Sex Plant Reprod (1990) 3:70–76

Sexual Plant Reproduction

Early post-pollination events in hexaploid wheat × maize crosses

D.A. Laurie[1] and M.D. Bennett[2]

[1] Institute of Plant Science Research, Maris Lane, Trumpington, Cambridge CB2 2JB, UK
[2] Jodrell Laboratory, Royal Botanic Gardens, Kew, Richmond, Surrey TW9 5DS, UK

Summary. Cytological events in the first 12 h after pollination were studied in crosses between the hexaploid wheat genotype Chinese Spring and the maize genotype Seneca 60. A pollen tube was first observed in the embryo sac 4 h after pollination, and maize sperm nuclei were first observed in the embryo sac after 5 h. On 29 occasions two, and on 1 occasion three, pollen tubes penetrated the embryo sac. Four categories of aberration limiting the frequency of fertilization were identified: (1) in 20% of florets no pollen tube reached the embryo sac; (2) in at least 1.9% the pollen tube severely damaged the wheat egg cell and polar nuclei; (3) in 33% the maize sperm nuclei were not released from the pollen tube; and (4) in 16% the sperm nuclei were released into the embryo sac but failed to move to either of the wheat gametes. In the remaining 29% sperm nuclei were more often found in the egg cell than at the polar nuclei. The results suggest that karyogamy occurs with very high efficiency when a sperm nucleus reaches the egg cell, but with only about 50% efficiency when a sperm nucleus reaches the polar nuclei.

Key words: Wheat – Maize – Hybridization – Fertilization – Karyogamy.

Introduction

Previous work on crosses between the hexaploid wheat genotype Chinese Spring and the maize genotype Seneca 60 found evidence of fertilization in 100 of the 343 florets dissected (29.2%). Eighty (23.3%) had only an embryo, eight (2.3%) had only an endosperm and in 12 (3.5%) double fertilization had occurred to give an embryo and an endosperm. The hybrids were karyotypically highly unstable, rapidly eliminating the maize chromosomes (Laurie and Bennett 1986, 1987, 1988a).

The main barrier to the recovery of plants from wheat × maize crosses appears to be the absence, or poor development, of the endosperm, which always has few nuclei in comparison to self-pollinated Chinese Spring caryopses grown under similar conditions (Bennett et al. 1975) and aborts early in seed development. In spite of these difficulties, haploid wheat plants can be recovered using spikelet culture (Laurie and Bennett 1988b), and this system is of interest both for wheat haploid production and for gene transfer experiments.

The recovery of plants has led us to study the cytology of Chinese Spring × Seneca 60 crosses further. In particular, we wanted to know why the frequency of fertilization was limited to about 30%. Failure of maize pollen tubes to reach the wheat embryo sac was one obvious reason, but previous work showed that a pollen tube often entered the embryo sac without subsequent fertilization (Laurie and Bennett 1987). Why was this so, and why did embryos develop at more than four times the frequency of endosperms?

We have addressed these questions by studying embryo sacs from Chinese Spring × Seneca 60 crosses fixed at hourly intervals between 3 h and 12 h after pollination. The timing of pollen tube arrival in the embryo sac and the timing of delivery, and the fate, of the maize sperm nuclei were recorded, and the results are discussed in relation to our previous work.

Offprint requests to: D.A. Laurie

Materials and methods

Glasshouse grown hexaploid wheat (*Triticum aestivum* L., $2n = 42$) plants of the genotype Chinese Spring were transferred to a controlled environment cabinet ($20 \pm 1°$ C with a 16-h day length, 300 μmol m^{-2} s^{-1} mean irradiance and 75% relative humidity) shortly before anthesis in the leading tiller. These plants were used as female parents in crosses with glasshouse grown plants of the single cross F_1 hybrid maize (*Zea mays* L., $2n = 20$) cultivar Seneca 60.

Emasculation of the wheat plants, pollen collection, crossing procedure, Feulgen staining and cytological examination of the ovaries were as described in Laurie and Bennett (1987).

