Tissue Culture Applications- Part I

Discipline: Botany

Paper: Plant Biotechnology

Lesson: Tissue Culture Applications- Part II

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Learning Outcomes

After reading the lesson the reader should be able to understand the

- Importance of haploid and hybrid plants.
- Mechanism of development of a complete plant from a microspore.
- Androgenesis, gynogenesis, somatic hybridization, protoplast fusion and embryo rescue.
- Basic steps involved in *in vitro* production of haploids and hybrids.

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Introduction

In the Chapter Tissue Culture Applications – Part I, we discussed three important aspects of tissue culture – micropropagation, virus elimination and endosperm culture. But does the story end here?

Well, far from it, we still have many topics left to cover the entire gamut of applications of this technique. In this part of the topic, we shall broadly discuss two types of plants – haploids and hybrids and address questions like, what is their importance, what are the methods of their production through tissue culture and what are their applications?

Haploid production

Principle

The life cycle of angiosperms (higher plants) is characterized by alternating generations of sporophytes and gametophytes.

- Gametophytic phase

  The diploid cells undergo meiosis (reduction division) to form gametes. It is a short lived phase as fertilization of the egg again results into the diploid sporophytic phase.
- Sporophytic phase

  Chromosome number (2n) is the product of fertilization of male and female gametes, containing the haploid (n) set of chromosomes from each parent.

Haploid is a generalized term for plants that contain the gametic chromosome number (n). They can be produced by forcing male or female gametes into a sporophytic pathway to develop into complete plants. Haploids are sterile; therefore, chromosome doubling is required, which produces doubled haploids or homozygous diploids, to produce fertile plants.

What is the difference between a haploid and a monoploid?

Some obvious questions may arise in your mind after reading this – how is it possible for a gametic cell to develop into a complete plant without becoming a zygote? And why do we need to produce haploids anyway? If haploid plants are produced, how will they differ from their normal diploid counterparts? The answers to all these questions lie ahead in the following section.

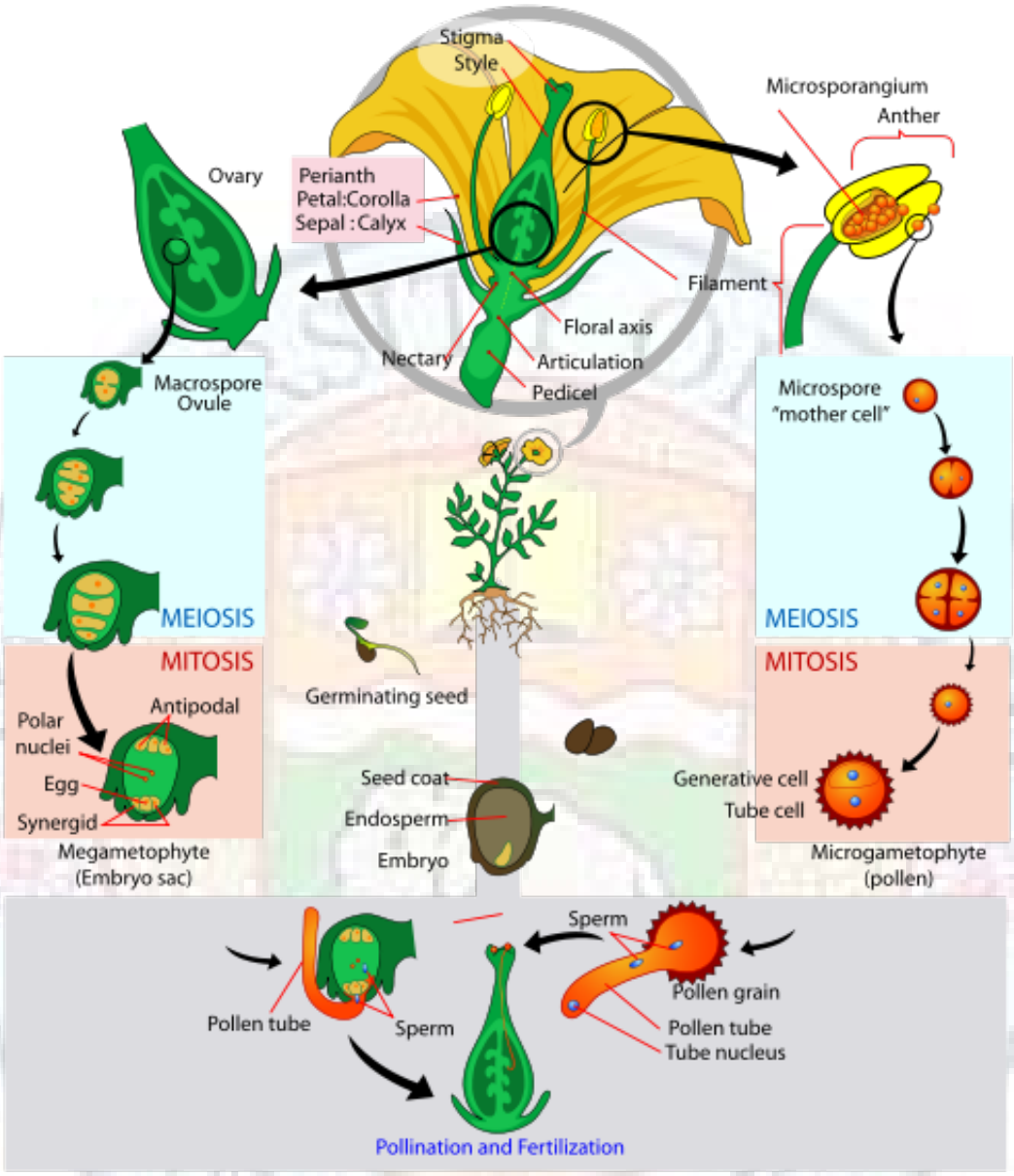

Figure: Life cycle of angiosperms showing alternation of generations. http://en.wikipedia.org/wiki/Flowering_plant#mediaviewer/File:Angiosperm_life_cycle_diagram.svg

Discovery

Dorothy Bergner was the first to describe the natural occurrence of sporophytic haploids in the weed species *Datura stramonium* in 1922.

Methodology

- ***In vivo* haploid production**

In vivo production of haploids may occur through parthenogenesis (development of embryo from an unfertilized egg). However, sometimes natural haploids show the characters of only the male parent, suggesting their origin through 'ovule androgenesis' (development of embryo inside the ovule by the activity of the male nucleus only where elimination or inactivation of egg nucleus occurs before fertilization). Although *in vivo* occurrence of haploids has been reported in several species, but it occurs at low and variable frequencies.

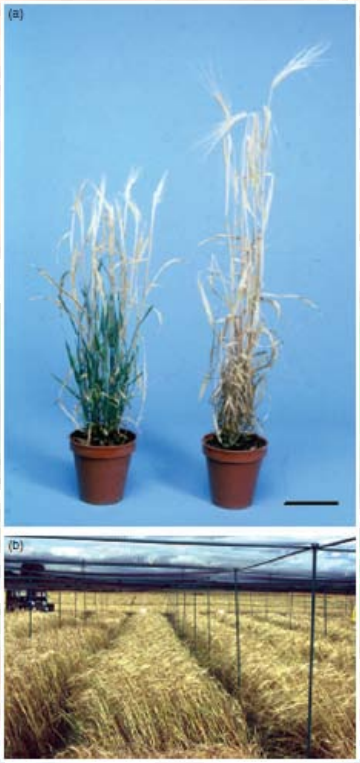

Figure: Plants of barley derived from microspore culture. (a) Microspore culture –derived haploid (left) and normal diploid (right). Scale bar is 10 cm. Haploid plant is sterile and therefore shows continued vegetative growth and tillering, and absence of monocarpic senescence. (b) Field trial of microspore culture - derived doubled haploid barley progeny plants.

