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Double Haploids for Vegetable Crop Improvement: A Review

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ABSTRACT

Vegetables are the richest source of vitamins and minerals and play a major role in nutritional security. The primary objective of vegetable breeding is enhancing production and productivity. Breeders use various conventional and non-conventional breeding approaches for genetic advance. Recent advances in biotechnology helps to enhance the efficiency and shorten the time required to achieve the fixed goals in a breeding program, as well as to address the economic and ecological goals. Among these, haploid (H) and doubled haploid (DH) production through gametic embryogenesis has long been recognized as a valuable tool to help crop improvement. Haploid plants are the sporophytes with a gametophytic chromosome number and doubled haploids (DH) are haploids that have undergone chromosome duplication. Haploids and double haploids occur spontaneously in nature. Haploids can also be induced artificially by various techniques such as wide hybridization, pollination with irradiated pollens androgenesis, gynogenesis and finally further chromosome doubling results in double haploids. Production of double haploids in vegetables will shorten the breeding cycle and also DH technology is useful in reverse breeding, CMS line production, gene stacking and various other genetic studies.

Key words: Androgenesis, Double haploid, Gynogenesis, Haploid.

Vegetables are the richest source of vitamins and minerals and play a major role in nutritional security. The most direct and affordable way to deliver better nutrition for all is to give more attention on vegetables. According to the recent reports by FAO, consumption of a minimum of 400 g of fruit and vegetables per day (excluding potatoes and other starchy tubers) is necessary for prevention of chronic diseases such as heart disease, cancer, diabetes and obesity, as well as for alleviating several micronutrient deficiencies, especially in under developed countries. So, the first and foremost step towards this is obviously the expansion in the production as well as productivity of fruits and vegetables (Schreinemachers *et al*., 2018).

The major challenge faced in vegetable production is the various abiotic and biotic stresses. Plant breeding serves as a tool for introducing host plant resistance, developing stress tolerant varieties and cultivars with the desired produce quality. Besides these, the genetic enhancement of vegetables aims primarily on achieving higher production along with quality demand in the market (Dias and Ortiz, 2019). Vegetable crop improvement can be done by various conventional and non-conventional breeding approaches. Recent advances in biotechnology helps to enhance the efficiency and shorten the time required to achieve the target in a breeding program, as well as to address economic and ecological goals. Among these, haploid (H) and doubled haploid (DH) production through gametic embryogenesis has long been recognized as a valuable tool to help plant improvement (Ren *et al*., 2017).

Haploid plants are the sporophytes with a gametophytic chromosome number and doubled haploids (DH) are haploids that have undergone chromosome duplication. Thus, in a diploid sporophytic (2n) species the haploids could also be called monoploid (x) as they have only one set of chromosomes. In polyploid species, the haploids (n) have more than one set of chromosomes and are polyhaploids. Dihaploid is a haploid plant from an autotetraploid (4*x*) and is not homozygous as it represents two chromosome sets selected from four sets in the autotetraploid (Germana, 2011).

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Double haploids

Haploids are the sporophytes that contain a gametic chromosome number (n) instead of normal somatic (2n) chromosome number (Murovec and Bohanec, 2012). Spontaneous occurrence of haploids was first ever reported in weed species, *Datura stramonium* (Blackeslee *et al*., 1922), followed by similar reports in several other crops (Chase, 2005; Dunwell, 2010; Maluszynski *et al*., 2003; Nanda and Chase, 1966; Riley, 1974). Even though the natural occurrences were reported in various species, the breakthrough in the field of haploid breeding was the production of haploids *in vitro* by anther culture in *Datura sp* (Guha and Maheshwari, 1964, 1966). Another milestone

was the production of haploids by wide hybridization followed by chromosome elimination and embryo culture (Kasha and Kao, 1970). Later in 1974, chromosome doubling techniques for the production of homozygous double haploids from sterile haploids were established (Jensen, 1974) which accelerated the haploid research. The research on haploid or double haploid system and their exploitation has been increasing remarkably owing to the establishment of protocols for the double haploid induction in over 250 plant species (Maluszynski *et al.*, 2003, Forster and Thomas, 2005).

