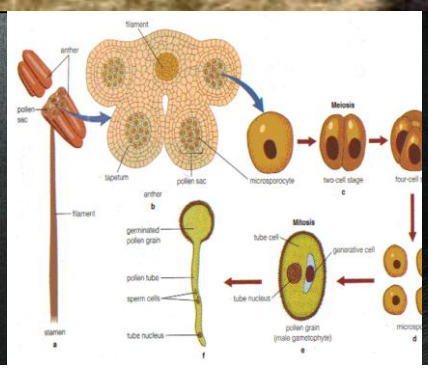
ICARDA
Science for Better Livelihoods in Dry Areas

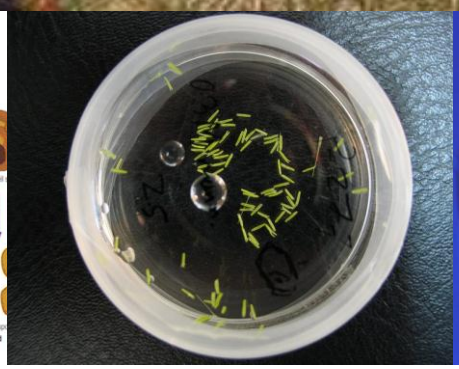
Tools & Guidelines

A Technical Manual

Methods and Applications of Doubled Haploid Technology in Wheat Breeding

W. Tadesse, S. Tawkaz, M.N. Inagaki, E. Picard, and M. Baum





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Executive Summary

The purpose of this manual is to be a useful guide for wheat breeders, geneticists, biotechnologists, students, and technicians. It will also serve as a reference for the wheat breeding programs of ICARDA's National Agricultural Research System partners seeking to establish their own doubled haploid labs and facilities.

In vitro haploid production followed by chromosome doubling greatly enhances the production of completely homozygous wheat lines in a single generation and increases the precision and efficiency of the selection process in wheat breeding. It enables the detecting of linkage and gene interactions, estimating of genetic variance and the number of genes for quantitative characteristics, and producing genetic translocations, substitutions, and chromosome addition lines, and also facilitates genetic transformation and mutation studies.

Wheat cultivars developed using doubled haploid systems have been released for cultivation in both developed and developing countries. In this manual, the origin and production of haploids, detailed procedures of anther culture for doubled haploid wheat production, and its application for wheat breeding at ICARDA are summarized clearly and systematically. The procedures are based on international standards, and have been refined through many years of use and experience at ICARDA.

Wheat is the most widely grown and consumed food crop in the world with a current annual production level of more than 651 million tonnes on a total production area of 217 million hectares. By the year 2050, the world population is estimated to be 9 billion and the demand for wheat will exceed 900 million tonnes. Fulfilling this demand is very challenging in the face of climate change, increasing drought, heat stress, and emergence of new virulent diseases and pests. Offsetting these challenges requires designing an effective wheat breeding strategy with the application of new technologies and tools in order to develop varieties with high yield potential and resistance/tolerance to abiotic and biotic stresses, and with acceptable end-use qualities.

1. Introduction

Wheat (*Triticum aestivum*; 2n = 6x = 42, AABBDD) is the most widely grown cereal crop in the world and one of the central pillars of global food security. About 651 million tonnes of wheat was produced on 217 million hectares in 2010 with a productivity level of 3 t ha^{-1} (FAO 2012). After the quantum leap of the Green Revolution, wheat yields have been rising by only 1.1% per year, a level that falls far short of the demand of a population that is growing 1.5% or more annually. According to some estimates, global wheat production must increase at least by 1.6% annually to meet a projected yearly wheat demand of 760 million tonnes by 2020 (Rosegrant et al. 2001).

Genetic improvement to develop varieties with high yield potential and resistance/tolerance to abiotic and biotic stresses, with acceptable end-use quality, is the most viable and environment-friendly option to sustainably increase wheat yield. Such improvement of crops requires creation and introduction of genetic variation, inbreeding coupled with selection, and extensive evaluation of breeding materials at multiple locations to identify adapted and stable genotypes with desirable agronomic traits.

Variation can be created by sexual crosses (usually single, three-way, double, or back crosses) and mutation breeding. Breeders have used different methods to fix and develop homozygous genotypes from such variation. Isolation of homozygous and homogeneous genotypes through conventional inbreeding methods – single seed descent (SSD), backcrossing, and selfing in the field, plus in some cases the use of off-season nurseries and shuttle breeding approaches – requires several cycles of inbreeding and selection making it the most tedious, time consuming, and expensive phase of any breeding program. Furthermore, in conventional plant breeding, truly homozygous lines are rare and most selections contain some heterozygous loci (Snape 1989; Raina, 1997; Baenziger et al. 2006).

Recent advances in plant tissue culture and its related disciplines opened an avenue that greatly facilitated the haplodiploidization breeding scheme, and this enables the extraction of instant homozygous lines/varieties from crop plants with any degree of heterogeneity in a single generation (Baenziger et al.1989; Wu et al. 2012). Haploid individuals are the sporophytes with gametic chromosome number (Ouyang et al. 1973; Riley 1974) and doubled haploids (DHs) can instantly be produced by doubling these haploid chromosome complements.

This review summarizes the origin and production of haploids, techniques in anther culture and isolated microspore culture and wheat × maize crosses, and their application in wheat breeding.

2. Doubled haploids: origin and production

All cultivated wheat belongs to the genus *Triticum*, which was divided by Schultz (1913) into three major taxonomic groups: einkorn, emmer, and dinkel. This classification was supported by the pioneering cytological study of Sakamura (1918), who found that Schultz's three wheat groups also differed in their chromosome number; the einkorns are diploids (2n = 2x = 14), emmers are tetraploids (2n = 4x = 28), and dinkels are hexaploids (2n = 6x = 42), all with the basic chromosome number x = 7. Soon after, based on cytogenetic analysis, Kihara (1924) designated the genome formulae for cultivated einkorn (*T. monococcum* L., 2n = 2x = 14), emmer (*T. turgidum* L., 2n = 4x = 28), and dinkel (*T. aestivum* L., 2n = 6x = 42) as AA, AABB, and AABBDD, respectively.

