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# Doubled haploid production in fruit crops

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## Abstract

The interest of fruit breeders in haploids and doubled haploids (DH), lies in the possibility of shortening the time needed to produce homozygous lines compared to conventional breeding. Haplo-diploidization through gametic embryogenesis allows single-step development of complete homozygous lines from heterozygous parents. In a conventional breeding programme, a pure line is developed after several generations of selfing. With fruit crops, characterized by a long reproductive cycle, a high degree of heterozygosity, large size, and, sometimes, self-incompatibility, there is no way to obtain haploidization through conventional methods. This paper reviews the current status of research on doubled haploid production in the main fruit crops: *Citrus*, *Malus domestica*, *Pyrus communis*, *Pyrus pyrifolia*, *Prunus persica*, *Prunus avium*, *Prunus domestica*, *Prunus armeniaca*, *Vitis vinifera*, *Actinidia deliciosa*, *Olea europaea*, *Morus alba*, *Actinidia deliziosa*, [*Musa balbisiana* (BB)], *Carica papaya*, *Annona squamosa*, *Feijoa sellowiana*, *Opuntia ficus-indica*, *Eriobotrya japonica*.

## Introduction

The most widely cultivated temperate and subtropical fruit trees in the world are citrus, bananas, grapes, apples, peaches, pears, plums, apricot and kiwis. World fruit production amounts to 497.4 million metric tons in 2004 (FAOSTAT, Database).

The main goals of research on fruit breeding are: to obtain new varieties with a shorter juvenile non-fruiting period, an increased yield, a longer ripening season, regular bearing, seedlessness and improved external and internal quality of the fruits. Another important aim in fruit tree improvement research is to make available new scions and rootstocks selected for resistance or tolerance to biotic and abiotic stresses.

Fruit species breeding is based on either conventional (hybridization, selection, mutation) or biotechnological methods employing embryo culture, regeneration from protoplasts, somatic hybridization, *in vitro* mutant selection, genetic transformation and haploid production. Using an integrated approach with both biotechnological tools and conventional ones it is possible to obtain good results in a short time.

## Importance of haploids and DH

Haploids are sporophytic plants with the gametophytic chromosome number because they originate from a single gamete. The importance of haploids in plant breeding and genetic research was recognized with the discovery of the first natural haploid in *Datura stramonium* and *Nicotiana* (Blakeslee et al., 1922; Blakeslee and Belling, 1924; Kostoff, 1929), but long before techniques for producing haploids by *in vitro* gametic embryogenesis became available. The discovery by Guha and Maheshwari (1964, 1966) that, by *in vitro* culture of immature anthers of *Datura innoxia*, a change in the normal

gametophytic development into sporophytic development can be induced and embryos with a haploid chromosome number can be obtained, led to further and extensive research on androgenesis.

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 (Evans, 1989), and DH are being routinely used in breeding programs for new cultivar development in many crops (Veilleux, 1994).

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 (Evans et al., 1984; Morrison and Evans, 1987), 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 (Morrison and Evans, 1987; Huang, 1996).

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).

## Haploids and DH production in fruit crops

Since 1970s, extensive research has been carried out to obtain haploids for fruit tree breeding through gametic embryogenesis (Chen, 1986; Ochatt and Zhang, 1996). However, as reviewed by Ochatt and Zhang (1996), this has not always given satisfactory results. Generally, haploids can mainly be induced by two strategies i.e. by regeneration from the female gamete or from the male gamete.

## Haploids from the female gametes

### *Development of spontaneous haploids*

Spontaneous haploids can occur either due to parthenogenesis, i.e. the production of an embryo from an egg cell without the participation of the male gamete, or due to apogamy, which means the production of an embryo from a gametophytic cell other than the ovum.

In 1974, Kasha reported spontaneously developed haploids in over 100 angiosperm species. According to Zhang et al. (1990), there are also several fruit tree species such as apple, pear, peach, plum, apricot, citrus, etc. capable to produce spontaneous haploids, but generally in very low numbers and with low viability.

### In situ *parthenogenesis induced by irradiated pollen followed by* in vitro *embryo culture*

Parthenogenesis induced *in vivo* by irradiated pollen, followed by *in vitro* culture of embryos, can be an alternative method of obtaining haploids in fruit crops. Gynogenesis by *in situ* pollination

with irradiated pollen has been successfully used for *Malus domestica* (L.) Borkh (Zhang and Lespinasse, 1991; Höfer and Lespinasse, 1996), *Pyrus communis* L. (Bouvier et al., 1993), *Actinidia deliciosa* (A. Chev) (Pandey et al., 1990; Chalak and Legave, 1997).

