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A study of regeneration and karyotypic variability in tissue cultures of *Hordeum vulgare*, *H. jubatum*, and their interspecific hybrid.

presented by

Thomas James Orton

has been accepted towards fulfillment
of the requirements for

Doctoral degree in Botany and Plant Pathology and Genetics

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A STUDY OF REGENERATION AND KARYOTYPIC VARIABILITY IN TISSUE CULTURES OF *HORDEUM VULGARE*, *H. JUBATUM*, AND THEIR INTERSPECIFIC HYBRID

By

Thomas James Orton

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

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Department of Botany and Plant Pathology
and Genetics Program

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ABSTRACT

A STUDY OF REGENERATION AND KARYOTYPIC VARIABILITY IN TISSUE CULTURES OF *HORDEUM VULGARE*, *H. JUBATUM*, AND THEIR INTERSPECIFIC HYBRID

By

Thomas James Orton

The range of cultivation of barley (*Hordeum vulgare*, HV) is limited by sensitivity to environmental factors. Earlier testing of the inheritance of certain such phenotypes for breeding purposes indicated the need for additional genetic variability in the HV gene pool to overcome this sensitivity. *H. jubatum* (HJ) was utilized for the introgression of genes into HV. All HVxHJ hybrids were sterile and unyielding to *in vivo* techniques to restore fertility. Tissue culture was investigated in this study as a means to achieve *in* vitro diploidization in HVxHJ and as a tool for the induction and recovery of new sources of genetic variability.

It was necessary to perfect techniques for callus induction, growth, and plant regeneration in *Hordeum* for this study. Numerous explanted tissues were placed on media containing 4 mg/l 2,4-dichlorophenoxyacetic acid for callus induction. Calli were then placed onto various media to test the effects of mineral nutrients, hormones, cAMP, and pH on growth and regeneration. Immature ovarian tissues, taken from florets between 10 and 50% of mature size, gave the highest frequency of primary callus formation. Cultures assumed one of five callus types, distinguishable by differences in cell

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size, tissue consistency, growth rate and habit, karyology, and organogenic potential. Regeneration was a quantitative phenomenon, requiring analyses of measured responses to ascertain treatment effects. Media containing gibberellic acid promoted primary root and shoot formation and inhibited crown root formation. All indoleacetic acid (IAA)-amino acid conjugates tested promoted callus growth and inhibited organogenesis more effectively than free IAA. Whole plants were regenerated from callus cultures of HJ and HVxHJ, but not of HV.

In previous studies, it was hypothesized that sterility of HVxHJ was due to the lack of chromosome pairing at meiosis. By doubling chromosome complements in vitro, it was hoped that fertile amphiploids could be regenerated. Calli of HVxHJ were treated with colchicine in solid media and regenerated into whole plants, 20% of which exhibited variable chromosome numbers with means slightly less than the expected amphiploid number of 42 (hypoamphiploids). All hypoamphiploids were sterile due to a breakdown of meiosis, despite a high degree of chromosome pairing.

Initial cytological observations of cultured Hordeum cells indicated chromosome number variability, perhaps encompassing an array of novel associations of chromosomes for genetics and breeding. In tissue-totipotent HVxHJ callus, mean chromosome numbers remained stable while aneuploidy accumulated over time. Polyploidy and chromosomal rearrangements were also detected in certain cultures. By physically separating callus tissue, it was possible to segregate and propagate chromosomal variants.

The agronomic and genetic significance of in vitro karyotypic variability depends on its expression in regenerated plants. Cytogenetic and isozymic analyses were used to trace the pathway of karyotypic variability into regenerates. Regenerating roots exhibited a complete loss of polyploidy, and an attenuation of aneuploidy and chromosomal rearrangements. Isozyme analysis of HVxHJ regenerates provided preliminary evidence for the *in vitro* quantitative segregation of parental genomes. One HV-like regenerate was a haploid exhibiting normal meiosis, and yet retained esterase isozyme hybridity; this constitutes preliminary evidence for *in vitro* introgression of HJ genes into HV.

Methods for callus induction, growth maintenance, and regeneration of whole plants of *Hordeum* are described. From plants recalcitrant to *in vivo* diploidization, it was possible to regenerate doubled plants from colchicine-treated calli. Finally, this study demonstrated the use of tissue culture as a means of rapid introgressive breeding, of recovering fertile intermediates of sterile interspecific hybrids, and of isolating haploids.

Dedication

For Delynn

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The following were important and often instrumental to the work presented in this dissertation:

T.B. Rice; who elucidated most of the preliminary details regarding the tissue culture of Hordeum.

R.P. Steidl; who synthesized the interspecific hybrids and cared for plant material.

M.D. Peterson; who provided media for testing of IAA-amino acid conjugates.

J.F. Fobes; who generously provided equipment, supplies, and training for starch gel electrophoresis.

P.S. Carlson, W. Tai, J.E. Grafius, L.W. Mericle, and C.J. Pollard, my guidance committee; who provided materials, equipment, critical evaluation, and served as models of scientific excellence.

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A special acknowledgment to W. Tai, my co-adviser, who believed and fought, to P.S. Carlson, my other co-adviser, for his exuberant concern, creativity, and passion for good science, and to my parents for their understanding and support.

A very special acknowledgment to my wife Delynn who made it all possible, and to whom this dissertation is dedicated.

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CHAPTER I

INTRODUCTION

The range of barley (Hordeum vulgare L. Emend Lam.) is restricted by plant sensitivity to high moisture, salinity, and cold temperatures. Further, the foliage of barley is susceptible to damage by the larvae of the cereal leaf beetle since the absence of epidermal trichomes allows the adult female to lay eggs on plant surfaces.

After an initial screen of available germ plasm with respect to these traits, it was determined that little or no additive genetic variance was available for purposes of breeding and selection (J. E. Grafius, personal communication). Two techniques were available for introducing new variability for these traits into the gene pool: mutation or the introgression of genes from sexually cross-compatible wild relatives. Mutations occur at random in the genome and rarely manifest genetic variability in the desired direction. Multiple lesions often mask rare desirable effects. Further, if a phenotype in question is complex, as with regulatory or developmental traits and agronomic characters, the probability of desirable mutants would require prohibitively large populations for screening. Similar problems in wheat and corn had proven quite amenable to the introgression approach. Hence, in the late 1960s, Dr. J. E. Grafius of the Dept. of Crop and Soil Sciences, Michigan State University, initiated an introgressive breeding program in barley with the objectives of transferring desirable genes for the above traits from wild gene pools into cultivated barley.

Hybridization between species and genera is becoming an increasingly important means of introducing genetic variation into the gene pools of crop plants (Smith 1971). Treating the genus or family taxa as potential gene pools is of particular interest to plant breeders. The possibility exists for substituting and adding genetic information from wild gene pools into the cultivated gene pool, as has been demonstrated in wheat (Sears 1972) and corn (Galinat 1977). In wheat, interspecific and intergeneric hybridization and backcross programs have introgressed such traits 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). Many interspecific and intergeneric hybrids have been successfully synthesized in the genus *Hordeum*, opening up the possibility of introgressive breeding in barley (Nilan 1964).

H. *jubatum* is a weedy perennial which embodies a desired range of phenotypes for moisture, salt, and cold sensitivity and epidermal trichomes. Further, it has been shown to be sexually cross-compatible with *H*. *vulgare*, the cross with *H*. *jubatum* as female parent culminating in relatively vigorous F_1 plants of intermediate morphology (Wagenaar 1960, Rajathy and Morrison 1959). Subsequently, Steidl (1976) has performed the reciprocal cross and obtained F_1 plants of much higher vigor, indicating a differential

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The F_1 hybrid between HV and HJ was sterile in both directions (Wagenaar 1960, Steidl 1976). HV is a diploid (VV, $2n=2x=14$) whereas HJ has been shown to be allotetraploid ($2n=4x=28$) with its two genomes possessing some degree of segmental homology (designated AAA'A', Starks and Tai 1974). The F_1 hybrid (VAA', $2n=3x=21$), however, has been shown to exhibit very little autosyndesis at meiosis, perhaps due to the presence of a homoeologous pairing inhibitor, as with $5B^L$ in wheat (Murry 1975). Hence, it was hypothesized that F_1 sterility was directly attributable to the lack of chromosome pairing.

Several attempts were made to circumvent this sterility. In specific examples with wheat, cross incompatibility has been overcome by interceding a bridge species with which both species were cross-compatible. Limited trials of this technique led to the conclusion that any juxtaposition of HV and HJ genomes culminated in F_1 sterility. Further, attempts were made to double chromosome numbers in tissues of the F_1 hybrid to induce amphiploidy. Intensive efforts with several proven techniques failed to give rise to somatically doubled tillers. It was concluded that in vivo interactions of F_1 tissue resulted in the suppression of doubled sectors, or that

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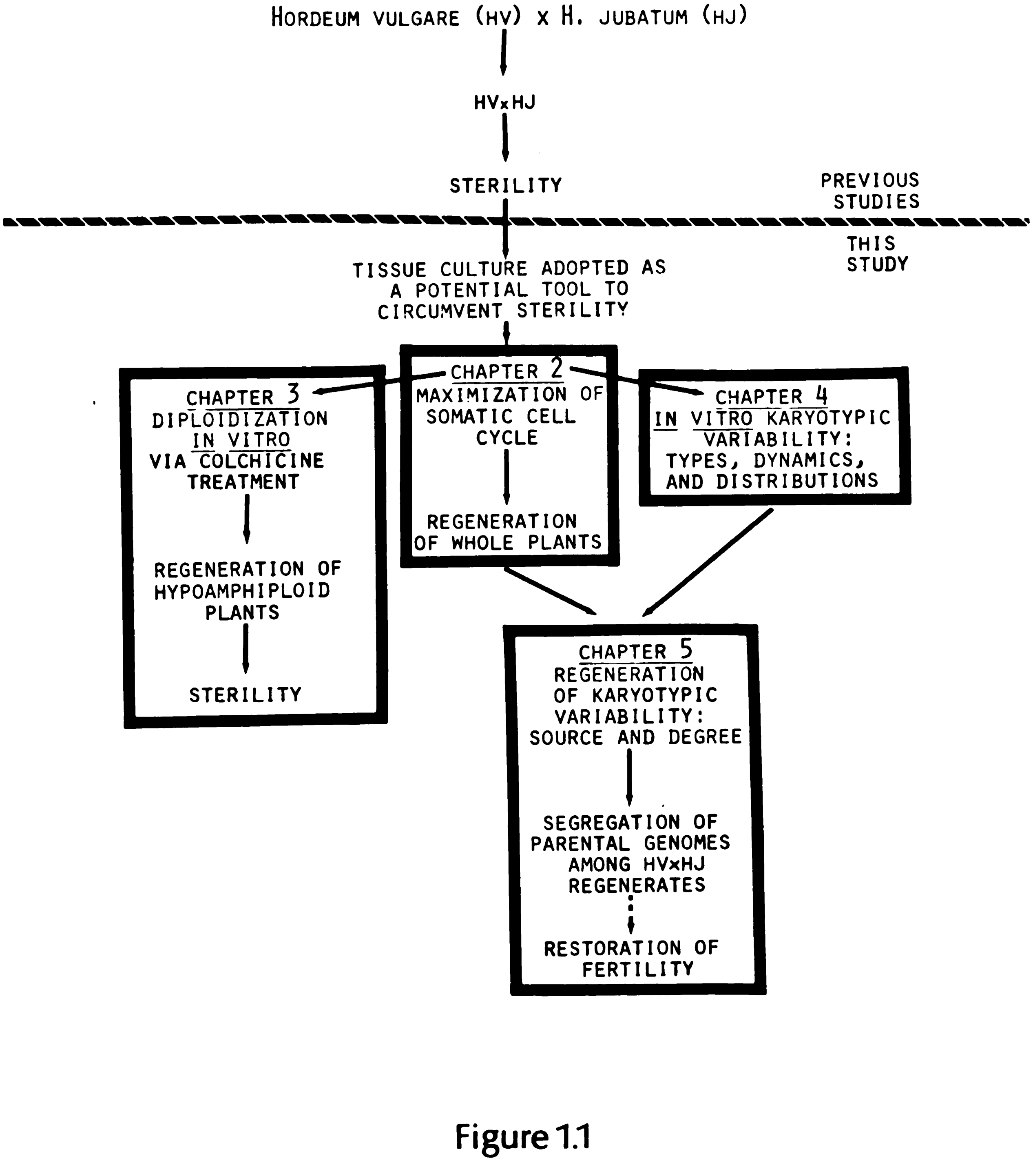
Tissue culture is a tool which provides the possibility of dealing with large populations of single cells as discrete organisms. Tissue culture was advanced as a possible means of isolating pure amphiploid sectors by regenerating whole plants from colchicine-treated cultures (Steidl 1976). In addition, the possibility existed for the induction of variability in vitro by mutagenesis or somatic fusion. Hence, studies on tissue cultures of Hordeum were initiated in the Fall of 1974 by Dr. T. B. Rice, then a Postdoctoral Fellow in the laboratory of Dr. P. S. Carlson, Dept. of Crop and Soil Sciences, Michigan State University.

This Dissertation will outline the steps taken in attempts to utilize tissue culture for introgression between HV and HJ and the isolation of fertile intermediates from this cross. Figure 1.1 illustrates these steps and their corresponding interrelationships. Chapter 2 will describe efforts to define the conditions which maximize growth and regeneration from callus cultures of HV, HJ, and HVxHJ. In Chapter 3, a technique of in vitro doubling of chromosome numbers will be described. This technique was used for the successful isolation of sterile 'hypoamphiploid' plants of the F_1 hybrid. Finally, these plants were analyzed cytogenetically to determine a possible cause of sterility. Previous studies indicate that cultured eukaryotic tissues exhibit variability in chromosome number and morphology. Chapter 4 will examine sources and degrees of in vitro karytypic variability in Hordeum, its possible sources, and the preliminary possibility of its utilization as genetic

Figure 1.1: Diagrammatic summary of research presented in the chapters of this dissertation, indicating stepwise relationships.


HORDEUM VULGARE (HV) x H. JUBATUM (HJ)
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THIS STUDY
TISSUE CULTURE ADOPTED AS A POTENTIAL TOOL TO CIRCUMVENT STERILITY
CHAPTER 2
MAXIMIZATION OF SOMATIC CELL CYCLE
REGENERATION OF WHOLE PLANTS
CHAPTER 3
DIPLOIDIZATION IN VITRO VIA COLCHICINE TREATMENT
REGENERATION OF HYPOAMPHIPLOID PLANTS
STERILITY
CHAPTER 4
IN VITRO KARYOTYPIC VARIABILITY: TYPES, DYNAMICS, AND DISTRIBUTIONS
CHAPTER 5
REGENERATION OF KARYOTYPIC VARIABILITY: SOURCE AND DEGREE
SEGREGATION OF PARENTAL GENOMES AMONG HVxHJ REGENERATES
RESTORATION OF FERTILITY

Figure 1.1

variability. The expression of *in vitro* karyotypic variability in regenerated whole plants of HVxHJ will be investigated in Chapter 5. Further, a technique will be introduced for the identification of parental segregants in populations of plants regenerated from callus cultures of HVxHJ. Collectively, these findings will be shown to constitute new preliminary techniques for *in vitro* introgression, isolating haploids, and circumventing hybrid sterility.

CHAPTER II

A QUANTITATIVE ANALYSIS OF GROWTH AND REGENERATION FROM TISSUE CULTURES OF *HORDEUM VULGARE*, *H. JUBATUM*, AND THEIR INTERSPECIFIC HYBRID

Introduction

Realization of the full potential of somatic cell genetics in higher plants is predicated on the ability to induce desired developmental states. Callus has now been induced in a large number of species, indicating that this phenomenon is not limiting (Narayanaswamy 1977). Various manifestations and degrees of organogenesis have generally been observed subsequent to callus formation. The culmination of organogenesis, plantlet formation, is sporadic, and is a major factor limiting progress. As a group, dicotyledonous plants are much more responsive to de- and redifferentiation than monocotyledonous plants. Among the monocots, the annuals, such as wheat, corn, rice, barley, sorghum, oats, etc., are most resistant to callus formation and regeneration. Although whole plants have been regenerated in all of these groups, it has been argued (for wheat) that plantlet regeneration is not a true consequence of induction (Bhowanji and Hayward 1977). From most reports, it is clear that regeneration in this group is not an "all or none" response with respect to experimental conditions.

There appear to be three possible reasons why it is not possible to consistently obtain organogenesis in annual grains: 1) different genotypes tend to vary with respect to their proficiency in de- and redifferentiation (Green and Phillips 1975, Jacobsen 1976), 2) callus is a term used to describe a wide range of developmental states which differ in morphogenic potential, and 3) depending on exogenous conditions, karyotypic abnormalities tend to accumulate over time in culture (Torrey 1967, Bayliss 1975, Sunderland 1977) concomitant with a decline in regenerative potential (Reinert and Backs 1968, Smith and Street 1974). The conclusions of past studies are difficult to compare because complete information regarding genotype, callus growth form, and karyotype were usually not reported.

The present study was undertaken with the objective of defining the conditions required to demonstrate the somatic cell cycle in _Hordeum_ _vulgare_ (cultivated barley), _H_. _jubatum_, and their interspecific hybrid. Because the regenerative response is not "all or none," I employed a quantitative analysis. The experiments reported in this chapter were designed to measure the significant effects of certain potential modifiers on tissue necrosis, callus growth, and regeneration. By a statistical treatment of data from a large number of trials, significant differences among treatments were detected.

Materials and Methods

Diploid _H_. _vulgare_ L. Emend. Lam. cv Coho (HV, 2n=2x=14), _H_. _jubatum_ L. (HJ, 2n=4x=28), and their interspecific hybrid (HVxHJ, 2n=3x=21, HV as maternal parent) were obtained from Dr. J. E. Grafius, Dept. of Crop and Soil Sciences, Mighigan State University. Plants were maintained in greenhouses at approximately 25 to 28°C during the day and 21 to 25°C during the night. These were the conditions under which all plants grew vigorously and karyotypes were invariant.

Viable, healthy tissue from various parts of whole plants were excised, surface-sterilized in 95% ethanol for 30 seconds, washed twice in sterile distilled water, and placed on agar-solidified media. If a high incidence of contamination was obtained after this treatment, tissues were sterilized in 10% chlorox for 10 minutes and washed twice in sterile distilled water. Callus induction media were those of Murashige and Skoog (1962)(MS, as modified by Linsmaier and Skoog 1965) supplemented with 5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 4% sucrose, and that of Gamborg and Eveleigh (1968)(B5) supplemented with 4 mg/l 2,4-D and 3% sucrose. All media were solidified with 0.9% Difco Bacto Agar. To characterize that developmental stage giving maximal callus induction from ovaries, the ratio of excised palea length to mature palea length was recorded.

After induction, calli were maintained on B5 medium supplemented with 3 to 4 mg/l 2,4-D and 2 to 3% sucrose in the dark at 25°C. Asceptic transfers were performed every four to five weeks. Suspension cultures were initiated by introducing vigorously growing, friable callus into the same medium lacking agar, in 125 ml Ehrlenmeyer flasks rotated at 120 RPM. Finely divided, rapidly growing suspension cultures (doubling time = 48 hrs.) were easily isolated from all three parental sources.

Initially, I hoped that growth rates would show the effects of media on callus growth. Uniform callus tissue was subdivided into six subcalli of equal size, plated onto fresh medium and transferred every two weeks. Growth rate was measured by determining the relative fresh weight (initial fresh weight = 100%) over the course of 40 days at four to seven day intervals. Linear regression equations and 95% confidence intervals of regression coefficients (relative fresh weight on time) were calculated. To determine fresh weight (FW): dry weight (DW) ratios, uniform callus tissue was subdivided into ten equal pieces, weighed, dried for 48 hrs. at 60°C and reweighed.

Individual and synergistic effects of certain physico-chemical modifiers were ascertained in attempts to determine the medium conditions which elicit specific tissue responses. Callus tissue from 11 different cultures* was plated in replicate onto a medium grid consisting of 8 'Basic' x 12 'hormonal' treatments. Basic treatments included the following: (1) MS vs. B5 salts, (2) casamino acids without NH_4^+ salts vs. NH_4^+ salts, (3) sucrose vs. sucrose + glucose, (4) 100 mg/l vs. 5.0 g/l inositol, and presence vs. absence of (5) coconut water, (6) a water extract of developing barley caryopses, and (7) dimethyl sulfoxide (DMSO) (see legend of Fig. 2.3 for more details). Hormonal treatments included: (1) indole-3-acetic acid (IAA), (2) kinetin (KIN), (3) benzyladenine (BA), and (4) gibberellic acid (GA_3)(see legend of Fig. 2.4 for more details). Callus and suspension culture tissues were drawn from

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*For the purposes of this paper, a culture is defined as uniform, stable tissue derived from the same point of initial callus formation.

cultures exhibiting uniform, stable growth habit. Within each culture tested, all tissue had identical transfer histories. Fresh weight of calli varied from 0.10 to 0.20 g at the time of plating. For suspension cultures, approximately 1 ml of settled cell volume was plated on each dish as a lawn. Callus type, age,and a profile of chromosome numbers were recorded for each culture at the time of plating.

Also, tissue was plated in a similar manner onto media with various concentrations of cyclic AMP (0.0, 0.0005, 0.00025, 0.0005, 0.0025, 0.005, and 0.025%, W:V), pH levels (4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5), and selected combinations and concentrations of free IAA and IAA-amino acid conjugates. Conjugates tested included the following: IAA-D-alanine, IAA-L-alanine, IAA-glycine, IAA-β-alanine, and IAA-D-aspartate (see Table 2.5 for more details). Petridishes were maintained at 25°C under 16 hrs./day of light (2000 to 3250 lux) from GE F96T10-CWX bulbs. After 50 to 60 days, the dishes were randomized and tissue responses were scored. Standards for relative comparison were established before the scoring procedure was initiated. These consisted of drawings of minimal and maximal response which were compared with experimental tissue to determine the relative response (0 = minimum, 5 = maximum). Scores were determined for the following tissue response classes: tissue necrosis (discoloration), callus (unorganized) growth, and crown root, primary root, and shoot formation. Crown roots were tentatively distinguished from primary roots on the basis of the following criteria: crown roots lacked root hairs, were pigmented, and exhibited a characteristic negative to positive geotropism, while primary roots bore root hairs, were unpigmented, and exhibited positive geotropism (Fig. 2.6).

The variance of the scores for tissue response was partitioned and tested for each response class (i.e. tissue necrosis, callus growth, etc.) for each culture on the 8 basic x 12 hormanal grid. If a given variance was significant ($\alpha = .05$), means for that response class and culture were contrasted in pairwise comparisons, in a 95% confidence family, according to the Tukey procedure (Neter and Wasserman 1974). Contrasts for each response class were grouped according to genotype for each culture in order to condense the analysis and identify recurrent phenomena. An arbitrary scale was constructed to report the frequency of given significant contrasts (e.g. medium A vs. medium B) for each genotype and response class as follows: 0 to 33% of contrasts significant, 33 of 66% of contrasts significant, and 66 to 100% of contrasts significant. To negate the effects of tissue potential (see Table 2.3 for definition), only cultures exhibiting an overall mean response intensity greater than zero were considered (e.g. if a given culture showed no shoot regeneration, it was not included in determining overall significant effects relative to shoot regeneration).

Results

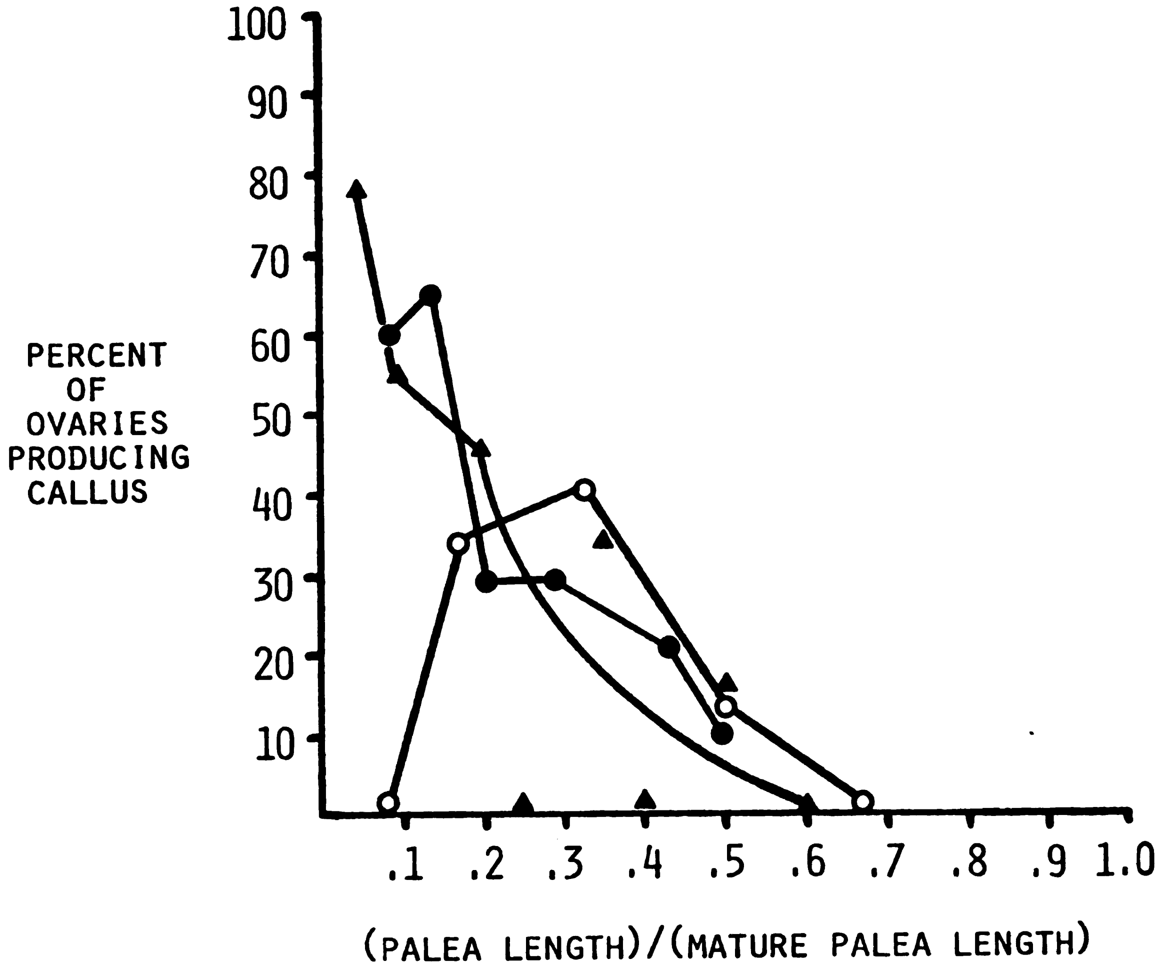
Previous studies dealing with annual grains have reported callus induction from embryo, endosperm, root, ovary, stem section (Yamada 1977), apical meristem (Cheng and Smith 1975, Koblitz and Saalbach 1977), microspore (Wilson 1977), and rachis (Dudits et al. 1975). In this study, callus was successfully induced from root meristems, immature ovaries and peduncles, and 3 day postpollination embryos and endosperms. Callus emanating from whole, immature ovaries was the most easily obtained and prolific, and was used exclusively for all growth and morphogenic measurements.

The frequency of callus induction (% of ovaries producing callus vs. developmental stage) was determined for all three genotypes. The highest frequency of callus induction occurred between 10 to 50% of mature palea length for all three genotypes (Fig. 2.1). Additionally, over all developmental stages, ovaries borne on the basal half of the rachis formed callus more frequently than those of the apical half.

