Review Article

Is Doubled Haploid Production in Sorghum Impossible?

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Abstract
Doubled Haploid (DH) is an efficient tool in plant breeding programs. This method reduces time to homozygosity. DH production has been developed and routinely used in several species but not sorghum. The objective of this review is to provide an overview of DH production and the barriers in DH production of sorghum from previous studies on three potential methods for haploid induction; androgenesis, gynogenesis, and wide hybridization. The information could be valuable in the development of DH protocol in sorghum which may be employed in genetics and molecular studies.

Keywords: Haploid, Anther culture, Chromosome doubling, Plant breeding, Sorghum

Abbreviations

2, 4-D	:	2, 4-dichlorophenoxyacetic acid	KN	:	Kinetin
BA	:	N6-benzyladenine	MS	:	Murashige and Skoog's medium
BAP	:	6-benzylaminopurine	QTL	:	Quantitative trait locus
DMSO	:	Dimethyl sulfoxide	TDZ	:	Thidiazuron
IAA	:	Indole-3-acetic acid	ZN	:	Zeatin

1 Introduction

Sorghum *(Sorghum bicolor* (L.) Moench) is the fifth most important cereal crop after wheat, maize, rice, and barley [1]. The crop is generally used for food, feed, and raw material for biofuel production. It is estimated that the grain is used as food for more than 500 million people, in 30 countries in tropical and subtropical Africa and South Asia [1], [2]. As feed, the grain is a major ingredient in cattle, poultry and swine feed [2]. Sorghum provides raw material for many industrial products, such as malt, beer, syrup, gruels, starches, adhesives, core binders for metal casting, ore refining, and grits as packaging material [2]. Recently, sweet sorghum has been promoted as a bioenergy crop. Sweet sorghum accumulates large amount of carbohydrates in its stalk and produces a total biomass as high as 30 Mg ha^{-1} [3]. Stalk carbohydrates are easily converted to ethanol via fermentation of stalk juice. The pressed stalk by-product after juice extraction can also be used for electrical energy generation [4]. New techniques which are efficient, reliable, practical, and rapid can be integrated into sorghum breeding programs to increase genetic gain. The doubled haploid technology is useful in plant breeding as a shortcut to homozygosity in plants [5].

Haploids are individuals possessing a single set

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of chromosomes of the species (n), which is the number of chromosomes in a gamete [6]. Doubled Haploids (DHs) are haploid plants which chromosomes are doubled in order to become homozygous and gain genetic stability. Efficient DH production is an important tool in plant breeding programs, germplasm collection, genetics, and molecular studies. The generation time of plants is the major bottleneck in conventional plant breeding programs [7]. Induction to homozygosity requires at least 6–8 generations. DH event accelerates breeding programs by reducing time to homozygosity. After crossing between two parental lines, F_1 plants are obtained and advanced to the F_2 generation where maximum segregation is present. F_2 segregating plants will be immediately induced to homozygosity which reduces selection time of desirable traits to develop new cultivars (Figure 1). It was demonstrated that DH could reduce the time of breeding programs by 3–5 years [8]. In hybrid seed production, inbred lines can also be developed rapidly by using the DH system and they may be valuable germplasms for studying quantitative inheritance. In genetic studies, DH lines are ideal to directly obtain the recombination value between genes [9]. Moreover, haploids are very useful for induction of mutations because mutants with desirable traits will not be masked by dominant alleles, as in diploids [10]. Furthermore, the DH lines with 100% homozygosity are the best population for gene mapping and QTL identifications. DH lines are excellent populations to obtain reliable information on the location of genes and QTLs related to desirable traits which are important for crop improvements [11].