The method is based on the *in vitro* culture of immature seeds or embryos obtained as a result of pollination with pollen irradiated by gamma rays from cobalt 60, and it should be tested in those species in which *in vitro* anther culture has not been successfully applied. Irradiation does not hinder pollen germination, but prevents pollen fertilization, stimulating the development of haploid embryos from ovules. The success of this technique is dependent on the choice of radiation dose, the developmental stage of the embryos at the time of culture, culture conditions and media requirements.

### In situ *or* in vitro *parthenogenesis induced by pollen from a triploid plant followed by* in vitro *embryo culture*

Pollen from a triploid plant, like irradiated pollen, germinates, but does not fertilize and stimulates the development of haploid embryos from ovules. For example, 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) (Oiyama and Kobayashi, 1993). Haploid and diploid embryos did not show any difference in their size, however, haploid seedlings grew very slowly in the soil. Restriction endonuclease analyses of both nuclear and chloroplast ribosomal DNA were used to determine the maternal origin of these haploids.

The *in vitro* stigmatic pollination technique consists of applying pollen to the apical part of the stigma of an excised gynoecium implanted in solid culture medium. This method was successfully applied in *Citrus clementina* Hort. ex Tan. (Germanà and Chiancone, 2001). Some ovaries were transformed into brownish and friable callus, sometimes breaking to reveal ovules. From this kind of ovary the gynogenic embryos emerged 4 or 5 months after *in vitro* pollination, which is practically the same time required for regeneration from anther culture. The pollination and mature stage of pistils were necessary for gynogenic embryo regeneration.

## Haploids from the male gametes

Both basic and applied studies have improved the knowledge of pollen biology and pollen biotechnology, making the manipulation of pollen development and function, a reliable tool for crop improvement (Mulcahy, 1986). The most important application of pollen biotechnology in breeding and genetic studies is the ability to obtain haploids and DH.

*In vitro* anther or isolated microspore culture, are usually the most effective and widely used methods of producing haploids and DH. Regeneration from male gametes has been reported in about 200 species belonging to some families, such as *Solanaceae*, *Cruciferae* and *Gramineae* (Dunwell, 1986; Hu and Yang, 1986). On the other hand, some of the members of *Leguminosae* family and many woody plants are more recalcitrant (Sangwan-Norrel et al., 1986; Bajaj, 1990; Raghavan, 1990; Wenzel et al., 1995).

The cellular, biochemical and molecular bases for the transformation of microspores into pollen embryos are not yet been completely understood. However, it is already possible to report some findings. For example, it is known that the androgenic character is genetic and inheritable, and that the stage of microspore development is critical for induction. Usually in the period around the first haploid mitosis (late uninucleate or early bicellular pollen stage), male gametes become competent to differentiate in a different way from the gametophytic pathway with continued growth and division. Moreover, external stresses need to be present to enable competent microspores to undergo androgenic development. The stress can be physical (wounding connected to the anther excision and culture), thermal (heat, cold) or chemical (water stress, starvation). The induced microspores are 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 the zygotic embryogenesis.

### *Haploidization by anther culture*

Research on haploidization by anther culture has been carried out on several fruit trees (Ochatt and Zhang, 1996). Floral buds, with the pollen grains at a specific stage of development, are collected

from the donor plant, usually from field trees. After pre-treatment, the buds are surface sterilized usually by immersion in 70% (v/v) ethyl alcohol, followed by immersion in sodium hypochlorite solution (about 1.5% active chlorine in water) containing a few drops of Tween 20, and finally rinsed three times for 5 min with sterile distilled water. Petals are aseptically removed with small forceps, and anthers are carefully dissected and placed into the medium.

The stage of pollen development is commonly determined by staining one or more anthers per bud with acetocarmine, Schiff's reagent, or DAPI stain.

*Haploidization by isolated microspore culture*

Pollen culture is performed by removing somatic anther tissue. This technique, although more difficult and laborious, is ideal for studying the mechanism of pollen embryogenesis, because it eliminates the unknown effects of the sporophytic anther tissue, thereby allowing a greater control over the culture process. The few reports about this method in fruit crops are regarding apple (Oldani, 1993; Höfer et al., 1999; Höfer, 2004), citrus (Germanà et al., 1996) and olive (Bueno et al., 2004). Research is in progress on microspore culture of several genotypes: prickly pear, cherry, olive, loquat, etc. (Germanà et al., unpublished).

