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Review

Technology for Production of Wheat Doubled Haploid via Maize Pollen Induction—Updated Review

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Abstract: Chromosome elimination resulting in haploids is achieved by rapid loss of chromosomes from one parent during the zygote stage and is an important procedure to produce doubled haploid (DH) lines in plants. During crosses between an emasculated wheat (*Triticum aestivum* L.) and maize (*Zea mays* L.) as pollen donors, the complete loss of maize chromosomes results in wheat haploid embryos. Through embryo rescue and chromosome doubling processes, pure lines with stable traits can be quickly obtained. The technique is called the "Wheat × Maize System". Although this technology is not new, it remains a practical approach to date. In order to optimize and improve this technology and to achieve its maximum potential in the winter wheat area of China, this paper reviews the previous and ongoing research and technical procedures for the production of wheat DH lines via the maize pollen induction and presents outlooks on DH research and its application in wheat breeding.

Keywords: wheat; speed breeding; maize pollen induction; chromosome elimination; doubled haploid (DH)

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1. Introduction

In many countries, distinctness, uniformity, and stability (DUS) are prerequisites for a new plant variety to obtain protection and registration [1]. In China, without exception, both the approval or registration of new varieties and the protection of variety rights require DUS tests [2,3]. DUS tests are essential attributes for new wheat varieties. A variety's uniformity and stability can be estimated by its proportion of homozygous DNA loci and seed purity; the higher the ratio of the homozygous DNA loci, the better the uniformity and stability of the variety. Varieties with a ratio of homozygous DNA loci less than 90% are poor in uniformity and stability [4]. Seed purity is an important indicator used to evaluate the quality of wheat seeds. The China National Standard (GB 4404.1-2008) [5] stipulates that the purity of wheat foundation seeds should not be less than 99.9%, and the purity of wheat variety seeds should not be less than 99.0%. Therefore, for a wheat line to qualify for testing, its ratio of homozygous DNA loci needs to be over 90%, and it has to be advanced to the F_5 or later generations. For wheat lines to reach the level of variety or foundation seeds, the ratio of homozygous DNA loci needs to be over 99.9%, and the generation should reach at least F_{11}. The breeding cycle is thus very long, which certainly limits the wheat seed industry.

Haploid induction and doubled haploid (DH) technology can obtain pure lines with genotypic homozygosity of 100%, bypassing the need for 6–10 generations of inbreeding through selfing or sibcrossing [6]. This is a significant breakthrough for developing new varieties [7]. This technology requires only one generation to obtain genetically homozygous lines and will substantially reduce the breeding course [8].

The first naturally occurring haploid plant was discovered in jimson weed in 1922 [9]. Thereafter, using anther and microspore culture techniques, haploid plants of jimson weed

and rice were obtained, respectively [10,11]. DH technology gradually came into the view of breeders, and varieties of oilseed rape and barley were successively developed [12]. Along with the improved haploid induction and chromosome doubling, doubled haploid technology has been applied in many plant species, including Arabidopsis, Avena sativa, pearl millet, maize, tomato, cucumber, melon, and so on [13].

DH plants rarely occur in nature but can be induced by in vitro or in vivo treatments [14]. In vitro culture includes androgenesis (anther or microspore culture) and gynogenesis (bud, ovary, or unfertilized ovule culture). In vivo methods include induction of parthenogenesis by inactive pollen pollination (ionizing radiation, chemical agents, and high temperature) and intra- or interspecific hybridization, also known as the chromosome elimination method [15]. The effectiveness of different methods depends on plant species [13].

In wheat DH breeding, the in vitro anther or microspore culture is limited due to its genotype dependence, time-consuming regeneration, unstable ploidy, and albino seedlings [16,17]. Ionizing radiation and chemical treatments of pollen barely induce haploid and may cause aneuploidy [18]. In contrast, wheat mutants of the pollen-specific phospholipase gene *TaMTL* can induce wheat to produce haploid seeds [19]. Using the wheat *Tamtl* mutant, Tang et al. [20] have established a visual screening to identify the haploid seeds. The Wheat × Maize System involves both in vivo and in vitro operations, alien chromosome elimination, and maternal chromosome doubling. The Wheat × Maize System is two to three times more efficient for producing green plantlets than the anther culture [21], shows little or no genotype dependence in wheat, does not require haploid identification, and thus becomes a popular method for producing DH wheat. The chromosome elimination method is well utilized in Yunnan, China, where both wheat and maize can grow in the same season, and several wheat varieties, such as Yunmai 110, have been developed [22].

Winter wheat area is about 2.17×10^7 ha and accounts for 92% of the total wheat acreage in China [23,24]. In the winter wheat region, wheat and maize are rotating crops; thus, they do not flower at the same season in nature. Therefore, it is unrealistic to conduct large-scale wheat haploid induction using maize pollen in the winter wheat region. However, newly constructed plant growth facilities can be used to coordinate the flowering time of winter wheat and maize, thus allowing industrialized production of wheat DH via maize pollen grains in the winter wheat region. To improve the Wheat × Maize System and to gain its full potential in the winter wheat region in China, in this paper, we review previous achievements and the technical procedure for the winter wheat region and then present outlooks on wheat DH research and application.

