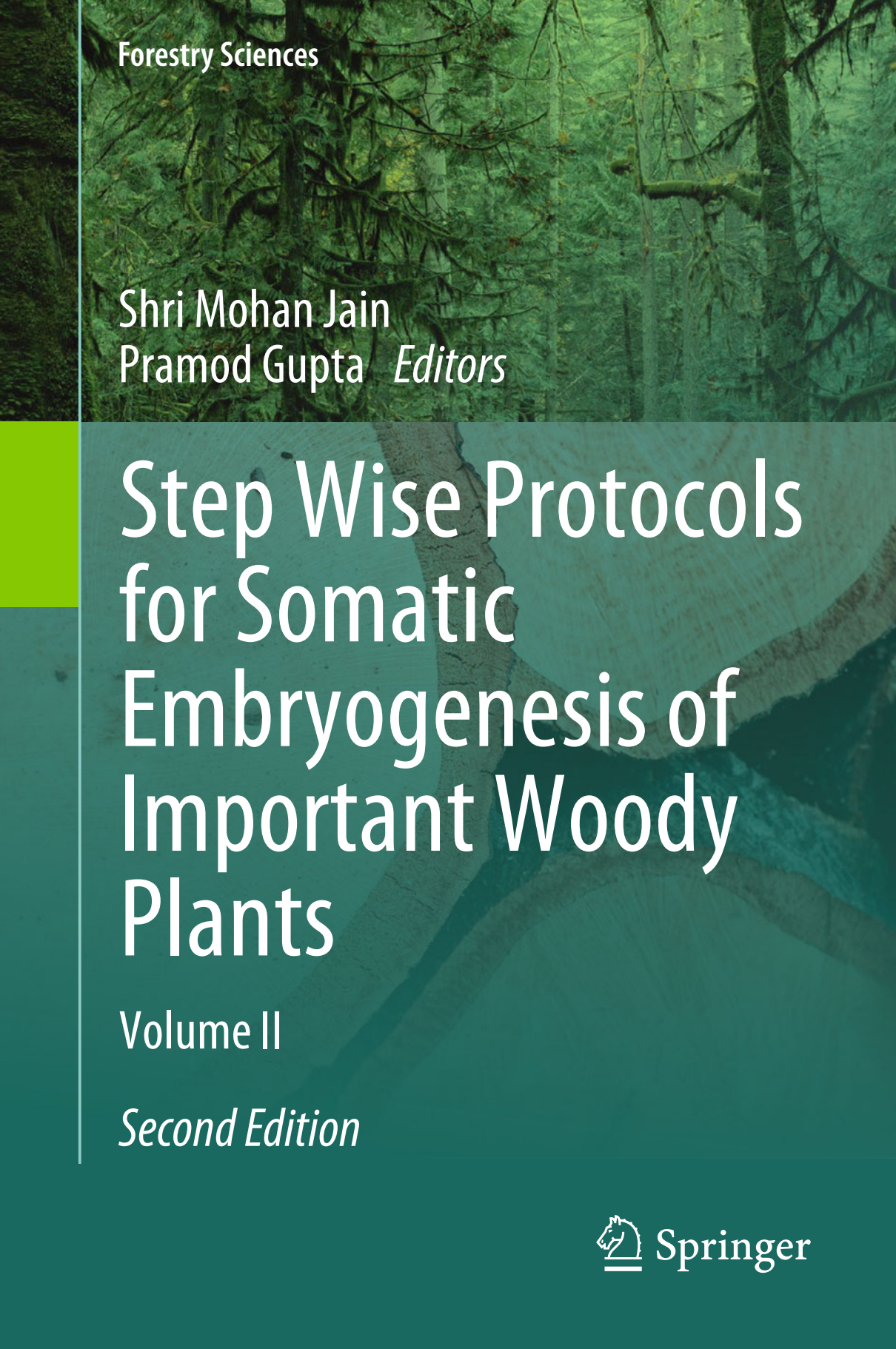
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Chapter 26
Haploid Embryogenesis in Tea

R. Bajpai and R. Chaturvedi

26.1 Introduction

Plants have been a boon to mankind, from serving as a source of food and energy for ever increasing world's population to generating employment to millions of people and being hub of potentially beneficial medicinal compounds (Namita et al. 2012). Thus, considerable efforts have been made to improvise the existing farming practices and to obtain new varieties with high quality traits that could meet the demands of the ever increasing population (Olmedilla 2010). Genetic improvement in commercially important woody perennials, such as Tea, Neem, Citrus, Populous etc., including grain crops, such as wheat, rice, rye, barley, is nearly impossible following traditional breeding approach, owing to their long reproductive cycle, self-incompatibility, high inbreeding depression and extreme heterozygous nature (Germana 2006, ; Srivastava and Chaturvedi 2008). Producing genetically modified (GM) crops using transgenic technique has been another option to achieve value added crop improvement but regulatory measures involved in propagation of GM crops restricts their usage (Comai 2014). Thus, in vitro haploid plant production serves as a legitimate solution to overcome the shortcomings of conventional breeding and, thereby, to develop genetically stable elite clones with high quality traits (Islam and Tuteja 2012; Mishra et al. 2017).

Haploids may be defined as sporophytic plants with gametic chromosomal constitution (Palmer and Keller 2005; Germana 2011; Shen et al. 2015). Doubling of haploid chromosomes, using chemical mutagens, results in 100% homozygosity in a single generation (Hazarika et al. 2013; Dwivedi et al. 2015). It proved to be the shortest possible route of attaining pure breeding lines, which otherwise requires

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several generations of selfing (Seran 2007; Srivastava and Chaturvedi 2008; Shen et al. 2015; Mishra et al. 2017) using conventional approaches.