Callus cultures of HJ and HVxHJ usually continued to grow rapidly subsequent to formation. Callus from HV, however, was very difficult to propagate. Typically, periods of several months to over a year elapsed before callus growth resumed, even with constant subculturing. Callus cultures adapted a stable, recognizable type soon after formation (Table 2.1, Fig. 2.2). The morphology and behavior of type A callus was very similar to the callus from scutellum in maize (Green _et al_. 1974) and from apical meristems of barley (Cheng and Smith 1975). Type E calli were generated at the original point of callus formation, or arose spontaneously from callus types A, B, or C. Type E calli spontaneously transformed very rarely to other callus types; (e.g. at a very low frequency, type A nodes were observed to arise within the martix of type E calli).

Table 2.2 displays regression coefficients for standardized FW vs. time. For all genotypes and callus types tested, significant

Figure 2.1: Graph of the percent of ovaries producing callus vs. developmental stage as determined by the ratio of palea length/mature palea length. ○ - HJ, ● - HVxHJ, ▲- HV.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(PALEA LENGTH)/(MATURE PALEA LENGTH)

Figure 2.1

TABLE 2.1 : Description of callus types

Type	Description	Observed in genotypes	Figure	Pigmentation	Relative growth	Organogenic potential
A	Very hard, large discrete nodes; small, unvacuolated cells occurring in closely associated meristemoids.	HV, HJ, HVxHJ	2A	yellow	linear, slow	shoots, crown roots, primary roots
B	Large, physically separated hard nodes (each a single meristem); gradient of cell size (small at center, large at cortex).	HV	2B	off-white to yellow	slightly exponential, moderate	crown roots, primary roots
C	Small hard nodes, loosely associated (relatively friable); consist of small unvacuolated cells	HJ	2C	off-white to yellow	slightly exponential, moderate	crown roots, primary roots
D	Consists of small cells which appear to exhibit some organization; relatively friable, impregnated with a muscilaginous exudate	HJ HVxHJ	2D	white	very slow	none
E	Highly friable; loosely associated single cells and clumps of large, amorphous parenchymal cells (highly vacuolated).	HV, HJ, HVxHJ	2E	white	exponential, very fast	none

Figure 2.2: Callus types in tissue cultures of *Hordeum*: (A) type A of HVxHJ, (B) type B of HV, (C) type C of HJ, (D) type D of HJ, (E) type E of HVxHJ (A to E X4.2), (F) comparison of the surface topology of type A vs. (G) type E callus (F and G X11), (H) histology of HVxHJ type A callus, squash preparation stained with feulgen, (J) Histology of HVxHJ type E callus, squash preparation stained with modified carbol fuchsin (H and J X178).

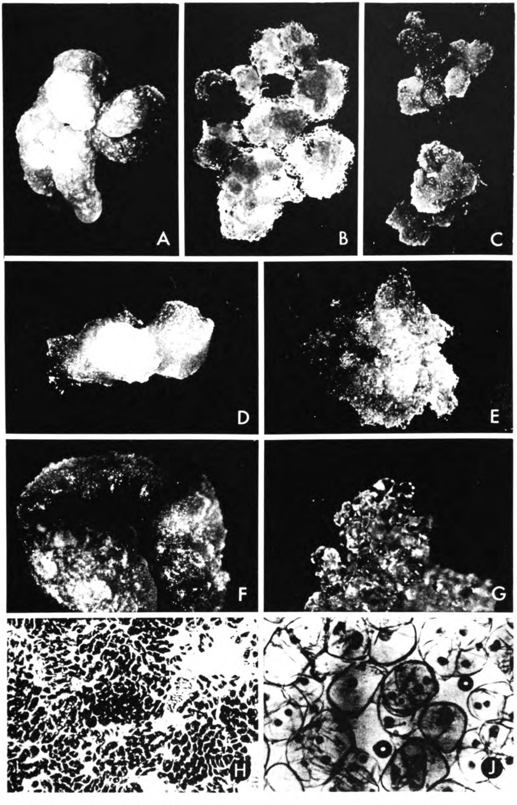

Figure 2.2

'between subcallus' differences were evident. Comparisons of mean growth regression coefficients indicated that type B callus had an intrinsic growth rate of approximately 2X that of type A callus. Growth rate of type C callus was intermediate between types A and B. Type E calli had not been observed at the time of these measurements, but has been observed to grow at a much faster rate than all other callus types. Note also that FW/DW ratios showed variability, especially in type E callus. Different sectors of the same callus, although morphologically uniform, showed intrinsically different growth potentials and degrees of hydration.

Pertinent characteristics regarding callus from the eleven cultures plated on the basic x hormonal grid are listed in Table 2.3. Cultures were grouped according to callus types to stress similarities within these types. No genome- or type-specific patterns were apparent for tissue necrosis. Relative callus growth was manifested similarly to the manner described above. Deviations from the expected hierarchy (i.e. type E > B > C > A > D) may have been due to the lack of 2,4-D in the test media. Type A calli exhibited a high spontaneous frequency of shoot formation, even when cultured on 4 mg/l 2,4-D. Type A callus cultures retained the ability to regenerate shoots up to 24 months after initiation. No regeneration of shoots was observed in types B and C calli although the extent of primary and, in most cases, crown root regeneration was comparable to type A callus. Type E calli and suspension lawns (which were derived from type E calli) exhibited little or no organogenesis. Insufficient amounts of callus type D precluded testing on this grid. In separate trials, however, type D callus demonstrated no

TABLE 2.2: Comparative growth rates and hydration among subcalli of morphologically uniform *Hordeum* callus

Parental explant	Subcallus	Callus type	Regression of fresh wt. on time			Mean FW/DW*	S.D. FW/DW*
			B_0	B_1	Mean B_1		
HJ	1	C	0.99	0.11	0.17	11.21	0.90
	2	C	1.05	0.06			
	3	C	-0.11	0.32			
	4	C	0.98	0.09			
	5	C	0.13	0.22			
	6	C	0.59	0.18			
HV	1	B	1.04	0.15	0.20	10.11	0.91
	2	B	0.67	0.19			
	3	B	0.30	0.30			
	4	B	1.01	0.15			
	5	B	0.90	0.15			
	6	B	0.00	0.27			
HVxHJ	1	A	0.82	0.07	0.11	8.69	0.51
	2	A	0.64	0.10			
	3	A	0.77	0.09			
	4	A	0.78	0.09			
	5	A	0.65	0.10			
	6	A	0.36	0.19			
HVxHJ	-	E	-	-	-	20.74	2.44

*Values not calculated from the same tissue used for growth rate determinations, but were taken from tissue of the same culture; N = 10.

TABLE 2.3: Summary of pertinent characteristics regarding callus tissue of cultures utilized in the Basic x Hormone grid.

Culture	Growth[a] habit	Tissue[b] plated	Callus type	Culture age	Reps per treatment	2n source plant	Mean chromosome number	M.S. chromosome number	Potential[c]				
									Tissue necrosis	Callus growth	Crown root	Primary root	Shoot
1) HJ-7	P	1^o callus[d]	C	14 Mo.	6	28	23.40	5.69	.32	.49	.19	.33	.00
2) HJ-4-3	P	1^o callus	C	24 Mo.	6	28	25.31	16.23	.77	.84	.23	.17	.00
3) HJ-4-3	P	1^o callus	C	28 Mo.	6	28	-	-	.55	1.22	.48	.42	.00
4) HJ-12	P	1^o callus	A	10 Mo.	6	28	27.56	2.17	.35	.25	.42	.13	.10
5) HJ-12	P	1^o callus	A	12 Mo.	6	28	26.37	6.47	.42	.25	.29	.17	.17
6) HVxHJ-57	P	1^o callus	A	9 Mo.	1	21	21.19	10.66	.43	.39	1.15	.30	.70
7) HVxHJ-3	P	1^o callus	A	24 Mo.	6	21	19.47	8.61	.76	.35	.53	.16	.05
8) HVxHJ-57	P	1^o callus	E	9 Mo.	6	21	54.56	863.13	.72	.17	.02	.00	.00
9) HVxHJ-57	P	2^o callus[e]	E	10 Mo.[f]	6	21	57.12	2693.18	1.16	.63	.00	.00	.00
10) HVxHJ-AE	P	suspension	-	26 Mo.[g]	1	21	42.35	256.87	.27	2.04	.00	.00	.00
11) HV-1	A	1^o callus	B	24 Mo.	6	14	31.46	80.43	.74	.42	.45	.20	.00

[a] of source plant; P = perennial, A = annual

[b] see Table 1 for an explanation of callus types

[c] Potential: overall mean response intensity of a given culture with respect to response class.

[d] primary callus: callus derived directly from serial subculturing of an explant on solid medium.

[e] secondary callus: callus derived from suspension culture.

[f] 6 Mo. 1^o callus, 2 Mo. suspension, 2 Mo. 2^o callus

[g] 8 Mo. 1^o callus, 18 Mo. suspension

organogenesis.

An analysis of variance was performed on response measurements for each culture and response class for the basic x hormonal grid. Nearly 100% of all partitioned variance proved to be significant for the following sources: between basic treatments, between hormone treatments, and basic-hormonal interactions. No further attempts were made to study the nature of these interactions. A composite of significant treatment contrasts is presented in Figs. 2.3, 2.4, and 2.5*. The conclusions of this analysis are summarized in Table 2.4. The most striking contrast among basic treatments was between MS and B5 salts. B5 salts tended to promote tissue necrosis, while MS salts promoted crown root formation in HJ.** The bulk of significant contrasts were apparent among hormone treatments. Media without hormones appeared to stimulate callus growth and inhibit tissue necrosis. IAA, alone or in combination with other hormones, promoted callus growth in HV. KIN alone had very little effect, but interacted with GA_3 as follows: it promoted callus growth and shoot formation in HVxHJ type A callus. BA, alone or in combination with KIN, promoted tissue necrosis in HV and HJ. In combination with IAA, and/or KIN, GA_3 suppressed crown root growth and stimulated primary root growth.

For quantitative ascertainment of the effects of cAMP and pH,

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*The bulk of raw data and analysis have been omitted due to the magnitude of material. This information can be supplied upon request.

**Conclusions concerning medium effects could also be interpreted conversely, e.g. 'treatment A promoted response x' could not be distinguished from 'all treatments except A inhibited response x.'

Figure 2.3: Significant pairwise contrasts for Basic treatments, broken down according to genotype and response class (row preferred, see example).

Treatment 1 - MS medium + 100 mg/l inositol

2 - MS medium

3 - B5 medium

4 - B5 medium, with NH_3 salts deleted, + 4.0 g/l casamino acids

5 - B5 medium + D-glucose (1%, W:V), + sucrose (1%, W:V)

6 - B5 medium + 20 ml/l coconut water

7 - B5 medium + 20 ml/l caryopsis extract

8 - B5 medium + 100 mg/l DMSO

Note: all media contain 2% sucrose (W:V) and 5.0 g/l inositol unless otherwise noted.

Key to Symbols Used:

Blank Cell) 0 - 33% of row means significantly different from column means.

◁) 33-66% of row means significantly less than column means.

▷) 33 - 66% of row means significantly greater than column means.

◀) 66 - 100% of row means significantly less than column means.

▶) 66 - 100% of row means significantly greater than column means.

Example:

	1	2	3
1		◁	▶
2			
3			

- For 33 - 66% of contrasts, the mean response of treatment 1 is significantly less than treatment 2.
- For 66 - 100% of contrasts, the mean response of treatment 1 is significantly greater than treatment 3.

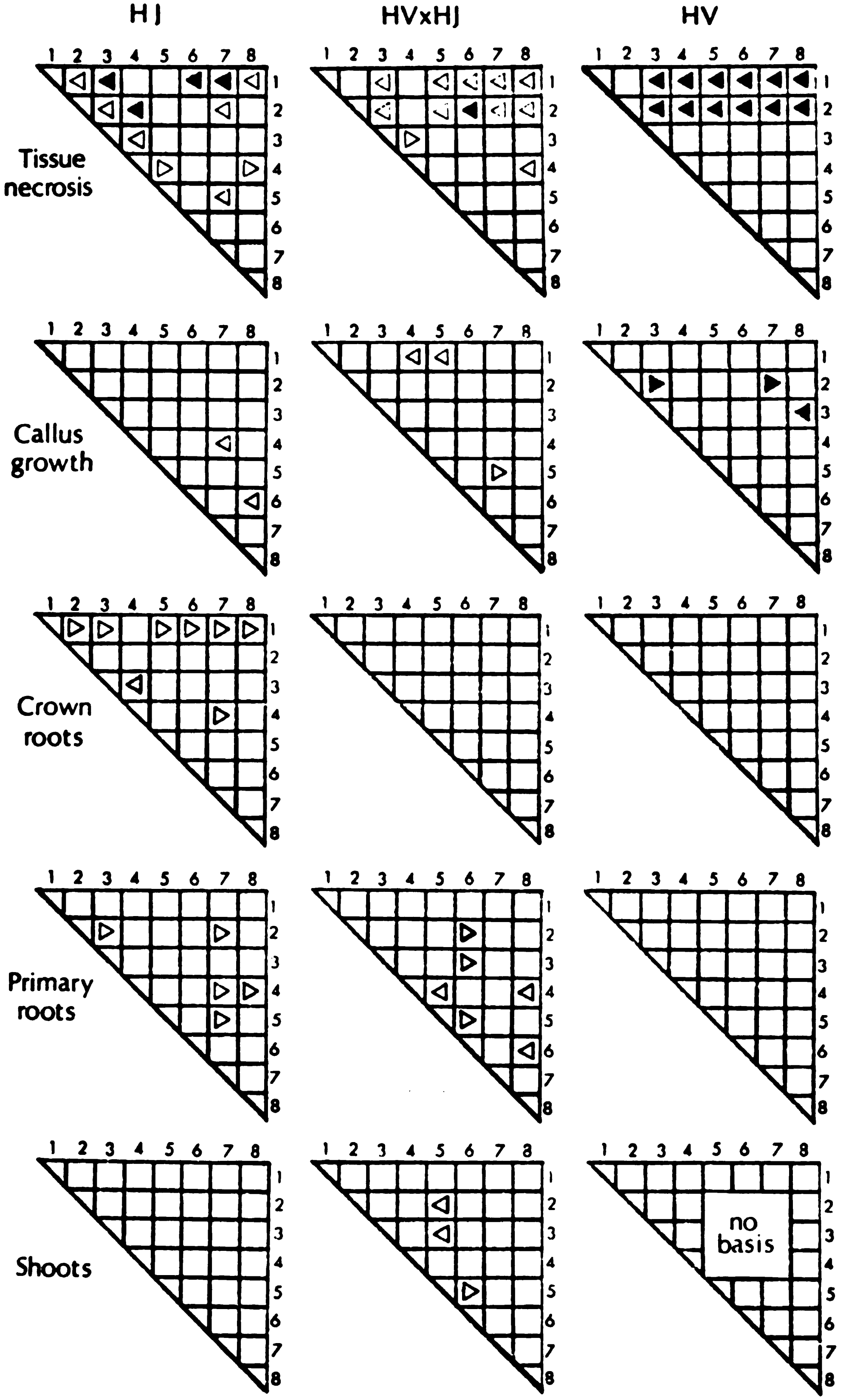
HJ
HVxHJ
HV
Tissue necrosis
Callus growth
Crown roots
Primary roots
Shoots
no basis
1 2 3 4 5 6 7 8

Figure 2.3

Figure 2.4: Significant pairwise contrasts for hormonal treatments, broken down according to genotype and response class (row preferred).

Treatment 1 - No hormones

2 - 0.5 mg/l IAA

3 - 0.5 mg/l IAA + 0.3 mg/l KIN

4 - 0.3 mg/l KIN

5 - 0.5 mg/l IAA + 3.0 mg/l BA

6 - 3.0 mg/l BA

7 - 6.0 mg/l BA

8 - 0.3 mg/l KIN + 3.0 mg/l BA

9 - 0.5 mg/l IAA + 1.0 mg/l GA_3

10 - 0.3 mg/l KIN + 1.0 mg/l GA_3

11 - 0.6 mg/l KIN + 1.0 mg/l GA_3

12 - 0.5 mg/l IAA + 0.3 mg/l KIN + 1.0 mg/l GA_3

(See legend of Fig. 4 for key to symbols used and example)

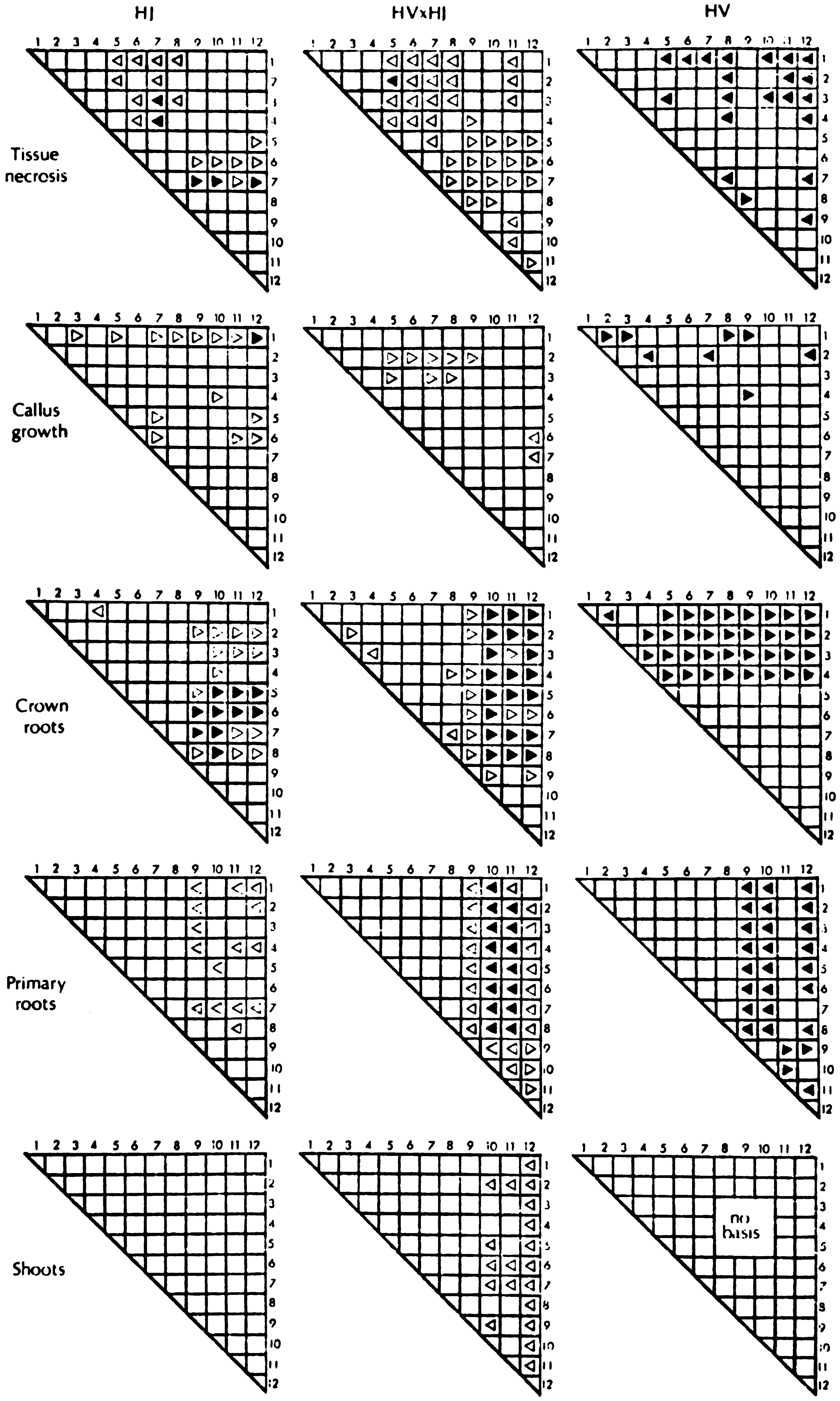
HJ
HVxHJ
HV
Tissue necrosis
Callus growth
Crown roots
Primary roots
Shoots
no basis
1 2 3 4 5 6 7 8 9 10 11 12

FIGURE 2.4

Figure 2.5: Comparison of significant contrasts for hormonal treatment between HVxHJ types A and E calli (see legend of Fig. 2.3 for key to symbols used and example).

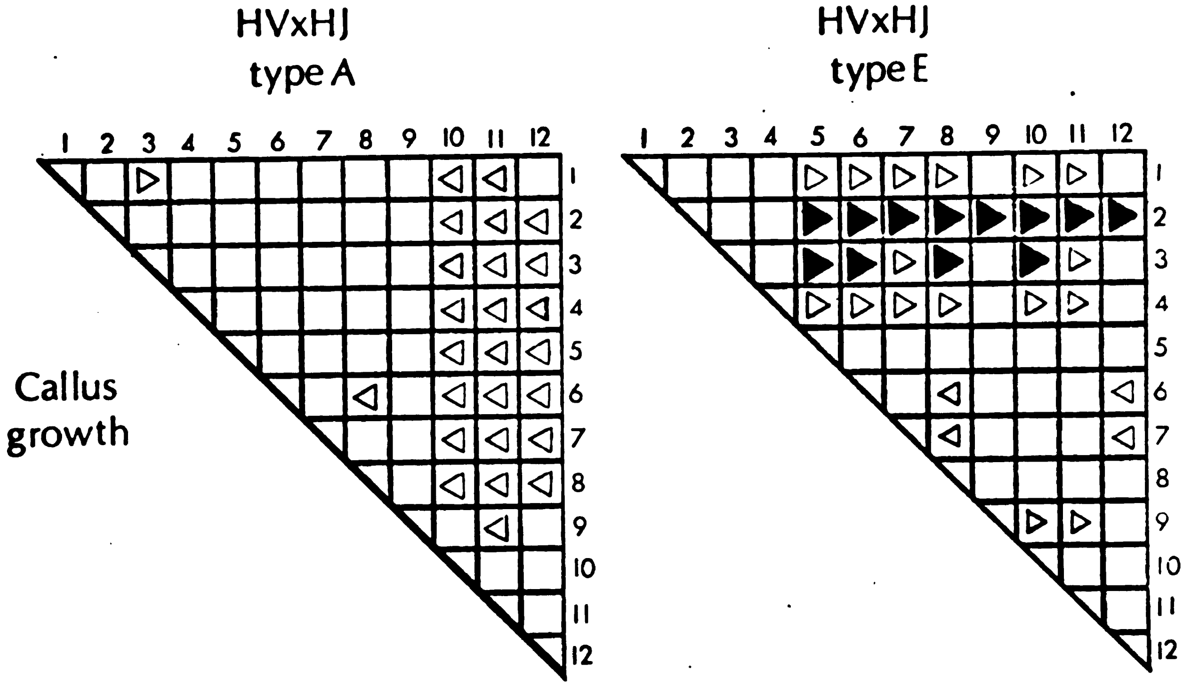
HVxHJ
type A
HVxHJ
type E
Callus growth
1 2 3 4 5 6 7 8 9 10 11 12
1 2 3 4 5 6 7 8 9 10 11 12

Figure 2.5

TABLE 2.4: Summary of significant Basic and hormone effects from Figs. 2.3, 2.4, and 2.5

		Basic treatments		Hormone treatments	
Response class	Genotype	inhibitors	promoters	inhibitors	promoters
Tissue necrosis	HJ	-	B5 salts	-	BA
	HV	-	B5 salts	null hormones	KIN + BA
	HVxHJ	-	B5 salts	IAA + GA_3	-
Callus growth	HJ	-	-	-	no hormones, possibly BA
	HV	-	-	-	no hormones
	HVxHJ type A	-	-	-	KIN + GA_3
	HVxHJ type E	-	-	-	no hormones IAA, IAA + KIN, KIN, IAA + GA_3
crown roots	HJ	high inositol	MS salts	KIN + GA_3	possibly BA
	HV	-	-	-	no hormones, IAA, IAA + KIN KIN
	HVxHJ	-	-	KIN + GA_3	-
primary roots	HJ	-	B5 salts + caryopsis extract	-	-
	HV	-	-	-	KIN + GA_3, IAA + GA_3,
	HVxHJ	B5 salts + coconut water	-	-	GA_3
shoots	HJ	-	-	-	-
	HV	no basis	no basis	no basis	no basis
	HVxHJ	-	B5 salts + 1% sucrose + 1% glucose	-	KIN + GA_3 IAA + KIN + GA_3

a single factor analysis of variance was performed on response intensities for each response class. Further, regression coefficients were calculated for $\log_{10}$ [cAMP] and pH vs. response intensity for each response class. All between-treatment variances and regression coefficients were insignificant.

Amino acid conjugates of IAA are found in numerous plant groups (Schneider and Wightman 1974). It was hypothesized that they may serve as pools for the storage and/or detoxification of IAA (Feung et al. 1977). Recent studies have indicated that IAA-amino acid conjugates may be more powerful auxins than free IAA by serving as slow-release auxin sources (Peterson 1978). Concentrations of 0.0, 0.01, 0.1, 1.0, and 10.0 mg/l IAA equivalents of various IAA-amino acid conjugates, 9 conjugate:1 free IAA mixtures, and free IAA controls were tested for their effects on the tissue response classes previously described. Two independent cultures were utilized (HVxHJ-AE suspension and HV type B). The mean intensity was calculated over four concentrations for each conjugate. The results are presented in Table 2.5. All conjugates promoted tissue necrosis in comparison with free IAA, IAA-glycine and IAA-L-alanine being the most effective. In addition, all conjugates stimulated callus growth, the strongest effects evident from IAA-L-alanine. Conversely, conjugates tended to suppress organogenesis, with the possible exception of IAA-D-alanine. No differences between 9 conjugate:1 free IAA mixtures and conjugate alone could be detected.

The regeneration of shoot meristems was the most limiting transformation in the barley somatic cell cycle. Once shoots were regenerated on calli, roots could easily be induced by transferring

TABLE 2.5: Effects of IAA-amino acid conjugates on mean response class intensity in *Hordeum* tissue cultures

Auxin[a]	Tissue necrosis		Callus growth		crown roots		primary roots	
	1[b]	2[c]	1	2	1	2	1	2
free IAA	.00	.00	.25	.50	.00	3.00	.00	1.00
IAA-D-alanine	.33	.00	1.33	.00	.00	3.00	.00	.67
9IAA-D-alanine: 1free IAA	.33	.33	1.33	.00	.00	3.00	.00	.67
IAA-L-alanine	.50	.50	2.00	1.75	.00	2.50	.00	.25
9IAA-L-alanine: 1free IAA	1.00	1.00	2.50	2.25	.00	2.00	.00	.25
IAA-glycine	1.25	.25	1.50	1.00	.00	2.00	.00	.75
9IAA-glycine: 1free IAA	1.75	.50	1.00	1.00	.00	2.00	.00	.25
IAA-β-alanine	.00	.25	1.50	.25	.00	3.25	.00	.00
9IAA-β-alanine: 1free IAA	.00	.25	2.00	.50	.00	2.25	.00	.75
IAA-L-aspartate	.50	.25	1.75	1.25	.00	2.00	.00	.50
9IAA-L-aspartate: 1free IAA	.50	.50	2.25	1.00	.00	2.50	.00	.50

[a] all media are MS salts + 2 mg/l BA

[b] culture 1) HVxHJ suspension lawn

[c] culture 2) HV-1 type B callus

to MS salts + 1 mg/l GA_3. Thus, over 95% of calli producing shoots could be successfully transformed into whole plants (Fig. 2.6). The results of these findings are summarized schematically in Fig. 2.7.

Discussion

Blakely and Steward (1964) commented on the morphological plasticity of long-term callus cultures of *Haplopappus gracilis* and *Daucus carota*. In their study, subcalli were isolated which frequently differred, both morphologically and metabolically, from parent strains. Variant strains often differred in chromosomal constitution, but it was not known whether this was a cause or an effect. The present study differentiates five morphologically distinct callus types in barley, exhibiting three different patterns of regenerative potential: no organogenesis, the ability to regenerate crown and primary roots, and tissue totipotency (Tables 2.1, 2.2). Historically, the primary distinction among callus types has been between friable and non-friable callus (Aitchison *et al*. 1977). Characteristic differences have included cell size, organization (Blakely and Steward 1964), and matrix polysaccarides (Grant and Fuller 1968). Further distinctions are apparent between friable (type E) and non-friable (type A) calli of barley: degree of hydration, growth rate, degree of karyotypic variability, and organogenic potential.

