Anther and Pollen Culture

Anther culture

- Culturing of anther obtained from unopened flower bud in the nutrient medium under aseptic condition. callus tissue or embryoids from anther, that give rise to haploid plantlets either though organogenesis or embryogenesis.

Pollen culture

- Pollen or microspore culture is an in vitro technique by which the pollen grains preferably at the uninucleated stage, are squeezed out aseptically from the intact anther and then cultured on nutrient medium. • The microspores develop into haploid embryoids or callus tissue that give rise to haploid plantlets by embryogenesis or organogenesis.

HISTORY of Anther and Pollen Culture

W.TULECKE(1953) First observed that mature pollen grains of Ginkgo biloba (gymnosperm) can be induced to proliferate in culture to form haploid callus.

S.GUHA AND S.C MAHESWARI(1964) First reported the direct development of embryos from microspores of Datura innoxia, by the culture of excised anther. This technique can be used in over 200 species, including tomato, rice, tobacco, barley and geranium.

J.P. BOURGIN AND J.P.NITSCH(1967) Obtained complete haploid plantlets from anther culture of Nicotiana tabacum.

Andreogenesis

Androgenesis is the in vitro development of haploid plants originating from totipotent pollen grains through a series of cell division and differentiation. It is of two types: 1)Direct androgenesis 2)Indirect androgenesis

1.) Direct androgenesis

- The microspores behaves like a zygote and undergoes change to form enbryoid which ultimately give rise to a plantlet.

