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A unique anther-mucilage in the pollination biology of *Tylosema esculentum*

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Summary. *Tylosema esculentum*, a perennial geophyte bearing yellow distylous flowers in racemes, maintains a high degree of outbreeding through reciprocal herkogamy. In addition, a viscous liquid, the anther-mucilage, is produced by the anther connective tissue and released concurrently with the pollen. The polysaccharide- and lipid-rich mucilage, which is functional in the shedding and transfer of pollen, is available for more than 1 day due to the gradual solidification of the mucilage. The assimilation of the pollen with the liquid substance significantly affects the pollination biology of *T. esculentum*. This is the first report on the unique phenomenon of wet pollen in the Caesalpiniaceae.

Key words: Wet pollen – Morama/Marama bean – Gemsbuck bean – Shedding – Pollen transfer

Introduction

Tylosema esculentum (Burch.) Schreiber is a perennial geophyte of the Caesalpiniaceae and has a large to very large tuber from which radiating runners emerge annually. Popularly known as Marama bean or Gemsbuck bean, *T. esculentum* is endemic to southern Africa and occurs in semi-arid to savanna regions (Coetzer and Ross 1977).

The plants are heteromorphic with pin and thrum flowers borne in racemes on separate plants. Except for distyly and reciprocal lengths of the styles and filaments, the two morphs are similar. Each flower has two fertile anthers and eight short staminodes.

The yellow flag flowers of *T. esculentum* are typical melittophylous (Faegri and Van der Pijl 1979) and the occurrence of pollenkitt was expected. During a study of the reproductive biology of *T. esculentum*, however, we noticed a dark, honey-textured substance that was released concurrently with the pollen. It seemed to be an abundant liquefied pollenkitt that swamps the pollen. To our knowledge, similar wet pollen has not yet been reported.

Together with the stigmatic secretion, the sporoderm and other components like the pollenkitt play important roles in the pollen-stigma interaction (Yamada 1988) as they are vital in the incompatibility recognition reaction. Pollenkitt is functional in entomophily for pollen attachment to the pollinator's body as well as for the protection of the pollen against abiotic factors such as a dry climate (Shivanna and Johri 1985) and irradiation (Brewbaker and Emery 1962). The reason for the production of anther-mucilage may reside in one or more of these factors.

In this paper we report on the origin and composition of this unique pollen-associated substance. An attempt is also made to establish its significance for the pollination ecology of *Tylosema esculentum*.

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Materials and methods

Pollen shedding, insect visits and the appearance of the anther-mucilage were studied under varying field conditions during the flowering season (October to January) in both pin- and thrum-flowered plants. Field observations were made on the timing, frequency and behaviour of insects visiting the flowers. Pre-anthesis stamens were collected, kept in distilled water and observed under controlled conditions in the laboratory.

For microscopical studies, anthers were collected at the following stages from both pin and thrum flowers:
stage 1, just prior to dehiscence; stage 2, at dehiscence or with a granular appearance; stage 3, after dehiscence and in the transition phase from granular to shiny-wet; and stage 4, well after dehiscence or with shiny-wet appearance.

Anthers of the four stages were fixed in 2% glutaraldehyde and embedded in Quetol according to the method of Kushida (1974) as adapted by Coetzee and Van der Merwe (1985). The material was sectioned at ± 2 µm for light microscopical (LM) studies. Ultra-thin sections for transmission electron microscopical studies (TEM) were contrasted with uranyl acetate and lead citrate (Reynolds 1963).

After determining the solubility of the mucilage in water and organic solvents we ascertained the nature of the exudate by staining for proteins with Coomassie Bright Blue R250 (Gahan 1984), for lipids with Sudan Black B (O'Brien and McCully 1981) and for polysaccharides (Courtoy and Simar 1974). The necessary controls for all of the tests were performed as described by Courtoy and Simar (1974).

Pollen availability was determined by two different experiments. The first was to imitate pollen removal during successive insect visits, and the second, to determine the effect of anther-mucilage as it dries out over a certain period of time.

