APPENDIX

Cytological features of the fresh, bee-collected and stored pollen of *Aloe greatheadii* var *davyana*

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Introduction

The pollen of *Aloe greatheadii* var *davyana* has excellent nutritional value and satisfies all the requirements of the developing honeybee brood (Chapter 1). Nutrients in the pollen cytoplasm are protected by the pollen grain wall which has an outer layer known as the exine, composed of, among other substances, sporopollenin (Heslop-Harrison, 1971; Stanley & Linskens, 1974). The exine is frequently perforated by pores leading to the inner layer or intine. These pores play an important role during dehydration, dispersal and rehydration of pollen grains and determine the amount of water loss or uptake during these processes (Roulston & Cane, 2000). The intine consists of cellulose, pectins, proteins and hemicellulose (Roulston & Cane, 2000), while the exine is an indigestible and chemically resistant bio-polymer (Nepi & Franchi, 2000).

Pollen grains can be digested either by the destruction of the outer wall or through the pores. Roulston and Cane (2000) reviewed the mechanisms used by pollen feeders to reach the substances contained in the pollen cytoplasm. Honeybees are different from other insects because they pre-digest pollen, which is a process of pollen manipulation that begins during foraging. Honeybees add nectar and glandular secretions to pollen for external transport as well as for preparation of larval food or "bee bread", thereby altering the composition and nutritional value of the pollen (Chapter 1; Herbert & Shimanuki, 1978; Roulston, 2005). Manipulation of the pollen not only makes it digestion easier but also leads to increased digestion efficiency of *A. greatheadii* var

davyana pollen by adult worker bees (Chapter 2). Few studies have focussed on the efficiency of pollen digestion in adult bees but have been studied mainly in larvae (see Human & Nicolson, 2003).

The determination of the chemical composition of pollen grain cytoplasm (Chapter 1) requires biochemical methods while histochemistry and cytochemistry provide detailed information about the localisation of these substances. This information is difficult to obtain and sometimes one can only determine the presence or absence of certain substances with different stains. Therefore the two methods combined may be useful in understanding both the cytochemical and structural modifications that occur in pollen grains.

Methods

Fresh, bee-collected and stored *A. greatheadii* var *davyana* pollen was collected using the same methods as described in Chapter 1. Pollen samples were fixed in 2% glutaraldehyde in phosphate buffer at pH 7.2 and dehydrated in an ethanol series with increasing concentrations and embedded in LR white (London Resin Co. Ltd). Semi-thin sections (1-2 µm) were obtained with an LKB 8800 microtome, mounted on slides and stained with the following stains: Toluidine blue (TBO) as a general stain (O'Brien & McCully, 1981); Auramine O for cuticle (Heslop-Harrison, 1977); Calcofluor for cellulose in intine (O'Brien & McCully, 1981); PAS (periodic acid Schiff reaction) for insoluble polysaccharides such as starch (O'Brien & McCully, 1981) and Alcian blue 8GX for pectins (Jensen, 1962). These sections were examined on a Zeiss Axiophot 200 inverted microscope (Carl Zeiss, Götingen, Germany) at different magnifications in the Department of Environmental Sciences, University of Siena, Italy.

Results

Mature pollen contains, among other substances, carbohydrate and lipid reserves. All insoluble polysaccharides can be detected by PAS (Franchi et al., 1996). The intine and cytoplasm of fresh, bee-collected and stored *A. greatheadii* var *davyana* pollen are visible with PAS staining (Fig. 1) but no starch was observed. Pseudo-germination was observed in a few stored pollen grains (not shown).

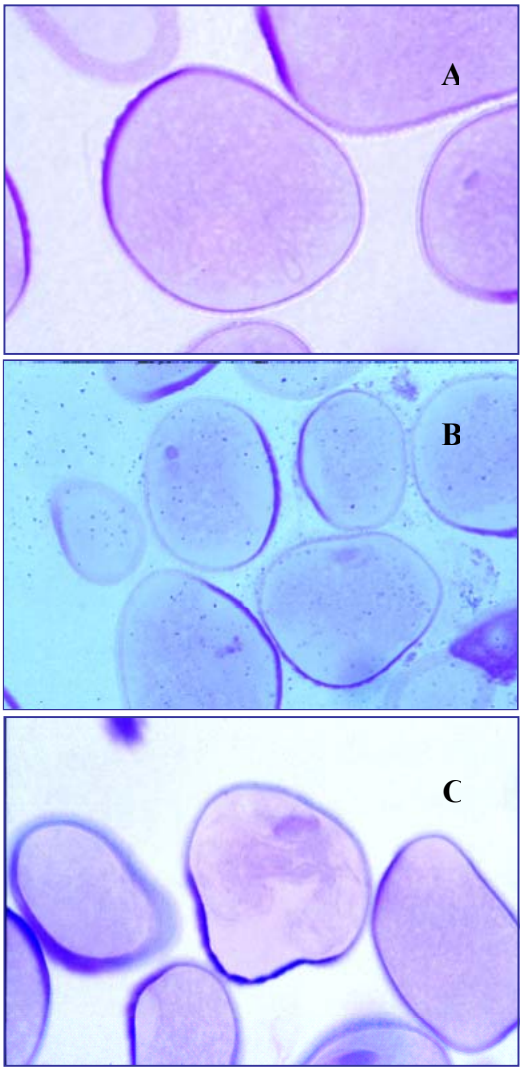

Figure 1. Indicates the intine and cytoplasm with PAS staining in **(A)** fresh, **(B)** bee-collected and **(C)** stored *A. greatheadii* var *davyana* pollen.