2. Origin, Principle, and Advantages of Wheat × Maize System

Chromosome elimination was first encountered in an interspecific hybridization in tobacco (*Nicotiana tabacum* × *N. sylvestris*), resulting in the *N. tabacum* haploids [25]. Similarly, Gaines and Aase [26] obtained wheat haploids after performing an intergeneric hybridization (*T. compactum* Host × *Aegilops cylindrica* L.). Barclay [27] also created wheat haploid plants when using *Hordeum bulbosum* as pollen donors, a method commonly termed the bulbosum technique. The *H. bulbosum* is sensitive to dominant crossability inhibitor genes *Kr1* and *Kr2*, which are located on chromosomes 5B and 5A. However, the bulbosum technique is invalid for wheat genotypes lacking dominant *Kr1* and/or *Kr2*. Later, Laurie and Bennett [28] established an intergeneric system between wheat and maize, representing an early study of the Wheat × Maize System, in which the elimination of maize chromosomes was independent of wheat *Kr* genes [29,30]. Thus, the Wheat × Maize System became popular and was often used to produce haploid plants from commercial wheat varieties [31–33]. Now, the Wheat × Maize System acts as one of the most practical methods for wheat [34–36].

Despite the popularity of the Wheat × Maize System, the underlying mechanism and/or genes causing chromosome elimination are still unclear. There are different hypothe-

ses, including asynchrony in nucleoprotein synthesis [37], asynchronous cell cycles [38], and parent-specific inactivation of centromeres [39]. In Arabidopsis, the centromeric histone H3 (*CENH3*) regulates the intraspecific genome elimination [40]. Similarly, *CENH3* controls chromosome elimination in the interspecific hybridization of *H. vulgare* × *H. bulbosum* [41]. Whether *CENH3* accounts for the maize chromosome elimination in wheat × maize remains to be answered. Chen et al. [42] constitutively expressed the maize *CENH3* in wheat 'Yangmai 158'; however, maize chromosomes were again eliminated in the case of *ZmCENH3* overexpressing wheat × maize. Likely, low contents of *ZmCENH3* in transgenic wheat may fail to suppress the chromosome elimination of maize.

Kapoor et al. [43] compared the haploid plant rate after crossing polyploid wheat (4× and 6×) or hexaploid triticale with maize or *Imperata cylindrica*. The hexaploid wheat showed a higher frequency of embryo and haploid formation than the tetraploid wheat when they were crossed with *Imperata*; thus, the wheat D genome may be prone to trigger chromosome elimination of *Imperata*. Gurtay et al. [44] also tested the wheat × maize programed haploid production and conducted wheat anther culture to acquire DH plants using spontaneous doubling in androgenesis, which includes the use of ancient, local, and modern types of polyploid wheat (4× and 6×). As a result, more haploid embryos were acquired in hexaploid wheat than those in tetraploid wheat, but the plant regeneration was comparable. Apparently, the D genome impacts wheat anther culture and wheat × maize hybridization. Obviously, centromeres, especially those in the wheat D genome, play an important role in inducing wheat haploids by eliminating maize chromosomes. However, more studies are needed to understand how the wheat D genome functions to eliminate the entire parental chromosomes.

Besides *H. bulbosum* and maize, other Poaceae species, such as sorghum [45], pearl millet [46], teosinte [47], *Tripsacum* [48], Job's tears (*Coix lacryma-jobi* L.) [49], *I. cylindrica* [32,50], and *Ae. Caudata* [51], also induced haploid formation in wheat. In comparison (Table 1), sorghum's effect highly depends on wheat genotypes, and thus, sorghum is not suitable for inducing wheat haploids [52,53]. Surprisingly, according to other studies, pearl millet [53–55], teosinte [47,56], and *Tripsacum* [48,57] can induce wheat haploids at a comparable or higher rate than maize. Only a few studies were conducted with Job's tears [49] and *Ae. Caudata* [51], and their applicability for wheat haploid induction needs to be further studied. Although *I. cylindrica* is a noxious weed [58], *I. cylindrica* is comparable to maize for inducing haploid embryos in common wheat [50]. But *I. cylindrica* outperforms maize when used in durum wheat, triticale, or their derivatives [59,60]. In practice, *I. cylindrica* should be handled with care due to its weedy nature. To gain first-hand experience, we tested maize hybrid, maize inbred line, sorghum, teosinte, and Job's tears for inducing wheat haploids. We found that pollen quantity and accessibility were critical for developing a large-scale and robust wheat haploid induction, for which maize outperforms all others tested (Figure 1). In addition, a variety of maize genotypes with large acreage are widely planted in the world, which makes it possible to screen for ideal genotypes with higher induction rates.

Table 1. Utilization of different pollen sources in interspecific crosses with common wheat.