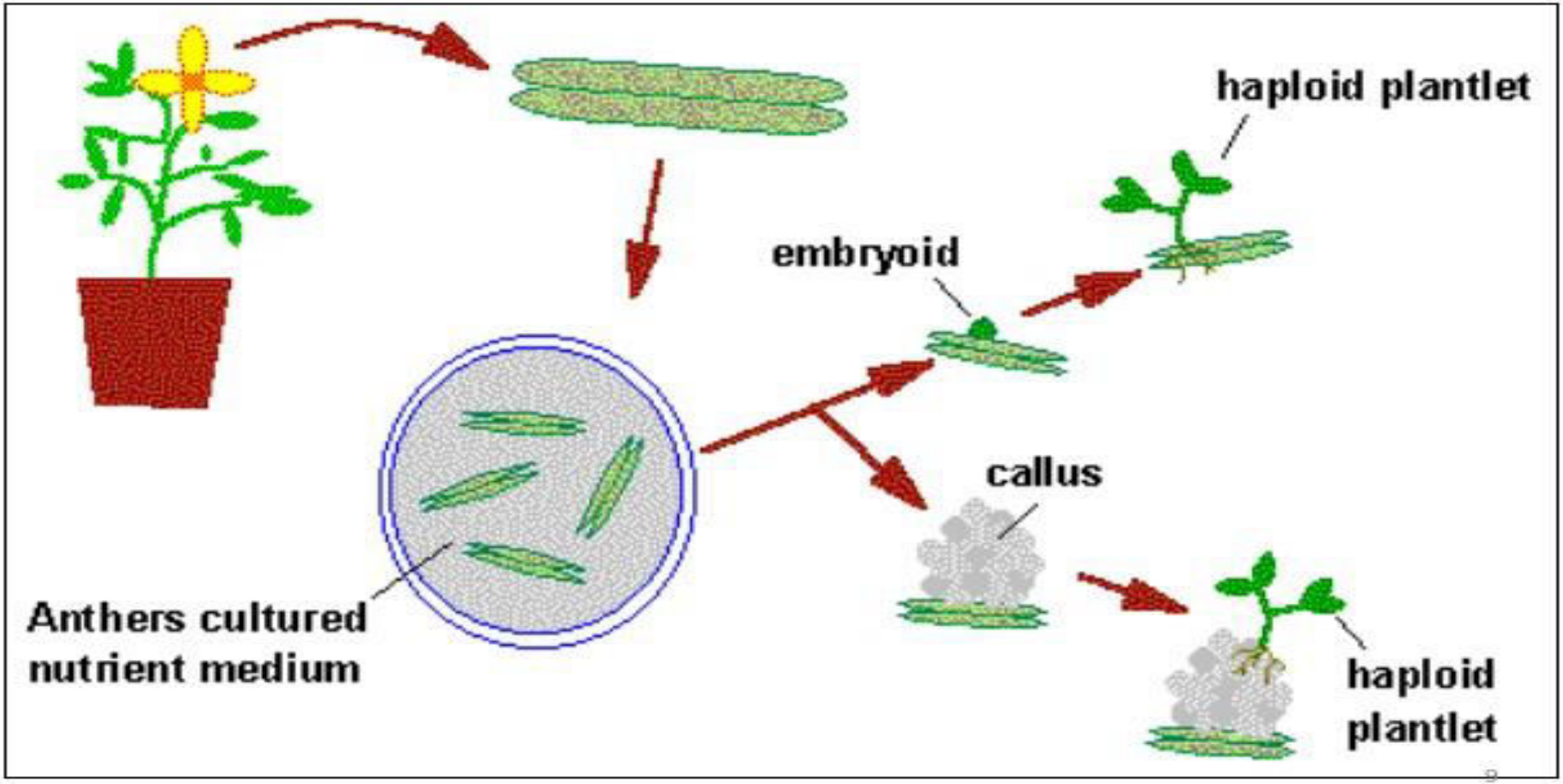
2.) Indirect androgenesis

- The microspores divide repeatedly to form a callus tissue which differentiates into haploid plantlets.

Principle of anther and pollen culture

The production of haploid plants is to exploit the totipotency of microspore .

In this process the normal development and function of the pollen cell to become a male gamete is stopped and is diverted forcely to a new metabolic pathway for vegetative cell division .


haploid plantlet
embryoid
callus
Anthers cultured
nutrient medium
haploid
plantlet

Pathways of Development of Pollen

Pathway I

- The microspores divide by an equal division and two identical daughter cells developed.
- Vegetative and generative cells are not distinctly formed in the pathway.
- Example: Datura innoxia

Pathway II

- The division of uninucleate microspores is unusual, resulting in the formation of vegetative and generative cell.
- The sporophyte arises through further division in the vegetative cell and generative cell does not divide.
- Examples: Nicotiana tabacum, Hordeum vulgare, Triticum aestivum

Pathway III

- The uninucleate microspore undergoes a normal division but pollen embryos are formed from generative cell alone.
- The vegetative cell does not divide.
- Examples: Hyoscyamus niger

Pathway IV

- Both generative and vegetative cell divide further to the development of sporophyte.
- Examples: Datura metal, Atropa belladona, Datura innoxia(occasionally).

