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REVIEW

Anther culture for haploid and doubled haploid production

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Abstract Haploids are plants with a gametophytic chromosome number and doubled haploids are haploids that have undergone chromosome duplication. The production of haploids and doubled haploids (DHs) through gametic embryogenesis allows a single-step development of complete homozygous lines from heterozygous parents, shortening the time required to produce homozygous plants in comparison with the conventional breeding methods that employ several generations of selfing. The production of haploids and DHs provides a particularly attractive biotechnological tool, and the development of haploidy technology and protocols to produce homozygous plants has had a significant impact on agricultural systems. Nowadays, these biotechnologies represent an integral part of the breeding programmes of many agronomically important crops. There are several available methods to obtain haploids and DHs, of which in vitro anther or isolated microspore culture are the most effective and widely used. This review article deals with the current status of knowledge on the production of haploids and DHs through pollen embryogenesis and, in particular, anther culture.

Keywords Anther culture · Doubled haploids · Haploids · Homozygosity · Pollen embryogenesis

Introduction

Haploid plants are sporophytes carrying the gametic chromosome number (n instead of $2n$). When spontaneous or induced chromosome duplication of a haploid occurs, the resulting plant is called doubled haploid (DH). In comparison, dihaploid plants ($2n = 2x$) are haploid plants obtained from an autotetraploid ($4x$) (Kasha and Maluszynsky 2003). Haploids occur spontaneously at a low frequency, or they can be induced by several methods, such as modified pollination methods in vivo (wide hybridization, chromosome elimination, pollination with irradiated pollen, etc.) and by in vitro culture of immature gametophytes. Gametic embryogenesis is one the different routes of embryogenesis present in the plant kingdom, and it consists in the capacity of male (microspore or immature pollen grain) or female (gynogenesis) gametophytes to irreversibly switch from their gametophytic pathway of development towards a sporophytic one. Differently from somatic embryogenesis, which provides the clonal propagation of the genotype (unless the somaclonal variation), gametic embryogenesis results in haploid plants (unless spontaneous or induced chromosome duplication occurs), because such plants are derived from the regeneration of gametes, products of meiotic segregation.

Microspore or pollen embryogenesis (also referred to as androgenesis) is regarded as one of the most striking examples of cellular totipotency (Reynolds 1997), but also as a form of atavism. It is an important survival adaptation mechanism in the plant kingdom that is expressed only under certain circumstances and as a consequence of an environmental stress (Bonet et al. 1998). In comparison to conventional breeding methods, gametic embryogenesis makes the production of homozygous lines feasible and shortens the time required to produce such lines, allowing the single-step development of completely homozygous lines from the heterozygous parents. Conventional methods performed to achieve homozygosity consist of carrying out

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several backcrosses; as such, they are time-consuming and labour-intensive procedures (Morrison and Evans 1987).

The production of haploids through gametic embryogenesis for breeding purposes has been studied by many research groups since the 1970s. There are many published reviews on the production of haploids and DHs, including those of Andersen (2005), Dunwell (2010), Germanà (1997, 2006, 2007, 2009), Jain et al. (1996–1997), Kasha (1974), Magoon and Khanna (1963), Maluszynski et al. (2003a, b), Palmer et al. (2005), Seguì-Simarro and Nuez (2008a), Seguí-Simarro (2010), Smykal (2000), Touraev et al. (2009), Zhang et al. (1990) and Xu et al. (2007). The DH techniques have been well established in a range of economically important crop species, including major cereals and cabbages (Wedzony et al. 2009). Gynogenesis is the least favoured technique at the present time because of its low efficiency, but it has been applied to species that do not respond to more efficient methods (Forster et al. 2007). The ability to obtain haploids and DHs is one of the most important applications of pollen biotechnology in plant breeding and genetics, involving the manipulation and reprogramming of pollen development and function (Testillano et al. 2000). Regeneration from male gametes has been reported in more than 200 species belonging to the Solanaceae, Cruciferae and Gramineae families (Dunwell 1986; Hu and Yang 1986), while many legumes and woody plants are rather recalcitrant (Sangwan-Norreel et al. 1986; Bajaj 1990; Raghavan 1990; Wenzel et al. 1995; Germanà 2006, 2009).

Embryogenesis in pollen is normally induced through anther or isolated microspore culture. Anther culture is often the method of choice for DH production in many crops because the simplicity of the approach allows large-scale anther culture establishment and application to a wide range of genotypes (Sopory and Munshi 1996). The technique of isolated microspore culture, performed by removing somatic anther tissue, requires better equipment and more skills compared to anther culture, although the former provides the better method for investigating cellular, physiological, biochemical and molecular processes involved in pollen embryogenesis (Nitsch 1977; Reinert and Bajaj 1977). Pelletier and Ilami (1972) introduced the concept of "Wall Factor", according to which the somatic tissues of the anther play an important role in the induction of sporophytic divisions in pollen, with the diffusion of nutrients through the anther walls often considered to be one of the factors affecting microspore embryogenesis. A number of studies on the role of the anther wall in pollen embryogenesis have shown that it not only acts as a barrier to nutrient flow but that it also provides both beneficial and inhibitory substances (Heberle-Bors 1985, 1989). Pulido et al. (2005), using both culture systems (anther and isolated microspore culture) to induce microspore embryogenesis in barley, showed that the initial phases of both processes are similar. These researchers as well demonstrated that the anther wall also served as a filter by preventing excessive concentrations of Fe around the microspores within the anther, even when the concentrations present in the culture medium were high. This protective role of the cell wall against toxic elements such as Cd, Zn and Ni was also shown by Krämer et al. (2000) and Kupper et al. (2000).

This review deals with the main aspects of H and DH production through in vitro anther culture.

Brief history of anther culture for haploid and DH production

The first natural sporophytic haploid was observed in 1921 by Bergner in a weed species *Datura stramonium* L. and reported by Blakeslee et al. (1922). The importance of haploids in plant breeding and genetic research was immediately recognized. The number of spontaneous haploids detected has steadily grown, and in 1974 Kasha recorded the occurrence of over 100 angiosperm species. A list of selected examples of occasional haploids in a range of species has been reported by Dunwell (2010). The frequency of spontaneous haploids is, however, too low for practical application in breeding. About 40 years after the identification of the first natural haploid, Guha and Maheshwari (1964) discovered that it was possible, by in vitro culture of immature anthers of the Solanaceous species *Datura innoxia*, to change the normal gametophytic development of microspores into a sporophytic one and that embryos and plants with a haploid chromosome number would then be produced. This discovery paved the way to further and extensive research on anther culture that was particularly successfully in the Solanaceae, Brassicaceae and Gramineae. However, not all of the angiosperm crops of interest efficiently respond to embryogenesis induction, and although barley (*Hordeum vulgare* L.), rapeseed (*Brassica napus* L.), tobacco (*Nicotiana* spp.) and wheat (*Triticum aestivum* L.) are considered to be model species to study microspore embryogenesis due to their high regeneration efficiency (Forster et al. 2007), other scientifically or economically interesting species, such as *Arabidopsis*, many woody plants or members of legume family, still remain recalcitrant to this type of in vitro morphogenesis (Sangwan-Norreel et al. 1986; Bajaj 1990; Raghavan 1990; Wenzel et al. 1995; Germanà 2006, 2009). Gamete embryogenesis is a particularly indispensable tool for obtaining homozygosity in woody plants, which are characterized by a high genome heterozygosity, a long generation cycle with a long juvenile period, a large size and, often, self-incompatibility, and for which it is not

possible to obtain haploidization through conventional methods, i.e., several generations of selfing (Germanà 2006, 2009).