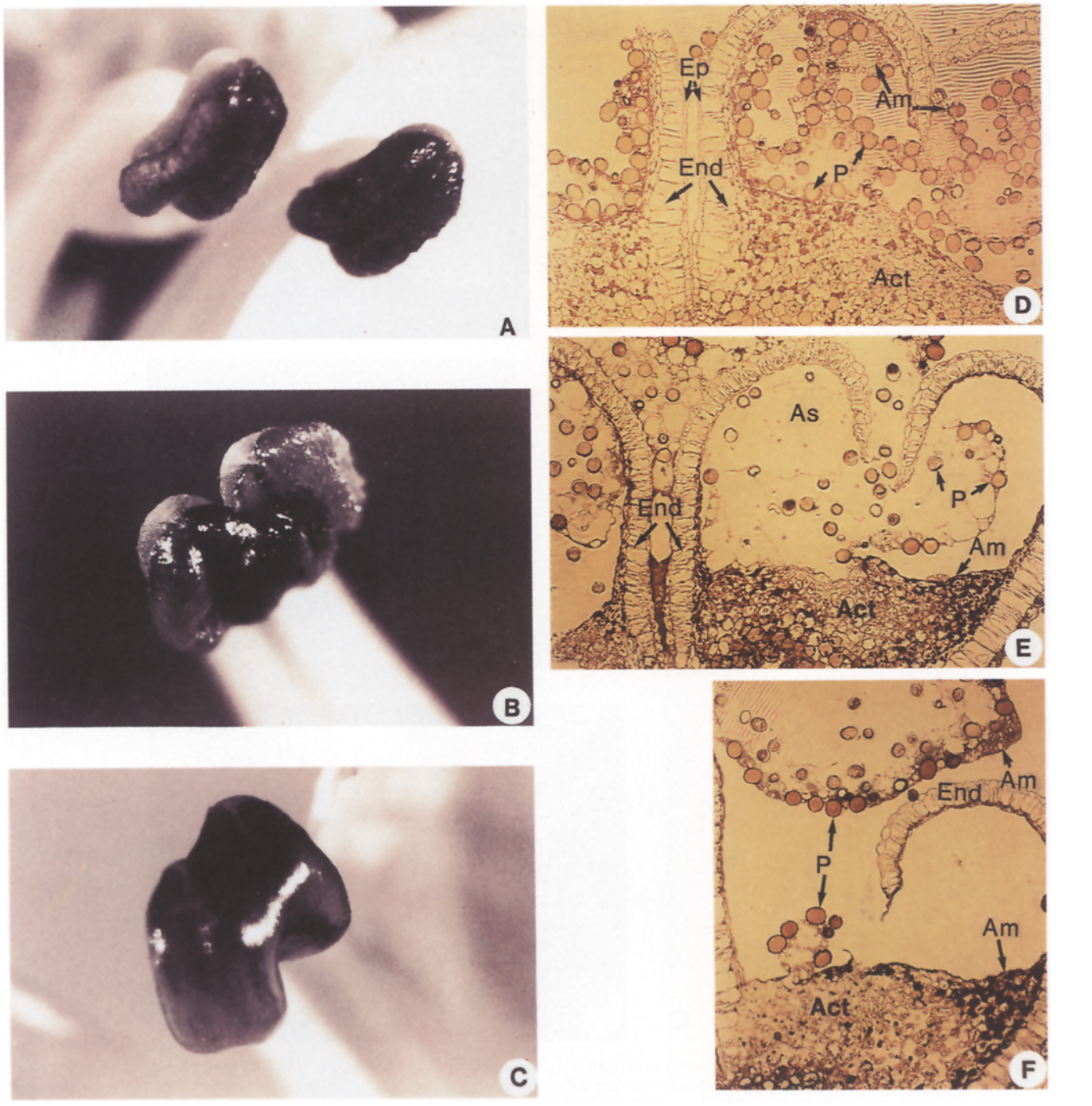

Fig. 1A–F. Anthers of *Tylosema esculentum* from thrum flowers illustrating the physical (**A–C**) and anatomical (**D–F**) changes that occur as the anther advances from the granular to the shiny-wet stage. *Am* anther-mucilage, *Act* anther connective tissue, *As* anther locule, *End* endothecium, *Ep* epidermis, *P* pollen. ×100

Experiment 1: Eight stage-4 anthers, collected at random from pin and thrum flowers, were separately dabbed on a clean coverslip every 2 h. The number of pollen grains deposited per coverslip was determined with a camera lucida drawing on graph paper. The average number of pollen grains on a single square of the graph paper was counted, and the average number of pollen grains for each successive pollen deposit was calculated. The average number of pollen grains per eight deposits per time interval was determined. Data were statistically processed using a quadratic log fitting.

