Research Article

Rapid Communications in Mass Spectrometry

Received: 15 May 2012 Revised: 28 June 2012 Accepted: 1 July 2012 Published online in Wiley Online Library

Rapid Commun. Mass Spectrom. **2012**, *26*, 2165–2172
(wileyonlinelibrary.com) DOI: 10.1002/rcm.6333

Practical considerations for the use of pollen $\delta^{13}C$ value as a paleoclimate indicator

D. Caleb King, Brian A. Schubert* and A. Hope Jahren

Department of Geology and Geophysics, University of Hawaii, Honolulu, HI 96822, USA

RATIONALE: Workers have shown a correlation between temperature and the pollen $\delta^{13}C$ value, and therefore suggested using pollen $\delta^{13}C$ values to reconstruct paleotemperature. To evaluate the potential for pollen $\delta^{13}C$ values to be used as a paleotemperature proxy, it is essential to quantify the variability in pollen $\delta^{13}C$ values and to evaluate the effect of temperature on pollen $\delta^{13}C$ values, in isolation, under controlled environmental conditions.
METHODS: Pollen was isolated from 146 *Hibiscus* flowers from 26 plants within a single climate environment to evaluate isotopic variability in pollen $\delta^{13}C$ values. The nearest leaf (n = 82) and flower phloem (n = 30) were also sampled to measure the $\delta^{13}C$ variability in carbon providing the raw material for new growth. To evaluate the correlation between temperature and pollen $\delta^{13}C$ values, we isolated pollen from 89 *Brassica rapa* plants grown in controlled growth chambers with temperatures ranging from 17 to 32°C.
RESULTS: The range in pollen $\delta^{13}C$ values collected from different flowers on the same *Hibiscus* plant was large (average = 1.6‰), and could be as much as 3.2‰. This amount of variability was similar to that seen between flower-adjacent leaves, and phloem extracted from styles of individual flowers. In controlled growth chamber experiments, we saw no correlation between temperature and the pollen ($R^2 = 0.005$) or leaf ($R^2 = 0.10$) $\delta^{13}C$ values.
CONCLUSIONS: We measured large variability in pollen $\delta^{13}C$ values. When temperature was isolated from other environmental parameters, temperature did not correlate with the pollen $\delta^{13}C$ value. These results complicate the supposed relationship between temperature and pollen $\delta^{13}C$ values and caution against using nanogram isotope analytical techniques for characterizing whole-plant individuals. Copyright © 2012 John Wiley & Sons, Ltd.

Angiosperm fossil pollen can be found abundantly in the geological record, from the time of the evolution of the first angiosperm plants (130 Ma)[1] up to and including Quaternary sediments, while fossil gymnospermous prepollen extends back 300 million years or more.[2] The great utility of fossil pollen as a biostratigraphic indicator based on its resistance to diagenesis has led to the identification of most palynomorphs to the taxonomic level of Family, and often Genus. In addition, the carbon isotope ($\delta^{13}C$) value of pollen has been suggested as a possible paleoenvironmental indicator (e.g.,[3,4]), based on the principle that plants respond to their environment mainly by modifying stomatal aperture, which, in turn, influences the ^{13}C:^{12}C ratio of the CO_2 fixed during photosynthesis.[5] Loader and Hemming[6] showed a correlation between the $\delta^{13}C$ values of *Pinus sylvestris* pollen and the average environmental temperature during the 1-month developmental period that preceded pollen dispersal. Their test of this particular correlation was motivated by the determination of a 1-month development period for species of the *Pinus*[7,8] and *Picea*[9] genera. Based on this correlation, they further suggested that variations in paleotemperature could be reconstructed from the $\delta^{13}C$ values of fossil pollen.[10]

* *Correspondence to:* B. A. Schubert, Department of Geology and Geophysics, University of Hawaii, Honolulu, HI 96822, USA.
E-mail: bschube@hawaii.edu

Two issues must be considered prior to the implementation of any paleotemperature proxy based on pollen $\delta^{13}C$ values. First, plant carbon isotope values have been shown to be highly variable, both with respect to composition[11] and to the time of formation.[12] Recent technical advances make possible the $\delta^{13}C$ analysis of extremely small amounts of material,[13] equivalent to just ~25 large-sized pollen grains.[4] A thorough characterization of the isotopic variability is necessary, however, for the potential application of these techniques to paleoenvironmental reconstructions to be evaluated. Second, because the $\delta^{13}C$ values of plant tissues have been shown to correlate with a wide variety of other environmental parameters (e.g., light levels,[14,15] water availability,[16–18] and nutrient availability[19,20]), the potential for the $\delta^{13}C$ values of fossil pollen to accurately reconstruct environmental temperature must be evaluated in isolation, with all other environmental parameters held constant. Here we address these two issues by documenting the intra- and inter-plant $\delta^{13}C$ variability in pollen by sampling hundreds of *Hibiscus* flowers growing on several plants all living at the same site. In addition, we quantify the relationship between temperature and the $\delta^{13}C$ values of pollen of *Brassica rapa* plants grown to flowering maturity within precisely controlled environmental growth chambers across a large range of environmental temperatures. We present here the results of these experiments, with their implications for use in paleoclimate reconstructions.