The great interest in haploids was apparent with the organization of the First International Symposium 'Haploids in Higher Plants', which took place at Guelph (Canada) in 1974 (Kasha 1974). In the early 1970s, cv. Maris Haplona of rapeseed (*Brassica napus*) was the first DH crop plant released (Thompson 1972), followed by cv. cultivar Mingo in barley (*Hordeum vulgare*) in 1980 (Ho and Jones 1980). Since then, a great deal of research has been carried out with the aim of establishing efficient techniques for haploid and DH production with an increasing number of genotypes. For a long time, many postulations regarding pollen embryogenesis protocols have been based on practical experience. However, recent scientific and technological innovations, a greater understanding of underlying control mechanisms and an expansion of end-user applications have induced a resurgence of interest in haploids in higher plants (Forster et al. 2007). This interest is shown by the establishment of the COST 851 programme, a European Union-funded research network entitled 'Gametic cells and molecular breeding for crop improvement', that ran from 2001 to 2006.

To date, almost 300 new superior varieties belonging to several families of the plant kingdom (particularly annual crops) have been produced. A variety of methods were used to obtain these DHs, such as chromosome elimination subsequent to wide hybridization, the "*bulbosum*" method by Kasha and Kao (1970), pollination with irradiated pollen, selection of twin seedlings, in vivo or in vitro pollination with pollen from a triploid plant, gynogenesis and pollen embryogenesis through in vitro anther or isolated microspore culture (Forster and Thomas 2005). The website http://www.scri.sari.ac.uk/assoc/COST851/Default.htm provides a list of haploid-derived varieties, mostly asparagus, barley, *Brassica*, eggplant, melon, pepper, rapeseed, rice, swede, tobacco, triticale and wheat. The application of intellectual property (IP) protection and the patenting system of haploid plants (where patents also include anther and microspore culture techniques) has been reviewed by Dunwell (2009), and the strong commercial interest in methods for the production and exploitation of haploid plants is exemplified by the extensive number of granted patents and patent applications from the USA and elsewhere, also reported by Dunwell (2009, 2010).

General approach to anther culture for haploid and DH production

The exploitation of haploid and DHs as a powerful breeding tool requires the availability of reliable tissue culture protocols that can overcome several methodology problems, such as low frequencies of embryo induction, albinism, plant regeneration, plant survival and the genotype- and season-dependent response, in order to improve the regeneration efficiency in a wider range of genotypes. Maluszynski et al. (2003a) published a detailed manual that describes 44 protocols for DH production, related to at least 33 plant species. Although different species, as well as different cultivars within a species, show very diverse requirements and there is no single standard condition or protocol for inducing pollen-derived plant formation, it is possible provide common guidelines for anther culture, as summarized in Fig. 1.

Numerous endogenous and exogenous factors affect the embryogenic response of anthers in culture (Atanassov et al. 1995; Datta 2005; Smykal 2000; Wang et al. 2000). Genotype, physiological state and conditions of growth of donor plants, stage of pollen development, pretreatment of flower buds or anthers and in vitro culture medium and conditions, together with their interactions, are all factors that greatly affect the response of anthers to in vitro culture.

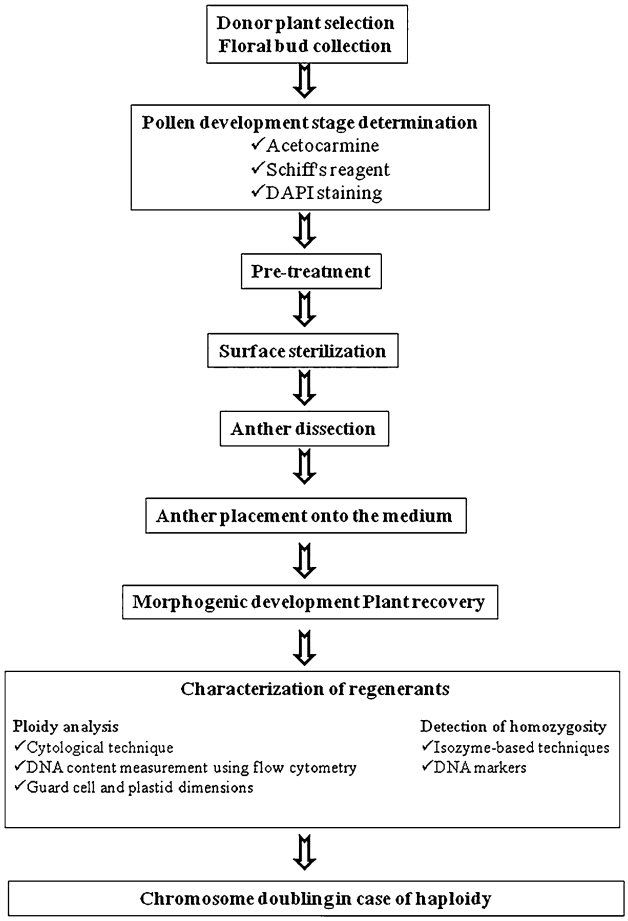

Fig. 1 Diagram describing common guidelines of the anther culture method

Genotype

Among the endogenous factors, the genotype plays a major role, as recognized by most of the researchers working on pollen embryogenesis. It has been repeatedly reported that different cultivars within a species exhibit diverse responses in anther culture. For example, of 21 cultivars of *Triticum aestivum*, haploid tissue could be obtained from anthers of only ten cultivars, while in rice, japonica subspecies have been found to be more productive than indica subspecies (Bajaj 1990). Also, research carried out simultaneously on anthers of a large number of *Citrus* genotypes (4 cultivars of clementine, 2 of mandarin, 4 of sweet orange, 4 of sour orange, 5 of lemon, 4 of grapefruit) using the same culture conditions and pretreatments and 11 different media, obtained haploid calli production in one cultivar of clementine and in one cultivar of lemon (Germanà 2007).

The formation of microspores competent to undergo embryogenesis ("E-grains", Sunderland 1978 or "P-grains", Heberle-Bors 1982) is controlled by an interaction between cytoplasmic and nuclear genes and modified by the environment (Heberle-Bors 1985). Studies carried out on *Solanum tuberosum* showed that the ability to undergo microspore embryogenesis is a heritable recessive trait controlled by more than one gene and that the genes are recessive (Chupeau et al. 1998; Rudolf et al. 1999; Smykal 2000). Foroughi-Wehr et al. (1982) distinguished four independently and differently inherited traits, namely, "callus induction", "callus stabilization", "plantlet regeneration" and "albino versus green plantlet formation". Petolino and Thompson (1987) demonstrated that breeding for improved responsiveness in maize is possible. The percentage of anthers producing microspore embryos and the number of regenerants produced per anther appear to be determined independently (Dunwell 2010).

