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Wide hybridization: pollination of *Zea mays* L. by *Sorghum bicolor* (L.) Moench

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Summary. Foreign pollen tubes in the stigma of *Zea mays* can be prevented from reaching the ovary cavity by the unusual length of the pollen tube pathway. A simple and rapid procedure is described for overcoming this difficulty by pollinating the basal parts of the stigmas without removing the ensheathing bracts ("husks"). The method maintains high humidity in the vicinity of the ovaries, and by conserving photosynthetic tissues probably also ensures a more normal O_2/CO_2 balance in the neighbourhood of the stigmas than do bagging procedures. It is shown that *Sorghum* pollen tubes readily reach the ovary after pollination by the method. Their presence induces some of the characteristic post-pollination effects caused by *Zea* pollen tubes, but they frequently also stimulate premature enlargement of the nucellus and lysis of nucellar cells. Although *Sorghum* tubes have been traced across the inner ovary wall, they have not been seen to enter the micropyle, and hybrid embryos have not yet been obtained.

Key words: *Zea mays* – *Sorghum bicolor* – Wide crossing – Pollination technique – Post-pollination effects

Introduction

It is well known that prezygotic barriers often form the main obstacles to interspecific and intergeneric hybridisation in the grasses (Heslop-Harrison 1982). The normal functioning of the pollination system may be prevented by a selective inhibition of tube growth in a manner similar to that observed in the self-incompatibility reaction, or it may be disturbed by a lack of physiological co-adaptation between pollen tube and pistil. In the latter circumstance there may be some hope that the barriers can be surmounted by suitable manipulative procedures, allowing at least the possibility of fertilisation. The cross *Zea mays* × *Sorghum bicolor* may fall in the latter category.

Dhaliwal and King (1978) found that *Sorghum* pollen will germinate on the *Zea* stigma in suitable conditions, and that the pollen tubes can penetrate the receptive trichomes, although these authors obtained no more than limited growth thereafter. In our own investigations, we have shown that in appropriate circumstances *Sorghum* tubes can reach the transmitting tracts of the *Zea* stigma and achieve growth rates there comparable with those attained by *Zea* tubes (Heslop-Harrison et al. 1984b). The principal governing factor for germination of the pollen on the stigma is the ambient atmospheric humidity, and in the stigma the limiting circumstance may well be the capacity of the *Sorghum* tube to achieve lengths greater than those required to reach the ovary in the *Sorghum* pistil and at the same time to cope with the various controls in the pollen-tube pathway in *Zea* which provide guidance and act to regulate normal pollination (Heslop-Harrison et al. 1984a, 1985).

In the present paper we outline a simple pollination method designed to allow the application of pollen in the lower parts of *Zea* stigmas without removing the leaf bases ("husks") that ensheath the ear, and describe the results of applying the method in *Zea* × *Sorghum* crossing. The most important reason for retaining the husks is that they establish a humid atmosphere throughout the lower parts of the silks, a circumstance which will undoubtedly favour the germination of foreign pollen in these sites. Furthermore, the photosynthetic activity of the husks may well be important in maintaining an O_2/CO_2 balance in the vicinity of the ovules favourable to tube growth, which is notoriously sensitive to oxygen tension.

Materials and methods

Plant material

Experimental pollinations with *Zea mays* L. were carried out during April, 1984, usind Hybrids 3147 and 304C from Pioneer

Overseas Corporation, Des Moines, Iowa, USA as female parents. The plants were grown throughout under glass, the stock of 304C having been sown 19th January, 1984, and that of 3147 on 1st February. The tassels were removed from the ears of the selected plants, which were then kept in isolation with the silks further protected by glassine bags until the pollinations were carried out.

Zea pollen was collected from plants of the same two stocks, and also from plants of a sweet corn hybrid, NK 199. Pollen of *Sorghum bicolor* (L.) Moench was from plants of an inbred line, Asgrow H674, sown 19th January.

