Accelerated Wheat Breeding: Doubled Haploids and Rapid Generation Advance

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Abstract New breeding objectives, evolving disease organisms, changing climate patterns and the need for quick genetic ameliorations make accelerated breeding an important aspect of wheat improvement work. The emergence and deployment of wheat x maize system of DH production over the last few decades are discussed as an important option for accelerated wheat breeding. The lack of acute genotypic specificity favours the application of this method in wheat breeding. A low-cost, high-throughput system based on detached tiller culture and pre-regeneration chromosome doubling is presented. Recent studies in wheat have focused on development of alternative accelerated breeding systems based on modulation of growth environments and compression of crop cycle duration. Multiple crop generations obtained in this manner allow homozygosity to be approached in a single year. Directed assemblage of recurrent parent background and selection in nontarget environments have been enabled by use of molecular markers and complement accelerated breeding through these means. The future prospects of accelerated breeding are enriched by recent advances in deciphering molecular genetic basis e.g. *CEN H3* and *Mtl*-1 genes of haploid induction.

Keywords Wheat · Embryo culture · Detached tiller culture · Chromosome elimination · Pre-regeneration colchicine treatment · Rapid cycle breeding

1 Introduction

The wheat-breeding strategy underlying the green revolution relied on simple genetic changes, complemented by improved management practices and higher inputs. Future genetic improvements in crop productivity may need to be made without enhanced use of fertilizer, irrigation and other inputs, in deference to

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natural resource depletion and environmental concerns. The list of target traits for breeding, particularly in relation to nutritional and quality concerns, is growing rapidly. Climate perturbations also pose an unprecedented challenge to the wheat improvement strategy. Thus there is a need for diverse and multiple genetic ameliorations, often accompanied by demanding time frames. All these add up to a tall order for a crop with a narrow genetic base, notwithstanding the earlier breeding successes. Breeding programmes in the conventional mould may not be able to measure up to these challenges. The need for an accelerated system which integrates a wide spectrum of the available and emerging molecular-genetic technologies is inevitable. Hallmarks of such a breeding programme would be:

- Working with a multitude of traits and objectives by virtue of depth of germplasm and access to fast developing gene and genomic resources
- Molecular marker tools for precise and rapid mobilization of genes to genotypes suitable for various cultivation contexts
- An information management system for allelic, haplotypic and genealogical data of the breeding programme
- Accelerated progress to homozygosity through doubled haploids (DH) or rapid generation advance

Accelerated breeding generally aims at cutting out the protracted segregating phase either through single step homozygosity as delivered by the DH systems or shortening the time for generational turnover. Selecting the desired chromosomal constitution through molecular markers provides another avenue of acceleration, particularly in context of backcrosses. All modes of acceleration strengthen the 'breeding option' in face of sudden biotic and abiotic challenges. Overall efficiency of the breeding programme also improves on account of precision of evaluation (being based on fixed, homozygous material) and exclusively additive nature of genetic variation – resulting in a much higher genetic advance. Application of such a system, however, requires considerable groundwork for development of an efficient DH technology.

2 Development of Wheat x Maize System of Doubled Haploid Production

2.1 Early Haploid Induction Systems

The first reports of haploidy in plants came from anther culture in *Datura* spp. by Guha and Maheshwari (1964, 1966). Later, Ouyang et al. (1973) attempted anther culture and obtained haploid plants in wheat for the first time. But the major constraint in this system was the poor plant regeneration in majority of wheat genotypes and a high frequency of albino plants in the haploid population. Chromosome elimination in wide crosses opened up an alternate route to haploidy in cereals when Kasha and Kao (1970) developed barley haploids from *Hordeum vulgare* x *Hordeum*

bulbosum crosses. This system was extended to wheat by Barclay (1975) who developed haploids by crossing wheat with *Hordeum bulbosum*. However, the system was compatible only with wheat genotypes carrying the crossability alleles *kr1* and *kr2* (Falk and Kasha 1981). Most of the improved germplasm relevant to breeding programmes lacked these genes.

2.2 Emergence of the Wheat x Maize System

Arrival of wheat x maize system was marked by the observation of microscopic, early stage embryos in crosses between hexaploid wheat and maize (Zenkteler and Nitzsche 1984). In this study a set of exploratory crosses between different Gramineae species including wheat, maize, pearl millet, rye, barley, etc. had been analysed for early post-pollination events. Later, Laurie and Bennett (1986) at Plant Breeding Institute (PBI), Cambridge, studied the early post-pollination events in wheat x maize crosses and demonstrated that both the wheat and maize chromosomes were present in the zygote, but the maize chromosomes were eliminated during the initial cell divisions. Another significant observation from their study was that in such crosses the endosperm was absent and resulted in embryo abortion before the embryo could develop to a rescuable size. Surprisingly, despite of the wide genetic difference between the parents, the frequency of fertilization was high (about 25%). Further, Laurie and Bennett (1987) showed that the wheat x maize system in contrast to the wheat x *H. bulbosum* system was free from the effects of non-crossability conferring alleles *Kr1* and *Kr2*. Later in 1988, Laurie and Bennett devised a system of in vitro culture of pollinated wheat spikelets and recovered the first haploid plant from wheat and maize crosses.

2.3 Crossing Technique

The idea that conventional crossing techniques used in wheat may not be suitable in case of wheat x maize crosses, because of greater genetic distance involved, led to the efforts towards modifications of these techniques. Laurie and Bennett (1988) modified the crossing techniques in such a way that one of the primary florets was cross-pollinated and the other was allowed to self-pollinate. They recovered more embryos in case of self-pollinated/cross-pollinated spikelets. It was suggested that the self-pollinated spikelets gave a feeder effect, may it be via release of a growth substance. The importance of keeping glumes intact was also emphasized by Laurie (1989). Once the potential of wheat x maize crosses for haploid breeding became evident, modifications that saved time and effort became important. Pollinations without emasculation were found to be effective as wheat ovaries become receptive 2–3 days ahead of anthesis (Matzk and Mahn 1994; Suenaga et al. 1997), but the pollinations must be timed close to date of anthesis (e.g. 1 day ahead) to have

minimum reduction in fertilization frequency. Differences in embryo formation frequency with respect to the position of the spikelet were indicated as spikelets from the middle of the spike gave higher embryo formation frequency (Martin et al. 2001).