Pollen Source	First Reported	Rates of Haploid Embryos (%)	Average Rate of Haploid Embryos (%)	Rates of Haploid Plantlets (%)	Average Rate of Haploid Plantlets (%)	Wheat Genotype Dependence	References
Maize	[28]	1.6–60.7	17.0	16.3–86.6	58.0	weak	[35,36,60–62]
Sorghum	[45]	0–42.1	18.0	56.4–63.3	59.9	strong	[52,53]
Pearl millet	[46]	0.3–39.4	18.1	44.6–72.2	53.1	weak	[53–55]
Teosinte	[47]	12.5–57.5	40.5	34.6–90.3	72.2	weak	[47,56]
Tripsacum	[48]	5.0–59.0	22.9	69.3–83.3	78.5	weak	[48,57]
Job's tears	[49]	10.6	10.6	26.1	26.1	unknown	[49]
Imperata cylindrica	[50]	0–64.7	27.4	18.1–84.9	48.9	weak	[50,59,60,63]
Ae. caudata	[51]	No data	No data	No data	No data	unknown	[51]

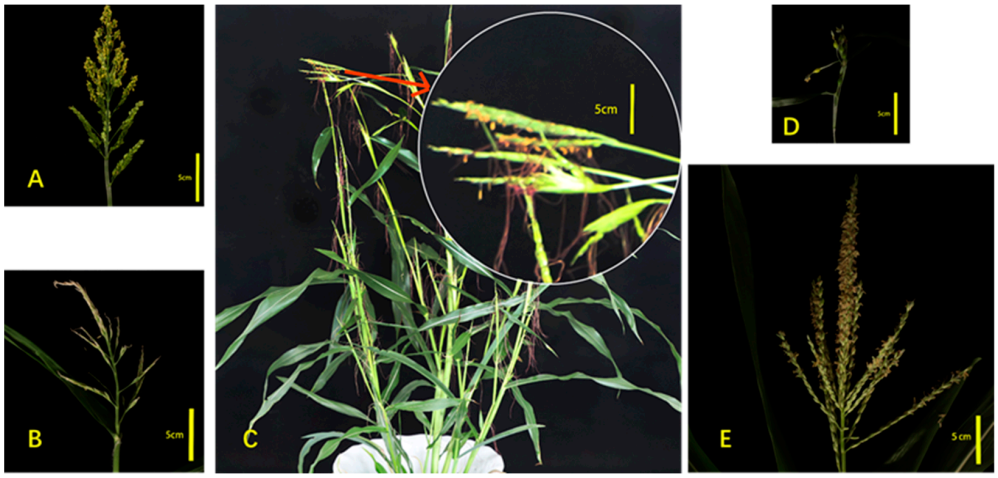

Figure 1. Tassel morphology of tested plants in greenhouse. The red arrow indicates the male inflorescences of Teosinte that are magnified in the circle. (**A**)—Sorghum; (**B**)—Maize inbred line; (**C**)—Teosinte; (**D**)—Job's tears; (**E**)—Maize hybrid.

3. Research Progress of the Wheat × Maize System

The main steps in the Wheat × Maize System are wheat and maize planting, wheat emasculation, maize pollen pollination, hormone treatment, embryo rescue, doubling treatment, and DH plant harvesting (Figure 2). Along the process, numerous factors control the ending wheat DH rate, which is actually attributed to pseudoseed formation frequency (*PFF*), embryo formation frequency (*EFF*), haploid regeneration frequency (*HRF*), haploid formation frequency (*HFF*), and haploid doubling frequency [59,60,64–66]. However, the inheritance of *PFF*, *EFF*, and *HRF* is independent [61,67], and they can ultimately be reflected in the embryo rate (obtained wheat haploid embryos after crossing and hormone treatment), the plantlet rate (obtained wheat haploid plantlets after haploid embryo rescue), and the doubling rate (obtained wheat DH plantlets after chromosome doubling). Over the years, the Wheat × Maize System has been gradually upgraded and now offers a powerful tool for research on wheat genetics and breeding. This includes applications such as molecular cytogenetic characterization [68], the development of thermophoto sensitive genic male sterile lines [36], QTL mapping [69], and so on.

3.1. Genotype Effects

The Wheat × Maize System functions independent of wheat *Kr1* and *Kr2* genes, behaves superior to the bulbosum technique [70,71], and thus is reliable and widely used in wheat. Over the years, there has been a nonstop passion for understanding how wheat and/or maize genotypes affect the efficiency of the Wheat × Maize System, and complex conclusions have been drawn.

For maize, most researchers believe that the maize genotype has a significant influence on the Wheat × Maize System [13,60,61,64,72–74], partially through modulating *EFF* and *HRF*. Niroula et al. [34] propose using more responsive maize genotypes to enhance wheat DH production. Specific genotypes account for haploid embryo induction and embryo regeneration, respectively [64]. In another case, the anther culture-responsive F_1 hybrids of hexaploid wheat were tested with three sweet corns, 'Baron', 'Challenger', and 'Merit', of which Challenger had the highest haploid embryo rate (3.5%), but not for the plantlet regeneration. Surprisingly, the use of a pollen mixture of multiple sweet corn genotypes enhanced haploid plantlet regeneration [74].

