Isolated microspore culture techniques and recent progress for haploid and doubled haploid plant production

Ferrie, A. M. R.; Caswell, K. L

Publisher's version / Version de l'éditeur:
https://doi.org/10.1007/s11240-010-9800-y
Plant Cell, Tissue and Organ Culture, 104, 3, pp. 301-309, 2010-07-15

Isolated microspore culture techniques and recent progress for haploid and doubled haploid plant production

A.M.R. Ferrie and K.L. Caswell

Plant Biotechnology Institute – National Research Council

110 Gymnasium Place

Saskatoon, SK

S7N 0W9

Phone: (306) 975-5993

Email: Alison.Ferrie@nrc-cnrc.gc.ca

NRCC# 50167

ABSTRACT

An isolated microspore culture provides an excellent system for the study of microspore induction and embryogenesis, provides a platform for an ever-increasing array of molecular studies, and can produce doubled haploid (DH) plants, which are used to accelerate plant-breeding programs. Moreover, isolated microspore cultures have several advantages over anther culture, wherein presence of the anther walls can lead to the development of diploid, somatic calli and plants. Although protocols for isolated microspore culture vary from laboratory to laboratory, the basic steps of growing donor plants, harvesting floral organs, isolating microspores, culturing and inducing microspores, regenerating embryos, and doubling the chromosomes, remain the same. Over the past few years, a large proportion of the research reports on isolated microspore culture have focused on cereal and *Brassica* species. For some of these species, isolated microspore culture protocols are well established and routinely used in laboratories around the world for developing new varieties, as well as for basic research in areas such as genomics, gene expression, and genetic mapping. Although these species are considered highly responsive to microspore culture, improvements in efficiency are still being made. However, with many species, isolated microspore culture is simply not yet efficient enough at producing DH plants to be cost-effective for breeding programs. There has been a recent resurgence of haploidy research with response being reported in some species once considered recalcitrant. Future research programs aimed at elucidating pathways involved in microspore induction and embryogenesis will be of benefit, as will novel approaches to improve the efficiency of microspore culture for DH production. With many species, anther culture has proven to be more effective than isolated microspore culture, necessitating more research to clarify the contribution of the anther wall to embryogenesis. The development of molecular markers for use in determining the gametic origin of regenerated plants, irrespective of their ploidy, would also be beneficial. In this review, we aim to provide an overview of the basic isolated microspore culture protocol with an emphasis on recent progress in several crop species.

Keywords: isolated microspore culture, microspore embryogenesis, protocol development

INTRODUCTION

Isolated microspores, when given the optimal combination of culture conditions and stresses, can be diverted from the normal gametophytic developmental pathway to a sporophytic pathway, and subsequently produce embryos and haploid or doubled haploid (DH) plants. The production of DH plants from microspores is an important technique used in plant breeding and basic research. DH technology is a rapid method for developing homozygous lines, which can be used to accelerate crop improvement programs. Commercial varieties developed through DH protocols have been reported for many crops, such as wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), triticale (x *Triticosecale* Wittm.), rice (*Oryza sativa* L.), *Brassica* spp., eggplant (*Solanum melongema* L.), pepper (*Capsicum annuum* L.), asparagus (*Asparagus officinalis* L.), and tobacco (*Nicotiana tabacum* L.) (Thomas et al. 2003). A plethora of other uses for isolated microspore culture has arisen and this subject has been recently reviewed by Dunwell (2010).

Maluszynski et al. (2003) co-edited an excellent book titled "Doubled Haploid Production in Crop Plants: a manual", which provides in-depth protocols for 33 important plant species and lists 226 other species along with significant papers relating to these species. This book covers several diverse methods for producing doubled haploids, including wide crosses, gynogenesis, anther culture, and isolated microspore culture, with the majority of the protocols relating to anther culture. In 2009, another book was published which focused on advances in haploid production in higher plants, based on the "Haploids for Higher Plants" conference held in 2006 (Touraev et al. 2009). This book contains reviews on some of the species considered recalcitrant to DH production (e.g. *Asteraceae*, medicinal plants, rice).

While most of the early DH work relied on anther culture, isolated microspore culture is usually the preferred method. Microspore culture is defined as isolating the microspores from the anther prior to culture, whereas anther culture involves culturing the whole anther. Microspore culture has a number of advantages over anther culture: (1) in anther cultures, the anther wall may negatively impact the microspores, or may produce diploid, somatic callus and subsequently embryos; (2) anther culture is extremely time-consuming, and depending on the species, may require micro-surgical skills; (3) the isolated microspore culture system allows for better nutrient availability to the developing microspores; and (4) isolated microspore culture provides a superior method for tracking and studying

microspore maturation and embryo development. For some species, isolated microspore culture protocols are well established and are routinely used in laboratories around the world for developing new varieties, as well as for basic research in areas such as genomics, gene expression, and genetic mapping.

In 1974, Nitsch (1974) cultured *Nicotiana* microspores that were free from anther tissue. These early studies used microspores shed naturally from cultured anthers. Eight years later, Lichter (Lichter 1982) mechanically isolated microspores from *Brassica* buds prior to culturing them, which launched the field of isolated microspore culture research. Since then studies have focused on increasing the frequency of embryogenesis with responsive species and on developing protocols for recalcitrant species. Despite the progress that has been made, many species are still considered recalcitrant: for example, even though there is abundant information available describing the Arabidopsis genome, there is currently no microspore culture protocol for this species.

