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16. Polyhaploid production in the Triticeae by sexual hybridization

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Contents

1. Introduction

Several workers have successfully crossed *Triticum aestivum* L. with *Zea mays* L. (Zenkteler and Nitzsche, 1984; Laurie and Bennett, 1986) and *Tripsacum dactyloides* (Riera-Lizarazu and Mujeeb-Kazi, 1993), which has led to documented production of polyhaploid plants. There have also been successful crosses between *Z. mays* and *T. turgidum* L. as well as other *Triticum* and *Aegilops* spp. (O'Donoughue and Bennett, 1988). Successful fertilizations have also been accomplished in crosses between wheat and *Sorghum bicolor* L. Moench, sorghum (Laurie and Bennett, 1988a,b); *Pennisetum glaucum* R. Br., pearl millet (Laurie, 1989); *Z. mays* ssp. *mexicana*, teosinte (Ushiyama *et al.*, 1991); *Hordeum vulgare* L., barley (Laurie and Bennett, 1988c); and *Secale cereale* L., rye (Laurie *et al.*, 1990).

Crosses between wheat and the above species (as the pollen parent) provide an alternative means of producing polyhaploid (haploid if the species

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is a diploid) wheat plants through the natural elimination of the pollen parent's chromosomes in the early stages of embryo development. Also, there is the possibility of exploiting the genetic variability of the diverse gene pools within these alien species for wheat improvement if, for instance, maize or *Tripsacum* chromosomes could actually be retained in a wheat background.

After fertilization occurs in any of the above crosses, chromosomes of the male parent are eliminated very early (Laurie and Bennett, 1988a), thus producing a polyhaploid embryo with the chromosomes of the female parent. Normally, the embryo soon aborts; however, exogenous treatment with the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D) promotes seed and embryo development until the embryo can be excised and plated onto a synthetic medium for continued growth and plantlet regeneration (Laurie *et al.*, 1990).

Using this methodology, polyhaploid cereal plants have been recovered from crosses of bread wheat (*T. aestivum*) × maize (Comeau *et al.*, 1988; Laurie and Bennett, 1988c; Suenaga and Nakajima, 1989; Inagaki and Tahir, 1990; Rines *et al.*, 1990; Riera-Lizarazu and Mujeeb-Kazi, 1990; Laurie and Reymondie 1991); durum wheat × maize (Riera-Lizarazu and Mujeeb-Kazi, 1993; O'Donoughue and Bennett, 1994); wheat × pearl millet (Ahmad and Comeau, 1990); bread wheat × sorghum (Ohkawa *et al.*, 1992); bread wheat × teosinte (Ushiyama *et al.*, 1991); barley × maize (Furusho *et al.*, 1991); and *T. aestivum* × *Tripsacum* (Riera-Lizarazu and Mujeeb-Kazi, 1993).

Polyhaploid plants are important in efforts to reduce the number of generations it takes to fix the homozygosity of wheat and other cereal plants. A homozygous plant is obtained when a polyhaploid's chromosomes are doubled. This homozygosity is required in basic research projects such as our collaborative work with Cornell University and the International Triticeae Mapping Initiative (ITMI) to produce RFLP maps of the wheat and barley genomes.

Until recently, polyhaploid production in the Triticeae had relied mostly on anther culture and sexual crossings with the perennial barley relative *Hordeum bulbosum* L. The occurence of somaclonal variation, aneuploidy, and genotypic specificity (Picard, 1989) are major limitations of anther culture in polyhaploid production. The homoeologous group 5 crossability loci (*Kr*1, *Kr*2, *Kr*3) are the major limiting factors of the *H. bulbosum* sexual crossings (Snape *et al.*, 1979; Falk and Kasha, 1981, 1983; Sitch and Snape, 1986, 1987; Mujeeb-Kazi and Asiedu, 1990). In order to avoid tissue culture-associated somaclonal variation that may occur because of the callus phase, the sexual route to polyhaploid production seemed to be more desirable; however, we needed a substitute for the troublesome *H. bulbosum* technique. So, we have been exploring *Zea mays* L. (Laurie and Bennett, 1986, 1988a,c; O'Donoughue and Bennett, 1988; Laurie *et al.*, 1990) and *Tripsacum dactyloides* (Riera-Lizarazu and Mujeeb-Kazi, 1993) as alternative sexual routes for polyhaploid production in the Triticeae.

Over the last four years we have been producing high frequencies of polyhaploid wheat plants in crosses using either maize or *Tripsacum* pollen. We believe that either or both of these polyhaploid production procedures for wheat have important advantages over anther culture or *T. aestivum* × *Hordeum bulbosum* crosses.

This paper is divided into two distinctive sections dealing with (i) *Triticum* species hybridizations with *Zea mays*, and (ii) hybridizations with *Tripsacum dactyloides*.

2. Maize-mediated wheat polyhaploid production

2.1. *Wheat* × Zea mays *hybridization*

Since maize pollen growth and fertilization activity appear to be insensitive to the *Kr* crossability alleles of wheat (Laurie and Bennett, 1987), polyhaploids can be recovered across different genotypes (Suenaga and Nakajima, 1989; Inagaki and Tahir, 1990). This makes it superior to the *H. bulbosum* system since it can be readily applied to wheat breeding programs. In addition, gametoclonal variation induced in doubled polyhaploid lines using the maize system was similar to that found in doubled polyhaploids obtained from wheat × *H. bulbosum* crosses (Laurie and Snape, 1990).

The use of 2,4-D appears to be critical in promoting seed set and embryo formation in wheat × maize crosses (Laurie and Bennett, 1988c; Inagaki and Tahir, 1990). Techniques using 2,4-D treatment include: floret culture (Laurie and Bennett, 1988c), tiller injection (Suenaga and Nakajima, 1989; Inagaki and Tahir, 1990), spike spraying (Rines *et al.*, 1990), and floret treatment (Riera-Lizarazu and Mujeeb-Kazi, 1990). Detached tillers (Riera-Lizarazu and Mujeeb-Kazi, 1990) and detached spikelets (Laurie and Bennett, 1988c) offer more flexibility because experimental material can be transferred to locations where conditions can be more easily controlled and monitored.