Nature bestows plants with a life cycle that alternates between two sequential phases, the dominant sporophytic (2n) phase involving formation of numerous haploid spores (n) as a result of meiosis. These haploid spores undergo mitotic divisions forming multicellular haploid gametophyte where each cell is haploid. The gametophytic phase on the other hand, involves formation of gametes by the process called mitosis, whereby this multicellular haploid gametophyte produces male gametes (sperms) and female gametes (eggs). The gametes (male and female) unite together and give rise to diploid zygote i.e. a sporophyte (2n), repeating the sporophytic phase (Germana 2011; Hazarika et al. 2013) (Fig. 26.1). Plants with

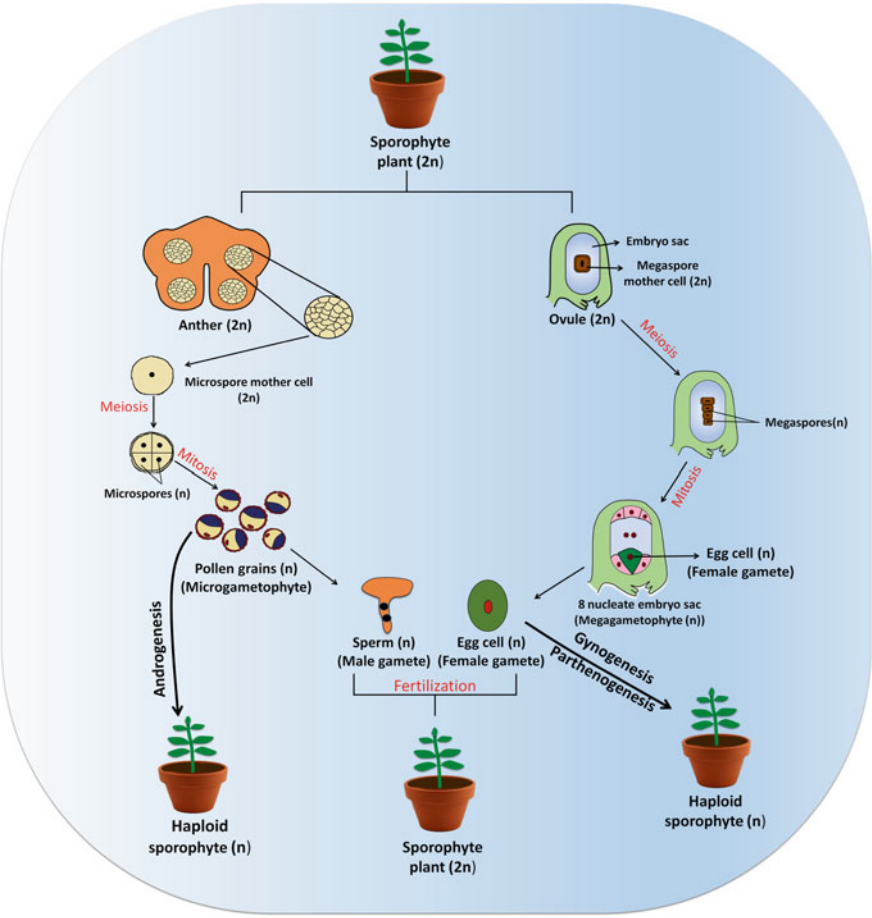


Fig. 26.1 Development of male and female gametes and their involvement in haploid plant development

gametophytic constitution (n) are sterile in nature and are considered to have reduced size compared to their diploid counterparts (Bhojwani and Razdan 1996). The breakthrough discovery of naturally occurring haploid plants in *Datura stramonium* L. (Blackslee et al. 1922), followed by remarkable journey of obtaining haploids through anther cultures in *Datura annoxia* (Guha and Maheswari 1964, 1966), induction of haploid embryos via interspecific crosses in barley (*Hordeum vulgare* L.) (Kasha and Kao 1970) and from unfertilized cells of female gametophyte (embryo sac) in barley (San Noeum 1976), revolutionised the definition of haploid plants and proved their utility in plant breeding.

Spontaneous occurrence of haploids is a rare phenomenon (0.001–0.1%) in nature, which limits their utility in breeding programs (Bhojwani and Razdan 1996; Hazarika et al. 2013; Karasawa et al. 2015). Therefore, adopting artificial methods of haploid induction using in vivo techniques (parthenogenesis, polyembryony, distant hybridization using chromosome elimination technique) and in vitro methods, by inoculating immature explants, like anthers bearing haploid microspores (n, microgametophyte, process referred as androgenesis) and ovary/ovules bearing unfertilized female gamete (n, egg cell; process referred as gynogenesis) into culture medium, becomes a necessity to uplift the agricultural productivity. In vitro methods of haploid production are the most preferred of all existing approaches and have been reported in more than 250 plant species till date, where most of them are legumes (Segui 2010; Dunwell 2010). However, it has gained limited success in woody plants, such as tea, owing to its recalcitrant nature (Mondal et al. 2004; Hazarika et al. 2013; Mishra and Goswami 2014; Mishra et al. 2017).

Tea is an economically important medicinal plant and a source of beverages consumed throughout world next to water (Katiyar and Mukhtar 1996). High demand and low rate of production necessitates development of high yielding elite varieties in tea (Akula et al. 2000). More than a decade of strenuous efforts, varietal improvement within the existing varieties of tea were attained following conventional breeding approaches, but, have been narrowed down due to the long reproductive cycle, extremely heterozygous nature and intense inbreeding depression within cultivated taxa (Mondal et al. 2004). In such a scenario, developing haploid plants followed by their diploidization will not only shorten the long gestation period but, will also aid in development of genetically stable clones. Current chapter represents a successful protocol of obtaining complete haploid plantlets in tea.

26.2 Methods for Haploid Inception

Many research groups attempted generation of haploids using in vivo and in vitro methods and the choice vary from crop to crop (Magoon and Khanna 1963; Kasha 1974; Maluszynski et al. 2003; Xu et al. 2007; Touraev et al. 2009; Dunwell 2010). In majority of the crops microspore mediated (androgenic) haploid development was obtained, while in case of barley distant hybridization was a method of choice.