BASIC PROTOCOL FOR ISOLATED MICROSPORE CULTURE

There is no universal protocol that will result in microspore embryogenesis in all species, as differences occur among species and among genotypes within a species in terms of embryogenic response. When initially screening species for microspore culture response, standard protocols namely *Brassica napus*, barley, and tobacco, are commonly used. Some factors to be considered when optimizing an isolated microspore culture protocol are listed in Figure 1. Despite slight variations in techniques, which will occur from laboratory to laboratory, the basic steps involved in isolated microspore culture are consistent and include the following: growing donor plants, harvesting floral organs, isolating microspores, culture and induction of microspores, regeneration of embryos, and doubling of chromosomes if required.

Growing donor plants

A prerequisite for successful and consistent microspore culture response is healthy, pest-free donor plants. Seeds are planted with adequate spacing to allow for vigorous growth, plants are fertilized and watered regularly, and screened and treated as required to minimize disease and insect infestations. Donor plants can be grown in the field, the greenhouse, or in environmentally controlled growth chambers, with the latter providing the most consistent and repeatable conditions. Growth chambers allow for the control of temperature, humidity, photoperiod, and light

intensity, and provide an enclosed space where the incidence of disease or insect infestation can be minimized and effectively treated when necessary. Field-grown donor material has been used in some studies, but contamination can be an issue and embryogenic potential can be adversely affected. A combination of growing conditions may prove to be useful, for example, with some perennial species like asparagus, seeds are initially planted in the greenhouse until the seedling stage, at which time the seedlings are transplanted to the field. At the end of the growing season, the crowns are dug up, vernalized at 4°C for up to 6 months, and then planted in a growth chamber (Wolyn and Nichols 2003). Donor plant conditions not only play a role in microspore culture response, i.e. the production of embryos, but also in regeneration of these embryos to plants. It has been reported that donor plants of barley grown under growth chamber conditions produced more DH green plants than donor plants grown under greenhouse conditions (Dahleen 1999). This is particularly important in cereal crops where albinism is a major problem.

The temperature at which the donor plants are grown plays a critical role in microspore culture response. For *Brassica* species, the donor plants are initially grown at 20/15°C, and just prior to bolting, the temperatures are reduced to 10/5°C (Ferrie and Keller 1995). This slows the growth of the plant and allows for a longer time period during which buds at the appropriate developmental stage can be selected. The cold temperature stress of the donor plants results in a higher frequency of microspore embryogenesis, and while embryos can still be obtained from greenhouse grown plants, the response is decreased. Cooler than normal temperatures are also beneficial for barley, with winter barley (15/12°C) requiring lower temperatures than spring barley (18/15°C) (Kasha et al. 2003b). However a cold temperature stress is not a requirement for other species such as asparagus (Wolyn and Nichols, 2003), pepper (Lantos et al. 2009), or *Saponaria vaccaria* L. (Kernan and Ferrie 2006).

Microspore culture response varies among genotypes within a species, as is commonly found with tissue culture techniques. There have been countless studies which have screened genotypes for embryogenic response and some genotypes stand-out as being highly embryogenic, and are therefore used as controls for that particular species. With *B. napus*, for example, Topas DH4079, a DH selection from the spring variety Topas, is highly embryogenic and is used for many basic research studies (Ferrie and Keller 1995). For *B. oleracea* ssp. *italica*, Shogun, SDB9, and Green Valiant are highly embryogenic cultivars (da Silva Dias 2001) and for *B. rapa*, CV-2 is highly

embryogenic (Ferrie et al. 1995). In wheat, the cultivars Chris, Pavon 79, and Bob White have proved to be highly responsive (Kasha et al. 2003a). The winter 2-rowed barley cultivar Igri is utilized as a highly responsive genotype for microspore culture experiments (Davies 2003).

Harvesting floral organs

The developmental stage of the microspores used for culture is crucial for success and varies depending on the species. Buds or tillers are typically harvested when the microspores are at the uni-nucleate to early binucleate stage. Acetocarmine and DAPI (4', 6-diamidino-2-phenylindole) are the stains most commonly used for determining the developmental stage of the microspore (Fan et al. 1988). For many species the plant material is collected and used immediately, while for most cereals, tillers are selected, placed in nutrient solution, media, water, or inducer chemicals and kept for up to several weeks prior to microspore isolation. Most temperature pretreatments are at 4 – 10°C, but short heat shock conditions of 33°C for 48 -72 hr can also be used (Liu et al. 2002).

The tillers and buds are surface sterilized prior to microspore isolation to eliminate bacterial or fungal contaminants. The most common surface sterilization protocols involve briefly (1-2 min) immersing the plant material in ethanol (70%), followed by immersion in sodium hypochlorite (6% or less) with a drop of tween for several minutes (up to 15 min), followed by several washes with sterile distilled water. Mercuric chloride has also been used, but should be avoided because of its toxic effects. In order to avoid deleterious effects from surface sterilization, treatments should be kept to the minimum which will provide contaminant-free plant material.

Isolating microspores

There are two main techniques used to isolate microspores for culture and many variations of these have been published. The first method involves mechanically crushing the surface-sterilized buds to release the microspores from the anthers, using a mortar and pestle or a blender. The resulting slurry is then passed through a filter, or a series of filters, so that the somatic anther wall and bud tissue is separated from the microspores. The microspores are subsequently collected by centrifugation. Percoll gradients (Fan et al. 1987, Joersbo et al. 1990), maltose density gradients (Kasha et al. 2001), and sucrose gradients (Maraschin et al. 2003) have been used to separate the developmental stages of the microspores to give a more uniform and debris-free sample. The second method is shed

microspore culture, wherein anthers are extracted from the buds, placed in a liquid medium, and microspores are allowed to dehisce. This has been used in barley (Ziauddin et al. 1990) and pepper (Supena et al. 2006).

