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Haploids and Doubled Haploids in Plant Breeding

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1. Introduction

Haploids are plants (sporophytes) that contain a gametic chromosome number (n). They can originate spontaneously in nature or as a result of various induction techniques. Spontaneous development of haploid plants has been known since 1922, when Blakeslee first described this phenomenon in *Datura stramonium* (Blakeslee et al., 1922); this was subsequently followed by similar reports in tobacco (*Nicotiana tabacum*), wheat (*Triticum aestivum*) and several other species (Forster et al., 2007). However, spontaneous occurrence is a rare event and therefore of limited practical value. 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). 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. There are several reasons for this. These might be categorized as *biological,* based on plant status (annual, biannual, perennial, authogamous, allogamous, vegetativelly propagated) and flower morphology or *technical,* which are the result of the feasibility and efficiency of DH induction protocol. Induction protocols substantially vary, in fact, not only among species but also among genotypes of the same species.

2. Production of haploids and doubled haploids

Haploids produced from diploid species (2n=2x), known as monoploids, contain only one set of chromosomes in the sporophytic phase (2n=x). They are smaller and exhibit a lower plant vigor compared to donor plants and are sterile due to the inability of their chromosomes to pair during meiosis. In order to propagate them through seed and to include them in breeding programs, their fertility has to be restored with spontaneous or induced chromosome doubling. The obtained DHs are homozygous at all loci and can represent a new variety (self-pollinated crops) or parental inbred line for the production of hybrid varieties (cross-pollinated crops). In fact, cross pollinated species often express a high degree of inbreeding depression. For these species, the induction process *per se* can serve not only as a fast method for the production of homozygous lines but also as a selection tool for

the elimination of genotypes expressing strong inbreeding depression. Selection can be expected for traits caused by recessive deleterious genes that are associated with vegetative growth. Traits associated with flower fertility might not be related and should be eliminated by recurrent selection among DH lines.

The production of pure lines using doubled haploids has several advantages over conventional methods. Using DH production systems, homozygosity is achieved in one generation, eliminating the need for several generations of self-pollination. The time saving is substantial, particularly in biennial crops and in crops with a long juvenile period. For self incompatible species, dioecious species and species that suffer from inbreeding depression due to self-pollination, haploidy may be the only way to develop inbred lines.

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.

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 heksaploid 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.

The main factors affecting haploid induction and subsequent regeneration of embryos are:

- genotype of the donor plants,
- physiological condition of donor plants (i. e. growth at lower temperature and high illumination),
- developmental stage of gametes, microspores and ovules,
- pre-treatment (i. e. cold treatment of inflorescences prior to culture, hot treatment of cultured microspores)
- 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),
- physical factors during tissue culture (light, temperature).

3. Haploid techniques

3.1 Induction of maternal haploids

3.1.1 *In situ* induction of maternal haploids

In situ induction of maternal haploids can be initiated by pollination with pollen of the same species (e.g., maize), pollination with irradiated pollen, pollination with pollen of a wild relative (e.g., barley, potato) or unrelated species (e.g., wheat). Pollination can be followed by fertilization of the egg cell and development of a hybrid embryo, in which paternal

chromosome elimination occurs in early embryogenesis or fertilization of the egg cell does not occur, and the development of the haploid embryo is triggered by pollination of polar nuclei and the development of endosperm.

Pollination with pollen of the same species

Maternal haploid induction in **maize** (*Zea mays* L.) is a result of legitimate crossing within one species with selected inducing genotypes (line, single cross or population). It results in a majority of regular hybrid embryos and a smaller proportion of haploid maternal embryos with normal triploid endosperms. The first recognized inducer line was the genetic strain Stock 6, with an haploid induction rate of up to 2.3% (Coe, 1959), which was subsequently improved by hybridization and further selection. Today, modern haploid inducing lines display high induction rates of 8 to 10% (Geiger & Gordillo, 2009). They are routinely used in commercial DH-line breeding programs due to their high effectiveness and lower genotype dependence. In contrast to other induction techniques, no *in vitro* culture is needed, since kernels containing haploid embryos display a normal germination rate and lead to viable haploid seedlings. Haploid embryos can be selected early in the breeding process, based on morphological and physiological markers.

Pollination with **irradiated pollen** is another possibility for inducing the formation of maternal haploids using intra-specific pollination. Embryo development is stimulated by pollen germination on the stigma and growth of the pollen tube within the style, although irradiated pollen is unable to fertilize the egg cell. It has been used successfully in several species (Table 1).