Pollen development stage

The pollen development stage is a complex factor that strongly affects the success of anther culture. The developmental window of embryogenic competence differs depending on the species tested but, generally, the period of sensitivity to inductive treatments is around the first pollen mitosis—that is, between the vacuolate microspore (Fig. 2a) to early, mid-bicellular pollen (Touraev et al. 2001)—probably due to the transcriptional status that at that time is still proliferative and not yet fully differentiated (Malik et al. 2007). After the pollen grains begin to accumulate storage reserves, they usually lose their embryogenic capacity and follow the gametophytic developmental pathway (Heberle-Bors 1989; Raghavan 1990). In *Brassica napus*, Telmer et al. (1992) reported that the best stages for induction are around the first pollen mitosis, from late-uninucleate to early-bicellular pollen grains, but Binarovà et al. (1997), applying an additional short and a more severe heat stress (41°C), were able to obtain the division of already formed vegetative cells. In *Nicotiana tabacum*, pollen grains at the bicellular stage are the best responding when starvation is used as pretreatment; when a heat shock is applied instead, younger uninucleate microspores can be employed (Touraev et al. 1996a, b). Sopory and Munshi (1996) reported that microspore stage would appear to affect the ploidy level of the plant produced in anther culture because, in their study, plantlets obtained from pollen at the uninucleate stage were found to be mostly haploids, whereas plants with higher chromosome numbers were produced by anthers at the later stages.

The stage of pollen development is usually tested in one anther per floral bud size by the acetic-carmine method (Sharma and Sharma 1972). The anthers are collected from flower buds at different stages of development and squashed in acetocarmine staining solution (1% acetocarmine in 45% acetic acid) for observation under an optical microscope to determine the stage of pollen development. DAPI (4′, 6-diamidino-2-phenylindole dihydrochloride) fluorescent staining has also been used. However, different developmental stages and, consequently, a non-uniform starting pollen population have been observed within a single anther as well as between different anthers of the same flower (Hidaka et al. 1979, 1981; Chen 1985; Vasil 1967; Shull and Menzel 1977).

Physiological state and growth conditions of donor plants

The physiological conditions of the donor plants, which affect the number of P-grains (Heberle-Bors 1985), the endogenous levels of hormones and the nutritional status of the tissues of the anther (Sunderland and Dunwell 1977) all determine the success of the technique. The in vivo and/or in vitro formation of embryogenic pollen grains or P-grains, characterized by thinner exine structure, weak staining with acetocarmine, presence of a vacuole and absence of starch grains, seems to be connected with a nitrogen starvation phenomenon (Heberle-Bors 1983, 1985, 1989). Significant seasonal variations in anther response have been observed in many genotypes. Vasil (1980) observed that anthers removed from field-grown plants gave a better response than those picked from greenhouse-grown plants. In addition, anthers from the first flush of flowers in the season were found to be more responsive (Sunderland 1971). In tobacco, the effect of preculture environment was investigated first by Dunwell (1981), who showed that both photoperiod and light intensity affected the yield of micropore embryos and plantlets. The frequency of P-grains is also increased by conditions (short days, low

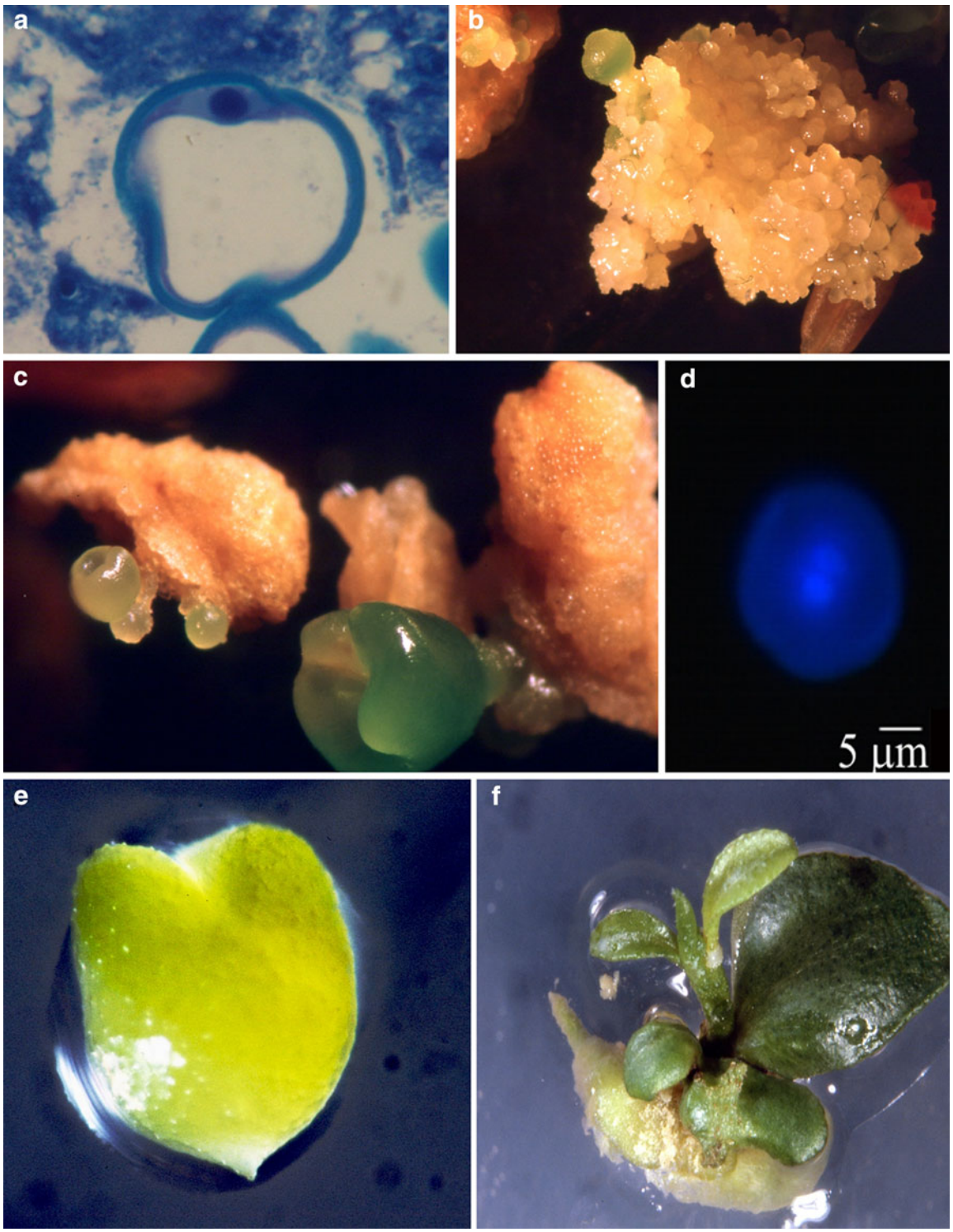

Fig. 2 **a** Vacuolated microspore of apricot (*Prunus armeniaca* L.) cv. Ninfa at the time of anther culture establishment (section stained with toluidine blue; photo taken at the laboratory of MC Risueño, C.S.I.S. Spain). **b** Embryogenic pollen-derived friable calli and embryos in different stages developing inside of *Citrus clementina* Hort. ex Tan. cv. Monreal anther, after 4 months of culture. **c** Direct microspore embryogenesis in *Citrus* anthers after 3 months of culture. **d** Symmetrical division of nucleus in an olive (*Olea europaea* L.) microspore after 3 weeks of anther culture. **e** Heart-shaped pollen-derived embryo of *C. clementina* cv. Nules. **f** Microspore-derived plantlet of clementine obtained from embryo germination

temperatures) that are unfavourable for plant growth (Heberle-Bors and Reinert 1981; Heberle-Bors 1989). The influence on the culture response of the temperature under which the donor plants are grown has been demonstrated by studies on barley (Foroughi-Wehr and Mix 1976), oilseed rape (Keller and Stringham 1978; Dunwell and Cornish 1985), turnip (Keller et al. 1983) and wheat (Lazar et al. 1984), despite the optimal growth conditions appearing to vary between species. The nitrogen status of plants also greatly affects the yield of microspore embryos (Sunderland 1978; Tsay 1981), with "nitrogen-starved" plants providing better results than those supplied with fertilizer.

