Haploid Production_Anther Culture

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Haploid Production

- Haploid culture is an in vitro technique used to produce haploid (cells have half the number of chromosomes) plants.
- Blackslee et al. (1922) first reported the natural occurrence of the haploid condition in *Datura* plants, due to parthenogenesis (embryo development from an unfertilized egg).
- The process of parthenogenesis is the only natural cause that leads to the spontaneous production of haploids.
- Until 1960, haploid production was achieved by hybridizing two species of the plants or pollen irradiation.
- Then, Guha and Maheswari (1964) introduced the first haploid embryos and plantlets by culturing the excised anthers of *Datura innoxia* in lab conditions.
- The study was then performed on the *Nicotiana tabacum*, which is considered a model species for the anther culture experiments.

Anther culture

- The technique of haploid production through anther culture ('***anther androgenesis***') has been extended successfully to numerous plant species, including many economically important plants, such as
  - cereals and
  - vegetable,
  - oil and
  - tree crops

THE TECHNIQUES_Anther Culture

- The experimental plants for anther and pollen culture should ideally be grown under controlled conditions
  - temperature,
  - light and
  - humidity,
- Anthers should be taken from young plants.
- Generally, with increasing age of the donor plants the androgenic response declines and abnormalities appear.
- The selected buds are surface sterilized with a suitable disinfectant.
- Anthers along with their filaments are excised under aseptic conditions and placed on a sterilized petri-plate.
- One of the anthers is crushed in acetocarmine to test the stage of pollen development and if it is found to be of the correct stage the anthers of the remaining stamens are gently detached from their filaments, without injuring the anthers, and placed horizontally on the medium (anther culture).
- Where growth hormones are essential to induce androgenic development of pollen grains the sporophytic tissues should be removed as far as possible.
- The gap between bud collection and anther/pollen culture should not exceed 2h.

Maintenance of culture

- The anther cultures are generally maintained in alternating periods of light (12-18 h; 5000-10 000 lx m 2) at 28~ and darkness (12-6 h) at 22~.
- However, optimal storage conditions need to be determined for individual systems.
- For example, the anther cultures of *Brassica* species are very sensitive to light and, therefore, should be maintained in the dark throughout.

Development and transplantation

- In responsive anthers, the wall tissues gradually turn brown and, depending on the species, after 3-8 weeks they burst open due to the pressure exerted by the growing pollen callus or pollen embryos.
- The embryos may germinate on the original medium or require transfer to another medium to form plantlets.
- After they have attained a height of about 3-5 cm, the individual plantlets or shoots are excised and transferred to a medium which would support good development of the root system.
- The rooted plants are transferred to sterilized potting-mix in small pots or seed trays.

THE TECHNIQUES_Isolated pollen culture

- There are many problems associated with raising haploids through anther culture.
- The pollen grains within an anther lobe being genetically heterogeneous, the plants arising from an anther would constitute a heterogeneous population.
- Mixing of calli of different pollen origin within an anther lobe enhances the chances of regenerating chimeric plants.
- Furthermore, if the proliferation of anther wall cells occurs concomitant with the callusing of pollen the tissue finally derived would not be purely of gametophytic origin.
- Isolated pollen culture cannot only circumvent these problems.

Advantages of Isolated pollen culture

- It is a haploid, single cell system.
- A homogeneous population of pollen grains at the developmental stage most suitable for androgenesis can be obtained by gradient centrifugation.
- Isolated microspores, can be genetically modified by exposing them to mutagenic treatments or insertion of foreign genes before culture.
- The new genotypes can be selected at an early stage.
- In rapid cycling *Brassica napus*, pollen culture is 60 times more efficient than anther culture in terms of embryo production.