Ovaries were fixed at hourly intervals from 3 h to 12 h after pollination. For each time interval 40 ovaries which showed adhesion of maize pollen grains to the stigma were dissected. A maximum of 10 ovaries were taken from each spike, giving a minimum of four replicate spikes for each sampling time. All times quoted in the Results are in hours after pollination. Gametes in maize pollen are haploid protoplasts (Dupuis et al. 1987; Cass and Fabi 1988) but since only the nuclei are visible in Feulgen-stained material we refer throughout this paper to sperm nuclei.

Results

Dissected embryo sacs were scored for the presence of a pollen tube and for the presence and location of sperm nuclei.

Timing of pollen tube arrival in the embryo sac

One ovary fixed at 4 h showed entry of a pollen tube into one of the synergids. The frequency of embryo sacs with one or more pollen tubes rose at each subsequent interval up to 9 h, when the frequency was 80% (Fig. 1). Ovaries fixed at 10–12 h showed similar frequencies, suggesting that all the pollen tubes that reached the embryo sac did so within 9 h of pollination. Thus, failure of fertilization in about 20% of florets can be attributed to cessation or malorientation of pollen tube growth.

Overall, 209 embryo sacs showed entry of a pollen tube, with 179 having one pollen tube, 29 having two and 1 having three. There was no obvious trend towards an increased frequency of entry of two pollen tubes at later fixation times. Pollen tubes were often highly coiled near the micropyle, indicating that they were growing under suboptimal conditions, and this made the path of their growth hard to interpret. This may mean that some instances where a second or third pollen tube entered the embryo sac were not recognized. The figure of 14.4% (30/209) for embryo sacs showing entry of two or more pollen tubes must therefore be regarded as a minimum estimate. This value assumes that all pollen tubes observed arose from separate pollen grains and not from forking during tube elongation, which may occur if maize pollen tubes are germinated in vitro under suboptimal conditions (J.S. Heslop-Harrison, personal communication).

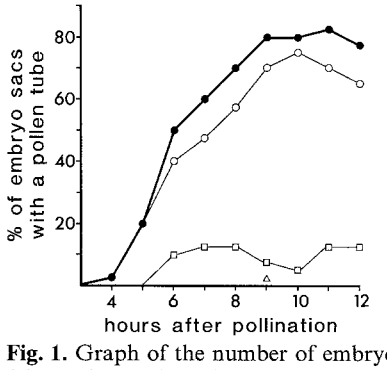


Fig. 1. Graph of the number of embryo sacs with one (○), two (□) or three (△) pollen tubes in material fixed 3 h to 12 h after pollination. The summed frequency is also shown (●)

In Chinese Spring × Seneca 60 crosses the pollen tube is large in relation to the components of the wheat embryo sac (Fig. 2), and in 2.4% (5/209) of ovaries the entry of the pollen tube damaged the base of the embryo sac so severely that the component parts were no longer recognizable. This appeared to be because the maize pollen tube continued to grow after entering the synergid. It is highly unlikely that fertilization would occur in such cases. Damage to the wheat egg cell and polar nuclei can account for failure of fertilization in at least 1.9% of all florets, assuming that 80% of embryo sacs receive a pollen tube (i.e, 2.4% × 0.8).

Pollen tube arrival in the embryo sac without release of the sperm nuclei

In 33% (69/209) of cases where one or two pollen tubes had entered the embryo sac no sperm nuclei were seen. It could be argued that they were overlooked but, as they were clearly visible in other preparations, it is more likely that they had not been discharged from the pollen tube. This was confirmed in one instance where two sperm nuclei were found in the pollen tube about 500 μm outside the micropyle.

In 9.1% (19/209) of cases, including some from the later fixation times, the sperm nuclei were in the synergid which had been penetrated by the pollen tube. Some of these might later be released, but this seems unlikely since examples of this behaviour could still be found at 48 h. These were therefore interpreted as cases where the pollen tube had entered the synergid and where the sperm nuclei had moved to the tip of the pollen tube but had not been released. If this is correct, failure

