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Anther culture and double-haploid production in fruit crops: Status & opportunities

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Abstract
Horticultural crops, especially fruit crops play a significant role in the food and nutritional security of the country. Conventional methods, involving several generations of selfing, are not applicable to produce homozygous lines in fruit crops, due to the high heterozygosity of the genomes, the long duration of the generation cycle, the large size, and, often, the self- incompatibility. 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. In addition, haploids constitute an important material for induction and selection of mutants, particularly for recessive genes, hybrid development, induction of genetic variability, cytogenetic research, genome mapping, *etc*. 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.

Keywords: Conventional, double haploids, embryogenesis, transcriptomic

Introduction
The term haploid refers to those plants which have gametophytic number of chromosome as compared to the sporophytes from which they have been developed. Spontaneously in natures they are produced through the process of apomixes and parthenogenesis. Looking at the wide and unevitable application of diploids in crop development and improvement many new artificial strategies to develop haploids such as through distant hybridization, chemical treatment, x - ray irradiation, hormone treatment and temperature shock methods have been developed. 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) [21]. 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.

Brief history of anther culture for haploid and Double Haploid 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) [3]. 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. 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) [12] 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) [6]. 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) [19]. 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) [6]. 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) [20], 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) [7].

Importance of Haploids and Double Haploids

The interest of breeders in haploids or, by doubling the chromosome numbers, DH, lies in the possibility of shortening the time needed to produce completely homozygous lines compared to conventional breeding. In fact, haplo-diploidization through gametic embryogenesis allows the single-step development of complete homozygous lines from heterozygous parents. In a conventional breeding programme, pure lines are developed after several generations of selfing and still may not be 100% homozygous. In woody plants, generally characterized by a long reproductive cycle, a high degree of heterozygosity, large size, and, sometimes, self-incompatibility, it is not possible to obtain haploidization through conventional methods. Actually, the absence of pure lines in woody plants makes genetic studies rather difficult to conduct. New superior cultivars produced *via* gametic embryogenesis (above all from the male gametes) have been reported for several genotypes, and DH are being routinely used in breeding programs for new cultivar development in many crops (Veilleux, 1994) [33]. Often *in vitro* regenerated plants show differences in their morphological and biochemical characteristics, as well in chromosome number and structure. ''Gametoclonal variation'', the variation observed among plants regenerated from cultured gametic cells (Morrison and Evans, 1987) [26], is another opportunity to use haploids in crop improvement. Unlike ''somaclonal variation'' which is related to the variation among plants regenerated from cultured cells or tissue (Larkin and Scowcroft, 1981), gametoclonal variation results from both meiotic and mitotic division. Moreover, because of their homozygosity, in the gametoclones it is possible to observe the direct expression of both dominant and recessive mutations. Several different sources of variation have to be considered in order to explain gametoclonal variation including 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 (Huang, 1996) [15]. Double haploids can also increase the efficiency of crop breeding programmes, particularly of genome mapping. They, in fact, provide excellent material to obtain reliable information on the location of major genes and QTLs for economically important traits (Khush and Virmani, 1996) [23].

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. 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 (Wang *et al.,* 2000) [34]. 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.

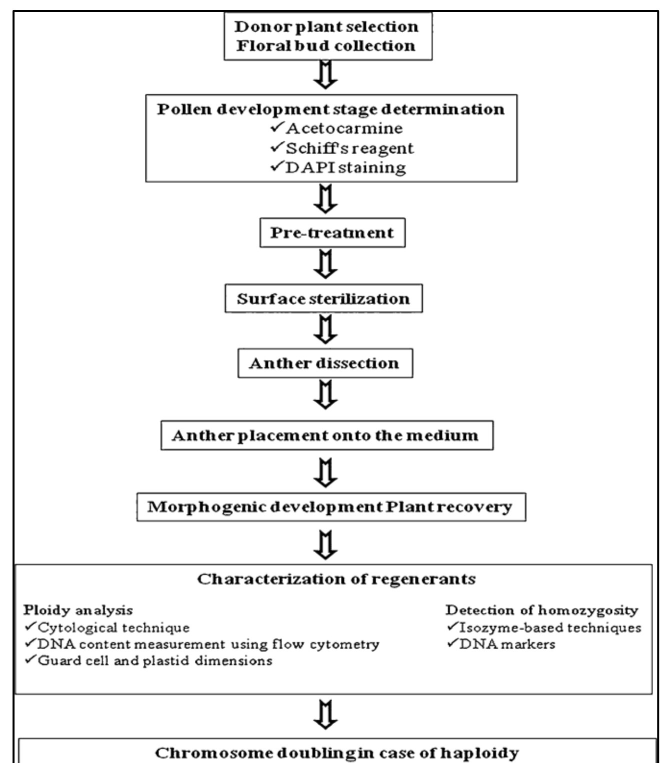

Fig 1: Diagram describing common guidelines of the anther culture method

Isolation of pollen

The pollen grains are released from the cultured anthers either mechanically. Or the cold treated anthers cultured on liquid medium burst open after 2-7 days liberating the pollen grains into the medium. This is called 'float culture method' which has proved better than mechanical isolation of pollen from fresh or pre-cultured anthers. To improve the efficiency of

