Behind the scenes of microspore-based double haploid development in *Brassica napus*: A review

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Abstract

Double haploids are extremely valuable for generating completely homozygous genotypes and have been used in plant breeding program of a number of crop species. This method is a much faster way of developing genetically pure breeding lines in one single generation. The main objective of this review is to describe in a clear and simple manner how microspore-based double haploids of rapeseed are produced and helps a reader to understand the amazing process of microspore embryogenesis, also referred to as androgenesis or pollen embryogenesis. This review will explain what double haploids are as well as their importance in both *Brassica* breeding and molecular studies. Additionally, a brief discussion of different factors affecting the double haploid production and a comprehensive explanation of the steps involved in the development of the double haploids will be covered.

Keywords: Double haploid, protocol, *Brassica*, plant breeding

Introduction

What is double haploid?

Double haploids are plants those carry two sets of chromosomes, similar to normal diploid plants. However, the main difference between them is that the double haploid is created from the haploid pollen grain (without fertilization) cultured on a nutrient medium. The haploid genome of the pollen grain is chemically doubled, produces a plant with a complete, fully homozygous genome. Usually, the toxic chemical 'Colchicine' is used for chromosome doubling to convert the sterile haploid plant into a fertile double haploid plant. Therefore, the double haploid is homozygous at every locus with a potential for having a combination of highly variable phenotypes. The technique of developing double haploids from pollen is also referred to as microspore culture, pollen embryogenesis or androgenesis. The first successful anther culture in *Brassica* was reported by [24] and [61]. However, [32] developed the first culture system to produce double haploid plants from microspores. In the late 1980s, studies on rapeseed (*Brassica napus*) have shown that embryos can be generated with high efficiency by culturing isolated microspores using a hormone free medium without callus phase [25,41].

Review

Importance of double haploid in plant breeding program

In classical plant breeding program a total of about eight inbreeding (selfing) generation is required to get an almost complete homozygous plant (99.2%) with traits of interest. Double haploid shows its superiority in achieving perfect homozygosity (100%) of all traits in only one generation (Figure 1). Plant selection from a double haploid population is extremely valuable and more efficient because the lines are already truly pure breeding and no need for several generations of self-pollination. In self-pollinating species, double haploid can reduce 4 generations in a breeding cycle to release a variety (Figure 1). It is clear that double haploid technology would benefit tremendously to produce inbred lines with 100% purity of any trait to increase significant breeding efficiency and effectiveness. In rapeseed, double haploids are usually produced from hybrids of crosses followed by chromosome doubling. Therefore, every double haploid line could be a potential cultivar/variety. Microspore derived double haploid technology have been used routinely in canola breeding and genetic program to obtain genetically true homozygous lines [28,63]. The double haploid lines not only reduce the breeding

Figure 1. A comparison of a standard time for a cultivar release using traditional and double haploid breeding methods (N.B.: The numbers within the bracket is the percentage of homozygosity).

cycle [4,16,57], but also increase the selection efficiency [15,16,46], and reduce the activity to maintain the breeding lines through selfing and selection [46].

Double haploid in genetic study, molecular marker development, and transformation

Double haploid lines are used with high efficiency in genetic study for traits of interest. In traditional bi-parental crossing population a simple trait controlled by a single dominant gene segregates in the F_2 with a 1:2:1 ratio for homozygous dominant, heterozygous, and homozygous recessive, respectively. However, the same trait will segregate in the double haploid population with a 1:1 ratio for homozygous dominant and homozygous recessive, respectively. In the case of multiple genes, double haploid technology could fix the genotypic recombinations for the homozygosity for many loci in a single generation. Thus, this method offers an advantage to use a smaller population for genetic study of quantitative traits.

In genetic map construction a segregating population, such as F_2 or back cross or near isogenic lines or double haploid is required. The double haploids are true-breeding lines, which can be used repeatedly in marker development program, where the F_2 or back cross populations will segregate in each generation. Studying complex traits to identify quantitative trait loci (QTL) require replicated trials with multiple years and multiple locations evaluation, where double haploid is an ideal population to use. Numerous research on various

crops in using double haploid populations for molecular marker development do exist. Double haploid brings a major advantage in using a dominant marker to select 100% homozygous dominant plants from segregating population, where in the F_2 , the dominant trait segregates as one-third homozygous dominant and two-third heterozygous dominant in the population. Therefore, the success rate of using a dominant marker for selecting homozygous dominant plants in the F_2 population is only 33%.

