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A review on double haploid breeding in modern crops

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Abstract
Classical breeding introduced a number of methods for increasing of genetic gain and reduction of resources required for efficiency improvement. In this regard, the discovery of double haploid breeding in the early 20th C. is one. Double haploids are plants generated through doubling haploids. Haploids can be induced through natural and artificial means including in vivo and in vitro techniques. Spontaneous induction is preferred to be the best for the reason of not using no rigorous methods but it happens to be very rear event. The artificial methods are better in generating a greater number of progenies but they applied in limited crops and challenges like lack of endosperm with low induction rate are observed in such methods. Doubling of chromosome mostly handled by chemicals especially colchicine is widely used but it is known to be carcinogenic. Other chemicals and methods are also used but colchicine remains to be widely used. The main application of haploid breeding is shortening the breeding cycle effectively and increasing selection efficiency. It also used in reverse breeding, QTL studies and CMS generations. Although DH come up with multiple benefits, it needs high establishment cost and challenged with small amount of induction rate. Therefore, using DH breeding in combination with molecular and conventional techniques of breeding will increase it efficiency of contribution in more number of cultivated modern crops.

Keywords: haploids, double haploid, genetics, classical breeding

Introduction

Double haploids are plant that their chromosome is doubled after their chromosome induced to be haploid which have a great advantage in shortening the cycle of breeding and easy selection of parents in the course of variety development. The discovery of haploid first observed in Datura stramonium specious in early 20th C. in 1922 (Segui-Simarro, 2015) [18]. Then this phenomenon is observed in number of other specious which revolutionized the conventional breeding in 1964 and the following years by improvement of this technique (Dwivedi *et al*., 2015) [8]. Wide range of techniques are used in double haploid induction both in invitro and in vivo. Also, natural phenomena existed in spontaneous haploid induction and doubling of chromosome. These options are preferred over the artificial methods because they used to reduce the resource and negative effects of chemicals. But they are not frequently used because of their rear occurrence (Maqbool *et al*., 2020) [11]. In in vivo techniques, different methods are developed using maternal and paternal parents. Mostly maternal parents are used for the induction and paternal parents are used as an inducer. Paternal induction used when maternal is not working. Chromosome elimination is one the possible explanation in in-vivo induction which most of the scientists agree. Also, irradiated, heated and nonfunctional pollens are used (Abenezer, 2017) [21]. This is method is first used in barley by using other specious called H. bolbousum where the method called by its name. It is also used in other specious in tobacco and potato. There are also other interspecific hybridizations in wheat, oat, maize, brassica and the hybridization maize with wheat and barley is very popular which resulted for the release of number of varieties (Begheyn & Studer, 2016) [4]. The intraspecific haploid induction by the parent called stock6 resulted in the development of varieties, haploids and more improved inducers. Beside that in vivo paternal induction contributed to production of cytoplasmic sterile male parents in addition to the haploid which has a great contribution in easing of the conventional breeding (Dwivedi *et al*., 2015; Abenezer, 2017) [8, 21]. The other induction method is invitro which used anther, pollen, microspores and ovary to produce haploids. In this method haploid induction is not needed, it simply uses the haploid sources to induce embryo followed by doubling by natural and artificial method like colchicine. In this method, embryo rescue is used in situation where endosperm formation is not possible which is also used in some of invitro inter specific hybridization like wheat and maize. Conversely to in vivo induction here mostly androgenesis or paternal induction is used and gynogenesis used when androgenesis is not possible. The success of this induction is dependent on genotype, stage of development, culture composition, pretreatment of flower and environmental conditions (Maqbool *et al*., 2020; Abenezer, 2017; Murovec & Bohanec, 2012) [11, 21, 14]. In addition, transgenic approach is used in haploid induction using centromere binding protein but till now its application is limited to Arabidopsis (Ren *et al*., 2017) [16]. Chromosome doubling is used both by natural and artificial means. The natural means very small but efforts are made for its improvement and some improvements are made. But the artificial chemical colchicine is the most used in chromosome even in animals but it is carcinogenic. The other chemicals used are oryzalin, amiprophosmethyl (APM), trifluralin and pronamide. In addition, high pressure gases are used (Maqbool *et al*., 2020; Murovec & Bohanec, 2012) [11, 14]. Double haploids have great contribution in aiding the conventional breeding in shortening the time, resource and increasing of efficiency. They contributed for variety release, cytoplasmic male sterility, mutation induction, Genetic transformation and QTL discovery.

Moreover, forward and reverse breeding are their other contribution in back tracing of the parents of hybrids (Maqbool *et al*., 2020; Abenezer, 2017; Weber, 2014) [11, 21, 24]. Although, DHs are come up with various advantage there are challenges in haploid induction, doubling, wide applicability in cultivated crops, high establishment cost and carcinogenic effects of chemicals used (Abenezer, 2017; Murovec & Bohanec, 2012 Baenziger, 1996) [21, 14, 1].

Reviewed Topics

What is Double Haploid?