Source: Dunwell, Jim M. "Haploids in flowering plants: origins and exploitation." *Plant biotechnology journal* 8.4 (2010): 377-424.

- ***In vitro* haploid production**

  In vitro, haploid plants can be obtained by triggering the male or female gametic cells to undergo sporophytic development.

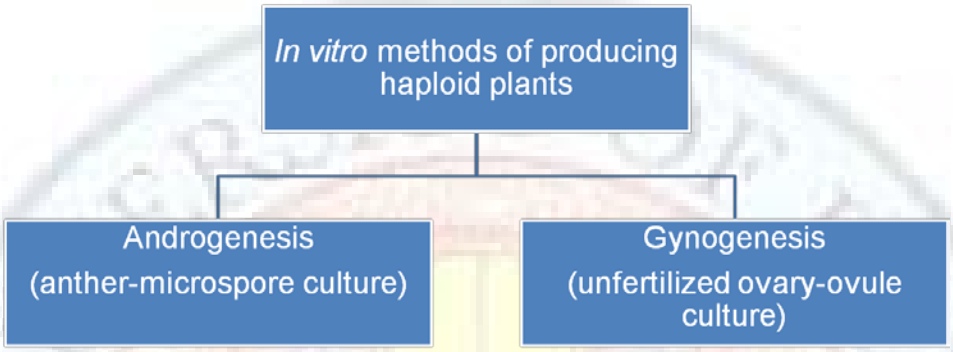

Figure: *In vitro* methods of haploid plant production

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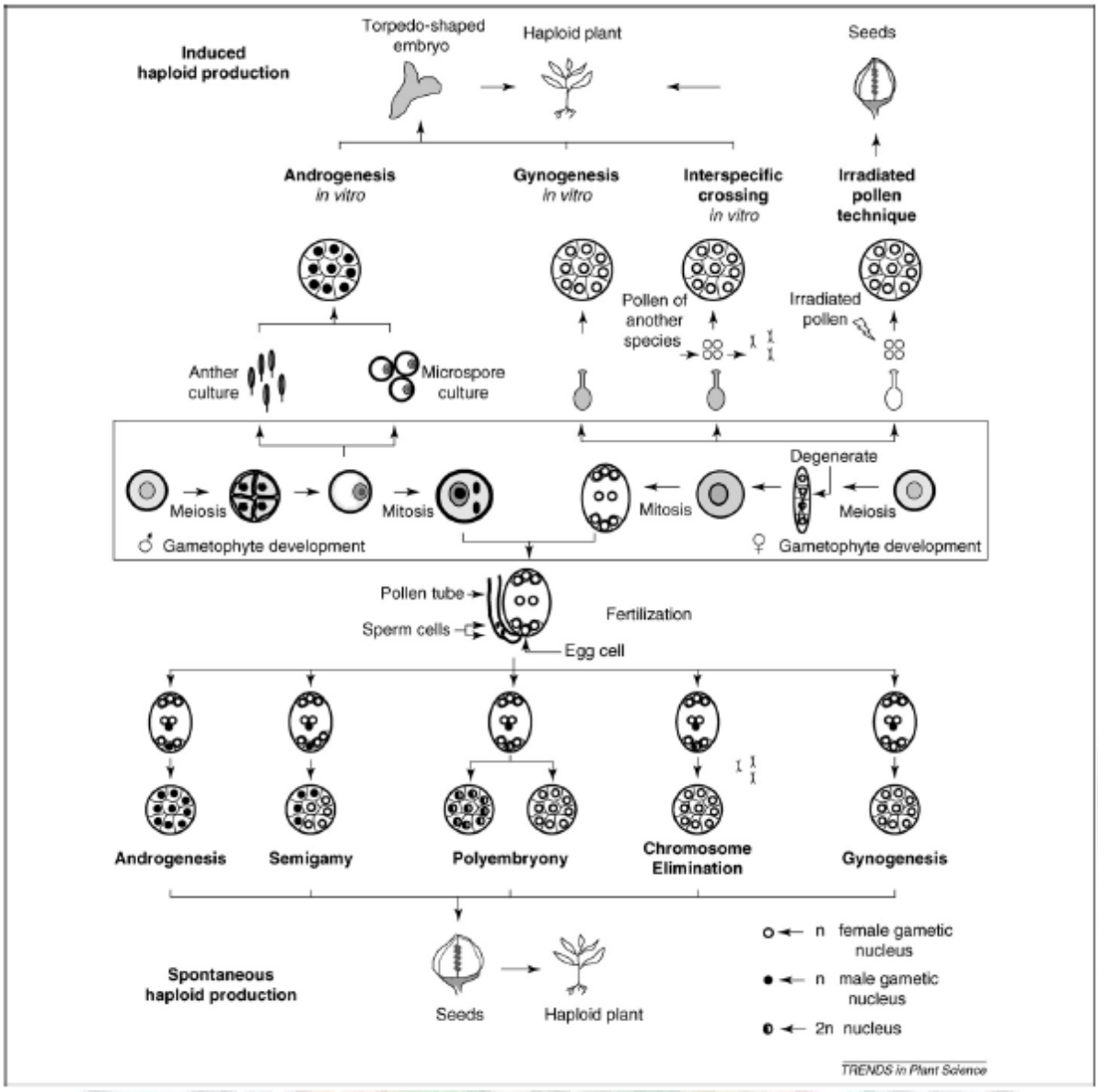

Figure: Methods of haploid plant production. Spontaneous haploids can be observed via semigamy, polyembryony, chromosome elimination, gynogenesis and androgenesis at extremely low frequencies. Haploids can be induced via androgenesis or gynogenesis and by distant hybridization using irradiated pollen or pollen from another species.

(Semigamy – an abnormal type of fertilization whereby either reduced or unreduced male and female gametes participate in embryo formation but fertilization does not occur. Polyembryony – The production of two or more embryos in one seed, owing either to the existence and fertilization of more than one embryonic sac or to the origination of embryos outside of the embryonic sac).

Source: Forster, Brian P., et al. "The resurgence of haploids in higher plants." *Trends in plant science* 12.8 (2007): 368-375.

http://www.sciencedirect.com/science/article/pii/S1360138507001598

- **Androgenesis**

  In androgenesis, the male gametophyte (microspore or immature pollen) produces haploid plants.

  - **Principle**

    The basic principle is to stop the development of pollen into a gamete (sex cell) and force it to develop into a haploid plant or sporophyte.

  - **Discovery**

    The discovery of the phenomenon that haploid embryos and plants can be developed by *in vitro* culture of anthers of Datura (Guha and Maheshwari 1964, 1966) brought reinvigorated interest in haploidy. This method of production of haploids by androgenesis was quickly tried in many species to hasten the breeding programme in several commercially important plants. A milestone was set with the release of the first DH crop plant, the cultivar Maris Haplona of *Brassica napus* in the early 1970s and Mingo in barley *Hordeum vulgare* in 1980.

