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Role of doubled haploids in vegetable crop improvement

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Abstract
A doubled haploid (DH) is a genotype formed when haploid cells undergo chromosome doubling. Artificial production of doubled haploids is important in plant breeding. Homozygous lines are of utmost importance in breeding programmes which are produced by doubled haploids. Doubled haploid techniques provide plant breeders with pure lines in a single generation, which may save considerable time in the breeding of new cultivars. Heterosis is fixed in case of doubled haploids. Many attempts have been made since then, resulting in published protocols for over 250 plant species belonging to almost all families of the plant kingdom. In fact, under optimal conditions, doubled haploids (DH) have been routinely used in breeding for several decades, although their common use is still limited to selected species.

Keywords: doubled haploid, *in vitro*, *in vivo*, chromosome doubling

Introduction
The first ambitious objective, among the millennium development goals, consists in the eradication of extreme poverty and food shortage by the 2015 target date. Now-a-days, for fighting hunger and malnutrition using a sustainable and low-input farming system, plant breeding rather than agro-chemistry and mechanization seems to be able to more efficiently increase food and feed production on less land and often in a more environment-friendly way. Particularly, recent advances in biotechnology represent a valuable and powerful tool to enhance the efficiency and shorten the time required to reach the fixed purposes in a breeding programme, as well as to address economic and ecological goals. Among the biotechnological methods, haploid (H) and doubled haploid (DH) technology has long been recognized as a valuable tool to help plant improvement.

Double haploid means a plant or line obtained by doubling the chromosome number of a haploid plant or individual. The genetic upgradation of crops through conventional breeding approaches require longer time so there is a need to assist these methods following certain biotechnological tools to shorten the breeding cycle and Double Haploid (DH) breeding is one such tool which has been widely used in breeding programmes. The potential of haploidy for plant breeding arose in 1964 with the achievement of haploid embryo formation from *in vitro* culture of Datura anthers (Guha and Maheshwari, 1964, 1966), which was followed by successful *in vitro* haploid production in tobacco (Nitsch and Nitsch, 1969). Many attempts have been made since then, resulting in published protocols for over 250 plant species belonging to almost all families of the plant kingdom (reviewed in Maluszynski *et al*., 2003). Double haploid technique is a valuable method for genetic cartography of complex traits *viz.* yield, transgenesis and genomics. In order to obtain a DH, two main steps should be usually considered

1. Induction of haploid
2. Doubling of chromosome number of the haploid individual

Haploid cells or the plants that contain a single complete set of chromosomes or individuals having gametophytic chromosome number in its sporophyte.

Why there is need to develop doubled haploids?
1. For development of homozygous lines which are used in hybrid seed production.
2. For fixation of heterosis:-doubled haploids are required because are more homozygous as compared to conventional breeding methods.

3. For mutational studies and easy to induce mutation by chromosome doubling which can be done by colchicine treatment so there is induction of mutation.
4. For production of biotic and abiotic stress resistant plants. Heterosis can be fixed. So by choosing parents of different biotic and abiotic stress resistance we can select resistant plants.
5. For cytogenetical research.
6. For induction of genetic variability at haploid level.
7. For evolutionary studies.
8. For genome mapping as genetic maps are very important to understand the structure and organization of genomes from which evolution patterns and syntenic relationships between species can be deduced. DH populations have become standard resources in genetic mapping for species in which DHs are readily available. Doubled haploid populations are ideal for genetic mapping. It is possible to produce a genetic map within two years of the initial cross regardless of the species. Map construction is relatively easy using a DH population derived from a hybrid of two homozygous parents as the expected segregation ratio is simple, *i.e.* 1:1.

Doubled haploid plant is a plant in which the plant cells contain two gene sets which are exactly identical. But in case of other plants cells contain two gene sets which are almost identical (but not exactly). Doubled haploids helps in accelerating breeding as plants which are selected from a doubled haploid population always breed true whereas those plants which are developed through conventional breeding method donot breed true. In case of conventional breeding method there is production of disease resistant high quality & poor quality, disease susceptible high quality & poor quality plants from disease resistant high quality plants. It takes 10-12 generations until all off-springs breed true to type whereas in case of doubled haploid plants there is production of disease resistant high quality plants and it takes only 3-5 years earlier than conventional breeding method.

