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Colchicine-Induced Double Haploid Production of Spring Bread Wheat (*Triticum aestivum* L.) via Wheat × Maize Hybridization

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Colchicine-Induced Double Haploid Production of Spring Bread Wheat (*Triticum aestivum* L.) via Wheat × Maize Hybridization

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Highlight

- 0.1% Colchicine solution is the optimist to produce double haploid plants;
- Double haploid approach is the easier-, time- and cost-saving method to develop a homozygous line than the conventional breeding method;
- A homozygous line of spring bread wheat can be obtained within 2-3 years.

Abstract: Bangladesh faces significant wheat grain deficiency compared to its demand due to the lengthy process (10-12 years) required to release a variety through a conventional breeding approach. Traditional breeding methods involve several years of various types of crosses between male and female parents and/or progenies, along with recurrent selection-crossing to obtain a homozygous line (F_1 to F_6 generations) with desired traits within almost 10 years. The double haploid (DH) method is also a time-saving, easier, and more reliable technique to achieve the desired traits in homozygous lines within 2-3 years. DH lines were produced by crossing two F_1 hybrids of spring wheat with maize. 0.1% solution of colchicine was applied to produce DH of F_2 progeny of wheat in the study. 80.8% (340) of the 421 pollinated florets from the two F_1 wheat hybrids were produced as green parthenocarpic caryopses (GPCs) (F_2). 35 of the 70 rescued embryos (or 20.6% of the GPCs) germinated. The germinated embryos regenerated 10 haploid green plants (14.3% of total embryos). After being applied colchicine, 8 of the regenerated haploid (H_1) plants made it. Out of the 8 plants that made it through, only one developed homozygous double haploid seed (DH_1 seed). These seeds were multiplied to produce DH_2, used in the preliminary yield in the next year. The results demonstrated the potential of this method to produce a homozygous line within a short period (2-3 years) by doubling the haploid line generated through pollination of spring bread wheat F_1 with maize.

Keywords: colchicine; conventional breeding method; double haploid; spring bread wheat; wheat × maize hybridization

Introduction

Wheat has faced greatly detrimental effects of biotic and abiotic stress, such as diseases, pests, heat, drought, salt, cold, and excessive rainfall in recent decades due to global climate change. These stresses weakened varietal inheritance and increased vulnerability to diseases and pests. Wheat is critical to the present food system and global food security, accounting for 21% of all calories [1,2]. Although wheat production has expanded significantly, the world's rising population has raised food supply demands. More efficient and novel wheat breeding technologies are required to meet this

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need. In 1980, there were 108 million cases of diabetes; in 2014, 422 million cases were reported. In the world in 2019, there were 2.0 million deaths from diabetes and related disorders. As one age, the death rate of @3% rises. Diabetes is becoming more common in low- and middle-income nations, most likely as a result of rising rice consumption. Bangladesh has the second-highest per capita rice consumption in Asia and the world (179.9 kg), with an average per-person consumption of 53.5 kg worldwide [3–6]. Bangladesh is facing a diabetes epidemic, which is indicative of a larger worldwide problem; in 2014, 13.1 million people were diagnosed with the disease [7,8]. There are still 37 million people in the nation who are insecure about food. Around the world, 783 million people will go to bed today hungry [4].

After rice, wheat is a staple food in Bangladesh. It provides 13.6% of the 51.4 million metric tons (MMTs) of grain required annually [9]. It's amazing how wheat consumption is rising by 5–10% per year for humans, and it's also used to prepare feed for fish, cattle, and poultry, diversify eating habits, and grow the bread sectors, among other things. While wheat consumption was 6.3 MMTs in 2022–2023, and imports accounted for 5.1 MMTs of the total, representing over 2.21 billion USD in exchange (grain price: 1 MT = 45600 BDT), the government set a target of 6.1 MMTs for the fiscal year 2023-2024 (5.8 MMTs of wheat was imported to meet the demand by government and public sectors) [10]. At the same time, domestic production was just 1.17 MMTs, accounting for 19.5% of total requirements. Wheat output peaked in the country in 1998-99 (1.99 MMTs), then dropped drastically, followed by a decade-long pause. Wheat production should be increased for a variety of reasons, including reducing groundwater pumping and greenhouse gas (GHG) emissions from Boro rice cultivation (which accounts for 34% of total agricultural GHG emissions), protecting environmentally friendly environments, reducing the amount of rice fed to people (179.9 kg per adult annually), combating foreign money trafficking, and other reasons. As a result, rapid variety discovery has evolved to address wheat production issues.