- The first report of callus formation in isolated pollen culture of an angiosperm (*Brassica oleracea* and the hybrid B. oleracea x B. alboglabra) was published in 1970 by Kameya and Hinata.
- Since then the technique of pollen culture has been considerably improved and androgenic plants through isolated pollen culture have been raised for many crop plants, including
  - Brassica carinata (Chuong and Beversdorf, 1985),
  - B. campestris (Ziemborska and Pauw, 1987; Baillie et al., 1992),
  - B. napus (Chuong et al., 1988; Takahata et al., 1991),
  - B. nigra (Lichter, 1989),
  - B. oleracea (Takahata and Keller, 1991),
  - B. rapa (Burnett et al., 1992),
  - Hordeum vulgare (Datta and Wenzel, 1988),
  - Oryza sativa (Chen et al., 1980a; Cho and Zapata, 1988, 1990; Datta et al., 1990),
  - Petunia (Sangwan and Norreel, 1975),
  - Nicotiana rustica, N. tabacum (Imamura et al., 1982),
  - Triticum aestivum (Datta and Wenzel, 1987) and
  - Zea mays (Pescitelli et al., 1989; Gaillard et al., 1991).

- The initial success with isolated pollen culture was based on the use of some kind of a nurse tissue or its extract.
- Sharp et al. (1972) raised haploid tissue clones from isolated pollen grains of tomato by plating them on small filter paper pieces placed over cultured anthers of the same species.
- Similarly, Pelletier and Durran (1972) used anther cultures of *Nicotiana glutinosa* or *Petunia hybrida* or the petal callus of the latter as an effective nurse tissue for isolated pollen culture of *N. tabacum*.
- Co-culture of ovaries has been used to nurse the formation of embryos from isolated pollen of wheat (Datta and Wenzel, 1987).
- For most of the cereals a pre-culture of anthers for 2-7 days is essential or promotory to obtain embryogenic response in isolated pollen cultures.
- The pollen grains are released from the cultured anthers either mechanically or the cold treated anthers cultured on liquid medium dehisce after 2-7 days liberating the pollen grains into the medium- 'float culture method'.
- Float culture method' has generally proved better than mechanical isolation of pollen from fresh or pre-cultured anthers .
- These observations suggest that anther wall provides a suitable environment necessary to trigger androgenesis.

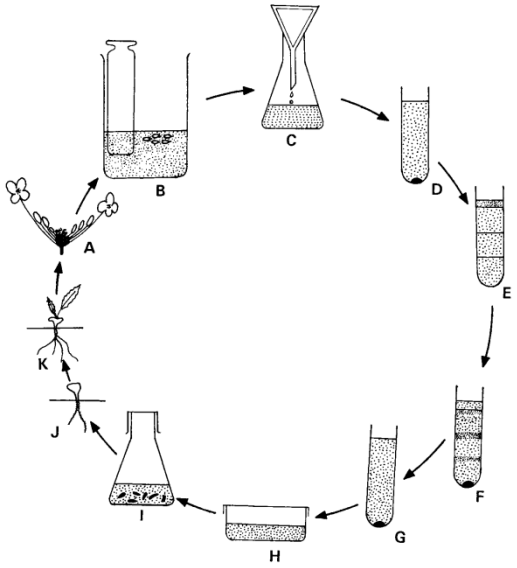
Protocol for isolated pollen culture of *Brassica napus*

- Grow the donor plants under controlled conditions; of 16 h photoperiod (approx. 200~mol m -2 s -~) and at 24+ 2~ 12~ day/night temperature.
- Water and fertilize the plants daily.
- Collect healthy buds at the late uninucleate stage (approx.4.5 mm long) and surface sterilize them in 5.6% sodium hypochlorite
- solution for 10-15 min (the buds should be processed for pollen culture immediately after plucking).
- Rinse the buds three times in cold, sterile distilled water for 5 min each time.
- Transfer the buds to the blender cup under aseptic conditions, add cold B5 liquid medium containing 13% sucrose (ca. 30 ml medium in a 50 ml blender cup) and blend them for a total of 20-30 s alternating between high and low speeds.
- Filter the contents of the blender through two layers of nested sterile filters (Nytex 63 ~m top and 44 #m bottom).
- Centrifuge the filtrate at 900-1000 rev. min -~ for 5-8 min.
- Discard the supernatant, resuspend the sediment in cold B5 medium and spin as above.
- Repeat this process four times.
- Finally, suspend the microspores in 50 ml of NLN 4 liquid medium and adjust the plating density to 7.5 • 103-104 m1-1 using a haemocytometer.
- Dispense 10ml of pollen suspension into petri plates using a sterilized pipet.
- Seal the petri plates with a double layer of parafilm.