Temperature and humidity measurements

Surveys of air temperature and humidity in, or in the vicinity of, various parts of pre-flowering and flowering plants of 3147 were carried out using a dual-function digital meter (Electrical Thermal Instruments, Worthing, Sussex), with a probe diameter of 18 mm. The response time of the humidity sensor of this instrument in the range 43–95% is stated to be 4 min, with an accuracy of 2% between 10 and 95% RH. For surface measurements, the probe was retained as closely as possible to the plant part, protected from direct air movement but unshaded, until steady readings were obtained, usually in 5–8 min. Measurements in the silks were made with the probe pushed into the pendant mass until completely covered, and those in the vicinity of the growing point of vegetative plants with the sensors pressed as far as possible into the funnel formed by the leaf bases. To obtain an estimate of the conditions in the vicinity of the stigmas in an enclosed ear, two or more outer husks were folded back, and a circular hole cut through the remainder until the probe could be placed at the level of the stigmas; the outer husks were then pressed into place so as to enclose the sensor completely. Comparable measurements were made on naked ears, and on naked ears enclosed in kraft-paper pollination bags. Vapour pressure deficits were calculated from the temperature and humidity data.

Pollen testing

Pollen quality was assessed by the fluorochromatic reaction (FCR), using a medium made up by the semi-empirical method (Heslop-Harrison and Heslop-Harrison 1970; Heslop-Harrison et al. 1984), osmotically buffered with 12–15% sucrose.

Pollination in vitro

The time-course of pollen germination and tube growth was followed in vitro using segments of ears carrying 20–50 florets. The freshly excised segments were placed on filter paper pads saturated in sterile water in 5 cm petri dishes, which were then mounted on the necks of conical flasks so that the stigmas could be draped in identifiable groups over the flask walls for pollination. The preparations were incubated in containers in 80–90% RH at 24–27 °C throughout the period of observation.

Pollination in situ

Control and experimental pollinations were carried out so far as possible with ears matched for developmental stage. The protruding silks were excised at the level of the top of the ensheathing husks, and the cut surfaces protected by foil caps. A longitudinal slit 5–10 cm in length was then made through the husks in the central zone of the ear until a column of florets was exposed (Fig. 2a), and the cut edges of the husks pressed away until access could be gained to the stigmas. Pollen was then transferred uniformly to the exposed stigmas with a fine artist's brush. After pollination, the cut husks were eased back into place, and the edges prevented from gaping by two parafilm bandages (Fig. 2b). The bandages were replaced as required during the course of the experiment to accommodate growth. RH measurements made by inserting the probe of the meter into the sealed slit showed that the humidity in the environment of florets pollinated in this manner never fell below 90%.

Sampling and tissue preparation

Florets were excised from the ear segments pollinated in vitro at successive intervals, and the progress of germination and tube growth in *Zea* × *Zea* and *Zea* × *Sorghum* pollinations observed using the procedure of Linskens and Esser (1957), which is based upon the use of decolourised aniline blue as a fluorochrome for identifying the callosic inner pollen tube wall. These observations provided an approximate guide for the sampling of the ears pollinated in situ.

The florets from the *Zea* × *Sorghum* "slit" pollinations which showed indications of ovary growth were excised, and either dissected directly to observe the state of the embryo sac and associated tissues, or fixed in 1.5% glutaraldehyde in 0.05 M phosphate buffer at pH 7.0 with 8% sucrose for 2–4 h. *Zea* × *Zea* pollinations were sampled at corresponding times as controls.

After fixation, material for microscopy was dehydrated through an alcohol series and embedded in HEMA resin (TAAB Laboratories). Semi-thin (1.5–2.0 μm) sections were cut with glass knives, and observed without staining in a medium of appropriate refractive index with a differential interference contrast (DIC) system.

Measurements were made using a camera lucida and Apple microcomputer with digitiser. Embryo sac volumes were computed by summation from serial sections cut at uniform thickness through whole ovules.

