Double Haploid Production in Wheat through Microspore Culture and Wheat X Maize Crossing System: An Overview

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Abstract

Double haploid (DH) technology ensures the production of complete homozygous wheat lines in a single year making the selection process efficient in plant breeding. This not only shortens the time period to release a variety, but dihaploids are also used for various aspects of genetic studies. Anther culture and wheat x maize hybridization are most commonly used methods for wheat double haploid production. However, recent studies indicate that wheat x maize hybridization is the most effective method for haploid production in wheat because of its higher efficacy, simplicity, less genotypic specificity, less somaclonal variation and less time consumption. Moreover, DH lines have applications in basic and applied research. DH lines are the ideal material for genetic studies. In this review paper, we have tried to explain the methodologies of DHs production in wheat and their comparison of both procedures in terms of their ease, efficiency and applicability. We have also summarized the possible application of DH lines in Plant Breeding.

Key words: Doubled haploid, Anther Culture, Wide hybridization, wheat x maize crossing system, Embryo rescue, Cultivar development, QTL mapping

Introduction

Conventional breeding methods for genetic improvement of crop plants involve introduction, hybridization, selection of desirable plants and evaluation. This is a tedious process which consumes at least 7-8 generations/years of inbreeding and selection to get a desired level of homozygosity. Recent advances in field of tissue culture have made it possible to get complete homozygous lines/varieties from crop plants in a single generation by haplodization via anther culture and wide hybridization. Haploid individuals possess only a gametic number of chromosomes, which makes them extremely useful for genetic and molecular studies, for example, for studying induced mutations. In haploid plants, even recessive mutations can be
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easily detected as there is no presence of contrasting dominant allele of that recessive allele in haploids. However, haploids (n) are sterile, more sensitive to biotic and abiotic stresses and less vigorous than normal diploid (2n) plants. Therefore, it is a general practice to double the chromosome complement of haploid plants by using a chemical called as colchicine to get doubled haploids (DHs). These doubled haploids are completely homozygous therefore their progenies will be homogeneous for all loci as double dose of single allele is present in doubled haploids; contrary to conventional breeding methods where even after a number of selection cycles, some heterozygous loci are present. Therefore, DH system accelerates a breeding program as complete homozygosity for all loci is obtained in a single year. In this way, time required to release a variety is reduced to half or less than half as compared to conventional breeding methods like back cross, pedigree or bulk method. Moreover, DH system ensures more accurate and efficient selection of desirable plants. Several researchers indicated that haploid breeding has several benefits over the conventional methods including their use in molecular and genetic mapping. Double haploid breeding also provides a way of combining the desirable characters of diverse wheat genotypes which in other case cannot be combined due to genetic barriers. At present, haploid breeding is being used throughout the world for development of inbred lines and cultivars of crops. For example, China has alone developed hundreds of rice and wheat varieties by using this technology within a period of 15 years. Hundreds of varieties of different crops have been developed and released for general cultivation by using DH technology across the globe.

Methods Available for Haploid Production:

At present, different methods such as chromosome elimination following wide hybridization, anther culture, ovule culture, haploid inducer gene/s and chemical treatments have been applied by researchers for production of double haploids in wheat. These methods have varying rate of accuracy and efficacy but interspecific/ interspecific hybridization e.g wheat x maize hybridization and anther culture has been routinely used in breeding programmes due to high efficacy and wheat x maize hybridization being the most suitable method. Here is the detailed description of these methods:

Anther/ Microspore Culture:

In 1964, Guha and Maheshwari were the first to produce haploid plants from anthers of Datura inoxia, since then this technique has been used in many plant species. Anther cultured plants have been successfully regenerated in most of the cereal crops like wheat, rice and maize. Plant anther culture is one of the tissue techniques that have been developed to produce new homozygous varieties within a relatively short time. For anther culture in wheat, spikes of F1 are selected before start of anthesis and stored at cool temperature in dark condition to inhibit anthesis. Sterilization of wheat spikes is done in 2 % sodium hypochlorite solution for 20 minutes by adding some props of “Tween 20” solution and rinsed with autoclaved water 2-3 times after sterilization. Anthers are isolated from these spikes in aseptic condition in Laminar Air Flow Cabinet. Anthers are cultured in test tubes containing N-6 medium, modified potato-2 medium or Potato-2 medium or modified W14 basic media having varying concentrations of