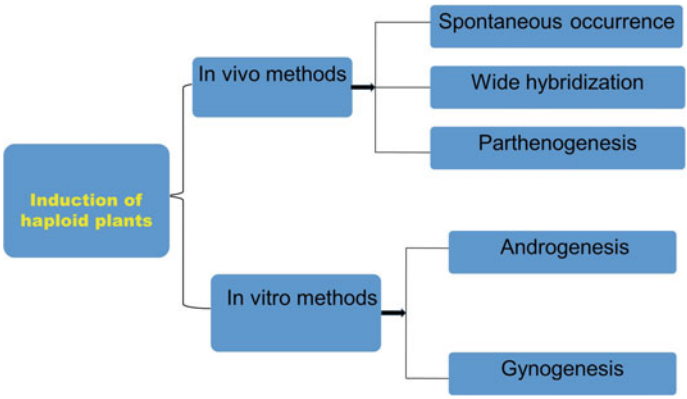


Fig. 26.2 Methodologies of haploid induction

In a few other plants, gynogenesis or parthenogenesis in unfertilized female gametophyte resulted in haploids. Occasionally, spontaneous occurrence of haploids was also reported (Fig. 26.2).

26.2.1 In Vivo Haploid Induction

This technique involves the spontaneously occurring haploids, haploids induced by wide hybridization and by parthenogenesis.

26.2.1.1 Spontaneous Occurrence of Haploids

The rare occurrences of haploids are noticed in nature but at low frequency of 0.001–1% (Bhojwani and Razdan 1996). After the discovery of first spontaneously occurring haploid plants in *Datura* with the cytological evidences of their existence by Dorothy Bergner in 1921 (Blackslee 1922), several other reports followed. Although spontaneously occurring haploids have been used for producing DH lines, but the frequency of homozygous development was low as compared to other methods (Palmer and Keller 2005).

26.2.1.2 Wide Hybridization

It is an effective method of achieving haploidy in plants via interspecific or intergeneric crosses between the cultivated species and their wild relatives. In addition to it, hybridization facilitates creation of new varieties through transfer of agronomically important characters (Zenkteler and Nitzsche 1984). This process is

prevalent mostly in cereal crops, such as wheat, rye and barley (Froster et al. 2007). In most of the cases, haploid formation precedes fertilization of female gametophyte via pollinators from distantly related species or an intergeneric plant, resulting in viable embryo and functional endosperm but in certain cases the endosperm may be absent or abnormal (Laurie and Bennet 1988). The other crossing over method, known as *Hordeum bulbosum* method, was described by (Kasha and Kao 1970) after haploid plants in *H. vulgare* were achieved during its cross with *H. bulbosum* following selective elimination of chromosome of the latter. Chromosomal elimination could be referred as an extension of wide hybridization process where the nuclei of the zygote formed after cross inherits the genome from both parents but subsequently gets eliminated; the elimination is a selective elimination (Dunwell 2010; Comai 2014). Generation of haploids using hybridization process has certain advantages over in vitro methods, which are sometimes encountered with albinism in microspore/egg cell derived haploid embryos and may also cause mortality. On the other hand, the wide hybridization method has limitations due to its dependency on simultaneous flowering in both the parents involved in crosses (Froster et al. 2007).

26.2.1.3 Parthenogenesis

Development of an embryo from an egg cell without involving the fusion of egg cell with the male/sperm nuclei is termed as parthenogenesis. This method is rarely observed in nature (Dunwell 2010), but could be induced via pollination through irradiated or inactive pollen or by using chemicals (Khush and Virmani 1996).

26.2.2 In Vitro Methods of Haploid Production

In vitro development of haploid plants is achieved by triggering immature male or female gametophyte with certain stimulus, generally a temporary stress treatment that diverts their mode of development from gametophytic to sporophytic giving rise to haploids (Srivastava and Chaturvedi 2008; Germana 2011). The process of gametogenesis could be termed as androgenesis or gynogenesis on the basis of gametic explants (male/female) chosen for culture initiation. These haploid explants undergo the gametic embryos development, directly or preceded by callusing. The embryos germinate into complete haploid sporophytic plants. Both the process have been reported to develop haploids efficiently, but, androgenesis is preferred over gynogenesis due to the presence of several anthers with indefinite number of haploid pollen grains within a flower in contrast to the presence of single ovary with limited number of ovules (Srivastava and Chaturvedi 2008; Srivastava et al. 2011; Soriano et al. 2013) and a single haploid egg cell inside each ovule. A brief description of both the methods is described in the subsequent sub-sections followed by a detailed protocol on tea haploid embryogenesis as case study.

This will provide a vision on the implementation of in vitro haploid technology and its intervention to generate homozygous diploid plants (pure breed lines) even in strictly cross pollinating tree species where conventional methods are difficult to implement.

26.2.2.1 Androgenesis

In androgenesis the male gametophyte (pollen grain/microspore) undergoes transition from its normal gametophytic mode of development to sporophytic mode under the influence of a stress pre-treatment that could be either a physical or chemical treatment (Srivastava and Chaturvedi 2008). The mechanism of androgenic haploid production (Fig. 26.3) involves either detachment of intact entire immature anthers from stamens or isolation of microspores from anthers by methods, like centrifugation, simple stirring, squeezing, and inoculating them into