Each group of wheat forms their own respective haploids. Haploids are sporophytes that contain gametic chromosome numbers (n). The haploids from einkorn, emmer, and dinkel possess n = x = 7, n = 2x = 14, and n = 3x = 21 chromosomes with genomic constitution of A, AB, and ABD, respectively (Quisenberry and Reitz 1967; Fehr 1993; Folling and Olesen 2002). Haploids can originate spontaneously in nature or as a result of various induction techniques. Spontaneous development of haploid plants has

been known since Blakeslee et al. (1922) first described it in *Datura stramonium* L.; however, spontaneous occurrence is rare and therefore of limited practical value. DHs are genotypes produced through induction of haploids and doubling of chromosomes.

2.1 Haploid induction techniques

The potential of haploidy for plant breeding arose in 1964 with the achievement of haploid embryo formation from *in vitro* culture of *Datura* anthers (Guha and Maheshwari 1964), which was followed by successful *in vitro* haploid production in tobacco (*Nicotiana tabaccum* L.) (Nataka and Tanaka 1968; Nitsch 1969) and rice (*Oryza sativa* L.) (Niizeki and Oono 1968). Subsequently, bread wheat (*Triticum aestivum* L.) haploids were also produced by anther culture (Ouyang et al. 1973; Picard and De Buyser 1973), isolated microspore culture (Wei 1982), and by using wide hybridization with wild barley *Hordeum bulbosum* L. and maize (*Zea mays* L.) (Barclay 1975; Laurie and Bennett 1986, 1988; Inagaki and Tahir 1990). The two systems of anther culture and wheat × maize cross are the most commonly used induction methods in wheat.

2.1.1 Anther culture

The first success in regeneration of bread wheat plants through anther culture was achieved in the early 1970s (Ouyang et al. 1973; Picard and De Buyser 1973). Anther culture exploits the fact that a certain proportion of pollen grains *in situ* are embryogenic. These pollen grains can develop into embryos only when they are placed on artificial medium. The process of anther culture begins in the selection of primary wheat spikes which contain anthers with pollen at the mid–late uni-nucleate stage of development as indicated in Fig. 1 (Ouyang et al. 1973; Picard and De Buyser 1973; Liu et al. 2002).

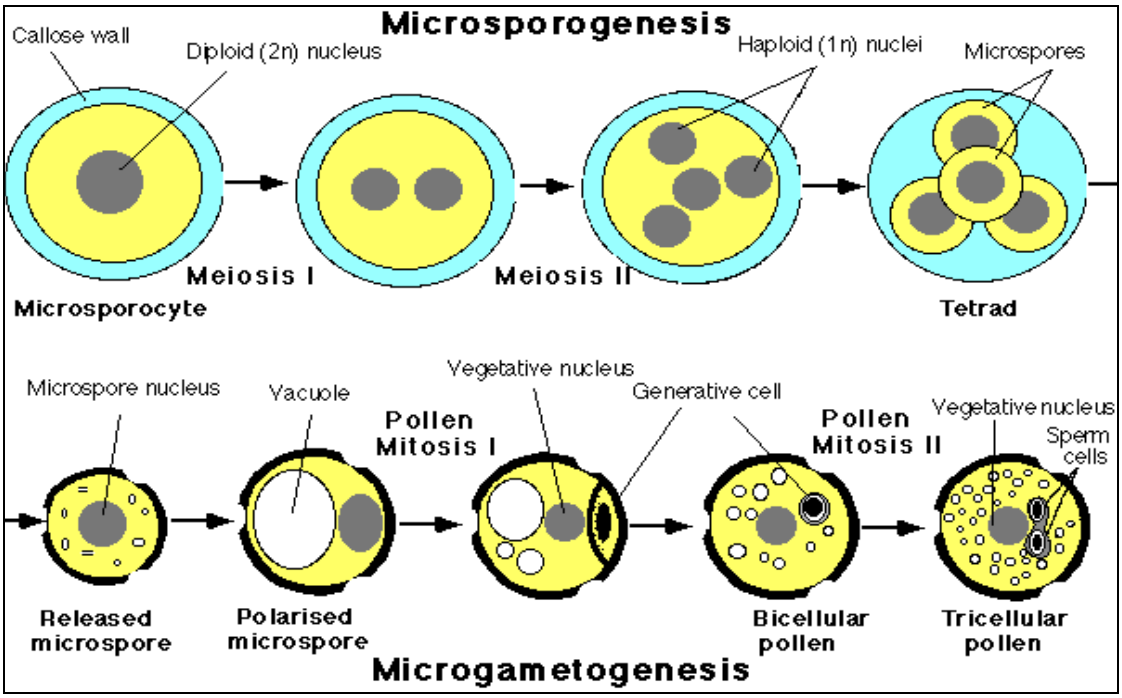

Fig. 1. Stages of pollen development

The stage of pollen development is very important as minor deviations can lead to major decreases in yield. Anthers are aseptically dissected and cultured on induction medium and incubated in darkness at 26–28°C for 4–6 weeks, after which calli are transferred to a solid plant regeneration medium and incubated in a culture room at 25°C and 16 h day length for about 30 d. Green plantlets are transferred to culture tubes containing 20 mL of modified plantlet regeneration medium (PLL). After 1–2 months of hardening, vigorous seedlings are transplanted into pots with 2:1:1 soil:sand:peat-moss mixture and kept in a plastic house (Tawkaz 2011).

