

Insights into a hydration regulating system in *Cupressus* pollen grains

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• **Background and Aims** Hydration, rupture and exine opening due to the sudden and large expansion of intine are typical of taxoid-type pollen grains. A hemispheric outgrowth external to the exine was observed on *Cupressus* and *Juniperus* pollen grains before the intine swelling and exine release. However, the actual existence of this permanent or temporary structure and its precise role in pollen hydration is still being debated. The aim of this paper is to collect information on the actual presence of this peculiar outgrowth on the surface of the *Cupressus* pollen grain, its structure, composition and function.

• **Methods** Pollen grains of several *Cupressus* species were observed using various techniques and methodologies, under light and fluorescence microscopy, phase-contrast microscopy, confocal microscopy, scanning electron microscopy, and an environmental scanning electron microscope. Observations were also performed on other species with taxoid-type pollen grains.

• **Key Results** A temporary structure located just above the pore was observed on *Cupressus* pollen grains, as well as on other taxoid-type pollens. It is hemispheric, layered, and consists of polysaccharides and proteins. The latter are confined to its inner part. Its presence seems to regulate the entrance of water into the grains at the beginning of pollen hydration.

• **Conclusions** The presence of a temporary structure over the pore of taxoid-type pollen grains was confirmed and its structure was resolved using several stains and observation techniques. This structure plays a role in the first phases of pollen hydration.

Key words: *Cupressus sempervirens*, *C. glabra*, exine breakage, intine swelling, bulge, calcofluor, aniline blue black, taxoid-type pollen.

INTRODUCTION

Hydration, rupture and exine opening due to the sudden and large expansion of the intine, have been observed in taxoid-type pollen grains of *Cupressus* and *Juniperus* (Duhoux, 1982; Kurmann *et al.*, 1994; Chichiriccò and Pacini, 2008). A minute pore that plays an important role in rupture of the exinic wall has been described (Duhoux, 1982; Bortenschlager, 1990; Pacini *et al.*, 1999; Grilli Caiola *et al.*, 2000). Water penetrates through the pore and hydrates the pollen grains, which become spherical in a few seconds. After several minutes, the swelling of the intine causes the exine to rupture and open in mature pollen grains. Exine rupture is considered to be a passive phenomenon, since it has been observed in both living and dead pollen grains (Duhoux, 1982).

Duhoux (1982) observed a smooth protuberance on the external surface of some living pollen grains under a scanning electron microscope (SEM). Despite the fact that the protuberance hydrated and swelled in water, this author equated it to the operculoid described by Wodehouse (1935 in Punt *et al.*, 1994), which is an ectexinous structure that plugs the ectoaperture. Duhoux (1982) also detected another hemispheric protuberance, which he named ‘bulge’, on the surface of pollen grains of *Juniperus communis* and *Cupressus arizonica* when stained

with calcofluor and observed under fluorescence. The fluorescent protuberance was observed to decrease in size and brightness, and to disappear within 2–3 min. The bulge is assumed to be an intrinsic part of the intine, which expands in water, thus causing the ejection of the operculoid. Duhoux also assumed that the operculoid is lost when the grain is in contact with water, which is independent of the stage of maturity, and that the partial dissolution of the so-called bulge could take place in a humid atmosphere or even inside the pollen sacs. However, the mechanism of the exine rupture described by Duhoux (1982) is not completely clear as the text, drawings and figure captions are somewhat conflicting. Chichiriccò and Pacini (2008) detected a structure similar to the operculoid (*sensu* Duhoux) in acetolysed pollen grains of *C. arizonica*. Under light microscopy, after calcofluor treatment, the same authors mention the presence of a persistent fluorescent, globular bulge in the pollen grains. Under an SEM, both a globose operculoid crossing the pore and a bulge were evident. The bulge appeared to be made of fibrous material coming from the inside of the pollen. At present, the actual existence of a permanent or temporary structure which covers the pore prior to the intine swelling and exine release in *Cupressus* pollen grains is still controversial.

During a previous study carried out on *Cupressus* pollen grains from different geographical areas, the occurrence of an outgrowth on the pollen surface was observed under fluorescence microscopy (Danti *et al.*, 2010). It was similar to the bulge observed by Duhoux (1982) and Chichiriccò and Pacini (2008).