For wheat itself, an early study failed to show the genotype effect on the Wheat × Maize System [72]. In contrast, Verma et al. [64] proved that wheat genotypes significantly influenced *PFF* and *EFF* but were not as good as those from the maize side. Today, wheat genotypes are primarily counted towards affecting the Wheat × Maize System [61,67,73,75]. When both winter and/or spring wheat were considered, the winter wheat (winter parents

and winter × winter F_1s) performed better than the nonwinter wheat (spring parents, spring × spring F_1s, and winter × spring F_1s) towards embryo formation. However, the winter × spring F_1s performed the best in acquiring regenerated plantlets [67].

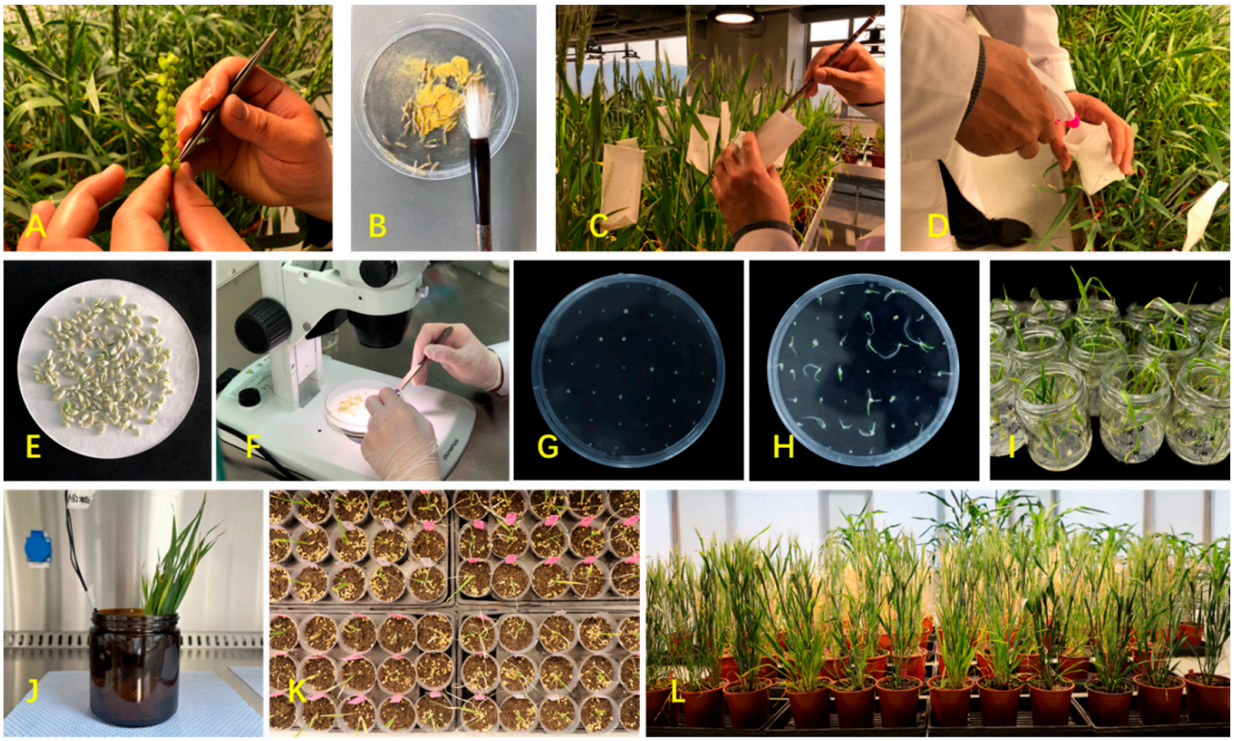

Figure 2. Main procedures of Wheat × Maize System. (**A**)—Manual emasculation of wheat; (**B**)—Collecting maize pollen grains; (**C**)—Manual pollination (applying maize pollen on wheat pistils); (**D**)—Auxin treatment; (**E**)—Immature seeds harvested; (**F**)—Embryo isolation; (**G**)—Embryo rescue; (**H**)—Embryo regeneration; (**I**)—Haploid plantlets; (**J**)—Chromosome doubling by colchicine; (**K**)—Transplanted DH plantlets; (**L**)—DH plants.

To study the interaction between wheat and maize, Singh et al. [73] compared winter wheat, spring wheat, and their F_1s in conjunction with specific maize. There was significant interaction on embryo formation and regeneration of plantlets; the wheat × maize interaction for embryo formation and regeneration was due to nonadditive gene action. In addition, the DNA heterozygosity in wheat and maize genotypes improved the haploid induction rate. Dhiman et al. [61] further demonstrated that the overall contribution of the maize induction line to embryo formation and regeneration was the highest, followed by the wheat line × maize induction line interaction.