Table 1: Comparison between doubled haploid breeding and conventional breeding

Particulars	Double haploid breeding	Conventional breeding
Time required for developing pure lines	One year or one crop season	6-7 years
Time required for developing cultivars	3-5 years	8-10 years or more
Fixation of heterosis	Possible	Not possible
Expenditure/cost involve	More	Lesser
Identification of recessive mutants	Very easy	Difficult
Mapping population	Permanent	Temporary

Methods for haploid production

1. *In vivo* method
2. *In vitro* method

In vivo **method:** This includes other methods like distant hybridization crosses followed by chromosome elimination, bulbosum technique, parthenogenesis and Inducer based approach. Again parthenogenesis include pseudogamy, semigamy and apogamy.The possibilities to use interspecific hybridisation between *Brassica napus* and some wild species in the family of Brassicaceae for introduction of resistance to the important fungal pathogens is presented. A large collection of wild relatives as resources of resistance to *Leptosphaeri amaculans*, *Alternaria brassicola*, *A. raphani* and *Plasmodiophora brassicae* has been screened. Successful hybridisation with *Brassica napus* has been achieved using *Brassica elongata*, *B. fructiculosa*, *B. souliei*, *Diplotaxis tenuifolia, Hirschfeldia incana, Coincya monensis* and *Sinapis arvensis.*

The 'bulbosum' method was the first haploid induction method to produce large numbers of haploids across most genotypes and quickly entered into breeding programmes. Inducer based approach means the haploid inducing lines have been used in maize to produce haploids by development of unfertilized egg cells. Haploid seed induction rate of Inducer lines – 8-12 %.

Apogamy: Development of sporophyte directly from gametophyte, without fusion of gametes; such sporophytes have the same chromosome number as the gametophyte from which they have been derived (apospory, diplospory).

Parthenogenesis: A haploid embryo develops from the haploid egg.

Pseudogamy: Pollination serves as a stimulus for embryo development but the egg and sperm nuclei do not fuse. Fusion of the polar nuclei with one of the sperm nuclei may occur to produce endosperm.

Semigamy: The haploid sperm nucleus enters the egg but does not fuse with the haploid egg nucleus. Each nucleus divides independently creating a haploid embryo that contains sectors of male and female origin.

In vitro **method:** This includes androgenesis and gynogenesis. Androgenesis is the process of induction and regeneration of haploids and double haploids originating from male gametic cells. Due to its high effectiveness and applicability in numerous plant species, it has outstanding potential for plant breeding and commercial exploitation of DH. For example, *Brassica spp. In vitro* induction of maternal haploids, so-called gynogenesis, is another pathway to the production of haploid embryos exclusively from a female gametophyte. It can be achieved with the *in vitro* culture of various un-pollinated flower parts, such as ovules, placenta attached ovules, ovaries or whole flower buds. Although gynogenetic regenerants show higher genetic stability and a lower rate of albino plants compared to androgenetic ones, gynogenesis is used mainly in plants in which other induction techniques, such as androgenesis and the pollination methods above described, have failed. Gynogenic induction using un-pollinated flower parts has been successful in several species, such as onion, sugar beet, cucumber, squash, gerbera, sunflower, wheat, barley etc. 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. In onion, for example, pronounced differences in embryo yields have been recorded among accessions and among plants within accessions.

Factors affecting haploid induction and subsequent regeneration of embryos

1. The genotype of the donor plants affects the haploid

induction and also the subsequent regeneration of embryos.
2. Physiological condition of donor plants that is growth at lower temperature and high illumination.
3. Developmental stage of gametes, microspores and ovules.
4. Pre-treatment that is cold treatment of inflorescences prior to culture, hot treatment of cultured microspores.
5. Composition of the culture medium including culture on “starvation” medium low with carbohydrates and/or macro elements followed by transfer to normal regeneration medium specific to the species.
6. Physical factors during tissue culture like light and temperature.