Experiment 2: Thirty anthers from pin as well as from thrum flowers were tagged at anthesis and left on the plants. Five anthers per flower morph were dabbed on a coverslip every 2 h and then discarded. Anthers were used once only to rule out the effect of repeated pollen removal from the same anther. Calculations and statistical processing were the same as for experiment 1. The results were compared using Bonferroni tables at $P<0.05$ significance.

Stamens from stage 4 were used to determine the number of pollen grains per anther by means of a haemacytometer and for pollen viability and germination tests. The potential pollen viability was tested according to the fluorochromatic (FCR) procedure of Heslop-Harrison and Heslop-Harrison (1970), and the germination potential was tested using the hanging drop technique of Van Tieghem (1869).

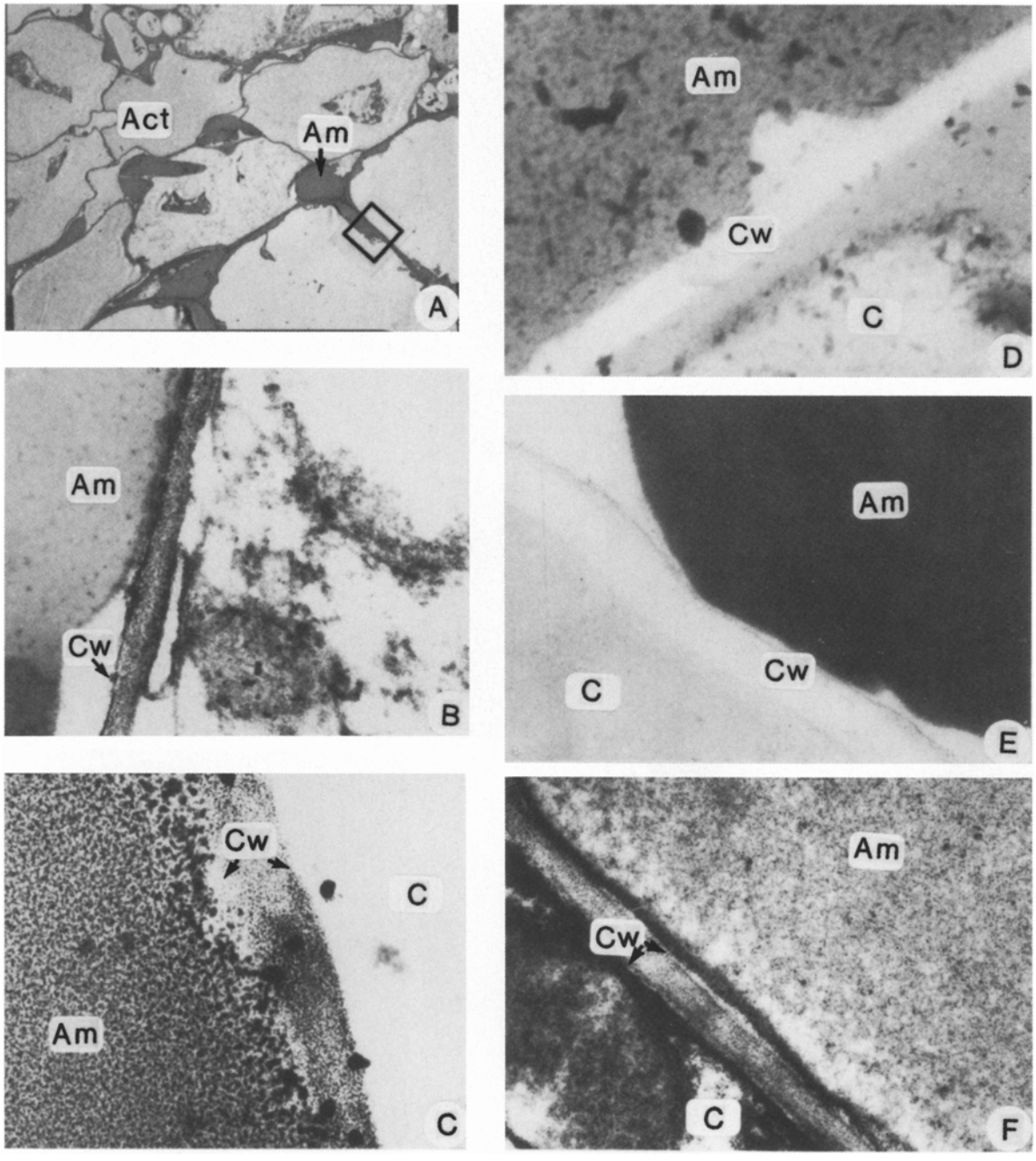

Fig. 2A–F. Electron micrographs illustrating anther-mucilage in the anther connective tissue and the results of the Thiery test for polysaccharides. **A** Anther-mucilage (*Am*) in the intercellular spaces of the anther connective tissue (*Act*). ×22000. **B** Anther-mucilage next to a cell wall (*Cw*), contrasted with uranyl acetate and lead citrate. ×59000. **C** Results of the Thiery test. Sections was oxidized and contrasted with Thiocarbohydrazide (TCH) and silver proteinate. The anther-mucilage is distinctly granular because of the electron-dense deposits. ×59000. **D** Control 1. Section oxidized and contrasted with TCH. The cytoplasm (*C*) did not stain. ×59000. **E** Control 2. Section oxidized and contrasted with silver proteinate. ×59000. **F** Control 3. Section without oxidation and contrasted with TCH and silver proteinate. ×59000

Results

Although anthesis took 21–28 h the androecium and gynoecium were exposed 3–4 h before the flowers were fully open. In both pin and thrum flowers, anther dehiscence occurred soon after exposure and usually between 09.00 and 10.00 a.m.

The anthers of *T. esculentum* open introrsely by means of longitudinal slits. Immediately after dehiscence, the pollen appear granular and slightly moist (Fig. 1A); no powdery stage occurs. Within 1 h the anthers change from the granular stage to the shiny-wet stage (Fig. 1B, C). This transformation is remarkable, particularly in the thrum flowers, and is affected by temperature and humidity. On hot, dry days, the change occurs in less than 1 h, but it takes much longer during rainy or overcast days. These field observations were substantiated by observing anthers under controlled

