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Review of Palaeobotany and Palynology 99 (1997) 17–24

REVIEW OF PALAEOBOTANY AND PALYNOLOGY

Stable carbon isotope composition of Poaceae pollen and its potential in paleovegetational reconstructions

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Received 7 August 1996; accepted 24 March 1997

Abstract

Stable carbon isotope differences between ecologically distinct groups of Poaceae (C_3 and C_4 photosynthetic groups) provide a means of isotopically subdividing grass pollen in paleovegetation studies. We examined the isotopic composition of bulk grass plant tissue, untreated pollen, and chemically treated pollen, from several C_3 and C_4 grass species. Based on our data, untreated pollen is isotopically similar to the host plant from which it is derived, although small, random differences between plants and pollen occur. Methods of pollen concentration involving carbon-bearing compounds can alter the isotopic composition of recovered pollen, and in some cases, make pollen from different grass types isotopically indistinguishable. We conclude that the isotopic composition of physically separated Poaceae pollen should be an important means of determining the proportion of C_3/C_4 grasses as long a carbon-bearing chemicals are not used in sample preparation. The carbon isotope composition of pollen should provide a new means of determining paleoclimatic conditions in grassland environments and aid in identifying the origin of the C_4 photosynthetic pathway in the geologic past. © 1997 Elsevier Science B.V.

1. Introduction

Palynological reconstructions of latest Pleistocene and Holocene vegetation patterns in North America have provided a detailed insight into the response of the terrestrial biosphere to climate change (e.g. Webb et al., 1987). One of the limitations of the palynological approach to vegetation reconstruction in areas that supported a significant Poaceae biomass is the difficulty in unambiguously assigning grass pollen to individual species, or important groups of species, using morphology (Moore et al., 1991). Of particular ecological interest in grasslands is a measure of the relative proportion of C_3 to C_4 grasses, since the proportion of these grasses at a given site is largely determined by climatic factors such as precipitation seasonality and growing season temperature (Fig. 1). C_4 grasses are sensitive to cold temperatures (Pearcy and Ehleringer, 1984). In tropical environments with little seasonal variation in temperature, C_4 grasses dominate warm temperature, low elevation sites. With increasing elevation, and decreasing temperature, there is a corresponding decline in C_4 grasses, and increase in C_3 grasses (Tieszen et al., 1979; Rundel, 1980). In the grasslands of the Great Plains of North America, the abundance of C_4 grasses is related to

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PII 0034-6667(97)00031-6

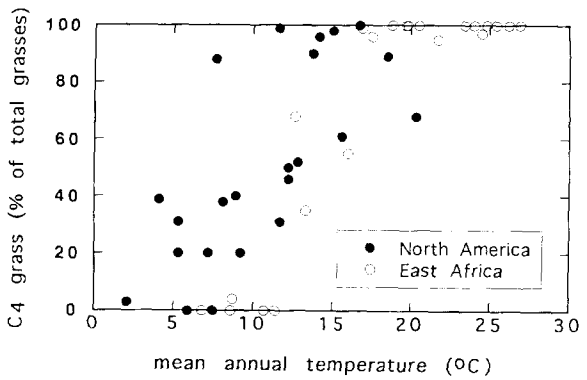

Fig. 1. The distribution of C_4 grasses as a function of mean annual temperature for the Great Plains of North America and eastern Africa. Data for the Great Plains are from Coupland (1979) and Teeri and Stowe (1976) while data for east Africa (Kenya) are from Tieszen et al. (1979). Estimates of temperature for the east African sites were made from lapse rates calculated using climate data from Griffith (1968) and Hedgeberg (1964).

the growing season temperature (Teeri and Stowe, 1976; Boutton et al., 1980; Kemp and Williams, 1980), and a general decline in C_4 grasses occurs with increasing latitude. Clearly, an understanding of the long-term changes in the proportion of C_3 to C_4 grasses at a site offers new perspectives into the paleoecology and climatology of a region.