In genetic transformation, haploid microspores could be targeted in transforming a gene prior to chromosome diploidization process that allow a stable fixation of homozygosity of the integrated gene. Haploid plants could also be used for gene transformation followed by the chromosome diploidization process. Drought tolerant gene (*HVA1*) was successfully transformed in haploid bread wheat followed by chromosome doubling to fix the homozygosity of the integrated gene [5].

A journey towards double haploid canola

It has been reported in previous research that the efficiency of the microspore embryogenesis of Brassica can be affected by many factors such as donor plants growing conditions, genotype, pretreatments, media ingredients, stage of pollen grains, as well as the conditions of the microspore culture [12]. Each of the factors that influence the regeneration of microspore must need to optimize to enhance embryoid production. The factors are briefly discussed here:

Genotypes for double haploid production

B. napus cultivars are more responsive for producing double haploid lines compared to other Brassica species [22]. However, the genotypic difference in microspore derived double haploid production is also reported, such as, *B. napus* cv. Topas is a highly embryonic line [22]. Variable embryogenic response is also identified in different growth habit types, such as the spring growth habit type of *B. napus* is more embryogenic than winter type [22,29]. The genotypes which are poorly responsive to microspore culture have a large variation of different stages of meiosis [29].

Donor plants growing condition

Donor plant growing condition is very important for successful double haploid production. It is recommended to grow the donor in a controlled environment where the stress conditions could be minimized [12]. Plants grown in cool temperatures of 12/10°C one week before microspore isolation was found to be the most optimal for *B. napus* [19]. [44] used 15/10°C for light and dark conditions in a plant growth chamber and observed a better performance of microspore culture. Lower growing temperature can support an equal development of the donor plant that can enhance the success of the microspore culture [2,10,26]. Most commonly the donor plants are grown in a growth chamber for microspore culture. The plants grown in the greenhouse or field are reported to reduce the efficiency in embryogenesis [12].

Cold treatment

Cold treatment on flower buds stimulates the embryogenesis process and improve the quality of the embryo. It significantly enhances the microspore embryogenesis (could be 7 times) over the commonly used microspore culture protocol in *B. napus* [19]. It was also reported that the cold treatment was less effective in *B. rapa* and negatively impact in *B. oleracea* microspore culture. In the cold treatment, the flower buds appropriate for microspore culture are treated with 4°C for 2-4 days in a liquid medium with 13% sucrose in the dark [19].

Bud selection and developmental stage of the pollen grain

Bud size is directly correlated with the meiotic stage of pollen grains. However, this correlation is variable for different genotypes and different species. It is possible to differentiate between higher and smaller amount embryogenic microspore from bud size, but is not possible to predict actual embryo production [41]. During the meiosis, the stage of the microspores can be identified by staining with hematoxylin. Buds at the mid to late uninucleate stage of microspore is the perfect size for double haploid production [12,27]. In the mid uninucleate stage the microspores are round in shape and the nucleus is located at the center of the cell, where the nucleus migrates to the cell wall in the late uninucleate stage [13]. It has been reported that the older stage of microspores may produce toxins and inhibitors during microspore culture

which could reduce the efficiency of the microspore derived double haploid production [29,30].

Microspore concentration

The concentration of microspores in the liquid media is important for normal development of embryos. The density of microspores in solution is determined by a haemocytometer. Study with varying concentrations of microspores have been conducted by various researchers to get higher embryo yield [8,43,58]. The best favorable density is $4-8 \times 10^4$ microspores mL^{-1} has been found in *B. napus* [8,58].

Chromosome doubling

In diploid (2n) species, the sporophyte undergoes meiosis to produce haploid cells which are called microspores. Haploid plants derived from microspores are infertile because of sexual fertility depends upon the meiotic division of the diploid chromosome number. Therefore, chromosome doubling is must to obtain fertile plants. Different methods for *in vitro* chromosome doubling have been used in various crop species. Colchicine is the most commonly used chemical for chromosome doubling in plants [40,56].