Haploid induction is the primary step of DH production followed by analysis of chromosome number to confirm haploidy and then chromosome doubling (Figure 2). The first haploid production with a cytological experiment was reported by A.D. Bergner in 1921 using Datura stramonium L. (Jimson weed) [5], [13]. Many methods for inducing haploidy have since been developed. The four major methods for induction of haploids are androgenesis (anther or microspore cultures), gynogenesis (unfertilized ovule and ovary cultures), wide hybridization (chromosome elimination from one parent of a cross) and parthenogenesis (development of an embryo from haploid cell of the embryo sac or the egg cell) [14], [15] (Figure 2). The efficiency of

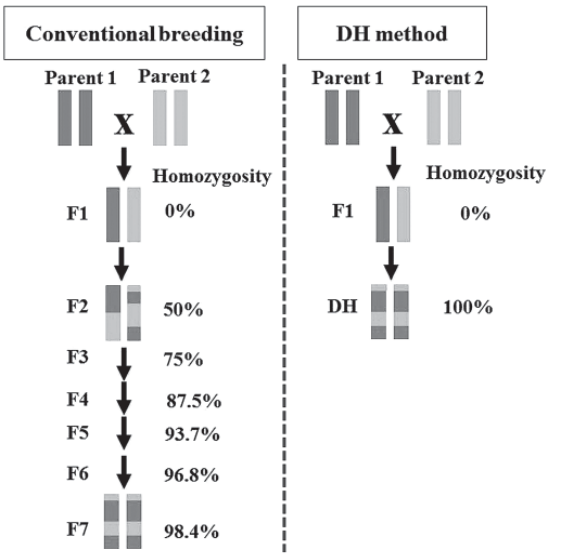

Figure 1: Strategy in applying DH production to plant breeding to obtain 100% homozygosity in one generation. Modified from [12].

each method varies depending on species [6]. Of the four available methods, parthenogenesis gives the lowest frequency of haploids, and the frequency is too low for plant breeding purposes [14]. The DH techniques have been well reported in several economically important crop species, including major crops in Poaceae and Brassicaceae families [9].

Common methods for DH production are anther culture and wide hybridization. Anther culture is often the method of choice for DH production because the simplicity of the approach allows large scale anther culture establishment and application to a wide range of genotypes. In comparison, the technique for isolated microspore culture requires sophisticated equipment and more skill than anther culture [16]. Development of DH protocols has been reported in almost all major crops, including important cereal crops in Poaceae family that are relative to sorghum such as wheat, maize, rice, and barley [17]. Surprisingly, DH protocols for sorghum are not available. This review provided three potential haploid inductions for sorghum; androgenesis, gynogenesis, and wide hybridization. Importantly, causes of barriers in DH production of sorghum from previous studies were discussed.