Collectively, genotypes of wheat and maize and their interaction all play roles in the Wheat × Maize System. In the future, more maize genotypes should be tested in conjunction with target wheat genotypes, which are designed to acquire specific maize and/or their interaction with specific wheat in conferring excellent *EFF* and *HRF*, and they will be applied to advance the Wheat × Maize System. What we need to do is to extensively screen a multitude of maize hybrid varieties against various wheat genotypes, selecting several maize varieties with high *EFF* and *HRF*. These maize varieties will then be cultivated in a greenhouse for pollen collection. The collected pollens will be thoroughly mixed. The mixed pollens will be used to pollinate the emasculated wheat spikes.

3.2. Environmental Factors

Gu et al. [76] achieved a haploid embryo rate of 31.6% in the Wheat × Maize System when cut plants were in vitro cultured in a nutrient solution (40 g/L sucrose, 10 mg/L silver nitrate, 3 g/L calcium phosphate, and 8 mL/L sulfurous acid) under controlled conditions

of 22–23 °C in light, 16–17 °C in dark, and an ambient humidity of 70%. However, the haploid embryo rate was only 9.6% using plants from fields. Khan et al. [77] further conducted an in vitro culture of 25 hexaploid wheat genotypes from fields using a tillering medium containing 100 mg/L 2,4-D, 40 g/L Sucrose, and 8 mL/L Sulfurous acid. They analyzed the controlled factors such as temperature during pollen collection, time of pollination, light intensity, and relative humidity towards haploid seed formation. As a result, the optimal factors are maize pollen from 21 to 26 °C, pollination at 24 h postemasculation, a light intensity of 10,000 Lux (140 μmol/m^2/s), and a relative humidity of 60–65% at 20–22 °C. Khan et al. [78] investigated the haploid induction rate between wheat F_1s and *Z. mays*/*I. cylindrica* under different conditions. The DH production rate of the F_1s in the greenhouse was considerably higher than that of the F_1s in the field. Thus, the growing condition of both wheat and maize plays a pivotal role in the Wheat × Maize System, and optimal environmental factors can be drawn for an improved Wheat × Maize System. The environmental factors proposed by Khan et al. [77] can serve as a reference for technical improvement.

In our laboratory, wheat breeding lines (≥F3 generation) and hybrid maize varieties are grown in environmentally controlled facilities: wheat under 20–24 °C, day/night of 20 h/4 h, light intensity > 420 μmol/m^2/s, and humidity 60–65%; maize under 22–24 °C, day/night of 12 h/12 h, light intensity > 140 μmol/m^2/s, and humidity 55–60%. These specific environmental parameters have led to a nearly 100% pseudoseed formation frequency (*PFF*) with the well-filled seeds.

3.3. Treatment of Wheat Spikes and Timing of Pollination

Growth condition, or controlled environment, is preferred for conducting the Wheat × Maize System. However, due to the limited space of any environmentally controlled facility, immature wheat spikes were harvested during heading and then subjected to in vitro culture [76,77,79]. Today, modern and spacious greenhouses are readily accessible, which allows to maintain enough wheat and maize plants continuously throughout the year. Therefore, it is not necessary to in vitro culture wheat immature spikes. According to Laurie [29], any accountable pollination is based on the wheat floret status, those with a feathery stigma being best. Martins-Lopes et al. [80] studied the spikelet's position effect on wheat × maize compatibility and found more success with middle spikelets. Thus, maize pollen should be applied to middle spikelets with a feathery stigma in order to obtain more haploid embryos under controlled conditions.

3.4. Hormone Treatment

Phytohormone treatment post wheat × maize pollination is crucial for haploid production. The applied hormones promote ovary growth and survival rate of haploid embryos, from which haploid embryo rescue on media becomes more practical and effective [31,45,81]. To improve the Wheat × Maize System, a variety of hormones were tested, including 2,4-dichlorophenoxyacetic acid (2,4-D), dicamba, picloram, indole-3-acetic acid (IAA), phenylacetic acid (PAA), silver nitrate, 1-naphthaleneacetic acid (NAA), kinetin, 6-benzyladenine (BA), and zearalenone [82]. Among them, 2,4-D is widely used to control organ regeneration and callus induction. The 2,4-D also regulates early and postembryogenic plant development involving both somatic and zygotic embryogenesis [83].

When applying a hormone in the Wheat × Maize System, the dosage, timing, and methodology of it should be determined. At 100 ppm, 2, 4-D effectively induces haploid embryos in hexaploid wheat [72,84,85]. At 250 ppm, 2, 4-D effectively promotes haploid production in tetraploid wheat [81]. Kaushik et al. [84] tested different application methods of 2,4-D, including spray, tiller injection, dipping, and spikelet culture, of which only the spikelet culture method behaved well in recovering embryos. Despite this, we adopted the spraying method because of its simplicity and high efficiency in our hand, and we have acquired an average embryo rate of 12.9%.