Investigation of isolated microspore culture of several *Citrus* species (lemon, orange, clementine, sour orange, grapefruit) and a related genus (*Poncirus*) has been carried out (Germanà et al., 1996). After various periods of time (1–4 months), the isolated microspores of almost all investigated *Citrus* species produced multinucleated structures and developed into small proembryos, which failed to develop any further. Formation of ''pseudobulbils'', white or green spherical bodies, described in *Citrus* by Button and Kochba (1977), has been obtained only in those genotypes (clementine and lemon) that had also produced haploids by anther culture.

Höfer et al. (1999) for the first time reported the induction of embryogenesis and plant formation from isolated microspore of the apple cultivar 'Rene'. In a succesive report (Höfer, 2004), the improvement of the induction phase through the study of the pre-treatment, the concentration of carbon source and the microspore density, has been achieved.

Recently, Bueno et al. (2004) obtained sporophytic division, multinucleate microspores and multicellular structures in isolated microspore culture of olive cultivars Arbequina and Picual.

## Haploids and DH in the main fruit crops

### Citrus

*Citrus* species represent the largest production of fruits worldwide, with over 105.4 million tons produced during 2005 (FAOSTAT database). All cultivated forms of *Citrus* and related genera (*Poncirus*, *Fortunella*, etc.) are diploid with a monoploid number of chromosomes ($n = x = 9$) (Frost, 1925). Triploid and tetraploid forms of *Citrus* also exist.

In *Citrus natsudaidai* haploid seedlings were first obtained by the application of gamma rays (Karasawa, 1971). One haploid embryo was obtained in an immature seed from a diploid (Clementine mandarin) $\times$ diploid (Pearl tangelo) cross (Esen and Soost, 1972).

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 (Ollitrault et al., 1996). 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'') $\times$ a triploid hybrid of ''Kawano natsudaidai'' (*Citrus natsudaidai* Hayata) (Oiyama and Kobayashi, 1993). 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 (Gemanà and Chiancone, 2001). 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.) (Soost and Cameron, 1980).

With regards to *Citrus* and their relatives (Germanà, 2003), haploid plantlets have been recovered, by anther culture, from *Poncirus trifoliata* L. Raf. (Hidaka et al., 1979) and *C. madurensis* Lour. (Chen et al., 1980); one doubled haploid plantlet has been obtained from the hybrid No. 14 of *C. ichangensis*×*C. reticulata* (Deng et al., 1992a); haploid plantlets and highly embryogenic haploid calli of *C. clementina* Hort. ex Tan. (Germanà et al. 1994, 2000a, 2005; Germanà and Chiancone, 2003); haploid, but albino embryos of 'Mapo' tangelo (*C. deliciosa*×*C. paradisi*) (Germanà and Reforgiato, 1997); haploid and diploid calli, embryos and leafy structures but no green plants of *C. limon* L. Burm. f. (Germanà et al., 1991); haploid embryos of *Clausena excavata* (Froelicher and Ollitrault, 2000) 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.

### *Culture medium in* Citrus *anther culture*

The diverse genotypes show very different basal medium, different carbon sources and plant growth regulators requirements to induce pollen-derived plant formation (Germanà, 1997). Usually, anther culture media are solidified by adding agar. Other gelling agents can be potato starch (Germanà, 1997; Germanà et al., 2000a, unpublished), gelrite (Froelicher and Ollitrault, 2000), agarose (Kadota et al., 2002; Assani et al., 2003) and gellan gum (Kadota et al., 2002). There are different opinions regarding the use of liquid media rather than solid media. Chaturvedi and Sharma (1985) obtained diploid plantlet regeneration by floating *C. aurantifolia* anthers on a liquid medium, then embedding them in a semisolid medium. Germanà et al. (unpublished) obtained better results in *Citrus* using a solid medium rather than a liquid one: in liquid medium, citrus anthers initially swell, and later turn brown and sometimes shrivell.

Activated charcoal was beneficial to androgenesis induction of *P. trifoliata* (Deng et al., 1992a). However, no positive effect of activated charcoal addition has been observed in anther culture of several *Citrus* species (Germanà et al., 1994; unpublished).

### *Conditions of incubation of* Citrus *anther culture*

Regarding the conditions of incubation, Chen (1985) observed that temperature seems to be more important than light in *Citrus* anther culture, and they obtained embryos at 20–25 °C, especially under dark conditions. The temperatures usually used in citrus anther culture are 25–28 °C (Germanà, 1997). After dark incubation, Petri dishes are usually placed under cool white fluorescent lamps with a photosynthetic photon flux density of 27–60 μmol $m^{-2}$ $s^{-1}$ and a 16–18 h light photoperiod (Germanà et al., 1994, 2000a; Germanà and Reforgiato, 1997; Germanà and Chiancone, 2003).