It is well known that C_3 and C_4 grasses differ significantly in their stable C isotopic composition (Bender, 1968; Smith and Epstein, 1971), suggesting that isotopic differences between pollen of the different groups may exist. Preliminary isotopic analyses have been made on fossil algal spores (Brooks, 1971), but the systematic study of modern pollen has not been attempted. Several questions must be investigated before isotopic studies of Poaceae pollen can be utilized since the carbon isotope composition of pollen recovered from treated sediments may not necessarily directly correspond to those of the plants on which formed. First, it is well known that the carbon isotope composition of different organic compounds within a plant, such as cellulose, lignin, or lipids (Parker, 1964; Benner et al., 1987) or occluded carbon in opal phytoliths (Kelly et al., 1991), may differ considerably from that of bulk plant tissue. The complex and unique chemical nature of pollen suggests its carbon isotope composition may also differ from that of the parent plant. Second, standard chemical extraction procedures used to separate and concentrate pollen from sediments may produce artifacts in the isotopic composition of the concentrated pollen. The effects of these treatments need to be evaluated before isotopic interpretations are attempted.

Here we report our results on the stable carbon isotope composition of whole plants, pollen, and chemically treated pollen from some representative C_3 and C_4 grass species. First, we discuss the effects of chemical treatment and diagenesis on the stable isotope ratios of pollen. Second, we discuss alternative ways of applying our findings to palynological reconstructions of grassland environments.

2. Methods

Eleven species of grasses, five C_3 and six C_4, were selected for study. The C_3 species *Bromus carinatus* Hook. and Avn., *Lolium multiflorum* Lam., *Avena barbata* Link and *Hordeum murinum* L. were collected from wild populations near Santa Rosa, California. The C_4 species *Hilaria cenchroides* H.B.K., *Orcuttia californica* Vasey and *Opizia stolonifera* J. Presl. were grown in a greenhouse at the Rancho Santa Ana Botanical Garden (samples provided by J.T. Columbus). The C_4 plant *Pennisetum clandestinum* Chiov. was collected from a Berkeley lawn. The remaining C_4 species, *Cynodon dactylon* (L.) Pers and *Zea mays* L., were collected from a cultivated field in Berkeley, California. Pollen was isolated by shaking inflorescences into plastic bags to dislodge pollen and anthers. Pollen and anthers were immersed in distilled water and poured through a 90 mm sieve to remove the anthers and other extraneous material. The untreated pollen samples were concentrated through centrifugation and checked for purity under a microscope.

The treated samples were subjected to standard pollen extraction and concentration techniques (Fægre and Iversen, 1975; Moore and Webb, 1978). This method, which contains C-bearing compounds, clearly will introduce the maximum possible isotopic change to the pollen samples and

was investigated specifically to determine the magnitude of this effect. Samples were placed in a polyethylene boiling tube, covered with 10% hydrochloric acid (HCl) to remove carbonates, and stirred for 5 minutes. Following two distilled water washes and centrifugations at 3000 rpm, tubes were filled with 10% potassium hydroxide (KOH) to remove some organic matter, and boiled in a water bath for 20 minutes. After centrifugation, the KOH was decanted and samples were washed with distilled water and centrifuged twice. Samples were then covered with 30% hydrofluoric acid (HF) to remove siliceous material, placed in a boiling bath for 10 minutes, and stirred occasionally. The HF was decanted and the samples washed with water and centrifuged twice. Acetolysis, a process for removing cellulose from pollen samples, followed. Dehydration was achieved by covering the samples with glacial acetic acid, centrifuging, and decanting. A 9:1 mixture of acetic anhydride and sulfuric acid was added and tubes were placed in a boiling bath for 3 minutes. After centrifuging and decanting, glacial acetic acid was again added, centrifuged, and decanted, followed by multiple distilled water washes until odor of acetic acid was no longer evident. Treated samples were checked for purity under a microscope.