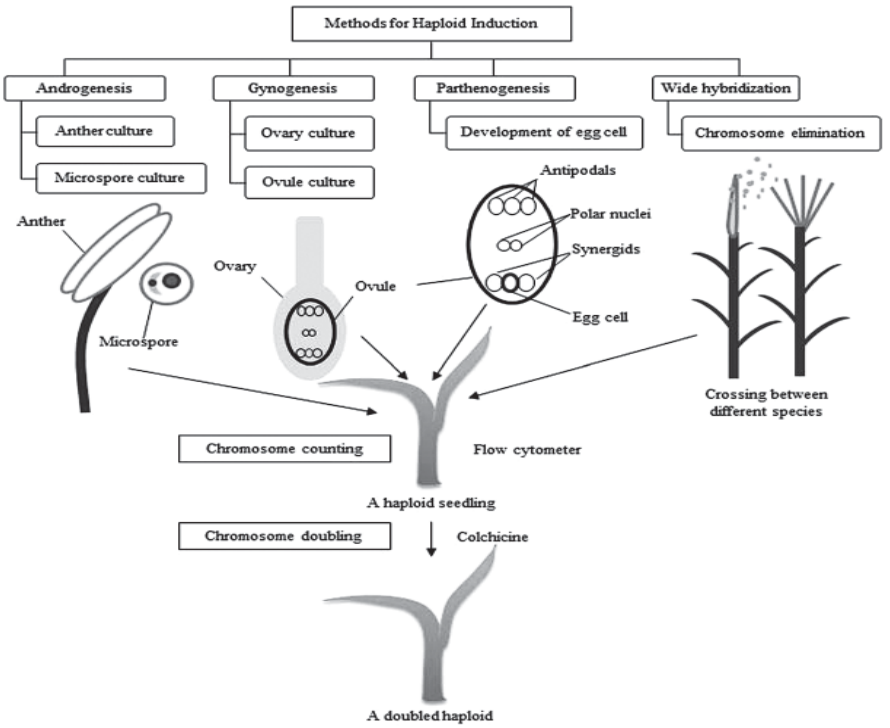

Figure 2: General DH production steps including; methods for haploid induction, chromosome counting, and chromosome doubling.

2 Methods of Haploid Induction

2.1 *Androgenesis: anther culture*

In vitro androgenesis via anther culture is the most preferred technique for haploid induction because of its simplicity [9]. Basically, anthers are cultured on a proper nutritive medium for callus induction. Then calli are transferred to a regeneration medium for the shoot and root development. In anther cultures of cereals, most of the regenerated plants are diploid at the high frequency of spontaneous doubling, so chromosome doubling application may be unnecessary [18]. Successful, regeneration from anther cultures has been reported in more than 200 species belonging to the Solanaceae, Cruciferae, and Poaceae families [16]. Haploids generated by anther culture of economic crops in Poaceae family were reported such as in rice [19], maize [20], barley [21], and wheat [22]. Many factors can affect the response of androgenesis via anther culture including genotype, microspore development stage, pretreatment of pollen, medium composition, and culture conditions [16]. Sorghum is one of the most difficult species for tissue cultures, especially using immature inflorescences as explants. The success has been restricted by low callus induction rate, low regeneration rate, pigment exudation or phenolic compounds from somatic cells, and albino plantlets [23]. Kumaravadivel and Sree Rangasamy [24] reported doubled haploid sorghum from anther culture using mid to late uninucleate pollen cultured in N_6 medium supplemented with 3.0% sucrose, 2.0 mg l^{-1} 2,4-D, 0.8% agar, and incubated at 30°C for callus induction. Then, calli were transferred to medium supplemented with 3.0% sucrose, 2.0 mg.l^{-1} BAP combined with 0.3 mg.l^{-1} IAA, and 0.8% agar for regeneration of embryos. The regeneration rate for that experiment was only 7.6%, and they were unable to reproduce their results [25]. Androgenesis

of sorghum is genotype dependent [24], [26]–[30]. Grain sorghum cv. C401, C2–97, and Tx403 were reported having a high response for tissue cultures [27], [31]. Moreover, sweet sorghum cv. R9188, Ramada, and Wray were identified as the three best from 32 sweet sorghum lines for embryogenic callus induction [32]. Genotype is an important factor for androgenesis not only in sorghum but also in most species. Ismaili and Mohammadi [20] tested embryo induction from anther culture of ten maize varieties in three media and found that each responsive genotype produced embryos in at least one of the three media used in the experiment. The result indicated that genotype background has more effect than medium [20]. Powell [33] reported large genotype by pre-treatment interactions for percentage responding anthers in barley. Siddique [34] investigated the impact of genotypes in four rice varieties and found that percentage of anther formed callus of the four varieties in N_6 medium varied from 1.0 to 4.5%.

The microspore development stage is an important step that has a strong influence on the success of anther culture [35]. The mid-uninucleate stage to the early binucleate stage is the optimum stage in most species for embryogenic response [14]. Sorghum panicles along with the enclosed leaf sheath is an indicator for mid to late uninucleate of microspore development [24]. Kumaravadivel and Sree Rangasamy [24] reported that the optimum incubation temperature for callus induction of sorghum anther culture was 30°C. Nguyen *et al*. [27] studied the physiological effects on anther culture of sorghum and reported that the rate of callus induction was high in young anthers, and decreased with maturation of the anther. They also pointed out that the physiological status of parent plants had a considerable effect on anther culture of sorghum. Callus induction was lower in the anthers collected from plants grown at 30°C than those from 20°C and 25°C.