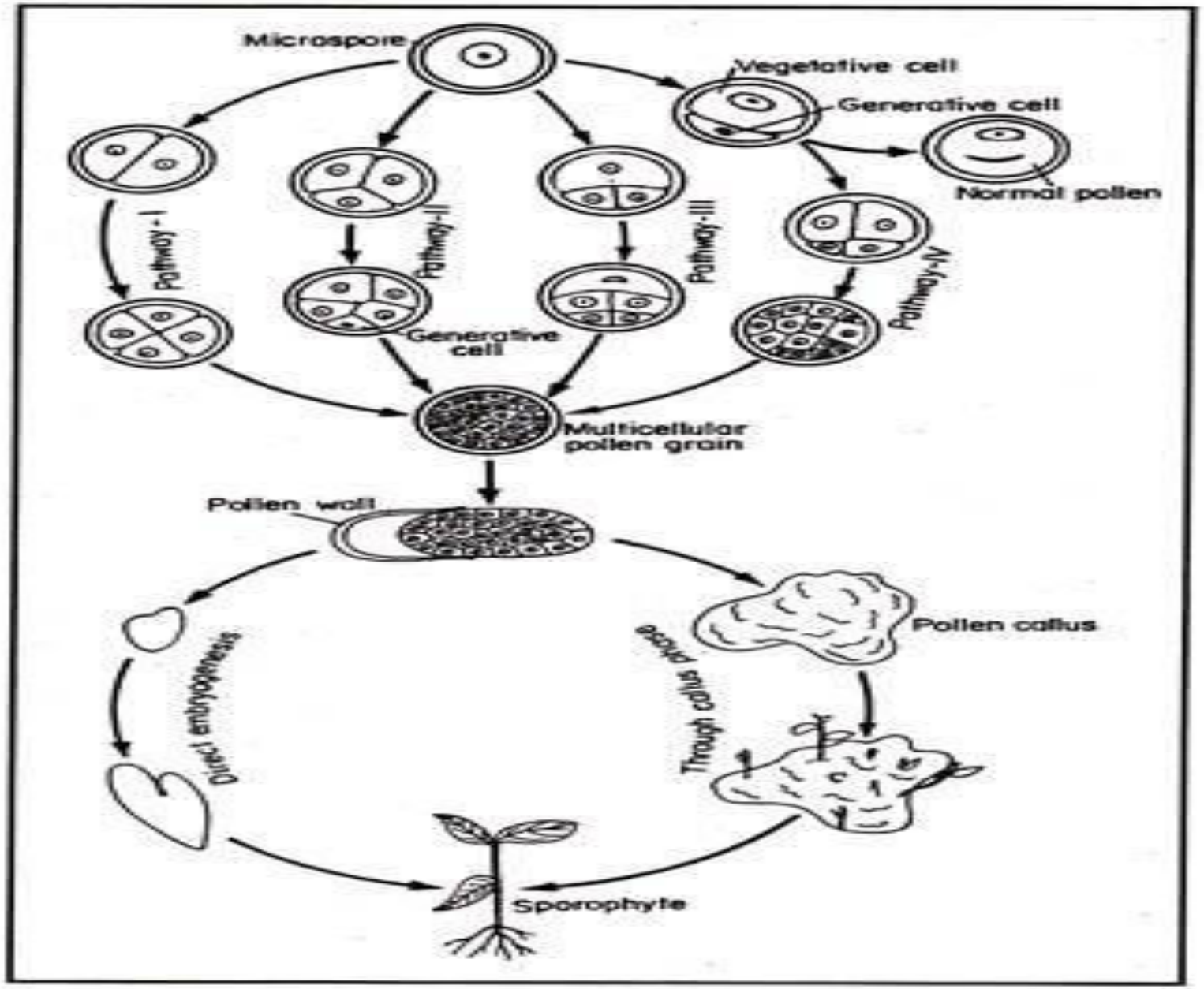
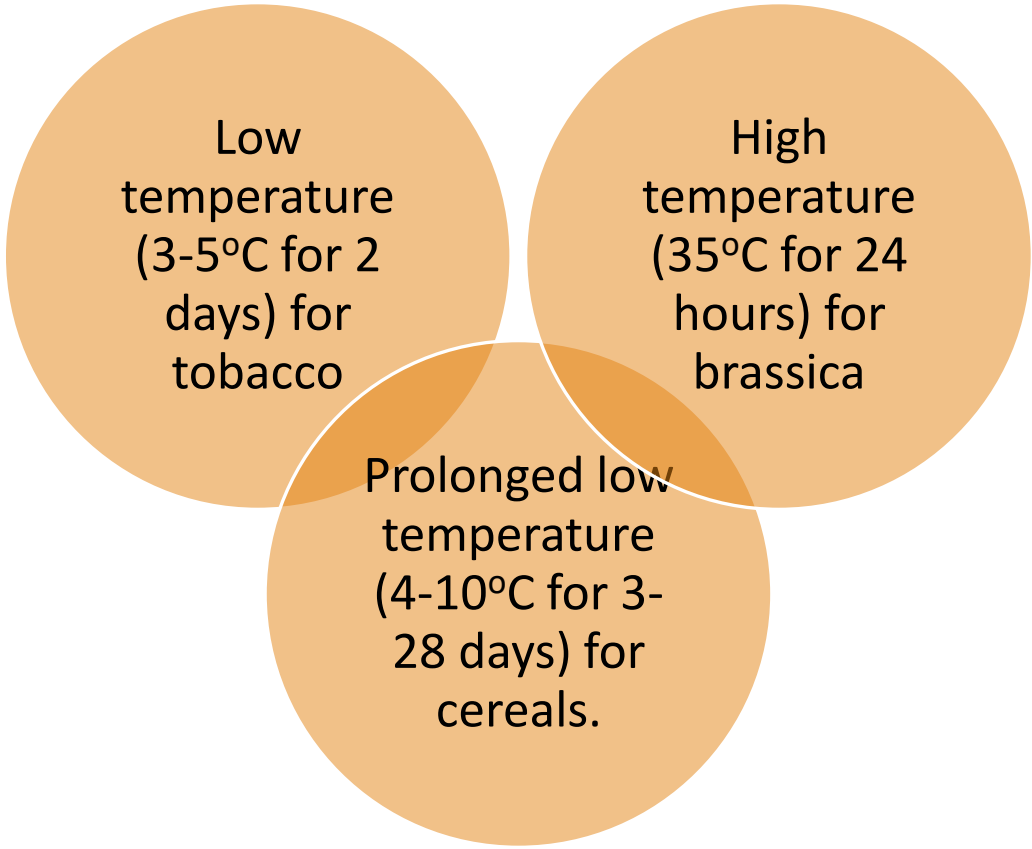


Fig 11.1

Diagram showing the origin of sporophytes from pollen gains in anther cultures. A microspore may follow any one of the four pathways to form a multicellular pollen grain. The latter may directly form an embryo or produce callus tissue (After Bhojwani and Razdan 1983)

Pretreatment



Media

MS+2,4D 1.5 mg +NAA 0.5 mg per litre of media for callus induction

MS+BAP 2 mg for shoot regeneration

½ MS + NAA 0.5 mg + IBA 1 mg per litre of medium for in vitro rooting.

