Theor. Appl. Genet. 57, 89-95 (1980)

Cytogenetic Analysis of Plants Regenerated from Colchicine-treated Callus Cultures of an Interspecific *Hordeum* Hybrid

T.J. Orton and R.P. Steidl
Department of Crop and Soil Sciences Michigan State University, East Lansing, Michigan (USA)

Summary. Hybrids of *Hordeum vulgare* (HV) × *H. jubatum* (HJ) were synthesized for purposes of introgressive breeding, but were sterile and the recovery of pure diploid tillers by colchicine applications in vivo was difficult. Plant regeneration from colchicine-treated callus cultures of the hybrid (HV × HJ) was investigated as a means to produce high numbers of pure diploid, fertile intermediates. 10 of 50 plants regenerated in this manner exhibited variable chromosome numbers with means of approximately 37 (expected diploid number = 42). Cytological examinations of microsporogenesis in all such plants revealed a high incidence of bivalent formation at metaphase I (as compared to nearly complete asynapsis in the F_1), but spindle and chromosome abnormalities in later meiotic stages led to complete sterility. Approximately 40% of HJ plants regenerated from colchicine-treated calli appeared to be pure tetraploids of high fertility. These techniques are hence useful for high frequency production of diploid or polyploid plants.

Key words: Cytogenetics – Plant regeneration – Introgressive breeding – Callus culture – Regenerates – Barley – *Hordeum* hybrids

Introduction

Hybridization between taxa is becoming an increasingly important means of introducing genetic variation into the gene pools of crop plants (Smith 1971). It is possible to substitute and add genetic information between related gene pools as in wheat (Sears 1972), corn (Galinat 1977), and tomato (Rick 1973). For example, traits such as stem rust resistance (Knott 1961; Knott et al. 1977), leaf rust resistance (Caldwell et al. 1956; Sears 1956), and wheat streak mosaic virus resistance (Larson and Atkinson 1973) have been introgressed into wheat from different species.

Prerequisite to such gene transfers is sexual fertility in intertaxon hybrids. These crosses generally result in depressed or complete loss of fertility, often attributable to functional haploidy at meiosis. Fertility can be restored in many such hybrids by doubling the chromosome number, but these techniques are often tedious or unyielding or the plant may be resistant to induced diploidization. New techniques are needed for the production of fertile intermediates in cases of hybrid sterility and recalcitrance to diploidization.

The amount of genetic variability available in barley (*Hordeum vulgare*) for selection in response to winterhardiness, salt tolerance, tolerance of wet soils, and epidermal trichomes (which confer resistance to oviposition by the cereal leaf beetle) is extremely low (J.E. Grafius, personal communication). *H. jubatum* is a weedy perennial which embodies a desired range of phenotypes for all of these traits. The F_1 hybrid (2n=3x=21, hereafter referred to as HV × HJ) was unconditionally sterile in both directions (Rajathy and Morrison 1959; Steidl 1976) and exhibited an extremely low frequency of bivalent formation and abnormal meiosis (Wagenaar 1960; Murry 1975). The two genomes of *H. jubatum* (an autoallotetraploid) have been shown to possess segmental homology and, in other cases, to exhibit autosyndesis to a much greater extent than in HV × HJ (Wagenaar 1960; Starks and Tai 1974). It was concluded that sterility in HV × HJ was caused by a lack of homeologous chromosome pairing, possibly attributable to an inhibitor system, as with the pH gene in wheat (Murry 1975).

Doubled tillers of HV × HJ were recovered at a very low frequency but failed to set seed. Moreover, tillers of such colchicine-treated plants often appeared to be mixoploid. Tissue culture was examined as a possible tool to circumvent the recalcitrance of HV × HJ to induced diploidization and recover fertile, pure diploid hybrid intermediates. After in vitro colchicine treatments, a large number of plants were regenerated from callus cultures of