2.4 Use of Growth Regulators

Growth regulators play an important role in caryopsis and embryo development. Gibberellic acid was used in wheat wide crosses primarily to delay endosperm abortion, but it did not seem to be useful in wheat x maize crosses (Laurie 1989). Application of growth regulators was not known to be an essential requirement for the success of wheat wide crosses. Laurie and Bennett (1988) had relied on in vitro culture of spikelets, 2 days after pollination, to ensure caryopses growth and embryo development. Thus it was unexpected when Suenaga and Nakajima (1989) demonstrated that injection of 2,4-D solution (100 ppm) in the uppermost internode was sufficient to sustain caryopsis growth and development on the plant itself for 15 days, till embryos reached a rescuable stage. Use of a growth regulator (auxin) and that too in an unconventional target tissue (hollow of the stem) turned out to be the single most important requirement for caryopsis development in case of in vivo embryo formation in wheat x maize crosses. This intervention involved two consecutive injections (0.3–0.5 ml each of 100 ppm 2,4-D), 24 and 48 h after pollination. The potential of this technique to bypass the need for cumbersome spikelet culture was quickly confirmed by studies (Inagaki and Tahir 1995). Laurie and Reymondie (1991) proposed an alternative mode of 2,4-D application by showing floret drops at relatively low concentration, to be equally effective. A single treatment of 10 ppm to the florets, one day after pollination or continuous availability of lower doses of 2,4-D (0.5–2.0 ppm) was also found to be effective for embryo formation. The 2,4-D application eased out haploid embryo formation to such an extent that it came to be perceived as a potential system for haploid breeding. The timing of 2,4-D application was also seen to be critical. The use of 2,4-D before pollination drastically reduced the embryo formation frequency (Suenaga and Nakajima 1989; Matzk and Mahn 1994). 2,4-D treatments immediately after and again 1 day after pollination increased the frequency of embryo formation to 11.8% as compared to 1% in the pre-pollination treatments. Delaying the application to 24 h or more after pollination gave higher embryo formation frequency. Many other plant growth regulators like NAA, IAA, zeatin, kinetin and ABA with various concentrations were tried, and the result from 100 ppm 2,4-D was significantly better (Suenaga 1994). Gracia-Llamas et al. (2004) proposed the application of dicamba alone or in combination with 2,4-D as a means for further improving the yield of haploid plants though other studies had not indicated its superiority over 2,4-D (Matzk and Mahn 1994; Knox et al. 2000). Use of 2,4-D thus continues to be an integral component of wheat x maize system.

2.5 Comparison of Wheat x Maize System with Other Haploid-Inducing Systems

Response in wheat to anther or microspore culture tends to be genotype specific, making this technique less suitable for breeding work though it has resulted in development of varieties like Florin in France (De Buyser et al. 1987). A few comparative studies on haploid induction systems in wheat are available. Inagaki and Tahir (1990) reported that the average embryo formation frequency was 0.2% in crosses with *H. bulbosum* as compared to 9.5% in crosses with maize. Kisana et al. (1993) found wheat x maize system to be better than anther culture in terms of efficiency. Another comparison with anther culture was made by Sadasivaiah et al. (1999). They concluded that wheat x maize system was better in terms of lower genotype specificity, absence of albinism and ease of application.

2.6 Alternative Pollinator Species for Induction of Wheat Haploids

Apart from maize, pearl millet (Laurie and O' Donoughue 1989) and sorghum (Ohkawa et al. 1992) have been found to be effective pollinators for inducing haploidy in wheat. Teosinte was found to be a better pollinator than maize, in a study by Ushiyama et al. (1991), when they evaluated 39 maize and teosinte genotypes. Matzk and Mahn (1994) reported higher frequency of embryo formation using pearl millet (27%) as compared to maize (22%), while Inagaki and Mujeeb-Kazi (1995) had compared maize, pearl millet and sorghum as potential pollinators and found maize to be better than others. Dusautoir et al. (1995) crossed various durum wheat varieties with maize and teosinte and found that teosinte pollen was more effective for inducing haploids. Li et al. (1996), while using tetraploid *Tripsacum dactyloides* as pollinator, obtained high frequency of fertilization and embryo formation in crosses with hexaploid wheat as female. An alternative pollinator species *Coix lacryma-jobi* (Job's tears) was identified by Mochida and Tsujimoto (2001). A perennial wild grass *Imperata cylindrica* was shown by Chaudhary et al. (2005) to have a haploid induction efficiency comparable to that of wheat x maize crosses. Wheat x maize system proved to be a ready template for uncovering several similar cross combinations offering the potential of haploid induction through chromosome elimination in wheat. The complete elimination of one of the parental genomes may not be very uncommon; it has been documented in 74 hybrids involving monocotyledonous species and 35 involving dicotyledonous species (Ishii et al. 2016). The choice of haploid-inducing pollinator in wheat however continues to be maize in most cases, primarily due to ease of access and abundance of pollen production besides absence of a major or qualitative advantage, if any, offered by alternative pollinator species.

2.7 Quantitative Variation in Haploid Induction Response of Maize and Wheat Lines

Suenaga (1994) crossed maize with 47 wheat varieties and observed a wide range for caryopsis formation and embryo development (33.3–99.1% and 2.2–50.0%, respectively). Similar results were obtained by Amrani et al. (1993) for tetraploid wheat. On the other hand Matzk and Mahn (1994) found little difference for embryo formation between wheat lines, when they used crosses between eight wheat and nine maize lines. Similarly significant differences for embryo formation and plant regeneration were not observed by Zhang et al. (1996). Laurie and Reymondie (1991) found differences for embryo formation between spring and winter wheat lines but not within the two sets. The genotypic differences, by and large, are not acute enough to prevent use of the system for target wheat genotypes. Efficacy of maize genotypes as pollinators was also investigated (Suenaga and Nakajima 1989; Zhang et al. 1996), and significant differences for embryo formation were observed. Differences in haploid embryo formation and regeneration frequencies on account of maize genotype can be exploited to improve output of the system by identification of superior pollinators. Superior maize genotypes reported by some of the early studies included CM75 (Suenaga 1994), ZML (Matzk and Mahn 1994) and Pearl Popcorn (Verma et al. 1999). In absence of information on genotypic responses, collection of pollen from a set of maize lines or populations may be a practical strategy (Inagaki and Tahir 1995; Lefebvre and Devaux 1996; Mangat 2000).

