

A SIMPLIFIED AND EFFECTIVE PROTOCOL FOR PRODUCTION OF BREAD WHEAT HAPLOIDS ($n = 3x = 21$, ABD) WITH SOME APPLICATION AREAS IN WHEAT IMPROVEMENT

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Abstract

Somaclonal variation, aneuploidy, and genotypic specificity are major limitations of anther culture and bread wheat \times *Hordeum bulbosum* crosses for producing haploids ($n=3x=21$, ABD). Sexual combinations of wheat \times *Zea mays* have since emerged as an alternate and significant procedure as wheat genotypic specificity does not exist. This procedure has been refined and simplified over the last decade. It is in extensive use in wheat breeding, cytogenetics, genetics, wide crosses, genetic transformation and molecular mapping. Described here is this simplified wheat \times maize haploid production protocol that is 100% effective across all bread wheat cultivars, generating data means of 25% for embryo excision, 90 to 95% for plantlet regeneration and between 95 to 100% for doubled haploid ($2n=6x=42$, AABBDD) outputs. Simplification steps that enhance efficiency involve hot water emasculation on detached tillers, bud-pollination, elimination of the use of several exogenous chemicals between post-pollination and embryo rescue with no cold shock given to the plated embryos; thus making the product costs significantly competitive and economical.

Introduction

Bread wheat (*Triticum aestivum*; $2n=6x=42$, AABBDD) haploids result from anther culture (Andersen *et al.*, 1987; Kisana *et al.*, 1993) and its crosses with diploid or tetraploid wild barley (*Hordeum bulbosum*) (Barclay, 1975). Wheat genotype specificity (Picard, 1989) is a major limitation for both these procedures, which led to the wheat \times maize hybridization discovery (Laurie & Bennett, 1986) of fertilization and subsequent maize chromosome elimination. Very soon thereafter polyhaploid ($n=3x=21$, ABD) wheat plants were produced (Laurie & Bennett, 1988) and the essential role of 2,4-dichlorophenoxyacetic acid (2,4-D) determined (Suenaga & Nakajima, 1989). Using these crucial discoveries, refinement of the standard protocols processes for wide scale use in wheat research aspects was initiated (Riera-Lizarazu & Mujeeb-Kazi, 1990) followed by innovative modifications that cumulatively have simplified the haploid production protocol and made it significantly economical (Inagaki & Mujeeb-Kazi, 1994) on pollen storage (Riera Lizarazu & Mujeeb-Kazi, 1993), on use of *Tripsacum dactyloides* as an alternative pollen source (Bains *et al.*, 1998) for basal lighting to detect hybrid haploid embryos in seed; elimination of all exogenous chemicals between pollination and embryo rescue, except for 2, 4-D (Mujeeb-Kazi, 2000).

This paper elucidates the current protocol in use by us for producing haploids and its application aspects associated with wheat improvement.

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Table 1. Bread wheat (BW) germplasms utilized in double haploid (DH) production targeted for output in cytogenetics, wide crosses, breeding, genetics, transformation and molecular mapping

DH target category	Germplasm detail	Purpose
Cytogenetics	BW/ <i>Thinopyrum bessarabicum</i> //BW BCI (<i>Phph</i>)	Induce translocations
Wide Crosses	BW/ <i>Th.bessarabicum</i> //n*BW mono or multiple chromosome addition lines	Obtain 21 chromosome haploids with alien addition chromosomes
Breeding	(a)BW/BW//BW derivatives (b)BW/Synthetics//BW	Homozygous plants for (a) drought and (b) high micronutrient/content
Genetics	D genome (1D to 7D) Glennson 81 monosomes/Resistant BW/SH lines where D genome is the resistance source	Partial D genome monosomic analysis
Transformation	Putative transformants (heterozygotes)	Obtain trans-formed homozygotes
Molecular mapping	<ul style="list-style-type: none"> Scab resistant lines x Susceptible BW cultivars Drought tolerant lines x Susceptible BW cultivar Opata 	100 DH / combination for molecular mapping

Materials and Methods

Germplasm: Generating haploids to facilitate research objectives in areas of wheat cytogenetics, wide crosses, breeding, genetics, transformation and molecular mapping required a wide array of germplasm to be utilized (Table 1). The studies were undertaken at CIMMYT, El Batan, Mexico over a decade (1994-2004) forming the basis of selective data to be presented here. Plants were field grown or originated from pot populations maintained under controlled glasshouse regimes of 24°C/14°C day/night and approximately 65% RH.