One variation of the above techniques involves excising anthers from buds or tillers, and subsequently crushing them with a mortar and pestle or in a blender. This technique reduces the amount of somatic tissue that must be separated from the microspores, resulting in cleaner microspore cultures with some species, although time and skill are required to isolate the anthers. Iqbal and Wijesekara (2007) reported an interesting microspore isolation method, wherein the large (1.2-1.5 cm) anthers were isolated from *Datura metel* L., pretreated with temperature pulses, and subsequently the anthers were sliced open and the microspores squeezed out. Debris was removed from the cultures using a stereo-microscope.

The removal of somatic tissue (debris) from the microspore preparation is critical because its presence may negatively affect the microspores through the release of phenolic compounds, and in some cases, it may produce diploid calli, embryos, and plants, complicating the search for haploid or DH embryos and plants. With the Compositae species, *Helianthus annuus* L. (sunflower), multicellular somatic hairs were difficult to eliminate during microspore isolation: the hairs were highly reactive and produced calli, necessitating the use of percoll gradients to purify the microspore preparations (Coumans and Zhong 1995). Similar problems have been found with other Compositae species (Ferrie and Caswell, unpublished).

Culture and induction of microspores

Isolated microspores must be provided with suitable, nutrient-rich medium and appropriate culture conditions. As with all tissue culture media, macro and micro-nutrients, vitamins and a carbohydrate source must be provided. Antibiotics, such as cefotaxime can also be added to the medium if contamination is a problem (Davis 2003, Lantos et al. 2006). A very effective medium for *B. napus* microspores, for example, is NLN with 13% sucrose and a pH of 5.8 (Lichter 1982), while a highly efficient barley isolated microspore culture system utilizes FHG (Hunter 1988) medium, which contains 6.2% maltose and has a pH of 5.8 (Kasha et al. 2001). A thorough compendium of DH protocols was published by Maluszynski et al. (2003), and details the specific media used with various species. There are many modifications of media formulations with the addition of growth regulators, amino acids, and other

components. Some of the additives, which have provided an enhancement of embryogenesis, are discussed later within the recent progress section.

Stress is considered to be the inducer of embryogenesis in microspores: without stress, microspores follow their normal gametophytic pathway to form pollen grains (Touraev 1997). The application of stress to the donor plants or isolated microspores can divert the microspores to a sporophytic pathway and the development of embryos and subsequently plants. Stress can be applied through the growing conditions provided for the donor plants, pretreatment of the buds, tillers or isolated microspores, and culture media or conditions. In fact, all aspects of the microspore culture protocol could be classified as stresses, i.e. harvesting the buds, surface-sterilization, and isolation of the microspores. Shariatpanahi et al. (2006) published an excellent and thorough review of stresses and divided them into three categories: widely used, neglected, and novel. The widely used stresses were cold or heat shock, sugar starvation, and colchicine treatment, while γ-irradiation, ethanol stress, hypertonic shock, centrifugal treatment, reduced atmospheric pressure, feminizing agents, and abscisic acid were considered neglected stresses. Novel stresses included high medium pH, carrageenan oligosaccharides, heavy metal stress, inducer chemicals, and 2,4-D pretreatment. Ochatt et al. (2009) found a combination of osmotic stress and electrostimulation of isolated microspores of some legume species to be effective for production of microcalli, and concluded that pyramiding stress agents may trigger the switch to sporophytic development of microspores in recalcitrant species. As with surface sterilization treatments, the mildest effective stress should be used to avoid deleterious effects of harsh treatments.

Regeneration of embryos

Microspores can take a direct or an indirect route to develop into an embryo. The indirect route involves a number of irregular, asynchronous divisions which results in a callus, the callus undergoes organogenesis, and subsequently haploid embryos are formed. The direct and preferred route is similar to zygotic embryo development, wherein the embryos develop directly and proceed through the globular, heart-shaped, torpedo, and cotyledonary stages. Direct embryogenesis is primarily observed in the *Brassica* and *Solanaceae* species. For the *Brassica* species, embryos can usually be detected 10 days after culture initiation. For both routes, once cotyledonary embryos have developed, they can be removed from the culture media for regeneration into plants. Microspore-derived embryos are usually

plated onto a solid media in the light for conversion of the embryos into plants. Regeneration of embryos into normal plants can be very difficult in some species with a number of factors influencing this developmental process. Developmental stage of the embryo, embryo culture medium, and culture conditions can be manipulated in order to enhance normal development (Huang et al. 1991, Tian et al. 2004, Belmonte et al. 2006, Haddadi et al. 2008).

Doubling of chromosomes, if required

The ploidy level of regenerated plants can be determined through chromosome counts (Maluszynska 2003) or using flow cytometry (Bohanec 2003, Ochatt 2008). For some species, there is a high percentage of spontaneous chromosome doubling, which results in homozygous DH plants. If spontaneous doubling does not occur or occurs at a low frequency, the haploid plants will need to be treated with a doubling agent in order to produce fertile, homozygous, DH plants. A recent report (Kahrizi and Mohammadi, 2009) showed differences between genotypes of barley with regard to spontaneous chromosome doubling, and interestingly, also showed a negative relationship between embryogenesis and spontaneous chromosome doubling. Chromosome doubling methodologies are discussed in another review article in this journal issue.

PROGRESS

Numerous research papers, review articles, and excellent books covering doubled haploidy techniques have been published over the last few years. In this review, we will focus on some notable progress reported since 2003.