2.8 Detached Tiller Culture

The wheat ear can be detached from the plant with a sharp, often underwater cut below first or second node and maintained for some days by placing the cut end in a solution carrying sucrose and basal salts. Grain development in ears cultured in this manner at around anthesis stage and under appropriate conditions is seen to progress well. The detached tiller culture system was initially developed to study nutrient translocation and seed development physiology in wheat spikes (Graham and Morton 1963; Donovan and Lee 1977; Singh and Jenner 1983). The culture solution in these studies was generally maintained at low temperature or under aseptic conditions through specially designed or improvised equipment to avoid microbial contamination of the nutrient-rich medium. Kato and Hiyashi (1985) attempted to forego sterilization and simplify the tiller culture system, but seed development was very poor due to contamination of the sucrose-containing medium. A remarkable modification involved the use of sulphurous acid to suppress contamination in the culture solution and culm decay as first introduced by Kato et al. (1990). Sulphurous acid (H_2SO_3) is a weak inorganic acid, which is considered an aqueous solution of sulfur dioxide in water. Kato et al. (1990) successfully cultured selfed wheat ears at room temperature on liquid medium containing 100 g/l sucrose and

0.075% sulphurous acid. The study aimed at conferring cold treatment to growing caryopsis on detached tillers, as a substitute to seedling vernalization. The relevance of detached tiller culture system for wheat x maize crosses was first indicated by Riera-Lizarazu and Mujeeb-Kazi (1990). Ushiyama et al. (1991) used detached spike culture system in wheat x teosinte and wheat x maize crosses and recommended MS-based medium containing 100 mg/l 2,4-D, 10 ml/l ethanol, 8 ml/l sulphurous acid and 40 g/l sucrose for tiller culture. Riera-Lizarazu et al. (1992) used detached tiller culture in triticale x maize crosses, while Inagaki (1997) applied it in studies on selection of efficient pollen donor for double haploid production and reported positive effect on embryo formation and plant regeneration frequency. Detached tiller culture system was also shown to be more efficient than the conventional on plant alternative by Cherkaoui et al. (2000). The tiller culture medium containing 100 mg/l 2,4-D, 40 g/l sucrose, 10 mg/l silver nitrate, 8 ml/l sulphurous acid and 3 g/l calcium phosphate was recommended by Jian et al. (2008), who obtained caryopsis formation in 95% of pollinated florets and an embryo formation frequency of about 30%. MS-based medium containing 40 g/L sucrose, 100 mg/L 2,4-D and 8 ml/L sulphurous acid was recommended by several studies (Inagaki and Hash 1998; Hussain et al. 2012). Detached tiller culture is primarily of interest to researchers working with field-grown wheat plants, whose tillers could be detached post-pollination and brought indoors to obtain embryo development under optimal environmental conditions.

2.9 Embryo Rescue Media and Culturing Techniques

The double fertilization norm is rarely fulfilled in fertilization of wheat with maize pollen, and embryo formation is mostly not accompanied by the endosperm development. The post-pollination caryopsis growth, unless supported by 2,4-D application or in vitro culture, is imperceptible, and the embryo degenerates. If the recommended growth regulator-based interventions are followed, a proportion (25–30%) of the well-developed caryopsis carry irregularly shaped embryos, floating in a watery fluid. About 15 days after pollination, these need to be rescued aseptically on artificial medium. Early removal of caryopses from spikes results in low number and small size of embryo. Delayed culture on the other hand adversely affects the regeneration potential of embryos. Ten- to eleven-day-old embryos gave higher regeneration percentage (78.3%, Suenaga 1994), while Kammholz et al. (1996) found 12–15-day-old embryos having better regeneration. Younger and older embryos gave a much lower percentage of regeneration. Fifteen days are optimum for developing a rescuable sized embryo without compromising its regeneration potential. Another important factor that is expected to impact regeneration is embryo rescue media. The common synthetic nutrient medium includes half-strength MS (Murashige and Skoog 1962) or B5 (Gamborg et al. 1968), and both have been extensively used for culture of wheat embryos from wheat x maize crosses (Suenaga and Nakajima 1989; Comeau et al. 1992; Kammholz et al. 1996; Cherkaoui et al.

2000; Dogramaci-Altuntepe and Jauhar 2001; Sourour et al. 2012; Niu et al. 2014). Medium-solidifying agents were studied by Morshedi and Darvey (1997) who identified Gelcarin GP812 as significantly better than other gelling agent like agar, agarose, wheat starch, etc. At present, agar or synthetic gelling agents like gelrite are employed without major implications for regeneration. Nurse culture, common with microspore culture protocols, was studied by Niu et al. (2014) where excised embryos were placed on a 20-day-old seed endosperm tissue and then cultured on the MS medium. This method helped small-sized embryos but is laborious and time-consuming compared with the regular embryo rescue method. Use of additional media components like hormones, vitamins, amino acids, etc. to enhance embryo regeneration frequency finds few mentions (Kammholz et al. 1996; Sourour et al. 2012; Niu et al. 2014). Various modifications have been studied by adding different organic supplements (Zenkteler and Nitzsche 1984; Zhang et al. 1996; Suenaga et al. 1997; Campbell et al. 2000; Singh et al. 2004; Ayed et al. 2011), but none seems to be a critical requirement. This is unlike wheat anther culture and isolated microspore culture protocols which require very specific medium composition besides pretreatment conditions. Such stringent conditions with respect to culture conditions have not been worked out for wheat x maize system. Embryo development conditions prior to rescue, age and size of embryo seem to be more important, and several basic media are known to support adequate regeneration.

2.10 Chromosome Doubling

Haploid plants derived from wheat x maize show almost no spontaneous chromosome doubling (Sadasivaiah et al. 1999). Chromosome doubling can be achieved through various compounds of which colchicine is the most widely used (Subrahmanyam and Kasha 1973; Hassawi and Liang 1991; Ouyang et al. 1994; Soriano et al. 2007). In wheat x maize crosses, colchicine treatment is normally given to haploid seedlings at 3–4 tiller stage for 5–8 h by submerging the whole root system in colchicine solution followed by washing with water and reestablishment of seedlings. Chromosome doubling efficacy depends upon colchicine dose and duration of treatment. Use of colchicine solution having concentration of 0.1% for 5 h (Inagaki 1997; Thiebaut et al. 1979), 0.2% for 5 h (Sadasivaiah et al. 2001; Maluszynski 2003) and 0.45% colchicine for 6–8 h (Niu et al. 2014) has been found effective for doubling with some differences. Higher doses are effective in chromosome doubling but may result in deformed plants, low survival rate and increased cost, while lower doses require prolonged exposure and may not result in doubling. The standard post-regeneration chromosome doubling treatment leads to formation of chimeric plants. These show partial seed production, and therefore, an additional growth cycle for seed multiplication before evaluation in the field is required (Chen et al. 1994; Islam 2010). A chromosome doubling technique integrated into the haploid induction procedures given prior to regeneration may be more effective. In this context, colchicine when applied to embryo rescue media reduced the rate of

embryo germination (Niu et al. 2014). In addition to colchicine, alternate doubling agents have also been studied for chromosome doubling in wheat like use of nitrous oxide (Hansen et al. 1998), caffeine (Thomas et al. 1997) and herbicides trifluralin, oryzalin, etc.(Dhooghe et al. 2011).