- **Methodology**

1. Anther culture

The basic methodology is summarized in flowchart below

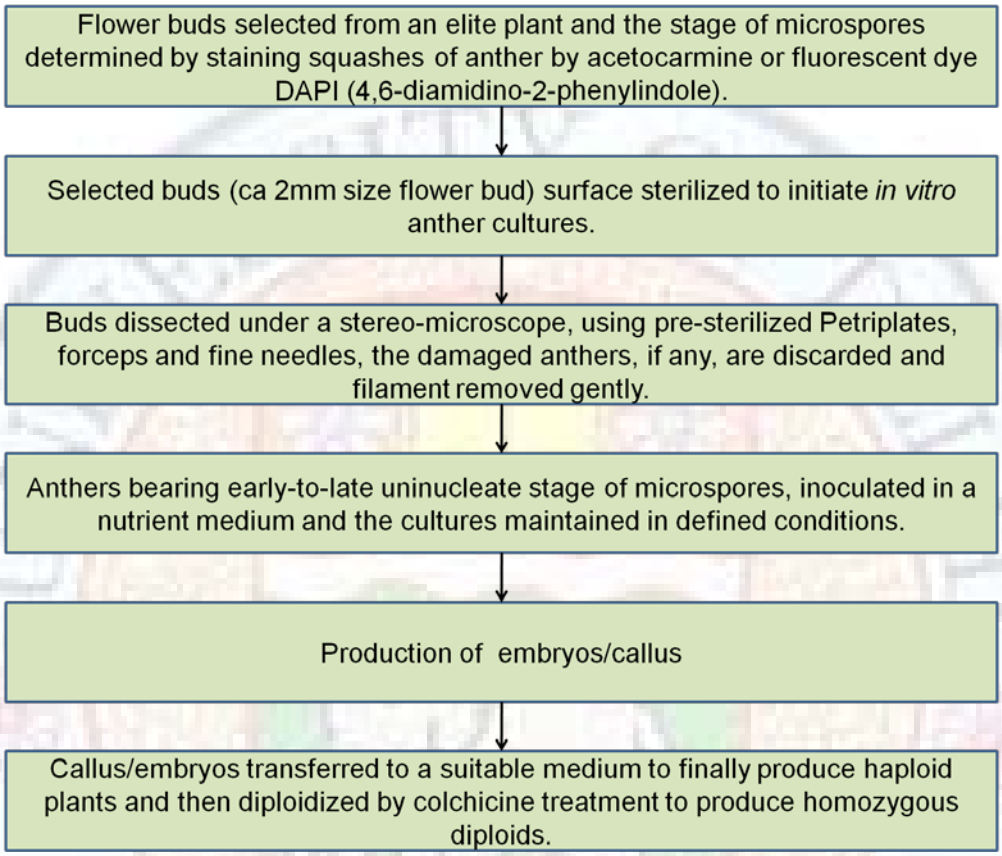

Figure: Flowchart showing steps in anther culture.

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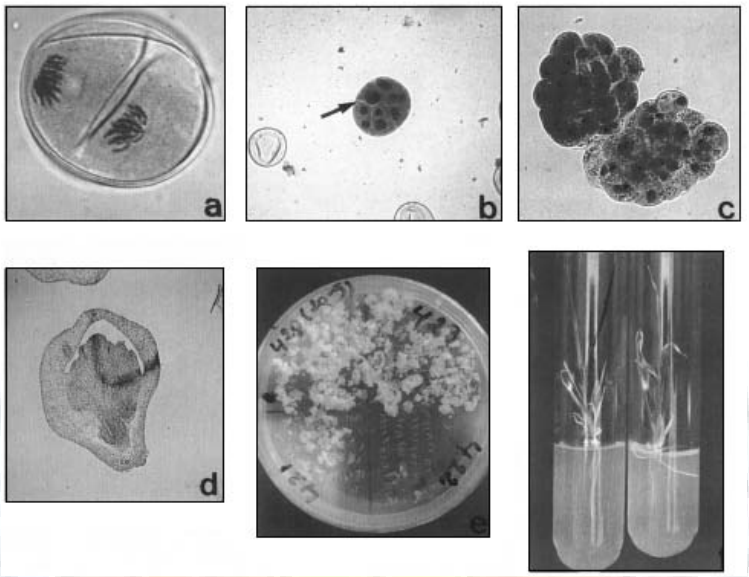

Figure: Different stages of microspore culture in barley (A) First stage of mitosis of a microspore (1250 x). (B) Multicellular pollen grain, (after12 days), it contains12 cells, separated by cell walls (see arrow) (200 x). (C) Pro-embryos released from inside the anthers after exine rupture (200 x). (D) Longitudinal section of an isolated embryo (100.8 x). (E) Petri plate with cultured anthers showing the induction of structures from which plantlets (see arrow) (F) Green plantlet.

Source: da Silva, A. L. S., M. I. Moraes-Fernandes, and A. G. Ferreira. "Ontogenetic events in androgenesis of Brazilian barley genotypes." Revistabrasileira de biologia 60.2 (2000): 315-319.

