations are ca. 600–2000-fold higher than in ambient air or in leaves and to our knowledge are among the highest concentrations ever reported in plants. We found no significant difference in CO₂ concentration when seeds were homogenized immediately or after 10 min ($P < 0.05$, Table 1), indicating that CO₂ levels inside seeds do not change during the handling of siliques and removal of seeds. Therefore, a reliable measurement is possible of several samples collected on the same sampling day. This lack of change in seed CO₂ during handling probably reflects the barrier properties of the seed coat [14]. The method described is applicable to a range of seed sizes and types by adjustment of the sample vial size. The only limitation we found is that the seeds to be measured must be soft enough to allow complete tissue homogenization. Mature or dry seeds are difficult to homogenize with glass beads and thus cannot be measured by this method, although more aggressive ho-

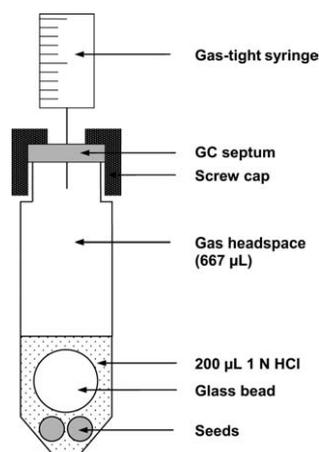


Fig. 1. Diagram of the vial system used to prepare seed samples for measuring CO₂ levels.

Table 1
Effect of processing time on seed CO₂ concentration

Treatment (min)	CO ₂ (mM)	S.D.
0	23.7 ^{NS}	1.8
10	22.4	2.1

^{NS} No significant difference at $P = 0.05$.

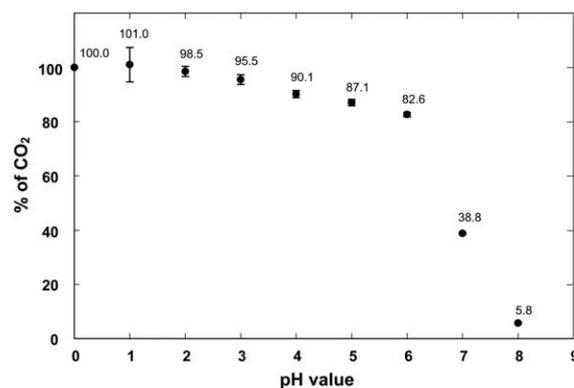


Fig. 2. Relative percentage of gaseous CO₂ (pH 0, 100% CO₂) in vial headspace at different pH values. Error bars represent the S.D. of the means. Data labels indicate the average % of CO₂.

mogenization using steel balls might be successful. The standard error (S.E.) of the present method for the analysis of seed CO₂ concentration in rapeseed ranged from ~2% at 40 mM to ~10% at 8 mM. The S.E. we found represents ca. 4.0% of the mean seed CO₂ concentration (19.9 mM) in rapeseed during development. The S.E. of the analysis of CO₂ levels in soybean seeds was also low (0.6 mM), and represented 5.2% of the average CO₂ concentration found in soybean seeds (11.6 mM). These results indicate that the method is highly reproducible for determining CO₂ levels inside the seeds.

Since 1 mol of NaHCO₃ will release 1 mol of CO₂ when acidified, we calculated the maximal theoretical CO₂ concentration in the headspace for different sodium bicarbonate concentrations in the described vial system, and we compared these values to actual measurements of vial headspace CO₂. Carbon dioxide concentration in the headspace was on average 89.6% (S.D. = 2.1%) of the maximal theoretical gaseous CO₂, indicating that most of the CO₂ produced is in the gas phase. The effect of pH on CO₂ concentration in the gas phase is presented in Fig. 2. As expected, at pH higher than 8 almost no CO₂ (aq. and g) is present in the biphasic (gas/liquid) system (5.8% of the inorganic carbon is present as CO₂), whereas at pH lower than three, aqueous plus gaseous CO₂ account for more than 96% of all inorganic carbon. Therefore, by reducing the homogenate pH to values lower than two, we ensure that almost all inorganic carbon is transformed into aqueous and gaseous CO₂, which allows a quantitative measurement of CO₂. Carbamate complexes are also liberated as CO₂ by acidification, but the levels of protein carbamylation sites are not high enough to account for a substantial part of the total CO₂ measured by this method.