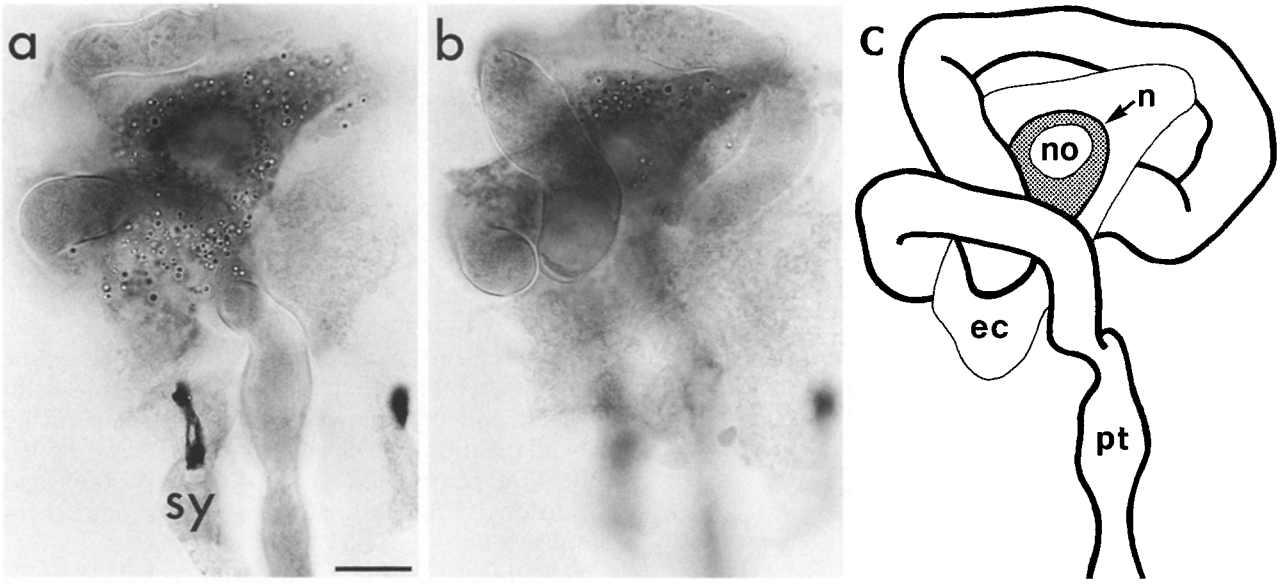


Fig. 2. a, b Maize pollen tube and wheat egg cell in two focal planes; **c** an interpretive drawing. *sy* Synergid, *pt* pollen tube, *ec* egg cell, *n* egg cell nucleus, *no* nucleolus, *Bar* 10 μm

to release the sperm nuclei into the embryo sac can account for failure of fertilization in a total of 33.7% of florets (assuming 80% of embryo sacs received a pollen tube).