Method of Anther culture

Collect the flower buds of Nicotiana tabacum at the onset of flowering (normally 3- 4 months old plant). Measure the length of each individual with a cm ruler. Select the flower bud of 17-22 mm in length when the length of the sepals equals that of the petals. Reject all flower buds which are beginning to open.

Transfer the selected flower buds to the laminar airflow. Each flower bud contains five anther and these are normally surface sterile in closed buds. The flower buds are surface sterilized by immersion in 70% ethanol for 10 seconds followed immediately by 10 minutes in 2% (v/v) sodium hypochlorite. They are washed three times with sterile distilled water. Finally, transfer the buds to a sterile petridish.

To remove the anthers, slit the side of the bud with a sharp scalpel and remove them. With a pair of forceps, place the five anthers with the filaments to another petridish. The filaments are cut gently. During excision of anthers, special care should be taken to ensure that they are not injured in any way. Damaged anthers should be discarded as they often form callus tissue from the damaged parts other than the pollen.

Anthers are placed on agar solidified basal MS or White or Nitsch and Nitsch medium.

The culture are kept initially in dark. After 3-4 weeks, the anthers normally undergo pollen embryogenesis and haploid plantlets appear from the cultured anther. In some cases, anther may undergo proliferation to form callus tissue which can be induced to differentiate into haploid plants.

At this stage, the cultures are incubated at 24-28° C in a 14 hrs. daylight regime at about 2,000 lux.

Approximately 50 mm tall plantlets are freed from agar by gently washing with running tap water and then transferred to small pot containing autoclaved potting compost. Cover each plantlet with a glass beaker to prevent desiccation and maintain in a well- lit humid green-house. After one week, remove the glass beakers and transfer to larger pots when the plants will mature and finally flower.

Method of Pollen culture

Selection of suitable unopened flower bud, sterilization, excision of anther without filament are the same as described previously in anther culture.

About 50 anthers are placed in small sterile beaker containing 20 ml of liquid basal medium.

Anthers are then pressed against the side of the beaker with the sterile glass piston of a syringe to squeeze out the pollens

The homogenized anthers are then filtered through a nylon sieve (pore diameter 40µ- 60µ) to remove the anther tissue debris.

The filtrate or pollen suspension is then centrifuged at low speed (500-800 revolution per minute) for 5 minutes. The supernatant containing fine debris is discarded and the pellet of pollen is suspended in fresh liquid medium and washed twice by repeated centrifugation and re-suspension in fresh liquid medium.

Pollens are mixed finally with measured volume of liquid basal medium.

2.5 ml of pollens suspension is pipetted off and is spread in 5 cm petridish. Pollens are best grown in liquid medium but, if necessary, they can be grown by plating in very soft agar added medium. Each dish is sealed with cello-tape to avoid dehydration.

Petridishes are incubated at 27-30° C under low intensity of white cool light

Young embryoids can be observed after 30 days. The embryoids ultimately give rise to haploid plantlets.

Haploid plantlets are then incubated at 27- 50°C in a 16 hrs. day light regime at about 2,000 lux. Plantlets at maturity are transferred to soil as described in anther culture.

Nurse Culture Technique for Pollen Culture by Sharp *et al.* (1972)

Selection of flower bud, sterilization, excision of anther, isolation of suitable pollen are the same as described previously.

In this method, the intact anthers are placed horizontally on the top of solid or semisolid basal medium within a conical flask.

A small filter paper disc is placed over the intact anther and about 10 pollen grains in suspension are then placed on the filter paper disc. Hence the intact anthers are considered as the nurse tissue. A control set is also prepared in exactly the same way except that the pollen grains on filter paper are directly kept on solid medium. Sometimes, callus tissue derived from any part of the plant is used as nurse tissue

With this method, pollen grains in the control set did not grow at all. The pollen grains kept on nurse tissue grow and form a culture of green parenchymatous tissue in two weeks. Such tissue ultimately form the haploid callus tissue.