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2,4-D, BAP, NAA or different combinations of BAP and NAA for callus induction\textsuperscript{12}. The cultured test tubes are kept in dark in incubation room for 15 days at 28-30\degree C to enhance reaction in case of 2, 4-D which increase the embryo development rate\textsuperscript{34,36}. Plant regeneration of the resultant embryonic calluses is carried out on the modified 190-2 Cu medium\textsuperscript{17} having mineral salt solution, sucrose, glycine, agar, myo-inositol, NAA, nicotinic acid, thiamine-HCl, pyridoxine-HCl, and kinetin. When after three weeks, green shoots attain a height of 5-10 mm; calluses having green shoots are shifted to the rooting medium for development of the roots in a semi-solid agar medium \textsuperscript{52}. This medium is same as 190-2 mineral solution with a reduced concentration of NAA and kinetin. The temperature of the growth chamber is fixed at 25-27\degree C during the period of plant regeneration and root development. The optimum light intensity is 2500-3000 lux with 14 hours of light photoperiod \textsuperscript{11}. After 3-5 weeks in regeneration medium, plants are hardened by shifting to a mixture of Peat and coconut crush in pots. At 3-5 tiller stage, colchicine treatment is done by dipping clipped roots of haploid plants in 0.1% colchicine solution along with 2% dimethyl sulfoxide (DMSO) and 0.05% tween 20 at 20\degree C for 5 hours \textsuperscript{32}. This results in double haploid production from haploid plants. The colchicine treated plants are again hardened for four weeks before transplanting into external environment. The seed setting in colchicine treated plants is the indicator of successful chromosomal doubling \textsuperscript{40}. A simplified procedure of anther culture is summarized in fig. 1.

\textit{Anther} \hspace{1cm} \textit{Microspores (1n)} \hspace{1cm} \textit{Embryos}

\textit{Haploid plant (1n, infertile)} \hspace{1cm} \textit{Plantlet}

\textit{Diploid plant (2n, fertile)}

\textit{colchicine}

Fig. 1, Simple procedure for doubled haploid plant production by anther culture

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Progress has been made in the anther culture technique through modifying the culture medium. However, this method may cause unpredictable genetic alterations due to gametoclonal variation. These factors could affect the means and genetic variation of a breeding population, thus affecting selection. Kondia- Spika et al. investigated efficacy of microspore culture for DHs production by growing anthers of 20 F1 combinations in vitro on a modified Potato-2 medium. All the 20 genotypes produced pollen calluses as well as regenerated green plants. As a result, 47.2 calluses were produced per 100 cultured anthers. The green plant regeneration ranged from 0.8 to 13.4 green plants per spike, with an overall mean of 5.8. From the total of 582 regenerated green plants, 47.9% (279) were spontaneous double haploids. This shows a low rate of green plant regeneration this is because of the fact that the embryos got from anther culture either failed to germinate or develop to albino plants even after germination as few studies reveal that the rate of embryo formation in anther culture is higher than wheat x maize crossing system. On the other hand, embryos got from wheat x maize hybridization system do not face these problems. Dogramacı-Altuntepe et al studied the regeneration ability of 10 wheat cultivars in four different media and three different growth conditions. From a total of 86,400 anthers cultured, 324 plants were obtained: 248 green and 76 albino plants. This shows very low success rate in anther culture. Moreover, anther culture in wheat has some other problems like high genotypic specificity and its efficiency is greatly affected by environmental conditions under which these plants are grown. This limits the use of anther culture in wheat as compared to wheat x maize crossing system.

**Inter-specific/ Inter-generic Hybridization:**

Several inter-specific/ inter-generic hybridization methods are used for haploid production in wheat. These methods include wheat x maize, wheat x sorghum, wheat x pearl millet, wheat x tripsacum, wheat x teosinte, wheat x barley and wheat x job’s tears. Such Inter-generic crosses have been found to be effective for the production of dihaploid plants in wheat. All these methods include embryo rescue technique and haploids are formed due to chromosomes elimination of the pollen parent during embryo development. However wheat x maize is the best method of haploid production in wheat.