Chromosome Doubling

Double haploids can occur spontaneously, but in most cases chromosome doubling of haploids is required to restore fertility. This is achieved by the use of anti-microtubuleagents. Haploid plant may grow up to a flowering stage, but viable gametes cannot be formed due to lack of pairing partner of homologous chromosomes in meiosis. Consequently there is no seed formation. Mechanisms of spontaneous doubling differ, with nuclear fusion being the most common cause. As first described by Sunderland *et al*. (1974) [12], synchronous division of two or more nuclei in early stages of embryo development might develop a common spindle. The nuclear fusion theory is supported by the frequent occurrence of a small proportion of triploid regenerants. Nuclear fusions might be associated with delayed cell wall formation, which, as reviewed by Kasha (2005). Chemical treatment might be avoided by using *in vitro* adventitious somatic regeneration, which itself frequently leads to increased ploidy. Such an approach was efficient in onion (Alan *et al*., 2007) [1]. The method has two advantages: the first being that no potentially damaging chemicals are used in the process and the second that regenerants do not for the most part show a mixoploid character. Up to 100% doubling efficiency in relation to individual line treatment can be achieved using this method (Jakse *et al*., 2010).

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.

Other chromosome doubling agents

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

Colchicine: It is analkaloid 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. Increase fertile plant and reduce albinism in anther culture. The Systematic (IUPAC) name for colchicine is $C_{22}H_{25}NO_6$.Colchicine inhibits microtubule polymerization by binding to tubulin. Availability of tubulin is essential to mitosis, and therefore colchicine effectively functions as a "mitotic poison" or “spindle poison”.

Methods of colchicine application:

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

Checking of ploidy level

After treating with colchicine checking the ploidy level of colchicine treated plant is important. This can be achieved by using ploidyanalyser, flow cytometry, morphological observation, indirect method based on guard cells. Several direct and indirect approaches are available for determining the ploidy level of regenerated plants. Indirect approaches are based on comparisons between regenerated and donor plants in terms of plant morphology (plant height, leaf dimensions and flower morphology), plant vigour and fertility, number of chloroplasts and their size in stomatal guard cells. They are fairly unreliable and subject to environmental effects but do not require costly equipment. Direct methods for ploidy determination are more robust and reliable and include conventional cytological techniques, such as counting the chromosome number in root tip cells (for a protocol, Maluszynska, 2003) [9] and measurement of DNA content using flow cytometry (for a protocol, Bohanec, 2003) [2]. The latter provides 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. Flow cytometry is a laser-based, biophysical technology employed in cell counting, cell sorting, biomarker detection and protein engineering, by suspending cells in a stream of fluid and passing them by an electronic detection apparatus. It allows simultaneous multiparametric analysis of the physical and chemical characteristics of up to thousands of particles per second.The latter provides 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.

In the past, evaluation of regenerants mainly relied on phenotypic markers, progeny testing after self-pollination and isozyme analysis. Nowadays, DNA molecular markers, such as AFLP (Amplified Fragment Length Polymorphism), RAPD (Random Amplified Polymorphic DNA), SCAR (Sequence Characterized Amplified Regions) or SST (Simple Sequence Repeat), are commonly used for homozygosity testing and assessment of plant origin. There is a considerable difference in interpretation between dominant or co-dominant electrophoretic profiles. Co-dominant molecular markers, as

well as isozyme markers, have the advantage that a single locus, when heterozygous in donor plants, might be used for homozygosity determination. In contrast, a more complex profile is analyzed with dominant markers. In such a case, bands missing from the donor profile indirectly indicate homozygosity. An approach is used in potato, in which selection is based on a homozygous dominant colour marker gene carried by the pollinator line (Maine, 2003) [8]. The purple spot embryo marker shows up on seeds whose embryos possess a genome from the pollinator. Those hybrid seeds are discarded, while spotless dihaploid seeds are included in breeding process. Selection can be repeated at the seedling stage, when a purple nodal band can be detected on the hybrid's stem. In the case of both maize and potato selectable markers, it is not possible to distinguish hybrid seeds resulting from unintentional self-pollination of donor plants. Selection has to be supplemented with other morphological or molecular markers. A fast and reliable haploid identification method is needed for large scale production of DHs. Morphological markers expressed at the embryo, seed or early seedling stages are preferentially used.