Anther culture is used in many cereal breeding programs, and is more cost-effective than intergeneric crosses in the production of DHs (Snape et al. 1986; Pratap et al. 2006). However, anther culture is dependent on genotype (Wehr and Zeller 1990; Pratap et al. 2006, Khiabani et al. 2008; Grauda et al. 2010; El-Hennawy et al. 2011; Tawkaz 2011). To counter the genetic effect, components of the system such as growth environment of donor plants, modification of the medium components, changing the physical state of the medium (liquid or solid), cold pre-treatment of anthers before culturing, increasing the incubation temperature during the first few days of culture, and subjecting anther explants to gamma irradiation have been investigated (Karsai et al. 1994; Karimzadeh et al. 1995; Xynias et al. 2001; Zamani et al. 2003; Shirdelmoghanloo et al. 2009; Tawkaz 2011).

A further disadvantage of anther culture in wheat breeding is the proportion of albino plants. In some bread wheat genotypes the frequency of albinism is in the range of 20–50% with a mean of 30% of all regenerated plants (Abd El-Maksoud and Bedö 1992; Abd El-Maksoud et al 1993;Tawkaz 2011). Durum wheat (*T. turgidum* L. subsp. *durum*) is very recalcitrant to anther culture with low regeneration rate and very high frequency of albino plants (Cistué et al. 2006, 2009; Labbani et al. 2007). In an effort to improve this problem in durum wheat, Ayed et al. (2010) applied different pre-treatments and found that cold treatment (4°C) of anthers for five weeks significantly improved embryogenesis induction and green plant regeneration – with green: albino plant ratio of about 75%. Mannitol pre-treatment is also effective in improving the efficiency of anther culture in durum wheat (Labbani et al. 2007; Ayed et al. 2010).

Recently, Grauda et al. (2010) reported increased DH production efficiency through the utilization of AM induction medium with added copper. The use of copper in anther culture reduces the number of albino plants and increases the number of green plants. These effects are related to improved survival of microspores during the different tissue culture stages and the synchronization of the first microspore symmetric division (Wojnarowiez et al. 2002; Jacquard et al. 2009).

DHs are also produced using microspore culture following similar methods and approaches to anther culture. However, isolation and purification of microspores requires different techniques and facilities. Microspores can be isolated either through shedding, magnetic-bar stirring, maceration, and/or blending methods. According to Gustafson et al. (1995), isolation by blending gives the highest initial microspore viability of 75%. Microspores released through blending usually have less damage and higher embryogenic capacity and reproducibility than those obtained through maceration. However, blending isolation can also damage the microspores, especially when the blending speed and length is not optimized. The blending parameters vary somewhat with the number of florets in the blender cup, toughness of the tissue surrounding anthers, and the relative volume of liquid medium vs. solid tissues.

2.1.2. Wheat × maize cross method of haploid induction

Wide crosses have been utilized for the production of haploids for crop improvement and genetic studies (Baum et al. 1992). Bread wheat DHs are produced by various intergeneric crosses with maize (Suenaga and Nakajima 1989; Laurie and Benett 1989; Inagaki and Tahir 1990; Laurie and Reymondie 1991), pearl millet [*Pennisetum glaucum* (L.) R. Br.] (Inagaki and Mujeeb-Kazi 1995), teosinte (*Z. mays* L. spp. *mexicana*) (Ushiyama et al. 1991), *H. bulbosum* (Barclay 1975), and sorghum [*Sorghum bicolor* (L.) Moeneh] (Ohkawa et al. 1992; Inagaki and Mujeeb-Kazi 1995).

The crossability of bread wheat *× H. bulbosum* depends on the wheat allelic composition for the *Kr* genes responsible for the incompatibility between these two species (Sitch and Snape 1987). Production of wheat haploids through wheat × maize crossing was reported as successful with no development of albino plants (Sadasivaiah et al. 1999; Ushiyama et al. 2007) and insensitivity of maize to the action of cross-incompatibility genes. Several reports demonstrated the success of DH plant production using maize pollen on bread wheat (Suenaga and Nakajima 1989; Amrani et al. 1993; Niroula et al. 2007) but relatively few durum wheat genotypes show such crossability with maize (Almouslem et al. 1998; Inagaki et al. 1998a; David et al. 1999; Garcia-Lilamas et al. 2004).

The superiority of wide crosses over other techniques includes higher efficacy (2–3 times more efficient for green plant production than anther culture), simplicity, less genotype dependent response, less gametoclonal variation, and being less time consuming (Niroula and Bimb 2009). In the wheat × maize system, the culture of wheat donor plants is carried out in climate chambers and in the greenhouse. At 1–2 d before anthesis, wheat florets are emasculated and 2 d later are pollinated with fresh maize pollen. Cryopreserved (–80°C) maize pollen can also be successfully utilized for pollination of wheat florets, although the efficiency of haploid formation is quite low compared with fresh pollen (Inagaki et al. 1997).