### *Embryo development from* Citrus *microspores and the origin of haploids*

Regarding the origin of haploids, Hidaka and Omura (1989) described cytologically the development of embryos from microspores in *C. aurantium* and *P. trifoliata*.

When the nucleus divides without cell division, a multinucleate pollen grain is initially formed which later gives rise to a multicellular structure, that develops into a proembryo and finally into an embryo, until the exine rupture. Moreover, nuclear fusion among vegetative and generative nuclei has been observed, and this might explain an increase in ploidy level.

Morphological and ultrastructural studies, at cellular and sub-cellular levels, of early microspore embryogenesis in several embryogenic varieties of *Citrus clementina* revealed very important aspects of this embryogenic process, indicating differences between *Citrus* microspores derived embryos and those derived from other embryogenic species, such as starch accumulation during the first embryonic stages. Moreover, different cellular types have been observed in these embryos after the exine breakdown (Ramirez et al., 2003).

The developmental process of a plant from a single microspore is referred to as microspore embryogenesis, although the route of regeneration may be via direct embryogenesis (Figure 1A), secondary embryogenesis or, organogenesis. In

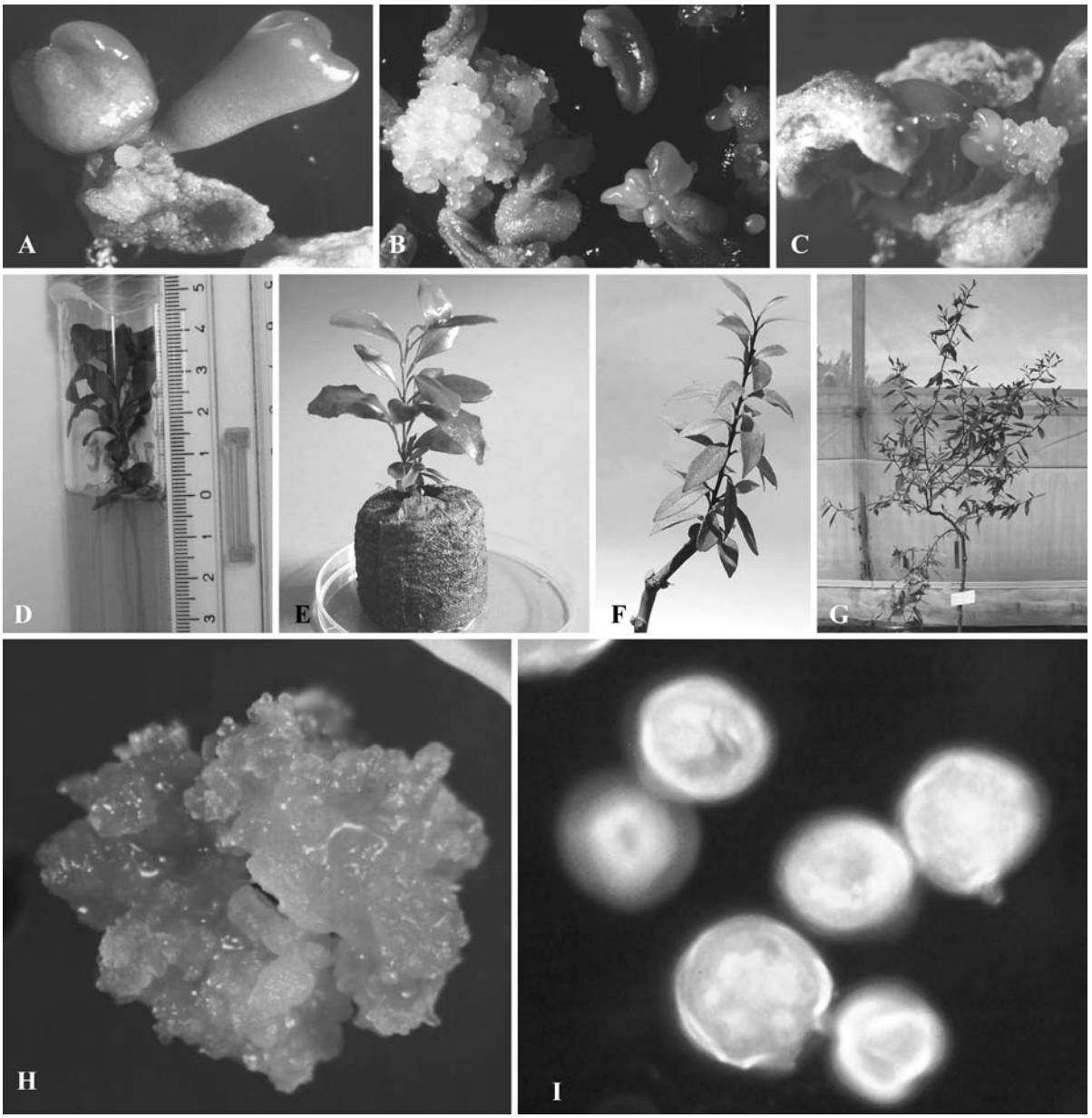


*Figure 1.* (A) Direct embryogenesis in *Citrus* anther culture. (B) Embryogenic, haploid, friable, callus emerging after 4 months of culture from anther culture of the cultivar Nules of *C. clementina* Hort. ex Tan, cv. Monreal. (C) Haploid embryogenic calli and embryos in different stages developing 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.

other cases microspores in culture produce undifferentiated calli, instead of embryos.

Usually, after 1 week of culture, most of the anthers are swollen and then they start to produce calli. Anther-derived calli can be non-morphogenic, or highly embryogenic, and they can maintain embryogenic potential for a long time. The morphogenic calli in citrus appear friable (Figure 1B) and white. Sometimes calli develop from two different lobes of an anther. The

embryogenic calli differentiate into a clump of embryos (Figure 1C).

### *Plant recovery, hardening and characterization of regenerants obtained by* Citrus *anther culture*

Plantlet formation from cultured anthers may occur either directly through embryogenesis of microspores or indirectly through organogenesis or embryogenesis of microspore-derived callus.

The embryogenic haploid callus is multiplied and, as the embryos appear, they are germinated in Petri dishes. They are later transferred to Magenta boxes (Sigma V8505) or to test tubes (Figure 1D).