2.2. CO₂ levels during seed development

The concentration of CO₂ inside developing *B. napus* seeds increased threefold from 20 days after flowering (DAF) to a maximum of 35 mM at 30 DAF, and decreased to 8.5 mM at 40 DAF (Fig. 3). We also measured the levels of CO₂ in developing seeds of soybean with seed fresh weights ranging

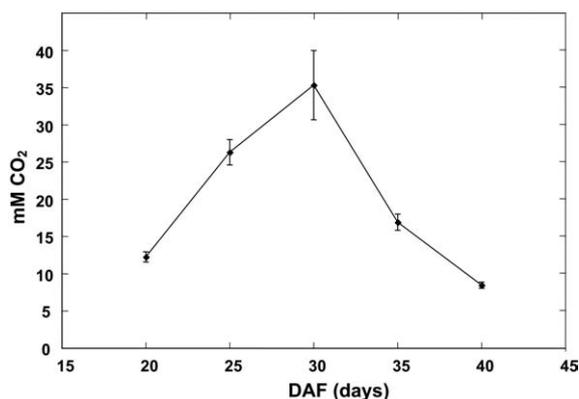


Fig. 3. Total inorganic CO₂ concentration in seeds of *B. napus* L. during seed development. Error bars represent S.D. of means.

from 50 to 120 mg. The values for CO₂ concentration were similar in all samples (mean = 11.6 mM, and S.D. = 1.1 mM). These CO₂ levels were two to threefold lower than those observed in *B. napus* seeds at 30 DAF. Seed oil content in this soybean variety is about 19% (w/w) of the total seed weight, substantially less than in rapeseed seeds (>45%, w/w). The higher inorganic carbon levels found in developing *B. napus* seeds may be related to their higher oil biosynthetic rate although additional investigations are needed to confirm this hypothesis. Seed CO₂ concentration is the result of a balance between CO₂ production, CO₂ refixation, CO₂ diffusion across the seed coat and possible export via the phloem/xylem. The relative contributions of these processes to oilseed CO₂ balance are not well understood. Eastmond et al. [8] concluded that seed CO₂ refixation is unlikely to make a net contribution to carbon economy, whereas King et al. [14] suggested that refixation of CO₂ in the seed may be even more important than in the endocarp. Based on the high CO₂ levels measured in the silique cavity (up to 2.5%), it is nevertheless likely that the quantity of CO₂ refixed does not completely accommodate the amount produced. Assuming that CO₂ diffusion remains low and relatively constant during development, CO₂ concentration within the seeds would be related to the synthesis rate of storage products, particularly lipids. We have found very high levels of CO₂ inside the seeds (up to 40 mM). The concentration of CO₂ in seeds is therefore between two and three orders of magnitude higher than in ambient air. By using Eqs. 3–9 we calculated the partial CO₂ pressure inside the seeds, and the CO₂/HCO₃⁻ ratios for the different subcellular structures. A 40 mM seed CO₂ concentration is comparable to a seed equilibrated with a 72% CO₂ v/v atmosphere. Thus, the concentration in seeds is much higher than would be expected if the seeds were in equilibrium with the ~2% CO₂ content of gas of the silique cavity (locule). This supports the conclusion of King et al. [14] that the seed coat is relatively impermeable to gases. To our knowledge, such high levels of CO₂ have not previously been reported in plant tissues. Other plant organs or tissues also accumulate large amounts of CO₂, but not in such high quantities as those of *B. napus* seeds. For example, CO₂

concentration within fruits, typically ranges between less than 0.3% (e.g. in tomato fruits) [7] to about 2% (e.g. in pepper) [2], and can reach 8% [3]. Stem CO₂ levels range from 1% to ca. 26% [6,12]. Xylem sap also exhibits high CO₂ concentrations, ranging from 1.6 to 10.3 mM [17].