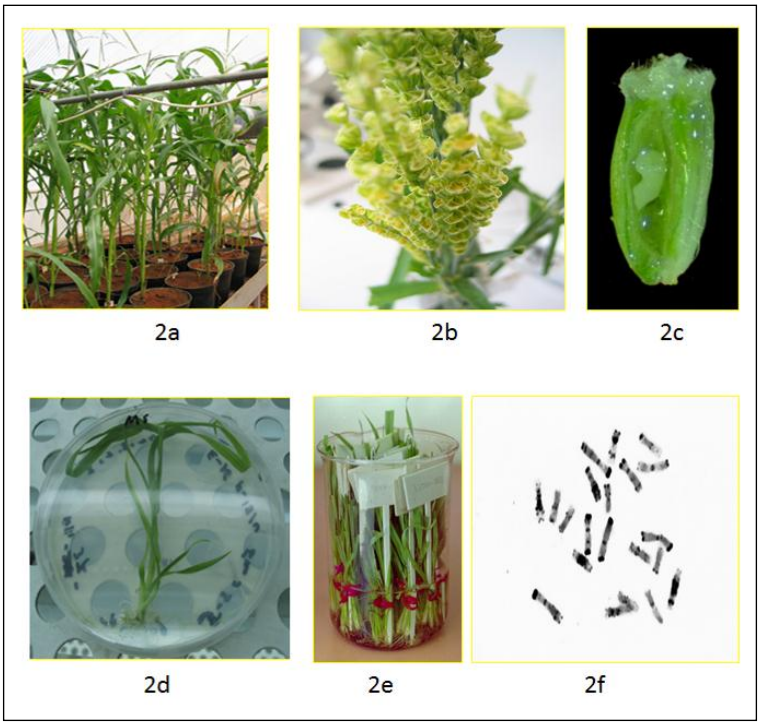

Fig. 2. Procedures of wheat × maize cross method for DH wheat production: 2a = growing maize plants; 2b = pollinated spikes with maize pollen; 2c = obtained seed from crosses with maize; 2d = plant developing from rescued embryo; 2e = haploid plants under colchicine treatment; 2f = chromosome counting (Inagaki 2003)

Use of alternative sources of stored pearl millet pollen, as well as fresh pollen, is effective for haploid production in wheat (Inagaki et al. 1997). This cryopreservation technique is quite advantageous where synchronization is a problem. Once pollination is complete, the application of auxin is essential for the successful recovery of haploid wheat embryos (Suenaga and Nakajima 1989; Niroula et al. 2007).

Recently, the frequency of polyhaploid embryo formation was greatly improved through the manipulation of Dicamba alone or in combination with 2,4-D (O'Donoughue and Bennett 1994; Almouslem et al. 1998; Garcia-llamas et al. 2004; Ahmad and Chowdhry 2005). Embryo rescue is essential to recover haploid plantlets from the wheat × maize system (Suenaga and Nakajima 1989; Zhang et al. 1996; Sood et al. 2003; Niroula et al. 2007; Niroula and Bimb 2009) 14–20 d after pollination. Excised embryos can be cultured on either full strength MS (Murashige and Skoog 1962) or ½ MS or B5 basal medium (Gamborg et al. 1968) containing various modifications of organic supplements (Zenkler and Nitzsche 1984; Zhang et al. 1996; Suenaga et al. 1997; Campbell et al. 2000; Singh et al. 2004; Ayed et al. 2011) and can be grown *in vitro* for 3–5 weeks at 20–25°C and 16 h day length. Generally seedlings are ready to transfer after that period and need to be hardened for one week in a growth chamber under the same environmental regime. The wheat × maize system is illustrated in Fig. 2.

2.2. Chromosome doubling

Haploid plants are infertile because sexual fertility depends upon meiotic division of the diploid chromosome number. Spontaneous rates of chromosome doubling among plants derived from microspores of wheat are relatively low. In many experiments only 15–20% of the plants obtained are capable of seed set by selfing without a treatment for chromosome doubling (Hansen et al. 1988; Navarro-Alvarez et al. 1994; Ouyang et al. 1994).

To regain fertility, the number of wheat chromosomes needs to be doubled following haploid embryo rescue and seedling formation. Colchicine is the most frequently used drug for chromosome doubling in plants (Ouyang et al. 1994; Soriano et al. 2007). The drug inhibits spindle function during mitosis and disturbs normal polar segregation of sister chromatids to form a restitution nucleus. Upon mitotic divisions of such affected cells, chromosome-doubled chimera sectors are formed which lead to partial fertility of the plant if they comprise sexual organs. Colchicine is traditionally administered to the young plants established in soil at the 3–5 tiller stages.

The colchicine treatment procedure of Inagaki (2003) is very efficient. According to his method, roots of the haploid seedling are pruned leaving a zone of 2–3 cm and submerged in a 0.1% colchicine solution supplemented with 2% dimethyl sulfoxide (DMSO) and ca. 0.05% Tween-20 at 20°C for 5 h. After this treatment the roots are washed free from residual colchicine and potted in peat soil. In large-scale production of DHs the colchicine treatment of individual plants after establishment in soil is expensive both in terms of chemical and labor costs. It causes high mortality rate and production of mixoploids or chimeric plants that leads to low seed production, and therefore an additional growth cycle for seed multiplication is required before evaluation in the field (Chen et al. 1994; Islam 2010).

Chromosome doubling techniques directly integrated into the haploid induction procedures may have potential for more cost efficient chromosome doubling of haploids for future wheat breeding. Different methods for *in vitro* chromosome doubling of wheat have been proposed. Colchicine has been added directly to the anther culture induction medium at concentrations around 0.2 g/L (500 mM). Anthers transferred to colchicine-free medium after 72 h resulted in up to 70% fertile plants (Barnabas et al. 1991; Navarro-Alvarez et al. 1994). Alternatively, Ouyang et al. (1994) cultured pollen calli on colchicine-containing medium during regeneration and reported an average increase in the frequency of fertile plants from 17% in the control to 54% with the best *in vitro* treatment. Recently, however, it was

reported that toxicity of high colchicine concentrations reduced the number of embryos derived from microspore culture (Barnabas et al. 1991; Navarro-Alvarez et al. 1994). Furthermore, the early chromosome doubling event may lead to a possible increased frequency of aneuploids among *in vitro* chromosome-doubled wheat haploids.

3. Detailed protocols of anther culture for doubled haploid wheat production at ICARDA

Of all the different DH wheat production techniques reviewed above, wheat breeding at ICARDA applies anther culture for production of DH wheat genotypes. Its detailed procedures and protocols are presented as follows.

3.1. Cultivation of donor plants

Targeted crosses are made by breeders and seeds are germinated in Petri dishes on a wet filter paper at room temperature and transferred to 4°C for one week for spring wheat, and 6–8 weeks for winter wheat because it needs vernalization. To enhance vigorous plant growth, the seedlings are planted in pots containing 2:1:1 soil:sand:peat-moss mixture and placed in a plastic house or growth room under controlled environmental conditions at 18–22/15°C day/night temperature for spring wheat and 15–18/10°C for winter wheat.