3 Application of Wheat x Maize System in Wheat-Breeding Programmes

The wheat x maize approach of doubled haploid production is finding use in several wheat-breeding programmes worldwide. The technique is an integral part of the wheat-breeding methodology at Australian Grain Technology Pty. Ltd., the largest wheat-breeding company in Australia (Kuchel et al. 2005). Longreach Plant Breeder, another wheat-breeding company in Australia, also employs doubled haploid breeding, wheat variety Longreach Reliant, being a DH product released in 2016. Public institutes in Australia, e.g. Plant Breeding Institute, Sydney, South Australian Research and Development Institute and Department of Agriculture and Food, Western Australia, have also employed this technique. In Japan, wheat cultivar Sanukioyume-2000 is one of the cultivars developed by using the wheat x maize system (Yuichi et al. 2002). In the USA, wheat variety Bond CL was developed by Colorado State University using wheat x maize crosses and released in 2004 (Haley et al. 2006). Oklahoma State University released Gallagher, a DH, in 2012 (Carver 2016). Elite crosses in the OSU wheat programme are processed with DH technology, often contributing about one fourth of the lines for advanced trials. Kentucky Small Grain Growers Association approved wheat DH lines BW 965 and BW 966 in 2015. CIMMYT, Mexico, worked towards refining the DH production protocol and used it to generate populations for gene mapping and genetic analysis but did not make it a part of the breeding programme as advantages were not perceived over the long-standing shuttle breeding system, involving two seasons of selection in large populations per year (Huihui et al. 2013). In Canada, the first wheat DH variety, McKenzie, was developed through anther culture and released in 1997. In the next about 12 years, 27 wheat DH cultivars were released in Canada mostly developed through wheat x maize crosses at wheat-breeding centres of Agriculture and Agri-food Canada and Universities of Saskatchewan and Manitoba. In 2009 DH cultivar Lillian, developed using marker-assisted selection to improve protein content with gene *Gpc-B1*/*Yr36*, occupied the largest area under any single variety in Canada (De Pauw et al. 2010); in 2010, DT801, first DH variety of durum wheat, was released in Canada. In India the wheat x maize system is being applied to selected crosses from wheat-breeding programmes at Punjab Agricultural University, Ludhiana (Singh et al. 2012), and at Himachal Pradesh Agricultural University, Palampur. India's first DH wheat cultivar, Him Pratham, released in 2013, has been developed at Palampur through wheat x *Imperata cylindrica* crosses (Chaudhary et al. 2014). Some of the wheat DH laboratories work in the service mode as well. Washington

State University, Pullman, produces wheat DH plants for $15 for on-campus and $25 for off-campus breeders (www.css.wsu.edu/facilities/dhlab/). One of the big service providers in wheat DH, Heartland Plant Innovations Inc. (HPI) in Kansas, USA, produces up to 50,000 DH plants per year and charges partner and commercial breeders $35 and $50 per plant, respectively (www.heartlandinnovations.com/our-programs/doubled-haploid-production).

4 Adapting the Wheat x Maize System to a Wheat-Breeding Programme

Having discussed above the studies which led to development of wheat x maize system as a potential wheat-breeding tool, we describe research aimed at adapting this system to needs of a wheat-breeding programme, located in a subtropical clime with extremes of winter and summer and modest controlled plant growth facilities. This programme at Punjab Agricultural University, Ludhiana, caters to the state of Punjab in particular and north-western plain zones in general, the largest and most productive wheat zone of India. The wheat breeding in the region is challenged by fast-evolving stripe rust races, abiotic stresses imposed by rising temperature and other climatic changes, urgent need for improving nutrient and water use efficiencies and taking up of nutritional and processing quality as hard core breeding objectives. The region is the mainstay of national food security, and acceleration in breeding outcomes can be highly rewarding. First demonstrations of wheat x maize system in India came from work at PAU, Ludhiana (Bains et al. 1995; Dhaliwal et al. 1995). The crosses were effective between field-grown wheat and maize plants raised in the polyhouse for a short (2–3 weeks), optimal environmental span in the main season. The window for crossing work needed to be expanded for DH work. The haploid plants generated in the main season had to be shifted to off-season location where chromosome doubling was not efficient because of long days and other conditions not conducive to tillering. Shifting wheat x maize crossing work to off-season location, solved the chromosome doubling issue, as plants were brought to main location and doubled during short day, winter conditions. The primary success of the wheat x maize crosses in terms of embryo formation frequency, however, was low (~10%) as night temperature at off-season location tended to fall below the required threshold of 15 °C. These major bottlenecks and several other issues were resolved within the framework of infrastructural capacity and environmental constraints. In fact, application and modification of the system went hand in hand leading to the current protocol development as discussed below.

Unlike conventional wheat crosses, wheat x maize crosses involve keeping the glumes intact (Laurie and Bennet 1988) to support better caryopsis growth and embryo development. This emasculation technique is skill and labour intensive. The pollination of spikes with intact glumes involves opening of individual florets for dispensing the pollen, generally requiring a team of two workers. Possibility of

employing a chemical hybridizing agent (CHAs) being used in hybrid wheat research for facilitating wheat x maize crosses was explored (Sandhu et al. 2002) and found to drastically lower haploid formation frequency, probably due to phytotoxic effects of CHA. Under an alternate strategy aimed at striking a favourable trade-off between caryopsis development and labour saving, unemasculated- clipped and emasculated-clipped florets were seen to be at par with standard (glumes intact) method in terms of haploid formation and plant regeneration (Sandhu et al. 2003). Application of this methodology on larger set in subsequent work, however, revealed that results may vary widely as ear health and other conditions have to be optimal for this strategy to work. We reverted back to use of standard method. Unemasculated florets with glumes intact could be useful to hasten up crossing work in the field, but pollinations have to be timed very carefully to 1 or 2 days prior to anthesis. Selfed caryopsis, which is expected in some proportion in unemasculated ears pollinated with maize, proved to be detrimental by competing for nutrition and starving out the crossed caryopsis. This observation is in contrast to the benefits reported for maintaining selfed florets in one part of the ear (Laurie and Bennet 1988). Later, when our DH protocol shifted to detached tiller culture system, it excluded the use of unemasculated spikes altogether, as the very process of detachment triggers anthesis resulting in high proportion of selfing. It was also observed that clipped florets were less suitable for detached tiller culture-based system as caryopsis health tended to fall below desirable level for regeneration ability. Thus different components of the protocol have to be compatible, and more instances of this requirement will come up in subsequent paragraphs. One emasculation shortcut which may be compatible with detached tiller culture approach is hot water dip as spikes with intact glumes are used and detached spikes can be very conveniently administered the hot water dip. Dipping of detached ears packed loosely in water-tight polythene bags in a water bath held at 43 °C for 3 min (Singh 2016) is partly successful and is being refined further to greatly enhance the throughput of the system. With respect to growth regulators, use of auxins like picloram and dicamba were also studied (Puja 2007, Kansal 2011), but use of 2,4-D was found to be optimal and cost-effective for embryo frequency and plant regeneration. Initially Pearl Popcorn, an open-pollinated maize cultivar released by PAU, Ludhiana, was picked up by us as pollinator (Verma et al. 1999) as it was found to confer comparatively high embryo formation and also high regeneration frequency, an unexpected outcome for a pollinator. Practical considerations that emerged over time made us to look for pollinators which flowered early and were thermo and photoperiod insensitive with respect to flowering time, so as to achieve synchronizations more predictably for maize plants grown in polyhouse or open field. A tassel which is resilient to environmental stress (cold in our case) in terms of tassel and floret size as well as pollen production is the second important requirement in this regard. Over the last few years, a local maize population adapted to Lahaul valley in the Himalayas (Himachal Pradesh, India) has met these requirements well for wheat x maize crosses at main as well as off-season location (also in Lahaul valley). It maintains a time to flowering of 35–40 days in various environmental conditions. Resource partitioning in the plant favours good tassel development even under stress.

