Pollen Tube Growth and Early Embryogenesis in Wheat × Maize Crosses Influenced by 2,4-D

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Pollen tube growth and embryogenesis were investigated in the intergeneric *Triticum aestivum × Zea mays* cross. Emasculated wheat florets were pollinated with maize pollen, and at one day after pollination, the wheat plants were injected with 2,4-dichlorophenoxyacetic acid (2,4-D). The influence of 2,4-D on pollen tube behaviour was determined applying callose staining in whole mount preparations of pistils. Changes in the embryo sac and early embryogenesis were analysed on sections after various histochemical stainings.

Maize pollen tubes germinated within 30 min and grew much slower through the pollen tube pathway compared with selfings of both maize and wheat. Deviations in pollen tube growth occurred such as coiling, widening and forking, irrespective of treatment with 2,4-D. Pollen tubes reached the micropyle between 5 and 24 h after pollination. 2,4-D treatment increased the number of the pollen tubes that reached the micropyle, and additionally, multiplication of sperm cells was found in the maize pollen tubes.

Embryo formation was analysed at 2 d after pollination. Only when pollen tubes were in the micropyle, zygotes and embryos were observed. This points to their hybrid origin. Moreover, the embryos are likely of zygotic origin since multicellular embryos exhibited micronuclei, a sign of chromosome elimination not occurring in parthenogenic embryos and leading to haploidy of the embryos. Treatment with 2,4-D increased successful intergeneric fertilization from 18·7 to 69·3%. Immature embryos were rescued by *in vitro* culture from 14% of pollinated florets when excised at 14 d after pollination.

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Key words: Intergeneric crossing, 2,4-D, pollen tube growth, haploid embryo, *Triticum aestivum*, wheat, *Zea mays*, maize.

INTRODUCTION

Wheat × maize crosses have been studied since Zenkteler and Nitsche (1984) described that embryos were formed after pollination of wheat with maize. Laurie and Bennett (1986) found that the embryos were initially hybrids between wheat and maize, but the embryos lost the maize chromosomes during the first cell divisions and became haploid embryos of wheat (Laurie and Bennett, 1988, 1989; Laurie and Reymondie, 1991). As such they are potentially important for breeding programmes (Laurie, O'Donoughe and Bennett, 1990; Kisana et al., 1993; Suenaga, 1994). In contrast, in some grasses and cereals (Matzk, 1991) and also in rice (Ishige et al., 1990), parthenogenic embryos were reported after treatment with 2,4-D. Laurie and Bennett (1988) developed the first *in vitro* method to rescue the haploid wheat embryos from the wheat × maize cross. On the day 2 after pollination (DAP) they placed spikelets on nutrition medium enriched with 0·1 mg l⁻¹ 2,4-dichloro-phenoxyacetic acid (2,4-D). After 3 weeks embryos were excised and subcultured to plants. Since then, various rescue protocols were developed to obtain haploid wheat plants after intergeneric crossings. 2,4-D is used in all of them, but usually at the surprisingly high concentration of 100 mg l⁻¹.

Without 2,4-D treatment embryos do not develop well enough to be excised for embryo rescue techniques. Solutions of 2,4-D were injected into the uppermost internode of the stem by Suenaga and Nakajima (1989). They treated plants twice: immediately after pollination and at 1 DAP. Subsequently Inagaki and Tahir (1992) used the same type of injection but at 1 and 2 DAP. Sun et al. (1992) applied the hormone by immersing spikes in the solution at 4 h after pollination (HAP). Comeau et al. (1992) initially succeeded to rescue haploid embryos by *in ovulo* culture but they improved the method applying the 2,4-D solution on spikes at 1 DAP. Riera-Lizarazu, Mujeeb-Kazi and William (1992) first incubated cut spikes in nutrition solution supplemented with 100 mg l⁻¹ 2,4-D during the first 2 DAP, and then in hormone-free nutrition medium until the embryos were excised. Matzk and Mahn (1994) found it most efficient to spray, apply drops, or dip spikes in 2,4-D solutions at concentration of 50–100 mg l⁻¹, and later than 20 HAP.

The embryology of wheat × maize crosses was studied by Laurie and Bennett (1986, 1987, 1988, 1989, 1990) on whole mount preparations stained with the Feulgen method and acetic carmine. Wang et al. (1991) and Sun et al. (1992) analysed the cross on paraffin-sectioned material. Neither of the authors studied the cross embryologically after 2,4-D treatment. Pollen germination rates and an early pollen tube

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growth in the stylodia were also not studied before although they are of particular interest since they could be influenced by the hormone treatments proposed in some protocols (Larter and Chaubey, 1965; Suenaga and Nakajima, 1989; Riera-Lizarazu *et al.*, 1992; Sun *et al.*, 1992). Therefore we followed pollen tube germination and growth from 15 min after pollination onwards and analysed the influence of 2,4-D on pollen tube behaviour in the ovary. We improved aniline blue staining for the visualization of pollen tubes with fluorescence microscopy. Toluidine blue was used as a blocking agent for autofluorescence following suggestions of Smith and McCully (1978). We also monitored and compared early embryogenesis from 2,4-D treated and untreated plants on glycol-methacrylate sections stained histochemically. The single injection of 100 mg l⁻¹ 2,4-D to the upper internode of the stem was applied in this study because this method has been used successfully to raise wheat haploids (Suenaga, 1994; Wędzony, Góral and Spiss, 1995).

MATERIALS AND METHODS

Plant material, pollination and 2,4-D treatment

The hexaploid ($2n = 42$) wheat (*Triticum aestivum* L.) cultivars 'Grana' and 'Minaret', were grown in the greenhouse under 16/8 h day/night regime at 23/18 °C, respectively. The maize (*Zea mays* L.) lines A188 and A632 were grown in a growth cabinet under a 16/8 h day/night regime, at 25/20 °C and at a relative humidity of 70%. The upper and basal spikelets were removed from spikes. From the remaining spikelets the central florets were dissected leaving the primary and secondary ones. These florets were emasculated 2–3 d before anthesis and to prevent desiccation the spikes were wrapped with parafilm. At anthesis the stigmatic hairs of wheat were covered with maize pollen within 10 min after the pollen was collected from the maize anthers. The spikes were wrapped again with parafilm until 1 DAP and then parafilm was replaced by crossing bags.

