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Maize (*Zea mays* L.) mediated polyhaploid production in some Triticeae using a detached tiller method

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ABSTRACT

A detached tiller culture procedure was investigated as an alternative method for cereal haploid production through sexual crossing of some Triticeae with maize (*Zea mays* L.). Polyhaploid embryo recovery frequencies for seventeen *Triticum aestivum* L. (n = 3x = 21, ABD) cultivars, five *Triticum turgidum* L. (n = 2x = 14, AB) cultivars and five synthetic hexaploids of *Triticum turgidum x Aegilops squarrosa* L. (n = 3x = 21, ABD) averaged 15.6, 16.9 and 19.8%, respectively. Colchicine doubling frequencies of polyhaploids produced for *T. aestivum*, *T. turgidum* and the synthetic hexaploids were 60.7, 69.5 and 63.6%, respectively. There was low chromosome pairing at metaphase I in polyhaploids of several *T. aestivum* cultivars and in those derived from the *T. turgidum x Ae. squarrosa* synthetics. The mean metaphase I chromosomal associations of *T. aestivum* polyhaploids were 18.5 univalents + 0.03 ring bivalents + 1.13 rod bivalents + 0.08 trivalents (chiasmata = 1.34/cell), while those for polyhaploids from synthetic hexaploids were 20.1 univalents + 0.44 rod bivalents (chiasmata = 0.44/cell). The use of the maize system for haploid production in the Triticeae is very encouraging since stringent genotype specificity is not apparent. Reaching homozygosity in earlier generations will accelerate breeding progress.

Key words: Allosyndetic pairing, Chromosome doubling, Chromosome elimination, Synthetic hexaploids, *Triticum*, Wheat x maize hybridization.

INTRODUCTION

Haploid production of the Triticeae cereals has mostly relied on anther culture and sexual crossing with *Hordeum bulbosum* L. Limitations to these haploid production strategies include aneuploidy, somaclonal variation and genotypic specificity in the former approach (for review see PICARD, 1989), with the homoeologous group 5 crossability loci (*Kr1*, *Kr2*, *Kr3*) being the essential limiting factor in the latter method (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, a sexual route to haploid production is seemingly more desirable. Recently, crosses of wheat and other members of the Triticeae with maize have been suggested as an alternative sexual route for haploid production (LAURIE and BENNETT, 1986, 1988a, 1988b; O'DONOUGHUE and BENNETT, 1988; LAURIE *et al.*,1990). Since maize appears 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), thus making it potentially superior to the *H. bulbosum* system. In addition, gametoclonal variation induced in doubled haploid lines using the maize system was similar to that found in doubled haploids obtained from wheat x *H. bulbosum* L. crosses (LAURIE and SNAPE, 1990).

The use of 2,4-D in promoting seed set and embryo formation in wheat x maize crosses is critical (INAGAKI and TAHIR, 1990). The 2,4-D treatment techniques include floret culture (LAURIE and BENNETT, 1988b), tiller injection (SUENAGA and NAKAJIMA, 1989; INAGAKI and TAHIR, 1990), spike sprays (RINES *et al.*, 1990) and floret treatment (RIERA-LIZARAZU and MUJEEB-KAZI, 1990). The use of detached spikes (RIERA-LIZARAZU and MUJEEB-KAZI, 1990) and detached spikelets (LAURIE and BENNETT, 1988b) offer more experimental flexibility because

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experimental material can be transferred to locations where environmental conditions can be monitored and controlled.

High frequency of wheat polyhaploid recovery was reported for crosses between the wheat cultivar Morocco and the maize population "Pool 9A" (RIERA-LIZARAZU and MUJEEB-KAZI, 1990). In this paper we report successful polyhaploid embryo production of additional *Triticum aestivum* and *T. turgidum* L. cultivars, and of a few *T. turgidum x Aegilops squarrosa* L. synthetic hexaploids using a detached tiller culture method. Cytological and biochemical features of these polyhaploids and their doubled progeny are also characterized.

MATERIALS AND METHODS

Plant material

Field grown plants of breadwheat (*Triticum aestivum*), durum wheat (*T. turgidum*), rye (*Secale cereale*), *T. turgidum x Aegilops squarrosa* amphiploids and maize (*Zea mays*) grown at El Batan, CIMMYT, Mexico, were used (Table 1). A bulk sample of pollen from several maize cross-pollinating populations was used for all crosses.

Crossing procedures, detached tiller culture, and embryo rescue

Spikes were hand emasculated before anthesis and covered with glycine bags. When the stigmatic surface was receptive (3 to 4 d after emasculation), the spikes were pollinated with fresh maize pollen. Tillers of pollinated spikes were detached 5 cm below the peduncular node and placed in a beaker with an aqueous solution of 100 mg/l 2,4-D for transport from the field. Culms 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 MURASHIGE and SKOOG's (1962) basal medium components with 100 mg/l 2,4-D without agar. The test tubes with detached tillers were placed 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. Detached tillers remained in 2,4-D containing medium for 48 h and were then transferred to growth regulator-free medium where they remained for 12 days. The embryo rescue, plant regeneration and transplantation procedures were described earlier (MUJEEB-KAZI *et al.*, 1987).