0040-5752/80/0057/0089/$ 1.40

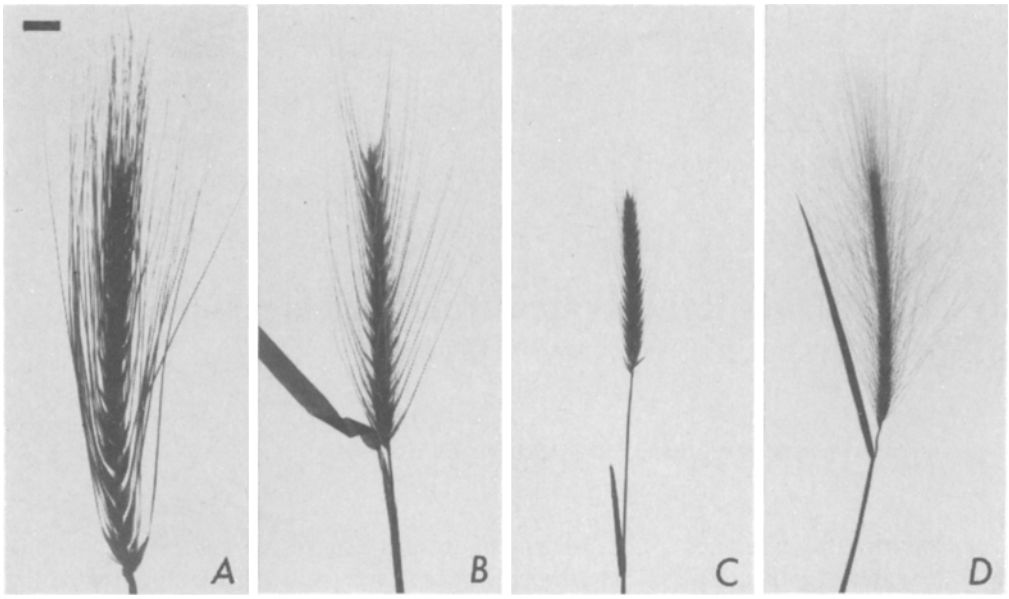

Fig. 1A-D. Comparison of floral morphology between (A) HV, (B) typical hypo-6x, (C) typical HV×HJ, and (D) HJ to show differences in size and morphology. (Bar = 10 mm)

HV × HJ, several of which possessed doubled chromosome complements. This paper will describe how the above techniques were utilized and present a cytogenetic analysis of certain plants recovered in this manner.

Material and Methods

The interspecific cross of *H. vulgare* (HV, 2n=2x=14) × *H. jubatum* (HJ, 2n=4x=28) (the 2n=3x=21 F_1 hybrid designated as HV × HJ) exhibited extremely low seed set. The use of embryo culture was necessary to recover whole plants (Steidl 1976). Immature embryos of HV × HJ isolated immediately after the cross was made were placed on agar-solidified MS medium (as modified by Linsmaier and Skoog 1965) supplemented with 5 mg/l 2,4-D + 4% sucrose (W:V) using method described previously (Orton 1979). The resulting callus cultures were maintained in the dark at 25°C on B5 medium (Gamborg et al. 1968) supplemented with 4 mg/l 2,4-D + 3% sucrose. Calli were transferred to the same medium containing 0.005% or 0.01% colchicine for four days, and then onto MS medium + 5.0 g/l inositol + 2% sucrose and exposed to 16 hr/day of light (2000 to 3250 lux from GE F96T10CXW bulbs) to induce regeneration (T.B. Rice, unpublished). Approximately 50 regenerating plantlets were isolated and placed on the same medium in deep petri dishes to facilitate continued growth. After sufficient growth, the plants were potted in soil and maintained in a greenhouse at 24 to 28° during the day and 21 to 24°C during the night.

Spikes of the proper stage were removed from the plant and fixed in fresh 1:3 acetic ethanol under vacuum for 24 hours at 25°C for cytological preparations of microsporocytes. Anthers were excised, macerated, and stained in aceto carmine for 30 seconds. Microsporocytes were then squashed with a coverslip, the slide was warmed gently, and sealed with dental wax. Associations of chromosomes at metaphase I were recorded for each floret (3 anthers). Various stages of microsporogenesis were photographed to reconstruct to progression of meiosis in these plants. Root tips were pretreated in 0.05% aqueous colchicine for 2 h, fixed as described above, hydrolyzed for 15 min. in 1N HCl (60°C), stained with Feulgen reagent for 2 h, and squashed with a coverslip in 45% acetic acid. Electrophoretic techniques will be described in a forthcoming publication (Orton 1980).