In wheat x maize crosses, caryopsis development results from 2,4-D application, irrespective of fertilization by maize pollen. Thus while caryopsis development is found in majority of the florets treated with 2,4-D, embryo formation can vary greatly (0–40%), particularly for crosses performed under variable or suboptimal conditions. The caryopses which carry an embryo are apparently indistinguishable from others. At low embryo formation frequencies, embryo rescue thus becomes highly labour intensive. A method to identify and sort out embryo carrying caryopsis can be useful in this regard; Lefebvre and Devaux (1996) attempted to identify embryo carrying caryopsis prior to dissection using X-ray radiography, but they were not successful. Bains et al. (1998) devised a simple method of screening a 15-day-old, harvested caryopsis in a glass petri dish against light incident from above, which makes the floating embryos settled at bottom of the caryopsis visible as dark spots. Using this technique, 98% of caryopsis with embryo could be detected prior to dissection. For chromosome doubling, the standard colchicine treatment is targeted to crown region of well-tillered, 3–5-week-old wheat plants, after uprooting them from soil. The treated seedlings need to be planted back in soil under tiller promoting conditions. The off-season conditions, to which haploids produced in main season are shifted, are essentially long day and favour flowering rather than tillering. For haploids produced in off-season location, better conditions prevail, but it is a relatively small period in the main season which is conducive. Further, the standard colchicine treatment is low throughput and was originally devised for wide hybrids in wheat, which are typically few in number. Responding to the local and general need for alternate chromosome doubling technique, two strategies aimed at doubling the chromosome number of haploid embryos prior to plant regeneration were pursued (Sood et al. 2003). In the first approach the haploid embryos were rescued on medium containing colchicine (at concentrations of 0.2%, 0.3%, 0.4% and 0.5%) and moved to a colchicine-free regeneration medium 48 h later. Embryos exposed to 0.5% colchicine had 91.67% of their regenerated plants showing chromosome doubling. In the second approach based on tiller injections, different concentrations (0.5%, 0.75% and 1.0%) of colchicine solution, which also contained 2,4-D (100 ppm), were injected into the uppermost internode of crossed tillers 48 and 72 h after pollination. The chromosome doubling efficiency was high for 1% treatment which became the most attractive alternative. The treatment targeted early zygotic divisions. Chimeras of doubled/haploid sectors were generally not observed in the case of the tiller injection treatment, and most of the florets showed seed set in the doubled plants. In absence of chimeras, stomatal guard cell length provided rapid, early-stage analysis of ploidy level. This approach found favour with our team for field-based crosses, though use of high colchicine concentration (1%) involved greater expense. The caryopsis were rounded (less plump) and opaque, which served as an indicator of an effective treatment but prevented the application of pre-dissection identification of embryo carrying caryopsis. The shift to detached tiller culture-based system for reasons discussed below, however, excluded the tiller injection of colchicine, as unlike whole plants, cultured tillers did not tolerate the 1% injections well and lower doses were less effective. The colchicine treatment compatible with detached tiller culture consists of two applications of 0.2%

colchicine (prepared in 100 ppm 2,4-D), administered as drops inside the pollinated florets, 24 and 48 h after pollination (unpublished results). While exploring APM and trifluralin as replacements of colchicine, we found that APM at low concentration (50 μM) enhanced the embryo formation, whereas trifluralin promoted plant regeneration. In context of chromosome doubling, initial results have shown that 150 μM of APM and 350 μM of trifluralin gave doubling in about 75% of the plants when applied as drops to florets as explained for colchicine above (Singh 2014).

Staggered planting and low-cost interventions like polyhouses make wheat spikes and maize tassels available for several months during both main and off-season, virtually covering the whole year. However, fertilization-promoting conditions for wheat x maize crosses are prevalent for a small part of the year. The detached tiller culture system thus widens the crossing window to several months by easily providing optimal conditions in relatively small controlled environment facility. We extended the tiller culture system to include indoor pollinations as well at a raised temperature (25–27 °C). Besides consistent and improved embryo formation frequency, the most laborious step, i.e. pollinations, could be conducted around the clock. The dehiscing maize tassels are also detached and maintained in culture to provide fresh pollen, which is critical for high embryo formation. The system augers well for the breeding objectives as it allow a wide and flexible choice of plant material from the field for inducing the doubled haploids. The administration of colchicine to the florets can be done more safely, precisely and effectively under detached tiller culture system. The system makes very efficient use of space as 10–20 times more wheat spikes being used for haploid induction can be accommodated as compared to potted plants. Batch handling for various steps in the protocol is facilitated and promotes high throughput. In our early work, however, issues of spike bleaching, inadequate embryo size and regeneration potential were faced in the tiller culture system. Bleaching was typically noticed in small reach-in growth chambers, obviously due to accumulation of sulphurous fumes. Larger chambers with ventilation and improved internal air movement were found to restore ear health. The problem of embryo size/regeneration was sought to be solved by extending the culture phase. This tended to improve embryo size, but regeneration could not be improved as ageing sets in. A solution was found in the form of cold treatment to ears completing the tiller culture phase. For this purpose, compact bunches of detached tillers with culm ends dipped in water are kept at 4 °C in refrigerator for 3–5 days. The regeneration frequency improves to a tune of 60–70%, and unexpectedly the embryo recovery shows an improvement of about 40–50%. The cold treatment can be extended in interest of phasing the embryo rescue work. Several embryo rescue media compositions were investigated over the years. These include use of casein (Kaur 2004), activated charcoal in addition to casein (Puja 2007), amino acids (Bains et al. 2009) and polyamines (Goyal 2016). A major impact of tissue culture constituents and conditions was not observed on haploid production parameters. Thus, the embryo size and developmental stage are primary determinants of regeneration ability and basic media composition suffices.

