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Potential of Tissue Culture Applications in Some Triticeae Via Callus Induced Variation, Alien Introgression and Amphiploid Production

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ABSTRACT

Tissue culture applications in the Triticeae associated with embryo culture and hybrid plantlet differentiation are routine in the production of intergeneric or interspecific hybrids, of which× Triticosecale WITTMACK is a notable example. Callus induction from immature embryos, its maintenance, with eventual plantlet regeneration is also recognized as a meaningful source of inducing heritable variability in *Triticum aestivum* for agricultural traits that are simply inherited or are under complex polygenic control.

Alien germplasm resources are unique source of genes that could be introgressed and pyramided in wheat breeding programs for the crops improvement. Such attempts have at least two operational constraints as associated with alien gene introgression and amphiploid induction. Callus culture methodology has significantly aided in overcoming both these aspects; the first by influencing chromosome pairing manipulation analogous to that characteristic of the *Ph* loucs on chromosome 5B and second by inducing amphiploidy in two intergeneric hybrid combinations mediated by a meiotic divisions restitution. The test system was *T. aestivum* and *T. turgidum*× *Aegilops variabilis*. The cytogenetical and practical implications of these observations are elucidated.

Key words : Tissue culture, Triticeal, Alien introgression, Amphiploid.

INTRODUCTION

Wheat (*Triticum aestivum* L.; 2n=6X=42, AABBDD) improvement methodologies have predominantly adopted conventional plant breeding procedures. However, over the last decade one does see modest inputs of novel techonology getting incorporated. The broadly labelled area of "biotechnology" has promisingly demonstrated a significant arsenal for wheat improvement, specifically by projecting unique opportunities in general for crop improvement (Mujeeb-Kazi and Asiedu, 1989). One such area; callus culture and regeneration; has been exploited to demonstrate the potentials of inducing variability within various groups of Triticeae for morphological, biochemical and cytological characteristics.

The present study focused on utilizing callus culture methodology to promote alien genetic transfer in an intergeneric hybrid test system; *T. aestivum* × *Aegilops variabilis;* that could be considered as a cytogenetic standard. The alien species accession (13E) has been reported to possess a remarkable level of karnal bunt (*Neovossia indica*) resistance (showing 0% infection under the boot inoculation procedure, Warham *et al*, 1986). We are cognizant of the fact that conventional cytogenetic procedures are being utilized, but this novel callus culture procedure was applied to a standardized system based upon global investigations of several cytogeneticists who had concluded that the F_1 hybrid between *T. aestivum* cultivar Chinese Spring × *Ae. variabilis* exhibits a metaphase I meiotic association frequency of less than 1 open bivalent per meiocyte (Sears, 1977; Jewell, 1983; Jewell and Mujeeb-Kazi, 1982). This Paper reports the studies on :

(a) long term callusing and regeneration with cytological analysis of some *T. aestivum* (bread wheat) and *T. turgidum* (durum wheat) cultivars, and

(b) similar aspects for *T. aestivum* × *Ae. variabilis* F_1 hybrids.

MATERIALS AND METHODS

(a) For the long term callusing and regeneration of *Triticum aestivum* (2n=6X=42, AABBDD) and *T. turgidum* (2n=4X=28, AABB) few cultivars of each (Table 1) were grown in pots in a 2 : 1 : 1 sterilized mixture of soil : sand : peat moss. The plants were maintained under greenhouse conditions of 24°C day/14°C night, 15 hours natural light and approximately 65% relative humidity. Immature embryos excised at 15 days post-anthesis were cultured on LS medium (Linsmaeier and Skoog, 1965) with 2, 4–Dichlorophenoxy–acetic acid for callus induction and maintenance. The calli from both *Triticum* species were maintained until the seventh, one month passage interval. At each monthly passage some embryogenic (E) callus was regenerated and plants transplanted in pots maintained under greenhouse conditions mentioned above. The regenerated plants were phenotypically observed, cytologically analyzed and individually harvested for obtaining R-1 seed.

(b) An additional planting of *T. aestivum* (including Chinese Spring), *T. turgidum* and *Aegilops variabilis* Accession 13 E, CIMMYT) were also maintained in the greenhouse. The *Triticum* species cultivars were hybridized with *Ae. variabilis* (as the pollen parent) and immature embryos were excised 15 days after pollination. The embryos were plated on Murashige and Skoog (1962) medium for plantlet differentiation; plantlets that served as the cytogenetic control. Remaining immature embryos were plated and maintained on LS medium (Linsmaeier and Skoog, 1965) upto 22 months with monthly transfer/s of embryogenic callus/calli. At each monthly passage a portion of the callus was regenerated into plants, transferred to the greenhouse

Table 1. Regenerated *Triticum aestivum* and *T. turgidum* plant data from callus maintained up to seven months.