Colchicine: Colchicine is a toxic alkaloid from "meadow saffron" also known as the autumn crocus (*Colchicum autumnale*) used for a long time to treat gout and familial Mediterranean fever [49]. In 1934 A.P. Dustin's laboratory in Brussels discovered the role of colchicine as a mitotic poison [11]. However, [34] was the one who concluded that the colchicine causes an arrest of mitosis due to a failure of the mitotic spindle to form and function in the normal manner. The mode of action of colchicine was well-studied and it was discovered that this chemical forms tubulin-colchicine-complex, which attaches to the ends of microtubules physically inhibiting the polymerization of the microtubule [38]. Colchicine could be incorporated into microspore culture media [66] or could be applied to shoot-tip using cotton wool balls at 5-6 leaf stage [35] in *B. napus*. Colchicine application rate may influence the rate of diploidization in microspore culture. [37] observed higher (80-90%) diploid embryo with 50 mg/L colchicine for 24 h or 10 mg/L colchicine for 72 h, but the lower diploidization rate (70-80%) with 100 mg/L rate for 24 h in microspore culture. [35] used various rates (0.10, 0.15, 0.20%) of colchicine on shoot-tip using cotton wool balls and reported that 0.15% gave the highest success (86%) of chromosome doubling in *B. napus*.

Alternatives to colchicine: [20] looked at the potential of colchicine and three microtubule depolymerizing herbicides trifluralin, oryzalin, and amiprophosmethyl (APM) for chromosome doubling in *B. napus* microspore culture. The study observed 94% chromosome doubling rate after 24 h treatment with 1 mM colchicine. The three herbicides worked similarly to colchicine but at 100x lower concentrations. Interestingly, APM showed lower toxicity than other herbicides making it the preferred chromosome doubling agent [20]. [67] also proposed trifluralin as an alternative treatment to colchicine.

Trifluralin showed faster than colchicine depolymerization of microtubules (30 min vs. 3-8 h of colchicine treatment). The study reported 60% of success in producing normally developing fertile plants when using trifluralin. [67] recommended 1 or 10 μ M trifluralin during initial 18 h of microspore culture as a superior method for generating double haploids.

Heat shock

High temperature heat shock treatment (32°C for 3-21d) is commonly used for developing microspore derived embryos, both in anther and microspore culture [7,13,42,44,50,51,64]. In *B. napus*, changes of cellular architecture from gametophytic to embryogenic pathway by heat shock treatment have been reported by many researchers [48,51,53,59,60,65]. Heat treatment at 32-35°C for 3-4 days in microspore culture of amphidiploid Brassica species, such as *B. napus*, *B. carinata* and *B. juncea* generated effective embryos [6,39,41]. Successful application of heat shock in anther/ microspore cultures of horticultural herbaceous and woody species and microspores reprogramming to embryogenesis induction have also been identified [17,55].

Growth regulators

Plant growth regulators are needed to enhance tissue culture conditions, such as callus tissue proliferation, root or shoot formation. The commonly used plant growth regulators are auxins, cytokinins, gibberellins. Auxins such as IAA (indoleacetic acid), IBA (Indolebutyric acid), NAA (naphthalenescetic acid), 2,4-D (2,4-dichlorophenoxyacetic acid) are used in rooting media which promote cell and root growth [47]. Cytokinins such as BAP (benzylaminopurine), zeatin, kinetin are used in shooting media which enhance cell division and shoot growth [33]. Gibberellins (GA) promote cell elongation and is a key component in GA-regulated stem elongation process [9,21]. Other stress hormones such as abscisic acid, jasmonic acid, salicylic acid play an important role to promote somatic embryogenesis and improve the quality of embryos [1]. Activated charcoal has been identified to be beneficial for embryo growth and development in both microspore and anther culture in *B. napus* [18,23]. However, the role of charcoal in inducing microspore embryogenesis is unknown [7,18].

Confirmation of the double haploid's ploidy level

The chromosome complement of the plants regenerated from microspore could be haploid, diploid or polyploid. These haploid or polyploid plants cause meiotic failure and infertility. Even, colchicine treated microspore derived plant may have haploid or polyploid chromosome complements. Thus, it reduces the efficiency of double haploid plant production and a number of infertile plants survive until flowering. Plant morphology, cytological analysis and flow cytometry could be used to confirm the ploidy level of microspores derived plants.

Morphological clues

Both haploid and double haploid plants generate flowering

buds and flowers. However, flowers from double haploid plants are fertile and they are sterile in haploid plants. A morphological distinction also identified between the haploid and double haploid plants. [62] reported a distinct morphological difference in *Chrysanthemum morifolium* between these two types where the haploid plant was shorter than the doubled haploid plants, and developed smaller leaves, flowers, and stomata. We also identified weak and thin plants with flower bud and flower size, and anther with no pollen grain in haploid plants of *B. napus* derived from microspore culture.