Although the physiological state of the donor plant can dramatically affect the response of the anthers to the in vitro culture, this parameter has been investigated only in herbaceous plants due to the difficulties involved with determining it in woody plants. In fact, the growth conditions and physiological status of donor plants can not be standardized in the anther culture of perennial plants cultivated in open-air and affected by climatic (temperature, photoperiod and light intensity), cultural (pruning, irrigation, fertilization, etc.) and pedological conditions (especially during flower induction and differentiation). This can explain why the response to the culture of woody plant anthers is very season dependent even when the same protocols are applied (Germanà 2009).

Pretreatment

It has been observed in many genotypes that physical or chemical pre-culture treatments applied to excised flower buds, whole inflorescences or excised anthers before culture act as a trigger for inducing the sporophytic pathway, thereby preventing the development of fertile pollen (gametophytic pathway). Pretreatments such as chilling, high temperature, high humidity, water stress, anaerobic treatment, centrifugation, sucrose and nitrogen starvation, ethanol, γ-irradiation, microtubuli disruptive agents, electrostimulation, high medium pH, heavy metal treatment are particularly popular approaches in anther and in microspore culture, as recently reviewed by Shariatpanahi et al. (2006), who classified them into three categories: widely used, neglected and novel.

Temperature shock is considered to be the most effective treatment to induce pollen embryogenesis development. The optimum temperature and duration of pretreatment vary with the genotype. For example, among three cultivars (Tower, Willi and Duplo) of spring rape (*Brassica napus* ssp. *oleifera*), Dunwell et al. (1983) found that the highest yield (equivalent to 1.1 embryo per cultured anther) was obtained from anthers of the cv. Duplo after a 3-day treatment at 35°C, while the yields from the other cultivars were much lower and relatively unaffected by the 35°C treatment. Cold pretreatment (4°C for 2–3 weeks) is employed routinely in the anther culture of many crops, and its effect is also genotype-dependent (Osolnik et al. 1993; Powell 1988). In *Brassica* species, a short, high temperature treatment (30–35°C) before further culture at 25°C is required to efficiently switch the developmental pathway. Nutrient starvation, especially for sugars and nitrogen, has been routinely used to induce pollen embryogenesis in tobacco (Kyo and Harada 1986). Touraev et al. (1996b, 1997) showed that it was possible to replace the starvation pretreatment by a heat shock treatment. Chemical and physical mutagens (e.g., gamma rays) applied to plants or to the seeds from which M1 plants were obtained resulted in an increase in the androgenic response, as reported by Aldemita and Zapata (1991) in recalcitrant rice varieties, by Vagera et al. (2004) in barley and by Kopecky and Vagera (2005) in *Solanum nigrum*. In addition to being used to induce chromosome doubling, colchicine has also been used to induce microspore division and to promote gametic embryogenesis in several species, including sugar beet (Levan 1945), sorghum (Sanders and Franzke 1962; Simantel and Ross 1964), maize (Hu et al. 1991), *Brassica* (Mollers et al. 1994), wheat (Barnabas et al. 1991) and rice (Alemano and Guiderdoni 1994). However, the stimulating effect of mutagenic treatment on anther culture efficiency in recalcitrant genotypes is also highly genotype and dose dependent. Centrifugation and exposure to reduced atmospheric pressure or water stress are other pretreatments used prior to anther culture (Sopory and Munshi 1996).

Although the mechanism of just how stress affects pollen differentiation has not yet been firmly established, it seems to act by altering the polarity of the division at the first haploid mitosis involving reorganization of the cytoskeleton (Nitsch and Norreel 1973; Reynolds 1997), delaying and modifying pollen mitosis (two equal-size vegetative-type nuclei instead of one vegetative and one generative), blocking starch production or dissolving microtubules (Nitsch 1977) or maintaining viability of the cultured P-grains (Heberle-Bors 1985).

Surface sterilization, anther excision

Before anther excision, it is necessary to remove surface contaminants (bacteria and fungi) through sterilization. Many sterilization protocols have been used to obtain contaminant-free anthers, and most of these can be found in *Doubled haploid production in crop plants: a manual*, edited by Maluszynski et al. (2003a, b).

In general, after pretreatment, the floral buds are surface sterilized by immersion in 70% (v/v) ethyl alcohol for few minutes, followed by immersion in a sodium hypochlorite solution (about 1.5% active chlorine in water) containing a few drops of Tween 20 for 10–15 min and then by three

5-min washes with sterile distilled water. In the last step, anthers are excised aseptically from the filaments and placed onto the medium. An exception are barley spikes, which are sterilized only by being sprayed with 70% ethanol (Cistué et al. 2003) or by immersion for 5 min in ethanol 70%, followed by rinsing in sterile distilled water (Jacquard et al. 2003).

Injures to anthers during excision should be avoided in order to prevent somatic callus production from anther-wall tissues (Reinert and Bajaj 1977). When the anther sizes are minute, such as in *Asparagus*, *Brassica*, *Trifolium* and *Olea*, their extraction can be performed under a stereoscopic microscope (Bhojwani and Razdan 1983; Cistué et al. 2003; Germanà et al., unpublished).

Medium composition

A pivotal role in the induction of microspore embryogenesis is played by the culture medium composition. The diverse genotypes show very different basal medium requirements to induce pollen-derived plant formation. The nutritional requirements of the excised anthers are much simpler than those of isolated microspores (Reinert and Bajaj 1977; Bajaj 1990).

The most commonly used basal media for anther culture are N6 medium (Chu 1978), (modified) MS medium (Murashige and Skoog 1962), Nitsch and Nitsch (1969) medium and B5 medium (Gamborg et al. 1968), but there are many others. Generally, half-strength MS salt mixtures are suggested for the Solanaceae, and N6 medium for the cereals (Chu 1978).