Results

Chromosome numbers in regenerated plants appeared to be quite variable, although counts on root tips HV × HJ invariably exhibited 21 chromosomes. Chromosome counts on root tip cells of regenerating plants from colchicine-treated callus indicated that 40 had approximately 21 chromosomes (3x) and 10 had chromosome numbers ranging from 30-44, designated as hypoautoallohexaploids[1] (henceforth referred to as hypo-6x). Distinct morphological differences were observed between the original HV × HJ hybrid and hypo-6x plants; the latter were more robust (Fig. 1). Esterase, peroxidase, and acid phosphatase isozyme activities were also greater in hypo-6x regenerates than in the original HV × HJ hybrid (Fig. 2). The entire population of hypo-6x plants was unconditionally self-sterile. Observations of mature florets revealed reduced, deformed, or missing anthers. No hybrid embryos were recovered when florets of hypo-6x regenerates were pollinated by HV and HJ (Steidl 1976).

1 The term hypoautoallohexaploid is used here to denote plants exhibiting variable chromosome numbers with a mean slightly less than the expected hexaploid number of 42

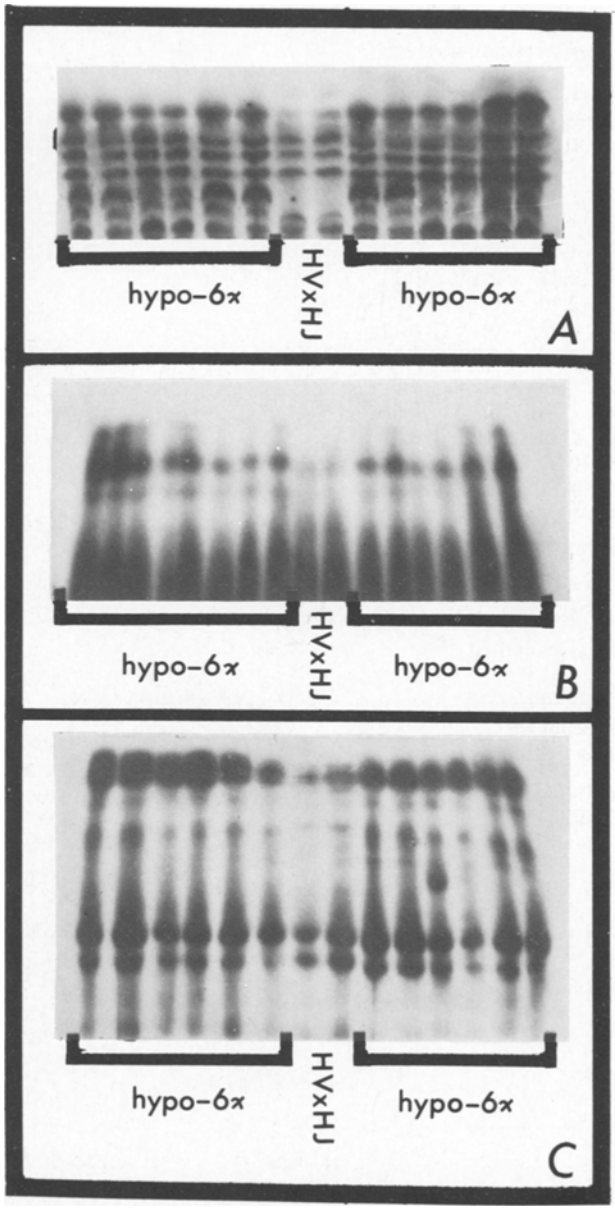

Fig. 2A-C. Comparison of (A) Cathodal peroxidase, (B) anodal acid phosphatase, and (C) anodal esterase zymograms showing quantitative differences in band staining intensity (enzyme concentration) between HVXHJ and regenerated hypo-6x plants (grouped in paired replicates)

Mean chromosome numbers varied from 35.43 to 37.49 among hypo-6x plants (Table 1). The range of counts was approximately the same among all plants, considering the numbers of cells counted. Chromosome counts of one plant (plant 1, Table 1) were further partitioned to spikes and florets to gain insight into the possible source of this variation. Means and ranges of chromosome numbers were generally similar among all tissues. Comparisons of mean associations per cell were also very similar among plants, tillers and florets (Table 1). The overall mean numbers of chromosome associations per cell were as follows: 6.45 I + 12.20 II + 1.74 III + 0.15 IV + 0.05 V. An example of typical metaphase I association is pictured in Figure 3 B.