Genetics of DH population

In DH method only two types of genotypes occur for a pair of alleles, A and a, with the frequency of ½ AA and ½ aa, while in diploid method three genotypes occur with the frequency of ¼ AA, ½ Aa, ¼ aa. Thus, if AA is desirable genotype, the probability of obtaining this genotype is higher in haploid method than in diploid method. If n loci are segregating, the probability of getting the desirable genotype is (1/2) n by the haploid method and (1/4) n by the diploid method. Hence the efficiency of the haploid method is high when the number of genes concerned is large.

Studies were conducted comparing DH method and other conventional breeding methods and it was concluded that adoption of doubled haploidy does not lead to any bias of genotypes in populations, and random DHs were even found to be compatible to selected line produced by conventional pedigree method.

Applications of DH in plant breeding

1. Development of homozygous inbred line and cultivars in self-pollinated crops it can be released directly as a cultivar and in a cross pollinated crop it can be used as an inbred line.
2. It gives an immediate product of stable recombinants from species crosses or fixation of heterotic combination.
3. There is no masking effects because of high homogenity.
4. High efficiency is seen in stacking specific targeted genes in homozygous line.
5. There is an increased performance per se due to selection pressure in the haploid phase or during first generation of DHs.
6. Simplified logistics for seed exchange between main and off season programmes since each line is fixed and can be represented by a single plant.
7. Development of substitution and addition lines.

The induction and regeneration of haploids followed by spontaneous or induced doubling of chromosomes are widely used techniques in advanced breeding programs of several agricultural species. They have been successfully used for commercial cultivar production of species such as asparagus, eggplant, melon, pepper and more than 290 varieties have already been released. Using DH technology, completely homozygous plants can be established in one generation thus saving several generations of selfing comparing to conventional methods, by which also only partial homozygosity is obtained. It should be noted that, following chromosome doubling, DH plants are normally selfed for maintenance and for further multiplication. In cross-pollinated species with strongly expressed self-incompatibility, various techniques are used to overcome the incompatibility reaction. For instance in Brassicas, bud pollination is enhanced by treatment in a CO_2 enriched atmosphere (Nakanishi & Hinata, 1973) [10] or by application of gibberelic acid, sodium chloride, urea or ammonium sulphate on stigmas. Alternatively, DH lines might be clonally propagated, in which case micropropagation is often the best choice.

Mutation breeding is another area of plant improvement for which doubled haploid techniques can help to accelerate the process. Homozygosity of regenerants and true breeding propagation enables the fixation of mutations in the first generation after mutagenic treatment. All mutated traits are immediately expressed, allowing screening for both recessive and dominant mutants in the first generation without the need for self-pollination. The first option is, that mutagenic treatment is applied to dormant seeds that, on germination and flowering, produce M_1 gametes, which are used as donor material for haploid culture. The second option relies on mutagenic treatment of haploid cells *in vitro*. The mutagenic agent is usually applied soon after microspore isolation at the uninucleate stage, before the first nuclear division in order to avoid heterozygosity and chimerism caused by spontaneous diploidization through nuclear fusion. *In vitro* mutagenic treatment can be followed by *in vitro* selection of desired traits, such as disease and herbicide resistance.

Applications of DH in genomics

1. DHs serve to recover recessives.
2. DHs ideal for the study of mutation frequency and spectra.
3. It helps in permanent mapping population.
4. It can be repeated at any time in different laboratories by different researchers.
5. It can be used to enhance the expression level of transgene.
6. Construction of genetic maps or Gene tagging / locating genes.
7. Identification of molecular markers for trait selection.
8. QTL analysis is facilitated by using DH mapping populations and enable accurate phenotyping.
9. In DH populations, Dominant Markers are as efficient as Co-Dominant Markers.

Advantages of DHs

1. The ability to produce homozygous lines after a single round recombination saves a lot of time for the plant breeders.
2. Studies conclude that random DH's are comparable to the selected lines in pedigree inbreeding.
3. The other advantages include development of large number of homozygous lines, efficient genetic analysis and development of markers for useful traits in much less time.
4. More specific benefits include the possibility of seed propagation as an alternative to vegetative multiplication in ornamentals, and in species such as trees in which long life cycles and inbreeding depression preclude traditional breeding methods, doubled haploidy provides new

alternatives.