Caplin (1947) demonstrated that subcalli of uniform callus cultures of tobacco exhibited widely divergent growth rates. He concluded that the differences were intrinsic. This study supports Caplin's conclusions (Table 2.2). This is an indication that techniques additional to end-point growth measurements may be needed to

Figure 2.6: Organogenesis from *Hordeum* callus: (A) comparison of regenerated primary root (top) vs. crown root (bottom) (X13), and (B) shoot regenerating from a type A callus of HVxHJ (X7).

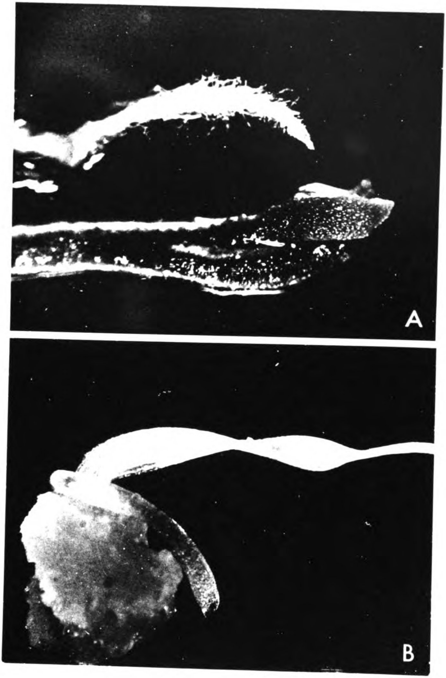

Figure 2.6

Figure 2.7: Summary of the optimal conditions for callus induction, callus growth, and regeneration in *Hordeum*. *IAA-amino acid conjugates may be substituted to avoid the possible undesirable side-effects of 2,4-D.

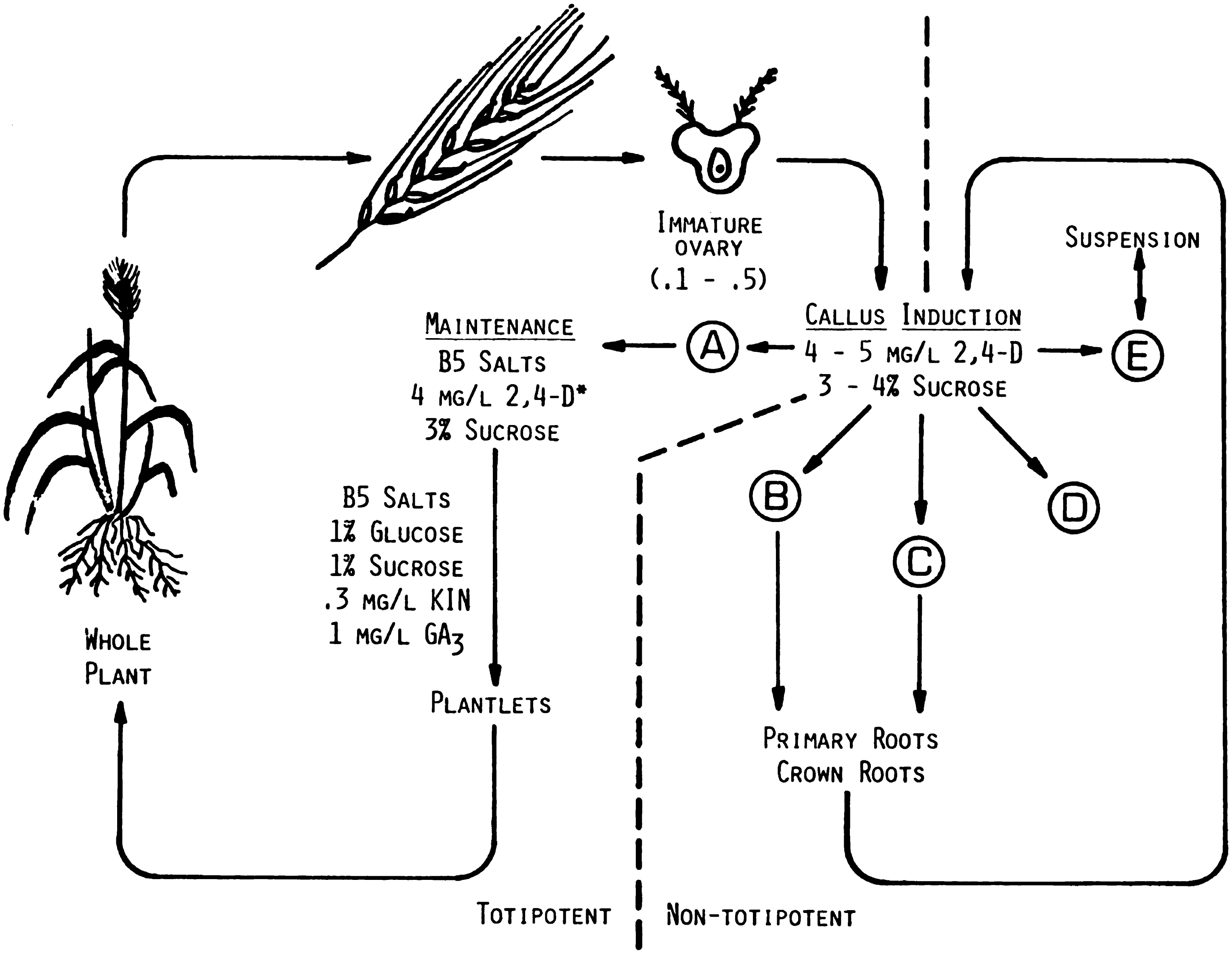
Immature
ovary
(.1 - .5)
Callus Induction
4 - 5 mg/l 2,4-D
3 - 4% Sucrose
Suspension
A
B
C
D
E
Maintenance
B5 Salts
4 mg/l 2,4-D*
3% Sucrose
B5 Salts
1% Glucose
1% Sucrose
.3 mg/l KIN
1 mg/l GA3
Plantlets
Whole
Plant
Primary Roots
Crown Roots
Totipotent
Non-totipotent

Figure 2.7

determine causative effects of test conditions on callus growth.

Among annual grains, a wide range of substances and growth conditions have been claimed to promote the initiation of shoot meristems. They vary from media lacking hormones, to auxin alone, and auxin + cytokinin (Yamada 1977). The results of this study indicate that GA_3, in combination with KIN or KIN + IAA, stimulated shoot formation compared to media lacking hormones, auxin alone, and auxin + KIN or BA (within the context of concentrations tested). Schraudolf and Reinert (1959) found that gibberellic acid inhibited root and shoot growth from Begonia leaf discs. Similarly, Vasil and Vasil (1972) concluded that gibberellins cause partial or complete inhibition of embryogenic cells. Nickell and Tulecke (1959) found that growth of monocot tissues are inhibited by GA_3. Conversely, Sangwan and Harada (1976) reported that IAA + BA + GA_3 maximized the induction of shoot primordia in Petunia. Plantlet formation from mesocotyl-derived callus of germinating barley embryos was enhanced by GA_3 and inhibited by IAA (Granatek and Cockerline 1978). Results of the present study indicated that, in combination with KIN, GA_3 inhibited the formation of crown roots and promoted primary root and shoot growth. It is possible that a KIN-GA_3 interaction has been overshadowed by the effects of GA_3 alone, or that the ratio of KIN:GA_3 in previous studies was out of the range of those studied previously.

B5 media promoted tissue necrosis as compared to MS media. The primary differences between MS and B5 media are in concentrations of NH_4^+ and NO_3: MS is 20.6 mM NH_4^+ and 39.4 mM NO_3 while B5 is 1.0 mM NH_4^+ and 25.0 mM NO_3. Perhaps callus cultures of Hordeum require high concentrations of NH_4^+ for sustained growth.

In animal cells, carbohydrate metabolism is pervasively linked to cellular concentrations of 3', 5'-cyclic AMP. Thorpe and Murashige (1968) discovered a strong correlation between starch accumulation and incipient shoot formation in tobacco. Cyclic nucleotide phosphodiesterases and cAMP have been reportedly isolated from numerous plant tissues (Amrhein 1974a). Further, in the moss _Funaria hygrometrica_, cAMP has been demonstrated to induce the differentiation of chloronema filaments (Handa and Johri 1976). In the present study, however, cAMP had no effect on callus growth or regeneration, thus supporting the conclusions of Palmer (1976) and Amrhein (1974b). Alternatively, free cAMP may have been impermeable to the cells. cAMP should have been supplied as a dibutyryl salt to facilitate uptake.

As to the biological effects of IAA-amino acid conjugates, this study is in general agreement with the conclusions of Feung _et al_. (1977) and Peterson (1978). The IAA-L-aspartate complex, however, was seen to stimulate growth and inhibit organogenesis, unlike the results in the tobacco and tomato assay systems (Feung _et al_. 1977, Peterson 1978). To initiate callus cultures of most monocots, high concentrations of synthetic auxins, such as naphthalene acetic acid (NAA) or 2,4-D are usually required. 2,4-D, however, has been associated with elevated frequencies of mitotic abnormalities (Bayliss 1973) and a loss of regenerative potential (Torrey 1967). Perhaps IAA-amino acid conjugates will prove to be powerful growth promoters without the undesirable side-effects of 2,4-D.

The two parents and their interspecific hybrid were tested separately in the hope that some insight would be gained into the effects of specific genomes on callus growth and regeneration. Cheng and Smith (1973) compared triploids and tetraploids of varying genome doses in *Nicotina glauca* x *N. langsdorfii* hybrids. They concluded that, under the proper conditions, a higher proportion of glauca genomes led to greater growth and shoot initiation. Using a similar approach, Ogura and Tsuji (1977) determined that the genetic determinants for shoot initiation in the amphiploid *N. tabacum* resided in the constituent *N. sylvestris* genome. In many cases, such as in tomato, cultures of wild species express more morphogenic potential than cultivated species (Tal *et al*. 1977). In most instances, all three genotypes reacted similarly to test media in this study (Figs. 2.3, 2.4). In the case of tissue necrosis, HVxHJ appeared to exhibit a response which approximated additivity between HJ and HV. Because the reactions of different callus types were grouped and compared in this composite, the analysis may have been weakened somewhat. Callus in grouped HVxHJ type A vs. type E callus showed extreme contrasts in growth rates resulting from hormone treatments (Fig. 2.5). Also, cultures were more similar when grouped according to callus type than to parental source (Table 2.3).

Among the Gramineae, species which exhibit a perennial growth habit often regenerate more readily, as with sugarcane (Heinz and Mee 1969). HJ and HVxHJ are both perennial as whole plants and exhibited the ability to regenerate shoots from long-term callus cultures. No shoot regeneration was observed from callus of HV, an annual. The results of this study suggest the use of sexually compatible perennial relatives as bridge species to confer regenerative capacity on recalcitrant grain crops as interspecific hybrids. Perhaps the genetic determinants for morphogenesis reside in the HJ genome in HVxHJ. Alternatively, perhaps the primary difference was in the propensity to generate highly organogenic type A callus. It can be advanced that tissue culture is a condition of indeterminant growth to which perennials possess a greater innate adaptivity than annuals.

Relatively minor genetic differences may play an important role in determining amenability to tissue culture methods. Different barley lines comprising single gene differences exhibited differences in auxin-induced callus formation from seedlings (Jacobsen 1976). Green and Phillips (1975) observed differences in callus induction and regeneration between inbred lines and hybrids of maize. Large numbers of genotypes should be screened to identify those which adapt most readily to the somatic cell cycle.

Plantlets produced from type A callus probably arose from multicellular meristemoids within the callus as with the adventive embryo callus derived from ovules in Citrus (Button _et al_. 1974). Consequently, regeneration of this type may be more accurately termed meristem propagation. True totipotency requires the demonstration of the single cell as the smallest functional unit. Very rarely, type A-like nodes were isolated from the matrix of type E secondary callus (i.e. type E callus generated from lawns of plated suspension cultures). This suspension line had been maintained for over six months and microscopic examination revealed only the presence of single cells and small clumps of large, parenchymal cells. These nodes exhibited root regeneration, but shoots have yet to be observed. Thus, single cells or small clumps of cells have demonstrated the ability to regenerate roots, assuming that these nodes arose de novo from constituent cells of secondary type E callus. It is hoped that further testing of this callus will result in shoot regeneration, thus opening the door to the use of single cells for genetic and biochemical studies in annual grains.

CHAPTER III

A CYTOGENETIC CHARACTERIZATION OF PLANTS REGENERATED FROM COLCHICINE-TREATED CALLUS CULTURES OF HORDEUM VULGARE x H. JUBATUM

Introduction

The amount of genetic variability available for selection in response to winterhardiness, salt tolerance, tolerance of wet soils, and epidermal trichomes is extremely low in the *Hordeum vulgare* gene pool. *H. jubatum* is a weedy perennial which embodies a desired range of phenotypes for all of these traits. Hence, attempts were made to introgress these genes from *H. jubatum* (an allotetraploid) into *H. vulgare* (a diploid). The F_1 hybrid (2n=3x=21, hereafter referred to as HVxHJ) has been successfully synthesized and is unconditionally sterile in both directions (Rajathy and Morrison 1959, Steidl 1976). Cytogenetic observations revealed an extremely low frequency of bivalent formation and abnormal meiosis in HVxHJ (Wagenaar 1960, Murry 1975). The two genomes of *H. jubatum* have been shown to possess segmental homology and, in other hybrids, to exhibit autosyndesis to a much greater extent than in HVxHJ (Wagenaar 1960, Starks and Tai 1974). It was concluded that sterility in the amphiploid was caused by a lack of homeologous chromosome pairing, possibly attributable to an inhibitor system, as with $5B^L$ in wheat (Murry 1975).

All attempts to induce amphiploidy on whole plants met with failure.

Tissue culture was adopted as a means of circumventing the recalcitrance of the hybrid to induced diploidization. It is now possible in some instances to regenerate whole plants from callus cultures of *Hordeum* (Cheng and Smith 1975, Chapter 2). After *in vitro* colchicine treatments, a large number of plants were regenerated from callus cultures of HVxHJ, some of which possessed doubled chromosome complements (Steidl 1976). This chapter will describe how the above techniques were utilized and present a cytogenetic analysis of certain plants that were regenerated.

Materials and Methods

The interspecific cross of *H. vulgare* (HV) x *H. jubatum* (HJ) exhibited extremely low seed set. The use of embryo culture was necessary to recover whole plants (Steidl 1976). Immature embryos, isolated immediately after the cross had been made, were placed on agar-solidified MS medium (Linsmaier and Skoog 1965) supplemented with 5 mg/l 2,4-D + 4% sucrose (W:V) using methods described in Chapter 2. The resulting callus cultures were maintained in the dark at 25°C on B5 medium (Gamborg and Eveleigh 1968) supplemented with 4 mg/l 2,4-D + 3% sucrose. Calli were transferred to the same medium containing .005% or .01% colchicine for four days. To induce regeneration, they were transferred onto MS medium + 5.0 g/l inositol + 2% sucrose and exposed to 16 hr./day of light (2000 to 3250 lux from GE F96T10-CWX bulbs). 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 25 to 30°C during the day and 21 to 25°C during the night.

Cytogenetic and electrophoretic techniques are fully described in Appendices A and B. Associations of chromosomes at metaphase I were recorded for each floret (3 anthers). Various stages of microsporogenesis were photographed in an attempt to reconstruct the progression of meiosis in these plants.

Results

Chromosome numbers in regenerated plants appeared to be quite variable, although counts on root tips of one of the original amphiploids invariably exhibited 21 chromosomes. Chromosome counts on root tip cells of regenerating plantlets from colchicine-treated callus indicated that 40 plantlets were undoubled and 10 were hypoamphiploid.*

Plants were grown to maturity in greenhouses. The entire population of hypoamphiploids was unconditionally self-sterile. Observations of mature florets revealed reduced, deformed, or missing anthers. In attempts to discern whether any female fertility existed, florets were pollinated by HV and HJ. No hybrid embryos were recovered (Steidl 1976, and personal communication).

Distinct morphological differences were evident between the original HVxHJ hybrid and the population of 10 induced hypoamphiploids; the latter plants were more robust (Fig. 3.1). Also, apparent

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*The term 'hypoamphiploid' will be used to denote plants exhibiting variable chromosome numbers and a mean slightly less than the amphiploid number of 42.

Figure 3.1: Comparison of spike morphology between hypoamphiploids and HV, HJ, and HVxHJ: (A) HV,)B) hypoamphiploid, (C) original HVxHJ hybrid and (D) HJ (all X.6).

Figure 3.1

differences in isozyme concentrations have been observed on electrophoretic zymograms of esterase, peroxidase, and acid phosphatase isozymes (Fig. 3.2).

Among hypoamphiploids, mean chromosome numbers varied from 35.43 to 37.49 (Table 3.1). The range of counts was approximately the same between plants, considering the numbers of cells counted. Chromosome counts of one plant (plant 1, Table 3.1) were partitioned to spikes and florets to gain further insight into the possible source of this variation. In general, the means and ranges of chromosome numbers were very similar.

Data of metaphase I chromosome associations is summarized in Table 3.1. Comparisons of mean associations per cell were very similar between plants, tillers, and florets, as with means and ranges of chromosome numbers. The overall mean numbers of chomosome associations per cell were as follows: 6.45 I + 12.20 II + 1.74 III + 0.15 IV + 0.05 V. Examples of typical metaphase I chromosome associations are pictured in Figs. 3.3B, 3.3C, and 3.3D.

Observations of various stages of microsporogenesis were made in an attempt to identify anomalous processes which could result in sterility. Meiosis proceeded in a normal fashion from prophase I (Fig. 3.3 A) to metaphase I (Figs. 3.3 B, 3.3 C, 3.3 D). At anaphase I, however, separation of chromosomes was clearly abnormal. Frequently, univalents appeared to attach to the spindle and migrate towards the poles (Fig. 3.3 E). Bivalents and multivalents failed to separate normally, often fractionating, forming bridges, or moving partially toward one pole. At telophase I, the spindle became invariably divergent, thus failing to coalesce chromatin at the

Figure 3.2: Comparison of isozymes from crude extracts of culm bases from hypoamphiploid population vs. HVxHJ. (A) cathodal PRX, (B) anodal acid phosphatase, and (C) anodal EST.

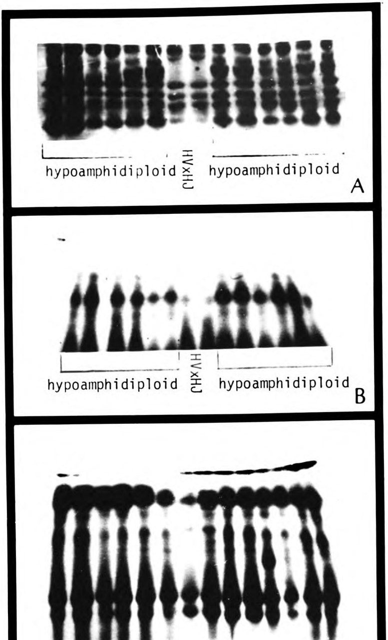

hypoamphidiploid
HVxHJ
hypoamphidiploid
A
hypoamphidiploid
HVxHJ
hypoamphidiploid
B
hypoamphidiploid
HVxHJ
hypoamphidiploid
C

Figure 3.2

TABLE 3.1: 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 no. 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

Figure 3.3: Microsporogenesis in hypoamphiploids: (A) zygotene (X 879), (B) metaphase I cell with 10 univalents and 16 bivalents (X1269), (C) metaphase I cell with 6 univalents, 12 bivalents, and 3 trivalents (X 976), (D) normal-appearing alingnment of chromosomes of a metaphase I division plate (X1017), (E) anaphase I showing abnormal movement of chromatin; note the movement of monad chromosomes toward the poles (X 814), (F) failiure of chromatin to coalesce at the poles of a telophase I microsporocyte, due to divergent spindle (X 944), (G) decondensation of chromatin and persistent bridge at late telophase I (X 879), and (H) formation of a transverse cell plate through a chromatin bridge at late telophase I (X 976).

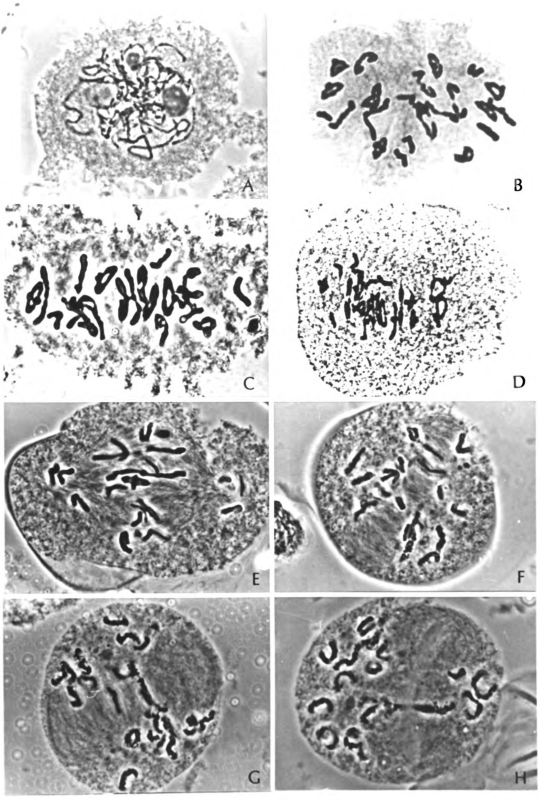


Figure 3.3

poles (Fig. 3.3 F). The dispersed chromatin then decondensed and a transverse wall formed across the cell (Fig. 3.3 G, 3.3 H). Immediately, a second set of spindles formed, usually perpendicular to the cell plate (Fig. 3.4 A, right side). Often, multiple spindles were evident (Fig. 3.4 B). Again, chromatin appeared to react abnormally to spindle forces, failing to divide and move toward the poles (Figs. 3.4 A, 3.4 B, 3.4 C). These anomalies culminated in the formation of cells with large numbers of micronuclei of various sizes (Fig. 3.4 D). Subsequently, each micronucleus was collectively or singularly packaged by a discrete cellular membrane (Fig. 3.4 E). Each of these units then separated (Fig. 3.4 F) and gave rise to inviable pollen-like structures (Fig. 3.4 G). No dyad chromosomes were observed at any stage of the process in this study or that of Murry (1975), leading to the speculation that no pre-meiotic DNA synthesis had occurred.

In a similar manner, type A calli of the HJ parent were treated with colchicine and regenerated. Six of ten tillers from regenerated plants had approximately the expected doubled chromosome complement of 56 (50 to 58, Fig. 3.4 H). Spikes of these tillers exhibited approximately 80% selfed seed set.

Discussion

Regeneration of polyploid plants from diploid-derived callus has been observed frequently (Murashige and Nakano 1966, D'Amato 1977, Sunderland 1977). In totipotent type A callus and regenerated plants of Hordeum, however, all chromosome numbers have been observed to be approximately diploid (Chapter 2). Thus, the recovery of

Figure 3.4: Microsporogenesis in hypoamphiploids (con't): (A) abnormal separation of chromatin at anaphase II (X793), (B) anaphase II; supernumerary spindles are perpendicular to the axis of the cell plate (X1036), (C) Late telophase II cells showing dispersed and bridged chromatin (X692), (D) formation of numerous micronuclei in posttelophase II microsporocyte (X 854), (E) Packaging of micronuclei by supernumerary cytokinesis (X1017), (F) the formation of separate cells of many different sizes, and containing different amounts of cromatin in postmeiosis microsporocytes (X159), (G) the formation of inviable pollen-like grains (X293), (H) a 25-25 anaphase I separation in a microsporocyte of a plant regenerated from colchicine-treated HJ callus (X793).

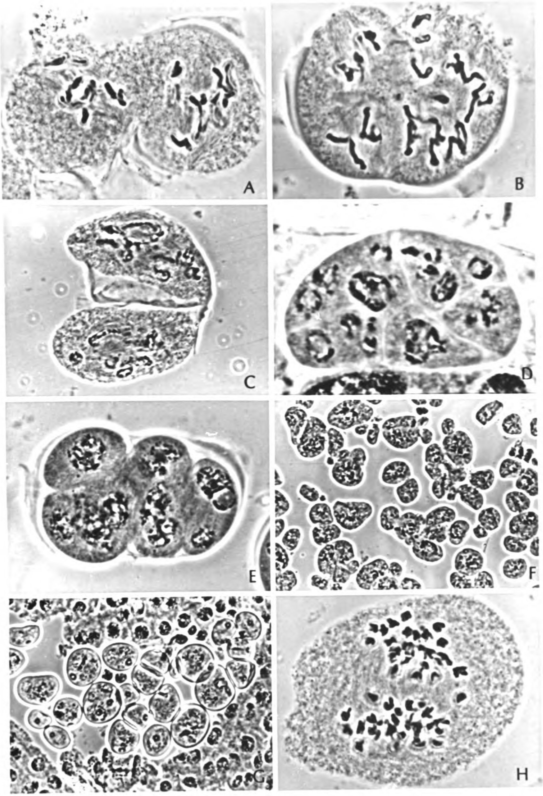


Figure 3.4

hypoamphiploid plants was most likely a result of in vitro doubling of chromosome numbers by colchicine treatments.

Table 3.2 summarizes pertinent observations regarding the generation of the observed range of chromosome numbers in the hypoamphiploids. Within the original HVxHJ hybrid, a large range was evident, but accounted for only 12% of the cell population (Murry 1975). The callus of this study, however, had been generated from immature embryos. For this reason, it is more reasonable to assume that most of the observed variation among cells of hypoamphiploids originated during the callus state (Table 3.2). It is interesting to note that the observed range limits among the hypoamphiploid plants were approximately 2X those observed in plants of the original HVxHJ hybrid, supporting the suggestion by D'Amato (1977) that polyploidy provides buffering capacity for tolerance of aneuploidy.

Past studies on HVxHJ have found an average of 0.54 to 1.20 bivalents per cell, the balance of 21 chromosomes essentially manifested as univalents (Rajathy and Morrison 1959, Wagenaar 1960, Murry 1975). It is impossible to predict the precise expected metaphase I profile of chromosome associations in hypoamphiploids from that of HVxHJ due to immeasureable interactions giving rise to univalents, trivalents, and pentavalents. Assume, however, that a hypothetical uniform amphiploid of HVxHJ should exhibit 19.85 II + 0.54 IV + 0.02 VI (based on data of Murry 1975). The composite mean chromosome number of 75 metaphase I microsporocytes was 37.02, or a loss of approximately 5 chromosomes from the expected amphiploid number of 42. Applying the loss of chromosomes proportionately to each association class results in the generation of the following

TABLE 3.2: 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 type A callus	21	16.92	13-22	97.0
PMC's[c] from regenerated plants of untreated type A calli.	21	20.60	12-28	87.5
PMC's from regenerated hypoamphiploid	42	37.04	31-44	97.0

[a] with reference to expected chromosome number

[b] after Murry 1975

[c] PMC: Pollen Mother cell

profile: 4.48 I + 15.26 II + 0.08 III + 0.41 IV + 0.01 V + 0.01 VI (adjusted for the random loss of homologues). The differences between these and observed mean associations per cell (Table 3.1) are more easily explained by competitive homeologous pairing than nonrandom loss of specific chromosomes. This evidence supported the conclusions that chromosomes were lost at random. Further, directional chromosome elimination is generally concomitant with a shift in phenotypic characters in the direction of the parent whose chromosomes remain. This phenomenon was not evident among hypoamphiploid plants.