Cruciferous species

Although microspore embryogenesis is routine in *B. napus*, improvements are still being reported. A recent study found a 2 – 4 fold increase in embryogenesis with the addition of 0.1 μg ml^{-1} bleomycin, a glycopeptide antibiotic (Zeng et al. 2010). Higher concentrations of bleomycin decreased embryogenesis. Two brassinosteroid compounds, 2,4-epibrassinolide (EBR) and brassinolide, increased embryogenesis in all *B. napus* genotypes evaluated, and EBR enhanced microspore culture response in *B. juncea* L. (Ferrie et al. 2005b). Prem et al (2008) reported a four-fold increase in *B. juncea* microspore embryogenesis when silver nitrate and activated charcoal were included in the culture medium. Other recent improvements include the addition of the anti-auxin, *p*-chlorophenoxyisobutyric acid (PCIB), to enhance microspore embryogenesis in *B. juncea* (Agarwal et al. 2006), and substitution of polyethylene

glycol for sucrose in *B. nigra* (L.) Koch*, Crambe abyssinica* Hochst.*, Raphanus oleifera* L., and several genotypes of *B. napus* (Ferrie and Keller 2007). The *B. napus* microspore protocol has been successfully extended to another cruciferous species with the report by Leskovšek et al. (2008) of DH plantlet production from rocket or arugula (*Eruca sativa* Mill.), a leafy, annual vegetable.

Cereal species

There have been a number of recent improvements to isolated microspore culture protocols for the cereal crops. Although doubled haploidy efficiencies are sufficient for breeding purposes in wheat and barley, several major cereal crops are considered recalcitrant e.g., oat (*Avena sativa* L.) and rice. Albinisim of the regenerated haploid/DH plants is a major problem for many of the cereal crops and several studies have been undertaken to alleviate this problem. The addition of copper sulphate to the anther pre-treatment medium was beneficial in increasing the number of green plants from barley anther culture (Jacquard et al. 2009). Zinc sulphate has also been used to increase the number of microspore-derived embryos and green plants produced from isolated microspores of barley (Echavarri et al. 2008). An increase of 43% in the number of embryos was observed with 90µM $ZnSO_4$ when compared to the control, which contained 30 µM $ZnSO_4$. Patel et al. (2004) found that the presence of wheat ovaries was essential for response from wheat microspores and determined that adding spent medium or paclobutrazol to the culture medium, and using gelrite rather than agarose as a gelling agent, resulted in high efficiency production of microspore-derived embryoids and green plants. Arabinogalactans (e.g. larcoll) and arabinogalactan protein were beneficial in wheat embryogenesis and regeneration (Letarte et al. 2006). Larcoll has been used to decrease the mortality of the microspores, and thereby increase the number of cells that are able to divide and develop into multicellular structures. There appeared to be a synergistic effect of the larcoll and the presence of ovaries, which are important for microspore embryogenesis in wheat. The arabinogalactan protein was also beneficial in inducing embryogenesis and producing green plants. For the variety Chris, it was possible to regenerate green plants without the addition of wheat ovaries when the arabinogalactan was present in the media. Soriano et al. (2007a) found that η-butanol at concentrations of 0.1- 0.2% (v/v) in the induction medium increased embryo production from anthers of two bread wheat cultivars, resulting in a 3-5 times increase in the number of green plants produced. Soriano et al. (2007b) also reported that the incorporation of colchicine in the culture medium during the first hours of culture of bread wheat isolated microspores and anthers significantly increased the

number of green, DH plants with three of four cultivars tested. Small numbers of fertile, green DH plants were produced from isolated microspores of eight cultivars of 6x triticale by Eudes and Amundsen (2005), who determined that 100 g/l Ficoll in the medium increased numbers of embryos and green plants for all cultivars.

Ovary co-culture has been used with many of the cereal crops (Li and Devaux 2001; Zheng et al. 2002). Barley ovaries were more effective than wheat ovaries in wheat microspore cultures (Bruins et al. 1996). As noted earlier, Patel et al (2004) cultured isolated wheat microspores with and without wheat ovaries and embryoids and green plants were only produced from treatments where wheat ovaries were present. Floret co-culture has also been used with success in barley microspore culture (Lu et al. 2008). When the two methods, floret co-culture and ovary culture, were compared, it was found that floret co-culture was more efficient.

Haploids and doubled haploids of oat have been produced; however, these are mostly through the oat x *Zea mays* technique, which is inefficient (Rines and Dahleen 1990, Rines 2003). Anther culture protocols have been published, but the response frequency is low, and therefore the method is not economically feasible for breeding programs (Kiviharju et al. 2000; Kiviharju et al. 2005). Recently, Sidhu and Davis (2009) reported regeneration of the first fertile, green plants from isolated microspores. Conditions important for microspore embryogenesis were a cold, dark pre-treatment (4°C) of the panicles for at least 6 weeks, a culture medium of pH 8.0, and the use of medium conditioned with barley microspores. Plants were regenerated but the efficiency was nonetheless low: in total, two green plants and 15 albino plants were regenerated.

Similarly in durum wheat (*Triticum durum* Desf.), the frequency of regenerated green plants from DH techniques is very low. There is a significant genotype and microspore culture protocol interaction which makes wide application of DH technology in breeding programs difficult. Intergeneric crosses with *Zea mays* L. have been successful in some cases, but are also genotype dependent (Almouslem et al. 1998). This method is very time consuming and results in haploid plants which need to be doubled with colchicine. Cistue et al. (2006) determined that ovary co-culture is critical for microspore culture of durum wheat, and more recently showed that the addition of glutathione and glutamine enhanced the development of embryo-like structures and the frequency of green plants (Cistue et al. 2009). Labbani et al. (2007) reported that pretreating durum tillers for 7 days at 4°C in 0.3M mannitol was an

important component of an efficient method for inducing embryos and regenerating green plants from isolated microspores.