EXPERIMENTAL

Field sampling

Pollen from 26 mature *Hibiscus* plants growing at two public gardens on Oahu, Hawaii, USA, was sampled, for a total sample set of 146 flowers. Sixty-eight flowers from eight individual *Hibiscus arnottianus* plants were sampled within Kapiolani Gardens (21°16′10″N, –157°48′56″W), and 78 flowers from 18 individual *Hibiscus rosa-sinensis* plants were sampled within Waialae Gardens (21°16′29″N, –157°46′54″W). The two gardens were located 3.5 km apart and therefore experienced the same mean daily temperature (26.7 ± 0.7°C) and total precipitation (9 ± 2 mm) during the periods of plant tissue collection. Both gardens were actively maintained using a daily irrigation system and all *Hibiscus* plants were subject to cosmetic pruning. Flowers were chosen for sampling based on their presentation of maximum floral development and lack of insect herbivory, and the style, stamen, anthers and associated pollen were removed from the flower using a razor blade. During manual dissection under 20× magnification, 70–200 pollen grains from each flower were manually deposited into a pure tin capsule using a dry sterile needle tip, in preparation for stable isotope analysis. In addition, we removed and collected the nearest leaf for a large subset (n = 82) of the flowers sampled. Because we wished to assess the isotopic variability within the carbon providing the raw material for new tissue growth, the $\delta^{13}C$ values of phloem were determined from the styles of 30 flowers from five different *H. arnottianus* plants within Kapiolani Gardens. We extracted phloem, which transports water and solutes to the flower,[21] by slicing across the length the style and transferring the exuding phloem into a pre-weighed tin capsule. Flowers sampled for phloem extraction were collected ~2 h after sundown in order reveal the variability across the plant individual after the cessation of photosynthesis.

Growth chamber experiments

We grew 89 genotypically identical *B. rapa* plants (Wisconsin Fast Plants, #158805, Carolina Biological Supply Company, Burlington, NC, USA) from seed to maturity. *B. rapa* (Family: Brassicaceae) is commonly used for studies of plant reproduction, given its remarkably short life cycle:[22] it commonly grows from seed to reproductive age in less than 1 month. All plant growth occurred within 0.5 m^3 positive-pressure Plexiglass chambers (after[23,24]) designed to precisely control light levels, temperature, water availability, and pCO_2 level within the plants' environment. Photosynthetic photon flux, measured at the leaf surface using a solar electric quantum meter (Spectrum Technologies, Inc., Plainfield, IL, USA), was maintained at 260 µmol m^{-2} s^{-1} (400–700 nm) using fluorescent grow lamps (33-W GE Brightstik). Each plant occupied a separate soil container and, within each, the potting soil (Jiffy-Mix seed starting soil, Jiffy Products of America, Inc., Lorain, OH, USA) was supplemented with Osmocote smart release plant food (Scotts-Sierra Horticultural Products Co., Marysville, OH, USA) in order to achieve consistently high availability of nitrogen, phosphorus, and trace minerals. Twice daily, soil was monitored and maintained at field-moist conditions as defined by the USDA Natural Resources Conservation Service Soil Survey Staff[25] using a manually operated watering system. As expected, plants grown at higher temperatures required more water than plants growing at cooler temperatures in order to maintain constant soil water availability across treatments.

The twelve growth chambers were kept at a stable temperature of 17 to 32°C for the entire growth period. In order to accomplish this, all chambers were operated within the same 7.3 × 3.7 × 2.7 m room maintained at 20°C by central air conditioning. The heat generated by the fluorescent grow lights was sufficient to increase temperatures within the growth chambers to ~35°C (i.e., to the highest temperatures reached in our study) if additional cool air was not applied directly to the chamber. By adjusting the output of small air conditioning units (Everstar portable air conditioner, model MPN1-095CR-BB6), each of the twelve growth chambers was dynamically stabilized to internal air temperatures ranging from 17.2 ± 0.5°C to 32.4 ± 2.1°C (mean ± 1σ). Air was exhausted through an upper pipe and vented through a fume hood; complete atmospheric turnover occurred approximately once every 10.4 min. In accordance with experimental conventions involving *B. rapa*, the plants were grown under continuous light, which eliminated all diurnal temperature variation. Although water availability was constant across the chambers, it is important to note that the relative humidity was not controlled. As expected, the average relative humidity correlated well with chamber temperature (Fig. 1) and varied between chambers by as much as 31%, but was stable within each chamber to <5% (1σ) (measured using a humidity monitor, model #06-662-4, Fisher Scientific, Waltham, MA, USA). Upon flowering (i.e., after 14–27 days of growth), each plant was harvested, and the pollen was isolated and homogenized via centrifugation in deionized water.[3] In addition, for each plant (n = 89), all the leaves were collected.

Stable isotope analysis

Leaf and pollen tissues were dried at 60°C in preparation for stable isotope analyses; dried leaf tissues from each plant were also homogenized together by grinding to a uniform powder using a mortar and pestle. The carbon isotope values were

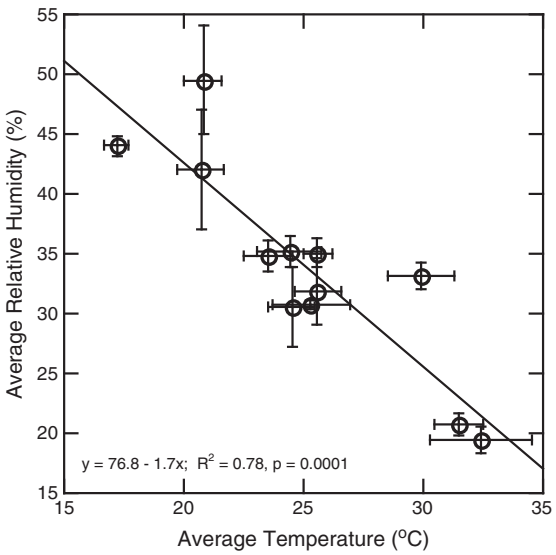

Figure 1. Correlation between average relative humidity and chamber temperature (±1σ) for *B. rapa* growth experiments.