Why double haploids?

The DHs obtained through various techniques are homozygous at all loci and they may be used as a new variety in the case of self-pollinating species whereas they serve as potential parental inbred line for the production of hybrids in cross-pollinated crops (Murovec and Bohanec, 2012). In conventional breeding, through inbreeding/ sib-mating it will take around 6-7 generations to develop a homozygous line especially in biennials as well as those crops having long juvenile phase whereas within a single generation an inbred line can be developed via double haploidization (Bohanec, 2009; Prigge *et al.*, 2012; Wedzony *et al.*, 2009). Besides these, double haploids are relevant in those species having self-incompatibility or higher inbreeding depression on continuous selfing (Thaneswari *et al.*, 2018). Double haploidy along with marker assisted selection can be used as an alternative option for backcross breeding which consumes more time. In mutation studies, double haploids provide the attractive features for fixing mutations. As double haploidy is a way to homozygosity with greater reliability, they can be used to fix mutations which are mostly recessive in nature or otherwise they can be directly used for mutation treatment so that we can capture the resulting mutation in pure homozygous condition (Szarejko and Forster, 2006).

In addition to the applications in crop improvement, double haploid systems aid in many genomic approaches like construction of genetic maps, QTL mapping and marker identification (Alheit *et al.*, 2011; Forster and Thomas, 2005; Forster *et al.*, 2007).

Double haploid technology and its applications in vegetables

1. Haploidization *via* wide hybridization

This involves both inter specific and inter generic hybridization followed by the parental chromosomal elimination (Murovec and Bohanec, 2012). It was first applied in barley, commonly known by the name, bulbosum technique. In barley, wide hybridization was done between cultivated barley, *Hordeum vulgare* (2n=2x=14) as the female and wild *H. bulbosum* (2n=2x=14) as the male which resulted in haploid. The hybrid embryo formed was containing the chromosomes of both parents. But, chromosomes of the wild relative were preferentially eliminated from the cells of developing embryo due to the failure of endosperm development, which lead to the production of a haploid embryo. The haploid embryo was then extracted and grown *in vitro* (Kasha and Kao, 1970).

Doubled haploids can be produced from tetraploid genotypes of *Solanum tuberosum* (cultivated potato) by pollination with the diploid potato species *Solanum tuberosum* L. *Phureja* Group (De Maine, 2003). In about 0.5% of pollinated ovules, both male sperm cells of *Solanum tuberosum* L. *Phureja* Group take part in formation of functional endosperm, which triggers the parthenogenic development of unfertilized egg cells. The best pollinator lines of *Solanum tuberosum* L. *Phureja* Group were bred for a dominant purple spot embryo marker, thus seeds containing haploid embryos can be easily distinguished from hybrid *S. tuberosum* × *S. phureja* seeds. Methods of chromosome duplication were developed more recently and production of potato can be obtained by androgenic methods with a better efficiency (Jacobsen *et al.*, 1993).

2. Haploidization *via* pollination with irradiated pollen

Maternal haploids can be obtained *in vivo* by using pollen that has been irradiated (using gamma rays from cobalt-60) or pollen collected from a triploid plant. As this pollen fail to fertilize the egg cell, there won't be fertilization. However, pollination stimulates the development of haploid embryo (Musial and Przywara, 1998). Here, the success relies on the donor plant genotype and its growing condition radiation source and dose, the developmental stage embryos, the culture conditions and the media composition and also chromosome doubling and ploidy identification (Zhang *et al.*, 2006; Lim and Earle 2008, 2009; Germana, 2011; Solmaz *et al.*, 2011; Nasertorabi *et al.*, 2012; Tas¸kın *et al.*, 2013; Baktemur *et al.*, 2013, 2014; Kaur *et al.*, 2019).