Results

Pollen quality

Earlier observations indicated that *Sorghum* pollen was extremely short-lived in glasshouse atmospheres with RH 45–55% and temperatures in the range 25–35 °C. As Fig. 1 shows, the FCR score of *Sorghum* pollen held

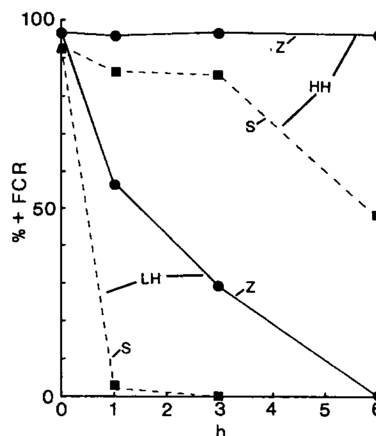

Fig. 1. Percentage pollen grains showing positive fluorochromasia (+FCR) following storage for periods up to 6 h in atmospheres with c. 55% RG (LH) and c. 95% RH (HH) at 24 °C. *Z* = *Zea mays*; *S* = *Sorghum bicolor*

at c. 55% RH at 24 °C after shedding also declined rapidly. *Zea* pollen stored in the same conditions showed a similar trend, but in this case the fall was less dramatic. The pollens of both species gave high FCR scores during storage for up to 3 h in an atmosphere with c. 95% RH at the same temperature. For the controlled pollinations, pollen was collected directly onto microscope slides from newly dehiscing anthers and either used immediately, or after brief storage in an atmosphere with RH c. 80–90%.

Water vapour pressure deficits in the vicinity of plant organs

Surveys were carried out on the afternoon of a typical day during the period of the pollination experiments on three *Zea* plants which had been watered to capacity c. 6 h earlier. The results are summarised in Table 1. It is evident that in plants not subject to water stress transpiration maintains high water vapour pressures within the mass of silks and in the vicinity of the ovules of the enclosed ears even in desiccating atmospheres, a predictable enough finding. Curtailment of the silks and removal of the ensheathing husks from the ear greatly increased vapour pressure deficit in the vicinity, creating conditions that would certainly be inimical to successful pollination (Heslop-Harrison et al. 1984b). Enclosing the stripped ears in kraft-paper bags if anything increased the vapour pressure deficit by raising the temperature in the neighbourhood of the florets.

Table 1. Temperatures and vapour pressure deficits (VPD) in the greenhouse air and in the vicinity of organs of vegetative and flowering plants of *Zea mays* hybrid 3147. The measurements were made at 14.40 h on April 11, 1984 with the glasshouse in full sun, the plants having been watered to capacity between 09.00 and 10.00 h. Sensors were placed as described in the text, and shaded during the period of equilibration (5–8 min)

Site	Temp. °C	VPD mm Hg
Free air, shade	36.0	20.9
Above the growing point, vegetative plant	37.7	14.9
In the silks, flowering plant	35.9	10.53
In contact with the ear, husks in situ	37.7	0.93
In contact with the ear, husks removed	38.0	22.79
In contact with the ear, husks removed; ear bagged	39.5	24.44

Zea × *Zea*

Samples taken at 3 h following the in vitro pollinations *Zea* 304C × *Zea* 304C and *Zea* 3147 × *Zea* 304C showed good pollen germination, with tubes in active growth in the stigmas. At 6 h the tubes were present in the ovary, and the stigma abscission zone had been activated (Heslop-Harrison et al. 1985). Coenocytic endosperm was present at 54 h, and normal embryos c. 0.3 mm in length at 5 days. No differences in the timing of pollen germination, tube penetration or fertilisation were observed in any of the combinations of *Zea* genotypes tested.

An ear of *Zea* 3147 extracted from the ensheathing husks 96 h after pollination by *Zea* NK199 using the "slit" method is illustrated in Fig. 2c, and a detail appears in Fig. 3a. The ovules in register with the slit through which the pollination was conducted virtually all show pollination-induced growth, demonstrating the effectiveness of the technique in localising fertilisation to a precisely identifiable column of florets. Embryo sacs in the pollinated ovules contained cellular endosperm at 96 h, with embryos 220–230 μm in length.

Zea × *Sorghum*

In confirmation of earlier findings (Heslop-Harrison et al. 1984b), samples from the pollinations made in vitro of *Zea* 3147 by *Sorghum* H674 under high-humidity conditions showed good pollen germination and a high proportion of tube penetrations into the receptive trichomes. Pollen tubes were observed in the stigma axis in the 6 h and later samples. Although no precise timings were obtained in the present experiments, there were indications that *Sorghum* tubes could activate the stigma abscission zone in much the same manner as do *Zea* tubes.