Meiosis in hypo-6x plants proceeded normally from prophase I (Fig. 3 A) to metaphase I (Fig. 3 B). At anaphase I, however, separation of chromosomes was clearly abnormal. Frequently, univalents appeared to have attached to the spindle and migrated towards the poles (Fig. 3 C) and bivalents and multivalents had failed to separate normally, often fractionating, forming bridges, or moving partially toward one pole. At telophase, the spindle invariably became divergent and migration of chromatin to the poles was not observed (Fig. 3 D). The dispersed chromatin then became decondensed and a transverse wall was formed across the cell. Subsequently, a second set of spindles was formed, usually perpendicular to the cell wall (Fig. 3 E, right side) which was often split or multipolar (Fig. 3 F). Abnormal chromatin separation at this stage culminated in the formation of cells with large numbers of micronuclei of various sizes (Fig. 4 B). Micronuclei were singularly or multiply packaged by cell membranes (Fig. 4 C). Each unit then separated (Fig. 4 D) and gave rise to infertile pollen-like structures (Fig. 4 E). No dyad chromosomes were observed at any stage of the process in this study or that of Murry (1975), suggesting (the possibilities) that no premeiotic DNA synthesis had occurred.

Type A calli of the HJ parent were treated with colchicine and regenerated in a similar manner as with HV × HJ. Six of ten tillers from regenerated plants had approximately the expected doubled chromosome complement of 56 (50 to 58, Fig. 4 F) and spikes of these doubled tillers exhibited approximately 80% selfed seed set.

Discussion

Regeneration of polyploid plants from diploid-derived callus has been reported frequently (Murashige and Nakano 1966; D'Amato 1977; Sunderland 1977). In type A callus cultures of *Hordeum* and corresponding regenerated plants, however, all chromosome numbers were approximately equal to that of the original explant (Orton 1980). Hence, the recovery of hypo-6x regenerates was most likely a result of in vitro doubling of chromosome numbers by colchicine.

The overall range of chromosome numbers among microsporocytes of the original HV × HJ hybrid was large, but aneuploids accounted for only 12% of the cell population (Murry 1975). The callus of the present study, however, was derived from young embryos which probably had not yet generated any such variability. It is hence reasonable to assume that most of the observed variation

Table 1. Summary of means, ranges and associations of chromosomes among regenerated hypoamphiploids, broken down according to source plant, tiller, and floret

Plant	Tiller	Floret	No. cells	Mean chromosome number	Range	Mean members, associations per cell				
						I	II	III	IV	V
1	–	–	55	37.49	31-44	6.53	12.20	1.84	0.15	0.07
	1	–	15	38.26	33-44	7.80	12.00	1.53	0.20	0.20
		1	12	38.83	33-44	7.59	12.50	1.58	0.17	0.17
	2	–	18	37.39	31-44	5.44	11.94	2.28	0.17	0.11
		1	5	37.20	33-43	5.60	11.20	2.20	0.40	0.20
		2	9	40.00	32-44	4.67	13.00	2.78	0.11	0.11
		3	4	31.75	31-32	7.00	10.50	1.25	0.00	0.00
	3	–	22	37.01	32-43	6.55	12.54	1.68	0.09	0.00
		1	8	35.88	32-39	7.88	10.38	2.25	0.13	0.00
		2	5	38.20	36-43	5.60	13.00	2.20	0.00	0.00
		3	9	37.44	34-41	5.89	14.56	0.89	0.11	0.00
2	–	–	7	35.43	32-39	6.43	12.00	1.71	0.00	0.00
3	–	–	13	36.00	32-40	6.14	12.31	1.31	0.23	0.00

Table 2. Comparison of means and ranges of chromosome numbers from tissues derived from HVXHJ

Tissue	Expected chromosome number	Mean chromosome number	Range	% aneuploid cells[a]
Original HVXHJ[b]	21	20-21	16-22	12.0
Type A callus of HVXHJ	21	18.10	8-24	90.0
Root tips regenerated from typa A callus	21	16.92	13-22	97.0
PMC's[c] from regenerated plants of untreated typa A calli	21	20.60	12-28	87.5
PMC's from regenerated hypo 6x	42	37.04	31-44	97.0

[a] with respect to expected chromosome number
[b] after Murry 1975
[c] PMC: Pollen Mother Cell

among cells of hypo-6x regenerates was a consequence of growth in vitro and was not pre-existent in explanted tissue (Table 2). The observed range limits of chromosome numbers among the hypo-6x plants were approximately 2x those observed in plants of the original HV x HJ hybrid, supporting the suggestion by D'Amato (1977) that polyploidy provides progressive buffering capacity for tolerance of aneuploidy.

