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DOUBLED HAPLOID PRODUCTION IN *BRASSICA OLERACEA* L. : A REVIEW

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Abstract

Brassica vegetables are of considerable interest to the breeders and seed companies since their area of production has increased in the recent years mainly due to improved nutritional qualities like anti-cancerous, antioxidants and various other properties which enrich their nutritional profile. In Vegetable breeding, private seed companies strive more and more to bring F_1 hybrid seeds onto the market. F_1 cultivars ensure high uniformity, offer high yields due to heterosis, allow rapid production and selection of desired genotypes and provide protection to plant breeder's rights and the markets of seed companies. The production of hybrid cultivars requires homozygous parental lines. Inbred lines in *Brassica* vegetables can be produced by recurrent selfing, a procedure that takes time (6 to 7 generations of selfing) and is labour intensive as these are highly cross pollinated and due to the presence of saprophytic self-incompatibility. An alternative way to obtain pure inbred lines in one generation is the production of doubled haploid (DH) lines by microspore culture. Hence, this review describes the different factors which are responsible for efficient DH production in *Brassica* vegetables.

Key words: *Brassica* vegetables, Doubled haploid, Embryogenesis, Microspore culture, Plant regeneration.

Introduction

Modern cultivars of most of the *Brassica* vegetables are F_1 hybrids. Development of F_1 hybrid differs from inbred ones, in having higher yields and better qualities. Such developed hybrids are considered as a priority in the modern breeding of agricultural crops. Development of pure/inbred parental lines for F_1 hybrid breeding is usually labour intensive, time intensive and takes around 6-7 generations to establish homozygosity (Yan *et al*., 2017). Therefore, for establishing the homozygous parental lines, development of doubled haploid (DH) plants from microspores can be a valuable alternative to combat conventional criteria. DH plants thus, obtained are the potent homozygous lines, useful for exhibiting desired agronomic traits (Maluszynski *et al*., 1995; Morrison and Evans, 1988). DH, broaden the spectrum of basic research involving gene transfer, biochemical and physiological studies and in the production of desired traits such as herbicide resistance and fatty acid modification through mutagenesis and selection (Gil humanes, 2009). This lays one of the most important areas of practical application of this technology in plant breeding. At present, most of the developed countries widely use DH technology for the production of completely homozygous parental lines to accelerate breeding (Dunwell, 2010). To this, broccoli breeders increasingly use the development of DH lines from anther or microspore culture (Kellar *et al*., 1975; Takahata and Keller, 1991). The production of DH lines in broccoli through anther culture has been reported (Keller and Armstrong, 1983), however the general problem encountered in this approach is that the resulting population contains mixture of haploids, DH, triploids and aneuploids individuals (Chiang *et al*., 1985). Because of mixed populations resulting from culture, breeder must identify diploids, since they are true DH individuals having potent homozygous lines to be further used for hybrid combinations. A new culture technique was initiated by extracting microspores from anthers (buds) and, in turn, the microspores are cultured, free of any diploid anther tissue. Initially, early 1980s are attributed for the first successful surveys on *Brassica* crop microspore (Lichter, 1982). Later, the microspore culture was applied forth to diverse species of Brassica, like Cauliflower (*Brassica. oleracea* var. *botrytis*), Broccoli (*Brassica. oleracea* var. *italica*), Tronchuda cabbage (*Brassica. oleracea* var. *costata*),

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Kohlrabi (*Brassica oleracea* var. *gongylodes*), White cabbage (*Brassica oleracea* var. *capitata*), Flowering cabbage (*Brassica oleracea* var. *acephala*) and Pak Choi (*Brassica rapa* ssp. *chinensis*) (Lichter, 1989; Takahat and Keller, 1991; Duijs *et al*., 1992; Cao *et al*., 1994; Zhang *et al*., 2008; Winarto and Teixeira da Silva, 2011 and Yuan *et al*., 2012).

Culture of isolated microspores has several advantages over anther culture, including the absence of possibly regenerating anther tissue and a significant reduction in labour (Swanson *et al.,* 1987). However, yield is one of the most important drive, as not only brassica but in almost every cultivar of different genus where microspore technique has been employed faces low embryo yields and this yield is affected by numerous factors like growth condition-constraints, genotype dependency, stages of microspore development, culture medium composition and cultivation conditions (Duijs *et al.,* 1992; Pink, 1999). In addition, the optimal value of the above listed factors is a core requirement for embryogenesis. So, it leads to a great room for researchers to carry out to intensively, some complex technical modification in order to obtain efficient number of regenerants from an efficient embryogenesis process. Despite this, little work has been done on the applicability of microspore culture to the different horticultural crop types of *B. oleracea*, such as Brussel's sprouts, cauliflower, curly kale, broccoli and various cabbage types.