Species	Reference
apple	Zhang & Lespinasse, 1991; Hofer & Lespinasse, 1996; De Witte & Keulemans, 1994
blackberry	Naess et al., 1998
carnation	Sato et al., 2000
cucumber	Przyborowski & Niemirowicz-Szczytt, 1994; Faris et al., 1999; Faris & Niemirowicz-Szczytt, 1999; Claveria et al., 2005
European plum	Peixe et al., 2000
kiwifruit	Pandey et. al., 1990; Chalak & Legave, 1997; Musial & Przywara, 1998, 1999
mandarin	Froelicher et al., 2007; Aleza et al., 2009
melon	Sauton & Dumas de Vaulx, 1987; Cuny et al., 1993; Lotfi et al., 2003
onion	Dore & Marie, 1993
pear	Bouvier et al., 1993
petunia	Raquin, 1985
rose	Meynet et al., 1994
species of the genus *Nicotiana*	Pandey, 1980; Pandey & Phung, 1982
squash	Kurtar et al. 2002
sunflower	Todorova et al. 1997
sweet cherry	Höfer & Grafe, 2003
watermelon	Sari et al., 1994

Table 1. Induction of haploid plants by pollination with irradiated pollen

The production of maternal haploids stimulated by irradiated pollen requires efficient emasculation, which has in some cases been shown to limit its use because the method is too laborious. To overcome such an obstacle in onion, for instance, only male sterile donor plants were used as donor plants, but such lines, possessing cytoplasmically inherited male sterility, are of very limited practical use. Apart from the factors affecting haploid production already mentioned, the dose of irradiation is the main factor controlling *in situ* haploid production. At lower doses, the generative nucleus is partly damaged and therefore maintains its capacity to fertilize the egg cell. It results in large numbers of obtained embryos but all of hybrid origin and abnormal (mutant) phenotype. An increase in the irradiation dose causes a decrease in the total number of developed embryos but the obtained regenerants are mostly of haploid origin.

For most plant species, *in vitro* embryo rescue is necessary to recover haploid plants. The collection of mature seeds has only been reported for kiwifruit (Pandey et al., 1990; Chalak & Legave, 1997), onion (Dore & Marie, 1993), mandarin (Froelicher et al., 2007) and species of the genus *Nicotiana* (Pandey & Phung, 1982). Even for the aforementioned species, *in vitro* germination of seeds enhanced the recovery of haploid plants.

Wide hybridization

Wide crossing between species has been shown to be a very effective method for haploid induction and has been used successfully in several cultivated species. It exploits haploidy from the female gametic line and involves both inter-specific and inter-generic pollinations. The fertilization of polar nuclei and production of functional endosperm can trigger the parthenogenetic development of haploid embryos, which mature normally and are propagated through seeds (e.g., potato). In other cases, fertilization of ovules is followed by paternal chromosome elimination in hybrid embryos. The endosperms are absent or poorly developed, so embryo rescue and further *in vitro* culture of embryos are needed (e.g., barley).

In **barley,** haploid production is the result of wide hybridization between cultivated barley (*Hordeum vulgare*, 2n=2x=14) as the female and wild *H. bulbosum* (2n=2x=14) as the male. After fertilization, a hybrid embryo containing the chromosomes of both parents is produced. During early embryogenesis, chromosomes of the wild relative are preferentially eliminated from the cells of developing embryo, leading to the formation of a haploid embryo, which is due to the failure of endosperm development. A haploid embryo is later extracted and grown *in vitro*. The 'bulbosum' method was the first haploid induction method to produce large numbers of haploids across most genotypes and quickly entered into breeding programs. Pollination with maize pollen could also be used for the production of haploid barley plants, but at lower frequencies.

Paternal chromosome elimination has also been observed after interspecific crosses between **wheat** (*Triticum aestivum*) and maize. After pollination, a hybrid embryo between wheat and maize develops but, in the further process, the maize chromosomes are eliminated so that haploid wheat plantlets can be obtained. Such haploid wheat embryos usually cannot develop further when left on the plant, because the endosperm fails to develop in such seeds. By applying growth regulator 2,4-dichlorophenoxyacetic acid *in planta*, embryo growth is maintained to the stage suitable for embryo isolation and further *in vitro* culture. The maize chromosome elimination system in wheat enables the production of large

numbers of haploids from any genotype. Pollination with maize is also effective for inducing haploid embryos in several other cereals, such as barley, triticale (x *Triticosecale*), rye (*Secale cereale*) and oats (*Avena sativa*) (Wędzony, 2009). Similar processes of paternal chromosome elimination occur after the pollination of wheat with wild barley (*H. bulbosum*), sorghum (*Sorghum bicolour* L. Moench) and pearl millet (*Pennisetum glaucum* L.R.Br.; Inagaki, 2003). In contrast to maize and pearl millet pollination, pollination with *H. bulbosum* is strongly influenced by the maternal genotype.

