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Confocal imaging of exine as a tool for grass pollen analysis

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A new method which utilises confocal optical imaging has been developed which can be expected to improve grass pollen analysis. Confocal microscopy, in reflection mode, was used to examine the exine morphology of unacetolysed pollen grains from the following species of common wild grasses: *Paspalum dilatatum*, *Setaria gracilis*, *Bromus catharticus*, *Dactylis glomerata*, *Lolium perenne*, *Poa pratensis*, and *Phalaris aquatica*. Variations in the surface texture patterns, similar to those hitherto only seen by scanning electron microscopy, were visualised. In contrast to the latter method, specimen preparation for this confocal microscopy based technique was characterised by its simplicity, permitting the use of fresh and chemically untreated pollen grains. This confocal imaging technique, with its capacity for optical sectioning of specimens, offered the additional advantage of allowing the examination of the sub-surface exine layers as well as the surface morphology of the pollen grains. Furthermore, three-dimensional reconstruction of these optical sections enabled visualisation of the identified sculptural and structural exine elements and layers. A number of differences in these patterns were found, which indicate that confocal microscopy, in combination with image analysis, may enable finer taxonomic distinctions to be made than those currently provided by other light microscope based methods.

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INTRODUCTION

The pollen grains of grasses (family Poaceae) are, in most cases, morphologically very homogeneous. The taxonomic identification of their pollen to the species or generic levels, has for a long time presented a problem, since all species are monoporate, with an operculum, an annulus, and a thin exine, with uniformly arranged supratectal processes or spinules (Wodehouse 1935, Fægri & Iversen 1989). Although relatively good distinction based on pollen morphological evaluation has been made between the cereal and wild grass species (Rowley 1960, Andersen & Bertelsen 1972, Köhler & Lange 1979), finding the appropriate methodology to distinguish between species of wild grasses, which are the main causative agents of hay-fever and asthma, has been less successful and has become the subject of much research (Watson & Bell 1975, Peltre et al. 1987, Driessen et al. 1989).

Numerous studies utilising conventional light microscopy (LM) aimed at differentiating grass pollen have been reported, but in general, these studies have enabled only very broad taxonomic groupings to be made (Firbas 1937, Grohne 1957, Erdtman & Praglowski 1959, Liem 1967, Liem et al. 1968, Andersen 1978, Peltre et al. 1987, Fægri & Iversen 1989). Whilst these previous studies based on features such as pollen shape, overall dimension, aperture diameter and pollen wall architecture, have been useful, most showed overlaps in the measured features between the grass genera. Over and above these simple morphological features, however, many of the grass pollen grains were found to exhibit

exine surface reticulate patterning (Grohne 1957, Erdtman & Praglowski 1959). To date such patterning characteristics have been too subtle and difficult to quantify to provide a reliable analytical means for taxonomic distinction using conventional light microscopy (Fægri & Iversen 1989).

Investigations using scanning electron microscopy (SEM) and transmission electron microscopy (TEM) revealed that the reticulate patterning of the exine surface seen under conventional light microscopy was due to the presence of tectal islands (or insulae) on which supratectal processes (spinules) were grouped (Andersen & Bertelsen 1972, Watson & Bell 1975, Peltre et al. 1987). These tectal islands were seen to be separated by grooves, either deep or shallow, at the bottom of which micro-perforations occurred (Rowley 1960, Andersen & Bertelsen 1972, Köhler & Lange 1975, Watson & Bell 1975, Driessen et al. 1989). These investigations allowed some broad distinction of grasses into groups based on the shape of insulae, the depth of the inter-insular spaces and the number of spinules on the insulae, however, once again these distinctions were very difficult to make and generally permitted only limited differentiation (Watson & Bell 1975, Peltre et al. 1987).

Recently, the study of grass exine morphology by conventional light microscope based techniques was enhanced by the use of ultraviolet microscopy by Driessen et al. (1989). This technique enabled 15 of the 21 grass pollen species examined to be identified on the basis of autofluorescence colour of grain cytoplasm, colour of exine layers, and of

autofluorescence intensity. Although the reported method was found to be promising, it was concluded that taxonomic identification may potentially be possible only by combining UV-microscopy with other techniques, such as conventional light microscopy morphological measurements and/or SEM. Another recently introduced microscope technique is confocal laser scanning microscopy (CLSM), which has emerged as a powerful tool yielding high resolution images in which the out-of-focus blur normally present in conventional light microscopy is virtually eliminated. The present study was initiated to examine whether this new technique of reflection confocal microscopy when applied to examine grass pollen surface and sub-surface features could provide exine morphology data which was useful in the process of differential identification of wild grass species.

MATERIALS AND METHODS

Sample preparation

A total of seven grass species were examined in this study. Samples of five of these species were collected from sites in the state of New South Wales, Australia, whilst samples of the remaining two species were obtained from commercial sources (Greer Laboratories, North Carolina, USA). A number of individual pollen grains of each species were selected for study from the collected material and from the commercial material (Table I). Pollen grains were dissected out from fresh or air-dried grass samples into a drop of 0.1 M phosphate buffer (pH 7.2). The buffer was used to preclude rupture of pollen grains. A large proportion of pollen from dry grass samples was found to show a reduction of the grain's cytoplasmic contents, resulting in folding or creasing of the exine. Since confocal imaging required a completely smooth and unwrinkled pollen surface to enable good quality images to be collected, such dried-out pollen samples were placed into a mounting solution (25 ml glycerine, 25 g gelatine, 1 g phenol and 50 ml phosphate buffer) for eight to twelve hours, which resulted in some swelling and the consequent smoothing of grain surface prior to mounting on slides. Confocal microscopic analysis of pollen grains was carried out after mounting grains on a slide in a drop of mounting solution and covering with a glass cover slip. A uniformly flattened grain surface was obtained by firmly pressing down on the cover slip. The slide was sealed with nail varnish.

