# Chapter 8
# In Vitro Haploid Production—A Fast and Reliable Approach for Crop Improvement

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## 1 Introduction

### *1.1 Overview of Haploidy*

Haploids have attracted great interest of plant physiologists, embryologists, geneticists and breeders ever since the discovery of the first natural haploid embryos and plants in *Datura stramonium* in 1922 by Blakeslee et al. Subsequently, haploidy has been reported in many species, but at low and variable frequencies and was regarded as a special biological phenomenon. The low frequency of spontaneously arising haploid plants severely limited the utilization of haploids for crop improvement and genetic studies. The remarkable discovery that haploid embryos and plants can be produced through in vitro culture of anthers of *Datura* (Guha and Maheshwari 1964, 1966) brought renewed interest to haploidy. This method of androgenic haploid production was quickly attempted in many species to hasten the breeding programme in several economically-important plants.

The life cycle of angiosperms (higher plants) is characterized by alternating generations of sporophytes and gametophytes. The gametophytic phase arises when the diploid cells undergo meiosis (reduction division) to form male and female gametes. This phase is shortlived as fertilization of the egg re-establishes the diploid sporophytic phase. The sporophytic phase characterized by diploid (2n) chromosome number is the product of fertilization of male and female gametes, containing the haploid (n) set of chromosomes from each parent (Forster et al. 2007). Therefore, haploid is a generalized term for plants that contain the gametic chromosome number (n). This is in contrast to diploid plants, which contain two sets (2n) of chromosomes. Haploids are sexually sterile and, therefore, doubling of the chromosomes is

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N. Tuteja, S. S. Gill (eds.), *Crop Improvement Under Adverse Conditions*,

DOI 10.1007/978-1-4614-4633-0_8, 

required to produce fertile plants which are called doubled haploids or homozygous diploids.

Spontaneous production of haploids usually occurs through the process of parthenogenesis (embryo development from an unfertilized egg). However, occasionally, they bear the characters of male parent only, suggesting their origin through 'ovule androgenesis' (embryo development inside the ovule by the activity of the male nucleus alone where elimination or inactivation of egg nucleus occurs before fertilization) (Bhojwani and Razdan 1996). Although in vivo occurrence of haploids has been reported in several species, the frequency is very low (Pochard and Dumas de Vaulx 1971; Lacadena 1974). Several methods have been employed for producing haploid plants. In 1970, Kasha and Kao reported haploid production in barley following wide hybridization and the subsequent preferential elimination of the chromosomes of wild species during early embryogenesis. This provided a system that produced a large number of haploids across most genotypes (Choo et al. 1985). However, this program has been restricted to only a few crops so far. In vitro haploid plants can be obtained by triggering the male or female gametic cells to undergo sporophytic development. In vitro androgenesis (anther-microspore culture) is one of the most preferred techniques for obtaining haploids, but in vitro gynogenesis (unfertilized ovary–ovule culture) can prove to be a complementary technique in species where anther culture is inaccessible or less productive. It means that not only the microspore, but also the megaspore of angiosperms can be triggered in vitro to undergo sporophytic development. However, production of haploids via gynogenesis is more tedious, less efficient in comparison to androgenesis. In addition to the above techniques, *in situ* parthenogenesis (pollen irradiation and chemical treatment) can be employed for generation of haploid plants (Kurtar and Balkaya 2010).

Haploid and diploid lines play a vital role in genomics and have been used for the purpose of physical mapping, genetic mapping and also for integration of genetic and physical mapping. Additionally, haploid and doubled haploid plants are adapted for mutagenesis and genetic transformation experiments, presenting the advantage of immediate production of homozygous lines. It is also expected that, in the near future, haploid and doubled haploid plants will play an increasingly important role in whole genome sequencing projects, where homozygosity is of particular advantage.

## *1.2 History of Haploids*

Dorothy Bergner in 1921 was the first to describe the natural occurrence of sporophytic haploids in the weed species *Datura stramonium* and this was reported by Blakeslee et al. in 1922. This report was followed by similar reports in several other crop species. The initial attempts to use haploidy in breeding were started by Chase in 1952 who selected the low frequency of parthenogenetic haploids in maize and then applied chromosome doubling treatments to produce inbred lines (Kasha and Maluszynski 2003).

Ever since the discovery of the first haploid in *Datura stramonium* in 1921, several researchers attempted to induce the unfertilized egg cell or other cells of the embryo sac to undergo parthenogenesis via in vivo means. In vivo induction of haploidy is carried out by the application of various physical, chemical or biological stimulants (Yang and Zhou 1982). This was followed by repeated attempts by various scientists to improve the frequency of parthenogenesis. While ovule and ovary culture suffered setbacks, Guha and Maheshwari (1964) made a breakthrough in anther culture (Yang and Zhou 1982). Pioneering work by Guha and Maheshwari in 1964 and 1966 opened a new vista for haploid breeding through in vitro means. It was a major advancement in haploid breeding of higher plants in which development of numerous pollen plantlets through in vitro anther culture of *Datura innoxia* was achieved. They wanted to study normal breeding development, but they found development of embryos in their cultured anthers (Maheshwari et al. 1980, 1982). These embryos developed into plants with a haploid chromosome number. However, the frequency of haploid production was significantly low. These studies led to further experiments to improve the frequency of haploids through in vitro anther culture. Plant regeneration via anther culture has been reported in more than 250 plants (Maluszynski et al. 2003a).

Consequently, attention shifted mainly to anther culture and culture of female tissues was neglected for about a decade. However, other researchers continued to work in the field of in vitro gynogenesis and in 1971, Uchimiya et al. observed the division of haploid cells in callus tissues obtained from cultured unpollinated ovaries of *Zea mays* and ovules of *Solanum melongena*. But this came into limelight only in 1976 when San Noeum reported development of haploid plantlets in unfertilized ovary cultures of *Hordeum vulgare*. Subsequently, Zhu and Wu (1979) obtained haploid plants from cultured unfertilized ovaries of *Triticum aestivum* and *Nicotiana tabacum.* This was followed by development of haploid plantlets via unfertilized ovary culture in several economically-important plant species such as *Zea mays* (Tang et al. 2006), *Psoralea corylifolia* (Chand and Sahrawat 2007), *Cucurbita pepo* (Shalaby 2007), *Guizotia abyssinica* (Bhat and Murthy 2007), *Cocos nucifera* (Perera et al. 2007), *Morus alba* (Thomas et al. 1999), etc.

## *1.3 Current Status*

There are a number of excellent reviews on the production of haploids and doubled haploids so far, including those of Andersen (2005), Dunwell (2010), Germanà (1997, 2006, 2007, 2009), Kasha (1974), Magoon and Khanna (1963), Maluzynski et al. (2003a, 2003b), Palmer et al. (2005), Touraev et al. (2009), Zhang et al. (1990) and Xu et al. (2007). The doubled haploid techniques have been well established in a range of economically-important crop species, including major cereals and cabbage (Wedzony et al. 2009). Regeneration via anther culture has been reported in more than 250 species belonging to Solanaceae, Cruciferae and Gramineae families (Dunwell 1986; Germanà 2011; Hu and Yang 1986), while there are limited reports

on many legumes and woody plants as they are rather recalcitrant (Sangwan-Norreel et al. 1986; Bajaj 1990; Raghavan 1990; Wenzel et al. 1995; Germanà 2006, 2009, 2011). Recent advances in anther culture have been reported by Dunwell (2010), Wedzony et al. (2009), Pratap et al. (2009), Srivastava and Chaturvedi (2008); and Touraev et al. (2009). Unfertilized ovary–ovule culture has been applied to species including sugar beet, tulip, cucumber, sweet potato, onion, squash, gerbera, rice, maize, niger and tea (Chen et al. 2011). Wide hybridization method for production of haploids is routinely used in wheat and other cereal breeding programmes. The general method followed involves a phase of embryo rescue in vitro, usually followed by chromosome doubling with colchicine (Maluszynski et al. 2003a).

## *1.4 Efficiency of Haploid Production*

Homozygous lines are of utmost importance in breeding programmes. Wide hybridization and anther/microspore culture are two of the most preferred techniques for doubled haploid production in crop plants. There is not much difference in the cost of haploid production or in the time and amount of labour required to produce haploids via these means. Currently, gynogenesis is the least favoured technique because of the low efficiency of production of haploids, but the value of doubled haploids in species that do not respond to other methods of haploid production makes this method worthwhile (Maluszynski et al. 2003a; Touraev et al. 2001). The limitation of wide hybridization is that it is restricted to the cereals where the chromosome elimination system appears to operate. Each crop has different requirements and, thus, there is need for extensive research to develop an efficient system. Wide hybridization has the advantage of being quite effective across genotypes and produces little or no induced variation from cultured embryos. However, anther/microspore culture scores higher over distant hybridization due to the fact that anthers harbour large numbers of haploid microspores per anther. In barley and wheat, where both systems have been comparatively well developed, the yield of green plants from isolated microspore culture can be up to 100 times higher than from wide hybridization in the most responsive genotypes (Kasha and Maluszynski 2003).

Anther culture is feasible in most species but it generally takes plenty of time to develop a competent system in some crops. Moreover, a good aseptic technique is a necessity even though the available methods are simple and reproducible (Maluszynski et al. 2003a). In general, haploid plants are regenerated in vitro from the microspores contained in the anther and require chromosome doubling treatments using chemicals such as colchicine, pronamide, trifluralin, oryzalin and amiprophos methyl (APM) (Wan et al. 1991). A few species such as barley regenerate a large number of doubled haploids as a result of induced chromosome doubling during early division of the microspores (Kasha 2005). Although the application of anther culture is widespread, the presence of the sporophytic anther wall serves as

a hindrance towards complete access of the microspores. There is a need to ensure direct embryogenesis to eliminate an intermediary callus phase that can promote gametoclonal variation among regenerants. The large number of successful reports on haploid production through anther culture stands as a witness of the immense benefits that this system offers. However, the presence of extraneous tissue in the form of anther wall makes this system complex for genetic studies where precision is inevitable.