Alternatively, F_1 plants can also be grown in the field under optimum management. It is always important to have 7–10 spikes emasculated and pollinated for a given cross in order to get enough F_0 seed and good size of F_1 plants. Optimum management of F_1 plants ensures vigorous plants with a good number of tillers. We undertake DH production from F_1 plants originated from elite × elite crosses instead of F_2 plants to increase the gain in time rather than the genetic gain which could be obtained by having one more generation of recombination. This is especially important for winter wheat as there is only one cycle of generation advancement per year compared to spring wheat for which growing of two generations per year is possible through shuttle breeding under irrigated and rain-fed schemes in winter and summer seasons or using SSD in greenhouse conditions.

3.2. Spike collection and pre-treatment

Anthers from the central floret are collected from F_1 plants at booting stage, squashed in 0.5% aceto-carmine and examined under a light microscope to determine the appropriate time of microspore development for spike collection. Tillers with microspores at the mid–late uni-nucleate stage are collected and then incubated at 4°C for at least 2 d and maximum 21 d before anther culture. It is important to synchronize the collected tillers with the collection date and cross number.

3.3. Anther culture

Spikes are marked and sterilized with 4% sodium hypochlorite solution for 5 min followed by rinsing them three times with sterilized distilled water for 5 min each. The sterilized spikes are then placed on sterilized filter papers and anthers from the central part are aseptically removed and placed in a 60 mm × 15 mm Petri dish containing 5 mL of induction medium (CHB3) (Table 1). Up to 45 anthers are cultured per Petri dish under sterilized conditions in a laminar flow bench. A few ovaries need to be co-cultured to create a natural environment for calli formation and regeneration. Petri dishes containing anthers should be firmly wrapped with Para-film (Pechiney Plastic Packaging Co. Chicago) and incubated in darkness at 26–28°C for 4–6 weeks. Evaporation of the liquid medium is reduced by placing a beaker containing water with the Petri dishes.

Induced calli are transferred to a solid plant regeneration medium (R9, Picard and deBuyser 1973) (Table 2), and incubated in a culture room at 25°C and 16 h day length. Green plantlets are scored after 4–7 d of incubation. One month later, they are transferred to culture tubes containing 20 mL of plantlet regeneration medium (Table 3). Green plantlets with poor root and shoot systems are sub-cultured on a new R9 Petri dish. Cultures are kept in the culture room under the same conditions as the calli. The number of green plantlets, albinos, date of transfer of calli to R9 medium, and date of transfer of green plantlets to PLL medium are recorded.

After about two months, green plantlets with good root and shoot systems are transplanted into small pots containing peat moss, and the transplants are first hardened in a growth chamber under 18/12°C day/night temperatures for 16 h day length with 70–80% relative humidity.

3.4. Colchicine treatment and doubled haploid plant production

Healthy plants are taken from the soil and their roots are thoroughly washed with tap water. Parts of the root and the shoot system are cut off. The trimmed plants are then immersed in 0.2% colchicine with DMSO and a few drops of Tween-20 for 4 h at room temperature. The plants are then taken out of the colchicine and washed with running tap water overnight, and transplanted into pots with 2:1:1 soil:sand:peat-moss mixture in a plastic house and covered with plastic bags for 1 d. Before anthesis, fertile heads are covered with bags to avoid outcrossing. The plants were left to grow until maturity. Seeds from each plant are harvested separately and kept as individual DH lines. Production of more than 100 DH lines per cross is important to increase the chance of getting genotypes with good combinations of desired agronomic traits. The DH seeds are multiplied and DH lines are tested in preliminary yield trials across different locations for different traits such as growth habit, cold tolerance, days to heading, maturity, plant height, resistance to diseases, yield, 1000 seed weight, and grain quality.

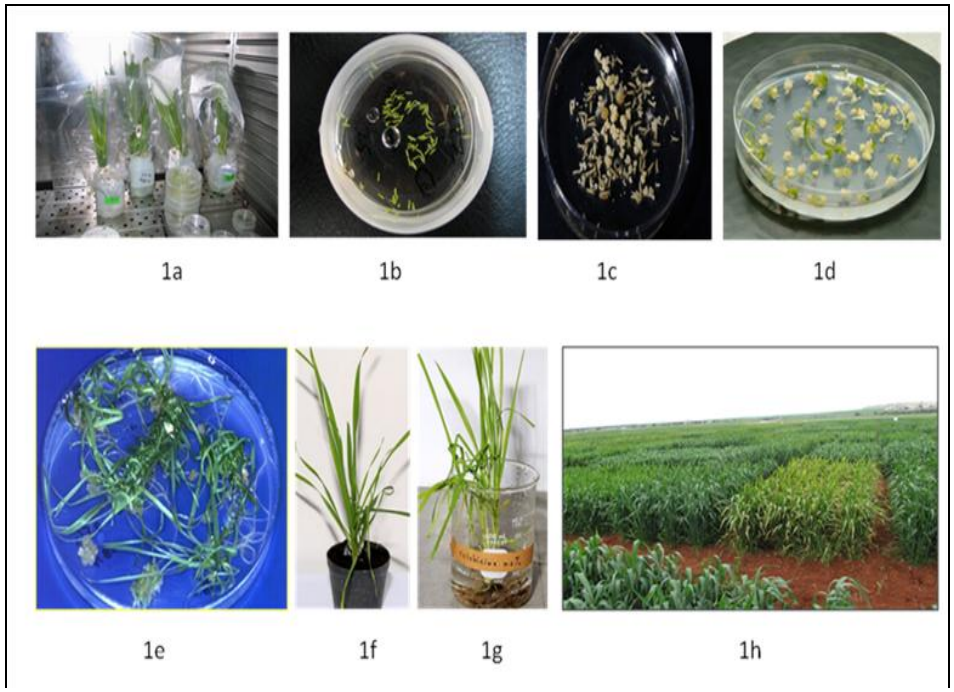

Fig. 3. Procedures of anther culture for DH wheat production: 1a = pre-treatment of the donor plants at 4°C; 1b = anthers in liquid induction medium; 1c = developing of embryos in liquid induction medium; 1d = embryo converting to green on solid regeneration medium; 1e = green plants in regeneration medium;1f = haploid plants at acclimatization stage; 1g = haploid plants under colchicine treatment (0.2%); 1h = DH lines in the field, showing uniformity within lines (Tawkaz 2011; Tadesse et al. 2012)

Table 1. Composition of the CHB3 induction medium (Picard and De Buyser 1973).