Cytological analysis

Direct method for ploidy determination through chromosome counting in mitotic cells is the best method to confirm the diploid chromosome number (for protocol, see) [36]. However, the chromosomes of *B. napus* are small and indistinct, which is complicated, challenging and time consuming to count under microscope during mitotic cell division stages [54].

Flow cytometry

Flow cytometry is commonly used in plant species to determine the DNA content of isolated nuclei. In the traditional method, the microspore derived double haploid progeny need to grow up to flowering stage without knowing the ploidy level or fertility of the plants. The flow cytometry is used to identify the ploidy level of the microspore derived double haploid plants at the youngest emerging stage of seedling which will allow us to identify and to grow only potential double haploid plants [58]. It is a simple method and provides a rapid detection of quantifying DNA in cells. In this process, a nuclear staining agent, propidium iodide (PI), is used which binds to double stranded DNA by intercalating between base pairs. PI is excited at 488 nm and emits at a maximum wavelength of 617 nm. The quantity of DNA in each nucleus is correlated with the degree of PI fluorescence of cell nuclei (for protocol, see) [45].

Laboratory protocol for double haploid production

The protocol for the production of microspore-derived double haploids of *B. napus* is has been shown in Figure 2 and described as follows:

Donor plant growth conditions

Optimal plant growth condition is important to get healthy plants which also enhance the embryogenesis process. Light, temperature, fertilizer and water were supplied to produce healthy plants. The donor plants were grown in a greenhouse room with a 16 h photoperiod and 24/18°C days/night temperature. At flowering state the plants were transferred into a plant growth chamber with a 16 h photoperiod and 15/10°C day/night temperature one week before microspores isolation. It is reported that the cold temperature during flowering time enhance the embryogenesis process [31]. The donor plants grown during winter season in a greenhouse responded much

better to embryo culture and produced a higher number of embryos in comparison to the donor plants grown during summer season in the greenhouse. Therefore, it is better to grow the donor plants in a controlled plant growth room during summer time.

Bud selection and sterilization

We have identified a relationship between a bud size and a meiotic stage of microspores through graded the buds into five bud sizes. Two anthers from each bud were crushed on a slide containing acetocarmine stain, and squeeze the microspores out of the anthers using a needle. The anther tissues were removed, the slide was warmed over a flame for 3-4 seconds and a cover slip was placed on. The bud size correlated with the highest number of microspores in a uninucleate stage under the microscope were selected for microspores isolation (Figure 3). Bud size and microspore development stage may vary depending on the genotype of donor parent and plant growing conditions.

Microspores isolation and culture

Buds containing uninucleate stage of microspores were surface-sterilized in cold 15% (v/v) commercial bleach for 10 min and washed for 4-5 times with cold sterile distilled water. The buds were crushed in a cold and sterilized mortar and pestle. Initially, the buds were rinsed with cold half-strength B5 medium (B5 Gamborg media with 130 g/L of sucrose, pH 5.8-autoclaved) [14], and macerate the buds in a fresh cold half-strength B5 for around 15 seconds. The suspension was filtered through 40 microns cell strainer and collected in a 50 ml falcon tube. The mortar, pestle and the strainer were rinsed with half-strength B5 medium to make up the volume to 30 ml. The falcon tube containing microspores suspension was centrifuged at 700 rpm for 3 min at 4°C. The supernatant was discarded and the pellet was re-suspended in 10 ml of

Figure 3. Microspores of canola at different stages of development. Microspores marked with red arrows are in the uninucleate stage that is used in the process of double haploid production.

cold half-strength B5 medium and centrifuged as mentioned before. The microspores washing process was repeated two times. The supernatant was discarded and the final pellet was suspended in cold NLN-13 (NLN-13 with 130 g/L of sucrose, pH 6.0-filter sterilized) [32] with a density of about 4×10^5 microspores ml^{-1} . Large sterilized petri plates (100x20 mm) were used for plating and labeled with the name of the genotype and the date of the isolation. Colchicine (200 μM) was added with 12.5 μl for every 10 ml of microspores solution. The plates were incubated for 48 h in 32°C in the dark. After the colchicine treatment and heat shock (incubation) about 20 ml microspores suspension was transferred into a new 50 ml Falcon tubes, and centrifuged at 700 rpm for 3 min. The supernatant was discarded and the pellet was re-suspended in an equal amount of fresh NLN-13. The plates were placed in the dark for another 11 days under room temperature at 22°C. Microspores in the mid-uninucleate stage are the most responsive to the double haploid production. Additionally, differential growth of embryos (Figure 5) carries higher contamination risk, and 35-40 days old embryos have a lower survival rate.

