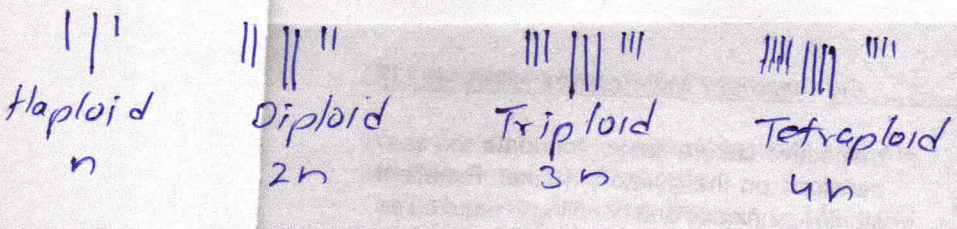
In flowering plants, the sporophyte comprises the whole multicellular body except the pollen and embryo sac

In general gametophytic — haploid — single set

In general sporophytic — diploid — double set from both parents

Polyploidy — Having more than two paired sets of chromosomes



Haploid n | Diploid 2n | Triploid 3n | Tetraploid 4n

Microspore → Immature pollen grain

8

In vitro Production of Haploids

The term haploid refers to those plants which possess a gametophytic number of chromosomes (single set) in their sporophytes. The interest in haploids stems largely from their considerable potential in plant breeding, especially for the production of homozygous plants and in their studies on the detection of mutations.

The significance of haploids in the fields of genetics and plant breeding has been realized for a long time. However, their exploitation remained restricted because of low frequency (.001–.01%) with which they occur in nature. Spontaneous production of haploids usually occurs through the process of apomixis or parthenogenesis (embryo development from an unfertilized egg). Artificial production of haploids was attempted through distant hybridization, delayed pollination, application of irradiated pollen, hormone treatment and temperature shock. However, none of these methods was dependable and repeatable. It was in 1964 that Guha and Maheshwari reported the direct development of embryos and plantlets from microspores of *Datura innoxia* by the culture of excised anthers. Later, Bourgin and Nitsch (1967) obtained complete haploid plants of *Nicotiana tabacum*. Since then, anthers containing immature pollen have been successfully cultured for a wide range of economically important species.

Haploids may be grouped into two broad categories: (a) monoploids (monohaploids), which possess half the number of chromosomes from a diploid species, e.g. maize, barley; and (b) polyhaploids, which possess half the number of chromosomes (gametophytic set) from a polyploid species, e.g. potato, wheat. Here, the general term haploid is applied to any plant originating from a sporophyte (2n) and containing (n) number of chromosomes.

Haploid production through anther culture has been referred to as androgenesis while gynogenesis is the production of haploid plants from ovary or ovule culture where the female gamete or gametophyte is triggered to sporophytic development.

ANDROGENIC METHODS

The androgenic method of haploid production is from the male gametophyte of an angiosperm plant, i.e. microspore (immature pollen). The underlying principle is to stop the development of pollen cell whose fate is normally to become a gamete, i.e. a sexual cell, and to force its development directly into a plant. Haploids can be obtained by the culture of excised anthers and culture of isolated pollen (microspore). Here it is worthwhile to explain the structure of anther and development of pollen *in vivo* (Fig. 8.1). An angiosperm stamen consists of a filament, connective tissue and anther. A typical anther shows two anther lobes and each lobe possesses