Haploid production in cultivated **potato** (*Solanum tuberosum* L. ssp. *tuberosum*, 2n=4x) can be achieved by inter-generic pollination with selected haploid inducer clones of *S. phureja* (2n=2x). The tetraploid female *S. tuberosum* produces an embryo sac containing one egg cell and two endosperm nuclei, all with the genetic constitution n=2x, while the diploid pollinator *S. phurea* produces two sperms of the genetic constitution n=x or 2x. After pollination, dihaploid (2n=2x) embryos can develop from un-fertilized egg cells, which are supported by a 6x endosperm formed by the fusion of polar nuclei with both reduced sperm cells. The frequency of dihaploid seeds is low; they have to be selected from hybrid seeds containing 3x or 4x embryos developed from egg cells (n=2x) fertilized with haploid (n=x) or diploid (n=2x) sperm cells. (Maine, 2003). Dihaploid potatoes can be used for breeding purposes, including alien germplasm introgression or selection at the diploid level, but such plants are not homozygous. Haploids have a significant role in potato breeding programs of quite a few companies, since they enable interspecific hybridization, which would not otherwise be possible due to differences in ploidy levels and endosperm balance numbers. The gene pool of potato can be broadened and certain valuable traits, such as disease resistance characters from the wild solanaceous species, can be more efficiently introgressed into cultivated potato (Rokka, 2009).

3.1.2 *In vitro i*nduction of maternal haploids - gynogenesis

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. (for a detailed list and protocols overview, see Bohanec, 2009 and Chen et al., 2011) 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. The average frequencies of induced embryos (calculated from ovaries possessing 6 ovules) varied between 0% in non-responding accessions to 18.6-22.6% in extremely responsive accessions, with individual donor plants producing up to 51.7% embryos. The high haploid production frequency was tested in two consecutive years and showed to be stable over years (Bohanec & Jakše, 1999).

Induction rates were even higher in preselected onion genotypes, achieving frequencies of 196.5%embryos from a doubled haploid line (Javornik et al., 1998) or 82.2% for an inbred line (Bohanec, 2003).

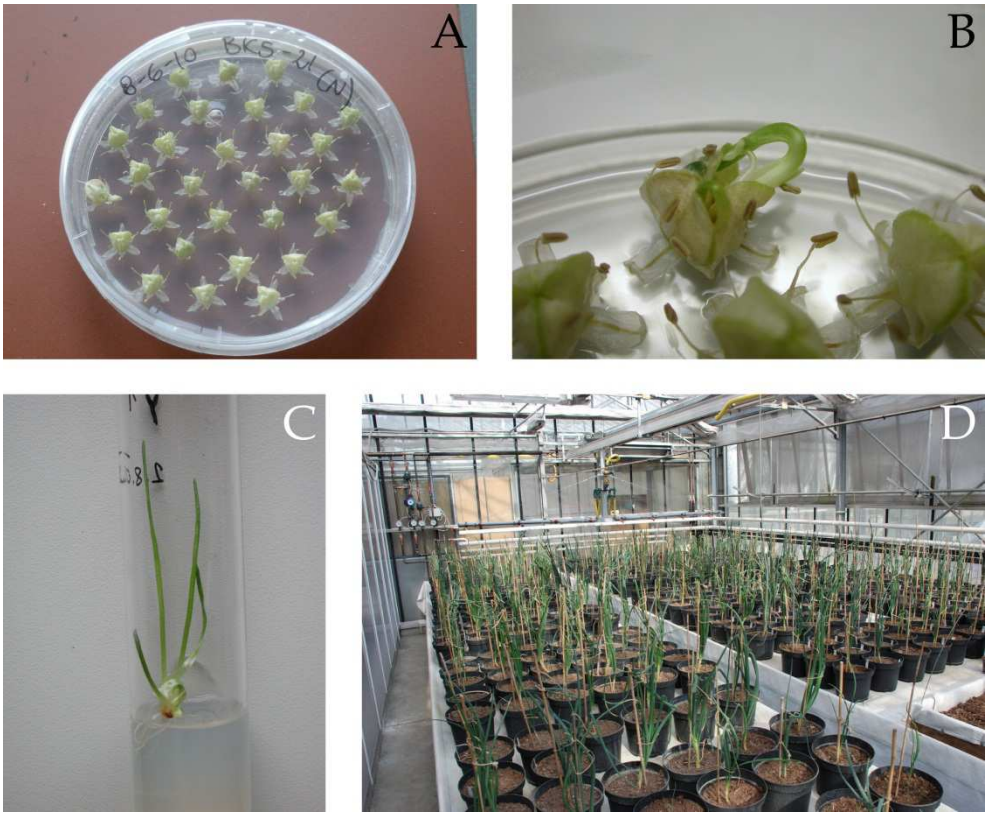

Fig. 1. Production of onion haploid plants with *in vitro* gynogenesis. (A) *In vitro* culture of un-pollinated flower buds on BDS medium (Dunstan and Short, 1977) supplemented with 500 mg/l myo-inositol, 200 mg/l proline, 2 mg/l BAP, 2 mg/l 2,4-D, 100 g/l sucrose and 7 g/l agar; (B) germination of haploid embryos after 60 to 180 days in culture; (C) elongation of haploid plantlets and (D) acclimatization of haploid plants in the greenhouse.