The aim of this paper is to collect new information about the actual presence, structure and composition of the outgrowth by means of observations carried out using the following techniques and methodologies on *Cupressus* pollen: light and fluorescence microscopy, phase-contrast microscopy, laser scanning confocal microscopy, SEM and environmental scanning electron microscopy.

MATERIALS AND METHODS

Pollen of *Cupressus sempervirens* L. and *Cupressus glabra* Sudw. was sampled from several trees belonging to the IPP-CNR cypress collection maintained at Antella, near Florence (Italy). Twigs bearing mature microsporophylls were cut and placed in plastic bags; then within 24 h, they were put in vases indoors that were arranged on a sheet of wrapping paper to collect the shed pollen. Pollen was collected at 2- to 3-d intervals. The pollen was then cleaned with a 300- μ m sieve and dehydrated under vacuum using silica gel at room temperature until it reached a relative humidity of 30–35 %. The pollen was then stored at -20°C in hermetic plastic tubes until needed. The tubes were removed from the freezer and kept at room temperature for 10 min. Pollen grains were then collected from the tubes, re-hydrated in a 2 % sucrose water solution and observed using various microscopy techniques.

Assessment of pollen grain viability

Two different staining procedures were followed to evaluate the viability of the pollen grains and to distinguish viable grains from non-viable ones: (1) fluorescein diacetate (FDA) (Gahan, 1984), on grains previously counterstained with 0.04 % neutral red in a 4 % sucrose solution for 10 min and then observed under a fluorescent microscope; and (2) propidium iodide (Molecular Probes), using a 3 $\mu\text{g mL}^{-1}$ water solution (Firstencel *et al.*, 1990), followed by observation under a confocal microscope using an excitation wavelength of 514 nm.

Identification and description of a pollen grain bulge

The structure and composition of the protuberance were observed on viable fresh pollen of *C. sempervirens* and *C. glabra*, after hydration but prior to exine breakage, using different methodologies and stains. The observation times of the pollen grains were sometimes different due to the variation in technical time necessary to set up the various instruments.

The following techniques were used, where light microscope = l.m. and confocal microscope = c.m.

Calcofluor (Hughes and McCully, 1975) at 0.02 % in water solution (pH 6–98), operating at a wavelength of 360 nm to highlight the β -glucans (l.m., c.m.)

Astra blue (Krauss *et al.*, 1998): to highlight the 1.4 β -glucans (l.m.)

Aniline blue black (Fischer, 1968): 1 % in acetic acid (pH 2–37), diluted to 0.5 % in water to highlight proteins (l.m., c.m.)

Combination calcofluor–aniline blue black: aniline blue black was added in a slide edge where grains stained with calcofluor were present (l.m.)

Combination calcofluor–astra blue: astra blue was added in a slide edge where pollen grains stained with calcofluor were present (l.m.)

Aniline blue (O'Brien and McCully, 1981): to highlight callose (l.m.)

Sudan black b (Ruzin, 1999): to highlight lipids (l.m.)

Dehydrated pollen grains were also acetolysed (Erdtman, 1960) and observed in a 50 % glycerol water solution under light microscope.

Observations were carried out using light, phase-contrast and epifluorescent microscopy utilizing a Laborlux S (Leitz) combined with a digital camera (Digital Sight DA-5M, Nikon) and dedicated software. Confocal imaging was performed using an upright Leica laser scanning confocal microscope SP5 and a $\times 63$ oil immersion lens. The excitation wavelength for calcofluor was 405 nm. For both species, the size of the protuberance was measured on at least 30 pollen grains.

Stainings with calcofluor, astra blue and aniline blue black were also performed on pollen grains of *C. macrocarpa* Hartw. ex Gordon, *C. dupreziana* A. Camus, *C. funebris* Endl., *C. guadalupensis* S. Watson, *C. torulosa* D. Don, *Juniperus communis* L. and *Taxus baccata* L., *Thuja orientalis* (L.) Franco, *Metasequoia glyptostroboides* Hu and Cheng, *Sequoia sempervirens* (Lamb.) Endl., which were collected at Antella (Florence) and in the Botanical Garden of the University of Florence. Calcofluor and aniline blue black staining were also performed on fresh pollen grains to validate the results of the observations on frozen pollen grains.