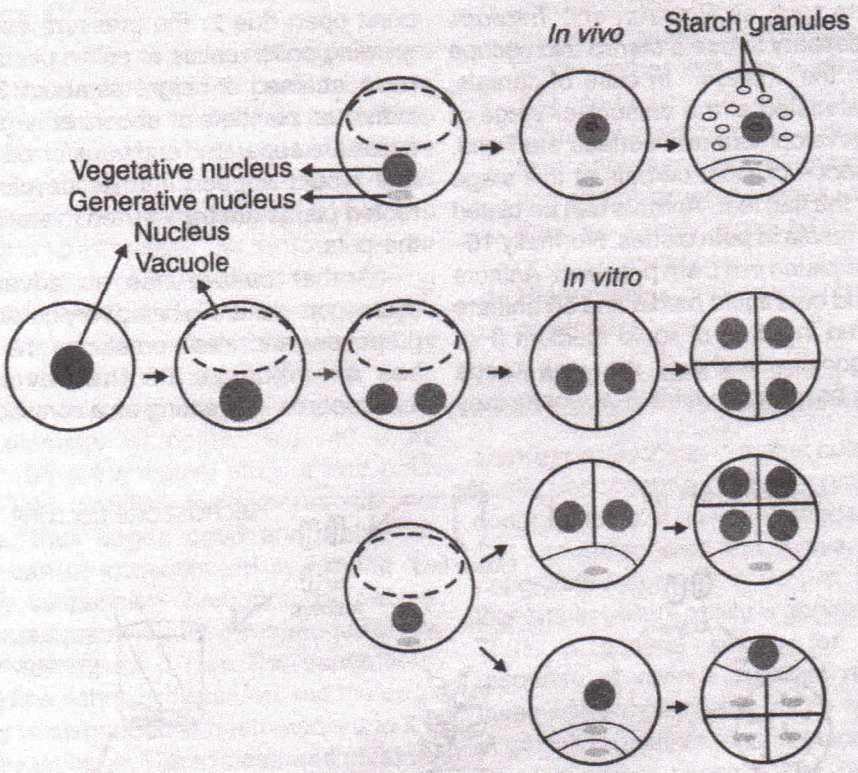


Fig. 8.1 Diagrammatic representation showing various modes of division of the microspores under *in vivo* and *in vitro* conditions.

two microsporangia or pollen sacs. During microsporogenesis in a young anther, there are four patches of primary sporogenous tissue, which either directly function as pollen mother cells (PMCs) or undergo several divisions. The PMCs form pollen tetrads by meiosis and when the callose wall of tetrad dissolves, the four pollen grains or microspores are liberated. The newly released microspore is uninucleate, densely cytoplasmic with the nucleus centrally located. As vacuolation occurs, the nucleus is pushed towards the periphery. At the first division or first pollen mitosis, the microspore nucleus produces a large vegetative and a small generative nucleus. The second pollen mitosis is restricted to generative nucleus and forms two sperms and takes place in either the pollen or pollen tube.

Anther culture

The technique of anther culture is rather simple, quick and efficient. Young flower buds with immature anthers in which the microspores are confined within the anther sac at the appropriate stage of pollen development are surface sterilized and rinsed with sterile water. The calyx from the flower buds is removed by flamed forceps. The corolla is slit open and stamens are removed and placed in a sterile petri dish. One of the anthers is crushed in acetocarmine to test the stage of pollen development. If it is found to be correct stage, each anther is gently separated from the filament and the intact uninjured anthers are inoculated horizontally on nutrient media. Injured anthers may be discarded because wounding often stimulates callusing of the anther wall tissue. When dealing with plants having

Calyx → Ring of small green leaves = Sepals
Corolla → Petals

Flag leaf → The last leaf to emerge → Apical meristem terminates growth

minute flowers such as *Brassica* and *Trifolium*, it may be necessary to use a stereo microscope for dissecting the anthers. In case of cereals, spikes are harvested at the uninuclear stage of microspore development and surface sterilized. The inflorescence of most cereals at this stage is covered by the flag leaf. Anthers can be plated on solid agar media in petri dishes. Normally 10–20 anthers are plated in a 6 cm petri dish. Anthers can be cultured on a liquid media and 50 anthers can be cultured in 10 ml of liquid medium (Fig. 8.2). In responsive anthers, the wall tissue gradually turn brown and within 3–8 weeks they burst open due to the pressure exerted by the growing pollen callus or pollen plants. After they have attained a height of about 3–5 cm, the individual plantlets or shoots emerging from the callus are separated and transferred to a medium that would support further development. The rooted plants are transferred to sterile soil mix in the pots.

Anther culture has an advantage over microspore culture in being very quick for practical purposes, and also sometimes the anther wall has an influence on the development of microspores in it, acting as a conditioning factor.

