

Developing wheat lines for rust resistance through *Imperata cylindrica* mediated chromosome elimination Doubled Haploidy technique: A Review

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Abstract

Emergent food security issues for the rising population are an alarming concern. Thus, enhancing the productivity of the major food crops like wheat against different biotic and abiotic stress are targets on priority throughout the world. For wheat, rust diseases (leaf rust, stem rust and stripe rust) have been a devastating threat. To manage the disease, cultivation of rust resistant wheat cultivars is economically and environmentally sustainable approach. Different conventional breeding approaches have been used for the development of rust resistant wheat varieties. However, these conventional breeding tools are lengthy so to develop rust resistant lines in short time with lesser number of breeding cycles double haploidy (DH) technique has turned up as a boon for the breeders. Among different DH techniques, so far wheat x maize wide hybridization is reported to be the most effective method. However, the unsynchronized flowering of maize with that of wheat makes it a second choice in front of *Imperata cylindrica* which is a perennial wild grass easily found around the wheat fields. Also, it has higher frequency of haploid embryo development and regeneration than that of maize. The wheat DH breeding research programme for development of rust resistant lines has speeded up with the development of genetic stocks like DH-1 at ICAR-IARI, regional Station, Shimla (H.P) and many other resistant lines added to the wheat breeding programme of the station. However, extensive research, industrial collaborations and efficient protocol integrating the conventional breeding programmes are required for the exploration of this wild grass for wheat rust resistance breeding via DH technique.

Keywords: *Imperata cylindrica*, double haploidy, wheat rust

1. Introduction

Wheat rust is a devastating fungal disease that spreads rapidly and reduces the yield and quality of crop. Wheat production is drastically threatened by three rust diseases namely; leaf rust caused by *Puccinia triticina* (Pt), stem rust by *Puccinia graminis* f.sp. *tritici* (Pgt) and stripe rust by

Puccinia striiformis f.sp. *tritici* (Pst). Global annual losses of wheat crop by rust pathogens have been estimated to be around 15 million tons valued at US\$ 2.9 billion (Huerta-Espino *et al.*, 2020). The yield losses due to the three rusts are depicted in table 1.



Table 1: Potential yield losses, symptoms, and favourable temperature of wheat rusts

Yellow rust		
Yield loss	40% in moderately susceptible varieties	(Amanov <i>et al.</i> , 2016)
Symptoms	orange-yellow colored pustules; arranged in lines on affected parts	(Akci, 2022)
Favorable temperature	2-15°C	(Huerta-Espino <i>et al.</i> , 2020)
Brown rust		
Yield loss	15% and upto 50% in very susceptible varieties	(Kaur <i>et al.</i> , 2023, Patial <i>et al.</i> , 2017c)
Symptoms	orange-brown pustules on leaves	(Akci, 2022)
Favorable temperature	10-30 °C	(Huerta-Espino <i>et al.</i> , 2020)
Black rust		
Yield loss	70% in susceptible cultivar	(Abdulridha <i>et al.</i> , 2023)
Symptoms	dark red brown pustules (turn black on maturity) on the affected parts	(Akci, 2022)
Favorable temperature	15-35°C	(Huerta-Espino <i>et al.</i> , 2020)

The main controlling strategies for wheat rust disease are: breeding resistant cultivars and chemical control. Sundry fungicides are available as a chemical control however; the use of fungicides is neither economical nor safe for the environment. On the contrary, use of resistant cultivars is the prominent strategy for controlling the rust disease in wheat. Therefore, to overcome this threat, deployment of rust resistant wheat cultivars has proved to be an effective strategy. Back in the few decades, use of rust resistant cultivars has significantly reduced the yield losses from rusts and thereby cultivation of resistant varieties proves to be a cost-effective and environment friendly approach to curtail the losses.

Wheat improvement for the development of high yielding resistant varieties *via* conventional breeding is limited by the long process of 6-7 season required for selfing and selection cycles (Patial *et al.*, 2019). Also, the rust pathogen is fast-mutating and the long-term conventional breeding strategies slow down the success of rust resistance breeding program in wheat. Therefore, the possible speeding programme to develop improved plant varieties involves techniques which are less tedious, time saving and less expensive. In this context, the speed breeding for supplementing/complementing conventional breeding with biotechnological tools is highly anticipated.