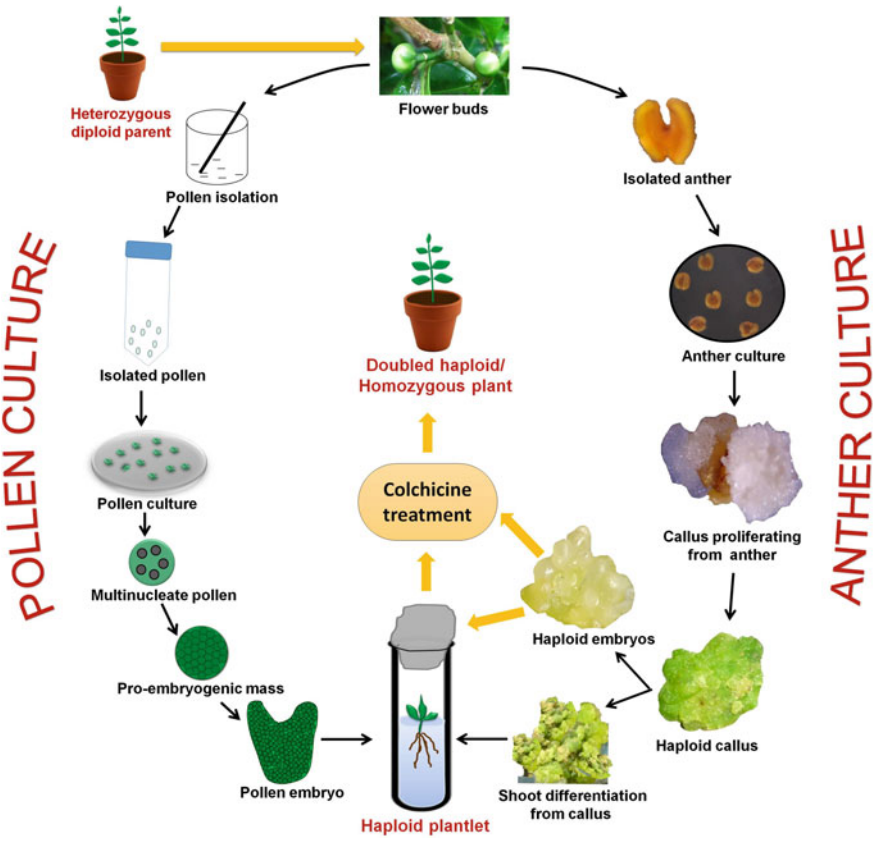


Fig. 26.3 Androgenesis mediated production of doubled haploid plants

the culture medium (Ferrie and Caswell 2011). Anther culture has been reported to be a simple, one step protocol for achieving pure breeding lines (Chaturvedi et al. 2003; Hazarika et al. 2013). Anthers in medium provide better growth conditions to the microspores and simultaneously prevent them from being directly exposed to the stress (Soriano et al. 2013). The culture of isolated microspores on the other hand is complex and requires more skill and highly enriched medium for androgenic induction (Germana 2011; Mishra and Goswami 2014).

Anther culture has been reported as an easier and more efficient method of producing haploid plants (Hofer 2004; Germana 2011), however it has certain drawbacks, such as delayed response time due to the presence of anther wall, which need to be burst open to release microspores (Foster et al. 2007; Soriano et al. 2013) and the presence of mixed stages of microspores development. The other limitation with anther culture is simultaneous proliferation of the somatic cells of the anther wall along with the microspore proliferation, which results into chimera formation. Thus crucial observation of the cultures has to be made to abstain any callusing from anther wall (Bhojwani and Razdan 1996). The problem of chimera formation during anther culture could be avoided, if the haploids would be induced from isolated microspores (Germana 2005; Ferrie and Caswell 2011; Germana 2011). Androgenic haploids have been obtained in more than 200 genera. In tea, however no report on complete plant development via androgenesis was available until before the report by Mishra et al. (2017).

26.2.2.2 Gynogenesis

Is an alternative strategy of attaining in vitro haploids in plants when anther culture is not successful and encountered with recalcitrance or white plant production (Bohanec 2009). Immature ovaries or ovules or unopened flower-buds bearing unfertilised egg cell are generally used to obtain gynogenic haploids. Families such as *Chenopodiacea*, *Liliaceae* and *Cucurbitaceae,* where androgenesis seems difficult, or in certain plants with male sterility or those with dioecious nature, gynogenic cultures serves as best possible route for achieving haploid plants (Thomas et al. 2000; Bhatt and Murthy 2007; Chen et al. 2010). The technique has equally been used in crops, like maize, rice, barley, where haploids have successfully been attained through anther or microspore culture (Sita 1996). However, this technique is considered to be very cumbersome and less efficient method for induction of haploids (Palmer and Keller 2005; Froster et al. 2007) though it favours genetic stability of doubled haploids and produces green plant production as compared to androgenesis (Palmer and Keller 2005). No literature on gynogenesis in tea was available until before Hazarika and Chaturvedi (2013). The transverse sections of the unpollinated ovaries were used in the author's laboratory to induce gynogenic haploids in tea (*Camellia sinensis* (L.) O. Kuntze) (Hazarika et al. 2013).

26.3 In Vitro Haploid Induction in Tea—*A Case Study*

The methodology on haploid plant development via androgenic embryos in tea has been adopted from the author's laboratory. Certain crucial parameters involved in successful regeneration of a haploid plantlet are elaborated in the below subsections.

26.3.1 Explant Selection

Fresh flower-buds, measuring 4 mm in diameter (Fig. 26.5a), from tea (*Camellia assamica* ssp. *assamica* (Masters) plantations were plucked in the early morning hours, between 6 am and 7 am, during the months from October to December. Buds were dissected and anthers were taken to determine the correct stage of microspores, at early-to-late uninucleate stages, using acetocarmine. Later, 4 mm sized buds were routinely used to initiate anther cultures.

26.3.2 Inception of Aseptic Cultures

Immature flower buds of appropriate size, as mentioned in above section, were collected from field. The selected buds were taken inside the laminar-air-flow cabinet and surface sterilized with 0.8% (v/v) sodium hypochlorite for 7 min, after which they were washed at least three times with sterile distilled water. Following sterilization, the buds were dissected under the stereo-microscope with the help of fine needles and forceps. Any of the anthers, if damaged were discarded. Twenty anthers from a single bud were cultured into pre-sterilised Petri plates (60 × 15 mm) containing 10 ml of callus induction medium mentioned in section below. After inoculation the plates were sealed with Parafilm and subjected to different conditions of light and temperature treatment mentioned in ensuing sections.