isolated pollen culture for the production of haploids, Wenzel and his colleagues introduced the technique of density gradient centrifugation which allows the separation of embryogenic grains from a mixture of embryogenic and non-embryogenic grains obtained after crushing the anthers. The anthers of Barley obtained at the proper stage of development and gently macerated to obtain a suspension of pollen grains. After removing the debris by repeated filtration and centrifugation, the suspension was layered on 30% sucrose solution and centrifuged at 1200 g for 5 min. The androgenic, vacuolated pollen grains formed a band at the top of the sucrose solution. Isolated pollen culture is not only more efficient but also more convenient than anther culture. The tedious process of dissection of anthers is avoided. Instead, the entire buds within a suitable size range are crushed and the embryogenic grains are then separated by gradient centrifugation.

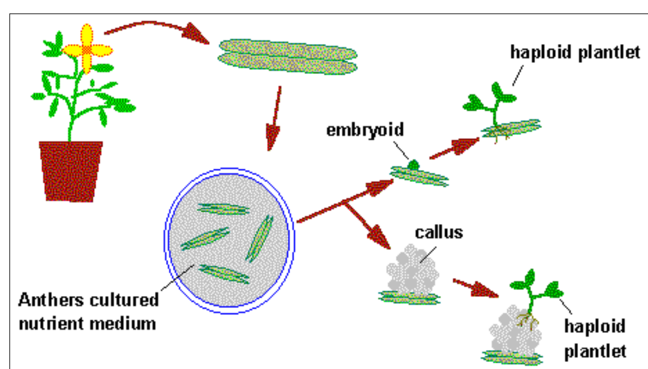

Fig 2: Anther isolation and culture

Factors affecting androgenesis

- **Physiological status of the donor plants:** The age of the donor plants and the environmental conditions under which it has been grown significantly affects the androgenic process. Generally, the buds from the first flush of flowers show better response than those borne separately. Exposures of donor plants to nutrient and water stresses reported to promote androgenesis.
- **Stage of pollen development:** The pollen grains around the first mitosis is most responsive. The uninucleate microspores produce haploids while the binucleate pollen form plants of higher ploidy.
- **Anther wall factors:** The pollen from one cultivar of tobacco would successfully develop into an embryo even if transferred into the anthers of another cultivar.
- **Genotype:** Hybrids are more androgenic than their parents.
- **Pretreatment of cultured anthers/pollen grains:** application of certain physical (temperature shock, centrifugation, γ irradiation) and chemical (auxins) treatments to cultured anthers or pollen grains prior to standard culture room conditions, has proved essential or promotory for *in vitro* androgenesis.
- **Culture medium:** Addition of ethrel (2-chloroethylphosphonic acid), sucrose, agar and other nutrients specific to certain genotype found to increase the success rate of androgenesis.
- **Culture density:** The frequency of pollen embryogenesis was enhanced if the anther culture density was increased from 3 anthers per ml to 12-24 anthers per ml in *Brassica oleracea*.
- **Effect of gaseous environment:** The composition of the gas mixture that surrounds the anthers has profound influence on the number of embryos produced in anther cultures. The removal of CO_2 from the culture vessel resulted in decline in anther culture response in *Nicotiana tobaccum*.
- **Effect of light:** Isolated pollen culture is more sensitive to light than anther culture.

Detailed Description of steps involved in anther culture

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 to early, mid-bicellular pollen—probably due to the transcriptional status that at that time is still proliferative and not yet fully differentiated (Malik *et al.,* 2007) [24]. After the pollen grains begin to accumulate storage reserves, they usually lose their embryogenic capacity and follow the gametophytic developmental pathway. The stage of pollen development is usually tested in one anther per floral bud size by the acetocarmine method (Sharma and Sharma, 1972) [32]. 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 micro- scope to determine the stage of pollen development. DAPI (40, 6-diamidino-2-phenylindole dihydrochloride) fluorescent staining has also been used.

Physiological state and growth conditions of donor plants

The physiological conditions of the donor plants, which affect the number of P-grains, the endogenous levels of hormones and the nutritional status of the tissues of the anther 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.

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, c-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) [31], 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. 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. 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. Centrifugation and exposure to reduced atmospheric pressure or water stress are other pretreatments used prior to anther culture. 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, delaying and modifying pollen mitosis (two equal-size vegetative-type nuclei instead of one vegetative and one generative), blocking starch production or dissolving microtubules or maintaining viability of the cultured P-grains.