3.5. Embryo Rescue

During the Wheat × Maize System process, maize chromosomes are eliminated not only in embryo cells but also in endosperm cells, which will cause seed abortion [50,77,86,87]. Therefore, wheat haploid embryos must be rescued by tissue culture to generate haploid plantlets. In practice, wheat embryo rescue is highly dependent on the plant regeneration media. Among B5, MS, and ½ MS tested, Cherkaoui et al. [88] found that B5 and ½ MS were superior to MS in obtaining young embryos for the tetraploid wheat × maize hybridizations. The supplement of putrescine and spermidine, each in 0.5 mg/L in the embryo rescue medium, SM (standard medium), resulted in a 69.3% regeneration rate of wheat plantlets but only 33.5% regeneration in the control group (SM) [89]. Most tests are needed with how to supply putrescine and spermidine in B5 and/or ½ MS medium.

During this phase, our methodology entails using a clean bench where haploid embryos are carefully isolated from sterilized immature seeds under a dissection microscope. The isolated embryos are then plated on ½ MS medium (½ MS + 20 g/L sucrose + 2.4 g/L plant agar, pH 5.8). These embryos are cultured under controlled conditions at a temperature of 20–24 °C with a 16 h light/8 h dark photoperiod and a light intensity of 67.2 μmol/m^2/s. Using this protocol, we have achieved an average plantlet rate of 51.8%.

3.6. Doubling Treatment

Wheat haploid plants obtained through the Wheat × Maize System naturally remain undoubled [90]. Chromosome doubling is essential to acquire homozygous and stable diploid plants. Antimitotic compounds are selected to double plant chromosomes [91], for which colchicine is the most applied agent. Colchicine inhibits spindle function during mitosis and stops the polar segregation of sister chromatids, ending with a doubled nucleus. In the process, chromosome-doubled chimera sectors are formed, which leads to partial fertility [92] and poor grain setting in DH plants (Figure 3). Colchicine treatment is partially lethal to plant haploids; thus, it only results in a low frequency of doubled haploids. It is necessary to optimize the dosage, processing time, and plant stages for an effective colchicine treatment, particularly when dealing with new plant genotypes.

Inagaki [93] trimmed roots by keeping 2–3 cm on haploid plantlets and soaked the trimmed roots in 0.1% colchicine (with 2% dimethyl sulfoxide/DMSO and fifteen Tween-20 drops per liter) at 20 °C for 5 h. At the 2–3 tiller stage, the colchicine application resulted in a 95.6% doubling rate. Khan et al. [94] treated haploids with 3–5 tillers in 0.1–0.2% colchicine for 3 h and provided continuous air flow in the solution. Niu et al. [95] also supplied air during colchicine treatment at 14–16 °C and achieved over 90% survival and chromosome doubling among the treated wheat plantlets. Sharma et al. [96] also studied the in vitro effect of colchicine. The wheat DH production was enhanced after four hours of treatment with 0.075% colchicine in hexaploid wheat and 0.15% colchicine in tetraploid wheat. However, in our cases, haploid plantlets at the 2–3 tiller stage were treated in 0.05% colchicine for 16 h, resulting in over 90% survival and chromosome doubling rates.

All in all, many studies have been conducted on the Wheat × Maize System in recent years. Genotypes of wheat and maize and their interaction all play roles in the wheat DH line production via maize pollen induction. Wheat breeding materials and hybrid maize varieties should be grown in environmentally controlled conditions for a high efficiency of DH line production. Optimization of the procedures, including treatment of wheat spikes and timing of pollination, hormone treatment, embryo rescue, and doubling treatment, could further enhance the efficiency of wheat DH line production in the Wheat × Maize System.