Fate of sperm nuclei in the embryo sac

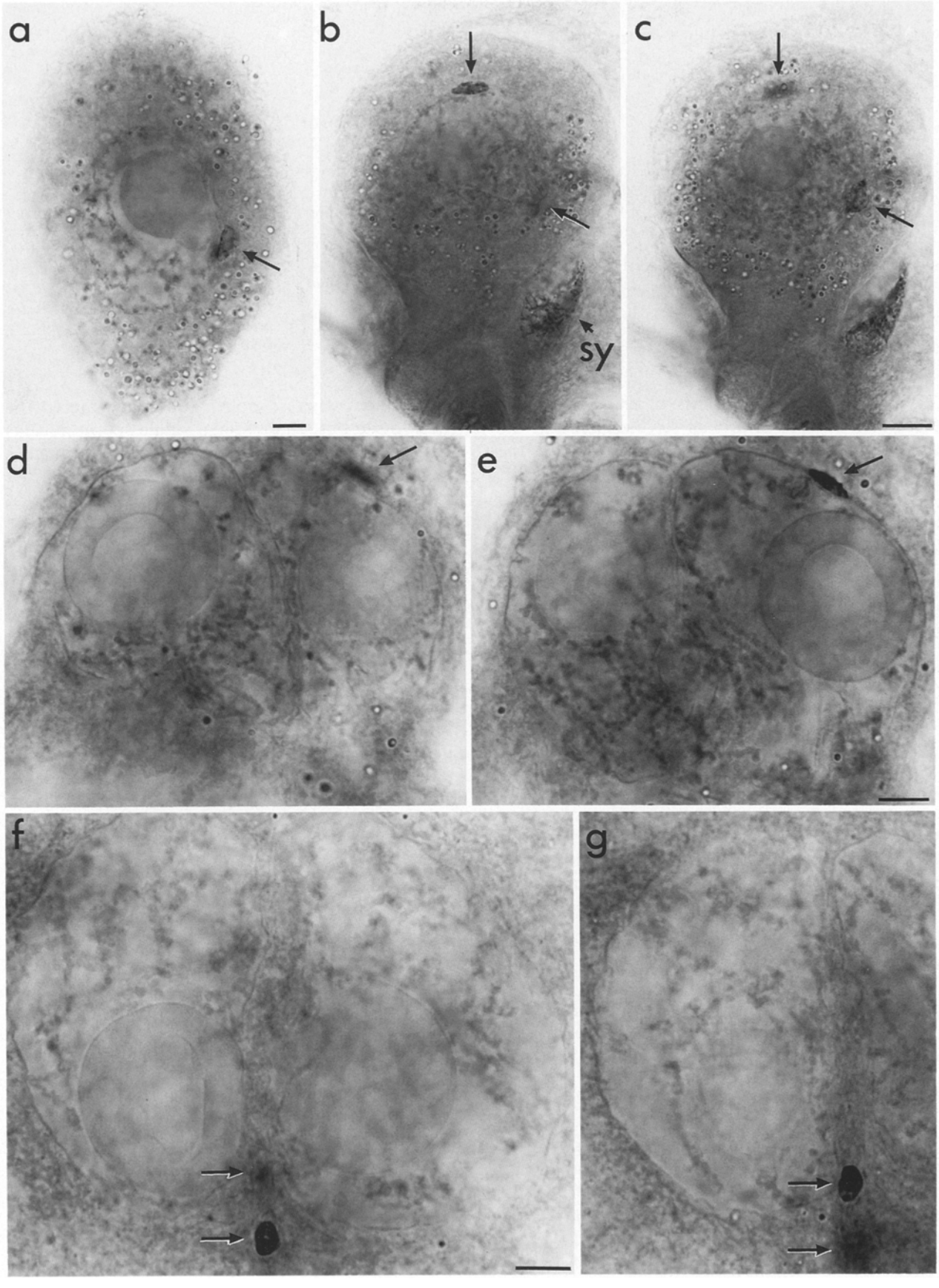
Sperm nuclei which had been released from pollen tubes were observed in two embryo sacs fixed at 5 h. In one, two sperm nuclei were on the outside of the egg cell. In the second, one sperm nucleus was adpressed to the egg cell nucleus and another was in the central cell cytoplasm between the egg cell and the polar nuclei.

Other examples of egg cells where a sperm nucleus was adpressed to the nucleus (Fig. 3a) were found at each subsequent fixation time. In four cases two sperm nuclei were involved (Fig. 3b, c).

A sperm nucleus adpressed to the polar nuclei (Fig. 3d, e) was first observed at 6 h, and further examples were found at each subsequent fixation time. In 16 cases one sperm nucleus was present on the polar nuclei, in 8 cases two were present, and in 2 cases there were three. Overall, only 4.3% (9/209) of the embryo sacs which received one or more pollen tubes showed the expected normal pattern of one sperm nucleus on the egg cell nucleus and one sperm nucleus on the polar nuclei. Of the 209 instances in which one or more pollen tubes had entered the embryo sac, 58 showed one or more sperm nuclei in the egg cell, and 26 showed one or more sperm nuclei on, or very close to, the polar nuclei. Assuming that 80% of embryo sacs received a pollen tube, 6.1% of all florets would have one or more sperm nuclei only on the polar nuclei, 3.8% would have sperm nuclei in the egg cell and on the polar nuclei and 18.4% would have one or more sperm nuclei only in the egg cell.