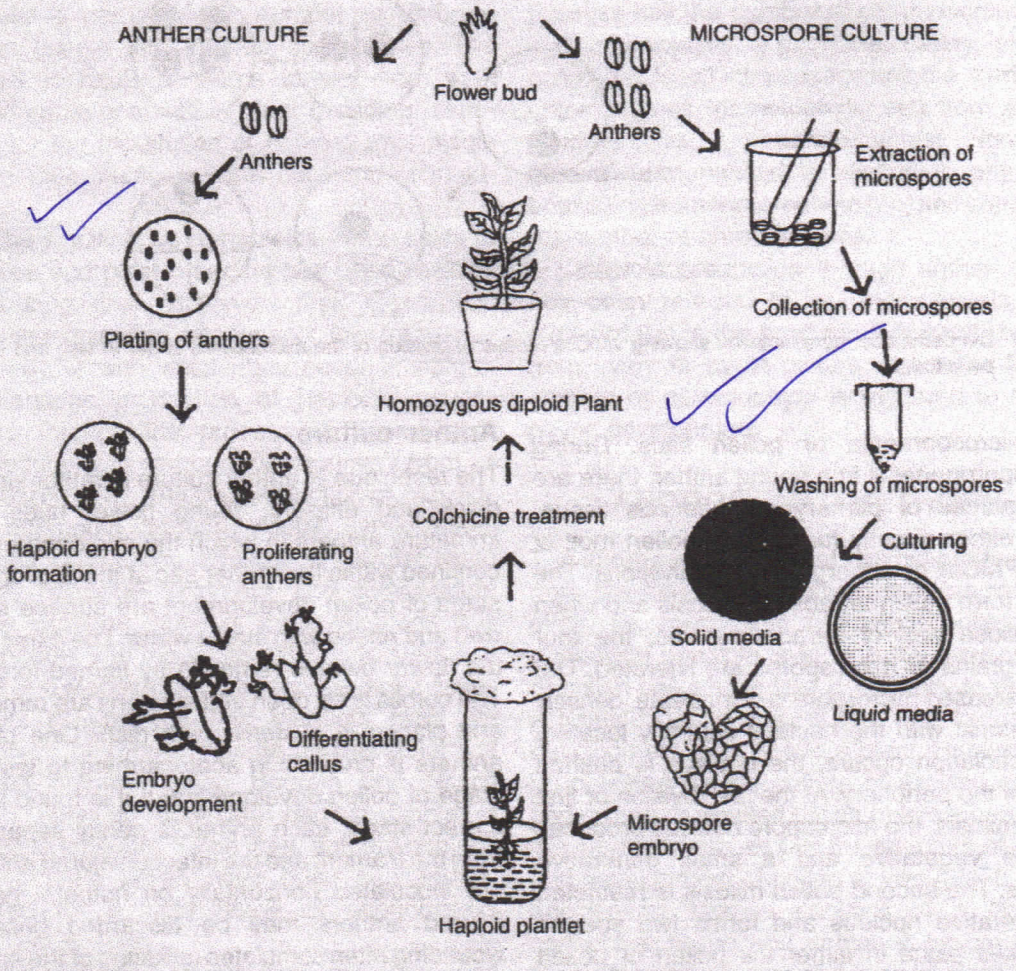


Fig. 8.2 Diagrammatic illustration of anther and microspore culture for production of haploid plants and diploidization

Microspore Culture

Haploid plants can be produced through *in vitro* culture of male gametophytic cells, i.e. microspores or immature pollen. In a general procedure for microspore culture, anthers are collected from sterilized flower buds in a small beaker containing basal media (e.g. 50 anthers of *Nicotiana* in 10 ml media). The microspores are then squeezed out of the anthers by pressing them against the side of beaker with a glass rod (Fig. 8.2). Anther tissue debris is removed by filtering the suspension through a nylon sieve having a pore diameter which is slightly wider than the diameter of pollen (e.g. 40 μ for *Nicotiana*, 100 μ for maize, etc.). It has been observed that smaller microspores do not regenerate, thus larger, good and viable microspores can be concentrated by filtering the microspore suspension through nylon sieves. This pollen suspension is then centrifuged at low speed ca. 150× g for 5 min. The supernatant containing fine debris is discarded and the pellet of pollen is resuspended in fresh media and it is washed atleast twice. The microspores obtained are then mixed with an appropriate culture medium at a density of 10^3 to 10^4 microspores/ml. The final suspension is then pipetted into small petri dishes. To ensure good aeration, the layer of liquid in the dish should be as thin as possible. Each dish is then sealed with parafilm to avoid dehydration and is incubated. The responsive microspores form embryos or calli and its subsequent development to plant formation can be achieved by transferring to suitable media.

Culture of anthers has proved to be an efficient technique for haploid induction. But it has one main disadvantage that plants not only originate from pollen but also from various other parts of the anther (especially in dicots) with the result that a population of plants with various ploidy levels are obtained. This difficulty can be removed by culture of isolated microspores which offer the following advantages:

1. Uncontrolled effects of the anther wall and other associated tissue are eliminated and various factors governing androgenesis can be better regulated. But disadvantageous where anther wall has a stimulatory effect.
2. The sequence of androgenesis can be observed starting from a single cell.
3. Microspores are ideal for uptake, transformation and mutagenic studies, as microspores may be evenly exposed to chemicals or physical mutagens.
4. Higher yields of plants per anther could be obtained.

The various factors governing the success of androgenesis have been discussed below.

Genotype: Success in anther culture is predominantly dependent on the genotype of the anther donor material. It has been repeatedly observed that various species and cultivars exhibit different growth responses in culture. It is now known that anther culture ability is genetically controlled. Thus, a general survey for tissue culture response of various cultivars must be undertaken with simple media, as complex media rich in growth regulators tend to favor proliferation of somatic anther tissue giving rise to callus of various ploidy levels.