Sorghum tubes were observed in the upper ovary wall in some but not all of the 24 h and later samples examined. In this site, they were often seen to be disoriented (cf. Reger and James 1982), but in a few cases tubes were traced throughout the transmitting tract in sectioned ovules, and observed at the point of entry into the ovary cavity (Fig. 4a) and between the inner ovary wall and the contiguous inner integument. In no instance, however, was a *Sorghum* tube traced to the micropyle, nor were hybrid embryos observed in any of the present experiments.

An ear of *Zea* 3147 extracted from the ensheathing husks 96 h after "slit" pollination by *Sorghum* H674 is illustrated in Fig. 3b. In comparison with Fig. 3a of a *Zea* 3147 × *Zea* NK199 pollination, there is no evidence of uniform pollination-induced growth. However, several florets in the two columns in register with the slit through which the pollination was conducted show hypertrophy of the ovaries, which were exserted beyond the paleae and glumes. Another ear sampled at 7 days following the same pollination appears in Fig. 3c;

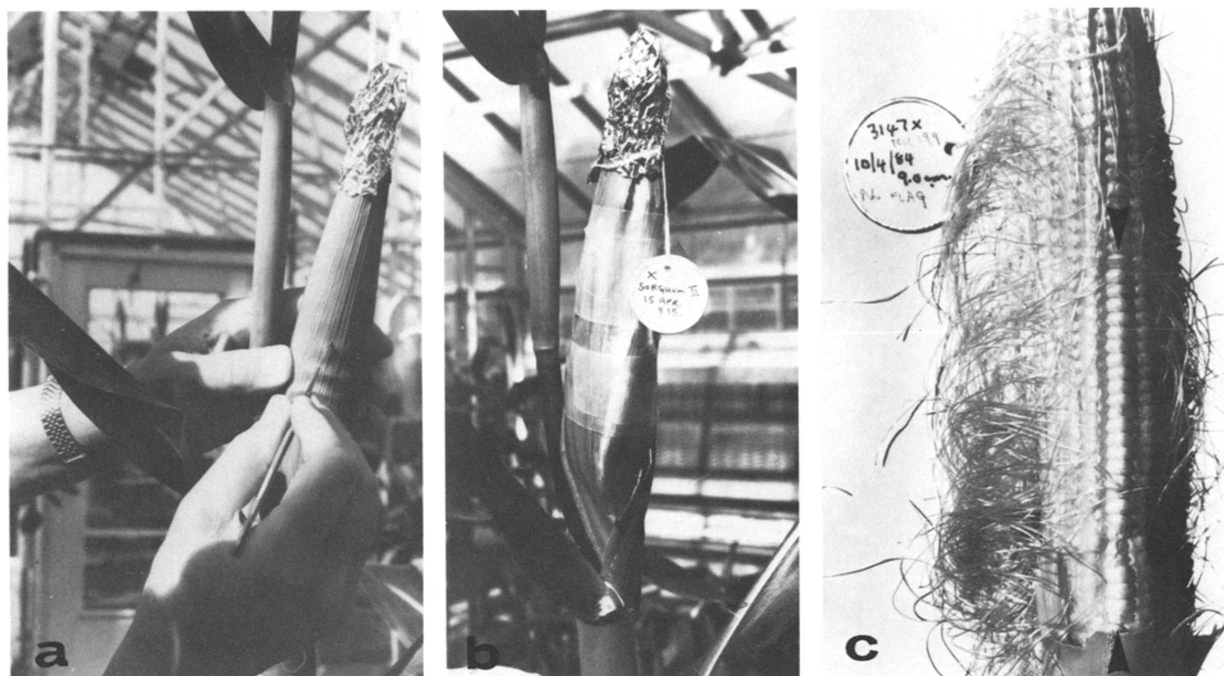

Fig. 2a–c. *Zea* 3147. **a** Preparation of the pollination slit. The tassel has been removed, and the emergent stigmas have been excised and capped; **b** Pollinated ear, with the husks replaced and held in position by parafilm bandages; **c** De-husked ear, 4 days after slit-pollination by *Zea* NK199. The almost complete column of developing florets between the arrows defines the site of the pollination slit. × ca. 0.6