In 20.1% (42/209) of florets one or more maize sperm nuclei were within the embryo sac but not in contact with the wheat gametes. Some may have been in the process of moving to the wheat gametes, but embryo sacs with an egg cell, polar nuclei and one or more sperm nuclei in the central cell cytoplasm could still be found 48 h after pollination. There was no evidence that endosperm development could commence later than 28 h after pollination (present authors' unpublished data), suggesting that most of these sperm nuclei never effected fertilization. Failure to transport the sperm nuclei to the wheat gametes can account for failure of fertilization in 16.1% of florets (assuming 80% of embryo sacs receive a pollen tube).

Fig. 3. a Wheat egg cell with a maize sperm nucleus (*arrowed*) adpressed to the nuclear membrane. **b, c** Wheat egg-cell with two maize sperm nuclei (*arrowed*) adpressed to the nuclear membrane. *sy* Synergid. **d, e** Maize sperm nucleus (*arrowed*) adpressed to one of the wheat polar nuclei. **f, g** Two maize sperm nuclei (*arrowed*) adpressed to, or very close to, wheat polar nuclei. *Bars* 10 μm


a
b
sy
c
d
e
f
g

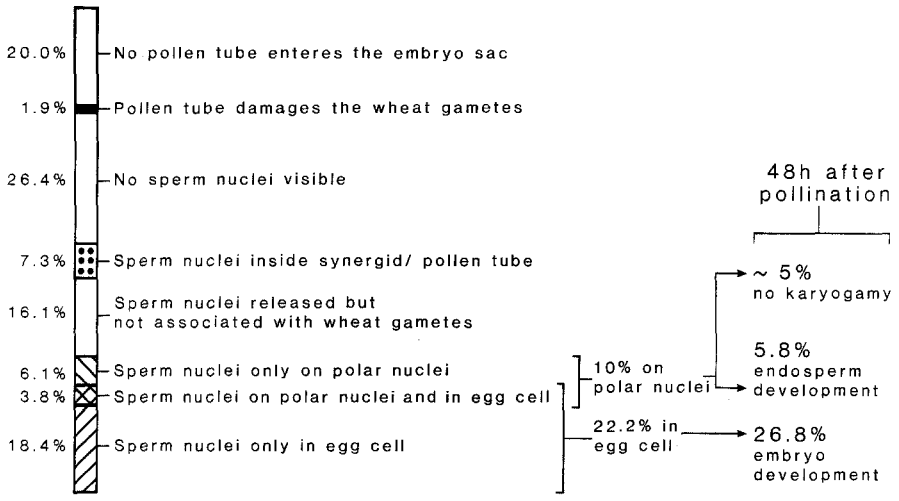


Fig. 4. Diagram summarizing the percentage of florets showing entry of a maize pollen tube into the embryo sac, presence of sperm nuclei and estimated frequencies of fertilization. Frequencies of embryo and endosperm development 48 h after pollination are from Laurie and Bennett (1988a)

The relative contributions of the aberrations described above to failure of fertilization and the frequencies with which sperm nuclei reached the wheat gametes are summarized in Fig. 4

Primary endosperm mitosis

The first endosperm division had occurred in two of the ovaries fixed at 12 h, but was not observed in material fixed at earlier times. In the first, the primary endosperm nucleus was at anaphase and, although the chromosomes could not be counted, at least 28 maize daughter chromosomes were present, suggesting that at least two maize sperm nuclei had fused with the polar nuclei. In the second, a single endosperm nucleus was found, which was bilobed and appeared to be a restitution nucleus formed at a mitosis where the chromosomes had not separated properly. Although only one pollen tube had entered this embryo sac, two sperm nuclei were found close to the endosperm nucleus, suggesting that division had been stimulated even though karyogamy had not taken place.

Discussion

The basis for the difference in the frequencies of embryo and endosperm formation observed 48 h after pollination

Movement of sperm nuclei to the wheat gametes. If all instances in which a sperm nucleus was observed in contact with a wheat gamete gave rise to an embryo or endosperm, the frequency of egg cell fertilization would be 22.2% (80% of 58/209) and the frequency of polar nuclei fertilization would be 10.0% (80% of 26/209), a ratio of 2.2:1. This discrepancy is largely accounted for by the 45 instances where a pollen tube had entered the embryo sac but where only a single sperm nucleus was visible. In 37 out of 45 cases the sperm nucleus was found in the egg cell, in 7 cases it was in the central cell cytoplasm, and in only 1 case was it on the polar nuclei. Assuming that the second sperm nucleus was not simply overlooked, this suggests that when only one sperm nucleus was released it usually moved to the egg cell.