The protocol

Initial standard procedure: At ear emergence, wheat spikes on intact plants were conventionally emasculated and pollinated with fresh CML 72 maize pollen; short (85 cms) and early (63 days to flowering); or pollen obtained from bulked maize pool populations the third day after emasculation. The pollinated wheat spike culms were needle-injected with a 100 mg/l solution according to the method of Inagaki & Tahir (1990). At 15 days after pollination, immature embryos were aseptically excised from all seed set on the wheat spikes, and transferred onto half-strength Murashige & Skoog (1962) culture medium supplemented with 20 g/l sucrose and 6 g/l agarose. Embryo regeneration, plantlet transfer, and colchicine-induced doubling procedures were similar to those of Mujeeb-Kazi *et al.*, (1987), cytological procedures for mitosis, chromosome banding and fluorescent *in situ* hybridization (FISH) adhered to those reported by Mujeeb-Kazi *et al.*, (1994), Jahan *et al.*, (1990), Islam-Faridi & Mujeeb-Kazi (1995) respectively.

The modified protocol: The initial variation was made in using detached tillers (Riera-Lizarazu & Mujeeb-Kazi, 1990) for haploid production. Briefly, wheat tillers with spikes approaching the conventional emasculation stage were cut off at the base of the growing plant and cultured in a flask with tap water. After pollination with maize on the emasculated spikes the tillers were cultured for 4 days in a solution containing 40 g/l sucrose, 8 ml/l sulfurous acid (6% SO₂) and 100 mg/l 2,4-D. After 4 days the tillers were transferred to a solution containing only sucrose and sulfurous acid until ready for embryo rescue after about 10 to 12 days *i.e.*, a total of 15 to 17 days after pollination. The procedures up to this stage were conducted under glasshouse-controlled regimes as described earlier. Steps associated with embryo rescue and subsequent stages up to doubled haploid (DH) seed harvest were similar to those of the '*standard procedure*'. Three variations were subsequently introduced to add efficiency. First, hot-water emasculation (43°C for 3 minutes) was a viable alternative to hand-emasculation. Second, all use of exogenous chemicals other than 2,4-D was eliminated until the embryo recovery/plating stage and substituted with distilled or tap water. Third, since the 2,4-D systemic action gives ovular enlargement for each floret pollinated, detecting the 25% seed with embryos on a spike is labor intensive. The basal lighting (Bains, 1998) approach was integrated to facilitate swift selection of only those enlarged seed of a spike that possessed an embryo.

Results and Discussion

Elucidation of the protocol: Haploid frequencies across all the bread wheat based areas (Table 1) are fairly consistent. In general the haploid mean embryo recovery frequencies are 25%, regeneration levels between 90 to 95%, with DH outputs of 95 to 100% across all wheat genotypes (cultivars) and growth habit (spring, winter or facultative). These inferences are drawn from data over several years where from 1000/year to 7000/year DH's have been produced. Steps involved in our current simplified/efficient modified detached tiller protocol are shown in Fig. 1 (a to d), Fig. 2 (a to f), Fig. 3 (a to c) and the schematic in Fig. 4.

Application of the doubled haploid protocol: Some application of the haploid methodology in areas of wheat cytogenetics, wide crosses, breeding, genetics, transformation and molecular mapping are elucidated using an appropriate research focus for each category.

Cytogenetics: *Gene transfers from alien species of the Triticeae.* In intergeneric hybrids where the wheat and alien species genomes are unrelated, intergenomic transfers are infrequent due to the presence of the *Ph* locus on chromosome 5BL of wheat. These hybrids are characterized by univalent meiosis (Mujeeb-Kazi *et al.*, 1987, 1989). Using the *ph* genetic stock is an option if some genomic homoeology is prevalent (Sears, 1977; Sharma & Gill, 1986; Jiang *et al.*, 1994). This *ph* involvement can occur when the F₁ hybrid is produced (Sharma & Gill, 1986) but its presence does not permit the high pairing F₁ hybrid to be advanced further.