We first obtained a high recovery of wheat polyhaploids from crosses between the wheat cultivar "Morocco" and CIMMYT maize population "Pool 9A". Subsequently, we achieved successful polyhaploid embryo production for additional *T. aestivum* and *T. turgidum* cultivars and for the *T. turgidum* × *T. tauschii* synthetic hexaploids, using a detached tiller culture method.

2.2. *Plant material*

We used two sets of plants that were field-grown at El Batan, CIMMYT, Mexico:

- *T. aestivum* cv. "Morocco" and *Z. mays* population "Pool 9A".
- *T. aestivum*, *T. turgidum*, *Secale cereale*, *T. turgidum*/*T. tauschii*-derived

amphiploids, and *Z. mays* (bulk pollen sample from several cross-pollinating maize populations).

2.3. *Crossing procedures and detached tiller culture*

We hand-emasculated spikes before anthesis and covered them with glassine bags. When the stigmatic surface was receptive (three to four days after emasculation), the spikes were pollinated with fresh maize pollen. The tillers of pollinated spikes were detached 5 cm below the peduncular node and placed in a beaker with an aqueous solution of 452 μM 2,4-D for 48 h. The basal halves of detached tillers were then surface-sterilized in a 20% (v/v) chlorine bleach (5.25% sodium hypochlorite) solution for 5 min, rinsed six times in sterile deionized water, and transferred to test tubes (45 ml) containing liquid MS (Murashige and Skoog, 1962) basal medium components amended with 452 μM 2,4-D (Riera-Lizarazu and Mujeeb-Kazi, 1990). We placed the test tubes with detached tillers in a Styrofoam box containing ice-water in the greenhouse under regimes of 25/12°C (day/night), 16 h photoperiod, and 45 to 60% relative humidity. The purpose of the ice water was to retard microbial growth in the culture medium at the base of the tillers, while the wheat spikes developed under favorable growth temperatures. Detached tillers were kept in the 2,4-D medium for 48 h and then transferred to a growth regulator-free medium for 12 days.

2.4. *Embryo rescue, plant regeneration, and transplanting*

The detached tiller culture method was compared to two other wide hybrid crossing techniques described previously, spraying of florets with a 2,4-D solution 24 h prior to pollination (Kruse, 1974) and injections of 2,4-D solution (452 μM) into the uppermost wheat stem internode 24 h post maize pollination (Suenaga and Nakajima, 1989). For each of the three crossing techniques, we collected seeds approximately 14 days after pollination and sterilized them in a chlorine bleach solution (20% v/v) for 15 min. Embryos were excised under a stereomicroscope (2×) in a laminar flow hood decontaminated with 75% ethanol. Excised embryos were transferred to vials containing half strength MS basal medium supplemented with 20 g sucrose/L, 2.2 μM indole-3-acetic acid (IAA), 0.4 μM 6-benzyladenine (BA), and 2 g Gelrite (Scott Laboratories, Inc., West Warwick, RI, USA)/L. Vials with embryos were kept in the dark at room temperature for 1 to 2 weeks. After germination, we transferred the regenerated 5 to 7 cm size plantlets to peat pots and eventually to soil in pots kept in the greenhouse.

2.5. *Cytology*

Somatic chromosome analysis of all regenerated plants was conducted according to the method of Mujeeb-Kazi and Miranda (1985). For meiotic

analysis, the young spikes were fixed in 6:3:1 (ethanol (99%):chloroform:glacial acetic acid) for 48 h and stored in 70% ethanol solution in the freezer (−10°C) until needed. Anthers at metaphase I were stained in alcoholic carmine (Snow, 1963), then processed according to the modified procedure of Mujeeb-Kazi *et al.* (1994) for high contrast, intense staining, and reduced stickiness. Mean metaphase I pairing associations were calculated from 25 meiocytes for some bread wheat and synthetic hexaploid polyhaploids.

2.6. *Colchicine treatment*

We treated cytologically-identified polyhaploid plants with colchicine (Mujeeb-Kazi *et al.*, 1987) in order to induce chromosome doubling. We presumed successful doubling had occurred if we observed seed set.

2.7. *Protein separation*

Some female wheat parents and their doubled polyhaploid progenies were analyzed by studying the banding profiles of their seed storage proteins (glutenin) and isozymes (esterase, E.C. 3.1; and β-amylase, α-1,4-glucan maltohydrolase E.C. 3.2.1.2). The endosperm halves of mature kernels were used to analyze protein separation and the isozymes.

The high molecular weight glutenin subunits were separated by using a slight modification of the SDS-polyacrylamide gel electrophoresis procedure of Ng *et al.* (1988). Stacking gels of 2 cm and 10% separation gels of 15.5 cm were used. Thickness and width of the gels were 0.15 and 16 cm, respectively. Each gel was run at 20 mA constant current for 1 h followed by 30 mA constant current for 4 h on a Bio-Rad Protean II electrophoresis unit. The temperature was maintained at 15°C during electrophoresis. Esterase and β-amylase isozymes were separated by isoelectric focusing using precast Pharmacia PAG plates with pH gradients of 3.5–9.5 for esterase and 4–6.5 for β-amylase. The running conditions and the staining protocols were similar to those of William and Mujeeb-Kazi (1992).