Cytology

Somatic cytology of all regenerated plants was conducted according to the method of MUJEEB-KAZI and MIRANDA (1985). For meiotic analysis young spikes were fixed in 6:3:1 (ethanol (99%):chloroform: glacial acetic acid) for 48 h and stored in 70% ethanol in the freezer (−10°C) until use. Anthers at metaphase I were stained in alcoholic carmine (SNOW, 1963), then processed according to the modified procedure of MUJEEB-KAZI *et al.* (1992) for high contrast, intense staining and reduced stickiness. Mean metaphase I pairing associations were calculated from 25 meiocytes for some of the bread wheat and synthetic hexaploid polyhaploids.

Colchicine treatment

Cytologically identified polyhaploid plants were treated with colchicine as described by MUJEEB-KAZI *et al.* (1987). Successful chromosome doubling was inferred from seed setting on the colchicine-treated polyhaploid plants.

Protein separation

Some female wheat parents and their doubled haploid progenies were analyzed by studying the banding profiles of seed storage proteins and isozymes. The storage protein glutenin, and isozymes of esterase (est., E.C. 3.1.1.1) and β-amylase (α-1,4-glucan maltohydrolase, E.C. 3.2.1.2) were evaluated. Endosperm halves of mature kernels were used for protein separation and isozyme analysis.

High molecular weight glutenin subunits were separated using SDS-Polyacrylamide gel electrophoresis as described by NG *et al.* (1988) with slight modifications. Stacking gels of 2 cm and 10% separation gels of 15.5 cm were used. Thickness and width of the gels were 0.15 cm 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 4.0-6.5 for β-amylase. The running conditions and the staining protocols were described by WILLIAM and MUJEEB-KAZI (1992).

RESULTS AND DISCUSSION

Sexual crossing between members of the Triticeae and maize offers a new alternate route to haploid production (LAURIE and BENNETT, 1986, 1987, 1988a, 1988b; O'DONOUGHUE and BENNETT, 1988; LAURIE *et al.*, 1990). This avoids somaclonal variation induced aneuploidy plus genotypic specificity associated with anther culture and the *H. bulbosum* system (for review see PICARD, 1989).

Caryopses produced from crosses between the Triticeae species and maize, when harvested at 14 days post pollination, lacked normal endosperms.

TABLE 1

Embryos produced, recovery percentage, plants regenerated and colchicine doubled of *Triticum aestivum* L., *T. turgidum* L. and *T. turgidum x Aegilops squarrosa* L. lines following crosses with maize

Cultivars and lines	Number of florets pollinated	Embryos		Plant number and percentage (%)	
		Produced No.	Recovery (%)	Regenerated	Doubled
Triticum aestivum[a] *cultivars*					
AGA/6*YR	195	32	16.4	28 (87.5)	19 (67.9)
ALD"S"/PVN"S"	225	44	19.6	34 (77.2)	21 (61.8)
BAU"S"	260	55	21.2	38 (69.0)	25 (65.8)
BOW"S"/PVN"S"	190	48	25.3	30 (62.5)	20 (66.7)
F12.71/COC/ /GEN	145	17	11.7	9 (52.9)	4 (44.4)
FUKUHOKOMUGI	210	35	16.7	29 (82.9)	17 (58.6)
GENARO 81	185	26	14.1	22 (84.6)	14 (63.6)
GLENNSON 81	167	16	9.6	10 (62.5)	6 (60.0)
GOV/AZ/ /MUS"S"	143	16	11.2	10 (62.5)	7 (70.0)
KAUZ"S"	215	40	18.6	27 (67.5)	15 (55.6)
MRL"S"/BUC"S"	139	31	22.3	19 (61.3)	7 (36.8)
OPATA	181	16	8.8	9 (56.3)	4 (44.4)
PAPAGO"S"	170	11	6.5	8 (72.7)	4 (50.0)
PVN"S"/BUC"S"	60	4	6.7	1 (25.0)	1 (100.0)
SERI 82	139	22	15.8	13 (59.1)	9 (69.2)
TESIA 79	95	7	7.4	6 (85.7)	4 (66.7)
THB"S"/KEA"S"	120	22	18.3	10 (45.5)	7 (70.0)
TOTAL	2839	442	15.6	303 (68.6)	184 (60.7)
Range		4-55	6.5-25.3	1-38	1-25
Triticum turgidum[a] *cultivars*					
CNDO/ENTE/ /MEMO/MEX	150	27	18.0	22 (81.5)	17 (77.3)
ALTAR 84	286	44	15.4	37 (84.1)	27 (73.0)
LARU"S"	153	18	11.8	13 (72.2)	6 (46.2)
CHEN"S"	108	21	19.4	12 (57.1)	9 (75.0)
MEMO/MEX	144	32	22.2	21 (65.6)	14 (66.7)
TOTAL	841	142	16.9	105 (73.9)	73 (69.5)
Range		18-44	11.8-22.2	13.37	6.27
T. turgidum x Ae. squarrosa lines[b] CNDO/R143/ /ENTE"S"/MEX /3/*Ae. squarrosa*	140	23	16.4	19 (82.6)	11 (57.9)
RUFF/*Ae. squarrosa*	178	43	24.2	35 (81.4)	27 (77.1)
YUK/*Ae. squarrosa*	115	20	17.4	13 (65.0)	6 (46.2)
GAN"S"/*Ae. squarrosa*	89	20	22.5	12 (60.0)	7 (58.3)
DOY1/*Ae. squarrosa*	73	12	16.4	9 (75.0)	5 (55.6)
TOTAL	595	118	19.8	88 (74.6)	56 (63.6)
Range		12-43	16.4-24.2	9-35	5-27

[a] *Triticum aestivum* cv. "Fukuhokomugi" was obtained from G. Fedak, Plant Research Centre, Agriculture Canada, Ottawa, Ontario, Canada. All other cultivars are part of CIMMYT's breeding germplasm.
[b] Obtained from CIMMYT's Wheat Wide Crosses program.