Double haploids are plants that have two sets of chromosomes created through doubling the haploid chromosome by natural or artificial mechanisms (Maqbool *et al*., 2020; Tefera *et al*., 2017; Begheyn & Studer, 2016) [11, 21, 4]. Different techniques are used to brought double haploids from different tissue sources of the plants including anther, pollen grain and others. This technique is used to attain homozygosity with in short period of time to accelerate the breeding and it is applied in a more than 200 plant specious. But its application is most notable in brassicas and cereals (Segui-Simarro, 2015) [18]. Also, some specious like tomato, cotton, grape, trees and medicinal plants considered recalcitrant (Begheyn & Studer, 2016) [4]. The plants generated from DHs are homozygous or fixed for both recessive and dominant alleles but before generating, selection and regeneration of haploids is required. Then doubling the chromosome and regeneration follows (Murovec and Bohanec, 2012; Ferrie and Caswell 2011; Baenziger, 1996) [14, 1]. Haploid plants are plants that contain single set of chromosomes resulted by spontaneous or artificial induction (Maqbool *et al*., 2020; Murovec & Bohanec, 2012) [11, 14]. Haploids are discovered first in the spontaneous development of Datura stramonium in 1922 followed by similar discoveries in tobacco, wheat and several others. Since spontaneous mutation is occurring rarely, their application is not reliable. Haploid breeding started with the same plant in 1964 by invitro induction of anther. After that through discoveries of different techniques of induction it is applied in different crops including brassicas, maize and barley. The discovery of interspecific hybridization followed by embryo culture widen the application of DH in plant breeding (Dwivedi *et al*., 2015; Burbulis & Kott, 2013; Murovec & Bohanec, 2012) [8, 5, 14].

Haploid induction/Generation Techniques

Haploid can be obtained from diploid specious which are called monoploid or from polyploid specious called poly-haploids. There are different possibilities of haploid induction. Haploid can occur naturally through spontaneous induction or different artificial induction techniques which can be invitro in the laboratories or in vivo with in the natural environment. In vitro induction method includes different techniques using culture of immature male or female gametophytes. Microspore embryogenesis is known to have high potential in invitro induction due to higher frequency of DH outputs compared to other techniques because of the availability of higher number of microspores with in single flower. But factors such as rate of embryogenesis, regeneration, frequency of albinism and chromosome doubling affects its application. In a condition or specious where androgenesis is not possible like sugar beet and onion, ovule culture is used. But its efficiency is much lower because of the availability of small number of ovules in a flower. Crops such as onion have a greater number of ovules that are suitable for gynogenesis (Abenezer, 2017) [21].

Spontaneous Induction

Spontaneous induction of haloids has been reported in crops like cotton and maize. Especially in maize lines, higher proportion of induction were observed specifically in Stock 6 which was extensively used in different maize breeding programs. The induction of meiosis in somatic cells was proposed to be the cause of the induction of haploid at cellular level. In maize, lines producing few pollens were found to be suitable for in situ gynogenesis. It was also observed that delayed pollination may enhance spontaneous parthenogenesis. Molecular studies indicate ggi1 (gynogenesis inducer 1) gene in maize is a major locus controlling in-situ gynogenesis which is linked to gynogenetic induction with incomplete penetrance. Another haploid inducing gene in maize is ig gene which inhibits embryogenic potential of the cell which results haploid maternal and paternal origin (Maqbool *et al*., 2020; Ren *et al*., 2017) [11]. Although spontaneous haploid induction has some application and contribution in the crop improvement, its occurrence is very limited. Therefore, artificial induction should be exploited more for wide range of application with more number of crops. For this, techniques such as in-vitro and in vivo can improve the efficiency of induction.

In-vivo Induction Method

In-vivo induction of haploids is paternal or maternal induction which involves uniparental elimination of genome by crossing the plant to be induced with inducers that are close (intra specific) or distant (inter specific). In addition, irradiated, heat treated and nonfunctional pollens are used to induce the artificial induction (Begheyn & Studer, 2016) [4]. The method called bolbosum was the first method used in the interspecific hybridization of barley. Other specious in the Hordeum are also reported to induce haploid by uniparental chromosome elimination in early development stages of hybrid embryo. It is also used in tobacco and potato. Moreover, more distance crosses were made by coupling crops like pear, apple, Avena sativa, maize, Triticum turgidum, Triticum aestivum, and pearl millet to generate double haploids. The preferred production of wheat DH lines was the wide cross between wheat with maize and barley (Dwivedi *et al*., 2015) [8]. It was reported that it is the most commonly used induction method in wheat which resulted for the releasing of heat tolerant lines. DH oats can also be generated by making crosses with maize which lack the formation of endosperm. After the cross dicamba spray, embryo rescue and colchicine treatment will follow (Abenezer, 2017) [21]. Embryo rescue also used in DH wheat made by crossing with maize (Begheyn & Studer, 2016) [4].
Other than interspecific hybridization, intra specific hybrids are also used for in-vivo haploid induction. Haploid inducer line that are routinely used to induce about 10% of the kernel of the parent is most common in maize breeding (Begheyn & Studer, 2016) [4]. A haploid line inducer called Stock6 revolutionized DH production in maize. The haploid produced could be induced in maternal or paternal parents where in paternal haploids, the pollinator is induced to be haploid and the female parent acts as inducer and vice versa (Maqbool *et al*., 2020). A number of haploid inducers have been developed from Stock6 in Russia (RWS) and Germany

(UH400) for temperate region. Also, CIMMYT with collaboration of university of Hohenheim developed tropical inducer with 8 to 10% haploid induction rate (Abenezer, 2017) [21].