The two most important factors affecting sorghum androgenesis are callus induction and regeneration media. A number of modified media for sorghum tissue culture have been reported to eliminate phenolics, resulting in improvement of callus regeneration rate. Recently, two potential protocols were reported. Dora *et al*. [26] reported an efficient callus induction protocol for sweet sorghum using immature inflorescence cultured on a modified MS-medium containing a combination of auxin and cytokinins. Using the MS-medium containing 2, 4-D 1.5 $mg.l^{-1}$, 0.5 KN $mg.l^{-1}$ and incubation in the dark at 25±2°C showed the best results with 100% of calli frequency in the genotype IS3477. For shoot regeneration, embryogenic callus was transferred onto regeneration medium containing 0.5–3.0 $mg.l^{-1}$ of BAP/ZN/KN/TDZ, which regenerated the embryogenic callus into plantlets. Liu *et al*. [36] established a robust tissue culture system for sorghum using immature seeds of three effective tissue culture cultivars including SA281, Tx430, 91419R. They optimized media to reduce phenolics, low generation frequencies, and short duration of callus generability by using various plant growth regulators. Callus induction rate was the greatest in Tx430 on medium M11AP (84.0%), which was a modified MS medium by Elkonin and Pakhomova [37] with 1 $g.l^{-1}$ L-asparagine, and 1 $g.l^{-1}$ L-proline. The best callus regeneration medium (72.6%) was B1I1C1 which was a modified MS with 1 $mg.l^{-1}$ BA, and 1 $mg.l^{-1}$ IAA.

2.2 *Gynogenesis: ovule and ovary cultures*

Gynogenesis is an alternative route to haploid plants using unfertilized egg cell or antipodal cells of female gametophyte [14]. Unfertilized ovule or ovary cultures can induce haploids arising either from the egg cell or the antipodal cells [38]. Comparing ovary and ovule cultures, ovary culture is simpler and gives greater regenerants than ovule culture because ovary culture reduces injury of the ovule from the dissection [38]. Unfertilized ovule and ovary cultures have been successfully applied to produce DH plants in many plant species, such as Beta vulgaris, Allium cepa, and Cerbera jamesonii, which are recalcitrant to androgenesis [38]. Unfortunately, the DH production from sorghum gynogenesis has never been reported. The failure of this method for sorghum may be attributed to inappropriate protocols. The most important factor affecting gynogenesis is genotype. The specific microspore stage (mid to late uninucleate) is very important for callus induction in androgenesis, but gynogenesis generally gives haploid regenerants cultured from a broad range stage of ovules [39]. However, there

have been reported that the optimal stage for gynogenesis in mulberry, barley, maize, and some species of onion, sugar beet, squash, sunflower, and gerbera is early flower developmental stages. In sorghum, fertilization occurs 2–4 hours after pollination [40]. Therefore the flowering stage before pollination may be an appropriate indicator to determine the unfertilized ovule stage of sorghum.

Considering media for gynogenesis, the media for gynogenesis vary among plant species [39]. In contrast to androgenesis, the macro and micro elements for gynogenesis are commonly used for micro-propagation [39]. The phytohormones that promote gynogenesis are general hormones like auxins and cytokinins, other hormones are less useful [39]. Mdarhri-Alaoui *et al.* [41] used ½ MS, 2 $mg.l^{-1}$ 2,4-D, 1 $mg.l^{-1}$ KN and 12% sucrose as a callus induction medium for ovary culture of durum wheat *(Triticum durum)*. The highest haploid induction frequency was obtained at 24.1% and 100% of haploids were generated. In lily (*Lillium longiflorum* L.), haploids were highly induced up to 45% on MS, 1 $mg.l^{-1}$ 2,4-D, 2 $mg.l^{-1}$ BA and 5% sucrose. Another difference from anther culture is that the majority of regenerants obtained via gynogenesis are haploid, so a treatment for chromosome doubling is needed [39].