26.3.3 Preparation of Culture Medium

Murashige and Skoog (1962, MS) medium in combination of different growth regulators and certain additives was used to initiate androgenic cultures in tea. The concentration of the stock solutions used for preparation of MS medium is described below:

- Macronutrient stock solution (10X); Micronutrients (20X); Iron stock (20X) and Vitamins (20X).
- All the growth regulators used during the experiment were at the concentration of 1×10^{-3} M. GA_3 (Gibbrellic acid) used at certain stages in medium was filter sterilised with 0.2 μM filter and added to medium after autoclaving.
- Additives like L-glutamine and L-serine were prepared freshly every time, filter sterilised and added to the medium following autoclaving.

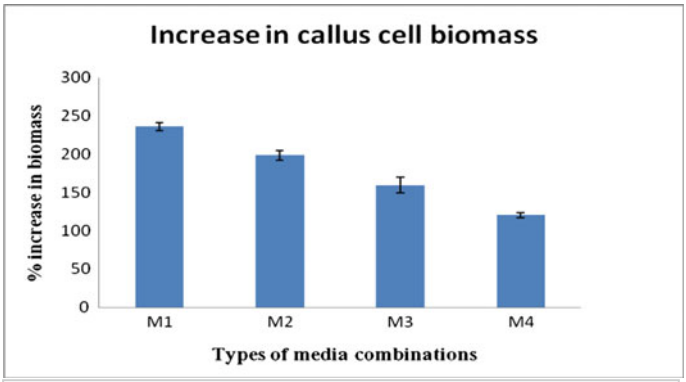
The stock solutions, growth regulators and the additives were refrigerated at 4 °C until use. Sucrose and myoinositol were added afresh while the medium was prepared. The pH of the medium was adjusted to 5.8 using 1 N NaOH or 1 N HCl as per requirement. Agar at a concentration of (0.8%) was used to solidify the media. The medium was autoclaved for 15 min at 121 °C at 15 psi and finally poured into the vessels (Petriplates or test-tubes used for experiment).

26.3.4 Culture Condition

Stress conditions, such as high temperatures, cold shock and sucrose starvation, have been reported to promote callusing in anthers (Chaturvedi et al 2003; Srivastava and Chaturvedi 2008). Therefore, following published reports on androgenesis in author's laboratory, the tea anthers in culture medium were exposed to temperature pre-treatment, either cold (5 °C) or heat (33 °C shock) in completely dark conditions. The incubation of cultures at 25 °C in light and dark served as control experiments. After the initial exposure to dark conditions for 5 days, all cultures were maintained at 25 ± 2 °C, humidity of 50–60% under (16/8 h) photoperiod. Cultures were observed regularly to monitor any changes in their morphology.

26.3.5 Callus Induction and Multiplication

The isolated anthers (Fig. 26.5b) were inoculated on MS (Murashige and Skoog 1962) medium supplemented with auxins, like 2,4 dichlorophenoxyacetic acid (2, 4-D), α-naphthalene acetic acid (NAA) and cytokinins, such as 6-furfurylaminopurine (Kinetin) and 6-benzylaminopurine (BAP), at different concentrations together with L-glutamine and L-serine that acted as additional sources of nitrogen in the medium. Two different carbon sources (glucose and sucrose) at varying concentration between 3%, 6% and 12% were tested to identify which of them induces callusing faster. Different media combinations were tested for haploid callus induction. Maximum callusing (96%) from inside anther locules was achieved on callus induction medium consisting of MS with glucose (6%), 2, 4-D (5 μM), Kinetin (5 μM), L-glutamine (800 mg/L) and L-serine (200 mg/L).



M1: MS + sucrose (3%) + 2,4-D (5 μM) + Kinetin (5 μM) + L-glutamine (800 mg/L) + L- serine (200 mg/L)

M2: MS + glucose (3%) + 2,4-D (5 μM) + Kinetin (5 μM) + L-glutamine (800 mg/L) + L- serine (200 mg/L)

M3: MS + sucrose (3%) + 2,4-D (3 μM) + TDZ (18 μM)

M4: MS + sucrose (3%) + 2,4-D (1 μM) + NAA (1 μM) + BAP (5 μM)

Fig. 26.4 Increase in callus cell biomass

Profusely growing light green callus (Fig. 26.5c) was attained on this medium. But, a significant increase in cell biomass proliferation was observed (Fig. 26.4) when glucose in the induction medium was substituted with sucrose (3%).

26.3.6 Callus Differentiation and Embryo Maturation

Highly proliferating callus on maintenance medium MS + Sucrose (3%) + 2,4-D (5 μM) + Kinetin (5 μM) + L-glutamine (800 mg/L) and L-serine (200 mg/L) showed variations in its morphology and developed as nodulated callus after 8 weeks (2 subcultures of 4 week each on same medium). The callus with nodules showing signs of regeneration was transferred to MS + BAP (10 μM) + GA_3 (3 μM) + L-glutamine (800 mg/L) and L-serine (200 mg/L) medium where it developed into embryos. Maturation of asynchronously growing embryos showing various stages of embryo development (Fig. 26.5d) was attained when the