Surface sterilization and 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.* (2003) [25]. 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 three5-min washes with sterile distilled water. In the last step, anthers are excised aseptically from the filaments and placed onto the medium. Injures to anthers during excision should be avoided in order to prevent somatic callus production from anther- wall tissues.

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. The most commonly used basal media for anther culture are N6 medium, (modified) MS medium and B5 medium, but there are many others. A carbohydrate source is essential for embryo production in anther culture because of their osmotic and nutritional effects. Sucrose is the major translocated carbohydrate in plant tissue, and it is the most common carbon source used in anther culture, normally at levels of 2–4%. High sucrose levels (6–17%) are required in those species (*e.g.,* Gramineae, Cruciferae) in which mature pollen is shed in the tricellular condition, whereas for those in which mature pollen is bicellular (*e.g.,* Solanaceae) lower levels, such as 2–5%, are usually beneficial.

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. Gibberellins and abscisic 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 embryo- genesis in several species. The addition of antioxidants 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 poly vinyl pyrolidone, has been reported.

pH is another factor which can influence the gametic embryogenic process. 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. ‘‘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.

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, but other culture conditions have been reported. However, optimal conditions need to be determined for each individual system (Bhojwani and Razdan, 1983) [2]. Light is an environmental signal that regulates pollen morphogenesis *in vitro*. 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. The incubation of anthers continuously in the dark has, on occasion, been found to be essential.

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). 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. 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.

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. 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.

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. Ploidy level can also be estimated by indirect methods, such as those based on chloroplast counts in stomatal guard cells and plastid dimensions. 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. Triploids regenerated from anther culture have been reported in *Datura innoxia*, *Petunia hybrida* and several fruit species (Germana, 2009) [8].

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. In some cases, the origin of non-haploids seems to derive from an incomplete cell-wall formation between the vegetative and generative nuclei. 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.

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. 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) [4], apple and citrus (Germana` and Reforgiato, 1997) [11], confirming the achievement of true homozygous regenerants. Microsatellites have been also employed to characterize regenerants obtained from citrus anther culture (Germana` and Chiancone, 2003) [9] and to assess homozygosity in pear (Bouvier *et al.,* 2002) [4] and apple (Kenis and Keulemans, 2000) [22].

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. 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*, but other doubling agents have also been used, such as oryzalin and trifluralin.

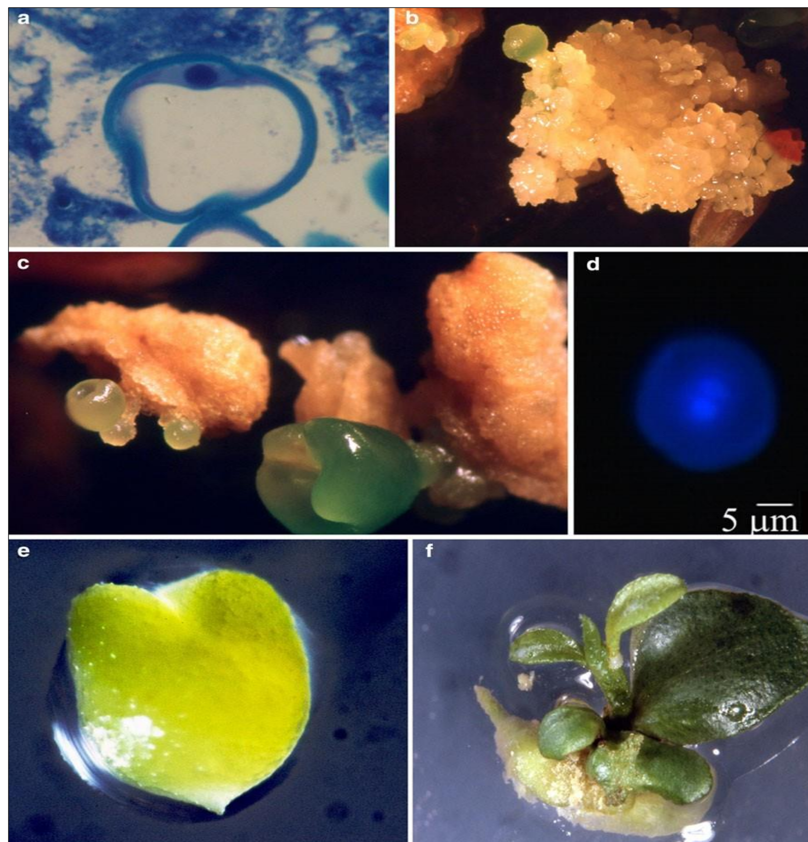

Fig 3: A. Vacuolated microspore of apricot (*Prunus armeniaca* L.) cv. Ninfa at the time of anther culture establishment (section stained with toluidine blue; photo taken at the laboratory of MC Risuen˜o, C.S.I.S. Spain). B. Embryogenic pollen-derived friable calli and embryos in different stages developing 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. Symmet-rical 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