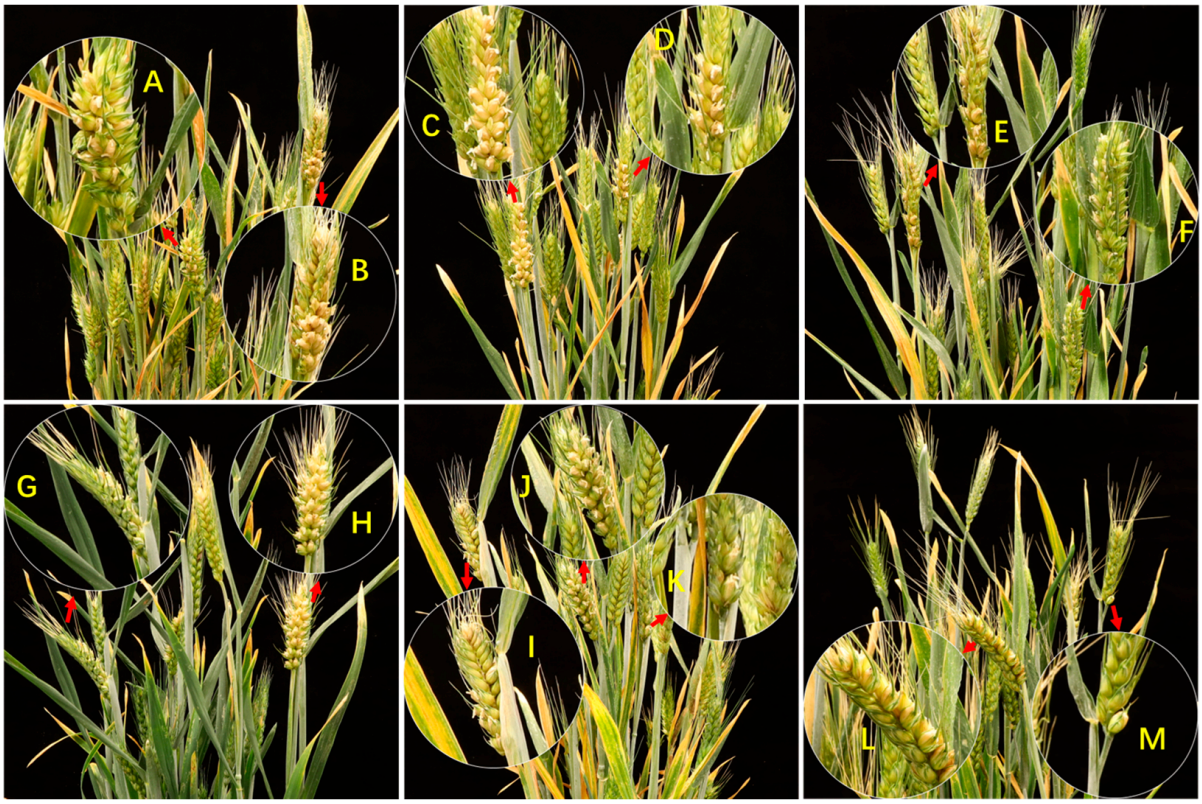

Figure 3. Grain setting in wheat DH plants in greenhouse. Spikes with doubled chromosomes were highlighted by red arrows and are magnified in the circle. (**A**)—Complete sterility at the base of the spike; (**B**)—Complete sterility at the apex of the spike; (**C**)—Absence (missing grains) at the apex of the spike; (**D**)—Complete sterility at both ends of the spike; (**E**)—Absence of grains throughout the spike; (**F**)—Absence of grains at the base of the spike; (**G**)—Complete sterility on one side of the spike; (**H**)—Normal seed set in the spike; (**I**)—Complete sterility at the apex of the spike; (**J**)—Absence of grains at the base of the spike; (**K**)—Only two grains set throughout the entire spike; (**L**)—Complete sterility on one side of the spike; (**M**)—Only one grain set throughout the entire spike.

4. Stability of Doubled Haploids

DH stability is crucial for agronomy, breeding, and research. In the Wheat × Maize System, maize chromosomes disappear during early embryo cell divisions [46] or later in chromosome doubling [97]. The genetic stability is then established after the doubling of wheat haploid chromosomes. Using six glutenin loci of the Wheat × Maize decedents, Kammholz et al. [98] proved their stable inheritability across generations. Furthermore, Chen et al. [99] and Brazauskas [100] demonstrated the predominant genetic stability from the wheat side, and there was little or no maize DNA in the Wheat × Maize decedents.

However, colchicine also causes chromosomal aberrations, such as aneuploidy [101–103]. Suenaga and Nakajima [104] evaluated 110 wheat DH lines, of which 15 DH lines exhibited irregular phenotypes, such as dwarfism, poor seed setting, spike variation, and leaf stripes. Likely, they were caused by colchicine treatment. Shrestha et al. [105] studied two wheat DH populations and found many chromosomal aberrations, including duplication, deletion, translocation, and aneuploidy, which were likely caused by unusual chemical exposure during haploid induction and chromosome doubling.

Apparently, the stability of DH lines is based on both haploid embryo induction and colchicine doubling. In the Wheat × Maize System, maize DNA barley remains in haploid embryos; thus, the chromosomal aberrations in the resulting wheat DH lines are considerably caused by colchicine treatment. In our study, however, there is no significant variation in spike traits and leaf morphology (Figure 4).