Carbonate dehydratase (carbonic anhydrase) (EC 4.2.1.1) is active in oilseeds [11] and is expected to equilibrate CO₂ with HCO₃⁻. Assuming that the CO₂ partial pressure is the same among all subcellular structures, in the plastid stroma, cytosol and vacuole or apoplast with pH of ~8, ~7 and ~5.5, respectively, the CO₂/HCO₃⁻ ratios at equilibrium would be 0.024, 0.24 and 7.59, respectively. The high total inorganic carbon and these ratios may have several consequences for seed metabolism and physiology. First, by mass action, high CO₂/HCO₃⁻ will influence the equilibrium of the large number of enzymes for which CO₂/HCO₃⁻ is either a substrate or product. For example, acetyl-CoA carboxylase (EC 6.4.1.2, HCO₃⁻ is substrate) would be favored in the forward direction, whereas the equilibrium of the condensing enzymes of FAS (CO₂ is product) would be shifted toward the reverse direction. Second, for enzymes which normally experience CO₂ or HCO₃⁻ concentrations below their K_m, the kinetics may also be impacted. For example, ribulose-1,5-biphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39) normally functions in leaves at substrate concentrations below its K_m for CO₂ but in seeds this substrate concentration will be >10 times the K_m. Third, CO₂ and HCO₃⁻ can act as inhibitors and activators of enzymes. For example, Rubisco is activated by carbamylation by CO₂ and the very high CO₂ levels reported here indicate that the enzyme should be in the fully active form in seeds. Fourth, the release of CO₂ into aqueous solution can reduce the pH by capturing OH⁻ during bicarbonate formation, which would trigger the export of H⁺ to counteract the acidification. In the stroma for example, pH is decreased by about 0.8 pH units on addition of 16% CO₂ in air [10]. This effect is even stronger at low levels of O₂ as those found in developing rapeseed seeds (0.8% v/v) [22]. Under anaerobiosis the cytosolic pH decreases as a result of acid influx from outside [4] and acid production inside the cytosol [19]. Although CO₂ is the substrate of carbon assimilation, excessive concentrations of CO₂ can inhibit photosynthesis [20,23].

Another consequence of high CO₂ levels in seeds is that plants have probably evolved strategies for improving their carbon efficiency by CO₂ re-assimilation. In fact, a variety of green fruits refix internally respired carbon [3]. In ripening conifer cones for example, CO₂ refixation can be as high as 85% of respiratory carbon ([15,16]; for a review on non-foliar photosynthesis see Ref. [1]). Because of the high availability of CO₂ and assuming that the endocarp is not able to refix all the CO₂ evolved by the seeds, in the case of rapeseed (and probably other green oilseeds), it appears that an increase in the seed CO₂ photosynthetic refixation capacity may positively contribute to the plant carbon budget.

3. Conclusion

A new method has been described for determining total inorganic CO₂ concentration within the seed tissue in developing seeds of rapeseed and soybean. The procedure involves the conversion of seed bicarbonates and carbonates into CO₂. The method is rapid, simple, requires only a commonly available IRGA and has high reproducibility, represented by a low S.E. By adapting the type of vial used to the size of the sample, the method appears to be suitable for measuring CO₂ levels of developing seeds from species other than the ones reported in this manuscript or to other samples such as fruits.