The well-structured pollen-derived citrus embryos develop normally like zygotic embryos, through the globular, the heart, the torpedo and the cotyledonary stages and often produce secondary embryos. In fruit crop anther culture, often teratomatal structures, cotyledonary-fused, pluricotyledonary and thickened embryos are observed (Höfer, 1995; Germanà, 1997). Green, compact and non-morphogenic calli emerging from anthers were also observed.

Haploid embryos often germinate vigorously *in vitro*; by contrast, haploid plantlets grow slowly in soil, presumably due to harmful recessive genes expressed in homozygosity. These plantlets, when transplanted *in vivo*, frequently die as a result of fungal contamination. In *Citrus*, better results have been obtained by grafting *in vitro* homozygous small shoots (2–3 mm) onto etiolated 20-day-old Troyer citrange seedlings. After 3–4 months, the grafted plantlets obtained were washed with sterile water to remove the medium from their roots and then transferred to sterilized pots containing peat moss, sand and soil in the ratio 1:1:1 or to Jiffy pots for the acclimation phase (Figure 1E). The new scions obtained were later grafted onto 2-year-old sour orange seedlings (Figure 1F and G). They showed a more compact habit and a decrease in vigour, with significantly smaller leaves, shorter internodes and more thorns when compared to the heterozygous parent of the same age of grafting (Germanà et al., 2000b).

Isozyme analyses have been employed to confirm the gametic origin of calluses and plantlets in citrus (Germanà et al., 1991, 1994, 2000a, b; Deng et al., 1992a; Ollitrault et al., 1996; Germanà and Reforgiato, 1997; Germanà and Chiancone, 2001).

Isozyme techniques allow us to distinguish between androgenetic and somatic tissue when the enzyme is heterozygotic in the diploid condition of the donor plant and the regenerants show a lack of an allele.

For example, *Citrus clementina* is heterozygous for PGI-1 and PGM. To identify the origin of calli, embryos and plantlets obtained, their crude extracts are analysed using two enzyme systems: phosphoglucoisomerase (PGI) and phosphoglucomutase (PGM), as reported by Grosser et al. (1988). Numbering for isozymes (PGI-1) and lettering for different allozymes are the same as those used by Torres et al. (1978).

According to Torres et al. (1978), the heterozygous clementine parent is FI (F = allele which specifies fast migration toward the anode enzyme; I = intermediate) in PGM, and WS (W = allele which specifies an enzyme migrating faster than F; S = allele which specifies a slowly migrating enzyme) in PGI. For analysis of calli and leaves obtained from anther culture, the presence of a single band was retained as the homozygous state (Figure 2) and both enzyme systems confirmed the androgenic nature of regenerants because of the lack of an allele.