2.8. *Morocco* × Z. mays *pool 9a*

Data were obtained for the number of florets pollinated, embryos rescued, and percentage of embryos regenerated for three 2,4-D treatment procedures: detached tiller culture, tiller injection, and floret spray (Riera-Lizarazu and Mujeeb-Kazi, 1990). Embryo recovery as a percentage of total florets emasculated was higher using the detached tiller method (28.7%) than from crosses that received 2,4-D tiller injections (12.8%) (Table 1). In turn, tillers treated with an injection of 2,4-D in the uppermost internode had significantly higher ($p < 0.05$) embryo recovery frequencies than plants that received 2,4-D sprays made 24 h prior to maize pollination (2.8%). Plant regeneration frequencies, as a percentage of embryos excised, did not differ significantly

Table 1. Total number and range per wheat spike emasculated of florets pollinated, embryos rescued, and percentage of embryos regenerated to give wheat polyhaploid plants from wheat × - maize crosses using three 2,4-D treatment procedures

2,4-D Treatment Procedure*	Florets pollinated	Embryos rescued	Plant regeneration %
Floret Spray	**216**	**6**	**67**
Range:	(20-24)	(0-2)	(0-100)
Tiller injection	**234**	**30**	**77**
Range:	(18-24)	(0-6)	(23-100)
Detached tillers	**221**	**64**	**81**
Range:	(20-24)	(3-10)	(20-100)

among embryos originating from spikes receiving different treatment procedures. Plant regeneration frequencies averaged 75% across the three techniques. Recovery of wheat polyhaploid plants as a percentage of florets emasculated on a spike ranged from 0 to 10%, 0 to 30%, and 14 to 42% for the floret spray, tiller injection, and detached tiller culture techniques, respectively.

Cytological analysis confirmed that the recovered wheat plants possessed the expected polyhaploid complement of $n = 3x = 21$ chromosomes. Chromosomes 1B and 6B were consistently identified by their characteristic secondary constriction; occasionally, a 5D chromosome with its secondary constriction was also identified.

Laurie and Bennett (1988c) reported that embryos in caryopses, when allowed to develop on the plants without growth regulator treatments, had poor viability, whereas spikelets cultured in agar-solidified MS medium supplemented with 2,4-D two days after pollination resulted in increased embryo recovery from 0.17 to 26.5%. Suenaga and Nakajima (1989) reported equal to better embryo recovery frequencies (18.0 to 31.9%) by injecting the uppermost stem internode with 452 μM 2,4-D. Exogenous treatments with 2,4-D appear to enhance embryo viability, although the mechanisms are not clear.

In our study, embryo recovery was unexpectedly low when we applied 2,4-D in the field with the tiller injection and floret spraying methods. Recovery was consistently high when we applied 2,4-D in the greenhouse with the detached tiller culture (Table 1). The field environment was exceedingly wet and cold during the experiment, which might have negatively affected cross fertilization and seed development in the injected or sprayed spikes. Spraying of the florets was particularly ineffective, probably due to the exposure of unfertilized ovaries to 2,4-D 24 h prior to pollination. Effective embryo recovery has been reported when 2,4-D spray applications were made 24 h post-pollination (Rines *et al.*, 1990). Also, reasonably good fre-

quencies of embryos were recovered (0.6 to 26.8%) when the spray procedure was used 24 h post-pollination in wheat × *Tripsacum* crosses described later.

Suenaga and Nakajima (1989) also observed a reduction in embryo recovery when tillers were injected one to two days before pollination. They speculated that 2,4-D treatments prior to pollination induced morphological and physiological changes in unfertilized florets that were detrimental to cross fertilization. On the other hand, 2,4-D treatments prior to pollination in wide crosses have been shown to improve embryo recovery frequencies (Kruse, 1974; Riera-Lizarazu and Dewey, 1988). In the latter case the 2,4-D was applied by the detached tiller culture method; hence, critical concentrations of 2,4-D may not have reached the florets until after fertilization. Also, other factors besides 2,4-D applications prior to pollination may have affected the differences in embryo recovery.

When donor wheat plants were produced in the greenhouse, detached tillers were drier and the caryopses larger than those obtained from the field material. Translocation and seed development were probably better under the greenhouse conditions as well. In another controlled experiment, we found the detached tiller procedure to be significantly better than tiller injection across several wheat genotypes (unpublished data). So, we conclude that the use of detached tillers offers the most practical and versatile alternative for wheat polyhaploid production when crossing wheat × maize. However, as we point out later in this chapter, some modifications may be in order to improve the detached tiller system, especially when crossing other *Triticum* and Triticeae species with maize.

Although plant production frequencies from recovered embryos did not dramatically vary among different treatments (frequencies ranged from 67 to 81%), we found embryo germination could be increased with improved embryo culture procedures or by enhancing embryo development on the crossed spikes. We rescued embryos 14 days after pollination. Allowing embryos to remain on the spikes longer might be appropriate if differentiated embryos are desired. Although polyhaploid frequency per floret pollinated using detached tillers averaged about 23% (average embryo recovery frequency of 28.7% × average plant regeneration frequency of 81%), it could potentially be as high as 42% if we consistently obtained 100% differentiation and high embryo recovery.

In summary, the embryo excision/plantlet regeneration/polyhaploid production frequencies (all percentages) obtained in our experiment were:

- 28.7/81.3/23.3 with detached tillers;
- 12.8/76.6/9.8 with tiller injection.

The success rates of two other laboratories not using the detached tiller procedure were:

- 25.1/83.6/20.9 (Suenaga and Nakajima, 1989);
- 21.7/43.7/9.5 (Inagaki and Tahir, 1990).

2.9. *Triticeae species* × *diverse pollen mixtures of* Z. mays

As reported by Inagaki and Tahir (1990) and Laurie and Reymondie (1991), we have also recovered polyhaploid embryos using an assortment of wheat genotypes. In addition, our results suggest that using detached tillers in the maize system (as described above) can be extended to recover polyhaploids in durum wheats and *T. turgidum* × *T. tauschii*-derived amphiploids.