As haploid plants in crop species obtained from microspore cultures through embryogenesis rather than from callus, the problem of extensive culture induced variability found in earlier reports on anther culture can be significantly done away with. With improvement of frequencies from microspores, the genotypic competence has also been reduced so that the system can be used in breeding programs. The feature of a high frequency of spontaneous chromosome doubling in some crops which results in completely fertile doubled haploid plants without subsequent doubling treatments, is another advantage of microspore culture. In breeding, the instant production of true breeding lines in diploid or allopolyploid species saves a number of generations in the breeding program. High density cultures of synchronized microspores can be set up containing more than thousand embryos per ml of culture media. Although a newer technique in comparison to anther culture, the potential of microspore culture has been realized quite early and efficient procedures have been successfully developed to produce doubled haploids in microspore cultures of tobacco, rapeseed, pepper, wheat, barley, rice, etc (Maluszynski et al. 2003a; Touraev et al. 2001).

## 2 Methodologies of In Vitro Haploid Production

### *2.1 Androgenesis*

In androgenesis, immature pollen grains are induced to follow the sporophytic mode of development with the application of various physical and chemical stimuli. There are two methods for in vitro production of androgenic haploids, viz anther culture and pollen culture.

#### 2.1.1 Anther Culture

Anther culture imparts an easy and one step protocol for haploid plant production (Chaturvedi et al. 2003).There is no single specific condition or protocol for inducing in vitro androgenesis in all plants since different species and even different cultivars within a species show diverse requirements. The following methods were adopted by Chaturvedi et al. (2003) to initiate in vitro androgenesis in neem plants (*Azadirachta indica* A. Juss.):

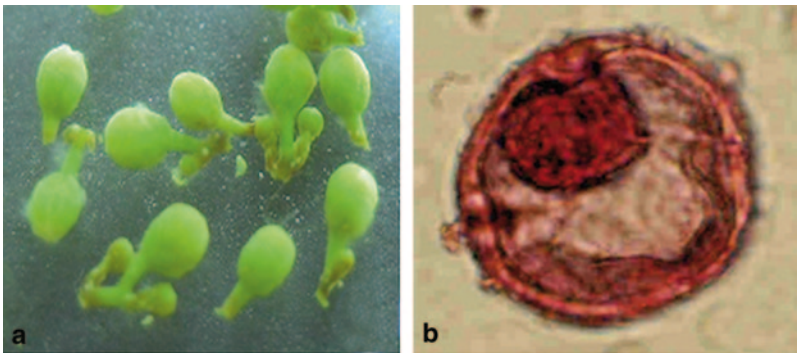


**Fig. 8.1** **a** Neem flower buds of 2 mm size bearing correct stage of microspores (6X) **b** An uninucleate microspore of neem stained with acetocarmine (2300X)

## Plant Material

For anther culture, 2 mm size flower buds (Fig. 8.1a) were collected early in the morning during the flowering season (April-May). The stage of microspore development was determined by acetocarmine squash preparations. Anthers containing early-to-late uninucleate stage of microspores (Fig. 8.1b) were cultured in the laboratory.

## Establishment of Aseptic Cultures

The flower buds, collected from a field, were rinsed with sterile distilled water (SDW) several times, followed by surface sterilization in a glass vial with 0.1 % mercuric chloride solution ($HgCl_2$) for 7 min. Finally, the buds were washed three to four times with SDW. The buds were dissected out with the aid of a binocular microscope using pre-sterilized Petriplates, forceps and fine needles. Damaged anthers, if any, were discarded. Twenty anthers, from two buds, were cultured in 55 × 15 mm pre-sterilized, disposable Petriplates containing 10 ml of MS (Murashige and Skoog 1962) medium. The Petriplates were sealed with parafilm and subjected to specific treatment conditions.

## Culture Media Preparation

A number of media compositions containing different concentrations of auxins and cytokinins were used for inducing in vitro androgenesis in neem. MS media, devoid of any growth regulator, did not induce any morphogenic response in anther cultures. For successful induction of callus from anthers, a combination of auxins and a cytokinin was found to be necessary in most of the cultures.

Analytical grade chemicals were used for preparation of stock solution and cultured media. Stock solution for MS/$N_6$ media preparation can be summarized as follows:

- Macronutrient – 10X; Micronutrient – 20X; Iron stock – 20X; Vitamins – 20X
- Growth regulator is used at a concentration of $1 \times 10^{-3}$ M

All constituents were stored at 4 ºC for further use. Myo-inositol and sucrose were added freshly at the time of media preparation. The pH of the medium was adjusted at 5.8 with 1N HCl or 1N NaOH. The media were solidified with 0.8 % agar and autoclaved for 15 min at 1.06 kg $cm^{-2}$ and 121 ºC, before pouring in 60 mm size Petriplates.

Culture Conditions

The anthers on each media were subjected to an array of pre-treatment conditions, like cold and heat shock for various duration under both dark and light incubation. Light and dark incubation at 25 ºC temperature served as a control in all the experiments. All the cultures were maintained continuously at 25 ± 2 ºC and 50–60 % relative humidity under 16/8 h (light/dark) photoperiod with diffuse light (1,000–2,000 lx) provided by cool daylight fluorescent tubes (Philips TL40 W). Anther cultures were initially kept continuously in the dark, but after eight weeks, the calli that had developed from these cultures were transferred to multiplication medium and maintained in light.

Twentyfour cultures were raised for each treatment, and each experiment was repeated at least three times. The cultures were observed periodically and the morphological changes were recorded at weekly intervals.

### 2.1.2 Pollen/Microspore Culture

Anther culture is beset with a number of problems. One of the major problems of anther culture is the concomitant callusing of anther wall along with the pollen, as a result of which the final tissue derived may not be of purely gametophytic origin. Moreover, the plants arising from an anther would constitute a heterogenous population. It has been observed in some species that anther cultures show asynchronous pollen development, the older grains may suppress the androgenic potential of younger grains by releasing toxic substances (Bhojwani and Razdan 1996). Kameya and Hinata (1970) reported for the first time callus formation in isolated pollen cultures of *Brassica oleracea* and the hybrid *B. oleracea* X *B. alboglabra* following which successful pollen-derived androgenic plants have been produced in many crop plants. There are numerous advantages of microspore culture over anther culture for haploid plant production. Unlike anther culture, isolated microspore culture allows haploid plant regeneration directly from microspores, assuring pure gametophytic origin and, thus, avoiding mixing of proliferating anther walls. A homogeneous prep-

aration of pollen at the developmental stage, most suitable for androgenesis, can be obtained by gradient centrifugation. Isolated pollen can be modified genetically by mutagenesis or genetic engineering before the culture and a new genotype can be selected at an early stage of development. Some of the important factors that affect induction of androgenesis in cultured pollen are the composition of the culture medium, pre-treatment and plating density. In most of the cereals, pollen culture involves pre-culture of the anthers for a few days or co-culture of pollen with a nurse tissue. Treatment of pollen-derived embryos and pollen derived callus to recover complete plantlets is similar to that of anther culture. However, the nutritional requirements of isolated pollen in culture are more complex than those of cultured anthers.

## *2.2 Gynogenesis*

As mentioned earlier, in vitro gynogenesis is used as a complementary technique in species where anther/pollen culture is inaccessible or unsuccessful. Young flowers, ovaries or ovules have been used as explants to produce gynogenic haploids where the plants develop from unfertilized cells of female gametophyte (embryo sac) either through direct embryogenesis or via callusing. The following techniques are generally used for production of haploids via in vitro gynogenesis.

### 2.2.1 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), *Pyrus communis* L. (Bouvier et al. 1993), *Actinidia deliciosa* (A. Chev) (Pandey et al. 1990; Chalak and Legave 1997). This method is based on in vitro culture of immature seeds or embryos, obtained as a result of pollination by irradiated pollen with gamma rays from cobalt 60. The method is useful in those species in which in vitro anther culture has not been successful. Irradiation does not hinder pollen germination but prevents pollen fertilization, thus 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.

### 2.2.2 Ovary Slice Culture

Ovary slice culture technique involves culture of transverse sections of unpollinated ovaries on culture media. The various steps of ovary slice culture of tea are sum-

marized below. The following methods were used to induce in vitro gynogenesis in tea plants (Hazarika and Chaturvedi; unpublished data).

### Plant Material

For ovary slice culture in tea, unopened and unpollinated mature flower buds (6–10 mm) size were collected early in the morning. Some of the buds were fixed in FAA (5:5:90 v/v/v Formaldehyde: Acetic acid: 70 % Ethanol) for 48 h and then stored in 70 % alcohol. Later, the appropriate developmental stage of the embryo sac was determined by histological analysis.

### Establishment of Aseptic Cultures

The mature unpollinated tea flower buds were surface sterilized with 0.1 % $HgCl_2$ for seven minutes, followed by rinsing with sterile distilled water at least thrice. Carefully dissected transverse sections of ovaries were cultured on Murashige and Skoog's (MS) media supplemented with varying concentrations of auxins and cytokinins. Six ovary slices containing unpollinated ovules were cultured in 60 × 15 mm pre-sterilized disposable Petriplates containing 10 ml MS medium. The sealed Petriplates were subjected to various regimes of temperature and light treatments.

### Culture Media Preparation

Murashige and Skoog (1962) basal medium was used throughout the studies to raise ovary slice cultures. MS basal medium was supplemented with a range of growth regulators, viz., 2, 4-Dichlorophenoxyacetic acid (2, 4-D), α-Naphthalene acetic acid (NAA), $N_6$- Furfuryladenine (Kinetin), 6-Benzylaminopurine (BAP) and Thidiazuron (TDZ) either individually or in combinations. The media contained 3 % sucrose and was gelled with 0.8 % Agar.

### Culture Conditions

The ovary slice cultures from each media were subjected to an array of pre-treatments, like cold (4 °C) and heat shock (33 °C), for various durations under both light and dark incubation. The light and dark incubation at 25 °C served as a control in all the experiments. After the application of treatments, the cultures were maintained continuously at 25 ± 2 °C condition with 50–60 % relative humidity under 16/8 h (light/dark) photoperiod irradiance (1000–2000 lx) provided by cool daylight fluorescent tubes. The cultures were observed periodically and the morphological changes were recorded at weekly intervals.