At 1 DAP we injected the synthetic auxin analogue 2,4-D into the stem cavity of the highest internode using a 1 ml syringe with a steel needle (diameter 0·5 mm). The highest possible volume of solution, at a concentration of 100 mg 2,4-D l⁻¹, was injected. The volume ranged from 0·5 to 1 ml. The hole made by the injection was immediately sealed with parafilm. Control plants were not injected. Plants injected with water did not differ from those not injected.

Examination of pollen tube growth in whole mount preparations

Pistils were fixed at 0·25, 0·5, 1·5, 2·5, 3·5, 5, 6·5, and 7·5 HAP, and at 1 and 2 DAP. At least 10 pistils from at least three different spikes were collected in each sample. Up to 1 DAP the plants were not treated with 2,4-D. Pistils collected at 2 DAP were partly from 2,4-D treated plants and partly from control plants. Some of them were processed for Technovit embedding (see below), others were examined in whole mount preparations stained with aniline blue, modified as follows:

Pistils were fixed in ethanol: acetic acid (3:1) solution and preserved in 70% ethanol at 4 °C. They were cleared in 5 N NaOH at room temperature. Samples collected up to 3·5 HAP were cleared for 2–5 h, and overnight clearing was applied for samples fixed at later stages. Pistils were then rinsed in distilled water (3 ×) and placed in an aqueous solution of 0·01% toluidine blue O (Merck, Darmstadt, Germany) for 1–3 h to quench autofluorescence of cell walls, and to reduce non-specific fluorescence as proposed by Smith and McCully (1978). Vacuum infiltration was applied to get rid of air bubbles which arose after clearing and to enhance toluidine blue penetration to deeper layers of the tissues. After rinsing in water (3 ×), callose was stained with 0·01% aniline blue (Sigma) in 0·015 M K_2HPO_4, pH 10. Samples were examined with a Labophot epifluorescence microscope (Nikon, Tokyo, Japan) equipped with UV light (Excitation filter 365 nm, Dichroic mirror 400, Barrier filter 420 nm). The number of emerging pollen tubes and pollen tube lengths were determined in all the samples fixed until 3·5 HAP. The percentage of germination cannot be calculated, because ungerminated and poorly germinated pollen was usually washed away during the staining procedure. The numbers of pollen tubes in the material fixed at later stages were counted at four sites in the pistil, i.e., (1) at the base of the stylodia, (2) in the transmitting tissue of the ovarian wall, (3) in the ovarian cavity between the ovarian wall and integuments, and (4) in the micropyle (Fig. 1).

Technovit embedding and staining of sectioned material

At 2 DAP some pistils were taken from the same spikes from which samples were collected for aniline blue staining. They were fixed in 3% paraformaldehyde and 3% glutaraldehyde in phosphate buffered saline (PBS: 135 mM NaCl, 2·7 mM KCl, 1·5 mM KH_2PO_4, 8 mM Na_2HPO_4) with 0·01% Triton X-100, pH 6·9, for 4 h. To enable better penetration of the fixative, the lateral sides of the ovaries were cut, and fixation was started under vacuum for at least 20 min. Ovaries were washed twice in PBS, dehydrated in ethanol (10, 30, 50, 70%), stored at 4 °C, and finally embedded in glycol methacrylate (Technovit 7100, Kulzer, Wehrheim, Germany) according to the manufacturers' procedure. They were sectioned (5–7 μm thick) on a rotary microtome (Microm HM340). For general anatomical studies sections were stained with a solution of 1% toluidine blue O in 1% sodium tetraborate ($Na_2B_4O_7$) and stored dry until use. They were examined in a drop of water with bright field microscopy.

To detect nuclear chromatin and chromosomes we used DAPI staining. Toluidine blue stained sections were destained in water at 45 °C until they were pale blue. Then a drop of 0·0001% DAPI in 0·1 M piperazine buffer (pH 7) was applied in a humid chamber for 15 min. Slides were briefly washed in deionized water and stored dry. To detect callose 0·01% aniline blue in 0·1 M phosphate buffer, pH 8, was applied by dipping slides in the solution for 1 min. Slides were washed (3 × 5 min) in deionized water and stored dry. Aniline blue and DAPI staining were combined
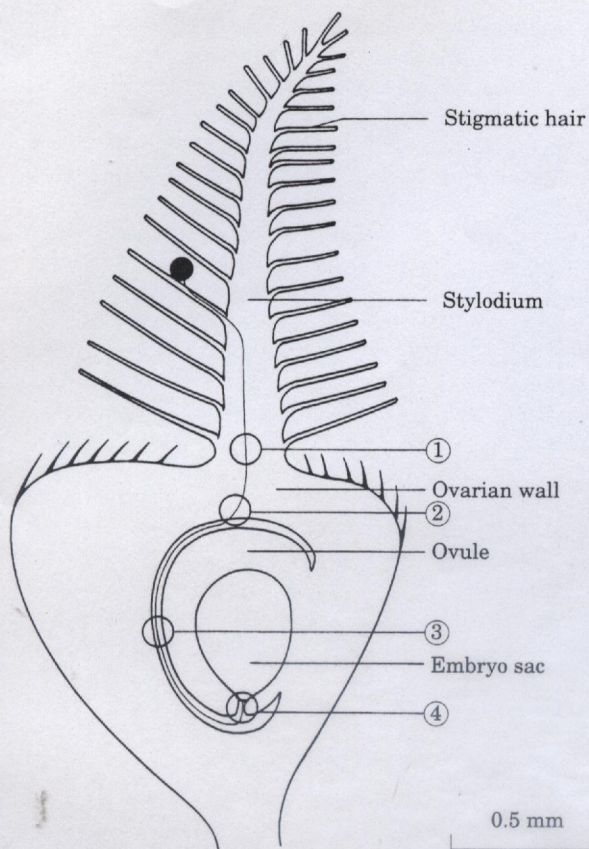
FIG. 1. Schematic drawing of the longitudinal median section of a wheat pistil with one stylodium showing the pollen tube path way of maize pollen from the stigmatic hairs towards the micropyle of the ovule. The four sites where maize pollen tubes were counted are indicated by numbers: 1, base of stylodium; 2, transmitting tract of ovarian wall; 3, ovarian cavity; 4, micropyle.

on many slides with DAPI as the second stain. Preparations stained as described above were examined in deionized water under UV light.