A doubled haploid (DH) is a genotype formed when haploid (n) cells successfully undergo either spontaneous or artificial induced chromosome doubling. Haploids are sporophytes that contain gametic chromosome numbers

(n). Doubled haploidy approach involving *in-vitro* or *in-vivo* techniques like anther/microspore culture, ovary/ovule culture, chromosomal elimination following wide hybridization, haploid inducer genes and chemicals has been used by different researchers to induce instant homozygosity and expediting conventional wheat breeding programmes (Zhang *et al.*, 2011; Grauda *et al.*, 2014; Bhattacharya *et al.*, 2015; Patial *et al.*, 2017b; Patial *et al.*, 2023, Bishnoi *et al.*, 2022). Of the number of DH inducing techniques employed in wheat, maize mediated and *I. cylindrica* mediated chromosome elimination approaches are reported to be the most effective. This review therefore, highlights the work done for rust resistance breeding in wheat using *I. cylindrica* mediated DH approach.

Boons of Double haploidy Approach

Doubled haploid lines are homozygous, and through the DH technique these doubled haploid lines are developed in shortest span of time (Kumar *et al.*, 2020). Doubled haploidy technology provides advantages like: induction of homozygosity in only one generation; elimination of unfavourable genes; enrichment of the genetic pool of favourable genes etc. Accelerating wheat breeding using DH technique is of great interest to geneticists and breeders due to complete homozygosity, short duration (1–2 years) of their production, smaller population sizes and more efficient selection. DHs are potentially helpful for genetic mapping of complex qualitative traits, linkage studies, estimation of recombination, unmasking recessive mutants, or, in reverse breeding (Gandhi *et al.*, 2023).



With the help of DH technology, less technical programmes with constrained resources might achieve remarkable outcomes while saving millions of dollars. In the Peruvian highlands, DH barley cultivars outperformed conventional lines in terms of yields, grain quality, and stripe rust resistance, according to Gomez-Pando *et al.* (2009). For these reasons, it is anticipated that DH lines will have a bigger economic impact than conventional lines and that their production cost was 26% less. Since the introduction of molecular markers for QTL identification, the possibility for employing DHs in the field of quantitative genetics has expanded.

Double haploidy: Historical perspective

Spontaneous haploid induction was discovered by Blakeslee *et al.* (1922) in *Datura stramonium*, and later the potential of haploid development for plant breeding *via in-vitro* culture of *Datura* anther by Guha and Maheshwari (1964) lead to the popularization of double haploidy approach. In wheat, haploid development was reported with maize by Barclay (1975) and later on number of techniques were reported *viz.*, anther culture (De Buyser *et al.*, 1987), microspore culture (Hussain *et al.*, 2013), intergeneric crosses with maize (Laurie and Bennett, 1987; Khan and Ahmad, 2011), sorghum (Ohkawa *et al.*, 1992), pear millet (Ahmad and Comeau, 1990), barley (Mihailescu and Giura, 1998) and *Imperata cylindrica* (Chaudhary *et al.*, 2005). The haploids from einkorn, emmer, and dinkel wheat possess $n = x = 7$, $n = 2x = 14$, and $n = 3x = 21$ chromosomes with genomic constitution of A, AB, and ABD, respectively (Fehr, 1993; Quisenberry and Reitz, 1967; Folling and Olesen, 2002).

Laurie and Bennett (1987) used maize for DH induction in wheat and reported maize pollen to be insensitive to the dominant crossability inhibitor *Kr1* and *Kr2* genes in wheat and identified wheat $\times$ maize to be more efficient technique to develop wheat haploids. However, the non-synchronization of flowering between the two species hinders the hybridization process. Number of other plant species *viz.*, jobs tear (*Coix lacryma-jobi*), pearl millet or sorghum, were also tested for DH induction in wheat and *Imperata cylindrica*, a wild grass remotely related to wheat (Chaudhary *et al.*, 2005) was found to be the most efficient one (Kishor *et al.*, 2011; Mahato and Chaudhary, 2015). In wheat, doubled haploidy breeding through chromosome elimination has been studied and

investigated by researchers and nowadays have large-scale practical applications especially for supplementing conventional wheat breeding programmes (Khan and Ahmed, 2011; Bhattacharya *et al.*, 2015).