SEM

Pollen grains were treated in accordance with the two following procedures: (1) acetolysis (Erdtman, 1960); and (2) fixation in acetic acid–alcohol (1:3, v/v) solution and dehydration in ethanol series according to Chichiriccò (2006). The treated grains were then critical-point-dried, gold coated (sputtering chamber: Emitech K550), and observed with a Philips XL20 SEM. Untreated air-dried *Cupressus* grains were also observed immediately after being gold coated.

Environmental scanning electron microscope (ESEM)

Dehydrated grains were directly observed in a vacuum; hydrated grains were observed immediately or after treatment with calcofluor while operating at a relative humidity of 60–70 %; and freeze-dried pollen grains were rehydrated and observed both immediately and after treatment with calcofluor at a relative humidity of 60–70 %. Observations were carried out with a Quanta 200 (Fei) ESEM.

RESULTS

Assessment of pollen grain viability

In hydrated pollen grains containing a round protoplasm, the plasmamembrane stained green-yellow (FDA positive), thus confirming the activity of the plasmamembrane esterases. However, the abnormal pollen grains, characterized by an irregular, star-like cytoplasm even when hydrated, stained red (FDA negative; Fig. 1). The propidium iodide test confirmed the integrity of the plasmamembrane of the FDA-positive grains, which showed unstained nuclei (Fig. 2).

Identification and description of a pollen grain bulge

Light and confocal microscopy. A prominent hemispheric protuberance (bulge) was observed on the exine surface of many pollen grains after treatment with calcofluor or aniline blue black. The bulge was observed on both viable and non-viable, fresh and frozen pollen grains. A second bulge was sometimes observed, although very rarely.

Directly after treatment with calcofluor, the bulge was very bright. After 3 min, the size of the bulge was about $3 \times 2 \mu\text{m}$ (basal diameter $\times$ height) in *C. glabra* and $5 \times 3 \mu\text{m}$ in *C. sempervirens* (Fig. 3). After several minutes, however, the size increased to about $8 \times 3 \mu\text{m}$ in *C. glabra* and $9 \times 5 \mu\text{m}$ in *C. sempervirens*. This protuberance could also be observed under both the confocal microscope in visible light (Fig. 4) and under the phase-contrast microscope (Fig. 5). Subsequently, the fluorescence decreased in size and brightness, and disappeared after about 15 min or sometimes even longer. In some cases, the bulge was observed to detach from the pollen surface (Fig. 6) and a new outgrowth was observed to protrude from the detachment point of the previous bulge (Fig. 7).

Aniline blue black deeply stained the bulge, which quickly increased in size up to about $13 \times 6 \mu\text{m}$ in *C. glabra* and $7 \times 3 \mu\text{m}$ in *C. sempervirens* (Figs 8 and 9). Subsequently, the outline of the bulge became ill-defined, and dark-blue

elongated crystals appeared on the site of the bulge (Fig. 10A, B). The bulge appeared to be layered when concurrently treated with calcofluor and aniline blue black (Figs 11 and 12). The calcofluor stained the entire structure, particularly the outer layer, while the aniline blue black stained the inner portion. The bulge was not revealed by staining with aniline blue, sudan black b and astra blue, nor when the grains were observed in water (MilliQ pH 6.86).

In *C. glabra*, a few minutes after staining with astra blue, a thin layer of the intine appeared to be coloured blue below the exine. Subsequently, a darkly stained outgrowth appeared which increased in size with time. After about 8–10 min, the protuberance measured $3 \times 2 \mu\text{m}$ (Fig. 13). A break in the exinic wall layer was then observed, the timing of which corresponded to the appearance of the protuberance. After the exine breakage, the intine and the cytoplasm were rapidly stained blue. In *C. sempervirens*, there was no protuberance revealed by staining with astra blue, and after exine breakage, the intine and the cytoplasm turned pale blue.

Calcofluor in combination with astra blue revealed that, in *C. glabra*, a hemispheric structure started to become visible under the bulge after about 3–5 min. With time, it grew in size, exactly like the one observed after staining only with astra blue, which developed right under the bulge. This structure was never observed in *C. sempervirens*.

Acetolysis, along with light microscopy, revealed the presence of only one well-defined circular pore in the pollen grains of *C. glabra*; however, the same pore was barely detectable in *C. sempervirens*.