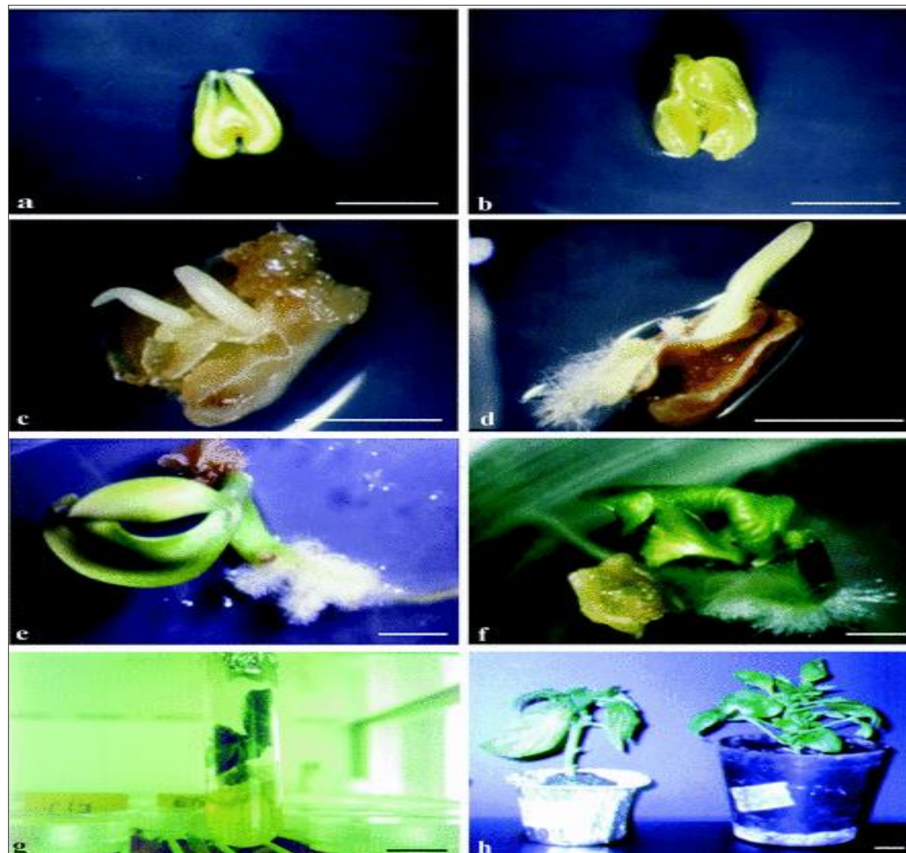

Fig 4: (a) Anther at the onset of the culture. (b) Anther after 6 days in culture. (c, d) Embryos emerging from the anthers after 30 days in culture, showing roots (c) and shoots (d). (e–g) Plantlets with cotyledons (e) and with leaves (f, g) subcultured in growing medium. (h) 80-dayold regenerated haploid plant from anther culture (left-hand side) and a diploid control of the same age (right-hand side). Scale bars in (a–d), 2.5 mm; in (e–h), 5 mm.

Status of Haploids and Double Haploids in major Fruit crops

Prunus persica (L.) Batsch

Haploids (1x=1n=8) reported in peach [*Prunus persica* (L.) Batsch] arose spontaneously. They show the typical haploid traits of thin shoots, narrow leaves, weak vegetative growth and small non-fertile or less fertile flowers. A study carried out by Scorza and Pooler (1999) [30] on the growth and yield of F_1 hybrid peaches developed from DH demonstrated that their productivity is similar to those of standard cultivars, but F_1 hybrids offer advantages in the production of uniform seedling scion cultivars. F_1 hybrids can be also useful in high-density production (HDP) systems where the cost of the trees proves to be a limiting factor. In fact, they eliminate the necessity of grafting and permit the direct sale of seeds or non-grafted seedlings. This can make HDP attractive by reducing production costs, especially where there is no need for a specific rootstock.