Confocal Reflection Microscopy

A Bio-rad MRC 600 confocal laser scanning microscope (Bio-rad Microscience Ltd., Hemel Hempstead, England) was used in conjunction with an optical light microscope (Zeiss Axiophot) and a

×100 oil immersion objective (Zeiss Plan-Neofluor) to image the pollen exine of individual grains. Confocal imaging was done in the epireflection mode using 488 nm light from a Krypton/Argon laser. To obtain an overall view of pollen surface patterning and shape for each species, an image of a whole grain surface was collected at a confocal zoom magnification setting of ×2 (94 nm/pixel). Image noise was reduced by averaging a number of individual frames. A more detailed qualitative analysis of exine patterning was subsequently made, at a higher confocal zoom magnification setting of ×6 (31 nm/pixel), by collecting a number of confocal images at incrementally increasing depths (i.e. approximately 0.2 µm increments) within the exine for each replicate grain from each grass sample. Collected images were stored and examined on a computer workstation (Silicon Graphics Indigo, Silicon Graphics Pty. Ltd., Mountain View, California, USA). A commercial software package (VoxelView Ultra 2.1.2, Vital Images Pty. Ltd., Fairfield, Iowa, USA) was used to produce three dimensional (3-D) reconstructions from these individual optical sections.

Terminology

Since the technique described in this report has not been previously applied to investigations of pollen exine structure, it is not at this stage known how closely the confocally imaged pollen surface and sub-surface patterns correspond to those previously identified by other microscopical methods. For this reason the use of common palynological terms applied to exine morphology has been avoided until further studies elucidate the relationships between the features identified by SEM and TEM and those obtained by confocal microscopy. The following nomenclature will be adhered to, until further structural correlations are made between imaging methodologies:

- 1. Imaged structures, either roughly oval or circular in shape and which appeared to be of a darker shade than the surrounding structures were interpreted to correspond to structural cavities or hollows. In general, these cavities were seen to be surrounded by either sharply delineated structural boundaries or gently sloping boundaries and were referred to as "reticulate lumina". A number of such lumina (i.e. 2-4) were frequently found fused to form elongated reticulate grooves or irregularly shaped meandering lumina.
- 2. Structures appearing to be imaged in a lighter shade were interpreted to be projecting above the plane of imaging. Such projecting elements were referred to as (i) knobs if they appeared as small unconnected granules, small hemispherical warts, tiny flakes or any other small elements. Similar structures, but of larger sizes, were referred to as (ii) mounds.
- 3. The network of reticulate ridges surrounding and joining the above-mentioned knobs or mounds were referred to as reticulate elements. Each such group of reticulate elements and mounds, with a central reticulate pit or a furrow, was referred to as a reticulate component. This terminology was applied both to surface and sub-surface structures.

RESULTS

General morphology of pollen grains

The thickness of the pollen exine as determined from the confocal microscope images was found to vary from 1.0 to 2.5 µm depending on the species examined. In general, all pollen grains examined were found to have exine showing pronounced patterning of both surface and sub-surface layers that consisted of a network of light reticulate elements surrounding dark reticulate lumina or grooves. Two main types of such patterning was seen in the present study, which

Table I. Species studied using confocal laser scanning microscopy (CLSM).

Species	Sample replicates	No. of grains imaged
<i>Bromus catharticus</i>	3	17
<i>Paspalum dilatatum</i>	3	18
<i>Setaria gracilis</i>	3	9
<i>Dactylis glomerata</i>	3	18
<i>Phalaris aquatica</i>	2	16
<i>Lolium perenne</i>	commercial	12
<i>Poa pratensis</i>	commercial	8

were distinguished on the basis of the size of the reticulate pits. The majority of pollen grains of grass species examined were found to have large reticulum (larger than $0.5\ \mu\text{m}$ in diameter) (Fig. 1A), while the exine of *Phalaris aquatica* and *Lolium perenne* were found to have small lumina (smaller than $0.5\ \mu\text{m}$ in diameter) (Fig. 1B).

Exine stratification

The examination of the patterning of pollen exine at different depths from the surface revealed a succession of distinct layers with variable sculpturing characteristics. The stratification was typically characterised by:

- (1) An uppermost surface layer of mounds and knobs.
- (2) A reticulate pattern of lumina bordered by ridge-like patterns of reticulate elements supporting the mounds, starting from a depth of $0.2\text{--}0.4\ \mu\text{m}$ below the surface, and extending to a depth of approximately $1.0\ \mu\text{m}$ below the surface. At the base of this layer the lumina often fused to form meandering grooves.
- (3) A sub-surface layer (Only present in some species) ranging from a depth of $0.8\ \mu\text{m}$ down to a depth of $1.8\ \mu\text{m}$ from the surface, in which the reticulate elements were angular, with or without internal projections constricting the resultant cavities. In some instances this resulted in the formation of a distinctive "star-shaped" cavity or lumina.
- (4) A base layer devoid of either reticulate elements at the deepest margin of the exine, which had a rough fibrous like texture but without any distinctive structural pattern. This layer varied in thickness, extending up to $2.0\text{--}2.5\ \mu\text{m}$ below the surface.

Variations in the size of both the observed reticulate elements, and the thickness of the layers described above, provided some qualitative criteria for taxonomic differentiation of the grass species examined in this study. In particular, serial imaging of the reticulate patterning at different depths of pollen grains with large lumina such as *Bromus catharticus* (Fig. 2A–F) showed considerable qualitative differences when compared to pollen grains having small reticulation as characterised by *Phalaris aquatica* (Fig. 3A–F).