In vitro *culture*

Ten spikes, pollinated and treated with 2,4-D were left *in planta* until 14 DAP. Embryos were excised under sterile conditions and cultured *in vitro* in Petri dishes on MS medium supplemented with N6 medium vitamins, 250 mg l^{-1} casamino acids (Sigma), and with 13% sucrose when embryos were very small, or with 6% sucrose for well-developed embryos. They were kept in a growth chamber with a 12/12 h day/night cycle at 21 °C.

Statistics

The significance of differences between non treated and treated samples was checked with the χ^2 test for each trait separately. Confidence values were estimated with the Fisher's exact probability test.
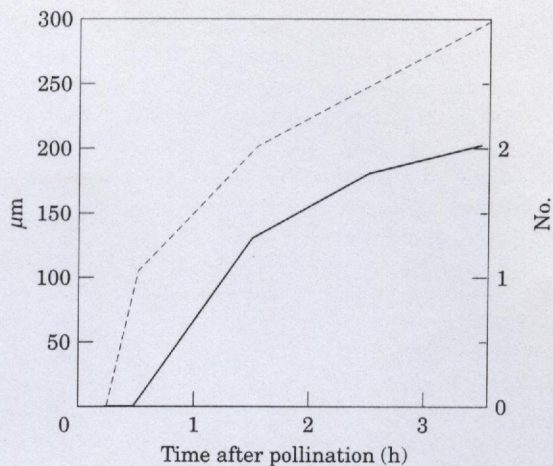

FIG. 2. *Triticum aestivum × Zea mays*. Pollen tube growth rates up to 3·5 h after pollination. Per sample 679 to 920 pollen tubes were scored. (–––), Average length of pollen tube; (——), average number of pollen tubes reaching base of stylodia.
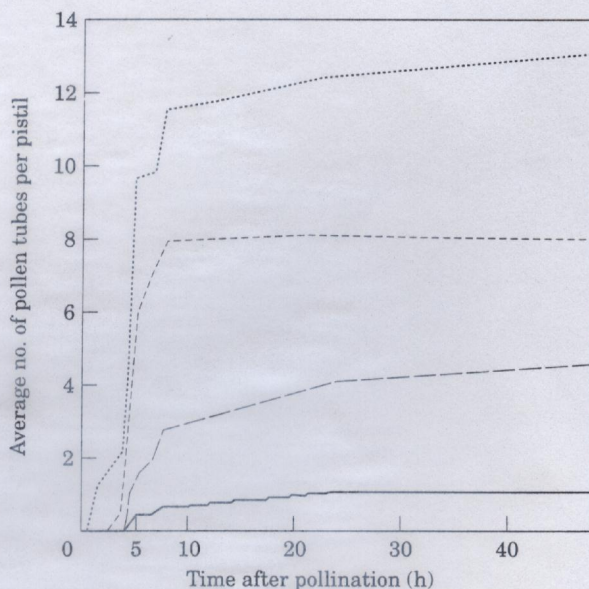

FIG. 3. *Triticum aestivum × Zea mays*. The average number of pollen tubes found at four scoring sites in the sequence of time up to 2 days after pollination. Per sample 30 to 47 pistils were scored. Number of pollen tubes at (...) base of stylodium, (– –) transmitting tract, (– — –) ovarian cavity and (——) micropyle.

RESULTS

Pollen tube growth rates

The growth of pollen tubes was investigated in plants not treated with 2,4-D. Maize pollen germination was successful in comparable rates independent of the parents' combinations. The first pollen tubes were detected not before 30 min after pollination. The lengths of several thousands of pollen tubes were determined up to 3·5 HAP (Fig. 2). Most pollen tubes grew slowly, reaching on average a length of 294 μm, i.e. 3·27 × the diameter of the pollen grain at 3·5 HAP. On average two pollen tubes per ovary, however,

TABLE 1. Triticum aestivum × Zea mays. *The influence of 2,4-D on maize pollen tube arrival at wheat micropyles. Injection of 2,4-D was at 1 d after pollination.*Significant at* P = 0·01

2,4-D treatment	No. of pistils		Average no. of pollen tubes per micropyle	No. of pollen tubes at micropyle				
	Total	Micropyles penetrated with pollen tube*		0	1	2	4–5	6
no	47 (100%)	32 (68%)	0·89	15 (32%)	26 (55%)	5 (11%)	0	1 (2%)
yes	43 (100%)	40 (93%)	1·19	3 (7%)	29 (67%)	11 (26%)	0	0

reached the base of the stylodia in this period (Figs 2, 3). These pollen tubes had a maximal length of 800 μm. Thus the average speed of pollen tube growth was 84 μm h^{-1} and the maximal 230 μm h^{-1}. We found a stepwise reduction in the number of pollen tubes at the successive scoring sites (Fig. 3). Pollen tubes were seen at the micropyle from 5 HAP onward. We have not seen pollen tubes penetrating embryo sacs another way than via the micropyle.

At 2 DAP pollen tube growth was also investigated in the sample treated with 2,4-D (Table 1). Independent of the treatment, all pistils fixed at 1 and 2 DAP had pollen tubes growing in the ovarian cavity. The 2,4-D treatment did not influence the number of pollen tubes at the three first scoring sites but the number of micropyles that received one or two pollen tubes increased by 25% after 2,4-D treatment (Table 1). The difference is significant with the χ^2 test at $P = 0·01$, and the level of confidence with Fisher's exact probability test is 0·002. Thus the probability that the observed values are a random variation in population, unaffected by treatment, is very low.