Citrus

All cultivated forms of Citrus and related genera (Poncirus, Fortunella, *etc.*) are diploid with a monoploid number of chromosomes (n=x=9). Triploid and tetraploid forms of Citrus also exist. In *Citrus natsudaidai* haploid seedlings were first obtained by the application of gamma rays. One haploid embryo was obtained in an immature seed from a diploid (Clementine mandarin) diploid (Pearl tangelo) cross. The production of nine haploid plantlets, which did not survive, and two embryogenic callus lines have been obtained in clementine (*Citrus clementina* Hort. ex Tan.), cv. SRA 63 after *in situ* parthenogenesis induced by pollen of Meyer lemon (*Citrus meyeri* Y. Tan.) irradiated at 300, 600 and 900 Gray (Gy) from a cobalt 60 source. Flowers of clementine SRA 63 were pollinated in the field with the irradiated pollen; fruits were picked at maturity and embryos were cultivated *in vitro*. Three haploid plants were obtained from *in vivo* crosses of two monoembryonic diploids (clementine and ‘‘Lee’’)a triploid hybrid of ‘‘Kawano natsudaidai’’ (*Citrus natsudaidai* Hayata). Haploid plantlet regeneration through gynogenesis in *Citrus clementina* Hort. ex Tan., cv. Nules, has been induced by *in vitro* pollination with pollen from a triploid plant. The pollen source chosen was ‘Oroblanco’, a triploid grapefruit-type citrus obtained in 1958 through a cross between an acidless pummelo (*Citrus grandis* Osbeck) and a seedy, tetraploid grapefruit (*Citrus paradisi* Macf.).

With regards to Citrus and their relatives, haploid plantlets have been recovered, by anther culture, from *Poncirus trifoliata* L. Raf. and *C. madurensis* Lour.; haploid plantlets and highly embryogenic haploid calli of *C. clementina* Hort. ex Tan.; haploid, but albino embryos of ‘Mapo’ tangelo (*C. deliciosax C. paradisi*); haploid and diploid calli, embryos and leafy structures but no green plants of *C. limon* L. Burm. f.; haploid embryos of *Clausena excavata* have been also achieved. *In vitro* pollen embryogenesis is affected by numerous factors: genotype, the pre-treatment applied to anthers or to floral buds, pollen developmental stage, donor plant growth conditions, culture media (macro and microelements, carbon source, and plant growth regulators), and conditions of incubation.