In-vivo Maternal Induction Method

Maternal haploids can be generated by in situ induction with the pollen of same specious like maize, irradiated pollen, pollen of wild relative like potato and barley or related specious in wheat. After pollination fertilization of egg cell and development of embryo follows with parental chromosome elimination (Murovec & Bohanec, 2012) [14]. There are two hypostases regarding chromosome elimination in maternal haploid induction. One says single fertilization while the other hypothesize postzygotic genomic elimination (Maqbool *et al*., 2020) [11]. Then the development of haploid embryo continues by pollination of polar nuclei and endosperm development. Irradiated pollen pollination is the other techniques of maternal haploid induction. In this technique, irradiated pollen is pollinated to the female flower and pollen germination and growth proceeds on the stigma and style respectively. But the pollen will not able to fertilized since it is sterile by irradiation. Haploid induction through this method labor intensive since it needs emasculation of the original parents. Cytoplasmic male sterility is used to overcome such challenges in crops like onions but it has very limited potential use. There are also issues related to the dose of irradiation which results complete loss of pollen or partial fertility (Murovec & Bohanec, 2012) [14]. Beside generation of haploids, in-vitro embryo rescue is needed for most specious because of inefficiencies in formation of endosperm. According to Murovec & Bohanec (2012) [14] the collection mature seed only reported in onion, mandarin, kiwifruit and specious of Nicotiana. Also, embryo rescue is reported to enhance the recovery of haploid plants. Intraspecific hybridization to induce in-vivo haploid is common in maize (Maqbool *et al*., 2020) [11]. Haploid inducers parents are used which are specialized genetic stocks resulting an average of 10% haploid maternal kernel (Abenezer, 2017) [21]. The first recognized inducer is Stock6 in 1959 with the induction rate of 2.3% which was subsequently improved through different techniques of hybridization and selection. The genetic improvement results the induction rate of 8 to 10% in temperate inducers lines such as WS14, RWS, UH400, BHI306 and CAU5 (Maqbool *et al*., 2020; Murovec & Bohanec, 2012) [11, 14]. Recently, tropically adapted haploid inducer lines (TAILs) such as TAIL7, TAIL8, TAIL9 and TAIL8 x TAIL 9 are also developed by the collaboration of CIMMYT and university of Hohenheim. The advantage of in-vivo maternal induction using inducer lines is it do not require any in-vitro method to rescue embryo (Maqbool *et al*., 2020; Abenezer, 2017) [11, 21].

In-vivo Paternal Induction Method

In paternal haploids, the pollinator is the genome donor and the female parent acts as the haploid inducer. It is the composition of paternal chromosome and maternal cytoplasm. Naturally haploid induction is a very rear event which occurs one in thousands. Its occurrence affected background of paternal and maternal parents. Such induction may result substitution or hybridization of cytoplasm which ultimately lead to cytoplasmic male sterility (CMS). Such inducer could be exploited in manipulation of CMS lines for genetic improvement (Maqbool *et al*., 2020) [11].

Interspecific Hybridization Induction

One of the effective in-vivo haploid artificial induction methods which found to effective is distant or wide cross hybridization of species. This method of induction has been successful in several modern cultivated specious. This process first discovered in barley in cross between H.vulgare and H. bulbosum. Then it was also found in the hybridization between wheat and maize. Maize is the most used and effective inducer plant in most of modern cultivated crops such as triticale, rye and oats. Also, interspecific induction was successful in the hybridization between other crops wheat X pearl millet, pear X apple and Triticum aestivum X Triticeae species. It uses gametic lines involved both in interspecific and intergeneric crosses (Ren *et al*., 2017) [16]. Parthenogenic development of haploid embryo occurs through fertilization of polar nuclei and development of functional endosperm. In case of postzygotic induction, after fertilization paternal chromosomal elimination will follow. The example crop for the former can be potato while for the latter barley could be the good one (Abenezer, 2017; Ren *et al*., 2017; Murovec & Bohanec, 2012) [21, 16, 14]. Interspecific induction could be supplemented by in-vitro germination in case of endosperm failure like the cross between maize/wheat and H.bulbosum/Barley. The mechanism that causes selective chromosome elimination in interspecific pollination not discovered till know but different in timing of mitotic process, parent specific inactivation of centromeres, asynchrony and many more hypostasis laid for explanation. Chromosome elimination also found to be happened in exra fertilization organ in rapidly dividing endosperm which results abortion of seed development (Ren *et al*., 2017) [16]. In addition, genetic and environmental conditions such as light and temperature found to affect the frequency of haploids.