### 2.2.3 Ovule Culture

Embryos are difficult to excise. To prevent damaging the embryos during the excision process, they are sometimes cultured while still inside the ovule. This technique is referred to as ovule culture or in ovulo embryo culture (Reed 2005). The unfertilized ovary is surface sterilized and the ovules are taken and placed into the culture. Excision of ovule, followed by culture on specific media may be either extremely easy to accomplish, as in case of large-seeded species in which only a single ovule is present, or time-consuming and intricate, as in small-seeded polyovulate species. Two types of ovule support systems have been developed. The filter paper support system involves culturing of the ovules on top of filter paper placed over liquid medium, whereas the vermiculite support technique demands placing the ovules on a sterile vermiculite/liquid media mixture (vermiculite support) with the micropylar side down. Unpollinated ovule culture has been used for haploid production in sugar beets and onions. Since there is usually only one egg cell per ovule, ovule culture has much less potential than microspore culture (Kasha and Maluszynski 2003). In *Nicotiana rustica* cv Rustica ovules with placenta were isolated from flower buds and were cultured on $N_6$ medium supplemented with growth regulators (Katoh and Iwai 1993).

## *2.3 Wide Hybridization*

Haploids can also be induced by a process of selective chromosome elimination that follows certain interspecific pollinations. This phenomenon was discovered first in barley with crossing between *Hordeum vulgare* and *H. bulbosum* (Kasha and Kao 1970) and is now used routinely in wheat and other cereal breeding programmes; haploids were induced in these species following pollination with maize pollen. High frequency gynogenic haploids of *Triticum aestivum* have been raised by crossing them with *H. bulbosum* followed by embryo culture (Barclay 1975; Zenketler and straub 1979; Inagaki 1990). The process involves a phase of embryo rescue in vitro, usually followed by chromosome doubling with colchicine. The term "embryo rescue" refers to an vitro techniques the purpose of which is to promote the development of an immature or weak embryo into a viable plant (Reed 2005). Embryo rescue has been widely used to raise plants from hybridization in which failure of endosperm development causes embryo abortion. In embryo rescue procedures, the artificial nutrient medium serves as a substitute for the endosperm, thereby allowing the embryo to continue its development. Embryo rescue techniques are among the oldest and most successful in vitro procedures. One of the primary uses of embryo rescue has been to produce interspecific and intergeneric hybrids. While interspecific incompatibility can occur for a wide variety of reasons, one common cause is embryo abortion. The production of small, shrunken seed following wide hybridization is indicative of a cross in which fertilization occurs but seed development is disrupted. Embryo rescue procedures have been very successful in overcoming

this barrier among wide hybridizations in a wide range of plant species (Collins and Grosser 1984). In addition, embryo rescue has been used to recover maternal haploids that have developed as a result of chromosome elimination following interspecific hybridization.

## 3 Factors Affecting Haploid Production

There are numerous endogeneous and exogeneous factors that affect in vitro haploid production. These factors can be genetic, physiological, physical and chemical may also interact amongst each other to divert the microspores/egg cells to enter into a new developmental pathway. Some of the crucial factors affecting haploid production in plants are discussed below:

### *3.1 Genotype of the Donor Plant*

The genotype of the donor plants has great influence on anther culture response and has been known since the early days of development of plants from pollen grains (Nitsch and Nitsch 1969; Maheshwari et al. 1980). However, it is only in recent times that the genetic factors affecting androgenesis have been studied more intensively. Many crop species are quite recalcitrant in their in vitro response which is governed by specific genes on the chromosomes (Datta 2005). Certain regions of the chromosomes appear to be associated with the formation of embryo like structures (Wan et al. 1992). As has been mentioned by Zhou in 1996, additive gene effects explain most of the variations observed across diverse genotypes, but cytoplasmic influences and non additive gene effects also play important roles in determining in vitro regeneration ability of anther-derived cultures. In earlier studies, significant difference in callus formation using varieties or crosses was observed. In some species, only a few genotypes have responded of the many that were tested. In fact, genetic factors contribute in a major way to the differences in the number of haploid plants produced (Custodio et al. 2005; Sopory and Munshi 1996).

As in anther culture, a difference in response also exists among donor cultivars in ovary and ovule culture. It was reported by Zhu et al. (1981) that the percentage of ovaries producing gynogenic calli in four wheat cultivars varied from 1.3 to 10.9 %. In *Nicotiana tabacum*, two cultivars had an induction frequency as high as 75 % and 80 %, but in another species, *N. rustica*, it was only 8 % (Wu and Chen 1982). Similar genotypic competence for gynogenesis has been reported in a number of crop species (Keller 1990; Lux et al. 1990; Tosca et al. 1999; Kobayashi et al. 1993; Sibi et al. 2001; Alan et al. 2004). It has been observed that genotypic competence exists even between different cultivar types within the same plant species, as has been reported in *Guizotia abyssinica* (Bhat and Murthy 2007).

## 3.2 Physiological Status of the Donor Plant

The physiological conditions of the donor plant, i.e., the environmental conditions and age of the donor plant, directly affect both in vitro androgenesis and in vitro gynogenesis in almost all plant species. A correlation between plant age and anther response has also been demonstrated by various scientists. Generally, the first flush of flowers yields more responsive anthers than those that are born later. Similar is the case with ovary culture. The frequency of androgenesis is usually higher in anthers harvested at the beginning of the flowering period and showed a gradual decline in relation to plant age (Bhojwani and Razdan 1996). However, it has been reported in *Brassica napus* and *B. rapa* that pollen from older, sickly looking plants yielded a greater number of embryos than those from young and healthy plants. Varying temperature and light conditions during the growth of donor plants also affect anther response. In anther culture of grape, the induction frequency of embyoids, derived from spring flowers, was higher than that derived from summer flowers (Zou and Li 1981). The microscopical observations showed that some varieties of rubber trees often have a lot of degenerated and sterile microspores in their anthers, in early spring or hot summer, due to the influence of unfavourable climatic conditions. As a result, no pollen embryoids were obtained from such anthers, but only somatic calli were obtained (Chen et al. 1982).

## 3.3 Stage of Explants Material at the Time of Inoculation

### 3.3.1 Stage of Microspores

The stage of microspores at the time of inoculation is one of the most critical factors for induction of androgenesis. Detailed cytological studies conducted on poplar, rubber (Chen 1986) and apple (Zhang et al. 1990) have shown that androgenic callus and embryos were mainly induced through a deviation of the first pollen mitosis to produce two undifferentiated nuclei. Besides affecting the overall response, the stage of microspore at culture also has a direct effect on the ploidy of plants produced in anther culture (Sunderland and Dunwell 1977). About 80 % of the embryos obtained from binucleate microspores of *Datura innoxia,* a highly androgenic species, were non haploids (Sunderland et al. 1974). In a vast majority of species where success has been achieved, anthers were cultured when microspores were at the uninucleate stage of microsporogenesis (Chaturvedi et al. 2003; Pedroso and Pais 1994; Sopory and Munshi 1996).

### 3.3.2 Stage of the Embryo Sac

It has been reported that the effect of ovule development on gynogenesis is profound as it harbours the embryo sac comprising the egg cell. The stage of embryo sac is an important determining factor for in vitro gynogenesis in various plant

species. However, it is difficult to know the stage of embryo sac at the time of inoculation. Several authors prefer to describe the inoculation stage according to the developmental stage of the flower bud or stage of pollen development. However, this could not be possible in several species, where male and female gametophytes do not mature simultaneously, a phenomenon known as protandry, the maturation of anthers before carpels (e.g., onion, leek, sunflower, sugar beet, carrot,) and the opposite protogyny (e.g., pearl millet). In such cases, the stage of embryo-sac at culture can be determined by histological preparations of ovary/ovules that are at identical stage with that of cultured ovary/ovules (Srivastava et al. 2009).

The unpollinated ovules collected two or three days before anthesis were non responsive on culture media as has been reported in Niger whereas unpollinated ovules collected one day before anthesis were most responsive with about 5 to 13.3 % embryogenesis recorded (Bhat and Murthy 2007). Although a wide range of embryo sac stages are responsive to gynogenic development, in most cases, nearly mature embryo-sac stage gave better results. This is quite contrary to anther culture in which mature pollen is nonresponsive to androgenesis. In barley and rice, unfertilized ovary cultures with late staged mature embryo sacs gave good results (San Noeum 1976, 1979; Wang and Kuang 1981) while others reported success with ovary cultures containing uninucleate to mature embryo sacs (Zhou and Yang 1981; Yang and Zhou 1982; Kuo 1982; Huang et al. 1982).

## *3.4 Culture Media*

### 3.4.1 Basic Media

The constituents of the basal medium and combinations of growth regulators serve as an important factor in eliciting successful androgenesis and gynogenesis. However, it is difficult to suggest one single culture medium with a particular growth regulator for all the systems. Regeneration of androgenic and gynogenic plants may occur directly via embryogenesis or via callus formation from pollen/egg cell, followed by organogenesis. In the later stages of plant development, the media constituents may vary according to culture conditions and requirement by the plant itself.

Most species exhibit androgenesis on a complete nutrient medium (mineral salts, vitamins and sucrose) with or without growth regulators. Most commonly used basal media for anther culture are $N_6$ (Chu 1978) medium, MS (Murashige and Skoog 1962) medium with slight modifications, Nitsch and Nitsch (1969) medium, and B5 medium (Gamborg et al. 1968). Half strength MS medium is suitable for Solanaceae and $N_6$ medium has been used for cereals (Chu 1978).

Most early work in 1950s used Nitsch medium for unpollinated ovule and ovary culture; however since the 1970s, Miller (1963), MS or $N_6$ media have been used in successful experiments. In *Gerbera*, MS seems better than the Knop's and Heller medium (Cagnet-Sitbon 1980). An increase in the content of B group vitamins and glycine in H medium has been reported to have promoted induction-frequency in tobacco ovary culture (Wu and Chen 1982).

### 3.4.2 Growth Regulators

The requirement of growth regulators and culture medium in terms of kind and concentration may vary with each and every plant system. Generally, there is an agreement that the source and amount of total nitrogen as well as combination of a cytokinin and an auxin is necessary for pollen embryogenesis and pollen callusing in several woody plants (Chaturvedi et al. 2003; Chen 1986; Nair et al. 1983). It has been reported in most members of Solanaceae, that addition of an auxin to the induction medium is not a pre-requisite for anther response, but the addition of auxins and cytokinins alone or in combination is crucial for microspore-derived embryo induction in majority of the plants, especially the recalcitrant ones (Maheshwari et al. 1982). The type and concentration of auxins seem to determine the pathway of microspore development (Ball et al. 1993), with 2, 4-D inducing callus formation, and indole-3-acetic acid (IAA) and Napthaleneacetic acid (NAA) promoting direct embryogenesis (Armstrong et al. 1987; Liang et al. 1987). Gibberellins and abscisic acid have been occasionally added to the media.