SEM and ESEM. Scanning electron microscopy allowed identification of a well-defined pore in the acetolysed grains of both species. The pore measured about $1 \mu\text{m}$ in *C. glabra*, but less in *C. sempervirens*.

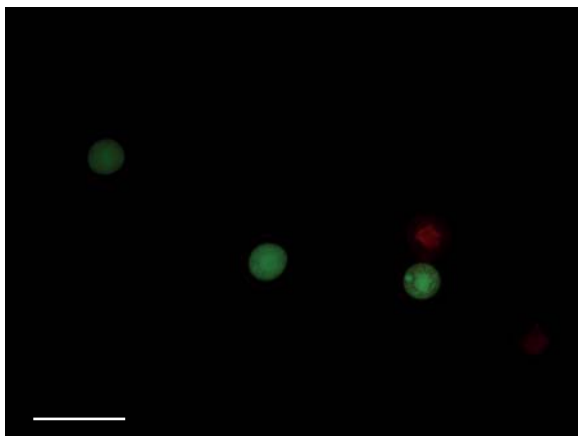


FIG. 1. FDA test for evaluating viability of *C. sempervirens* pollen grains. Pollen grains were previously counter-stained with neutral red and observed under the fluorescent microscope. The viable grains were green-yellow (FDA positive); the non-viable grains remained red (FDA negative). Scale bar = $50 \mu\text{m}$.

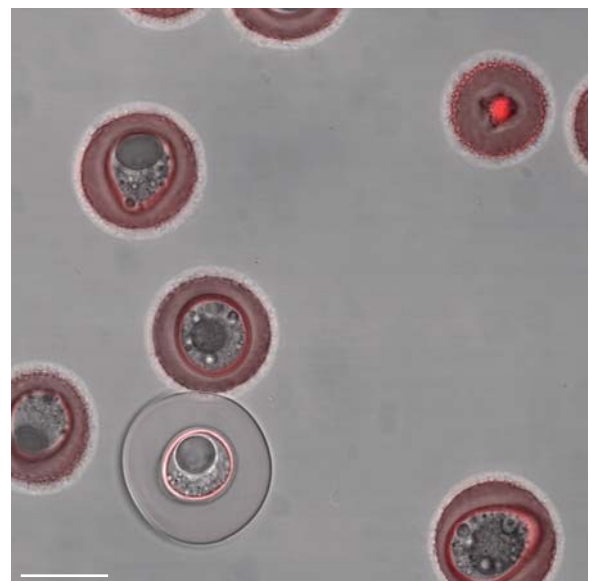


FIG. 2. Propidium iodide test for evaluating viability of pollen grains, observed under the confocal microscope. In the non-viable pollen grains, the dye passed through the membrane and the nuclei were stained red, while in the viable pollen grains the dye remained outside the membrane. Scale bar = $25 \mu\text{m}$.

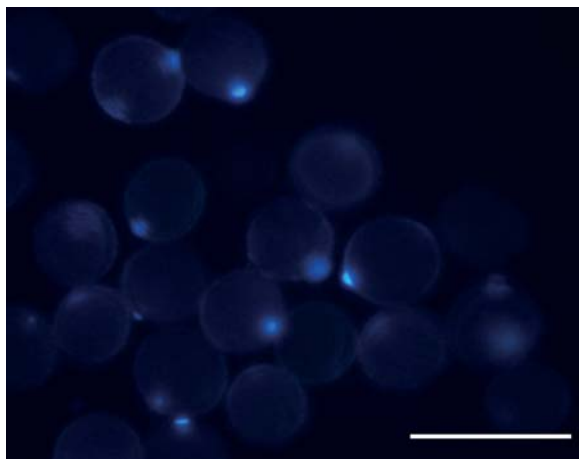


FIG. 3. Bulges on the external surface of the exine of *C. sempervirens* pollen grains made visible with calcofluor and observed under the fluorescent microscope. Scale bar = 50 μm .



FIG. 4. A pollen grain of *C. sempervirens*. The bulge is stained with calcofluor and observed under the confocal microscope in visible light.

The occurrence of one (rarely two) small irregularly shaped outgrowth was often observed (Fig. 14). The outgrowth was occasionally observed either in acetolysed grains or, more frequently, in grains that were fixed and dehydrated, or in grains treated with calcofluor and in freeze-dried grains. It was not detectable in the grains immediately observed at SEM and ESEM as such.