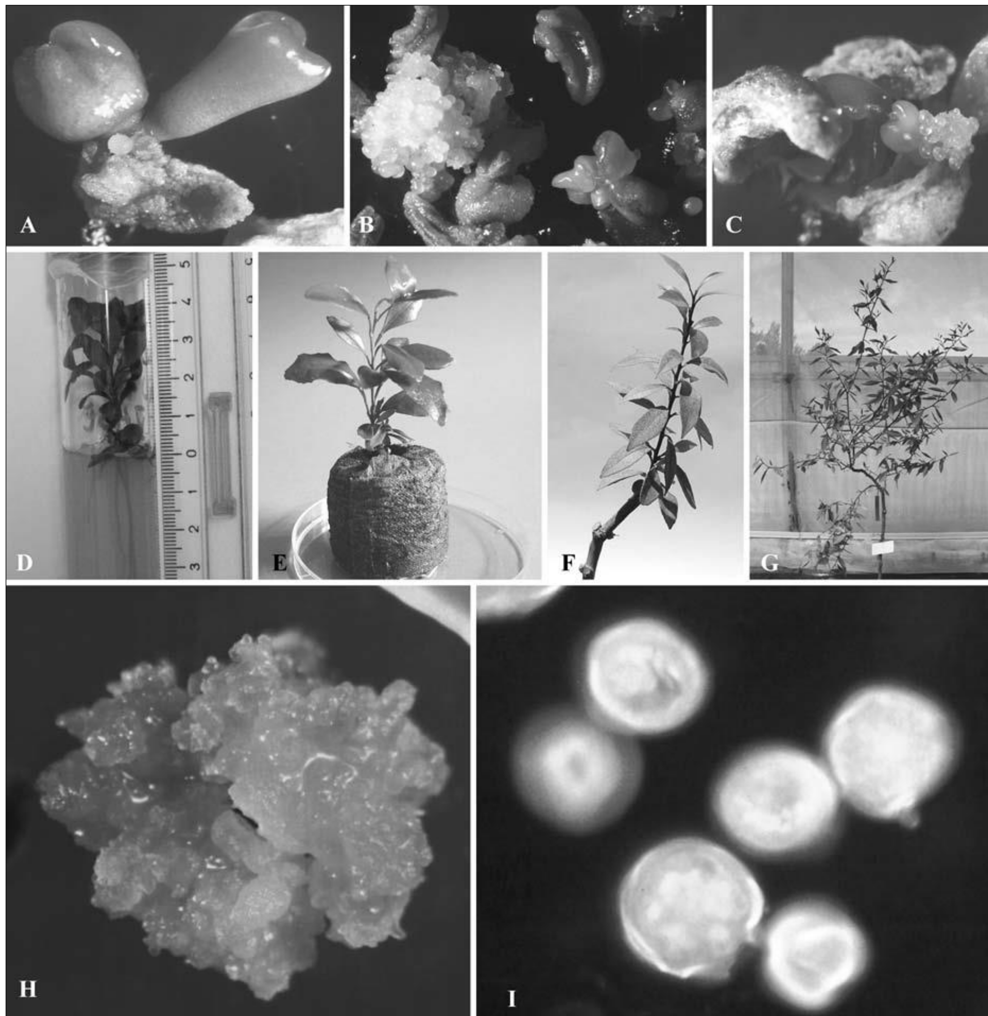

Fig 5: (A) Direct embryogenesis in Citrus anther culture. (B) Embryogenic, haploid, friable, callus emerging after 4 months of culture from anther culture of the cultivar Nules of *C. clementina* Hort. ex Tan, cv. Monreal. (C) Haploid embryogenic calli and embryos in different stages developing from inside a clementine SRA 63 anther in culture. (D) Haploid plantlet of Nules clementine obtained from embryo germination. (E) A haploid plantlet of *Citrus clementina*, cv. Nules transferred to soil. (F) and (G) Doubled haploid Nules grafted onto sour orange seedlings 1 year (F) and 4 years (G) after grafting. (H) Non-morphogenic callus from *Vitis vinifera* L., cv Lacrima di Maria, anther culture. (I) A multinucleated pollen grain of *Eriobotrya japonica* Lindl., cv. El Buenet.

***Prunus avium* L.**

The regeneration of four homozygous lines in sweet cherry (*Prunus avium* L.) has been obtained by *in situ* parthenogenesis induced by pollination with irradiated pollen, followed by embryo and cotyledon culture in the cultivar 'Altenburger' (Hofer and Grafe, 2003) [14]. Isozyme analyses have been employed to confirm the gametic origin of calluses in cherry (Hofer and Grafe, 2003) [14].

***Prunus armeniaca* L.**

The formation of calluses on cultured anthers of apricot 'Harcot', as well as the differentiation of nodular structures have been reported by Peixe *et al.* (2004) [28]. A heat pre-treatment of 28 °C for 8 days provided the best results when compared with 36 or 24 °C (Peixe *et al.,* 2004) [28]. After initiation, temperature used was 24/22 °C day/night (Peixe *et al.,* 2004) [28]. The ploidy of calluses, evaluated by flow cytometry, ranged from haploid to octoploid.

***Olea europaea* L.**

Olive is among the most typical crops and the most important oil-producing plants of the Mediterranean basin, characterized by a very long juvenile phase, a large plant size and often by self-incompatibility. It is a diploid (2n=2x=46), outcrossing long-living species. Sporophytic division, multinucleate microspores and multicellular structures have been successfully induced in isolated microspore culture of two olive cultivars (Arbequina and Picual).

***Morus alba* L.**

Because of the dioecious nature of mulberry, inbreeding to obtain haploids and homozygous plants is not applicable. Gynogenic haploids of a female clone of mulberry (*Morus alba* L. Cv.K-2) were obtained by *in vitro* culture of unpollinated ovaries from *in vitro* developed inflorescences. Anther culture has not been successful in producing haploids of this tree crop.

***Feijoa sellowiana* Berg.**

Multinucleated pollen grains were obtained in anther culture of feijoa, but attempts to regenerate pollen plants were unsuccessful.

***Opuntia ficus-indica* (Mill.)**

Opuntia ficus-indica (Mill.) breeding to obtain new cultivar development has been hampered by some reproductive aspects such as cleistogamy, nucellar embryony and low seed germination. Research has been carried out to study the

correlation of sequential floral and male gametophyte development and to investigate the response to *in vitro* culture of anthers collected from flower buds of two different stages of development of prickly pear, *Opuntia ficus-indica* L. Mill.

***Eriobotrya japonica* Lindl.**
Loquat, originated in China, has adapted well to the Mediterranean climate and grows in the same areas where citrus species are cultivated. Very often, current varieties are selected as seedling variations resulting from natural hybridization and not very much attention has been paid to use of biotechnology as a tool to create new variability in this species. Preliminary research is in progress to apply anther culture and haploid production to loquat, resulting in callus production and multinucleated pollen grains.

***Carica papaya* L.**
The main breeding systems of papaya, a polygamous species, using true-bred lines, benefit greatly from haploid induction through anther culture. Haploid plantlets and pollen-derived embryos were obtained from papaya anthers cultured at the uninucleate stage. Haploid plantlets were induced through anther culture in a medium without any growth regulators and under dark conditions.

***Actinidia deliciosa* (A. Chev)**
Parthenogenetic tri-haploids were induced in kiwifruit, cv. Hayward, a hexaploid species (2n=6x=174), by irradited pollen. The best results were obtained with a dosage of 500–1500 Gy and the genotype of the pollen parent greatly influenced the ability to obtain both seedlings and tri-haploids.