The degree of bivalent pairing in the hypoamphiploids should not have precluded fertility (Table 1.1). Cytological observations of microsporogenesis provided evidence for a breakdown of meiosis culminating in sterility. This breakdown was manifested in two cytologically distinct phenomena: 1) Chromosomes did not interact normally to spindle forces; they tended to form bridges and/or fragment (Figs. 3.3 E, 3.3 F, 3.3 G, 3.3 H, 3.4 A, 3.4 B, 3.4 C). No dyad chromosomes were observed, leading to the speculation that premeiotic S phase was missing. This observation could explain the abnormal behavior observed at anaphase II by assuming that spindle forces had been applied to unineme chromosomes. 2) The spindles themselves appeared to be abnormal. At anaphase I they were invariably divergent (Fig. 3.3 F). At anaphase II, supernumerary spindles were frequently observed (Fig. 3.4 B). A breakdown of meiosis in interspecific hybrids, concomitant with abnormal spindle expression, has been reported in a Lolium-Festuca derivative (Darlington and Thomas 1937).

In previous studies of HVxHJ, supernumerary cytokinesis and pollen formation occurred immediately after the abortive meiosis I division. No evidence could be found for the existence of any meiosis II-like divisions, similar to observations of certain wheat-rye intergeneric hybrids (Bennett *et al*. 1972). It was proposed that abnormal meiosis in HVxHJ was a consequence of physio-genetic incompatibilities (Murry 1975). An anomalous meiosis II was observed in microspore mother cells of hypoamphiploids of this cross. Perhaps chromosome doubling, loss, or pervasive aneuploidy obviated some of this incompatibility thus permitting meiosis II to proceed.

The sterility of hypoamphiploid plants of this study was attributed to a breakdown in the process of meiosis itself, and not a lack of sufficient chromosome pairing. Mass pollinations of florets with fertile pollen from both parental sources yielded no embryo formation. It was concluded that these plants were female sterile as well, the causes of which remain unknown. It is likely that this phenomenon is a consequence of incompatible genome interactions in the cell cycle (Murry 1975). Steidl (1976) has speculated that all interspecific hybrids which juxtapose the genomes of HV and HJ might be sterile for this reason.

The induction of amphiploidy has been shown to be extremely difficult among F_1 interspecific and intergeneric hybrids of the genera *Hordeum* and *Agropyron* (Steidl 1976). In this study, however, approximately 60% of regenerated tillers from colchicine-treated calli of allotetraploid HJ were octaploids of high fertility. Hence, in the absence of genomic incompatibilities which cause sterility, the use of tissue culture is a potentially powerful tool for the production of fertile allo- and autopolyploids.

CHAPTER IV

KARYOTYPIC VARIABILITY IN TISSUE CULTURES OF HORDEUM

Introduction

Variation of chromosome number and morphology in cultured plant cells is a widely documented phenomenon (Partanen 1965, D'Amato 1975, Sunderland 1977). No patterns are discernible in the generation and distribution of this variability among and between plant groups. Even callus derived from different tissues of the same plant often give rise to divergent karyotypic profiles (D'Amato 1975).

Karyotypic variability has ambivalent consequences. The phenomenon is disadvantageous in many respects. Genetic and biochemical studies generally assume that somatic tissues are genetically uniform. Often, the conclusions of such studies are weakened by the possibility of variable chromosome constitution. Karyotypic variability also has potential advantages. New avenues may be opened to the production of novel gene associations and chromosomal mutations useful in basic sicence and plant breeding (Sunderland 1977).

Most of the preceding reports concerning karyotypic variability have utilized material that is convenient, such as *Nicotiana tabacum* (Cooper *et al*. 1964, Shimada and Tabata 1967, Kallak 1968, Shimada 1971) and *Daucus carota* (Bayliss 1973, 1975). *Haplopappus gracilis* has been used extensively due to lower chormosome numbers

(2n=4), larger chromosome size, and morphological differentiation (Mitra and Steward 1961, Singh *et al*. 1975, Singh and Harvey 1975). *Crepis capillaris* has the advantage of exhibiting only 2c nuclei in differentiated tissues, unlike most other plant groups, making studies of *in vitro* polyploidization more tenable (D'Amato 1975). A paucity of reports exists concerning karyotypic variability in cell and tissue cultures of cereal grains, probably due to difficulties in elucidating the conditions for callus induction, growth, and regeneration. Such variability has been reported in callus cultures of wheat (Kao *et al*. 1970, Shimada 1971), rice (Nishi and Mitsuoka 1969), and rye (Asami *et al*. 1976). Cytogenetically, a number of advantages are apparent in these plants. Chromosomes are generally large, well differentiated, and cytogenetically characterized. In many cases marker genes have been mapped to chromosomes, providing a means of correlative cytogenetic and genetic studies. Also, the possibility exists to identify chromosome arms via C-banding techniques (Vosa 1975).

In barley, the use of introgressive breeding to introduce genetic variation has been hindered by the pervasive sterility of interspecific and intergeneric hybrids (Huang 1975, Steidl 1976). It has been concluded that sterility is due to a genetic incompatibility leading to a breakdown in gametogenesis (Murry 1975, Chapter 3). It was hypothesized that the induction of karyotypic variability among cells of such a hybrid may result in the non-equational distribution of chromosomes and chromosomal segments, giving rise to cells regenerable into potentially fertile plants. This chapter will attempt to demonstrate the preliminary feasibility of this hypothesis by examining the following: 1) the rate of spontaneous *in vitro* polyploidization and aneuploidization vs. differentiated state, 2) the distribution of chromosome numbers *in vitro* vs. the differentiated state, 3) spontaneous *in vitro* chromosomal rearrangements, and 4) the isolation and clonal propagation of karyotypic and genetic variability.

Materials and Methods

Plants or seed stocks of *Hordeum vulgare* (HV, 2n=2x=14), *H. jubatum* (HJ, 2n=4x=28), and their interspecific hybrid (HVxHJ, 2n=3x=21) were provided by Dr. J. E. Grafius, Dept. of Crop and Soil Sciences, Michigan State University. All plants were produced or maintained as described by Steidl (1976). Callus and suspension cultures were isolated and maintained as described in Chapter 2. All tissue was maintained on B5 basal medium (Gamborg and Eveleigh 1968) supplemented with 4 mg/l 2,4-D and 3% sucrose (W:V) at pH 6.0. Cultures derived from immature ovaries were used exclusively for this study.

Cells were prepared for cytological observation as described in Appendix B. It was more difficult to make accurate counts on cells with progressively higher chromosome numbers. Thus, if all cells with uncertain metaphase chromosome counts had been excluded, the data may have become biased in favor of lower chromosome numbers. To alleviate this possibility, the following procedure was adopted: each cell was tallied three times. If the difference of the highest and lowest of three counts exceeded 10% of the mean, the datum was discarded. Means and ranges, to the nearest whole number, were recorded for each cell within tolerance. For each experiment, a coefficient of the degree of certainty (DC) was calculated as follows: $DC = 1 - \Sigma(\text{high} - \text{low count})/\Sigma(\text{mean})$. With three exceptions, this value ranged from 0.9 to 1.0.

Crude extracts of type E subcalli were prepared, electrophoresed, and stained for peroxidase and esterase isozymes to detect genetic variability as described in Appendix A. Equal amounts of tissue were crushed and the crude extract taken up into Beckman electrophoresis paper wicks cut into equal sizes. Gels were scored qualitatively and quantitatively for peroxidase (PRX) and esterase (EST) bands using bands from source callus extracts as a control and reference for relative intensities. The following arbitrary scale was used: 0 - band absent relative to control, .5 - band present at 1/2 the intensity of the control, 1 - band present at equal intensity of the control, and 2 - band present at greater than or equal to 2X the intensity of the control.

Results

Of 20 chromosome counts performed on root tips cells of HVxHJ, no variation from the expected 21 was observed. In previous studies on microsporogenesis of this hybrid, however, chromosome numbers were observed to vary from 12 to 22 (Rajathy and Morrison 1959), Murry 1975) and exhibit 12% aneuploidy (Murry 1975). This could have been a consequence of developmental or environmental factors. Further, it is conceivable that *in vitro* variability of chromosome numbers was, in part, pre-existent in the original explants of HVxHJ. Chromosome counts from root tips of HV and HJ were invariably 14 and 28, respectively.

Differences in mean chromosome number and coefficient of variation (V) were found to exist between cultures and over time (Table 4.1). Figure 4.1 illustrates the dynamics of chromosome numbers over time in cultures of HVxHJ. The mean of type A callus remained stable at approximately 18 to 21 over the period studied (16 months, starting from callus induction). After six months of subculture, type E callus was spontaneously generated from type A nodes of the culture studied. Mean chromosome numbers in this type E callus increased dramatically to approximately 54, then decreased to about 35. Subsequent observations indicated that the mean had stabilized at 35 to 36. Initially, the mean chromosome number was seen to increase even more dramatically over time in suspension cultures generated from type E calli. As with type E callus, this rapid increase was followed by a plateau, and rapid decrease. When these suspension cultures were plated onto solid media, secondary type E calli were produced. In these cultures, chromosome numbers continued to increase to approximately 58, then levelled off and decreased gradually. Examples of typical metaphase plates are pictured in Fig. 4.2.

In addition, coefficients of variation (V) for each datum point in Fig. 4.1 were calculated and plotted vs. time to gain insignt into the dynamics of chromosome number dispersion (Fig. 4.3). In type A calli, a gradual exponential increase in V was observed over the 16 month period. Type E callus exhibited a rapid increase in V that levelled off after 4 months and subsequently remained stable. In type E-derived suspension cultures, the pattern was similar, only more rapid, as with corresponding mean chromosome numbers (Fig. 4.1). Further, variability was observed to decrease rapidly.

TABLE 4.1: A comparison of means and distributions of chromosome numbers among cultures derived from HVxHJ-57

Culture	Age months	Mean chromosome number	DC	S.E.	V	N
Type A	6	19.03	.92	.317	.090	30
Type A	10	21.19	.92	.748	.154	20
Type A	15	18.10	.93	2.579	.767	30
Type E, primary	10(6)*	54.56	.92	6.260	.538	23
Type E, primary	15.5(6)	34.95	.93	.710	.541	710
Suspension	8(7,6)*	48.00	.88	5.564	.478	18
Suspension	10(7,6)	50.38	.92	3.758	.365	25
Suspension	12(7,6)	38.43	.94	1.004	.131	25
Type E, secondary	11.5(9, 7,6)*	57.12	.95	5.565	.390	16
Type E, secondary	13(9, 7,6)	52.67	.92	4.795	.490	30

*Age of initial callus induction, followed in parentheses by the ages at which preceeding transformations were induced or occurred spontaneously (e.g. 11.5(9,7,6) implies type E secondary callus 11.5 mo. old from original callus induction, transformation to type E callus at 6 mo., to suspension at 7 mo., and to secondary type E at 9 mo.).

Figure 4.1: Dynamics of mean chromosome number from callus induction (time = 0) to 16 months, vs. culture state.

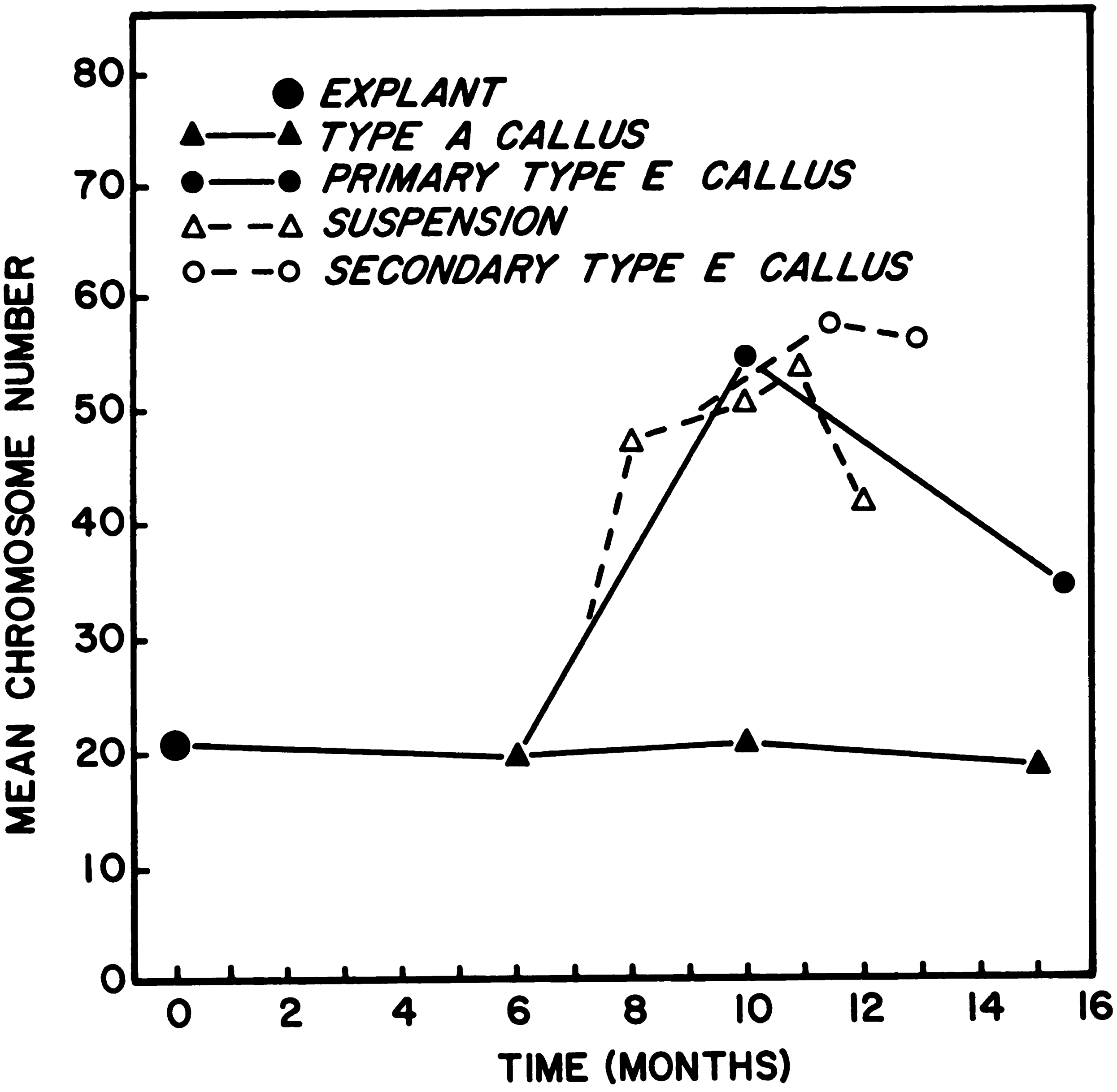

● EXPLANT
▲—▲ TYPE A CALLUS
●—● PRIMARY TYPE E CALLUS
△- -△ SUSPENSION
○- -○ SECONDARY TYPE E CALLUS
MEAN CHROMOSOME NUMBER
0
10
20
30
40
50
60
70
80
0
2
4
6
8
10
12
14
16
TIME (MONTHS)

FIGURE 4.1

Figure 4.2: Karyotypic variability in tissue cultures of Hordeum. (A) metaphase cell of HVxHJ type E callus with 17 chromosomes (X 936), (B) metaphase cell of HJ type A callus with 22 chormosomes (X1067), (C) metaphase cell of HVxHJ suspension with approximately 36 chromosomes (X1076), (D) Highly polyploid metaphase call of HVxHJ type E callus (X1017), (E) Chromosome size differential in metaphase HVxHJ type E callus cell (X4393), (F) Dicentric chromosome in metaphase HVxHJ type E callus cell (X4296).

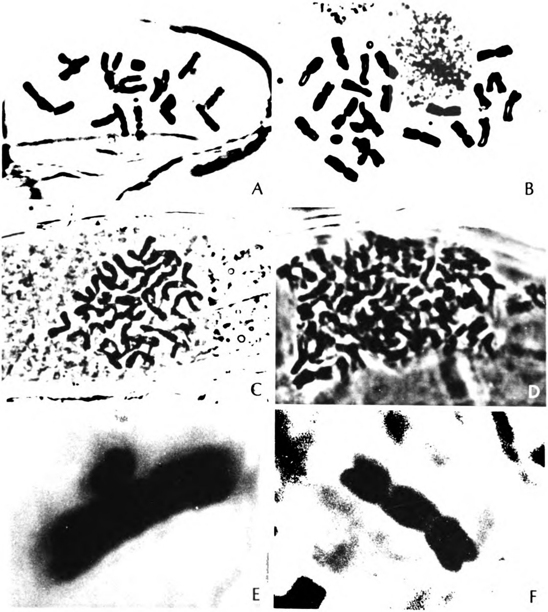
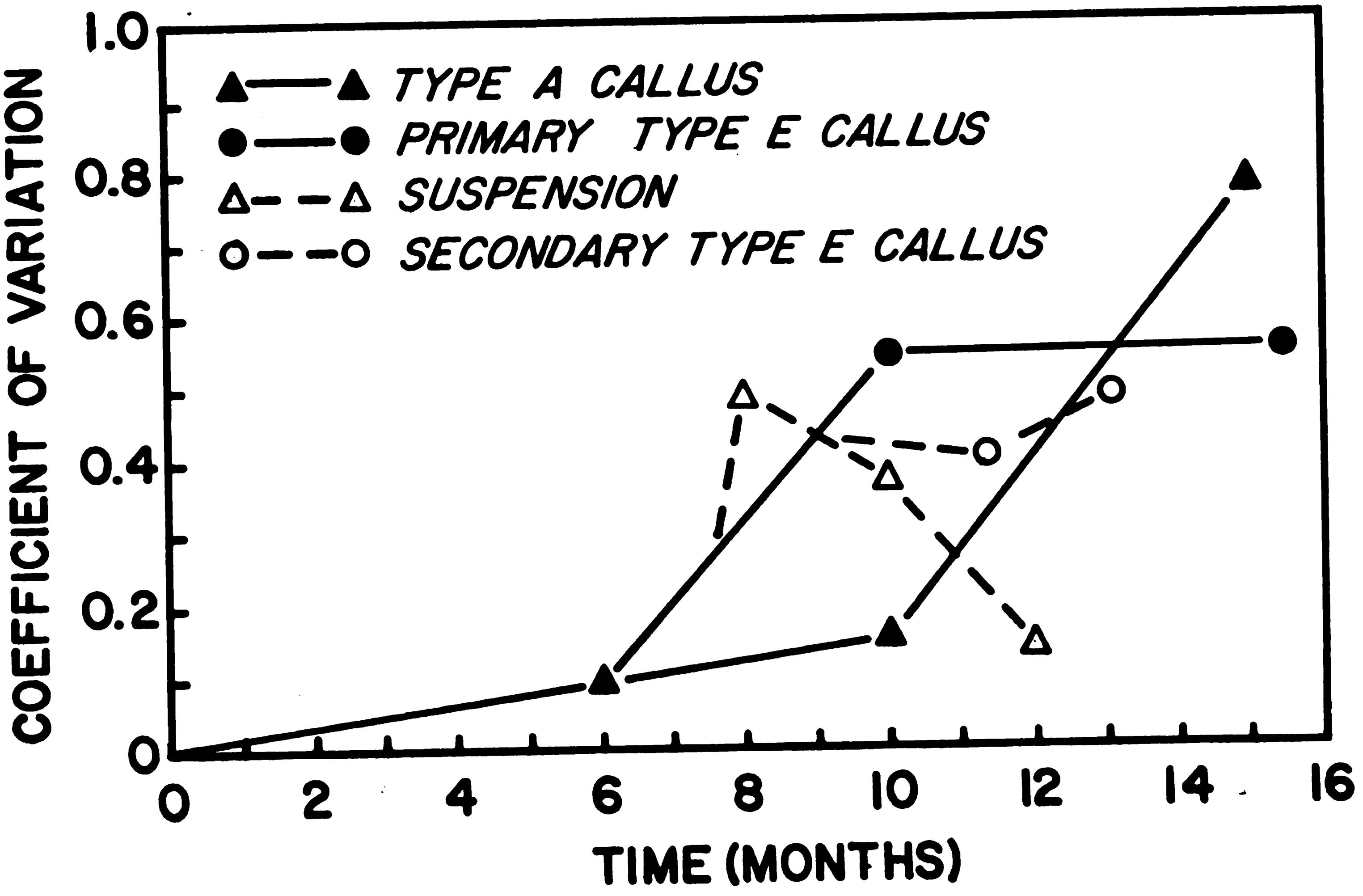


Figure 4.2

Figure 4.3: Dynamics of the coefficient of variation (V) of chromosome numbers from callus induction (time = 0) to 16 months, vs. culture state.



▲—▲ TYPE A CALLUS
●—● PRIMARY TYPE E CALLUS
△- -△ SUSPENSION
○- -○ SECONDARY TYPE E CALLUS
COEFFICIENT OF VARIATION
0
0.2
0.4
0.6
0.8
1.0
0
2
4
6
8
10
12
14
16
TIME (MONTHS)

FIGURE 4.3

In secondary type E calli, V decreased at a slower rate than the source suspension culture, followed by a gradual increase.

The power of comparing means and coefficients of variation would be weakened if the distributions of chromosome numbers were not approximately normal. The distributions of chromosome counts of type A callus were approximately normal (Figs. 4.4 A, B). A comparison of the distributions at 6 and 15 months illustrated the above observation that the mean was stable, while variability tended to increase. Type A callus, therefore, exhibited aneuploidy, but no polyploidy. The distribution of chromosome numbers for type E callus at 16 months (see Fig. 4.1), however, was not normal (Fig. 4.4 C). Rather, the distribution appeared to be three to four modal. An approximately equal number of counts were centered on clusters at 20 to 22 and 38 to 40. Minor clusters were apparent at 70 to 75 and 140 to 150. It was concluded that the pattern of these clusters approximated a hypoeuploid series with flanking aneuploidy. Polyploidy and aneuploidy were also prevalent in suspension cultures (Fig. 4.4 D). These data were taken from a relatively young culture which had probably not reached steady state (see Discussion section for explanation). An insufficient number of counts of secondary type E callus were recorded to give an accurate overall picture of the distribution (Fig. 4.5). Once again, polyploidy and aneuploidy were evident. Clusters of cells at 20 to 25 and 54 to 63 were noted.

It is conceivable that *in vitro* karyotypic variation in cultured cells of HVxHJ could have arisen purely as a consequence of parental genome interactions rather than as a result of the cultured

Figure 4.4: Composite distributions of chromosome numbers in tissue cultures of HVxHJ. (A) HVxHJ type A 6 mo., (B) type A 16 mo., (C) HVxHJ type E 15 mo., and (D) suspension 10 mo.

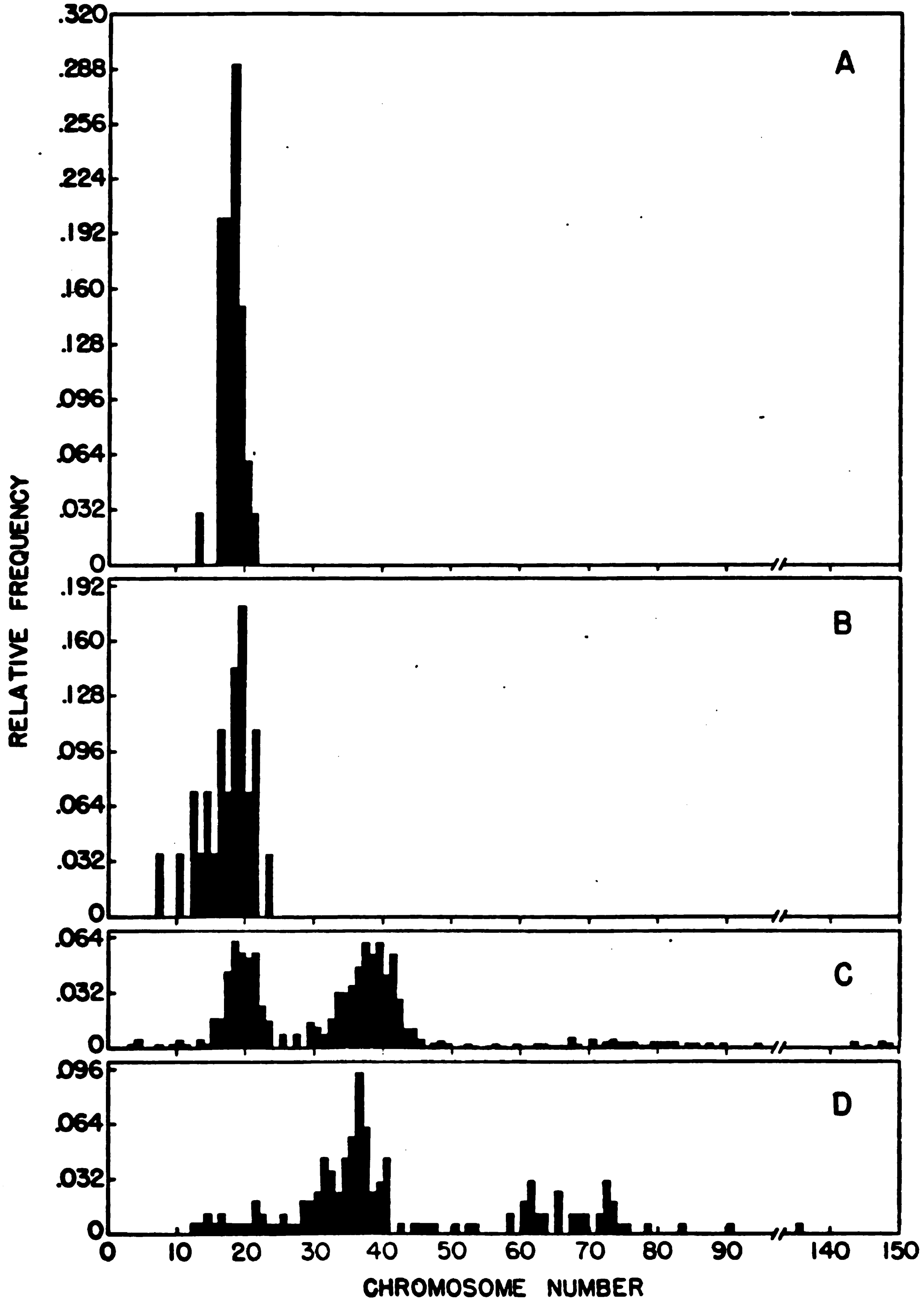

RELATIVE FREQUENCY
A
.320
.288
.256
.224
.192
.160
.128
.096
.064
.032
0
B
.192
.160
.128
.096
.064
.032
0
C
.064
.032
0
D
.096
.064
.032
0
0
10
20
30
40
50
60
70
80
90
140
150
CHROMOSOME NUMBER

FIGURE 4.4

Figure 4.5: Distribution of chromosome counts in HVxHJ secondary type E callus

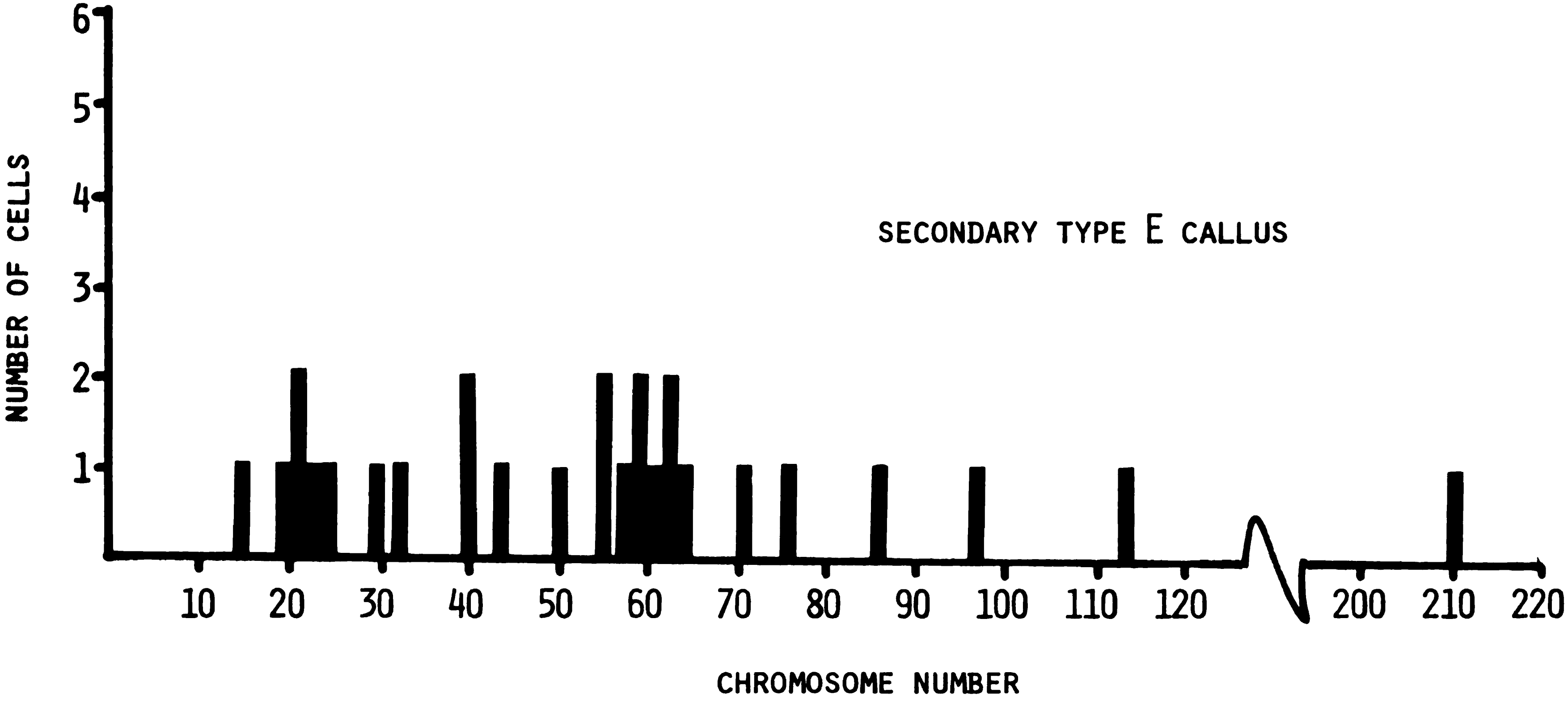

SECONDARY TYPE E CALLUS
NUMBER OF CELLS
6
5
4
3
2
1
10
20
30
40
50
60
70
80
90
100
110
120
200
210
220
CHROMOSOME NUMBER

FIGURE 4.5

state itself. Variation in chromosome numbers has been observed in pollen mother cells of HVxHJ (Rajathy and Morrison 1959, Murry 1975), but not root tips of HV or HJ. Immature ovaries of both parental types were introduced into culture in a manner identical to HVxHJ, yielding callus types described in Chapter 2. Chromosome counts for selected cultures are presented in Table 4.2. All callus types and suspension cultures exhibited aneuploidy and/or polyploidy. These data support the conclusion that the observed variation of chromosome numbers in HVxHJ cultures was a consequence of growth *in vitro*, and not of genome interactions.