Pollen tube morphology in the pistil

Pollen tubes frequently grew on the surface of pollen grains and often coiled several times before entering a stigmatic hair (Fig. 4A). Many pollen tubes failed to enter stigmatic hairs, others grew from the stigmatic hair into the stylodium and towards its base (Fig. 4B), and sometimes in the opposite direction. Frequently finger-like outgrowths, short side branches and spiky tips were seen in stylodia. Callose was noticed in wheat cells bypassed by maize pollen tubes.

Pollen tubes grew through the transmitting tissue of the upper ovarian wall (Fig. 4C) to the ovarian cavity and then towards and into the micropyle (Fig. 4D). From 496 pollen tubes seen in ovaries, only 6 (1·2%) deviated from this normal way growing away from the transmitting tissue into the carpellar tissue and ceased growth. At the base of the stylodia and in the transmitting tract many pollen tubes formed bulb-like widenings (Fig. 4C) which sometimes stopped growing and occasionally burst. More often pollen tubes continued growth but their diameters were larger than before. The callose pattern of their walls was uneven with callose knobs of different size (Fig. 4D).

Independent of the treatment, all flowers fixed at 1 and 2 DAP had pollen tubes growing in the ovarian cavity.

Out of 397 pollen tubes seen there, 20·1% were normal and 79·9% showed abnormalities such as branches of various length and pattern (Fig. 4E) and coiled structures (Fig. 4F). Many pollen tubes were seen bypassing the micropyles and growing further around the ovules, sometimes making more than one loop (Fig. 4G). This also happened when pollen tubes were not present in the micropyle. After treatment with 2,4-D, pollen tubes made more turns around the ovule forming a basket-like structure. The entrance of the pollen tube to the micropyle was usually straight forward, however, deposits of callose in the micropyle region were seen in 31% of the ovules that received pollen tubes.

Pollen tubes in the embryo sac

Post pollination events were studied on sections of 16 untreated and 13 2,4-D treated ovaries fixed at 2 DAP (Table 2). Despite the treatment, pollen tubes entered the micropyle and penetrated one of the synergids (Fig. 4H) while the second synergid usually started degeneration. Pollen tubes often forked in the micropyle and the protrusions penetrated between the integuments (Fig. 4H). Some pollen tubes remained intact in the synergid, sometimes swollen pollen tube tips destroyed the egg apparatus, and in two ovaries from the 2,4-D treated sample the pollen tubes passed through the synergid and the central cell, finally discharging their content into the antipodals (Fig. 4I).

Multiple maize nuclei

In the samples treated with 2,4-D, we have found three cases of multiple maize nuclei: (a) Serial sections revealed that one pollen tube widened close to the transmitting tissue in the ovarian wall and contained up to 12 nuclei (Fig. 5B). The nuclei were small and compact resembling sperm nuclei by size and shape. (b) Another ovule contained at least 30 similar nuclei in the embryo sac (Fig 5A, C). They were in and close to one of the synergids and some of them were stacked in the micropyle. The adjacent cell of the egg apparatus contained a nucleus with three nucleoli and an arrangement of cytoplasm characteristic for zygotes (Fig. 5D). (c) In the third case, similar multiple nuclei were found near the egg apparatus, but outside the embryo sac. Staining with DAPI confirmed that these compact structures contained DNA.
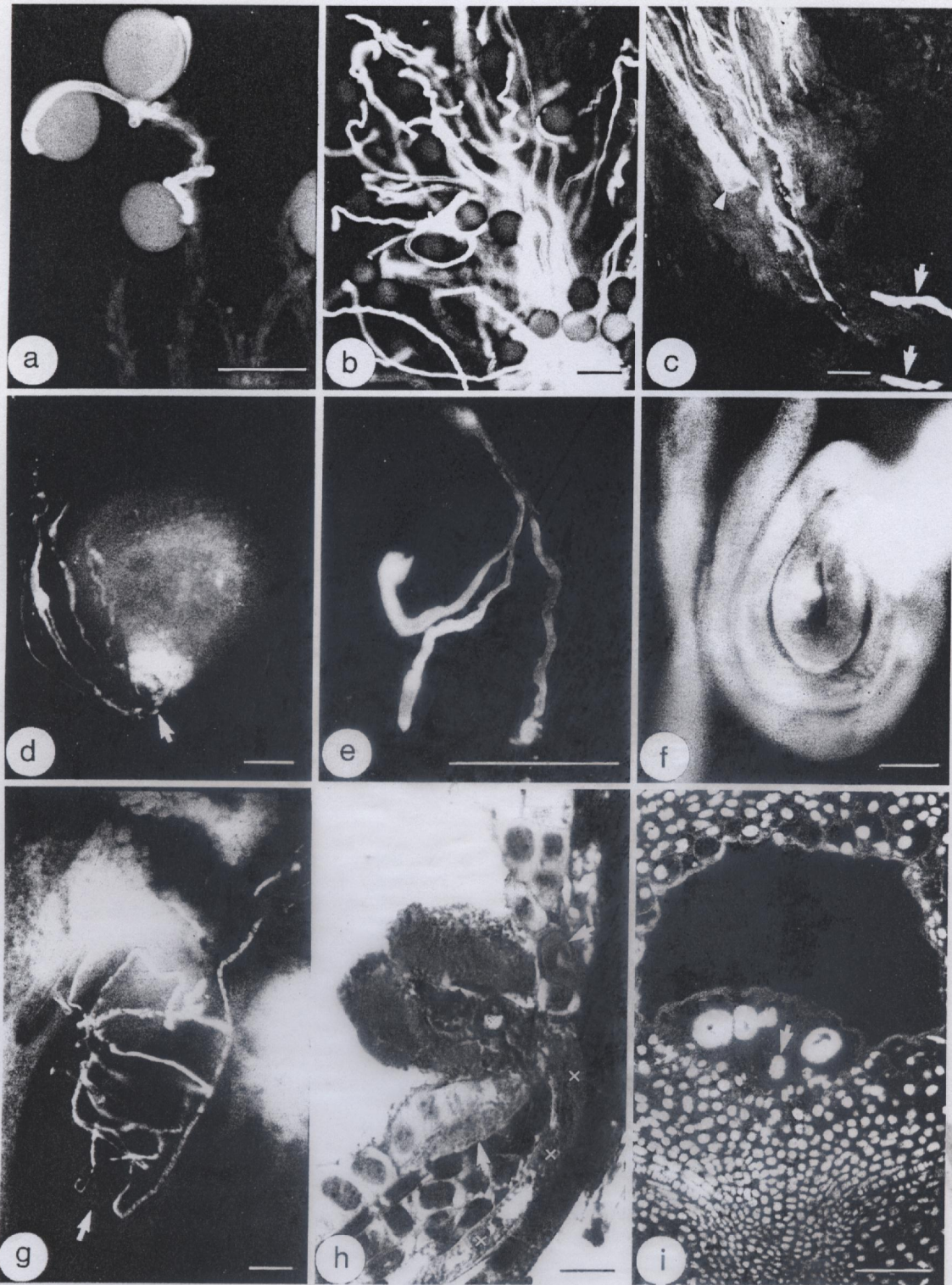
FIG. 4. *Triticum aestivum × Zea mays.* Progamic phase. Micrographs of maize pollen tubes in wheat pistils. Pollen tubes are visualized by fluorescence microscopy after a modified aniline blue staining (A–G, whole mount preparations; I, section) and with bright field microscopy after toluidine blue staining (H, section). Bars in F, H = 10 μm; A–E, G, I = 100 μm. A, Germinated pollen on stigma at 0·5 HAP. Note that pollen tubes grow on the surface of the pollen grain and coil before they enter the stigmatic hairs. B, Pollen tubes in stigmatic hairs and stylodium at 5 HAP. C, Numerous pollen tubes entering transmitting tissue of the ovarian wall. Some of them are growing further into ovarian cavity (arrows). Note pollen tube widening (arrowhead). D, Pollen tubes in the ovarian cavity growing towards the micropyle of the ovule. One pollen tube enters the micropyle (arrow). Note callose deposition in the micropylar region. E, Branching pollen tube in the ovarian cavity. F, Pollen tube forming a coiled structure in the ovarian cavity of pistil. G, Pollen tubes growing in ovarian cavity, making loops around the ovule. Micropylar side is indicated by an arrow. H, Section of embryo sac showing a pollen tube (x) in the micropyle, entering the synergid. Note the finger-like protrusions of the pollen tube penetrating between the nucellus and the integument (arrows). I, Section of ovule stained with DAPI and aniline blue showing nuclei of antipodals which were stained blue, and pollen tube in cross section which was stained yellow (arrow).