Wheat Varieties	Months in callus					Total
	3	4	5	6	7	
	Number of plants regenerated					
Triticum aestivum						
MRL "S"/BUC "S"	21	33	26	6	0	86
BOW "S"	1	17	12	0	0	30
ALD "S"/PVN "S"	24	36	21	15	11	107
GH "S"	30	68	33	6	7	144
PAVON	0	0	3	0	0	3
					Total	370
T. turgidum						
CHEN "S"	0	8	0	0	0	8
LARU "S"	12	12	4	10	0	38
CNDO/R143/ENTE"S" MEXI"S"	9	0	0	0	0	9
MEMO "S"/MEXI "S"	22	3	0	0	0	25
ROK "S"/KMLI "S"	2	0	0	0	0	2
					Total	82
					Grand Total	452

growing condtions, cytologically analyzed and advanced by backcrossing to appropriate wheat cultivars. The procedures for hybridization, mitotic cytology and meiotic analysis were similar to those described earlier (Mujeeb-Kazi and Miranda, 1985; Mujeeb-Kazi *et al*, 1987, 1989).

RESULTS AND DISCUSSION

Embryo culture leading to plantlet differentiation has been routinely adopted for either hastening seed increase generations or for aiding interspecific and intergeneric hybrid production where the distant hybrids require nutrient support since the interpolid endosperm of divergent hybrids is either absent or rudimentary. Its extensive use in the production of primary hexaploid/octoploid × Triticosecale WITTMACK has become highly oversimplified with the complexities now being extended to more diverse hybrid combinations.

Though there are a wide array of media that can be employed, a base exploitation of Murashige and Skoog (1962) and Taira and Larter (1978) media offer significant promise. This can be gauged by the success of the range of complex intergeneric

hybrids produced so far (Mujeeb-Kazi and Bernard, 1985; Mujeeb-Kazi *et al* 1987, 1989). There is considerable merit to utilize other media since wheat hybrids with several alien species (Table 2) undrwent embryo formation devoid of plantlet differentiation. Would it be benficial to invest efforts in exploring media inputs or exploit other revolutionary metodologies requires a critical consideration. The success of polyhaploid production for *T. aestivum*×*Zea mays* crosses (Laurie and Bennett, 1986, 1987, 1988b; Laurie and Reymondie, 1991; Suenaga and Nakajima, 1989; Inagaki and Tahir, 1990; Riera-Lizarazu and Mujeeb-Kazi, 1990) high fertilization events of *T. aestivum*×*Sorghum bicolor* (Laurie and Bennett, 1988a), success of *T. aestivum*×*Pennisetum americanum* (Ahmad and Comeau, 1990), *T. aestivum*×*Teosinte* (Ushiyama *et al*, 1991), *T. aestivum*×*Tripsacum* (Riera-Lizarazu and Mujeeb-Kazi, Unpublished) as well as the recovery of hybrid plants (Ahmad and Comeau, 9991) from the unique *T. aestivum*×*Elymus scabrus* species (source of accessions with apomictic genes) may change research application concepts of the near-future quite dramatically as to employing embryo culture differently from its present simplistic form. One must however, consider that hybrization successes may vary to a significant degree across diverse location for a multitude of ill-defined reasons. A simple cross in one location (Chinese Spring wheat×*A intermedium*) successful to about 60% under field crossing, without embryo culture (Mujeeb-Kazi *et al*, 1987) warranted special inputs elsewhere to yield hybrid progeny at a frequency level of less than 1% (A. Mujeeb-Kazi personal Communication with G. Liang; see Ann. Wheat Newsletter, 1984 pp. 114-115)

Table 2. Some *Triticum aestivum*×Alien hybrid combinations where embryos were excised but could not be differentiated into hybrid plants*.

Alien species (Male parent)	Percentage seed Set	Embryos Excised
Agropyron cristatum	1.35	1
A. smithii	3.85	1
A. strigosum	8.33	3
A. tauri	9.43	4
Elymus salina	11.11	2
E. junceus	6.67	1

*Mujeeb-Kazi *et al*. 1987.