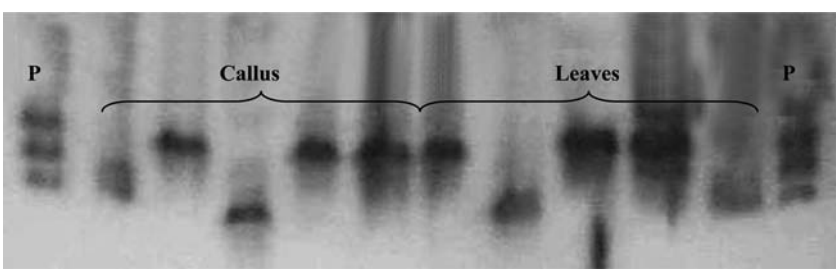


*Figure 2.* Isozyme pattern of phosphoglucoisomerase (PGI) of calli and leaves. The first lane on the left and the last lane on the right are the zymogram of heterozygous Nules parent, the other ones are those of homozygous regenerants.

The aberrant transmission of random amplified polymorphic DNA (RAPD) markers, due to the presence of a band found in DH and not present in the parental, has been observed in homozygous clementine (Germanà et al., 2000b) as well as in peach (Pooler and Scorza, 1995a).

Microsatellites have been also employed to characterize regenerants obtained from citrus anther culture (Germanà and Chiancone, 2003; Germanà et al., 2005).

### *Production of triploids in* Citrus

The importance of triploids in some fruit crops improvement, like *Citrus* or table grape, derives from the seedlessness of their fruits. This is a desirable trait of commercial importance and one of the main goals in many breeding programmes.

Triploids can be conventionally produced by 2x×4x and 4x×2x crosses.

Triploid plants can also be obtained through *in vitro* culture of endosperm, which, being the fusion of three haploid nuclei, is triploid. Triploid hybrid *Citrus* plants were recovered by *in vitro* embryogenesis from endosperm-derived calli (Gmitter et al., 1990).

One of the most interesting applications of haploids in *Citrus* breeding is the possibility of obtaining triploid somatic hybrids by fusion between haploid and diploid protoplasts (Kobayashi et al., 1997; Ollitrault et al., 2000). Recently (Germanà et al., 2005), ploidy analysis by flow cytometry of 94 regenerants from clementine anther culture, showed as many as 82% of them were tri-haploids, rather than haploids or doubled-haploids as expected. Regeneration from anther culture was therefore proposed as a rapid, and attractive method of obtaining new triploid varieties in clementine, which could be of great interest for the fresh fruit market that now requires fruit to be seedless.

### *Gametosomatic hybridization in* Citrus

A preliminary study on gametosomatic fusion between *Poncirus trifoliata* tetrads and somatic protoplasts of *Citrus sinensis* cv 'Jincheng' was reported by Deng et al. (1992b). One chimeric plantlet was regenerated, but not further reports on this subject have been published indicating that this method is probably not useful for citrus breeding.

## Malus domestica (*L.*) *Borkh*

Apples, with over 63.4 million tons produced during 2005 (FAOSTAT database), are after citrus, bananas and grapes, the most produced fruits in the world.

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 authors (Fei and Xue, 1981; Xue and Niu, 1984; Zarsky et al., 1986; Zhang et al., 1987; Höfer and Hanke, 1990, 1994; Verdoodt et al., 1998; Höfer, 2003). The induction of embryogenesis from cultured apple anthers is still low and highly genotype-dependent (Höfer, 1995, 1997).

Zhang and Lespinasse (1988) reported the induction of gynogenesis through *in vitro* culture of unpollinated ovaries and ovules, without plant regeneration. Haploid plant have been obtained through i*n situ* parthenogenesis induced by pollination of cv. Erovan with pollen irradiated at 500–1000 Gy, followed by *in vitro* embryo culture (Zhang, 1988). This technique has been successfully applied to other apple cultivars also with different γ-rays from Cobalt 60 (Zhang et al., 1987; Zhang and Lespinasse, 1991; Zhang et al., 1992; De Witte and Keulemans, 1994).

The induction of embryogenesis and plant formation from isolated apple microspores has been reported in the cultivar 'Rene' (Höfer et al., 1999). Further improvement of the induction phase allowed to obtain induction of androgenic embryos in the following cultivars: 'Alkmene', 'Remo', 'Rene' and 'Realka'; for all of them, except for 'Alkemene', the results obtained from microspore culture were up to 10 times better than those from anther culture (Höfer, 2004).