Bromus catharticus exine was found to be covered with large knobs, of variable sizes, each protruding from a medium sized mound (Fig. 2A). A network of reticulate elements joined each mound, forming tall ridges around medium sized deep lumina, which were either oval, polygonal or irregularly shaped (Fig. 2B). In places, these lumina were found fused to form grooves and were particularly common on parts of pollen grains with excessively undulate surfaces. The reticulate elements occurred from a depth of approximately $0.2\ \mu\text{m}$ below the pollen grain surface, encircling the pits which extended deep into the exine, to form a characteristic "star-shaped" layer at approximately $0.8\text{--}1.6\ \mu\text{m}$ below the surface (Fig. 2C, D). This "star-shaped" layer distinguished *Bromus catharticus* from the pollen of other grass species investigated. The lumina ended at about $1.8\ \mu\text{m}$ forming a layer of angular elements (Fig. 2E), followed by a base layer with fibrous elements extending to approx. $2\text{ to }2.5\ \mu\text{m}$ depth (Fig. 2F).

In contrast, the exine of *Phalaris aquatica* was found to have distinctly different patterning characteristics than those found in *Bromus catharticus*. Numerous mounds and knobs of varying sizes were present on the surface of exine and these were often almost too small to distinguish by confocal microscopy (Fig. 3A). Their arrangement differed in different grains and parts of each grain analysed, but they frequently formed pentagonal or hexagonal clusters. The knobs were more numerous and occurred much closer together than in *Bromus catharticus* and it was also more difficult to distinguish the mounds from the knobs. The luminae, separating the mounds, were also more numerous and were very small ($<0.5\ \mu\text{m}$ in diameter). This was the main feature which distinguished this species from most of the other grasses examined (Fig. 3B). The lumina were roughly round or oval in shape and relatively shallow, extending to a depth of approximately $0.4\text{--}0.8\ \mu\text{m}$ below the pollen surface. The reticulate elements underlying and joining all the surface elements were thick, roughly circular and also relatively small (Fig. 3B, C). A very characteristic deeper layer feature of this species was the fusing of the lumina into elongated and narrow grooves at approximately $0.5\text{--}0.8\ \mu\text{m}$ depth (Fig. 3C, D), which linked to form x-shaped grooves at about $0.8\text{--}1.2\ \mu\text{m}$ (Fig. 3D). Below this layer was a very rough base layer extending to a depth of $1.4\text{--}1.6\ \mu\text{m}$ (Fig. 3E, F).

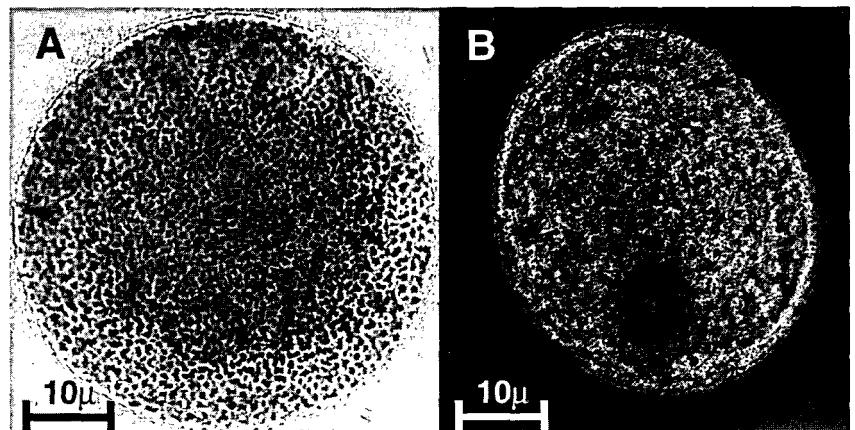


Fig. 1. Whole pollen grains viewed by confocal microscopy. A. *Dactylis glomerata* pollen grain with a pronounced reticulate network of large single, fused and meandering lumina (dark areas), surrounded by angular reticulate elements (light areas); B. *Phalaris aquatica* pollen grain with small lumina (dark areas), surrounded by a network of thick reticulate elements (light areas).

The difference in background shading is due to confocal glare being present in A.