Legume species

The legume species are considered recalcitrant to DH production. Croser et al. (2006) reviewed DH progress with the Fabaceae and stated that DH technology was not in use in breeding programs for any leguminous species and that anther culture was a common factor in legume protocols developed to date. There are, however, a few reports of isolated microspore culture with legumes. Bayliss et al (2004) cultured isolated microspores of several species of lupin (*Lupinus* spp.), which are grown in Australia as crop and pasture plants, and reported the production of pro-embryos. They concluded that further development of the pro-embryos was restricted by the presence of the intact microspore exines. Recently, there have been some positive initial results on isolated microspore culture of field pea (*Pisum sativum* L.), grasspea (*Lathyrus sativus* L.), and *Medicago truncatula* (Ochatt et al. 2009). Uninucleate microspores were found to be the most responsive, and flower buds required a dark, cold pretreatment of at least 2d at 4°C. Osmotic stress was applied by initially culturing the microspores in medium with 17% sucrose and after 7d reducing the sucrose concentration to 10%. A combination of osmotic stress and electrostimulation was more beneficial than a single stress treatment. Ochatt et al. (2009) concluded that pyramiding stress treatments on microspores greatly enhanced response. Haploid/doubled haploid plants were regenerated from field pea, grasspea, and *Medicago truncatula* microspores.

Flax

A recent review on doubled haploidy in flax (*Linum usitatissimum* L.) states that anther culture is the most successful method for generating DH (Obert et al. 2009), and most flax DH research has focused on this method. DH lines generated from isolated microspore culture were reported as early as 1993 (Nichterlein and Friedt 1993), but the frequency of success was too low for breeding purposes. Bud preparation and microspore isolation improvements are required to increase efficiency of the protocol, so that the advantages of isolated microspore culture can be gained with flax.

Fruit and vegetable species

Germanà (2006) reviewed DH production in fruit crops and noted that there were relatively few published reports of isolated microspore culture. Although fruit species are considered to be recalcitrant to DH production, some progress has been made with apple (*Malus domestica* Borkh.). Höfer (2004, 2005) compared anther and isolated microspore culture of apple and found that the total number of regenerated plants was higher from anther culture, even though embryo induction was higher in microspore cultures of some genotypes. Microspore induction and regeneration were also slower in microspore cultures than anther cultures. Bueno et al. (2005) cultured isolated microspores of olive (*Olea europaea* L.) and pro-embryos were produced. Tomato (*Solanum lycopersicum* L.) is considered to be recalcitrant to DH technology, however plants have been derived from anther tissue and globular embryos have been reported from isolated microspores (Segui-Simarro and Nuez 2007). Cultured tomato anthers produced both gametophytic and sporophytic calli, and regenerated plants were mostly mixoploid, although there were also small numbers of haploid and diploid plants. Suspena et al. (2006) developed a successful shed microspore protocol for Indonesian hot pepper (*Capsicum annuum* L.), which utilized a 2 layer culture medium consisting of a liquid upper layer and a solid lower layer containing activated charcoal. Cultured anthers floated to the surface of the upper liquid layer, whereupon microspores dehisced and embryos formed from microspores which were free from the anther wall tissue. In contrast, isolated microspore culture produced higher numbers of sporophytically dividing microspores, but very few embryos. Kim et al. (2008) achieved embryogenesis from hot peppers using NLN medium with 9% sucrose. They also noted that microspore plating density was extremely important with the optimum plating density being 8×10^{-4} to 10×10^{-4} microspores/ml. Recently, improvements in isolated microspore culture of pepper were achieved utilizing co-culture with wheat or pepper ovaries (Lantos et al. 2009). Cultures with pepper ovaries produced multicellular structures but development stopped at this stage, while microspore cultures with wheat ovaries continued embryo development.

Species with medicinal properties

Reviews of DH production in nutraceutical and medicinal species were published in 2007 (Ferrie 2007) and 2009 (Ferrie 2009). There has been very little DH research conducted on this wide range of plants, although progress has occurred with a few species. The majority of the DH response has been reported with anther culture, while isolated microspore culture has elicted response, for example, from laceflower (*Ammi majus*), dill (*Anethum graveolens*), angelica (*Angelica archangelica*), borage (*Borago officinalis*), caraway (*Carum carvi*), fennel (*Foeniculum vulgare*),

lovage (*Levisticum officinale*), anise (*Pimpinella anisum*), (Ferrie et al. 2005a), gingko (*Gingko biloba*) (Laurain et al 1993), and cowcockle (*Saponaria vaccaria*) (Kernan and Ferrie 2006). Microspore-derived plants produced from the Apiaceae species, dill, caraway and fennel, were evaluated in the field and lines with different agronomic characteristics and beneficial biochemical profiles were identified (Ferrie 2009). Recently there has been heightened interest in and greater use of medicinal and nutraceutical species. Very little plant breeding and few genetic studies have been conducted with these species, so there is great potential for DH technology to be of benefit.