TABLE 2. *Triticum aestivum × Zea mays. Comparision of embryological data in samples fixed 2 d after pollination i.e., 1 d after 2,4-D injection. The differences between samples significant with χ^2 test at the level of confidence: *0·05, **0·02, ***0·01*

Number of ovules examined:		2,4-D injection		Probability of random variation between samples†
		No 16	Yes 13	
Degeneration of ovule	*	5 (31·2%)	0	0·037
Pollen tube at micropyle	***	9 (56·3%)	13 (100%)	0·007
Embryos + zygotes at mitosis	***	3 (18·7%)	4+5 (69·3%)	0·008
Endosperm	*	1 (6·3%)	4 (30·8%)	0·09
Embryo + endosperm	**	0	2 (15·3%)	0·02
Egg apparatus damaged by pollen tubes	—	4 (25%)	4 (30·8%)	—
Multiple sperm nuclei of maize	—	0	3 (15·4%)	0·07

† Calculated with the Fishers' exact probability test

Influence of 2,4-D on embryo and endosperm formation

One day after injection ovaries from 2,4-D treated spikes were about twice as large as those from spikes not treated. There were many histological changes within the ovary which will be studied in more detail elsewhere. The treatment with 2,4-D prevented degeneration in ovules and promoted significantly the number of developing embryos and endosperms (Table 2). Zygotes at prophase were found along with three to six celled embryos in the treated sample while only embryos consisting of four to six cells were found in the sample not treated. All the embryo sacs containing zygotes and embryos were penetrated by pollen tubes. Regardless the treatment, embryos were regular in shape (Fig. 5E) and showed many micronuclei in the cytoplasm when stained with DAPI (Fig. 5F). The endosperms contained at least 16 nuclei each and some nuclei were irregular in shape. From the treated sample two embryo sacs contained both an embryo and endosperm (Fig. 5E).

Embryo rescue *in vitro*

Ten spikes of 'Minaret' with 256 flowers were pollinated with pollen of the maize line A188, injected with 2,4-D and left *in planta* until 14 DAP. We dissected 36 embryos (14% of pollinated florets) out of 114 (44·5%) enlarged ovaries. The remaining 142 ovaries were desiccated. Enlarged ovules lacked endosperm, except one with poor traces of it. Embryos were surrounded by translucent fluid and varied significantly in size and shape from almost normal to poorly developed globular ones. Four embryo sacs contained two embryos each. The twin embryos were of similar size and attached to each other in the coleorrhiza and suspensor zone. From the 36 excised embryos 18 (7%) developed to haploid seedlings after 1 month of *in vitro* culture.

DISCUSSION

Maize pollen needed approximately 30 min to germinate on the wheat stigma whereas the whole progamic phase in wheat selfings takes only 25 min (Gao *et al.*, 1992). The growth rate of the fastest maize pollen tubes in our experiment (0·23 mm h^{-1}) was low in comparison with the 10 mm h^{-1} found in maize self pollinations *in vitro* (Booy, Krens and Bino, 1992) and the 5 mm h^{-1} found *in vivo* (Sheridan and Clark, 1993). Variability of growth rates within the population of pollen may be caused by variations among the pollen grains or differences in sites at the stigma.

The number of pollen tubes reaching the micropyle between 4–12 HAP was studied previously by Laurie and Bennett (1990). Our data support their results. The minor differences that occurred, may be caused by variability of genotypes or growing conditions. The stepwise reduction of the number of pollen tubes found in the present investigation (Fig. 3) might be an effect of a high excess of maize pollen on the stigma. In wheat self pollinations there is reduction only at the transition from the transmitting tissue to the ovarian cavity (Vishnyakova and Willemse, 1994) while in maize at the basal part of the silk (Heslop-Harrison, Heslop-Harrison and Reger, 1985).