A carbohydrate source is essential for embryo production in anther culture because of their osmotic and nutritional effects (Powell 1990). The influence of carbohydrate concentration is probably related to osmotic pressure regulation during the induction phase (Sunderland and Dunwell 1977; Sangwan and Sangwan-Norreel 1990) as later on in the culture period, high concentrations of the carbon source seem to be deleterious (Keller et al. 1975). Sucrose is the major translocated carbohydrate in plant tissue (Powell 1990), and it is the most common carbon source used in anther culture, normally at levels of 2–4% (Reinert and Bajaj 1977). High sucrose levels (6–17%) are required in those species (e.g., Gramineae, Cruciferae) in which mature pollen is shed in the tricellular condition (Dunwell and Thurling 1985), whereas for those in which mature pollen is bicellular (e.g., Solanaceae) lower levels, such as 2–5%, are usually beneficial (Dunwell 2010). Sucrose is heat labile, and autoclaved media contain a mixture of sucrose, D-glucose and D-fructose (Powell, 1990). Maltose has successfully been used to replace sucrose in barley anther culture, usually at a concentration of 62 g/l in the induction medium and at half this amount in the regeneration medium (Wedzony et al. 2009). Maltose has also been added to anther culture medium of wheat, triticale, rye and rice at concentrations ranging from 60 to 90 g/l (Wedzony et al. 2009). Fructose and glucose have both been shown to be inhibitory to pollen embryogenesis in *Petunia* anther culture (Raquin 1983). Lactose at 18 g/l and galactose at 9 g/l are regularly used in clementine anther culture (Germanà 2003). Sucrose was found to be the best carbon source, in comparison to glucose, in the anther culture of two clementine and two mandarin cultivars (Germanà et al. 1994), while glucose rather than sucrose has proved to be stimulatory in rye (Wenzel et al. 1977). Glycerol in combination with sucrose was found to stimulate callus production in clementine (Germanà et al. 2000a).

The effects of plant growth regulators have been widely investigated in anther culture. Although a few model species (e.g., most members of the Solanaceae) do not require the addition of an auxin to the induction medium, and induction does occur on simple media, the presence of growth regulators (auxins, cytokinins or a combination) is crucial for microspore-derived embryo production in the majority of plant species, particularly the recalcitrant ones (Maheshwari et al. 1982). The type and the concentration of auxins seem to determine the pathway of microspore development (Ball et al. 1993), with 2,4-dichlorophenoxyacetic acid (2,4-D) inducing callus formation (Fig. 2b) and indole-3-acetic acid (IAA) and α-naphthaleneacetic acid (NAA) promoting direct embryogenesis (Fig. 2c) (Armstrong et al. 1987; Liang et al. 1987). Giberellins and abscissic acid have been occasionally added to the media.

The addition of activated charcoal (0.5–2 g/l) to the medium increases the efficiency of microspore embryogenesis in several species (Bajaj 1990). Bajaj et al. (1977) obtained an increase in responsive tobacco anthers, from 41 to 91%, by supplemented the basal medium with 2% charcoal. It would appear that the charcoal acted by removing inhibitory substances from the medium and, presumably, from the anther wall and by regulating the level of endogenous and exogenous growth regulators (Reinert and Bajaj 1977; Vasil 1980; Heberle-Bors 1985). The addition of anti-oxidants and activated charcoal is often useful with some genotypes as it reduces the tissue browning caused by the phenols.

The supplement of other substances, such as glutamine, casein, proline, biotin, inositol, coconut water, silver nitrate (ethylene antagonist) and polyvinylpyrolidone, has been reported (Reinert and Bajaj 1977; Powell 1990; Achar 2002). Moreover, the addition of exogenous aliphatic polyamines (PAs) to the culture medium has been found to increase the number of pollen-derived embryos in potato (Tiainen 1992), in some Indian wheat cultivars (Rajyalakshmi et al. 1995), in cucumber (Ashok Kumar et al. 2004) and in clementine (Chiancone et al. 2006). PAs,

such as putrescine, cadaverine, spermidine and spermine, are low-molecular mass polycations, present in all living organisms; they are classified as growth regulators and involved in in vitro organogenesis and embryogenesis (Bagni and Tassoni 2001; Kumar et al. 1997). In anther culture, amino acids, related to the degeneration of wall tissues, were observed to improve the induction rates.

In the anther culture of many cereal species, a beneficial effect of co-cultivation with ovary tissues has been found (Broughton 2008), due to stimulatory role of arabinogalactans (Letarte et al. 2006).

pH is another factor which can influence the gametic embryogenic process (Stuart et al. 1987). In anther culture, the pH of the media is in the acid range and usually adjusted to 5.7–5.8 before autoclaving.

Anther culture media are generally solidified by adding agar, but the beneficial effect of other solidifying agents, such as starch (potato, wheat, corn or barley starch), gelrite, agarose and ficoll, has been reported. Liquid, semisolid and two-phase systems in which anthers are floated on liquid medium overlying an agar-solidified medium have been tested with different results (Dunwell 2010).

"Shed microspore" culture is a simple modification of anther culture in which anthers are stimulated to dehisce and to release their microspores, usually into a liquid medium of high osmolarity. The method has been found to be successful in several species, including barley (Ziauddin et al. 1990), wheat (Touraev et al. 1996b, 1997), tobacco (Dunwell 1985) and pepper (Supena et al. 2006).

Culture conditions

Anther cultures are usually incubated at 24–27°C and exposed to light at an intensity of about 2,000 lux for 14 h per 24-h day (Reinert and Bajaj 1977), but other culture conditions have been reported. For example, Vasil (1973) reports using alternating periods of light (12–18 h; 5,000–10,000 lux/m^2) at 28°C and darkness (12–6 h) at 22°C. However, optimal conditions need to be determined for each individual system (Bhojwani and Razdan 1983). Light is an environmental signal that regulates pollen morphogenesis in vitro (Reynolds and Crawford 1997). With respect to the effect of light quality on anther culture, the embryogenic induction of microspores is inhibited by high-intensity white light, whereas darkness or low-intensity white light are less inhibitory (Nitsch 1981; Wenzel and Foroughi-Wehr 1984; Bjørnstad et al. 1989). The incubation of anthers continuously in the dark has, on occasion, been found to be essential. An alternating light and dark period has also been shown to be beneficial after the induction period in several species: *Hyoscyamus niger* (Corduan 1975), *Datura innoxia* (Sopory and Maheshwari 1976), *Nicotiana tabacum* (Sunderland 1971) and *Citrus clementina* Hort. ex Tan. (Germanà et al. 2005b).

The composition of the atmosphere in the culture vessel has not been the focus of much attention, although experiments on tobacco have indicated its importance (Dunwell 1979). The density of the culture (i.e. number of anthers plated per volume of culture vessel or per unit volume of medium) and the manner of explant placement on the medium have been also found to be critical in anther culture (Sopory and Munshi 1996). Earlier studies did examine the influence of anther orientation in tobacco (Sunderland and Dunwell 1972; Misoo et al. 1981), *Datura innoxia* (Sopory and Maheshwari 1976), rice (Yangn and Zhou 1979) and barley (Shannon et al. 1985).

Morphogenic development, reprogramming of gene expression and plant recovery

After the pretreatment and during the culture period, the microspores can follow different routes, namely, to arrest their development and/or to die, to become a mature pollen grain, to divide forming a multicellular callus-like structure or to turn into a microspore-derived embryo (MDE) (Hosp et al. 2007; Seguì-Simarro and Nuez 2008a). Several changes, ranging from morphology to gene expression, distinguish microspores after induction and during embryogenic development. The acquisition of embryogenic potential by stress is accompanied by the stress-related cellular response, the repression of gametophytic development, the acquisition of a totipotent status and the dedifferentiation of the cells with cytoplasmic and nuclear rearrangements. Morphological and biochemical changes involve enlargement, cytoplasm dedifferentiation and clearing, the presence of a large central vacuole and a pH shift toward alkalinization (Huang 1996; Hoekstra et al. 1992; Maraschin et al. 2003; Touraev et al. 2001). The large central vacuole is subsequently divided into fragments, interspersed by radially oriented cytoplasmic strands, resulting in a structure denoted as "star-like" in which cytoskeleton rearrangements are involved (Barnabas et al. 1991; Zaki and Dickinson 1995; Zhao et al. 1996; Gervais et al. 2000; Obert and Barnabás 2004). After induction, the microspores are also characterized by an altered synthesis and an accumulation of RNA and proteins, and it seems that the genes involved in this reprogramming are stress related and/or associated with zygotic embryogenesis (Seguì-Simarro and Nuez 2008a).