The outgrowth, which was always particularly bright, was either a compact mass about 4 μm wide, or was slightly elongated with a shallow or even deep depression on the surface. Sometimes the outgrowth was funnel-shaped, with fringed edges and a central circular hole (Fig. 15). The pore was never observed in the grains that showed the outgrowth.



FIG. 5. *Cupressus glabra* pollen grains stained with calcofluor and observed under the phase-contrast microscope. The bulge is highlighted under visible light. Scale bar = 50 μm .

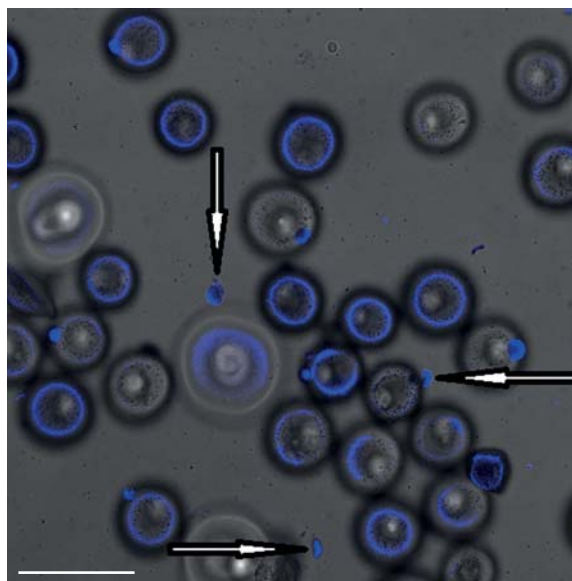


FIG. 6. Pollen grains of *C. glabra* hydrated and stained with calcofluor and observed under the confocal microscope. Note the presence of some bulges that have detached from the pollen surface (arrows). Scale bar = 50 μm .

DISCUSSION

The evaluation of pollen viability may have several applications. It is very important in horticulture for breeding experiments and in ecology for evaluating successful male reproduction (Dafni, 1992). Various methods for assessing pollen viability have been developed that are based on indirect features (Rodríguez-Riano and Dafni, 2000), enzymatic procedures (Ariano *et al.*, 2006), or fluorochromatic reactions (Nepi *et al.*, 2005), and some of these are very time-consuming.

In this work, the FDA test (Gahan, 1984) and a counterstain with neutral red were utilized. This addition made it possible to distinguish unequivocally which pollen grains were



FIG. 7. A *C. sempervirens* pollen grain stained with calcofluor observed under the confocal microscope at an excitation wavelength of 405 nm and under visible light (the images were superimposed). Sometimes, a few minutes after hydration, a new outgrowth protruded from the detachment point of the bulge.



FIG. 9. A pollen grain of *C. sempervirens* stained with aniline blue black showed a well-defined bulge on the external surface of the exine under the confocal microscope. Scale bar = 10 μm .



FIG. 8. *Cupressus glabra* pollen grains stained with aniline blue black and observed under the light microscope. Bulges were immediately visible as deeply stained protuberances. Scale bar = 25 μm .

viable and which ones were not. It was possible to verify that pollen grains with a rounded protoplasm and thick wall were viable and that those with an irregular, star-like protoplasm and thin wall were not viable. The staining with propidium iodide confirmed these results.

On many pollen grains, both viable and non-viable, it was possible to identify a hemispherical protuberance, a bulge, on the surface of the exine. The dimensions varied rapidly over time, probably as a result of hydration, with the diameter even exceeding 10 μm in some cases. The bulge is an extremely labile structure that was identified with the use of several stains which interacted with the protein and

polysaccharide components. The two components were not mixed homogeneously, as shown by observations under both the light and confocal microscopes, where a sort of stratification was visible. The polysaccharide component prevailed in the peripheral zone, while that of the protein was limited to the internal part.

By staining with astra blue and with calcofluor in combination with astra blue, it was possible to determine the absence of hemicellulose in the bulge of both *C. glabra* and *C. sempervirens*. Furthermore, the treatment with aniline blue excluded the presence of callose. It was found that calcofluor is able to reveal the presence of β glucans other than the ones stainable with astra blue (1-4- β glucans) and aniline blue (1-3- β glucans and 1-4- β glucans) under fluorescence as well as in the visible light and with the use of confocal and contrast phase microscopes. Thus, a polysaccharide is presumably present in the bulge that cannot be identified with the other histochemical stains that were used.