The exine contains sporopollenin that is fluorescent; therefore with autofluorescence microscopy one can distinguish between two non-continuous exine layers (Fig. 2A) without the use of any stains. The Auramine O stain intensifies the differences between parts of the exine such as the columellar structures in the outer exine layer (Fig. 2B, C).

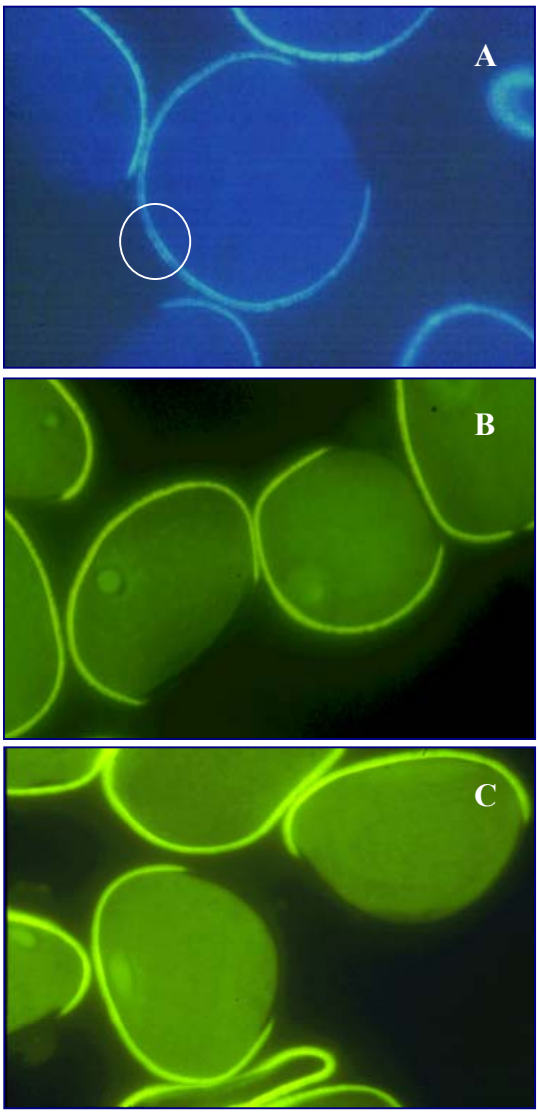

Figure 2. (A) With auto-fluorescence microscopy two non-continuous layers of the exine and a pore is visible in fresh *A. greatheadii* var *davyana* pollen. **(B)** Differences between parts of the exine are intensified with Auramine O in bee-collected pollen. **(C)** Stored pollen stained with Auramine O clearly showing the swollen intine.

The intine consists of cellulose and pectins; in this case a very thin layer of cellulose is observed in the inner part of the intine after staining the pollen grains with calcofluor (Fig. 3). Cellulosic intine appears with a more irregular profile in pollen grains stored in the hive compared to pollen from flower and bee corbiculae. Alcian blue stain showed the localisation of pectin in the outer intine layer. The surface of the outer intine layer became more irregular in pollen grains stored in the hive compared to grains in bee collected pollen (Fig. 4).