FIGURE 1 - Spikes of wheat polyhaploids (n = 3x = 21, ABD).
A. Side and frontal views of a sterile polyhaploid (n = 3x = 21) spike without seeds.
B. Side and frontal views of a fertile spike as a consequence of colchicine treatment of a n = 3x = 21 sterile polyhaploid.

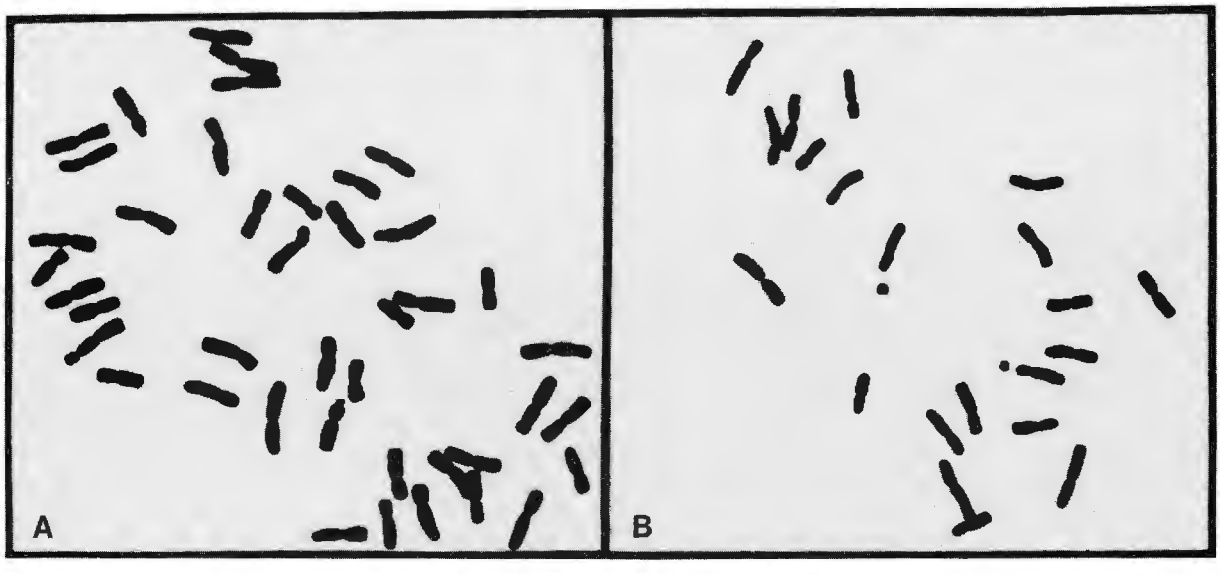

FIGURE 2 - A. Mitotic metaphase spread of *Triticum aestivum* L. ($2n$ = 6x = 42, AABBDD).
B. Mitotic spread of a *T. aestivum* L. polyhaploid (n = 3x = 21, ABD).