Rapid Communications in Mass Spectrometry

determined using a Thermo Finnigan Delta V Advantage stable isotope ratio mass spectrometer (Thermo Scientific, Bremen, Germany) coupled to a Costech automated elemental combustion system with a zero-blank autosampler attached (Costech Analytical, Valencia, CA, USA). All samples were introduced into the combustion system in pure tin capsules; the stable isotope values are reported in standard δ-notation (‰). The reporting standard is Vienna Pee Dee Formation limestone (VPDB) with $R = {}^{13}C/{}^{12}C = 0.011224$. Individual $\delta^{13}C$ values are reported as the average and standard deviation of three replicate samples; the analytical uncertainty for each measurement did not exceed ±0.09‰.

RESULTS

The *Hibiscus* pollen collected from Waialae and Kapiolani Gardens showed large variability in $\delta^{13}C$ values, both between individual flowers on the same plant, and between plants growing adjacently, within the same garden. For the 18 *H. rosa-sinensis* plants sampled within Waialae Gardens, differences in the $\delta^{13}C$ values of pollen between flowers located on a single plant ranged from 0.4 to 2.9‰ (Table 1; Fig. 2). The two most intensively sampled plants at this garden (ten flowers were sampled from both plant numbers 12 and 18) both displayed large ranges in pollen $\delta^{13}C$ values between flowers (0.9 and 2.1‰, respectively). Similarly, for the eight *H. arnottianus* plants sampled within Kapiolani Gardens, differences in the $\delta^{13}C$ values of pollen between flowers located on a single plant ranged from 0.7 to 3.2‰ (Table 2; Fig. 2). At this garden, three plants were sampled intensively: eleven flowers were sampled from plant 1, 20 from plant 5, and ten from plant 6. All three plants displayed large ranges in pollen $\delta^{13}C$ values between flowers (1.7, 2.8 and 1.3‰, respectively). No relationship was observed, however, between the number of flowers analyzed within a single plant and the range in $\delta^{13}C$ values seen across flowers ($R^2 = 0.05$, $n = 26$, $p = 0.36$). A similar level of isotopic variability was observed between individual flower-adjacent leaves growing on the same plant, and between flower-adjacent leaves on plants growing within the same garden. For the *H. rosa-sinensis* plants sampled within Waialae Gardens, differences in the $\delta^{13}C$ values of three flower-adjacent leaves located on a single plant ranged from 0.2 to 3.7‰ ($n = 10$) (Table 1; Fig. 3). Similarly, for the eight *H. arnottianus* plants

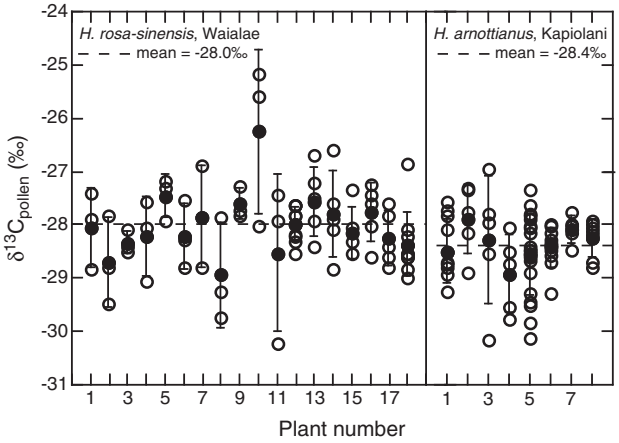

Figure 2. The $\delta^{13}C$ values of pollen from different flowers on individual, numbered *H. rosa-sinensis* plants sampled within Waialae Gardens, and *H. arnottianus* plants sampled within Kapiolani Gardens near Honolulu, Hawaii. Each open circle represents a single flower; closed circles show average pollen $\delta^{13}C$ value for each plant; error bars are one standard deviation of the mean.

Table 1. *Hibiscus rosa-sinensis*, Waialae Gardens

Plant no.	No. of flowers	Average $\delta^{13}C_{pollen}$ values (‰)	Range $\delta^{13}C_{pollen}$ values (‰)	Average $\delta^{13}C_{leaf}$ values (‰)	Range $\delta^{13}C_{leaf}$ values (‰)[a]
1	3	−28.0	1.5	−28.2	n.a.
2	3	−28.7	1.7	−29.1	3.7
3	3	−28.3	0.4	−28.5	0.8
4	3	−28.2	1.5	−29.0	0.7
5	3	−27.5	0.7	−28.6	1.0
6	3	−28.2	1.2	−28.8	2.6
7	3	−27.8	1.9	−28.8	2.6
8	3	−28.9	1.9	−29.4	1.3
9	3	−27.6	0.6	−28.7	1.6
10	3	−26.2	2.9	−26.2	0.2
11	3	−28.5	2.8	−29.4	1.2
12	10	−28.0	0.9	n.c.	n.c.
13	5	−27.6	1.7	n.c.	n.c.
14	5	−27.8	2.2	n.c.	n.c.
15	5	−28.2	1.2	n.c.	n.c.
16	5	−27.8	1.4	n.c.	n.c.
17	5	−28.2	1.2	n.c.	n.c.
18	10	−28.4	2.1	n.c.	n.c.

n.a. = not applicable (only 1 sample analyzed).
n.c. = not collected.
[a]Values are reported as the range of three leaves.