The overall mean chromosome number of 75 metaphase I microsporocytes was 37.02 (Table 1), or a loss of approximately 5 chromosomes from the expected doubled number of 42. Non random chromosome loss is a common phenomenon among interspecific and intergeneric hybrids of the Gramineae (Kasha 1975). HV-like plants have been observed to arise spontaneously from perennial HV x HJ stocks (Steidl 1976) and haploid HV-like plants have been regenerated from HV x HJ callus cultures (Orton, 1980) suggesting that HJ chromosomes may be selectively eliminated from the hybrid, albeit slowly. Attempts to develop a technique to distinguish HV and HJ chromosomes by banding have been unsuccessful. Morphologically, hypo-6x plants are more robust than HV x HJ, but exhibit qualitative intermediacy. It is hence likely that chromosomes were not lost selectively, but further experiments were needed to resolve this possibility.

Cytological observations of microsporogenesis in hypo-

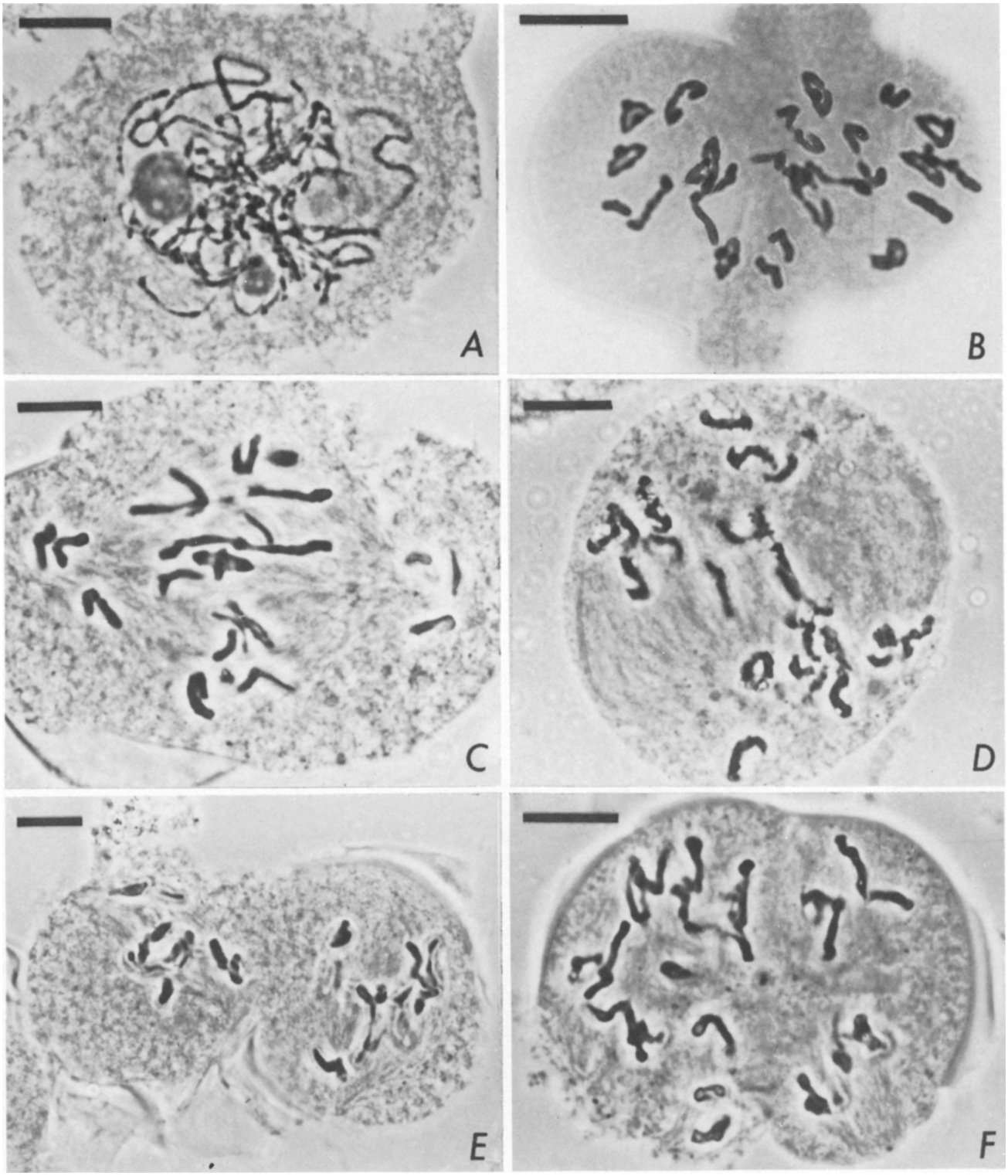

Fig. 3A-F. Microsporogenesis in hypo-6x plants: (A) zytogene, (B) metaphase I cell with 10 univalents and 16 bivalents, (C) anaphase I showing abnormal movement of chromatin; note the movement of monad chromosomes toward the poles, (D) decondensation of chromatin and persistent bridge at late telophase, (E) abnormal separation of chromatin at anaphase II; supernumerary spindles are perpendicula to the axis of the cell plate (Bar = 10 μm)