In this study, we obtained a wide range of embryo recovery frequencies among 16 hexaploid wheats, 5 tetraploid wheats, and 15 synthetic hexaploids, averaging 15.6, 16.9, and 19.8%, respectively (Table 2). Mean plant regeneration frequencies for bread wheats, durum wheats, and the synthetic hexaploids were 68.5, 73.9, and 74.5%, respectively. Successful chromosome doubling with colchicine averaged 64% for *T. aestivum* cultivars, 69.5% for *T. turgidum* cultivars, and 63.6% for the synthetic hexaploids (Table 2).

Production frequencies of 1 to 4% have been considered to be acceptable for the economic production of polyhaploids (Comeau *et al.*, 1988). In our study, the average doubled polyhaploid recovery for *T. aestivum*, *T. turgidum*, and the synthetic hexaploids (based on florets pollinated) ranged from 6.5 to 9.4% (Table 2). Although the polyhaploid plant frequencies we obtained for wheat in this study more than adequately meet economic threshold levels, Suenaga and Nakajima (1989), Inagaki and Tahir (1990), and Riera-Lizarazu and Mujeeb-Kazi (1990) have reported higher frequencies across genotypes.

We attribute our lower recovery compared to earlier results of Riera-Lizarazu and Mujeeb-Kazi (1990) to continuous rainfall during tiller collection in the field and presumably to some genotypic variation. This led to a lack of complete tiller microbial decontamination sterilization, which resulted in progressive decay of the spike culm base in the culture medium, in turn

Table 2. Embryos produced, recovery percentage, plant regeneration and colchicine induced doubling frequencies of *Triticum aestivum* L., *T. turgidum* L.and *T. turgidum* × *T. tauschii* lines following crosses with maize (*Zea mays*)

Cultivars and lines		Embryos produced	Percent Embryo recovery	Plants regenerated	Plants doubled
Triticum aestivum					
Total		**442**	**15.6**	**303**	**184**
	Range	(4-55)	(6.5-25.3)	(1-38)	(1-25)
Triticum turgidum					
Total		**142**	**16.9**	**105**	**73**
	Range	(18-44)	(11.8-22.2)	(13-37)	(6-27)
T. turgidum x T. tauschii lines					
Total		**118**	**19.8**	**88**	**56**
	Range	(12-43)	(16.4-24.2)	(9-35)	(5-27)

affecting normal nutrient translocation and seed development. For such situations in the future, it may be best to use intact spikes (Suenaga and Nakajima, 1989) or to modify the detached tiller process.

The detached tiller system was specifically designed to study nutrient translocation and seed development physiology in wheat spikes (Jenner, 1970; Donovan and Lee, 1977; Singh and Jenner, 1983); hence, modifications may be needed before we can apply the procedure to other *Triticum* and Triticeae species. One modification involves the use of sulfurous acid to suppress contamination in the culture solution and culm decay as reported by Kato *et al.* (1990). Also, we can avoid humid/wet environments by making crosses in environmentally controlled greenhouses.

In another example, we observed severely reduced detached tiller viability when tillers of *Secale cereale* cv. Prolific and Sardev were used. No embryos were recovered in this rye × maize combination. It appears that, in this particular case, an *in vivo* approach may hold more promise. If the detached tiller method prevails, then the constitution of the nutrient solution and the place of tiller detachment plus the constraints expressed earlier will have to be addressed.

In our studies, seeds produced from crosses between the Triticeae species and maize lacked normal endosperm. In addition, the embryos were found floating in a watery solution inside the seeds. Generally, any embryo recovered from seed lacking normal solid endosperm is a polyhaploid. This lack of endosperm development could serve as a morphological diagnostic tool for screening selfed versus cross-pollinated products.

Cytological analysis of plants recovered from wheat × maize crosses showed them to possess the expected polyhaploid complement of $n = 3x = 21$ chromosomes for *T. aestivum* (Fig. 1a) and $n = 2x = 14$ chromosomes for *T. turgidum* (Fig. 1b), where each wheat parent had the euploid number of $2n = 6x = 42$ or $2n = 4x = 28$, respectively. Two *T. aestivum* polyhaploids were aneuploids with 20 chromosomes of which one died at the seedling stage. Another anomaly was a *T. aestivum* polyhaploid that possessed 21 chromosomes including a telocentric.

Polyhaploids of *T. aestivum* cultivars and the synthetic hexaploids showed very low A, B, and D genome association, i.e., allosyndetic pairing. Ring bivalents were rare; the chiasmata ranged from 0.44 to 1.72/meiocyte. Riley and Chapman (1958) reported chromosome associations of wheat polyhaploids ($n = 3x = 21$) to be 18.05 univalents +1.38 bivalents +0.07 trivalents. Subsequently, Kimber and Riley (1963) reported a mean frequency for bread wheat of 19.18 univalents +0.90 bivalents +0.008 trivalents from analyses of eight euhaploids – mean chromosome pairing values indicating very low allosyndetic pairing. These chromosome pairing relationships are consistent with our data where the *T. aestivum* polyhaploids of several cultivars gave a mean metaphase I chromosome association frequency of 18.6 univalents + 0.01 ring bivalents +1.24 rod bivalents +0.06 trivalents (Table 3). Values for the synthetic (*T. turgidum* × *T. tauschii*) polyhaploids were 20.1