Fig. 1. Detached tiller utilization in haploid production showing in (a) selected F₃ plant tillers from breeding populations (b) emasculated spikes covered with a plastic bag (c) spikes 15 days after pollination and (d) a close-up of developing spikes with seed set and 2,4-D induced enlargement

Fig. 2. (a) A normal diploid embryo in a well-formed seed with endosperm (left) and a haploid embryo (right) floating in the endosperm cavity (b) haploid embryos plated in a agar-base culture media, (c) embryos regenerating, (d) completely regenerated haploid seedling with green leaves and root presence, (e) seedlings from (d) transplanted into jiffy-7 peat pots and seen in (f) in a close-up.

Fig. 3. (a) Haploid seedlings ready for colchicine treatment, (b) the aerated root treatment process (Mujeeb-Kazi *et al.*, 1987) and (c) the end-product showing C-O seed set on a colchicine treated plant.

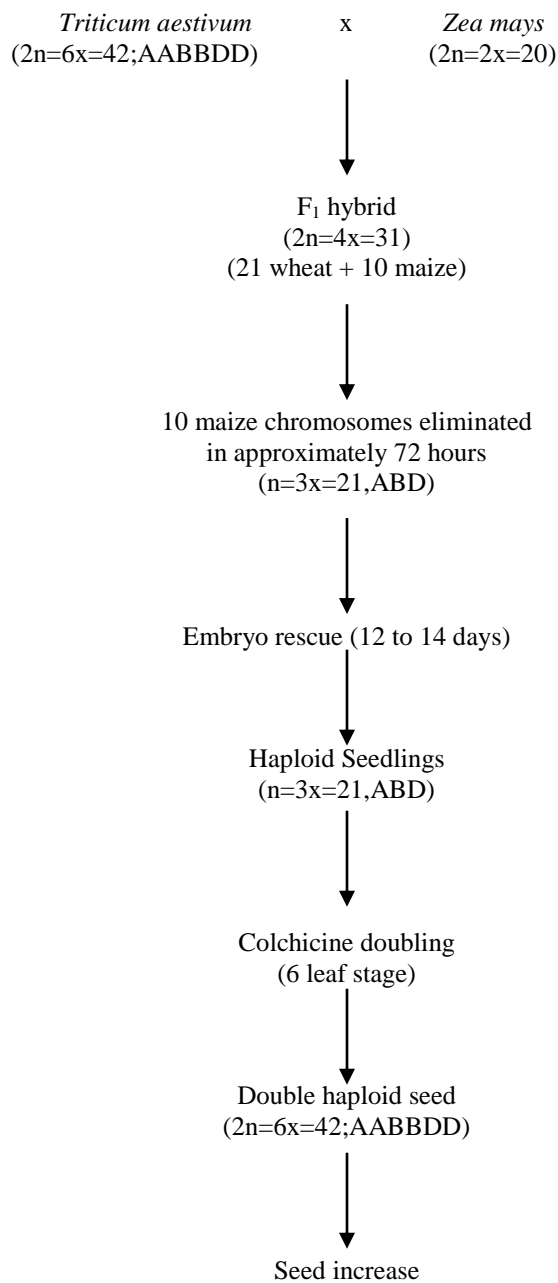


Fig. 4. The schematic of the standard haploid and doubled haploid production protocol for bread wheat x maize crosses.