Physiological status of the donor plants: The physiological status of the plants at the time of anther excision influences the sporophytic efficiency of microspores. In order to allow for the *in vitro* development of pollen into an adult plant it is very important to start with healthy pollen cells. Therefore, it is good to culture anthers from plants grown under the best environmental conditions. The donor plant should be taken care of from the time of flower induction to the sampling of pollen. The use of any kind of pesticide, whether externally applied or systemic should be avoided for 3–4 weeks preceding sampling. The response in culture is predominantly influenced by the different growth conditions during various seasons. The variation in response of anthers from plants grown under different environmental conditions may be due to the differences in the endogenous level of growth regulators. Critical environmental factors

are light intensity, photoperiod, temperature, nutrition and concentration of carbon dioxide. It has been generally observed that plants grown outdoors during natural growing seasons are more responsive than greenhouse-grown material. Flowers from relatively young plants at the beginning of the flowering season are more responsive. It is therefore of prime importance that plants be grown under optimal growth conditions, watered with minimal salt solutions periodically, and that relatively young plants be used.

Stage of pollen: It has been established that selection of anthers at an appropriate stage of pollen development is most critical. Anthers with microspores ranging from tetrad to the binucleate stage are responsive. But as soon as starch deposition has begun in the microspore, no sporophytic development and subsequently no macroscopic structure formation occurs. Data has established that uninucleate microspores are more prone to experimental treatment for culture just before or during first mitosis. There is an optimum stage for each species. For example, pollen at or just after the pollen mitosis in *Datura innoxia*, *Nicotiana tabacum*, early bicellular stage in *Atropa balladonna* and *Nicotiana sylvestris* and mid- to late uninucleate stage of microspore development in cereals has been found to give the best results.

Pretreatment of anthers: The underlying principle of androgenesis is to stop the development of the pollen cell whose fate is normally to become a gamete, and to force its development directly into a plant. This abnormal pathway is possible if the pollen cell is taken away from its normal environment in the living plant and placed in other specific conditions. This induction of androgenesis is enhanced by giving certain treatments to the whole spike, or flower bud or to the anthers.

i) ***Cold pretreatment:*** In general, cold treatment between 3 and 6°C for 3 to 15 days gives good response. Maize responds better to a temperature of 14°C. The degree of cold that should be given is dependent on the species. As a result of cold treatment, weak or non-viable anthers and microspores are killed and the material gets enriched in vigorous anthers. It is possible that cold pretreatment retards aging of the anther wall, allowing a higher proportion of microspores to change their developmental pattern from gametophytic to sporophytic.

ii) ***Hot treatment:*** Floral buds or entire plants in some species when subjected to 30°C for 24 h or 40°C for 1 h stimulates embryogenesis (e.g. *Brassica*). The temperature shock appears to cause dissolution of microtubules and dislodging of the spindle which causes abnormal division of the microspore nucleus.

iii) ***Chemical treatment:*** Various chemicals are known to induce parthenogenesis. 2- Chloro ethylphosphonic acid (ethrel) has a pronounced effect in increasing the haploid production in various species. Plants are sprayed with an ethrel solution (e.g. 4000 ppm in wheat) just before meiosis in PMCs which results in multinucleated (4–6) pollen with fewer starch grains. It is possible that multinucleated pollen might be induced to form embryos when cultured.

Culture media: The composition of medium is one of the most important factors determining not only the success of anther culture but also the mode of development. Normally only two mitotic divisions occur in a microspore, but androgenesis involves repeated divisions. It is difficult to draw a conclusion as to which medium is most suitable as species or even genotypes may demand different nutritional conditions. Basal medium of MS, White (1963), Nitsch and Nitsch (1969) and N_6 for solanaceous crops; B_5 and its modifications for *Brassica*; and B_5, N_6, LS (Linsmaier and Skoog, 1965) and Potato2 medium (Chuang *et al.*, 1978) for cereals have been used. Thus, no general recommendations can be given but some general observations have been mentioned.

Sucrose is essential for androgenesis. Sugars are indispensable in the basal medium as they are not only the source of carbon but are also involved in osmo-regulation. The usual level of sucrose is 2–4%; however, higher concentration (6–12%) favors androgenesis in cereals. Chelated iron has been shown to play an important role in the differentiation of globular embryos into heart-shaped embryos and further into complete plants.

Nitrogen metabolism is quite an important feature. The presence of nitrate, ammonium salts as well as amino acids appear to play a very special role at different stages of the developmental process. However, glutamine is probably beneficial for most plant species as an aid to achieving the *in vitro* differentiation of a cell to a complete plant.

Pollen embryogenesis can be induced on a simple mineral-sucrose medium in plants like tobacco, yet for androgenesis to be completed, addition of certain growth regulators is required. For example, cereal anthers require both auxins and cytokinins and optimal growth response depends on the endogenous level of these growth regulators. However to promote direct embryogenesis simple media with low levels of auxins are advisable. When the response of cereal cells and tissues to different phytohormones concentrations and combinations are compared with dicots, generally growth hormones are needed in the former whereas high amount of phytohormone autotropy is present in the dicots.