The focus of the present study is to summarize general review regarding the development of DH in *Brassica* vegetables using microspore culture technique and to direct the focus on certain approaches which help to improve the microspore embryogenesis in *Brassica* genus.

Factors Affecting Embryo Formation

Due to the intra and interspecific differences in response to androgenesis, no universal protocol is available for isolated anther/ microspore culture. Basic steps used for initial screening to define response for androgenesis remains constant, they include donor plant growth, bud selection, microspore isolation and cultivation, embryogenesis induction, plant regeneration, chromosome doubling, ploidy conformation and hardening. The fundamental protocol which forms the basis of microspore isolation and the steps described since so far till date, have been optimized regularly by scientists. In this context, the present report results from a program, aimed at the development of a microspore culture and the attention has been paid to factors influencing microspore embryogenesis in *Brassica oleracea*.

Genotype and donor growth conditions

Brassica microspore culture is highly genotype-dependent, as reported in most of the *Brassica* species (Ferrie *et al.,* 1995; Lichter, 1989; Phippen, 1990). This variability was studied by Barro and Martin, (1999) in which only 10 lines out 16 of *B. carinata* tested showed cell division and embryo formation. The conditions under which donor plants are grown are an important consideration for successful culture of *brassica* microspores. To minimize stress donor plants have to be grown in an environmentally controlled growth chamber. Optimal growth conditions produce healthy plants and enhance embryogenic responses. Factors such as temperature, light, water and nutrients are important in order to obtain healthy plants. It is known that the most responsive microspores are obtained from plants grown at low temperatures (Dunwell, 1985). For example, best results were obtained in *B. carinata* plants, in which plants are grown at 25°C/15°C day/night temperature cycle for 1 month and then at 15°C/10°C for 2 months. Plants can be used as donors for a period of up to 2 weeks after the first flower has opened. Da silvadias, (2003) stated that the growth temperatures of donor plants in *Brassica oleracea*, should not exceed 20°C. Like temperature regimes of 20°C day/15°C night or constant l8°C, with a 16/8 h photoperiod and a photosynthetic photon flux density of 150-200 $\mu E\ m^{-2} s^{-1}$ given by 'warm-white' tubular fluorescent lamps are adequate.

Microspore development stage

The proper evaluation of male gametophyte of a donor plant, at its right stage, marks the success of efficient embryogenic induction. In *Brassica* genus plants, the microspores at late uninucleate stage with variation of 10-30% bi-nucleate microspores were capable of embryogenesis, (Pechan and Keller, 1988; Huang *et al.,* 1990). Kott *et al*., (1988) saw a reduction of the conversion of microspores to embryos in microspore cultures of *Brassica napus*, caused by a toxin generated by the cultured microspores themselves. The negative effects of the toxin were correlated with the presence of bi-nucleate microspores in the culture. Baillie *et al*., (1992) assessed the length of flower buds for embryoid development, out of two sizes taken, 2.2-2.9 mm and 3.0-3.9 mm in *Brassica campestris,* the embryoids development was observed from microspores isolated from flower buds having size 2.2-2.9 mm. The strong variations in optimal size of flower buds in *Brassica campestris* and *Brassica napus* were also established (Pechan and Keller,1988). In *B. oleracea var botrytis* L. the optimized bud size late uninucleate to early binuclete contained highest percentage of viable microspores

(Bhatia *et al.,* 2016). The proper determination of optimal bud sizes lays the prerequisite provision for a successful microspore conduction experiment. The microspores having late uninucleate stage with smallest size in *Brassica nigra*, medium size in *Brassica napus* and largest one in *Brassica oleracea* resulted in better embryogenic response (Lichter, 1989). For accessing proper microspore development stage bud length is said to be more easy reference and it can be further correlated by microspore staging, using fluorescent microscopy and DAPI staining which allows good visualization of the nucleus.