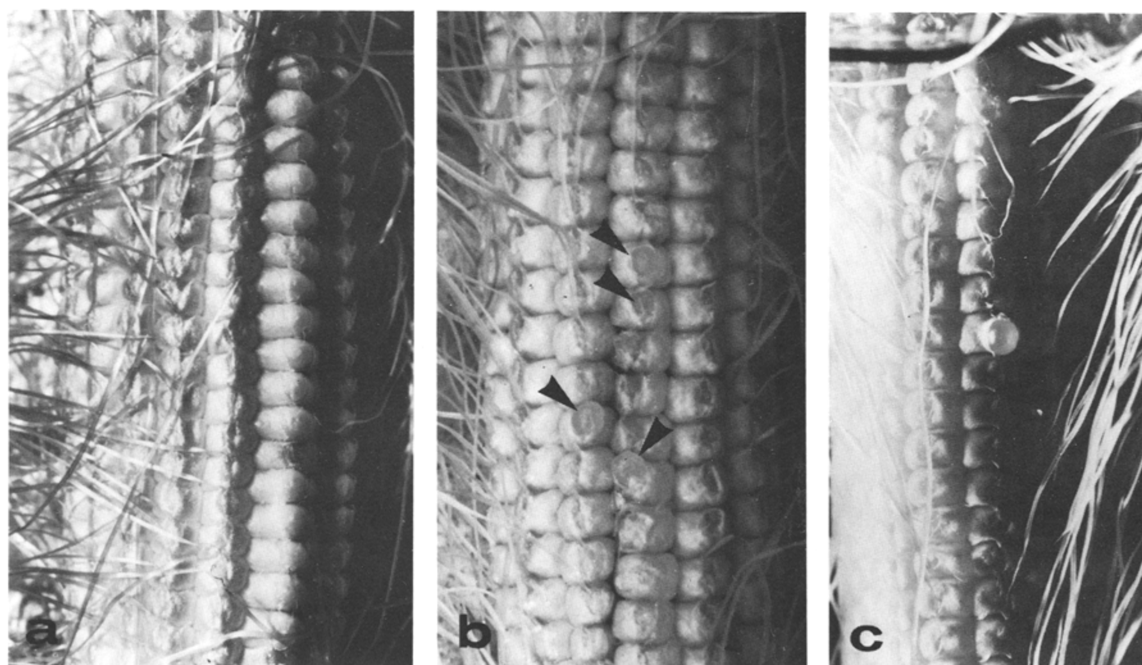

Fig. 3a–c. Slit-pollinated ears of *Zea* 3147, all × ca. 1.4. **a** The column of developing florets illustrated in Fig. 2c at higher magnification, showing that the enlarging ovaries are not greatly exserted beyond the glumes; **b** Ear four days after slit-pollination by *Sorghum* H674. Examination showed that *Sorghum* tubes had penetrated into most of the stigmas in register with the slit. The arrows point to the small number of florets in which hypertrophy of the nucellus had taken place with consequent exsertion of the ovary; **c** As Fig. 3b, 7 days after slit-pollination. In this ear only a single floret has responded to *Sorghum* pollination, but the ovary of this has enlarged very conspicuously