In previous studies of *J. communis* and *C. arizonica* pollen grains, a similar protuberance was observed under the light microscope by means of calcofluor staining; however, a detailed description was not provided in either case (Duhoux, 1982; Chichiricò and Pacini, 2008).

In this work, an irregularly shaped protuberance occasionally also showed up under SEM and ESEM. According to the observations carried out, this could correspond to the bulge, which takes on different shapes when observed under different conditions. The irregular shape could be a result of the difference in hydrophobicity of the proteins that constitute it. This structure very closely recalls what Duhoux (1982) illustrated as an 'opercule'. Therefore, it can be assumed that Duhoux's opercule (1982) and the bulge are actually the same structure. SEM observations did not point out any occurrence of fibrous materials similar to those detected by

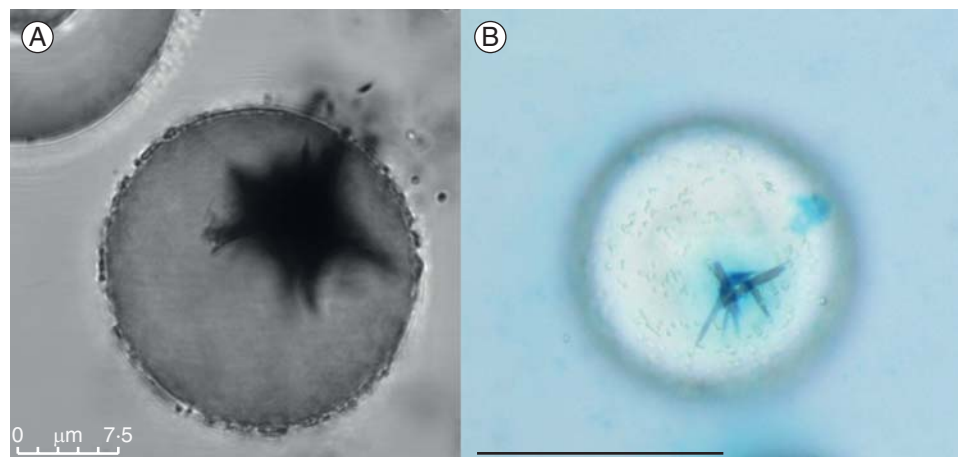


FIG. 10. *Cupressus glabra* pollen grains stained with aniline blue black and observed under the light microscope (A) and the confocal microscope (B) several minutes after the dyeing. Dark-blue elongated crystals were visible. Scale bar = 25 μm .

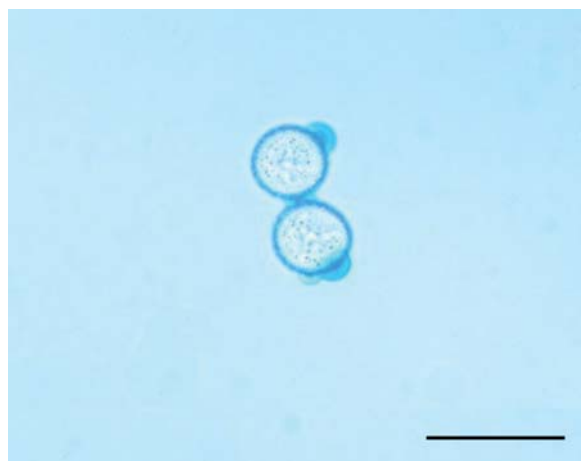


FIG. 11. Pollen grains of *C. sempervirens* stained concurrently with calcofluor and aniline blue black. The bulge was stained entirely with calcofluor, while aniline blue black marked the inner portion. Scale bar = 50 μm .