Developmental stage of gametes, the pre-treatment of flower buds prior to inoculation, *in vitro* culture media and culture conditions are other factors affecting the embryogenic response of gametes in culture. The female gametophyte is usually immature at inoculation and, in contrast to androgenesis, its development continues during *in vitro* culture, leading to a mature embryo sac (Musial et al., 2005). Mature embryo sacs contain several haploid cells theoretically capable of forming haploid embryos, such as the egg cell, synergids, antipodal cells and non-fused polar nuclei. However, under optimal conditions, the egg cells in most gynogenetic responsive species undergo sporophytic development (haploid parthenogenesis) (Bohanec, 2009). They can develop into haploid plants directly, avoiding the risk of gametoclonal variation, or through an intermediate callus phase.

Media components, mainly the type and concentration of carbohydrates and plant growth regulators, play an important role in reprogramming haploid cells from gametophytic to the sporophytic pathway. The requirements are species and genotype dependent and no universal protocol for *in vitro* gynogenesis exists.

3.2 Induction of paternal haploids - androgenesis

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. It is well established for plant breeding, genetic studies and/or induced mutations of many plant species, including barley, wheat, maize, rice, triticale, rye, tobacco, rapeseed, other plants from *Brassica* and other genera (for protocols, see Maluszynski et al., 2003). Its major drawbacks are high genotype dependency within species and the recalcitrance of some important agricultural species, such as woody plants, leguminous plants and the model plant *Arabidopsis thaliana*. The method relies on the ability of microspores and immature pollen grains to convert their developmental pathway from gametophytic (leading to mature pollen grain) to sporophytic, resulting in cell division at a haploid level followed by formation of calluses or embryos.

Androgenesis can be induced with *in vitro* culture of immature anthers, a technically simple method consisting of surface sterilization of pre-treated flower buds and subsequent excision of anthers under aseptic conditions. The anthers are inoculated and cultured *in vitro* on solid, semi-solid or liquid mediums or two-phase systems (liquid medium overlaying an agar-solidified medium). Anther culture was the first discovered haploid inducing technique of which efficiency was sufficient for plant breeding purposes (Maluszynski et al., 2003). It is still widely used, although isolated microspore culture is an improved alternative. During isolation of microspores, the anther wall tissues are removed, thus preventing interference of maternal sporophytic tissue during pollen embryogenesis and regeneration from somatic tissue. Moreover, basic research of haploid embryogenesis can be performed directly at the cellular, physiological, biochemical and molecular levels.

Androgenesis, like other haploid inducing techniques, is influenced by several biotic and abiotic factors. The developmental stage of male gametes at the time of anther or microspore isolation, in combination with suitable stress treatments, are the main factors determining the androgenetic response. It can be triggered within a relatively wide developmental window around the first pollen mitosis, when uninucleate microspores divide asymmetrically resulting in a generative cell embedded in a vegetive cytoplasm (Touraev et al., 1997; Maraschin et al., 2005).

The application of suitable physiochemical factors promotes a stress response, which arrests the microspores or young pollen grains in their gametophytic pathway. Their development is triggered through embryogenesis by promoting cell divisions and the formation of multicellular structures contained by the exine wall. Finally, the embryo-like structures are released from the exine wall (Maraschin et al., 2005). The most widely used triggering factors are temperature pre-treatment, sucrose and nitrogen starvation and osmotic stress. Depending on the plant species and genotype, temperature stress can be applied by subjecting excised flower buds, whole inflorescences or excised anthers to low (barley,

wheat, maize, rice, triticale, rye) or high (rapeseed, *Brassica* species, tobacco, wheat) temperatures for several hours or days. As demonstrated in rapeseed and tobacco, different triggering factors can promote embryogenesis from microspores or immature pollen cells at different developmental stages. In rapeseed, early binucleate pollen grains can be converted to the embryologic pathway by applying a heat shock treatment at 32°C, while late binucleate pollen needs an extra heat shock treatment at 41°C (Maraschin et al., 2005). In tobacco, a heat shock treatment is effective in triggering unicellular microspores but not in triggering immature bicellular pollen grains, which successfully start embryogenesis after sucrose and nitrogen starvation (Touraev et al., 1997).

