

IS PINE (*PINUS DENSIFLORA*) POLLEN ANOTHER EXAMPLE OF POTASSIUM ACCUMULATION AT SULCUS AREA?

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Abstract

Microscopic studies revealed that pine (*Pinus densiflora*) pollen grain body consists of a pair of air sacs and has a narrow sulcus (furrow) between two sacs. It is suggested that hydration and emergence of pollen tube take place through this furrow. Macallum's staining solution, energy dispersive X-ray analysis (EDX) and confocal laser microscopy using PBFI provide convincing evidence that potassium is concentrated only in the sulcus area of pollen grains. These results demonstrate that the consistent appearance of potassium at the sulcus area of pine pollen may regulate water uptake. Moreover, these results support the idea that the potassium accumulation at the aperture area may be a general pattern in all kinds of pollen grains.

Introduction

It has been reported that potassium (K) was heavily present only at the aperture area of mature pollen regardless of number of apertures of some angiosperms studied, as in case of single aperture barley (*Hordeum vulgare* L.) (Rehman *et al.*, 2004; Rehman & Yun, 2006) and 12 apertures sesame (*Sesamus indicum* L.) pollen (Rehman *et al.*, 2002). The consistent appearance of K at the aperture area of pollen was attributed as one of the factors that govern the faster uptake of water and rapid swelling of pollen. Macallum's staining (1905) solution and energy dispersive X-ray analysis (EDX) are used for K investigation in plant (Fischer, 1971) and in pollen (Rehman *et al.*, 2004). However, confocal laser scanning microscopy has been rarely applied for K sensing probes like potassium-binding benzofuran isophthalate (PBFI) to study K in pollen (Rehman *et al.*, 2005).

Morphologically pine pollen, a conifer is far different from previously reported other angiosperms pollen e.g., barley and sesame. The pine pollen grains have a narrow sulcus (furrow) rather pore(s) which is used for hydration and emergence of pollen tube (Iwanami *et al.*, 1988). Furthermore, in conifers like pine the flower and flower dehiscence are not same as in case of angiosperms e.g., barley. Therefore, present work was designed to investigate whether pine pollen grains which are distantly different from angiosperms, exhibit same phenomenon of K accumulation at sulcus area or not.

Materials and Methods

Pollens grains were collected from pine (*Pinus densiflora*) trees growing in the Chonbuk National University campus, Jeonju, South Korea. Pollen grains were collected from mature open male cones by dusting them in a bag. Pollen grains were passed through fine sieves to remove chaff. They were then stored at 4°C, in an airtight plastic container and were subsequently used for various experimental trials.

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A light microscope was used to study the changing size and shape of pollen. Pollen was hydrated with sterile double deionized distilled water and swelling phenomenon was observed directly under light microscope. Sodium cobaltinitrite solution was prepared as described by Macallum (1905). Pollen was placed in 5% Ammonium sulfide rather 100% concentrated solution as used by Macallum (1905) and Fischer (1971) because higher concentrations of Ammonium sulfide resulted in very deep dark stain, making it impossible to view the pollen. The presence of dark micro-crystals or crystal aggregates of the salt adequately identified the K rich areas in pollen. Light microscope was used to observe K stains in pollen.

In order to verify the K staining results, K was traced in pine pollen by using a scanning electron microscope (SEM) (JEOL JSM-6400) with the energy dispersive X-ray analysis (EDX) attachment. A thin layer of carbon was coated on pollen surface before EDX study.

PBFI a K⁺-sensing fluorescent probe (P1267) was purchased from Molecular Probes (Eugene, OR, USA). The presence of K was examined by loading PBFI as described by Rehman *et al.*, (2005). Pollen grains were placed in 200 µl micro-centrifuge tube. The PBFI was dissolved in Dimethyl sulfoxide (DMSO). Twenty microliter of 20 µM of PBFI was pipetted in the micro-centrifuge tube containing pollen grains. The micro-centrifuge tube was incubated in dark at 4°C for 1 h followed by incubation at 20°C for 1 h in dye free solution. In addition, pollen grains were treated with PBFI and directly observed under confocal microscope without incubating at 4°C. The results of the two methods were same. The samples were observed under a confocal laser scanning microscope (Carl Zeiss LSM 510, Jena, Germany) with an optical filter BP 385-470 (excitation 364 nm). The intensity of K specific fluorescence was measured in pixels (Fig. 5) by using GAIA Blue image analyzer (<http://www.gaia-zone.com/>). Each data point represents the accumulated value of randomly chosen eleven points and their corresponding standard deviation values.

Results

Scanning with electron microscope showed that the pine pollen body consisted of a pair of air sacs (Fig. 1). In dehydrated condition (pollen in 'normal' condition at the time of dispersal), pollen shrunk and the sacs lay close to each other. It is believed that the primary function of these sacs is to keep pollen afloat and carried to great distances by the wind (Owens *et al.*, 1998). Pollen has a narrow sulcus (furrow) between the two sacs. It is suggested that hydration and emergence of pollen tube take place through this furrow (Iwanami *et al.*, 1988).