Activated charcoal in the medium enhances the percentage of androgenic anthers in some species presumably by removing the inhibitors from the medium.

Process of androgenesis

The anthers normally start undergoing pollen embryogenesis within 2 weeks and it takes about 3–5 weeks before the embryos are visible bursting out of the anthers. In rice this may take up to 8 weeks. Haploid plantlets are formed in two ways: (i) direct androgenesis: embryos originating directly from the microspores of anthers without callusing or (ii) indirect androgenesis i.e. organogenic pathway: microspores undergo proliferation to form callus which can be induced to differentiate into plants.

The process of androgenesis has shown that microspores undergo divisions, which continues until a 40–50-celled proembryo is formed. The embryos, mostly at globular stage, burst out of the exine and are released. The embryos undergo various stages of development, simulating those of normal zygotic embryo formation. However, when the microspore takes organogenetic pathway, it looks to be larger than embryonal type of micropspore after 2–3 weeks and contain only a few cells. These cells increase in size, exerting pressure on the exine which bursts open and the contents are released in the form of a callus. These calluses then differentiate into plantlets. The plants with well developed shoots and roots are then transferred to pots. The plantlets originating from the callus generally exhibit various levels of ploidy.

The physical environmental conditions in which the cultures are to be placed can enhance the differentiation. The cultures are incubated at 24–28°C. In the initial stages of induction of morphogenesis, darkness is normally more effective or cultures should be kept in low light intensity (500 lux). After induction, macroscopic structures are transferred to a regeneration medium (in cereals with reduced sucrose and auxin concentration) and kept at 14 h day–light regime at 2000–4000 lux.

The ploidy level and chromosome doubling

The ploidy of plants derived from anther or microspore culture is highly variable. The wide range of ploidy levels seen in androgenetic plants has been attributed to endomitosis and/or fusion of various nuclei. Variations in ploidy observed in anther cultures appears to be a function of the developmental stage of anthers at the time of excision and culture, with higher ploidy levels more prevalent in anthers cultured following

microspore mitosis. Moreover, haploid tissues are quite susceptible to changes in ploidy level during cell proliferation and growth *in vitro*. For obtaining homozygous lines, the plants derived through anther culture must be analysed for their ploidy status.

1. ***Counting of plastids in the stomata:*** The ploidy level of a plant may be determined by counting the number of plastids in the stomata of a leaf. For example, in potato monohaploids have 5–8, dihaploids 10–15, and tetraploids 18–24 chloroplasts per guard cell.
2. ***Chromosome number:*** It can be counted from pollen mother cells of buds which can be collected from the regenerated plants and fixed in Carnoy's solution. Acetocarmine or propionocarmine can stain cells. Root tips are also utilized for chromosome counting. Fixed root tips are normally hydrolyzed in 1 N HCl at 60°C for 10 min followed by staining with acetocarmine.
3. ***Number of nucleoli:*** Haploid plants contain one nucleolus while diploids contain 2 nucleoli. Number of nucleoli is directly related to the ploidy status of a plant. Leaves can be incubated overnight with orcein and number of nucleoli can be counted.
4. ***Flow cytometric analysis:*** Leaves of potential haploid plants are finely chopped and intact interphase nuclei are freed from the cells. At this stage nuclear DNA content reflects the ploidy state of the donor which is determined by flow cytometry. This method is very quick.

Diploidization

Haploids can be diploidized to produce homozygous plants by following methods:

1. ***Colchicine treatment:*** Colchicine has been extensively used as a spindle inhibitor to induce chromosome duplication. It can be applied in the following ways:

i) The plantlets when still attached to the anther are treated for 24–48 h with 0.5% colchicine solution, washed thoroughly and replanted.

ii) Anthers can be plated directly on a colchicine-supplemented medium for a week and when the first division has taken place, these are transferred to colchicine-free medium for androgenesis process to take place. This method can be followed in maize where male and female flowers are borne separately and diploidization is a problem.

iii) Colchicine–lanolin paste (0.4%) may be applied to the axils of leaves when the plants are mature. The main axis is decapitated to stimulate the axillary buds to grow into diploid and fertile branches.

iv) Repeated colchicine treatment to axillary buds with cotton wool plugs over a period of time (e.g. 14 days in potato).

v) In cereals, vigorous plants at 3–4 tiller stage are collected, soil is washed from the roots and are cut back to 3 cm below the crown. The plants are placed in glass jars or vials containing colchicine solution (2.5 g colchicine dissolved in 20 ml dimethyl sulfoxide and made up to a liter with water). The crowns are covered with colchicine solution. The plants are kept at room temperature in light for 5 h, the roots are washed thoroughly with water and potted into light soil.