Cytological examination of chromosomal morphology in cultured cells revealed an array of novel types, particularly of increased size differentials (Fig. 4.3 E) and multicentrics (Fig. 4.3 F). The alarming frequency of their occurrence in the karyotypes of cultured cells prompted the hypothesis that chromosomal rearrangements constituted yet another source of karyotypic variability. A method was devised to demonstrate the existence of chromosomal rearrangements, in cultured tissues, based on comparative ratios of longest to shortest (L/S) chromosomes (illustrated in Fig. 4.6). This procedure was first suggested by Shimada and Tabata (1967). It was assumed that the degree of chromosomal contraction may have varied from cell to cell, but was consistant over all chromosomes within a given cell. Lengths were measured with an ocular micrometer. The control ratio, determined from metaphase plates of 20 root tip cells in the original HVxHJ F_1 hybrid was 2.04±0.046 (mean ± standard error, S.E.). If the L/S ratio was significantly less than 2.04 in a sampled population, it could be concluded that either chromosomal rearrangements

TABLE 4.2: Summary of chromosome numbers in tissue cultures of HV and HJ

Source plant	Culture type	Mean chromosome number	DC	S.D.	Range	N
HV	B	35.44	.89	-	26 - 51	9
HV	B	31.46	-	-	12 - 44	13
HV	suspension	60.12	.95	33.45	24 - 171	25
HJ	A	27.56	-	2.17	25 - 30	24
HJ	A	26.37	-	2.54	20 - 30	19
HJ	C	23.40	.89	5.69	21 - 28	15
HJ	suspension	43.26	.91	14.44	19 - 83	23

Figure 4.6: Summary of procedure for mathematical proof of chromosomal rearrangements by comparison of ratios of longest to shortest chromosomes (L/S).

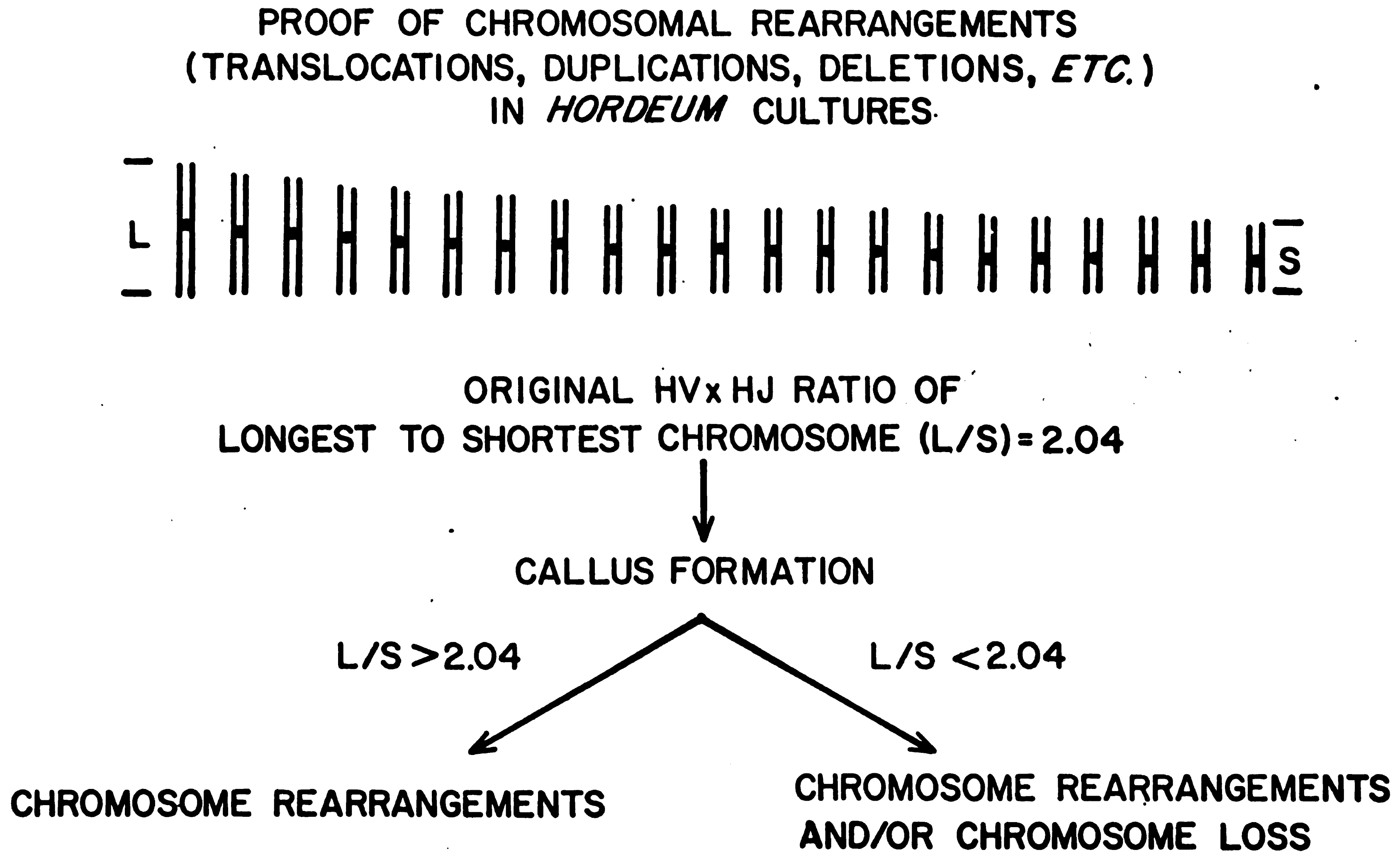

PROOF OF CHROMOSOMAL REARRANGEMENTS
(TRANSLOCATIONS, DUPLICATIONS, DELETIONS, *ETC.*)
IN *HORDEUM* CULTURES
L
S
ORIGINAL HVx HJ RATIO OF
LONGEST TO SHORTEST CHROMOSOME (L/S) = 2.04
CALLUS FORMATION
L/S >2.04
L/S <2.04
CHROMOSOME REARRANGEMENTS
CHROMOSOME REARRANGEMENTS
AND/OR CHROMOSOME LOSS

FIGURE 4.6

or whole chromosome loss were responsible (e.g. the loss of a longest or shortest chromosome would depress this ratio). If the L/S ratio was significantly greater than 2.04, however, chromosomeal rearrangements (i.e. translocations, deletions, duplications, etc.) must have occurred (Fig. 4.6). Data of four tested cultures are presented in Table 4.3. For all cultures, i.e. type A and E callus and 11 and 27 month old suspension cultures, mean L/S ratios were significantly greater than the control. Note also that the magnitude and variability of L/S was greater than that of type A callus in both type E callus and suspension cultures.

Three classes of karyotypic variability have been characterized: polyploidy, aneuploidy, and chromosomal rearrangements. Cytological observations of untreated tissues were made to identify phenomena which could explain their existence. Endoreduplication and nuclear or spindle fusion in multinucleate cells have been advanced as possible means of polyploidization (D'Amato 1975, Sunderland 1977). Both multinucleated cells and endoreduplicated chromosomes have been observed frequently in type E calli and suspension cultures (Figs. 4.7 A, B). Ploidy ranges of 5x, 6x, 7x, etc. (i.e. non-geometric), however, were not observed in clusters. It was therefore concluded that endoreduplicated chromosomes were the primary means of polyploidization in the cultured tissues of this study. It is not known whether this endopolyploidy was generated *in vitro*, or existed in tissues of the original explant as suggested by D'Amato (1977). Spindle abnormalities are common in dividing cultured plant cells and are manifested primarily by lagging chromosomes and multipolar divisions (Sunderland 1977). These phenomena were observed in

TABLE 4.3: Comparison of L/S ratios in cultured tissues of HVxHJ-57

Tissue	Mean chromosome number	DC	Mean L/S	S.D. L/S	Mean no./cell		
					dicentrics	tricentric	fragments
Root meristem, orig. HVxHJ hybrid	21.00	1.00	2.04	0.20	0	0	0
Type A callus	18.10	.93	2.15*	0.61	.17	0	0
Type E callus	34.95	.93	2.30*	0.56	1.12	.06	.47
Suspension 11 Mo.	57.74	.92	3.04*	1.57	1.87	.06	.35
Suspension 27 Mo.	32.35	.94	2.86*	1.17	.91	.15	.11

Figure 4.7: Cytological observations of mitotic anomalies in tissue cultures of *Hordeum* possibly responsible for variable chromosome numbers. (A) tetranucleate HVxHJ suspension culture cell (X163), (B) metaphase cell of HVxHJ type E callus exhibiting endoreduplicated chromosomes (X 936), (C) Mitotic anaphase cell of untreated HVxHJ type E callus exhibiting lagging chromosomes (X1383), (D) Mitotic anaphase cell of untreated HVxHJ suspension culture exhibiting multipolar spindles (X 976).

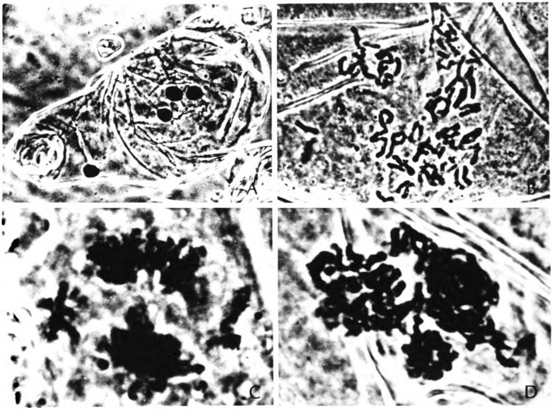


Figure 4.7

cells of untreated cultures in this study as well (Figs. 4.7 C, D). By hypothesizing reciprocal or nonreciprocal chromosome loss or gain based on these processes, the generation of a wide array of aneuploids is quite conceivable. It was only possible to observe secondary phenomena in attempts to discern the source of chromosomal rearrangements. This is because the primary event was necessarily molecular. Chromatin bridges were observed, but no efforts were made to quantify their frequency. Bridges could have resulted from nonreciprocal translocations leading to the formation of dicentric chromosomes, or from dicentric chromosomes perpetuated by a breakage-fusion-bridge cycle (McClintock 1939). Micronuclei were observed requently in all cultured tissues. Type A callus HVxHJ exhibited 9.27% of cells with one or more micronuclei (Table 4.4). They could have been generated by lagging chromosomes, multipolar cell divisions, or acentric fragments.

TABLE 4.4: Micronuclei in type A callus tissue of HVxHJ-57

	No. micronuclei per cell			
	0	1	2	Total
No. cells	509	48	4	561
% cells	90.73	8.56	0.71	

Experiments were formulated to determine the possibility of isolating and propagating karyotypic variability. Subcalli of 200 to 400 cells each were physically removed from a lawn of HVxHJ type E callus with a pair of fine forceps and propagated separately for six weeks. Cells were removed from each of the 35 subcalli and prepared for cytological examination as described in Appendix B. Twenty chromosome counts were taken from each culture. Of the remaining cells of each subcallus, six subsubcalli of 200 to 400 cells each were isolated, and cultured separately for another six weeks. Of the 210 subsubcalli, equal aliquots of tissue were removed and prepared for starch gel electrophoresis as previously indicated (see Fig. 4.8 for an illustration of the procedure). Table 4.5 lists the means and standard errors for 35 subcalli. In addition to variation in mean chromosome number, a large range of standard errors was evident. To test the hypothesis that all of the means were equal, a single factor analysis of variance was performed, the results of which are presented in Table 4.6. Variance attributable to 'between subcalli' was highly significant at P = .001. Hence, it was concluded that all means were not equal.

Figure 4.9 introduces the nomenclature system used for identifying cathodal PRX and anodal EST bands. A zymogram of plumule tissue from a HVxHJ tiller was included for comparison. Differences in band expression and intensity were evident between these differentiated states. Figure 4.9 also illustrates typical examples of band scoring. Instances of null expression were encountered, but most of the variability could be attributed to comparative differences in band intensity (Fig. 4.10, Table 4.8). The mean relative band intensities of the 35 subcalli are presented in Table 4.5. Overall mean relative intensity and coefficient of variability of relative intensity were calculated for each of the five PRX and four EST bands (Table 4.7). Note that coefficients of variability were not equal for most bands.

Figure 4.8: Procedure used for isolating and propagating karyotypic variability.

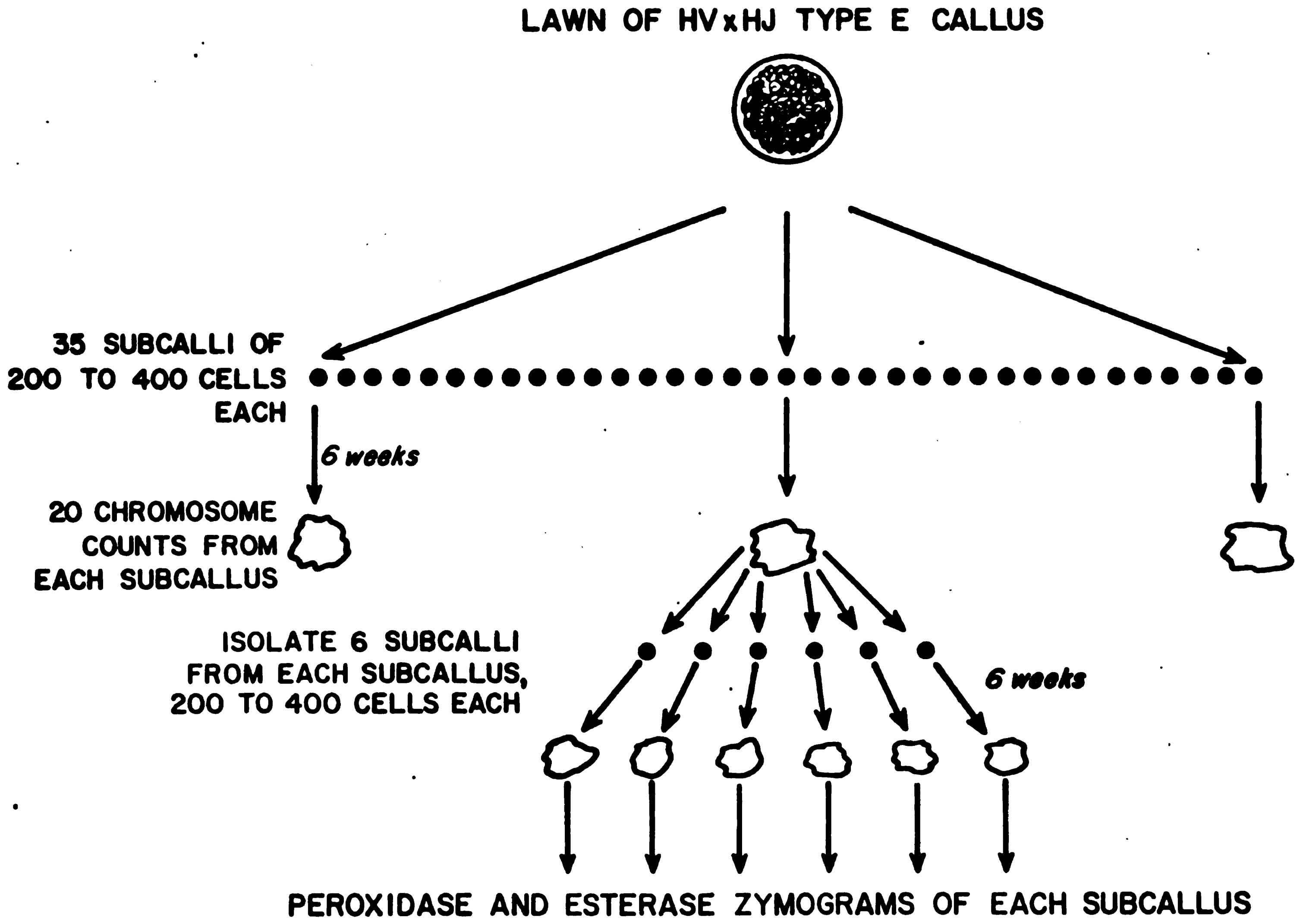

LAWN OF HVxHJ TYPE E CALLUS
35 SUBCALLI OF 200 TO 400 CELLS EACH
6 weeks
20 CHROMOSOME COUNTS FROM EACH SUBCALLUS
ISOLATE 6 SUBCALLI FROM EACH SUBCALLUS, 200 TO 400 CELLS EACH
6 weeks
PEROXIDASE AND ESTERASE ZYMOGRAMS OF EACH SUBCALLUS

FIGURE 4.8

TABLE 4.5: Means of chromosome numbers and PRX and EST isozyme band intensities among 35 subcalli of HVxHJ-57 type E callus.

				Means of 6 subsubcalli								
				PRX band					EST band			
Subcallus	Mean chromosome number	DC	S.E. chromosome number	1	2	3	4	5	1	2	3	4
1	53.05	.96	6.58	0.0	0.0	0.0	0.5	0.5	0.8	1.0	1.2	1.2
2	41.47	.93	2.30	0.0	0.1	0.3	0.6	0.7	1.0	0.7	1.5	1.5
3	33.00	.93	6.34	0.2	0.1	0.5	0.5	0.4	0.7	1.3	1.3	1.7
4	41.32	.94	1.63	0.3	0.3	0.8	0.8	0.8	1.0	1.0	1.7	1.5
5	42.56	.94	2.75	0.2	0.2	0.8	0.5	0.5	1.2	1.2	1.0	0.8
6	37.30	.92	2.97	0.0	0.0	0.6	0.3	0.4	0.5	1.0	1.0	0.7
7	48.76	.94	6.25	1.0	0.5	2.0	0.5	0.5	0.5	1.7	1.3	1.7
8	32.53	.95	2.60	1.0	0.0	0.3	0.5	1.0	0.8	1.7	1.7	0.8
9	35.90	.93	1.72	0.6	0.2	0.9	0.5	0.8	0.8	1.0	1.2	0.8
10	41.06	.94	4.64	0.3	0.1	0.1	0.5	0.5	0.5	0.8	0.8	0.8
11	37.33	.93	2.83	1.0	0.2	0.0	0.9	0.4	0.5	1.0	1.0	0.8
12	20.16	.96	1.76	1.5	0.0	0.4	0.7	0.5	0.2	0.8	1.2	1.1
13	33.53	.92	3.17	0.0	0.0	0.4	0.4	0.5	0.7	1.7	1.7	1.7
14	19.50	.96	1.03	0.0	0.1	0.9	0.5	0.3	0.5	0.8	1.5	0.5
15	24.11	.93	1.43	1.0	0.2	0.7	1.0	0.1	0.5	0.9	0.9	0.8
16	20.55	.96	1.27	-	-	-	-	-	-	-	-	-
17	42.53	.91	2.90	0.8	0.8	0.8	0.7	1.0	0.8	1.2	1.2	0.6
18	43.12	.90	6.50	0.0	0.3	0.8	0.6	0.4	0.2	0.6	0.9	1.2
19	37.27	.94	1.17	0.0	0.1	0.9	0.3	1.0	0.8	0.8	1.0	1.0
20	59.95	.94	7.96	0.8	0.4	1.5	0.9	0.9	0.5	1.8	1.8	1.5
21	25.30	.96	2.00	1.8	0.3	1.8	0.4	0.9	0.2	0.9	1.0	0.7
22	23.51	.95	2.99	0.8	0.0	1.7	0.4	0.7	0.5	1.0	1.5	1.7
23	50.05	.93	6.36	1.0	0.0	0.4	0.5	1.0	0.2	0.9	1.0	0.7
24	49.19	.91	4.70	0.0	0.1	0.4	0.3	0.5	0.4	1.5	1.5	1.5
25	23.03	.94	1.56	1.7	0.2	0.8	0.6	0.5	0.8	0.8	0.8	0.8
26	41.16	.92	3.93	1.0	0.0	0.8	0.6	0.5	0.5	0.7	0.7	0.5
27	50.00	.93	7.39	1.0	0.0	0.8	0.4	1.3	0.6	1.5	1.4	1.7
28	31.81	.93	3.18	1.0	0.2	0.8	1.0	1.0	0.3	1.5	1.5	1.5
29	28.29	.92	2.79	0.0	0.0	0.8	0.3	0.4	0.3	1.7	2.0	2.0
30	40.11	.92	3.08	1.0	0.1	0.0	0.6	0.0	0.5	0.5	0.9	0.2
31	38.67	.92	2.65	1.0	0.0	1.7	0.5	0.9	0.5	0.5	0.5	0.5
32	37.50	.94	3.98	1.9	0.2	1.5	0.9	0.8	0.8	0.8	0.8	1.7
33	37.61	.94	2.32	1.5	0.0	1.5	0.5	1.0	0.5	2.0	2.0	1.3
34	21.11	.95	0.61	1.2	0.2	1.3	1.0	0.5	0.2	1.0	1.0	1.7
35	26.13	.94	1.82	0.9	0.3	0.1	0.7	0.1	0.6	0.6	0.6	1.3

Figure 4.9: Nomenclature system and examples of typical scoring for cathodal PRX and anodal EST bands from crude extracts of HVxHJ type E callus.

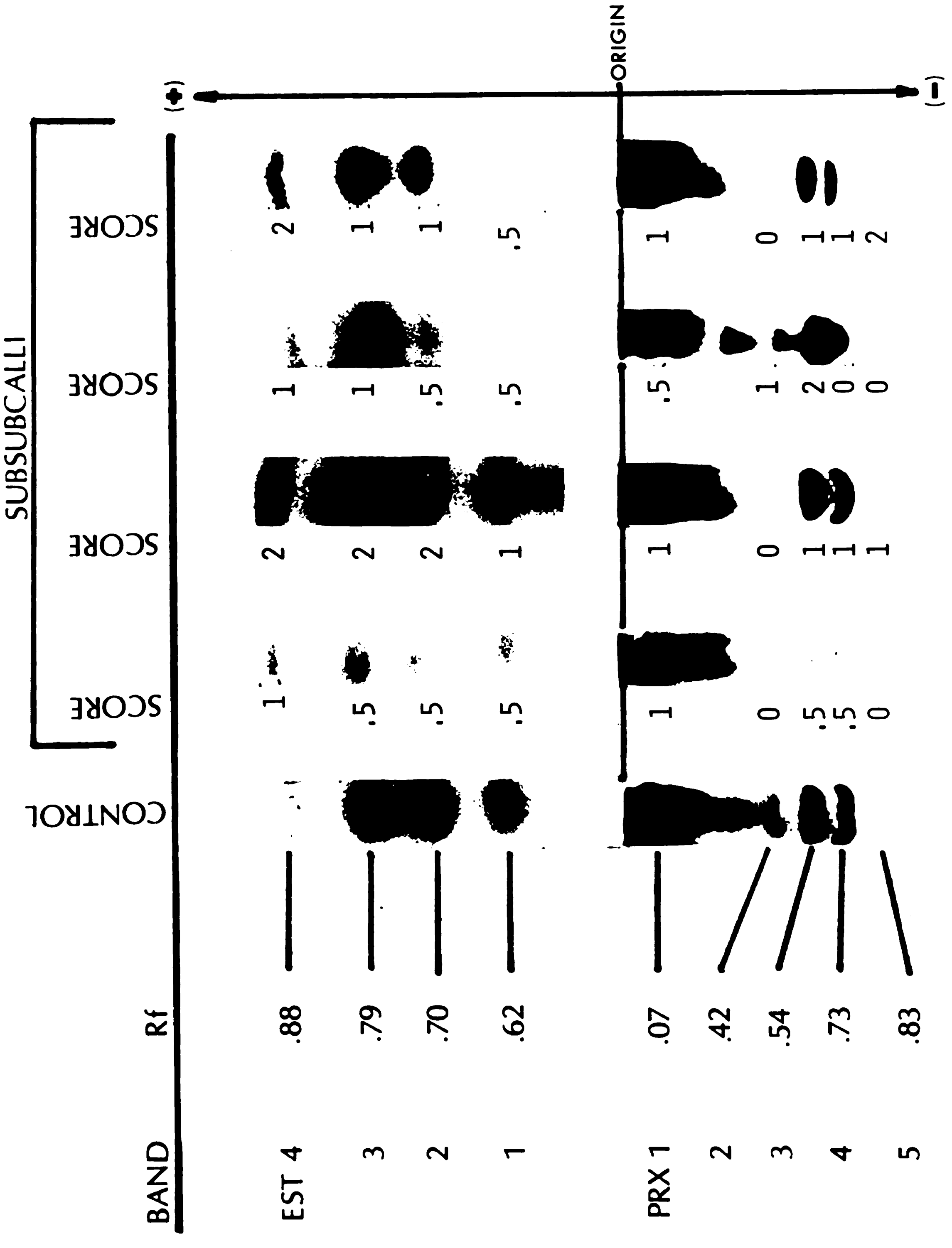

BAND
Rf
CONTROL
SUBSUBCALLI
SCORE
SCORE
SCORE
SCORE
(+)
(−)
ORIGIN
EST 4
3
2
1
PRX 1
2
3
4
5
.88
.79
.70
.62
.07
.42
.54
.73
.83
1 .5 .5 .5 1 0 .5 .5 0
2 2 2 1 1 0 1 1 1
1 1 .5 .5 .5 1 2 0 0
2 1 1 .5 1 0 1 1 2

Figure 4.9

Figure 4.9: Nomenclature system and examples of typical scoring for cathodal PRX and anodal EST bands from crude extracts of HVxHJ type E callus.

