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## RESEARCH/REVIEW ARTICLE

# UV-B absorbing pigments in spores: biochemical responses to shade in a high-latitude birch forest and implications for sporopollenin-based proxies of past environmental change

Wesley T. Fraser,<sup>1,2</sup> Mark A. Sephton,<sup>3</sup> Jonathan S. Watson,<sup>4</sup> Stephen Self,<sup>1</sup> Barry H. Lomax,<sup>5</sup> David I. James,<sup>6</sup> Charles H. Wellman,<sup>7</sup> Terry V. Callaghan<sup>7,8</sup> & David J. Beerling<sup>7</sup>

<sup>1</sup> Department of Earth and Environmental Sciences, The Open University, Milton Keynes MK7 6AA, UK

<sup>2</sup> School of Geosciences, University of Edinburgh, West Mains Road, Edinburgh EH9 3JN, UK

<sup>3</sup> Department of Earth Science and Engineering, South Kensington Campus, Imperial College London, London SW7 2AZ, UK

<sup>4</sup> Planetary and Space Sciences Research Institute, The Open University, Milton Keynes MK7 6AA, UK

<sup>5</sup> School of Biosciences, Division of Agriculture and Environmental Sciences, University of Nottingham, Nottingham NG7 2RD, UK

<sup>6</sup> Thermo Fisher Scientific, Stafford House, Boundary Way, Hemel Hempstead HP2 7GE, UK

<sup>7</sup> Department of Animal and Plant Sciences, University of Sheffield, Sheffield S10 2TN, UK

<sup>8</sup> Abisko Scientific Research Station, SE-981 07 Abisko, Sweden

## Keywords

FTIR; spores; ferulic acid; *p*-coumaric acid; shade; sporopollenin.

## Correspondence

Wesley T. Fraser, School of Geosciences, University of Edinburgh, West Mains Road, Edinburgh EH9 3JN, UK. E-mail: wesleyfraser@gmail.com

## Abstract

Current attempts to develop a proxy for Earth's surface ultraviolet-B (UV-B) flux focus on the organic chemistry of pollen and spores because their constituent biopolymer, sporopollenin, contains UV-B absorbing pigments whose relative abundance may respond to the ambient UV-B flux. Fourier transform infrared (FTIR) microspectroscopy provides a useful tool for rapidly determining the pigment content of spores. In this paper, we use FTIR to detect a chemical response of spore wall UV-B absorbing pigments that correspond with levels of shade beneath the canopy of a high-latitude Swedish birch forest. A 27% reduction in UV-B flux beneath the canopy leads to a significant ( $p < 0.05$ ) 7.3% reduction in concentration of UV-B absorbing compounds in sporopollenin. The field data from this natural flux gradient in UV-B further support our earlier work on sporopollenin-based proxies derived from sedimentary records and herbaria collections.

Concern over the effects of anthropogenic emissions on the chemistry of Earth's atmosphere has been increasing for the past three decades. The role of ozone-depleting compounds operating in the stratosphere has been recognized since 1974 (Molina & Rowland 1974). The most common anthropogenic ozone depleting substances are chlorofluorocarbons (CFCs), the photodissociation of which is driven by energy from incoming solar ultraviolet (UV) radiation. The resultant free radical species in the upper atmosphere, Cl and ClO, catalyse ozone destruction reactions.

The effect of anthropogenic CFC emissions on stratospheric ozone destruction was demonstrated in the 1980s when observations revealed large decreases in springtime

ozone column depth above Antarctica (Farman et al. 1985). Subsequent attempts to reduce CFC production and the associated ozone loss were encapsulated in the Montreal Protocol that came into force in 1989 (UNEP 2000). Yet despite these efforts of mitigation, stratospheric ozone depletion events continue to occur in polar regions owing to the long residence times of CFCs in the atmosphere. Stratospheric ozone attenuates harmful incoming UV-B radiation ( $\lambda = 280\text{--}315$  nm) but, due to atmospheric absorption, wavelengths below 288 nm are not detected at Earth's surface. Negative changes in ozone abundance, termed ozone depletion events, pose an increased threat to terrestrial life because more UV-B is allowed to reach Earth's surface. As a result, ozone