Developing a variety using conventional wheat breeding takes a long time (≥10 years). Selfing is necessary to achieve homozygosity, necessitating recurrent selection from the F1 to F6 generations for more than 10 years [11,12]. Yet wheat breeding operations have advanced thanks to cutting-edge biotechnology advancements. Wheat breeding operations can be improved within a year or two using cutting-edge technologies like the DH technique. In haploid cells, chromosomal doubling produces the DH genotype, characterized by perfect homozygosity at all gene loci in the plant. In plant breeding, the artificial generation of DH is significant because it speeds up the formation of pure lines necessary to create consistent and reliable crop types. Breeders can swiftly produce genetically stable and true-bred plants by inducing chromosomal doubling by procedures like colchicine treatment. This greatly reduces the time required for the breeding cycle and increases the productivity of creating new kinds. It is possible to create DH both in vitro and in vivo. Using procedures like parthenogenesis, pseudogamy, or chromosome removal after extensive crossings, haploid embryos are produced in vivo. These haploid embryos are rescued and cultured to produce DH, and chromosomes are doubled. Gynogenesis (ovary and flower culture) and androgenesis (anther and microspore culture) are two in vitro methods for producing DH, with the latter being preferred. Androgenesis is growing male gametophytes to produce haploid plants, which are doubled to achieve homozygosity [13]. Another method of producing haploids is wide crossing, which takes time and effort. Colchicine is essential for the production of doubled haploids because it induces chromosome doubling, which speeds up the development of homozygous, stable wheat varieties. This approach greatly benefits wheat breeding projects by facilitating the quick creation of new varieties that can satisfy Bangladesh's expanding wheat demand and the needs of other areas dealing with comparable difficulties [14].

Barley (*Hordeum bulbosum*) was the pollen parent used in the initial use of DH technology [15]. However, because their dominant alleles cause poor cross-ability, the *Kr1* locus on chromosome 5B and the *Kr2* locus on chromosome 5A cause most wheat varieties to display no cross-ability with barley (Snape et al. 1979). However, because maize chromosomes are destroyed early and the *Kr* genes have little effect on haploid production efficiency, wheat × maize cross-pollination has successfully produced haploid wheat plants [16–18]. Other species, such as sorghum and pearl millet,

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can also act as pollinators in producing haploid wheat [19,20]. Homogenous breeding lines can be developed in as little as 2-3 years thanks to DH technology, which delivers 100% homozygosity in a single generation. Wheat DH is mainly developed through androgenesis (anther and microspore culture) and wheat-maize broad hybridization [21]. Despite their efficacy, these strategies have limits and necessitate additional techniques for DH induction [22,23]. In addition to the quick generation of new homozygous lines, additional culture may result in unforeseen genetic changes due to gametoclonal differences [24,25], influencing selection [26]. Wheat DH can also be created through interspecific crosses, which result in haploid embryos due to selective chromosomal removal during embryogenesis. The ideal colchicine dose for increasing DH production is 0.075% for 4 hours in hexaploid wheat and 0.15% for 4 hours in tetraploid wheat [14]. Colchicine induces chromosomal doubling, accelerating the creation of homozygous, stable wheat types. This strategy greatly benefits wheat breeding efforts, allowing the rapid production of new varieties to fulfill the expanding demand for wheat in Bangladesh and other places facing similar issues [14].

Bangladesh, the world's most densely populated country, has significant food grain deficiency, particularly wheat, with only 8.5 million hectares of arable land [10]. In 2023, the annual wheat demand was 7.0 MMTs, while output was only 1.13 MMTs, with demand rising by 5-10% per year [10,27]. There are significant fallow lands in salinity-prone areas (1.10 million hectares), drought-prone areas (2.7 million hectares), and charlands (0.83 million hectares) [28,29]. These lands can't be cultivated due to a lack of biotic and abiotic stress-tolerant varieties. Expanding wheat cultivation in these areas with high-yielding stress-tolerant varieties using DH technology is a faster and more efficient solution (Figure 2).

The BWMRI released 38 varieties that produced 4.5 - 5.5 t ha^{-1} in research fields but had an average yield of 3.65 t ha^{-1} in farmer fields [27]. As a result, a quickly evolving, high-yielding, biotic, and abiotic stress-tolerant variety is urgently required. Wheat production could be increased with the quick development and diffusion of DH technology wheat cultivars. The study aimed to quickly generate homozygous spring wheat lines using DH production technology.