This raises the possibility that the destination of sperm nuclei is predetermined, as proposed by Roman (1948) from data on the inheritance of B-chromosomes in embryos and endosperms of maize and by Russell (1985) from data on the transmission of plastids in *Plumbago*. Such functional differentiation may be accompanied by morphological differentiation, since McConchie et al. (1987) have reported that sperm nuclei in mature unhydrated maize pollen grains differ in size and shape. If maize sperm nuclei are pretargetted, our results could be explained if the maize sperm nucleus programmed for fusion with the egg cell was preferentially released from the pollen tube. Alternatively, the first sperm nucleus to be released from the pollen tube may fertilize the egg cell.

Failure of karyogamy. The figures for the expected frequencies of embryo and endosperm formation are minimum estimates. They exclude florets from early fixation times where sperm nuclei have not yet been delivered to the embryo sac, and florets from later fixation times where sperm nuclei may have fused with wheat gametes and become too diffuse to be identifiable. Nevertheless, the estimated value for the egg cell (22.2%) is close to the 26.8% observed in material fixed 48 h after pollination (Laurie and Bennett 1988a). This, together with the observation that sperm nuclei were

not seen in egg cells at 48 h, suggests that karyogamy is very efficient if a sperm nucleus reaches the egg cell.

In contrast, the estimated frequency of endosperm development (10.0%) is slightly higher than the 5.8% found 48 h after pollination. Furthermore, sperm nuclei could still be observed in association with the polar nuclei at 48 h, suggesting that karyogamy was less efficient than in egg cells. Thus failure of karyogamy is an additional factor which reduces the frequency of endosperm formation (Fig. 4).

Comparison with other wide-crosses. Crosses between Chinese Spring and the grain sorghum genotype S9B also showed a marked discrepancy between the frequency of embryo formation (67%) and the frequency of endosperm formation (12%) (Laurie and Bennett 1988c). However, this was not so in crosses between the tetraploid wheat genotype Kubanka and Seneca 60 maize (O'Donoughue and Bennett 1988) or between Chinese Spring and the pearl millet genotype Tift 23BE (Laurie 1989). In both these hybrids most embryos were accompanied by an endosperm. Furthermore, the frequency of endosperm formation may differ when different hexaploid wheat genotypes are pollinated with Seneca 60 maize. For example, Chinese Spring (Hope 5B), in which the Chinese Spring 5B chromosome had been replaced by the 5B chromosome from the variety Hope, gave endosperm formation in 11.6% of florets. This was significantly higher than the 6.0% found in Chinese Spring (Laurie and Bennett 1987). These results strongly suggest that genetic differences are important in determining the relative frequencies of embryo and endosperm formation.

Cell/cell interactions are likely to be important in determining these varied responses. Glycoproteins in the *zona pellucida* are known to be important in egg cell/sperm interaction in placental mammals (Wassarman 1988), and it is reasonable to suppose that recognition signals are also necessary in plants (Knox and Singh 1987). If so, the results from our wide-hybridization experiments suggest that egg cell/sperm nucleus signals tend to be more conserved than polar nuclei/sperm nucleus signals.

Does fertilization always involve one sperm nucleus?

Fertilization of the egg cell or polar nuclei normally involves one sperm nucleus, but in wheat × maize crosses this may not always be the case. In 4 ovaries there were two sperm nuclei adpressed to the wheat egg cell nucleus, while in 48 ovaries there was only one. Thus 7.7% (4/52) zygotes could have two haploid complements of maize chromosomes. There is no direct evidence that this occurs, as all the zygotes so far observed in which chromosomes could be counted had the expected F_1 combination of 21 wheat and 10 maize chromosomes (Laurie and Bennett 1986, 1988a). However, a zygote with twice the expected number of chromosomes from the male parent was found in both Chinese Spring × S9B sorghum (Laurie and Bennett 1988c) and Chinese Spring × Tift 23BE pearl millet crosses (Laurie 1989). All embryos with four or more cells from crosses between wheat and maize, sorghum or pearl millet have had micronuclei, indicating that karyogamy is essential for embryo development. Results from endosperm suggest that fertilization by two sperm nuclei can occur and also that the polar nuclei can commence development without karyogamy.