Light microscopic studies revealed that, upon hydration, pine pollen grains fully expanded and air sacs stretched apart from each other (Fig. 2). It was noticed that pollen usually swirled upon addition of water. It is assumed that the pollen swirl is due to rapid expansion of the pollen sacs and/or rapid moving of the furrow towards water.

Macallum's solution (1905) stained pollen grains dark at the K rich areas. Heavy dark micro-crystals or crystal aggregates were found only at the sulcus of pollen grains (Fig. 3). The position of dark stain, mainly at the sulcus area, in individual pollen varied due to sitting of pollen at certain angles (Fig. 3).

Scanning electron microscope with the EDX attachment was used to trace only K at the sulcus area of pollen grains (Fig. 4). It was found the K peaks were higher when the electron beam was passed through the both outer margins of sulcus while lower K peaks were observed at the inner area that is the point at which two sacs lie close to each other.

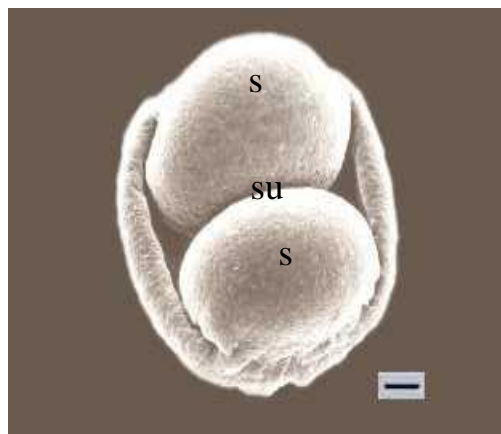


Fig. 1. Scanning electron micrographs of pine pollen grain with a visible narrow sulcus (su) between two air sacs (s). Bars: 10 μ m.



Fig. 2. Light microscopic micrographs of pine pollen grains before hydration that is 'normal' dry pollen (A) and swelled upon hydration (B). Bars: 10 μ m.

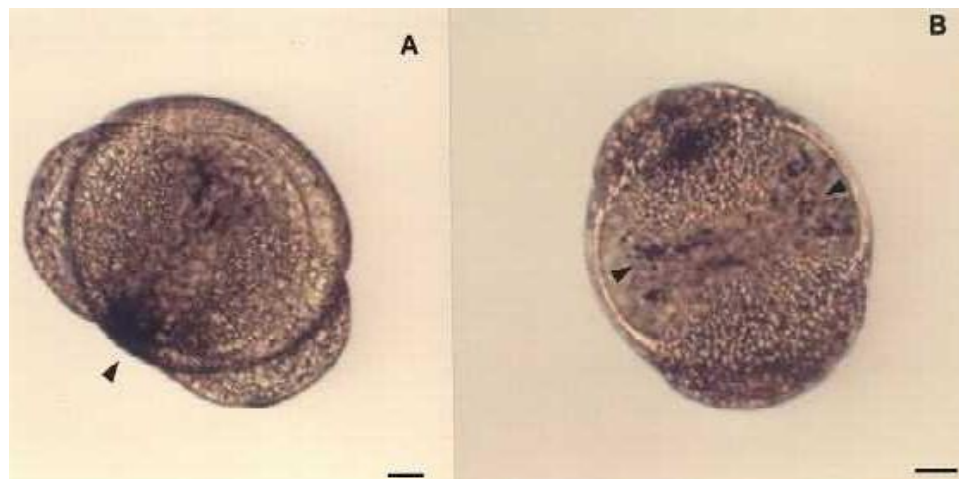


Fig. 3. Pine pollen grains stained with Macallum's solution. Arrowheads indicate the K staining (black stain) concentrated at the both ends of sulcus area of pollen grain. Pollen (Figs. A & B) photographed sitting at different angles (Bars: 10 μ m).

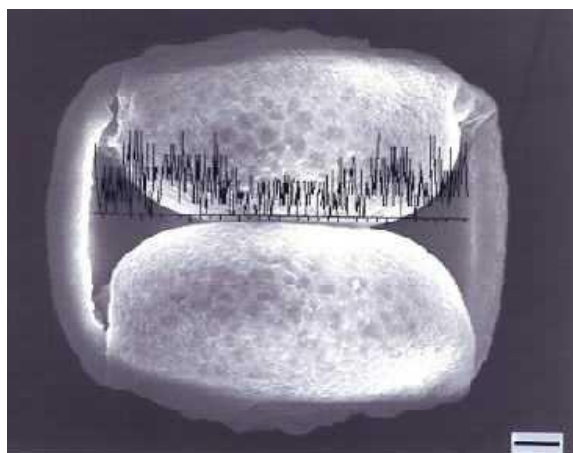


Fig. 4. The EDX spectrum of the sulcus area of pine pollen grain. Lines show the chart of K peak after electron beam was passed across the sulcus and the sulcus is shown below the chart of K traces. Higher peaks indicate the concentrated areas of K at the outer margins of sulcus while lower peaks were observed at the centre of sulcus where two sacs sit close each other. Bar: 10 μ m.