Potential DH techniques

Production of DH wheat cultivars involves two major steps: a) haploid plant development and b) Chromosome doubling for DH plant development.

Haploid development

In higher plants, haploids can occur spontaneously; as in case of first report of sporophytic haploid found in *Datura stramonium* L. (Blakeslee *et al.*, 1922). Also, formation of haploids can be induced artificially through *in-vitro* or *in-vivo* methods. Through parthenogenesis, pseudogamy, or chromosomal elimination after wide hybridization, haploid embryos can be developed *in-vivo*. There are currently several methods that use both male and female gametophytes to produce haploids, and eventually DHs (after a process of chromosome doubling). Haploids can be created from the female gametophyte by inducing gynogenesis and uniparental genome deletion. Crossing two sexually incompatible species, using intraspecific crosses where one genitor bears a specific mutation, or genetically altering CENH3, a centromeric variation of the H3 histone are the usual methods for achieving uniparental genome deletion (Ravi and Chan, 2010). Gynogenesis is the process of inducing the development of a haploid embryo, typically from the egg cell, from unfertilized ovules, ovaries, or even entire flowers that have been cultivated *in-vitro*. Haploid induction through gynogenesis was first reported in barley in 1976 (San Noeum, 1976). Since 1976, in more than 20 angiosperm species; *in-vitro* culturing of ovule and ovaries have been successfully practiced for the development of haploids (Wu *et al.*, 2004). Despite this, gynogenesis is not widely accepted on large scale for haploid production due to tedious isolation of female haploid cells and presence of small number of megaspores (Kristof and Imre, 1996). It is also possible to produce haploids from the male gametophyte. By androgenesis, haploids can be produced from the male gametophyte. Microspore/pollenembryogenesis which reprogrammes microspores/pollen towards embryogenesis, is the most prevalent and practical androgenic mechanism. This process, which Guha and Maheshwari first discovered more than 40 years



ago (Guha and Maheshwari, 1964), has grown significantly in practical because it makes it possible to produce DH lines in more species and at a lower cost than the other methods previously used.

Practically, anther culture has been successfully used in breeding and many cultivars have been developed. Wheat cultivars such as “GK Delibab” (Pauk *et al.*, 1995), “AC Andrew” (Sadasivaiah *et al.*, 2004), “GK Deva” (Pauk *et al.*, 2020) have been developed through androgenesis followed by DH. Due to some limitations of anther culture such as low responsive genotypes, high rates of albinism and long-term induction and regeneration processes; wide hybridization followed by chromosome elimination have come into limelight. This procedure involves hybridization of wheat with other species such as maize, *Imperata cylindrica* and *Hordeum bulbosum*. In wide hybridization, pollinated florets are sprayed with various chemicals and auxins, such as 2,4-dichlorophenoxyacetic acid (2,4-D), 3,6-dichloro-2-methoxybenzoic acid (dicamba), 4-amino-3,5,6-trichloropicolinic acid (picloram), indole-3-acetic acid (IAA), phenyl acetic acid (PAA), and silver nitrate. Theses growth regulator administration is crucial for DH breeding since it influences the rate and percentage of embryo development (Knox *et al.*, 2000; Patial *et al.*, 2021).

Chromosome doubling for DH plant development

For haploid plants to become fertile doubled haploids, their chromosomes must double. An essential component of DH induction is the survival rate of haploid after

colchicine treatment, which provides a measurement of how many green plants grown on culture media survive till maturity. Anti-mitotic/anti-microtubule drugs have been utilised for the artificial chromosomal doubling, with colchicine being the most popular and frequently used at various concentrations (Marcinska *et al.*, 2013; Nowakowska *et al.*, 2015). Colchicine disturbs normal polar segregation of sister chromatids by inhibiting function during mitosis; forming a restitution nucleus. However, scientists have experimented with many other microtubule depolymerising agents such as trifluralin (Zhao and Simmonds, 1995), oryzalin and amiprophos (Hansen and Andersen, 1996). Use of charcoal and amino acids as antimitotic agents has also been reported in few studies (Pintos *et al.*, 2007, 2010). Large scale colchicine administration to individual plants established in the soil is tedious and expensive task. Alternatively, colchicine is added into the anther culture induction medium (Barnabas *et al.*, 1991) or regeneration medium for culturing of pollen callus (Ouyang *et al.*, 1994). However, the concentrations of colchicine must be optimised carefully as its high concentrations have been reported to highly toxic which eventually leads to reduction in the number of embryos derived from anther culture and ultimately number of DH plant produced (Barnabas *et al.*, 1991; Navarro *et al.*, 1994). Hence, determining the appropriate colchicine dose is pre-requisite to obtain the greatest number of DH plants with the lowest mortality.