CONCLUSION

Over the past few years, a large proportion of the isolated microspore culture research reports have focused on cereal and *brassica* species, with only a few reports of progress with horticultural crops and other plants of interest. Recently there has been a revival in doubled haploidy protocol development and application. Dividing microspores, microspore-derived embryos, or haploid plants have been reported in some of the species considered recalcitrant. With many species, isolated microspore culture is simply not yet efficient enough at producing DH plants to be cost-effective for breeding programs. Future research programs aimed at elucidating pathways involved in microspore induction and embryogenesis will be of benefit, as will creative approaches to improvement in efficiency of microspore culture for DH production. With many species anther culture has to date proven more effective than isolated microspore culture, necessitating more research to clarify the contribution of the anther wall to embryogenesis. The development of molecular markers for use in determining the gametic origin of regenerated plants, irrespective of their ploidy, would also be beneficial. The ever-expanding variety of innovative end-use applications for DH technology has led to a renewed interest by researchers in advancing this technology.

REFERENCES

Agarwal P, Agarwal P, Custers J, Liu CM, Bhojwani, S (2006) PCIB an antiauxin enhances microspore embryogenesis in microspore culture of *Brassica juncea*. Plant Cell, Tissue and Organ Culture 86:201-210

Almouslem AB, Jauhar PP, Peterson TS, Bommineni VR, Rao MB (1998) Haploid durum wheat production via hybridization with maize. Crop Sci 38:1080-1087

Bayliss KL, Wroth JM, Cowling WA (2004) Pro-embryos of *Lupinus* spp. produced from isolated microspore culture. Australian Journal of Agricultural Research 55:589-593

Belmonte MF, Ambrose SJ, Ross ARS, Abrams SR, Stasolla C (2006) Improved development of microspore-derived embryo cultures of *Brassica napus* cv Topaz following changes in glutathione metabolism. Physiologia Plantarum 127:690-700

Bohanec B (2003) Ploidy determination using flow cytometry. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled Haploid Production in Crop Plants: A Manual. Kluwer Academic Publishers, pp 397-403

Bruins MBM, Rakoczy-Trojanowska M, Snijders CHA (1996) Isolated microspore culture in wheat (*Triticum aestivum* L.): the effect of co-culture of wheat or barley ovaries on embryogenesis. Cereal Research Communications 24:401-408

Bueno MA, Pintos B, Höfer M, Martin A (2005) Pro-embryos induction from *Olea europaea* L. isolated microspore culture. Acta Physiologiae Plantarum 27:695-701

Cistue L, Romagosa I, Batlle F, Echavarri, B (2009) Improvements in the production of doubled haploids in durum wheat (*Triticum turgidum* L.) through isolated microspore culture. Plant Cell Reports 28:727-735

Cistue L, Soriano M, Castillo AM, Valles MP, Sanz JM, Echavarri B (2006) Production of doubled haploids in durum wheat (*Triticum turgidum* L.) through isolated microspore culture. Plant Cell Reports 25:257-264

Coumans M, Zhong D (1995) Doubled haploid sunflower (*Helianthus annuus*) plant production by androgenesis: fact or artifact? Part 2. *In vitro* isolated microspore culture. Plant Cell, Tissue and Organ Culture 41:203-209

Croser JS, Lülsdorf MM, Davies PA, Clarke HJ, Bayliss KL, Mallikarjuna N, Siddique, KHM (2006) Toward doubled haploid production in the Fabaceae: progress, constraints, and opportunities. Critical Reviews in Plant Sciences 25:139-157

da Silva Dias JC (2001) Effect of incubation temperature regimes and culture medium on broccoli microspore culture embryogenesis. Euphytica 119:389-394

Dahleen LS (1999) Donor-plant environment effects on regeneration from barley embryo-derived callus. Crop Science 39**:**682-685

Davies PA (2003) Barley isolated microspore culture (IMC) method. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled Haploid Production in Crop Plants: A Manual. Kluwer Academic Publishers, pp 49-52

Dunwell JM (2010) Haploids in flowering plants: origins and exploitation. Plant Biotechnology Journal 8:377-424

Echavarri BA, Soriano M, Cistué L, Vallés M, Castillo A (2008) Zinc sulphate improved microspore embryogenesis in barley. Plant Cell, Tissue and Organ Culture 93:295-301

Eudes F, Amundsen E (2005) Isolated microspore culture of Canadian 6x triticale cultivars. Plant Cell, Tissue and Organ Culture 82:233-241

Fan Z, Armstrong KC, Keller WA (1988) Development of microspores *in vivo* and *in vitro* in *Brassica napus* L. Protoplasma 147:191-199

Fan Z, Holbrook L, Keller WA (1987) Isolation and enrichment of embryogenic microspores in Brassica napus L. by fractionation using percoll density gradients. 7th International Rapeseed Congress, Poznan, Poland, 11 – 14 May 1987.

Ferrie AMR (2007) Doubled haploid production in nutraceutical species: a review. Euphytica 158:347-357

Ferrie AMR (2009) Current status of doubled haploids in medicinal plants. In: Touraev A, Forster BP, Jain SM (eds) Advances in Haploid Production in Higher Plants. Springer Science + Business Media, B.V pp 209-218

Ferrie AMR, Bethune T, Kernan Z (2005a) An overview of preliminary studies on the development of doubled haploid protocols for nutraceutical species. Acta Physiol Plant 27:735-741

Ferrie AMR, Dirpaul J, Krishna P, Krochko J, Keller WA (2005b) Effects of brassinosteroids on microspore embryogenesis in *Brassica* species. In Vitro Cellular and Developmental Biology - Plant 41:742-745

Ferrie AMR, Epp DJ, Keller WA (1995) Evaluation of *Brassica rapa* L. genotypes for microspore culture response and identification of a highly embryogenic line. Plant Cell Reports 14:580-584

Ferrie AMR, Keller WA (1995) Microspore culture for haploid plant production. In: Gamborg O L, Phillips GC (eds) Plant Cell, Tissue and Organ Culture. Fundamental Methods. Springer-Verlag, Berlin, pp 155-164