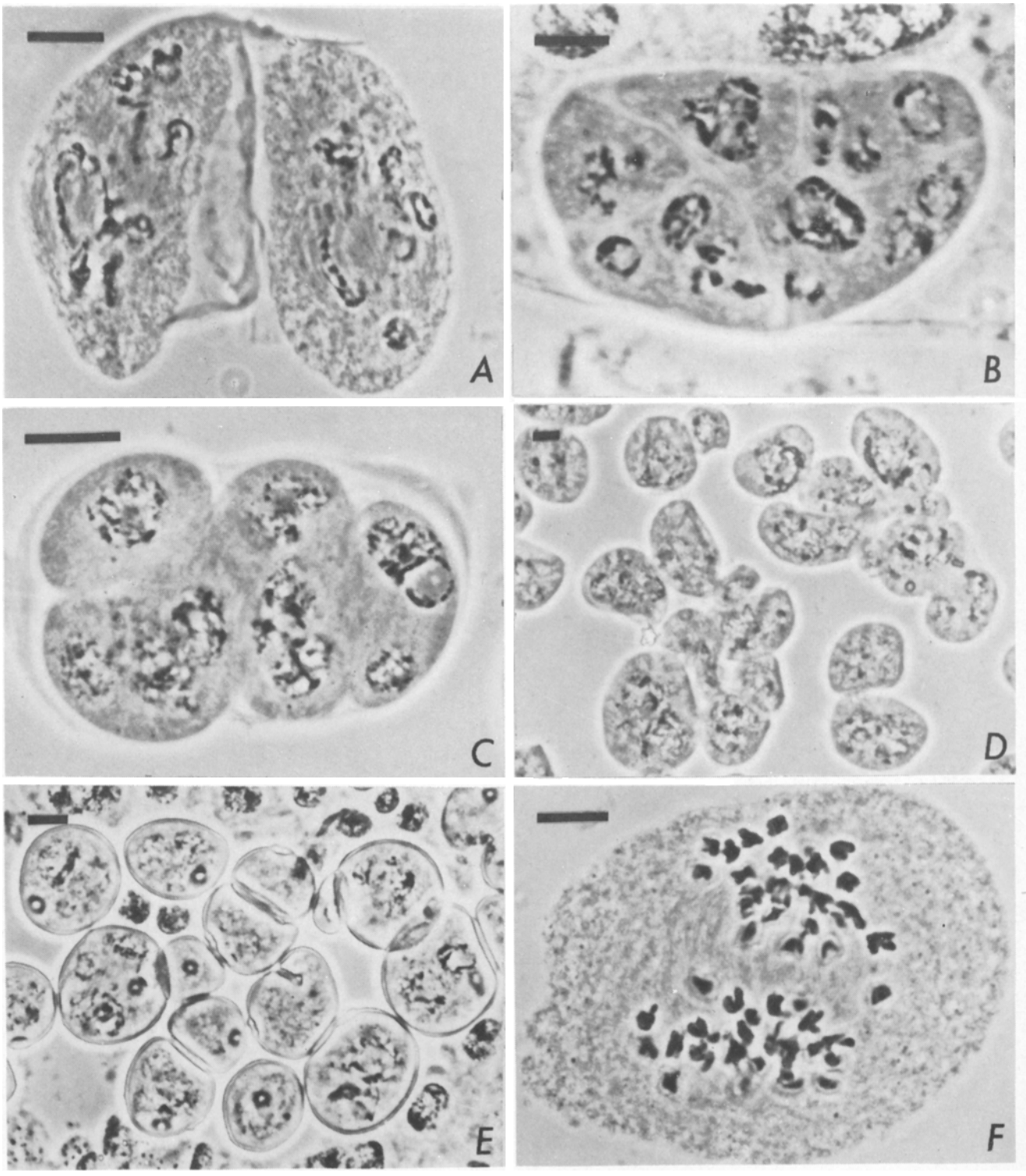

Fig. 4A-F. Microsoprogenesis in hupo-6x plants (con't): (A) Late telophase II cells showing dispersed and bridged chromatin, (B) formation of numerous micronuclei in posttelophase II microsporocyte, (C) 'packaging' of micronuclei by supernumerary cytokinesis, (D) formation of separate cells of many different sizes, and containing different amounts of cromatin in postmeiosis microsporocytes, (E) formation of inviable pollen-like grains (F) 25-25 anaphase I separation in a microsporocyte of plant regenerated from colchicine-treated HJ callus (Bar = 10 μm)

6x regenerates revealed a breakdown of meiosis manifested in two cytologically distinct phenomena: 1) Chromosomes/chromatin did not interact normally with spindle forces; chromatin bridges and fragments were observed frequently (Figs. 3C-F, 4 A). No dyad chromosomes were observed, a phenomenon possibly related functionally to spindle-chromatin abnormalities. 2) Spindle expression appeared to be abnormal. At anaphase I, spindles were divergent (Fig. 3 D) and at anaphase II, supernumerary spindles were frequently observed (Fig. 3 B). A similar breakdown of meiosis has been reported in a *Lolium-Festuca* hybrid concomitant with abnormal spindle expression (Darlington and Thomas 1937).

The sterility in hypo-6x plants of this study was probably due to the observed breakdown in meiosis, and not the lack of sufficient chromosome pairing. Mass pollinations of florets with fertile HV and HJ pollen yielded no seed set suggesting that these plants were female sterile as well, the causes of which remain unknown. Murry (1975) has proposed that this phenomenon is a consequence of incompatible interactions between parental genomes at meiosis. Steidl (1976) has speculated that all interspecific hybrids which juxtapose the genomes of HV and HJ might be sterile for this reason.