4. Methods

4.1. Plant material

B. napus L. cv. Reston were planted in 30-cm plastic pots in a 1:1 (v/v) mixture of peat moss/vermiculite (Therm-O-Rock Inc., New Eagle, PA) and were grown in a greenhouse with an average light irradiance of ~1200 μE m⁻² s⁻¹, a 16-h day/night cycle, and at 20 °C day/15 °C night temperature. Seedlings were thinned to two per pot after 2 weeks. Buds from the main stem were tagged at the date of flowering and seeds were sampled at 20, 25, 30 and 35 DAF. At each sampling day, three siliques were harvested, placed into plastic bags and kept in an ice bath to slow metabolism. After collection, the pods were immediately opened and the seeds from each silique were separated. The seed samples were kept in an ice bath until analysis, which was done as described below. Soybean plants [*Glycine max* (L.) Merr.] cv. Vinton 81 (USDA-ARS, National Genetic Resources Program, Germplasm Resources Information Network, GRIN, accession no. **PI 548625**) were cultivated under the same conditions as the rapeseed plants. (Accession description available at <http://www.ars-grin.gov/cgi-bin/npgs/html/acchtml.pl?1443561>.) Soybean pods were harvested at the reproductive stage R 5.5 of development as described by Fehr et al. [9], which corresponds to a period of rapid, steady seed dry weight accumulation.

4.2. Theoretical basis of CO₂ determination in seeds

Inorganic carbon in seeds may occur in different forms, i.e. as bicarbonates, aqueous and gaseous CO₂ (aq. and g), and soluble and insoluble carbonates. The total content of inorganic carbon in seeds can be formulated as:

$$\text{Inorganic C} = \text{CO}_2 (\text{g}) + \text{CO}_2 (\text{aq.}) + \text{HCO}_3^- (\text{aq.}) + \text{CO}_3^{2-} (\text{aq.}) + \text{MCO}_3 (\text{s}) \quad (1)$$

The abundance of each species is pH dependent. At pH higher than 8, almost no CO₂ (aq. and g) is present, whereas

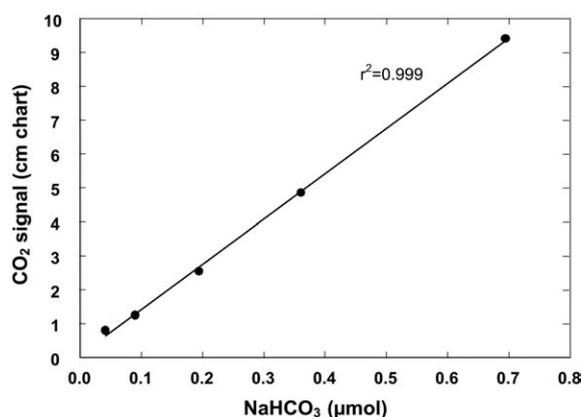


Fig. 4. Calibration curve of sodium bicarbonate standards for the determination of CO₂ concentration in seeds.

at pH lower than 4, aqueous plus gaseous CO₂ account for almost all the inorganic carbon. For total inorganic carbon quantification with an IRGA, the different forms must be converted to gaseous CO₂, which is directly measurable. By homogenizing the seeds with 1 N HCl, the pH of the homogenate is reduced to less than 1, and therefore, >96% of all inorganic carbon forms are converted to CO₂ (aq. and g). Inside the vial (gas-tight) an equilibrium is reached between the gas and liquid phase for carbon dioxide. In order to determine the fraction of CO₂ that is dissolved, we use Henry's Law, which states that the concentration of a solute gas (CO₂) in a solution is directly proportional to the partial pressure of that gas above the solution [21]. It can be formulated as:

$$p\text{CO}_2 = X_s\text{CO}_2 \cdot k_{\text{H}}\text{CO}_2 \quad (2)$$

where $p\text{CO}_2$ = partial pressure of CO₂ in air phase; $X_s\text{CO}_2$ = molar fraction of CO₂ in solution; $k_{\text{H}}\text{CO}_2$ = Henry's Law constant for CO₂.