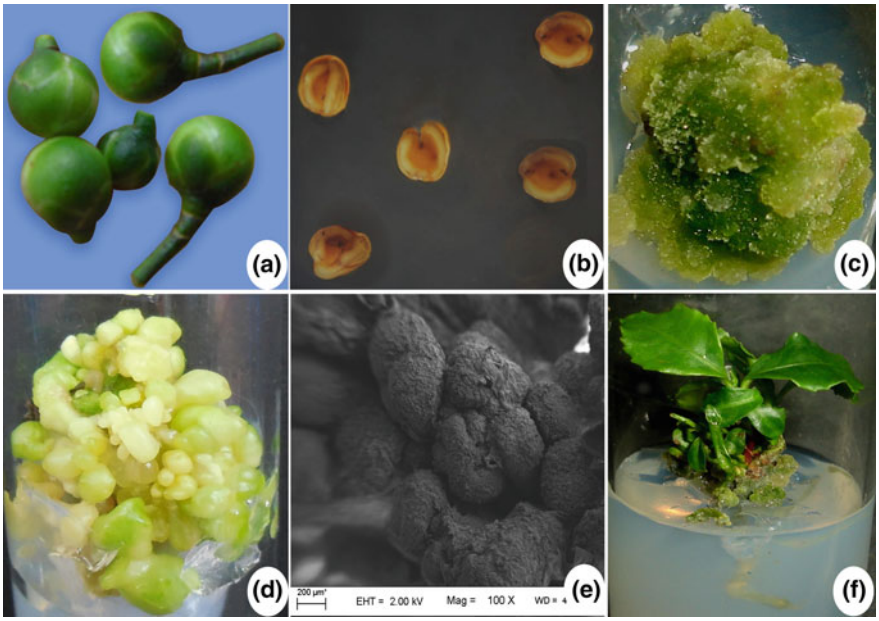


Fig. 26.5 Anther culture in tea. **a** Flower buds bearing anthers at correct stage of microspores **b** Isolated anthers inoculated on callus induction medium **c** Highly proliferating callus induced from anthers in culture on induction medium. **d** Asynchronously growing cluster of haploid embryos at different stages of development. **e** Same as **d**, scanning electron micrograph image of embryos in cluster. **f** Complete developed haploid plantlet

concentration of growth regulators and supplements in the embryogenesis medium were reduced by 10 folds. The structure of embryos obtained was determined through scanning electron micrographic technique (Fig. 26.5e).

26.3.7 Embryo Germination and Development of Haploid Plantlet

The germination of embryos into complete haploid plantlets (Fig. 26.5f) was attained when the embryos were transferred to MS + BAP (10 µM) + IBA (1 µM) + GA_3 (0.5 µM) + L-glutamine (80 mg/L) and L-serine (20 mg/L) medium. Further growth of the plantlets was attained when the concentration of the major salts in the above mentioned germination medium was reduced to half (1/2). Cytological squash preparation from root-tips of the regenerated plants and the flow cytometric analysis of their leaves, discussed in section below, confirmed the haploid status of the regenerated plants.

26.4 Factors Affecting Haploid Embryogenesis

The in vitro haploid induction is governed by certain crucial factors, such as the physiological condition of the donor plant, genotype, stage of the explant, stress pre-treatments to explants, composition of the medium and culture conditions used to initiate cultures.

26.4.1 Physical Condition of the Donor Plant

Successful induction of gametic embryogenesis greatly relies on availability of flower-buds from diseases free plants. The quality of light and temperature conditions provided to the donor plants affect the responses from the explants, anthers/ovaries (Ferrie and Caswell 2011). Growing donor plants in green house would reduce the contamination that is often high in experimental material collected from field (Ferrie and Caswell 2011). Age of the donor plant plays a vital role in promoting gametogenesis (Maheswari et al. 1982; Jacquard et al. 2006; Mishra and Goswami 2014).

26.4.2 Genotype of Anther/Ovary Donor

Numerous studies revealed that genotype of the donor plant is a key player in deciding the efficiency of embryogenesis (Olmedilla 2010). The response of different cultivars within same species varies in terms of embryo induction ability; for example in *Brassica napus*, Topas, DH 4079 cultivars are highly embryogenic while the other members of the same family i.e. *Brassica olerecea* ssp *italica*, Shogun is the variety with high embryogenic potential (da Silva Dias 2001). The exact mechanism as to how the genotype influences the rate of embryogenesis is not yet clear. However, several studies conducted on micropspore embryogenesis reveal that the frequency of normal green plant formation via anther culture technique varies with genotype and is genetically controlled by additive effect of nuclear genes whose expression is largely influenced by environmental factors and also by certain cytoplasmic factors (Larsen et al.1991; Datta 2005; Olmedilla 2010).

26.4.3 Stage of the Explant

Stage of the explant is one of the most crucial factors to induce haploid embryogenesis (Bhojwani and Razdan 1996; Olmedilla 2010). Touraev et al. (1997) indicated that microspores are liable to vary their developmental pattern within

wide developmental window. The immature anthers with early-to-late uninucleate stages of microspores have been suggested to be most responsive stage for attaining androgenic haploids for example in Barley (Clapham 1971); *Azadirachta indica* (Chaturvedi et al. 2003; Srivastava and Chaturvedi 2008; Srivastava et al. 2011); *Eribotrya japonica* (Germana 2006); *Camellia assamica* (Mishra et al. 2017). In a few others, like *Arabidopsis thaliana* (Gress hoff and Doy 1972), *Albizzia lebbek* L (Gharyal et al. 1982), *Camellia assamica ssp assamica* (Gharyal et al. 1983), Maize (Gaillard et al. 1991), only the late uninucleate stage of microspore was responsive for haploid induction. Similarly, determining the correct stage of embryo sac appears to be a crucial parameter while initiating haploid generation through gynogenesis. It is quite difficult to identify the stage of embryo sac, but, this could be done through histological sections of the ovule (Hazarika and Chaturvedi 2013).