The recovery of pure doubled tillers following in vivo colchicine treatments is relatively infrequent in species and hybrids of *Hordeum* and *Agropyron* (Steidl 1976). In this study, however, 20 to 60% of regenerated tillers from colchicine-treated callus cultures of this study had approximately doubled chromosome complements and those of HJ exhibited high fertility. Hence, in the absence of genomic incompatibilities which cause sterility, tissue culture is a potentially powerful tool for the production of fertile intertaxon hybrid intermediates.

Acknowledgment

The authors wish to thank P.G. Carlson and J.E. Grafius for support and critical evaluation. Most of the details of tissue culture, in vitro colchicine treatments, and regeneration of HV × HJ were elucidated by T.B. Rice.

Literature

Caldwell, R.M.; Schafer, J.F.; Compton, L.E.; & Patterson, F.L. (1965): Resistance to leaf rust derived from *Agropyron elongatum*. Int. Wheat Rust Conf. **3**, 95-96

D'Amato, F. (1977): Cytogenetics of differentiation in tissue and cell cultures. In: Plant cell, tissue, and organ culture (eds.: Reinert, J.; Bajaj, Y.P.S.), pp. 343-357. Berlin-Heidelberg-New York: Springer

Darlington, C.D.; Thomas, P.T. (1937): The breakdown of cell division in a *Festuca-Lolium* derivative. Ann. Bot. **1**, 747-761

Galinat, W.C. (1977): The origin of corn. In: Corn and corn improvement. (ed. Sprague, G.F.), pp. 1-48. Madison, Wis. Amer. Soc. Agron.