The next phase is characterized by cell divisions with the formation of multicellular structures (MCSs) inside the exine wall. Cytological and ultrastructural observations have shown that the formation of MCSs from star-like microspores involves different developmental pathways that are defined by the symmetry of the first division and

the fate of the daughter cells (Maraschin et al. 2005a). According to Raghavan (1997), there are different routes of pollen-derived embryo formation:

1. Repeated division of the vegetative cell ("A" pathway). Nuclear division without cytokinesis can produce a multinuclear pro-embryo. Callus rather than embryo can be obtained.
2. Division of generative cell or both generative and vegetative cells ("E" pathway). Cell fusion can result in non-haploid chromosome numbers (Sunderland 1974).
3. Symmetrical division of microspores ("B" pathway) (Fig. 2d), a major pathway of embryo formation when microspores are collected prior to the first pollen mitosis (Smykal 2000).

The predominance of one pathway over the others seems to be dependent upon different factors, such as the developmental stage and the type of stress applied as pretreatment (Zaki and Dickinson 1995; Rihova and Tupy 1999; Kasha et al. 2001).

In the following phase, the embryo-like structures (ELS) are released out of the exine wall, with the rupture point at the generative domain, located on the side opposite the pollen germ pore (Seguì-Simarro and Nuez 2008a). Periclinal divisions of the cells that surround the ELS subsequently occur, leading to epidermis differentiation (Telmer et al. 1995; Yeung et al. 1996), and thereafter the ELS proceed through the heart- (Fig. 2e) and torpedo-shape stages in similar way to zygotic embryos (Hause et al. 1994).

Even when calli and embryos have been obtained, their conversion into plantlets (Fig. 2f) is not a foregone conclusion. There have been many reports of low regeneration rates even with a high induction rate (Wedzony et al. 2009). Therefore, further studies are required to raise the overall efficiency of the available protocols. In general, plant regeneration is mostly preceded by callusing, which increases the chances of gametoclonal variation. Often, depending on the genotype, duplication of the haploid genome is observed, more frequently during the first divisions of the embryogenic microspore and above all through a nuclear fusion mechanism (Gonzalez-Melendi et al. 2005; Shim et al. 2006; reviewed in Kasha 2005; Seguì-Simarro and Nuez 2008a).

The switch from the gametophytic to the embryogenic pathway is also characterized by an extensive remodeling of gene expression, with an upregulation of genes involved in primary metabolism and the biosynthesis of lipids, carbohydrates and protein (Seguì-Simarro and Nuez 2008a). In global terms, most of the genes differentially expressed can be ascribed to three main categories: (1) cellular response to the stress with the synthesis of heat-shock proteins (HSPs); (2) suppression of the gametophytic programme (cytoplasmic cleaning) (programme to return to an undifferentiated status, downregulation of genes involved in starch biosynthesis and accumulation); (3) expression of the embryogenic programme (symmetric division, randomly oriented divisions within the exine coat, polarity establishment, exine rupture, protoderm formation) (Maraschin et al. 2005a). Renewed DNA synthesis during the division of pollen cells and a decrease in RNA content during starvation are characteristic features of pollen embryogenesis (Bhojwani et al. 1973). Although the role of most of the markers during androgenesis remains to be determined, some marker genes, such as BABY BOOM (BBM), have been isolated during rapeseed pollen embryogenesis, representing the first identified androgenic-related genes (Boutilier et al. 2002; Maraschin et al. 2005a). Mitogen-activated protein kinase (MAPK) cascades seem to be involved in the process. In barley pollen embryogenesis, programmed cell death (PCD) takes place during the transition from multi-cellular structures to globular barley embryos at the late stages of microspore embryo development (Maraschin et al. 2005a, b).

Characterization of regenerants: ploidy analysis

Chromosome numbers from root-tip cells of regenerated embryos and plantlets have been counted using conventional cytological techniques. Ploidy level can be more easily assessed by flow cytometry analysis (Bohanec 2003). Ploidy level can also be estimated by indirect methods, such as those based on chloroplast counts in stomatal guard cells and plastid dimensions (Lee and Hecht 1975; Qin and Rotino 1995; Yuan et al. 2009).

Not only haploids or DHs have been obtained by in vitro anther culture. Non-haploid (diploid, triploid, tetraploid, pentaploid, hexaploid) embryos and plantlets have been obtained from anther culture of various genotypes (D'Amato 1977; Dunwell 2010). Triploids regenerated from anther culture have been reported in *Datura innoxia* (Sunderland 1974), *Petunia hybrida* (Raquin and Pilet 1972) and several fruit species (Germanà 2006, 2009). Non-haploids may arise from: (1) somatic tissue of anther walls, (2) the fusion of nuclei, (3) endomitosis within the pollen grain, (4) irregular microspores formed by meiotic irregularities (D'Amato 1977; Sunderland and Dunwell 1977; Narayanaswamy and George 1982; Sangwan-Norreel 1983). In some cases, the origin of non-haploids seems to derive from an incomplete cell-wall formation between the vegetative and generative nuclei (Dunwell and Sunderland, 1974a, b, 1975, 1976a, b, c). The dynamics and mechanisms of diploidization at early stages of microspore-derived embryogenesis have been studied in barley by Gonzalez-Melendi et al. (2005) and reviewed in Seguì-Simarro and Nuez (2008b). Duplication of the haploid

genome of pollen-derived individuals has been thought to occur through three mechanisms, namely, endoreduplication, nuclear fusion and c-mitosis (mitosis that takes place after treatment with colchicine), whereas triploids originate from a process of endoreduplication occurring during the early divisions of the microspores in culture (Sunderland 1974). It has been suggested that a spindle fusion mechanism operates in the very frequent production of triploid plants in *Petunia hybrida* and *Datura innoxia*: the endoreduplicated generative nucleus (*n* diplochromosomes) and the vegetative nucleus (*n* chromosomes) divide on a common spindle, giving origin to two triploid daughter nuclei. The formation of a hexaploid plant in *D. innoxia* (Sunderland 1974) and of triploid–hexaploid mosaics in *P. hybrida* (Raquin and Pilet 1972) was considered to be a failure of the spindle mechanism or chromosome doubling. The diploid or polyploid plants originating in this way are completely homozygous.

Because of the spontaneous chromosome doubling that occurs in the haploid calli and embryos, ploidy level analysis cannot always identify pollen-derived plants. In fact, diploid plants can be homozygous DHs or heterozygous somatic diploids produced by the anther-wall tissue. In fact, anther culture can be also used to obtain somatic embryos and plant clonal propagation in many genotypes (Germanà 2003, 2005).