Androgenesis and gynogenesis methods are based on tissue cultures. Protocols for sorghum tissue cultures have been developed for decades. Fortunately, efficient protocols have been recently reported which can minimize phenolics and increase regeneration rates [26], [36]. However, the efficient protocols were based on immature embryos [36] or immature inflorescence [26] as the explants. The efficient tissue culture protocols should be tested for separated anther, ovule, and ovary cultures. Moreover, genotype-independent media should be developed for sorghum tissue culture.

2.3 *Wide hybridization*

Wide hybridization or interspecific hybridization is a cross between different species. Haploids induced by wide hybridization is the result of the elimination of pollinating parent chromosome in early cell divisions following syngamy (the fusion of two cells) [42]. Wide hybridization method is sometimes referred to the bulbosum method because the first haploid produced using wide hybridization was induced from a cross between barley (*Hordeum vulgare*) with a wild species, *H. bulbosum* as the pollinator [5], [14]. Use of wide hybridization is one of the most effective methods for haploid production and has been used successfully in various species such as wheat [43], barley [44] and oat [45]. In Poaceae family, using maize as the pollinator has a reduced effect of genotype dependency, a high efficiency of haploids and few albinos [14], [23].

DH production using wide hybridization in sorghum has never been reported. However, a polyhaploid obtained from a hybrid derivative of *Sorghum halepense* (2n = 40) x *S. vulgare* (2n = 20) was reported by Duara and Stebbins Jr [46]. Sorghum was used as a pollinator in a cross between wheat 'Chinese Spring' (2n = 42) and grain sorghum 'S9B' (2n = 20) by Laurie and Bennett [47]. They found cytological evidence that sorghum chromosomes were eliminated and embryos contained only 21 wheat chromosomes. Inagaki and Mujeeb-Kazi [48] also reported that hybridization between wheat and sorghum resulted in elimination of sorghum chromosomes, which indicated that sorghum is a potential pollinator for wheat polyhaploid production.

Treatments after pollination have been reported to improve haploid induction rate. Bidmeshkipour *et al.* [30] reported that application of 2,4-D and silver nitrate after 24 hours of pollination improved frequency of embryos and haploid plants in haploid production of wheat via maize pollination. DH production using wide hybridization may need embryo rescue and embryo culture after fertilization, because endosperm may not be developed, and colchicine treatment is usually used for chromosome doubling [14], [23]. In DH production, wide hybridization is preferred to androgenesis and gynogenesis because it is the most practical procedure and cost effective. Haploids may occur without requirement of embryo rescue [49]. However, screening for effective haploid inducing pollinators in sorghum should be more tested. Wide hybridization between sorghum and species in Poaceae family may be the best choice.

3 Chromosome Counting

Chromosome counting is the step to confirm the success of haploid induction. An efficient ploidy

measurement method is required. There are various methods for identification of haploids including direct and indirect measurements [15].

Haploids can be directly determined using a cytological technique or flow cytometry. The cytological technique is a traditional method used for counting mitotic chromosomes on slides under microscopes. Basic steps of this method are collection of material, fixation and chromosome staining [50]. Young buds, leaves or callus can be used. However, root tips are the most convenient source for collection of mitotic cells [50]. This method is simple but tedious and time-consuming. The reliability of results decreases for species with very small genomes.