Table 2: Different Colchicine treatment doses and effects in wheat

S.No.	Variety	Dose	Treatment stage	Effect	Reference
	Rossini/FD91147, Texel/ FD91091, Trbmie/ Avital, Sid6ral/Eridane, FD91072/Soissons, Fertil/FD91072, FD91072/FD91147, FD0830/Corsaire, Vivant , Ritmo/FD627, Rialto/Tr6mie, Texel/ Sid6ral, FD5566/ FD502, FD90050/ FD627, Contra/Soissons, Cordial/Soissons, Ritmo/Sidbral, Soissons/ FD91143.	0.1%	At the three to five tiller stage	70 % (60-81%) of the colchicine treated haploid plants were fertile	Lefebvre and Devaux, 1996



Petrus, Certo, Dream and History	300 and 900 mg/l	Colchicine was incorporated into media. Also, some plants were treated after regeneration.	30.6% plants survived which were treated with colchicine followed by plants regenerated on medium with 300 mg/l colchicine (19.9%) and 900 mg/l colchicine (18.4%).	Daniel <i>et al.</i> , 2005
DH 100 and DH 40	100-10000 ppm	Colchicine doses ranging from 100 to 10 000 ppm with and without 2, 4-D application were administered <i>in vivo</i> to the uppermost internodes of <i>Imperata cylindrica</i> -pollinated wheat plants at various intervals	2000 ppm was found to be the most effective dose	Tayeng <i>et al.</i> , 2012
A-9-30-1 and PDW 314	500 to 7000 ppm	Various concentrations of colchicine ranging from 500 to 7000 ppm were injected to the pollinated spikes at different time interval <i>viz.</i> , 24 h, 48 h and 72 h after pollination.	Application of 5000–7000 ppm colchicine at 48 h after pollination was established as the optimum colchicine dose for chromosome doubling	Mahato and Chaudhary, 2015
CT 14259, Mo 35957, Mo 35981, Mo 36082 and Mo 36229, PJ 486, PJ 525, TJ 15033, TJ 15035 and TJ 15042	1 mg/l	1 st treatment: Anthers were incubated in liquid media containing colchicine for 24 hrs and then transferred to same media without colchicine 2 nd treatment: Embryo like structures developed from anthers on normal media were shifted to colchicine containing media for 24 hrs	About 30 embryo like structure were formed	Slusarkiewicz and Ponitka 2017
PDW-314, A-9-30-1, DH-40 and C-306	Lower doses (0.01, 0.025, 0.05% and higher doses (0.05, 0.075, 0.10, 0.15, 0.20, 0.25%	Lower doses for longer durations (0.01, 0.025, 0.05% each for 5, 7, 9, 11 hrs) and higher doses for shorter durations	In hexaploid and tetraploid wheats, colchicine doses of 0.075% for 4 hrs and 0.15% for 4 hrs, respectively were established as optimum for enhanced doubled haploid production.	Sharma <i>et al.</i> , 2019



Wheat rust resistance breeding using Imperata cylindrica mediated DH technology

Change in the pathogen population and development of new variants of rust pathogen affects wheat production globally. To counter these wheat rust, breeding climate resilient high yielding wheat varieties resistant to rust diseases is the compelling need which is the most economical and ecofriendly approach (Patial *et al.*, 2020).