Hence a viable alternate is to use the *Ph* based F_1 hybrid as a female parent and pollinate with the *ph* genetic stock which would give a backcross I (BC_1) progeny that is *Ph ph* heterozygote (Fig. 5). A similar BC_1 will also result if a *Ph Ph* amphiploid is crossed by the *ph* source. Such BC_1 's (*Ph ph*) are a source that can yield haploid derivatives which are *ph* in nature leading to progeny that possesses alien genetic transfers. In the *T.aestivum* x *Th.bessarabicum* combination ($2n=4x=28,ABDJ$) there is a predominance of meiocytes with 28 univalents (Mujeeb-Kazi *et al.*, 1987). Its BC_1 with the *ph* stock possesses $2n=7x=49,AABBDDJ$ chromosomes and still does not allow J genome chromosomes to pair with those of wheat due to the dominant control of *Ph* locus in the heterozygote (*Ph ph*). Crossing the BC_1 with maize enables the recovery of *Ph* or *ph* based haploids which have 21 wheat chromosomes (ABD) and up to 7 *Th. bessarabicum* chromosomes. The haploid production fits a 1:1 ratio. The haploids with the *ph* locus are detected by PCR (Qu *et al.*, 1998), doubled with colchicine, selfed a few times with the progeny then becoming a source of homoeologous and/or non-homoeologous translocations between ABD and J genome chromosomes (Mujeeb-Kazi, 2003). A similar output of alien exchange will also occur if the amphiploid is first produced and utilized (Fig. 5). The plants with translocations are *ph ph* and generally have more than 42 chromosomes comprised of unaltered wheat, translocated wheat/alien and unaltered alien. After the wheat/alien chromosome translocations are detected repeated backcrossing with a *Ph Ph* wheat cultivar complimented with cytological diagnosis to ascertain presence of the translocation takes place. The end product of this process will yield a *Ph Ph* derivative homozygous for the translocation chromosome to be further utilized in wheat improvement.

Wide crosses: In cytogenetic studies when alien chromosomes are to be added to wheat to yield disomic addition lines a constraint identified is the paternal transmission of the alien chromosome from 42+1 chromosome plants (Islam *et al.*, 1981). Hence 44 chromosome derivatives occur rarely requiring laborious cytology to recover 44 chromosome plants ($42+1$ alien pair = 44). Incorporation of maize based haploidy enhances the efficiency of obtaining disomic addition lines (Fig. 6 schematic) from $42+1$ alien = 43 chromosome plants. Apart from enhancing the frequency of recovering individual disomic addition lines, there may occur greater stability of such doubled haploid addition line genetic stocks since total allelic homozygosity may likely be a contributing factor. This strategy has also been affective for intergeneric derivatives possessing more than one alien chromosome *i.e.*, plants with 44 or 45 chromosome in which 2 to 3 different monosomes are present (eg., 42 wheat + any 2 or 3 of the $1E^b$ to $7E^b$ chromosomes) which permits a faster route to complete the addition series set. There also is the possibility of recovering multiple disomic addition lines comprised of various pair combinations of the $1E^b$ to $7E^b$ which are a novel source of cytogenetic curiosity for studying cumulative gene action affecting polygenic traits.

Breeding: Conventional wheat breeding programs essentially are based upon (a) presence of genetic recombination for enlarging variation, (b) observing and selecting recombinants considered desirable for eventual cultivar release, and (c) advancing the selected derivatives in order to achieve homozygosity thus fixing to considerable extent all allelic *loci*; a phase that requires several generations beyond the F_2 or F_3 . The latter final breeding steps leading to homozygosity can be hastened via the artificial production of haploids where a selected promising F_2 or F_3 heterozygote can be made uniform in a