The study on barley shows CENH3 has an important role in chromosome elimination by replacement the standard histone H3 by histone variant in centromeric nucleosomes which determines the position of centromeres necessary for segregation during cell division. The H. bulbosum centromere has no activity during anaphase leading to chromosome elimination and H. vulgare haploid embryo development (Watts *et al*., 2016) [23].

Intraspecific Hybridization Induction

The hybridization within specious has also found effective in induction of haploid in some species. This method is very predominant in maize after the discovery of stock6 which was discussed in the previous sections. Haploid induction largely relies on dominant marker gene called R1-nj (purple scutellum and aleurone). An oil content marker for rapid identification, high throughput system was also proposed (Wang *et al*., 2016; Melchinger *et al*., 2013) [22, 25]. Different genes and QTLs had been reported in intraspecific induction. A major locus ggil causing in situ gynogenesis and segregation distortion that showed the potential of pollen in maternal induction. Also, two QTLs (qhir1 and qhir8) with the larger effect that shows segregation distortion was also found by Prigge *et al*. (2012) [15]. A narrowed down genome region study by Xu *et al*., (2013) and Dong *et al*., (2013) [7, 25] showed sed1 expression among

pollen grain from sed1/sed1 and postzygotic frame shift mutation in MTL (gene contributing for haploid induction) by Kelliher *et al*., (2017) [10].

Invitro Induction Method

In-vitro culture inductions involves the use of anther, microspores and ovaries in the media with high totipotency for production of haploid plant through use of unfertilized gametes (Maqbool *et al*., 2020) [11]. Androgenesis is the process of induction and regeneration of haploids and double haploids originating from male gametic cells. It is well established procedure in genetic improvement of modern crops like barley, wheat, maize, rice, triticale, rye, rapeseed and brassica specious. The production of haploid embryo that can also be achieved with in-vitro culturing of unpollinated flower parts such as ovule, placenta attached ovules, ovaries and whole flower bud is called gynogenesis and it is exclusively from female gametophyte (Murovec & Bohanec, 2012) [14]. Anther and microspore culture is a popular approach in number of specious but gynogenesis is used in crops where the previous methods are not effectively functional. Gynogenesis is not frequently used for the reason lower frequency of haploid induction because of lower number of female reproductive organ compared to pollen or anther (Maqbool *et al*., 2020) [11]. But gynogenic regenerant show higher stability and lower rate of albino plants. It is successful in crops like onion, sugar beet, squash, sunflower, wheat and barley (Murovec & Bohanec, 2012) [14]. Generally, in-vitro generation involves for major stapes for induction first microspore extraction or female flower part, then embryogenesis and embryo germination and finally chromosome doubling (Burbulis & Kott, 2013). The regeneration of plant brought through haploid tissues works for some species but for some others none of the methods are not effective (Dwivedi *et al*., 2015) [8]. For example, haploid rapeseed can be produced through culturing of pollen grain or microspore in the laboratory. Isolated was also successfully initiated in Brassica napus (Burbulis & Kott, 2013). Also, androgenesis method is preferred in most of the oil seeded crops. In the production of DH flax, culturing through gyno and androgenesis yielded calli which is regenerated to haploid and finally to DH plant but further improvement was suggested (Ferrie & Caswell, 2016) [9]. The success of in-vitro culture induction influenced by genotype, growth condition, stages of development, culture composition, pre-treatment of flower buds and environmental condition (Ferrie & Caswell, 2016) [9]. For example, genotype dependent regeneration was observed in onion which varies between 0 and 51% (Murovec & Bohanec, 2012) [14]. The application of physiochemical factors such as temperature, nitrogen starvation and osmotic stress that induce stress are found to trigger embryogenesis. The application of temperature for several hours/days used in crops like barley, wheat, maize, rice and rye using low temperature and high temperature used in rape seed, wheat, brassica specious and tobacco. In tobacco heat shock is effective in triggering microspores followed by nitrogen and sucrose starvation for formation of embryo. In addition, several triggering factors such as irradiation, colchicine and auxin used for reprograming of microspores (Murovec & Bohanec, 2012) [14]. Beside stress, culture media composition of micro and macro nutrients are systematically used.

Although in-vitro culture mainly used for haploid induction, it is also the source of soma clonal variation where the incidence of such case can be increased by the application of mutagens to the haploid cells. The effective stage for optimal application is shortly after extraction in single cell stage which insures homogeneity of embryo (Burbulis & Kott, 2013).