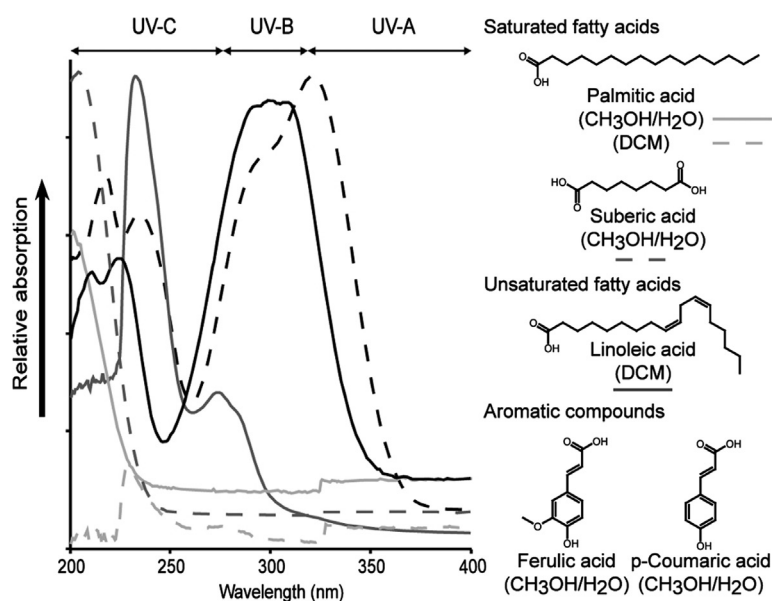
depletion raises the likelihood of deleterious effects on plants, animals, ecosystems and particularly human health (Diffey 2004).

As with all environmental effects of anthropic activity, providing historical context is difficult owing to the lack of long-term instrumental records. Instrumental records of total atmospheric ozone date back to 1926 and since this time the only continuously measuring station has been that at Arosa, Switzerland. The ozone layer over the Antarctic has been monitored less extensively and records began in the mid-1950s (Farman et al. 1985). To extrapolate these instrumental records back in time, geochemists must identify proxies that host a record of environmental change.

A promising proxy for stratospheric ozone levels and corresponding UV-B flux is the pigment contents of pollen and spore wall materials (Rozema et al. 2001; Watson et al. 2007; Lomax et al. 2008). The outer walls (exine) of pollen and spores are constructed from the biopolymer sporopollenin. This material is made up of two main building blocks: aliphatic acids and phenolic (aromatic) acids. Fig. 1 reveals how aliphatic acids have little capacity to absorb UV-B but aromatic compounds are highly effective UV-B screening entities. Current theories suggest that plants can adapt their biochemistry to increase pollen and spore phenolic pigment concentrations during high levels of ambient UV-B thereby

protecting the cytoplasm and organelles from damage (Rozema et al. 2001) during this crucial period of the plant's life cycle. Characterizing the biochemical response in pollen and spores from herbaria collections and relatively recent sedimentary records would provide a potential proxy for ozone levels and UV-B flux through time.

The potential of using sporopollenin composition as a proxy for UV-B radiation has been explored in some recent publications (Rozema et al. 2001; Blokker et al. 2005; Watson et al. 2007; Lomax et al. 2008). Examination of spore wall chemistry utilizing thermally assisted hydrolysis and methylation (thermochemolysis) gas chromatography-mass spectrometry reveals the individual UV-absorbing phenolic acids. The UV absorbing pigments appear to be the only components in the sporopollenin that contain aromatic rings (Watson et al. 2007; Fig. 1) and these aromatic rings are readily identified by spectroscopic methods. Based on this reasoning, we have utilized Fourier transform infrared (FTIR) microspectroscopy to measure the relative abundance of aromatic units and, thereby, determine pigment contents (Watson et al. 2007; Lomax et al. 2008). Our method was previously tested using spores obtained from five populations of the tropical lycopsid (a clubmoss) *Lycopodium cernua* growing across an altitudinal gradient (650–1981 m a.s.l.) in south-east Asia with the assumption that plants would experience an



**Fig. 1** Representative monomer components of sporopollenin, after Watson et al. (2007), showing the ultraviolet (UV) absorbance of each compound. Analysis was conducted at the University of Sheffield using a PU8720 UV/Vis scanning spectrophotometer (Philips, Eindhoven, The Netherlands). A wavelength range of 200–400 nm, corresponding to the mid-UV-C through to far-UV-A range, was selected for investigation. Samples were dissolved in either a 50:50 mix of methanol:MilliQ (>18.2 M $\Omega$ ) water or dichloromethane (DCM), depending on their solubilities. Palmitic acid was dissolved in both methanol:MilliQ and DCM to confirm comparable results using different solvents.