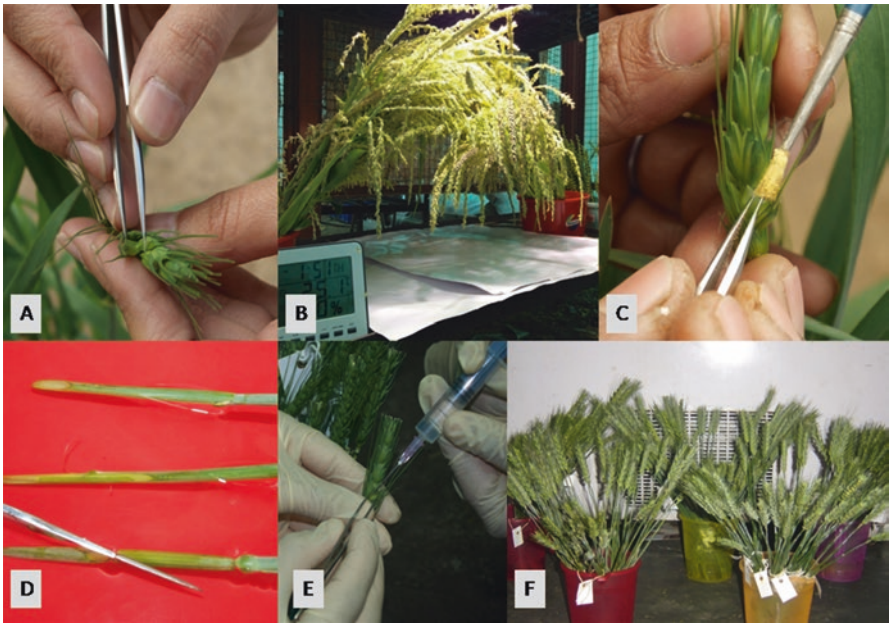

Fig. 1 Steps in wheat x maize system. (**a**) Emasculation of wheat ears without removing the glumes. (**b**) Collection of maize pollen after giving artificial light to maize tassels. (**c**) Pollination of emasculated ears with maize with the help of paint brush. (**d**) Underwater cut given to tillers prior to detached tiller culture. (**e**) 2,4-D drops being given to florets 24 h after pollination. Pre-regeneration colchicine, if given, is added to 2,4-D solution. (**f**) Tillers being maintained under detached tiller culture

Aspects of experimentation and observation above were aimed at adapting the DH production to local requirements. It has resulted in a low-cost, high-throughput protocol as given below, which can be useful in several situations:

1. Emasculation of wheat ears on field-grown plants with removal of central florets and keeping glumes of retained florets intact (Fig. 1).
2. Detachment of wheat tillers 2–3 days after emasculation, followed immediately by underwater cut with sharp scalpel on the stems, retaining uppermost node. The cut tillers are kept with ends dipped in water in small open-top buckets (each containing 100–200 ears) for about the next 24 h.
3. Pollination of detached ears maintained in water with freshly collected maize pollen from detached tassels in the same chamber under controlled conditions (25 °C, 70% relative humidity).
4. Colchicine (0.2%) + 2,4-D (100 ppm) + DMSO (2%) solution administered as drops to inside of florets 24 h after pollination.
5. Shifting of detached tillers to culture medium (1/2 MS + 40gm sucrose/L + 0.8% H_2SO_3).
6. Second dose of colchicine (0.2%) + 2,4-D (100 ppm) + DMSO (2%) to florets, 48 h after pollination.

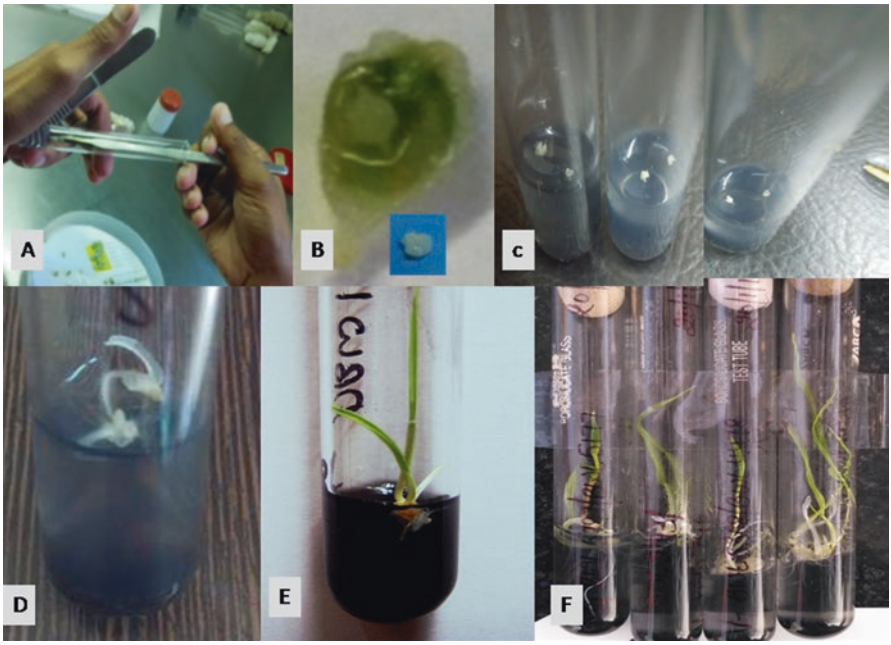

Fig. 2 Steps in wheat x maize system continued. (**a**) Embryo rescue being carried out. (**b**) Dissected caryopses showing free floating embryo without endosperm. (**c**) Embryos being kept on artificial medium for regeneration to plants. (**d**) Initiation of regeneration; plants are shifted to 16 h light and 8 h dark after this stage. (**e**) Regenerated green haploid plant. (**f**) Regenerated haploids ready to be transferred to soil for hardening

7. Detached tillers maintained at approximately 18–22 °C, 16 h light/day, 70–75% relative humidity and good air circulation in a spacious chamber (e.g. walk-in rather than reach-in) for 15 days after pollination.
8. Shifting of detached tillers in compact bunches with ends dipped in water to 4 °C for 3–5 days in refrigerator.
9. Embryo rescue under aseptic conditions on solid media (½ MS+ 20gm sucrose+1 ppm kinetin+100 mg myoinositol+2.6 gm Gelrite+2gm activated charcoal powder) (Fig. 2).
10. Cultures kept in dark till shoot emergence and shifted to 8 h light/16 h dark.
11. Regenerated plants transferred to vermiculite for hardening (15 days) and shifted to soil till maturity and seed set (Figs. 3 and 4).