Reagent*	Amount (mg/L)
Macroelements	
KNO_3	1415
$CaCl_2.2H_2O$	83
$MgSO_4.7H_2O$	93
KH_2PO_4	200
$(NH_4)SO_4$	232
Microelements	
KI	0.4
$ZnSO_4.7H_20$	5
$MnSO4.4H_2O$	5
H_3BO_3	5
$NaMoO_4.2H_2O$	0.012
$CuSO_4.5\ H_2O$	0.025
$CoCl_2.6\ H_2O$	0.025
Iron stock	40
Vitamins and hormones	
Thiamine HCl (B1)	2.5
Ac. Nicotinic acid B53	0.5
Pyridoxine HCl (B6)	0.5
Glycine	1.00
d-Biotin (H)	0.25
Ac.Panth.1/2Ca	0.25
Ascorbic acid (C)	0.25
2,4-Dichlorophenoxyacitic acid	0.5
Kinetin	0.5
L-Glutamine	993.5
Myo-inositol	300
Maltose 90000	

* All components were sterilized using Nalgene filter units with membrane size of 0.2 µm, 75 mm diameter, and vacuum pump after adjusting the pH to 5.4

Table 2. Composition of the R9 regeneration medium (Picard and De Buyser 1973).

Reagent*	Amount (mg/L)
Macroelements	
KNO_3	1000
$MgSO_4.7H_2O$	71.5
NH_4NO_3	1000
KH_2PO_4	300
KCl	65
$Ca(NO_3)_2.4H_20$	500
Microelements	
KI	0.75
$CoCl_2.6H_2O$	0.05
$Na_2MoO_4.2H_2O$	0.20
H_3BO_3	1.60
$MnSO_4.H_2O$	4.90
$CuSO_4.5H_2O$	0.076
$ZnSO_4.7H_20$	2.70
Iron stock	40
Vitamins	
Myo-inositol	100
Carbohydrate source	
Sucrose	20
Solidifying agent	
Agar	7 g
Vitamins added after autoclaving	
Thiamin HCl (B1)	1
Nicotinic acid (B3)	5
Pyridoxine HCl (B6)	5
Glycine	2
IAA (indole-3-acetic acid)	1

* All components were autoclaved at 121°C and 15 kg/cm^2 pressure for 20 min, except the hormones which were added after autoclaving. The medium was poured into 90 mm × 15 mm sterilized plastic Petri dishes and stored in darkness at 4°C. pH was adjusted to 5.9 before autoclaving

Table 3. Composition of PLL plantlet regeneration medium (Picard and De Buyser 1973).

Phase	Reagents*	Amount (mg/L)
Acid phase	NH_4NO_3	164
	$CaCl_2.2H_2O$	440
	$MnSO_4$	22.3
	H_3BO_3	6.2
	$ZnSO_4.7H_2O$	8.6
	KI	8.3
	Na_2MoO_4	0. 25
	$CuSO_4.5H_2O$	0.025
	Iron Stock	40
		pH = 4.2
Neutral phase	KNO_3	1900
	$MgSO_4$	370
	KH_2PO_4	170
	Glutamine	750
	Myo-inositol	100
	Biotin	0.4
	Thiamin	0.4
	Nicotine	0.4
	Pyridoxine	0.4
	Sucrose	20 000
	Agar	6000
		pH = 5.8
Added after autoclaving	IAA	0.5 g

* The components were divided into two phases: neutral and acidic phases, prepared separately, mixed, and autoclaved at 121°C. Hormones were added after autoclaving. The medium was poured (20 mL) into test tubes when it was still warm (50°C) and kept at 4°C

4. Stability and agronomic performance of doubled haploids

Xu et al. (1997) reviewed RIL (Recombinant Inbred Lines), DH, BC (backcrosses), and F_2 populations for segregation distortion with molecular markers. RIL populations had the highest distortions, DH (by anther culture) and BC had similar distortion, and F_2s were the least distorted populations. Some regions in the genome are distorted because they are linked to fertility/sterility genes. Some distorted regions contain genes that are related to the androgenetic procedure.

To be used in a breeding program, DH plants must be genetically stable with no aberrant genetic variation arising during the process. Therefore, it is important to determine if any genetic variation is introduced during the production of DH lines; however, very few studies have investigated this area.

Suenaga and Nakajima (1993) evaluated 110 wheat DH lines derived from wheat × maize crosses and found that 15 DH lines were variable for traits like extreme dwarfism, low seed fertility, and alteration of spike type. Similarly, Kammholz et al. (1998) found that expected normal segregation pattern for six glutenin loci across seven crosses indicated that the wheat × maize system was stable across the generations. Laurie and Snape (1990) assessed the agronomic performance of wheat DH lines derived from wheat × maize crosses. They compared the performance of various DH lines of 'Chinese Spring'/ 'Hope' cross, and lines of a single chromosome substitution of 'Chinese Spring' and their respective parents under field conditions. No significant variation was detected in either population of 'Chinese Spring/Hope' DH lines and neither population differed significantly from its parent.

Kisana et al. (1993) compared wheat DHs produced through anther culture and wheat × maize crosses, and found that anther-derived plants were cytologically unstable, whereas all plants regenerated from wheat × maize crosses were stable. In contrast, in other experiments, where the extent of variation from intergeneric cross and anther-culture methods were compared, there were no significant differences in agronomic characters between the methods of DH production (Bjornstad et al. 1993).