Ferrie AMR, Keller WA (2007) Optimization of methods for using polyethylene glycol as a non-permeating osmoticum for the induction of microspore embryogenesis in the *Brassicaceae*. In Vitro Cellular and Developmental Biology - Plant 43:348-355

Germanà MA (2006) Doubled haploid production in fruit crops. Plant Cell Tissue and Organ Culture 86:131-146

Haddadi P, Moieni A, Karimzadeh Gh, Abdollahi MR (2008) Effects of gibberellin, abscisic acid and embryo desiccation on normal plantlet regeneration, secondary embryogenesis and callogenesis in microspore culture of *Brassica napus* L. cv. PF704. International Journal of Plant Production 2:153-162

Höfer M (2004) *In vitro* androgenesis in apple – improvement of the induction phase. Plant Cell Reports 22:365-370

Höfer M (2005) Regeneration of androgenic embryos in apple (*Malus x domestica* Borkh.) via anther and microspore culture. Acta Physiologiae Plantarum 27:709-716

Huang B, Bird S, Kemble R, Miki B, Keller W (1991) Plant regeneration from microspore-derived embryos of *Brassica napus*: Effect of embryo age, culture temperature, osmotic pressure, and abscisic acid. In Vitro Cell Dev Biol 27P:28-31

Hunter CP (1988) Plant regeneration from microspores of barley, *Hordeum vulgare* L. Ph.D. Thesis. Wye College, University of London, London

Iqbal MCM, Wijesekara KB (2007) A brief temperature pulse enhances the competency of microspores for androgenesis in *Datura metel*. Plant Cell, Tissue and Organ Culture 89:141-149

Jacquard C, Nolin F, Hecart C, Grauda D, Rashal I, Dhondt-Cordelier S, Sangwan RS, Devaux P, Mazeyrat-Gourbeyre F, Clément C (2009) Microspore embryogenesis and programmed cell death in barley: effects of copper on albinism in recalcitrant cultivars. Plant Cell Reports 28:1329-1339

Joersbo M, Jørgensen RB, Olesen P (1990) Transient electropermeabilization of barley (*Hordeum vulgare* L.) microspores to propidium iodide. Plant Cell, Tissue and Organ Culture 23:125-129.

Kahrizi D, Mohammadi R (2009) Study of androgenesis and spontaneous chromosome doubling in barley (*Hordeum vulgare* L.) genotypes using isolated microspore culture. Acta Agronomica Hungarica 57:155-164

Kasha KJ, Simion E, Miner M, Letarte J, Hu TC (2003a) Haploid wheat isolated microspore culture protocol. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled Haploid Production in Crop Plants: A Manual. Kluwer Academic Publishers, pp. 77-81

Kasha KJ, Simion E, Oro R, Shim YS (2003b) Barley isolated microspore culture protocol. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled Haploid Production in Crop Plants: A Manual. Kluwer Academic Publishers, pp 43-47

Kasha KJ, Simion E, Oro R, Yao QA, Hu TC, Carlson AR (2001) An improved *in vitro* technique for isolated microspore culture of barley. Euphytica 120:379-385

Kernan Z, Ferrie AMR (2006) Microspore embryogenesis and the development of a double haploidy protocol for cow cockle (*Saponaria viccaria*). Plant Cell Reports 25:274-280

Kim M, Jang I-C, Kim J-A, Park E-J, Yoon M, Lee Y (2008) Embryogenesis and plant regeneration of hot pepper (*Capsicum annuum* L.) through isolated microspore culture. Plant Cell Reports 27:425-434

Kiviharju E, Moisander S, Laurila J (2005) Improved green plant regeneration rates from oat anther culture and the agronomic performance of some DH lines. Plant Cell, Tissue and Organ Culture 81:1-9

Kiviharju E, Puolimatka M, Saastamoinen M (2000) Extension of anther culture to several genotypes of cultivated oats. Plant Cell Reports 19:674-679

Labbini Z, De Buyser J, Picard E (2007) Effect of mannitol pretreatment to improve green plant regeneration on isolated microspore culture in *Triticum turgidum* ssp. *durum* cv. 'Jennah Khetifa'. Plant Breeding 126:565-568

Lantos C, Juhasz AG, Somogyi G, Otvos K, Vagi P, Mihaly R, Kristof Z, Somogyi N, Pauk J (2009) Improvement of isolated microspore culture of pepper (*Capsicum annuum* L.) via co-culture with ovary tissues of pepper or wheat. Plant Cell Tissue and Organ Culture 97:285-293

Lantos C, Páricsi S, Zofajova A, Weyen J, Pauk J (2006) Isolated microspore culture of wheat (Triticum aestivum L.) with Hungarian cultivars. Acta Biologica Szegediensis 50:31-35

Laurain D, Trémouillaux-Guiller J, Chénieux J (1993) Embryogenesis from microspores of *Ginkgo biloba* L., a medicinal woody species. Plant Cell Reports 12:501-505

Leskovšek L, Jakše M, Bohanec B (2008) Doubled haploid production in rocket (*Eruca sativa* Mill.) through isolated microspore culture. Plant Cell Tissue Organ Culture 93:181-189

Letarte J, Simion E, Miner M, Kasha KJ (2006) Arabinogalactans and arabinogalactan-proteins induce embryogenesis in wheat (*Triticum aestivum* L.) microspore culture. Plant Cell Reports 24:691-698

Li H, Devaux P (2001) Enhancement of microspore culture efficiency of recalcitrant barley genotypes. Plant Cell Reports 20:475-481