In callus culture the variation that emanates from long term callusing and regeneration has correlated significance for wheat breeding programs. We are however, not overly sure how callus induced variability (Scowcroft, 1989) would differ in quality and quantity from that obtained through applications of ionizing, non-ionizing and chemical mutagenic sources. Nevertheless, the findings of Larkin and Scowcroft (1981), Lorz *et al* (1988), Scowcroft (1989) demonstrate the induced

variation effects quite distinctly, effects that the former classiffed as "Somaclonal variation". This aspect has been exploited in the first phase of our study with several tetraploid and hexaploid wheat cultivars where the callusing plus regeneration responses were measured (Table 1). The ensuing progeny from each cultivar was observed for morphological, cytological plus biochemical responses.

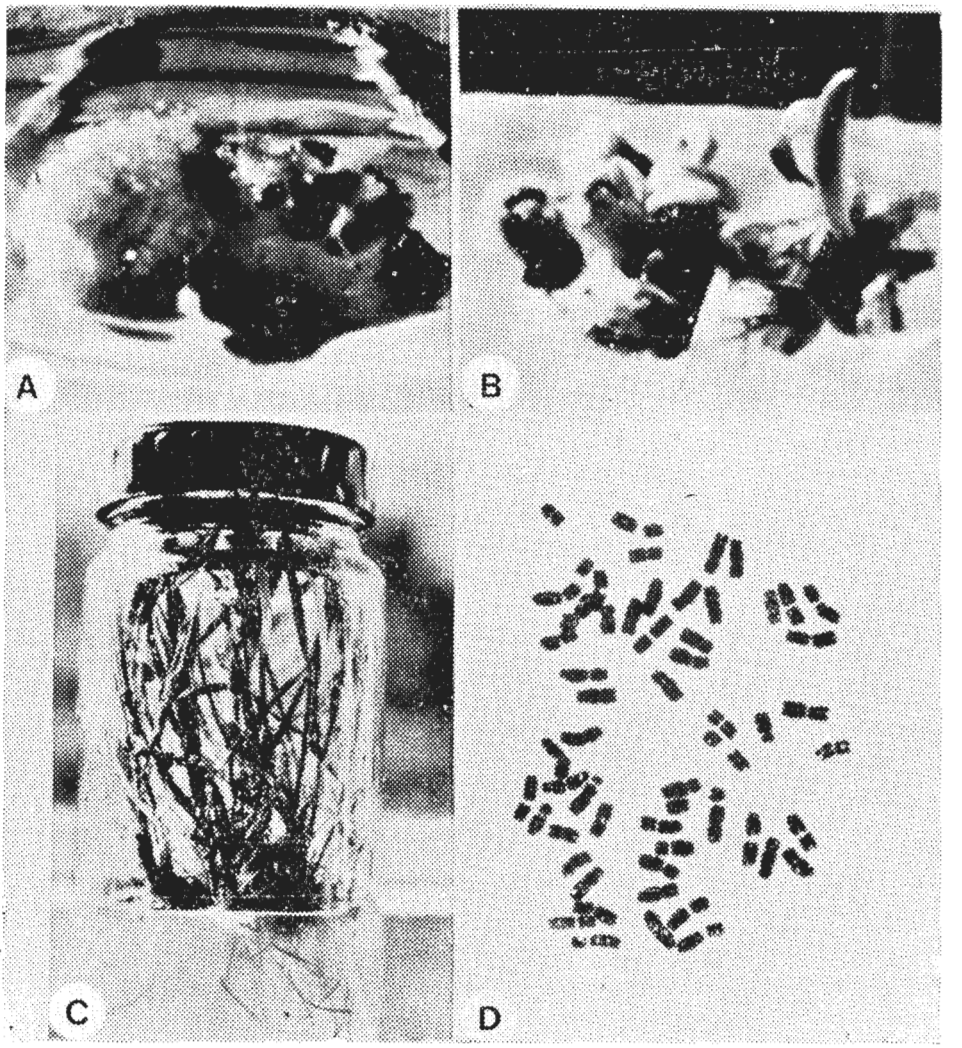

Figure 1. (A) Embryogenic callus formation in *Triticum aestivum* cultures;
(B) Early regeneration in *T. aestivum* cultures;
(C) Advanced regeneration in *T. aestivum*;
(D) A spontaneously doubled (2n=8x=56) *T. turgidum* somatic cell