Imperata cylindrica is a synchronously flowering perennial wild grass ($2n = 20$) with wheat that is commonly found around the wheat fields. It does not need to be grown in a polyhouse or greenhouse like that in maize. In contrast to maize-mediated system, *I. cylindrica* has a substantially higher frequency of haploid embryo development and regeneration. *I. cylindrica* pollen is abundant throughout the wheat hybridization period, and it is genotype nonspecific and hence can hybridization with any species of wheat such as triticale, or their derivatives. This weedy perennial grass grows naturally in almost all regions of the world and does not need to be sown again.

The *I. cylindrica* method of DH induction has been used in bread wheat (Patial *et al.*, 2015, 2016, 2022), durum wheat (Mahato and Chaudhary, 2015), triticale $\times$ wheat (Pratap *et al.* 2005) as well as wheat $\times$ rye derivatives (Kishore *et al.*, 2011). Cytological analysis of the *I. cylindrica*-mediated chromosome elimination mechanism in wheat highlighted that there is no endosperm formation and *I. cylindrica* chromosomes are eliminated during the first zygotic division, enabling the development of seeds that carry embryos. According to Chaudhary *et al.* (2013), the use of molecular cytogenetic methods like GISH (Genomic *in-situ* hybridization) and FISH (Fluorescence *in-situ* hybridization) in conjunction with *I. Cylindrical* mediated DH method can speed up alien introgression-mediated wheat breeding programmes.

Researchers compared the effectiveness of the wheat $\times$ maize and wheat $\times$ *I. cylindrica* systems for developing wheat embryos and concluded that the former system was superior to the latter (Patial *et al.*, 2022). The system of inducing double haploidy in wheat using the *I. cylindrica* approach of double haploidy breeding and the development of DH lines in crosses between triticale-wheat and wheat-rye hybrids were first described by Chaudhary *et al.* in 2019. For the creation and regeneration of the haploid embryo, different haploid induction

parameters were standardized. The induction of DH using this method was also reported by Sharma *et al.* (2019), who highlighted *Imperata cylindrical* as the improved DH induction technology with greater efficiency, time savings, and less expensive technique in wheat than maize. By using cytological data, Komeda *et al.* (2007) revealed the effective fertilisation of parental gametes and demonstrated complete elimination from nuclei at the at first cell division. Later, the backcrosses between the F_1 hybrids of winter wheat genotypes (Saptdhara and Tyari) and spring wheat genotypes (HS 375 and C 306) with Indian rye were highlighted by Kishore *et al.* (2011). Intergeneric hybridization was carried out in wheat-rye derived backcrosses utilising the pollens of maize and *Imperata cylindrica* to compare the relative efficacy of maize and *I. cylindrica* chromosome elimination techniques in the production of haploid plants and reported no embryo carrying seed in maize while the relative frequency of embryo carrying seeds in *I. cylindrica* ranged from 8% to 30%.

The use of *I. cylindrica* mediated chromosome elimination technique for rust resistance breeding in wheat was initiated at Indian Council of Agricultural Research-Indian Agriculture Research Institute, Shimla (H.P). By using the technique, Patial *et al.* (2015) created haploid wheat plants from intergeneric hybridization of F_1 winter and spring wheat with *Imperata cylindrica*. Most of the crosses resulted in the production of DH plants. In a different study, Patial *et al.*, (2017a) used two spring wheat genotypes and three winter wheat genotypes that were crossed in a Line $\times$ Tester method to produce six F_1 's, as well as three spring wheat genotypes and one winter wheat genotype to produce two three-way F_1 's. Wheat F_1 s and three-way F_1 s were crossed with *I. Cylindrical* resulted in the mean frequency for embryo and haploid plantlet regeneration were 3.54% to 8.13% and 0 to 11.11%, respectively.

Patial *et al.* (2021) improved upon and standardised the *I. cylindrica*-mediated chromosomal elimination approach for enhancing the effectiveness of doubled haploid production in wheat and developed the first genetic stock "DH-1" (Patial *et al.*, 2022) The developed wheat doubled haploid genetic stock is reported to be resistant to yellow and brown rust which was tested against a mix of eight races of yellow rust and eighteen races of brown rust at the



seedling stage and adult plant stages. DH-1 demonstrated resistance to all pathotypes of yellow and brown except for the 77-5 race of brown rust at seeding stage.