Advantages of pollen culture over anther culture

- Overcrowding of pollen grain in anther is eliminated and isolated pollen grains are equally exposed to nutrient medium.
- Unwanted growth of the anther wall and other associated tissue are eliminated.
- The steps of androgenizes can be observed starting from single cell.
- Various factor governing androgenizes can be better regulated.
- Pollen is ideal for uptake, transformation and mutagenic studies as pollens can be uniformly exposed to chemicals and physical mutagens.
- Pollen may be directly transformed into an embryoid. So it is very suitable for understanding biochemistry and physiology of androgenizes.
- Higher yields of haploid plants per anther could be expected in pollen culture than the anther culture.

Importance of anther and pollen culture

Pollen culture has great importance in mutagenic studies.

By anther and, pollen culture many haploid plants can be produced very rapidly.

Homozygous diploid plants obtained by doubling the chromosomes of haploids have great importance in plant breeding and crop improvement.

Ovary Culture

Ovary culture is a technique of culture of ovaries isolated either from pollinated or un-pollinated flowers.

Ovary is a ovule bearing region of a pistil. Excised ovaries can be cultured in vitro.

Jensen and Bonper (1949) cultured ovary of *Lycopersicum* on a culture medium. Nitsch (1951) successfully cultured ovaries of tomato, tobacco, Phaseolus, Fragaria, etc.

Methods of Ovary Culture

Collect the pollinated or un-pollinated flowers from a healthy plant.

Wash them thoroughly with tap water, dip into 5% Teepol solution for 10 minutes and again wash to remove the trace of Teepol.

Transfer the flowers to laminar air flow cabinet. Surface sterilizes the flowers by immersing in 5% sodium hypochlorite solution for 5-7 minutes. Wash them with sterile distilled water.

Transfer the flowers to a sterile petri dish. Using a flamed force and a surgical scalpel, dissect out the calyx, petals, anther filaments etc. of the flower to isolate the pistil. During isolation of pistils care should be taken to ensure that the ovaries are not injured in any way. Damaged pistil, should be discarded as they often form callus tissue from the damaged parts.

Place the ovaries on agar solidified nutrient medium.

Incubate the cultures at 25°C in a 16 hrs, daylight regime at about 2000 lux. The light is provided by fluorescent lamp.

Application of ovary culture

It is useful to study the early development of embryo development, fruit development, different aspects of fruit physiology including respiration, maturation and disease.

The effect of phytohormones on parthenocarpic fruit development can be studied from the culture of un-pollinated pistil.

Floral organs play a significant role in fruit development. Role of floral organs can be studied from the in vitro culture of ovary. In some cases it has been found that pollinated ovary produce the normal fruits in vitro if the sepals are not removed before culture.

In hybridization, the plant breeders face many problems such as the failure of pollen germination on the stigma or the slow and insufficient growth of pollen tube as well as precocious abscission of flowers. Ovary or pistil culture, in vitro fertilization (test tube pollination) has been used to circumvent these obstacles. In many cases, successful results of in vitro fertilization and seed formation has been obtained.

Ovary culture has also been successful in inducing polyembryony. Poly-embryo may develop in culture from the various parts of the ovary. These poly-embryos give rise to many shoots instead of a single plantlet.

The process of double fertilization not only brings about the formation of embryo and endosperm, but also stimulates the development of ovary into fruit. In most apomictic plants, although there is no fertilization, pollination alone stimulates the growth of the ovary and seed. The culture of ovaries of apomicts may, therefore, help in understanding the nature of stimulus provided by pollination.

Ovule Culture

Ovule culture is an elegant experimental system by which ovules are aseptically isolated from the ovary and are grown aseptically on chemically defined nutrient medium under controlled conditions.

An ovule is a mega sporangium covered by integument. Ovules are attached with placenta inside the ovary by means of its funiculus. An ovule contains a megaspore or an egg cell. After fertilization, a single cell zygote is formed which ultimately leads to form a mature embryo possessing shoot and root primordia.

Methods of Ovule Culture

Collect the open flower (unfertilized ovules). If fertilized ovules are desired, collect the open flower where the anthers are dehisced and pollination has taken place. To ensure the fertilization, collect the flower after 48 hrs. of anther dehiscence.

Remove sepals, petals, androecium etc. from the ovaries containing either fertilized or unfertilized ovules.

Soak the ovaries in 6% NaOCl solution.

Rinse the ovaries 3-4 times with sterile distilled water.

Using sterile techniques, ovules are gently prodded with the help of spoon shaped spatula by breaking the funicles at its junction with placental tissue.

