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Production of wheat haploids through embryo rescue from wheat × maize crosses

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Appropriate hormone treatment using 2,4-D and gibberellic acid led to a high frequency (34.17%) of embryo formation in crosses of field-grown wheat strains with maize. Twenty-five days after culture on MS medium supplemented with casein (2000 mg/l) and amino acids, 30.95% of the embryos showed plant formation, 44.05% resulted in callus-like structures and the remaining failed to respond. The haploid nature of some of the regenerated plants was confirmed cytologically. Implications for wheat breeding are discussed.

WHEAT is a premier food crop of worldwide importance. It is also a crop where conventional plant breeding has paid rich dividends, as epitomized by the green revolution. However, the current impasse in yield levels calls for application of newer techniques. A major hindrance to rapid genetic turnover in self-pollinated crops, such as wheat, is the inordinately long time (generally 8–10 years) it takes to develop stable, homozygous and ready-to-use material from a fresh cross. In this context, anther/microspore culture has often been proposed as a method for producing instant homozygous lines via haploidy. In wheat, wide hybridization followed by chromosome elimination serves as an alternative route to haploidy, e.g. wheat × *Hordeum bulbosum* and wheat × *Zea mays* crosses. The wheat × *Z. mays* cross is a relatively new system which offers distinct advantages such as freedom from genotypic specificity, the bane of anther culture approach as well as the wheat × *H. bulbosum* system (which is restricted to wheat genotypes carrying the kr crossability genes). Moreover, the system is less prone to gametoclonal variation owing to the absence of a dedifferentiated phase.

Zenkteler and Nitzsche[1] were the first to report microscopic, early-stage embryos in crosses between wheat and maize. However, the frequency of embryo formation was not specified, nor was evidence presented for their hybrid origin. Intrigued by this report, Laurie and Bennett[2] set out to study early post-pollination events in wheat and maize crosses under a CIMMYT project. They demonstrated the presence of both wheat and maize chromosomes in the zygotes and observed further that maize chromosomes were eliminated during initial cell divisions. Endosperm development ceases early or never occurs and embryos fail to develop to a size that can be readily rescued. Two years later, they reported[3] the recovery of the first haploid plants using the wheat × maize system by employing *in vitro* culture of wheat spikelets, 2 days after pollination. A method that bypassed spikelet culture was devised by Suenaga and Nakajima[4], who injected 2,4-D solution (100 ppm) into the uppermost internodes of wheat stems to sustain embryo growth on the plant itself, till the appropriate stage for embryo rescue.

The present study was conducted as a preliminary assessment of the applicability of the system to indigenous material grown under field conditions and to judge its potential as a plant breeding tool. The wheat material used represented advanced, agronomically relevant homozygous lines. (For actual plant breeding purposes, however, heterozygous material would be required.) Wheat ears were emasculated 3–5 days prior to anthesis by opening up the glumes and removing the anthers, unlike the conventional practice, which involves the cutting of the glumes to expose the androecium. Freshly collected pollen from a winter maize variety, Partap 1, was used for pollinations, 3–5 days after emasculation. The pollinated tillers were administered injections of 2,4-D solution (125 ppm) into the uppermost internode, daily in the evening, for three days after pollination. On the fourth day the ears were momentarily dipped in a solution of 2,4-D (100 ppm) + gibberellic acid (75 ppm). On an average, seed-like structures (Figure 1 *a*) were seen to develop in 86.71% of the wheat ovaries pollinated with maize (Table 1). Closer examination of the ears revealed that ovaries which failed to develop into seed-like structures were damaged by severe fungal infection. The presence of a large amount of maize pollen in the floret and moisture provided by hormonal (2,4-D) ear dip might have favoured this fungal growth. Thus, seed-like structures develop in all the viable florets. Obviously, all such seeds are not the result of fertilization and are probably formed due to hormone induced enlargement. Thus, actual seed setting could be assessed only after dissecting the seed-like structures and observing the presence/absence of embryos. Twenty developing seeds each of six genotypes 15 days after pollination with maize were specifically dissected for this purpose. No visual differentiation seemed to exist between seeds that carry an embryo