Characterization of regenerants: detection of homozygosity

Isozyme analyses, random amplified polymorphic DNA (RAPD) markers and microsatellites can be utilized to assess homozygosis and to confirm the gametic origin of calluses and plantlets (Germanà 2006). Isozyme techniques allow androgenetic and somatic tissue to be distinguished when the enzyme is heterozygotic in the diploid condition of the donor plant and the regenerants show a lack of an allele. Isozyme analyses have been employed to confirm the gametic origin of calluses and plantlets in pear (Bouvier et al. 2002), apple (Höfer and Grafe 2000) and citrus (Germanà et al. 1991, 1994, 2000a, b; Deng et al. 1992; Germanà and Reforgiato 1997), confirming the achievement of true homozygous regenerants. Microsatellites have been also employed to characterize regenerants obtained from citrus anther culture (Germanà and Chiancone 2003; Germanà et al. 2005a, b) and to assess homozygosity in pear (Bouvier et al. 2002) and apple (Kenis and Keulemans 2000; Höfer et al. 2002). The single multi-allelic self-incompatibility gene has been used in apple by Verdoodt et al. (1998) to discriminate homozygous from heterozygous individuals obtained by anther culture as well as by parthenogenesis in situ.

Chromosome doubling in the case of haploidy

Chromosome doubling can occur spontaneously during in vitro anther culture, and the genotype, developmental stage of the microspores, type of pretreatment and pathway of development affect the percentage of doubling (Castillo et al. 2009). As examples of spontaneous doubling, average percentages of 70–90% have been reported in barley, 25–70% in bread wheat, 50–60% in rice, 50–90% in rye (Maluszynski et al. 2003a, b) and 20% in maize (Martin and Widholm 1996). For those species with low doubling percentages, an efficient chromosome doubling protocol is required to convert sterile haploids regenerated from the in vitro cultured anthers into fertile, homozygous doubled haploid plants. Colchicine is the most widely anti-microtubule agent used in vivo and in vitro (Castillo et al. 2009), but other doubling agents have also been used, such as oryzalin and trifluralin. For example, oryzalin DNA duplication coupled with high embryo survival was the most efficient approach to chromosome doubling of haploid embryos in *Quercus suber* (Pintos et al. 2007). The success of the chosen protocol depends on the compromise between toxicity and genome doubling efficiency, which can vary according to the genotypes (80% barley, 60–70% bread wheat, 40–70% durum wheat, 50–80% triticale, 40% rice, 40% maize; Castillo et al. 2009; Maluszynski et al. 2003a, b.

Progress in anther culture for the production of haploids and DHs

After several decades of DH research in plants, a lot of species have been studied, but haploid plants have not been obtained for all of them (Guha and Maheshwari 1964; Maluszynski et al. 2003a, b). Where this approach has been successful, the resulting DHs were used in plant breeding programmes to produce homozygous genotypes (Jain et al. 1996–1997; Maluszynski et al. 2003a, b).

Mainly, progress in technology has been achieved by empirical, time and cost consuming testing of protocols (particularly media, stress and environmental conditions) and, although this continuous range of improvements, there is still no method that can be universally recommended with a new species of interest (Dunwell 2010). However, in the most frequently studied crops (barley, wheat, triticale, maize, rice, and rapeseed) improved protocols of anther culture are now available (Wedzony et al. 2009). Significant advances have also been achieved in vegetable, fruit, ornamental, woody and medicinal species, though in many of them the response remains low, with legume species being particular recalcitrant (Wedzony et al. 2009). Despite that, the improvement in the haploidization protocol through the study on factors affecting microspore

embryogenesis (particularly, starvation and stress techniques), resulted in the increase of induction and regeneration rate of double haploids through anther culture, even also in legumes as recently reported in chickpea (*Cicer arietinum* L.) (Grewal et al. 2009) and in pasture lupin (*Lupinus* L.) (Skrzypek et al. 2008). An improvement to overcome albinism (occurrence chlorophyll deficient plants), that is a major problem in monocot anther culture (Wedzony et al. 2009), has been obtained adding $CuSO_4$ to the pretreatment "starvation" mannitol medium in barley (Wojnarowiez et al. 2002; Cistué et al. 2003) and supplementing ficoll to "starvation" mannitol medium at 4°C in winter triticale (Immonen and Robinson 2000).

Lists of recent advances in anther culture are reported by Dunwell (2010), Wedzony et al. (2009), Pratap et al. (2009), Srivastava and Chaturvedi (2008) and Touraev et al. (2009).

Application of haploids and DHs in plant breeding, genetics and functional genomics

Breeders have long recognized the advantages of DH technologies based on the knowledge that several theoretical and practical aspects of plant biology and genetics can take advantage of haploidy technology (Forster and Thomas 2005). For crop improvement purposes, DH lines are developed mainly to achieve homozygosity in diploid or allopolyploid species, saving several generations in a breeding programme and producing new homozygous cultivars or parental lines for F_1 hybrids (Veilleux 1994).

More than 280 varieties have been produced with the use of various DH methods in several crops (http://www.scri.sari.ac.uk/assoc/COST851/COSThome.htm), with the majority of the protocols referred to as anther culture. For example, in Europe, it has been estimated that 50% of the currently available barley (*Hordeum vulgare*) cultivars have been produced via a DH system, while in Canada, in 2007, three of the five most widely grown cultivars in all grades of the Canada Western Red Spring (CWRS) wheat class were DH cultivars (Dunwell 2010).

In vegetable crops, one of the main uses of DHs is as parents for F_1 hybrid seed production. Due to inbreeding depression, these lines often cannot be used directly but only as parental inbred lines for the production of hybrid varieties via crosses between selected homozygous males and females. The F_1 plants often exhibit so-called hybrid vigour (heterosis) (Maluszynski et al. 2001; Hochholdinger and Hoecker 2007), consisting in a dramatic increase in yield compared with their parents. DHs provide a unique system to attempt the "fixing" of hybrid performance in homozygous lines and to avoid the step of hybrid seed production.

There is currently a large interest in applying DH technology to high-value crops, such as medicinal and aromatic plants (Ferrie 2009), or to species suffering from inbreeding depression, such as rye (*Secale cereale*) (Immonen and Anttila 1996) and forage grasses (*Festuca* and *Lolium*; Nitzche 1970) in which it is difficult to produce fertile homozygous lines by self pollination.

Homozygous DHs also provide new opportunities for genetic studies and plant breeding in woody plants. In tree species, generally characterized by a long reproductive cycle, a high degree of heterozygosity, large size and, sometimes, self-incompatibility, it is not possible to obtain homozygous breeding lines through conventional methods involving several generations of selfing (Germanà 2006, 2009). Moreover, the size reduction of haploid and homozygous plants compared with diploid and heterozygous ones may be of horticultural interest in terms of, for example, ornamental plants or dwarfing rootstocks for fruit crops.

Another opportunity to use haploids in crop improvement is also the "gametoclonal variation", consisting of differences in morphological and biochemical characteristics as well in chromosome number and structures that are observed among plants regenerated from cultured gametic cells (Evans et al. 1984; Morrison and Evans 1987). Different sources of variation can explain gametoclonal variation, such as new genetic variation induced by the cell culture procedures, new variation resulting from segregation and independent assortment, new variation induced by the chromosome doubling procedure and new variation induced at diploid level, resulting in heterozygosity (Morrison and Evans 1987; Huang 1996).