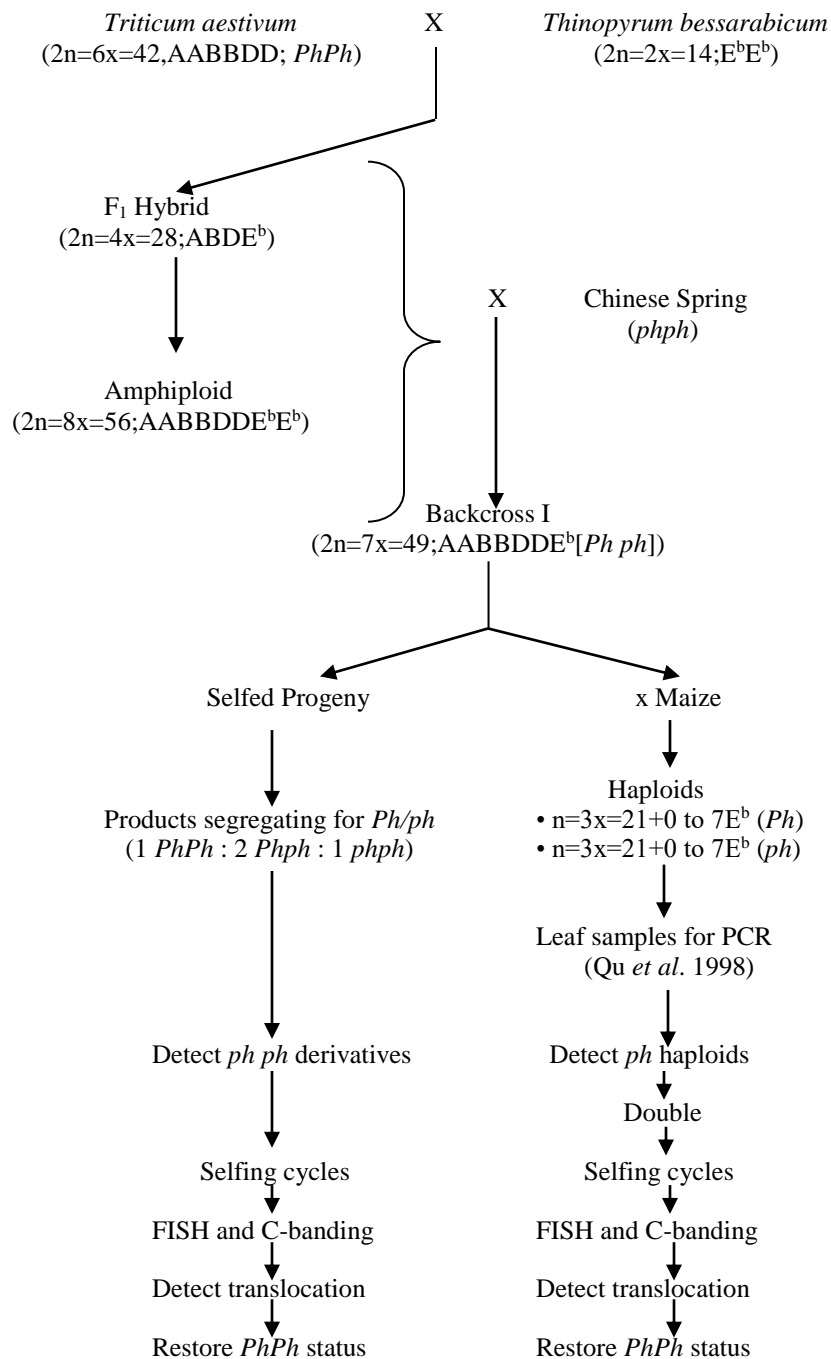


Fig. 5. Utilization of the maize based haploid protocol in an integrated *ph* manipulation strategy to generate wheat/alien chromosome translocations.

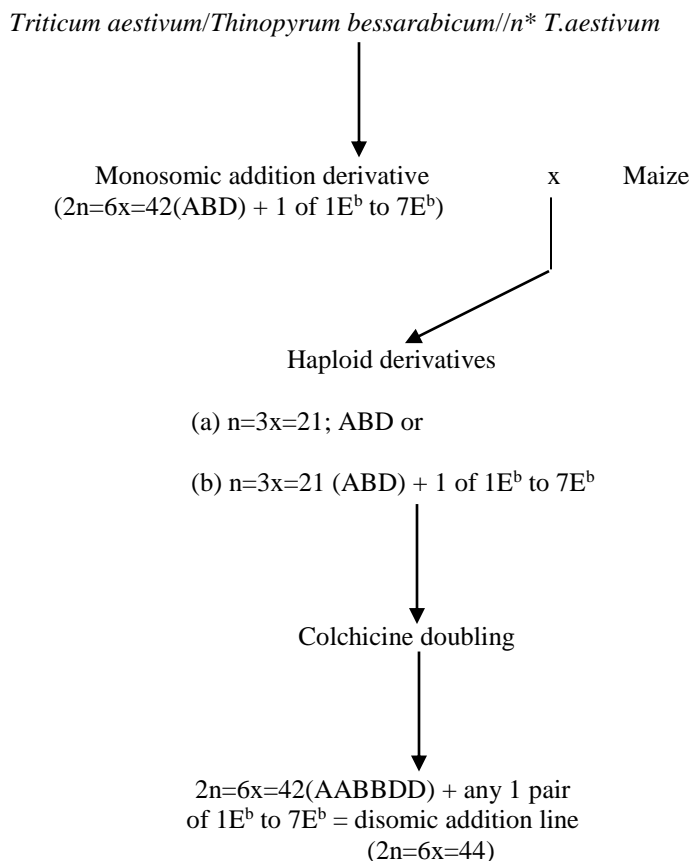


Fig. 6. Crosses between a monosomic alien addition line and maize to recover 21 wheat + 1 chromosome haploids as a means of promoting efficiency for the production of stable disomic addition line derivatives.