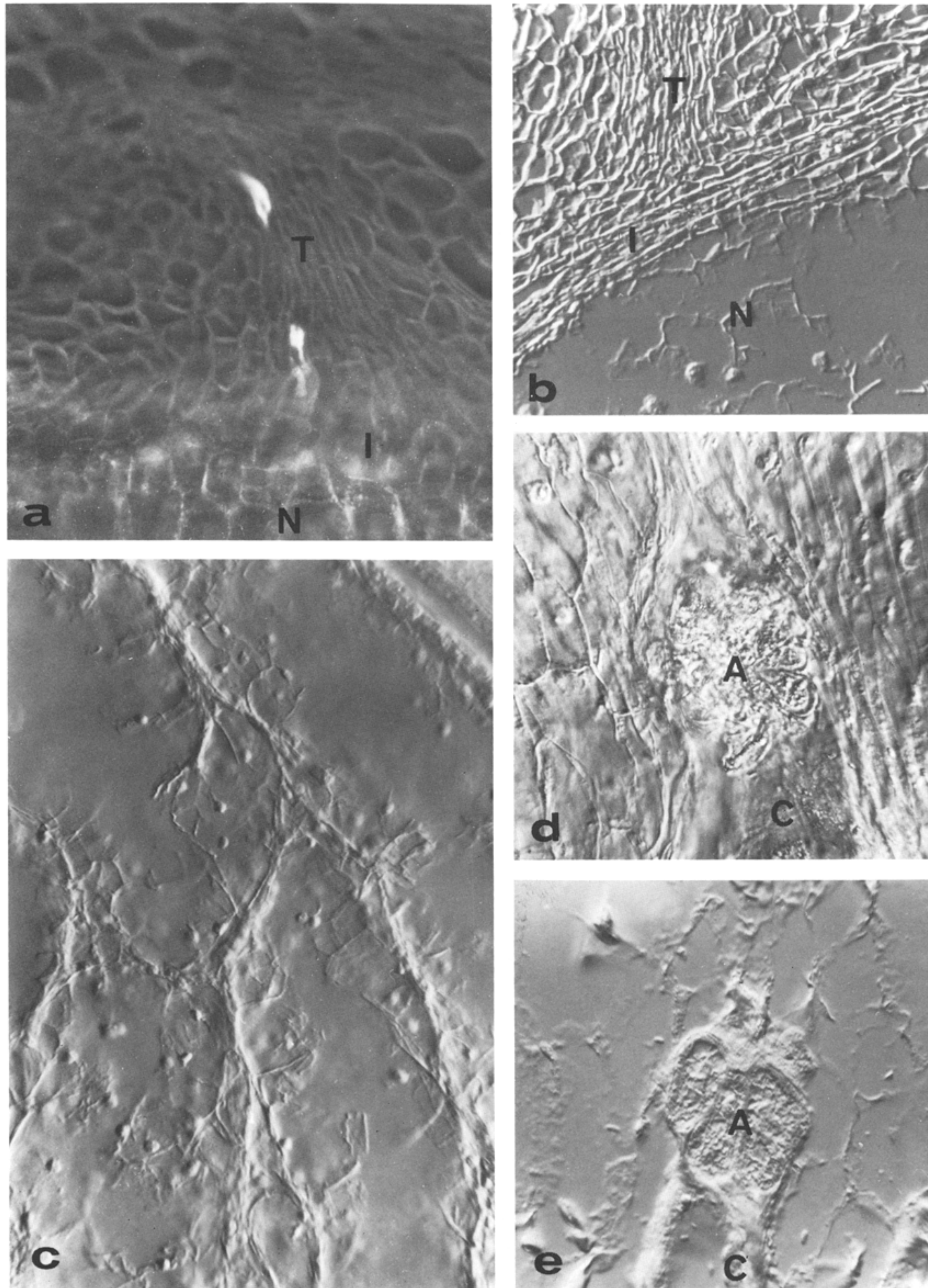

Fig. 4a–e. Micrographs of ovules of *Zea*, sectioned at 1.5–2 μm following GDA fixation and HEMA embedding. **a** Pollination in vitro of *Zea* 304C by *Sorghum* H674. The pollen tube, transected twice by the section plane, is seen in the transmitting tract (*T*) of the upper ovary wall and again passing through the inner epidermis into contact with the contiguous inner integument (*I*), which closely invests the nucellus (*N*). Fluorescence micrograph, decolourised aniline blue staining for callose. × ca. 300; **b** Ovary of *Zea* 3147 7 days after slit-pollination by *Sorghum* H674. The section transects in a longitudinal plane the transmitting tract (*T*) in the upper ovary wall and the adjacent inner integument (*I*), and shows the lysis of the inner cells of the greatly enlarged nucellus (*N*). DIC micrograph, × ca. 210; **c** As Fig. 4b, detail of partly lysed inner nucellar cells. × ca. 1500; **d** Antipodals (*A*), nucellar tissue and upper part of the central cell of the embryo sac (*C*) in an ovule of *Zea* 3147. DIC micrograph, × ca. 800; **e** Section corresponding to that of Fig. 4d from on ovule of *Zea* 3147 7 days after slit-pollination of the ear by *Sorghum* H674. The nucellus of this ovule had enlarged in a manner comparable with that of the ovule of Fig. 3c. The antipodals (*A*) have not proliferated, and the central cell (*C*) of the embryo sac is partly crushed. The nucellar tissue in the vicinity of the embryo sac is almost completely lysed. DIC micrograph, × ca. 800

this carried a single floret showing gross hypertrophy of the ovule.