**Wheat x Maize System of Crosses:**

It was reported by Zenkteler and Nitzsche first time that when wheat spikes are pollinated by maize anthers this results in the formation of embryos. Later Laurie and Bennett showed that when maize pollen pollinates the wheat floret, wheat egg is fertilized and zygote is formed. Initially the resultant zygote has haploid number (n) of wheat and maize but karyotypes of wheat ands maize are different which makes these zygotes unstable. Centromeres of maize chromosomes fail to attach to spindle fibers so maize chromosomes are unable to move to spindle poles during cell division. This non-attachment of chromosomes is due to loss of centromeric activity of maize chromosomes. As a result, maize chromosomes are rapidly lost and after a few cell divisions haploid embryo having 21 wheat chromosomes are formed and no endosperm is formed as result of wheat x maize cross instead a whitish fluid is formed along with embryo formation.
Khan and Ahmad described the method of wheat x maize system. In this method (fig. 2), F1 spikes of wheat are emasculated and pollinated by fresh or cryo-preserved maize pollens. Hand emasculation is tedious job so hot water treatment may be use as alternative. A comprehensive emasculation method has been detailed by Hussain et al. Fresh pollens are more efficient for haploid production as compared to cryo-preserved pollens but later are useful if synchronization of flowering in case of wheat and maize is problem. These pollinated spikes are now placed in tiller culture media having sucrose (40 g/L dH2O), sulphuric acid (ml/L dH2O) and auxin hormone and auxin application is required for seed setting in this crossing system. Different hormones are used for the purpose including 2,4-dichlorophenoxyacetic acid (2,4-D), indole acetic acid (IAA), naphthalene acetic acid (NAA), Zeatin, GA3, 6-benzyl aminopurine (BA) and kinetin at various concentrations. Whereas treatment with 2,4-D at a concentration of 100 mg/L increased embryo formation in crosses of wheat x maize and 2,4-D gives the best results than all other hormones mentioned above. The pollinated spikes are dipped in tiller culture media having 2,4-d at 20° C for 14-16 days after pollination and 2,4-D treatment is essential as it increase seed setting along with embryo formation. Development of embryo is also enhanced by spraying 2, 4-D 3 mg/l plus 120 mg/l AgNO3 in durum wheat. Embryo rescue is done 14-16 days after pollination. As wheat x maize embryo has no endosperm so these rescued embryos have to be cultured on nutrition medium like full strength of MS or ½ MS or agar B5 basal medium. The cultured embryos can be kept in vitro for a period of 3-5 weeks at specific relative humidity and temperature during this period. Khan and Ahmad studied different levels of light intensity, relative humidity and temperature during this period of growth and concluded that 10,000 Lux light intensity was sufficient for proper growth. Light intensity below 10,000 Lux results in slowing the pollen tube formation during embryo development. They also concluded that a temperature of 21-26° C and relative humidity of 60-65% were the best for regeneration of plants from rescued embryos. Humidity below 60% results in shriveled grains and RH above 70% results in greater occurrence of fungal diseases.

When the regenerated plants attain a sufficient height after 3-5 weeks in regeneration medium, these are hardened by shifting to a mixture of Peat and coconut crush in 1:2 ratio taken in pots. The seedlings are provided with Hoagland solution as nutrition medium in pots. At 3-5 tiller stage, colchicine treatment is done. For this purpose, haploid seedling roots are cut except 1-1.5 inches and dipped in a 0.1% colchicine solution along with 2% dimethyl sulfoxide and ca. 0.05% tween 20 at 20° C for 5 hours. Colchicine inhibits the spindle formation during cell division and hence stopping the chromosomes from moving to opposite spindle poles. This results in double haploid production from haploid plants. The colchicine treated plants are again hardened under the same conditions for a period of 4 weeks before transplanting into the pot at external environment. The seed setting in colchicine treated plants is the indicator of successful chromosomal doubling. The DH plants derived by culturing do not have any cytoplasmic variation as compared to normal wheat plants in terms of any chemical.
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Why We Prefer Wheat x Maize over Anther Culture Technique:

Suenaga 41 made comparison of efficacy of wheat embryo development between wheat x maize and wheat x H. bulbosum systems of crosses. He concluded that crossibility among most of the wheat varieties and H. bulbosum was very low due to occurrence of dominant genes naming Kr1 and Kr2. Hence this limits the use of H. bulbosum system of crosses for haploid production in wheat. Later other researchers 31,42,43 reported that wheat x maize crossing system has less genotype dependent response than anther culture and H. bulbosum method.