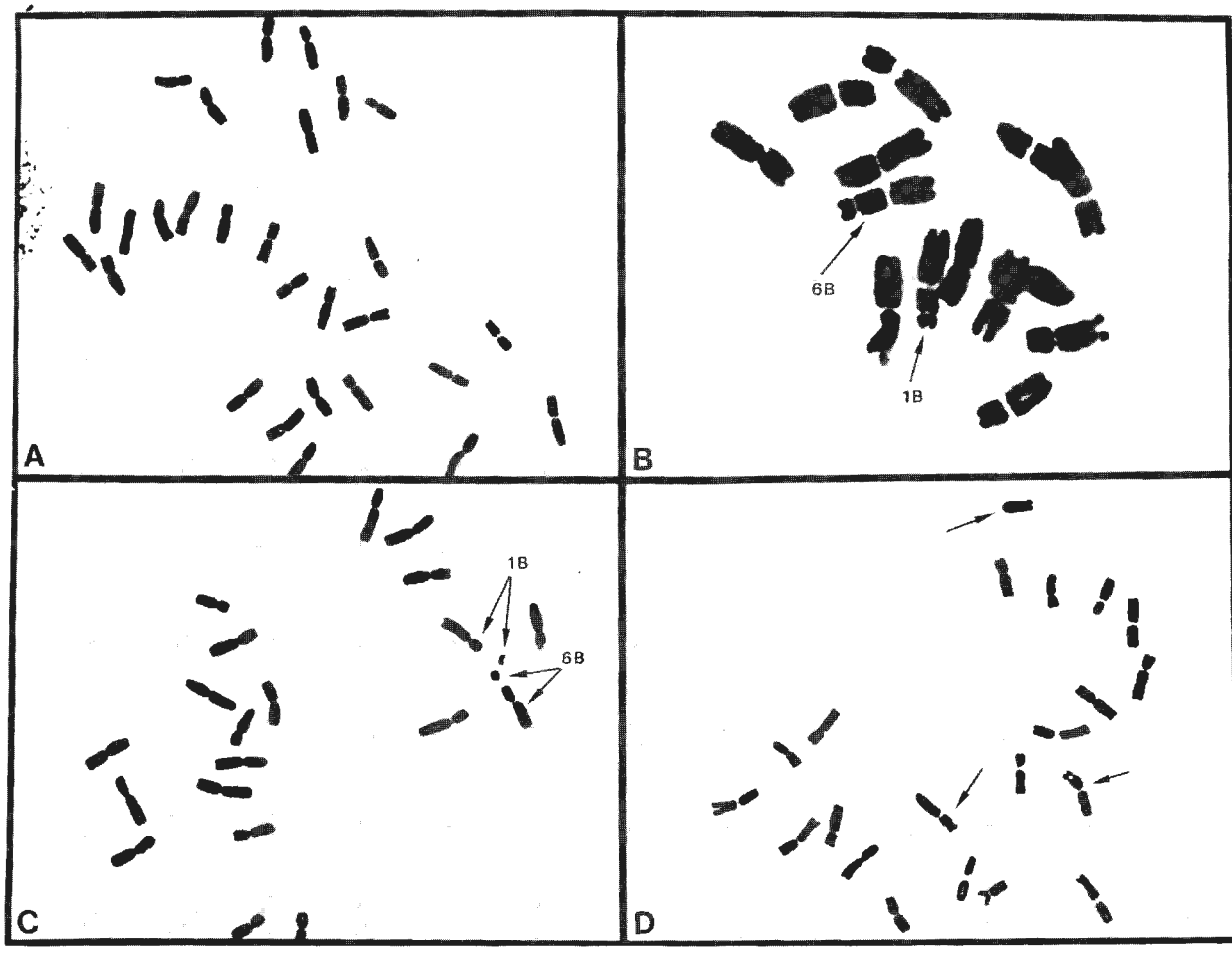

FIGURE 3 - A. Mitotic spread of *Triticum turgidum* cv. Altar 84 ($2n = 4x = 28$, AABB).
B. Mitotic spread of a *T. turgidum* cv. Altar 84 polyhaploid ($n = 2x = 14$, AB) with the 1B and 6B satellited chromosomes arrowed and identified.
C. A polyhaploid of *Triticum aestivum* with 20 chromosomes. The 1B and 6B satellites separated from their short arms are arrowed.
D. A *Triticum aestivum* polyhaploid with 21 chromosomes. The telocentric, 1B and 6B satellite chromosomes are arrowed.

Embryos floated inside the seeds in a watery solution. Generally, any embryo recovered from a caryopsis lacking normal solid endosperm is a haploid, thus serving as a morphological diagnostic for screening selfed-versus cross-pollinated products.

As reported by others (INAGAKI and TAHIR, 1990; LAURIE and REYMONDIE, 1991) we also recovered haploid embryos from many different wheat genotypes following pollinations with maize. In addition, our results obtained by using detached tillers indicate that the use of the maize system can be extended to recover polyhaploids in durum wheats and *T. turgidum x Ae. squarrosa* amphiploids (Table 1).

There was a wide range of embryo recovery frequencies in this experiment among hexaploid wheats, tetraploid wheats and the *T. turgidum x Ae. squarrosa* synthetic hexaploids with recovery averages of 15.6, 16.9, and 19.8%, respectively (Table 1). Since the earlier documentation (LAURIE and BENNETT, 1987) that wheat x maize crossing success was independent of the *Kr* locus and maize pollen donor source, we did not design this study for further establishing significant differences among genotypes. The focus was essentially upon assessing the magnitude of haploid induction amongst our genotypes for applications in wheat breeding and cytogenetics. No embryos were recovered from *S. cereale* x maize crosses. Mean plant regeneration frequencies calculated as a percentage of embryos recovered for bread wheats, durum wheats and *T. turgidum x Ae. squarrosa* amphiploids were 68.5, 73.9 and 74.5%, respectively. Successful chromosome doubling (Fig. 1) of polyhaploid plants treated with colchicine averaged