Examination of the stigmas of florets from the ears of Fig. 3 b, c in register with the pollination-slits showed that in most instances the *Sorghum* pollen had germinated, and that tubes had successfully entered the stigmas. However, as Fig. 3 b, c show, not all of the ovules responded by enlargement. The indications are that the hypertrophy is only induced when a small group of tubes successfully traverses the lower stigma and enters the upper ovary wall. However, the sample that could be examined to determine this was very small, so that the interpretation must remain speculative until further evidence is obtained.

The nature of the ovule hypertrophy illustrated in Fig. 3 b, c was investigated in serial sections. The "growth" proved to be entirely due to gross enlargement of the nucellus, the cells of which showed evidence of dissolution in the 4 day samples, and were wholly dissociated and fragmented in 7 days (Fig. 4 b, c). The enlargement of the nucellus was accompanied by a stretching of the ovary wall and a compression of the embryo sac. The volume of the sac in a 4-day *Zea* × *Sorghum* ovule was estimated as 3.4×10^6 µm^3, compared with 5.6×10^6 µm^3 in a neighbouring unpollinated ovule. In normally fertilised ovules the nucellar tissue is compressed by embryo sac growth, but the tissues do not undergo lysis until late in the development of the embryo. The point is illustrated by comparison of Fig. 4 d, e.

Discussion

The foregoing observations show that pollination through a slit cut in the husks provides a simple and satisfactory method for transferring foreign pollen to the lower parts of *Zea* stigmas while preserving essentially normal conditions of atmospheric humidity in the vicinity of the pistils. Since the photosynthetic tissues associated with the ear are conserved, it is also to be expected that a normal O_2/CO_2 balance will be maintained over the stigmas during the critical period of pollen germination and early tube growth. The method also offers another important advantage: not only does it reduce the opportunities for accidental pollination from unwanted sources, but by providing precise demarcation of the location of the pollinated stigmas it allows an immediate and unambiguous identification of responding ovaries.

The experiments were performed both to develop methods allowing the pollination of the lower parts of the *Zea* stigma not normally freely accessible to pollen, and also to test further the potential for producing *Zea* × *Sorghum* hybrids. The present experiments yielded no hybrid embryos from this cross, but they did provide new information bearing on the potentiality for producing them. As earlier observations have suggested (Reger and James 1982; Heslop-Harrison et al. 1984b), *Sorghum* pollen will function on the *Zea* stigma, and can produce tubes capable of passing through a long enough stretch of the transmitting tract to reach the ovary cavity. It can therefore be said that in the correct environmental conditions no inherent barrier exists for *Sorghum* tubes in this part of the pollen-tube pathway, notwithstanding the various restrictions (Heslop-Harrison et al. 1985). Furthermore, there is evidence that *Sorghum* tubes activate some of the physiological responses induced by *Zea* tubes, including stigma abscission.

Nevertheless, we have not as yet observed the entry of *Sorghum* tubes into the micropyle, nor have we any unambiguous evidence of fertilisation. The scale of the present trials has been quite limited, however, and now that a simple and unambiguous method is available for ensuring that tubes do reach the ovary cavity there is an obvious invitation to extend the experiments to incorporate greater numbers and other genotype combinations.

An unusual feature observed in these experiments was the hypertrophy of the nucellus of the *Zea* ovule induced by *Sorghum* tubes. This is the consequence of a rapid transfer of water into the nucellar cells after the entry of the tubes into the upper ovary wall, a process which culminates in the lysis of the tissue. It is noteworthy that the response has no parallel in the normal behaviour of the nucellus in the period immediately following upon the entry of *Zea* tubes. In fact the nucellar cells were found to be intact in ovules with 6-day embryos, although somewhat crushed in the vicinity of the sac. The inner tissue does, however, undergo lysis during the later development of the embryo sac and embryo, a process described in detail for barley by Norstog (1974). The response to *Sorghum* tubes may perhaps be looked upon as a premature induction of the lytic process. Whether by destroying the embryo sac this could itself form an obstacle in the way of obtaining hybrid embryos remains to be seen.

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