Although in-vitro culture has wide application and successful across specious, it should be noted that it is highly technical, labor intensive, costly, genotype and species dependent. Moreover, lower rate of embryogenesis, high frequency of albinism, segregation distortion and low frequency of doubling are the constraints related to this method (Dwivedi *et al*., 2015) [8]

Haploid induction by Genetic Engineering

A transgenic approach for induction haploids has been developed centromere chromosome of Arabidopsis. Centromere in the chromosome used as binding of kinetochore proteins which spindle microtubules bind during chromosomal cycle. In the new method Cenh3 kinetochore protein was disabled by genetic transformation. Also, CENH3 protein was disabled and substituted by similar mutant which cause the elimination of chromosomal set of the inducer during mitotic division of zygotic cell. In activation of CENH3 was applied by RNAi interference. The concept of this method resembles the chromosomal elimination of interspecific wide crosses of wheat/maize (Maqbool *et al*., 2020) [11]. The inducer line developed by this method used to induce both maternal and paternal haploids. The procedure is successful in induction of up to 50% F1 haploids. Although some efforts are ongoing in some crop specious, it is demonstrated successful only in Arabidopsis thaliana. The issue of transgenic in this method could be a possibly raised but it should be noted that the transgenic is only the inducer which used to induce the normal non transgenic plant. The final product is completely transgenic free haploid plant (Maqbool *et al*., 2020; Ren *et al*., 2017; Murovec & Bohanec, 2012) [11, 16, 14].

The other discovery on B chromosome by Zhao *et al*. (2013) showed extra chromosome can be transferred of haploid inducer of maize can be transferred to haploid progenies. This method also applied wide crosses of oat and maize.

Factor Affecting Haploid Induction

Although haploid breeding has known to have numerous advantages that cannot be applied by other breeding methods, its success is limited to the induction of 8 to 10% in some methods up to 50%. This is very small compared to the expected with all the investment including human and physical capitals. There are a plenty of factors limiting its success. It depends on developmental stage of flower, culture media, genotype, doner parent growth condition and haploid detection methods (Abenezer, 2017) [21]. In invitro induction the genotype of the doner plant determines the efficiency and the response varies within and among specious. Barassica napus is more responsive for microspore culture compared to Brassica juncea even with in napus winter genotypes are more responsive in comparison with spring genotype. Similarly, hexaploid bread wheat is more responsive than tetraploid durum wheat. Also, in bread wheat winter genotypes are more responsive. Moreover, genotype by bud size and by doner growth interact has an impact on haploid. Even androgenesis responsive for few specious controlled by pollen specific gene. The kr inhibiter

gene in wheat genotypes inhibit pollen tube growth Hordeum bulbosum whereas this phenomenon is not observed in wheat/maize hybridization. Plant age and physiology have an impact, plant at the beginning of flowering is suitable. Conversely, in Brassica napus and Brassica rapa old pollen yield will produce more embryo than young and heathy plant. Anthers from primary tiller in most cereals more responsive except rice. Growth condition of both doner and inducer has also an impact. Temperature and light influence the induction and DH regeneration as well (Dwivedi *et al*., 2015) [8].

In invitro culture, the induction and regeneration of embryo is dependent of genotype, physiological conditions, developmental stages of gametes, microspore and ovules, pre-treatments, media composition and physical factors like light and temperature (Murovec & Bohanec, 2012) [14]. Also thickness anther and bud size also influence the development and induction (Dwivedi *et al*., 2015) [8]. Also, filial generation, population size and competitive advantage of DH determine the cost and success of conventional breeding. In addition, early-stage homozygosity may also limit the opportunity of recombination. The study on different filial generation of barley found induction at F2 and F3 brings more genetic gain but population size should also be determined (Tadess *et al*., 2019) [19].

Development of Haploid Inducers

After the observation of spontaneous induction of haploid in 1969 by Chase which was a rear event (0.001%), Coe, (1959) [6] developed an inbred line called stock6 which has a much higher induction rate (3.23%) than the spontaneous counterpart. The development of Stock6 in maize open doors for the development more inbred line inducers suitable for temperate and tropical agricultural zones in India, Soviet Union, France, China and Germany. A German cross made by KEMS (Russian) and WS14 (French) resulted the most effective inducer line called RWS which was developed at University of Hohenheim, Stuttgart. It has about 8% haploid induction rate (HIR) which was a breakthrough. An induction rate increased to 9 -10% through reciprocal crosses of the same parent. The explanation for haploid induction through in vivo inducers was, one is abnormal fertilization which results due to the failurity of one sperm to fertilize an egg and the creation endosperm due to the fusion two polar nuclei and the other sperm. The other is chromosomal elimination as explained earlier in wide crosses (Maqbool *et al*., 2020; Abenezer, 2017; Weber, 2014) [11, 21, 24]. Evidence of chromosome elimination in the first 20 days was observed in self-pollination of RWS lines. Lagging chromosomes and micronuclei was observed in mitotic cells ovule of HZ11 pollinated by Stock6. The phenomena of chromosome elimination were also observed the cross of RWS with five other lines. In some instances, there may be a possibility of having a small portion chromosome of the inducer in some induced haploids. It was found 1-2% of haploid maize produced by RWS. This was also found in barley with Hordeum bulbosum after several days of pollination (Weber, 2014) [24].