www.scielo.br/pdf/rbbio/v60n2/a16v60n2.pdf

2. Microspore / Pollen culture

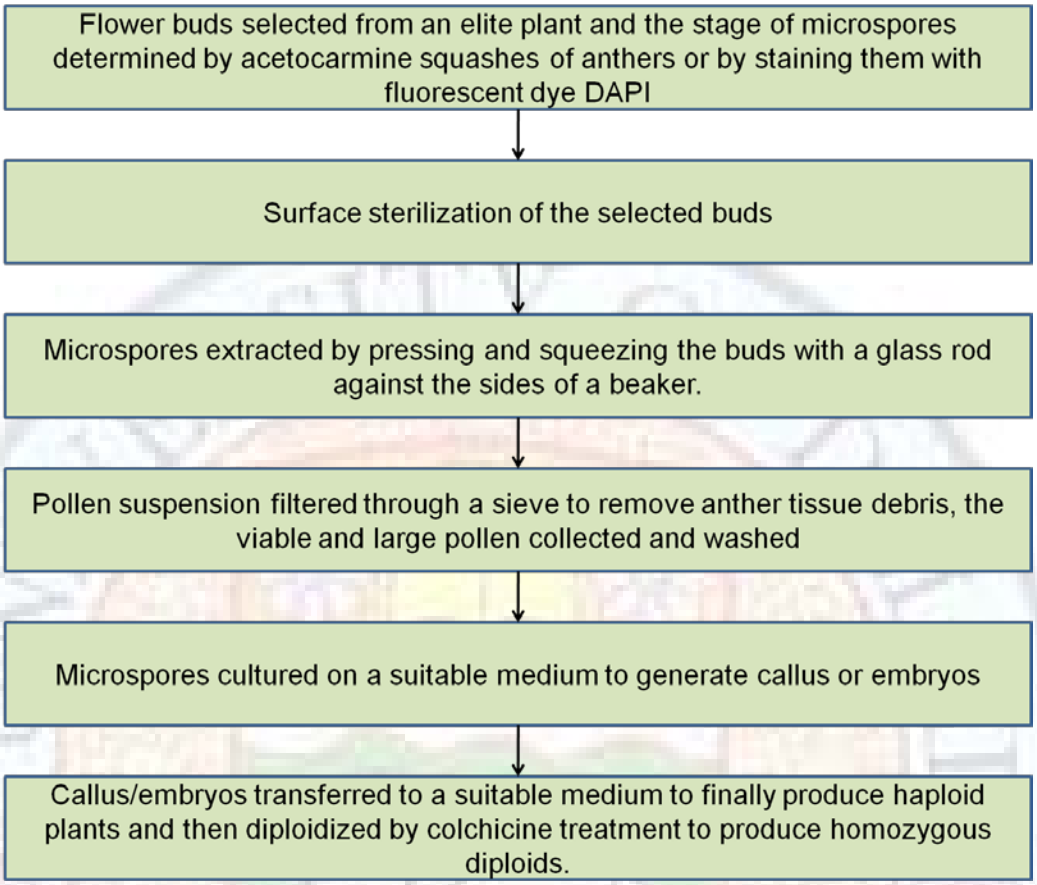

Figure: Flowchart showing steps in pollen culture

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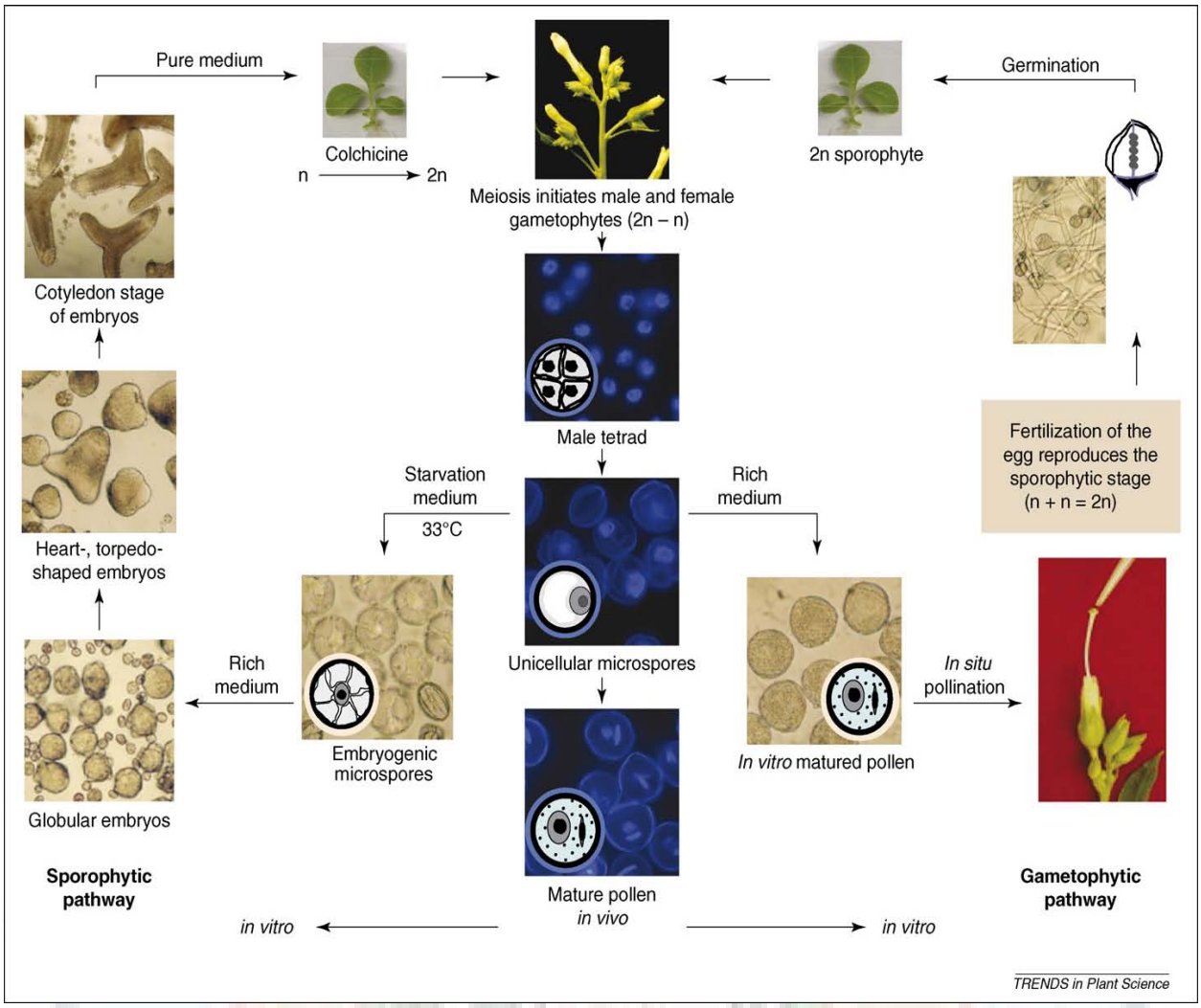

Figure: Developmental pathways of tobacco microspores *in vivo* and *in vitro*. Tobacco microspores develop *in vivo* into mature bi-cellular pollen (middle of the panel).Isolated microspores can be cultured *in vitro* until the formation of mature, fertile pollen in a rich medium with high sucrose concentration (right panel). However, microspores subjected to starvation at high temperatures form embryogenic cells that develop into haploid embryos and plants when cultured in a rich medium at normal temperatures (left panel). The haploid plants obtained can be diploidized by treatment with colchicines or other anti-mitotic drugs.

Source: Forster, Brian P., et al. "The resurgence of haploids in higher plants." *Trends in plant science* 12.8 (2007): 368-375.

http://www.sciencedirect.com/science/article/pii/S1360138507001598

- **Pathways of development**

The early divisions in pollen grains responding to culture may occur in any one of the following four pathways:

1) Pathway A - The uninucleate pollen grain may undergo symmetric division to yield two equal daughter cells, both of which undergo further divisions e.g. *Datura innoxia*.

2) Pathway B - The uninucleate pollen undergoes unequal division. The generative cell degenerates and callus/embryo originates because of successive divisions of the vegetative cell. E.g. *Nicotiana tabacum*, barley, wheat etc.

3) Pathway C - The pollen embryos may also originate from the generative cell; the vegetative cell may either not divide or divide only to a limited extent to form a suspensor like structure.

4) Pathway D - The uninucleate pollen grains may divide unequally, producing generative and vegetative cells and both these cells divide repeatedly to form the developing embryo/callus.

