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Cedrus atlantica pollen morphology and investigation of grain size variability using laser diffraction granulometry

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ABSTRACT

The morphology and size variability of pollen grains of *Cedrus atlantica* were investigated using a novel approach employing laser diffraction granulometry. We provide new insights into size variability and present high-quality light microscopy (LM) and scanning electron microscopy (SEM) imagery of *Cedrus atlantica* pollen. Grains have an average size of 59.1 ± 4.0 μm, measured on millions of grains from 91 samples. Analysis showed there is high variability of grain size within individual samples, although variability between samples is not significant. We found no significant relationships between grain size and climate (including temperature, precipitation and aridity), and suggest that grain size of fossil *Cedrus* pollen would not be a good proxy for climate reconstruction. Grain size may be influenced by a number of complex factors such as genome size or adaptations to support wind pollination, while variability within individual samples may result from the irregular development of pollen. The laser diffraction method produced repeatable, robust measurements on millions of pollen grains which are highly correlated with measurements taken using LM ($r = 0.91$, $p = 0.002$). Where grain size information is crucial for pollen identification, for developing isolation techniques for geochemical analysis, for investigating climatic and environmental influence, or for investigating links between genomes and grain size, particle size analysis by laser diffraction provides a reproducible and robust method for quickly determining pollen grain size on many samples.

KEYWORDS

Cedrus atlantica; pollen morphology; pollen size; grain size methods; laser diffraction granulometry; climate influence; moisture availability

1. Introduction

Detailed information on pollen morphology and grain size is critical for palynologists to accurately identify vegetation from fossil pollen assemblages for pollen analysis. Grain size may be studied to improve the taxonomic resolution of pollen identification, for example with *Pinus* pollen (Desprat et al. 2015), *Poaceae* pollen (Radaeski et al. 2016), and indeed with *Cedrus* pollen (Fujiki et al. 2003). Pollen size has also been correlated with genome size and may indicate polyploidy (three or more chromosome sets) in plants (Gould 1957; Kapadia & Gould 1964; Bennett 1972; Tate et al. 2005; Knight et al. 2010; De Storme et al. 2013). Increasingly, geochemical studies utilising pollen, such as stable isotope analysis for palaeoclimate reconstructions (Amundson et al. 1997; Loader & Hemming 2004; Nelson et al. 2006, 2007; King et al. 2012; Nelson 2012; Bell et al. 2017) and biomarker analysis for UV-B reconstructions (Rozema et al. 2001, 2002; Fraser et al. 2011; Willis et al. 2011; Lomax et al. 2012; Jardine et al. 2017), require detailed knowledge of grain size for developing techniques to isolate specific grains from fossil assemblages. For example, the use of micro-sieving to concentrate pollen from sediment (Heusser & Stock 1984; Brown et al. 1989) can be modified to target grains within a specified size range to facilitate the isolation of pollen for specific species.

Traditional pollen analysis relies on visual identification of pollen grains to determine vegetation composition, while geochemical studies undertake further analysis of the grains to gain insight into environmental or climate conditions. It has also been hypothesised that the size and shape of the pollen grain itself may be influenced by climate (Ejsmond et al. 2011). Temperature has been previously linked to pollen grain size (Kurtz & Liverman 1958), and Schoch-Bodmer (1936) proposed that grain size variability in pollen is a result of fluctuations in moisture availability in ambient air during pollen development. Ejsmond et al. (2011) analysed eight *Rosaceae* species and found grain size increased under desiccation stress (determined by temperature, potential evapotranspiration and altitude). A positive relationship was also found between temperature and pollen size in 232 plant species from 11 taxonomic groups, suggesting possible climatic influence on size during the flowering period (Ejsmond et al. 2015) and offering potential for pollen grain size to aid in reconstructions of past environments.

Measurements of grain width on modern *Nothofagus* spp. pollen showed a significant relationship with mean annual precipitation (MAP), where grain size increased with reduced MAP ($r^2 = 0.66$, $p < 0.0001$). Applying this relationship to fossil pollen samples from Antarctica, where average grain size increased by 23% from the late Eocene to the mid Miocene, suggests a decrease in precipitation during this period (Griener & Warny 2015). This is supported by an earlier study on the same fossil samples which found stable carbon isotope discrimination

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($\Delta^{13}C$) values of pollen decreased during the same period, which the authors also linked to a decrease in moisture availability (Griener et al. 2013). However, the validity of the relationship between grain size and moisture availability in this study has been questioned, citing a lack of theoretical and empirical support, with a view that it may be premature to use grain size as a moisture availability proxy (Jardine & Lomax 2017).

Atlas cedar (*Cedrus atlantica* (Endl.) Manetti ex Carrière) is a moisture-sensitive (Rhanem 2011; Linares et al. 2013; Ilmen et al. 2014) montane conifer endemic to semi-arid and humid areas of Morocco and Algeria (Farjon 1990), with pollen records indicating a presence in the area since at least the Last Glacial Maximum (Lamb et al. 1989; Magri et al. 2017; Zielhofer et al. 2017). The earliest work on *Cedrus atlantica* pollen morphology found the average size of the grain to be 61.5 ± 1.8 μm based on 100 grain measurements of pollen from a single tree in Ifrane, Morocco (Aytug 1961), while a later study recorded a size of 45.8 ± 2.4 μm measured on pollen collected from one tree in Marseille, France (Fujiki et al. 2003). In both studies, pollen samples had been collected and stored 15 years prior to measurements being taken. *Cedrus atlantica* pollen grains have also been measured at 75 μm on samples from Turkey (Altuner et al. 2012), and 58 μm in Vancouver, Canada (Ho 1972). In Algeria, samples from the Tell Atlas ranged in size from 60 to 63 μm, while samples from the eastern margins of the Saharan Atlas and Aurès Mountains measured 54 to 60 μm (Derridj et al. 1991).

The literature suggests that pollen from *Cedrus atlantica* varies in size significantly, which could possibly result from climate differences between sample locations. However, variations noted in these studies may also be due to different methodological approaches undertaken for measurement which could have affected grain size – e.g. changes resulting from chemical pre-treatments, pollen hydration, mounting media, cover-slip pressure and storage methods (Andersen 1960; Aytug 1960; Faegri & Deuse 1960; Cushing 1961; Reitsma 1969; Praglowski 1970) – so it is not possible to determine from current literature whether there is a climatic influence.

In this study, we provide a comprehensive analysis of grain size variability of *Cedrus atlantica* pollen from 91 modern samples across the Middle Atlas, Morocco (Figure 1), and a wider