Figure 1. Methods of Double haploid production. (A-B) Maize pollen donor plant and F_1 hybrid wheat plant in the field and greenhouse, C) Emasculation of the F_1 hybrid wheat spike, D) Pollination with maize pollen, E-F) Growth hormone application after pollination, G) Haploid wheat green parthenocarpic caryopsis, H) Haploid embryo, I) Regenerating haploid wheat plantlet, J) Transplanted haploid plant in the soil, K) Colchicine treatment of haploid plants, and L) Double haploid seed-produced spike.

Figure 2. Comparison of conventional breeding methods (CBM) and anther culture breeding methods (ACM). In traditional methods, at least 10 years are needed to produce homozygous wheat lines. Oppositely, only three years is required for the anther culture methods. This double haploid (DH) method enables the production of a homozygous wheat plant with just chromosome doubling of F_2 in one generation, one additional year for seed increase, and the possibility to undertake screening and preliminary yield trials in the third year (The figure was drawn with Microsoft Office 2019).

Materials and Methods

Experimental Location, Plant Materials, and Producing F_1 Hybrid of Wheat

The study was carried out at the BWMRI's Regional Stations (RS) in collaboration with the Biotechnology Division of the Bangladesh Agricultural Research Institute (BARI), using the research field, greenhouse, and laboratories of both institutes situated at Joydebpur, Gazipur, Bangladesh. In the first year, seeds of Kanchan, Barkat, and Bijoy (BARI Gom 23) varieties of spring bread wheat (*Triticum aestivum* L.) were sown in the research field of RS, BWMRI on 20 November. In January, i.e., in the flowering stage, Kanchan was crossed with Barkat and Bijoy, respectively to get F_1 progenies which Kanchan used as a female plant (Table 1). In the next years, in November, seeds of F_1 hybrids (Kanchan/Barkat and Kanchan/Bijoy) were sown separately three times at 10-day intervals for wheat × maize crossing in the greenhouse of BARI. BARI Maize 7 was also sown in October on four dates at

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7-day intervals in the field for flowering synchronization among both species' plants, and to assure maize pollen availability during the flowering stage of wheat (Figure 1).

Table 1. The main features of the wheat varieties.

Variety	Cross/Pedigree	Released Year	Saline Features
Kanchan	UP301/C306	1983	Yield: 3.3–4.0 t ha^{-1} and susceptible to leaf rust (LR) and *Bipolaris* leaf blight (BpLB) diseases
Barkat	BB/GLL//CARP/3/PVN	1983	Yield: 3.4–4.0 t ha^{-1} and susceptible to LR and BpLB diseases
Bijoy	NL297*2/LR25	2005	Yield: 4.3–5.0 t ha^{-1}, Heat tolerant, resistant to LR, and tolerant to BpLB diseases

Emasculation

Healthy tillers at the booting stage of spring wheat were selected, and the central floret of each spikelet with forceps to provide more space for lateral florets. Anthers were removed manually with the help of fine forceps, and spikes were covered with plastic bags to prevent cross-pollination (Figure 1C).

Pollination

Fresh maize pollen grains were collected in petri dishes, and the emasculated spikelets were pollinated with then maize pollen grains after 48 hours of emasculation (Figure 1D). Immediately after pollination, a solution containing 100 mg/L 2,4-dichlorophenoxy acetic acid (2,4-D) was injected 1.0 cm above the uppermost node of the leaf sheath of the pollinated spike (Figure 1E), as suggested by Pienaar et al. (1997) [31]. After 24 hours, the spikelets of the pollinated spikes were flooded with the same solution using an injection syringe (Figure 1F). Treatment of spikes with 2,4-D enabled the embryos to remain alive until they were large enough to survive the shock of conventional embryo rescue procedures [32,33].

Embryo Rescue

After 15-16 days of pollination, the spikes were collected, and the green parthenocarpic caryopsis (GPCs) were removed from the florets by bending them backward with forceps (Figure 1G). White and necrotic caryopses were removed because they contained no embryos. The GPCs were surface sterilized, and embryos from the disinfected GPCs were excised under aseptic conditions. These embryos were then placed on MS basal media supplemented with 20 g/L sucrose and 8 g/L plant agar for regeneration (Figure 1H) [34,35]. All the cultures were incubated at 25±1°C under complete darkness until the regenerating shoots were 1.0 cm long (Figure 1I).

Transplanting

Regenerating plantlets were transferred to a 25°C growth cabinet with cool white fluorescent tubes on a 10 h light/14 h dark cycle. After four to six weeks, when they reached the three-leaf stage, they were transplanted to pots in an 18°C greenhouse (Figure 1J).