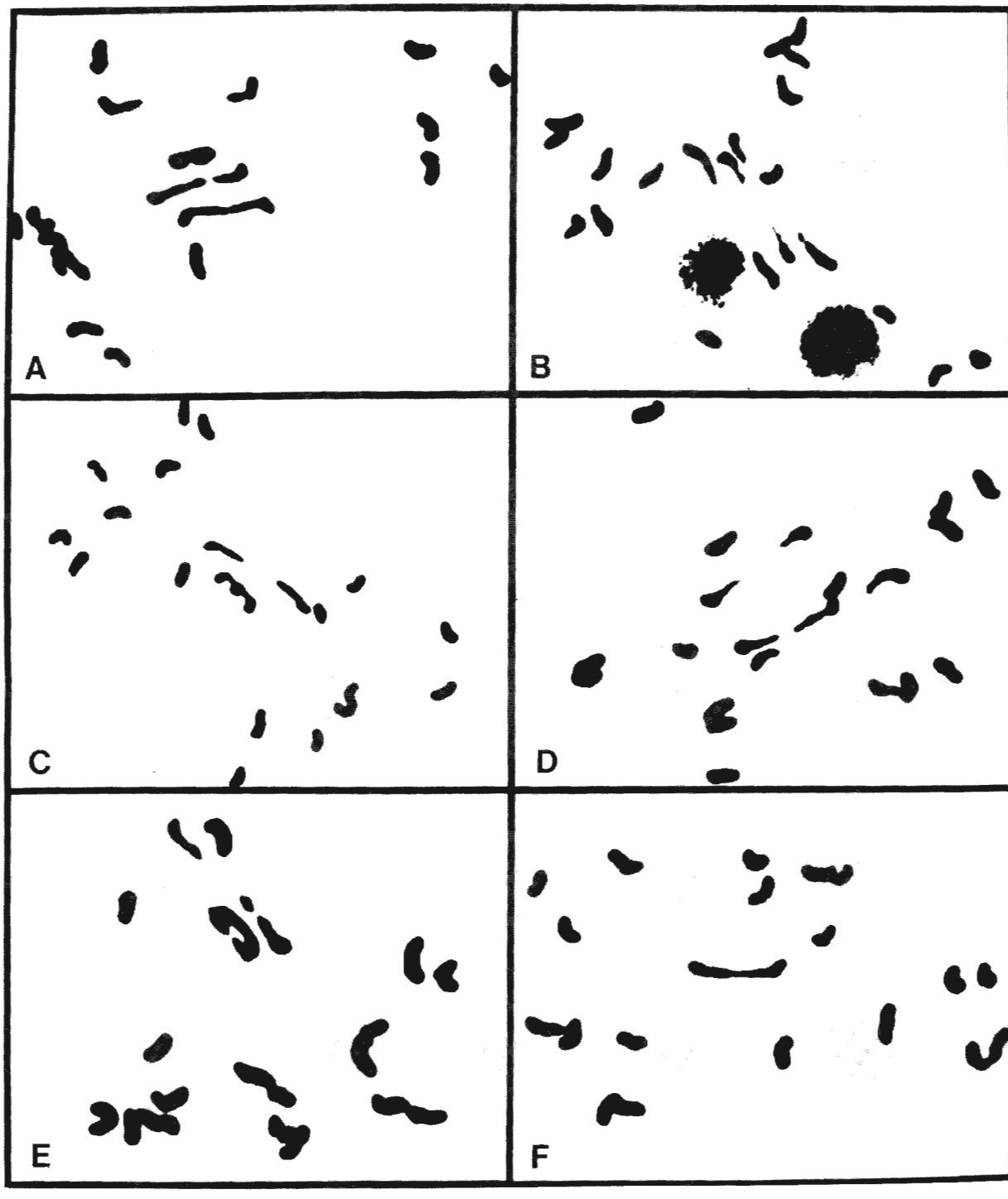

FIGURE 4 - Polyhaploid chromosome configurations of *Triticum aestivum* L. at Metaphase I of meiosis showing variable univalents and bivalents:

A. 13 univalents + 3 rod bivalents + 1 ring bivalent
B. 15 univalents + 3 rod bivalents
C. 17 univalents + 2 rod bivalents
D. 15 univalents + 3 rod bivalents (1 separated)
E. 17 univalents + 1 rod bivalent + 1 ring bivalent
F. 19 univalents + 1 rod bivalent.