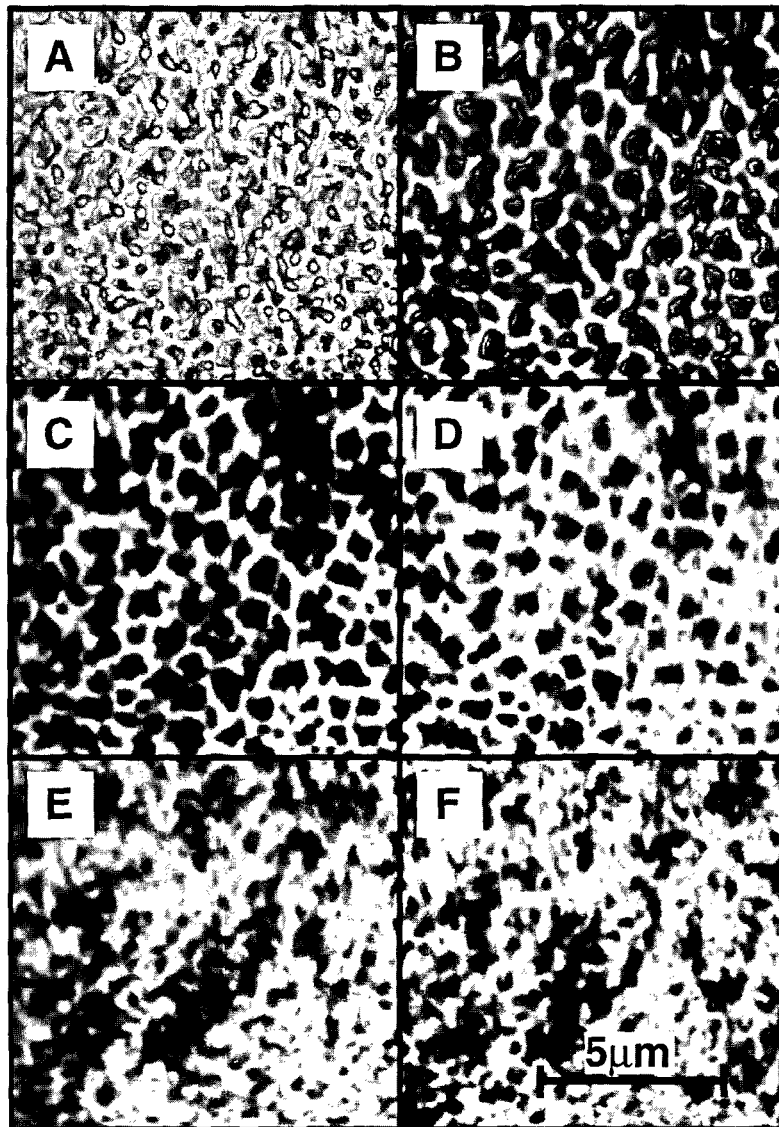


Fig. 2. Confocal microscopy of *Bromus catharticus* exine. A. The uppermost surface of pollen, showing the tips of large knobs protruding singly from mounds on the exine surface and separated by oval, roughly polygonal or hexagonal deep lumina (Imaging depth 0.2 μm). B. Large oval, pentagonal or hexagonal lumina surrounded by reticulate elements connecting and supporting the surface mounds and knobs (Imaging depth 0.4 μm). C. Deeper part of the reticulate exine layer with numerous mounds connected by thick, angular reticulate elements, which surround the roughly pentagonal or hexagonal lumina. This results in the characteristic "star-shaped" cavities of this layer (Imaging depth 0.8 μm). D. The lower-most portion of the layer having "star-shaped" cavities (Imaging depth 1.2 μm). E. Sub-surface layer with angular patterning (Imaging depth 1.8 μm). F. Base layer of the exine (Imaging depth 2.2 μm).

An overview of pollen exine patterning in different grass species

Qualitative observations derived from confocal microscopy of two of the most distinguishing or typical layers for each species examined in this study are given below. Features identified in these layers which appear to have species specificity may be useful for taxonomic identification of pollen grains.

Bromus catharticus. – The relatively thick exine (1.8 to 2.2 μm) was found to be covered with large knobs. Medium sized and regularly spaced reticulate elements were found to surround deep lumina (Fig. 4A). A sub-surface distinguishing layer in which the reticulate components had a distinct "star-shaped" angular pattern was imaged at about 0.6–1.2 μm (Fig. 4B). The mounds, supporting only 1 knob each, were arranged uniformly around large cavities in a polygonal or hexagonal arrangement. Thick reticulate elements were present at 0.4 μm depth. Pollen grains with

uniform grain surface generally had single component reticulate cavities, while those grains with an "undulating" or "dented" exine were found to have reticulate cavities fused to form furrows.

Paspalum dilatatum. – Small groups of pointed knobs (1–5) were imaged on surface mounds connected by a network of fine reticulate elements. Confocal imaging at a depth of 0.2 μm from the surface revealed the thin reticulate elements surrounding the lumina, the latter frequently much wider than in other grass species (Fig. 5A). The reticulate elements were found to increase in thickness with depth and had a granular appearance in many pollen samples (Fig. 5B).

Setaria gracilis. – The exine patterning of this species of pollen was found to be very similar to that of *Paspalum dilatatum*, with a pronounced reticulate pattern (Fig. 6A, B). The lumina were large and frequently fused to form meandering grooves, surrounded by very fine reticulate elements.

Fig. 3. Confocal microscopy of *Phalaris aquatica* exine. The uppermost surface of pollen, showing a finely reticulated surface layer with numerous pointed knobs (Imaging depth 0.2 μm). B. Numerous mounds joined by thick and extremely short reticulate elements, forming a retipilate network, connecting the mounds to form pentagonal or hexagonal components around deep lumina (Imaging depth 0.4 μm). C. Continuation of mounds and appearance of the deep and narrow grooves between the lumina (Imaging depth 0.6 μm). D. Fusion of reticulate lumina into X-shaped grooves (Imaging depth 0.8 μm). E. Rough patterning of the base layer (Imaging depth 1.8 μm). F. Lower limit of the base layer (Imaging depth 2.2 μm).