Table 2. *Hibiscus arnottianus*, Kapiolani Gardens

Plant no.	No. of flowers	Average $\delta^{13}C_{pollen}$ values (‰)	Range $\delta^{13}C_{pollen}$ values (‰)	Average $\delta^{13}C_{leaf}$ values (‰)[a]	Range $\delta^{13}C_{leaf}$ values (‰)
1	11	−28.5	1.7	−28.7 (4)	0.6
2	5	−27.9	1.6	−27.5 (5)	3.2
3	5	−28.3	3.2	−28.3 (5)	3.4
4	5	−28.9	1.7	−28.9 (5)	0.9
5	20	−28.5	2.8	−28.6 (13)	1.5
6	10	−28.4	1.3	−28.9 (10)	0.9
7	5	−28.1	0.7	−28.5 (5)	1.2
8	7	−28.3	0.9	−28.8 (3)	1.4

n.a. = not applicable (only 1 sample analyzed)
n.c. = not collected
[a]Value in parentheses indicates number of leaves analyzed.

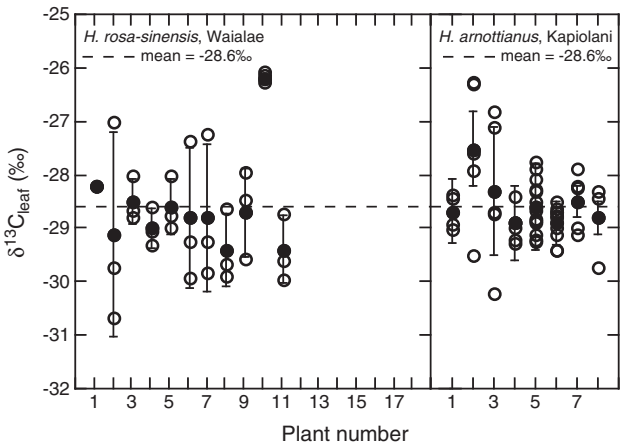

Figure 3. The $\delta^{13}C$ values of leaves adjacent to flowers on individual, numbered *H. rosa-sinensis* plants sampled within Waialae Gardens, and *H. arnottianus* plants sampled within Kapiolani Gardens near Honolulu, Hawaii. Each open circle represents a single leaf; closed circles show average leaf $\delta^{13}C$ value for each plant; error bars are one standard deviation of the mean. Plant numbers correspond to the same plants described by Fig. 2.

sampled within Kapiolani Gardens, differences in the $\delta^{13}C$ values of flower-adjacent leaves located on a single plant ranged from 0.6 to 3.4‰ (Table 2; Fig. 3). At this garden, two plants were sampled intensively: thirteen leaves were sampled from plant 5, and ten from plant 6. Both plants displayed large ranges in flower-adjacent leaf $\delta^{13}C$ values (1.5 and 0.9‰, respectively). No relationship was observed between the number of leaves analyzed within a single plant and the range in $\delta^{13}C$ values seen across flower-adjacent leaves ($R^2 = 0.002$, $n = 18$, $p = 0.76$). Finally, large ranges in the $\delta^{13}C$ value of phloem extracted from the styles of flowers within the same plant were observed for the six *H. arnottianus* plants sampled within Kapiolani Gardens (Table 3; Fig. 4). The $\delta^{13}C$ values of extracted phloem ranged from 1.3 to 3.6‰ across the five styles sampled from each plant.

Very large ranges in $\delta^{13}C$ values were seen between the flowers and leaves of *B. rapa* grown within the growth chambers described above (Table 4). Between plants growing

Table 3. *Hibiscus arnottianus*, Kapiolani Gardens

Plant[a]	Average $\delta^{13}C_{phloem}$ values (‰)	Range $\delta^{13}C_{phloem}$ values (‰)
A	−28.9	1.8
B	−28.8	2.4
C	−28.3	2.7
D	−29.0	1.3
E	−29.7	3.6
F	−29.1	2.7

[a]Phloem was sampled at the middle of the style, below the stamens of five different flowers from each plant.

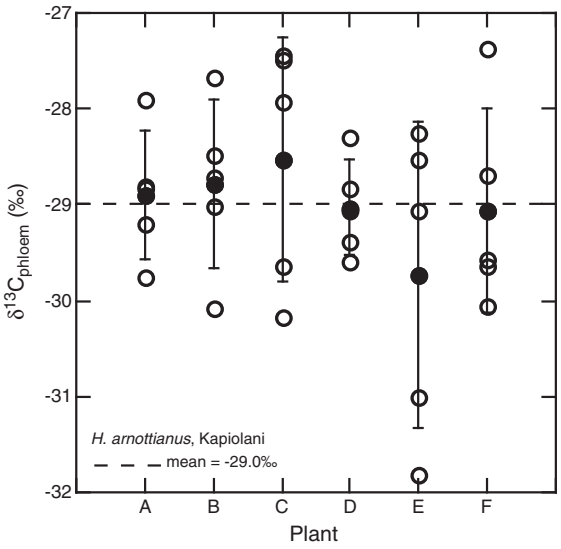

Figure 4. The $\delta^{13}C$ values of phloem extracted from the styles of different flowers on individual, lettered *H. arnottianus* plants sampled within Kapiolani Gardens near Honolulu, Hawaii. Each open circle represents a phloem $\delta^{13}C$ value for a single style; closed circles show average phloem $\delta^{13}C$ value for each plant; error bars are one standard deviation of the mean.