We established a standard curve for the IRGA using known concentrations of sodium bicarbonate under the same liquid/gas headspace volume ratios and at pH lower than 3 (Fig. 4). Assuming that the seed tissue does not have a significant effect on the $k_{\text{H}}\text{CO}_2$, the same equilibrium between CO₂ (aq.) and CO₂ (g) should be achieved in both the standards and the seed homogenates. At pH lower than 2, 1 mol of NaHCO₃ releases ca. 1 mol of CO₂, allowing calculation of the total inorganic CO₂ concentration in seeds.

4.3. Calculation of CO₂ species and CO₂ partial pressure inside the seeds

Henry's Law constants for CO₂ in water [5] were used to convert measured total inorganic carbon concentration to CO₂ partial pressure, and to estimate the concentration of the different CO₂ species inside the subcellular structures. We used the following equations:

$$\text{Total [Inorganic carbon]} = [\text{CO}_2]_{\text{aq.}} + [\text{HCO}_3^-] + [\text{CO}_3^{2-}] \quad (3)$$

where total [Inorganic carbon] is the total dissolved inorganic carbon (mol l⁻¹), and [CO₂]_{aq}, [HCO₃⁻] and [CO₃²⁻] are the different dissolved forms, and:

$$[\text{CO}_2]_{\text{aq}} = K_0 \cdot p\text{CO}_2 \quad (4)$$

$$[\text{HCO}_3^-] = K_1 \cdot K_0 \cdot p\text{CO}_2 / 10^{-\text{pH}} \quad (5)$$

$$[\text{CO}_3^{2-}] = K_2 \cdot K_1 \cdot K_0 \cdot p\text{CO}_2 / (10^{-\text{pH}})^2 \quad (6)$$

where K_0 , K_1 and K_2 are solubility constants, and $p\text{CO}_2$ is the partial pressure of CO₂ (atm.). The constants are temperature dependent and can be calculated for temperatures between 0 and 50 °C with the following equations:

$$K_0 = 0.0114 + 0.0661 \cdot e^{-0.0433T} \quad (7)$$

$$K_1 = (2.5764 \times 10^{-7}) + (3.3742 \times 10^{-7})(1 - e^{-0.0318T}) \quad (8)$$

$$K_2 = (2.3777 \times 10^{-11}) + (9.0041 \times 10^{-13})T \quad (9)$$

where T is temperature (°C).

4.4. Procedure for CO₂ measurement in developing rapeseed seeds

The seeds of each silique were separated into subsamples of two seeds. For each subsample, the fresh weight was measured. The two-seed samples were immediately placed into a 1 ml limited volume polypropylene screw cap vial (12 × 32 mm), with a 6 mm diameter glass bead. In order to avoid any CO₂ loss the vials were immediately sealed with an open hole cap containing a GC septum (3.175 mm thickness, gas-tight). Two hundred microliter of 1 N HCl were injected through the septum into the vial using a semi-micro syringe. A diagram of the vial system used is shown in Fig. 1. The samples were then completely homogenized using a paint shaker located in a cold room (4 °C) for 5–10 min. The samples were then brought to room temperature. A 200 µl gas sample was withdrawn from the vial headspace using a 1 ml syringe, and injected into an IRGA (model 225-MK3; Analytical Development Co., Hoddesdon, UK). The output signal from the IRGA detector was recorded and the CO₂ peak height was used to determine the CO₂ headspace concentrations. A certified gas mixture (5% CO₂, 2% O₂, balanced with N₂) was used to calibrate the instrument. CO₂ headspace concentrations and inorganic carbon levels in seeds were calculated using a calibration curve developed with sodium bicarbonate standards (0.83–13.9 mM sodium bicarbonate in 10 mM KOH). Potassium hydroxide (10 mM) was used for the preparation of the bicarbonate standards to increase the pH and prevent any loss of CO₂ during handling. Standards were prepared as follows: 50 µl of each bicarbonate standard was pipetted into the same type of vials used for the seed samples. A 6 mm glass bead was placed inside the vials. The