Growth regulators, especially, auxins are widely used for induction of gynogenesis and their optimum concentrations have been reported to vary considerably from species to species (San Noeum and Gelebart 1986). In sunflower, gynogenesis occurs only when 2,4-D or NAA is added to the medium (Gelebart and San Noeum 1987). As observed in many species, a combination of auxin and cytokinin was also reported to be useful for gynogenesis in allium species (Alan et al. 2003) and mulberry (Thomas et al. 1999). In mulberry, gynogenic haploids are also produced on BA or Kinetin medium (Lakshmi Sita and Ravindran (1991). These researchers observed gynogenesis in *ab initio* ovary cultures of mulberry. Thomas et al. (1999) obtained maximum gynogenic response when excised ovaries from inflorescence segments of mulberry were cultured on MS supplemented with BAP (8.5 μM) + 2,4-D (4.5 μM), followed by transferring them to MS + 2,4-D (4.5 μM) + Glycine (6660 μM) + Proline (1738 μM).

### 3.4.3 Growth Additives

The supplement of other substances, such as free amino acids (glutamine, proline, glycine), biotin, myo-inositol, casein hydrolysate, coconut water, silver nitrate (ethylene antagonist) and polyvinylpyrrolidone has been reported to enhance gynogenic response (Reinert and Bajaj 1977; Powell 1990; Achar 2002). Moreover, the addition of exogenous aliphatic polyamines (PAs) to the culture medium has been found to increase the number of pollen-derived embryos in potato (Tiainen 1992), in some Indian wheat cultivars (Rajyalakshmi et al. 1995), cucumber (Ashok kumar et al. 2004 and Chiancone et al. 2006). Polyamines such as putrescine, cadaverine, spermidine and spermine are low molecular mass polycations which are involved in in vitro organogenesis and embryogenesis (Bagni and Tassoni 2001; Kumar et al. 1997). Similarly, the use of additives has also been reported in unfertilized ovary cultures. Thomas et al. (1999) obtained maximum gynogenic response in mulberry

when excised unfertilized ovaries from inflorescence segments were transferred from MS + BAP (8.5 μM) + 2,4-D (4.5 μM) to MS + 2,4-D (4.5 μM) + Glycine (6660 μM) + Proline (1738 μM).

### 3.4.4 Carbon Source

Sucrose has generally been used as the major carbohydrate source in the culture medium. Sucrose concentration in induction medium has a major effect on osmosis, and the development of embryos is apparently influenced by osmosis (Wakizuka and Nakajima 1975). The effect of sucrose on anther culture has been investigated in a number of species. The necessity of sucrose for successful androgenesis was first demonstrated by Nitsch in 1969 for tobacco and later by Sunderland in 1974 for *Datura innoxia*. Generally, sucrose is supplied at 2–3 % concentration. However, increase in its concentration can lead to beneficial morphogenic potential (Agarwal et al. 2006; Sopory and Munshi 1996) by suppressing the proliferation of anther wall (somatic tissues) (Ouyang et al. 1973). For potato, 6 % sucrose proved distinctly superior than 2–4 % sucrose in terms of the number of anthers forming pollen embryos (Sopory et al. 1978). High sucrose levels (6–17 %) are required in Gramineae and Brassicaceae families in which mature pollen is shed in the tricellular condition (Dunwell and Thurling 1985), whereas for those in which mature pollen is bicellular (e.g., Solanaceae), lower level of sucrose, such as 2–5 %, is usually beneficial (Dunwell 2010). All *Brassica* species require 12–13 % sucrose for androgenesis in anther and pollen cultures. According to (Dunwell and Thurling 1985), high sucrose concentration favours better survival of pollen grains, thus improving the frequency of androgenesis in *Brassica napus* (Last and Bretell 1990). Wedzony et al. (2009) have reported the beneficial effects of maltose on androgenesis in anther cultures of wheat, triticale, rye and rice.

The type and concentration of sucrose used in the medium for inducing in vitro gynogenesis varies from species to species. High sucrose concentration (8–10 %) in the culture medium has been shown to be helpful in some species, like sweet potato (Kobayashi et al. 1993) and onion (Campion et al. 1992), whereas in summer squash, 9 % sucrose is detrimental for production of any embryos (Shalaby 2007). Sucrose concentration used in unfertilized ovary and ovule culture was 3 to 10 % in barley, 4 to 8 % in wheat, 3 to 6 % in rice, 2 % in tobacco and 3 to 6 % in *Gerbera* (Yang and Zhou 1982).

## *3.5 Culture Conditions*

Pre-treatments, such as chilling, temperature shock, high humidity, water stress, anaerobic treatment, centrifugation, sucrose, nitrogen starvation, ethanol, gamma radiation, microtubule disruptive agents, electrostimulation, high medium pH, heavy

metal treatments are particularly popular approaches in anther and microspore cultures (Shariatpanahi et al. 2006).

Temperature treatment is considered to be the most effective treatment to induce pollen embryogenesis and it may be applied to excised flower buds or whole inflorescences before culture or cultured anthers or pollen grain, prior to their transfer to standard culture room conditions, in order to divert the gametophytic pathway to sporophytic mode of development. The optimum temperature and duration of pretreatments vary with the genotype. Cold pre-treatment (4 °C for 2–3 days) is employed routinely in the anther culture of many crops and its effect is also genotype-dependant (Osolnik et al. 1993; Powell 1988). In *Brassica* species, a short-duration high temperature pre-treatment at 30–35 °C to cultured anthers before shifting them to 25 °C is required to efficiently switch the developmental pathway.

Light does not seem to be necessary for the induction of androgenesis. For pollen culture of *Datura innoxia* (Sangwan-Norreel 1977), *Nicotiana tabacum* (Sunderland and Roberts 1977) and *Annona squamosa* (Nair et al. 1983), an initial incubation of cultures in dark, followed by diffuse light was found to be suitable. Isolated pollen cultures are more sensitive to light than anther cultures (Nitsch 1977). In *Brassica juncea* (Sharma and Bhojwani 1989) and *Hordeum vulgare (*Xu 1990) species, light is detrimental even for anther cultures. Likewise, neem callusing from microspores requires continuous dark incubation of anthers (Chaturvedi et al. 2003).

A beneficial role of cold treatment on gynogenesis has been reported in some species whilst in others, no significant effects on gynogenesis were observed. Pretreating the capitula of sunflower at 4 °C for 24–48 h before culture, significantly increases the induction frequency (Yan et al. 1987). Cai et al. (1988) observed a promotory effect of cold treatment to young panicles of rice at 7 °C for one day prior to ovary culture in gynogenesis. In *Cucumis melo,* pollination of pistils with irradiated pollen is essential to obtain ovules capable of forming gynogenic haploids (Katoh et al. 1993).

## *3.6 Other Miscellaneous Factors*

### 3.6.1 Anther Wall Factor

One of the important research subject in anther culture for woody plants is to avoid the over-proliferation of callus from anther wall tissues and to achieve a high yield of pollen embyoids and pollen calli. In anther culture of most woody plants, both pollen calli or embryoids and somatic calli from anther wall tissues grow simultaneously. The development of callus from somatic tissues of anther can be avoided by culture of isolated microspores. However, there are not many successful reports on microspore culture in woody plants (Chaturvedi et al. 2003). Pelletier and Ilami (1972) had introduced the concept of “wall factor”, according to which the somatic tissues of anther play an important role in the induction of sporophytic divisions in pollen. In anther culture of rubber, 47 % of the microspores in close contact with

the surrounding somatic cells could develop into multicellular masses as compared to only 5 % of microspores away from the wall (Chen 1986). Anther wall callusing is regarded as a pre-requisite for the formation of androgenic haploids (Chaturvedi et al. 2003; Chen et al. 1982; Chen 1986).

### 3.6.2 Microspore Culture Density

The culture density is a critical factor in isolated pollen culture. Huang et al. (1990) made a detailed study on the effect of culture density on embryogenesis in pollen cultures of *Brassica napus*. According to this report, the minimum density required for embryogenesis is 3000 pollen/ml of the culture medium but highest embryo yield was obtained at 10,000 to 40,000 pollen/ml.This high plating density is crucial only for the initial couple of days. Dilution of the density from 30,000–40,000 to 1,000 pollen after two days of culture did not reduce the embryogenic frequency. Arnison et al. (1990) reported the effect of culture density in anther cultures of *B. oleracea*. The frequency of pollen embryogenesis was enhanced if the anther culture density was increased from three anthers per 4 ml to 12–24 anthers per ml of the medium. Cardy in 1986 reported that in *B. napus*, the response was better when anthers were cultured at a density of two anthers per ml.

### 3.6.3 Effect of Female Flower Position

Position of female flowers on the plant stem affected induction of embryos from ovule cultures of *Cucurbita pepo* L (Shalaby 2007). One of the possible explanations for enhancing responses of tissue culture could be attributed to indigenous hormonal level (Johansson 1986).

## 4 Haploid Production in Economically Important Dicotyledonous Species

### *4.1 Azadirachta indica A. Juss (Family: Meliaceae)*

*Azadirachta indica* A. Juss, commonly referred to as neem, is an adaptable, tropical, evergreen tree of the family Meliaceae. It thrives best in hot, dry climates where shade temperatures often reach 50 °C and annual rainfall ranges from 400 to 1,200 mm. The neem plant is a native of South and Southeast Asia and grows well in tropical and subtropical areas of the world. The plant is well known for its numerous medicinal, agrochemical and economic uses, which can be attributed to the presence of azadirachtin, a highly oxidized limonoid (triterpenoid) found prominently in the seed kernels. It possesses insect repellent, antifeedant, larvicidal,

growth inhibiting properties against a wide range of pests and thus, has been well accepted as an eco-friendly, biodegradable biopesticide. Today, due to the remarkable properties shown by azadirachtin and other related triterpenoids, the tree has attained universal significance. Almost each and every part of this tree, particularly the leaves, bark and seeds, has manifold applications. Besides being a popular avenue tree, with a large crown, the wood of this tree is used as timber for house building, furniture and other domestic and agricultural tools.