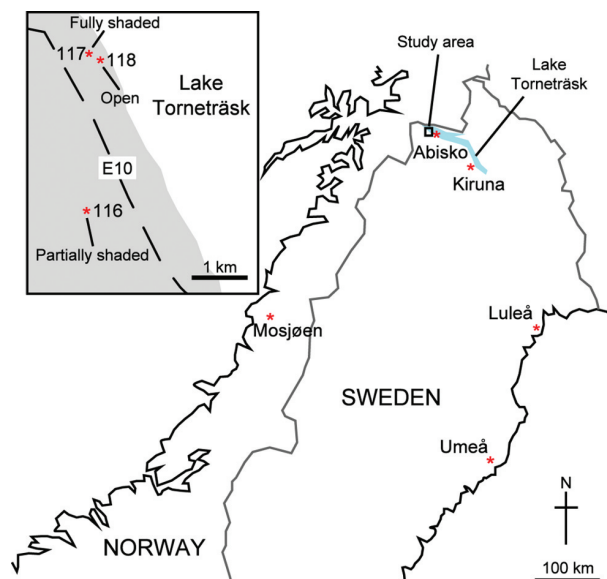
increase in the UV-B radiation flux with altitude. The data support this assumption and suggest that observed increases in phenolic pigment content with altitude reflect a biochemical response to higher UV-B flux (Watson et al. 2007). A second test exploited the relationship between changes in stratospheric ozone over Greenland and UV-B flux, whereby spore chemistry acted as a proxy of UV-B through historical time (Lomax et al. 2008). Although promising, these field-based observations require further investigation. In particular, examples are needed of present-day correlations of spore chemistry and UV-B flux to build confidence in sporopollenin-based proxies of past environmental change.

In this paper we extend our previous work and apply FTIR microspectroscopy to study the correlation of *Lycopodium annotinum* sporopollenin constitution with the degree of shading provided by birch forest canopy cover in northern Sweden. Work by Brown et al. (1994) demonstrates a reduction of UV-B to 1%–2% of incident flux beneath tree canopy cover compared with UV-B flux at the top of the canopy in an undisturbed forest. We therefore hypothesize that in naturally forested areas greater canopy cover would attenuate the UV-B flux (as well as radiation at other wavelengths) reaching the ground surface and reduce the concentration of phenolic UV-screening pigments in spore walls. Our findings provide further support for theories suggesting that spore biochemistry is responsive to ambient UV-B flux.

## Materials and methods

### Sample locations and collection

To test our hypothesis, we investigated three sets of naturally occurring *Lycopodium annotinum* spores collected during mid-September 2006 from well-established plants located at varying distance from the tree canopy and, therefore, experiencing different UV-B conditions at ground level. These samples constitute a natural UV-B manipulation experiment, equivalent to those conducted under artificially manipulated conditions in other studies (Phoenix et al. 2001). All samples were collected from northern Sweden in the local vicinity of the high-latitude Abisko Scientific Research Station (68° 21' N, 18° 49' E; Fig. 2). At each location spores were collected from three individual plants, resulting in three samples per location and totalling nine samples overall. Prior to analysis, all samples underwent sequential extraction with acetone in order to remove the majority of material that is not bound to the spore wall.



**Fig. 2** Location map of collection sites in northern Sweden. Area is located approximately 200 km north of the Arctic Circle, with collection sites adjacent to the shore of Lake Torneträsk. Marker 118 marks the location of the open-sky site (68° 25.902 N, 18° 40.278 E, 341 m a.s.l.); marker 117 is the fully shaded forest site (68° 25.833 N, 18° 40.370 E, 351 m a.s.l.); marker 116 is the partially shaded forest margin site (68° 24.578 N, 18° 40.805 E, 474 m a.s.l.).

### FTIR microspectroscopy

Fourier transform infrared microspectroscopy is used to determine functionality of sample material via vibrational excitation of bonds (within functional groups) that absorb characteristic wavelengths of infrared radiation. The position of the absorbance band identifies the type of bond/functional group present, whilst variations in band height and area represent changes in the relative abundance of such bonds/groups (Williams & Fleming 1980; Coates 2000).

The FTIR spectra can be used semi-quantitatively by normalizing all spectral bands within each individual spectrum to an internal stable absorption band, thus enabling inter-comparison of spectra by investigating relative changes in abundance of bonds/functional groups. The absorbance band due to hydroxyl (OH) groups is chosen for normalization because of its stability and absolute infrared (IR) absorption is proportional to quantity of sample analysed for each spectrum. A result of this normalization procedure is that the aromatic signal is quoted as a unit-less ratio. The FTIR analysis was conducted using a Nicolet Nexus FTIR bench unit (Thermo Scientific, Waltham, MA, USA), with integrated continuum IR microscope in transmission mode using a Refflachromat 15 × objective lens (Thermo Scientific).