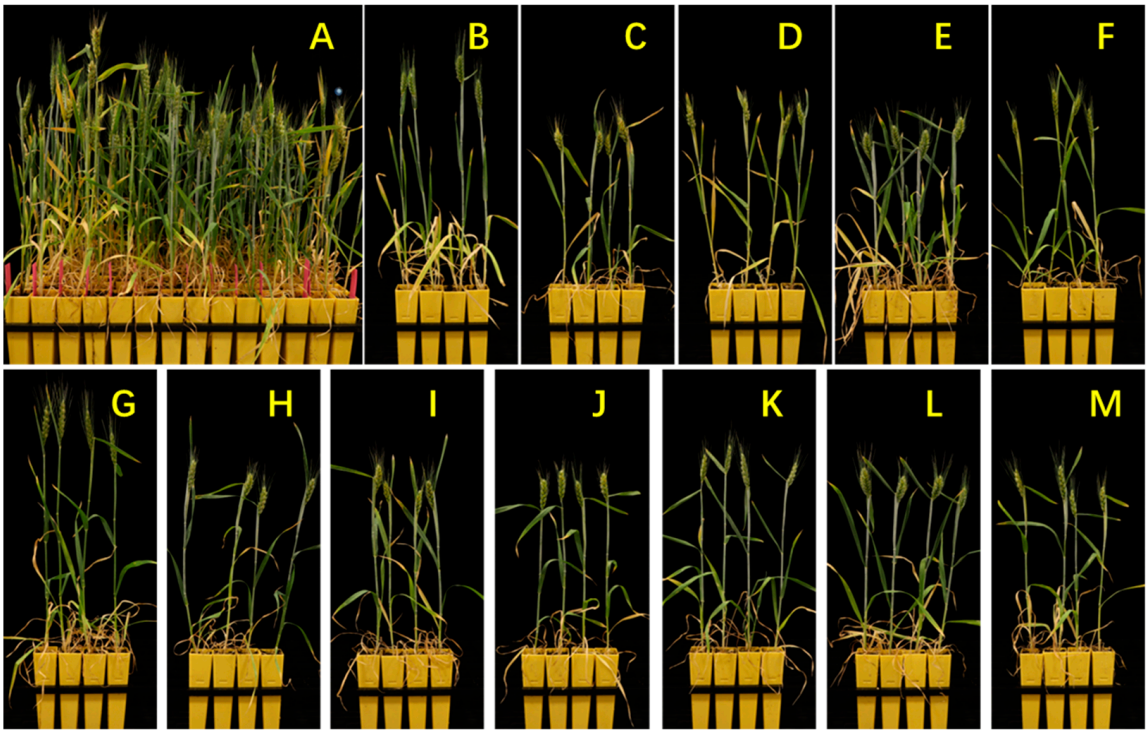

Figure 4. Cultivation of individual wheat DH plants of different families. (**A**)—Fourteen DH individual plants were harvested in the greenhouse, and ten seeds from each plant were randomly selected for germination. Seven germinated seeds were planted in a detachable plastic container (7 × 14). (**B**–**M**)—The plants around the periphery of the detachable plastic container complex from group A were culled. From the remaining progeny of 12 DH families with 5 plants each, 4 individual plants were selected for phenotypic observation.

5. Conclusions and Prospects

In recent years, genome editing has been used to develop novel wheat DH technologies, for example, the *TaMTL*-based maternal haploid induction [19] and the *TaCENH3α*-based paternal haploid induction [106]. This indeed opens a new path to upgrade wheat DH technologies, but its application to the industrialized production of wheat DH lines still requires time for verification.

Wheat DH technologies, including anther culture, microspore culture, and Wheat × Maize System, have been widely applied to speed up research and breeding. The fast growth in research and facility allows a full exploration of the Wheat × Maize System, which in the future might become a high throughput system for wheat DH lines. Nevertheless, there is still considerable room for improvement in the Wheat × Maize System. Many factors, including genotype, environment, pollination and hormone treatment, embryo rescue, and doubling treatment methods, can influence the production efficiency. Thus, it is necessary to improve and optimize the Wheat × Maize System around these factors. Screening the best maize induction lines (varieties) for specific types of wheat and identifying the optimal growth environment conditions, pollination times, hormone treatments, embryo rescue, and doubling treatment plans could further improve DH production efficiency. Moreover, the exact mechanism or gene for chromosome elimination in this technique is still unclear and needs further study. This will provide guidance for continuous improvement of the Wheat × Maize System and extend its application to other crops.

Based on these advancements, our laboratory has made an effort to optimize the maize genotype selection and haploid plantlet doubling treatment within the Wheat × Maize System. For maize genotype selection, we have transitioned from inbred lines to hybrid varieties and, after extensive screening, identified two hybrids suitable for greenhouse cultivation (Di Tian Nuo 336 and Wan Nong Tian Nuo 158) with high pollen production

and induction rate, achieving a maximum embryo rate of up to 40%. The haploid plantlet doubling process has been enhanced by treating them with 0.05% colchicine solution for 16 h, resulting in survival and doubling rates both exceeding 90%. The remainder of the procedure adheres to methodologies established by the previous researchers. With this updated procedure, we have provided excellent service to satisfy the breeding and research needs of Spring Valley Agriscience Co., Ltd., Jinan, Shandong, China. In the second half of 2023, we achieved an average embryo rate of 12.9%, an average plantlet rate of 51.8%, and an average doubling rate of 86.0%.

In the future, with the advancement of technology and in-depth research, the Wheat × Maize System will be further developed and applied. Firstly, the continuous optimization of DH line production techniques, driven by ongoing in-depth research, will render the DH line production process more efficient and stable. Secondly, this technique, in conjunction with greenhouse generation advancement technology and modern biotechnology such as marker-assisted selection, gene editing, mutation induction, transgene, genomic selection, etc., will further improve selection efficiency and accuracy, shorten breeding cycles, greatly enhance the efficiency of genetic improvement, and provide more possibilities for wheat genetics and breeding. Lastly, the improvement of equipment and facilities and the establishment of an industry-scale production procedure for the Wheat × Maize System will meet the demands of scientific research on wheat genetics and breeding and wheat production. In conclusion, the Wheat × Maize System possesses tremendous potential for development and will play, as a routine technique, a more significant role in research on wheat genetics and breeding.

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