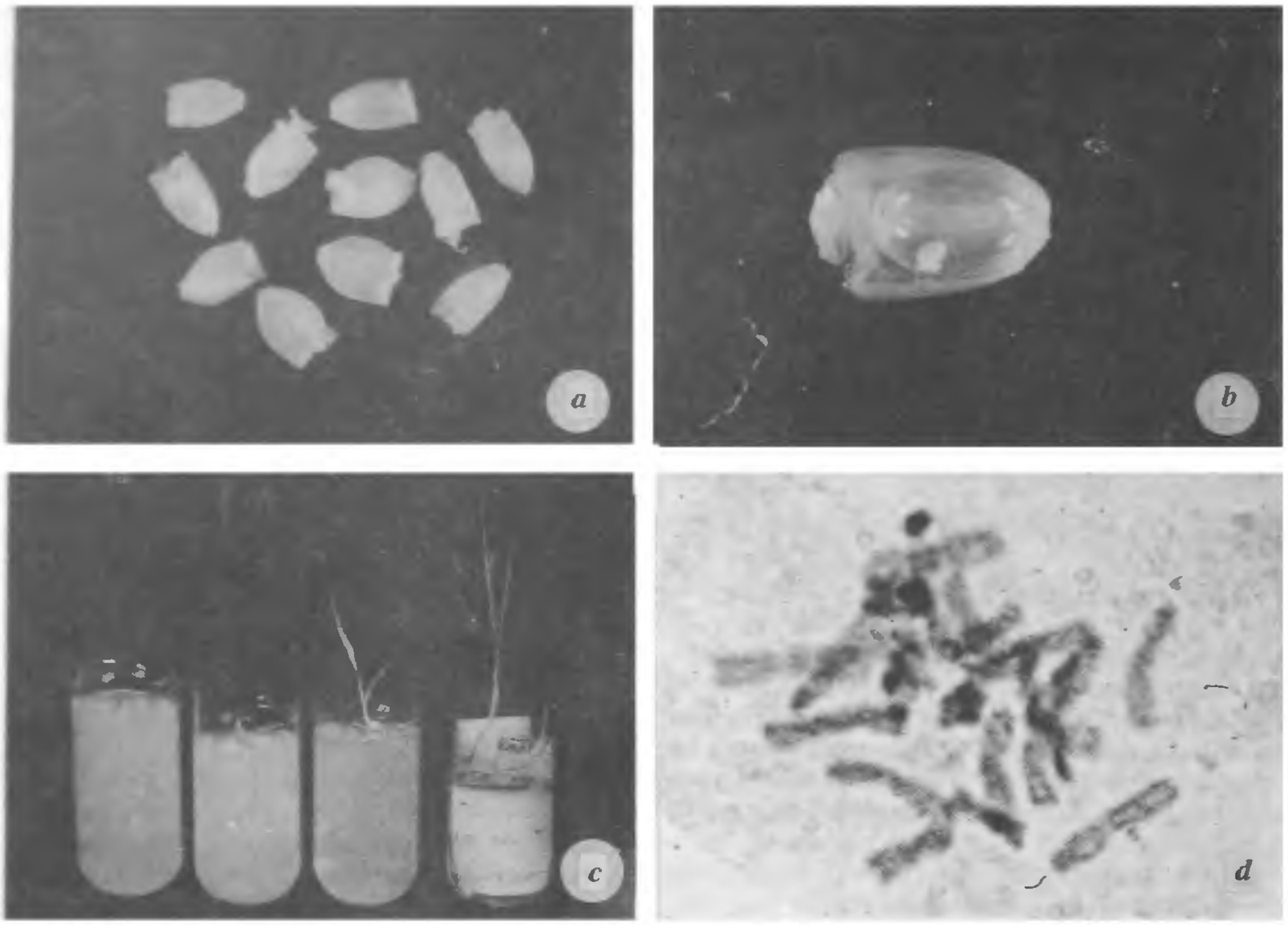

Figure 1. Wheat × maize crosses: *a*, seed-like structures observed in the crossed florets; *b*, haploid embryo floating in the watery endosperm; *c*, regeneration of haploid plants; *d*, root tip cell from embryo-derived plant showing haploid chromosome number ($2n = 21$).