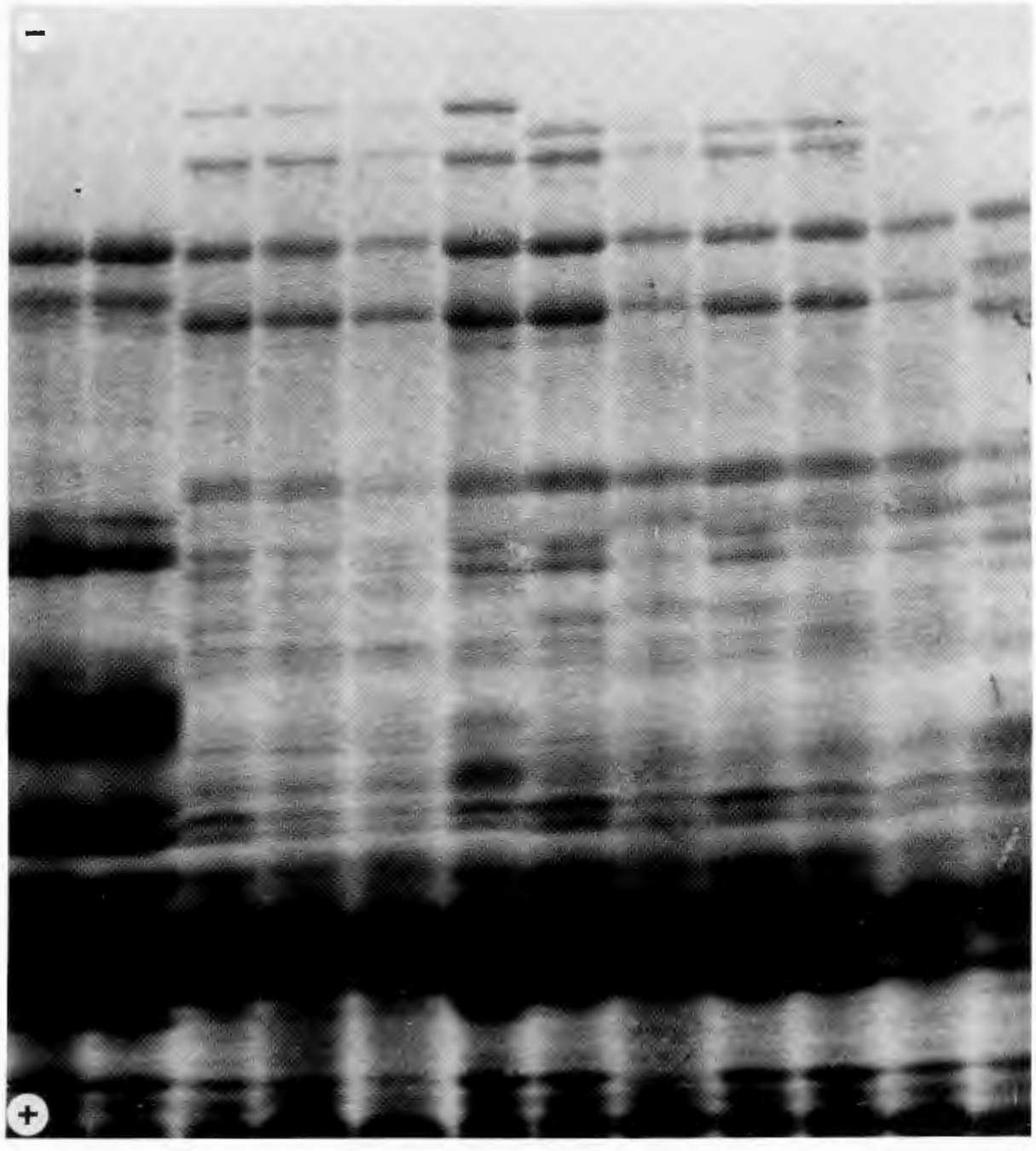

FIGURE 5 - SDS-PAGE separation of seed proteins from durum and bread wheat cultivars and their extracted doubled haploids. From left to right:
Altar 84 (A) and a doubled haploid (1); Seri 82 (S) and three doubled haploids (1, 2, 3); Kauz "S" (K) and four doubled haploids (1, 2, 3, and 4); and Chinese Spring (CS).

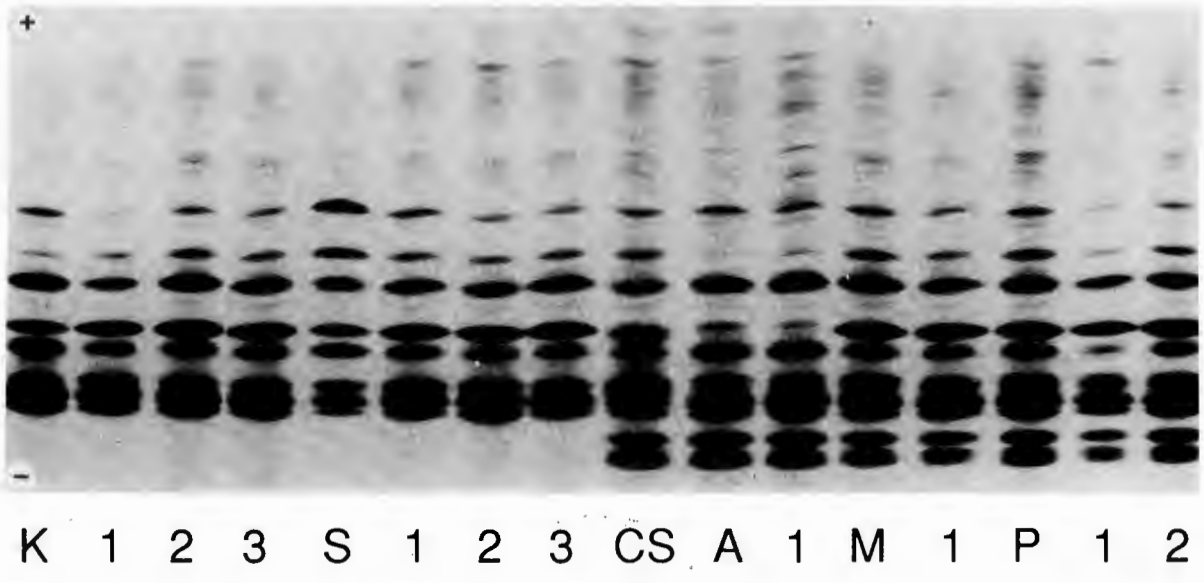

FIGURE 6 - Grain esterase profiles of durum and bread wheat cultivars and their extracted doubled haploids. From left to right: Kauz "S" (K) and three doubled haploids (1, 2, and 3); Seri 82 (S) and three doubled haploids (1, 2, and 3); Chinese Spring (CS); Altar 84 (A) and its doubled haploid (1); Mrl "S"/Buc "S" (M) and its doubled haploid (1); and Papago "S" (P) with two doubled haploids (1, 2).