Androgenesis is a very useful technique for resolving the problem of self-incompatibility, heterozygosity and long gestation period in this tree species. However, limited attempt has been made for the overall improvement of this valuable tree through in vitro haploid production. Gautam et al. (1993) made the initial attempts to produce haploids of neem for which they cultured anthers at the uninucleate stage of microspores and observed formation of multicellular pollen. However, all the plants regenerated from anther callus were diploids. Chaturvedi et al. (2003), for the first time, generated androgenic haploids of neem by anther culture, at early-to-late uninucleate stage of pollen. Callusing from anthers was induced on MS basal medium (with 9 % sucrose) supplemented with 2, 4-D (1 μM), NAA (1 μM), and BAP (5 μM). The calli multiplied best on MS medium (with 3 % sucrose) supplemented with 2, 4-D (1 μM), and Kinetin (10 μM). MS supplemented with BAP (5 μM) was optimum for regeneration from younger calli (75 % cultures differentiated shoots), but older calli showed the best regeneration at 7.5 μM BAP. As per histological investigations, in four-week-old cultures the anther-wall cells had started dividing, while the microspores appeared unchanged. However, in eight-week-old cultures, it was observed that the entire anther locules were filled with microcalli. Calli maintained on MS medium containing 2, 4-D exhibited good regeneration potentiality, but those calli that were maintained on MS medium containing 2, 4-D (1 μM) and Kinetin (10 μM), retained the regeneration potential for a longer period. Elongation of shoots was achieved at a lower concentration of BAP at 0.5 μM. These shoots were multiplied by forced axillary branching and were rooted through in vitro on ¼ strength of MS medium supplemented with IBA (0.5 μM). The plants were subsequently established in soil. Of the plants that regenerated from anther callus, 60 % were haploids (2n = x = 12), 20 % were diploids (2n = 2x = 24) and 20 % were aneuploids (2n = 2x–2 = 22). Srivastava and Chaturvedi (2011) reported a new improved method of haploid production from androgenic cultures of *Azadirachta indica* A. Juss. In this investigation, the best callus induction response was obtained in the induction medium with 12 % sucrose concentration on MS + 2,4-D (1 μM) + NAA (1 μM) + BAP (5 μM). Maximum shoot regeneration frequency obtained was 98.5 % with an average of 8.5 shoot-buds/ explant on MS + BAP (2.2 μM) + NAA (0.05 μM), as against 75 % with cultures forming an average of 4.5 shoot-buds/ explants on MS + BAP (5 μM) in the earlier report of anther culture of neem by Chaturvedi et al. (2003). Cytological analysis of the calli and regenerants confirmed their haploid status with the chromosome number as 2n = x = 12.

To date, there is only one single report on in vitro ovary culture of neem by Srivastava et al. (2009). However, the regenerated plants were diploid in nature. Un-

fertilized ovaries obtained from closed flower buds of an adult 54-year-old neem tree were used as explants. Maximum shoot regeneration (78 %) was observed when calli, induced from ovaries of 4 mm size flower buds and proliferating on MS + 2,4-D (0.5 μM), were transferred to MS containing BAP (5 μM). Histological analysis revealed that 4 mm sized flower buds corresponded to a 2-nucleate stage of the embryo sac. The best medium for inducing calli from unfertilized ovaries was MS medium with 9 % sucrose, 2,4-D(1 μM) + BAP(5 μM). The shoots were multiplied by forced axillary branching on MS medium supplemented with BAP (1 μM) and Casein hydrolysate (250 mg/l). The shoots were rooted on ¼ strength MS medium supplemented with IBA (0.5 μM) at a frequency of 79 %. The plants were subsequently hardened with transplantation rate of 81.8 %. Although all the regenerated plants were diploid, an efficient protocol for ovary culture of neem was established which can be used as a platform to produce gynogenic haploids of neem in future.

## *4.2 Camellia spp (Family: Theaceae)*

*Camellia sinensis* (L.) O. Kuntze commonly referred to as tea, belonging to the family Theaceae, is an evergreen, perennial, cross-pollinating plant. It grows naturally as tall as 15 m; however, it is usually trimmed to below 2 m when cultivated for its leaves (Mondal and Parathi 2003). The cultivated taxa of tea consists of three main natural hybrids. They are *Camellia sinensis* (L.) O. Kuntze or China type, *Camellia assamica* (Masters) or Assam type and *Camellia assamica* sub spp *lasiocalyx* (Planchon ex Watt.) or Cambod or Southern type. Tea is the oldest non-alcoholic caffeine containing beverage in the world. The medicinal properties of tea are also widely acclaimed. It is an important socio-economic crop that plays a significant role in the foreign exchange of developing countries like India. Polyphenols account for about 25–35 % of the total dry weight of freshly plucked tea leaves of which two-thirds is contributed by catechins alone (Saravanan et al. 2005). Research progress on tissue culture of tea has been rather slow for the last 20 years because tea is less amenable to these techniques, due to the high level of polyphenols, the presence of systemic bacterial contamination and its recalcitrant nature in tissue culture medium (Dood 1994).

The initial attempts to produce haploids through anther culture of tea were pioneered by Katsuo (1969) and Okano and Fuchinone (1970). They obtained roots from anther-derived callus. Later, Hoken Toi (1981) also achieved similar response on medium supplemented with NAA (9.67 μM) and Kinetin (10.1 μM) (Mondal et al. 2004). However, it was Chen and Liao in 1982, who produced complete plantlets from tea anthers of cultivar Fuyun No-7 out of nine different tea cultivars on which they worked. The plantlets were obtained when the anthers were cultured on $N_6$ medium supplemented with Kinetin (9.3 μM), 2,4-D (2.26 μM), L-Glutamine (800 mg/l), and Serine (100 mg/l), followed by sub culturing on $N_6$

medium supplemented with Zeatin (9.12 µM), adenine (148 µM) and Lactoalbumin hydrolysate (10 mg/l). On this medium, the calli continued to proliferate either into shiny mass or shoots. These shoots were subsequently rooted on medium containing IAA (0.57 µM). While three out of four plants were haploids, the rest were aneuploids with a chromosome number 2n = 18. Later, in (1992, Saha and Bhattacharya reported formation of globular structures in tea which failed to differentiate further on MS medium (with 7 % sucrose). This was supplemented with NAA (0.53 µM), 2, 4-D (0.45 µM), Kinetin (0.46 µM), and Glutamine (400 mg/l). However, the differentiation of true pollen embryos and regeneration of haploid plants were described by Raina and Iyer in (1992 and Shimokado et al. (1986). Pedroso and Pais (1994) tested 17 different media combinations based on MS and $N_6$ with various concentrations of carbon source, growth regulators and amino acids, such as Serine and Glutamine for *C. japonica*. The embryogenic calli from microspores were obtained when isolated microspores were cultured on 2, 4-D (4.53 µM), and Kinetin (0.46 µM) and subsequently on MS medium supplemented with BAP (2.22 µM). However, further growth ceased at maturation stage. Seran et al. (1999) reported the highest response in terms of micro calli formation in a Sri Lankan tea clone TRI-2043 (78–98 %) out of five different clones selected for study. On ½ MS + 2, 4-D (9.06 µM) + Kinetin (4.65 µM) + IAA (5.71 µM) under dark condition, 98 % anther response was achieved. Determination of ploidy levels in the callus cells showed that the frequency of haploid cells was greater (68 %) in comparison to diploid cells (6 %). However, plantlets could not be regenerated. Mishra and Chaturvedi (unpublished) has obtained for the first time microspore embryogenesis in anther cultures of TV clones of tea (TV19 and TV21) via an intervening callus phase. Within three weeks of culture, the anthers were swollen and after six weeks longitudinal dehiscence occurred along the anther wall (Fig. 8.2a), followed by emergence of transparent white callus from within (Fig. 8.2b). In both TV19 and TV21, calli were obtained when anthers were subjected to dark incubation at 25 °C. After successful induction of callus, embryogenesis in these calli and embryo germination occurred when cultures were transferred to light conditions (Fig. 8.2c, d).

So far, there have been no reports on in vitro gynogenesis in tea. Hazarika and Chaturvedi (unpublished data) have obtained for the first time, successful callus induction from unpollinated ovules of tea when thin sections of ovary cultures were subjected to various regimes of temperature and light treatments. Within a week of the culture, the ovules swelled to almost double their original size (Fig. 8.3a) and callusing was observed. In a few cultures, white, friable callus tissue emerged from within the cultured ovules (Fig. 8.3b) whereas in others profuse callusing from the entire sections occurred. The nature of callus obtained from within the ovules was friable and it was white in colour. Histological study of the ovary slice sections at the time of culture showed the presence of a mature egg cell within the embryo sac of the unpollinated ovules. Following successful callus induction, formation of dark green nodulated structures occurred on various media compositions. Histological investigations revealed the formation of tracheids in the callus tissue.

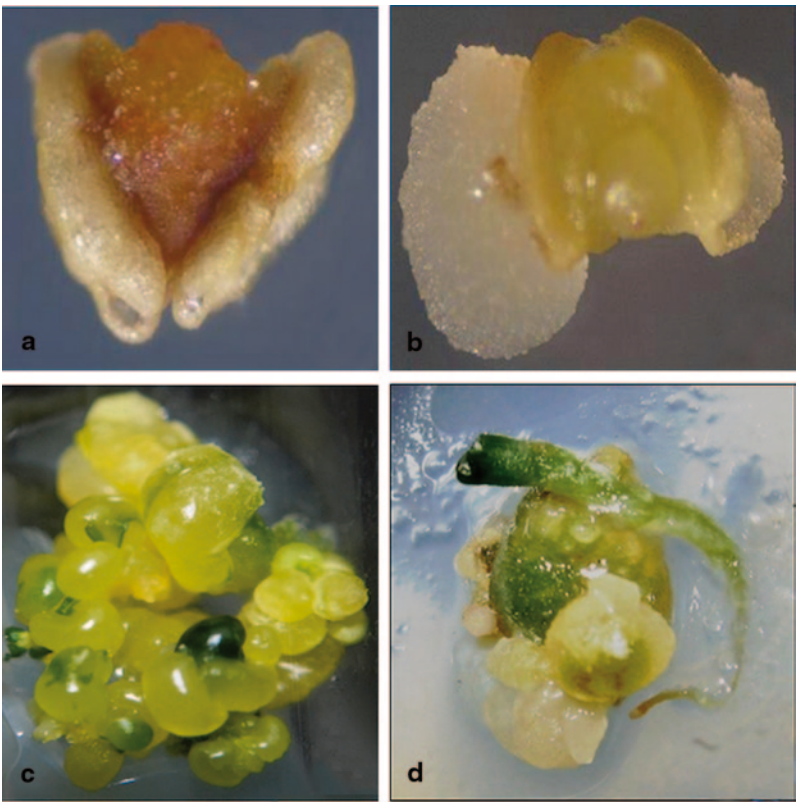


**Fig. 8.2** **a** Six-week-old anther culture of tea, showing longitudinal rupturing of anther walls (220X). **b** 10-week-old anther culture of tea. Note the emergence of calli from inside the anther walls (90X). **c** Embryogenesis occurring in anther-derived callus of tea, after eight weeks of culture. Embryos at various stages of development can be seen in the Fig. (4.5X). **d** A germinated embryo of tea (6X)

## *4.3 Coffea spp (Family: Rubiaceae)*

The coffee plant is a woody perennial, evergreen, dicotyledon that grows relatively high, therefore, more accurately described as a coffee tree. Coffee is a brewed drink prepared from roasted seeds, called coffee beans, of the coffee plant. While there are several different coffee species, the two main cultivated species are, *Coffea arabica,* known as Arabica coffee accounting for 75–80 % of the world's production and *Coffea canephora*, known as Robusta coffee, accounting for about 20 % of the total production. Genetic improvement of coffee, being an important commercial crop, is essential for improving the production and quality of coffee. Development of haploids via anther culture facilitates the production of homozygous plants in one generation and opens the way for new breeding strategies.