The pattern of pollen tube growth found in present study is similar as in the tetraploid wheats pollinated with maize (O'Donoughue and Bennett, 1994). In both types of crosses there was no apparent inhibition of pollen tube growth, however similar aberrations were found like swelling and branching in the ovarian cavity. Also, accumulation of several pollen tubes close to the micropyle and pollen tubes bypassing the micropyle were found. Images of pollen tubes looping around the ovule were described previously by Ahmad and Comeau (1990) in wheat × pearl millet crosses. In the above cases of wide hybridization, the first tube present near the micropyle was not necessarily the one successful in fertilization. It is important therefore that 2,4-D treatment at 1 DAP increases the number of pollen tubes reaching the micropyle (Tables 1 and 2 of the present study). Gibberellic acid had a similar effect in barley–rye crosses (Larter and Chaubey 1965). Laurie (1989), however, did not find the positive influence of GA on wheat × maize crosses.

Both in hexaploid (Laurie and Bennett, 1990 and present study) and tetraploid wheats (O'Donoughue and Bennett, 1994) the maize pollen tubes followed the way usual for wheat pollen tubes described in detail by Vishnyakova and Willemse (1994). This shows that maize pollen tubes are sensitive to the attraction signals leading them to the wheat micropyle. Such attraction is also likely to exist in other
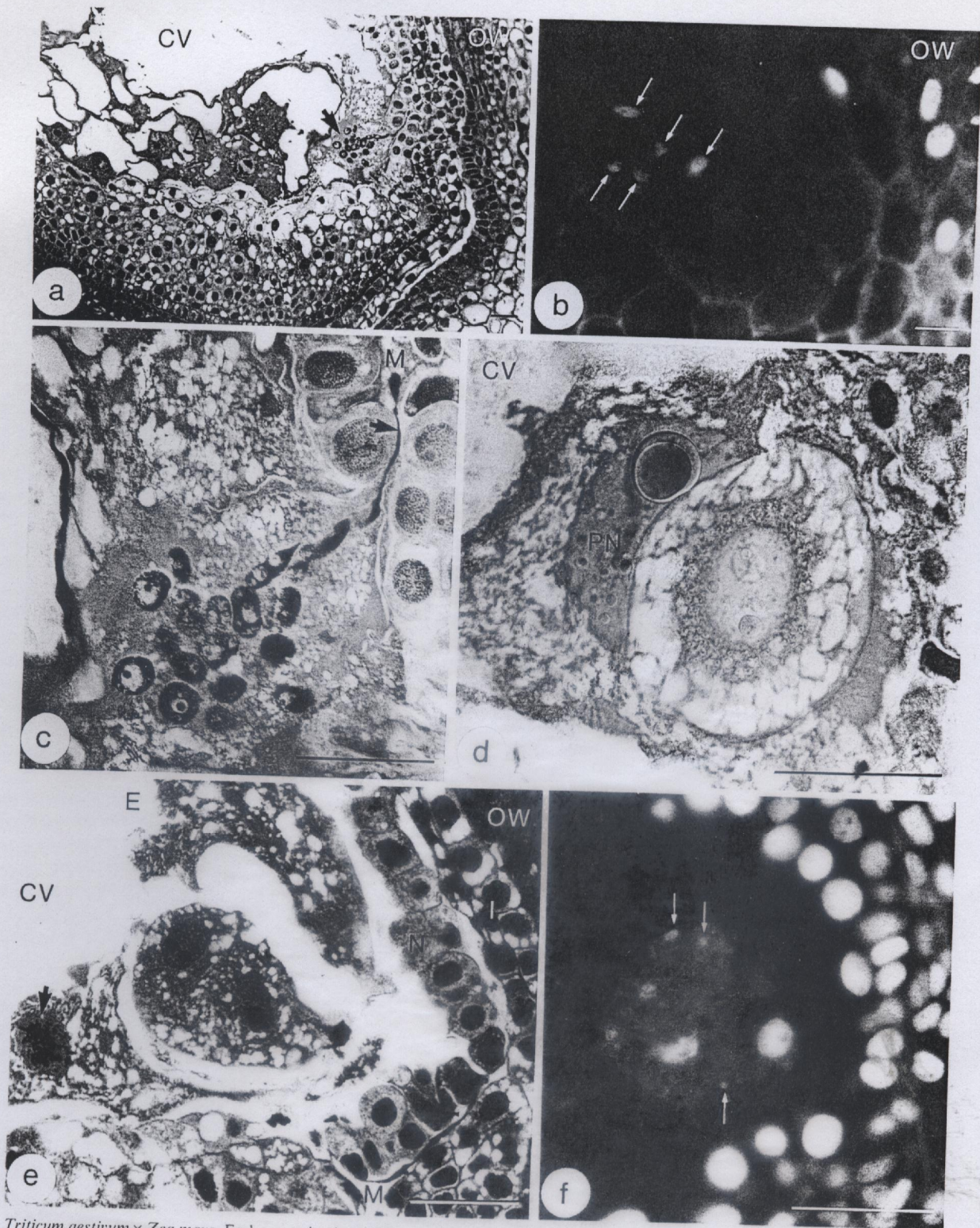
FIG. 5. *Triticum aestivum × Zea mays*. Embryogenic phase. Micrographs of semithin sectioned wheat ovules (A, C–F) and ovary (B) stained with toluidine blue (A, C, D, E) or DAPI (B, F). (A, antipodals; CV, central vacuole of the embryo sac; E, endosperm; N, nucellus; OW, ovarian wall; PN, polar nuclei). Bars = 100 μm for all figures. A, Overview of ovule showing antipodals, and multiple nuclei in the micropylar region of the embryo sac (arrow). B, Cross section through the ovarian wall with widened pollen tube containing multiple nuclei stained with DAPI (arrows). C, Differential interference contrast image of synergid containing many nuclei which come from a pollen tube. Note that the nuclei near the micropyle still have a spindle shape whereas those further away are spherical. Arrow points to DNA positive material still present in the micropyle. D, Overview of zygote and central cell of the same embryo sac as shown in Fig. 5C. Polar nuclei are not yet fused. Note that the nucleus of the zygote is in central position and contains three nucleoli. E, Overview of micropylar region of embryo sac with 3 cellular embryo (two nuclei are visible) and endosperm showing one nucleus at prophase. F, A six-celled embryo showing several micronuclei in apical and basal cells (arrows).

related crosses of, e.g. wheat × pearl millet (Ahmad and Comeau 1990), wheat × sorghum (Ohkawa, Suenaga and Ogawa, 1992), oat × maize (Rines and Dahleen, 1990) and barley × maize (Furusho, Suenaga and Nakajima, 1991). Thus in the intergeneric crosses of cereals the growth of the alien pollen tube is retarded and aberrant but not inhibited and still depends on the same pathway information as in selfed combinations.