Bulk host plant samples were ground with a coffee mill, acidified with 1 N HCl to remove carbonates, rinsed with deionized water by centrifugation and freeze dried. The plant tissue and pollen samples were combusted in sealed tubes containing Cu, CuO and Ag (Minagawa et al., 1984). The released CO_2 was purified cryogenically, its yield measured manometrically, and its $^{13}C/^{12}C$ ratio measured by mass spectrometry. Accurate measurements of yields were possible only on several pollen samples due to difficulties in transferring, and accurately weighing, micro quantities of pollen from small polyetheylene vials to glass combustion tubes. For approximately 75% of the measurements (the method used in later stages of this study), the pollen was introduced to the combustion tube as a water/pollen slurry and the water was removed via lypholization. This sample introduction method did not allow for accurate measurements of pollen mass.

All isotope ratios are expressed in the δ notation where:

$$\delta^{13}C = [(^{13}C/^{12}C)_{sample}/(^{13}C/^{12}C)_{std} - 1] \times 1000$$

The isotopic standard is the PDB carbonate (Craig, 1957). The precision of the determination, assessed from replicate analyses of the same plant tissue sample, was ±0.40‰.

Scanning electron microscopy was performed on both treated and untreated *Cynodon dactylon* pollen samples. Prior to microscopy, samples were sputter-coated with Ag according to standard preparation techniques. Secondary electron imaging was done on an ISI DSI 130 instrument, with the accelerating voltage set a 10 kV.

3. Results and discussion

The results of the isotopic measurements are presented in Table 1. The $\delta^{13}C$ values of the plant tissue within the C_3 and C_4 groups show some variability, but all fall within specified ranges representative for the two grass groups ($C_3 = -27 \pm 4$‰; $C_4 = -12 \pm 3$‰; Bender, 1968; Smith and Epstein, 1971). The $\delta^{13}C$ values of untreated pollen are closely related to that of their parent plant (Fig. 2), but ranged from values 6.3‰ more positive (*Hordeum murinum*) to 2.5‰ more

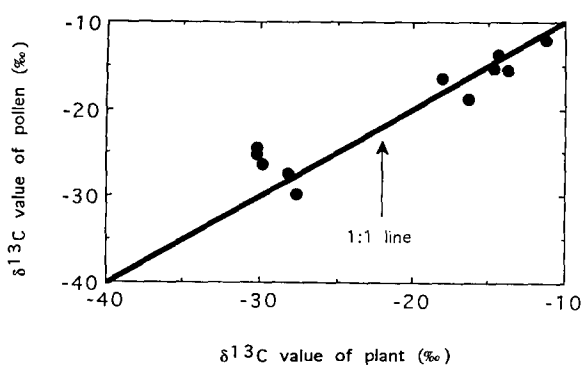

Fig. 2. The relationship between the $\delta^{13}C$ value of the host plant and that of untreated pollen (data from Table 1). Comparisons made only for samples where pollen and plant tissue were collected from the same plant.

Table 1
Stable carbon isotopic composition of selected Poaceae plants, fresh pollen, and chemically treated pollen

Grass species	Photosynthetic pathway	Plant $\delta^{13}C$ (‰ PDB)	Untreated pollen $\delta^{13}C$ (‰ PDB)	Treated pollen $\delta^{13}C$ (‰ PDB)
Cynodon dactylon	C_4	−14.4 (1)	−13.7 (1)	−17.6 (1)
		−13.8 (2)	−15.5 (2)	
		−14.0 (3)		
		−13.6 (4)		
		−13.3 (5)		
		−13.7 (6)		
Hilaria cenchroides	C_4	−18.1	−16.5	−18.9
Orcuttia californica	C_4	−14.6	−15.3	−23.0
		−14.8		
Pennisetum clandestinum	C_4	−12.8	−18.2 (1)	−28.8 (1)
		−12.4	−17.2 (2)	−23.3 (2)
		−12.7	−18.8 (3)	−25.2 (3)
			−21.9 (3)	−23.9 (4)
			−18.2 (4)	
			−19.8 (4)	
Zea mays	C_4	−11.3	−12.1	−14.4
		−11.6		
Opizia stolonifera	C_4	−16.3	−18.8	−25.5
Lolium multiflorum	C_3	−30.2 (1)	−25.1 (1)	−27.5 (1)
		−30.2 (1)	−24.6 (1)	−25.6 (3)
			−23.9 (1)	−23.1 (4)
			−26.1 (2)	−25.0 (5)
			−25.8 (2)	
			−24.9 (4)	
			−25.8 (5)	
			−26.8 (5)	
Lolium multiflorum	C_3	−28.5	−27.5	−30.1
		−28.1		
		−28.1		
Hordeum murinum	C3	−29.8	−25.3	
		−30.3		
		−30.6		
Bromus carinatus	C3	−30.0	−26.4	
		−29.6	−26.4	
Avena barbata	C3	−27.8	−29.8	
		−27.6		
		−27.4		