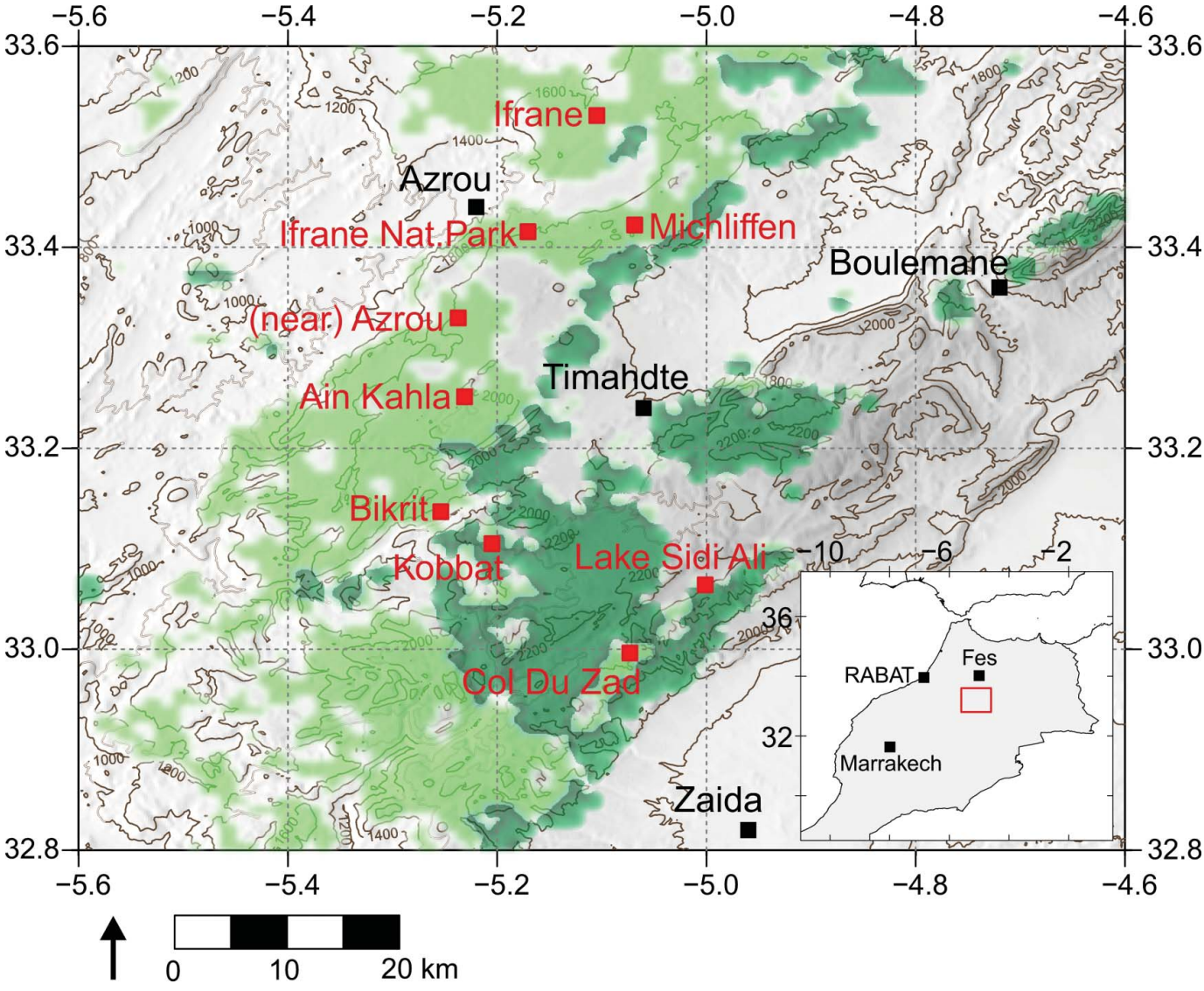

Figure 1. Map of the Middle Atlas and forest cover: dark green is predominately needleleaf evergreen, and light green is mixed broadleaf evergreen and needleleaf evergreen. Sample areas are shown in red and towns in black. Map created using Global Multi-resolution Terrain Elevation Data 2010 (GMTED2010) and forestry data extracted from Global Land Cover Characterization (GLCC) imagery. Data available from US Geological Survey (2017).

environmental gradient incorporating botanical garden samples from Europe and the USA. We employ a novel approach to determining pollen size by using non-destructive laser diffraction granulometry to consistently measure thousands to millions of individual pollen grains. We compare this technique to traditional measurements taken under light microscopy (LM), and we additionally measure samples following chemical treatment to analyse its effect on grain size. We describe the morphology and aim to determine the overall size of *Cedrus atlantica* pollen, and determine whether grain size variability is influenced by climate or environmental factors.

2. Material and methods

2.1. Sample collection and preparation

Pollen samples were collected from *Cedrus atlantica* trees from nine locations (Table 1) across the Middle Atlas, Morocco (n = 72), with additional samples collected from sites in Spain, France, UK and USA (n = 19). Samples were collected between September and October 2015, apart from the UK samples which were collected in 2014. Multiple strobili from each tree were collected and placed in paper envelopes on site, and freeze-dried back in the laboratory. Grains were extracted by vigorous shaking of the strobili, and collecting in sieves. Non-pollen contaminants were removed by visual inspection, and grains were stored in glass vials at 4 °C.

Measurements were carried out on untreated samples using both high-power transmitted LM and laser diffraction granulometry, with additional measurements under LM on samples treated with 10% potassium hydroxide (KOH) in a water bath at 90 °C for 15 minutes.

2.2. Light microscopy

Pollen was mounted on glass slides with silicone oil following dehydration with ethanol and tert-butyl alcohol. Two hundred and forty grains from eight samples (30 grains per sample) were measured with a Zeiss Axioscope A1 microscope and Zeiss Axiocam ERC5S camera using AxioVision 4 software. Grains in equatorial view were selected randomly and measured live on-screen following microscope calibration at 400× magnification.

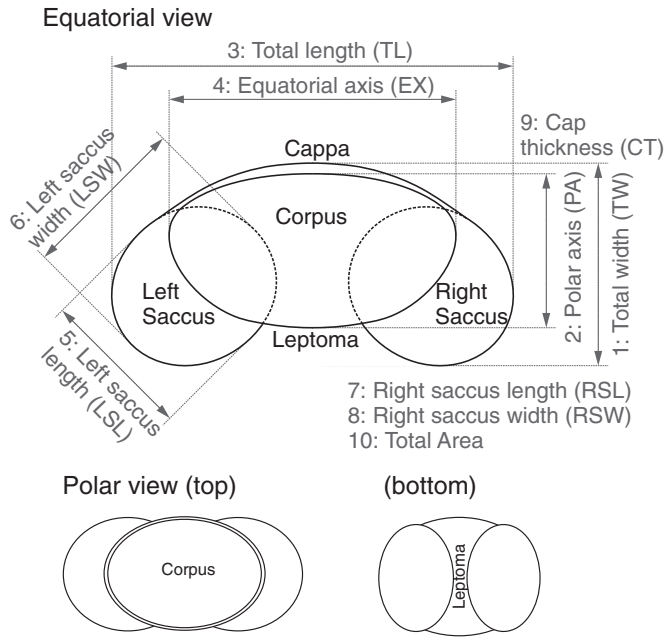

Figure 2. Schematic diagram of *Cedrus atlantica* pollen as it appears under light microscopy (LM), and the different measurements taken under LM.

Ten different properties of each grain were measured (Figure 2), adapted from Nakagawa et al. (1996) and Tiwari et al. (2012). Pollen terminology follows Erdtman (1943) and Punt et al. (2007).

2.3. Scanning electron microscopy

Scanning electron microscopy (SEM) images were obtained using a 120-kV FEI Tecnai 12 Twin Transmission Electron Microscope in The University of Manchester Life Sciences Faculty. Untreated and treated pollen samples were dehydrated in graded ethanol stages, then dried with Hexamethyldisilazane (HMDS) following (Chissoe et al. 1994). Grains were stuck to spurs with doubled-sided tape and sputter-coated with gold palladium for 5 minutes prior to SEM.

Table 1. Details of the sampling areas where pollen was collected.

Location[1]	No. of samples	Longitude	Latitude	Altitude (m asl)[2]	Mean annual precipitation (mm)[3]	Temperature range (°C)[4]
Ifrane	2	−5.11	33.43	1653	474 (836)	−1.8 to 28.0
Michliffen	15	−5.08	33.34	1940	516 (714)	−3.0 to 28.5
Ifrane National Park	6	−5.17	33.39	1723	516 (747)	−1.7 to 29.8
Col Du Zad	7	−5.07	33.07	2106	470 (515)	−3.8 to 29.6
Lake Sidi Ali	10	−4.99	33.08	2150	470 (408)	−3.3 to 29.2
Bikrit	9	−5.26	33.24	1611	516 (738)	−1.0 to 30.5
Kobbat	11	−5.21	33.19	2074	516 (667)	−3.8 to 27.7
Ain Kahla	6	−5.23	33.30	1939	516 (695)	−3.0 to 28.5
(near) Azrou	6	−5.24	33.35	1814	516 (696)	−2.2 to 29.3
Westonbirt, UK	5	−2.21	51.61	132	854	0.9 to 20.7
Manchester, UK	3	−2.21	53.41	43	890	1.4 to 19.6
Boston, USA	2	−71.12	42.30	45	1236	−9.2 to 27.1
Paris, France	4	2.36	48.84	35	618	1.8 to 24.8
Bordeaux, France	3	−0.60	44.85	23	958	2.4 to 25.2
Pyrenees, Spain	2	−0.55	42.57	820	753	−1.9 to 26.0

[1]Location within Morocco unless otherwise indicated.
[2]Average altitude (metres above sea level [asl]) of samples collected in the location.
[3]CRU (East Anglia Climate Research Unit) data averaged over 30 years (1986–2015). Values in parentheses indicate interpolated precipitation values (Bell et al. 2017).
[4]Mean annual minimums and maximums.