Treatment with 2,4-D just after pollination influences pollen tube growth in an unfavourable way in those ovaries where pollen tubes are not yet present near the micropyle (Matzk, 1991). To obtain highest embryo yields Matzk and Mahn (1994) suggest to apply the 2,4-D treatment not earlier than 20 HAP. We have found a positive influence of the 2,4-D treatment at 1 DAP not only on fertilization processes, but also on embryo survival. Our results show that the progamic phase in wheat × maize crosses lasts several hours up to 1 DAP but 2,4-D treatment significantly increased pollen tube arrival at the micropyle and the rate of successful egg cell and/or central cell fertilization (Table 2). Thus the progamic phase is extended in 2,4-D treated plants. Zygotes and embryos were found in 69·3% of pollinated flowers from the 2,4-D treated sample. That is the highest proportion reported. However, high percentages of embryo formation were also found earlier without 2,4-D treatment. For instance by Laurie (1989) in the wheat cv. 'Highbury' pollinated with maize cv. 'Seneca 60' where 55·8% of the ovaries contained embryos at 4–6 DAP. Laurie and Reymondie (1991) applied 2,4-D treatment at 1 DAP in Minaret × 'Seneca 60' crosses and isolated embryos from 46·5% of the ovaries at 14 DAP whereas Amrani, Sarrafi and Alibert (1993) found up to 25·3% embryos in such cross. Tested as pollinator, the maize line A188 with 'Fukuo-komugi' wheat gave embryos only in 17·3% of the crosses done by Suenaga (1994).

Embryos were isolated from only 14% of pollinated pistils because most of the embryos were lost before they were mature enough to be excised for culturing. Additionally, only half of the excised embryos gave seedlings. The above data show also that fertilization and embryo survival are two different events, both probably influenced by 2,4-D.

After the first divisions of the zygote, embryos always exhibited micronuclei. These micronuclei point to the elimination of the chromosomes of the maize genome, as reported by Laurie and Bennett (1988, 1989) and are an additional proof for real gamete fusion. The simultaneous presence of pollen tubes in such embryo sacs again points to the zygote nature of these embryos that makes the possibility of a parthenogenetic origin of the embryos less probable. In four ovaries we found twin embryos connected to each other at the coleorrhiza and suspensor zone. They are probably an effect of 2,4-D action early at embryogenesis in a comparable way as Ferguson and McEwan (1970) reported about the chemical induction of supernumerary shoots in wheat after treatment with 2,4-D and comparable to the polyembryony found by Erdelska and Vidovencova (1994).

We do not know other reports about numerous nuclei or cells in pollen tubes *in vivo*, neither in wheat nor in any other dicotyledonous plant. We call them 'nuclei', as we cannot see the plasmalemma and the sperm cell wall in the light microscope. Because at least some nuclei maintained the spindle shape characteristic for sperm cells and caused by microtubules present in cytoplasm (Palevitz and Cresti, 1988; Theunis and Van Went, 1989), we assume that the nuclei belong to real cells. It is not clear whether they are still functional as gametes. As they were only found in 2,4-D treated crosses, we suppose that the hormone, in combination with the abnormal conditions of growth during the wheat-maize interaction, provoked additional divisions of maize sperms. Other explanations for the presence of the numerous nuclei might be that several pollen tubes fused or that more than one pollen tube entered the micropyle. However, fusion of tubes has not been seen. On the contrary, pollen tubes branched in the ovarian cavity, and only incidentally more than one pollen tube entered the micropyle, not enough to discharge up to 30 sperm at a time.

In conclusion, wheat × maize intergeneric crosses are successful, because the maize pollen tube can grow along the wheat pollen tube pathway. The hybrid origin of the embryos in the cross is confirmed. The application of 2,4-D as presented increases the successful fertilization rate in wheat × maize crosses with a potential of up to 70%. Maize sperm sometimes are provoked to divide in the pollen tube and might still be functional.

ACKNOWLEDGEMENTS

LITERATURE CITED

Ahmad F, Comeau A. 1990. Wheat × pearl millet hybridization: consequence and potential. *Euphytica* **550**: 181–190.

Amrani N, Sarrafi A, Alibert G. 1993. Genetic variability for haploid production in crosses between tetraploid and hexaploid wheats with maize. *Plant Breeding* **110**: 123–128.

Booy G, Krens FA, Bino RJ. 1992. Analysis of pollen tube growth in culture maize silks. *Sexual Plant Reproduction* **5**: 227–231.

Comeau A, Nadeau P, Plourde A, Simard R, Maes O, Kelly S, Harper L, Lettre J, Landry B, St-Pierre C-A. 1992. Media for *in ovulo* culture of proembryos of wheat and wheat-derived interspecific hybrids or haploids. *Plant Science* **81**: 117–125.

Erdelska O, Vidovencova Z. 1994. Cleavage polyembryony *in vivo* and *in vitro*. *Biologia Plantarum* **36(3)**: 329–334.

Ferguson JD, McEwan JM. 1970. The chemical induction of supernumerary shoots in the developing embryos of wheat. *Physiologia Plantarum* **23**: 18–28.

Furusho M, Suenaga K, Nakajima K. 1991. Production of haploid barley plants from barley maize and barley Italian ryegrass crosses. *Japanese Journal of Breeding* **41**: 175–179.