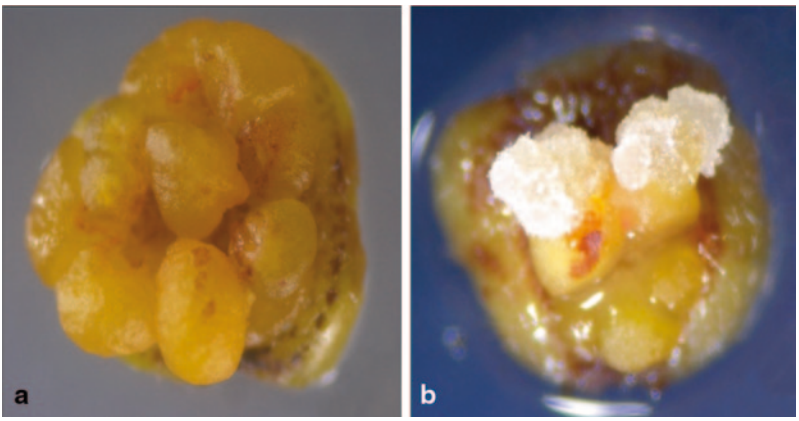


**Fig. 8.3** **a** A slice section of tea ovary culture, showing swollen ovules, after two weeks of culture (8X). **b** Four-week-old cultures showing bursting of ovules and emergence of callus from within (8X)

Anther culture studies are quite limited in coffee. The first attempt to produce haploid coffee plants was made in *Coffea arabica* (Sharp et al. 1973). Successful reports of haploid and dihaploid plants from anther culture of coffee have been published (Montes 1981; Ascanio and Arcia 1987). Ascanio and Arcia (1987) reported the existence of a correlation between different developmental stages of anthers, the size of flower buds and the quantity of calli obtained after 90 days of culture. Further in 1994, the authors studied the effect of developmental stage and heat shock on the formation of embryogenic calli from *C. arabica* var. guernica anthers.

Unlike anther culture, isolated microspore culture allows haploid plant regeneration directly from microspores, assuring pure gametophytic origin. Colonies of haploid cells have been successfully obtained via mechanically isolated microspore cultures of *C. arabica* varieties Catuai and Catimor in either liquid or solid media (Carneiro 1997). Herrera et al. (2002) described, for the first time, a new approach for embryo induction and plant regeneration from *C. arabica* cv caturra isolated microspores, pre-treated with colchicine. Their study clearly demonstrated that colchicine could activate the androgenic response in coffee microspores. A positive androgenic development was observed only when late uninucleated or early binucleated microspores were cultured. Such response was evidenced by microspore divisions beginning after 25 days of colchicine exposure. The best androgenic response was found when microspores were pre-cultured in 100 mg/l colchicine for 48 h. Flow cytometry and morphological analyses revealed that 95 % of regenerated plants were dihaploids (2n = 2x = 22). However, some doubled dihaploid plants (2n = 4x = 44) were also obtained, suggesting that not only androgenic induction but also chromosome doubling could be expected as a result of colchicine exposure of coffee microspores.

Lanaud in 1981 reported somatic embryogenesis in ovules from *C. canephora* and established conditions for rapid multiplication and differentiation of embryo-

genic mass to produce haploid plants. In brief, anther and ovule cultures are least developed coffee tissue culture techniques despite their substantial potential in reducing the time required to produce new varieties.

## *4.4 Citrus sp. (Family: Rutaceae)*

*Citrus* species represent the largest production of fruits worldwide. They are valued for their antioxidant properties and are one of the richest sources of vitamin C and essential oils.

The first report of development of haploid seedlings of *C. natsudaidai* was reported by Karasawa (1971) by the application of gamma rays. Thereafter, there have been several reports on production of haploids via in vivo crossing (Oiyama and Kobayashi 1993), gynogenesis induced by in vitro pollination with pollen from a triploid plant (Germanà and Chiancone 2001) and by in vivo parthenogenesis (Germanà 2006). Anther culture technique has been used to obtain haploid calli, embryoids and plantlets with limited efforts in a few *Citrus* species like *C. madurensis, C. limon, C. deliciosa* x *C. paradise* (Chen et al. 1980; Germanà et al. 1991; Germanà and Reforgiato 1997). However, extensive research has been carried out on *C. clementina*. Since the first embryogenic calli and haploid plantlets were obtained by anther culture in *C. clementina* Hort. Ex Tan. cv Nules (Germanà et al. 1994), many studies have been carried out to improve the androgenic response in *Citrus* species by the use of different combinations of plant growth regulators (Hidaka et al. 1979; Chaturvedi and Sharma 1985; Geraci and Starrantino 1990; Germanà et al. 1994, 2000a, b, 2005). Germanà and Chiancone (2003) proposed an improved and detailed protocol for haploid induction through anther culture of *C. clementina* Hort. Ex Tan. cv Nules by evaluating a number of factors that affect androgenesis. They observed that temperature pre-treatment of flower buds between 4 °C and 25 °C for 14 days was more favourable to induce embryogenic callus and embryoids in anther cultures. Anthers were excised from pre-treated flower buds and cultured on $N_6$ medium (Chu 1978) supplemented with Nitsch and Nitsch vitamins (Nitsch and Nitsch 1969), galactose (9000 mg $l^{-1}$), lactose (18,000 mg $l^{-1}$), coconut water (5 % v/v), casein hydrolysate (500 mg $l^{-1}$), L-glutamine (200 mg $l^{-1}$), biotin (0.5 mg $l^{-1}$), ascorbic acid (500 mg $l^{-1}$), NAA (0.11 μM), 2,4-D (0.09 μM), Kinetin (4.6 μM), BAP (2.2 μM), Zeatin (2.28 μM), TDZ (0.45 μM), and $GA_3$ (1.45 μM) in dark for 15 days before being shifted to diffuse light at 25 ± 2 °C. With this protocol, 1.9 % anther cultures showed embryoid development and a total of 570 and 1,000 embryoids developed in Nules and SRA 63 cultivars, respectively. Direct gametic embryogenesis without callus formation was observed in 7 % responsive anther cultures of the cv Nules and in 45 % of the responsive anther cultures of the cv SRA 63. The embryoids were later germinated on MS medium containing $GA_3$ (2.89 μM) and NAA (0.05 μM). Recently, Chiancone et al. (2006) studied the effect of polyamines, spermidine and putrescine, with an aim to further improve the rate of embryogenic callus and embryoid induction in anther cultures of *C. clementiana*

cv Nules. The addition of 2 mM spermidine to the suggested medium of Germanà and Chiancone (2003) stimulated gametic embryogenesis in 4 % cultures whereas putrescine did not influence embryo production. Flow cytometric analysis revealed that the regenerants were mostly trihaploids; few were doubled-haploids while none of them were haploids.

## *4.5 Malus domestica Borkh. (Family: Rosaceae)*

*Malus domestica* Borkh. (Family: Rosaceae) or apple is an important temperate fruit tree. It is one of the most widely cultivated tree fruits in the world. In vitro approaches to induce haploids in apple have been rather limited in comparison to other plant species (Höfer and Lespinasse 1996). Anther culture in apple was pioneered by Japanese scientists at the beginning of the 1970s. They induced calli capable of root formation. Subsequently, several working groups initiated haploid induction in apple by anther culture. Induction of embryogenesis and plant formation has been reported in apple from anther cultures (Fei and Xue 1981; Xue and Niu 1984; Höfer 1995). Although regeneration from embryos is reproducible via adventitious shoot formation, the induction of embryogenesis from cultured apple anthers is still low and highly dependent on genotype (Höfer 1995, 1997). Kolova et al. (1994) obtained multinuclear structures and Bouvier (1993) obtained callus formation from isolated microspore cultures. Höfer et al. (1999) reported, for the first time, the induction of embryogenesis and plant formation from isolated apple microspores at late uninucleate stage of development. The authors reported that several factors were responsible for successful androgenesis in apple, especially a combination of starvation and cold treatment. The effect of starvation of buds or microspores for 1–2 days at 4 °C or 27 °C was found to be very important for androgenesis in apple. Starvation is an effective stress treatment which has yielded successful embryogenic induction in tobacco (Kyo and Harada 1986; Touraev et al. 1996a) and wheat microspores (Touraev et al. 1996b). The requirement of heat or cold pre-treatment has been shown to vary from plant to plant (Höfer et al. 1999). The induction medium and genotype of the donor plant strongly affect embryogenic capacity of isolated microspores. “Alkmene” and “Rene” are the cultivars of apple in which the highest embryo induction via anther culture was achieved. Success with “Rene” has been reflected in many experiments related to androgenesis in apple. They attained 17 % embryo induction from microspore cultures as compared to only 7 % induction from anther cultures on modified $N_6$ basal medium devised by Chu et al. (1990) in wheat. The embryos were germinated on MS + TDZ (0.45 μM). Höfer (2004) reported that an increase in the frequency of embryo induction is possible up to 10 times by microspore culture depending on the genotype. Starvation treatment, induction medium, maltose concentration, type of culture vessel, microspore density and genotype influenced embryo induction in apple Höfer (2004).