26.4.4 Composition of the Medium

The culture medium not only acts as the source of nutrient but also routes the development of embryos (Datta 2005). There is no subscribed medium for induction of haploids via androgenesis or gynogenesis since the nutritional requirement of each plant species varies (Mishra and Goswami 2014). Thus, supplementing the medium with proper concentrations of mineral salts, carbon source, suitable growth regulators and certain additives regulates the fate of haploid explants in culture (Olmedilla 2010). Usually a combination of cytokinin and auxin has been found to successfully induce haploids in woody plants (Nair et al. 1983), using BAP in combination with 2,4-D at varying concentrations resulted in haploid callus induction in Neem (Chaturvedi et al. 2003), *Allium* spp. (Alan et al. 2003), *Vitis labruscana* (Nakajima et al. 2000), *Camellia sinensis* (L.) O Kuntze (Hazarika and Chaturvedi 2013); *Camellia assamica* ssp *assamica* (Masters) (Mishra et al. 2017). Optimization of type and concentration of carbon source is another vital factor that regulates androgenic response (Bhojwani and Razdan 1996). Sucrose is mostly the preferred carbon source in nutrient media for in vitro culture (Mishra and Goswami 2014), but the concentration at which it is used varies in different plant species (Olmedilla 2010). Sucrose at higher concentrations up to 12% was used for callus induction from anthers in Neem, but for further regeneration of callus into embryos a lower concentration of sucrose 3% was suitable (Chaturvedi et al. 2003; Srivastava and Chaturvedi, 2008). Similar observation was made in *Camellia assamica* ssp *assamica* (Masters) (Mishra et al. 2017); both carbon sources, glucose and sucrose, were used in the range of 3–12% but, best response in terms of callus induction from anther locules was achieved when (6%) glucose was used while higher concentrations of carbohydrate showed inhibition of callus proliferation. Following this, embryogenesis from calli and germination from these haploid embryos was attained on MS medium with 3% sucrose content (Mishra et al. 2017).

26.4.5 Stress Pre-treatment for Culture Initiation

Application of stress treatments is a pre-requisite for inducing haploids as it facilitates reprogramming of gametic explants and deviates their mode of development from gametophytic to sporophytic (Chaturvedi et al. 2003; Olmedilla 2010; Germana 2011; Karawasa et al. 2016; Mishra et al. 2017). The kind of stress pre-treatment varies from plant to plant; it could be temperature, osmotic stress, gamma irradiation, addition of ethanol, heavy metal and hypertonic environment (Islam and Tuteja 2012) or a combination of them. Cold temperature pre-treatment provided to the anthers promoted androgenesis in Dhatura (Sapory and Maheswari 1976), Wheat (Hu and Kasha 1997), Neem (Chaturvedi et al. 2003; Srivastava and Chaturvedi 2008); Tea (Mishra et al. 2017). In a few other plants, such as *Brassica campestries* (Keller and Armstrong 1979), *Brassica napus* ssp *oleifera* (Dunwell 1985), *Brassica napus* (Custers et al. 1994), Wheat (Touraev et al. 1996), high temperature pre-treatment was used to induce haploids. Starvation conditions maintained in the medium by depriving the cultures with carbon and/or nitrogen source have also proven beneficial for inducing haploids in *Brassica campestries* (Keller and Armstrong 1979), Wheat (Touraev et al. 1996), *Quercus suber* (Bueno et al. 1997). The starvation conditions have often been applied in combination of high temperature to induce haploid plants in *Brassica compestries* (Keller and Armstrong 1979), *Quercus suber* (Bueno et al. 1997). In addition to the temperature stresses, certain reports reveal the application of osmolytes, such as maltose in enhancing haploid production in Barley (Scott and Lyne 1994, 1995), Tobacco (Touraev et al. 1996), Wheat (Touraev et al. 1996) and Fabaceae (Ochatt et al. 2009).

26.5 Screening Haploids and Doubled Haploid Plants

Spontaneous diploidization that may occur in in vitro haploid cultures and somatic cell proliferation along with pollen callusing in anther cultures may cause mixing of haploid and diploid calli. Therefore, it is very essential to screen the plants regenerated from these calli (Germana 2011). The ploidy status of the regenerated plants can be determined by methods, such as cytological squash preparation of cells, flow cytometric analysis, isoenzyme analysis and utilization of molecular markers, such as RAPD (Randomly amplified polymorphic DNA), SCAR (sequence characterized amplified region) and SSR (simple sequence repeats) (Chen et al. 1998; Bartosova et al. 2005; Srivastava and Chaturvedi 2008; Mishra et al. 2017). Ploidy of the androgenic haploid plants was determined using flow cytometry and chromosomal counting via cytological squash preparation.

26.5.1 Flow Cytometry

Fresh, young leaves from in vitro regenerated haploid plantlets and the field grown plant (control) were utilized to estimate the ploidy through flow cytometer. The leaves were chopped in nuclear isolating buffer consisting of 0.2 M Tris HCl, 4 mM $MgCl_2.6H_2O$, 2.5 mM EDTA $Na_2.2H_2O$, 86 mM NaCl, 10 mM Sodium Metabisulfite, 1.5% Triton-X-100 and 2% PVP-10. The pH of the buffer was adjusted to 7.5. The nuclear suspension thus obtained was filtered using 30.0 μm nylon net filter membrane. Rnase A and propidium iodide, both at a concentration of 50 μg/ml were added to the obtained filtrate. The prepared samples were analysed using photomultiplier voltage of the instrument. The peak position of the reference (control) was determined following which the ploidy of the unknown sample was analysed keeping the instrument settings constant. The flowcytometric analysis from leaves of in vitro regenerated haploid plant shown in (Fig. 26.6a) exhibit major G1/G0 peak at channel position 100 and a smaller G2 peak at channel position 200 while (Fig. 26.6b), is the histogram obtained from the leaves of parent plant showing major G0/G1peak at channel position 200 and a smaller G2 peak at channel 400. The flowcytometry analysis confirms haploid nature of in vitro regenerated plants.