Plants should be handled with extra care after colchicine treatment for few days and should be maintained under high humidity.

2. ***Endomitosis:*** Haploid cells are in general unstable in culture and have a tendency to undergo endomitosis (chromosome duplication without nuclear division) to form diploid cells. This property of cell culture has been exploited in some species for obtaining homozygous plants or for diploidization. A small segment of stem is grown on an auxin-cytokinin medium to induce callus formation. During callus growth and differentiation there is a doubling of chromosomes by endomitosis to form diploid homozygous cells and ultimately plants.

Significance and uses of haploids

1. *Development of pure homozygous lines*: In the breeding context, haploids are most useful as source of homozygous lines. The main advantage is the reduction in time to develop new varieties. A conventional plant breeding program takes about 6–8 years to develop a pure homozygous line, whereas by the use of anther/microspore culture, the period can be reduced to few months or a year. Thus, homozygosity is achieved in the quickest possible way making genetic and breeding research much easier. Homozygosity is still more important for those plants which have a very long juvenile phase (period from seed to flowering) such as fruit trees, bulbous plants and forestry trees. Even if repeated self-pollination is possible, achievement of homozygosity in this group of plants is an extremely long process.
2. *Hybrid development*: As a result of complete homozygosity obtained from diploidization of haploids, one can rapidly fix traits in the homozygous condition. Pure homozygous lines can be used for the production of pure F_1 hybrids.
3. *Induction of mutations*: Haploid cell cultures are useful material for studying somatic cell genetics, especially for mutation and cell modification. Majority of mutations induced are recessive and therefore is not expressed in the diploid cells because of the presence of dominant allele. Single cells and isolated pollen have the advantage over the entire plant in that they can be plated and screened in large numbers, in a manner similar to microbiological technique. Mutants which are resistant to antibiotics, herbicides, toxins, etc. have been isolated in a number of plant species. By subjecting haploid *Nicotiana tabacum* cells to methionine sulfoximine, Carlson (1973) regenerated mutant plants which showed a considerably lower level of infection to *Pseudomonas tabaci*. Wenzel and Uhrig (1981) developed mutants in potato through anther culture which were resistant to potato cyst nematode. Mutants have also been isolated for various temperatures, radiosensitivity to ultraviolet light and gamma radiation, amino acid, e.g. valine, and various antibiotics and drugs.
4. *Induction of genetic variability*: By anther culture not only haploids but also plants of various ploidy levels and mutants are obtained and can be incorporated into the breeding programs.
5. *Generation of exclusively male plants*: By haploid induction followed by chromosome doubling it is possible to obtain exclusively male plants. For example, in *Asparagus officinalis* male plants have a higher productivity and yield earlier in the season than female plants. If haploids are produced from anthers of male *Asparagus* plants (XY) these are either X or Y, chromosome doubling of Y results in super male plants YY which can subsequently be vegetatively propagated.
6. *Cytogenetic research*: Haploids have been used in the production of aneuploids. Monosomics in wheat, trisomics with $2n = 25$ in potato and in tobacco nullisomics were derived from haploids obtained from monosomics which could not produce nullisomics on selfing. Haploids also give evidence for the origin of basic chromosome number in a species or a genus. For example in pearl millet (*Pennisetum americanum*), occurrence of pairing (upto two bivalents in some cells) suggested that the basic chromosome number may be $x = 5$.
7. *Significance in the early release of varieties*: Based on anther culture many varieties have been released which are listed in Table 8.1. In Japan, a tobacco variety F 211 resistant to bacterial wilt has been obtained through anther culture. In *Brassica napus*, anther-derived doubled haploid lines had low erucic acid and glucosinolate content. Similarly, in sugarcane, selection among anther culture derived haploids led to the development of

Point 1 and 7 are same

Table 8.1 Varieties released through anther culture

Crop	Varieties
Wheat	Lunghua 1, Huapei 1, G. K. Delibab, Ambitus, Jingdan 2288, Florin, Zing Hua-1, Zing Hua-3
Rice	Hua Yu-1, Hua Yu- 2, Zhong Hua 8, Zhong Hua 9, Xin Xiu, Tanfong 1, Nonhua 5, Nonhua 11, Zhe Keng 66, Aya, Hirohikari, Hirohonami
Tobacco	Tan Yuh No.1, Tanyu 2, Tanyu 3, F 211, Hai Hua 19, Hai Hua 30

superior lines with tall stem and higher sugar content. In bell peppers, dihaploid lines exhibited all shades of color ranging from dark green to light green. These reports have encouraged many plant breeders to incorporate anther culture in breeding methods.