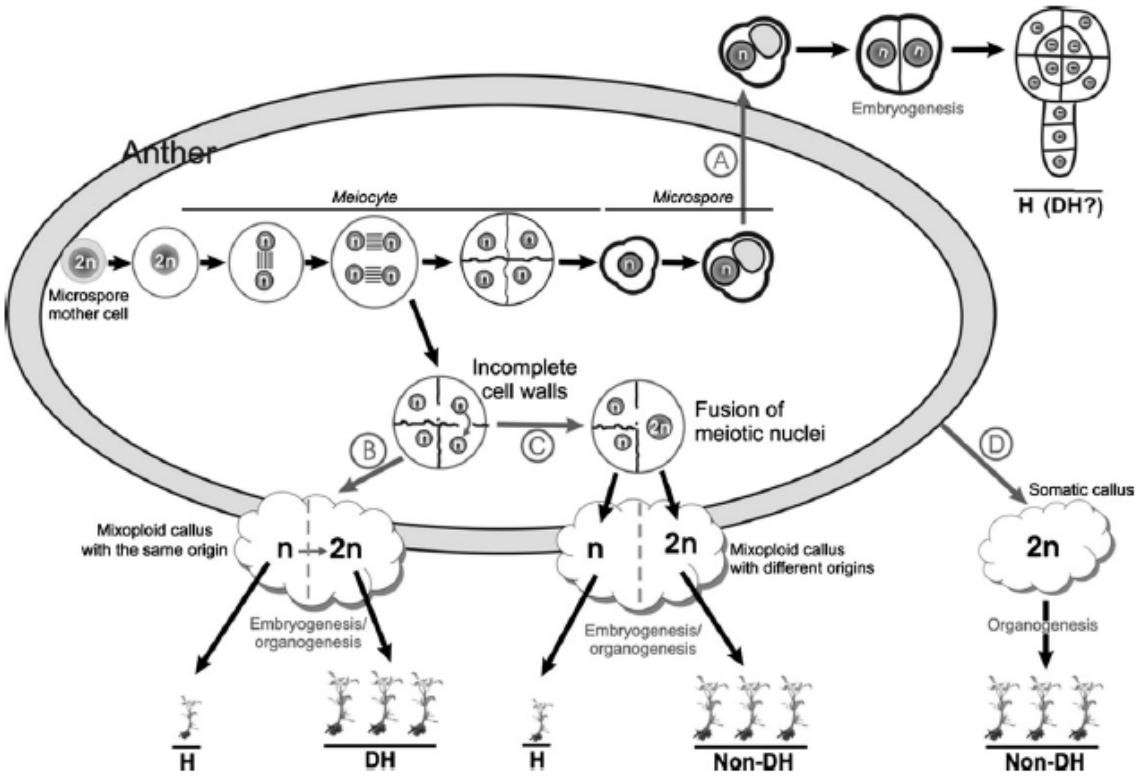

Figure: Diagram showing different possible *in vitro* pathways of development - A pathway, (from vacuolate microspores), B and C pathways (from meiocytes within cultured anthers) and D pathway (from somatic tissue of the anther walls). These pathways may result in formation of haploid (H) or doubled-haploid (DH) embryos and/or plants.

Source: Seguí-Simarro, José M., and Fernando Nuez. "Embryogenesis induction, callogenesis, and plant regeneration by *in vitro* culture of tomato isolated microspores and whole anthers." Journal of experimental botany 58.5 (2007): 1119-1132.

http://jxb.oxfordjournals.org/content/58/5/1119.short

- **Factors affecting androgenesis**

  A number of factors affect *in vitro* haploid production, which are listed in the figure ahead. Some of the factors are discussed below:

  1) Genotype, age and physiological conditions of plants may increase or decrease the chances of androgenesis. E.g. for most plants frequency of androgenesis is higher if buds are taken from the first flush of flowers, hybrids are usually more androgenic than their parents.

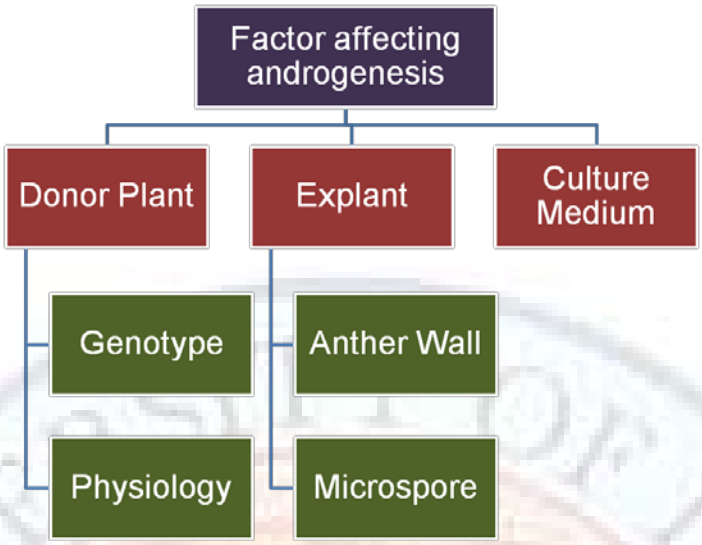

Figure: Different factors that affect androgenesis

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2) Anther wall has been shown to be important for development of somatic embryos from pollen. The concept of 'anther wall factor' was introduced by Pelletier and Ilami (1972) who conducted transplantation experiments to show that pollen from one cultivar of tobacco successfully develops into an embryo even if it is transferred into the anthers of another cultivar.
3) Presence of specific components in culture medium may be essential for androgenesis or increase is frequency. For example presence of sucrose is essential for androgenesis in most species.

An excerpt from the article by Sipra Guha Mukherjee about the discovery of haploids

'My first postdoctoral position was in the same department with S. C. Maheshwari, to study the biochemistry of meiosis. For this purpose, I had placed in culture the large anthers of *Datura innoxia*. After 6 weeks, on a hot day in June 1964, I decided to discard my cultures as they all appeared to be dead. I was greatly surprised, therefore, to observe small plantlets arising from the anthers in some of the cultures. I removed one such anther showing a small plantlet with a slight amount of callus and dissected it. The plantlet appeared to arise from inside the now dead anther wall. No other living tissue was visible. Although I did not have any direct evidence at the time, I was intrigued by the possibility that the plantlets may have arisen from pollen grains'

Readers are suggested to read the full paper for an engaging discovery about the discovery of haploids.

Guha-Mukherjee, Sipra. "The discovery of haploid production by anther culture." *In Vitro Cellular & Developmental Biology-Plant* 35.5 (1999): 357-360.

http://link.springer.com/article/10.1007%2Fs11627-999-0048-3?LI=true#page-1

- **Gynogenesis**
  - **Principle**

    The basic principle is to force unfertilized cells of female gametophyte to follow a sporophytic pathway and develop into haploids.
  - **Discovery**

    It was first reported by San Noeum (1976) in barley.

- **Methodology**

The basic method is summarized in the flowchart below:

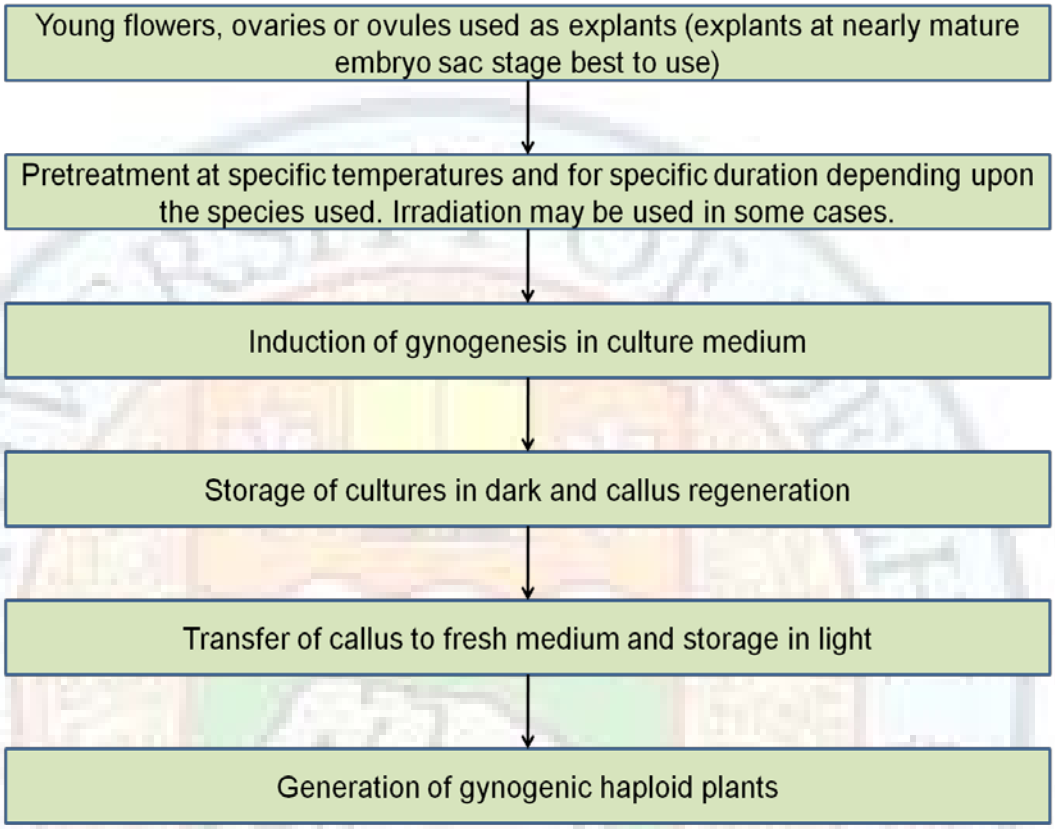

Figure: Flowchart showing steps in gynogenesis.