Pollination with irradiated pollen is the potent way to obtain haploids in the case of cucurbits. First ever haploid induction was successfully done in musk melon, *Cucumis melo*, by using irradiated pollen from *C. ficifolius*. This technique was successfully used to obtain haploid embryos by *in vitro* culturing (Sauton and de Vaulx, 1988). Later this technique was successfully employed in other cucurbits *viz.* cucumber (Lei *et al.*, 2006; Dolcet-Sanjuan *et al.*, 2006; Lofti and Salehi, 2008 and Shariatpanahi and Ramezanpour, 2019), watermelon (Sari *et al.*, 1994), summer squash (Kurtar *et al.*, 2002; Kurtar *et al.*, 2017), pumpkin (Kurtar *et al.*, 2009) and winter squash (Kurtar and Balkaya, 2010), melon (Ari *et al.,* 2010; Godbole and Murthy, 2012; Pamuk *et al.*, 2018; Hooghvorst *et al.*, 2020;), carrot (Rode and de Vaulx 1987).

3. Haploidization *via* gynogenesis

In vitro induction of maternal haploids, so-called gynogenesis, is induction of haploid embryos from the haploid cells, especially the unfertilized egg cell inside female gametophyte. Usually, *in vitro* culture of un-pollinated flower parts, such as ovules, placenta attached ovules, ovaries or whole flower buds is done to obtain the haploid plants. Gynogenic induction using unpollinated flower parts has been successful in several species, such as onion, sugar

beet, cucumber, squash, gerbera, sunflower, wheat, barley *etc*. (Forster *et al*., 2007, Thaneswari *et al*., 2018). But its application in breeding is mainly restricted to onion and sugar beet. The success of the method and its efficiency is greatly influenced by several biotic and abiotic factors. The genotype of donor plants, combined with growth conditions, is the crucial factor (Badu *et al*., 2017).

In onion, anther culture is not successful and gynogenesis is the only rule for haploid induction (Keller and Korzun, 1996; Musial *et al*., 2001). It is made possible using the flower buds or unfertilized ovaries (Campion and Azzimonti, 1988). The success of recovery of the haploid regenerants in onion depends mainly on the genotype/variety, stage ovule development, pre-treatments given and the composition of culture media (Juokevieiene *et al*., 2005; Mathapati *et al*., 2018; Khar *et al*., 2019). The response to gynogenesis also depends up on the length of flower bud taken for *in vitro* culture (Michalik, 2000; Fayos *et al*., 2011). Among the Indian short day cultivars, gynogenic potential was found to be highest in Bhima Shubhra (Anandhan *et al*., 2014).

In tomato, gynogenic haploid induction is an alternative way for anther culture and a successful report on gynogenesis in tomato were given by various researchers (Bal and Abak, 2007). Gynogenesis might become a potential tool for haploidization in tomato in the coming era (Zhao *et al*., 2014).

Gynogenesis is a potential route for haploid breeding in cucurbits (Dong *et al*., 2016). Successful protocols on *in vitro* culture of unfertilized ovule/ovary have been established in squash (Metwally *et al*., 1998; Zou *et al*., 2020), pumpkin (Sun *et al*., 2009; Min *et al*., 2016), cucumber (Gémes-Juhász *et al*., 2002; Sorntip *et al*., 2017) and melon (Beharav and Cohen 1995; Ficcadenti *et al*., 1999). In cucumber, it has been reported that unpollinated ovule culture could be done in cucumber basal medium containing thidiazuron and silver nitrate (Li *et al*., 2013). Further, the effect of various genotypes and culture media were studied various researchers in cucumber (Ozsan *et al*., 2017), onion (Fayos *et al*., 2011) *etc*.

Gynogenesis is the least favoured technique for induction of haploids as it is having low efficiency and is limited to only those species which fail to respond to other efficient methods (Forster *et al*., 2007).