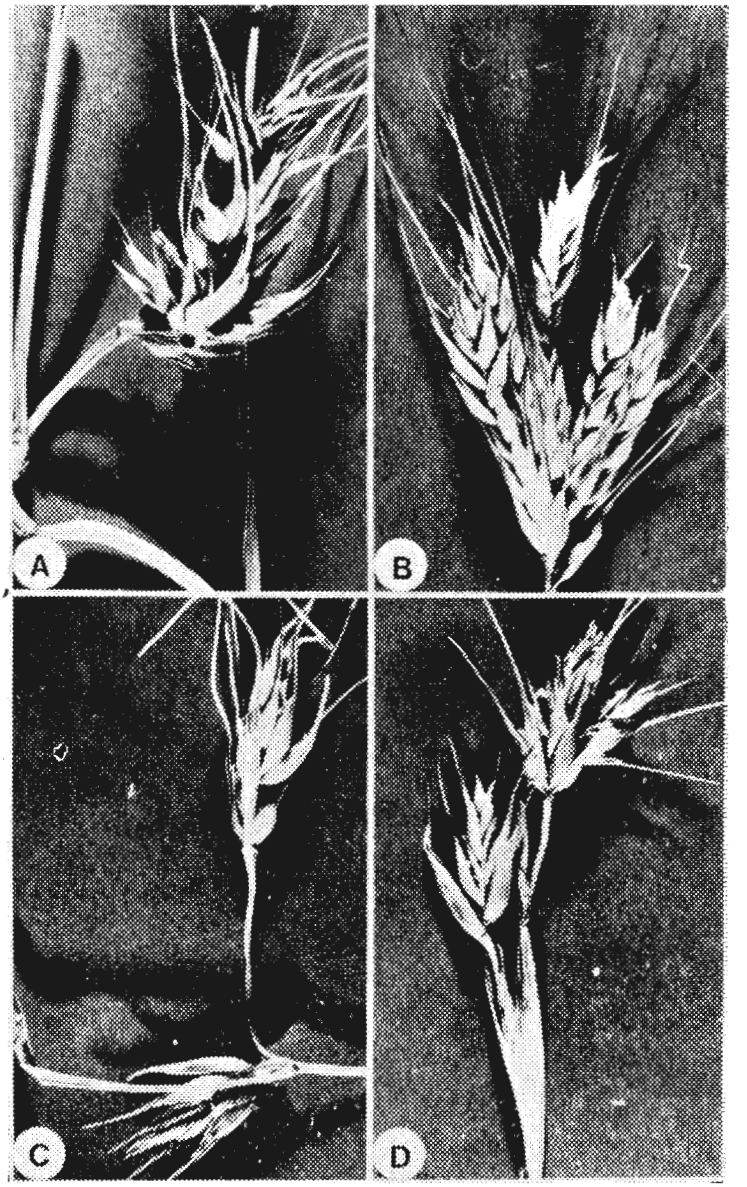

Figure 2. (A) to (D) Spike morphology variations in R–0 plants (callus regenerated) of *Triticum aestivum*.

Bread wheats (*T. aestivum*) were more profilic in producing E callus (Fig. 1A) and possessed a higher number of regenerated plants (370) than durum wheats (*T. turgidum*) (82) (Table 1). Figures 1B and 1C show the early and advanced regeneration stages for *T. aestivum*. There were several spike developmental

abnormalit'es (Figs. 2A to D) that at the R_0 stage may well be transient changes. The heritable changes require anlyses in advanced generations.