Identification of ploidy level

One of the challenges during the development of double haploids is identification the haploids induced since a mixture of the two is produced by the induction process. There are a number of methods of identification including morphological and molecular markers but morphological markers found to be less effective because of their nonexistence in some of the genotypes. Using phenotypic markers, early-stage identification is preferred for production of DHs to apply the doubling as early as possible (Abenezer, 2017) [21]. Anthocyanin color of R1-nj gene is a popular marker used especially in maize. Haploid shows pigmentation only in the endosperm while diploid shows the color on both the embryo and endosperm. Vanous *et al*. (2017) used red/purple coloration marker on the root where haploid showed colorless white root while diploids showed both colors in their root. Combining other morphological markers such as seed size and molecular marker could bring more accurate identification since the expression of these color depends on the genetic background of the genotype. Because complete inhibition of R1-nj gene was found frequently (Maqbool *et al*., 2020; Abenezer, 2017) [11, 21]. Seed weight and oil content are the other morphological markers where haploid kernels have less oil and seed weight (Baenziger, 1996) [1]. A similar approach in potato also used based on homozygous dominant color marker gene by pollinator. Purple spot embryo shows the possession of genome of the pollinator but it is not able to identify the hybrid (Murovec & Bohanec, 2012) [14]. Beside the above, morphological markers such as plant height, leaf dimension, flower morphology, radicle length, plant vigor, pollen viability, stomatal count and other seedling traits are used. These methods are unreliable because their high subjectivity to environmental effects but they are advantageous in terms of their cost-effective identification (Maqbool *et al*., 2020; Dwivedi *et al*., 2015; Murovec & Bohanec, 2012) [11, 8, 14]. A use of herbicide is also another option which the haploids are very sensitive compared to the F1 hybrid and diploids (Maqbool *et al*., 2020) [11].

In addition to morphological markers, direct ploidy identification though chromosome counting and flow cytometry identification are found reliable and fast methods (Maqbool *et al*., 2020; Dwivedi *et al*., 2015) [11, 21]. Measurement of DNA content using flow cytometry provides a rapid and simple option for large scale determination in early in vitro culturing phase (Murovec & Bohanec, 2012) [14]. Green florescence is also used in identification of transgenic markers to detect genes in haploid induction (Maqbool *et al*., 2020) [11]. Also, DNA markers are fast, easy and accurate in identification. The study by Battistelli *et al*., (2013) [3] shows the result obtained by SSR markers equally comparable with flow cytometry. The study on maize using SSR markers effectively discriminated the haploids, the induced homozygous and diploid individuals in the haploid induction processes (Ribeiro *et al*., 2018). Similar studies confirm the validity of SSR markers. Although, such markers are highly efficient, they require specialized skilled, cost and specialized techniques (Maqbool *et al*., 2020) [11].

Although diploids are not need in the haploid induction spontaneous doubling may also occur which can avoid the need of chromosome doubling. For this reliable technique is needed which can increase the efficiency of DH formation especially for commercial production. Depending on their availability, markers can be used to asses this. DNA markers such as AFLP, RAPD, SCAR, and SST are used to asses plant origin and homozygosity test. Co-dominant molecular markers will be useful to discriminate the heterozygous

(Murovec & Bohanec, 2012).

Genetics of Haploids

Genetic studies indicated that haploid induction is polygenic traits which is controlled by few major gens and lots of miner genes. The study on Stock6 showed the induction is controlled by a few major dominant genes. Multigenic trait control of maternal in vivo haploid induction was also reported in maize (Dwivedi *et al*., 2015) [8]. QTLs located on chromosome 1 in ggil locus was found to be involved in situ gynogenesis and segregation distortion. QTLs found chromosome 1and 3 qmhir1 and qmhir2 contributed the highest variation for maternal genotypic induction in maize. Also, in brassica specious Loci with additive effects known to control segregation distortion. A major and minor effect of QTLs was also found in genetic study of haploid maize. Among those qhi1 and qhir8 explained the highest genetic variations. The cross-validation study in different populations indicated the major effects of the two genes across population. qhir1 showed higher segregation distortion associated with failure to transmit the inducer gamete and also fixed nature of qhir1 gene in the parent also explains the genetics haploid induction. Genes that restrict the first and meiotic division reported in chromosome 4A, 3A and 6A of Langdon background durum wheat (Maqbool *et al*., 2020) [11].

Earlier study indicates ig1 gene in maize produces high frequency of both paternal and maternal haploids. The paternal haploid has more significance other than haploid aquation in generation CMS lines (Weber, 2014) [24]. A number of QTLs androgenic and gynogenic responsiveness of crosses wheat, rye, barley and other modern crops have been studied (Dwivedi *et al*., 2015) [8]. Also, it was reported that frame shift mutation in MTL gene during the postzygotic activity are one of the causes for haploid induction (Maqbool *et al*., 2020) [11].

One of the challenges of haploid induction is albinism in small grain cereals like wheat and barley. Nuclear genome which interacts with plastid development is the cause for the lack chlorophyll in albino plants. QTLs for green plant lets found in barley, rye, triticale and wheat (Dwivedi *et al*., 2015) [8].