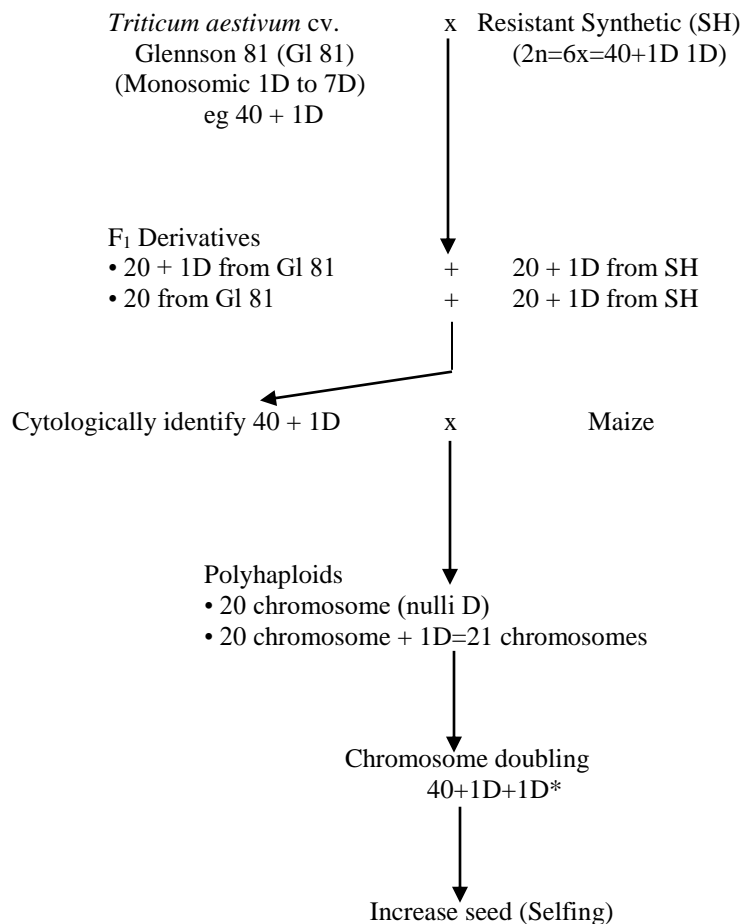
single generation (Nei, 1963). The wheat x maize technique has been perfected to the extent where any recombinant heterozygote can generate a haploid leading to double haploids irrespective of the wheat genotype and habit. Greater preference in breeding programs has been for using F_3 selections since observations can be combined for good agronomic plant type, and stringent initial testing can be made for biotic stresses where rusts usually are a priority. A common constraint in several country programs is that wheat is a winter crop and maize thrives in summer. Controlled environment wheat culture is usually prohibitive due to the hot environment. For such situations stored pollen (-196°C) is an option, for which pearl millet is ideal due to its viability and longevity under cold conditions (Inagaki & Mujeeb-Kazi, 1994). Obtaining four to five doubled haploids per spike has been a norm across all breeding materials over our programs past decade of service activities and similar outputs are realized by using pearl millet stored pollen; thus assuring wide applicability of the haploidy protocol across diverse country breeding programs.