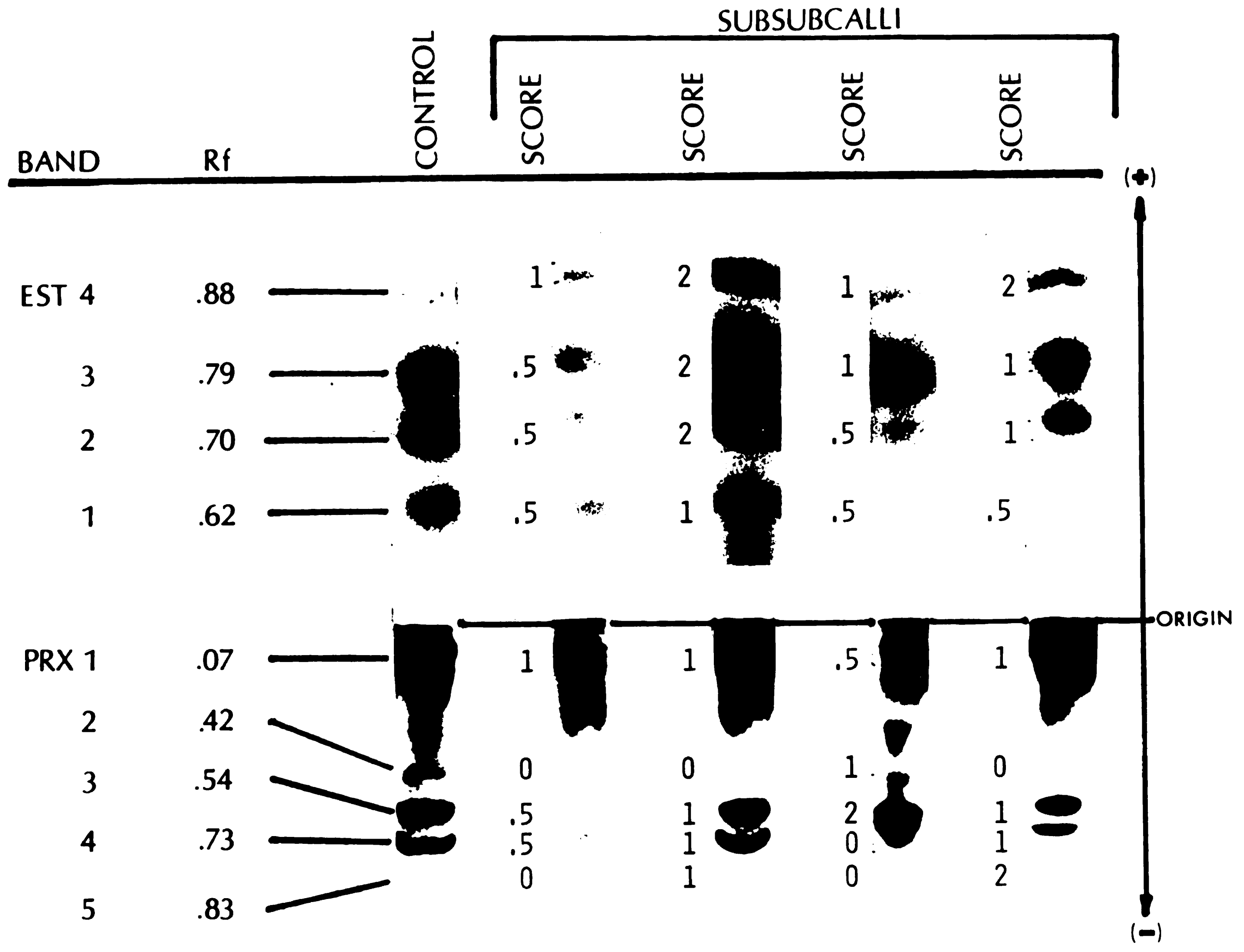

SUBSUBCALLI
BAND
Rf
CONTROL
SCORE
SCORE
SCORE
SCORE
(+)
EST 4 .88 1 2 1 2
3 .79 .5 2 1 1
2 .70 .5 2 .5 1
1 .62 .5 1 .5 .5
ORIGIN
PRX 1 .07 1 1 .5 1
2 .42 0 0 1 0
3 .54 .5 1 2 1
4 .73 .5 1 0 1
5 .83 0 1 0 2
(−)

Figure 4.9

Figure 4.10: Comparative cathodal PRX (A) and anodal EST (B) zymograms to show quantitative and qualitative differences in band expression among subsubcalli of HVxHJ type E callus.

[1] HV type E callus

[2] HJ type E callus

*plumule extract of original HVxHJ hybrid

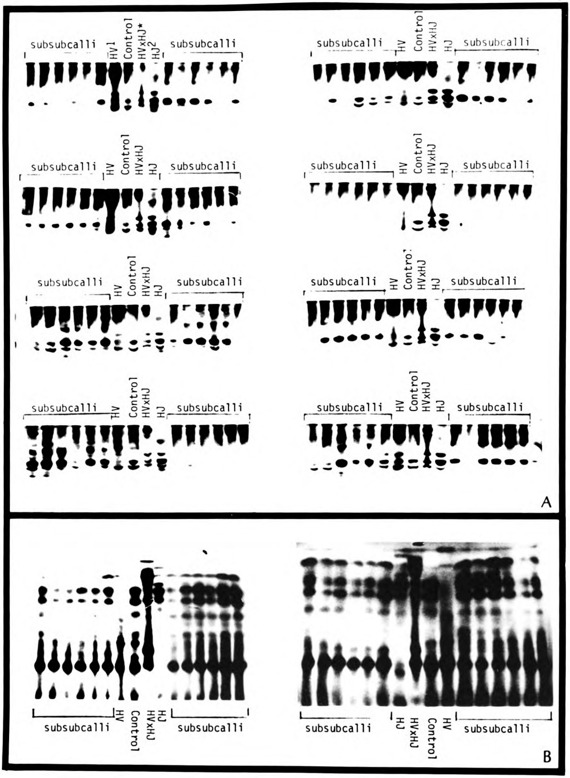

subsubcalli
HV1
Control
HVxHJ*
HJ2
subsubcalli
HV
Control
HVxHJ
HJ
A
subsubcalli
HV
Control
HVxHJ
HJ
B

Figure 4.10

TABLE 4.6: Analysis of variance; chromosome numbers of 35 subcalli of HVxHJ-57 type E callus

Source	SS	df	MS	F*
Between subcalli	48306.83	34	1420.79	5.01***
Within subcalli (error)	188457.94	664	283.82	
Total	236764.77	699		

TABLE 4.7: Means and coefficients of variation (V) of isozyme intensities among PRX and EST isozyme bands

Band	Overall mean	Range	V	Percent of cases band missing
PRX 1	0.69	0 - 2	0.80	31.90
2	0.14	0 - 2	1.21	74.76
3	0.80	0 - 2	0.69	26.67
4	0.58	0 - 1	0.36	8.57
5	0.62	0 - 2	0.51	19.05
EST 1	0.57	0 - 2	0.44	18.10
2	1.08	0 - 2	0.38	1.90
3	1.20	0 - 2	0.33	1.90
4	1.13	0 - 2	0.42	7.14

TABLE 4.8: Distributions of isozyme band intensities

Band	No. subsubcalli exhibiting a band intensity of: 0	.5	1.0	2.0
PRX 1	65	15	96	16
2	148	37	5	2
3	45	54	56	37
4	36	106	50	0
5	41	63	83	5
EST 1	40	103	48	1
2	3	42	105	42
3	3	31	96	62
4	14	41	83	54

In particular, PRX band 2 appeared to exhibit far more variability than the other four PRX or EST isozymes. The distribution of relative band intensities, for all tested subsubcalli, was approximately normal with exception of PRX 1 (Table 4.8). Among zymograms of replicate controls, no variability of band expression was observed.

Discussion

Observations of in vitro karyotypic variation in plant tissue cultures were first made by Straus (1954) on cultured endosperm of maize. Since that report, nearly every plant system observed in culture has been shown to exhibit some degree of in vitro karyotypic variability (Partanen 1965, D'Amato 1975, Sunderland 1977). Among grain monocots, plants regenerated from scutellum-derived cultures of maize (Green and Phillips 1975) and shoot tip of barley (Cheng and Smith 1975) were karyotypically identical to the original explants, although no counts were taken directly from the source callus. Variable chromosome numbers, however, have been reported in tissue cultures of wheat (Kao et al. 1970) and rice (Nishi and Mitsuoka 1969). In barley, Saalbach and Koblitz (1977) regenerated plants with highly variable chromosome numbers form haploid-derived callus. Further, the results of this study have conclusively demonstrated the existence of karyotypic variability in tissue cultures of Hordeum.

Callus cultures of this study adapted stable 'types' shortly after callus induction (Chapter 2). These types expressed differences in means and dispersion of chromosome numbers. This suggested that in vitro karyotypic variability may have been a consequence of the cultured state. Type A callus cultures were characterized by a stable mean chromosome number over time, usually eudiploid or slightly hypodiploid, in an approximately normal distribution. Aneuploidy accumulated dramatically in these cultures over time (Figs. 4.3, 4.4). Further, at 16 months, a significant degree of chromosomal rearrangements was detected (Table 4.3).

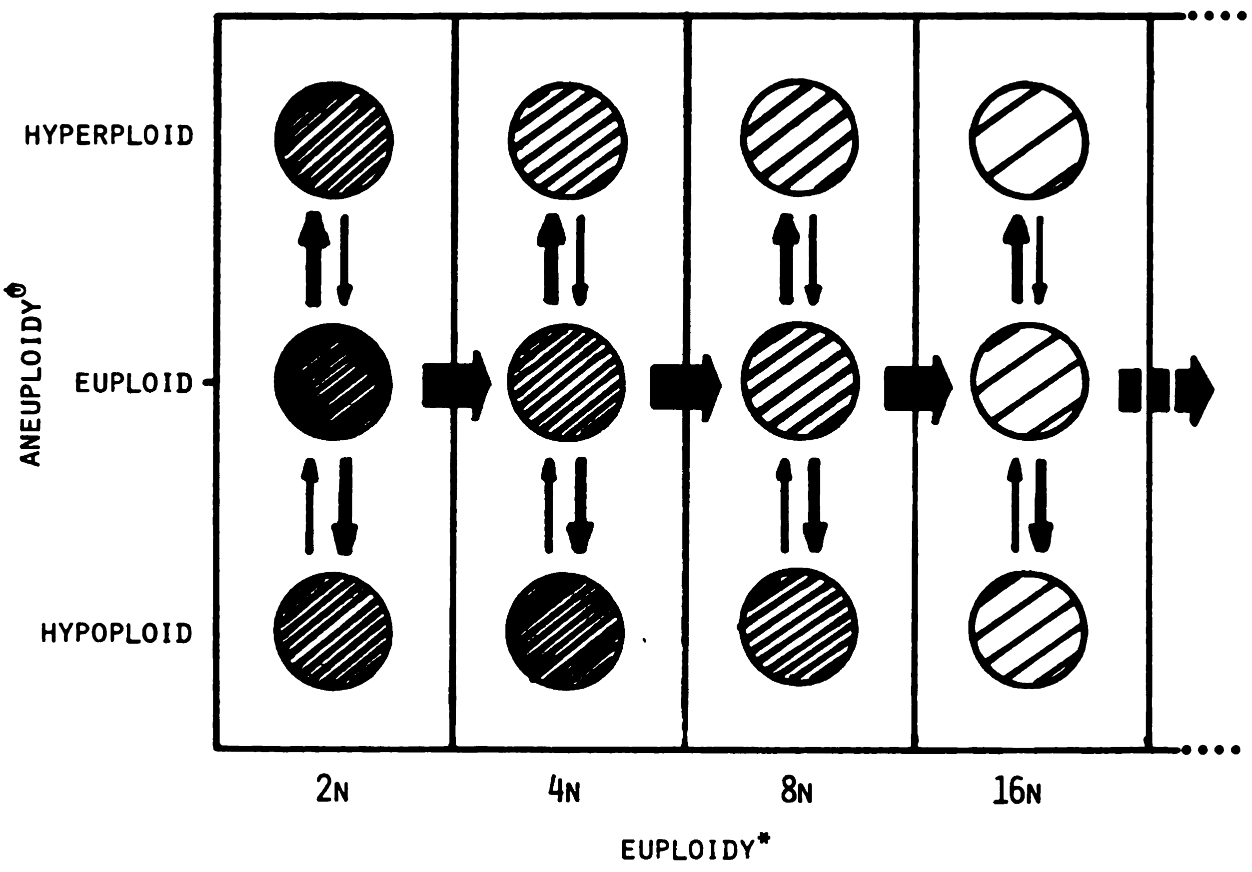
Type E calli frequently arose spontaneously on the cortex of type A callus nodes. Concomitant with this event was a dramatic alteration in the rate of aneuploidization, and the distribution of chromosome numbers. After increasing and reaching a plateau, mean chromosome numbers attenuated, and appeared to stabilize at 35 to 36 while variability remained stable. A distribution was generated with clusters in a hypoeuploid series, with triploid and hypohexaploid nodes predominating (Fig. 4.4 C). Chromosomal rearrangements, as manifested by mean L/S ratios, increased slightly in comparison to type A callus (Table 4.3). Suspension cultures behaved quite similarly to type E callus, from which they were derived. The process, however, appeared to have been accelerated. Chromosomal rearrangements were observed to increase dramatically in these cultures (Table 4.3). Concomitant with the regeneration of secondary type E callus from suspension culture was an apparent deceleration of the same sequence of phenomena outlined above. An insufficient number of counts were taken, however, to rigorously characterize the count distribution in these cultures.

It is proposed that types A and E calli are distinct differentiated states which differ in two cytogenically observable respects: 1) Chromosomes of type A calli are not endoreduplicated while those of type E are or may be. 2) The tissue of type A callus is quasi-organized (Chapter 2); only certain highly adapted karyotypes have the potential to exist in the matrix of this callus. Type E callus exhibits no such intrinsic organization. Hence, any cell which is capable of proliferating has the potential to exist. The first hypothesis should be testable by comparing cytogenetic and microspectrophotometric data. A direct test of the second hypothesis is less straightforward. At a low frequency, type A-like nodes arose de novo from within the matrix of type E callus. If the hypothesis is true, the karyotypic profile of these nodes should be approximately diploid (or euploid), with flanking aneuploidy in a normal distribution.

The observed distributions of chromosome numbers in these cultures is visualized as an interaction of tissue potentialities, as described above, with a challenging environment. Karyotypically variant cells are generated by the culture at a constant rate per cell division (assuming randomness), the rate being an intrinsic property of the callus/culture type. After a period of time at a constant growth rate, a steady-state distribution of chromosome numbers is generated. The constant rate of karyotypically variant cell production is balanced by the constant rate of selection for proliferative types. Selection for specific constituents of a variable cultured plant cell population has been demonstrated previously (Demoise and Partanen 1969, Singh _et al_. 1975, Bayliss 1975, Singh and Harvey 1975). A changed environment may select for a different cell type, thus resulting in a different stable steady-state distribution. This explanation is compatible with observations in time-course studies (Figs. 4.1, 4.3) and of distributions of chromosome numbers in derivatives of type E callus (Figs. 4.4, 4.5). Patterns of chromosome number fluctuation, following developmental/environmental changes (e.g. callus induction, generation of type E callus, etc.) depend upon source chromosome number profiles. The process is illustrated in Fig. 4.11 as an analogy to chemical equilibrium in a closed system. Growth in type A callus was extremely slow (Chapter 2), possibly explaining why a steady state distribution had not been observed.

Equivalent subcalli derived from stable uniform callus subcultures of Nicotiana tabacum (Caplin 1947) and Hordeum (Chapter 2) exhibited widely divergent growth rates. The results of this study offer a plausible explanation for these observations: the existence of spatially distinct tissues with different chromosomal constitution. These differences were a consequence of the generation and propagation of karyotypic variability. Mean chromosome numbers of 35 subcalli ranged from 19.50 to 59.95. Further, it was possible to isolate and propagate genetic variability, as indicated by zymograms of PRX and EST (Figs. 4.9, 4.10, Table 4.5). The use of horizontal starch gel electrophoresis was a relatively crude technique for quantitative assays. Hence, the results of the analysis must be treated as preliminary. More rigorous techniques, employing controlled extractions, polyacrylamide gel electrophoresis, and densitometer determinations of enzyme activity, are currently being developed for confirmation and extension of these results. Cytoplasmic concentrations of proteins were shown to be directly proportional to the dose of chromosomes carrying the corresponding structural genes in tomato (Fobes 1977). Carlson (1972) first suggested the use of aneuploids

Figure 4.11: Hypothetical mechanism to explain the generation of a steady state distribution of chromosome counts in HVxHJ type E callus cultures. The size of the arrows are proportional to rates of conversion; intensity (darkness) of the circles are proportional to fitness (= rate of mitotic proliferation, e.g. the darkest circles represent chromosomal complements with the greatest intrinsic cell division capacity for HVxHJ type E callus on maintenance medium).



^A CONSEQUENCE OF SPINDLE ANOMALIES
*A CONSEQUENCE OF ENDOREDUPLICATED CHROMOSOMES

Figure 4.11

to locate genetic loci. The results of this study suggest the use of genetic loci (e.g. isozyme expression) to identify aneuploids. Further, by measuring the covariation of phenotypes, it may be possible to establish linkage relationships. Such a relationship may exist for EST 2 and 3 (Tables 4.7, 4.8), or their transcription or translation may be coordinately controlled. No speculation was made as to the significance of differences between isozyme bands in overall mean, V, and % null expression aside from possible implications of mandatory vs. non-mandatory gene functions (Table 4.7).

Karyotypic variability was spontaneously generated as a consequence of the interactions of Hordeum tissues with culture conditions. The encountered variability was manifested in three distinct classes: polyploidy, aneuploidy, and chromosomal rearrangements. It is possible that novel genotypes were produced, of potential use in molecular or genetic studies. One such possibility is the regeneration, from tissue cultures of an interspecific hybrid, of plant populations embodying continuous mixtures of parental genomes. Aside from the economic advantages of conserved space and time, this technique has the potential of generating entities unobtainable via classical introgressive methods. In this study, progressive aneuploidy was demonstrated in cultures of type A callus, which have been shown to be totipotent. Further studies have been formulated to determine how much of the karyotypic variability generated in vitro can be expressed in corresponding populations of regenerated plants.

CHAPTER V

KARYOTYPIC VARIABILITY IN PLANTS REGENERATED FROM TISSUE CULTURES OF *HORDEUM*

Introduction

Karyotypic variability is a ubiquitous phenomenon in plant tissue cultures (Sunderland 1977). An increase in *in vitro* variability of chromosome numbers has been associated with a loss in regenerative potential (Muir 1965, Murashige and Nakano 1965, Torrey 1967). Further, regenerating plants express less karyotypic variability than precedent tissue cultures, prompting the conclusion that regeneration selects for certain cell types (Sacristan and Melchers 1969, Novak and Vystot 1975). In other reports, however, populations of regenerated plants exhibited a magnitude of variability comparable to source cultures (Ogura 1975, 1976, Heinz and Mee 1971). It is apparent that differences in karyotypic plasticity for regeneration requirements exist between species, explants, and culture systems.

This heterogeneity extends to comparisons among tissue culture systems of annual grains as well. Only eudiploid regenerates were observed in specific cases involving corn (Green and Phillips 1975), wheat (Shimada *et al*. 1969), and barley (Cheng and Smith 1976). Conversely, instances of variable morphology and abnormal meiotic configurations in regenerated oats (Cummings *et al*. 1976) and mixoploidy in barley regenerates (Saalbach and Koblitz 1977) have been reported.

In an earlier report, the use of in vitro karyotypic variability as a potential source of novel chromosomal variants was advanced (Chapter 4). Amphiploids of Hordeum vulgare x H. jubatum were sterile due to a genome incompatibility leading to a breakdown in meiosis (Chapter 3). In a population of cells exhibiting random (within the range of viability) chromosome complements, cells may exist with the potential of regenerating into fertile intermediates of this cross. Aneuploidy and chromosomal rearrangements have been shown to accumulate with serial subculturing in totipotent type A callus cultures of Hordeum, lending credence to this possibility (Chapter 4).

This chapter will examine the comparative karyology of tissue cultures and corresponding regenerated structures. The degree of morphological and genetic variability among regenerated plants will be ascertained. Finally, techniques will be used to detect and measure the in vitro segregation of parental genomes expressed in populations of regenerated plants.

Materials and Methods

Seeds or perennial stocks of Hordeum vulgare (HV, 2n=2x=14), H. jubatum (HJ, 2n=4x=28), and their interspecific hybrid (HVxHJ, 2n=3x=21) were obtained from Dr. J. E. Grafius, Dept. of Crop and Soil Sciences, Michigan State University. All plants were maintained by or as described by Steidl (1976). Techniques and media used for the induction and maintenance of callus and suspension cultures were described in Chapter 2. To induce regeneration, 65 type A calli of HVxHJ were transferred from maintenance medium to MS medium

(Linsmaier and Skoog 1965) supplemented with .3 mg/l KIN, 1 mg/l GA_3, 1% sucrose, 1% D-glucose, at pH 6.0. These petri dishes were maintained at 26°C under 16 hr./day of 2000 to 3250 lux from GE G96T10-CWX lamps. Approximately 50 of these calli gave rise to whole plants.

Pertinent cytogenetic methodology is described in Appendix B. To determine the in vivo expression of in vitro karyotypic variability, chromosome counts were categorized according to source. For regenerating root tips, counts were classified according to callus and root as follows: root tips from primary callus; 4 calli x 3 roots per callus x 3 counts per root: root tips from secondary type E-derived type A callus; 5 calli x 3 roots per callus x 3 counts per root. Counts from ovary wall tissue were partitioned as follows: 8 plants x 2 tillers per plant x 3 florets (ovaries) per tiller x 3 counts per ovary.

Zymograms of esterase (EST) and glutamate-oxaloacetate transaminase (GOT) were used to measure genetic variability. Crude extracts were obtained from fresh tissue of young tillers (3 leaf stage) between the base of the culm and the first leaf base by crushing equal amounts of tissue in weight boats with a pestle. The crude extract was taken up into Beckman electrophoretic paper wicks of equal size. Extract elution, running, slicing, esterase staining, and fixing were performed as described in Appendix A.

Results

Figure 5.1 displays the composite distribution of chromosome numbers in root tip meristems regenerating from 16 month old primary

Figure 5.1: Comparative distributions of chromosome numbers from (A) HVxHJ type A callus, (B) roots regenerating from HVxHJ type A callus, and (C) immature ovary wall tissue in HVxHJ regenerates.

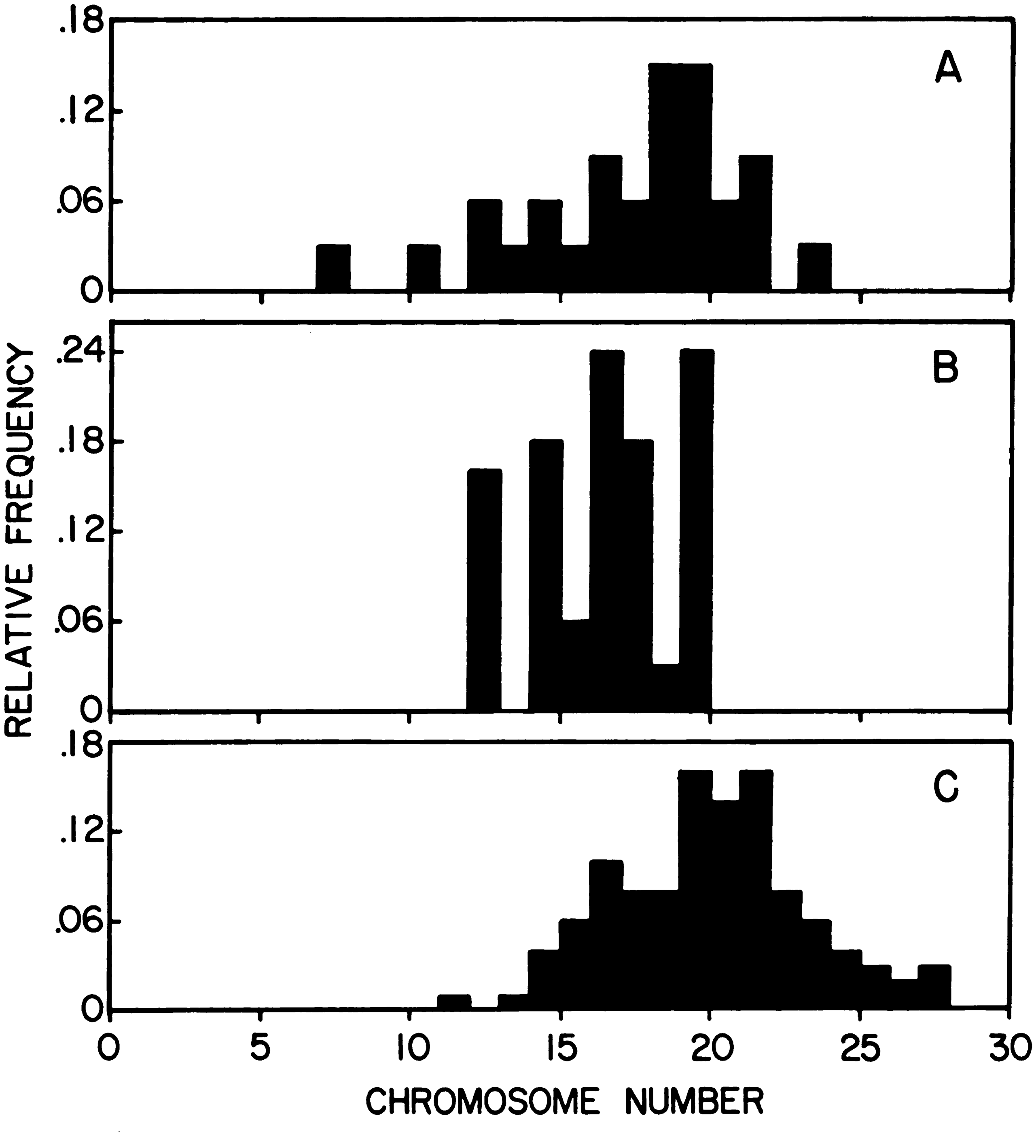

A
B
C
.18
.12
.06
0
.24
.18
.12
.06
0
.18
.12
.06
0
0
5
10
15
20
25
30
RELATIVE FREQUENCY
CHROMOSOME NUMBER

Figure 5.1

type A callus. These roots exhibited a mean of 16.94 compared to 18.10 in the source callus and 21.00 in the original explant (Table 5.1). Among 36 cells, counts ranged from 13 to 20, or 100% aneuploid relative to the original explant. In addition, the statistical variance exhibited by regenerating roots was significantly less than that of the source callus. Composite data from immature ovary wall tissues of mature plants regenerated from the same culture are presented in Fig. 5.1 C. The distribution is approximately normal around a mean of 20.47. In this sample of 144 cells, counts ranged from 12 to 28, and the statistical variance was significantly greater than that of regenerating root tips (Table 5.1). Upon regeneration, an immediate attenuation of karyotypic variability was observed, manifested by the truncation of extremes from the normal distribution of type A callus (Fig. 5.1). During progressive stages of development, the distribution had spread and normalized about a mean of 20.47, nearly that of the original explant. This _in vivo_ increase in karyotypic variability could have been a consequence of anaphase abnormalities such as lagging chromosomes and multipolar separations (similar to those observed _in vitro_, Chapter 4), which were observed frequently in ovary wall tissue of regenerated HVxHJ plants (Fig. 5.2).