Colchicine Treatment

Haploid plants were lifted from the pots as soon as they developed two to three sprouts (usually after a month), and their washed roots were trimmed to 10 cm. Then, the plants were treated in a solution of 0.1% colchicine for 3.5 hours at 22°C (Figure 1K), rinsed in running water for 3 hours, and

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returned to the 16°C growth room. After 7-10 days, the plants were transferred to a greenhouse, and a few weeks later, the colchicine-treated plants produced double haploid seeds (Figure 1L).

Results

Two F_1 hybrids of spring wheat (*Triticum aestivum* L.) were obtained by crossing Kanchan with Barkat and Bijoy (BARI Gom 23) (Table 1) in January. These two F_1 plants were sown in November on three dates at ten-day intervals and used for wheat × maize crossing. BARI Maize 7 was sown in October on four dates at seven-day intervals to synchronize wheat anthesis and ensure maize pollen availability throughout the reproductive stage of the wheat crop (Figure 1). The following procedures were adopted for doubled haploid production. 421 wheat florets from two F_1 wheat hybrids were pollinated with maize pollens, from which 340 GPCs (F_2) were developed, accounting for 80.8% of the total pollinated florets. A total of 70 embryos (F_2) (20.6% of GPCs) were rescued from the GPCs of two F_1 hybrids and transferred to vials containing MS basal media. 35 embryos (F_2) (50%) were germinated from 70 cultured embryos. Germinated embryos were produced from all wheat × maize crosses, indicating successful fertilization. Ten haploid green plants (F_2) (14.3% of the total embryos) regenerated from the germinated embryos (Table 2).

Table 2. Haploid production by wheat X maize hybridization in two F_1 hybrids of wheat.

Pedigree of F_1 hybrid of wheat	No. of florets pollinated	No. of GPCs developed	No. of embryos rescued	No. of embryos germination	No. of haploid green plants	No. of abnormal plants	Percent of GPCs developed	Percent of embryo formation	Percent of embryo germination	Percent of haploid green plant	Percent of abnormal plants
	A	B	C	D	E	F	B/A	C/B	D/C	E/C	F/C
Kanchan/Barkat	205	166	32	16	4	12	81.0	19.2	50	12.5	37.5
Kanchan/Bijoy	216	174	38	19	6	13	80.6	21.8	50	15.8	34.2
Total	421	340	70	35	10	25	80.8	20.6	50	14.3	35.7

GPCs = Green parthenocarpic caryopses.

This parameter indicated the efficiency of embryo rescue operations, similar to the findings of Khan et al. (2017). Regenerated plants were treated with colchicine. Eight out of 9 colchicine-treated haploid plants (H_1) survived. Colchicine treatment resulted in diploidization, and 90% of the plants survived. However, only one of the eight surviving plants produced double haploid seeds (DH_1 seeds) that were homozygous to their parents (Table 3).

Table 3. Double haploid production by wheat X maize hybridization in two F_1 hybrids of wheat.

Pedigree of F_1 hybrid of wheat	No. of Plants Transplanted	No of Colchicine Treated Plants	No. of survived plants	No. of DH seed-produced plants	Percent of survived plants
	G	H	I	J	I/H
Kanchan//Barkat	4	4	4	0	100
Kanchan/Bijoy	6	5	4	1	80

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Total	10	9	8	1	Mean= 90

In the second year, DH_1 seeds were multiplied. Thus, DH_2 seeds, which were the homozygous line, were produced. This line was taken in the preliminary yield trial (PYT) for variety release (Figure 2). After conducting the PYTs, the wheat plants that have similar height, spike length, grain number in spike, and uniform size will be released as a variety.

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Discussion

The wheat × maize system is commonly used to produce DH wheat because of its high efficiency and advantages over traditional procedures such as anther culture [11,30]. Unlike other cultures, the wheat × maize system demonstrates little to no dependence on the genotype. This makes it a versatile strategy that may be used with a wide range of wheat cultivars. Due to these advantages, the technology has become a popular and favored method of generating DH wheat. This streamlines the breeding process, allowing for the rapid production of novel wheat varieties with desirable features [30,36].