Nicolet Omnic software (Thermo Scientific) automatically converts spectra to absorbance once analysis is completed. Each analysis was replicated five times per sample, with 500 scans collected for each replicate at a resolution of four data points per reciprocal centimetre. Absorption band height (peak height) was measured relative to a linear baseline as defined using Omnic software and automated using TQ Analyst (Thermo Scientific); no other processing or correction steps were applied to these spectra. Peak height is favoured for semi-quantitative analysis here because a strong correlation is found between peak height and peak area ( $r^2 = >0.99$ ) and peak height measurements can be made more efficiently than peak area. All band positions are quoted in wavenumbers ( $\text{cm}^{-1}$ ).

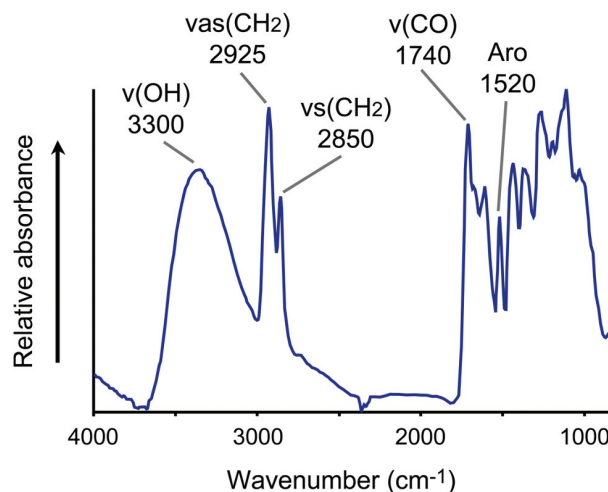
### UV-B field measurements

Ultraviolet-B flux was measured using a UV sensor (SKU 430 SiC detector, Skye Instruments Ltd., Powys, Wales, UK) in the research grounds of the Abisko Scientific Research Station, parts of which remain naturally forested and are identical to those found in the surrounding area. Measurements were taken within the birch forest and in adjacent non-forested areas in order to compare the shading effects of birch canopy on UV-B flux as experienced at ground level. Irradiance was measured in  $\mu\text{mol m}^{-2} \text{s}^{-1}$  and subsequently converted to  $\text{W m}^{-2}$ . Therefore, unweighted UV-B values are reported in this study rather than biologically effective UV-B. However, the sign and magnitude of biologically effective UV-B will correspond to the measurements of the ambient unweighted UV-B, allowing relationships to be identified. The UV-B flux is given as a percentage of ambient UV-B flux as measured at an adjacent, open-sky area; that is, completely beyond the shading influence of the birch canopy and is given as  $\%_{\text{amb}}$ . Two days of measurements were recorded: one with clear sky (15 June 2009) and the other with full cloud cover (17 June 2009). Readings were taken at hourly intervals during the period from 09:00 to 17:00 on each day.

## Results and discussion

### Typical spore response

A typical IR spectrum for *L. annotinum* is shown in Fig. 3. The prominent peak centred at ca.  $3300 \text{ cm}^{-1}$  is due to absorption by hydroxyl groups (OH). Aliphatic  $\text{CH}_2$  groups give rise to twin peaks at  $2925$  and  $2850 \text{ cm}^{-1}$ , corresponding to asymmetric and symmetric stretching of  $\text{CH}_2$  bonds, respectively. Carbonyl groups (most probably



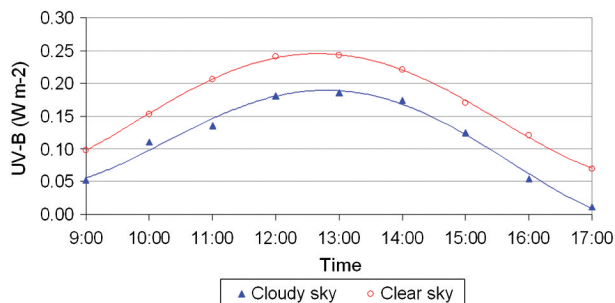
**Fig. 3** Fourier transform infrared (FTIR) microspectroscopy spectrum of *Lycopodium annotinum* spores wall with interpretation of spectral absorbance peaks. Absorption peaks are labelled as follows:  $\nu(\text{OH})$  denotes hydroxyl,  $\nu_{\text{as}}(\text{CH}_2)$  denotes asymmetrical stretching of  $\text{CH}_2$ ,  $\nu_{\text{s}}(\text{CH}_2)$  denotes symmetrical stretching of  $\text{CH}_2$ ,  $\nu(\text{CO})$  denotes carbonyl (in ester linkages) and aromatic (UV-B pigment) ring structure. Values below the labels are the wavenumbers of the target peak.