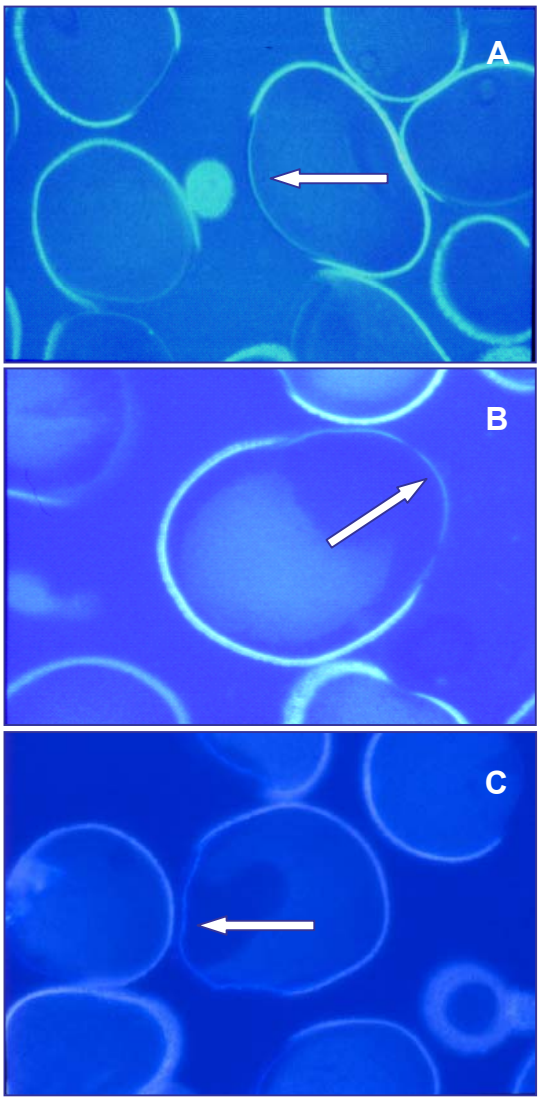

Figure 3. The obvious thin layer of cellulose in the intine of **(A)** fresh, **(B)** bee-collected and **(C)** stored *Aloe greatheadii* var *davyana* pollen.

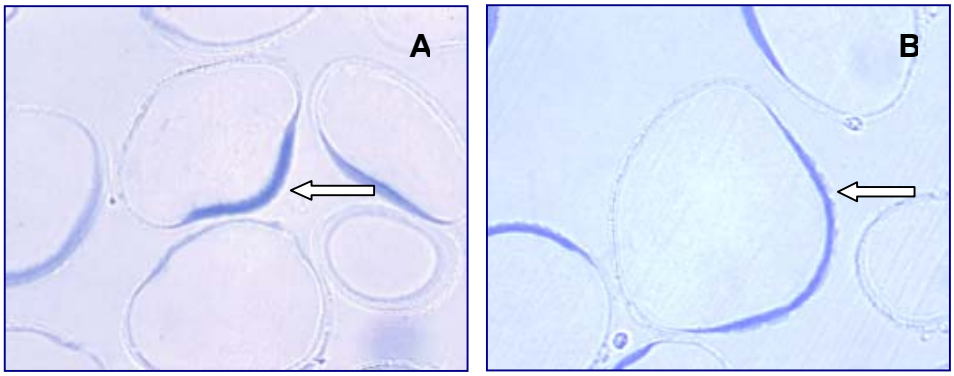

Figure 4. Alcian blue stain showed the localisation of pectin in the intine layer of **(A)** bee-collected and **(B)** stored *A. greatheadii* var *davyana* pollen

Discussion

Pollen shape changes during development, dispersal and arrival on the stigma due to loss and uptake of water in equilibrium with the surrounding environment. Mature pollen becomes dehydrated just before or during anther opening, thus increasing pollen fitness enabling it to withstand changes in environmental conditions. Ambient relative humidity and the number of pores may also have an effect on overall pollen volume. Upon landing on a compatible stigma, pollen rehydrates and germinates. This causes mechanical stress that must be sustained by the pollen walls, plasma membrane and protoplast (Nepi et al., 2001).

Aloe greatheadii var *davyana* flowers in winter, when relative humidity is low, which contributes to the dehydrated status of the fresh pollen grains. In this dehydrated state, the pollen wall is folded in the aperture regions and the indigestible exine is the only pollen wall component exposed to the external environment. Upon collection the nectar and glandular secretions added by honeybees supply moisture for pollen rehydration. During rehydration the intine absorbs water and increases in volume and surface area, especially in the furrow area, while the exine stretches. This demonstrates how the elasticity of the walls plays a role in the changes of volume and shape (Pacini, 1986). The pectin that is located mainly in the intine of *A. greatheadii* var *davyana* pollen may add to the hydration effects due to its hygroscopic properties (Aouli et al., 2001; De Halac et al., 2003).

The study by Suarez-Cervera et al. (1994) reported no ultrastructural changes in the pollen grain walls of stored pollen compared to fresh pollen. Added to this Klungness and Peng (1984 a, b) did not observe morphological changes in the pollen walls and protoplasm of stored pollen grains. Changes only occurred in the honeybee gut during digestion. In *A. greatheadii* var *davyana,* the only modification that occurred in the structure of the pollen wall, between fresh and stored pollen, appeared to be a slight change in the appearance of pectin and cellulose in the exposed intine. The suggestion that grains become compressed in the rectum as a result of the removal of certain structural components from the pollen wall through digestion (Klungness & Peng, 1984 a, b) may explain the occurrence of *A. greatheadii* var *davyana* pollen grains in the gut of honeybees (Chapter 2). The thin, exposed intine may contribute to the high digestion efficiency observed in honeybees for *A. greatheadii* var *davyana* pollen (Chapter 2).

The physiological state of *A. greatheadii* var pollen grains are deeply changed through hydration in that the intine is exposed, presenting a region for enzyme penetration during the digestive process. Thus pollen handling by honeybees probably "prepares" the pollen grains for efficient digestion.

Acknowledgements

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