Several other triggering factors such as irradiation, colchicine, auxin and water stress are also used for reprogramming microspores, but to a limited extent. The androgenetic response can be enhanced by *in vivo* pre-treatments of donor plants with nitrogen starvation, short days and low temperature culture conditions.

In addition to stress treatments, the majority of studies have focused on culture media constituents. In general, the concentration of salts in the culture media is lower compared to micropropagation media, but there is no general rule. Several commonly used recipes of macro and micro elements are often used, such as potato-2 (Ying, 1986), W-14, (Jia et al., 1994), NLN (Lichter, 1982), A2 medium (Touraev et al., 1996) and others. The same media are often used for systematically diverse taxa, for instance NLN medium developed for *Brassica napus* was also efficient in *Apiaceae* (Ferrie et al., 2011), while A2 medium developed

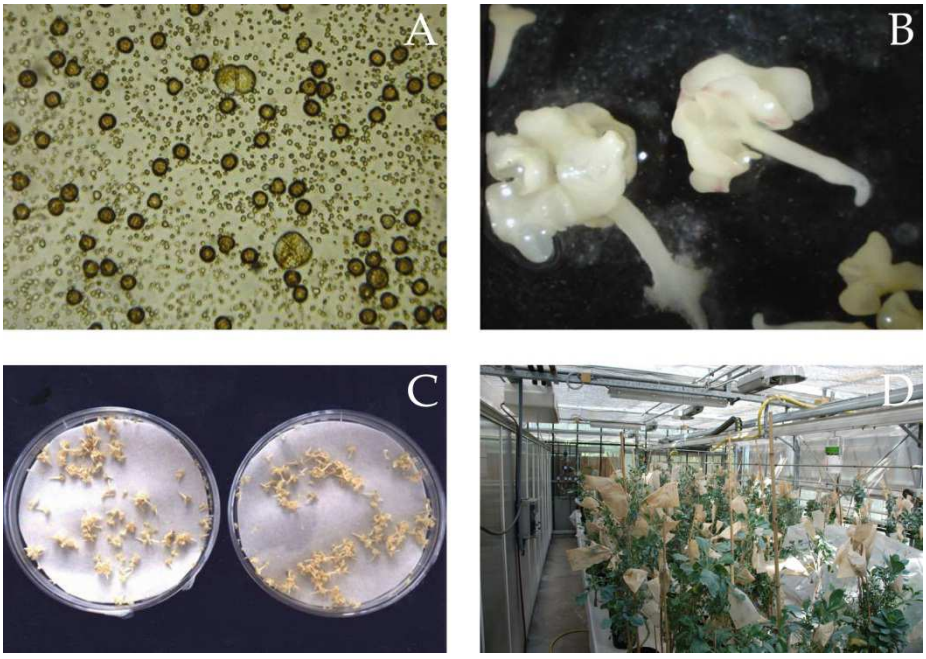

Fig. 2. Microspore culture of cabbage: (A) first divisions of microspores in NLN medium, (B) regenerated embryos, (C) embryos at desiccation treatment needed for regrowth, (D) selfing of DH lines

for tobacco was also optimal for wheat. Occasionally, even an increased concentration of micronutrients might be of high value, for instance elevated copper concentration reduced albinism in cereals (Jacquard et al., 2009). Choices and concentrations of carbohydrates are often essential. The most commonly used carbohydrate is sucrose, particularly in microspore media, and is added in high concentrations (i.e., 13%), while substitution of sucrose by maltose (Hunter et al., 1988) has been an important innovation, first discovered for barley anther culture. Although not always required, plant growth regulators might be essential. The influence of all groups of growth regulators has been tested, with positive effects of polyamines being among the latest studied.

Under optimal *in vitro* culturing conditions, androgenetic plants are regenerated from embryo-like structures (direct microspore embryogenesis) or from microsporial callus cells (organogenesis). Direct embryogenesis is preferred, since regeneration through the callus stage might induce undesired gametoclonal variation and might also cause albinism.

4. Identification of (doubled) haploids: ploidy level determination and homozygosity testing

After a successful haploid induction and the regeneration procedure, evaluation of regenerants is needed to distinguish between desired haploids (or spontaneously doubled haploids) and redundant heterozygous diploids. The haploid inducing techniques presented here differ in haploid induction rates and in the type of undesired regenerants which can be obtained. In this regard, isolated microspore culture is superior to other techniques because the filtering of microspore suspensions during isolation for the most part prevents diploid plant residuals from entering into the *in vitro* culture and later the regeneration of heterozygotes. Moreover, a relatively high proportion of induced haploids spontaneously double their chromosome number, leading to regeneration of homozygous doubled haploids. The phenomenon of spontaneous diploidization of microspore derived embryos has been studied in more detail in barley. The study revealed that, in microspores pre-treated with mannitol, chromosome doubling is caused by nuclear fusion after the first nuclear division (Kasha et al., 2001). In contrast to microspore culture and androgenesis in general, gynogenic regeneration leads to predominantly haploid regenerants in the majority of species.