The spatula with ovules is gently lowered into the sterile solid or liquid medium as the culture vial is slanted about 45°.

Damaged or undersized ovules are rejected when possible, during transfer.

Incubate the ovule culture in either dark or light (16 hrs. 3,000 lux) at 25°.C

Importance of Ovule Culture

Through ovule culture, test tube pollination and fertilization can be done. By technique, it may be possible to germinate pollen in the same culture as the excised ovule and to induce a vitro fertilization leading to the formation of mature seeds containing viable embryos.

Ovule culture has been successfully employed to obtain hybrid seedlings in Interspecific and Intergeneric crosses. In several interspecific crosses, the hybrid embryo of Abelmoschus fails to develop beyond the heart or torpedo-shaped embryo. By ovule cultures, viable hybrids have been obtained in three out of five crosses attempted in Abelmoschus species.

It is possible to obtain haploid callus by culturing unfertilized ovules.

In nature, the seeds of orchid germinate only in association with a proper fungus. As a result numerous seeds are lost due to unavailability of proper fungus. Besides this the seed capsule of many orchids takes a long time to mature. To overcome such problems, several attempts have been made to culture the fertilized ovule of orchid in vitro.

In horticultural practises, the artificial induction of polyembryos holds a great potential. It has been observed that the nucellus of mono-embryonic ovules of citrus can be induced to form adventive embryos in culture.

In the varieties of citrus which are impossible to free of virus by other means, the ovule culture has proved decisively advantages to make them virus free.

Embryo Culture

The embryo of different developmental stages, formed within the female gametophyte through sexual process, can be isolated aseptically from the bulk of maternal tissues of ovule, seed or capsule and cultured in vitro under aseptic and controlled physical conditions in glass vials containing nutrient solid or liquid medium to grow directly into plantlet.

The underlying principle of the method is the aseptic excision of the embryo and its transfer to a suitable nutrient medium for development under optimum culture conditions. In general, it is relatively easy to obtain pathogen-free embryos, since the embryo is lodged in the sterile environment of the ovule or seed or capsule or fruit. So, surface sterilization of the embryos as such is not necessary. Thus the entire seeds or fruits containing the ovule are surface sterilized and the embryos are then aseptically separated from the surrounding tissues.

Hanning (1940) first removed mature embryos from the seeds of carrot amd cultured them on a nutrient medium inyo plantlets.

Methods of Embryo Culture

Capsules in the desired stages of development are surface sterilized for 5-10 minutes in 0.1% $HgCl_2$, either in a closed small room previously illuminated by UV lamps or in a Laminar air flow.

Wash repeatedly in sterile water.

Further operations are carried out under a specially designed dissecting microscope at a magnification of about 90X. The capsules are kept in a depression slide containing few drops of liquid medium.

The outer wall of capsule is removed by a cut in the region of the placenta; the halves are pushed apart with forceps to expose the ovules.

A small incision in the ovule followed by slight pressure with a blunt needle is enough to free the embryos.

The excised embryos are transferred by micro-pipettes or small spoon headed spatula to standard 10 cm petridishes containing 25 ml of solidified standard medium. Usually 6-8 embryos are cultured in a petridish.

The petridishes are sealed with cello-tape to prevent desiccation of the culture.

The cultures are kept in a culture room at 25 ± 1oC and given 16 hrs. illumination by cool white fluorescent tube.

Subcultures into fresh medium are made at approximately four weeks intervals.

Applications of Embryo Culture

- Embryo culture is now days used in alot of fields such as agriculture ,Industry etc
- Its use is made more specific in the area of agriculture foccussing mostly on plant tissue culture.
- Embryo culture is being routinely used in some crop improvement programmes.
- Production of Monoploids helps in achieving the desired trait in less time as normally several generations of testing required before desired trait achieved in selective breeding
- Micropropagation is done to rapidly produce large number of progeny plants by multiplying stock plant material
- This is done to achieve virus free stock

Shortening breeding cycles and ovecomming dormancy thus reducing the time for the next generations which may be grown two or three weeks earlier by embryo culture than from seeds.

Example of Iris whose breeding cycle is shortened to less than one year from two years through this .

Seed sterility is another problem faced in the field of crop production .

Early ripening fruit cultivars have seeds that donot germinate as embryo is immature.

Thus using this seedlings are raised from sterile seeds and the embryos are recovered.

Plants derived from tissue culture techniques are monoploid and can be treated with chemicals to double chromosome number.

To multiply plants whose multiplication rate is too slow

For germplasm conservation and genetic transformations