Rapid Communications in Mass Spectrometry

Table 4. *B. rapa* results[a]

Temperature (°C)	No. of plants	Relative humidity (%)	Water[b] (mL/day/plant)	Average $\delta^{13}C_{pollen}$ values (‰)	Range $\delta^{13}C_{pollen}$ values (‰)	Average $\delta^{13}C_{leaf}$ values (‰)	Range $\delta^{13}C_{leaf}$ values (‰)
17.2 ± 0.5	5	44 ± 1	22	−30.4	2.4	−30.2	2.8
20.7 ± 1.0	7	42 ± 5	24	−30.4	2.7	−31.0	2.6
20.8 ± 0.8	7	50 ± 5	24	−30.7	4.1	−30.7	2.7
23.5 ± 1.0	7	35 ± 1	27	−31.0	4.5	−31.8	5.2
24.4 ± 1.3	8	35 ± 1	25	−30.0	2.2	−30.8	3.1
24.5 ± 1.0	8	31 ± 3	24	−31.4	3.4	−31.8	4.1
25.3 ± 1.6	8	31 ± 0.4	30	−29.7	4.6	−31.3	2.9
25.6 ± 0.6	9	35 ± 1	32	−29.7	7.7	−30.8	5.7
25.6 ± 1.0	8	32 ± 3	26	−30.3	5.9	−32.2	2.2
29.9 ± 1.4	8	33 ± 1	30	−30.8	4.6	−32.1	4.6
31.5 ± 1.0	9	21 ± 1	33	−30.6	4.1	−32.0	3.4
32.4 ± 2.1	5	19 ± 1	32	−29.0	5.9	−31.8	2.2

[a]All plants were grown under ambient pCO_2 and PAR = 260 μmol/m^2/s.
[b]Plants were watered to maintain field moist conditions.[25]

at the same temperature, differences in the $\delta^{13}C$ values of pollen ranged from 2.2 to 7.7‰. Similarly, differences in the $\delta^{13}C$ values of leaves between individual plants ranged from 2.2 to 5.7‰. No relationship was observed between the number of plants sampled within a single chamber and the range in $\delta^{13}C$ values seen in pollen ($R^2 = 0.07$, $p = 0.42$) or leaves ($R^2 = 0.21$, $p = 0.13$). Most importantly, neither pollen $\delta^{13}C$ values ($R^2 = 0.005$, $n = 89$, $p = 0.51$) nor leaf $\delta^{13}C$ values ($R^2 = 0.10$, $n = 89$, $p = 0.003$) were at all correlated with the environmental temperature of growth (Fig. 5). Similarly, the pollen $\delta^{13}C$ values were not correlated with chamber relative humidity ($R^2 = 0.006$, $n = 89$, $p = 0.47$) or amount of water applied ($R^2 = 0.04$, $n = 89$, $p = 0.05$); these two measures were well correlated with growth temperature ($R^2 = 0.78$ and 0.73, $p = 0.0001$ and 0.0004, respectively; $n = 12$; Fig. 1). The leaf $\delta^{13}C$ values were not correlated with chamber relative humidity ($R^2 = 0.10$, $n = 89$, $p = 0.003$) or amount of water applied ($R^2 = 0.005$, $n = 89$, $p = 0.50$).

DISCUSSION AND CONCLUSIONS

The most striking result of this work is that no correlation can be found between the $\delta^{13}C$ values of *B. rapa* pollen and the environmental temperature during plant growth and pollen formation. For *P. sylvestris* trees sampled from 28 sites spanning more than 20 degrees of latitude across Europe, Loader and Hemming[6] observed a "strong positive linear relationship" ($R^2 = 0.68$) between the $\delta^{13}C$ values of pollen and the development period temperature (see Fig. 2 in Loader and Hemming[6]). The authors postulated that the relationship could be due to increased intercellular pCO_2 values resulting from decreased stomatal conductance, or increased carbon assimilation, at elevated temperatures, citing the theoretical relationship described within Farquhar *et al.*[26] Loader and Hemming[6] further claimed that a "direct or indirect" link existed between environmental temperature

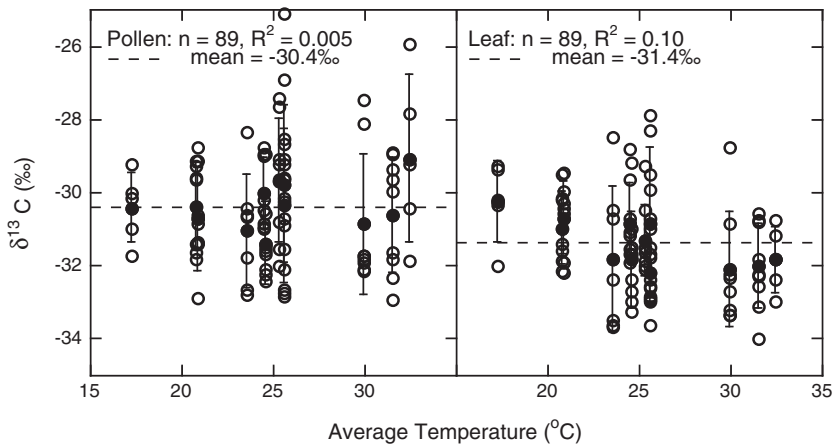

Figure 5. Relationship between the $\delta^{13}C$ values of pollen and leaf tissue and the temperature of the growth chamber for *B. rapa* plants. Each open circle represents a single pollen or leaf $\delta^{13}C$ value; closed circles show average pollen or leaf $\delta^{13}C$ value for a given temperature; error bars are one standard deviation of the mean.