Table 1. Seed setting, embryo formation and response to embryo culture in wheat × maize crosses

Wheat genotype used as female parent	Percentage of pollinated florets showing formation of seed-like structures (15 days after pollination)	Percentage of seed-like structures showing embryo formation* (15 days after pollination)	Percentage of cultured embryo (25 days after culture) showing		
			plant regeneration	callus formation	no response
PBW 299	91.86 (79/86)**	20.00 (4/20)	12 50 (1/8)	50 00 (4/8)	37.50 (3/8)
PBW 343	86.73 (85/98)	—	10.00 (1/10)	50.00 (5/10)	40 00 (4/10)
PBW 352	82.41 (89/108)	55.00 (11/20)	8.33 (1/12)	66 67 (8/12)	25.00 (3/12)
PBW 362	93 75 (60/64)	5.00 (1/20)	—	—	—
PBW 373	80.36 (90/112)	—	41.67 (5/12)	50.00 (6/12)	8.33 (1/12)
PBW 377	93.40 (99/106)	5 00 (7/20)	25 00 (2/8)	37.50 (3/8)	37.50 (3/8)
PBW 391	82.73 (91/110)	—	41.67 (5/12)	16.67 (2/12)	41 67 (5/12)
PBW 396	87.50 (98/112)	60.00 (12/20)	54 54 (6/11)	45.45 (5/11)	0.00 (0/11)
PBW 398	86.06 (105/122)	30.00 (6/20)	45 45 (5/11)	36.36 (4/11)	18.18 (2/11)
Mean	86.71 (796/918)	34.17 (41/120)	30 95 (26/84)	44 05 (37/84)	25 00 (21/84)

*Based on dissection of 20 seeds per genotype under a stereomicroscope.
**Values in parenthesis are the actual numbers of observations.

and those that do not. The frequency of embryos ranged from 5 to 60% (Table 1). Small sample size makes it difficult to say whether this denotes genotypic specificity. However, remarkably high overall frequency of 34.17% indicates the scope for applying this system to plant breeding situations.

The second important factor for the success of this system is the frequency of plant emergence from the cultured embryos. For culturing, 15-day-old (days after pollination with maize) seed were surface-sterilized in 70% alcohol for 30 s followed by a 10 min treatment with 0.15% mercuric chloride and three rinses in sterile water. Embryos were excised aseptically under a stereo microscope and cultured in a medium containing MS basal salts supplemented with casein (2000 mg/l), L-glutamine (400 mg/l), L-alanine (50 mg/l), L-cysteine (20 mg/l), L-arginine (10 mg/l), L-leucine (10 mg/l), inositol (100 mg/l) and sucrose (50 g/l). The embryos were atypical in shape, having microcalli-like appearance, and were found floating in the watery endosperm (Figure 1 *b*). The cultures were incubated in total darkness for 10 days at 25 ± 1°C and then shifted to 16 h/8h light/dark regime.

The response of the embryos was recorded 25 days after the culture and is shown in Table 1. Out of the total 84 embryos cultured, 26 showed plant emergence (Figure 1 *c*), giving a frequency of 30.95% averaged over genotypes. A considerable proportion (44.05%) of the embryos formed callus-like structures probably due to the carryover effect of 2,4-D treatments. Transfer to regeneration medium in the subsequent culture can result in plant formation from these embryo-derived calli. About 25% of the embryos showed no response. Further experimentation for fine-tuning of the medium composition can greatly enhance the recovery of haploid plants.

For confirming the haploid nature of the regenerated plants, mitotic chromosome analysis of root tips was conducted. Some randomly taken plants were analysed. The roots were pretreated in a saturated solution of α-bromonaphthalene for 2 h and fixed in Carnoy's solution for 24 h. The roots were then hydrolysed in 1 N HCl at 60°C for 10 min and stained with 2% aceto-carmine. The dividing cells carried a chromosome complement of 21 chromosomes (Figure 1 *d*) in all the plants as revealed by light microscopy. The normal somatic chromosome number of hexaploid wheat is 42.

The wheat × maize system holds promise for revolutionizing wheat breeding methodology. Homozygosity is approached in one step, thereby circumventing a period of 6–8 years in the breeding cycle. This system can be very valuable for situations requiring immediate genetic amelioration, e.g. incorporation of resistance to major crop diseases. In addition, doubled haploids from crosses of divergent wheat parents is a short cut in obtaining recombinant inbreds, which are very useful for construction of genetic linkage maps using molecular markers. Haploids can also be useful for induction and recovery of mutants as well as for basic studies involving genomic relationships.

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