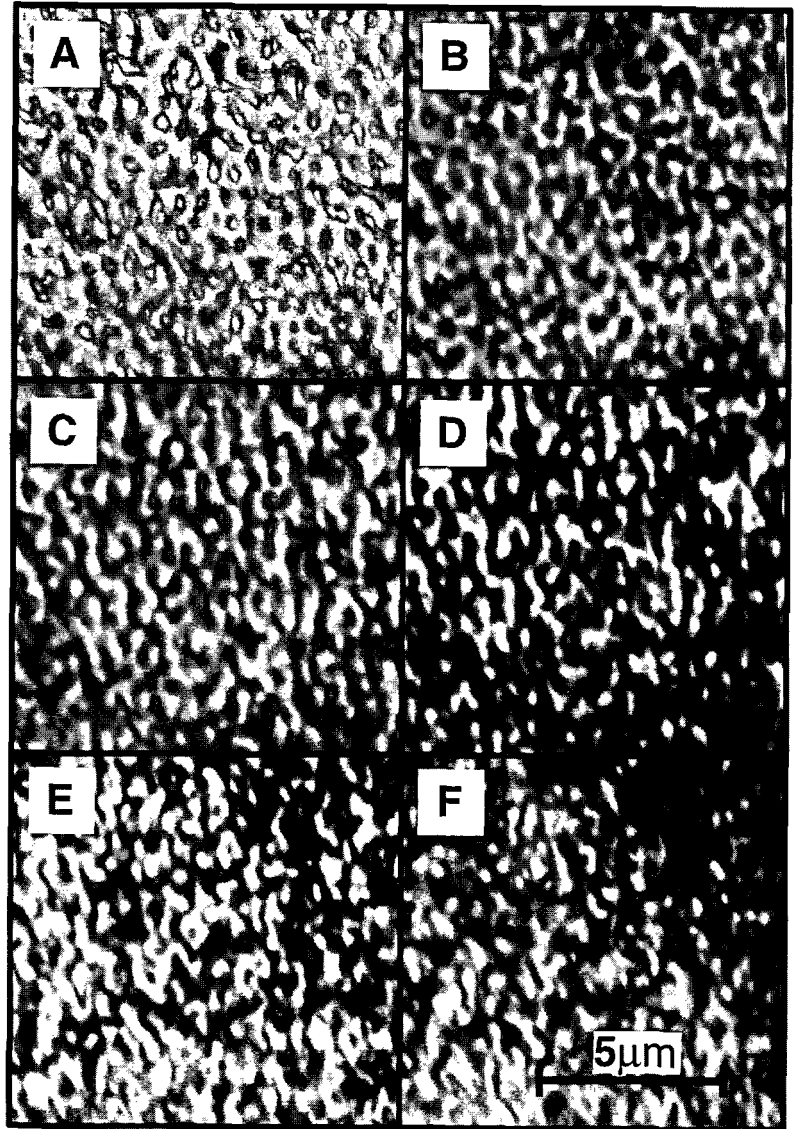
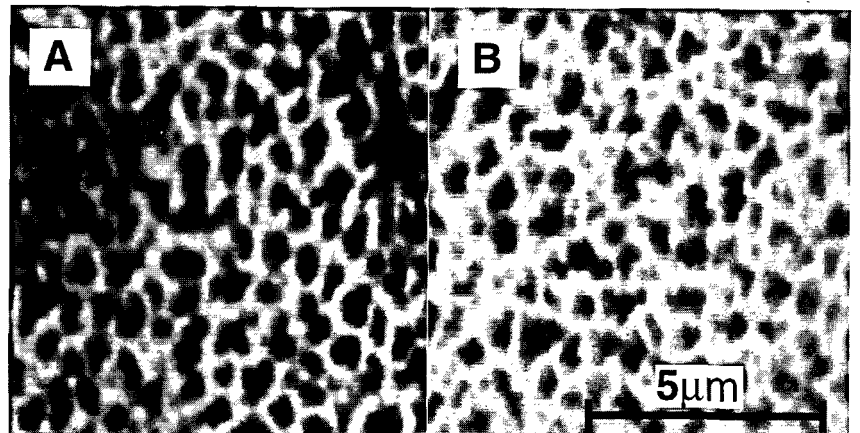


Fig. 4. Distinctive features of *Bromus catharticus* exine. A. Reticulate elements which were rarely fused, were oval, angular, polygonal or hexagonal in shape with a bumpy appearance (Imaging depth 0.4 μm). B. The characteristic “star-shaped” sub-surface layer, in which small side projections from the reticulate elements resulted in highly angular hexagonal appearance of the bottom of the lumina (Imaging depth 0.9 μm).



Dactylis glomerata. – Pollen exine was of medium thickness 1.4–1.8 μm . Small and pointed knobs were found to occur on the periphery of the exine surface mounds, which were in

turn supported by thin, large reticulate elements, that formed angular, oval or polygonal components with relatively little fusing (Fig. 7A). Reticular component shape varied between

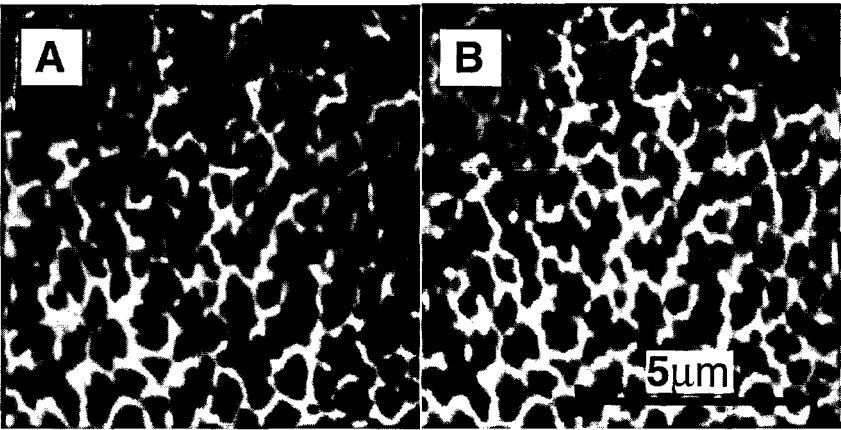


Fig. 5. Distinctive features of *Paspalum dilatatum* exine. A. Very large surface reticulate lumina (Imaging depth 0.2 µm). B. Granular thickening of fine reticulate elements with increasing depth (Imaging depth 0.6 µm).

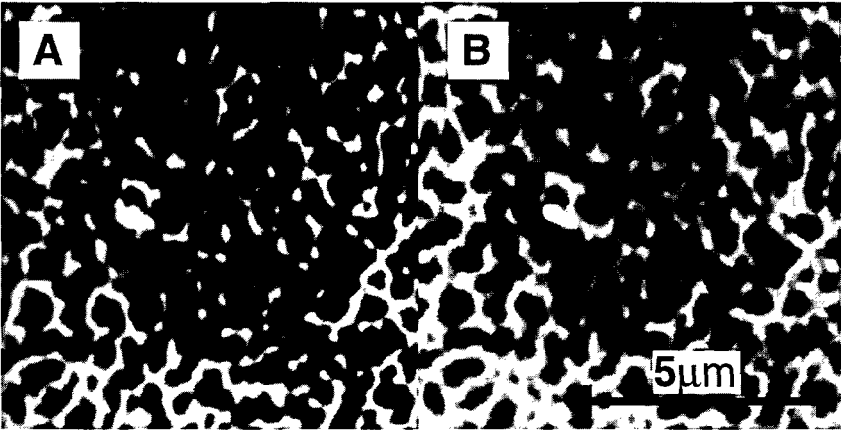


Fig. 6. Distinctive features of *Setaria gracilis* exine. A. Pronounced mounds on undulating exine surface, joined by reticulate elements (Imaging depth 0.4 µm). B. A deeper layer of large lumina, fused into grooves (Imaging depth 0.9 µm).