2.4. Laser diffraction particle size analysis

Untreated pollen samples were measured using a Malvern Mastersizer 2000, fitted with a Hydro 2000μP liquid sample dispersion unit for small particles (e.g. Sperazza et al. 2004) in The University of Manchester Geography laboratories. The machine was calibrated prior to measurements on pollen, using a spherical glass bead standard supplied by the manufacturer. The system was configured using the following method: Sample material was characterised as sporopollenin with a refractive index of 1.475 (Traverse 2007). Dispersant used was water with a refractive index set to 1.33 (Hecht 2002). Sample measurement time and background measurement time were set to 30 seconds. Pump speed was set to 2000 rpm, and ultrasonic treatment set to 75% with a pre-measurement period and delay of 30 seconds. Measurement repeats were set to three per aliquot with a 10-second delay. Using this method, the dispersion unit (Hydro 2000μP) was filled with deionised water using the anaerobic fill option (to remove air bubbles, which would otherwise affect the result). Once full, the pollen sample was added to the dispersion unit to reach a laser obscuration which ideally fell between 10 and 20% (this is shown on screen as the sample is added). Once the desired laser obscuration was reached, the measurement cycle was run. On completion of measurements, the system was drained, and flushed with deionised water 3 times to remove the sample. The sample can be recovered at this stage if required.

Particle size distribution of the sample was computed from the diffraction measurements using a model based on Fraunhofer diffraction theory (Syvitski 1991). The resulting analysis is reported as the relative distribution of the volume (%) of pollen grains by size (μm) (Figure 3). Each grain measurement is placed within a size class (which can be defined by the user), and the distribution therefore shows the percentage of grains within a given size range. For example, in Figure 3, 16.8% of the individual grains measured within the entire sample were between 56 and 63 μm in size. The D_{10} value represents the size of the grains below which 10% of the sample lies (i.e. 10% of the individual grains measured in the sample are smaller than the D_{10} value), D_{90} is the size of the grains below which 90% of the sample lies, and D_{50} is the median of the particle size distribution. The D_{50} value is the measurement we use to describe the size of each pollen sample.

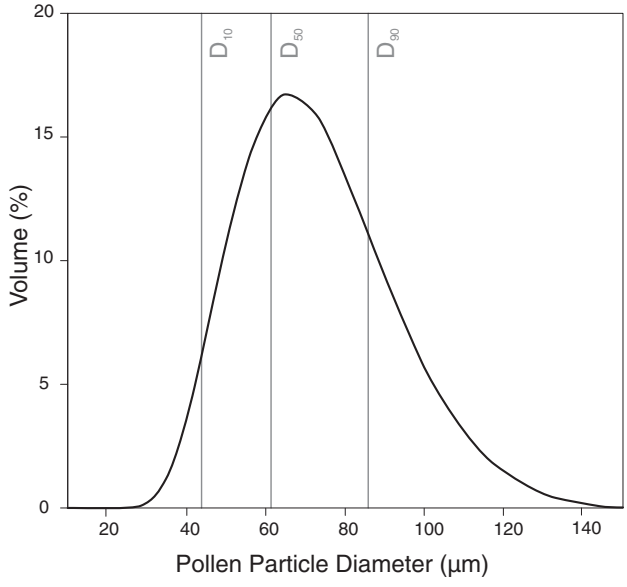

Figure 3. Results from laser diffraction granulometry. Size data for each sample is presented as the particle size distribution of the sample.

All laser particle size distribution models (Fraunhofer and Mie theory) assume the analyte particles are spherical (Syvitski 1991), and the machine will record a measurement of a particle in whichever orientation it passes through the laser. As *Cedrus atlantica* pollen grains are not completely spherical, and they may pass the laser in different orientations, the model will in practice provide a weighted average of the diameter of the pollen grain. Since the pollen morphology of *Cedrus atlantica* is consistent, an empirical relationship can be derived between the particle size measurement and LM measurements. For *Cedrus atlantica* pollen, the D_{50} value is effectively equivalent to the size of the equatorial axis (EX) as measured under LM (see Results section 3.2.2.).

2.5. Data processing and analysis

Data and statistical analyses were carried out using R (R Core Team 2016). Climate data was extracted from CRU TS v3.24.01 high-resolution (0.5°) gridded datasets (Harris et al. 2014). Precipitation for Middle Atlas locations was interpolated using local climate stations described in Bell et al. (2017). Aridity data was extracted from the self-calibrating Palmer Drought Severity Index (scPDSI) (Dai 2011).

3. Results

3.1. Description of grains (Plates 1 to 4)

Cedrus atlantica pollen are large diploxylonoid-type bisaccate grains. The grains are elongated, the corpus is typically prolate, and the cap wall is thick. Sacci are spheroidal in equatorial view, appearing more oblate in polar view. The junction of the sacci and corpus lacks a strong defining ridge, with the two parts appearing seamlessly together. Under LM the surface ornamentation of the corpus is a rough, reticulate pattern, while ornamentation is somewhat smoother on the sacci. Under SEM, surface ornamentation of the corpus is rough, appearing fossulate, with groups of irregular, spheroidal to elongated elements protruding, interspaced by deep groves. The sacci appear smooth with a scabrate–perforate surface. Surface ornamentation appears more defined following chemical treatment under both LM and SEM.

3.2. Grain size

3.2.1. Light microscope measurements

LM measurements of grain size on eight untreated pollen samples, with 240 individual grains measured (Table 2), found the average total grain size including sacci and corpus (TL) was 71.4 $\pm$ 7.6 μm, while the EX measured an average 52.2 $\pm$ 4.0 μm. There was large variation in the size of grains observed: For TL, there was a 40-μm difference between the smallest and largest grains, and for EX, there was a 20-μm difference. Full LM