Microspore isolation

Generation of broccoli embryos using microspore culture were first accomplished using procedures described by Duijs *et al*., (1992). However, stress patterns play an efficient role in embryogenesis to occur. The type of stress can vary from heat to gamma irradiation and colchicine treatment (Pechan and Keller, 1988; Zaki, 1995; Zhao *et al*., 1996). Nevertheless, heat treatment is mostly used in *Brassica* species in order to induce embryogenesis in microspore culture. Pechan, (2001) reported that 32°C temperature treatment is an absolute temperature requirement for inducing androgenesis in *B. napus*. In *B. carinata*, embryogenic response has seen within 1-4 days of pre-treatment at 32°C. Further, incubation in this temperature didn't improve cell division and embryo yield was drastically reduced (Barro and Martin, 1999) later the microspores are to be subjected to 25°C for further incubation resulting in embryogenesis. Generally, temperature stress treatment is given either before first haploid mitosis or during it. The incubation at first eight hours under high temperature stress is most critical. The regulatory processes of induction and embryogenesis of microspores are activated in stated time frame. Simmond and Keller, (1999) laid the observation that the first division of microspores is sysmetric at high temperature stress, contrary to the normal asymmetric division. Formation of heat shock proteins (HSPs) in microspores is caused by the effect of high temperature on microspores (Pechan *et al.,* 1988; Cordewener *et al.,* 1995; Pechan and Smykal, 2001). These proteins are directly linked to embryogenic induction. Various scientists designed a temperature regime for best heat shock. Bhatia *et al*., (2016, 2017) stated that in *Brassica oleracea*, temperature pre-treatment significantly affects the embryo yield. No microspore embryogenesis has been observed in the cultures maintained continuously at 25°C. The post culture temperature pretreatment of 32°C for 24 h followed by continuous maintenance at 30°C, induced maximum microspore embryogenesis, whereas pretreatment of 32°C for 48 h induced less embryonic response in broccoli microspore culture (Da silvadias, 2000). Post heat shock treatment, the maintenance of microspore cultures at continuously 20°C, reduced the microspore embryogenesis (Bhatia *et al.,* 2016, 2017). The repository of accumulated facts, led to the assumption, that cytoskeleton actively participates in regulating the mitosis and is involved in embryogenesis (Hause *et al.,* 1993).

A contradiction arose to previous surveys, as, low temperature treatment of microspores for embryogenesis induction in Brassica genus is not often. The effectiveness of low temperature treatment of flower buds to produce embryos using the microspore cultures in *Brassica. napus* (Lichter, 1982), *Brassica. oleracea* (Osolnik *et al.,* 1993) and *Brassica. rapa* (Sato *et al.*, 2002), as well as the direct pretreatment of isolated microspores in *Brassica napus* (Charne and Beversdorf, 1988) have also been reported. In direct pretreatment, use of cold pretreatment (4°C) of the *Brassica napus* surface sterilized flower buds into the NLN nutrient medium containing 13 percent of sucrose and reported positive embryogenesis. The negative effects of cold treatments in *Brassica napus* and *Brassica rapa* have also been reported earlier by Dunwell *et al.,* (1985) and Sopory and Munshi, (1996), respectively. The above findings underline the facts that each variety requires a prerequisite single inducible factor, this factor may have dependency on a plant's tolerance either to heat pretreatment or cold mediated pretreatment.

Microspore culture density

The other major factor affecting the microspore yields is microspore culture density. There is an indicatory assertion that, the optimal density cannot be applied to all *Brassica* crops. A microspore culture density of 100000 cfu mL is reported to be inhibitory to embryogenesis (Huang *et al.,* 1990). Density which is not surpassing the count, 50000 cfu mL is reported to have positive effect, on embryogenesis in *Brassica oleracea* (Ferrie *et al.,* 1999). Bhatia *et al*., (2017) established a relationship of optimal densities at different concentrations for embryogenesis in *brassica oleracea* with microspore densities of 2×10^4 per ml, 4×10^4 per ml, 6×10^4 per ml, 8×10^4 per ml and 10×10^4 per ml and to their findings only few embryos were reported with the microspore density of 2×10^4 per ml. The microspore culture density of 4×10^4 per ml induced the maximum embryos per plate and further increase in the microspore density beyond 4×10^4 per ml drastically reduced the embryo yield per plate. Overall, the density of 10-40 thousand cells per mL is mostly preferable for a good deal of varieties and species.

Osmotic pressure

One of the critical factor for developing proper embryoids using microspore technology is osmotic pressure. The early stages of development usually carries a requirement of high osmotic pressure, while low requirements at late developmental stage. The use of Sucrose at concentration of 13% is reported as, *in-vitro* source of carbohydrates and regulator of osmotic pressure in different species of the genus of *Brassica* (Palmer *et al.,* 1996). Moreover, higher embryo yields were ensured at initial stages with higher concentration of sucrose in number of surveys. Baillie *et al.,* (1992); Ferrie *et al.,* (1999) and Lionneton *et al.,* (2001) worked with *B. campestris*, *B. oleracea* and *B. juncea*, respectively, reported higher frequency of microspores embryogenesis at in a medium containing 17% sucrose solution during 48 hours of cultivation. Evidence regarding the double effect of sucrose on the development of embryoid bodies have been provided by Ilic Grubor *et al.,* (1998) in *Brassica napus*. Ferrie *et al.,* (1999) in *B. oleracea* showed that sucrose is one of the most important medium components affecting and regulating embryogenesis.