Developed by: Author

- **Comparison between androgenesis and gynogenesis**

Haploid production through gynogenesis is more tedious than androgenesis. The reasons are:

1) There are a large number of microspores within the anther wall for androgenesis as against single egg cell per flower for gynogenic haploid production. Since the probability of development of a cell into a somatic embryo is extremely low, a large number of experiments have to be set up for gynogenesis in contrast to androgenesis.

2) The embryo-sac is too is deep seated within, thus making the process of gynogenes is very cumbersome.

3) But gynogenesis may still be useful where anther culture has been unsuccessful, plants are male sterile or androgenesis is problematic due to the production of albino or non-haploids.

Applications

The basic application of haploid production is in crop improvement programmes. There are various scenarios where haploid production is useful.

1) Development of pure lines

Pure lines or true breeding homozygous lines are important components of breeding programmes. They are required for screening of high yielding lines and producing hybrids to capture hybrid vigor. The conventional methods of development of pure lines require many years as they require selfing of the lines for a number of generations. Tissue culture can help in achieving this homozygosity in a single generation.

2) **Genetic dissection of desirable traits**

It requires construction of mapping populations of plants segregating for desired traits. Doubled haploid populations raised through tissue culture are frequently used for genetic mapping studies. Such studies enable breeders to find genomic location of genes which govern important traits and incorporate those genes into cultivars to improve traits in crops.

Figure: Genetic segregation for a gene shown from tobacco anthers. Plantlets derived through microspore culture, developing from an anther of a plant heterozygous for the semi dominant sculpture nuclear mutation, Su /us (light green). The haploid plantlets are segregating in 1:1 ratio as green (su) or yellow (Su). Scale bar is 1 mm.

Source: Dunwell, Jim M. "Haploids in flowering plants: origins and exploitation." *Plant biotechnology journal* 8.4 (2010): 377-424.

http://onlinelibrary.wiley.com/doi/10.1111/j.1467-7652.2009.00498.x/full

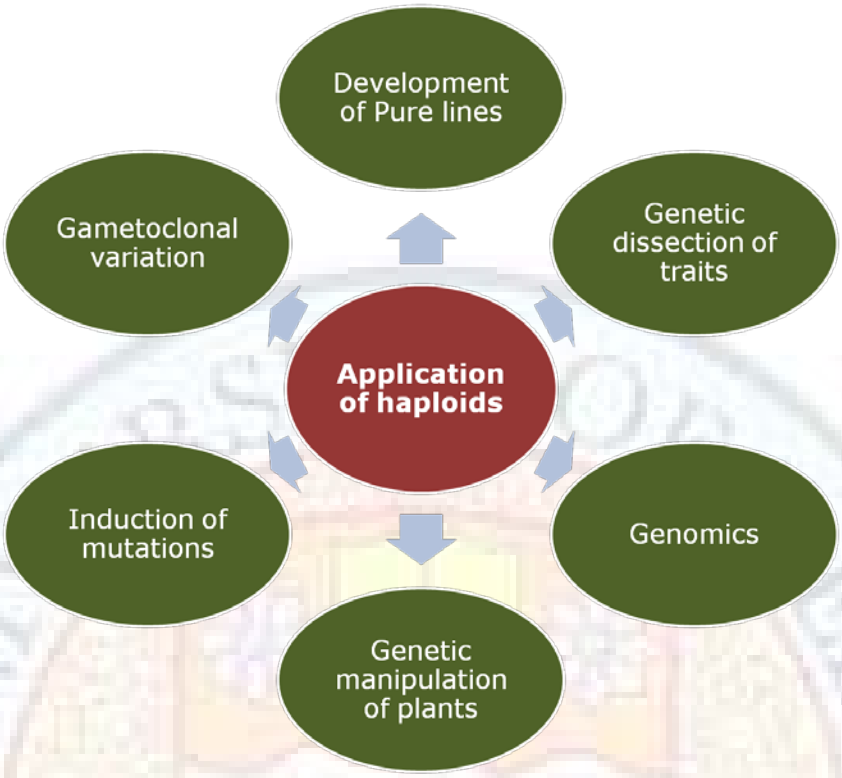

Figure: Applications of haploid plants

Developed by: Author

3) Genomics

The genetic maps constructed using doubled haploid plants are employed for integration of genetic and physical maps, which is an important part of genome sequencing projects.

4) Genetic manipulation of plants

Direct gene transfer by microinjection can be used for development of transgenic plant by using isolated pollen culture. If transgenes can be incorporated into the haploid microspore genome, prior to DNA synthesis and chromosome doubling, the doubled haploids may also be homozygous for the transgenes. For example, Rhoda et al., (2013) showed microspore transformation of *xylanase* gene to generate transgenic doubled haploid lines in wheat.

http://www.plosone.org/article/fetchObject.action?uri=info%3Adoi%2F10.1371%2Fjournal.pone.0080155&representation=PDF

5) Induction of mutations

In haploid plants, recessive mutations can be expressed as the effect will not be masked by dominant allele. This is important for selection of desirable traits which are attributed to recessive mutations. For example, anther culture technique has been successfully used to generate tobacco mutants resistant to black shank disease and wheat lines resistant to *Fusarium graminearum*.

6) Gametoclonal variation

Plants cultured from gametic cells may exhibit altered chromosome number or structure which may be a source of useful variation. For example, Witherspoon et al., (1991) identified a new gene for resistance to Potato Virus Y in anther culture derived doubled haploids from a susceptible cultivar.

Hybrid Production

Principle

Hybrids can be defined as offspring of two different genotypes of plants. They are useful for crop improvement because of 'hybrid vigor' or 'heterosis' associated with them, which implies that hybrids may show improved biological quality than either of the parents.

Figure: Figure showing a variety of hybrid corn.

Source: http://biology200.gsu.edu/houghton/2107%20%2714/lecture13.html

The credit for discovery of the phenomenon of hybrid vigor goes to G. H. Shull. In the early twentieth century, he crossed two varieties of corn, and the yield went from 20 to 80 bushels per acre. But it was first observed by Charles Darwin in 1876 and actually rediscovered by Shull years later!

Traditionally hybrids have been developed through sexual hybridization by crossing two contrasting varieties to yield superior offspring. But this method has certain limitations:

- It is very long, labor-intensive procedure requiring many years to develop a variety.
- The F1 seeds have to be procured each year by farmers, which is an expensive proposition.
- Hybrids can be developed only between closely related varieties or species which are compatible.

Based on above learning, can you suggest a less time taking method for developing hybrids?