Phytohormone treatment after wheat-maize pollination is critical for haploid generation, boosting ovary size and embryo survival for effective media rescue [16,37,38]. Various hormones were evaluated, including indole-3-acetic acid (IAA), kinetin, 2,4-dichlorophenoxyacetic acid (2,4-D), and dicamba (Shubham et al., 2023). 2,4-D is often utilized to stimulate organ regeneration and callus formation while also controlling early and postembryonic development [39]. In the wheat × maize system, 100 ppm of 2,4-D causes haploid embryos in hexaploid wheat [40–42], while 250 ppm induces haploid embryos in tetraploid wheat [43]. According to Kaushik et al. (2004), the anther culture method was the most effective, but we chose spraying since it was simple and efficient, resulting in an average embryo rate of 12.9%. Maize chromosomes are destroyed in both embryo and endosperm cells, resulting in seed abortion [44,45]. As a result, wheat haploid embryos must be saved by tissue culture. Cherkaoui et al. (2000) [46] found that B5 and ½ MS media were more effective for tetraploid wheat × maize hybridization. Adding 0.5 mg/L putrescine and spermidine to the regular medium raised the regeneration rate to 69.3%, up from 33.5% in the control group.

Our approach included isolating haploid embryos from sterilized immature seeds on a clean bench and plating them on ½ MS media (½ MS + 20 g/L sucrose + 8 g/L plant agar, pH 5.8). The embryos were grown at 25±1°C, with a 16 h light/8 h dark photoperiod and a light intensity of 67.2 μmol/m²/s. This approach yielded an average plantlet rate of 51.8%. Wheat haploid plants from the wheat × maize system doesn't duplicate their chromosomes [47], while homozygous, stable diploids do. The most often used antimitotic drug is colchicine, which inhibits spindle function and causes chromosomes to double during mitosis. However, colchicine can be partially deadly and cause low doubling rates, demanding optimization of dosage, timing, and plant stage [48,49]. Inagaki (1985) [49] demonstrated a 95.6% doubling rate by immersing clipped roots of haploid plantlets in 0.1% colchicine, Dimethyl sulfoxide, and Tween-20 for 5 hours at 20°C. Khan et al. (2012) and Niu et al. (2014) [36,50] both found high success rates with continuous airflow during treatment. Sharma et al. (2019) found that four hours of treatment with 0.075% colchicine in hexaploid wheat and 0.15% in tetraploid wheat increased DH production. In the protocol, haploid plantlets in the 2-3 tiller stage were treated with 0.1% colchicine for 16 hours, resulting in over 90% survival and chromosome doubling rates (Table 3; Figure 1).

Post-pollination treatments successfully produced haploids in conjunction with maize pollen grains. The study's successful seed set rate of 80.8% was nearly identical to the number reported by Chalyk [51] using twelve different wheat genotypes. The proportion of the seed set indicated the effectiveness of the emasculation and pollination processes. According to Zhang et al. (1996) [34] and Brazauskas et al. (2005) [52], the use of 2,4-D greatly enhanced the growth of the embryo. The most crucial elements influencing success were the 2,4-D concentration, how it was applied, and the timing of pollination [53]. 2005; Singh et al. Up to 14–17 days after pollination, 2,4-D causes caryopsis enlargement and the following haploid embryo development, according to Brazauskas et al. (2005) [52]. A critical concentration of 2,4-D treatment is probably required to induce caryopsis formation; additional increases in its concentration did not significantly impact it. Numerous investigations have examined the effects of maize and wheat genotypes on the development of embryos in crossings between wheat and maize [34,54]. The wheat × maize system for producing wheat DH lines by pollen induction has been the subject of recent studies. Important roles in this process are played by the genotypes of maize and wheat and their interactions. To get great efficiency in DH line production, hybrid maize varieties, and wheat breeding materials should be cultivated under carefully regulated environmental conditions. The development of spring wheat DH lines in the wheat × maize

hybridization can be made even more efficient by optimizing processes, including chromosome doubling, hormone therapy, pollination timing, wheat spikes treatment, and embryo rescue.

Conclusion

Using DH technology to hybridize wheat and maize speeds up the development of new spring wheat varieties (Figure 2). Of the 70 cultured embryos, 35 (50%) germinated, while 10 haploid green embryos (14.3%) regenerated. These plants were treated with 0.1% colchicine for three and half hours at 22°C, with 8 plants surviving out of 9. Colchicine treatment resulted in diploidization, with 90% survival; however, only one plant (12.5% of 8 regenerated plants) produced double haploid seed (DH_1). The DH_1 seeds were replicated and used in the preliminary yield trial in the following year. As a result, DH technology may produce homozygous wheat lines within 2-3 years (Figure 2), whereas the conventional breeding method takes almost 10 years. This technique must reduce the time required for homozygous lines to develop while simultaneously streamlining, speeding up, and smoothing the release path of a high-yielding, stress-tolerant variety suitable for growing under challenging conditions.

Contribution of Authors: MNA and MFA planned and designed the experiments. MFA conducted the experiments. GF and MNA supervised it. MFA and MNA wrote the manuscript. GF, MMK, and MMR revised, edited, and improved it.

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