Flow cytometry analyzes the DNA content in cell related to the ploidy level using a flow cytometer which converts fluorescence signals of particles in the DNA suspension into digital values [51], [52]. Sample preparation for flow cytometry is easy by using beads to pulverise leaf samples then adding extraction buffer [9], [51]. The suspension is then filtered and incubated for 10–15 minutes before measurement by flow cytometer [9]. Flow cytometry is the predominant method among direct and indirect protocols for ploidy determination as it also gives detailed information about the existence of mixoploid tissues and their proportions. Furthermore, a great number of samples (200 or more per day) can be accurately analyzed. [53]. Johnston *et al*. [54] used sorghum as one of 14 potential reference standards for plant DNA content determination. DNA content of sorghum cv. Pioneer 8695 for a diploid genome (2C) is 1.74 picograms (pg) which is five-fold smaller than maize cv. VA35 (5.73 pg) [54].

Indirect determination of haploids is based on leaf stomatal density and size or pollen fertility. Stomatal density has a negative relationship with genome size and guard cell length [55]. Hight density of small stomata shows low ploidy level [56]. Pollen fertility is referred to pollen size and viability. Mishra and Gowswami [9] reported that pollen grains of haploid rice were smaller than DHs and did not take stains of potassium iodide indicating zero viability.

However, chromosome counting is not a drawback of DH production in sorghum. Haploid induction and plant regeneration are the major obstacles in sorghum. Several methods for chromosome counting mentioned above can be utilized for all plant species.

4 Chromosome Doubling

The efficient doubling of a set of chromosomes to reach homozygosity and genetic stability is very important for the success of DH production [18]. There are several agents used for chromosome doubling such as colchicine, oryzalin, amiprophos methyl, nitrous oxide, and growth regulators. Combinations of using the agents and pretreatments with low or high temperature can improve the frequency of chromosome doubling [18], [57]. Antoine-Michard and Beckert [58] reported more than 50% of fertile DHs of maize derived from using colchicine combined with cold shock pretreatment at 7°C in the dark for two weeks of anthers. Guo and Pulli [59] pretreated cultured microspores of *Phleum pratense* by heat shock at 31°C for 24 hours and used colchicine for chromosome doubling and the efficiency of chromosome doubling was high as 66%.

Almost all doubling chromosome protocols for all crop species are based on colchicine [6], [18]. Colchicine is an alkaloid extracted from plants of the genus Colchicum. [18], [60]. Colchicine is a spindle inhibitor, which acts on dividing cells. It inhibits the polymerization of the spindle fibers in metaphase during cell division, avoiding the migration of the chromosomes to the cell poles, resulting in chromosome duplication after cell division [6]. The frequency of diploidization varies with the concentration used, plant parts/cells treated, and developmental stage of the tissue [18]. For chromosome doubling, the roots of haploid plants were treated for 7.5 hours with a 0.1% colchicine solution supplemented with 40 g dm-3 (DMSO), a drop of Tween and 0.025 g dm-3 (GA3 dimethyl sulfoxide gibberellic acid). Then the plant roots were washed in running water for 48 hours [61]. Usually, colchicine treatment, for doubling chromosome, is applied by soaking roots under the 0.06% colchicine solution along with 0.50 to 0.75% of DMSO for 12 to 17 hours [6]. However, colchicine is a high biotoxin [60], [62]. Amiprophos methyl and pronamide were recently reported as colchicine alternative in maize [63], [64]. Chromosome doubling using those chemicals should be considered for sorghum. Spontaneous chromosome

doubling usually occurs on regenerated plants from tissue cultures. In anther cultures of cereals, most of the regenerated plants are diploid [18]. So, chromosome doubling treatment may be unnecessary.

5 Conclusions

Sorghum is one of the extremely recalcitrant species for tissue culture. Development of efficient protocols for plant regeneration is the first strategy for the success of the DH production. Anther culture and ovary culture are the potential alternative procedures for the haploid induction under the appropriate culture conditions. The optimum stage of microspore and ovule development, and pretreatment of inflorescences with stresses should be considered. Wide hybridization is another potential method. However, more screening for effective pollinator species to induce haploids of sorghum is necessary. To improve haploid induction via wide hybridization, studies on effective treatments for pre and post-pollination are required.

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