Gao X, Francis D, Ormrod J, Bennett MD. 1992. An electron microscopic study of double fertilization in allohexaploid wheat *Triticum aestivum* L. *Annals of Botany* **70**: 561–568.

Heslop-Harrison Y, Heslop-Harrison J, Reger BJ. 1985. The pollen-stigma interaction in the grasses. 7. Pollen-tube guidance and the regulation of tube number in *Zea mays* L. *Acta Botanica Neerlandica* **34(2)**: 193–211.

Inagaki MN, Tahir M. 1992. Production of haploid wheat through intergeneric crosses. *Hereditas* **116**: 117–120.

Ishige T, Tamaki M, Suenaga K, Nakajima K. 1990. Production of rice pure lines by embryo culture. 1. Pollen germination by rice × sorghum and the effect of hormone on embryo development. (abstract) *Japanese Journal of Breeding* **40 (Suppl. 1)**: 120–121.

Kisana NS, Nkongolo KK, Quick JS, Johnson DL. 1993. Production of doubled haploids by anther culture and wheat × maize method in a wheat breeding program. *Plant Breeding* **110**: 96–102.

Larter E, Chaubey C. 1965. Use of exogenous growth substances in promoting pollen tube growth and fertilization in barley-rye crosses. *Canadian Journal of Genetics and Cytology* **7**: 511–518.

Laurie DA. 1989. Factors affecting fertilization frequency in crosses of *Triticum aestivum* cv. 'Highbury' × *Zea mays* cv. 'Seneca 60'. *Plant Breeding* **103**: 133–140.

Laurie DA, Bennett MD. 1986. Wheat × maize hybridization. *Canadian Journal of Genetics and Cytology* **28**: 313–316.

Laurie DA, Bennett MD. 1987. The effect of crossability loci Kr1 and Kr2 on fertilization frequency in hexaploid wheat × maize crosses. *Theoretical and Applied Genetics* **73**: 403–409.

Laurie DA, Bennett MD. 1988. The production of haploid wheat plants from wheat × maize crosses. *Theoretical and Applied Genetics* **76**: 393–397.

Laurie DA, Bennett MD. 1989. The timing of chromosome elimination in hexaploid wheat × maize crosses. *Genome* **32**: 953–961.

Laurie DA, Bennett MD. 1990. Early post-pollination events in hexaploid wheat × maize crosses. *Sexual Plant Reproduction* **3**: 70–76.

Laurie DA, O'Donoughue S, Bennett MD. 1990. Wheat × maize and other wide sexual hybrids: their potential for genetic manipulation and crop improvement. In: Gustavson JP, ed. *Gene manipulation in plant improvement II*. New York: Plenum Press, 95–126.

Laurie DA, Reymondie S. 1991. High frequencies of fertilization and haploid seedling production in crosses between commercial hexaploid wheat varieties and maize. *Plant Breeding* **106**: 182–189.

Matzk F. 1991. A novel approach to differentiated embryos in the absence of endosperm. *Sexual Plant Reproduction* **4**: 88–90.

Matzk F, Mahn A. 1994. Improved techniques for haploid production in wheat using chromosome elimination. *Plant Breeding* **113**: 125–129.

O'Donoughue LS, Bennett MD. 1994. Comparative responses of tetraploid wheats pollinated with *Zea mays* L. and *Hordeum bulbosum* L. *Theoretical and Applied Genetics* **87**: 673–680.

Ohkawa Y, Suenaga K, Ogawa T. 1992. Production of haploid wheat plants through pollination of sorghum pollen. *Japanese Journal of Breeding* **42**: 891–894.

Palevitz BA, Cresti M. 1988. Microtubule organization in the sperm of *Tradescantia virginiana*. *Protoplasma* **146**: 28–34.

Riera-Lizarazu O, Mujeeb-Kazi A, William MDHM. 1992. Maize (*Zea mays* L.) mediated polyhaploid production in some Triticeae using a detached tiller method. *Journal of Genetics and Breeding* **46**: 335–346.

Rines HW, Dahleen LS. 1990. Haploid oat plant produced by application of maize pollen to emasculated oat florets. *Crop Science* **30**: 1073–1078.

Sheridan WF, Clark JK. 1993. Fertilization and embryogeny in maize. In: Freeling M, Walbot V, eds. *The maize handbook*. Springer-Verlag: Heidelberg, 3–10.

Smith MM, McCully ME. 1978. Enhancing aniline blue fluorescent staining of cell wall structures. *Stain Technology* **53**: 79–85.

Suenaga K. 1994. Doubled haploid system using the intergeneric crosses between wheat (*Triticum aestivum*) and maize (*Zea mays*). *Bulletin of the National Institute of Agrobiological Resources* **9**: 83–139.

Suenaga K, Nakajima K. 1989. Efficient production of haploid wheat (*Triticum aestivum*) through crosses between Japanese wheat and maize (*Zea mays*). *Plant Cell Reports* **8**: 263–266.

Sun J, Liu H, Lu T, Wang X, Zhen R, Wang J, Fang R, Yang C. 1992. The production of haploid wheat plants via wheat × maize hybridisation. *Acta Botanica Sinica* **34**: 817–821.

Theunis CH, Van Went JL. 1989. Isolation of sperm cells from mature pollen grains of *Spinacia oleracea*. *Sexual Plant Reproduction* **2**: 97–102.

Vishnyakova MA, Willemse MTM. 1994. Pollen-pistil interaction in wheat. *Acta Botanica Neerlandica* **43**: 51–64.

Wang J, Sun J, Lu T, Fang R, Cui H, Cheng S, Yang C. 1991. Fertilization and embryo development in wheat × maize crosses. *Acta Botanica Sinica* **33**: 674–679.

Wędzony M, Góral H, Spiss L. 1995. The attempt to obtain haploids of wheat and triticale through crossing with maize. (Abstract in English) *Proceedings of 7th National Conference of in vitro culture*. Katowice-Ustroń, 21–23 Sep. 1994. (in press.)

Zenkteler M, Nitsche W. 1984. Wide hybridization experiments in cereals. *Theoretical and Applied Genetics* **68**: 311–315.