5. The induction of DH lines in dioecious plants, in which sex is determined by a regulating gene, has an additional advantage. Such a case is well studied in Asparagus, in which sex dimorphism is determined by a dominant gene M. Female plants are homozygous for the recessive alleles (mm), while male plants are heterozygous (Mm). Androgenically produced DH lines are therefore female (mm) or 'supermale' (MM). An advantage of supermales is that, when used as the pollinating line, all hybrid progeny are male.

Disadvantages of DH breeding technique

1. Frequency of haploid occurrence is low.
2. Success of DH method is genotype dependent.
3. Some techniques (e.g. Inducers line in maize) are proprietary and not available to all interested breeders.
4. Success is unpredictable and can consume valuable resources.
5. Health and legal concerns related to handling the doubling chemical agent.
6. Haploids from polyploid species have more than one set of chromosomes and are polyhaploids. For example dihaploids(2n=2x) from tetraploid potato (*Solanum tuberosum ssp. tuberosum*, 2n=4x), trihaploids (2n=3x) from hexaploid kiwifruit (*Actinidia deliciosa*, 2n=6x) etc. Dihaploids and trihaploids are not homozygous like doubled haploids, because they contain more than one set of chromosomes. They cannot be used as true-breeding lines but they enable the breeding of polyploid species at the diploid level and crossings with related cultivated or wild diploid species carrying genes of interest.

Approaches for DH production: There are two approaches for double haploid production. They are anther culture and ovary culture.

Anther culture: The *in vitro* culturing of anthers containing microspores or immature pollen grains on a nutrient medium for the purpose of generating haploid plantlets. Culturing anthers for the purpose of obtaining Double Haploid is not easy with many field crop species, particularly with the cereals, cotton, and grain legumes.

Procedure for Anther Culture: The immature anthers should be collected during morning time, then inoculation can be done in protected condition (laminar air flow). After inoculation, there is proliferation of anthers occur which results in formation of callus. Then there is development of embryo which leads haploid plant production. After treating with colchicine transplanting of plants can be done. It results in doubled haploid plant production.

Ovary Culture: Production of haploid individual by culture of unfertilized ovaries to obtain haploid plants from egg cells or other haploid cells of the embryosac. The plants produced are referred as Gynogenic Haploids. In vitro fertilization for the production of distant hybrids avoiding style and stigmatic incompatibility that inhibit pollen germination and pollen tube growth.

Novel approaches combining DHs and molecular genetics

A simplified scheme for backcrossing has been proposed (Forster *et al*., 2007), aimed at shortening the period needed for the introduction of a particular trait from donor to recipient germplasm. According to the scheme, DHs are produced from the BC1 generation. Segregation of parental chromosomes into the filial generation is followed by molecular markers to identify lines with only recipient chromosomes. The gene of interest should thus be introduced into the recipient chromosome by a random crossing over event in the BC1 generation.

A protocol for "reverse breeding" was proposed by Wijnker *et al*. (2007). According to this invention, superior hybrid genotypes are first identified among the segregating population. Using genetic transformation, a gene for induced suppression of meiotic recombination is then introduced, and several DH lines are produced. Segregation of chromosomes is followed by chromosome specific molecular markers and a final combination of two lines represents complementary sets constituting the original heterozygous superior hybrid.

Conclusion

Doubled haploidy is and will continue to be a very efficient tool for the production of completely homozygous lines from heterozygous donor plants in a single step. Since the first discovery of haploid plants in 1920 and in particular after the discovery of *in vitro* androgenesis in 1964, techniques have been gradually developed and constantly improved. The method has already been used in breeding programs for several decades and is currently the method of choice in all species for which the technique is sufficiently elaborated. Species for which well-established protocols exist predominantly belong to field crops or vegetables, but the technique is gradually also being developed for other plant species, including fruit and ornamental plants and other perennials. It should be mentioned that, in addition to breeding, haploids and doubled haploids have been extensively used in genetic studies, such as gene mapping, marker/trait association studies, location of QTLs, genomics and as targets for transformations. Furthermore the haploid induction technique can now-a-days be efficiently combined with several other plant biotechnological techniques, enabling several novel breeding achievements, such as improved mutation breeding, backcrossing, hybrid breeding and genetic transformation.

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