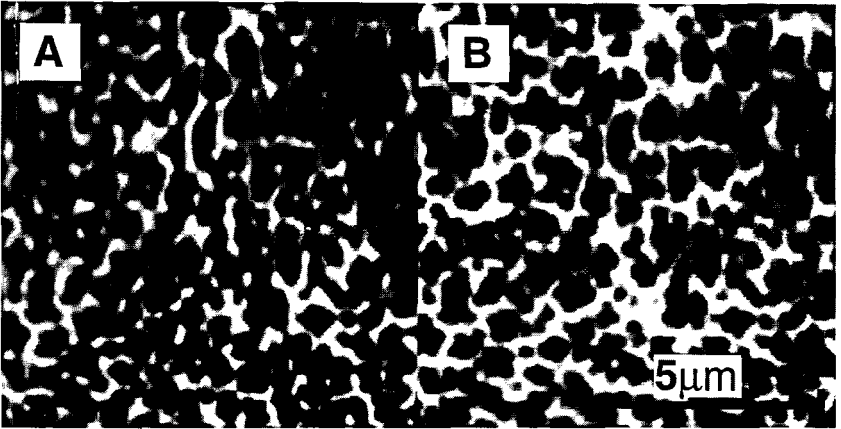


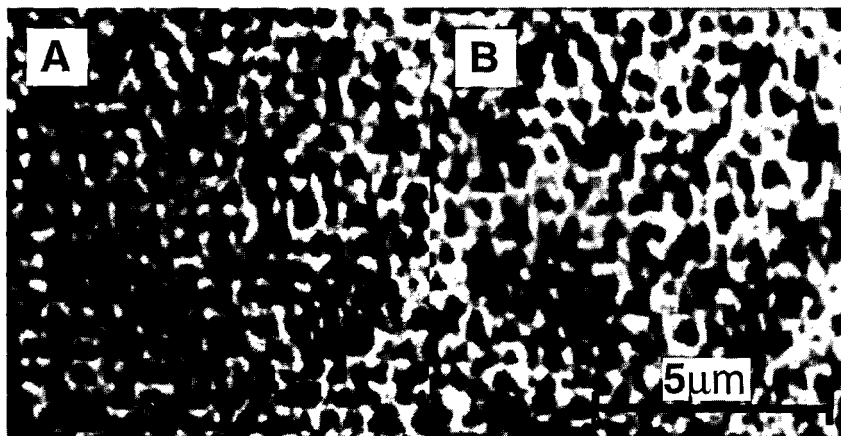
Fig. 7. Distinctive features of *Dactylis glomerata* exine. A. Sub-surface lumina joined to form a meandering “brain-like” pattern of grooves (Imaging depth 0.4 µm). B. A layer with the reticulate elements being crowned with protruding granular processes resulting in the “star-shaped” appearance of the lumina (Imaging depth 1.2 µm).

grains, from round to polygonal or almost “star-shaped”. Some grains were also found to have many smaller round components interspersed between the large reticular ones. The lumina were frequently found to coalesce to form a meandering “brain-like” pattern of grooves in deeper exine layers. A pronounced sub-surface layer was noted at 1.0–1.4 µm below the pollen surface, with the reticulate elements being crowned with protruding short processes. This gave the lumina the “star-shaped” appearance mentioned above which was similar to that found in *Bromus catharticus* (Fig. 7B). This layer was, however, much thinner

than in *Bromus catharticus* and was followed by a pronounced base layer that was flat and devoid of lumina.

Phalaris aquatica. – A relatively thick exine to 1.4–2 µm in depth, with a pronounced base layer at about 1.4 µm, was recorded for pollen of this species. As described previously, *Phalaris aquatica* was easily distinguished from other species by having very small lumina (Fig. 8A). Its mounds and knobs were found to be arranged in a pentagonal or hexagonal distribution. The reticular elements were short and

Fig. 8. Distinctive features of *Phalaris aquatica* exine. A. Small lumina surrounded by numerous mounds (Imaging depth 0.2 μm). B. Very short and thick reticulate elements (Imaging depth 0.6 μm).



thick, and appeared to increase in thickness with increasing depth (Fig. 8B).

Lolium perenne. – The exine patterning of this species was found to be very similar to *Phalaris aquatica*, with an outer surface layer covered with crowded knobs (Fig. 9A) and with a layer of very small lumina extending deep into the exine (Fig. 9B). The shape of the lumina was found to vary significantly between different grains of this species, from being almost circular (As in Fig. 9B) to forming larger angular cavities. Its main distinguishing features, when compared to *Phalaris aquatica*, was the absence of a distinct base layer underlying the reticulate elements and a generally thinner exine of less than 1 μm thickness.

Poa pratensis. – The pollen of this species was characterised by the presence of a moderately thick exine extending to 0.4–1.6 μm below the pollen surface. The exine surface was seen to have distinct knobs and mounds (Fig. 10A) and sub-surface reticulate elements forming large single components, which were either oval, angular or fused into short beandering grooves (Fig. 10B).

Three-dimensional reconstruction of serial sections

The reconstruction of the stacks of optical sections made through the exine permitted the visualisation and the exam-

ination of the imaged sculptural elements. Pointed or blunt knobs were found to project from the surface and their number on each mound varied, depending on the species examined. *Bromus catharticus* was found to have the largest knobs, generally with only one knob per mound. Both *Lolium perenne* and *Phalaris aquatica* had very short, small and pointed knobs while the other species had a variable number of knobs per mound (Usually between 1 and 6). There did not appear to be a sharp distinction between knobs and mounds except in *Bromus catharticus*, but the former smoothly merged into the latter (Figs. 11 & 12).