8. *Hybrid sorting in haploid breeding*: One of the essential steps in haploid breeding involves selection of superior plants among haploids derived from F_1 hybrids through anther culture. It is properly described as hybrid sorting and virtually means selections of recombinant superior gametes. The haploid method of breeding involving hybrid sorting is considered superior to pedigree and bulk methods, firstly, because the frequency of superior gametes is higher than the frequency of corresponding superior plants in F_2 generation, and secondly, because haploid breeding reduces significantly the time required for the development of a new variety. For instance if one assumes that the frequency of superior F_1 gametes is one in one hundred, then the frequency of homozygous F_2 plants derived from the fusion of two such superior similar gametes will be one in ten thousand. Therefore, under the haploid method a smaller population of doubled haploids derived from haploids will need be handled.
9. *Disease resistance*: Haploid production has been used for the introduction of disease resistance genes into cultivars. An established cultivar is crossed with a donor for disease resistance. Either F_1 or F_2 anthers are plated and haploids are developed. These haploids are screened for resistance and then diploidized. Resistance to barley yellow mosaic virus has been introduced into susceptible breeding lines by haploid breeding (Foroughiwehr and Friedt, 1984). A barley accession Q 21681 was found to be resistant to various diseases. This line was crossed to susceptible breeding lines and anthers of F_1 plants were cultured to develop double haploid lines which were resistant to stem rust, leaf rust and powdery mildew (Steffenson *et al.*, 1995). Rice varieties ZhongHua No. 8 and No. 9 have been developed with blast resistance genes, high yield and good quality using haploids integrated in conventional breeding approaches. Hwasambye, a rice variety bred through anther culture showed resistance to leaf blast, bacterial leaf blight and rice stripe tenui virus (Byeong-Geun *et al.*, 1997). In tobacco, a variant that showed resistance to a highly necrotic strain of potato virus Y (PVY) from a population of doubled haploids was reported by Witherspoon *et al.* (1991).
10. *Insect resistance*: A medium-late maturing rice variety 'Hwacheongbyeo' derived from anther culture showed resistance to brown plant hopper. This variety was also resistant to blast, bacterial blight and rice tenui virus, and showed cold tolerance (Lee *et al.*, 1989). In rice, promising anther culture lines have been developed which show resistance to rice water weevil (N'Guessan *et al.*, 1994), pests (Zapata *et al.*, 1991).
11. *Salt tolerance*: Salt tolerant breeding lines have been developed in different crop species which have been integrated in conventional breeding. Miah *et al.* (1996) developed doubled haploid salt tolerant rice breeding line that showed tolerance at the level of 8–10 decisiemens/m (ds/m). Sathish

et al. (1997) established stable NaCl-resistant rice plant line (EC 16–18 ms) by *in vitro* selection of anther derived callus exposed to NaCl. Likewise *in vitro* screening of wheat anther culture derived callus to NaCl resulted in line Hua Bain 124-4 which showed salt tolerance as well as high yield and desiccation tolerance (Zao *et al.*, 1994; 1995).

12. *Doubled haploids in genome mapping*: A rather recent application of DH lines are their use in genome mapping. For molecular screening studies a much smaller sample of doubled haploids is required for desirable recombinants. In a population of DH lines, the identification of markers is much more secure, as most intermediate phenotypic expressions are excluded due to heterozygosity. A gene will segregate in a 1 : 1 ratio for both molecular marker and the phenotype at the plant level. DH is used for genome mapping for major genes and/or quantitative traits in barley, rice, oilseed rape, etc.

Problems

1. High level of management and expertise is required to operate the tissue culture production of haploids.
2. Diploids and tetraploids often regenerate at the same rate as the haploids.
3. Selective cell division must take place in the haploid microspores and not in other unwanted diploid tissues. This selective cell division is often impossible.
4. Callus formation whether it has arisen spontaneously or has been induced by regulators is usually detrimental.
5. The relatively high incidence of albinism in some types of anther and pollen culture.
6. The lack of selection of traits during the derivation of haploid material.
7. There is little chance of isolating a haploid from a mixture of haploids and higher ploidy levels since latter ones are easily outgrown.
8. The doubling of a haploid does not always result in the production of a homozygote.

GYNOGENIC HAPLOIDS

Recent advances in plant tissue culture have resulted in the successful induction of haploid plants from ovary and ovule culture. This means that megaspores or female gametophytes of angiosperms can be triggered *in vitro* to sporophytic development. These plants have been described as gynogenic as compared to androgenic plants derived from microspores. Figure 8.3 shows the various parts of a female reproductive organ. *In vitro* culture of unpollinated ovaries and ovules represents an alternative for the production of haploid plants in species for which anther culture has given unsatisfactory results (e.g. albino plants) or has proven insufficient, e.g. *Gerbera*. The first successful report on the induction of gynogenic haploids was in barley by San Noeum (1976). Subsequently haploid plants were obtained from ovary and ovule cultures of rice, wheat, maize, sunflower, sugar beet, tobacco, poplar, mulberry, etc.