and the $\delta^{13}C$ values of pollen that might be exploited in the paleorecord but they do[4,10] suggest that analysis of a single genus, or even species, might be necessary. Similarly, Jahren[3] showed that pollen $\delta^{13}C$ values measured across diverse species, including angiosperms and gymnosperms, were not straightforwardly controlled by environmental temperature and concluded that the relationship reported by Loader and Hemming[6] might be specific only to *P. sylvestris* or other widespread conifers. Our new results show that the relationship reported by Loader and Hemming[6] between developmental temperature and *P. sylvestris* pollen $\delta^{13}C$ values does not hold for *B. rapa*, a model angiosperm widely invoked for experimental studies of flower and pollen development (e.g.,[27,28]). Our experiments encompassed a wide range in temperature (range = 15.2°C; 17.2 to 32.4°C) that was more than twice the range in the study by Loader and Hemming[6] (range = 6.50°C; 8.57 to 15.07°C), which reported a 0.61‰ increase in pollen $\delta^{13}C$ per °C. Had our *B. rapa* plants followed this relationship, their $\delta^{13}C$ values would have systematically increased by 9.3‰ as the temperature increased across our experiments. In contrast, the average $\delta^{13}C$ values of *B. rapa* pollen collected from each temperature-controlled growth chamber varied between −29.0 and −31.4‰ (n = 12) and showed no correlation with chamber temperature ($R^2 = 0.10$; slope = 0.05‰/°C). We note, however, that all our plants were grown under higher temperatures than those recorded by Loader and Hemming,[6] and therefore we cannot rule out a possible hyperbolic response between temperature and pollen $\delta^{13}C$ value within which the isotopic response levels off at temperatures greater than ~15°C. Nevertheless, it is more likely that the results obtained by Loader and Hemming[6] might be specific to gymnosperms (or only to *P. sylvestris*) or that environmental conditions other than temperature were affecting pollen $\delta^{13}C$ values (e.g., moisture, humidity, sunshine[4]). Although the timing and length of pollen development might vary among species, the response of $\delta^{13}C$ values in other plant tissues (e.g., wood) to temperature is also variable[29] and is probably driven by concurrent changes in moisture status, humidity, and sunshine.[30]

A second, related result of this study was the documentation of the very large range of $\delta^{13}C$ values seen across pollen and within other tissues near flowers, within a single plant. Although previous studies did not systematically document this variability, prior results hint at this high level of isotopic heterogeneity from flower to flower. For example, the $\delta^{13}C$ values of pollen from 2–3 different flowers on single Poaceae grass plants ranged from 0.3 to 3.1‰.[31] The difference in the $\delta^{13}C$ values of pollen between individual plants was found to be 0.7‰ for Cupressaea (gymnosperm) and 3.1‰ for *Platanus acerifolia* (angiosperm).[32] These numbers are in keeping with the intra-plant variability that we observed for the relatively large number of *H. rosa-sinensis* (0.4–2.9‰) and *H. arnottianus* (0.7–3.2‰) samples, as well as with the inter-plant variability that we observed for *B. rapa* (2.4–7.7‰).

The $\delta^{13}C$ values of flower-adjacent leaves and style-phloem lend insight into the probable source of this isotopic variability. For both *H. rosa-sinensis* (0.2–3.7‰) and *H. arnottianus* (0.6–3.4‰), as well as for *B. rapa* (2.2–5.7‰), the magnitude of $\delta^{13}C$ variation between flower-adjacent leaves in the same plant was similar to the magnitude of $\delta^{13}C$ variation in pollen between flowers on the same plant, suggesting that the variable carbon isotope signature of the extremely ephemeral tissues involved in reproduction might reflect the effects of short-term changes in plant carbon balance on tissue construction. This is further reinforced by our observations that the $\delta^{13}C$ values of phloem extracted from the styles of five different flowers from each of six *H. arnottianus* plants ranged from 1.3 to 3.6‰. Thus the maximum $\delta^{13}C$ variability seen within a single *Hibiscus* plant was similar for pollen, flower-adjacent leaf, and phloem extracted from flower-styles: 3.2, 3.7 and 3.6‰, respectively.

It is interesting to note that despite the large ranges in $\delta^{13}C$ values that we observed within and between plants growing in identical environmental conditions, each system tended towards ecosystem-average $\delta^{13}C$ values of pollen and leaf. The whole-garden averages of pollen $\delta^{13}C$ values in *H. rosa-sinensis* and *H. arnottianus* were very similar (−28.0 and −28.4‰, respectively), and the standard deviations in these datasets were small ($1\sigma = 0.80‰$, n = 78, *H. rosa-sinensis*; $1\sigma = 0.67‰$, n = 68, *H. arnottianus*), suggesting the possible existence of a species average $\delta^{13}C$ value within a given ecosystem that could be estimated provided that a sufficient number of samples were analyzed. In order to quantify how many flowers would need to be sampled to achieve a high-precision, average pollen $\delta^{13}C$ value for the ecosystem, we used the following equation:

$$n = 16\sigma^2/W^2 \quad (1)$$

where n is the number of samples (e.g., flowers), σ is the standard deviation of our sample, and W is the width of the confidence interval. This equation assumes a normal distribution and a 95% confidence interval (±2σ) such that $W = (4\sigma)(n^{-0.5})$. Using Eqn. (1), we calculate that in order to achieve a 1‰ confidence interval width (W), 7–10 *Hibiscus* flowers need to be sampled. In order to decrease W to 0.5‰, however, would require at least 29–41 flowers. The convergence of flower-adjacent leaf tissues toward ecosystem averages (mean = −28.6‰, $1\sigma = 1.2‰$, n = 32, *H. rosa-sinensis*; mean = −28.6‰, $1\sigma = 0.78‰$, n = 50, *H. arnottianus*) suggests that such an approach might also be applicable to studies attempting to correlate leaf $\delta^{13}C$ values with environmental parameters.