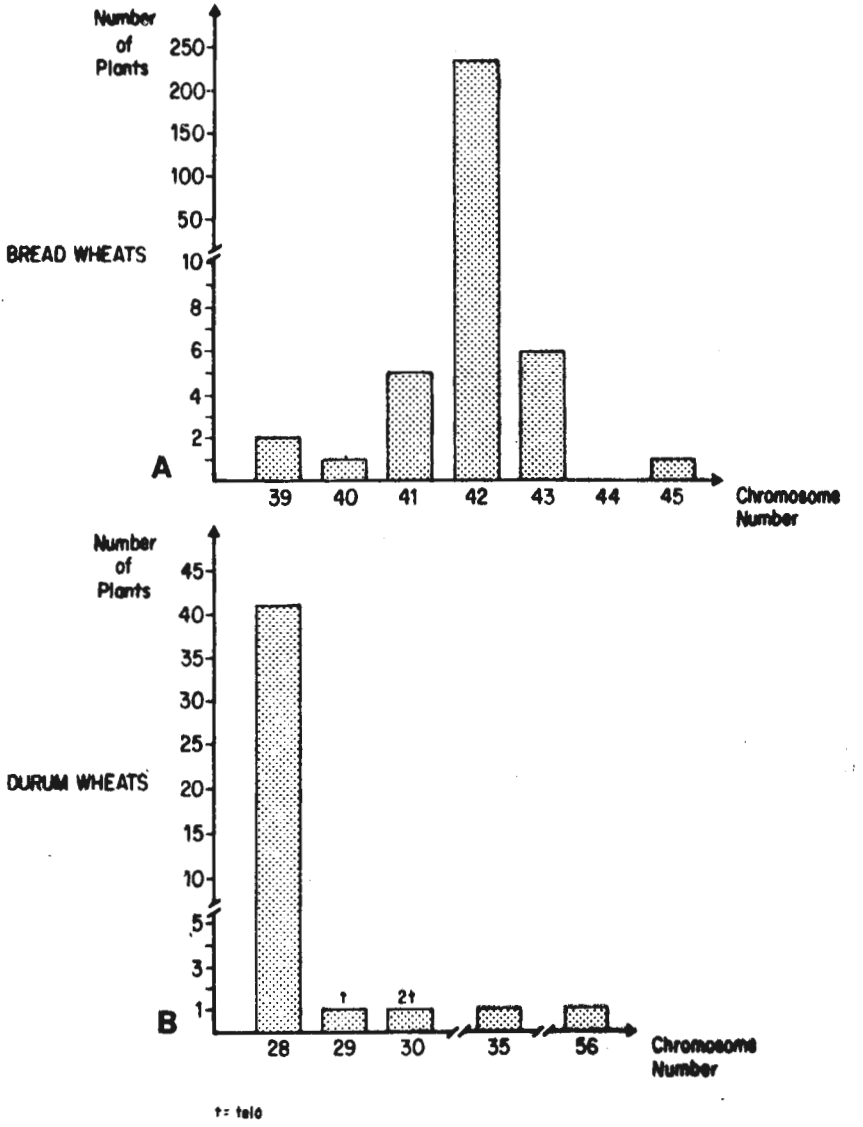

Figure 3. (A) Number of *Triticum aestivum* regenerated plants and their somatic chromosome numbers;
(B) Number of *T. turgidum* regenerated plants and their somatic chromosome number (t=telocentric chromosome).

Somatic root tip cytology was done on a limited number of plants of both wheat groups (255 for bread wheat and 45 for durum wheat) with some spikes being randomly sampled for meiotic anlyses. Somatic root-tip count data predominantly expressed counts of 42 for *T. aestivum* and 28 for *T. turgidum* (Figs. 3A and B). The range for *T. aestivum* chromosome number was from 39 to 45 and from 28 to 56 for *T. turgidum* with one to two telocentric chromosomes. There was a single *T. turgidum* plant with 56 chromosomes (Fig. 1D) attributed to spontaneous doubling. The plant was male sterile but female fertile and set seed upon backcrossing to *T. turgidum*. The meiotic analyses of a few 42 and 28 chromosome plants provided evidence of cytological variations where apart from the normal bivalent formation, meiocytes possessed several univalents, trivalents and quadrivalents. All R_0 derivatives have been advanced as there existed the potential of selecting stable varirnts in advanced generations because of the observed intrinsic R_0 meiotic changes. The vnriations partially selected in advanced generations so far are for plant height, days to anthesis plus maturity, solid stem and isozymic electrophoretic banding differences.