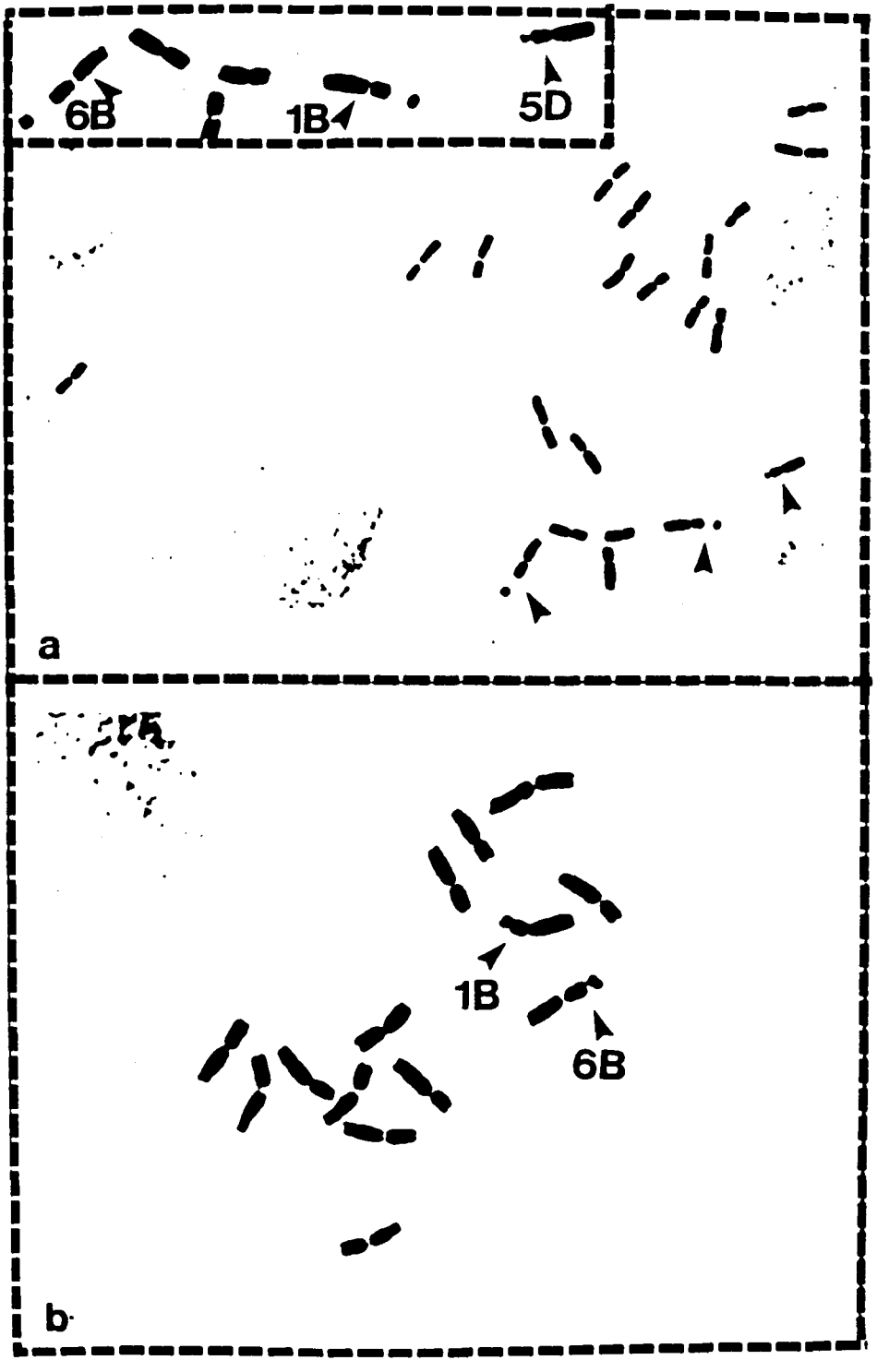

Figure 1. Somatic metaphase polyhaploid cells showing in: (a) $n = 3x = 21$ chromosomes of *T. aestivum* with the 1B, 6B and 5D secondary constriction chromosomes marked; with the same chromosomes magnified and inserted. (b) $n = 2x = 14$ chromosomes of *T. turgidum* with the 1B, 6B secondary constriction chromosomes marked.

Table 3. Mean chromosome pairing at metaphase I in some polyhaploids of *Triticum aestivum* L. and *T. turgidum* × *T. tauschii* synthetic hexaploids

Polyhaploid cultivars	Metaphase I chromosomal associations (25 meiocytes)						
	I	Bivalents		Total II	Trivalents		Total III
		Rings	Rods		Chain	Pan	
T. aestivum[a]	18.6	0.01	1.24	1.25	0.05	0.01	0.06
Synthetic hexaploid	20.1	0	0.44	0.44	0	0	0

[a] Chromosome pairing values are means of six cultivars

univalents +0.44 bivalents. This low pairing occurred because the wheat cultivars and the synthetic hexaploids used had the dominant *Ph* locus (one that remains intact over the polyhaploid induction process), which restricts homoeologous pairing.

Genes for high molecular weight glutenins have been located on the long arms of homoeologous group 1 chromosomes (Payne and Lawrence, 1983); grain esterase genes are on the long arms of homoeologous group 3 chromosomes (Ainsworth *et al.*, 1984); and genes for β-amylase are on group 4 and 5 chromosomes (Ainsworth *et al.*, 1983). Extensive allelic variations have also been reported for all three systems. We biochemically analyzed the banding profiles of high molecular weight glutenins, isozymes of seed esterase, and isozymes of β-amylase and observed extensive variations in the banding profiles for all the above three systems among different cultivar families – probably as a consequence of allelic variation. Parental banding profiles of HMW glutenin and esterase were identical to those present in the doubled polyhaploid progenies. For β-amylase, there were some minor differences in the banding profiles within some families. This isozyme variation may be partially attributed to post translational modifications (Ainsworth *et al.*, 1983), whereas some of the band intensity differences may also be accounted for by variation in endosperm protein concentration. The close similarity in the banding profiles of the doubled polyhaploid progenies and their parents "suggests stable transmission" of genetic information by this procedure. It also indicates that the parental genetic information for the evaluated enzyme systems is fixed in the doubled polyhaploid progeny without alteration.