Ovaries can be cultured as pollinated and unpollinated. For haploid production flower buds are excised 24–48 h prior to anthesis for

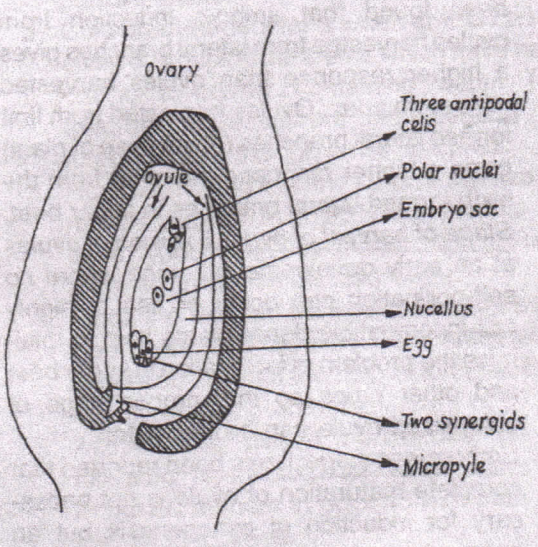


Fig. 8.3 A sectional view of an ovary.

unpollinated ovaries. The calyx, corolla, and stamens are removed and ovaries are then surface sterilized. Before culturing the tip of the distal part of the pedicel is cut off and the ovary is implanted with the cut end inserted in the nutrient medium. The normal Nitsch's (1951), or White's (1954) or MS or N_6 inorganic salt media supplemented with growth substances are used. When liquid medium is to be employed, the ovaries can be placed on a filter paper raft or float with the pedicel inserted through the filter paper and dipping into the medium. Sucrose as a carbon source is essential, although maltose and lactose have been shown to be equally favorable. The various species studied so far seem to have few requirements in common for growth *in vitro*. Some species require only the basal medium and sucrose for growth, although addition of an auxin brought about greater stimulation of growth.

Factors affecting gynogenesis

1. *Genotype*: Genotype of the donor plant is one of the most important factors since each genotype shows a different response. For each genotype a specific protocol must be followed for maximal efficiency.
2. *Growth condition of the donor plant*: It has been found that embryo induction from ovules harvested from lateral branches gives a higher response than ovules harvested from stem apex. Ovules harvested from first formed lateral branches (at the base of plant) gives a higher response than that from the sixth formed lateral branches in sugar beet.
3. *Stage of harvest of ovule*: Excision of ovules at an early developmental stage where no self-pollination can occur or use of highly self-incompatible donor plants help to minimize the problem of fertilization in sugar beet and other crops so that proper stage of unfertilized ovule can be harvested.
4. *Embryo-sac stage*: It has been reported that complete maturation of ovule is not necessary for induction of gynogenesis but an appropriate stage of embryo-sac is more important.
5. *Culture conditions*: Although solid media have been used more frequently for gynogenic culture, few investigators have used liquid medium for the induction of gynogenic calluses followed by dissection and transfer of embryogenic structures to solid medium for differentiation. MS, B5, Miller's basal medium have been most commonly used. The amount of sucrose has been reported to be important for embryogenesis. In rice 3–6% sucrose was effective, whereas in onion 10% sucrose gives maximum response.
6. *Seasonal effects*: Seasonal variation is an important factor. In sugar beet the highest embryo yield was obtained from the summer grown plants. Callus induction from ovules of Gerbera occurred at a higher frequency during autumn as compared to spring.
7. *Physical factors*: Certain physical factors (treatments) given to explants or plant parts from which explants are taken prior to culture may have a strong influence on embryo induction. For *Beta vulgaris* cold pretreatment of flower buds at 4°C for 4–5 days increases embryo yield from cultured ovules. Similarly, cold pretreatment of inflorescence prior to ovule isolation increased haploid callus frequency in *Salvia selarea*. Other physical factors influencing ovary and ovule culture are light and temperature of incubation.

In vitro ovary culture has been used to develop haploid plants and has the potential to overcome the problem of albino plant formation in anther culture. The technique has also got superiority over anther culture due to its potential use in the male sterile genotypes.

The origin of gynogenic haploids differs in species as reported to be from synergids in rice, whereas egg or antipodal cell develops into embryos in barley. *In vitro* culture of unfertilized ovules has been the most efficient and reliable technique for the production of haploid and doubled haploid plants of sugar beet. In tree species, gynogenic plants were reported in mulberry (*Morus indica*), an important tree for nourishing silkworms for use in the silk industry.