**Wheat x Maize - System**

![Diagram](image)

Fig. 2, Simple procedure for doubled haploid plant production by wheat x maize hybridization system

The wheat x maize system is preferred over anther culture or microspore culture because it has less gametoclonal variations 40,44, higher efficacy (3-4 times) 35,42 and less genotype specificity 31, 40, 41. The less gametoclonal variation in wheat x maize crossing system is due to occurrence of fewer spontaneous regenerations and presence of fewer chromosomal abnormalities as compared to anther culture 35. The higher efficiency of haploid green plant regeneration in wheat x maize crossing system than microspore culture is due to four major factors including absence of albinism, higher regeneration rate and higher embryo formation rate 42 and it saves 4-6 weeks in obtaining same aged haploid plants as compared to anther culture as it escapes the time consumed in callus induction during anther culture 35. Embryo development rates in some studies are better in microspore/ anther than wheat x maize crossing system but majority of these embryos of anther culture either do not regenerate or even if regenerated results in albino plants.
resulting in less regenerated green plants. On the other hand, no albinism is present in wheat x maize crossing system 18,45. Hence, higher rate of embryo development, absence of albinism and higher regeneration rate from embryo makes this wheat x maize crossing system efficient and easy to use than anther culture.

Uses of Double Haploids in Basic and Applied Plant Breeding Research

Double haploid technology helps is decreasing the selection cycles required for obtaining the desired homozygosity from 6-7 years to single year so cultivar development period is reduced to 6-7 years rather than 12-13 years in conventional breeding 34. Hundreds of wheat varieties having performance comparable to commercial varieties have been released in world 7,34,46,54. Both microspore culture and wheat x maize system has been utilized globally for the release of commercial varieties 55. Wheat cultivars developed by DH technology have superior traits like high yield 34,54, salt tolerance 40, good milling traits and early maturity 54. DH lines can be directly released as variety if they have better performance than commercial varieties other wise DH lines as being 100% homozygous lines make a valuable part of germplasm 34,46.

Back cross breeding is used to transfer a desired trait like disease resistance to a high yielding genotype. Cycles of back crossing and selection needs 5-6 years to restore the genetic content of commercial variety along with desired trait. If molecular markers are used to identify the gene of interest and DH technology is used to attain the homozygosity hence reducing the time to release a variety 56.

Quantitative traits are controlled by more than one gene so are complex in nature and their understanding is a serious problem for plant breeders. DH lines are the best material for quantitative genetic studies due to their complete homozygous nature 57. DH lines are used to study the components of quantitative genetics including gene linkage, number of genes governing the trait, interactions, additive variances and location of genes on chromosomes 46,58.

Complete homozygous nature of DH lines makes them ideal material for gene mapping 59. DH lines in wheat have been used for identifying and mapping QTLs associated with resistance to foliar disease of wheat 60, important agronomic traits 61, leaf rust (LR) resistance 62,63, adult plant stripe or yellow rust resistance (YR) and stem rust (SR) resistance 63, number of tillers 64. In this way, double haploid populations help in understanding the inheritance of quantitative traits which has been a challenge for plant breeders since long.

Genome mapping has been very important procedure which helps in detecting the position of desired genes on different chromosomes in genome of an individual and DH population are ideal for the purpose due to complete homozygous nature 59. Wheat whole genome was mapped by using DH population and fig. 3 shows A genome mapping 65.

Conclusion: The review concludes that wheat x maize crossing system for double haploid production helps in shortening the time to release a variety significantly. Hence it helps in accelerating the wheat breeding programmes. Anther culture and wheat x maize crossing are the

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most common methods of double haploid production in wheat but wheat x maize crossing system is superior to anther culture due to its less genotypic specificity, simplicity, efficacy, less somaclonal variation, less occurrence of albino plants, time saving ability and higher regeneration rate as compared to anther culture. Double haploid populations have a lot of applications in plant breeding like cultivar development, germplasm development, transferring traits from wild types by back crossing in less time, studying the components of quantitative genetics, QTL mapping and whole genome mapping. However, further studies focusing on advancement in wheat x maize crossing system in terms of regeneration under different environmental conditions for culturing are badly needed in future so that DH breeding can help the cultivar development, genetic improvement and understanding of genetic phenomena.

Fig. 3: Genome mapping by using wheat DH lines (Zhang et al., 2012)

This shows how DH production finds very important applications in basic and applied Plant Breeding research.

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