During the production of homozygous lines, various undesired heterozygous plantlets can be obtained. In anther culture and *in vitro* gynogenesis, such plants can be regenerated from the somatic tissue of inoculated plant organs such as anther wall cells, somatic cells of flower buds, ovaries or ovules. Moreover, heterozygous hybrids, produced after wide hybridization or after pollination with irradiated pollen, are another class of regenerants that could negatively affect breeding progress, if not discarded. Reliable and fast selection of regenerants is therefore necessary before further employment of putative haploids and doubled haploids.

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 vigor 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, see Maluszynska, 2003) and measurement of DNA content using flow cytometry (for a protocol, see Bohanec, 2003). 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.

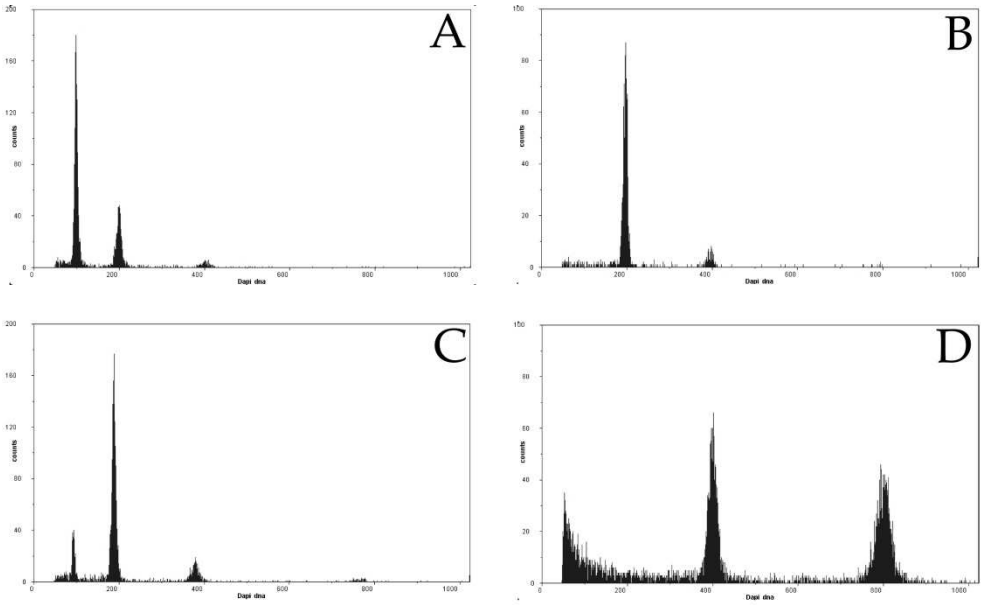

Fig. 3. Determination of ploidy using flow cytometry according to position of peaks representing size of nuclei as determined for microspore derived regenerants of rocket, *Eruca sativa*; (A) haploid, (B) diploid, (C) mixoploid and (D) tetraploid. Note: position of the first peak on the left determines the ploidy, the rest are G2 nuclear stages, G1 and G2 in mixoploids or endoreduplicated nuclei.

Regeneration of diploid plants is not always caused by unwanted adventitious regeneration from somatic cells or germination of hybrid embryos. Spontaneously doubled haploids may also occur, thus eliminating the need for chromosome doubling (see next section). Several markers can be used for assessing the origin of diploids, depending on their availability for a particular plant species. 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.

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. In **maize,** the most efficient haploid identification marker is the 'red crown' kernel trait, which causes deep pigmentation of the aleurone layer in the crown region (endosperm) and scutellum (embryo tissue) (Geiger & Gordillo, 2009). In a haploid inducing cross, the marker should be homozygous recessive in the female parent and homozygous dominant in the pollinator inducer line. After pollination, kernels with a red aleurone crown (resulting from regular fertilization of polar nuclei) containing a non-pigmented scutellum are visually selected from the hybrid kernel of regular fertilization with both aleurone and scutellum pigmented. A similar approach is used in **potato,** in which selection is based on a homozygous dominant color marker gene carried by the pollinator line (Maine, 2003). 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.

5. Chromosome doubling

Following regeneration, haploid plants obtained from either anther or ovule culture may grow normally under *in vitro* conditions or can even be acclimatized to form vital mature plants. Such plants often express reduced vigor but in some crops such as onion, even haploid plants might grow vigorously. At the flowering stage, haploid plants form inflorescences with evident malformations. Due to the absence of one set of homologous chromosomes, meiosis cannot occur, so there is no seed set. Duplication of the chromosome complement is therefore necessary.