In studies comparing the agronomic performance of best wheat genotypes selected through DH, SSD, and pedigree methods, no significant differences in grain yield were found among any populations when the parental varieties were closely related in their pedigrees (Abd El-Maksoud et al. 1993; Inagaki et al. 1998b). In two crosses with low coefficients of parentage and large progeny variation, grain yield of selected DH lines was significantly lower than grain yield of SSD and pedigree selected lines (Inagaki et al. 1998a). Recently, El-Hennawy et al. (2011) evaluated the agronomic performance of anther culture-derived DH wheat genotypes and identified genotypes that were highly stable and superior to the best checks in grain yield performance.

5. Factors to be considered in doubled haploid wheat breeding

For successful implementation of a DH wheat breeding program, different factors such as the filial generation from which DHs are made, the population size, and the comparative advantage of DH with other conventional breeding methods need to be determined. Conventionally, in an effort to shorten the breeding cycle, most breeders prefer to produce DH from the F_1 generation (Nei 1963; Tadesse et al. 2012). Inducing of homozygosity at such an early stage may limit the opportunity for recombination events that create potentially useful genetic variation for breeders.

Snape and Simpson (1981) examined the theoretical and practical effects of linkage on traits of DH lines derived from F_1, F_2, F_3, and intermated F_2 (S_3) generations of barley, and found a significant gain in genetic variation for spike emergence time, height, grain number/spike, and spikes/plant from delaying the production of DHs to the F_2 generation due to the breakup of repulsion linkages and creation of new allelic configurations at unlinked loci (Snape and Simpson 1981; Choo et al. 1985; Patel et al. 1985; Yonezawa et al. 1987). In contrast, Iyamabo and Hayes (1995) quantified the effects of an additional round of recombination when comparing F_1- and F_2-derived barley DH lines and showed the additional round of recombination did not lead to large performance differences between the two populations.

Population size plays an important role in the success of any breeding program. Theoretically, if a hybrid has *n* pairs of independently segregating genes, the chance of selecting a particular homozygous genotype from the F_2 population in a conventional breeding program is $(1/2)^{2n}$, whereas in haploid breeding it is $(1/2)^n$ (Chu 1982). Thus the selection efficiency in haploid breeding is *2n* times better than the conventional methods.

SSD is similar to the DH method in that both methods provide rapid generation advancement for producing homozygous lines (Grafius 1965). In DH breeding there is only one opportunity for recombination if F_1 plants are used as donors – while in SSD, recombination can occur in every generation of inbreeding (Grafius 1965). Minimum population sizes depend upon how many unlinked loci are to be fixed, for example, to be sure of fixing two unlinked loci, 16 DHs are needed. To obtain five unlinked loci, a DH population of 203 is needed. In the presence of linkage, minimum population sizes have to increase (Yonezawa et al. 1987; Jansen 1992) or alternatively, the production of DHs be delayed a generation. In general, to improve quantitatively inherited traits such as yield, it is very important to increase population size by nominating a limited number of elite × elite crosses for DH production.

6. Application of doubled haploids

The induction and regeneration of haploids followed by spontaneous or induced doubling of chromosomes are widely used techniques in advanced breeding programs of several agricultural species. In traditional plant breeding, after crossing it generally takes at least six generations before a sufficiently homozygous population is obtained to undertake screening and preliminary yield trials in the F_7 generation. The DH method enables production of a homozygous plant in one generation, and with one additional year for seed increase (DH_2), it is possible to undertake screening and preliminary yield trials in the third year (DH_3) as indicated in Fig. 3. Therefore, DH technology dramatically increases the speed of the inbred developmental processes by reducing several time-intensive generations of inbreeding, and by making phenotyping and genotyping more predictive. The gain in time through the DH system is especially important in winter wheat as there is only one cycle of generation advancement per year compared to spring wheat where advancing two generations per year might be possible by using SSD or a shuttle breeding approach.

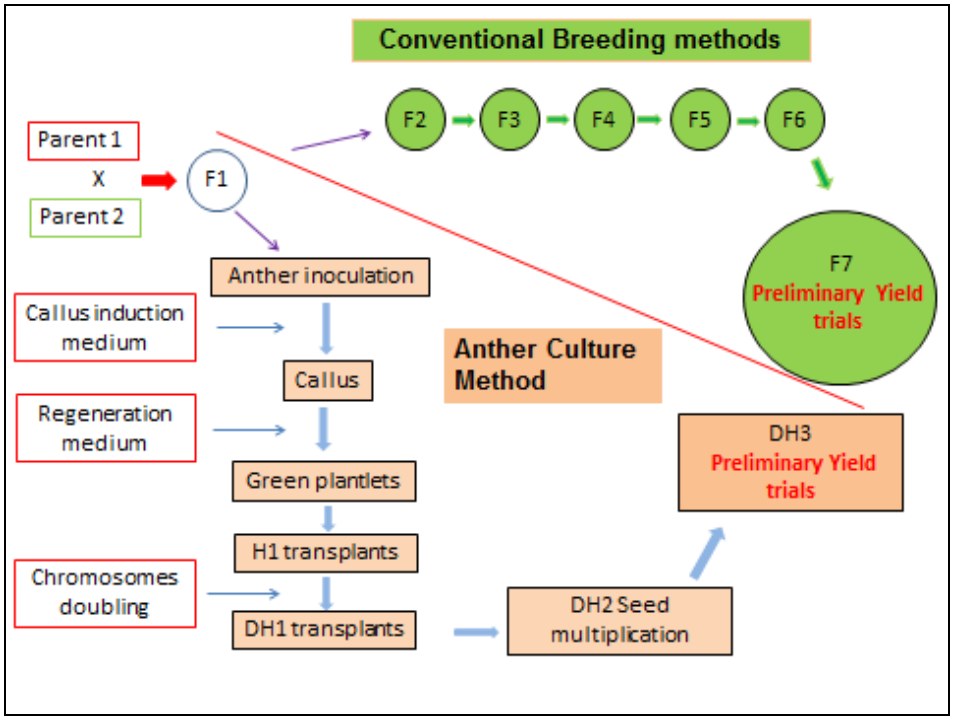

Fig. 3. Comparison of conventional and anther culture breeding methods. Conventional methods take at least 8–9 years for production of homozygous lines. Anther culture takes only 3 years. This double haploid (DH) method enables production of a homozygous plant in one generation, one additional year for seed increase, and the possibility to undertake screening and preliminary yield trials in the third year.