DISCUSSION

The use of CLSM in this study permitted the investigation of not only the surface but also of sub-surface exine layers. Using a single easily prepared sample, the confocal technique enabled the examination of both the surface exine morphology, for which SEM is commonly used, as well as the sub-surface structure, which is conventionally elucidated by TEM. As a further advantage, all of the gathered images were in digital form, which enabled both the three-dimensional qualitative studies to be undertaken, as well as the quantitative comparison of exine patterns (Data not reported here).

This preliminary qualitative investigation of the pollen exine by confocal microscopy revealed that the common

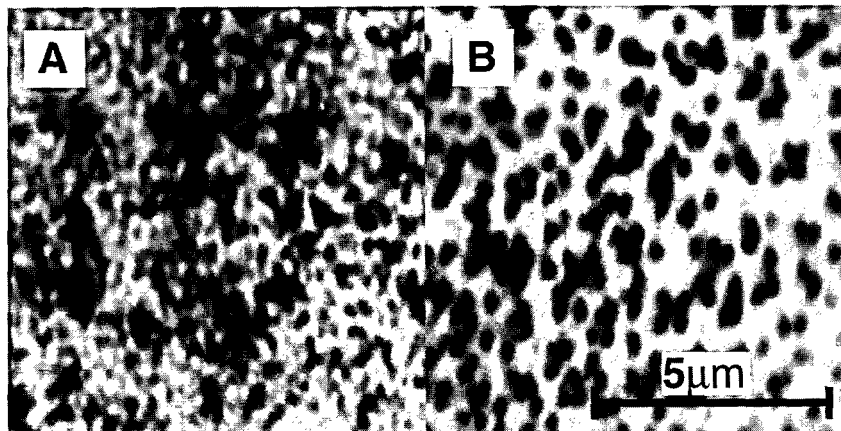


Fig. 9. Distinctive features of *Lolium perenne* exine. A. Crowded knobs and mounds (Imaging depth 0.2 μm). B. Circular lumina (Imaging depth 0.4 μm).

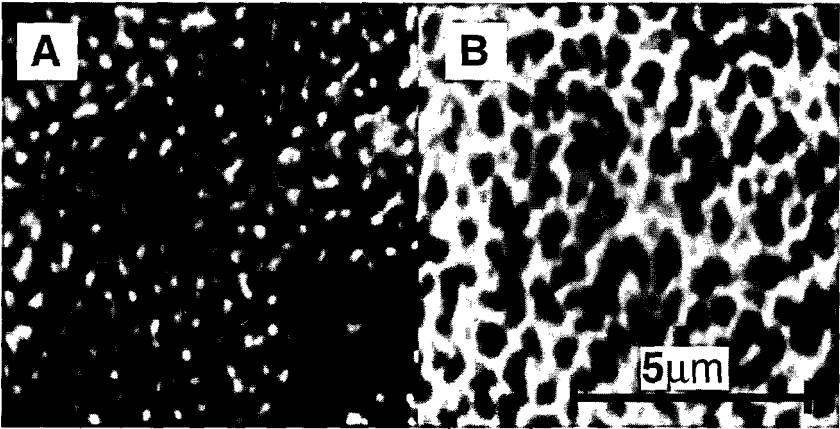


Fig. 10. Distinctive features of *Poa pratensis* exine. A. Surface protuberant knobs and mounds of large size (Imaging depth 0.2 µm). B. Medium sized lumina, frequently fused into short meandering grooves (Imaging depth 0.5 µm).

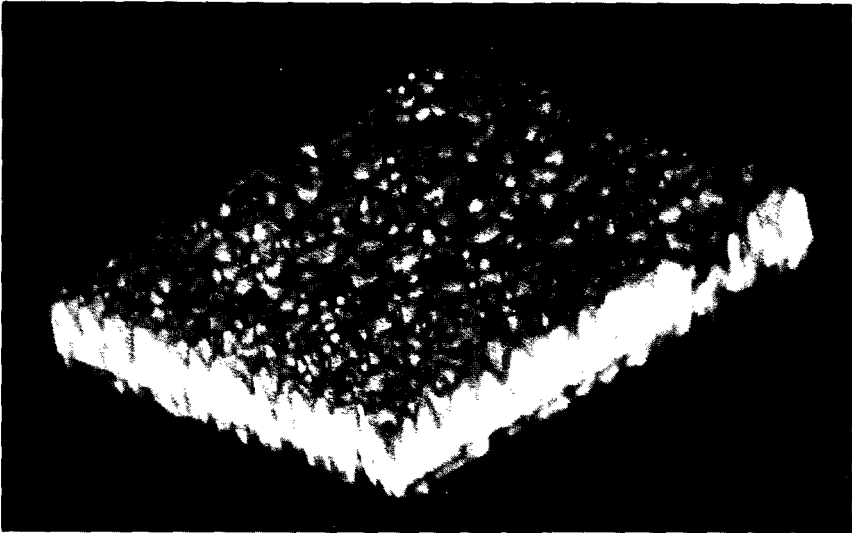


Fig. 11. Three-dimensional reconstruction of *Bromus catharticus* exine. Three-dimensional reconstruction of 18 interpolated optical sections through the exine of a *Bromus catharticus* pollen grain (Area approximately 10 µm × 10 µm). Note the characteristic large, blunt knobs on the surface of relatively few large mounds and the deep lumina between these mounds.