At a very low frequency among secondary lawns of type E callus, interstitial type A-like nodes were observed to arise spontaneously. These lawns had been generated from rapidly growing (doubling time = 48 hrs.), finely divided suspension cultures. Hence, these nodes must have arisen de novo from secondary type E callus tissue. Type E calli exhibited vastly different karyotypic profiles

TABLE 5.1: Comparison of chromosomal rearrangements between *in vitro* and corresponding regenerated *in vivo* tissue

Tissue	Mean chromosome number	DC	V chromosome number	Mean L/S	V L/S	Mean no./cell		
						dicentric	tricentric	fragment
Root meristem, source plant[a]	21.00	1.00	0.00	2.04	0.10	0.0	0.0	0.0
Type A callus	18.10	.93	0.77	2.15*	0.28	0.17	0.00	0.00
Regenerated root, primary type A	16.94	.96	0.13	2.03	0.25	0.00	0.00	0.00
Suspension,[a] 11 month	57.74	.92	0.66	3.04*	0.52	1.87	0.06	0.35
Regenerated root, secondary type A	20.00	.94	0.16	2.14*	0.27	0.13	0.08	0.00

[a] Chapter 4.

Figure 5.2: Mitotic anaphase cells in immature ovary wall tissue of regenerated HVxHJ plants exhibiting lagging chromosomes (A) X1154, (B) X1480

Figure 5.3: Distribution of chromosome counts from root tips regenerating from secondary HVxHJ type A callus and from HVxHJ progenitor suspension culture

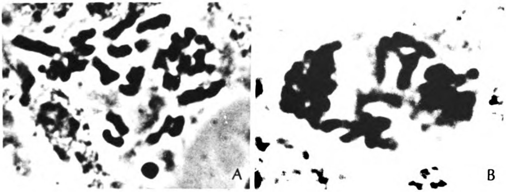


Figure 5.2

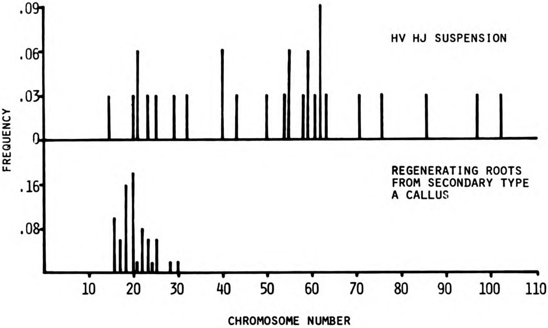


Figure 5.3

as compared to type A calli. Chromosome counts were recorded from root tips regenerated from these nodes. The distribution approximated assymetric normality around a mean of 20.00 (Fig. 5.3). In the course of differentiation, selection for a specific range of chromosome numbers had occurred (Fig. 5.3). Prohibitively small amounts of this de novo nodular callus precluded a comprehensive karyotypic analysis. Only three counts could be obtained, ranging from 18 to 21. Hence, it was impossible to determine conclusively whether the attenuation of karyotypic variability in these regenerating root tips was a consequence of the type E-type A or type A-regenerated root transformation juncture.

Previous studies of barley tissue cultures conclusively demonstrated the existence of structurally rearranged chromosomes (Chapter 4). Are cells with karyotypes containing these rearrangements capable of differentiation and fixation into somatic plant tissues? Longest:shortest chromosome ratios (L/S) were calculated from cells of primary and secondary root tips as described in Chapter 4. These ratios are displayed in Table 5.1 with those of progenitor cultures. In root tips regenerated from primary type A callus, the mean L/S ratio had decreased from 2.15 to 2.03, indicating possible selection for cells with karyotypes without rearranged chromosomes. Data from root tips regenerated from secondary type E-derived type A callus demonstrated a dramatic decrease in mean L/S from 3.04 to 2.14. Chromosomal rearrangements must have been present in the karyotypes of some of the cells of these regenerated root tips since the mean of this sample was significantly greater than 2.04. Further, the corresponding frequencies of multicentric chromosomes were consistent with these conclusions.

Cytogenetically, regeneration was a differentiative process characterized by the following karyological phenomena in this study: 1) The dispersion of chromosome numbers decrease following regeneration, 2) Only mixodiploid cells were present in regenerated tissues, and 3) Regeneration tended to select for karyotypes with a lower frequency of rearranged chromosomes than that of the source culture. To realize the full potential of genetic studies and selection, the regenerated pool of karyotypic variability must be expressed between plants, and not within plants. Potentially, three processes can be envisioned: 1) A small number of cell initials with a lower karyotypic variability relative to the source culture gave rise to each regenerative entity, which then propagated clonally giving rise to karyotypically 'uniform' plants, 2) A large number of initials exhibiting karyotypic variability approximately equal to the source culture gave rise to each regenerative entity, which then propagated clonally, giving rise to chimeral plants, and 3) After regeneration, new karyotypic variability was generated, giving rise to mixoploid tissues in conjunction with cases 1 or 2. To gain an insight into the pathway of karyotypic variability into regenerated plant tissues, chromosome counts were categorized within and between regenerated structures. For roots regenerated from primary and secondary type A callus, the variances attributable to between calli and within calli between roots were highly significant. Likewise, for ovary wall tissues of mature regenerated plants, all partitioned variances (i.e. between plant, within plants between tillers, and within tillers between ovaries) were highly significant (Tables 5.2, 5.3, 5.4).

TABLE 5.2: Analysis of variance, chromosome counts from immature ovary wall tissue of plants regenerated from HVxHJ type A callus

Source	SS	df	MS	F*
Between plants	721.08	7	103.01	75.74***
Within plants, between tillers	283.18	8	35.40	26.03***
Within tillers, between ovaries	188.30	32	5.88	4.32***
Error	130.67	96	1.36	
Total	1323.23	143		

TABLE 5.3: Analysis of variance, chromosome counts from root tips regenerating from primary type A callus of HVxHJ

Source	SS	df	MS	F*
Between calli	105.64	3	35.21	35.21***
Within calli, between roots	58.30	8	7.29	7.29***
Error	24.00	24	1.00	
Total	187.94	35		

TABLE 5.4: Analysis of variance, chromosome counts from root tips regenerating from secondary type A callus of HVxHJ

Source	SS	df	MS	F*
Between calli	116.43	4	29.11	7.89***
Within calli, between roots	220.60	10	22.06	5.98***
Error	110.67	30	3.69	
Total	447.70	44		

In previous reports, it was established that karyotypic variability could be attributed to polyploidy, aneuploidy, and chromosomal rearrangements (Chapter 4). The loss or gain of single chromosomes implied a quantitative change in the ratio of the number of HV to HJ chromosomes. Taking this notion of parental imbalance further, it was hypothesized that continuous mixtures of parental genomes existed in cultured populations of karyotypically variable HVxHJ cells. Each genome possesses all of the necessary functions for life, making the existence of pure parental types feasible. A normal curve was postulated regarding the stoichiometry of parental mixtures, based roughly on a binomial probility function (i.e. $\{7HV + 14HJ \text{ chromosomes}\}^2$). This hypothetical process is diagramatically illustrated in Fig. 5.4. The most direct test of the hypothesis would have been a comparison of predicted with actual distributions of parental chromosomes in the karyotypes of regenerated HVxHJ plants. Plant chromosome banding techniques are widely utilized and would provide a concise tool for the identification of the parental origin of specific chromosomes (Vosa 1975). As stated in Chapter 4, all attempts to perfect such a technique met with failure. Further, heterochromatic bands in Hordeum are generally centro- and telomeric, exhibiting very little linear differentiation (Linde-Larusen 1975). It can be speculated that such patterns would have been impotent for precise parental identification. Also, chromosomal rearrangements would have had the effect of defacing established banding patterns, further nullifying the analysis.

The isolation and propagation of genetic variability was demonstrated by comparing assays of isozyme concentration in crude extracts of test calli (Chapter 4). Within a given genotype, isozyme patterns were observed to differ significantly between callus types.

Figure 5.4: Illustration of the hypothesis regarding the generation of a continuum of mixtures of parental chromosomes in tissue cultures of interspecific hybrids (see text for a more detailed explanation).

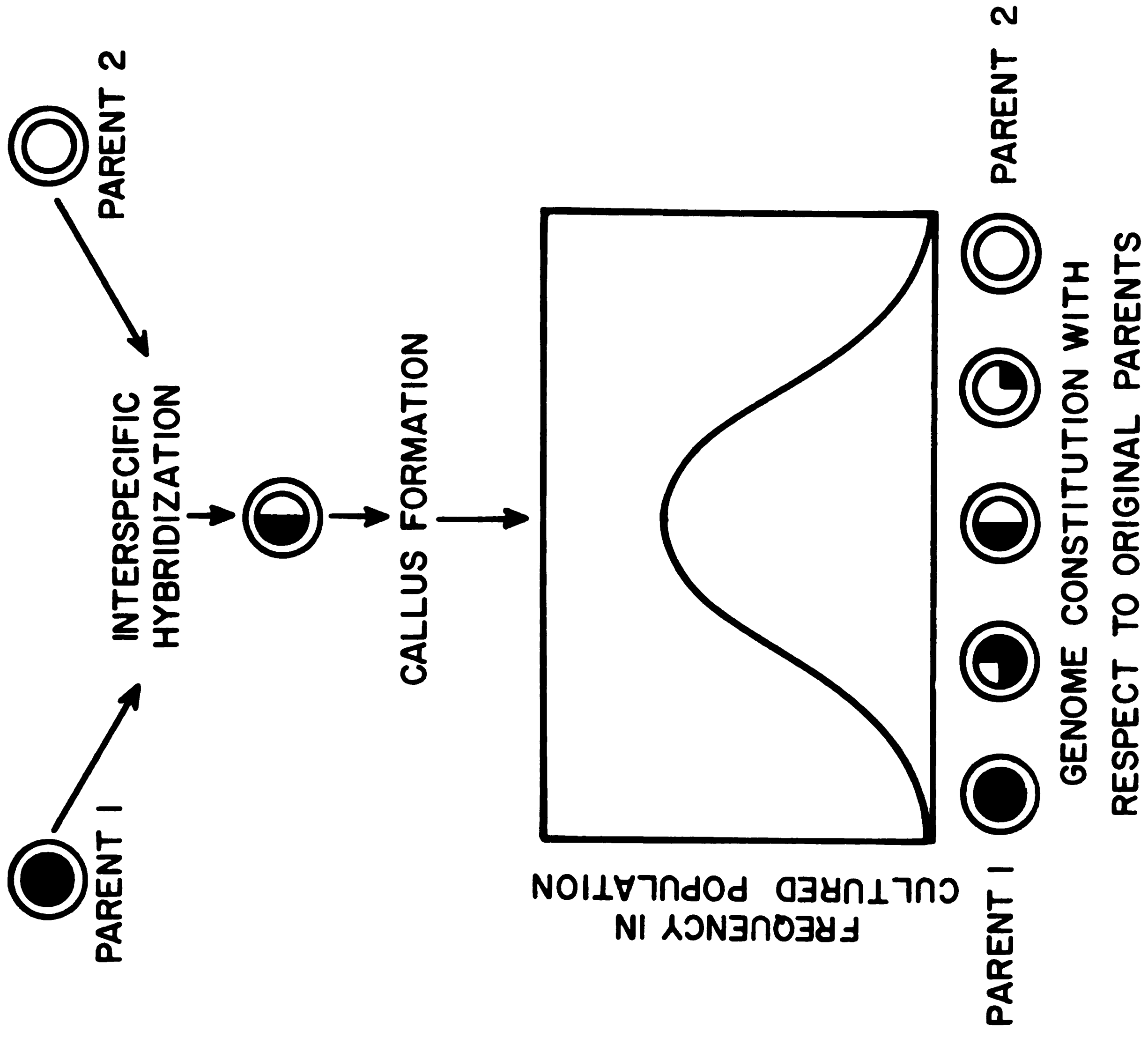

PARENT I
PARENT 2
INTERSPECIFIC HYBRIDIZATION
CALLUS FORMATION
FREQUENCY IN CULTURED POPULATION
PARENT I
PARENT 2
GENOME CONSTITUTION WITH RESPECT TO ORIGINAL PARENTS

FIGURE 5.4

This tended to make the identification of specific HV and HJ isozymes quite difficult. In whole plants, however, it was much easier to make comparisons between genotypes because these uncontrolled tissue differences were obviated. Comparisons of anodal EST and GOT zymograms from crude extracts of young culms yielded 5 HV-specific, 3 HJ-specific,1 comigrating (e.g. present in both parents), and 1 hybrid band (e.g. present in neither parent). The observation of three GOT bands in the hybrid supports the conclusion that this particular enzyme is a homodimer (Fig. 5.5).

Forty-three plants regenerated from HVxHJ type A callus culture were scored for EST and GOT isozyme intensity, as described in Chapter 4, vs. those of the HVxHJ F_1 hybrid (control). Replicate samples generally did not differ from each other (Fig. 5.6). Examples of the scoring procedure are illustrated in Fig. 5.5. In general, the variation of band intensity was much less for GOT than for EST (Table 5.5). Further, it was observed that HV-specific bands tended to be expressed in higher intensities than those of HJ. The distributions of all band intensities were approximately normal (Table 5.6). For each plant, the genome mixture was estimated by calculating a ratio of (ΣHJ band intensities:ΣHV band intensities). In control plants, this ratio was 0.6 (3/5). The distrubution of this ratio was bimodal (Fig. 5.7), but was manifested in a continuum from 0 to 1.0.

Morphological variability in phenotypes such as growth habit, growth rate, size (Fig. 5.8), head morphology, and auricle expression (Fig. 5.9) were also evident between these regenerated plants. Regarding head morphology, variability was manifested in awns

Figure 5.5: Nomenclature and examples of scoring for GOT and EST bands of crude extracts from basal culm tissue of HVxHJ regenerates.

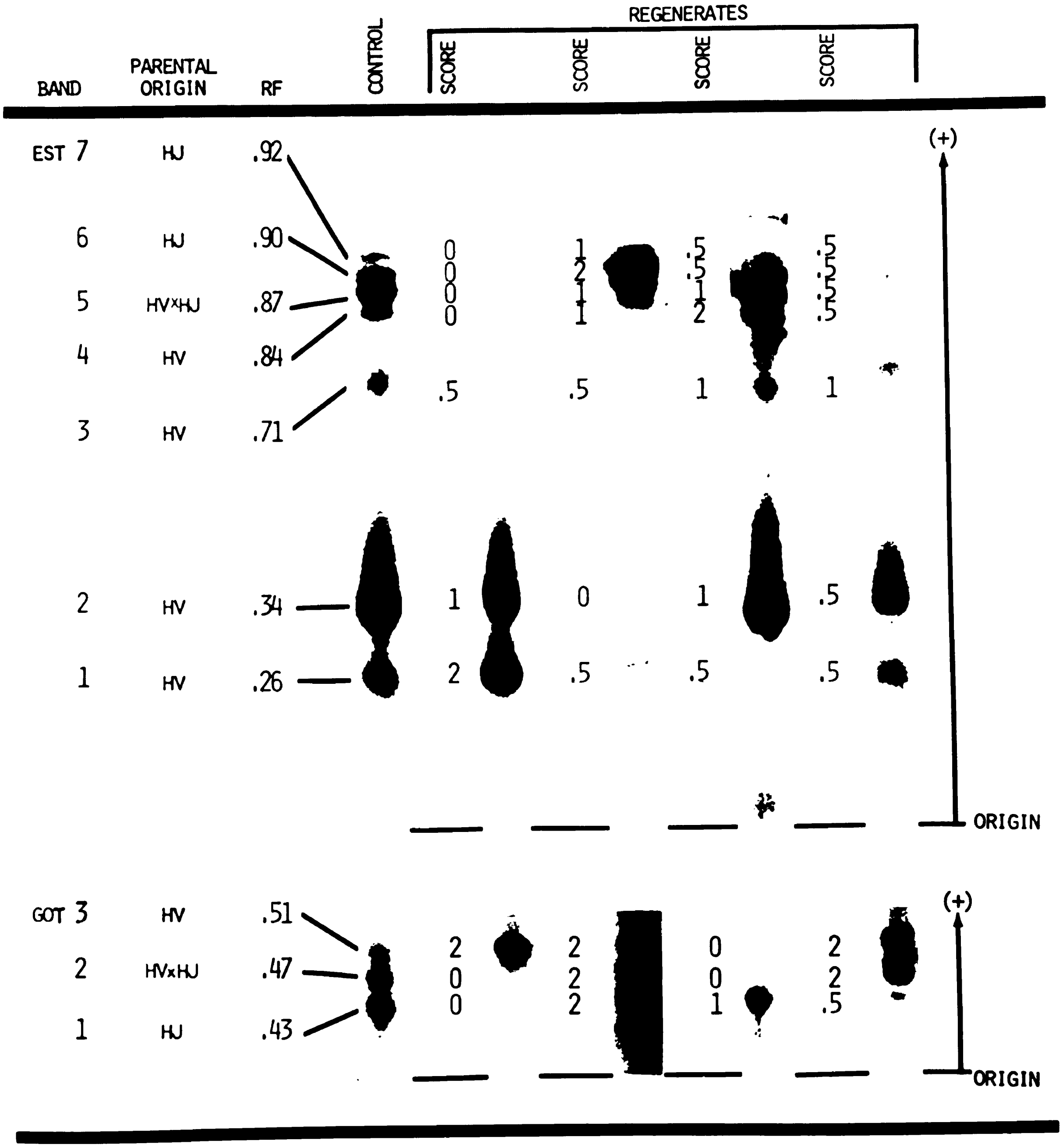

BAND
PARENTAL ORIGIN
RF
CONTROL
REGENERATES
SCORE
SCORE
SCORE
SCORE
EST 7 HJ .92
6 HJ .90
5 HV×HJ .87
4 HV .84
3 HV .71
2 HV .34
1 HV .26
0 0 0 0
.5
1
2
1 2 1 1
.5
0
.5
.5 .5 1 2
1
1
.5
.5 .5 .5 .5
1
.5
.5
(+)
ORIGIN
GOT 3 HV .51
2 HV×HJ .47
1 HJ .43
2 0 0
2 2 2
0 0 1
2 2 .5
(+)
ORIGIN

Figure 5.5

Figure 5.6: Typical examples of (A) EST and (B) GOT zymograms from crude extracts of culm bases of HVxHJ regenerates. Paired replicates of regenerates are juxtaposed.

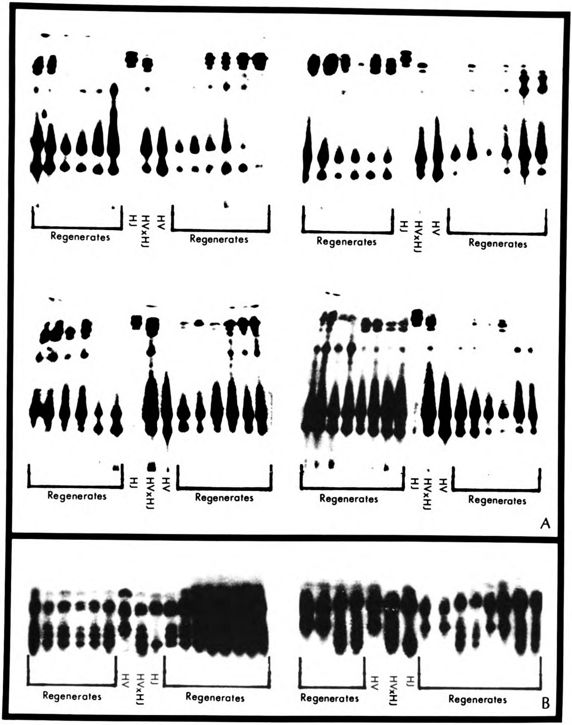

Regenerates
HJ
HVxHJ
HV
Regenerates
Regenerates
HJ
HVxHJ
HV
Regenerates
Regenerates
HJ
HVxHJ
HV
Regenerates
Regenerates
HJ
HVxHJ
HV
Regenerates
A
Regenerates
HV
HVxHJ
HJ
Regenerates
Regenerates
HV
HVxHJ
HJ
Regenerates
B

Figure 5.6

TABLE 5.5: Comparative intensities of EST and GOT isozymes among 43 regenerated HVxHJ plants.

Band	Parental origin	Mean intensity	S.D. intensity	Range intensity	Percent of cases band absent
GOT 1	HJ	0.97	0.35	0 - 2	6.98
2	HVxHJ (hybrid)	0.97	0.37	0 - 2	9.30
3	HV	1.08	0.37	0 - 2	2.32
EST 1	HV	1.30	0.48	1 - 2	0.00
2	HV	1.13	0.43	0 - 2	11.63
3	HV	1.00	0.43	0 - 2	9.30
4	HV	0.90	0.55	0 - 2	18.60
5	HVxHJ (co)	0.94	0.50	0 - 2	4.65
6	HJ	0.94	0.51	0 - 2	11.63
7	HJ	0.80	0.58	0 - 2	20.93

TABLE 5.6: Distribution of isozyme band intensities for 43 regenerated HVxHJ plants.

	Number of plants of intensity			
Band	0	.5	1	2
GOT 1	3	4	33	3
2	4	1	35	3
3	1	0	33	9
EST 1	0	1	31	11
2	1	2	33	7
3	4	2	30	5
4	13	14	20	7
5	2	12	23	6
6	5	9	25	4
7	9	13	17	4

Figure 5.7: Histogram of the distribution of ΣHJ:ΣHV isozyme band intensities, grouped as follows: 0 to .1, .1 to .2,, etc.

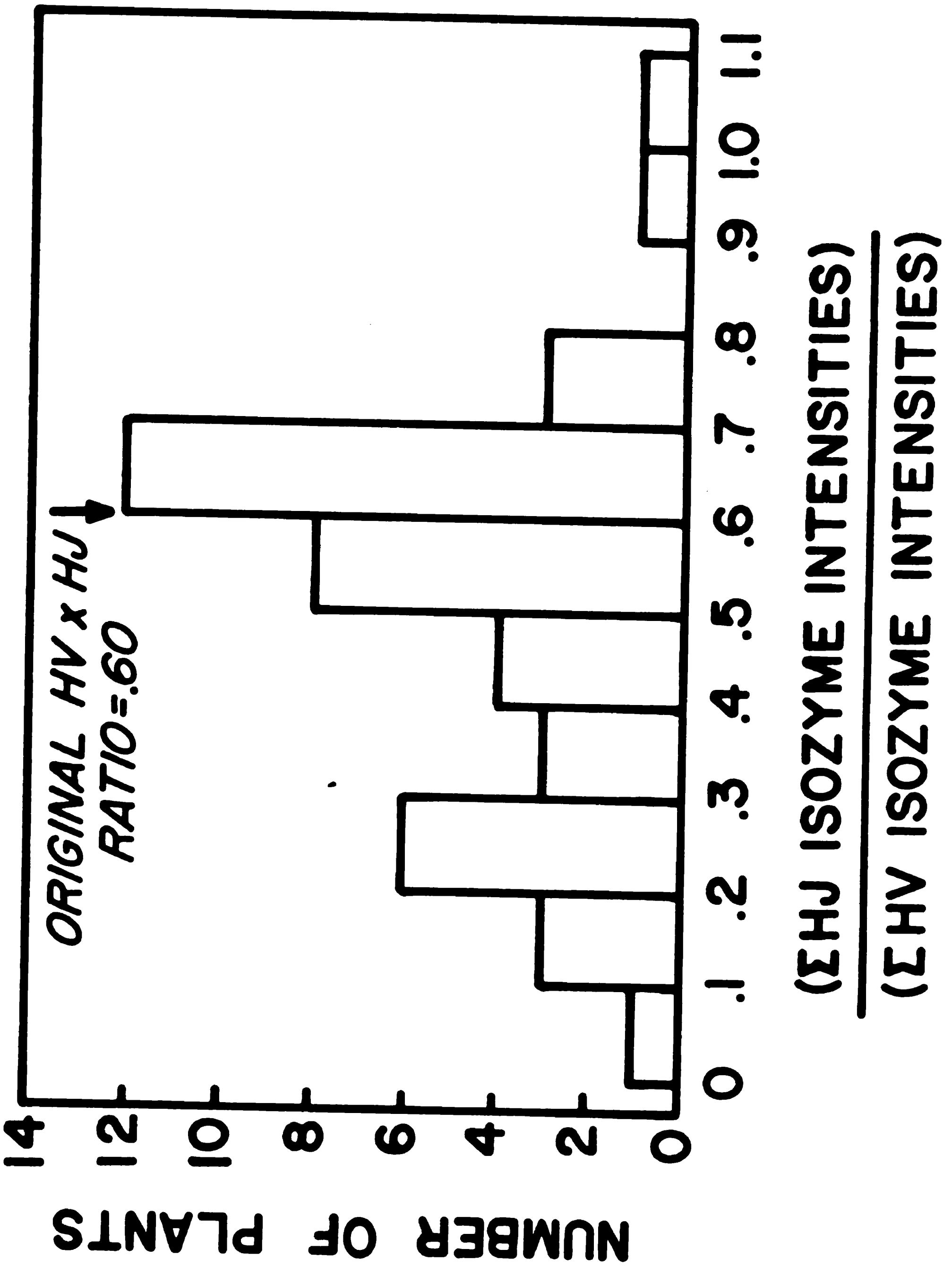

ORIGINAL HV x HJ
RATIO=.60
NUMBER OF PLANTS
0
2
4
6
8
10
12
14
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
1.1
(ΣHJ ISOZYME INTENSITIES)
(ΣHV ISOZYME INTENSITIES)

FIGURE 5.7

Figure 5.8: A comparison of plant types among plants regenerated from HVxHJ type A callus. (A) plant type, mature HV, (B) regenerated HVxHJ, vegetative, multi-tillering perennial-type (left) vs. reproductive, low heading, erect, annual-type (right, (C) plant type, mature HVxHJ, (D) differing foliage size between two HVxHJ regenerates, (E) plant type, HJ, (F) differing in vivo growth rates of regenerated HVxHJ. All regenerates were planted at the same time and maintained under identical conditions.

Figure 5.8

Figure 5.9: A comparison of floral structures and auricle morphology between HV, HVxHJ, HJ, and plants regenerated from HVxHJ type A callus cultures. (A) head type, mature HV, (B) auricle type, HV, (C) head type, HVxHJ regenerate, (D) auricle, HVxHJ regenerate, (G) head type, original HVxHJ hybrid, (H) auricle, original HVxHJ hybrid, (J) head type, HVxHJ regenerate, (K) auricle, HVxHJ regenerate, (L) head type, mature HJ, (M) auricle, HJ (all head types X.8, all auricles X4.8).

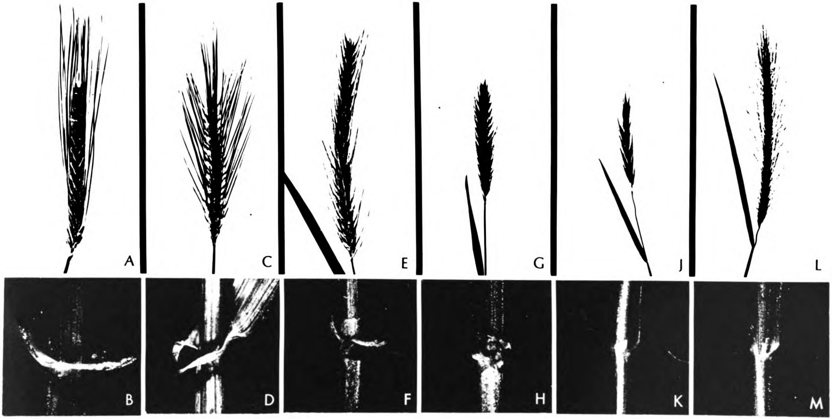


Figure 5.9

length and spines), number of rows per rachis (from 2 to 6), and average palea length (Fig. 5.9). Auricle expression ranged from HV-like, through the entire spectrum, to HJ-like (Fig. 5.9). This evidence is further support for the existence of a continuum of parental genome mixtures. No quantitative measurements were made, however, due to the large environmental component and unknown extent of genetic control of these traits.