2.10. *Conclusions*

The use of the maize system for polyhaploid production in the Triticeae is very encouraging since genotype specificity does not exist. Reaching homozygosity in earlier generations will certainly accelerate work in cereal breeding programs. Despite the current, presumably situation-specific, microbial

contamination problem we encountered with the detached tiller method, the potential for its application in polyhaploid production research in cereals looks promising. Laurie and Reymondie (1991) corroborate the use of the wide cross approaches, as they have reported high frequency polyhaploid production in spring and winter wheat × maize crosses. They however, did not use the detached tiller method. More durum wheat and rye genotypes need to be tested to further evaluate the detached tiller method.

3. *Tripsacum*-mediated wheat polyhaploid production

3.1. *Wheat* × Tripsacum dactyloides *hybridization*

The taxonomic proximity of eastern gammagrass (*Tripsacum dactyloides* L.) to maize (Doebley, 1983) has encouraged us to evaluate cross combinations involving wheat (*T. aestivum* and *T. turgidum*) and *T. turgidum* × *T. tauschii* amphiploids with *Tripsacum* as a novel and alternate sexual route for the production of cereal polyhaploids. It may also facilitate extending the wide crossing cycle in Mexico by at least eight weeks.

3.2. *Plant materials*

Eight cultivars of *T. aestivum*, 3 of *T. turgidum*, and 8 amphiploids derived from *T. turgidum*/*T. tauschii* were grown in outdoor pots at El Batan, CIMMYT, Mexico, and used as female parents in crosses with *Tripsacum dactyloides* also grown outdoors.

3.3. *Crossing, embryo rescue, plant regeneration, and transplanting procedures*

Spikes were hand-emasculated before anthesis and covered with glassine bags. When the stigmatic surface was receptive (three to four days after emasculation), the spikes were pollinated with fresh *Tripsacum* pollen. One day after pollination, the emasculated floral cups were flooded with an aqueous solution of 226 μM 2,4-D and 433 μM gibberellic acid (GA_3) (Mujeeb-Kazi *et al.*, 1987). To evaluate the effect of 2,4-D on embryo recovery, crosses involving the hexaploid wheat cultivar Ciano T 79 and the tetraploid wheat cultivar Altar 84 were given three treatments:

- Some spikes did not receive 2,4-D;
- Some spikes received 2,4-D, but were not pollinated; and
- Other spikes were pollinated and treated with 2,4-D (Table 4).

Embryo rescue, plantlet regeneration, and transplantation procedures were similar to those reported in the section on wheat × maize hybrids. The cytological processes for mitosis and meiosis were also identical to those

Table 4. Embryo recovery status in crosses between *Triticum aestivum* cv. Ciano 79 and *T. turgidum* cv. Altar 84 with *Tripsacum dactyloides* after various treatments

Cultivars	Florets emasculated	Florets pollinated	2,4-D applied	Embryos recovered
Ciano T 79	144	Yes	No	No
	148	No	Yes	No
	126	Yes	Yes	Yes
Altar 84	102	Yes	No	No
	156	Yes	Yes	Yes

earlier reported except we integrated a modified step (Mujeeb-Kazi *et al.*, 1994).

3.4. *Importance of 2,4-D treatment*

As mentioned earlier, Suenaga and Nakajima (1989) and Inagaki and Tahir (1990) found that 2,4-D treatment of the spikes is critical to recovering seeds and embryos from wheat × maize crosses. Our preliminary trials show that 2,4-D is also important for embryo recovery in *T. aestivum* × *Tripsacum* crosses. In crosses involving *T. aestivum* cv. Ciano T 79 and *T. turgidum* cv. Altar 84, we recovered embryos only from pollinated florets treated with 2,4-D. We did not recover embryos from unpollinated pistils after 2,4-D treatment or from pollinated florets without a 2,4-D treatment (Table 4). Exogenous 2,4-D treatments may be important in early stages of embryo development in *T. aestivum* × *Tripsacum* crosses.

3.5. *Results*

In all crosses receiving 2,4-D and GA_3 treatments 24 h after pollination, we obtained a wide range of embryo recovery frequencies. The mean frequencies were 20.6% for *T. aestivum*, 26.8% for *T. turgidum*, and 23.5% for the synthetic hexaploids (Table 5). There was no apparent genotype specificity, implying that *Tripsacum*, like maize and other species of the Panicoideae, is also insensitive to the *Kr* crossability alleles of wheat. A more detailed study is needed to reveal the extent of this insensitivity in different *Tripsacum* accessions because Suenaga and Nakajima (1989) observed variation among maize cultivars.

Embryos were smaller (averaging 0.5 mm long) than those resulting from

Table 5. Embryo recovery and plant regeneration from hybridization of some synthetic hexaploids (*Triticum turgidum* × *T. tauschii*) and *T. aestivum* and *T. turgidum* cultivars with *Tripsacum dactyloides*

Cultivar or line		Florets pollinated	Embryos recovered	Plants regenerated
Synthetic hexaploids				
	Total	450	106	80
Range over cultivars		(40-86)	(7-18)	(5-13)
Percentage			23.5	75.5
***T. aestivum* cultivars**				
	Total	654	135	106
Range over cultivars		(40-156)	(9-31)	(7-24)
Percentage			20.6	78.5
***T. turgidum* cultivars**				
	Total	168	45	30
Range over cultivars		(40-88)	(12-19)	(8-13)
Percentage			26.8	66.7

wheat × maize crosses (averaging 1 mm). In order to reduce the number of daily applications, we doubled the GA_3 concentration to 433 μM (Suenaga and Nakajima, 1989; Furusho *et al.*, 1991). This doubling might have been detrimental to normal embryo development. The GA_3 variable needs further evaluation to determine whether embryo size could be improved by using a lower GA_3 concentration or by omitting it altogether. We anticipate that with normal embryo development better germination frequencies will result as has been frequently observed in many wide cross hybrids (Mujeeb-Kazi *et al.*, 1987, 1989; Sharma and Gill, 1983).