The wheat x *I. cylindrica* crossing system is more effective and convenient to use than other methods used to produce rust resistant wheat doubled haploids due to the faster rate of embryo development, absence of albinism, and higher rate of regeneration from embryo. This innovation has globally opened new vistas for commercial utilization of the new system for large scale development of DHs in wheat for their direct use as improved varieties or gene mapping populations. Hence, the development of doubled haploids by *I. cylindrica* mediated chromosome elimination technique is an effective tool for the development of wheat DH lines in shortest timeframe (Patial *et al.*, 2021).

Factors affecting wheat DH production

Embryo rescue medium is a crucial *in-vitro* requisite which promotes the growth of immature embryos. Without endosperm, there is a lack of nutrition, and as a result, developing embryos may abort when left on spike. Consequently, to avoid embryo degeneration, weak, immature, and hybrid embryos are typically cultured *in-vitro* to produce haploid plantlets on artificial nutrient media. The type of media used for embryo rescue has an important role on haploid production. Different medias for haploid wheat embryos have been used: the MS (Murashige and Skoog, 1962), half-strength MS (Khan and Ahmad, 2011), and B5 media (Gamborg *et al.*, 1968). So far, researchers have used various modified media in the wheat X *I. cylindrica* technique (Patial *et al.*, 2015). Patial *et al.* (2021) reported the best media for inducing haploids in the wheat x *I. cylindrica* system, was half-strength MS media, while modified B5 media produced the fewest haploid plants. Cherkaoui *et al.* (2008) effectively cultured immature embryos on MS, half-strength MS, and B5 media and revealed the superiority of half-strength MS media over other medium and indicated that reducing the macroelements causes germination of big, well-developed embryos.

Another crucial *in-vitro* aspect for advancing haploid embryo to haploid plant development is the time of embryo rescue. Numerous authors have reported embryo rescue times ranging from 10 days, 14 days, up to 18-21 days after pollination. Also, have reported that if the embryo was not cultured at the appropriate time,

the recovery rate of the green plant would be reduced. Although no in-depth research has been done in this area, most papers on embryo rescue days identify days 16–18 as the ideal time for embryo rescue. Patial *et al.* (2021) reported 24-hour embryo rescue time days after pollination (DAP) as the ideal period for embryo culturing. Younger embryos (15-18 DAP) were more challenging to culture and excise and produced weak, lean plantlets, whereas the older embryos (27 DAP) began to discolour, shrivel, and displaying signs of ageing. Optimization of the process for development of rust resistant wheat DH involving *I. Cylindrical* mediated approach and to check the effectiveness of the procedures used till date is a milestone in DH induction in wheat using *I cylindrica* grass.

Conclusion

The doubled haploidy approach has been extensively used in wheat breeding programmes and several novel wheat cultivars developed *via* DH technology have been reported in Europe, the United States, Canada, Brazil, China and India (Patial *et al.*, 2022). In different research programmes like the development of molecular maps, the localization of loci responsible for qualitative and quantitative traits (QTLs), and in genomics and other molecular investigations, DH populations are commonly utilised. Microspore-derived embryos can be the focus of genetic modification and DHs are commonly utilised in the mapping of plant genomes also.

Without a doubt, the DH technology is a potent tool for modernising wheat breeding to derive fully homozygous lines. However, its successful adoption necessitates consideration of some important points. The first and most important step in ensuring the success of DH production is to properly train the workforce in the various aspects of DH production (haploid induction, haploid identification, chromosomal doubling, and DH line recovery). Second, the method used to produce haploid plants must be standardised because they are frequently feeble and susceptible to varied environmental conditions. Although many efforts have been made to produce DH using various methods, the production of DH plants is less. The addition of DH technology to wheat genetics and breeding programmes is very helpful to shorten the breeding cycle, create new cultivars, and increase our understanding of genetic features that are crucial for agronomic performance. In many plant



genera DH procedures has been standardized with the new technological advancements. In just a few decades, there are now 250 species that can be used for doubled haploidy. Also, the development of the optimum methods for deploying DH system for actual use in wheat breeding requires integration and collaborative efforts of industries, researchers from other disciplines, and breeders.

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Author contributions

The review was written and enriched by MP, RV. Both authors read, edited, and approved the final manuscript.

Conflict of interest

No

Declaration

The authors declare no conflict of interest.

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