Methodology

The above mentioned limitations can be overcome using tissue culture based methods. Following approaches can be used for development of hybrids:

a) Haploid production

b) Embryo rescue

c) Micropropagation

d) Somatic Embryogenesis

a) Haploid production

A detailed overview of use of haploids to generate doubled haploids has been given in previous section. An overview is of hybrid development through microspore culture is given in figure below.

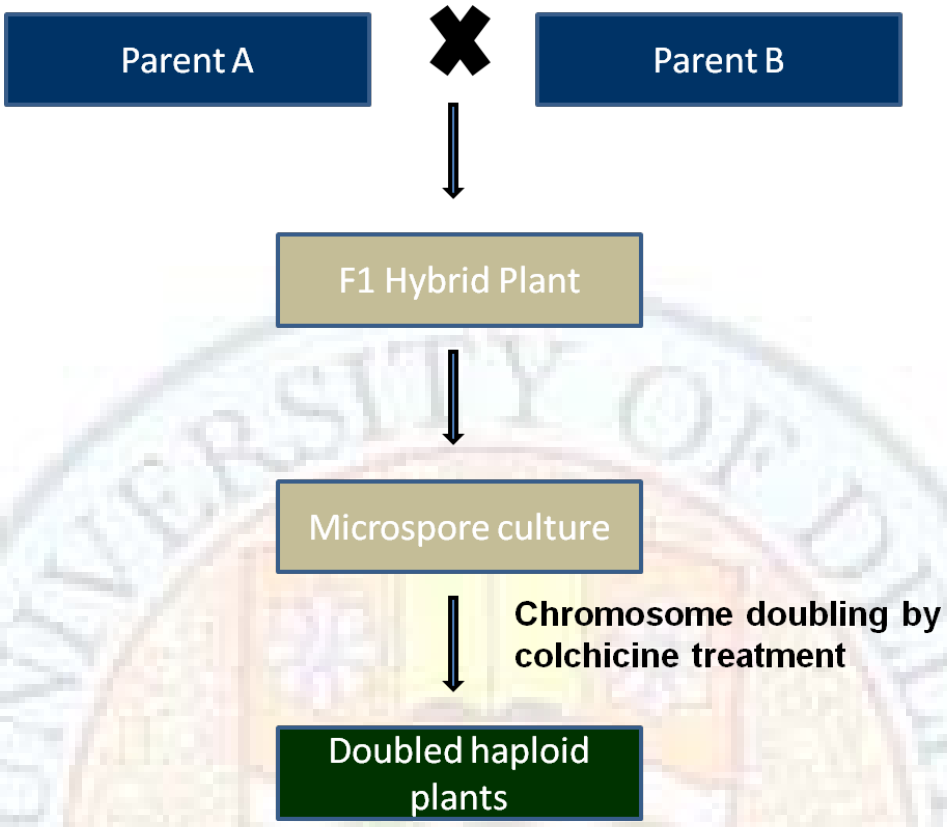

Figure: Steps in production of F1 doubled haploid plants.
Developed by: Author

b) Embryo rescue

This is the technique used in case of interspecific crosses where embryo forms after hybridization but fails to grow. In such cases, embryo can be dissected and grown in culture medium. For example: development of crop *Triticale* as shown in figure below.

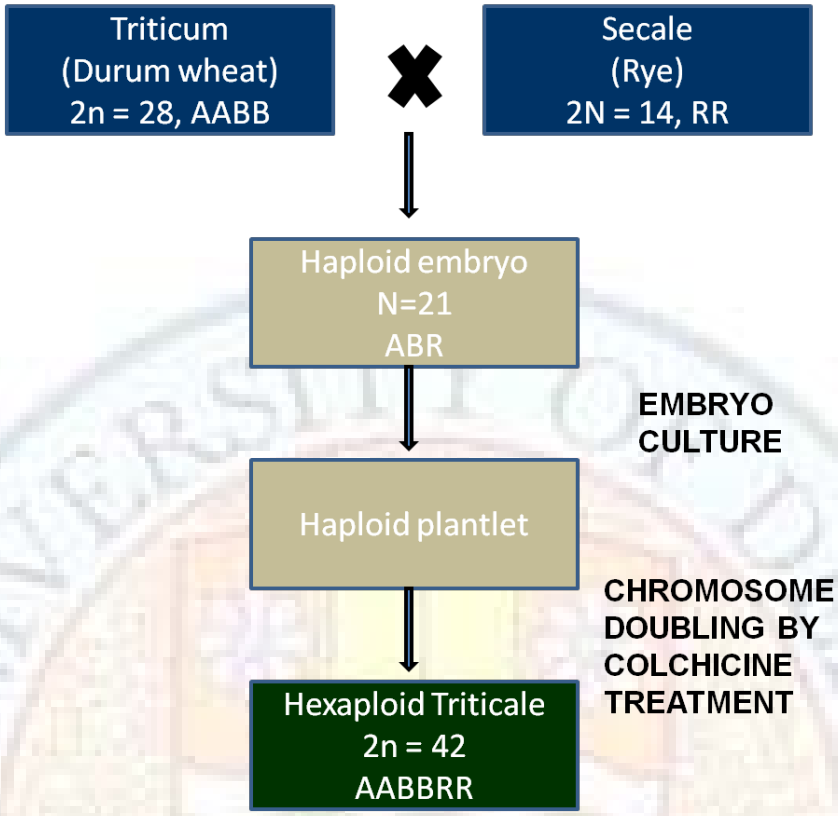

Figure: Overview of development of the hybrid crop *Triticale* using embryo rescue.

Developed by: Author

c) Micropropagation

Hybrid plantlets once obtained can be grown in *vitro* and propagated by micropropagation. This is of special importance in case of tree crops where evaluation of a tree crop would take many years and would require a large space.

d) Somatic Embryogenesis

This technique involves development of hybrids by protoplast fusion. The protoplasts of contrasting cultivars are fused and *in vitro* culture allows regeneration of hybrids via protoplast culture. This allows combination of nuclear as well as cytoplasmic genomes of both parents. Asymmetric hybrids, also called cybrids can also be formed which have nuclear genome from one parent and cytoplasmic genome from the other.