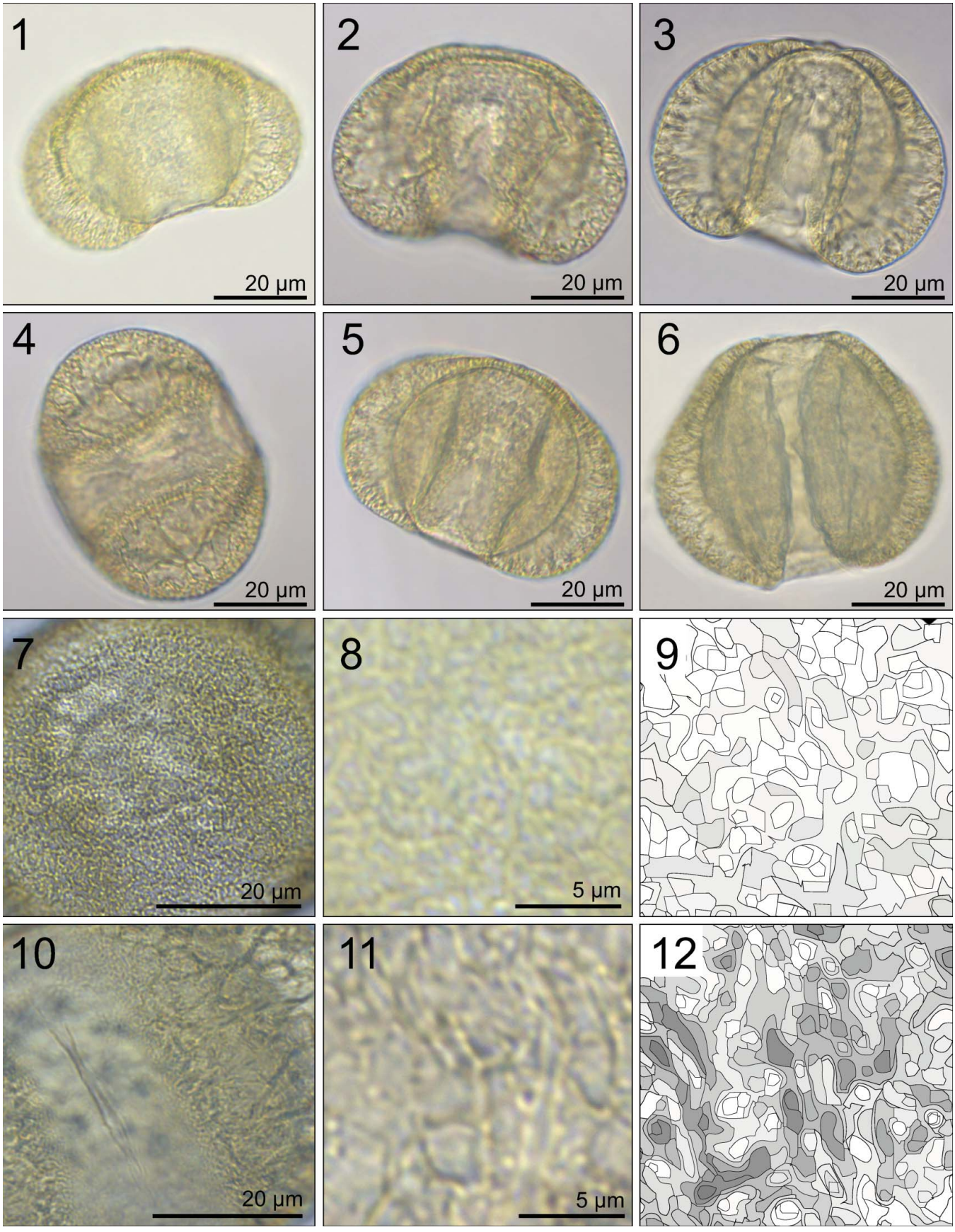

Plate 1. Photographs taken under light microscopy (LM) of *Cedrus atlantica* pollen grains. Scale indicated on each image. Figures 1–3. Equatorial view showing complete grain. Figures 4–5. Polar view of complete grain. Figures 7–8. Close-up of corpus surface. Figure 9. Corpus surface pattern (extracted using CorelDraw X8). Figures 10–11. Close-up of saccus surface. Figure 12. Saccus surface pattern.

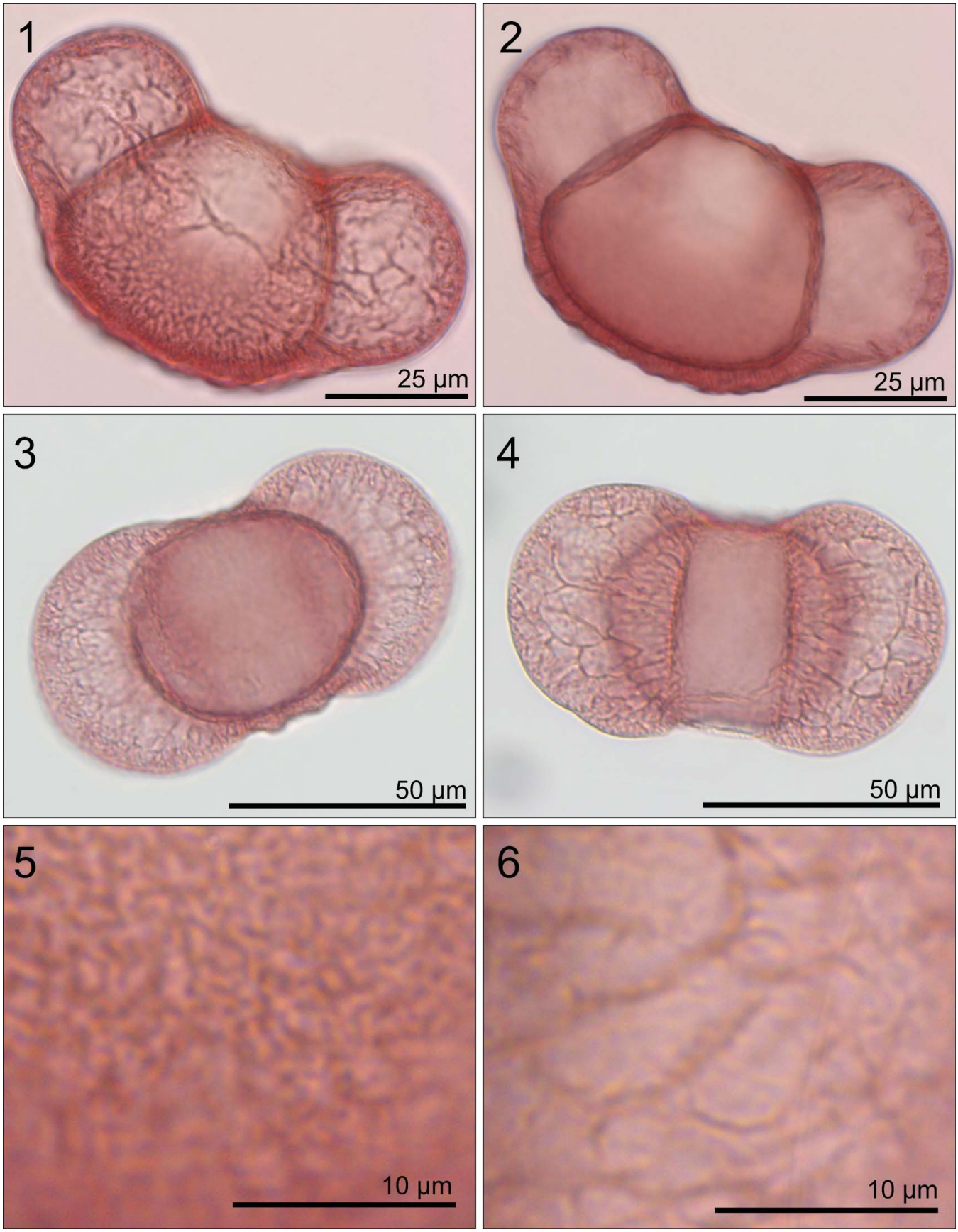

Plate 2. Photographs taken under light microscopy (LM) of treated *Cedrus atlantica* pollen grains stained with Safranin. Scale indicated on each image. Figures 1–2. Equatorial view showing complete grain at different foci. Figures 3–4. Polar view of complete grain. Figure 5. Close-up of corpus surface. Figure 6. Close-up of saccus surface.