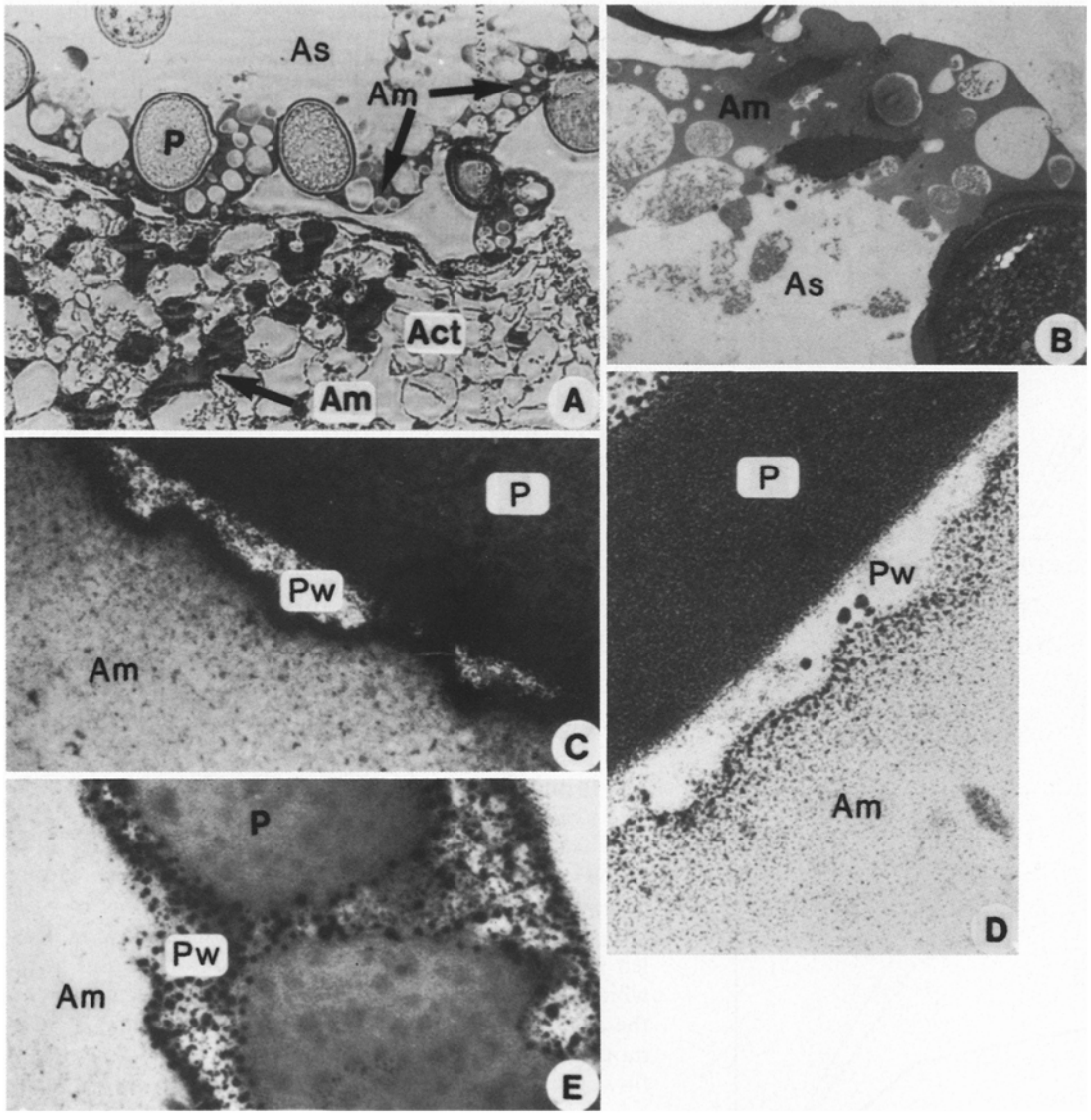

Fig. 3A–E. Anther-mucilage (*Am*) in the anther locule (*As*) and results from the Thiery test for polysaccharides. **A** Release of anther-mucilage (*dark spots*) from the anther connective tissue (*Act*) into the anther locule. ×200. **B** Anther-mucilage next to a pollen grain in the anther locule, contrasted with uranyl acetate and lead citrate. ×200. **C** The same as **B** but at high magnification. The pollen wall (*Pw*) stained more deeply. *P* Pollengrain. ×59000. **D** Results of the Thiery test. Section was oxidized and contrasted with TCH and silver proteinate. The anther-mucilage is distinctly granular because of the electron-dense deposits. ×59000. **E** Control. Section without oxidation and contrasted with TCH and silver proteinate. ×59000

conditions in a desiccator where the anthers became shiny-wet in about 90 min; the control anthers under ambient laboratory conditions had only reached the slightly wet stage (Fig. 1B) after the same period of time. The 'wet' anthers of the thrum flowers usually stuck together (Fig. 1C).

Shortly before anther dehiscence the anther-mucilage was present in the anther connective tissue and locules (Fig. 1D). During the change from the granular (Fig. 1E) to the shiny-wet stage (Fig. 1F), more anther-mucilage diffused into the anther locule, mixing with the pollen (Fig. 3A).

The TEM study revealed that the anther-mucilage in the connective tissue, in particular at the stage shortly before anther dehiscence, occurred mostly in the intercellular spaces close to the anther locule (Fig. 2A). Many of the cells from the middle layers and the tapetum were degenerating at this stage. Before the tapetum started to degenerate, it contained a substance, vesicular in origin (Coetzer 1982), which resembled the anther-mucilage.

The mucilage contained no perceivable solid substances such as the viscin threads reported by Hesse (1981, 1984). It appeared to be liquid-like, but viscous and homogeneously dark in the anther connective tissue and among the pollen grains (Fig. 3B). The granular appearance only showed up after the histochemical tests. The anther-mucilage was insoluble in water but dissolved in organic solvents such as acetone and chloroform. The histochemical tests showed that polysaccharides occurred in both the anther connective tissue (Fig. 2C–F) and in the anther locule (Fig. 3C–E). The