- Place the petri plates in a light-proof box and place in an incubator at 30~ for 14 days.
- After 2 weeks shift the box to a slow shaker (60 rev. min-1), for 7 days to ensure proper development of the embryos.
- Transfer individual embryos to B5 solid medium (ca. 10 embryos/ plate), seal the plate with parafilm and incubate at 4~with 8 h photoperiod for 10 days.
- Transfer the plates to a 27~ incubator with 12 h photoperiod.
- Under these conditions the embryos should develop into plantlets which can be transferred directly to soil.
- For mutant selection, after step 10 expose the pollen to mutagenic treatments and after step 11 transfer the embryos to a 250 ml flask containing 50-75 ml of B5 liquid medium (1% sucrose) containing 0.1 mg 1-1 GA3 and incubate them on a shaker (80 rev. min -~) in light.
- In 3-5 days the embryos turn green.
- Replace the medium with the BsG1 medium containing the filter sterilized selection agent (e.g. herbicide).
- The embryos which remain green and survive are selected and go for plant regeneration.

Isolated pollen culture by density gradient centrifugation

- To improve the efficiency of isolated pollen culture for the production of haploids, Wenzel et al. (1975) introduced the technique of density gradient centrifugation.
- This allows the separation of embryogenic grains from a mixture of embryogenic and non-embryogenic grains obtained after crushing the anthers.
- The anthers of barley were collected at the proper stage of development and gently macerated to obtain a suspension of pollen grains.
- After removing the debris by repeated filtration and centrifugation the suspension was layered on 30% sucrose solution and centrifuged at 1200 g for 5 min.
- The androgenic, vacuolated pollen grains formed a band at the top of the sucrose solution.
- Rashid and Reinert (1980) slightly modified the technique and used 55% Percoll and 4% sucrose solution, instead of 30% sucrose, for the separation of starch-free, embryogenic grains of tobacco.
- Percoll gradient centrifugation was found very useful to collect highly embryogenic grains of maize (Gaillard et al., 1991).
- The grains collected at the interface of 40/50% Percoll showed maximum androgenic response.
- Isolated pollen culture is not only more efficient but also more convenient than anther culture.
- The tedious process of dissection of individual anthers is avoided.
- Instead, the entire buds within a suitable size range are crushed and the embryogenic grains are then separated by gradient centrifugation.



Summary diagram of a protocol for isolated pollen culture of *Brassica napus*.

A. The surface sterilized buds (A) of suitable size are crushed to release the pollen grains in B 5 medium containing 13% sucrose (B5-13) in a glass homogenizer (B) and the medium is filtered through 42 ~m nylon mesh to remove large debris (C).

B. The filtrate is centrifuged at 1000 rev. min -1 for 3 min (D) and, after discarding the supernatant solution, the pellet is suspended in the B5-13 medium and gently loaded on the 24%/32%/40% Percoll gradient solution (E) and centrifuged at 1000 rev. min -1 for 5 min.

C. The two upper layers (F) are pipetted out and mixed with the B5-13 medium.

D. The suspension is again centrifuged at 1000 rev. min -1 for 5 min (G) and the supernatant medium is pipetted out and the pollen grains are suspended in medium adjusting the plating density of the pollen grains to 2-5 x 104 m1-1.

E. The suspension is plated as thin layer in petri plates (H) and incubated in the dark at 32^0 C for 3-5 days and then at 25^0 C.

F. The regenerated tissue/embryos are transferred to 18 ml of hormone-free medium in conical flasks (I) maintained on a shaking machine at 60 rev. min -1 at 32^0 C.

G. Finally, the mature embryos are transferred to solidified B 5 medium containing 2% sucrose for germination (J,K).

Stage of pollen development

- Selection of appropriate age of pollen grains is very critical in the induction of androgenesis.
- Generally, the pollen grains around the first mitosis are most responsive.
- The anthers of *Datura innoxia*, *Nicotiana tabacum* and *Paeonia hybrida* gave best response when the pollen were just before, at or just after the pollen mitosis.
- The early bicellular stage of pollen is best for *Atropa belladonna* and *Nicotiana sylvestris*.
- The pollen of rice (Raghavan, 1990) and most *Brassica* species (Leelavathi et al., 1984; Sharma and Bhojwani, 1985; Dunwell et al., 1985) are most vulnerable for embryogenic division at the late uninucleate stage.