### *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 (Höfer et al., 1999; Höfer, 2004). Activated

charcoal was beneficial to anther culture of apple (Johansson et al., 1987; Zhang et al., 1990).

*Conditions of incubation of apples anther culture*

Temperatures used for apple anther culture are 23–30 °C (Höfer and Lespinasse, 1996; Kadota et al., 2002). In apple anther culture, Höfer and Hanke (1990) obtained better results in the dark, while Fei and Xue (1981) achieved embryo induction under continuous light. Dark was used also in apple isolated microspore culture (Höfer et al., 1999; Höfer, 2004).

*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 (Höfer and Lespinasse, 1996).

Regarding the origin of haploids, Zhang (1988) observed three different routes in apple androgenesis: formation of two identical nuclei after an abnormal pollen mitosis; division of the vegetative nucleus after a normal pollen mitosis; and division of the generative nucleus after a normal pollen mitosis.

*Characterization and propagation of regenerants obtained by apple gamete embryogenesis*

Triploids regenerated from anther culture have been reported in apple (Höfer, 1994; Höfer et al., 2002). Isozyme analyses have been employed to confirm the gametic origin of calluses and plantlets in apple (Höfer and Grafe, 2000). 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* (Höfer and Grafe, 2000) and by simple sequence repeats (SSRs) (Kenis and Keulemans, 2000; Höfer et al., 2002). The single multiallelic self-incompatibility gene has been used in apple by Verdoodt et al. (1998) 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 (Patat-Ochatt et al., 1993).

Pyrus communis *L.*

Haploids and DH have been obtained by *in situ* parthenogenesis induced by irradiated pollen or by seedling selection (Bouvier et al., 1993) from three pear cultivars: 'Doyenné du Comice', 'Williams' and 'Harrow Sweet'.

Doubled haploids were obtained by either spontaneous chromosome doubling or by oryzalin treatment (200–300 μM) (Bouvier et al., 2002). 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).

Temperature used during anther culture is 25 °C for pear (Kadota et al., 2002).

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). 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 (Bouvier et al., 2002). Microsatellites have been also employed to assess homozygosity in pear (Bouvier et al., 2002), confirming the achievement of true DH clones of pear.

Pyrus pyrifolia *Nakai*

Triploid plants were obtained by anther culture from the diploid Japanese pear *Pyrus pyrifolia* Nakai cultivar Shinko ($2n = 2x = 34$) (Kadota and Niimi, 2004). 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).

No positive effect of activated charcoal addition has been observed in anther culture of Japanese pear (Kadota and Niimi, 2004).

Temperature used for Japanese pear anther culture is 24 °C (Kadota and Niimi, 2004).

Triploids regenerated from anther culture have also been reported in Japanese pear (Kadota and Niimi, 2004).

## Prunus persica (*L.*) *Batsch*

Haploids ($1x = 1n = 8$) reported in peach [*Prunus persica* (L.) Batsch] arose spontaneously (Pratassenja, 1939; Hesse, 1971; Toyama, 1974). They show the typical haploid traits of thin shoots, narrow leaves, weak vegetative growth and small non-fertile or less fertile flowers (Hesse, 1971; Toyama, 1974; Pooler and Scorza, 1995a). Haploids, DH and $F_1$ hybrids recovered by Toyama were the object of a study by Pooler and Scorza (1995a, b), who observed unreduced gamete occurrence in haploid trees and aberrant transmission of RAPD markers, likely due to somatic rearrangements (bud sports), often observed in tree fruit species.

A study carried out by Scorza and Pooler (1999) 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.

Michellon et al. (1974), Seirlis et al. (1979) and Hammerschlag (1983) obtained haploid callus from peach anther culture, but no plant regeneration.

## 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' (Höfer and Grafe, 2003). Production of haploid callus from anther culture has been also reported (Seirlis et al., 1979; Höfer and Hanke, 1990; Germanà et al., unpublished).

Isozyme analyses have been employed to confirm the gametic origin of calluses in cherry (Höfer and Grafe, 2003).

## Prunus domestica *L.*

Studies carried out by Peixe et al. (2000) in European plum (*Prunus domestica* L., cv. "Rainha Clàudia Verde"), showed that gamma-irradiated (200 Gy) pollen can induce the formation of $2n$ endosperm and abnormal embryo development showing the possibility of haploid embryo formation in this genotype by *in situ* parthenogenesis. Unfortunately, only heart-shape embryos were obtained, because no further development was observed.