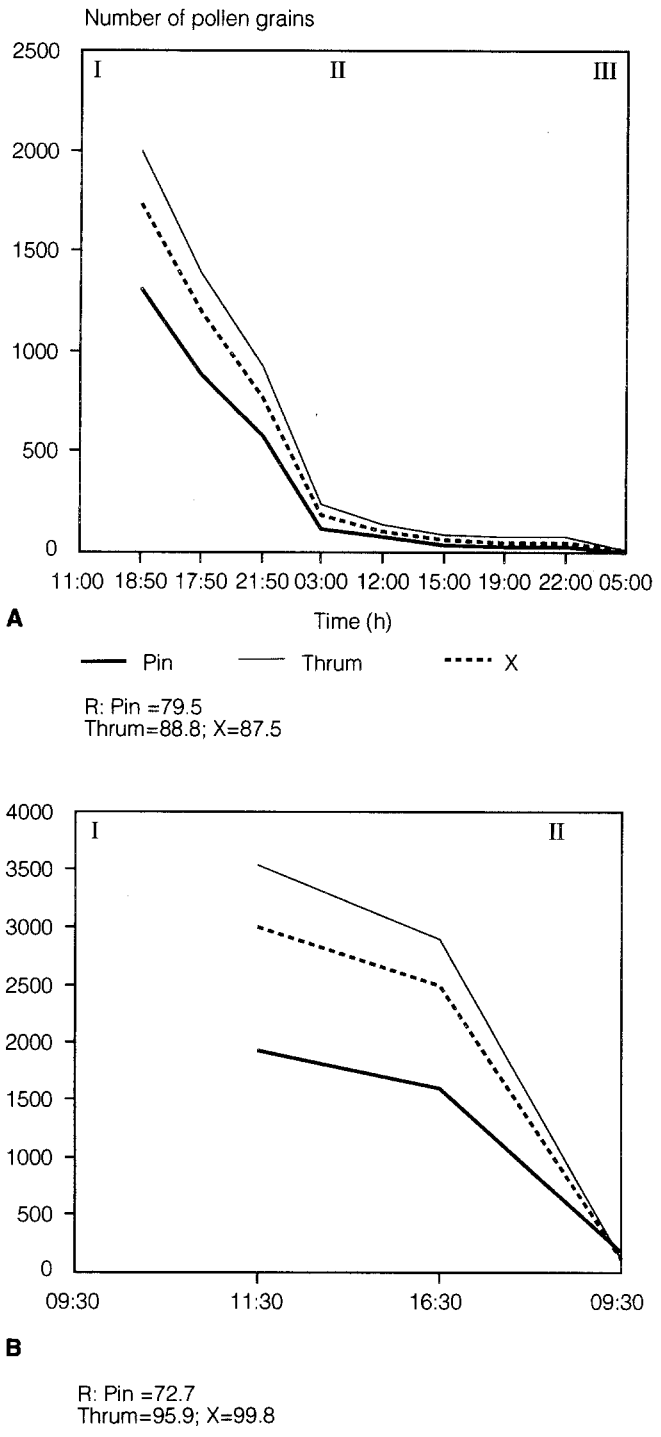

Fig. 4A, B. A quadratic log-fitted regression graph of the decline in pollen availability under laboratory conditions (**A**) and field conditions (**B**) over a period of 1 (*I*), 2 (*II*) and 3 (*III*) days. The decline in availability is directly proportional to the drying-out of the mucilage

protein test was negative, but the mucilage tested positive for lipids. No data is available on the water content, if any, of the exudate.

The viscous nature of the anther-mucilage and changes in its viscosity as well as changes in pollen availability over a period of time became evident during the two experiments. There was a negative correlation between the number of pollen grains on the coverslip and the number of dabs, indicating a decline in "available" pollen (Fig. 4A). There was also a sharp decline in the amount of pollen that rubbed off on the 1st day; less pollen rubbed off on day 2 and very little pollen rubbed off on day 3, even though there was still pollen present on the anther. The mucilage became very glutinous. However, viable pollen, according to the viability test, was available for more than 1 day.

In the second experiment the decline in pollen availability during the first day seemed to be much more gradual (Fig. 4B) than in experiment 1. The anther-mucilage remained more viscous because the anthers stayed on the plants. However, the Bonferroni test showed that there was a significant difference ($P<0.05$) in the decline of pollen available between pin and thrum flowers. During the first 5 h the decline in pollen available on pin flowers was more gradual. In these flowers the anthers are situated close to the gynophore and are partly covered by the staminodes, which makes them much less exposed than those of the thrum flowers. However, from both pin and thrum flowers very little pollen, if any, was available late on day 2. This shows that under field conditions, pollen could still be available on the 2nd day after anthesis.

The number of pollen grains per anther for the pin and thrum flowers did not differ significantly ($P<0.05$). The average number was 14544 per anther.