General conclusions

Pollen tube growth, release of sperm nuclei from pollen tubes, movement of sperm nuclei to female gametes and karyogamy probably require programmed intercellular or internuclear interactions between the male and female reproductive structures. If so, the results from wheat × maize crosses, and others which span comparable taxonomic distances, suggest that many of these signals are conserved in the grasses, since fertilization readily occurs if sperm nuclei are successfully delivered to the embryo sac. Thus, the scope for sexual hybridization may be much greater than has previously been thought, which has interesting implications for the role of sexual wide-hybridization in plant evolution and for the production of novel wide-hybrids in plant breeding. Conservation of cell recognition signals between sperm and egg cells, together with recent advances in regenerating plants from single cells (i.e. cereal protoplasts, Abdullah et al. 1986; Rhodes et al. 1988), may also enable novel hybrids to be produced by in vitro fertilization using isolated gametes.

Acknowledgements. This work was funded by the United Kingdom Overseas Development Administration, project R3797. We thank Miss Linda Tiller for technical assistance.

References

Abdullah R, Cocking EC, Thompson JA (1986) Efficient plant regeneration from rice protoplasts through somatic embryogenesis. Bio/Technology 4:1087–1090

Bennett MD, Smith JB, Barclay IR (1975) Early seed development in the Triticeae. Philos Trans R Soc London Ser B 272:199–227

Cass DD, Fabi GC (1988) Structure and properties of sperm cells isolated from the pollen of *Zea mays*. Can J Bot 66:819–825

Dupuis I, Roeckel P, Matthys-Rochon E, Dumas C (1987) Procedure to isolate viable sperm cells from corn (*Zea mays* L.) pollen grains. Plant Physiol 85:876–878

Knox RB, Singh MB (1987) New perspectives in pollen biology and fertilization. Ann Bot (London) 60 [Suppl 4]:15–37

Laurie DA (1989) The frequency of fertilization in wheat × pearl millet crosses. Genome (in press)

Laurie DA, Bennett MD (1986) Wheat × maize hybridization. Can J Genet Cytol 28:313–316

Laurie DA, Bennett MD (1987) The effect of the crossability loci Kr1 and Kr2 on fertilization frequency in hexaploid wheat × maize crosses. Theor Appl Genet 73:403–409

Laurie DA, Bennett MD (1988a) Chromosome behaviour in wheat × maize, wheat × sorghum and barley × maize crosses. In: Brandham PE (ed) Kew Chromosome Conference III. Her Majesty's Stationery Office, London, pp 167–177

Laurie DA, Bennett MD (1988b) The production of haploid wheat plants from wheat × maize crosses. Theor Appl Genet 76:393–397

Laurie DA, Bennett MD (1988c) Cytological evidence for fertilization in hexaploid wheat × sorghum crosses. Plant Breeding 100:73–82

McConchie CA, Hough T, Knox RB (1987) Ultrastructural analysis of the sperm cells of mature pollen of maize, *Zea mays*. Protoplasma 139:9–19

O'Donoughue LS, Bennett MD (1988) Wide hybridization between relatives of bread wheat and maize. In: Miller TE, Koebner RMD (eds) Proceedings of the 7th International Wheat Genetics Symposium. Institute of Plant Science Research, Cambridge, pp 397–402

Rhodes CA, Pierce DA, Mettler IJ, Mascarenhas D, Detmer JJ (1988) Genetically transformed maize plants from protoplasts. Science 240:204–207

Roman H (1948) Directed fertilization in maize. Proc Natl Acad Sci USA 34:36–42

Russell SD (1985) Preferential fertilization in *Plumbago*: ultrastructural evidence for gamete-level recognition in an angiosperm. Proc Natl Acad Sci USA 82:6129–6132

Wassarman PM (1988) Zona pellucida glycoproteins. Annu Rev Biochem 57:415–442