Numbers in parentheses indentify samples from the same plant collected at one location. Multiple values for a given sample are results of replicate analyses.

negative (*Cynodon dactylon*) than the $\delta^{13}C$ values of the respective parent plant.

SEM micrographs of untreated pollen from *Cynodon dactylon* revealed a variety of tissue types in addition to pollen (Fig. 3A, B). The chemical nature of these various compounds was not investigated. Manometric measurements of CO_2 produced during preparation of the plants and pollen for isotopic analysis indicated that the host plants (with the exception of *Cynodon dactylon*, which had C percentages between 21 and 27%) contained 29 to 48% carbon and untreated pollen from 43

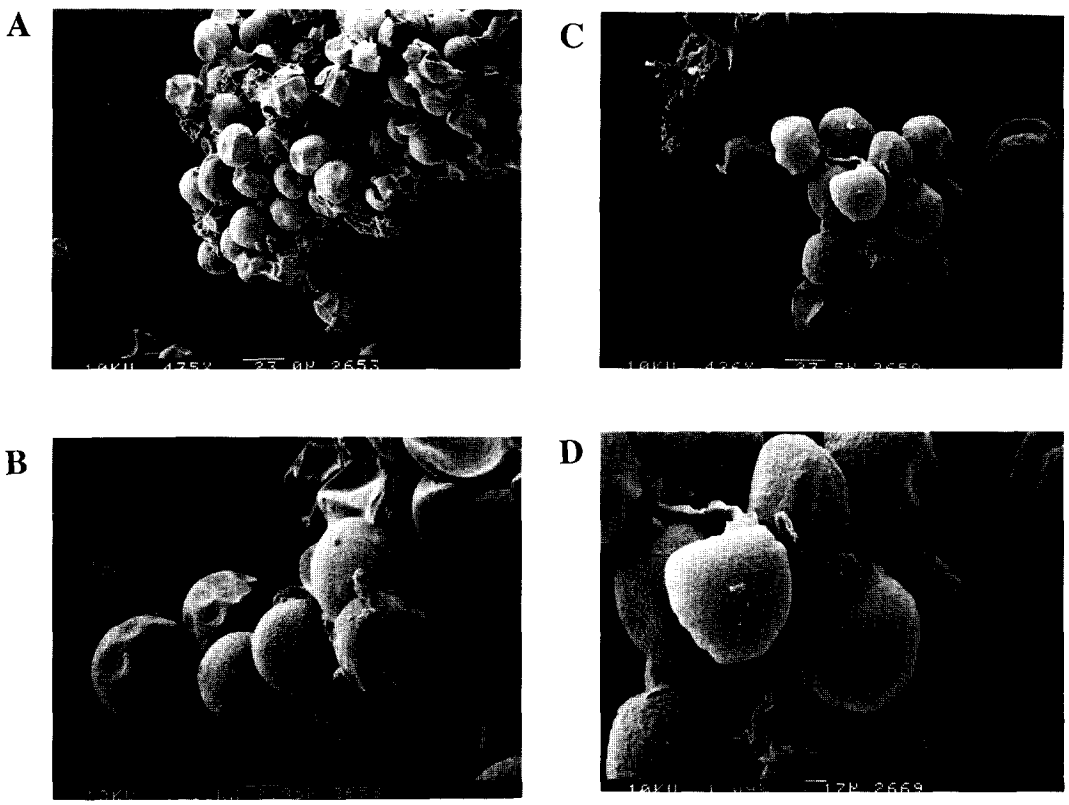

Fig. 3. SEM micrographs of Poaceae pollen. Untreated *Cynodon dactylon* pollen (A) at 436× and (B) at 1130× magnification. Chemically treated *Bromus carinatus* pollen (C) at 426× and (D) at 1090× magnification. Scale bars located at the lower center of the micrographs represent approximate length in μm.