Anther wall factor(s)

- The anther wall plays an important role in pollen-embryo development
- The pollen from one cultivar of tobacco would successfully develop into an embryo even if transferred into the anthers of another cultivar.
- Nursing effects of whole anthers for androgenic development of isolated pollen of the same species as well as of different species were reported.
- Even the extract of anthers stimulated pollen-embryo production.
- The role of anther wall factor(s) in pollen embryogenesis is also suggested by the histological studies of cultured anthers.

Pre-treatment of cultured anthers/pollen grains

Application of certain physical and chemical treatments to cultured anthers or pollen grains, prior to their transfer to standard culture room conditions, has proved essential or promotory for in vitro androgenesis.

- *Temperature shock*.
  - In many species the incubation of anther/pollen cultures at a low temperature (4-5^{0}C for various periods before shifting them to 25^{0}C enhanced the androgenic response in *Nicotiana tabacum* up to 58% of the anthers yielded embryos if the buds were pre-treated at 5^{0}C for 72 h as against 21% anthers from buds maintained at 22^{0}C for the same period.
  - In some plants, such as *Capsicum*, oat, and some genotypes of wheat an initial high temperature shock has proved beneficial. A high temperature shock (30-35^{0}C for the initial 1-4 days of culture is essential to induce androgenesis in most *Brassica* species

- *Centrifugation*.
  - *In Datura innoxia* the centrifugation of anthers at 40 g for 5 min after cold treatment of buds at 3^0C for 48 h improved the percentage of androgenic anthers.
- *γ-Irradiation*.
  - *Judicious application* of γ -irradiation to anthers before culture has been reported to promote pollen callusing and pollen embryogenesis in *Nicotiana* and *Datura, wheat, rice and B. napus*

APPLICATIONS

- **Shortening of breeding cycle**:
  - The most important application of androgenic haploids is in the production of stable, homozygous dihaploids (DH) in a single generation equivalent to the F_{∞} generation of pedigree breeding and, thus, considerably shortening the breeding cycle.
- **Gametoclonal variations**
  - Besides yielding haploids, in vitro androgenesis provides a unique opportunity to screen the gametophytic variation, caused by recombination and segregation during meiosis, at the sporophytic level.
  - The gametoclonal variants being hemizygous express even the recessive traits unlike somaclonal variants which require selfing and progeny analysis.
  - A gametoclone of tomato, which bears fruits with higher solid content than the parent cultivar, has been selected.

- **Mutagenesis**
  - Detection and isolation of recessive mutants in the haploid state and rapid obtainment of the mutated gene in a homozygous diploid state is a special merit of haploidy in higher plants.
  - Application of mutagenic treatment at the microspore stage, which is a single celled structure, has the added advantage of obtaining solid mutants.
  - Ethyl nitrosourea (20 mM) and γ -irradiation (0.5 Krad) have been successfully used to create herbicide resistant mutants of *Brassica napus*.
- **Genetic transformation**
  - *Agrobacterium* is a superior vehicle for transformation of dicots but its use with monocots is very limited.
  - Therefore, alternative methods, such as microinjection, electroporation etc., are being tried.
  - Another problem with somatic protoplasts/cell transformation in most monocots and some dicots is the poor regenerability of plants after DNA insertion.
  - In such cases immature pollen embryos, which exhibit high regenerative capacity offer excellent recipient cells.
  - The pollen embryos being haploid the proof of a successful transformation is facilitated because it is expressed homozygously.

- Neuhaus et al. (1987) produced transgenic plants of *B. napus* by microinjection of DNA into individual cells of immature embryos. The 12-cell stage of the embryo was optimum for this purpose, considering the optical control during microinjection, high survival rates and overall efficiency until plant regeneration.

- **Production of super-males of *Asparagus officinalis***
  - In *Asparagus officinalis*, an inbred population is produced through sib crosses between pistillate (XX) and staminate (XY) plants which yield 50% males and 50% females.
  - However, the commercially desirable features of this crop are uniform male population with spears having low fibre content.
  - Thevenin (1974) and Tsay et al. (1982) reported the production of haploids of this species by anther culture which could be diploidized to raise homozygous males (YY), also called super-males. The advantage of having super-males is that when such plants are crossed with females they yield a homogeneous male population