Observations of microsporogenesis among certain regenerates from HVxHJ type A callus revealed meiotic configurations exhibiting a high degree of chromosome pairing (Fig. 5.10, Table 5.7). Independent studies of the original F_1 hybrid demonstrated extremely low frequencies of bivalent formation (.55 to .86 per cell, Rajathy and Morrison 1959, Murry 1975). In four plants, χ^2 tests were performed to detect significant differences between observed and established frequencies of univalents, bivalents, and trivalents (Table 5.7). The data of Murry (1975) were used to calculate expected values, since they were taken from the same progenitor clone as that of the test plants. In two cases, these tests were difficult to interpret due to differences in mean chromosome numbers between the source and regenerated plants. Even extrapolation of expected numbers to conform to the observed mean chromosome numbers did not change the corresponding level of significance.

In five independent cases of over 250 plants regenerated from HVxHJ type A callus, complete segregation for HV morphological characteristics was observed. Of these, only one has thus far flowered. The spike was extremely HV-like and bore florets in 4 rows, compared to the 2- and 6-rowed HV parental varieties. Auricles were

Figure 5.10: Comparison of metaphase I chromosome association between the originan HVxHJ hybrid and HVxHJ regenerates; (A) original HVxHJ hybrid, 22 I + 1 II (X830), (B) HVxHJ regenerate, 2 I + 2 II + 1 IV + 1 V (X1178), (C) HVxHJ regenerate, 10 I + 2 II + 1 III (X1178), (D) HVxHJ regenerate, containing a large multivalent (X1178)

Figure 5.11: Plant type and spike morphology of a HV-like segregant regenerated from HVxHJ callus. (A) plant type of a HV-like segregant regenerated from HVxHJ type A callus, (B) head type of above, four-rowed rachis, (C) head type of HV parent, two-rowed rachis.

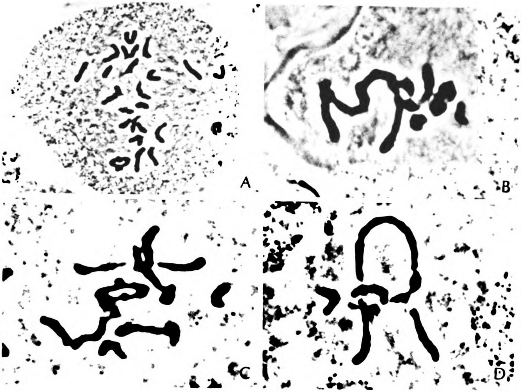


Figure 5.10

Figure 5.11

TABLE 5.7: Comparisons of chromosome associations among four regenerated HVxHJ plants

Plant	No. cells	Mean chromosome number	Mean No. per cell I	II	III	IV	V	$\chi^{2\,a}$
1	13	17.46	15.77	0.62	0.15	0.00	0.00	22.69***
2	6	21.50	9.33	3.67	0.67	0.50	0.17	267.50***
3	6	21.50	12.17	4.00	0.67	0.00	0.00	276.31***
4	5	16.20	10.40	0.80	1.40	0.00	0.00	499.22***

[a] expected numbers calculated from the frequencies observed by Murry (1975) on the original HVxHJ hybrid.

indistinguishable from those of HV. Further, the leaves and culms were glabrous, as in HV. Growth habit, however, was more similar to the multiple tillering perennial type of HJ (Fig. 5.11). Recently, the plant has begun to show signs of senescence, a character of annuals as in HV. GOT zymorgrams of this plant showed segregation for the HV isozyme. In EST zymograms, however, HJ isozymes were present, indicating some degree of retained hybridity (Fig. 5.12). Chromosome counts on immature ovary wall tissue ranged from 6 to 9 with a mean of 7.11, extremely close to the haploid number in HV (n = 7) and the basic number in the genus Hordeum (x = 7). At metaphase I of microsporogenesis in this plant, an average of 7.10 univalents were observed, ranging from 6 to 8 (Figs. 5.10 A - C). Despite the complete lack of chromosome pairing, meiosis appeared to proceed quite normally, exhibiting a random segregation of chromosomes at anaphase I - telophase I (Figs. 5.13 D, E), and an equational disjunction on bipolar spindles with normal orientation (Fig. 5.13 F), culminating in the formation of normal-appearing pollen quartets (Fig. 5.13 G) and non-functional pollen grains (Fig. 5.13 H). Cytogenetic analysis has not yet been performed on the other HV-like segregants, nor HJ-like segregants. Preliminary electrophoretic studies indicated intermediate levels of segregation of EST isozyme bands (Fig. 5.12).

Discussion

A comparison of chromosome numbers between tumorous and nontumorous callus cultures of tobacco and corresponding regenerated plants showed that selection played an important role in the process

Figure 5.12: Zymograms of HV-like and HJ-like parental segregant(s) in regenerated HVxHJ population of plants compared to HV, HJ, and HVxHJ. Note expression of EST 6 and 7 in HV-like segregants and of EST 1 and 2 in HJ-like segregant, indicating plant hybridity (*EST zymogram of plant pictured in Figs. 5.11 and 5.13).

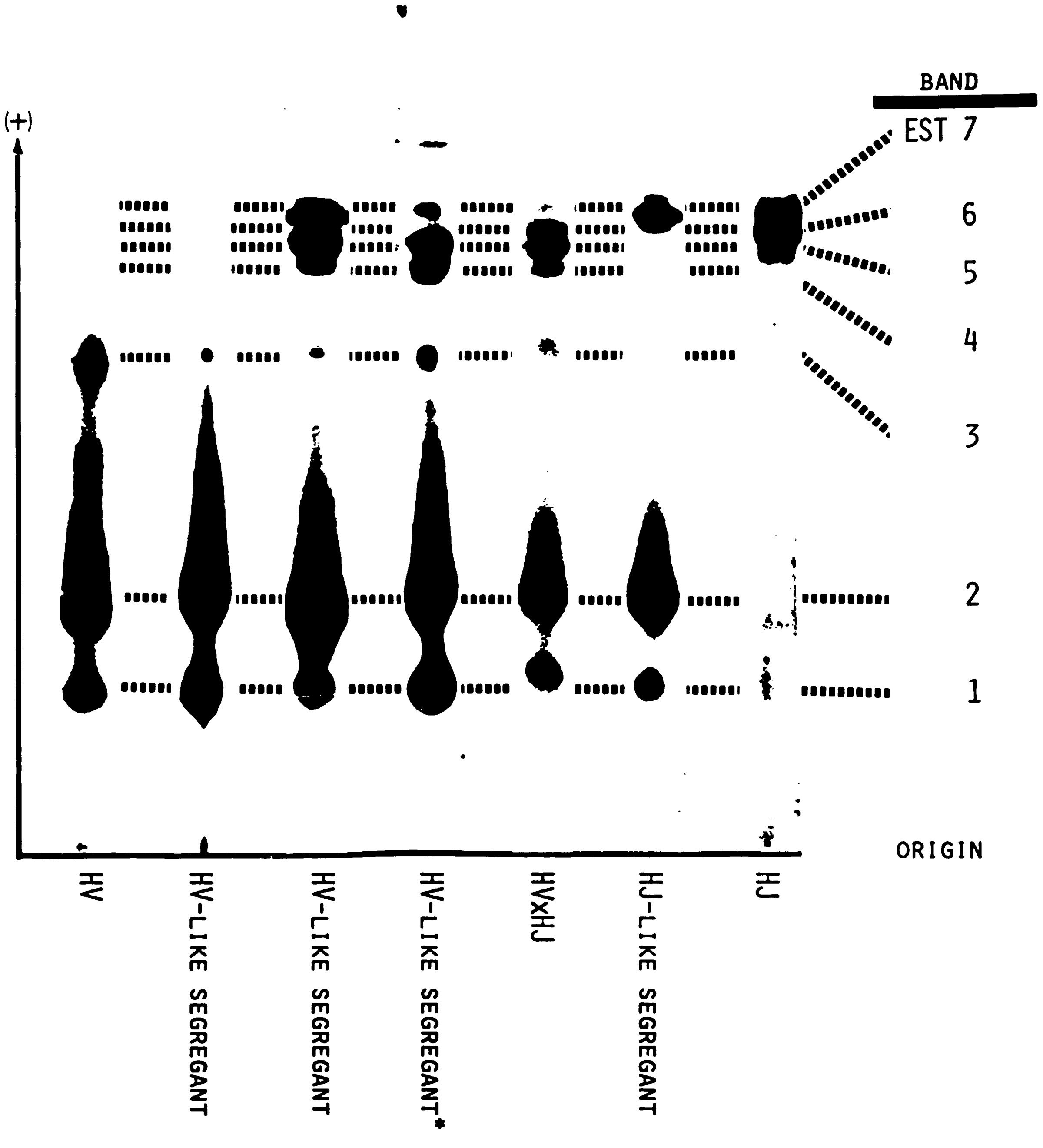

BAND
(+)
EST 7
6
5
4
3
2
1
ORIGIN
HV
HV-LIKE SEGREGANT
HV-LIKE SEGREGANT
HV-LIKE SEGREGANT*
HVxHJ
HJ-LIKE SEGREGANT
HJ

Figure 5.12

Figure 5.13: Meiosis in HV-like plant regenerated from HVxHJ type A callus. (A) seven univalents at diakinesis (X1728), (B) six univalents at late diakinesis (X 943), (C) eight univalents at metaphase I (X1005), (D) distributive 4-3 separation of univalents at anaphase I (X 943), (E) telophase I cell, 4-3 segregation (X 974), (F) equational separation at anaphase II (X746), (G) normal-appearing pollen quartets (X707), (H) culmination of process in abnormal, shrunken 'pollen' like grains (X283).

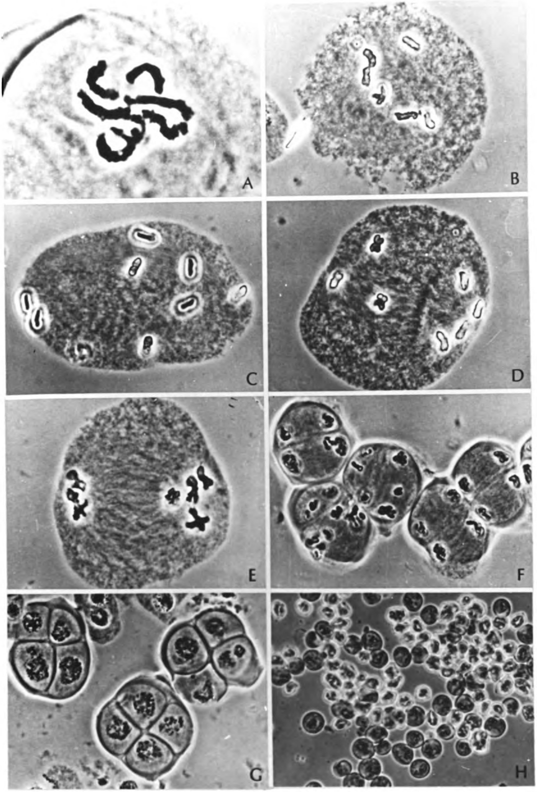


Figure 5.13

(Sacristan and Melchers 1969). Reports on the regeneration of karyotypic variability from tissue cultures differ, ranging from very little (Ogura 1975, 1976), to intermediate levels (Sacristan and Melchers 1969, Novak and Vystot 1975), to complete degrees of selection (Shimada *et al*. 1969, Yamane 1974). Recently, Ogura (1977) investigated progeny of crosses between karyotypically variable and normal tobacco plants and concluded that the generation of karyotypic variability is under genetic control.

This study has demonstrated an intermediate degree of karyotype selection from tissue cultures of HVxHJ. Attenuation was shown for all manifestations of variability, including polyploidy, aneuploidy, and chromosomal rearrangements. This is an indirect indication that constitutents of a karyotypically variable population of cells have different degrees of intrinsic totipotency. Further, progressively abnormal karyotypes *in vitro* may indeed be responsible for the observed progressive loss of regenerative potential in plant cell and tissue cultures (Reinert and Backs 1967, Smith and Street 1974).

It is difficult to conclude that any single process accounts for the channelling of *in vitro* karyotypic variability into regenerated plants. The analysis of variance of *in vivo* chromosome numbers demonstrated, in all cases, that between-structure variability was highly significant. At the initial regeneration event, variability was highly significant. At the initial regeneration event, variability was expressed between root meristems, evidence for a small number of cell initials with low karyotypic variability relative to the cell population at large. Nonetheless, within-root variability was present (Table 5.3). At a later point in development, within-plant variability between tillers, and ovaries within tillers was highly significant. These findings implicated the generation of in vivo karyotypic variability subsequent to regeneration. An important point to note is that karyotype/putative genetic segregations occurred at the point of regeneration, and in vivo processes could only generate variability within the context of that available subsequent to regeneration. Although all regenerated plants studied exhibited intra-tissue variability, cytogenetic, morphological, and molecular (i.e. isozymic) differences were evident between plants.

Cytogenetic observations and analysis of meiotic configurations in certain regenerated HVxHJ plants showed significant deviations from the data of Murry (1975) of the original F_1 HVxHJ hybrid. In three cases, the number of pairing configurations had increased dramatically. This increase could have been a result of loss or inactivation of a homoeologous pairing inhibitor or the selective loss or diploidization of certain chromosomes/segments of the original complement. This study found no detectable chromosome rearrangements in roots regenerated from primary type A callus. Further, multivalent configurations were found in cells with hypoploid chromosome complements. Thus, the loss of a homoeologous pairing inhibitor, possibly via karyotypic variability, appears the most likely explanation of this phenomenon.

The analysis of zymograms has been shown to be an effective tool for the elucidation of genetic differences in barley, such as cultivar identification (Fedak 1974, Bassiri 1976) and the quantification of genetic variability and isozyme polymorphism (Allard et al.

1970, Babbel and Wain 1977). Further, isozymes were used to distinguish somatic and sexual hybrids of *Nicotiana glauca* x *N. langsdorfii* (Wetter and Kao 1976). Tang and Hart (1975) have demonstrated the utility of isozymes in the identification of wheat-rye addition lines, and in determining the genetic identity of triticale lines. In most of the isozyme systems tested, a direct correlation has been observed between chromosome dose and corresponding isozyme concentration, although contrary cases are apparent (Nakai 1974, Fobes 1977). To test the validity of the analyses in this study, requests for seeds of barley trisomic lines were made to Dr. T. Tsuchyia at Colorado State University, Fort Collins, but were unanswered. Autotetraploids of HV and HJ generally exhibited increased doses for all EST and GOT isozymes, as compared to diploids. Further, karyotypically variable populations of hypoamphiploids regenerated from colchicine-treated HVxHJ calli showed increases in specific isozyme doses as compared to the original hybrid (Chapter 3).

In two clones of sugar cane, it was observed that differences between regenerated and source plants regarding morphological and isozymic variability were not directly correlated (Heinz and Mee 1977). In this study, however, electrophoretic data were in general agreement with morphological observation regarding variable expression and the segregation of parental types (Figs. 5.8, 5.9). Further, ratios of HV:HJ isozyme intensities substantiated the hypothesis of an *in vitro* continuum of parental genome mixtures (Fig. 5.7). Rather than a normal distribution of types, as postulated, a bimodal distribution was obtained, with a major cluster centered at HVxHJ values (approximately 0.6) and a minor cluster in the direction of

HV (i.e. less than 0.6). Further experiments are needed to confirm this finding and determine its generality. The segregation of genomes in interspecific hybrids has been previously observed in oats (Ladizinsky and Fainstein 1978). Further, meiosis-like reduction divisions have been observed in somatic tissue (Wilson and Cheng 1949). Perhaps this phenomenon is related functionally to directional chromosome elimination, as observed in embryos from certain interspecific crosses in Hordeum (Kasha 1974). If the chromosomes of parental genomes disjoin at random, the occurrence of pure parental cells should be defined by a probability function. Multipolar anaphase separations could have segregated whole parental genomes in a directed, non-random fashion, as postulated by Tai (1970).

Intensive analysis of one HV-like segregant in the regenerated HVxHJ population led to the following conclusions: 1) The plant was primarily constituted of HV genes, with a minor component of HJ, and 2) the genomic incompatibilities manifested by a breakdown of meiosis and sterility in HVxHJ, and corresponding hypoamphiploids of HVxHJ (Chapter 3) were not detected. Since the plant had approximately 7 somatic chromosomes and possessed both HV and HJ proteins, it is likely that interspecific chromosome substitution or segment transfer had occurred. Alternatively, in cells with greater than 7 chromosomes, the extra chromosome(s) could have been of HJ origin, producing enough isozyme for detection in the staining assay. Further studies are needed to substantiate this hybridity more conclusively. Efforts are currently in progress to diploidize this plant in vitro and in vivo. The observation of 'normal' meiosis in this plant spawns optimism that induced diploids will be fertile (Fig. 5.13).

The results of this study have shown that regeneration selects for certain karyotypes. This selection, however, is incomplete, allowing some variability to be expressed within regenerates. Further, some segregation of variability was apparent from different chromosome number profiles among HVxHJ regenerates. Karyotypic variability may be further manifested as a genomic continuum constituted of parental mixtures from pure HV to pure HJ; segregation was not random, appearing to favor that in the direction of HV. Finally, it was possible to isolate a haploid HV-like plant which expressed some HJ phenotypes; a preliminary example of *in vitro* introgression. Using conventional means, introgressive breeding programs require a great deal of time and space for their perpetration. Alternatively, the production of regenerated plants from callus cultures requires very little input beyond the establishment of conditions for induction, maintenance, and regeneration. Further, *in vitro* karyotypic variability may give rise to entities unobtainable by conventional crosses due to incompatibilities, sterility (as with HVxHJ), or rapid chromosome elimination, as with HV x *H. bulbosum* crosses (Kasha 1974). Hence, *in vitro* karyotypic variability is a potentially powerful tool for science and agriculture, and certainly warrants further investigation.

CHAPTER VI

CONCLUSIONS

Callus Induction, Growth, and Regeneration

1. Morphologically uniform callus cultures of Hordeum possess spatially distinct sectors which exhibit different growth rates and degrees of hydration.

2. Callus cultures in Hordeum can be classified into stable differentiated types which are characterized by differences in growth rate, consistency, intercellular associations, karyology, and regenerative capacity.

3. Type A callus cultures of HJ and HVxHJ could be regeneraated into whole plants, whereas no plant regeneration was observed under identical conditions for all other callus types and explant sources.

4. B5 salts and cytokinins promoted tissue necrosis. KIN + GA_3 inhibited crown root formation. GA_3, in combination with IAA and/or KIN, stimulated primary root and shoot formation. Also, B5 salts + 1% sucrose + 1% glucose promoted shoot formation in HVxHJ type A callus.

5. Neither pH nor exogenously supplied cAMP had any effect on tissue necrosis, callus growth, or regeneration.

6. All IAA-amino acid conjugates tended to serve as more

powerful promoters of callus growth and inhibitors of organogenesis than free IAA alone. IAA-L-alanine exerted the most prominent effect.

In Vitro Diploidization

1. By treating type A callus cultures with .005 to .01% colchicine in agar for 4 days, it was possible to double chromosome numbers in 20% of plants regenerated from cultures of HVxHJ and 60% from HJ.

2. Doubled plants regenerated from HVxHJ had variable chromosome numbers (31 to 44) with hypoamphiploid means (35.43 to 37.49). Of three such plants, means and ranges of chromosome numbers were approximately equal.

3. The differences between observed and expected chromosome pairing configurations for doubled plants were more easily explained by competitive autosyndesis than non-random chromosome loss.

4. Despite a high degree of chromosome pairing, regenerated hypoamphiploid plants remained completely sterile. Observations of meiosis revealed a breakdown manifested in two distinct processes: 1) all anaphase chromosome movements were abnormal, exhibiting numerous chromatin bridges and fragments, and 2) the spindle morphology was abnormal, often divergent or supernumerary. Hence, sterility in HVxHJ was most likely a consequency of incompatibile genome interactions at meiosis, and not the lack of chromosome pairing.

In Vitro Karyotypic Variability

1. Mean chromosome numbers in type A callus cultures of HVxHJ remained stable at 18 to 21 over time, while the frequency of aneuploid cells increased progressively.

2. The mean and variance of chromosome numbers in HVxHJ type E callus both increased after initially formation, then leveled off, after which mean chromosome numbers decreased and stabilized at a lower level.

3. HVxHJ suspension cultures derived from type E callus cultures exhibited a pattern similar to type E callus cultures of dynamics for mean and coefficient of variability of chromosome numbers. This pattern of mean and variability of chromosome number change, however, occurred much faster than type E callus cultures.

4. In secondary type E callus, the dynamics of mean and variability of chromosome numbers reverted to those similar to primary type E callus.

5. The patterns of change and stabilization in mean, variability, and distribution of chromosome numbers may have been a consequence of rates of aneuploidization and polyploidization vs. selective advantage (i.e. intrinsic mitotic division rate in a given environment) of cells in the population.

6. Three origin-distinct types of karyotypic variability have been identified in tissue cultures of Hordeum: polyploidy, aneuploidy, and chromosomal rearrangements. In general, type E callus and suspension cultures exhibited more variability for all categories than type A callus cultures.

7. Karyotypic variability in HVxHJ tissue cultures was primarily

a consequence of growth *in vitro*, and not of interactions of parental genomes.

8. It was possible to isolate subcalli which exhibited variable karyotypic profiles and isozyme expression. Hence, variants of the variable karyotypic array can be isolated and propagated *in vitro*.

Regeneration of In Vitro Karyotypic Variability

1. Among root tips regenerating from type A callus, an attenuation of the variability of chromosome numbers was observed, manifested as the selection of karyotypes without rearranged chromosomes and the truncation of extremes from the normal distribution of chromosome numbers in the source callus.

2. Selection for diploidy and fewer chromosomal rearrangements was evident among root tips regenerated from type A-like nodes in secondary type E callus.

3. Variability of chromosome numbers increased after a period of growth and development in regenerated HVxHJ plants.

4. Plants regenerated from type A callus cultures exhibited variability in karyology, growth habit, and head and auricle morphology.

5. In certain regenerated HVxHJ plants, a significantly higher degree of chromosome pairing was detected as compared to the original HVxHJ hybrid. This result was most easily explained by the loss of a homoeologous pairing inhibitor.

6. Preliminary evidence for the segregation of parental genomes among regenerated plants of HVxHJ was obtained by the quantification

of EST and GOT isozyme expression. Mixtures of parental genomes in regenerated HVxHJ plants were manifested in a continuum, from HV-like to HJ-like, and were distributed bimodally. One cluster of parental genome mixtures occurred around the expected HVxHJ values, and another in the direction of HV, indicating preferential loss of HJ chromosomes.

7. Studies of meiosis in one HV-like segregant showed a somatic chromosome number of approximately 7, the basic number of *Hordeum*. Meiosis in this plant was relatively normal, despite the complete lack of chromosome pairing, indicating that the HV-HJ genome incompatibility which had caused a breakdown in meiosis had been lost. Some HJ genes were expressed in this plant, possibly indicating that interspecific chromosomal substitution or subchromosomal addition had occurred.

8. This study has demonstrated the possibility of utilizing *in vitro* karyotypic variability for (1) rapid introgression, (2) producing haploids, and (3) circumventing intertaxon hybrid sterility.

APPENDICES

APPENDIX A

ELECTROPHORETIC TECHNIQUES

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Starch Gels

Starch gels were prepared by pouring 750 ml of .015 M tris + .003 M citrate buffer at 100°C into 250 ml of the same buffer at 25°C emulsified with 120 g of Sigma potato starch hydrolyzed for electrophoresis, under agitation. The resulting solution (12% starch) was aspirated and immediately poured onto plexiglas plates to a thickness of 8 mm.

Sample Preparation

Crude extracts were obtained by excising a specific amount of test material and crushing in plastic weigh-boats with a pestle. The extract was taken up into 3 x 8 mm Beckman electrophoretic paper wicks. The wicks were placed on a microscope slide, on moist filter paper in petri dishes, and stored at 2 to 4°C until elution (no longer than one hr.).

Elution and Running of Proteins

A transverse cut was made through the gel, approximately 25% from one end (e.g. resulting in two zones, 25:75 in length ratio). Using a pair of fine forceps, wicks were inserted into the cut, spaced at approximately 8 to 10 mm intervals. The gel was positioned such that the shortest zone was juxtaposed with the cathode. The tray buffer, .30 M boric acid at pH 7.8, was conducted onto the gel through pellon. For elution, a potential of 100V was applied to wicks for 30 min. The wicks were then removed and eluted proteins were subjected to 8 watts/gel until the anodal borate front had migrated 10 cm from the origin. During the entire process, gels were cooled to 0 to 2°C with ice water and circulated air.

Staining and Fixing

After running, gels were removed immediately, sliced in half horizontally, and stained. PRX: The bottom cathodal slice was immersed in .5 mg/ml 3-amino-9-ethyl carbazole, .045% H_2O_2, .03 M $CaCl_2$ in .05 M sodium acetate buffer at pH 4.5 for one hr. EST: The bottom anodal slice was placed in a solution of .03% α napthyl acetate, .03% β napthyl acetate, .03% α napthyl valerate, and 1.5 mg/ml fast blue RR salt in .1 M tris buffer at pH 7.0 for 2 hr. (in the dark). GOT: The top anodal slice was immersed in 2 mg/ml L-aspartic acid, 1 mg/ml L-glutamic acid, 0.1 mg/ml pyridoxal phosphate, and 1.5 mg/ml fast blue BB salt in .1 M tris buffer at pH 8.0 for 2 to 3 hrs. Acid phosphatase: The bottom anodal slice was immersed in .03% α napthyl phosphate, .02 M $MgCl_2$, and 1.0 mg/ml fast black KK salt in .05 M sodium acetate buffer at pH 4.5 for 2 to 3 hrs. (in the dark). After staining was completed, the solutions were discarded, the gels were fixed in 50% aqueous glycerin, and stored at 2 to 4°C until pictures were taken.

APPENDIX B

CYTOLOGICAL TECHNQUES

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CYTOLOGICAL TECHNIQUES

Root Tips and Type A Callus Cultures

These were pretreated optionally with .05% aqueous colchicine for 1 to 2 hrs. The tissues were fixed in fresh 1:3 acetic ethanol, under vacuum for 24 hrs. at 25°C. If required, storing was performed at -12°C. For staining, root tips were hydrolyzed in 1N HCl for 12 to 15 min. and type A callus for 25 to 35 min. at 60°C. Tissues were immersed in Feulgen reagent for 60 min. in the dark. Tissues were then immersed in 45% acetic acid, squashed with a coverslip, tapped lightly to remove excess, and sealed with dental wax. All Feulgen preparations were observed immediately due to rapid stain deterioration.

Microsporocytes and Immature Ovary Wall Tissue

Spikes of the proper stage were removed from the plant and fixed as described previously, using no pretreatments. For observations of meiosis, anthers were excised, macerated, excess debris removed, and stained in aceto carmine for 30 seconds. Stained microsporocytes were then squashed with a coverslip, the slide was warmed gently, and sealed with dental wax. For ovary wall tissue, whole ovaries were excised, stained in aceto carmine for two min., macerated, squashed with a coverslip, and sealed with wax. These preparations remained stable for one to two days.

Type E Callus and Suspension Cultures

Tissues were pretreated optionally with .05% aqueous colchicine. Fixing and staining were performed concomitantly by immersing live tissue in modified carbol fucshin stain (Kao 1975) for five min. The tissue was then macerated, squashed with a coverslip, and sealed with sticky wax. This technique gave rise to highly stable preparations (up to two weeks at 25°C) with the nucleus or chromosomes selectively stained an intense magenta.*

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*This technique was kindly provided by Dr. P. J. Bottino, Univ. of Maryland.

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