***Musa balbisiana* (BB)**
The production of 41 haploid (n=x=11) plants from anther culture of banana [*Musa balbisiana* (BB)], was reported by Assani *et al.* (2003): 18 from the genotype Pisang klutuk, 12 from Pisang batu, 7 from Pisang klutuk wulung and 4 from Tani. The frequency of callus induction was 77% and about 8% of anthers developed embryos after 6 months of culture. The frequency of embryo formation was genotype-dependent. Temperature used during anther culture was 27° C under dark conditions (Assani *et al.,* 2003) [1].

***Vitis vinifera* L.**
For grapevine, (2n=2x=38), one of the most cultivated plants in the world, haploids would be a powerful tool for increasing knowledge about the species and for dealing with the difficult task of its genetic improvement. Anther culture is usually employed to establish diploid somatic embryogenic cultures of Vitis. Embryogenic callus is valuable for propagation or genetic improvement and can be used for somatic hybridization by protoplast fusion, genetic transformation, synthetic seed production and germplasm storage. Temperatures used during anther culture were 24–26 °C for grape.

***Malus domestica* (L.) Borkh**
Several methods have been set up to obtain haploid plants in *Malus domestica* (L.) Borkh, 2n=2x=34. Induction of embryogenesis and regeneration of pollen-derived plants from anther culture in this species has been reported by several researchers. The induction of embryogenesis from cultured apple anthers is still low and highly genotype- dependent. Haploid plant have been obtained through in situ parthenogenesis induced by pollination of cv. Erovan with pollen irradiated at 500– 1000 Gy, followed by *in vitro* embryo culture. This technique has been successfully applied to other apple cultivars also with different c-rays from Cobalt 60.

Culture medium in apple anther culture
In fruit tree anther culture, the pH of the media is usually adjusted to 5.7–5.8 before autoclaving. A higher pH (6.2) has been employed for gametic embryogenesis in apple isolated microspore culture. Activated charcoal was beneficial to anther culture of apple.

Conditions of incubation of apples anther culture
Temperatures used for apple anther culture are 23–30 °C. Dark was used also in apple isolated microspore culture.

Embryo development from apple microspores and the origin of haploids
In apple, the androgenic plants are usually obtained not *via* direct gamete-derived embryo germination, but through the occurrence of adventitious shoots also from secondary embryos. Regarding the origin of haploids, three different routes in apple androgenesis were observed: formation of two identical nuclei after abnormal pollen mitosis; division of the vegetative nucleus after normal pollen mitosis; and division of the generative nucleus after normal pollen mitosis.

Characterization and propagation of regenerants obtained by apple gamete embryogenesis: Triploids regenerated from anther culture have been reported in apple. Isozyme analyses have been employed to confirm the gametic origin of calluses and plantlets in apple. Homozygous lines of apple have been analysed by the sequence characterized amplified region (SCAR) marker ALO7 linked to the *Vf* gene for scab resistance from *Malus floribunda* and by simple sequence repeats (SSRs). The single multiallelic self-incompatibility gene has been used in apple to discriminate homozygous from heterozygous individuals obtained by parthenogenesis *in situ* or by anther culture. Protoplasts were isolated from the stem and leaf of a haploid golden delicious apple clone and protoplast-derived shoots were successfully propagated *in vitro* via organogenesis.

***Pyrus pyrifolia* Nakai**
Triploid plants were obtained by anther culture from the diploid Japanese pear *Pyrus pyrifolia* Nakai cultivar Shinko (2n=2x=34). In previous anther culture experiments carried out on three Japanese pear cultivars, nine embryos were obtained from cultivar Gold Nijisseiki (at 0.12% rate) and 10 from cv. Shinko (at 0.13% rate), but plant regeneration was not established (Kadota *et al.,* 2002) [17]. No positive effect of activated charcoal addition has been observed in anther culture of Japanese pear (Kadota and Niimi, 2004) [16]. Temperature used for Japanese pear anther culture is 24° C (Kadota and Niimi, 2004) [16]. Triploids regenerated from anther culture have also been reported in Japanese pear (Kadota and Niimi, 2004) [16].