present as ester linkages) produce a strong absorbance at ca.  $1740 \text{ cm}^{-1}$  and phenolic (aromatic) ring structures absorb at  $1520 \text{ cm}^{-1}$ . Based on previous work (Watson et al. 2007), any changes in absorption peak height due to aromatic rings (at  $1520 \text{ cm}^{-1}$ ) measured using FTIR can be regarded as a change in abundance of these sporopollenin UV-B absorbing pigments. Nitrogen-containing compounds are widely documented to have the potential to contribute towards the absorption band at ca.  $1520 \text{ cm}^{-1}$  (Williams & Fleming 1980; Coates 2000); however, previous work using pyrolysis gas chromatography–mass spectrometry shows no evidence of such compounds in spore walls (Blokker et al. 2005; Watson et al. 2007), which is supported by an elemental analysis of *Lycopodium* spores (unpublished results).

### UV-B regime at the sample site

The vegetation around Abisko is a well-established birch forest experiencing a predominantly continental climate, shielded from maritime influence by mountains to the west. Because the ambient UV-B flux is not constant, we first constrained the ambient UV-B flux and its dependence on factors such as time of day, cloud cover and degree of shade using a hand-held UV sensor.

Ultraviolet-B profiles were measured for two days characterized by substantial differences in cloud cover (Fig. 4). As expected, UV-B flux is greatest at midday. The data reveal that the differential between UV-B flux on a



**Fig. 4** Daily ultraviolet-B (UV-B) profile as measured at the Abisko Scientific Research Station. The graph shows a typical profile for a cloudless sky in an open position (on 15 September 2006) and a profile for a cloudy day in an open position (on 17 September 2006). Trend lines are polynomial fit (order =4).

cloudy day and that on a day free from cloud cover is consistent throughout the measured period. The absolute content of UV-B absorbing and non-UV-B absorbing pigments may be subject to change due to variations in cloud cover altering UV flux over the course of a growing season. However, we believe that within our study area the difference in relative abundance of these compounds between shaded and non-shaded positions will be dominated by the longer term effects of shade rather than any shorter term variations in cloud cover.

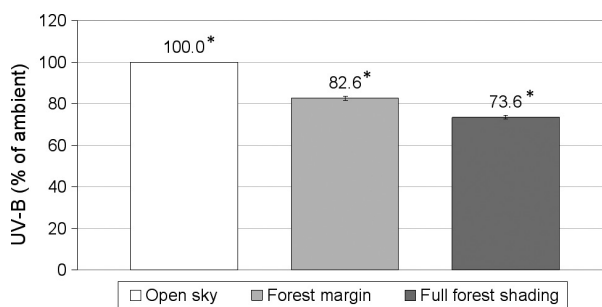
Three different UV-B settings are reported here: open sky, partially shaded birch forest margin and fully shaded birch forest (Fig. 5). Comparison of the open-sky (100%<sub>amb</sub>) and fully shaded forest (73.6%<sub>amb</sub>) sites reveal that there is a clear shading effect at ground level owing to the mature birch canopy. Incident UV-B at ground level (i.e., growth level for *Lycopodium* species) beneath the birch canopy is reduced to 73.6%<sub>amb</sub>. These data suggest that UV-B flux reduction at this location is not as pronounced as that reported by Brown et al. (1994). The partially shaded margin data is represented by the effect of an individual mature birch tree.