As described above, in pollen derived plants, spontaneous duplication of chromosomes may occur in cultures, often in a sufficient proportion, thus eliminating the need for chemically induced doubling. Spontaneously doubled plants are sometimes preferred because of the fear that the duplication process might induce undesired mutations. Mechanisms of spontaneous doubling differ, with nuclear fusion being the most common cause. As first described by Sunderland et al. (1974), 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), is typical of cereals. Other mechanisms such as endomitosis are another possible mechanism that is currently less understood. For maternally derived haploid plants, the rate of spontaneous doubling is often much lower or entirely absent, so a doubling procedure is essential.

Various methods have been applied over several decades and are still in development. The most frequently used application is treatment with anti-microtubule drugs, such as

colchicine (originally extracted from autumn crocus *Colchicum autumnale*), which inhibits microtubule polymerization by binding to tubulin. Although colchicine is highly toxic, used at a millimolar concentration and known to be more efficient in animal than in plant tissues, it is still the most widely used doubling agent. Other options are oryzalin, amiprophos-methyl (APM), trifluralin and pronamide, all of which are used as herbicides and are effective in micromolar concentrations. Anti-microtubule drugs might be applied at various stages of androgenesis, such as being incorporated into microspore pretreatment media. Colchicine application on anther culture medium, for instance, showed a significant increase in embryo formation and green plant regeneration in wheat (Islam, 2010). More often, duplication treatments are applied after regeneration at either embryo, shoot or plantlet level. Similarly, treatments of gynogenically derived embryos with colchine have also been found to be appropriate (Jakše et al., 2003). The treatment of plants at later developmental stages has the advantage that only already tested haploid regenerants are treated either *in vit*ro (for instance at the shoot culture stage) or *in vivo* following acclimatization.

The concentration and duration of treatments must be always determined in relation to two effects: the percentage of doubled plants and the percentage of survival. Optimization treatments often require large experimental units (such as 300 explants per treatment) due to the substantial variation of response. High doses/durations can lead to tetraploidization.

Treatment with nitrogen oxide (N_2O), which was developed for maize seedlings (Kato & Geiger, 2002) is a special case. Plantlets are treated at a high pressure of 600 kPa for two days at the six-leaf stage, in which plants develop flower primordia. The mechanism of action was studied by Kitamura et al. (2009) in *Lillium* and depolymerization of microtubules was found to be the cause.

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). 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 (Jakše et al., 2010).

6. Applications of doubled haploids in plant breeding

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, barley, *Brassica juncea*, eggplant, melon, pepper, rapeseed, rice, tobacco, triticale, wheat and more than 290 varieties have already been released (http://www.scri.ac.uk/assoc/COST851/Default.htm). 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.

Another feature that should be considered is the breeding strategy. Within the breeding process, DH lines can be induced as soon as from F_1 generation (note that gametes on F_1 plants represent the F_2 generation), although some breeders prefer to induce DH lines from later generations. Induction in the F_2 generation was proposed as an option because lines

originated from F_3 generation gametes had passed through another recombination cycle. However, Choo et al. (1982), comparing DH and single seed descent methods showed that there was no difference in the sample of recombinants.

The role of DH in the breeding process largely depends on the plant mode of reproduction. In self-pollinated species, they can represent final cultivars or they can be used as parental lines in hybrid production or test-crosses of cross-pollinated species. The basic breeding scheme in self-pollinated species starts with crossings of desired genotypes, leading to hybrids containing chromosome sets of both parents. During gamete formation, recombinations enable new gene combinations, which are fixed in the process of doubled haploid production. Doubled haploids thus represent recombinant products of parental genomes in a completely homozygous state. They can be propagated as true breeding lines, facilitating large-scale testing of agronomic performance over the years. Due to complete homozygosity, the efficiency of selection for both qualitative and quantitative characters is increased since recessive alleles are fixed in one generation and directly expressed. Additionally, doubled haploids can be used in a recurrent selection scheme in which superior doubled haploids of one cycle represent parents for hybridization for the next cycle. Several cycles of crossing, doubled haploid production and selection are performed and gradual improvement of lines is expected due to the alternation of recombination and selection.