Based on our assessment of isotopic variability, we hypothesize that the $\delta^{13}C$ values of sugars exported to plant phloem are variable up to several per mil due to the dynamic nature of carboxylation within chloroplasts, which has been shown to vary by as much as threefold over the course of 24 h.[33] Other researchers have observed temporal variation in $\delta^{13}C$ values of up to 6‰ within the phloem stream of *Ricinus communis*;[12] our results showed a somewhat reduced level of phloem $\delta^{13}C$ variability across several flowers on a single *H. arnottianus* plant (1.3–3.6‰). We note also that a poor correlation was observed between the $\delta^{13}C$ value of a flower's pollen and that of its most adjacent leaf in both *H. rosa-sinensis* ($R^2 = 0.31$) and in *H. arnottianus* ($R^2 = 0.35$), suggesting that flower construction does not act to homogenize the isotopic composition of the sugars acquired from the phloem stream. Detailed studies of the flower development in *Arabidopsis*[34] have revealed that critical steps of flower tissue construction, such as the extension of pollen-filled anthers, take place over less than 18 h. This same study determined that the average lifespan of a flower was just 60 h from bud opening to the

complete withering of petals and sepals. These measurements highlight the hasty time-frame of plant reproduction and suggest that the immediate demands of the reproductive cycle may give rise to deviation from the ecosystem $\delta^{13}C$ average value within individual reproductive tissues such as pollen. Such potential for variation is an important consideration during any discussion of a paleoenvironmental proxy development using the carbon isotope values of pollen. As stated above, each of our pollen sample $\delta^{13}C$ values represented the combustion of hundreds of pollen grains from one flower within one tin capsule. Given the standard deviation associated with our pollen $\delta^{13}C$ dataset for *Hibiscus* spp. ($1\sigma = 0.80‰$, n = 78, *H. rosa-sinensis*; $1\sigma = 0.67‰$, n = 68, *H. arnottianus*), in order to capture the ecosystem average $\delta^{13}C$ value with a precision that approaches that of the mass spectrometer itself ($W = \pm 0.1‰$), Eqn. (1) suggests that the pollen from approximately 700 to 1000 flowers must be sampled and analyzed. The large variability in flower-to-flower and plant-to-plant pollen $\delta^{13}C$ value as well as the lack of correlation between temperature and pollen $\delta^{13}C$ value raises concerns about the efficacy of fossil pollen $\delta^{13}C$ values for the reconstruction of paleotemperature, and calls into question the utility of the application of nanogram carbon isotope analytical techniques[35,36] to terrestrial paleoclimate reconstruction using plant substrates.

Acknowledgements

We thank W. M. Hagopian and Hannah Shelton for laboratory assistance. Support was received from NSF Grant EXE-CBET08-54754 and DOE/BES Grant DE-FG02-09ER16002.