Activated charcoal (AC)

Chemically, AC is used for the removal of gaseous substances and solid solutes as it is a strong adsorbent. Gland *et al.,* (1988) gave an assumption that AC usually adsorbs toxins which are produced by inactive microspores and in return improves the efficacy of embryo-genic development of microspores. Johansson, (1983) showed one such toxin is the ethylene, among other gaseous substances produced by cells in, *in-vitro* culture. The other assumption regarding the adsorptive property of AC is that it adsorbs the phenolic compounds produced by the damaged tissues during the isolation process (Fridborg *et al.,* 1978). Generally, in the *Brassica* microspore cultures, low levels of phenolic compounds are expected, as microspore suspension mainly lacks somatic tissues; but some amounts of phenolics could be released into the nutrient medium. Thus, adsorption of phenolic compounds by AC, is reported to have stimulatory improved effects on the embryogenesis, in anther culture of datura and anemone (Johansson, 1983). So, to increase the microspore efficiency, activated charcoal (AC) is generally incorporated along with the nutrient media in *B. rapa* ssp. *oleifera* (Guo and Pulli, 1996), *B. oleracea* (Dias, 1999) and *B. juncea* (Prem *et al.,* 2008). Gland *et al*., (1988) reported that AC solely not only increase embryogenesis but also improves the regeneration of plants from embryoids in rape. Margale and Chevre, (1991) revealed that AC at 1% concentration, improves the embryo yields, while the unavailability of AC resulted in haulting of embryoid development at the level of 4-8 cells only. The effects of AC added in the nutrient media showed variation depending on the genotype in broccoli and cauliflower microspores of Dias, (2001). Experiments stating the positive effects on embryogenesis were apparent when AC was added with low melting agarose, whereas the negative effects were reported in experiments when AC was added without agarose (Guo and Pulli, 1966). The dependency of AC upon the concentration of macro salts in nutrient medium was reported (Takahashi *et al.,* 2012). It was found by Wang *et al*., (2009) that embryo induction to occur in a medium containing half strength NLN macro salt without AC, which was apparently better than full strength medium.

Other factors affecting embryogenesis

The physiological role of nutrient medium pH is just as important as above factors discussed. The survey of Yuan *et al*., (2012) indicated the pH value (6.2-6.4) in the nutrient medium to be more effective for microspore embryogenesis in the number of *Brassica* genotypes, when compared to the pH 5.8. The best effect in this survey were obtained with the NLN-13 (pH 6.4) nutrient medium with arabinogalactan protein and 2-(N-morpholino)-ethanesulphonic acid (MES) when added as a buffer, leading to increase in the efficiency of embryogenesis induction rate from 4.5 to 22.9 embryoids per flower bud.

Exogenic growth regulators play a very important role in embryoid production in *Brassica* species. The use of 6-benzylaminopurine (BAP) and α-naphthylacetic acid (NAA) at low concentrations tends to increase the effectiveness of embryo production in microspore cultures, which is concordance to the work of Charne and Beversdorf, (1988) in *B. napus*, Takahashi *et al.,* (2012) in *Brassica rapa*, Lee and Kim, (2000) in *Brassica campestris* ssp. *Pekinensis* and Na *et al.,* (2011) in *Brassica oleracea* var. *italica.*

During the process of microspore suspension culture the disintegration of anther cell memberane is resulted producing ethylene causing negative effects on embryogenesis. The inhibitors of ethylene synthesis like silver nitrate, silver thiosulfate, cobalt chloride and aminoethoxyvinylglycine are generally added to the nutrient media to enhance the embryogenesis (Prem *et al.,* 2005, 2008; Na *et al.,* 2011). Pretreatment of plants of the *Brassica* genus, with PCIB anti-auxins (3-chlorophenoxy isobutyric acid), reduced the production of ethylene, which is auxin induced (Agarwal *et al.,* 2006; Ahmadi *et al.,* 2012). Ethylene reduction is also reported with the addition of a Pluronic F-68 polymer to the nutrient media for the induction of embryogenesis in *Brassica. napus* irresponsive genotypes (Barbulescu *et al*., 2011).