Apart from inducing variability, callus induction procedures may be exploited in effecting alien transfers in intergeneric hybridization. Generally these divergent hybrids express low chromosomal recombination frequencies (Sharma and Gill, 1983; Mujeeb-Kazi *et al*, 1987, 1989) and several hybrids do not respond positively to amphiploid induction procedures (Islam *et al*, 1981). Tho gh there are cytogenetic means to facilitate wheat/alien genetic recombinations, callus culture offers an alternative convenient means as well, as demonstrated for wheat×rye combinations (Lapitan *et al*, 1984, 1986, 1988). The callus mediated approach to introgress alien genes into *Triticum* species also seems workable for crosses with *Ae. variabilis* both for the goals of basic research and agricultural practicality as related to karnal bunt resistance plus aluminum tolerance in the 13E accession tested. The alien accession hybridizes with relative ease to *T. aestivum* and *T. trugidum*, is positive for C- or N-banding and has several characteristic biochemical markers. Ii is unequivocally accepted as a cytogenetic standard where its hybrids of 35 chromosomes with *T. aestivum* cv. Chinesse Spring express a mean chromosomal association frequency of less than 1 open bivalent per meiocyte at meiotic metaphase I. Additionally, no fertile amphiploid derivatives have been obtained, whereas BCI derivatives obtained by pollinating the F_1 hybrids wit'1 *T. aestivum* were highly aneuploid (Jewell, 1980, 1983; Jewell and Mujeeb-Kazi, 1982; Mujeeb-Kazi and Asiedu, 1989) with negligible normal 56 chromosome BC1 derivatives being produced. For this study, intergeneric hybrids between *T. aestivum* cv. Chinese Spring and *Ae. variabilis* (13E) were produced in a high frequency; an influence of the homozygous *Kr1*, *Kr2*, *Kr3* genes on homoeologous group 5. The endosperm was well formed but despite this all embryos were excised for direct regeneration (control) or plated in LS media for callus induction and

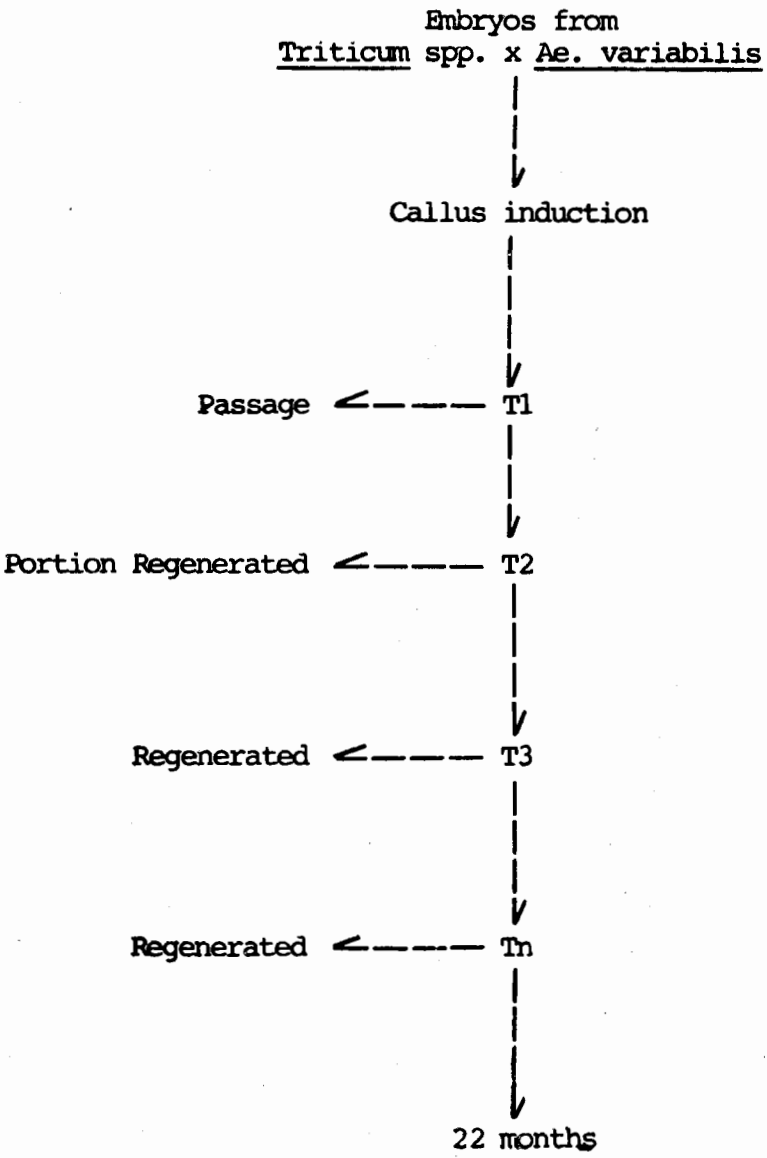

Figure 4. Schematic showing callus induction, transfer and regeneration protocol from *Triticum* species × *Aegilops variabilis* crossing.