Chromosome Doubling

Doubling chromosome happen to be the natural means which is spontaneous by its self or artificial doubling agents. Each of them has their own advantages and drawbacks let us see them one by one. Androgenesis has shown potential in spontaneous chromosome doubling (SCD) with the rate up to 46% in maize. In female in-vivo induction up 94% was observed in one study (Maqbool *et al*., 2020) [11]. Culturing is known to be one of the sources of haploid induction but sometimes undesirable mutation may result. Maternally derived haploid always has less SCD (Murovec & Bohanec, 2012) [14]. Early DH in vivo generation in vivo which exhibit complete fertility was found in maize. Such event could be exploited in practical breeding by increasing the rate, using the genetic diversity and determining the process (Ren *et al*., 2017; Dwivedi *et al*., 2015) [16, 8]. A three step was successful in SCD of wheat by using miotic restriction of interspecific hybridization and SCD by selfing F1 (Dwivedi *et al*., 2015) [8]. The other more interesting event in hypoploidy is haploid male fertility (HMF) which occur more frequently in female. Studies indicate that pollination of female haploid with diploid pollen results kernel development. Initially, HMF was not more than 10% but recent studies reported up to 65% fertility of haploids in certain genotypes. This can also be exploited by using the present genetic diversity and improvement to avoid the effect of chemical and reduce the cost of DH breeding (Maqbool *et al*., 2020) [11]. Although SCD and HMF known to reduce of breeding and undesirable chemical effect, there application is limited and impractical due to the infrequent occurrence.

The use of artificial chromosome doubling becomes feasible because spontaneous chromosome doubling (SCD) is a rear event in realizing the exploitation of double haloids in plant breeding. Various methods have been applied in doubling of chromosome but the most used chemical is colchicine an anti-microtubule drug extracted from crocus that inhibits microtubules polymerization by binding to tubulin (Dwivedi *et al*., 2015) [8]. Also, it happens to be more efficient in animal than plant tissues and widely used. Other options are oryzalin, amiprophosmethyl (APM), trifluralin and pronamide, all of which are used as herbicides and are effective in micromolar concentrations. Anti-microtubule drugs might be applied at various stages of androgenesis, such as being incorporated into microspore pretreatment media (Maqbool *et al*., 2020; Abenezer, 2017; Murovec & Bohanec, 2012) [11, 21, 14] Colchicine is applied after regeneration of embryo, shoot or plantlet level. Application after acclimatization of regenerant also has an advantage. The percentage of survival and the percentage of doubled plant also determines the concentration and duration of the treatment of colchicine. Increasing the number of regenerant will increase the chance of actualization of double haploid plants. High use of this chemical may cause death or tetraploidization in some cases. Other than colchicine nitrogen oxide (N_2O) treatment and plant treatment of high-pressure gases also used in DH generation. Also, adventitious somatic regeneration in in vitro was found efficient in onion which avoid potentially damaging chemicals and can bring up 100% doubling efficiency (Dwivedi *et al*., 2015; Murovec & Bohanec, 2012) [8, 14].

Application of DH in Breeding

The continuous demand of improved cultivars in different aspects needs a technology like double haploids which can shorten the whole variety development to two to three generation which was taking up to 10 years in a conventional way (Abenezer, 2017; Ferrie & Caswell, 2016; Dwivedi *et al*., 2015) [21, 9, 8]. The combination this method with other molecular techniques improves the both precision genetic gain. The combination of the two methods increases the efficiency of backcrossing, introgression elite genome, Cost per variety. The application of DH contributed for the releasing of varieties in rice, barley, rapeseed, wheat, maize and brassica specious. Also, they used as a potential parent in heterosis breeding. They also have commercial value in ornamental breeding (Abenezer, 2017; Begheyn & Studer, 2016; Dwivedi *et al*., 2015) [21, 4, 8]. Furthermore, they have greatly contributed for QTL discovery, creation of mapping population using at least one DH parent, in genome sequencing to reduce complexity of assembly. The other application is creation of mutagens in micro spore of tobacco, rapeseed, wheat and barley which can be fixed by DH induction. In Brassicas, DH technology enabled modification of disease resistance, cold tolerance and fatty

acid composition using microspore mutation. Additionally, DH contributed to the detail study of embryogenesis, cell fate study and totipotency (Begheyn & Studer, 2016; Ferrie & Caswell, 2016; Burbulis & Kott, 2013) [4, 9, 5].

Forward breeding is the other application of DH which can take more than five years to back trace the parents of hybrids. DH can have the homozygous parents in two to three generation which is an alternative to backcross conversion. This method used in maize to get the hybrid progeny of normal and yellow dent line which back traced by crossing the hybrid with the inducer and recovered 50% homozygous waxy lines and 50% homozygous yellow dent lines (Maqbool *et al*., 2020) [11]. Similarly, DHs are used in reverse breeding in the combination of molecular markers to identify the original parents of hybrid or backcross. They are also induced in BC1 and segreant with specific traits followed by molecular markers then the gene of interest introduced to the recipient by random crossing over by random crossing over (Murovec & Bohanec, 2012) [14].