### Biochemical response to changes in UV-B flux

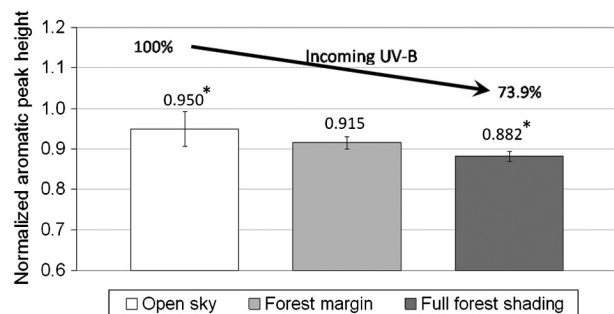
A negative relationship exists between spore wall aromaticity (UV-B absorbing compounds) and degree of shading due to the presence of a birch canopy (Fig. 6). The *L. annotinum* growing in the shade of a birch forest experiences the lowest UV-B flux (Fig. 5) and shows the smallest aromatic-signal ratio ( $0.882 \pm 0.012$ , standard error [SE]). By contrast, *L. annotinum* collected from a nearby position that is beyond the shading influence of any birch canopy and that received 100%<sub>amb</sub> UV-B (Fig. 5) exhibits the largest aromatic-signal ( $0.950 \pm 0.043$ , SE). The fully shaded forest and open-sky data form distinct groups with errors that do not overlap. A third set

of *L. annotinum* samples collected from a partially shaded, forest margin position with limited cover provided by the birch canopy show an aromatic-signal ( $0.915 \pm 0.015$ , SE) that is intermediate compared to the fully shaded and open-sky samples. This value is within the error range of the open-sky *L. annotinum* samples. This graphical interpretation of the results is confirmed by statistical analysis of the data using single factor ANOVA. Statistical analysis reveals that samples collected from the open-sky and forested positions are statistically discernable from one another ( $p < 0.05$ ), as would be expected if the pigment-based UV-B response mechanism within plants is active. The intermediate shading regime at the forest margin does show spore chemistries that are transitional between those in the open-sky (open vs. margin,  $p = 0.348$ ) or forest positions (margin vs. forest,  $p = 0.101$ ).

Our data suggest that *L. annotinum* responds to increased incident UV-B flux by incorporating a greater abundance of UV-B screening compounds into its spore wall structure, which is evident by the relationship of FTIR aromatic-signal observed in the spore samples with the UV-B regime pertaining to the growth habitat of the plants. Such an interpretation is consistent with previous studies of biochemical responses to UV-B flux (Rozema et al. 2001; Blokker et al. 2005; Watson et al. 2007; Lomax et al. 2008). An important point to note is that UV-A and photosynthetically active radiation (PAR) has the potential to also play a role in the stimulation of aromatic-based pigmentation within plants, however, these radiation wavelengths were not measured during this study. Brown et al. (1994) show that UV-B:PAR ratios vary greatly due to changes in shading by tree canopy, thus this could be a potentially important addition to the UV-B signal reported here. Future work should address



**Fig. 5** Degree of shading with respect to different canopy type, where “open sky” denotes no canopy ( $n = 10$ ), “forest margin” is the shade under a single mature birch tree on the margin of the forest ( $n = 10$ ) and “full forest shading” is the mature birch forest canopy ( $n = 10$ ). The percentage of ambient ultraviolet-B (UV-B) experienced at ground level in the respective position is given as %<sub>amb</sub>; error bars are one standard error. Asterisks indicates significant difference between means ( $p < 0.05$ ).



**Fig. 6** Comparison of hydroxyl-normalized aromatic response from open ( $n=5$ ), partially shaded ( $n=15$ ) and fully shaded sites ( $n=15$ ). The decreasing trend in ultraviolet-B (UV-B), with increasing shading, is indicated as measured at the Abisko Scientific Research Station (Fig. 5). Aromatic UV-B pigment compounds appear to be responding to the relative UV-B flux experienced at each site. Error bars are standard error. Asterisks indicates significant difference between means ( $p < 0.05$ ).

this matter further by investigating UV-A, UV-B and PAR simultaneously in order to more completely understand the UV-B response of spore wall chemistry. That being said, we believe that the data presented above demonstrates a clear relationship between UV-B flux and spore wall chemistry.

## Conclusions

Data presented here strengthen the use of a biochemical proxy as a monitor for UV-B flux by application to a natural UV-B manipulation experiment. This study expands on previous work by applying the proposed proxy to naturally occurring samples from known UV-B conditions that were measured during the same growing season. The UV-B absorbing pigment concentration is monitored by measuring the relative absorption intensity of a single, characteristic absorbance band in FTIR spectra that can be exclusively assigned to aromatic-based UV-screening pigments. The positive relationship between the UV-B regime and aromatic pigment content suggests that these plants biochemically adapt to their local UV-environment. These findings add weight to the proposition that spores and pollen hold the potential to act as palaeo-monitors of UV-B flux.

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