Lichter R (1982) Induction of haploid plants from isolated pollen of *Brassica napus*. Z. Pflanzenphysiol 105:427-434

Liu W, Zheng MY, Polle EA, Konzak CF (2002) Highly efficient doubled-haploid production in wheat (*Triticum aestivum* L.) via induced microspore embryogenesis. Crop Science 42:686-692

Lu R, Wang Y, Sun Y, Shan L, Chen P, Huang J (2008) Improvement of isolated microspore culture of barley (*Hordeum vulgare* L.): the effect of floret co-culture. Plant Cell, Tissue and Organ Culture 93: 21-27

Maluszynska J (2003) Cytogenetic tests for ploidy level analyses – chromosome counting. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled haploid production in crop plants: A Manual. Kluwer, Dordrecht, pp 391-395

Maluszynski M, Kasha KJ, Forster BP, Szarejko I (2003) Doubled Haploid Production in Crop Plants: A Manual. Kluwer Academic Publishers, Dordrecht

Maraschin SF, Lamers GEM, de Pater BS, Spaink HP, Wang M (2003) 14-3-3 isoforms and pattern formation during barley microspore embryogenesis. Journal of Experimental Botany 54:1033-1043

Nichterlein K, Friedt W (1993) Plant regeneration from isolated microspores of linseed (Linum usitatissimum L.). Plant Cell Reports 12:426-430

Nitsch C (1974) La culture de pollen isolé sur mileu synthétique. C. R. Acad. Sci. Paris 278:1031-1034

Obert B, Žáčková Z, Šamaj J, Pret'ová A (2009) Doubled haploid production in flax (*Linum usitatissium* L.). Biotechnology Advances 27:371-375

Ochatt SJ (2008) Flow cytometry in plant breeding. Cytometry 73A:581-598

Ochatt S, Pech C, Grewal R, Conreux C, Lulsdorf M, Jacas L (2009) Abiotic stress enhances androgenesis from isolated microspores of some legume species (Fabaceae). Journal of Plant Physiology 166:1314-1328

Patel M, Darvey NL, Marshall DR, Berry JO (2004) Optimization of culture conditions for improved plant regeneration efficiency from wheat microspore culture. Euphytica 140:197-204

Prem D, Gupta K, Gautam S, Agnihotri A (2008) Activated charcoal induced high frequency microspore embryogenesis and efficient doubled haploid production in *Brassica juncea*. Plant Cell Tissue Organ Culture 93:269-282

Rines HW (2003) Oat haploids from wide hybridization. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled haploid production in crop plants: A Manual. Kluwer, Dordrecht, pp 155-159

Rines HW, Dahleen LS (1990) Haploid oat plants produced by application of maize pollen to emasculated oat florets. Crop Sci 30:1073-1078

Segui-Simarro JM, Nuez F (2007) Embryogenesis induction, callogenesis, and plant regeneration by *in vitro* culture of tomato isolated microspores and whole anthers. J. Exp. Bot. 58:1119-1132

Shariatpanahi ME, Bal U, Heberle-Bors E, Touraev A (2006) Stresses applied for the re-programming of plant microspores towards *in vitro* embryogenesis. Physiologia Plantarum 127:519-534

Sidhu PK, Davis PA (2009) Regeneration of fertile green plants from oat isolated microspore culture. Plant Cell Reports 28:571-577

Soriano M, Cistué L, Castillo AM (2007a) Enhanced induction of microspore embryogenesis after *n*-butanol treatment in wheat (*Triticum aestivum* L.) anther culture. Plant Cell Reports 27:805-811

Soriano M, Cistué L, Vallés MP, Castillo AM (2007b) Effects of colchicine on anther and microspore culture of bread wheat (*Triticum aestivum* L.). Plant Cell Tissue Organ Culture 91:225-234

Supena EDJ, Suharsono S, Jacobsen E, Custers JBM (2006) Successful development of a shed-microspore culture protocol for doubled haploid production in Indonesian hot pepper (*Capsicum annuum* L.). Plant Cell Reports 25:1-10

Thomas WTB, Forster BP, Gertsson B (2003) Doubled Haploids in Breeding. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled Haploid Production in Crop Plants: A manual. Kluwer Academic Publishers, Dordrecht, pp 337-349

Tian H, Yao CY, Sun MX (2004) High frequency conversion of microspore-derived embryos of *Brassica napus* cv. Topas by supplemental calcium and vitamins. Plant Cell, Tissue and Organ Culture 76:159-165

Touraev A, Forster BP, Jain SM (2009) Advances in haploid production in higher plants. Springer.

Touraev A, Vincente O, Heberle-Bors E (1997) Initiation of microspore embryogenesis by stress. Trends in Plant Science Reviews 2: 297-302

Wolyn DJ, Nichols B (2003) Asparagus microspore and anther culture. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled Haploid Production in Crop Plants: A Manual. Kluwer Academic Publishers, Dordrecht, pp 265-273

Zeng X, Wen J, Wan Z, Yi B, Shen J, Ma C, Tu J, Fu T (2010) Effects of bleomycin on microspore embryogenesis in *Brassica napus* and detection of somaclonal variation using AFLP molecular marker. Plant Cell, Tissue and Organ Culture 101:23-29

Zheng MY, Weng Y, Liu W, Konzak CF (2002) The effect of ovary-conditioned medium on microspore embryogenesis in common wheat (*Triticum aestivum* L.). Plant Cell Reports 20: 802-807

Ziauddin A, Simion E, Kasha KJ (1990) Improved plant regeneration from shed microspore culture in barley (*Hordeum vulgare* L.) cv. Igri. Plant Cell Reports 9: 69-72