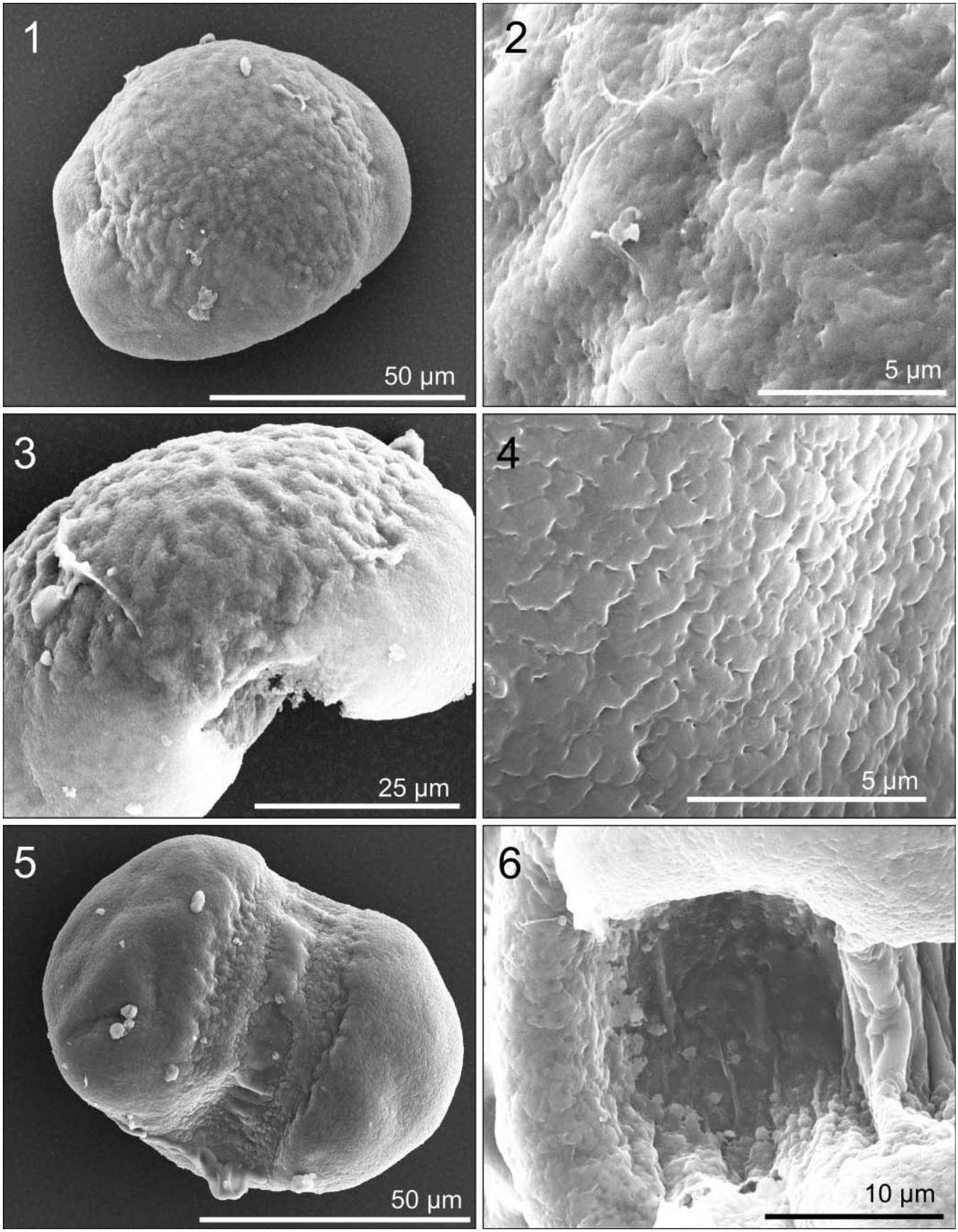

Plate 3. Scanning electron microscopy (SEM) images of *Cedrus atlantica* pollen grains. Scale indicated on each image. Figure 1. Polar view from the top. Figure 2. Close-up of corpus surface. Figure 3. Equatorial view. Figure 4. Close-up of saccus surface. Figure 5. Polar view from underneath the grain showing the leptoma. Figure 6. Close-up of leptoma, pollen wall visible to the left.

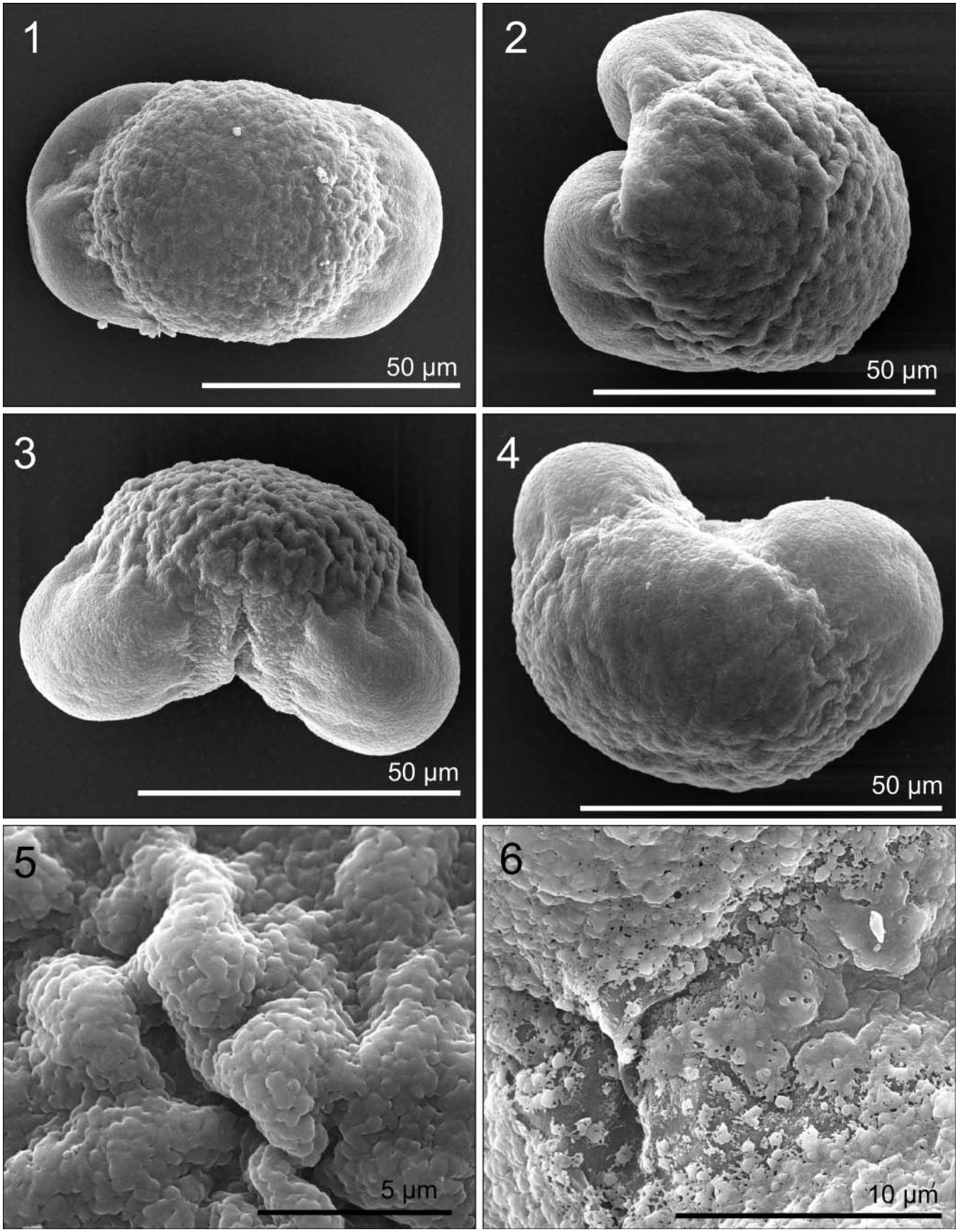

Plate 4. Scanning electron microscopy (SEM) images of treated *Cedrus atlantica* pollen grains. Scale indicated on each image. Figures 1–2. Polar view from the top and side. Figures 3–4. Equatorial view. Figure 5. Close-up of corpus surface. Figure 6. Close-up of leptoma.

Table 2. Grain size of each measured property (please refer to Figure 2) under light microscopy (LM; eight samples, 240 grains measured).

	1: TW (μm)	2: PA (μm)	3: TL (μm)	4: EX (μm)	5: LSL (μm)	6: LSW (μm)	7: RSL (μm)	8: RSW (μm)	9: CT (μm)	10: area (μm^2)	P/E ratio (EX/PA)
Mean	53.6	38.8	71.4	52.2	31.8	28.6	31.3	27.9	3.1	2774.9	1.4
Standard deviation	4.8	5.5	7.6	4.0	3.9	3.6	3.9	3.4	0.7	472.6	0.2
Minimum	35.5	25.5	49.8	43.8	19.1	20.1	21.5	19.7	1.4	1580.5	0.9
Maximum	68.0	54.0	89.1	63.8	42.5	39.7	43.0	37.3	5.2	4180.9	1.9

Table 3. Percentage change (%) in size for different properties (please refer to Figure 2) of the grain measured under light microscopy (LM) following chemical treatment with KOH.