## 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).

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). After initiation, temperature used was 24/22 °C day/night (Peixe et al., 2004).

The ploidy of calluses, evaluated by flow cytometry, ranged from haploid to octoploid.

Previously, Harn and Kim (1972) obtained callus formation from apricot anther culture, but ploidy level was not reported.

## 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.

One case of haploid was reported by Zou and Li (1981) and haploid callus line production has been reported by Gresshoff and Doy (1974), Kim and Peak (1981) and Cersosimo (1986). Regeneration of plants has been obtained by Rajasekaran and Mullins (1979), Bouquet et al. (1982), Hirabayashi and Akihama (1982), Mauro et al. (1986) and Cersosimo et al. (1990). Anther culture (Figure 1H) is usually employed to establish diploid somatic embryogenic cultures of *Vitis* (Mauro

et al., 1986; Cersosimo et al., 1990). 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.

A histological study on callused anthers of *Vitis rupestris* du Lot showed androgenic development of the microspores (Altamura et al., 1992). These embryos did not develop further to plants, probably due to the many deleterious genes leading to genetic disorders (Cersosimo, 1996).

In grape, Cersosimo (1987) obtained better results in a solid medium rather than liquid, while Rjasekaran and Mullins (1979) obtained embryogenic callus production in continuously agitated liquid medium.

Temperatures used during anther culture were 24–26 °C for grape (Cersosimo, 1996).

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) (Bueno et al., 2004).

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 (Dennis Thomas et al., 1999).

Anther culture has not been successful in producing haploids of this tree crop (Sethi et al., 1992; Jain et al., 1996).

Actinidia deliciosa (*A. Chev*)

Parthenogenetic tri-haploids were induced in kiwifruit, cv Hayward, an 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 (Chalak and Legave, 1997). Spontaneous doubling was also observed. Pandey et al. (1990) previously induced parthenogenesis by pollination with irradiated pollen.

Kiwifruit anther culture produced only somatic embryogenesis (Fraser and Harvey, 1988).

[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).

Previously, Kerbellec (1996) reported successful haploid plant regeneration in banana [*Musa acuminata* (AA)].

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 (Litz and Conover, 1978; Tsay and Su, 1985). Rimbeira et al. (2005) greatly increased up to about 4.0% the embryo induction rate (rate of anthers forming embryos) by investigating the effects of pre-cultural conditions. For example, the pre-treatment of 35 °C was more efficient for embryo induction than 25 °C and liquid media were more effective as pre-treatment than solid media in papaya anther culture (Rimbeira et al., 2005). They also applied a papaya sex-diagnostic PCR technique to plantlets obtained.

Haploid plantlets were induced through anther culture in a medium without any growth regulators and under dark conditions (Tsay and Su, 1985).

Annona squamosa *L.*

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

Feijoa sellowiana *Berg*.

Multinucleated pollen grains were obtained in anther culture of feijoa, but attempts to regenerate pollen plants were unsuccessful (Canhoto and Cruz, 1993).

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 (Chessa et al., 2000; Chessa and Nieddu, 2002).

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. (Gonzáles-Melendi et al., 2005)

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 (Figure 1I).

## Conclusions

The great potential of employing haploidy, doubled haploidy and gametic embryogenesis in fruit crop 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 *in vitro* culture of gametes is more or less a standard tool for plant breeders in many crops, particularly *Brassicaceae* and cereals, this has yet to be achieved in fruit crop breeding since the deployment of gametic embryogenesis in fruit crops improvement is still hampered by low frequencies of embryo induction, albinism, plant regeneration, plant survival and the genotype-dependent response. A better understanding of the gametic embryogenesis process, the improvement of currently available techniques and the development of new technologies could make haploid production a powerful fruit crop breeding tool in the future, enabling in these genotypes the effective exploitation of the potential of gamete biotechnology. In order to make this possible, the fundamental goals are: to enlarge the number of respondent genotypes, to improve the induction rate (the frequency with which gametes form embryos) and to increase the survival rate (the percentage of regenerated haploid and doubled haploid plants successfully transferred from *in vitro* to *in vivo* culture conditions). Further goals are to characterize and to deploy haploids and DH in fruit crop breeding (protoplast fusion, triploid production, transformation, etc.). A better knowledge of the gametic embryogenesis process in fruit crops is needed to transform this frontier of plant biotechnology into practical applications in these crops, as has already been achieved in some families (*Cruciferae*, *Gramineae* and *Solanaceae*).

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