As with the wheat × maize crosses, seeds produced from *T. aestivum* × *Tripsacum* lacked a normal endosperm. Embryos were lodged at the micropylar end of shrivelled seeds or were floating in a watery solution (probably translocated solutes) in more plump seeds. In spikes treated with 2,4-D after pollination, the ovary tissues were enlarged as happens in normal seed development, turgid but filled with liquid rather than endosperm (Suenaga and Nakajima, 1989; Inagaki and Tahir, 1990; Riera-Lizarazu and Mujeeb-Kazi, 1990). Sometimes embryos were found, other times not.

Cytological analyses showed the *T. aestivum* polyhaploids possessed 21 chromosomes, the *T. turgidum* polyhaploids possessed 14 chromosomes, and

Table 6. Mean chromosome pairing at metaphase I in polyhaploids of *Triticum aestivum* L. and *T. turgidum* × *T. tauschii* synthetic hexaploids

Polyhaploid n=3x=21 entries	Metaphase I Configurations			
	I	Bivalents		Total II
		Rings	Rods	
T. aestivum cultivars[a]	17.7	0	1.6	1.6
Synthetic hexaploids[b]	20.9	0	0.02	0.02

[a] = Mean of three cultivars; [b] = Mean of five hexaploids

polyhaploids from the synthetic hexaploids possessed 21 chromosomes. The secondary constriction site resolution readily identified the 1B and 6B chromosomes in all samples and occasionally the 5D chromosome's secondary constriction site in some $n = 3x = 21$ samples (Fig. 1a,b).

Meiotic analyses of some ABD polyhaploids ($n = 3x = 21$) demonstrated very low allosyndetic chromosome pairing at metaphase I. Riley and Chapman (1958) and Kimber and Riley (1963) reported similar low chromosome pairing relationships – data fairly consistent with our observations (Table 6). We detected no chromosome abnormalities.

Plant regeneration frequencies from recovered embryos were 66.7% for durum wheats, 78.5% for bread wheats, and 75.5% for the synthetic hexaploids (Table 5) – similar to the earlier regeneration frequencies of 73.9, 68.5, and 74.5%, respectively, of polyhaploids from maize crosses (Riera-Lizarazu *et al.*, 1992). In the maize studies, we found colchicine doubling ranged between 63.6 and 69.5% – an aspect we did not incorporate into the *Tripsacum* investigation.

Because of our diversified research interests in the synthetic hexaploids, we placed their polyhaploids in a glasshouse where we bagged each spike. We obtained spontaneous seed set on seven *T. turgidum* cv. Ruff"S" × *T. tauschii* polyhaploids and somatic analyses supported the anticipated chromosome count of $2n = 6x = 42$, AABBDD (Table 7). As mentioned earlier, each polyhaploid possessed $n = 3x = 21$ chromosomes; hence, a meiotic restitution-related process seems to have produced the doubled seed progeny – an event of frequent occurrence in intergeneric and interspecific hybrids.

3.6. *Conclusions*

Crosses between *Triticum* species and *Tripsacum* resulted in the production of wheat polyhaploids of various genotypes. Unlike wheat anther culture or sexual hybridization of wheat with *H. bulbosum*, troublesome genotypic specificity and aneuploidy were absent. As with maize, this makes *Tripsacum*-

Table 7. Spontaneous doubling in polyhaploids of *T. turgidum* cv. Ruff"S" × *T. tauschii*; spikes, seed number, and somatic chromosome counts of root tips from three seeds per doubled plant

Polyhaploid identification	Spike number	Total seeds	Somatic root-tip counts	
			Polyhaploid	Doubled seed
B91-7086	6	29	n=3x=21	42
B91-7087	9	5	n=3x=21	42
B91-7088	7	7	n=3x=21	42
B91-7089	5	20	n=3x=21	42
B91-10327	8	40	n=3x=21	42
B91-10328	8	12	n=3x=21	42
B91-10329	6	20	n=3x=21	42

mediated polyhaploid production a superior system for producing polyhaploids.

The merits of using *Tripsacum* instead of maize or a combination of both are worthy of consideration and further evaluation. In the field at El Batan, Mexico, *Tripsacum dactyloides* flowers six to eight weeks earlier than maize, which would allow a prolonged crossing cycle if both maize and *Tripsacum* are used as pollen donors. Regardless of which of these are used as male parents, polyhaploid production through such hybridizations will aid in accelerating progress in cereal breeding programs; other cytogenetic applications will be enhanced as well (Mujeeb-Kazi *et al.*, 1991). Easier production of doubled polyhaploid populations of different genotypes will facilitate genetic and genome mapping studies in cereals.

Finally, a long-term utility of *Triticum* × *Tripsacum* hybridizations is the possibility of transferring to wheat some of *Tripsacum*'s desirable traits, such as drought tolerance and insect resistance. Earlier, Laurie and Bennett (1986) theorized a similar concept for transferring the more efficient C-4 photosynthetic pathway from maize to wheat. Retention of the alien chromosomes in wheat will be a crucial step if such introgressions are ever to materialize.

4. Polyhaploidy: some specific applications

4.1. *RFLP genome mapping in wheat*

F_1 recombinants of inbred doubled polyhaploids can shorten the time it takes to obtain valuable homozygous lines. In the process commonly known as haplo-diploidization, a homozygous line is instantly obtained when the chromosomes of a polyhaploid plant are doubled. In wheat, where polymorphism

Table 8. Polyhaploid embryo production, plants regenerated, and doubled polyhaploid plants recovered using F_1 plants from crosses between *Triticum aestivum* L. (cvs. Buc, Opata M 85, Ciano T 79) and a synthetic hexaploid (*T. turgidum* L. × *T. tauschii*) as female plants with the maize polyhaploid induction system

Characteristic observed	Buckbuck synthetic	Opata 85 synthetic	Ciano 79 synthetic
Number of embryos	245	260	207
Plants regenerated	172	180	154
Plants doubled	107	136	115

at the DNA level is relatively low (Sorrells, pers. comm.), this system can be used to obtain polyhaploid plants from a cross that shows polymorphism. Upon doubling the chromosomes of these polyhaploids using colchicine treatment, we can produce a population of homozygous plants that represents the variation in the initial cross. These progeny can then be used for RFLP mapping of the cereal genomes (Mujeeb-Kazi *et al.*, 1993).