REFERENCES

[1] S. Lidgard, P. R. Crane. Quantitative analyses of the early angiosperm radiation. *Nature* **1988**, *331*, 344.
[2] G. W. Rothwell. Evidence of pollen tubes in Paleozoic pteridosperms. *Science* **1972**, *175*, 772.
[3] A. H. Jahren. The carbon stable isotope composition of pollen. *Rev. Palaeobot. Palynol.* **2004**, *132*, 291.
[4] N. J. Loader, D. L. Hemming. The stable isotope analysis of pollen as an indicator of terrestrial palaeoenvironmental change: a review of progress and recent developments. *Quat. Sci. Rev.* **2004**, *23*, 893.
[5] G. D. Farquhar, J. R. Ehleringer, K. T. Hubick. Carbon isotope discrimination and photosynthesis. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1989**, *40*, 503.
[6] N. J. Loader, D. L. Hemming. Spatial variation in pollen $\delta^{13}C$ corelates with temperature and seasonal development timing. *The Holocene* **2001**, *11*, 587.
[7] A. G. Gordon. *Seed Manual for Forest Trees*, HMSO, London, **1992**.
[8] J. N. Owens, M. Molder. Seed-cone differentiation and sexual reproduction in western white pine (*Pinus monticola*). *Can. J. Bot.* **1977**, *55*, 2574.
[9] S. Lü, Y. Li, Z. Chen, J. Lin. Pollen development in *Picea asperata* MAST. *Flora* **2003**, *198*, 112.
[10] N. J. Loader, D. L. Hemming, in *Study of Environmental Change Using Isotope Techniques*, vol. *13/P*, (Eds.: P. Aggarwal, J. J. Gibson, P. Povinec), IAEA, Vienna, **2002**.
[11] M. J. Lockheart, P. F. V. Bergen, R. P. Evershed. Variations in the stable carbon isotope compositions of individual lipids from leaves of modern angiosperms: implications for the study of higher land plant-derived sedimentary organic matter. *Org. Geochem.* **1997**, *26*, 137.
[12] A. Gessler, G. Tcherkez, A. D. Peuke, J. Ghashghaie, G. D. Farquhar. Experimental evidence for diel variations of the carbon isotope composition in leaf, stem and phloem sap organic matter in *Ricinus communis*. *Plant Cell Environ.* **2008**, *31*, 941.
[13] P. J. Polissar, J. M. Fulton, C. K. Junium, C. C. Turich, K. H. Freeman. Measurement of ^{13}C and ^{15}N isotopic composition on nanomolar quantities of C and N. *Anal. Chem.* **2009**, *81*, 755.
[14] I. Aranda, M. Pardos, J. Puértolas, M. D. Jiménez, J. A. Pardos. Water-use efficiency in cork oak (*Quercus suber*) is modified by the interaction of water and light availabilities. *Tree Physiol.* **2007**, *27*, 671.
[15] J. M. Kranabetter, S. W. Simard, R. D. Guy, K. D. Coates. Species patterns in foliar nitrogen concentration, nitrogen content and ^{13}C abundance for understory saplings across light gradients. *Plant Soil* **2010**, *327*, 389.
[16] C. Y. Yin, X. Wang, B. L. Duan, J. X. Luo, C. Y. Li. Early growth, dry matter allocation and water use efficiency of two sympatric *Populus* species as affected by water stress. *Environ. Exp. Bot.* **2005**, *53*, 315.
[17] A. F. Diefendorf, K. E. Mueller, S. L. Wing, P. L. Koch, K. H. Freeman. Global patterns in leaf ^{13}C discrimination and implications for studies of past and future climate. *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 5738.
[18] M. J. Kohn. Carbon isotope compositions of terrestrial C3 plants as indicators of (paleo)ecology and (paleo)climate. *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 19691.
[19] L. A. Cernusak, K. Winter, B. L. Turner. Physiological and isotopic ($\delta^{13}C$ and $\delta^{18}O$) responses of three tropical tree species to water and nutrient availability. *Plant Cell Environ.* **2009**, *32*, 1441.
[20] C. Y. Yin, X. Y. Pang, K. Chen. The effects of water, nutrient availability and their interaction on the growth, morphology and physiology of two poplar species. *Environ. Exp. Bot.* **2009**, *67*, 196.
[21] E. De la Barrera, P. S. Nobel. Nectar: properties, floral aspects, and speculations on origin. *Trends Plant Sci.* **2004**, *9*, 65.
[22] P. H. Williams, C. B. Hill. Rapid-cycling populations of *Brassica*. *Science* **1986**, *232*, 1385.
[23] A. H. Jahren, N. C. Arens, S. A. Harbeson. Prediction of atmospheric $\delta^{13}CO_2$ using fossil plant tissues. *Rev. Geophys.* **2008**, *46*, DOI: 10.1029/2006RG000219.
[24] B. A. Schubert, A. H. Jahren. Fertilization trajectory of the root crop *Raphanus sativus* across atmospheric pCO_2 estimates of the next 300 years. *Agr. Ecosys. Environ.* **2011**, *140*, 174.
[25] Soil Survey Staff, *Soil Conservation Service, Keys to Soil Taxonomy*, Pocahontas Press, Inc., VA, **1999**.
[26] G. D. Farquhar, M. H. O'Leary, J. A. Berry. On the relationship between carbon isotope discrimination and the intercellular carbon dioxide concentration in leaves. *Aust. J. Plant Physiol.* **1982**, *9*, 121.
[27] T. C. Osborn, C. Kole, I. A. P. Parkin, A. G. Sharpe, M. Kuiper, D. J. Lydiate, M. Trick. Comparison of flowering time genes in *Brassica rapa*, *B. napus* and *Arabidopsis thaliana*. *Genetics* **1997**, *146*, 1123.
[28] T. S. Sarkissian, L. D. Harder. Direct and indirect responses to selection on pollen size in *Brassica rapa* L. *J. Evolutionary Biol.* **2001**, *14*, 456.
[29] G. H. Schleser, G. Helle, A. Lücke, H. Vos. Isotope signals as climate proxies: the role of transfer functions in the study of terrestrial archives. *Quat. Sci. Rev.* **1999**, *18*, 927.
[30] D. McCarroll, N. J. Loader. Stable isotopes in tree rings. *Quat. Sci. Rev.* **2004**, *23*, 771.

[31] R. Amundson, R. R. Evett, A. H. Jahren, J. Bartolome. Stable carbon isotope composition of Poaceae pollen and its potential in paleovegetational reconstructions. *Rev. Palaeobot. Palynol.* **1997**, *99*, 17.
[32] C. Descolas-Gros, M. Calleja, P. Cour, P. Richard, C. Perruchietti, P. Jame. Carbon isotopic ratios of pollen: interest for palaeovegetations reconstructions. *Comptres Rendus de l'Academmie des Sciences, Serie III: Fascicule A-Sciences de la Terre et des Planetes* **2001**, *332*, 755.
[33] N. Nassoury, L. Fritz, D. Morse. Circadian changes in ribulose-1,5-bisphosphate carboxylase/oxygenase distribution inside individual chloroplasts can account for the rhythm in dinoflagellate carbon fixation. *Plant Cell* **2001**, *13*, 923.
[34] D. R. Smyth, J. L. Bowman, E. M. Meyerowitz. Early flower development in *Arabidopsis*. *Plant Cell* **1990**, *2*, 755.
[35] A. L. Sessions. Moving-wire device for carbon isotopic analyses of nanogram quantities of nonvolatille organic carbon. *Anal. Chem.* **2005**, *77*, 6519.
[36] D. Nelson, F. Hu, J. Mikucki, J. Tian, A. Pearson. Carbon-isotopic analysis of individual pollen grains from C_3 and C_4 grasses using a spooling-wire microcombustion interface. *Geochim. Cosmochim. Acta* **2007**, *71*, 4005.