Triploid plants derived via anther culture may have great commercial potential in crops, where the consumers desire the seedlessness of fruits. Triploids regenerated from anther culture have been reported in apple (Höfer 1994; Höfer et al. 2002), *Pyrus pyrifolia* Nakai (Kadota and Niimi 2004), *Carica papaya* L. and *Citrus clementina* Hort. ex Tan. (Germanà et al. 2005a; Germanà 2007, 2009). The ploidy analysis of 94 regenerants from clementine anther culture by flow cytometry showed that about 82% of the regenerants were tri-haploids, rather than haploids or DHs as expected (Germanà et al. 2005a).

In addition to increasing the efficiency of crop breeding programmes, haploids and DHs have been useful in such research areas as mutation studies, gene mapping and genomics and as targets for transformation. They provide excellent material to obtain reliable information on the location of major genes and quantitative trait loci (QTLs) for economically important traits (Khush and Virmani 1996), and several genome sequencing programmes are using a haploid genome because of its simplified assembly, such as those involving many perennial plants (e.g., peach, coffee, pear, apple and citrus) (Dunwell 2010).

In vitro selection during microspore embryogenesis can particularly function as an efficient and early screening

procedure for desired mutant traits, thereby saving time and space. For example, in rapeseed, not only mutants for herbicide, disease resistance and salt tolerance have been selected, but also mutants for seed quality traits (Turner and Facciotti 1990; Wong and Swanson 1991; Huang 1992; Rahman et al.1995; Kott 1998).

In transformation protocols, uni-cellular microspores as well as cells or haploid embryos at all stages of microspore embryogenesis and regeneration have been used as recipients for gene delivery, with the aim of directly obtaining DH plants that are homozygous for the transgene. Microinjection, electroporation, particle bombardment and *Agrobacterium tumefaciens*-mediated transformation are transformation techniques already tested (Touraev et al. 2001). Primarily in cereals, where Kasha et al. (2001) observed that the doubling of the chromosome number occurs by nuclear fusion after the first nuclear division, it is important that gene incorporation is prior to this stage to obtain DHs homozygous for the transgene.

Doubled haploidy combined with marker-assisted selection provides a short cut in backcross conversion, a plant breeding method for improving an elite line defective in a particular trait (Toojinda et al. 1998).

DHs play also a vital role in genomics, in integrating genetic and physical maps, thereby providing precision in targeting candidate genes (Kunzel et al. 2000; Wang et al. 2001). Moreover, DHs have been a key feature in establishing chromosome maps in a range of species, notably barley, rice rapeseed and wheat (Forster and Thomas 2005).

By targeting the mutation treatment at single gametic cells and then inducing embryogenesis and DH plant production, it is possible to create directly a population of homozygous mutant lines (Szarejko and Forster 2006), avoiding chimeras or heterozygosity and enabling the expression and identification of both recessive and dominant traits in the haploid cells, tissues and plants. Application of doubled haploidy to gametes of M1 plants (plants originating from mutagenized seeds) has been successfully used in barley, rice and wheat, increasing the efficiency of selection for desired traits in a mutated DH population (Forster et al. 2007). The uni-nucleate microspore is therefore an ideal target for mutagenic treatment, and treatments occur shortly after microspore isolation or after pretreatment before the first nuclear division. As well as in transformation, the timing is critical, as treatment after the first division will generate chimeric and heterozygous plants; this is particularly relevant for cereals such as barley, where spontaneous doubling occurs shortly after the first divisions in culture (Chen et al. 1984; Kasha et al. 2001). Microspores, but also haploid cells, tissues, organs and explants, have been used as targets for mutagenic treatments in protocols involving the application of both chemical and physical mutagens. Through the chemical mutagenesis of anther and microspores cultures, Castillo et al. (2001) and Lee and Lee (2002) produced developmental mutants in *Hordeum vulgare* and *Oryza sativa*, respectively. In rice, Chen et al. (2001) obtained improved DH lines applying gamma rays to anthers in culture. DH protocols are particularly effective when they are combined with genetic markers to select for desired genotypes (e.g., Tuvesson et al. 2006; Werner et al. 2007).

DH systems are particularly interesting in terms of inducing and fixing mutations, especially when induced mutations are recessive and detected in the second or third (M2 and M3) generation after the mutagenic treatment. About 30% of mutant cultivars have been developed through cross-breeding programmes in which mutants or mutant varieties served as a source of desirable alleles. The mutant traits commonly exploited are earliness, dwarfness, lodging resistance, biotic and abiotic stresses resistance, higher yield and better quality, and these have led to new cultivars. Doubled haploidy continues to be important in basic and applied genetic studies and has an important role in the development and exploitation of structured mutant populations for forward and reverse genetics (Szarejko and Forster 2007). The reverse genetics strategy, called TILLING (Targeting Induced Local Lesions In Genomes; McCallum et al. 2000; Perry et al. 2003), provides an alternative method of linking genes to phenotypes, inducing a high frequency of mutations by chemical mutagen application combined with the high throughput screening method for single nucleotide polymorphisms (SNPs) in the targeted sequence. To avoid the detection of false positives owing to inherent variation in the starting material, it is much better if the mutant populations derive from a homozygous line.

Conclusions and future perspectives

The great potential of employing haploidy, doubled haploidy and gametic embryogenesis in breeding is clearly evident. Haploids can improve the efficiency and the speed of the usually cumbersome, time-consuming, laborious and sometimes rather inefficient conventional breeding methods. Although the application of pollen embryogenesis is widespread and many species respond very well to anther culture, many others of interest are still recalcitrant, and the cellular, biochemical and molecular bases for the transformation of microspores into pollen embryoids are still poorly understood. For these reasons, it is urgent to develop new genotype-independent methods through the study and improvement of existing protocols and by obtaining a deeper understanding and control of the microspore embryogenesis process, particularly of the two main developmental switches: the induction of microspore cell division and their commitment to the embryogenic

pathway. In recent years, studies on the molecular basis of microspore embryogenesis have profited from the development of advanced genomic, transcriptomic, proteomic and imaging tools, and these tools will likely (and hopefully) result in the identification of many interesting genes involved in microspore reprogramming and embryogenesis in the near future. This should pave the way to a better understanding of these processes and to more efficient protocols, enabling the effective deployment of gametic embryogenesis and haploid technology in the improvement of all plant species. However, the recent increasing number of reports on gametic embryogenesis and haploid and DH production is evidence of the great interest in this useful breeding tool and fascinating research field, and we can expect its future applications in many other important crops.

Acknowledgments Due to restrictions in the limit of the article and the large number of research papers on in vitro anther culture, it is impossible to cite all published papers, and the author has had to leave many of these out of the article. Apologies are extended to those authors who have not been mentioned. For more extensive reviews on haploids and doubled haploids, it is possible to consult volumes 1–5 of *In vitro haploid production in higher plants* (Jain et al. 1996–1997) and the recent volume *Advances in haploid production in higher plants* (Touraev et al. 2009). For the protocols, the interested reader is referred to *Doubled haploid production in crop plants: a manual* (Maluszynski et al. 2003a, b).

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