The optimal doubled haploid efficacy parameters based on the above protocol, viz. caryopses formation frequency (>80%), embryo formation frequency (~40%), plant regeneration frequency (> 60%) and chromosome doubling frequency (~80%), jointly confer doubled haploid production of about 15 DH plants per 100 florets.

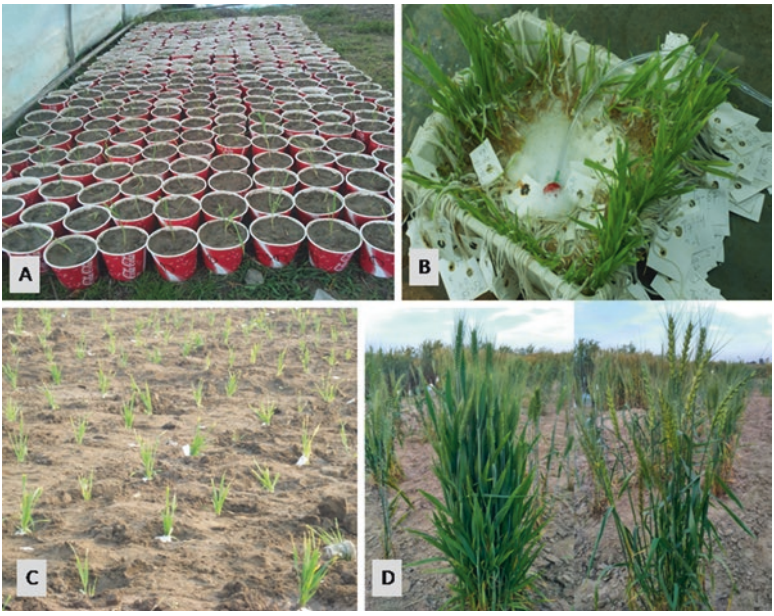

Fig. 3 Steps in wheat x maize system continued. (**a**) Regenerated haploids being grown in medium of soil+ vermicompost for hardening. (**b**) Colchicine treatment being given to haploid seedlings (in case where pre-regeneration colchicine was not given). The colchicine solution is kept aerated with the use of air bubblers. (**c**) Post colchicine treatment or post-hardening (pre-regeneration colchicine was applied), the seedlings are shifted to field under tiller promoting conditions. (**d**) Mature plant having seed set

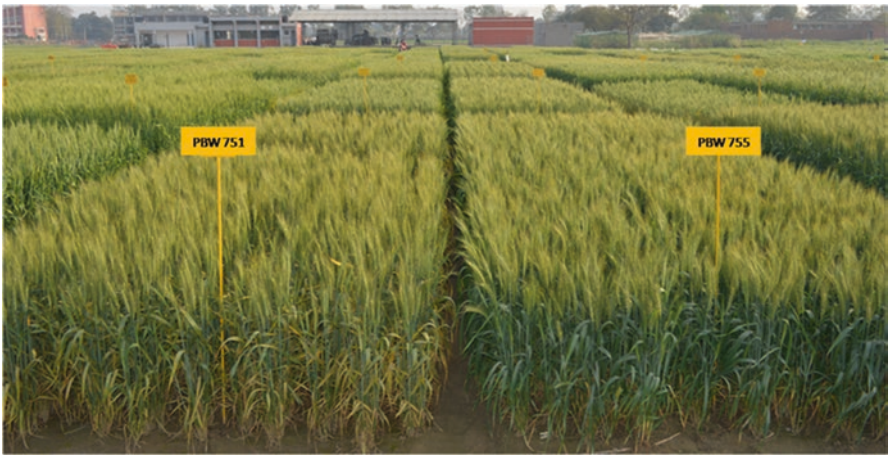

Fig. 4 Evaluation of DH lines in trials at Punjab Agricultural University, Ludhiana, India

5 Prospects of Developing Same Species Haploid Inducers

Chromosome elimination in interspecific crosses is seen as an established route to haploidy for crop improvement (Kasha and Kao 1970; Laurie et al. 1990; Forster et al. 2007; Wezdzony et al. 2009). Uniparental chromosome elimination can occur due to difference in the timing of mitosis, asynchrony in nucleoprotein synthesis, formation of multipolar spindles, and a spatial separation between genomes at interphase and/or at metaphase. The complete elimination of one of the parental genomes is not uncommon. It has been documented in 74 wide hybrids involving monocotyledonous species and 35 involving dicotyledonous species (Ishii et al. 2016). Haploid production by intraspecific hybridization is less frequent (Chang and Coe 2009; Geiger 2009), and one of the first reports came in 1959 with the discovery of maize haploid inducer Stock 6, producing 2–3% maternal haploids when outcrossed as a male. Over the years, improved inducer lines such as WS14, RWS, UH400, BHI306 and CAU5 with haploid induction rates (HIR) of 8–10% were developed. Combined with a haploid identification system based on a dominant scutellum and aleurone pigmentation gene *R1-nj*, the current levels of haploid induction have been successfully adapted for commercial DH production. In recent years this has fuelled dramatic increase in inbred line development in hybrid maize programmes. The advantage of intraspecific inducers is that the haploid production is an in vivo system with no requirement for the throughput lowering embryo rescue step. The important question for future developments in case of a naturally available intraspecies haploid inducer is the genetic basis and transferability of the haploid induction trait to another target species. Molecular genetic advances have narrowed down to underlying basis of chromosome elimination in interspecies crosses (most likely centromere-related genes) as well as intraspecies crosses (e.g. *Mtl1* gene in maize haploid inducers) as described briefly below.

The path-breaking report for development of haploid inducers (through CENH3 manipulations in *Arabidopsis*) was published by Ravi and Chen (2010). Soon after, CENH3 was also implicated in chromosome elimination in bulbosum system (Sanie et al. 2011). Distant CENH3s (e.g. *Zea mays*) were seen to complement the *Arabidopsis* null mutants but cause haploid induction (HI) in crosses (Maheshwari et al. 2015) on account of a divergent CENH3 N-terminal tail. Kuppu et al. (2015) showed that point mutations including preexisting ones in 'histone-folding domain' could confer HI in *Arabidopsis*. In crop species, CENH3 modifications were seen to generate HI trait in maize (Kelliher et al. 2016). Britt and Kuppu (2016) called CENH3 an emerging player in haploid induction technology and strongly recommended non-transgenic approaches involving alien gene introgression and mutagenesis for creating haploid inducers.