Fluorescent images of pine pollen grains resulting from the dye distribution of PBFI, illuminated with 364 nm light are shown in Fig. 5. Pseudocolours were used to enhance the visualization of PBFI distribution and to indicate fluorescence intensity. The higher intensity of fluorescence was detected only at the sulcus area of pollen grains (Fig. 5A), which indicate the heavy presence of K. Fig. 5B further elaborate these results which shows significantly higher presence of K at the sulcus compared to pollen sacs area.

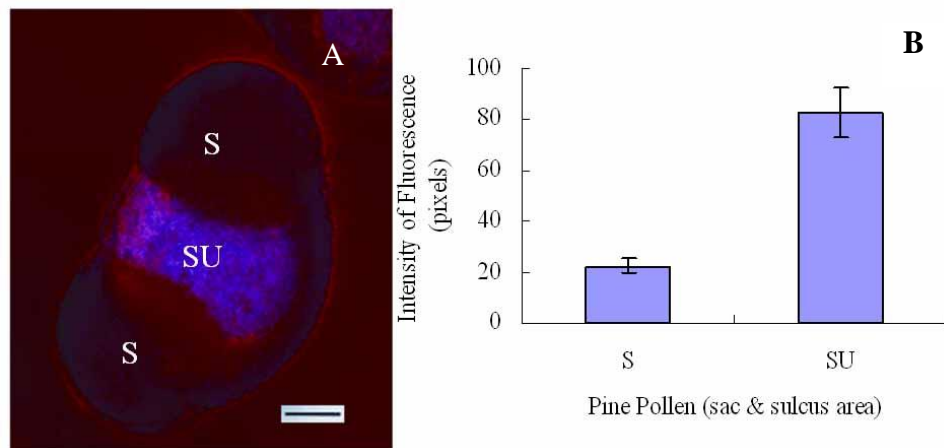


Fig. 5. Confocal potassium imaging: fluorescent image of pollen loaded with PBFI and excited with 364 nm light. Pseudocolour was used to enhance the visualization of PBFI distribution. The blue colour at the sulcus (su) area illustrates the accumulation of dye, which is localized at the sulcus area (A). Higher fluorescence indicates the higher presence of K which is measured in pixels (B) by using an image analyzer. The pollen grain was observed with a 40 x water immersion lens (C-Apochromat, NA=1.2, Carl Zeiss) and an image was captured by a confocal microscope, equipped with a 10 x ocular lens. Bar: 10 μ m.

Discussion

It was observed that pine pollen grains swell rapidly upon hydration. The observed rapid hydration and swelling phenomenon of pine pollen grains was very much same as was previously observed in barley and sesame pollen grains (Rehman *et al.*, 2004; 2002).

Macallum's staining (1905) and EDX techniques have shown that K was heavily present only at the sulcus area of pollen grains. The consistent appearance of K at the sulcus area of pine pollen grains was consistent with the previous findings reported by Rehman *et al.*, (2002; 2004). They reported that K was heavily present only at the aperture area of pollen grains of angiosperms, regardless the number of apertures e.g., 3 or 4 apertures in tobacco (*Nicotina tabaccum*); 3 apertures in *Eucalyptus* (Rehman *et al.*, unpublished data), single and 12 apertures in barley and sesame respectively. Furthermore, confocal laser scanning microscopy, using PBFI verify the existence of K at the sulcus area of pollen grains as obtained by using Macallum stain and EDX. The high intensity of fluorescence resulting from the dye distribution of PBFI was detected only at the sulcus area of pollen grains, which indicates the heavy presence of K. Similarly, fluorescent signals were specifically observed at the aperture area of single aperture barley (Rehman *et al.*, 2005) and 3 or 4 apertures of tobacco pollen grains (Rehman *et al.*, unpublished data).

Potassium is widely known for its rapid action as an osmotic regulator (Fischer, 1971; Heslop-Harrison & Heslop-Harrison 1996). The present results lead to the assumption that K could be one of the key factors regulating the imbibition of water and swelling of pine pollen grains due to the osmotic gradient created by K across pollen membranes. Furthermore, K accumulated at aperture area may also play an important role

in pollen germination and tube growth because it is an important constituent of pollen germinating medium (Fan, 2001).

Pine (conifer) pollen grains are far different from previously reported pollen grains e.g., barley (angiosperm), in terms of morphological features and the type and number of pollen apertures but despite these differences the heavy presence of K at the sulcus area of pine pollen grains is same as observed in barley and other pollen grains. Therefore, the consistence appearance of K at the sulcus area of pine pollen grains shows that it could be a general pattern of K accumulation in all kind of pollen grains.

In conclusion, these results provide convincing evidence that K is heavily present only at the sulcus area of pine pollen grains, which could govern hydration and furthermore, play important role in pollen germination and tube growth. Moreover, K accumulation in pine pollen grains may signal a common phenomenon existing in all kind of pollen grains.

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