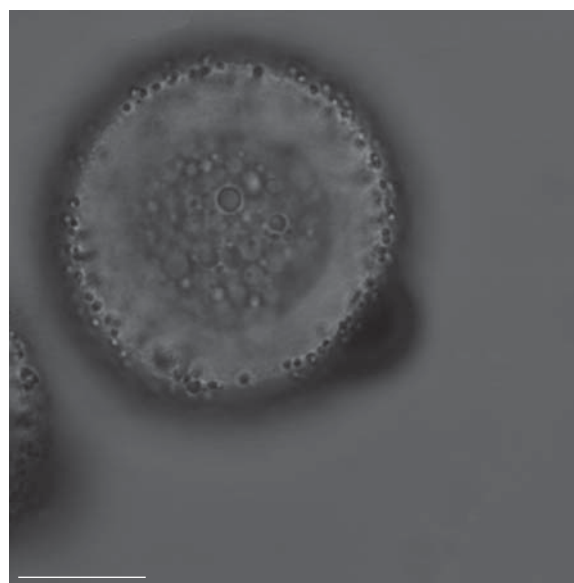


FIG. 12. A pollen grain of *C. sempervirens* stained with calcofluor and aniline blue black and observed under the confocal microscope at visible light. The bulge appeared layered, and the inner portion was darker than the outer one. Scale bar = 10 μm .

Chichiriccò and Pacini (2008), which were interpreted as coming from inside the pollen.

The bulge observed by Duhoux (1982), which differs from the opercule that he described, should be an intrinsic part of the intine because, when water penetrates through the pore, the part of the intine in contact with the water expands locally, forming a protuberance highlighted with calcofluor in *C. arizonica*. However, the bulge that we observed cannot be intinsic, otherwise it would not have shown up in *C. sempervirens* pollen grains, which have an intine that does not stain immediately with calcofluor in its outer part (Danti et al., 2010). The staining with astra blue never revealed any sort of protuberance, and in *C. glabra* an intinsic outgrowth was stained only after a few minutes had elapsed. In this work, but only in *C. glabra*, which has a larger-size pore, an intinsic-type protuberance was revealed, that became everted from the pore after staining with astra blue. Staining with calcofluor combined with astra blue allowed identification of the intinsic outgrowth developing just under the bulge. Under the confocal

microscope, an outgrowth (purported intinsic) protruding from the pore at the moment at which the detachment of the bulge occurred was observed. Intinsic outgrowth represents a temporary and very brief phase that immediately precedes the breakage in the exine wall and the swelling of the pollen grain.

The present observations showed that the bulge is positioned on the external surface of the exine, in correspondence with the pore. Prolonged observation over time made it possible to follow the evolution of the bulge, which dissolved or detached itself from the exine, as was observed directly using confocal microscopy.

The protein material identified with aniline blue black, formed acicular-shaped crystals after a few minutes. These could derive from the breaking of molecular bindings caused

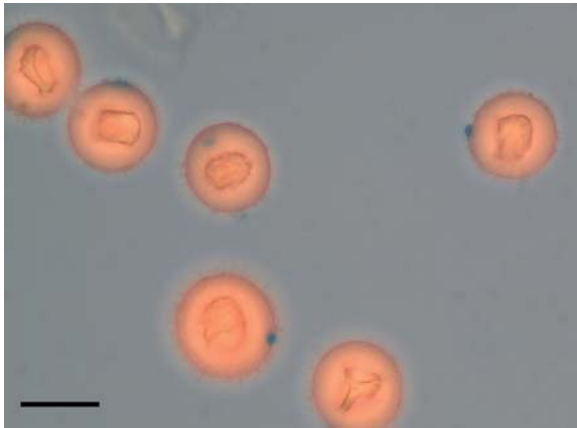


FIG. 13. *Cupressus glabra* pollen grains stained with astra blue observed under the light microscope. A few minutes (8–10 min) after dyeing, a blue-coloured outgrowth appeared on the surface of the pollen grains. Scale bar = 25 μm .

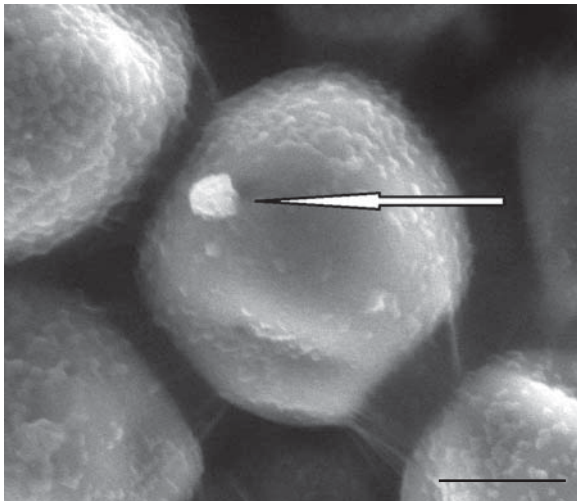


FIG. 14. *Cupressus glabra* pollen grains observed under ESEM after treatment with calcofluor, operating at 60–70 % relative humidity. Note the presence of a light protuberance on the surface (arrow). Scale bar = 10 μm .