Embryo regeneration into plants

After 11 days in the dark, the petri dishes were wrapped with aluminium foil and place them on a horizontal shaker with 60 rpm under room temperature at 22°C. The plates were kept there for 8-20 days or until the embryos turned into green and have developed coleoptiles parallel to the stem. NLN media was changed in every 2 weeks. Embryo development is variable for different genotypes (Figure 4). About 10 embryos were plated per petri plate (100x20 mm) containing full strength B5 solid medium (20 g/L of sucrose, pH 5.8, 2.8 g of gellan gum and autoclaved). After autoclaving, GA3 (250 $\mu\text{L/L}$) and Plant Preservative Mixture [PPM] (1 ml/L) were added when the medium temperature was cool down to 50°C. The embryos were grown at 25°C with a 16-h photoperiod. The embryos should be at least 20 days old from the day of extraction and the younger embryos will not or will take longer time to turn

Figure 4. Differential response of three different genotypes to microspore culture.

Figure 5. Canola embryos growing at different rates.

into plantlets. ABA could add in the media to synchronize the embryos growth and prevent embryo degeneration [3]. A cold shock with 4°C under 8-h photoperiod was given for 10 days. The petri dishes with plantlets were moved to 27°C under light till they develop both roots and healthy leaves. The plantlets without root were transferred into rooting media containing the same full strength B5 solid medium with auxin. Age of the microspores culture media and the size of the embryos to transfer to solid media are very important for successful regeneration of double haploid plants. Embryos

younger than 21 days will not recover into plants and similarly embryos with small and unopened coleoptiles will not thrive in further steps of the culture. Therefore, only embryos older than 21 days with coleoptiles opened perpendicularly to the stem are the best for double haploid production. Contamination is a major problem in microspore culture and causes many embryo losses. We could reduce the number of infections by using antimicrobial additive such as Plant Protective Mixture (PPM) in the medium. The PPM provides protection from both bacteria and fungi without compromising embryo growth and embryo regeneration into plantlets.

Planting the double haploids

Healthy plantlets (Figure 6) were transferred into the soil (Figure 7) in a plant growth chamber with a 16 h photoperiod and 24/18°C day/night temperature. The plantlets were planted in a tray, covered with a transparent tray lid and keep them well lighten in the growth chamber. The lid was covered for a week and ensured to have sufficient moisture inside the tray to adapt the seedling in natural conditions. The properly grown and strong seedlings were transferred into pots with soil and fertilizer. Evaluation and selection of double haploid plants were conducted at flowering stage.

Evaluation and selection of double haploid plants

The double haploid inducing technique may regenerate undesirable haploid plantlets. Therefore, it is necessary to evaluate and select the potential regenerated plantlets. Several methods such as morphological characterization, ploidy determination are available to differentiate the haploid and double haploid plants. The morphological characterization is based on characteristics between regenerated and donor

Figure 6. Plantlets with healthy leaves and roots ready to transfer into soil media.

Figure 7. Plantlets are transferred into pots containing soil media.

plants for pollen fertility, flower morphology, leaf shape, plant height, plant vigor etc. The morphological characterization does not need costly equipment. Therefore, the regenerated potential double haploid plantlets transferred into pot were evaluated for various developmental stages until the flowering time. Plants with sterile flower (usually, weak plant, smaller flower and no pollen grain) were identified as haploid plants and discarded from our inventory.

Conclusion

Double haploid production is an emerging technology in many plant breeding programs. It is an efficient tool for the production of completely homozygous lines from heterozygous parents in a single step. This technology was first applied in *Brassica* in 1975. The protocol for double haploid production in *B. napus* is well-established. Although different laboratories have developed different protocols, however, the major chemicals and the procedure remain same across the laboratories. In addition to using the technology successfully in breeding program, it has also been used in genetic studies, gene mapping, marker development, location of QTL, gene transformation research. This technology can efficiently be combined with other plant biotechnological methods to improve the research efficiency.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Authors' contributions	MR	MM
Research concept and design	✓	--
Collection and/or assembly of data	✓	✓
Data analysis and interpretation	✓	✓
Writing the article	✓	✓
Critical revision of the article	✓	--
Final approval of article	✓	✓
Statistical analysis	--	--

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