Since polymorphic loci in hexaploid wheat appear to be rare, RFLP linkage mapping can be achieved by using populations of wild progenitors where polymorphisms are more prevalent. Of these wheat relatives, *Triticum tauschii* accessions, which share complete homology with the D genome of hexaploid (bread) wheat, have been found to be highly polymorphic at the DNA level. RFLP mapping of hexaploid wheat is now feasible with the use of these synthetic wheat hexaploids – the result of crossing *T. turgidum* (AABB) with *T. tauschii* (DD). When the chromosomes are doubled, a reconstituted hexaploid wheat is produced (AABBDD). Our procedure is the following. First, we cross the durum cultivar Ruff with *T. tauschii* to produce a highly DNA-polymorphic synthetic hexaploid. We then cross this synthetic with hexaploid bread wheat cultivars such as Buckbuck, Opata M 85, and Ciano T 79. We cross the resulting F1 derivatives with maize to produce the polyhaploids. We then double the chromosomes of these polyhaploid plants to produce homozygous lines.

Our polyhaploid production procedure has been routinely effective, so we have not emphasized recording the number of embryos excised from pollinated florets. Typically, enough embryos can be excised to allow a regeneration frequency of between 70 and 80% and a doubling frequency of between 60 and 70% (Table 8).

4.2. *Production of alien chromosome addition lines*

In wheat wide crosses, polyhaploidy can be further exploited for the production of alien chromosome addition lines from populations that have varying

Table 9. Disomic *Thinopyrum elongatum* additions to wheat variety Goshawk"S" ($2n = 6x = 42 + 2$) developed by the maize haploid induction methodology, and identified by isozyme analysis for homoeology

Homoeologous group additions	Isozyme marker	Identification numbers
1	HMW-Glu	INVO 92-6704 6721
3	EST	INVO 92-6870 6876
5	ß-Amylase	INVO 92-6899 6911
7	α-Amylase	INVO 92-6840 6854

chromosome numbers. Preferably, plants with 22 chromosomes (21 chromosomes of wheat plus 1 alien chromosome) are recovered. The final product after colchicine treatment is a plant with 44 chromosomes (42 wheat plus an alien pair). This process not only simplifies our production of disomic addition lines, but also resolves the constraints of paternal transmission of alien chromosomes. In addition, it reduces the analyses necessary for recovering 44-chromosome disomic derivatives following the selfing of a 43-chromosome plant containing 21 bivalents plus 1 univalent.

If a wide cross program were built exclusively around the wheat cultivar Chinese Spring, the *Hordeum bulbosum* procedure (40 to 45% polyhaploid recovery) would be satisfactory. However, in our program where commercial wheat cultivars are used, the *H. bulbosum* technique is ineffective and we logically favor the wheat × maize methodology. We have initially applied the procedure to derivatives of *Thinopyrum elongatum* × *T. aestivum* crosses. From 180 backcross derivatives, with somatic chromosome numbers of 43, 44, and 45, we have obtained seed set after colchicine treatment on 62 plants. Doubled haploids with 44 chromosomes were stable and have so far allowed diagnostics of four homoeologous group additions through isozyme applications (Table 9). More 44-chromosome plants will be produced for completing the addition set. The wheat cultivar Goshawk"S" – involved in the hybrid, its amphiploid, and in its backcrosses – has poor crossability with *S. cereale*, indicative of a dominant crossability *Kr* locus.

4.3. *Other sources of polyhaploid production*

Observations of Laurie and Bennett (1988b), Laurie (1989), Ahmad and Comeau (1990) suggest that *Pennisetum glaucum* and *Sorghum* pollen are

also potential sources for wheat polyhaploid production. In a recent study (unpublished data) these sources were compared with maize as to their haploid production efficiency. In this study polyhaploid production utilizing maize was more consistent than utilizing pearl millet or *Sorghum* as pollen sources. Significant differences in embryo formation frequencies were found between pearl millet genotypes as well as among wheat cultivars. Because pearl millet is normally cross-pollinated it may be advantageous to select favorable lines from pearl millet populations in order to obtain higher and stable frequencies with diverse wheat varieties. In crosses with *Sorghum*, wheat genotypic specificity emerged as a factor that requires further investigation to validate current conclusions that are based upon a limited germplasm sample.

5. Some closing impressions

- The potential of utilizing stored maize and *Tripsacum* pollen needs to be explored because it could be a significant factor in extending the use of the methodologies discussed in this chapter to countries where cropping cycles are separated or where adequate facilities are lacking for growing plants under controlled conditions. Optimistic results have initially been obtained with stored maize pollen (Inagaki and Mujeeb-Kazi, 1994).
- Simplification of genetic studies, pyramiding of simple genes (e.g., for leaf rust resistance), and applications in wide crosses to homozygosity and molecular mapping populations are just a few avenues that could be further exploited and diversified.
- Equally promising is the development of doubled polyhaploids from F_1 combinations for traits like salt tolerance where the soil heterogeneity makes genetic studies almost prohibitive.
- Just as 2,4-D is unequivocally recognized as being an essential exogenous growth regulator in this methodology, we argue that the quality and quantity of maize or *Tripsacum* pollen are equally critical.
- Can maize and *Tripsacum* chromosomes be retained in a wheat background? If so, will any characters be expressed? Only future research will provide the answer. Currently the observations of Riera-Lizarazu *et al.* (1992) suggest that maize chromosomes are retained in oat polyhaploids. Further Riera-Lizarazu *et al.* (1993) also report that maize DNA is introgressed in 21 chromosome oat haploids. These findings provide an impetus to anticipate such events in a wheat system.

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