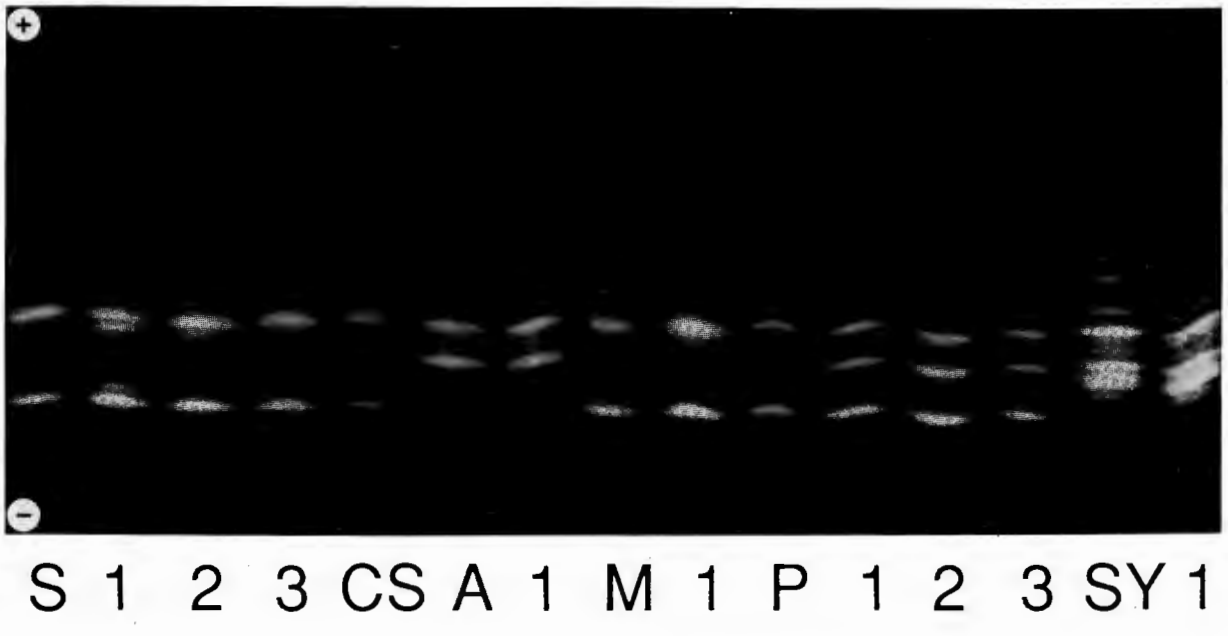

FIGURE 7 - β-amylase profiles of durum and bread wheat cultivars and their extracted doubled haploids. From left to right: Seri 82 (S) and three doubled haploids (1, 2, and 3); Chinese Spring (CS); Altar 84 (A) and its doubled haploid (1); Mrl "S"/Buc "S" (M) and its doubled haploid (1); Papago "S" (P) and three doubled haploids (1, 2, and 3); and the synthetic hexaploid Ruff/*Ae. squarrosa* (SY) and its doubled haploid (1).

60.7% for *T. aestivum* cultivars, 69.5% for *T. turgidum* cultivars and 63.6% for the synthetic hexaploids (Table 1).

Haploid plant production frequencies of 1 to 4% have been considered acceptable for the efficient production of haploids (COMEAU *et al.*, 1988). Our average doubled haploid plant recovery frequencies for *T. aestivum*, *T. turgidum* and the synthetic hexaploids ranged from 63.6 to 69.5%, average embryo excision frequencies were 15.6 to 19.8%, and the mean plant regeneration frequency range was 68.5 to 74.5% (Table 1). Though the wheat polyhaploid plant production frequencies obtained in this study more than adequately meet the economical threshold level (COMEAU *et al.*, 1988), higher frequencies across genotypes have been reported (SUENAGA and NAKAJIMA, 1989; INAGAKI and TAHIR, 1990; RIERA-LIZARAZU and MUJEEB-KAZI, 1990). The low haploid embryo recovery frequencies in this study as compared with earlier findings of RIERA-LIZARAZU and MUJEEB-KAZI (1990) are attributed to problems inherent to the detached tiller culture system. These constraints include the impossibility of total tiller sterilization, since samples were collected from the field at a time when disease factors and continuous rainfall had set in. This sterilization constraint resulted in progressive tiller culm base decay in the culture medium, thus affecting normal nutrient translocation and seed development. For such situations it may be appropriate to use intact spikes (SUENAGA and NAKAJIMA, 1989) or modify the detached tiller process. For the latter, the use of sulfurous acid to suppress contamination in culture solution and culm decay as reported by KATO *et al.* (1990) may be an alternative. Additionally, it may help to use less humid environments in controlled greenhouse crossing and to use stored maize pollen to facilitate crossing in preferred environments.

The detached tiller culture system was specifically designed for the study of nutrient translocation and seed development physiology in wheat spikes (JENNER, 1970; DONOVAN and LEE, 1977; SINGH and JENNER, 1983); hence, modifications of this technique may be needed for wider application to other species. For example, severe culm decay was observed when detached tillers of *Secale cereale* cv. Prolific and Sardev were used and no embryos were recovered in these rye x maize crosses, although LAURIE *et al.* (1989) have reported embryo initiation in rye x maize hybridizations. It appears that with rye an *in vivo* approach may be preferable. If the detached tiller method is to be commonly used, then the constitution of the nutrient solution, point of tiller detachment, plus the constraints expressed earlier must be addressed.