samples were then closed with caps and 150 µl of 1 N HCl were injected through the septum. Standards were treated exactly in the same way as the samples, but measured against a blank of 50 µl of 10 mM KOH, since potassium hydroxide may contain CO₂ traces. CO₂ seed levels were expressed in mM total inorganic carbon (mmol inorganic C per l of seed volume), assuming that 1 mg seed fresh weight is equivalent to 1 µl seed volume. Seed CO₂ concentration per sample (silique) was the average of five replicate analyses.

4.5. Analysis of CO₂ concentration in developing soybean seeds

Due to their large size, the protocol used for measuring CO₂ levels in developing soybean seeds was a slight modification of the one used for rapeseed. After harvest, each pod was immediately opened and the fresh weight of each individual seed was determined. A single seed was placed into a 2.5 ml limited volume polypropylene screw cap vial (15 × 45 mm), together with two 6 mm diameter glass beads. The vials were immediately sealed with an open hole cap containing a 12 mm TFE/silicone liner (0.254/2.286 mm thickness) to avoid any CO₂ loss. Five hundred microliter of 1 N HCl were injected through the septum into the vial using a semi-micro syringe. For the calculation of the seed CO₂ levels, the seed volume was subtracted from the total headspace volume, assuming that 1 mg seed fresh weight is equivalent to 1 µl volume. Bicarbonate standards were prepared as follows: 50 µl of each bicarbonate solutions (2.2–36.1 µM sodium bicarbonate in 10 mM KOH) was pipetted into the same type of vials used for the soybean seeds (2.5 ml). Two 6 mm glass beads were placed inside the vials. The vials were then closed with caps and 450 µl of 1 N HCl was injected through the septum. Standards were treated in the same way as the samples, but measured against a blank of 50 µl of 10 mM KOH. CO₂ seed levels were expressed in mM total inorganic C. Seed CO₂ concentration was the average of two replicate analyses.

4.6. Evaluation of the procedure for seed CO₂ determination

The reproducibility of the method was determined by calculating the S.E. of the analysis of the seeds of two siliques, using the equation proposed by Windham et al. [24]:

$$\text{S.E.} = \{\sum_i [\sum_j (x_{ij} - x_j)^2 / (R - 1)] / n\}^{1/2}$$

where x_{ij} is the j th replicate on the i th sample, x_j is the reference method mean value of all replicates of an i th sample, R is the number of replicates, and n is the number of samples.

For a quantitative measurement of CO₂, most of the CO₂ has to be driven off to the vial headspace. For testing our vial system, we calculated the average molar fraction of CO₂ from the sodium bicarbonate standards used for the CO₂

calibration. We also studied the relationship between CO₂ species and pH by measuring the concentration of CO₂ in the vial headspace generated by 20 µl of a 50 mM sodium bicarbonate solution in 10 mM KOH after adding 180 µl of different buffers. The buffers used ranged from pH 1 to 8 (pH 1–3, 50 mM phosphoric acid titrated with 1 N HCl buffers; pH 4–8, 50 mM sodium phosphate monobasic/dibasic buffers). To compare the different pH treatments, the levels of CO₂ were expressed as the relative percentage of the CO₂ concentration obtained after adding 180 µl of 1 N HCl (pH 0, 100% CO₂) to the bicarbonate solution. Furthermore, since seed CO₂ levels may rapidly change over time due to metabolism or diffusion, we analyzed the effect of sample preparation time (the period of time from silique opening until seed homogenization) on seed CO₂ levels. The seeds from one silique (25 DAF) were separated from the silique walls and placed in an open Petri dish at 4 °C. One set of seeds was immediately weighed and homogenized, whereas a second set of seeds was kept at 4 °C for 10 min in the Petri dish, being further processed afterwards. This analysis was done in triplicate.

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