Factors affecting plant regeneration

Since the frequency of adequate plant production is usually low and variable, so protocol enhancing the regeneration of plants for embryoids also needs random exercises for DH production.

According to the data compiled from the literature, embryoid pretreatments with gibberellic acid (GA) and abscisic acid (ABA), were reported as an enhancer for plant regeneration (Kott and Beversdorf, 1990; Huang *et al.,* 1991; Cegielska Taras *et al.,* 2002; Zhang *et al.,* 2006). Harvesting of embryos on a plate containing a filter paper over an agar medium or medium containing a jellifying substance also reported to enhance plant regeneration at high concentration (Takahata and Keller, 1991; Takahashi *et al.,* 2012).

Hormones play an important key in proper regulatory development pathway, like Auxins, which are generally reported to be responsible for a symmetric transition from a globular body to heart shaped embryoid body. The lack of a proper auxin level creates a void preventing hypocotyl elongation during its transition from the torpedo shape stage to the cotyledon stage (Yeung and Ramesar Fortner, 2006). The other hormone with a significant morphogenic role is ABA, which is normally synthesized in the vegetative parts of a plant and is transported into the seeds where it is accumulated in the endosperm. It has been also reported that proper levels of ABA and IAA are having relatedness to the somatic embryogenic response in certain plant species (Feher *et al.,* 2003). ABA not only has a distinctive role in maintainence of morphological integrity of early embryoids during zygotic embryogenesis, but also it has role in last stages of development and preservation of nutrient elements. The absolute level of ABA is generally lower in microspore derived embryoids when compared to zygotic embryoids in the same species (Hays *et al.,* 2001). The deficiency of ABA in microspore derived embryoids was compensated with the addition of the exogenous hormone, which in turn reported the improvement in number of normally developing embryoids in *B. oleracea* (Rudolf *et al.,* 1999; Yeung and Ramesar Fortner, 2006; Yadollani *et al.,* 2011). Generally, after proper microspore isolation and culture, haploid embryos are transferred to solid regeneration medium. Proper plant development from androgenic embryos depends at which stage of the embryos are transferred to solidified medium (Niu *et al.,* 1999) and is generally regarded critical for proper regeneration. Burnett, (1992) reported that embryos which were transferred at the cotyledonary stage only resulted in the highest frequency of plant regeneration from isolated microspores cultures. Medium composition determines the success of plant regeneration on solid medium. The B5 medium (Gamborg *et al.,* 1968) supplemented with 0.8% agar (w/v) and 0.1 mg/l of gibberellic acid (GA3) gives excellent results. However, some authors have reported that ½ MS (Murashige and Skoog, 1962) or ½ B5 media have better effect on the plantlet formation than full MS or B5 media (Gland-Zwerger, 1995). After 3 weeks on a solid medium, well developed rooted plants are transferred to soil for further growth and development after ploidy conformation using flow cytometer.

Conclusions

Microspore culture provides the opportunity of producing haploid embryos at high frequencies in many *Brassica* species and their commercial cultivars. When it is combined with other biotechnologies such as marker assisted selection and induced mutations, can speed up breeding programmes, thereby combating the 12-15 years of cultivar development in just one generation. Because DH lines so developed can either be released as cultivars or they may be used as parents in hybrid seed production or more conveniently be used in creation of breeder's lines and in germplasm conservation. Thus, DH production system can reduce certain generations in a breeding cycle to release a variety. The genus *Brassica* has always been of significant interest to breeders on commercial point of view as mainly *brassica* are major source of not only oilseeds found in *B. napus, B. juncea, B.rapa* and *B. carinata*, but also of various bio-chemicals which are routinely used in human diet in the form of vegetables like., *Brassica. oleracea* var. *botrytis*, *Brassica oleracea* var. *italica, Brassica oleracea* var. *costata, Brassica oleracea* var. *gongylodes, Brassica oleracea* var. *acephala, Brassica oleracea* var. *capitata, Brassica rapa* ssp. *Chinensis,* since centuries, So DH technology renders a pathway for breeders to achieve uniformity and offers various routes of broadening breeding research parameters such as to study the genetic makeup of species (molecular markers) to accelerate breeding, which is less laborious and less time consuming and moreover more precise, thereby incorporating resistance to various pathogen can also be achieved. Although, a very good progress in the production of haploids in *Brassica* genus has been ascertained till date, but still there is a need of profound fundamentals to optimize and improve DH technologies, which should ensure high yields in microspore embryogenesis induction and DH plant production in low responding recalcitrant genotypes of *Brassica oleracea*.

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