by a variation in pH and/or due to a modification in the tertiary structure of the proteins as a consequence of the activation of enzymes which also break the bonds that hold the bulge to the surface of the grain. This mechanism could result in the complete detachment of the bulge from the exine.

In conclusion, whereas Duhoux (1982) and Chichiriccò and Pacini (2008) believe that the bulge is an intinsic protuberance produced as an artefact of calcofluor, we show that the bulge can be revealed by various staining techniques. This speaks in favour of its existence and makes it possible to define its structure. The bulge is neither a structure that plugs the pore (operculoid), nor an intinsic outgrowth. It consists of compounds that are different from those of the exine, and probably have a well-defined and stratified structure. It is made up of (unknown) polysaccharides which are distributed prevalently on the outer part and proteins that are prevalently in the inner part. This arrangement could constitute a structure

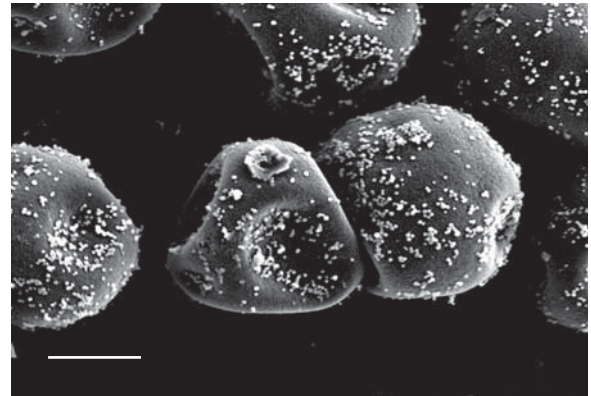


FIG. 15. A funnel-shaped outgrowth is present on the surface of a *C. glabra* pollen grain observed under SEM after fixation in acetic acid–alcohol solution and dehydration in ethanol series. Scale bar = 10 μm .

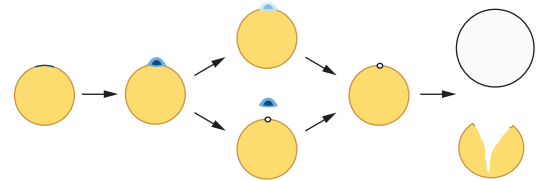


FIG. 16. Sequential phases, from the pollen grain hydration to the swelling and exine rupture (see text).

suitable for regulating the flow of water into the grain through the underlying pore during the initial phases of hydration.

Finally, we propose a model, based on the sequence of events leading to the swelling and breaking of the exine (Fig. 16):

- (1) The hydrated pollen takes on a rounded shape without any particular swelling but with a portion that has a different optical density compared with the surface of the exine;
- (2) The stratified hemispheric structure, that we call a bulge, develops very rapidly;
- (3) The bulge can (a) dissolve or (b) detach itself from the surface of the exine, thus making the pore visible;
- (4) An intinsic protrusion begins to come out of the pore immediately prior to the next phase;
- (5) Breakage occurs in the exine wall, caused by the rapid entry of water.

The duration of the different phases depends on the observation conditions, stains used, age and storing method of the pollen. It is possible to argue that, *in vivo*, the described mechanism takes place in the pollination drop, which essentially consists of a weak (1–10 %) sugar solution (Owens *et al.*, 1998), which corresponds to the staining media used in this investigation.

In addition to several *Cupressus* species (*C. macrocarpa*, *C. dupreziana*, *C. funebris*, *C. guadalupensis* and

C. torulosa) the bulge has been observed on the surface of pollen grains of *J. communis*, *Th. orientalis*, *M. glyptostrobooides*, *S. sempervirens* and *T. baccata*, thus it can be hypothesised that the entire mechanism is common to all species that have a taxoid-type pollen grain.

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