maintenance upto 22 months with callus portions being regenerated at each monthly passage (T_2 to Tn; Fig. 4 and Table 3). All control F_1 hybrids possessed the normal 35 chromosomes (ABDUS) characteristic of the hybrid combination and were with low pairing. The regenerated F_1 hybrids did express a certain

degree of aneuploidy (hyper–or hypo–ploid coupled with inclusion of telocentric chromosomes) which could not be corrated with the length of time in callus. In the 35 chromosome regenerated plants aneuploidy involving structural chromosomal change was prevalent that in one plant influenced the 5B chromosome. When N-banded, both short and long arms of chromosome 5B had chracteristic banding sites that were stable across different cells and varied chromosome contraction stages (Fig. 5). The regenerated plant with the 5B structural change indicated total absence of bands on the 5B short arm. It serves as an ideal cytological marker for this critical chromosome where by backcrossing and selection it should be possible to obtain derivatives homozygous for the marker 5B chromosome. This then could be exploited in transfers of the Chinese Spring *phlb* mutant stock quite simplistically. So far backcross derivatives from this 5B modified F_1 hybrid have not been obtained.

Table 3. Plants regenerated from *Triticum aestiuum* × *Aegilops variabilis* callus during different passages (T).

Passages (T)	Plants Regenerated
T 2	54
T 3	5
T 4	15
T 5	12
T 6	64
T 7	32
T 8	9
T 9	10
T 11	12
T 12	11
T 13	5
T 15	20
TOTAL :	249

The meiotic anlysis of the regenerated plants revealed intersting variation from the standard low pairing characteristic at metaphase I (Table 4). Several plants had highly paired multivalent associations indicative that chromosomal pairing cotrol mechanisms have been influenced (Table 4) some even upto the level of multivalency that prevails in Chinese Spring *phlb* hybrids with *Ae. variabilis* (Asiedu *et al*, 1989). Though the chromosome 5B dominant *Ph* genes suppresses homoeologous chromosome pairing, there are othere suppressors like those on 3AS, 3DS with a minor contribution of 4D (Mello–Sampayo and Canas, 1973; Driscoll,

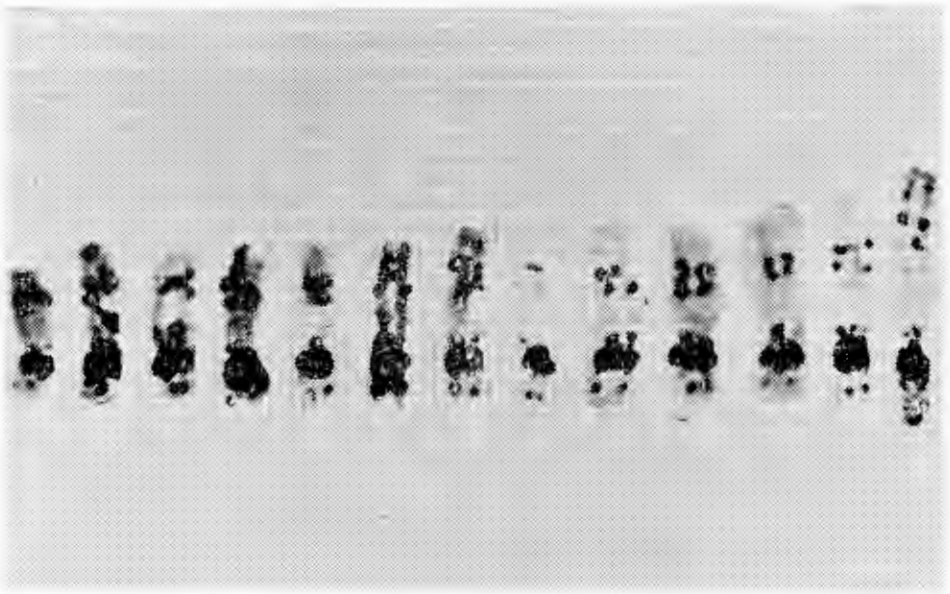

Figure 5. N-banded 5 B chromosome from *Triticum aestivum* L. cv. Chinese Spring showing consistency of banding sites on both arms despite stage of contraction of the chromosomes and their extraction for the figure from different cells and root tips.

Table 4. Meiotic associations in hybrids of Chinese Spring (CS) *Ph*×*Aegilops variabilis* (13 E) with low pairing; of CS *phlb*×*Ae. variabilis* (13 E) with high pairing; in a CS *Ph*×*Ae. variabilis* (13 E) callus regenerated F 1 with modified increased pairing.