1: TW	2: PA	3: TL	4: EX	5: LSL	6: LSW	7: RSL	8: RSW	9: CT	10: area	P/E ratio (EX/PA)
−2.8	8.5	9.3	−1.5	4.1	12.5	5.9	16.2	9.0	7.6	−7.1

measurement data can be found in the online Supplementary material.

Treated pollen samples measured under LM were on average 6.9% larger in size overall compared to untreated grains (Table 3). However, the increase in size was not consistent across all the measured properties of the grain, with the largest increases to the sacci width and cap thickness (CT), while the total grain width (TW) and EX decreased in size. This resulted in the shape of the grain changing slightly, becoming subprolate.

3.2.2. Laser diffraction particle size analysis

Pollen grain size measurements taken using laser diffraction granulometry on 91 untreated pollen samples, where millions of individual grains were measured, recorded an average grain size of 59.1 $\pm$ 4.0 μm. Particle size distribution data for each sample can be found in the online Supplementary material.

The reliability of the laser diffraction particle size measurements was tested by correlation analysis with the LM measurements (Figure 2) for the samples measured using both methods (Table 4). The strongest and most significant correlation was found between the D_{50} median size value (laser diffraction) and the LM measurement for the EX ($r = 0.91$, $p = 0.002$). During the measurement cycle for laser diffraction granulometry, pollen grains float freely in water as they pass the laser beam and are not subject to external pressure (e.g. from a cover-slip); consequently, the 'natural shape' of the grain is measured. In *Cedrus atlantica* pollen, the sacci lie underneath the corpus, protruding a short distance; effectively, the total length of the grain is slightly bigger than the equatorial axis of the corpus. Under LM, the protrusion of the sacci can appear to be greatly inflated (particularly in equatorial view) resulting in the total length of the grain appearing larger. Consequently, the best correlation with the grain size reported by laser diffraction granulometry for *Cedrus atlantica* pollen is with the equatorial axis as measured under a light microscope.

Table 4. Correlation analysis between the laser diffraction granulometry D_{50} grain size value, and light microscopy (LM) measurements.

Grain property	Pearson's r	*p*
Total width (TW)	0.68	0.062
Polar axis (PA)	0.39	0.345
Total length (TL)	0.66	0.076
Equatorial axis (EX)	0.91	0.002
Total area	0.67	0.069

3.3. Grain size variability

The eight samples measured under LM show large variability in grain size (in all measured properties) between individual grains within the same sample (Table 2). Between samples, analysis of variability (ANOVA) of the equatorial axis (measured under LM) suggests this is significant ($df = 7$, $F = 14.97$, $p < 0.0001$); however, Tukey's test reveals that six of these samples do not vary significantly in size from each other.

Laser diffraction granulometry results likewise show large grain size variability between grains within the same individual sample. Samples from the same geographical area also show variability in grain size between samples, and, lastly, there is variability of grain size between sampling areas (Figure 4). Pollen samples within the same geographical area had an average grain size range of 5.7 μm. The largest range was in the Pyrenees (12.2 μm), followed by Michliffen (9.2 μm), and the smallest range was found within Paris samples (2.0 μm). However, ANOVA shows that the variation in grain size between different sampling areas is not significant ($df = 14$, $F = 1.054$, $p = 0.412$).

3.3.1. Climatic controls on grain size variability

Regression analysis was performed on pollen grain size and possible influences including climate (temperature, precipitation, aridity and potential evapotranspiration), altitude, and carbon isotope discrimination values, which are an indicator of environmental moisture availability (Bell et al. 2017). Given that samples from within the same geographical area will experience the same climate conditions, and fossil pollen assemblages will comprise grains from several trees within the surrounding area (Bell & Fletcher 2016), analysis was performed testing the average grain size for each geographical sampling area, as well as individual sample grain size values (Table 5).

The regression analysis found no significant relationships with any of the climate variables or altitude to suggest a link to grain size. Similarly, multiple-regression analysis testing every possible combination of climate predictors using all subsets regression found no significant relationships or models to support a climate influence on grain size. Furthermore, there was no significant relationship with stable carbon isotope discrimination when samples were averaged by geographical location ($r^2 = 0.10$, $p = 0.234$) although there was a very weak significant association with individual samples ($r^2 = 0.05$, $p = 0.029$). However, 10-fold cross-validation of this model found the r^2 value could be as low as 0.003.

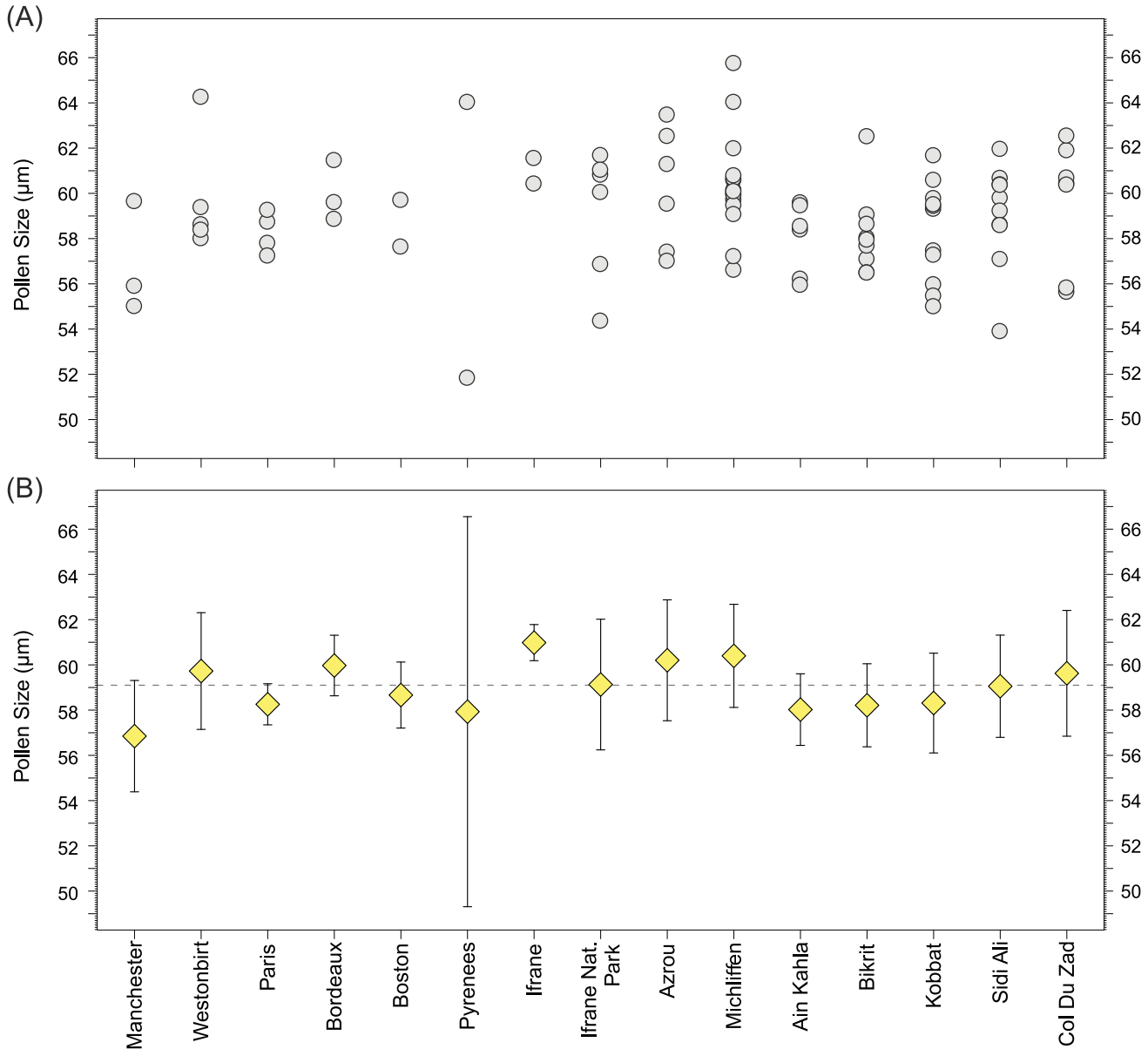

Figure 4. Results of laser diffraction granulometry showing: (A) Dot plot of D_{50} values for each sample by geographical sampling area, and (B) mean D_{50} values for each area. Error bars show standard deviation, and dashed horizontal line represents the mean grain size of all samples.