4. Haploidization *via* androgenesisa

a. Haploidization *via in vitro* cultured anthers

The technique of production of double haploids using anther culture is the mostly adapted in most of the crop species (Maluszynski *et al*., 2003). This is relatively a simple and less expensive method, even though precise study is hindered by the presence of extraneous sporophytic tissues such as anther wall (Forster *et al*., 2007). Anther culture involves the collection of flower buds followed by pretreatment, surface sterilization, dissection of anthers and finally placing the anthers into the medium (Germana, 2011). Similar to gynogenesis, anther culture is highly influenced by various factors, such as genotype (Dumas de Vaulx *et al*., 1981; Ltifi and Wenzel, 1994), donor plant culture conditions (Kristiansen and Andersen, 1993)., pretreatment of flower buds or anthers (Dumas de Vaulx, 1981; Oskum *et al*., 2001; Supena *et al*., 2006), stage of development of microspore (Lantos *et al*. 2009; Kim *et al*. 2004), culture medium composition and culture conditions (Gudeva *et al*. 2007). Shed microspore culture is a slight modification in the anther culture in which the anther is stimulated to dehisce and the released microspores are cultured. This is successfully employed in pepper (Supena *et al*., 2006).

List of vegetable crops in which anther culture is possible

Vegetables	References
Asparagus	Tsay, 1996; Peng and Wolyn, 1999
Broccoli	Qin *et al*., 2015
Cauliflower	Arnison and Keller, 1990; Bhattacharya *et al*., 2017
Brinjal	Kumar *et al*., 2003; Basay *et al*., 2013; Bhattacharya *et al*., 2019
Capsicum	Hegde *et al*., 2017
Tomato	Bal and Abak, 2007; Kumar *et al*., 2020
Chilli	George and Narayanaswamy, 1973; Kristiansen and Andersen, 1993; Kim *et al*., 2008; Gudeva *et al*., 2009;
Summer squash	Metwally *et al*., 1998
Carrot	Anderson, 1985
Potato	Aboshama and Atwa, 2020
Musk melon	Song *et al*., 2007
Cucumber	Song *et al*., 2007

b. Haploidization *via* isolated microspore culture

It has got several advantages as compared to that of anther culture. Removal of anther wall helps to avoid the embryogenesis from saprophytic tissues and more precise studies could be conducted as single cell is being manipulated (Touraev *et al*., 2001). Two alternative methods of microspore isolation are commonly used. Flowers at the optimal developmental stage are cut by hand or blended, microspores are then separated from the debris by a series of washes, sieving and/or centrifugation steps (Zaki and Dickinson, 1990). In the second method, anthers are manually isolated from flowers and placed in liquid medium containing mannitol where the microspores are spontaneously released into the medium and then filtered or separated by centrifugation and re-suspended in the induction medium (Touraev *et al*., 1996).

List of vegetable crops in which isolated microspore culture is possible

Vegetables	References
Turnip	Zhang *et al*., 2012; Shumilina *et al*., 2020
Carrot	Kiszczak *et al*., 2017; Shmykova *et al*., 2021
Chinese cabbage	Sato *et al*., 1989
Brinjal	Corral-Martínez *et al*., 2012
Radish	Tuncer, 2017
Cauliflower	Bhatia *et al*., 2017
Broccoli	Dias, 2001

Chromosome doubling

Double haploids can occur spontaneously, but in most cases. Chromosome doubling of haploids is required to restore fertility.

Methods of chromosome doubling (Diploidization)

1. **Endomitosis:** Endomitosis is described as chromosome multiplication and seperation but failure of spindle leads to one restitution nucleus with chromosome number doubled. It has also been called 'Nuclear Restitution'.
2. **Endoreduplication:** Endoreduplication is a phenomenon of DNA or chromosome doubling without Cytokinesis.
3. **C-mitosis:** C-mitosis is nothing but endomitosis under the influence of colchicine.
4. **Nuclear fusion:** it occurs when two or more nuclei divide synchronously and develop a common spindle. Thus, two or more nuclei could result with doubled, polyploid or aneuploid chromosome number.