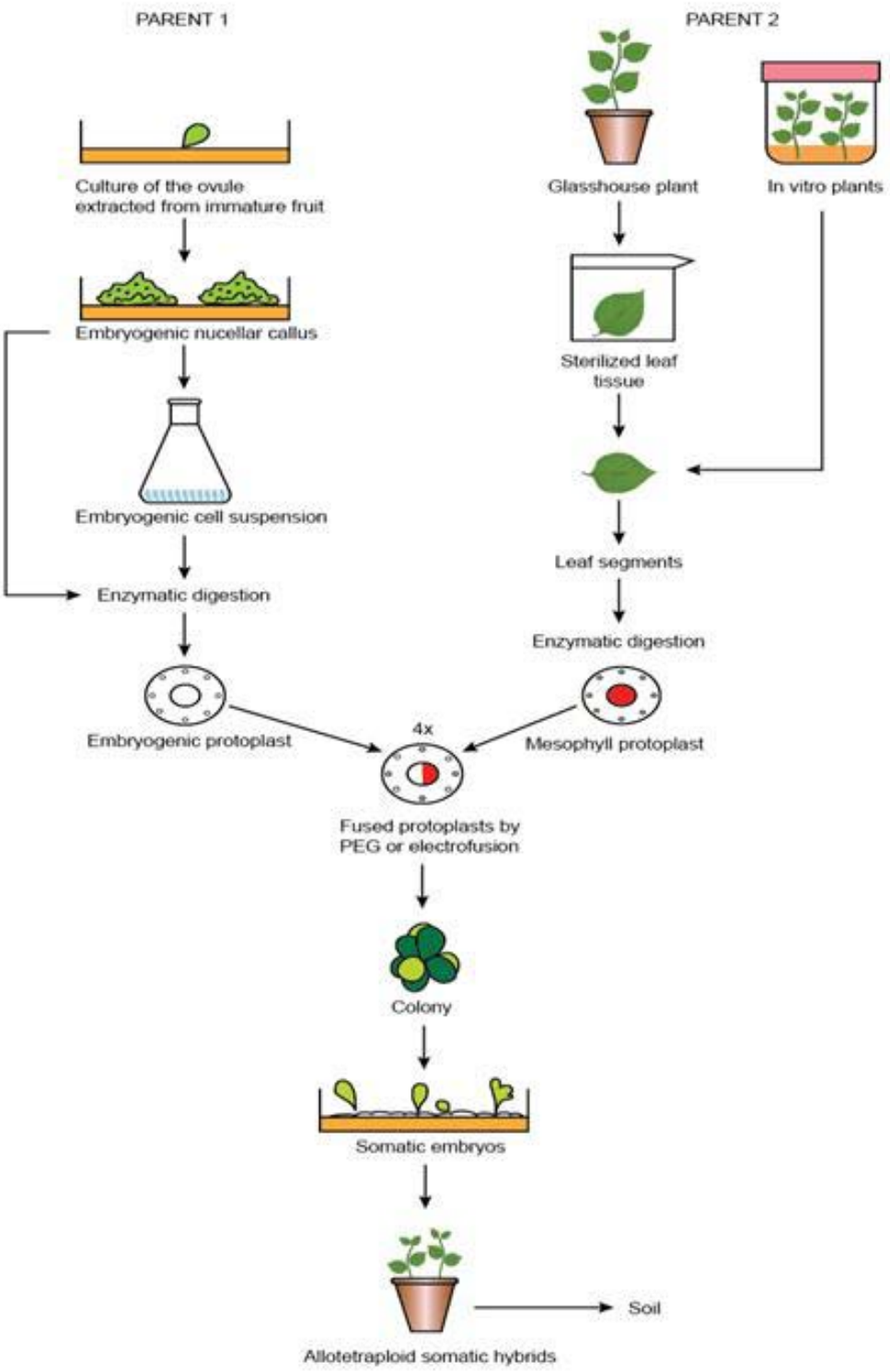

Figure: Schematic view of symmetric protoplast fusion producing somatic hybrids.

Source: www.nptel.ac.in

Method of somatic Embryogenesis

Protoplasts from both are first isolated by mechanical or chemical treatment which degrades the cell walls of tissue releasing the protoplasts. This is followed by fusion of protoplasts resulting in formation of a heterokaryon, which is mediated by following means

i. $NaNO_3$ - A hypotonic solution of $NaNO_3$can be used to fuse protoplasts.

ii. High pH and Ca^{++} treatment

iii. Polyethylene glycol (PEG) – This is the most widely used fusogen as it results in generation of a high frequency of heterokaryons which are mostly heterokaryons. A 15 – 45% solution is used for a period of 15 to 30 minutes followed by washing in an alkaline medium. But a major disadvantage of chemical fusogens is that they may be toxic to the cell, cause random aggregation and their removal is necessary for further culturing.

iv. Electrofusion – It does not suffer from the disadvantages of chemical fusagens and also the somatic hybrids produced by electrofusion are more fertile as compared to other methods. In this method electric pulses are passed through a fusion chamber in which protoplasts have been introduced. This results in reversible breakdown of plasma membranes, fusion of protoplasts and reorganization of the membrane.

The heterokaryons thus formed are selected on the basis of their morphology – they should have morphological markers of both parent protoplasts. After this the heterokaryons are cultured to raise calli and developed into hybrid plants as discussed in previous techniques.

Summary

Plant tissue culture techniques are important for *in vitro* production of haploids and hybrids. *In vitro* production of haploid plants is useful as homozygous diploids (doubled haploids) can be produced in a single generation via chromosome doubling by colchicines treatment in contrast to traditional ways which require many years of selfing. This can be done by androgenesis i.e. anther or pollen culture or gynogenesis i.e. ovule culture. While doubled haploids have many uses in plant breeding genomics and other fields, haploid production as such can be used for mutation detection, as they express recessive mutations.

Hybrid plants are important because they exhibit hybrid vigor. It has been observed widely in not just plant breeding, but also animal breeding .i.e. improved characters as compared to either of their parents. Conventional production of hybrids has several disadvantages which can be overcome by *in vitro* procedures like embryo rescue, haploid culture, micropropagation and somatic hybridization.

Exercise

1. What is the importance of hybrid plants?
2. How are haploid plants different from their diploid counterparts?
3. What do you mean by the term ‘embryo rescue’?
4. What is hybrid vigor?
5. What are the different methods of fusing protoplasts?
6. What are the applications of haploid plants?
7. What is the difference between
   - Androgenesis and gynogenesis
   - Symmetric and asymmetric hybrids
8. What is the use of ‘doubled haploids’ in plant breeding?
9. What is selfing?

Glossary

Androgenesis: Development of plants from male gametophytes.

Anther: The terminal portion of a stamen which contains pollen in pollen sacs.

Anther culture: Culture of excised anthers, to obtain haploids.

Cybrid: A cell or plant with nucleus of one parent and extranuclear genes of another or both parents, from the fusion of protoplasts.

Diploid: Having two copies of each chromosome characteristic for the species.

Gynogenesis: Development of plants from female gametophytes.

Haploid: Having single copy of each chromosome per cell characteristic of the species.

Heterokaryon: A cell in which two or more nuclei of unlike genetic make-up are present, usually derived as a result of cell fusion.

Homozygous: Diploid or polyploid individuals having identical alleles on the homologous chromosomes. Self-fertilization of homozygous individuals would give a homogeneous population.

Hybrid: An organism resulting from a cross between genetically unlike parents.

Hybridization: Any process by which hybrids are created.

Microspore: A uninucleate, haploid cell which develops into a pollen grain (male gametophyte).

Mutation: The occurrence of a heritable variation in an individual due to a change in genes or chromosomes.

Pollen: Male gametophyte, which is haploid and bears the male gametes (sperms).

Protoplast fusion: Technique in which related or unrelated protoplasts are fused to form a homokaryon or a heterokaryon.

References

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Chrispeels, Maarten J., and David E. Sadava. *Plants, genes, and crop biotechnology*. Jones & Bartlett Learning, 2003.

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Simarro, José M., and Fernando Nuez. "Embryogenesis induction, callogenesis, and plant regeneration by in vitro culture of tomato isolated microspores and whole anthers." Journal of experimental botany 58.5 (2007): 1119-1132.

Web links

https://www.uoguelph.ca/plant/research/biotech/haploid/anther.htm

http://theagricos.com/tissue-culture/anther-or-pollen-culture/

http://www.uoguelph.ca/plant/research/biotech/haploid/microsp.htm

http://agriinfo.in/default.aspx?page=topic&superid=3&topicid=1945

http://publishing.cdlib.org/ucpressebooks/view?docId=ft796nb4n2&chunk.id=d0e23941&toc.id=d0e23941&brand=eschol