26.5.2 Cytological Squash Preparation

Chromosome counting is one of the most commonly used, reliable method for determining the ploidy of regenerated plants (Mishra and Goswami 2014). It has been reported as a best method representing exact origin of plants (Maluszynska 2003). Cytological analysis of haploid plantlets obtained via anther culture in tea and the field grown *Camellia assamica* ssp *assamica* plant (Control) was performed. The root tips from in vitro developed haploid plantlets and the shoot tips from the field grown plant were washed with sterile distil water. The roots were then treated with 8-hydroxyquinoline and refrigerated at 4 °C for 4 h. Following this the treated roots were fixed in modified Carnoy's solution (7:3:3:1 v/v/v/v absolute ethanol: chloroform: methanol: glacial acetic acid) and kept at 4 °C for 48 h. The material was stored in 70% ethanol until use (Chaturvedi et al 2003). The material to be analysed was stained with 1% (w/v) aceto-orcein dye and 1 N HCL mixture and heated gently. It was transferred to a glass slide, covered with coverslip and squashed. The results were analysed under the Nikon 80i microscope. The cytological analysis of the root-tips from in vitro regenerated haploid plants (Fig. 26.6c) revealed the haploid chromosomal constitution as (2n = 2X = 15) which was exactly half of the chromosomal count from shoot-tip of (control) parent plant (2n = 2X = 30) (Fig. 26.6d). The study further confirms the haploid status of regenerated plants.

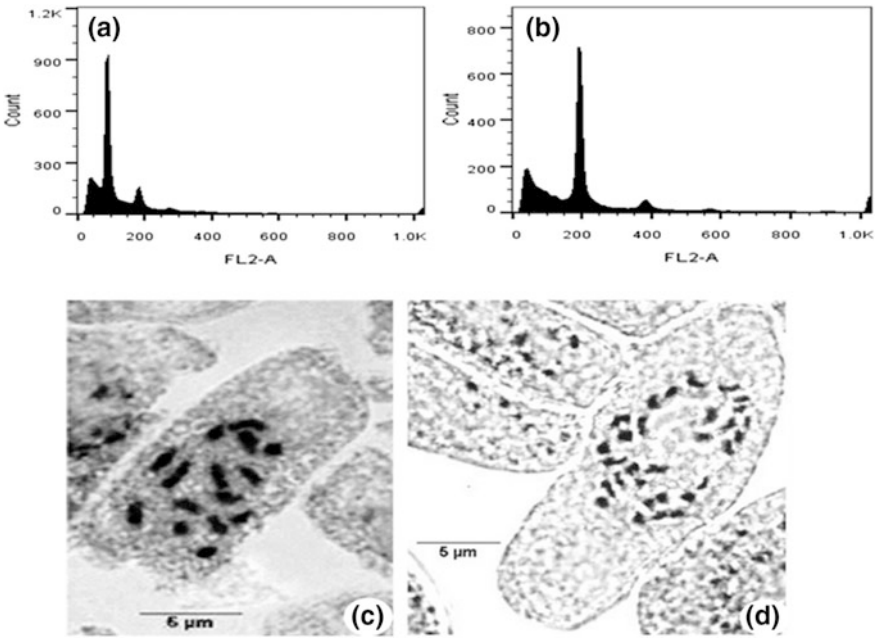


Fig. 26.6 The main objective is to determine the ploidy of the haploids and of control plants. The two methods for the ploidy analysis are (i) Flow cytometry that determines the total DNA count, and (ii) Cytological squash preparation that determines the total number of chromosomes in a given ploidy. Since the two techniques are followed for the same objective of ploidy analysis, hence, all the four figures, which belongs to this objectives are placed together and have been given the same figure number. This is as per the norms

26.6 Uses of Haploids and Doubled Haploid Plants

Haploid embryogenesis becomes a pre-requisite of the present day plant breeders providing speedy generation of pure breeding lines in heterozygous plants with long reproductive cycle (Srivastava and Chaturvedi 2008). Regeneration of doubled haploid plants would facilitate easier detection of recessive mutations at plant level which otherwise cannot be noticed in the presence of dominant alleles (Germana 2011; Mishra et al. 2017) in heterozygous diploid plants. The totipotent nature of microspores has been recognised as a readily available single origin target that could be utilised for transformation (Touraev et al. 1997). The genetic stability within the doubled haploid population makes it easy to mark QTLs within them and, thus, to study phenotype of plants in different environmental locations (Datta 2005). Although QTLs are of great importance in trait related studies, but gathering information about the genes for those traits is a tricky process. Use of recombinant chromosome substitution lines in such instances provide accurate mapping for both

QTLs and doubled haploids towards specific target (Thomas et al. 2000). Doubled haploid production is of great benefit as the seeds produced by them could be grown again and harvested for faster analysis using marker system (Maluszynski 2003). Generation of aneuploids during anther culture in certain species, such as rice, neem, help in studying extra chromosome or the chromosomal behaviour and thereby aid in genome construction (Datta 2005). The developed doubled haploids may overcome the limitations of conventional breeding methods and lead to the generation of elite homozygous breeding lines in strictly cross-pollinating, heterozygous plants with high inbreeding depression and, thereby, to mediate their overall genetic improvement (Mishra and Goswami 2014; Mishra et al. 2017).

26.7 Conclusion

Potential application of gamete biology in the field of plant breeding has revolutionised its status and has led to the development of genetically stable populations in variety of plant species, including vegetables, cereal crops and other difficult to cultivate woody perennials. Haploid embryogenesis is a cost effective, rapid method of attaining pure breeding lines with increased crop productivity in a short span of time. This book chapter summarises widely available literature on haploid induction and provides details on crucial parameters that needs to be addressed while implementing haploidy in plants. Although molecular aspects related to gametogenesis and analysis of regenerated doubled haploid populations has covered long journey. However, exact mechanism behind reprogramming of microspores to switch over from gametogenesis to embryogenesis has yet to be deciphered.

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