to 48% carbon. One treated pollen contained 64% carbon. Although we have only one accurate measurement of the C content of treated pollen, it is in close agreement with independent measurements of treated pollen by Heslop-Harrison (1968). The results of the chemical treatments on pollen morphology, relative to its untreated state, are illustrated in Fig. 3C and D. At low magnification, there is some evidence of a loss of non-pollen debris. The major difference, when compared to the untreated grains, is observable at high magnifications (Fig. 3D), where the grains appear to be more fragile and most show some evidence of collapse.

Untreated pollen is a complex mixture of organic compounds. An outer, sporopollenin-rich exine layer overlies an inner cellulose-rich intine layer (Heslop-Harrison, 1971). Additionally, lipids and proteins have also been detected (Heslop-Harrison, 1968). Relative to whole plant tissue, cellulose is enriched in ^{13}C by approximately 1 to 2‰ while lignin is depleted by about 2 to 4‰ (Benner et al., 1987). Lipids, relative to whole tissue in grasses, are depleted in ^{13}C by up to 8‰ (Parker, 1964). Pollen is composed of approximately 2–10% cellulose and 2–24% of sporopollenin, a complex, highly resistant bipolymer of carotenoids and carotenoid esters (Brooks and Shaw, 1971). Each species has different proportions of these pollen components (Brooks and Shaw, 1971), probably accounting for the range in $\delta^{13}C$ values observed for untreated pollen in our study (Table 1).

Most fossil pollen is subjected to diagenetic processes that rapidly remove lipids, cellulose and proteins. In addition to these natural decomposi-

tion processes, chemical extractions used to concentrate the fossil pollen removes alkali-soluble organics, silicates and carbonates. Additionally, the acetolysis step removes the intine, cellulose and cytoplasmic contents of the pollen grain, leaving only the sporopollenin-rich exine (Hemsley et al., 1992). The obvious disadvantage of this method is the use of C-bearing chemicals, which introduce the likelihood of C isotope changes in the remaining pollen.

The isotopic composition of the chemically treated C_4 pollen is clearly depleted in ^{13}C by several permil relative to untreated pollen while the few C_3 pollen samples appear to vary randomly around the value of the untreated samples (Fig. 4). Most importantly, the chemical pretreatments drive the $\delta^{13}C$ value of the treated C_4 pollen toward values characteristic of C_3 pollen. These isotopic changes and differences between grass types are likely due to (1) effects of chemical treatments and/or (2) inter-specific variation in the chemical composition of sporopollenin (Brooks and Shaw, 1971). Acetic anhydride, the C-bearing chemical used in the acetolysis step, has a $\delta^{13}C$ value of $-19.5‰$ ($n=2$) and glacial acetic acid, the final compound used before the water rinse, has a $\delta^{13}C$ value of $-20.4\pm0.1‰$ ($n=3$). These values are not entirely consistent with the magnitude of the observed shift in the pollen isotopic composition of the C_4 grasses, but incomplete reactions between these chemicals and the pollen could presumably produce such isotopic change. The acetolysis treatment involves the solubilization of cellulose through the reaction with acetic anhydride to produce cellulose acetate (Fægre and Iversen, 1975). Incomplete removal of cellulose acetate or the reaction of the acetic anhydride with the remaining pollen could both contribute to the isotopic shifts that appear following the treatment. The alternative hypothesis is that all chemically resistant pollen approaches the same isotopic composition, regardless of the photosynthetic pathway of the plants. Such an interpretation is highly unlikely given the fact that specific organic compounds (lipids, proteins, etc.) from C_3 and C_4 plants all reflect photosynthetic pathway-specific differences (Tieszen and Fagre, 1993) and because the untreated pollen shows such distinctive isotopic differences.