Table 5. Results of regression analysis on pollen grain size. Analysis was tested using individual sample grain size values, and the average grain size for each geographical sampling area.

		Individual sample		Location average	
Variable	Data source	r^2	*p*	r^2	*p*
Summer precipitation (2015)[1]	CRU TS v3.24.01	0.00	0.812	0.03	0.515
Mean annual precipitation (2015)[2]	CRU TS v3.24.01	0.00	0.441	0.10	0.246
Mean annual precipitation (30-year average)[3]	CRU TS v3.24.01	0.00	0.509	0.07	0.331
Mean annual precipitation (interpolated values)	Bell et al. (2017)	0.00	0.890	0.00	0.789
Mean annual temperature (2015)	CRU TS v3.24.01	0.00	0.747	0.06	0.380
Mean summer temperature (2015)	CRU TS v3.24.01	0.00	0.598	0.07	0.319
Mean annual temperature (30-year average)	CRU TS v3.24.01	0.00	0.779	0.06	0.375
Mean summer temperature (30-year average)	CRU TS v3.24.01	0.00	0.628	0.06	0.352
Aridity (scPDSI)[4]	Dai (2011)	0.00	0.586	0.07	0.324
Annual potential evapotranspiration (PET) (2015)	CRU TS v3.24.01	0.00	0.396	0.01	0.396
Summer PET (2015)	CRU TS v3.24.01	0.01	0.551	0.00	0.551
Carbon isotope discrimination ($\Delta^{13}C$)[5]	Bell et al. (2017)	0.05	0.029	0.10	0.234
Altitude	This study	0.00	0.439	0.07	0.316

[1]Summer corresponds to the development period for *Cedrus atlantica* pollen.
[2]Values for the year of pollen collection.
[3]Values based on a 30-year average between 1986–2015.
[4]Aridity values from self-calibrating Palmer Drought Severity Index (30-year average).
[5]Carbon isotope discrimination calculated from $\delta^{13}C$ values measured directly on the same pollen samples.

4. Discussion

4.1. Influences on pollen grain size variability

Our study has found no relationship between climate and the grain size of modern *Cedrus atlantica* pollen. This is supported by the lack of relationship between grain size and $\Delta^{13}C$ when grain size was averaged by geographic location ($r^2 = 0.10$, $p = 0.234$), where the $\Delta^{13}C$ value of the same pollen samples were previously shown to be significantly affected by moisture availability (Bell et al. 2017). However, there was a very weak association between individual sample grain size measurements and $\Delta^{13}C$ ($r^2 = 0.05$, $p = 0.029$). Further cross-validation of this model shows that less than 5% (in some cases, as little as 0.3%) of the variance in grain size is explained by carbon isotope discrimination. As no relationship was found between $\Delta^{13}C$ and grain size averaged by geographical area, this suggests that factors other than climate must influence grain size, as these samples would experience the same climate conditions. Although there may be microscale climate variations in these areas (Bell et al. 2017), variations in isotopic composition of samples from the same area may also be associated with the intrinsic water-use efficiency of the tree (Körner et al. 1991). Accordingly, the results suggest that while samples have a strong geochemical climate signature, there is no equivalent strong morphological climate signature (Figure 5).

In another study, summer moisture availability was also ruled out as influencing pollen grain size on *Cedrus atlantica* populations in Algeria. Analysis showed that pollen grain size discriminated trees into different genetic and ecological groups, suggesting genetics influenced pollen size (Derridj et al. 1991). The genetic control on pollen size may stem from genome size (Bennett 1972; De Storme et al. 2013), in particular the effect of an increased number of chromosomes due to polyploidy (Kapadia & Gould 1964; Dyer et al. 2013). Knight et al. (2010) found a significant positive trend between pollen width and genome size across 464 species. However, the trend was not significant when phylogeny was taken into account, suggesting that pollen size would not be a good proxy for genome size in the fossil record. The phylogenetic relationships among *Cedrus* have been debated (Bou Dagher-Kharrat et al. 2001), but DNA evidence suggests *Cedrus atlantica* separated from a common ancestor of *Cedrus libani* and *Cedrus brevifolia* around 23 to 18 Ma BP (Qiao et al. 2007), and DNA analysis of all four *Cedrus* (the 'true' cedars) species (including *Cedrus deodara*) found that genome size is homogeneous among the species (Bou Dagher-Kharrat et al. 2001). Genetic diversity of *Cedrus atlantica* trees from Morocco was shown to be high within populations and between populations (Renau-Morata 2005; Terrab et al. 2006), particularly between populations from the Rif, and High Atlas, when compared to Middle Atlas populations (Cheddadi et al. 2009). It is possible that the variations in grain size we observe between samples could relate to genome size; however, further research is needed to confirm this in *Cedrus atlantica* pollen.

The influence of temperature on grain size as demonstrated by Ejsmond et al. (2015) was suggested to relate to pollen performance, whereby a trade-off exists between the size of the grain and the quantity of grains produced (Vonhof & Harder 1995). The competitive ability of pollen increases with temperature during the flowering period, and this increased competition promotes larger pollen grains (Ejsmond et al. 2015). The lack of any temperature influence on *Cedrus atlantica* pollen size may be due to it being a wind-pollinating species. The apparent trade-off between pollen size and quantity may not be necessary, as priority is on the quantity of grains produced in order to increase the chances of successful pollination (Whitehead 1983; Cruden 2000). Larger grains are heavier, giving increased chance of reaching ovules as they can more easily break from the airstream, while smaller, lighter grains can travel greater distances (Niklas 1985). Pollen size in wind-pollinated species consequently reflects an equilibrium between these demands (Lu et al. 2011). Large grain size variation within individual samples could therefore possibly reflect an adaptation to facilitate both demands. The observed grain size variation also implies that smaller grains found within fossil assemblages from geological archives might be an indicator of long-distance pollen transport, with larger grains indicating nearby pollen sources rather than reflecting a climate signal.

Smith (1923) noted 'irregularity' with the development of *Cedrus atlantica* pollen, where newly forming grains exist alongside mature grains, and long retention of grains after they reached maturity. Strobili typically form 2–3 months prior to pollen release, with grains developing in the later parts of this period. Pollen release is a phenological response lasting a few days, with the timing varying between individual trees and between geographical locations, depending on optimal environmental conditions including temperature, humidity levels and wind speed (Whitehead 1983; Khanduri & Sharma 2009).

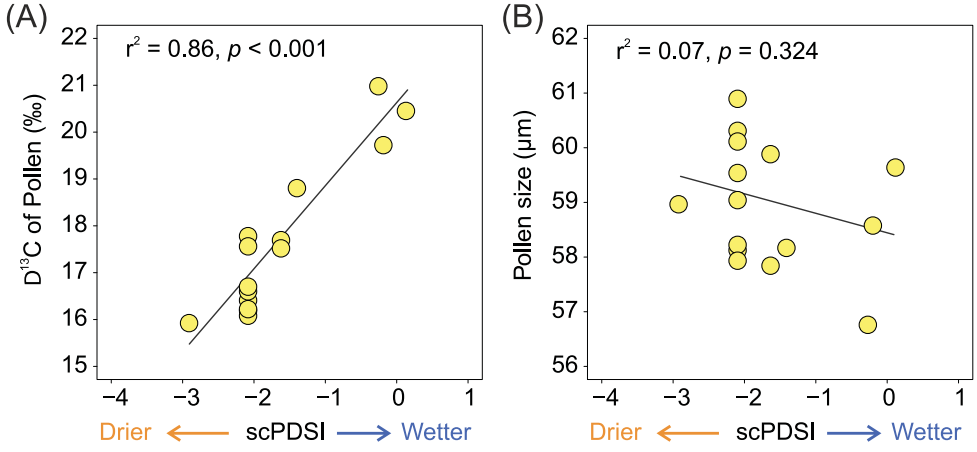

Figure 5. Biplots showing: (A) carbon isotope discrimination ($\Delta^{13}C$) (Bell et al. 2017) versus aridity (self-calibrating Palmer Drought Severity Index, scPDSI) (Dai 2011), and (B) pollen grain size versus aridity (scPDSI), with values averaged by each sampling area.