Chromosome doubling agents

1. Acenanaphthene
2. Chloramphenicol
3. Nitrous oxide
4. Parafluorophenyl alanine
5. 8- hydroxyquinone
6. Colchicine

Colchicine

It is the most commonly used doubling agent which can be applied *in vivo* (Kasha *et al.*, 2005). It is an alkaloid isolated by French chemists P.S. Pelletier and J. Caventon in 1820. It is a toxic natural alkaloid and secondary metabolite, extracted from plants of the Genus *Colchicum* (autumn crocus, *Colchicum autumnale*, also known as "meadow saffron"). It is extracted from seeds and corms of Colchicum. It increases fertile plant regeneration and reduce albinism in anther culture. The systematic (IUPAC) name for colchicine is $C_{22}H_{25}NO_6$. Colchicine acts by inhibiting microtubule polymerization by binding to tubulin. Mitosis will not take place without the availability of tubulin and therefore colchicine effectively functions as a "mitotic poison" or "spindle poison".

Methods of colchicine application

The optimum concentration as well as time of application of chromosome doubling may have negative impacts on embryogenesis, regeneration rate, as well as on the percentage of green plants (Castillo *et al.*, 2009). The different methods adopted are:

1. Seed treatment (0.001 to 1%; 0.2% is more common).
2. Germinating seed treatment.
3. Growing shoot apex (0.1 to 1% Colchicine).
4. Treatment of growing point in the cotyledonary stage.
5. Colchicine in glycerine (0.2-0.4% colchicine in 10% glycerine).
6. Colchicine in emulsion (0.2-0.4% colchicine).
7. Colchicine in agar (1% colchicine and 2% agar mixed in equal parts).

Among all methods of colchicine application, shoot apex treatment at the seedling stage is most effective.

Checking of ploidy level

Finally, after treating with the chromosome doubling agent the ploidy level of treated plant should be checked. There are several direct and indirect methods for determining the ploidy level. Indirect approaches based on comparisons between regenerated and donor plants in terms of plant morphology (plant height, leaf dimensions and flower morphology), plant vigour and fertility. Though the indirect methods are less expensive, much unreliable as it is influenced by environmental effects. Direct methods are more robust and reliable and include conventional cytological techniques, such as counting the chromosome number in root tip cells (Maluszynska, 2003) and measurement of DNA content using flow cytometry (Bohanec, 2009). Direct methods provide a rapid and simple option for large-scale ploidy determination as early as in the *in vitro* culturing phase. It also enables detection of mixoploid regenerants (having cells with different ploidy) and the determination of their proportion.

Applications of DHs in vegetable breeding

- Cultivar development.
- Mutation studies.
- Mapping quantitative trait loci.
- Backcross breeding.
- Production of biotic and abiotic stress resistant plants.
- Construction of genetic maps.
- Genomic studies.

Limitations of DHs

- Haploids cannot be obtained in high frequency required for selection.
- Selection cannot be imposed a double haploid population.
- The cost benefit ratio in haploid breeding is often very high, discouraging the use despite of its obvious advantages.
- Haploids will express recessive deleterious traits.
- Deleterious mutations may arise during anther culture.
- In haploids produced from anther culture, it is observed that some plants are aneuploids and some are mixed haploid-diploid types.
- The over-usage of doubled haploidy may reduce genetic variation in breeding germplasm.
- Other constraints associated with use of this technology are the low rate of embryogenesis and regeneration, high frequency of albinism, segregation distortion and the low frequency of chromosome doubling to obtain DH (Dunwell, 2010).

CONCLUSION

Double haploid is a plant or line obtained by doubling the chromosome number of a haploid plant or individual. *In vivo* and *in vitro* methods have been used to produce DH. The genetic upgradation of crops through conventional breeding approaches takes longer period. So, there is a need to assist these methods with certain biotechnological tools to shorten

the breeding cycle. Double Haploid (DH) breeding is one such tool which has got great potential in breeding programmes in the upcoming future.

Conflict of interest

All authors declared that there is no conflict of interest.

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