Cytologically analyzed plants possessed the expected haploid complement of 21 chromosomes for *T. aestivum* (Fig. 2b) and 14 chromosomes for *T. turgidum* (Fig. 3b), where each wheat parent had the euploid number of $2n = 6x = 42$ (Fig. 2a) or $2n = 4x = 28$ (fig. 3a). Two *T. aestivum* polyhaploids were aneuploids with 20 chromosomes (Fig. 3c), one of which died in the seedling stage. Another anomaly was a *T. aestivum* polyhaploid with 21 chromosomes (Fig. 3d) including a telocentric.

Polyhaploids of *T. aestivum* cultivars and the synthetic hexaploid showed very low allosyndetic pairing (Table 2 and Fig. 4). Ring bivalents were rare with chiasmata ranging from 0.44 to 1.96 per meiocyte (Table 2).

The earlier reported wheat polyhaploid ($n = 3x = 21$) meiotic associations were 18.05 univalents + 1.38 bivalents + 0.07 trivalents (RILEY and CHAPMAN, 1958). Subsequently, KIMBER and RILEY (1963) reported for bread wheat a mean frequency of 19.18 univalents + 0.90 bivalents + 0.008 trivalents from analyses of eight euhaploids; values indicating very low allosyndetic pairing. This degree of chromosome pairing is consistent with our data where the *T. aestivum* polyhaploids of several cultivars had a mean metaphase I chromosome association frequency of 18.6 univalents + 0.01 ring bivalents + 1.24 rod bivalents + 0.06 trivalents (extracted from Table 2). Values for the synthetic (*T. turgidum x Ae. squarrosa*) polyhaploid were 20.1 univalents + 0.44 bivalents. This low pairing indicates that the wheat cultivars and the synthetic hexaploid used had the dominant *Ph* locus that restricts homoeologous pairing and that the locus remained intact over the haploid induction process. Recently in *T. aestivum Ph1* euhaploids derived from wheat x barley hybrids, JAUHAR *et al.* (1991) also observed very low chromosomal pairing. Only 6.5 to 11.0% of the complement paired homoeologously with 0.7 to 1.2 chiasmata per cell.

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 those for β-amylase on group 4 and 5 chromosomes (AINSWORTH *et al.*, 1983). Extensive

TABLE 2

Mean chromosome pairing (with ranges in parenthesis) at metaphase I in some polyhaploids of *Triticum aestivum* L. and of *T. turgidum* X *Aegilops squarrosa* synthetic hexaploids

Polyhaploid cultivars	Metaphase I chromosomal associations (25 meiocytes)							Chiasmata per meiocyte
	I	Bivalents		Total II	Trivalents		Total III	
		Rings	Rods		Chain	Pan		
Fukuhokomugi	18.8 (15-21)	0	1.12 (0-3)	1.12	0	0	0	1.12
Bau"S"	18.4 (16-21)	0.08 (0-1)	0.96 (0-2)	1.04 (0-2)	0.08 (0-1)	0.08 (0-1)	0.16 (0-1)	1.52
Bow"S"/Pvn"S"	17.7 (15-21)	0.04 (0-1)	1.48 (0-3)	1.52 (0-3)	0.08 (0-1)	0	0.08 (0-1)	1.72
Genaro 81	19.0 (17-21)	0	1 (0-2)	1 (0-2)	0	0	0	1
Kauz"S"	18.7 (16-21)	0	0.96 (0-2)	0.96 (0-2)	0.12 (0-1)	0	0.12 (0-1)	1.2
Mrl"S"/Buc"S"	19.0 (17-21)	0.04 (0-1)	0.96 (0-2)	1 (0-2)	0	0	0	1.04
Cndo/R143//Ente"S"/ Mexi/3/*Ae. squarrosa*	20.1 (17-21)	0	0.44 (0-2)	0.44 (0-2)	0	0	0	0.44

allelic variations have also been reported for all three systems.

Banding profiles of high molecular weight glutenins, and isozymes of seed esterase and β-amylase are shown in Figs. 5 to 7. Extensive variations in the banding profiles were observed for all three systems among the different parental lines used in this study, probably as a consequence of allelic variation. Banding profiles of high molecular weight glutenin and esterase in the doubled haploid progenies were identical to those present in their respective parents. For β-amylase there were some minor differences in the banding profiles within some families (Fig. 7). 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 the endosperm protein concentration variation. In general, the close similarity in the banding profiles of the doubled haploid progenies and their parents suggests stable transmission of genetic information by this procedure of doubled haploid production. It also indicates that the parental genetic information for the enzyme systems evaluated is fixed in the doubled haploid progeny without alteration.

The use of the maize system for haploid production in the Triticeae is very encouraging since stringent genotype specificity is not apparent. Reaching homozygosity in earlier generations will accelerate cereal breeding progress. Recent results (INAGAKI and TAHIR, 1990; LAURIE and REYMONDIE, 1991) corroborate this contention where high frequency haploid production has been reported in spring and winter wheat x maize crosses. Despite the current, presumably location specific, contamination problem with the detached tiller procedure encountered in this study, the potential for its application in haploid production research in cereals is promising. More durum wheat and rye genotypes need to be tested to further evaluate the detached tiller procedure for haploid production using sexual crossing with maize.

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