For *Cedrus atlantica* this typically occurs from early to late September and early October. This irregular development of grains may also contribute to the size variation observed, if pollen release occurs while some grains are still developing and others are fully developed.

Soil nutrient availability has also been linked to pollen grain size and production, in squash (*Cucurbita pepo*) plants. Grain size and the number of grains produced both increased where plants were in soil which had higher nutrient contents; this effect was greatest with increased nitrogen (Lau & Stephenson 1993), but also evident with increased phosphorus (Lau & Stephenson 1994). If soil nutrient availability influenced grain size for *Cedrus atlantica* pollen, then we might expect to see larger grains in the botanical garden sites and smaller grains in the relatively nutrient-poor Middle Atlas locations. However, grain size is smaller than average at two of the three botanical garden sites (Westonbirt, Paris and Boston), while grain size is larger than the average at the most southerly Middle Atlas site (Col Du Zad), an environment characterised by sparse open forest and semi-arid conditions where nutrient availability is poor. This suggests that nutrient availability does not influence grain size for *Cedrus atlantica* pollen.

Overall, the large variability in pollen grain size we observe within individual samples, in some cases by as much as 20 μm, suggests that grain size is influenced by a number of complex factors. The grain size variability is also not unexpected, and is well documented in other pollen (e.g. Bell 1959; Clausen 1962; Bragg 1969; Cruzan 1990; Desprat et al. 2015). Consequently, we propose that due to the size variability in *Cedrus atlantica* pollen, and lack of evidence for climatic influence, it would not be possible to use this as a proxy for climate or environmental reconstruction, as differences in the size of fossil pollen may simply result from the observed natural variation in size between pollen grains. Our findings contrast the suggested climatic influence on grain size observed in other species (Ejsmond et al. 2011 2015; Griener & Warny 2015), and underscore the need for further investigation of the complex controls on pollen grain size (Jardine & Lomax 2017).

4.2. Methodological approaches to grain size measurements

We demonstrate the importance of a consistent methodological approach to grain size measurement, through the differences in grain size reported in the literature, as a result of the technique used, and between untreated and chemically treated pollen grains. It is notable that the size changes we observe in *Cedrus atlantica* pollen grains treated with KOH are not uniform across the entire grain, suggesting that morphological differences in pollen have different susceptibilities to chemical treatment. For example, Reitsma (1969) found the size-altering effects of KOH treatment depended on pollen type and exposure time to the treatment, while effects on pollen size from chemical pre-treatment have also been noted in Faegri & Deuse (1960), Praglowski (1970) and Charman (1992). In *Cedrus atlantica* the largest size increase in the pollen grain occurred in the sacci, which have a perforate surface in contrast to the rest of the grain. The small apertures allow the pollen to quickly dehydrate following pollen release (Tekleva et al. 2007), reducing its weight to assist long-distance transport (Lu et al. 2011). In a reversal of this process, the apertures may allow greater penetration of chemical treatment into the sacci compared to other parts of the grain, and may explain why they exhibited a greater increase in size. This suggests that morphological variations within pollen grains and between pollen types are affected by treatments in different ways, so relationships of grain size should always be established to specific pollen types following the same preparation and methodological protocols.

Fossil pollen grain size may also be affected by diagenesis. Although potential size-altering effects are not fully known, they are likely to differ depending on species and sedimentary setting (Mäkelä 1996), with resistance to these effects likely stemming from morphological traits of the pollen grains. Due to these effects, it may be difficult to compare grain size between fossil pollen from different sedimentary settings, implying that apparent grain size changes in a fossil sequence could result from changes to the sedimentary setting, unless it remains homogeneous throughout. Consequently, pollen grain size relationships and comparisons between pollen from a fossil setting and modern pollen samples may not be straightforward (Mäkelä 1996). Indeed, before comparison, the effects of diagenesis and sedimentary setting on fossil pollen size should first be established. Overall, all possible influences on grain size should be considered in the interpretation of results (Desprat et al. 2015), and a consistent methodological approach to measurements should be taken.

4.3. Practical considerations for laser diffraction particle size analysis of pollen

We have shown that laser diffraction granulometry provides a reliable and consistent method of determining pollen grain size, and produces results in line with existing LM methods of grain size measurement. The main benefit of laser diffraction granulometry is that it can measure thousands to millions of pollen grains (dependent on sample size) in just a few minutes, providing a very robust assessment of the typical grain size (here, D_{50} or median) which can be repeated on multiple samples quickly. The method is also non-destructive, allowing recovery of the sample after the measurement cycle. Measurement of grains is not affected by operator subjectivity or potential inaccuracies between measurements, nor is it affected by size changes caused by mounting medium or cover-slip pressure on the grain, which can affect LM measurements. Provided the morphology of the pollen grains is consistent, an empirical relationship between laser diffraction granulometry measurements and light microscope measurements can be established. In additional testing of the method, we found similar results with *Pinus* pollen between the grain size reported by laser diffraction granulometry and LM measurements; however, further testing of the method is needed on other pollen types with different morphologies, and pollen types with smaller grains.

The primary use of laser diffraction granulometry for pollen size determination would be on modern samples, due to the relatively large quantity of sample material required. There is a range of possible applications, including investigating the influence of climate and environmental factors, nutrient availability, and genome size. In testing, approximately 15 mg of pollen

was used to reach 10% laser obscuration, which could easily equate to millions of grains (dependent on the grain size/weight of the specific pollen type), based on estimates of pollen weight. For example, Brown & Irving (1973) weighed several pollen types, including *Quercus robur*, suggesting 93 grains weigh 1 μg, or that there are 1,395,000 grains per 15 mg. There are reportedly 404 maize grains per 1 μg, or 6,072,874 grains per 15 mg (Miller 1982). Bunderson & Levetin (2015) weighed different *Juniperus* species where approximately 273 grains weigh 1 μg, or there are 4,109,589 grains per 15 mg (averaged across species at 60% relative humidity).

We found that it is possible to use a reduced sample size, where laser obscuration only reached 0.4%, which produced grain size results in line with those at 10% laser obscuration. This equates to approximately 0.6 mg of pollen material required, which could equate to several thousand grains. Though perhaps this is still out of reach for measurements on fossil samples, it does allow measurements to be taken on modern pollen for a range of species, from small insect-pollinated flowers to large wind-pollinated trees.

5. Conclusions

Our study finds that while there is large variability in the pollen size of *Cedrus atlantica*, it is not significant between samples. We found no significant relationships between climate and grain size, testing temperature, precipitation, aridity and potential evapotranspiration (PET). Grain size of *Cedrus atlantica* may be influenced by a number of complex factors, and variability within individual samples may result from the irregular development of pollen. Our study confirms that a consistent methodological approach to grain size measurement must be taken, as there are many aspects of pollen handling and preparation stages that may influence the observed size. Size comparisons between values reported in literature, between different species/pollen types, and from different sedimentary settings may not always be possible or advisable. We have also shown a method utilising laser diffraction granulometry which can be used on modern pollen samples to accurately determine pollen size, and which produces results consistent with LM measurements. This non-destructive method of determining pollen size provides reproducible, robust results, from potentially millions of pollen grains, quickly and easily.

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Supplementary materials

Explanatory notes for the supplementary data

Cedrus atlantica pollen size measurement data under light microscopy.

Cedrus atlantica particle size distribution data from laser diffraction granulometry.

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