## 4.6 *Morus sp. (Family: Moraceae; Common name: Mulberry)*

Mulberry is a vital crop for sericulture industry and is native to warm temperate subtropical regions of Asia, Africa, Europe, and the Americas, with the majority of the species native to Asia alone. Mulberry leaves are ecologically important as the only food source for the silkworms (*Bombyx mori*), the pupa/cocoon which are used to make silk. Apart from its application as a food source for the silkworms, the medicinal properties of mulberry sp. are also widely acclaimed. Like many other woody genera, mulberry is highly heterozygous and, therefore, production of haploids and doubled haploids through anther culture is highly beneficial.

Several authors have reported androgenesis in mulberry which dealt with studies performed on induction of division in pollen culture (Katagiri 1989), effects of sugars and alcohols on pollen division (Katagiri and Modala 1991), embryo differentiation (Sethi et al. 1992) and production of haploid plantlets from anther culture (Shoukang et al. 1987). However, detailed studies were conducted by Jain et al. (1996) who evaluated the effect of temperature and Kinetin pre-treatment on induction of androgenic callus in anther cultures of mulberry. It was revealed that cold pre-treatment given to flower buds at 4 °C for 24 h increased the percentage of callus originating from anther cultures. The anthers split and produced embryogenic callus on Modified Bourgin (MB) medium (Qian et al. 1982) with 8 % sucrose and supplemented with NAA (2.68 μM) and BAP (4.44 μM). Upon transfer of the calli to MB basal medium supplemented with NAA (2.68 μM), BAP (2.22 μM), 2,4-D (4.12 μM) and Polyvinyl pyrrolidone (PVP) (1.0 mg/l), embryos were induced which later developed roots upon removal of 2, 4-D from the medium. Finally, the embryoids germinated precociously without developing cotyledons, but forming elongated shoots. Rhizogenesis was induced when calli were subcultured on MB medium containing NAA and BAP (each 0.5 mg/l with reduced myoinositol (75 mg/l). The cytological study of the induced roots from the calli revealed the haploid nature of the callus tissue.

To raise haploids from female clones of mulberry, ovary culture is the only possible approach because inbreeding and anther culture are not applicable (Thomas et al. 1999). In *ab initio* individual ovary cultures of *Morus alba,* the growth was very poor and gynogenic plants were never formed, suggesting that unfertilized ovaries required some stimulus from the parental tissue for the initial growth. This is in contrast to the observations of Lakshmi Sita and Ravindran in 1991 who observed gynogenesis in *ab initio* ovary cultures of *Morus indica*. These authors cultured individual ovaries before or after fusion to form sorosis under field conditions without taking any measure to prevent chance pollination. The inflorescence of mulberry is a catkin in which ovaries are loosely arranged. After fertilization, the ovaries enlarge and fuse to form a solid looking fruit called ‘sorosis’. It was possible that some of the ovaries cultured by them were fertilized. This could be the reason that some of the gynogenic plants were haploid, others were diploid. (Thomas et al. (1999) followed a two-step protocol for ovary culture of mulberry. They raised nodal segment cultures on MS+BAP (5 μM) which developed axil-

lary shoots and one or more inflorescence from the axil or leaves in three weeks. Inflorescence segments, each bearing 4–5 florets, from four-week-old cultures of nodal segments, were planted on MS + BAP (8.5 μM) + 2,4-D (4.5 μM) and after three weeks individual ovaries were transferred to MS + 2,4-D (4.5 μM) + Glycine (0.5 mg/l) + Proline (0.2 mg/l). In this treatment, 16 per cent of the ovaries developed a gynogenic seedling. After the gynogenic plants are transplanted in soil, out of 20 plants examined, 12 showed haploid number of chromosome (2n = x = 14) and the other eight were aneuploids with 13–17 chromosomes in their root tip cells.

## *4.7 Populus sp. (Family: Salicaceae)*

Poplars form a very important part of basic forest biology and are economically important trees cultivated for their high wood quality that finds use in paper industry and energy production. The genus Populus consist of more than 30species, occurring throughout the forests of temperate and cold regions of northern hemisphere. The species *P. ciliata*, the only native poplar of India, is endemic to the Himalayan belt and has been an important tree for the forest breeders in India. In 1950, a large number of exotic clones of *Populus* were introduced and grown in North India, mainly, for increasing the wood availability for match and plywood industries. The study of poplars is essential to complement the ever increasing knowledge database of this important tree species.

First successful report on anther culture in *Populus* is by Wang et al. (1975), who observed callus proliferation from pollen and subsequent formation of haploid plants via organogenesis from the pollen-derived calli. Later, several reports were published on production of pollen plantlets in anther cultures of poplar (Zhu et al. 1980; Ho and Raj 1985; Kim et al. 1986; Mofidabadi et al. 1995). Induction of haploids via embryogenesis occurring from cultured anthers of *P. Maximowiczii* was obtained by Stoehr and Zsuffa (1990). The scientists applied cold pre-treatment to the flower buds (at 4 °C for four days) prior to culture the anthers at uninucleate stage of microspores, on MS medium containing 2,4-D (2.26 μM) and Kinetin (0.46 μM). Globular calli were developed from anthers after 4–8 weeks of dark incubation in the medium at 20 °C. When the anthers with globular calli were cultured on MS medium supplemented with NAA (0.54 μM) and BAP (4.4 μM), microspore division and embryoidal structures resembling globular–to-heart-shaped embryoids were obtained. However, the embryoids germinated precociously without developing cotyledons. After transfer to MS medium with BAP (4.4 μM), adventitious shoots developed mainly from the roots. Shoots were rooted on half strength MS medium supplemented with NAA (0.13 μM). Out of 34 plants analyzed cytologically, 22 showed haploid chromosome number (n = 19), one was aneuploid and the rest were diploids (Srivastava and Chaturvedi 2008). Kiss et al. (2001) reported further improvement in the rate of haploid plant regeneration by increasing the rate of induction from the anthers and with sustained shoot regeneration frequency in five

different genotypes of two poplar species, viz., *P. nigra* L. and *P. deltoides* Bartr. They reported that frequency of callus induction, shoot regeneration and number of shoots developed per calli are highly dependent on the genotype. The anthers were taken from floral buds which were subjected to cold pre-treatment at 4 °C for 8–14 days. For callus induction and shoot regeneration, they used the media suggested by Stoehr and Zsuffa (1990) with slight modifications. The anthers were incubated in dark for 6–7 weeks at 25 °C. Calli for shoot organogenesis were transferred to both MS or WPM medium (Lloyd and McCown 1980) supplemented with BAP (4.4 μM), NAA (0.54 μM), and 2.5 % sucrose and incubated in light at 23 °C. Shoots were rooted on hormone-free WPM. Of the 208 rooted plants, eight haploids, 179 diploids, four tetraploids and 17 aneuploid plants were obtained. Deutsch et al. (2004) reported haploid plant regeneration via embryogenesis from isolated immature pollen culture of two poplar hybrids (*Populus nigra* L.× hybrid 'Aue1' and 'Aue2') and employed microsatellite marker analysis to confirm the ploidy level of the regenerants.

## 5 Analysis of Ploidy Level

Analysis of Ploidy level is an integral part of haploid production programme which can be carried out efficiently either by chromosome counting during mitotic and meiotic cell division or by flow cytometry.

### *5.1 Chromosome Counting*

Counting of mitotic chromosomes is easier and faster. Root tips are the most convenient source of mitotic cells. When roots are not available, young axillary buds, leaves or callus can be used. The cytological procedures of chromosome preparation and staining are different and need to be modified depending on the plant species. The cytological procedures for visualizing chromosomes in woody plants may not be the same as in herbaceous species. Nevertheless, basic principles for handling the mitotic chromosomes of all plant species are similar and consist of collection of material, fixation and chromosome staining. The most critical step for chromosome counting involves proper chromosome squash preparation. It is very important to obtain sufficient well spread metaphase plates and proper physical separation of the chromosomes. However, method of chromosome staining applied for ploidy level analysis depends on plant species and chromosome size (Maluszynski et al. 2003a).

Protocol for cytological investigation in neem (Chaturvedi et al. 2003)
Fixation:

- Healthy root-tips (1 cm) from plantlets were collected at 10.30 am and rinsed with tap water.

- The root-tips were pre-treated with 0.02 % 8-Hydroxyquinoline at 4 °C for 4 h.
- Following which, the root-tips were fixed in a modified Carnoy's fluid containing absolute alcohol-chloroform-Glacial acetic acid-Methanol (7:3:1:1) for 48 h.
- Finally, the root-tips were preserved in 70 % ethanol at 4 °C.

## *5.2 Chromosome Staining*

- The root-tips from fixed roots were excised and placed in a mixture of nine drops of 2 % aceto-orcein and one drop of 1N HCl in a watch glass and warmed gently.
- After cooling, the individual root-tips were placed in a drop of aceto-orcein on a glass slide, covered with a cover slip, warmed gently and squashed.

## *5.3 Flow Cytometric Analysis*

Flow cytometry using DNA selective flourochromes has been considered to be a fast and reliable method for the measurement of nuclear DNA content in recent years (Muirhead et al. 1985; Thorthwaite et al. 1985; Dolezel et al. 2007). Unfortunately, its application in plant biology has been overdue, largely owing to the fact that flow cytometry requires single cell suspension (Shapiro 1985). As plant cells usually exist in complex tissues, methods had to be developed for the preparation of such suspensions. Although flow cytometry is an extremely efficient technique with high degree of accuracy, the preparation of high quality plant samples for ploidy analysis remains a vital issue. For determination and interpretation of the haploid status of the regenerants, firstly tissue of known ploidy is analyzed followed by the unknown tissue whose ploidy is to be analyzed.

## *5.4 Protocol for Flow-Cytometric Analysis in Tea*

### 5.4.1 Preparation of Nuclear Sample

- Well developed calli obtained after two months of culture initiation were used for ploidy analysis.
- Extraction of nuclei and the analysis were carried out via fine chopping of the hard calli placed in 1 ml ice-cold woody plant buffer. The woody plant buffer was prepared by mixing 0.2 M Tris HCl, 4mM $MgCl_2.6H_2O$, 2mM EDTA $Na_2.2H_2O$, 86mM NaCl, 10 mM Sodium Metabisulfite, 1 % Triton X-100 (v/v), and 2 % PVP-10 (w/v) according to the protocol of Loureiro et al. 2007 with

slight modifications. The pH of the buffer was adjusted to 7.5, filtered through 0.22 µm Polyvinylidene fluoride (PVDF) membrane filter and stored at 4 °C.
- The suspension containing the nuclei was mixed gently by pipetting up and down, softly, several times, followed by filtering of the homogenate through a 30 µm nylon mesh.
- The nuclear suspension was stained with Propidium iodide at a concentration of 50 µg/ml.
- Simultaneously RNAse at a concentration of 50 µg/ml was also added to the nuclei.