DH methods have been employed in wheat breeding programs, and new wheat cultivars of DH origin have already been released in many countries such as China, France, Hungary, Romania, and Canada (Hu et al. 1983; De Buyser et al. 1987; DePauw et al. 2011: Săulescu et al. 2012). Wheat DH populations have also been used in the creation of molecular marker maps and identifying quantitative trait loci (Chauhan and Khurana 2011; Wu et al. 2012).

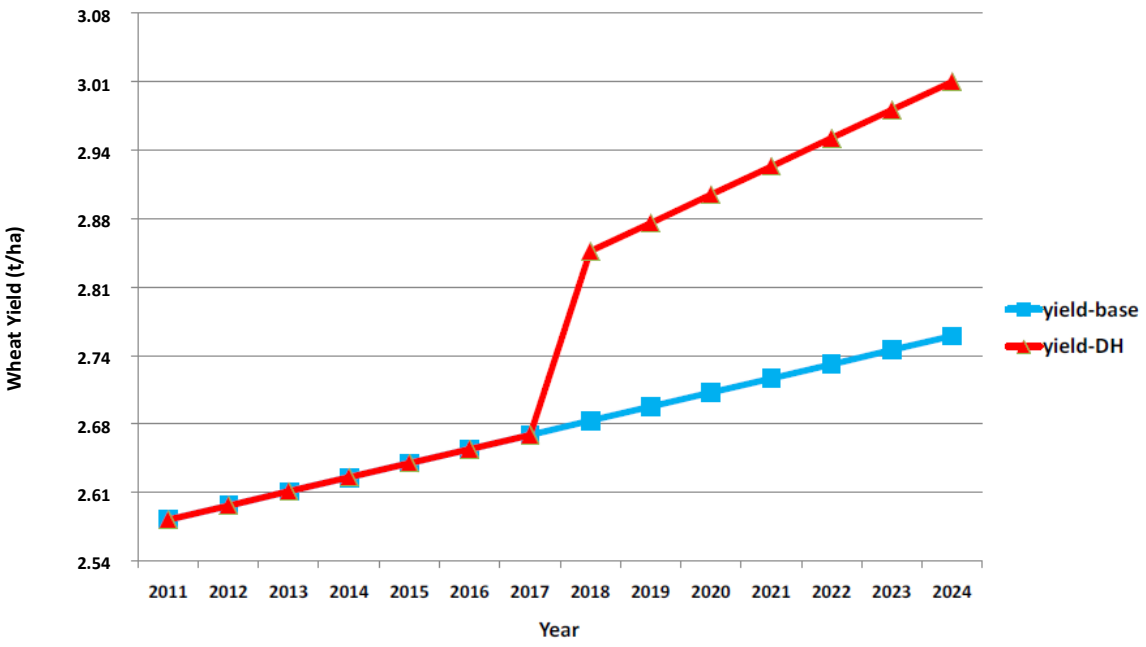

Fig. 4. Comparison of conventional and DH wheat variety development (Barkley and Chumley 2011)

Due to complete homozygosity, the efficiency of selection for both qualitative and quantitative characters is increased by using DHs since recessive alleles are fixed in one generation and directly expressed. Additionally, DHs can be used in a recurrent selection scheme in which superior DHs of one cycle represent parents for hybridization in the next cycle. Several cycles of crossing, DH production, and selection are performed and gradual improvement of lines is expected due to the alternation of recombination and selection. Barkley and Chumley (2011) demonstrated the advantages of a DH laboratory for a Kansas wheat breeding program using economic model analysis (Fig. 4) – the graph is drawn assuming that the rate of change in yield potential is 150% greater with the use of DH, relative to the baseline scenario of a conventional breeding program. If a DH laboratory were to be built in 2011, a new variety could be released seven years later in 2018 with increased yield potential and/or the same level of genetic potential as varieties released by the conventional breeding program four years later in 2022. The large discrete change in 2017 reflects the advantage due to the gain in time, and the steeper slope trend indicates the enhanced rate of genetic gain (Fig. 4).

DH systems are also the method of choice for mutant selection, due to the ease of selection and fixation of mutations and the desired recombinants, especially when quantitative traits are concerned (DePauw et al. 2011; Wu et al. 2012). Haploid technology has tremendous potential for accelerating breeding technologies when combined with Marker Assisted Selection (MAS). In combination with doubled haploidy, MAS is a time saving method of performing backcross conversion to select an elite line with a particular trait. By using molecular markers and DHs together it is possible to stack resistance genes. DH populations can be used as permanent mapping populations because they are stable and constant.

7. Conclusions

Double haploid breeding is increasingly developing into a core technology in crop improvement. The DH technology platform offers a rapid mode of truly homozygous line production that helps to expedite crop breeding programs where homogeneity is an absolutely essential parameter for rapid crop development. Integration of the haploidy technology with other available biotechnological tools such as MAS, induced mutagenesis, and transgene technologies can also effectively expedite wheat improvement programs. Direct incorporation of cloned genes at the haploid level following subsequent chromosome doubling may help accelerate stable integration of target gene(s) into wheat. Considering the cost and the fact that over-usage of doubled haploidy may reduce genetic variation in breeding germplasm, it is advisable to use DHs for very limited and highly desirable crosses in wheat breeding programs.

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