The two long-juxtaposed filaments of the thrum flowers resemble the style of the pin flowers in providing a landing place for visiting insects. Honey bees were the most abundant visitors to the *Tylosema* flowers. Pollen was observed to adhere to their abdomen and head, which corresponded to the positions of the anthers in the respective flowers. The distyly is functional in promoting outcrossing because the anthers of the thrum flowers are positioned the same distance away from the ovary as the stigma of a pin flower, where it corresponds to a certain position on the abdomen of a honey bee. The same applies to the position of the thrum stigmas and pin anthers. The visiting bee usually tries to reach the nectar inside the hypanthium. This brings its head and mouth parts in contact with the anthers of the pin flowers or with the stigma of the thrum flowers. The transfer of pollen in relative large numbers is enhanced by the anther-mucilage.

Discussion

No description of any pollen-associated component that corresponds with the anther-mucilage of *T. esculentum*, could be found in the literature. The anther-mucilage described in the present paper is totally different from the viscin threads described by Hesse (1981, 1984). The mucilage is also not excess pollenkitt as pollenkitt derives mainly from the degenerating tapetum (Hesse 1981).

It can be said, however, that the anther-mucilage is analogous, in terms of pollen ecology, to viscin threads and pollenkitt because it also determines the attachment and transfer of the pollen. No more pollen was available

for transfer by insects on the 3rd day because the anther-mucilage had dried out during the first 2 days after anthesis. On overcast days with a high relative humidity, the change from the granular to the shiny-wet stage was much slower. This delay coincided with the later visiting of pollinators when it became hotter. On days of continuous rain, no pollen was shed at all.

Hesse (1983) described a feature of *Delonix* spp. that is similar to the anther-mucilage. It differs, however, from anther-mucilage in its fibrous nature. Hesse (1983) does not mention the origin of this fibrous material in *Delonix* spp. The anther-mucilage corresponds with certain types of secretions described by Fahn (1979). These secretions are complex or neutral polysaccharide polymers with a high molecular mass. They have several functions, such as serving as a nutritional or as a connecting medium.

According to the TEM study (Fig. 3A), the anther-mucilage derives lisogenically from the anther connective tissue. Intercellular spaces in the anther connective tissue containing some exudate are more conspicious in sections of anthers at the shiny-wet stage than in those from pre-anthesis flowers.

The viscosity of the anther-mucilage of *T. esculentum* is functional in the initial attachment of the pollen to the pollinator. It also helps to attach the pollen to the stigma, which is very important in the pollen-stigma interaction (Heslop-Harrison and Heslop-Harrison 1982). The stigma of *T. esculentum* is funnel shaped with a very moderate stigmatic secretion (De Frey 1990) and will benefit from the wetting agent supplied with the pollen. The anther-mucilage may also protect the pollen against dehydration (Coetzer 1982) and radiation (Brewbaker and Emery 1962) in extreme high temperatures that usually prevail during flowering in the semi-arid habitats of *T. esculentum* (De Frey 1990). Mattsson (1983) has shown that a lipid-like sporoderm and coating also serve as a buffer against the too rapid equilibration of pollen grains with atmospheric water. Brewbaker and Emery (1962), using the term "wet" pollen when they referred to fresh pollen, state that desiccated pollen is twice as sensitive to irradiation as "wet" pollen. The anther-mucilage of *T. esculentum* most probably protects the pollen against the prolonged irradiation it is subjected to during the 2–3 days after anthesis.

To what extent the anther-mucilage plays a role in the germination of the pollen on the sigma still has to be ascertained. Shivanna and Johri (1985) have shown that factors determining the germination of pollen in species with a hollow style such as *T. esculentum*, are usually situated in the stigmatic exudate.

It is likely that the anther-mucilage has a determining role in the pollen germination because: (a) *T. esculentum* does not have a copious stigmatic secretion (De Frey 1990) and the mucilage contributes a "wetting agent" for that purpose; (b) De Frey (1990) found that during pollen viability tests, germination was equally good on 1-day- and 2-day-old stigmas; (c) no problem was encountered during in vitro germination of the pollen in the absence of a stigmatic secretion; (d) a much better ($P<0.05$) pollen germination occurred with pollen from the shiny-wet stage than from the granular stage; and (e) longevity of the pollen is assured by a moist coating that protects it against too rapid dehydration and excessive natural irradiation in arid habitats.

All of the above-mentioned factors contribute towards the success of the reproductive biology of *Tylosema esculentum* by promoting its outcrossing and fitness for survival.

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