Genetic analysis: The doubled haploid approach is being effectively used to conduct genetic analyses to identify the physical location of genes through complete or partial monosomic analysis of which the latter is exemplified in Fig. 7. Partial analyses are conducted when resistance is associated with any 1 of the three genomes of wheat *i.e.*, A, B and D; shown in Fig. 7 for the D genome chromosomes contributed by *Aegilops tauschii* ($2n=2x=14$; DD) to a stress resistant synthetic hexaploid wheat ($2n=6x=42$; AABBDD). For the analysis monosomic stocks of the D genome (1D to 7D or $2n=6x=40+1D$ to 7D) are each crossed by the resistant synthetic to yield F_1 progeny seed to be comprised of (a) 20 wheat + 20 wheat + 1D to 7D of synthetic origin = 40 wheat + 1D to 7D = 41 chromosomes or (b) 20 wheat + 1D to 7D + 20 wheat + 1D to 7D of synthetic origin = 40 wheat + a pair of 1D to 7D = 42 chromosomes. From each F_1 progeny 41 chromosome plants are cytologically extracted with each 1D to 7D synthetic chromosomes being represented (Fig. 7). These F_1 monosomes of 1D to 7D ($2n=6x=40+1D$ to $40 + 7D$) when crossed with maize yield 21 and 20 chromosome haploids. The 21 chromosome haploids are selected and doubled with colchicine to yield 42 chromosome double haploids (DH). Each DH now possesses the homozygous 1D to 7D chromosomes contributed to the synthetic wheat by *Ae. tauschii*. Upon stress screening the non-segregating resistant DH's are attributed with having the gene/genes in them for that particular stress. Such stable monosomic based DH's simplify conventional monosomic analyses and facilitate global distribution of stable germplasm allowing experimental repetition without having to re-build the analytical germplasm as is necessary when the conventional strategy is followed.

Transformation: The stability of doubled haploid products can also be applied in conferring this attribute to wheat transformants that are often considered to be unstable in the sense that an introduced genes expression may change due to gene silencing commonly explained by methylation coupled with incipient heterozygosity over several selfing cycles.

However, having a homozygous transformant initially positive for the transgenes expression may over-ride the introduced genes silencing constraint during selfing advance (Fig. 8); an aspect warranting more study beyond 3 selfing generations evaluated so far where both the normal and the DH transformants continued so far to exhibit positive transgene expression (Bar-Gus germplasm).

Molecular mapping populations: The development of molecular mapping populations (MP) based upon conventional or distant species involvement has been approached by the DH route as an alternate to the standard single seed descent (SSD) procedure. Desired parents are crossed to produce F_1 seed, which are then crossed with maize to generate haploid progeny. Instead of using F_1 often backcross I (BC_1) is also an alternate source for haploid production. Depending upon the genetic nature of the trait (*i.e.* simple to polygenic inheritance) the mapping population size varies between 100 to about 250 doubled haploids per population. The MP's are utilized in phenotyping and genotyping areas and due to their stability are considered more suitable by several researchers. Mujeeb-Kazi (2003) further suggested that MP's should be built for stress factors where one population could fit the needs of several stress traits. In order to make this functional parental choices should utilize lines with multiple resistances (Mujeeb-Kazi & Van-Ginkel, 2004) and susceptibilities at the F_1 developmental phase.



*7 such DH's result ie 40+1D1D to 40 + 7D7D

Now segregating resistant 1D to 7D DH's possess the resistant genetic control

Fig. 7. Steps involved in conducting a partial D genome based monosomic analysis that utilize the wheat x maize haploidy protocol.

Constraints and conclusions: All bread wheat germplasms irrespective of growth habit respond to haploid induction using maize, or other species as pollen sources. This has made its application attractive and routine for bread wheat improvement. There is no genotypic specificity existent, and the current production frequencies plus costs render the utilization of the technique for crop improvement across many diversified scenarios. A similar trend, however, is not present for durum wheats or Tritico-Secale (Almousslem *et al.*, 1998; Inagaki *et al.*, 1998) but some manipulation strategies have shown a positive response (Inagaki *et al.*, 1998). Haploid generation in bread wheat is independent of the source of maize pollen, but subtle preferences for the maize source do exist as for Seneca 60 and sweet corn. Researchers are using this approach extensively in various programs but are keenly waiting to replace the process by the promise of microspore culture

methodology if and when it becomes readily available (Liu *et al.*, 2002) and is genotype non-specific. The interest also remains in research circles to find haploid events in which maize chromosomes are present as seen in oat haploids derived from oat x maize hybridizations leading to extensive scientific information generation (Phillips *et al.*, 2004). The closest such a phenomenon occurred in wheat haploids was the retention of one millet maize chromosome up to meiosis (Ahmed & Comeau, 1990) leaving a stimulus for continuing to identify haploids retaining pollen donor chromosome/s. Currently however the role of haploidy is being put to significant use in bread wheat breeding, cytogenetics and molecular mapping which presumably will stay prosperous up to at least another decade.

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(Received for publication 10 May 2005)