Similarly as with self-pollinated species, the use of doubled haploids in cross-pollinated species improves selection efficiency and can be used at any or each cycle of recurrent selection. Cross-pollinated species are known to possess numerous deleterious recessive alleles that are not expressed in heterozygous states. They are gradually fixed during self-pollination, causing inbreeding depression and difficulties in producing homozygous lines during conventional breeding. Doubled haploid technology helps to overcome these problems through the rapid fixation of genes in one generation and early elimination of deleterious alleles from populations. The recovered recombination products thus represent more viable combinations of genes. Their complete homozygosity enables true breeding and stable field performance over generations of progeny, although the complete lack of heterozygosity and heterogeneity in varieties is thought to be more vulnerable to environmental changes and altered cropping systems.

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) or by application of gibberelic acid, sodium chloride, urea or ammonium sulphate on stigmas (Sun et al., 2005). 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 M1 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.

7. 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 BC_1 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.

Both methods described above are at present predominantly theoretical and one of their obvious limitations is the number of chromosomes of a particular species. As described by Dirks (2009), the probability of finding two lines with a complete set of homologous chromosomes sharply decreases with the number of chromosomes of a particular species. For instance, the probability of identifying such complementary lines is 1 in 47 for *Arabidopsis* (n=x=5) but as high as 1 in 532 in a species with n=x=12.

Genetic transformation at haploid level has been studied in several ways. The most common approach has been for haploid plants to be transformed using established transformation methods. To give just one example, Chauhan et al. (2011) transformed haploid bread wheat with an HVA1 gene to obtain drought tolerance. Chromosome doubling thus enabled stable fixation of the integrated gene, and this feature was tested for 14 generations. Another approach has been for haploid cells themselves, mainly microspores, to be targets of transformation prior to haploid induction. Touraev et al. biolistically transformed tobacco microspores, induced maturation and pollinated to achieve transformed progeny. Eudes and Chugh (2008) and Chugh et al. (2009) transformed triticale microspores using the coupling of cell-penetrating peptides with plasmid DNA and regenerated haploid transformed plants.

A completely novel approach for haploid induction has recently been developed by the genetic engineering of the centromeric region (Ravi & Chan, 2010). Centromeres are chromosomal regions in which DNA sequences serve as binding sites for kinetochore proteins, on which spindle microtubules bind during mitosis and meiosis. In this novel method, a kinetochore protein (Cenh3) was first disabled by mutation and the altered version was then inserted by genetic transformation. In such plants, this novel CENH3 protein is also disabled but only to such an extent that its chromosome segregating function is maintained, while defective kinetochores cause elimination of this chromosomal set during mitotic divisions in zygotic cells. To achieve haploid induction, therefore, the

method requires inactivation of the endogenous *CENH3* gene by mutation or RNAi interference and the insertion of an additional gene coding for the *CENH3* variant. This method has some resemblance to the genome elimination described previously in wide crosses or in the case of maize intra-specific crosses and potentially allows its use in any plant species. The authors claim that another feature of this system is that the 'inducer line' (line with the altered centromeric gene) can be used to induce either maternal or paternal haploids by crossing the mutant with female or male wild-type plants. The procedure has so far been demonstrated in *Arabidopsis thaliana*, causing up to 50% of the F_1 progeny to be haploid. At the same time, this protocol is the first demonstration of haploid induction in this model species, which has been recalcitrant to all haploid induction protocols available so far. Attempts to test this procedure are currently ongoing in other species.

Protocols involving genetic engineering in agricultural applications have given rise to opposition in several countries, thus limiting their availability in breeding programs. It should be noted that, in the case of the presented 'transgenic inducer technology', the final haploid line would not possess any transgenic elements, because the chromosomes of the inducer line are outcompeted by a wild-type parent. It remains to be resolved how such a process will be regulated under GMO legislation. At least at the EU level, legal regulation/deregulation of such new techniques is already under discussion.

8. 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 nowadays 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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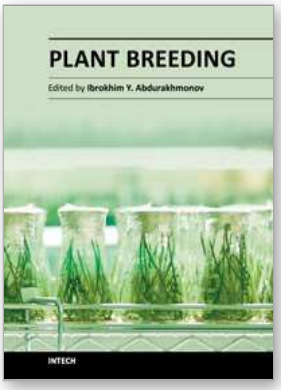

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Modern plant breeding is considered a discipline originating from the science of genetics. It is a complex subject, involving the use of many interdisciplinary modern sciences and technologies that became art, science and business. Revolutionary developments in plant genetics and genomics and coupling plant "omics" achievements with advances on computer science and informatics, as well as laboratory robotics further resulted in unprecedented developments in modern plant breeding, enriching the traditional breeding practices with precise, fast, efficient and cost-effective breeding tools and approaches. The objective of this Plant Breeding book is to present some of the recent advances of 21st century plant breeding, exemplifying novel views, approaches, research efforts, achievements, challenges and perspectives in breeding of some crop species. The book chapters have presented the latest advances and comprehensive information on selected topics that will enhance the reader's knowledge of contemporary plant breeding.

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