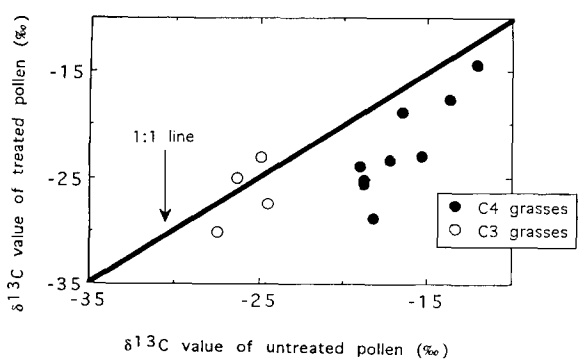

Fig. 4. The relationship between the $\delta^{13}C$ value of untreated and chemically treated pollen. The data, particularly for the C_4 samples, suggests that the chemical pretreatment utilized (acetolysis—see text for description) isotopically alters the remaining pollen.

The large isotopic shifts apparently induced by the chemical pretreatment tested here (chosen specifically because of the its large possible isotopic effects and the fact that it is commonly used in palynological studies) suggests that pollen collected for isotopic analyses be processed by alternative methods. The most desirable method would not involve any C-containing compounds. Therefore, a process involving the dissolution of mineral components with hydrofluoric acid and the removal organic detritus with oxidants would seem a viable approach. Fægre and Iversen (1975) suggest short oxidations, in cold solutions, to remove lignin without removing significant amounts of exine. The isotopic effects of this treatment were not examined in this study, but should be examined as an alternate means of removing extraneous organic materials from sediment samples being processed for pollen concentration.

How might these observations of the relationship between plants and chemically untreated pollen be applied to palynological reconstructions in grassland environments? The most direct approach would be to use a micro-pipetting technique to isolate a sufficient number of Poaceae pollen grains for isotopic analysis. Brown et al. (1989) report that spruce (*Picea*) pollen weighs approximately 9 mg. The amount of CO_2 required for auto runs varies with mass spectrometer instru-

ment characteristics. The mass spectrometer in our study required approximately 20 mmol of CO_2, requiring 50 or more *Picea* grains. Other continuous-flow instruments, with elemental analyzers, can conveniently analyze 0.5 mmol of CO_2, requiring approximately two *Picea* grains. Because of their generally smaller size, a larger number of Poaceae pollen grains might be needed for accurate isotopic measurements.

An alternative, but more ambiguous, approach would be to isotopically analyze bulk pollen retrieved from sediments and use mass balance calculations to estimate the percentage of C_4 grass pollen. The problems with mass balance methods include (1) differences in proportion of Poaceae grain numbers vs. mass to remaining pollen types in bulk pollen samples, (2) small variations in the isotopic composition of both grass and non-grass pollen from one location to another, and (3) the presence of non-graminoid C_4 pollen in certain environments. Clearly, mass balance approaches will be, at best, a semi-quantitative means of establishing the percentage of C_4 grass at a site.

4. Conclusions

The isotopic composition of untreated Poaceae pollen has been shown in this study to be closely related to that of the parent plant. Certain chemical pretreatments, used to concentrate pollen, obscure this relationship and are to be avoided in isotopic studies of pollen.

One application of these findings that seems particularly interesting is the use of pollen isotopes in the search for the development of C_4 photosynthesis. Given the ability of pollen to resist degradation and persist in the geologic record (Brooks, 1971), it is possible that this approach may address the intriguing issue of the timing of the evolution of C_4 grasses (Cerling et al., 1993, 1994; Morgan et al., 1994a,b). In this application, the isotopic analysis of grass pollen will provide a more direct link to the composition of grass flora than the analysis of paleosols, mammal teeth, or other compounds, all of which contain homogenized isotopic signatures of much, or all, of the flora of an ecosystem. While technical problems remain to be investigated regarding pollen concentration or preparation, the isotopic analysis of pollen could prove to be a useful technique in Quaternary and Tertiary paleovegetation studies.

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