Gamborg, O.L.; Miller, R.A.; Ojima, K. (1968): Nutrient requirements of suspension cultures of soybean root cells. Exper. Cell Res. **50**, 148-151

Kasha, K.J. (1975): Haploids from somatic cells. In: Haploids in higher plants, advances and potential. (ed.; Kasha, K.J.), pp. 67-90. Guelph, Ont. (Canada): University of Guelph

Knott, D.R. (1961): The inheritance of rust resistance 6: The transfer of stem rust resistance from *Agropyron elongatum* to common wheat. Can. J. Plant Sci. **41**, 109-123

Knott, D.R.; Dvorak, J.; Nanda, J.S. (1977): The transfer to wheat and homoeology of an *Agropyron elongatum* chromosome carrying resistance to stem rust. Can. J. Genet. Cytol. **19**, 75-79

Larson, R.I.; Atkinson, T.G. (1973): Wheat-*Agropyron* chromosome substitution lines as sources of resistance to wheat streak mosaic virus and its vector, *Aceria tulipae*. Proc. Int. Wheat Genet. Symp. **4**, 200-214

Linsmaier, E.M.; Skoog, F. (1965): Organic growth requirement of tobacco tissue cultures. Physiol. Plant. **18**, 100-127

Murashige, T.; Nakano, R. (1966): Tissue culture as a potential tool in obtaining polyploid plants. J. Hered. **57**, 115-118

Murry, L.E. (1975): A cytogenetic investigation of × *Agrohordeum pilosilemma*. E. Lansing, Mich.: Ph.D. Thesis, Michigan State University

Orton, T.J. (1979): A quantitative analysis of growth and regeneration from tissue cultures of *Hordeum vulgare, H. jubatum*, and their interspecific hybrid. Environm. Exper. Bot. (in press)

Orton, T.J. (1980): Chromosomal variability in tissue cultures and regenerated plants of *Hordeum*. Theor. Appl. Genet. **56**,

Rajathy, T.; Morrison, J.W. (1959): Cytogenetic studies in the genus *Hordeum 4*: Hybrids of *H. jubatum, H. brachyantherum, H. vulgare*, and a hexaploid *Hordeum* sp. Can. J. Genet. Cytol. **1**, 124-132

Rick, C.M. (1973): Potential genetic resources in tomato species: clues from observations in native habitats. In: Genes, enzymes and populations. (ed.: Srb, A.M.), pp. 235-269. New York: Plenum Press

Sears, E.R. (1956): The transfer of leaf-rust resistance from *Aegilops umbellulata* to wheat. Brookhaven Symp. Biol. **9**, 1-22

Sears, E.R. (1972): Chromosome engineering in wheat. Stadler Symp. **4**, 23-38

Smith, H.H. (1971): Broadening the base of genetic variability in plants. J. Hered. **62**, 265-276

Starks, G.D.; Tai, W. (1974): Genome analysis of *Hordeum jubatum* and *H. compressum*. Can. J. Genet. Cytol. **16**, 663-668

Steidl, R.P. (1976): The hybridizations of barley (*Hordeum vulgare* L. Emend Lam.) with its wild relatives. E. Lansing, Mich.: Ph.D. Thesis, Michigan State University

Sunderland, N. (1977): Nuclear cytology. In Plant cell and tissue culture. Vol. 2, pp. 177-206. Berkeley, Calif.: University of California Press

Wagenaar, E.B. (1960): The cytology of three hybrids involving *Hordeum jubatum* L.: The chiasma distributions and the occurrence of pseudoring bivalents in genetically-induced asynapsis. Can. J. Bot. **38**, 69-85

Accepted October 30, 1979
Communicated by F. Mechelke

Prof. Dr. T.J. Orton
Department of Vegetable Crops
University of California
Davis, CA 95616 (USA)

Dr. R.P. Steidl
Cargill Seeds
Monticello, IL 61856 (USA)