Ploidy analysis:

- The ploidy level was determined using a FACs Calibur cytometer (Becton-Dickinson, USA).
- All measurements were carried out in triplicate using fresh tea leaves as an external standard. Using instrument gain (photomultiplier voltage and amplitude gain), the position of peak $G_1$ nuclei of the reference sample was established on channel 200 on a 1,024 scale following which the instrument settings were kept constant and the unknown samples were run under the same parameters. The mean channel number of the unknown sample $G_1$ peak was determined and the DNA Ploidy was calculated according to the relationship:
  Sample Ploidy (integer) = Reference Ploidy X (mean position of the $G_1$ sample peak/mean position of the $G_1$ reference peak)

## 6 Diploidization of Haploids

Doubled haploids are of immense importance for genetic studies and in crop breeding programmes. Selected doubled haploid lines are used for production of commercial hybrids (Chase 1974). To obtain fertile, homozygous diploids for analyzing the progenies and the breeding behaviour of the pollen plants, the chromosome complement of the haploids must be duplicated. This is because haploids may grow normally up to the flowering stage, but in the absence of homologous chromosomes, meiosis is abnormal and consequently, viable gametes will not be formed and, hence, they are sterile. Spontaneous duplication of chromosomes in pollen-derived plants has been observed, but its frequency is very low.

In vegetable crops, doubled haploids are used prominently as parents for $F_1$ hybrid seed production. Similarly, in medicinal and aromatic plants, doubled haploids for $F_1$ production have the potential to make significant advances in providing high, stable and predictive yields of raw biochemicals to be processed by pharmaceutical and neutraceutical industries (Ferrie 2007). In case of cross pollinating species and hybrids, pure homozygous lines are highly desirable. Although conventional breeding method to produce homozygous diploids is well

established, it requires 7–8 repeated inbreeding cycles which in turn is time-consuming and labour intensive. Moreover, this approach is difficult in self-incompatible male sterile plants and tree species with long gestation period. Also, the pure lines obtained after several generations of self-pollination may not be 100 % homozygous (Germanà 2006). Production of fertile doubled haploid lines in *S. cereale* (Immonen and Anttila 1996) and *Festuca and Lolium* (Nitzche 1970) are of immense importance as these species suffer from inbreeding depression. One of the most efficient techniques for production of homozygous diploid plants in a single generation is through diploidization of the haploids. Doubled haploids have a key feature in establishing chromosome maps in a number of species, notably barley *Hordeum vulgare*, *Oryza sativa, Brassica napus, Triticum aestivum* (Forster and Thomas 2005), apart from providing a vast majority of mapped genetic markers. Molecular marker maps provide a platform for trait mapping, which is of particular interest to plant breeders. Doubled haploidy is also useful in rapid isolation and purification of selected mutants in subsequent generations. It is possible to create a population of homozygous mutant lines directly by targeting the mutation treatment at single gametic cells and then inducing embryogenesis, followed by subsequent chromosome doubling for homozygous diploid plant production (Szarejko and Forster 2006).

Doubled haploidy can be significantly enhanced by artificial means using chemicals such as colchicine, pronamide, trifluralin, oryzalin and amiprophos methyl (APM) (Wan et al. 1991). Colchicine treatment is one of the most preferred techniques for chromosome doubling, which in turn is one of the most critical steps in the doubled haploid breeding process. For *Nicotiana tabacum,* a 0.4 % solution of colchicine is recommended to diploidize the pollen plants. In practice, the young pollen derived plantlets are immersed in a filter sterilized solution of colchicine for about 96 h and then transferred to a culture medium to allow their further growth. Alternatively, the treatment is given in the form of a lanolin paste (Bhojwani and Razdan 1996). It is applied to the axils of the upper leaves and the main axis is decapitated to stimulate the axillary buds to grow into diploid and fertile branches. Besides bringing about chromosome duplication, colchicine treatment may also result in chromosome and gene instabilities (Burk 1970). Therefore, the frequent occurrence of spontaneous duplication of chromosomes in differentiated plant cells like cortex and pith and callus cells in long-term cultures has been exploited to raise homozygous, fertile diploids from haploid plants. In Apiaceae, for diploidization the plantlets grown in Petri dishes were taken out and the agar was removed from the roots. Subsequently, the roots were submerged in a 0.34 % (w/v) solution of colchicine for 1½ h. The roots were rinsed in water and the plantlets were transferred to a soil-less mix and grown in the greenhouse (Ferrie et al. 2005). For *Brassica* species, the roots of 25–30 pollen-derived plantswere immersed in a bunch in 0.25 % (w/v) colchicine solution for 5 h in light, for diploidization. After rinsing the treated roots with distilled water, the plants were transferred to a potting mix for hardening and further growth (Bhojwani and Dantu 2010).

# 7 Application of Haploid Production

## *7.1 Development of Pure Homozygous Lines*

Homozygous true breeding cultivars are highly important for screening of high yielding lines and to produce hybrid vigour as a method of crop improvement. Obtaining homozygous diploid plants by conventional methods is difficult in perennials. From several decades to over a hundred years are required to obtain a pure line by means of successive inbreeding throughout many generations. The seed set by inbreeding in many trees is very low, usually only a few of ten thousandth or sometimes no seed can be obtained at all; therefore, it is impractical to obtain pure lines by inbreeding (Chen 1986). Moreover, conventional method of haploid production by inbreeding is impossible if the plant is strictly cross-pollinating in nature. On the other hand, homozygous diploid plants can be achieved in a single generation by diploidization of in vitro raised haploids through colchicine treatment.

## *7.2 Genetic Studies*

Because of the lack of accurate material in research work, the progress in the study of genetics in trees is much slower than that in annual herbaceous plants. The genetics of a lot of important traits in economically important plant species has not been clearly demonstrated as yet. As a result, it is still unknown whether the desirable characters of the parents will appear in their progenies. Only when crossing between different homozygous diploid plants is carried out, we can gain a clear idea of dominance of genes controlling various characteristics and that these characteristics are either monogenic or polygenic (Chen 1986). Furthermore, if we can use the haploid plants as samples of gametes, then we can obtain directly the recombination value between genes. Moreover, we can also use the haploid plants to study chromosome homology within genome or between genomes.

## *7.3 Gametoclonal Variation*

The "gametoclonal variation" arises among plants regenerated from cultured gametic cells consisting of differences in morphological and biochemical characteristics as well as in chromosome number and structures that are observed. Besides yielding haploids, in vitro androgenesis helps in the screening of gametophytic variation at plant level. Pollen grains within an anther form a highly heterogeneous population because they are the product of meiosis which involves recombination and segregation. Therefore, each pollen plant is genetically different from the other. The gametoclonal variants being hemizygous in nature, express also the recessive characteristics in the $R_0$ plants (Bhojwani and Razdan 1996). Different sources of

variation can explain gametoclonal variation such as new genetic variation induced by cell culture procedures, from segregation and independant assortment, chromosome doubling procedures, etc., (Morrison and Evans 1987; Huang 1996).

## 7.4 Induction of Mutations

In general, a majority of induced mutations are recessive and, therefore, are not expressed in diploid cells due to the presence of dominant allele. Since haploid plants have only one set of chromosomes, their dominant and recessive characteristics can be seen simultaneously on separate plants. It is extremely advantageous to provide a convenient system for the induction of mutations and selection of mutants with desirable traits in the absence of their dominant counterparts (Bhojwani and Razdan 1996).

## 7.5 Obtaining New Genotypes with Alien Chromosomes

The technique of interspecific and intergeneric hybridization can be combined with anther culture techniques (Thomas et al. 2003) for obtaining new genotypes with alien chromosomes. Thus new genotypes with various reconstructed chromosome complements can be obtained after their successful chromosome doubling.

## 7.6 Genetic Manipulation

As microspore culture is a single cell system, it makes selection at the single cell level possible and, furthermore, offers new prospects for genetic manipulation like mutagenesis and transformation. Direct gene transfer by microinjection offers the possibility of transgenic plant formation by using isolated pollen culture having high regeneration efficiency (Kasha and Maluszynski 2003). Moreover, if transgenes can be incorporated into the haploid microspore genome prior to DNA synthesis and chromosome doubling, the doubled haploids may also be homozygous for the transgenes. Thus isolated microspores not only provide a good target for bombardment, but also are readily amenable to transgene in vitro selection. Jahne et al. (1994) were the first to achieve plants homozygous for the transgenes using biolistic bombardment of barley microspores.

## 7.7 Genomics

Doubled haploids play a vital role in genomics, especially, in the integration of genetic and physical maps, thereby, providing precision in targeting candidate genes

(Kunzel et al. 2000; Wang et al. 2001). Doubled haploids, combined with marker-assisted selection, provide a short-cut in backcross conversion, a plant breeding method for improving an elite line defective in a particular trait (Toojinda et al. 1998). Expressed sequence tags may help in identification of genes that determine any trait of interest.

## 8 Conclusion

The haploid-derived true breeding lines provide a rapid means of achieving homozygosity, thereby speeding up the usually cumbersome and protracted conventional breeding methods for crop improvement. Today, haploids and doubled haploids have been reported in over 200 plant species (Forster et al. 2007), however, less than 10 % of these reports encompass tree species. Thus much work needs to be done in the field of in vitro haploid production. This may be feasible only with the development of novel genotype-independent methods through the study and improvement of existing protocols and by obtaining a better understanding of the haploid induction process, especially of the two main developmental switches: the induction of the male/female gametophyte to undergo division, and subsequent occurrence of embryogenesis. The recent spurt in the development of ultra high throughput technologies of advanced genomic, transcriptomic, proteomic and imaging tools hold great promise for identification and analysis of genes that might be playing important roles in the haploid induction process. This will immensely help in the understanding of these processes and towards development of highly efficient protocols for production of haploids, resulting in the overall improvement of highly desirable and recalcitrant plant species. Through this chapter, we intend to provide a clear and simple overview of haploidy with the purpose to stimulate interest among scientists working in the related area and the potential application of the various in vitro techniques used in haploid production intended for crop improvement.

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