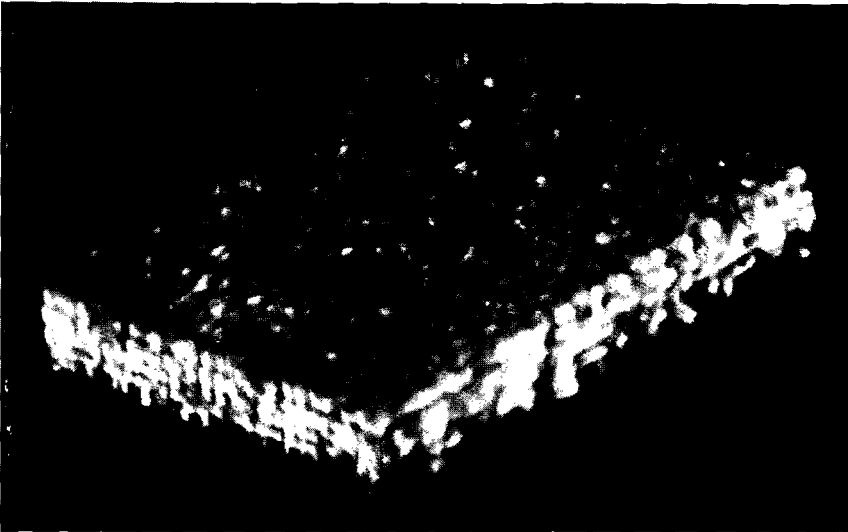


Fig. 12. Three-dimensional reconstruction of *Phalaris aquatica* exine. Three-dimensional reconstruction of 15 interpolated optical sections through the exine of a *Phalaris aquatica* pollen grain (Area approximately 10 µm × 10 µm). Note the very small and pointed surface knobs, giving a serrated appearance to the surface, as well as the complex sub-surface structure of exine layers.

characteristic found in all of the seven grass species examined was the presence of a distinct surface topography, made up of lumina of varying sizes and shapes, and surrounded by reticulate elements of differing thicknesses. The latter formed

distinct components and were found to be thickened in places to form surface mounds. The mounds so formed were generally found in pentagonal, hexagonal or triangular arrangements, supporting either single or multiple surface

knobs, which in turn were either blunt or sharply pointed, depending on the species examined. Although the aim of the study was an overall qualitative assessment of the exine architecture in different pollen species by confocal microscopy, the observed reticulate patterns allowed some taxonomic distinctions to be made between the various species. Of particular note, was the taxonomic division of the seven grass species into two groups: (1) *Bromus catharticus*, *Paspalum dilatatum*, *Setaria gracilis*, *Dactylis glomerata*, *Poa pratensis* and (2) *Phalaris aquatica* and *Lolium perenne*, simply based on the size of their reticulate lumina. Within these two groups other potentially diagnostic morphological features were recorded for all of the grass species examined. These features included the overall exine thickness, the size and shape of the reticulate components (comprising reticulate elements, mounds and knobs), the number and morphology of the knobs present, and the presence or absence of distinctive sub-surface exine layers.

Whilst only limited distinction between the species was achieved due to the qualitative basis of this initial application of confocal microscopy to the study of pollen morphology, the presence of these cited features does suggest that their quantitative evaluation could result in accurate identification of pollen species. Certainly, the features of *Bromus catharticus* which included the presence of single large blunt knobs on individual mounds, a thick and pronounced sub-surface layer having a "star-like" reticular pattern, and a thick base layer were considerably different from the features seen for the other four grasses with large reticulation. Similarly, *Paspalum dilatatum* was distinguishable from the other grasses by its very large lumina and relatively large mounds. In contrast, *Setaria gracilis* was found to have an overall thinner exine layer than the other species with large reticulate lumina and a distinct tendency for these lumina to fuse into meandering grooves at deeper exine layers. Similar qualitative distinctions were made for two grass species with small reticulate lumina. *Phalaris aquatica* had a thick exine with a pronounced base layer, whereas *Lolium perenne* had a very thin exine, lacking pronounced base layer.

Many of these identified features obtained with confocal microscopy were found to correlate with findings from more standard microscopic techniques currently used to identify the species of pollen grains, such as SEM. Confocal microscope imaged knobs on the surface of the mounds appear to correspond to spinules grouped on islands (or insulae) projecting from pollen surfaces, as identified by numerous SEM studies (e.g. Andersen & Bertelsen 1972, Page 1978, Watson & Bell 1975). The observed reticular patterns present in all of the species examined, may correspond to the intrareticulate columellae, which are known to form a reticulate pattern below the exine surface (Pragłowski & Punt 1973, Fægri & Iversen 1989). Confocal imaging, however, indicates that the surface mounds are separated by lumina of varying sizes, shape and depth – a feature which does not appear to correlate with sculpturing characteristics recorded by SEM, which only shows the presence of narrow incisions or grooves separating the surface sculpturing elements. One possible explanation for this difference is the potential shrinkage of some of the surface structures as a result of the fixation techniques used for preparation of samples for SEM analysis.

Further, the results of this study indicate that the size and shape of the identified reticulate components may provide a means for taxonomic differentiation of grass pollens. A similar conclusion has been reached by the authors of various SEM studies of pollen, with suggestions that the shape and the size of the protruding island-like structures or insulae (Andersen & Bertelsen 1972), the number of spinules per insula, and the width and sharpness of the incisions between the insulae are features having a certain amount of species specificity (Watson & Bell 1975, Köhler & Lange 1979, Peltre et al. 1987, Rowley et al. 1988). Confocal microscopy, however, has an advantage over SEM in terms of the methods of sample preparation, which permitted imaging of fresh grass pollen grains that were unacetolysed or otherwise chemically treated, in an aqueous environment. This is in contrast to the lengthy and chemically harsh preparation protocols which may significantly affect the specimen morphology required for SEM or TEM investigations (Ridgway & Skvarla 1969, Hanks & Fairbrothers 1970, Cusma Velari 1983). This simplicity of preparation suggests that confocal microscope imaging has a great potential as a routinely applied technique for grass pollen species analysis.

CONCLUSION

This investigation indicates that confocal microscope imaging has great potential in practical applications for grass pollen analysis. The study of various exine layers observed via this technique has shown that distinctive patterns may be observable for individual grass species. More extensive studies and the quantification of these patterns by application of image analysis techniques, as has been shown with SEM images (Langford et al. 1990, Vezey & Skvarla 1990, Vezey et al. 1992), will no doubt add significantly to the qualitative results presented in this report. Even as a purely qualitative technique, the application of confocal microscopy to pollen analysis is an addition to existing analysis protocols and offers an avenue for future research into methodologies for rapid and accurate identification.

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