The genetic basis of haploid induction behaviour in the commercially successful Stock 6-based maize system has been recently reported by three independent research teams within the space of 1 month (Gilles et al. 2017; Kelliher et al. 2017; Liu et al. 2017). It was shown that a 4 bp frameshift mutation in a pollen-specific phospholipase gene is responsible for haploid induction. RNAi, TALENS, and

CRISPR-Cas suggest of a loss of function in this gene (named *MATRILINEAL1* or *NOT LIKE DAD*) as the trigger for haploid induction behaviour. Wheat sequences showing partial homology to this gene have been identified in the wheat genome (Kelliher et al. 2017). These studies represent major recent breakthroughs that chart a feasible path to haploid induction, and newer gene targets are expected to emerge shortly.

6 Rapid Generation Advancement

CIMMYT's long-standing shuttle breeding system between Obregon and Toluca sites within Mexico is well known, whereas wheat breeders in England and Canada often move plant material to New Zealand for generation advancement. Wheat-breeding programmes in India rely on high-altitude locations such as Lahaul-Spiti (Himachal Pradesh) and Wellington (Tamil Nadu) for obtaining acceleration of breeding cycle by taking two generations per year. Enhancing the number of seed to seed cycles beyond two requires special techniques and controlled conditions. Preliminary experiments showed that 6–8 crop cycles can be achieved within a calendar year. Recently such methods have been referred to as 'faster generation cycling system' (FGCS, Yan et al. 2017) or rapid cycle breeding (RCB) or simply rapid generation advancement (RGA) by different workers (Wang et al. 1999, 2003; Ochatt et al. 2002; Zheng et al. 2013; Forster et al. 2014; Liu et al. 2016; Yao et al. 2016, 2017). Seed to seed crop duration is shortened by culturing young embryos and managing plants (through induced stress) to greatly reduce the time for flowering and seed maturity. FGCS involves two steps: firstly, plants are grown in a controlled environment where specific irrigation and nutrient management practices accelerate the vegetative growth and flower differentiation. Secondly, culturing of young or immature embryos is taken up for reducing the time required for seed maturation (Wang et al. 1999, 2003) or even to growing caryopsis through detached tiller culture, prior to embryo rescue. Immature embryo culture is advantageous, as when seed matures inhibitors to germination or promoters of dormancy set in (Chawla 2002) and seed dormancy in wheat develops before the hard dough stage (Lan et al. 2005). For rapid generation advancement in winter wheats, vernalization treatment may be given to germinated embryos before transplanting them into soil (Wang et al. 1999, 2003). Rapid generation advance system application revealed that only older embryos (more than 20 days post-anthesis) required vernalization to initiate flowering, whereas plants generated from younger embryos (15 dpa) flowered without vernalization (Qin and Wang 2002). In addition, in vitro protocol for faster generation cycle has also been reported for wheat (Yao et al. 2016).

Successful development of rapid generation advance systems have been reported in several major crops, which significantly shortened the generation time and enabled 6–9 generations per year, viz. barley and wheat (Zheng et al. 2013; Yao et al. 2017); maize (Pioneer 2008); oat and triticale (Liu et al. 2016); *Brassica* spp. (Yao et al. 2016) and legumes (Ochatt and Sangwan 2010; Ribalta et al. 2014). The

key advantage of rapid generation advance over DH lies in the greater opportunity for genetic recombination through multiple segregating generations. Also, selection can be incorporated in any generation, and NILs or RILs can be developed (Yan et al. 2017). Zheng et al. (2013) demonstrated a rapid generation advance system based on immature embryo culture and raising of miniaturized plants under controlled conditions. Plates containing the newly cultured embryos were kept at 20–22 °C constant temperature without any extra lighting. When the cultured embryos started to germinate (which took between 24 and 72 h), the plates were transferred into an incubator with 16 h lighting (fluorescent lamps) and 25 °C day/22 °C night temperatures. When the coleoptiles of the young seedlings reached about 1.5–2.0 cm in length, the young seedlings were transferred into trays having potting mix. Seed to seed cycle was shortened by providing water stress during plant growth. Seedlings were watered only when wilt symptoms appeared. This protocol offers the potential of producing up to eight generations of wheat and nine generations of barley per annum. A speed breeding protocol which does not involve embryo culture has been established as a result of an international collaborative study (Watson et al. 2018) and uses extended photoperiod with specific light spectrum and harvesting of immature caryopses. The protocol gives six generation of wheat in a year as demonstrated for spring bread wheat, durum wheat, barley and the model grass *Brachypodium distachyon*. These species were grown under controlled environment room with extended photoperiod (22 h light/2 h dark). Plants grown under speed breeding progressed to anthesis (flowering) in approximately half the time of those from glasshouse conditions. Depending on the cultivar or accession, anthesis was reached in 37–39 days (wheat – with the exception of Chinese Spring) and 37–38 days (barley), while it took 26 days to reach heading in *B. distachyon*. While in the same duration, same species grown in glasshouses with no supplementary light or heating reached the early stem-elongation growth stage or three-leaf stage, respectively. In wheat, grains per spike decreased to some extent in the speed breeding chamber as compared to the glasshouse with no supplementary light, but both wheat and barley plants produced a healthy number of spikes per plant, despite the rapid growth. Viability of mature seeds was unaffected by speed breeding with similar seed germination rates observed for all species. The method can be used for carrying out wheat x wheat crossing and backcrossing and phenotyping for different disease as well. In Australia, DS Faraday is the first variety to be developed using speed breeding method and is likely to be released during 2018. Completely controlled state-of-the-art facility may not be available everywhere; also these processes limit the number of crosses that can be handled at time. At PAU, a field-scale, inexpensive method for raising an extra crop generation between the off-season (May to mid-September) and the main wheat season (November–May) is being followed. A protocol based on raising of seedlings in growth chamber (in propagation trays at 25 °C during mid-September) and then transplanting to field in the first week of October. This accelerated caryopsis development by post-anthesis culture of tillers under high temperature and long photoperiod, harvest of 13-day-old caryopsis followed by drying at 38 °C (4–5 days) and cold treatment at 4 °C (2 days) is followed (Gill 2017). An ideal rapid generation advance sequence

using this method involving fresh crosses in off-season as a starting point would offer selection opportunities in main season during F_2 and F_5 generation. It would take two to two and a half years from a fresh cross to reach bulking stage. Though not as rapid as the other studies (Yan et al. 2017; Watson et al. 2018), the proposed protocol has higher applicability and scale of operation with fewer resources.

In conclusion, both wheat x maize crosses and rapid generation advance systems offer excellent opportunities for accelerated breeding in wheat, which is likely to become the norm rather than a rarely used option in wheat improvement.

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