***Pyrus communis* L.**
Haploids and DH have been obtained by in situ parthenogenesis induced by irradiated pollen or by seedling selection from three pear cultivars: ‘Doyenne´ du Comice’,

'Williams' and 'Harrow Sweet'. Doubled haploids were obtained by either spontaneous chromosome doubling or by oryzalin treatment (200–300 lM). Two embryos were produced by pear anther culture, cv. Le Lectier, but their origin was not established and plant regeneration was not obtained (Kadota *et al.,* 2002) [17]. Temperature used during anther culture is 25° C for pear (Kadota *et al.,* 2002) [17]. In pear, as well as in apple, regeneration from embryos was obtained after a cold treatment (12 weeks) through rooting of shoots developed from embryos (Kadota *et al.,* 2002) [17]. The adventitious shoots lacked vigour and for the most part died. In some genotypes hyperhydricity was observed. Isozyme analyses have been employed to confirm the gametic origin of calluses and plantlets in pear. Microsatellites have been also employed to assess homozygosity in pear, confirming the achievement of true DH clones of pear.

***Annona squamosa* L.**

Haploid embryos from male gametes were produced through anther culture of *Annona squamosa* L. (Nair *et al.,* 1983) [27].

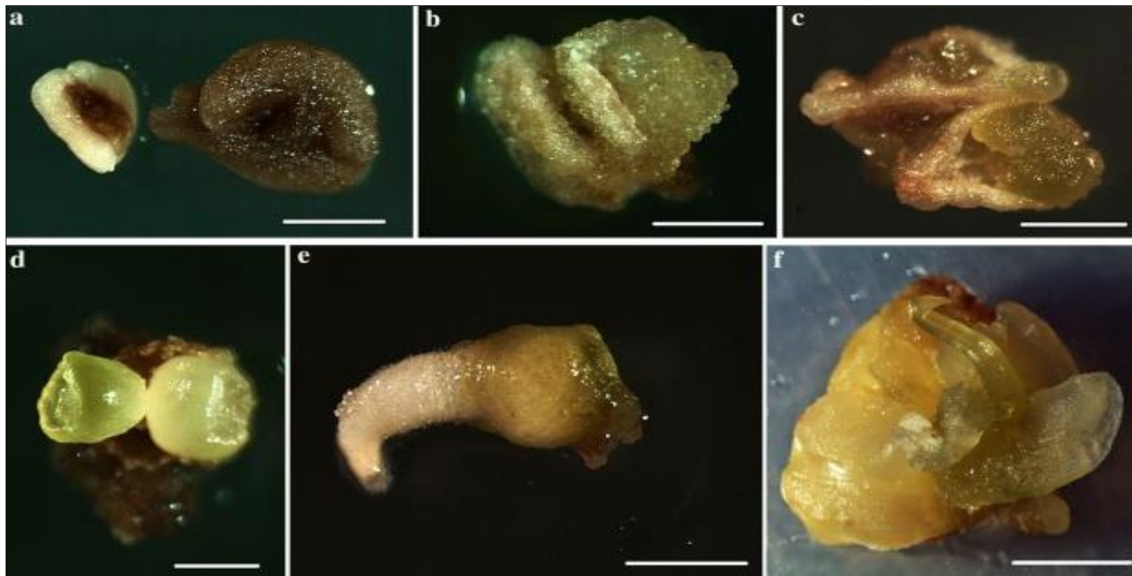

Fig 6: A. Not developed (left) and swollen (right) anthers of 'Filippo Ceo' after 3 months in culture. B. Anther with friable white callus. C. Anther with hard green callus. D. Early embryos of 'Filippo Ceo' coming out from the anther. E. Embryo of 'Filippo Ceo' developing the root axis. F. Abnormal embryos of 'Lauranne' (Bars represent in a, b, c: 1 mm, in d: 0.75 mm, in e: 1.5 mm, in f: 1 cm)

Application of haploids and DHs in fruit 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) [7]. 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. More than 280 varieties have been produced with the use of various DH methods in several crops, with the majority of the protocols referred to as anther culture. 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) (Hochholdinger and Hoecker, 2007) [13], 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. 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. 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 Triploid plants derived via anther culture may have great commercial potential in crops, where the consumers desire the seedlessnesss of fruits. Triploids regenerated from anther culture have been reported in apple (Hofer *et al.,* 2002) [14], *Pyrus pyrifolia* Nakai (Kadota and Niimi, 2004) [16], *Carica papaya* L. and *Citrus clementina* Hort. ex Tan. (Germana *et al.,* 2005) [10]. 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 (Germana *et al.,* 2005a) [10]. 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, 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) [5]. *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. 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. DHs play also a vital role in genomics, in integrating genetic and physical maps, thereby providing precision in targeting candidate genes (Wang *et al.,* 2001) [35].

Limitations

- Low Yield- generally 5-8% of the total pollen grains in a responding anther undergo androgenic development.
- 70-80% of the embryos are incapable of normal germination due to structural, physiological and biochemical abnormalities of pollen.
- Occurrence of high frequencies of albinos.
- Instability of genetic material during androgenesis.

Conclusions and future perspectives

The great potential of employing haploidy, doubled haploidy and gametic embryogenesis in fruit 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. 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 fruit crops.

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