Double haploid breeding used to improve selection efficiency both in cross pollinated and self-pollinated crops and can be used at any cycle of recurrent selection. They also easily used to avoid deleterious recessive alleles which are difficult to trace in early generation of conventional line development. They are also used to avoid self-incompatibilities in cross pollinated crops like Brassicas (Murovec & Bohanec, 2012) [14]. Application of DH significantly reduces population size that are required to find desirable genotype (Ren *et al*., 2017) [16]. The other most important application of DH during paternal haploid induction is cytoplasmic male sterility (CMS) which is inherited maternally by the male parent through the alternation of mitochondrial DNA. This induction two advantage at time in having the haploid plant with male sterility. This can avoid a cost of development, improve quality and cost of detasseling which is heavily laborious (Weber, 2014) [24].

Economic Benefits of Double Haploid

A study on economic benefit of double haploids showed the huge advantage of double haploids over the conventual breeding development and the hybrids as well especially for the farmers who are incurring their cost regularly every year for purchasing of hybrids. DH technique provides unique opportunity to develop lines that are truly homozygous and have excellent yields approaching hybrid cultivars. Moreover, genetic transformation in combination in-vitro DH technologies increase the competitiveness of DH to hybrids. Therefore, double haploids avoid the cost of farmers for the seed. In addition, the study shows the economic benefits by calculating the cost of creating pure lines of white cabbage compared to isolated in-vitro microspores in creating cabbage hybrids. The result shows the production time reduced from 12 to 6 years and the financial cost reduced by half (Mineikina *et al*., 2019) [13].

Challenges of Double Haploid Development

The use of double haploid is unquestionable. Recently, huge research institutions like CIMMYT are establishing large facilities for exploitation of double haploids. But development of double haploids comes with challenges. The establishment cost double haploids is huge compared to conventional plant breeding. In addition, highly skilled experts, materials and consumables are regularly required. They are genotype specific, complexity of genetic background, low haploid fertility, low induction rate, low spontaneous doubling rate and deleterious effect is one of the concerns also gametoclonal variation may result unplanned variations (Maqbool *et al*., 2020; Baenziger, 1996) [11, 1] The other is especially in maize the use of temperate inducers is not suitable for tropical germplasms. Also, reduction in haploid induction rate was observed during induction (Maqbool *et al*., 2020) [11]. Additionally, climatic conditions such as temperature, light, humidity and other abiotic stress influence the haploid induction rate especially during pollination. The other big challenge is chromosome doubling. The dependence of colchicine treatment needs skilled professional and modernized facility. Moreover, low chromosome doubling rate of colchicine and carcinogenic effect of it is the other concern. Also undesired plantlets could be obtained during homozygous induction and homozygous lines could negatively affect the breeding progress if not discarded as early as possible. (Maqbool *et al*., 2020; Murovec & Bohanec, 2012) [11, 14].

Summary and Conclusion

Since the discovery of genetics in late 19th century during Mendelian time crop improvement had been ongoing to address the demand of human being for wide range of applications. Classical breeding resulted the finding of improvement of crops and contributed for food security but the development of cultivar using this method takes very long time and too many resources. In reversion of this challenge a finding of spontaneous haploids in early 20th century in plant called datura showed a possibility of improvement of the existing method.

Double haploids are plants that are generated through doubling the chromosome of haploid plants. Double haploids become homogenous and homozygous in one to two generation of recovery. The main application of double haploid induction is shortening of breeding cycle of improved crops that used to take longer for generation of improved varieties through conventional methods. The induction of double haploid started with the rigorous selection of haploids generated through natural and artificial means. The natural means are used to avoid the laborious, resource input and technical revival aspects of haploid induction. But these events used to occur very rarely. Due to this, efficient method that can induce more number haploids with high induction rate is needed. For this, different methods are developed using invitro and invivo techniques with distant and within specious crosses. Although successes are made in number of crops including variety release and development, the induction is still very limited to some crop specious, low rate, not resource efficient and need skilled technicians. To manage this, effectors are made in different aspects by combining the induction techniques with molecular and other techniques. The discovery of interspecific hybridization with embryo rescue improved the application of this technology. In this regards, new developments are achieved in development of cytoplasmic male sterile lines, increasing of spontaneous haploid induction and spontaneous doubling.

The other challenge finding of efficient chromosome doubling. Other than colchicine a number of chemicals are used but the are not as efficient as colchicine. High gaseous pressure is also used with better efficiency of induction. But

more improvement should be done in this aspect.

Although double haploids found to have number of applications, low rate of induction, high establishment cost, the need of skilled technicians, the scarcity of efficient doubling agent and its limited application to small number of crops are some of the challenges.

Therefore, the use of double haploid crops in the combination of molecular and conventional breeding techniques by improving some of its limitations and widening its application to more number of specious will contribute to improved food and nutrition security, advance in science and resource use efficiency.

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