	Mean Meiotic Metaphase I Chromosomal Association						
	I	II Rings	II Rods	III	IV	V	VI
CS (Normal)*	34.67		0.16				
CS (*phlb*)*	9.50	1.6	7.2	2.1	0.25	0.08	0.04
CS (Regenerated)	13.20	0.70	6.60	1.60	0.40	0.20	

*(ASIEDU *et al.* 1989)

1973). Since the callus induced influence, is random, suppressor genetic changes could be similarly influenced not discounting the fact that pairing promoter genes may also cotribute positively to the observed meiotic associations of some regenerated plants (Table 4). These modifier gene/s may also be present in the 13 E accession an aspect that would require critical anlysis under stringent environmental control.

Amphiploids have significant advantages in germplasm distribution, maintenance and cytogenetic manipulation. Efforts of at least the past three decades, though limited, have not led to production of an amphiploid of the Chinese Spring hexaploid wheat × *Ae. variabilis* hybrid combination. In our studies this seemed rather crucial to achieve because of the practical significance of *Ae. variabilis* and also due to the fact that backcross I progeny derived by pollinating the CS × *Ae. variabilis* F_1 was highly aneuploid (Jewell and Mujeeb-Kazi, 1982). As a consequence it seemed rather improbable that all 14 alien disomic additions may be obtained, thus chances of not transferring the alien chromosome/s bestowing major effects for karnal bunt resistance or aluminum tolerance were quite high. Efforts of Jewell (1980, 1983) using F_1 based backcrossing led to an incomplete set of alien disomic additions that stressed upon us to :

(i) Obtain an amphiploid from CS × *Ae. Variabilis* with near normalcy at 70 chromosomes, and then

(ii) To derive the alien additions by backcrosses onto the amphiploid which may complete the alien disomic addition set.

Colchicine induced doubling attempts via direct treatment of F_1 hybrids eluded all our efforts but towards the advanced growth stages of the callus regenerated F_1 hybrids of CS × *Ae. variabilis* and *T. turgidum* × *Ae. variabilis*, occasional seed set on a few plants was observed in both combinations (Tables 5). The seed setting apparently was a random event. These seeds upon germination and cytological analysis possessed either 70 or 56 chromosomes and were meiotically quite regular plus being self-fertile as evident from the C–1 plus C–2 derivatives produced (Table 5). The intial seed setting on F_1 regenerants is attributed to meiotic restitution since the plants setting seeds possessed either 35 (*T. aestivum* × *Ae. variabilis*) or 28 (*T. turgidum* × *Ae. variabilis*) chromosomes. One meiotic division would lead to chromatid separation in the 35 or 28 chromosome hybrids

Table 5. Regenerated plants of F_1 hybrid with *Ae. variabilis* (13 E) of *Triticum aestivum* and *T. turgidum* showing cytologically doubled progeny.

Female Parent	Somatic Chromosome Number	Passages	C 0 Seed Number	Somatic Chromosome Number	C-1 Seed Number	C–2 Seed Number
T. aestivum	35	12	1	70	14	Not advanced
T. turgidum	28	7	23	56*	57	Not advanced
T. turgidum	28	8	4	56	51	69
T. turgidum	28	10	9	56	56	633

*Some aneuploidy (Mixoploidy)

that if coupled with meiotic restitution would subsequently produce male and female gametes of 35 or 28 chromosomes that upon fusion are capable of forming progeny with 70 or 56 chromosomes. Such cytological events were indeed observed and now the 70 chromosome *T. aestivum*×*Ae. variabilis* progeny (Table 5) is to serve as the base for hopefully developing normal BCI dervatives leading to a more complete alien chromosome addition set.

CONCLUSIONS

Embryo culture applications have been crucial for production of complex hybrids within the Triticeae and their manipulations will presumably even widen the existent range of hybridization possibilities. Embodied in such extensions of rerearch applications is the integration of callus culture methodlogy. This is not only advantageous to induce variability in euploid wheat cultivars or to facilitate *in vitro* screening for stress or toxin producing pathogens, but it has the capacities to structurally alter chromosomes. additionally the process may modify recombination frequencies in otherwise low pairing complex hybrids as well as facilitate recovery of chromosomally doubled hybrid derivatives that could be critically significant in cytogenetic inputs for practical crop improvement areas.

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