FACULTY OF MEDICAL SCIENCES

MICROPROPGATION

- Plants can be propagated by sexual (through generation of seeds) or asexual (through multiplication of vegetative parts) means.
- Clonal propagation refers to the process of asexual reproduction by multiplication of genetically identical copies of individual plants, where the term clone is used to represent a plant population derived from a single individual by asexual reproduction.
- In vitro clonal propagation through tissue culture is referred to as micro propagation.

RAMA UNIVERSITY

- Micropropagation is the practice of rapidly multiplying stock plant material to produce a large number of progeny plants, using modern plant tissue culture methods.

- Micropropagation is used to multiply noble plants such as those that have been genetically modified or bred through conventional plant breeding methods. It is also used to provide a sufficient number of plantlets for planting from a stock plant which does not produce seeds, or does not respond well to vegetative reproduction.

RAMA
UTTAR PRADESH
KANPUR

TECHNIQUE OF MICROPROPAGATION

STAGE 0

Preparative stage

- Initial step in micropropagation
- Involves the selection and growth of stock plants for about 3 months under controlled conditions.

STAGE 1

Establishment of explants

- Initiation and establishment of culture in a suitable medium is achieved.
- Selection of appropriate explants is important.
- The most commonly used explants are organs, shoot tips and axillary buds.

STAGE 2

Shoot multiplication

- In this stage, the major activity of micro propagation occurs in a defined culture medium.
- This stage mainly involves multiplication of shoots or rapid embryo formation from the explant.

Contd.

STAGE 3

Rooting

- Transfer of shoots to a medium for rapid development into shoots.
- Sometimes, the shoots are directly planted in soil to develop roots.
- In vitro rooting of shoots is preferred while simultaneously handling a large number of species.

STAGE 4

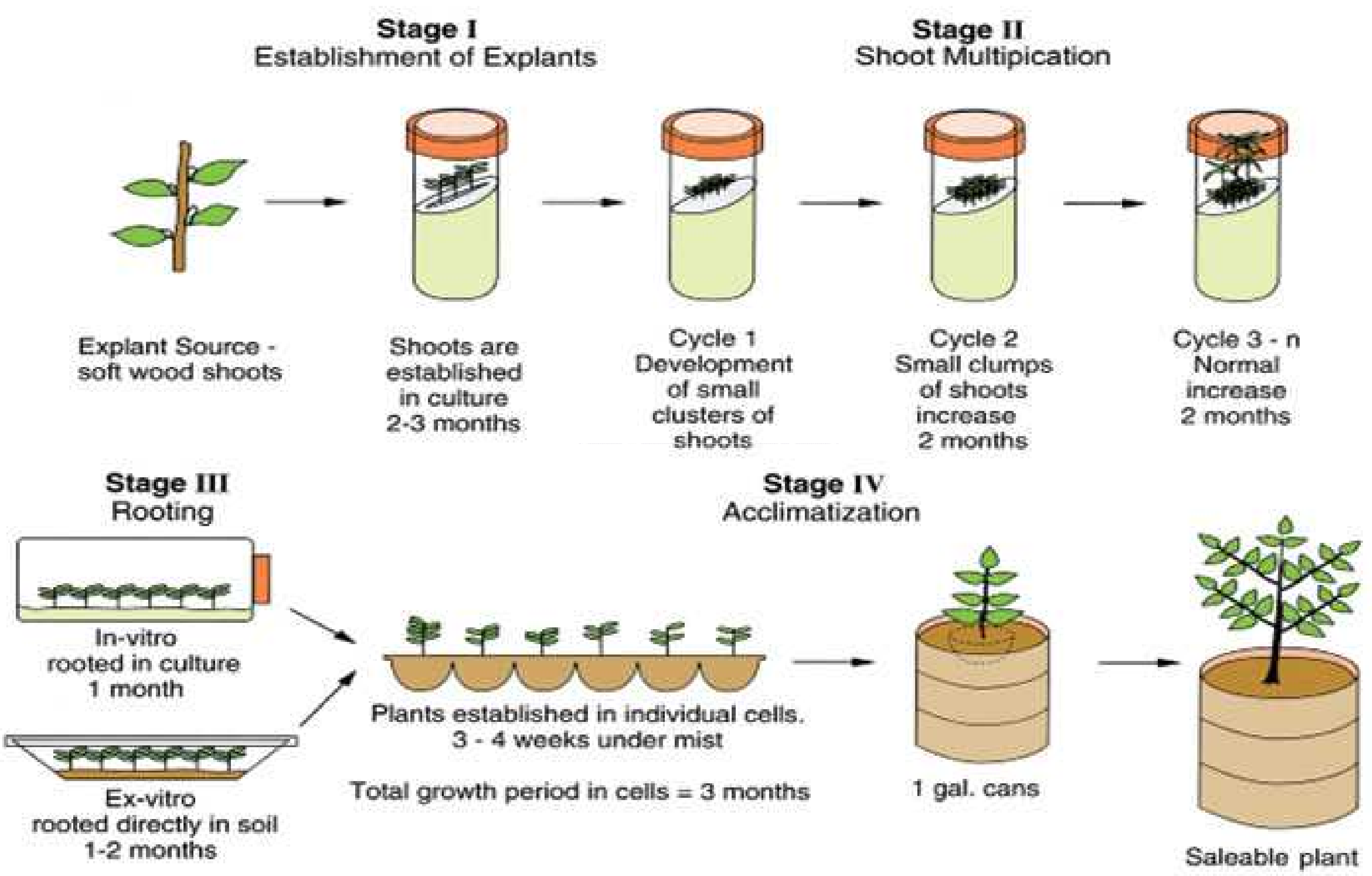
Acclimatization

- Establishment of plantlets in soil.
- Done by transferring the plantlets of stage 3 from the laboratory to the environment of greenhouse..

STAGE 5

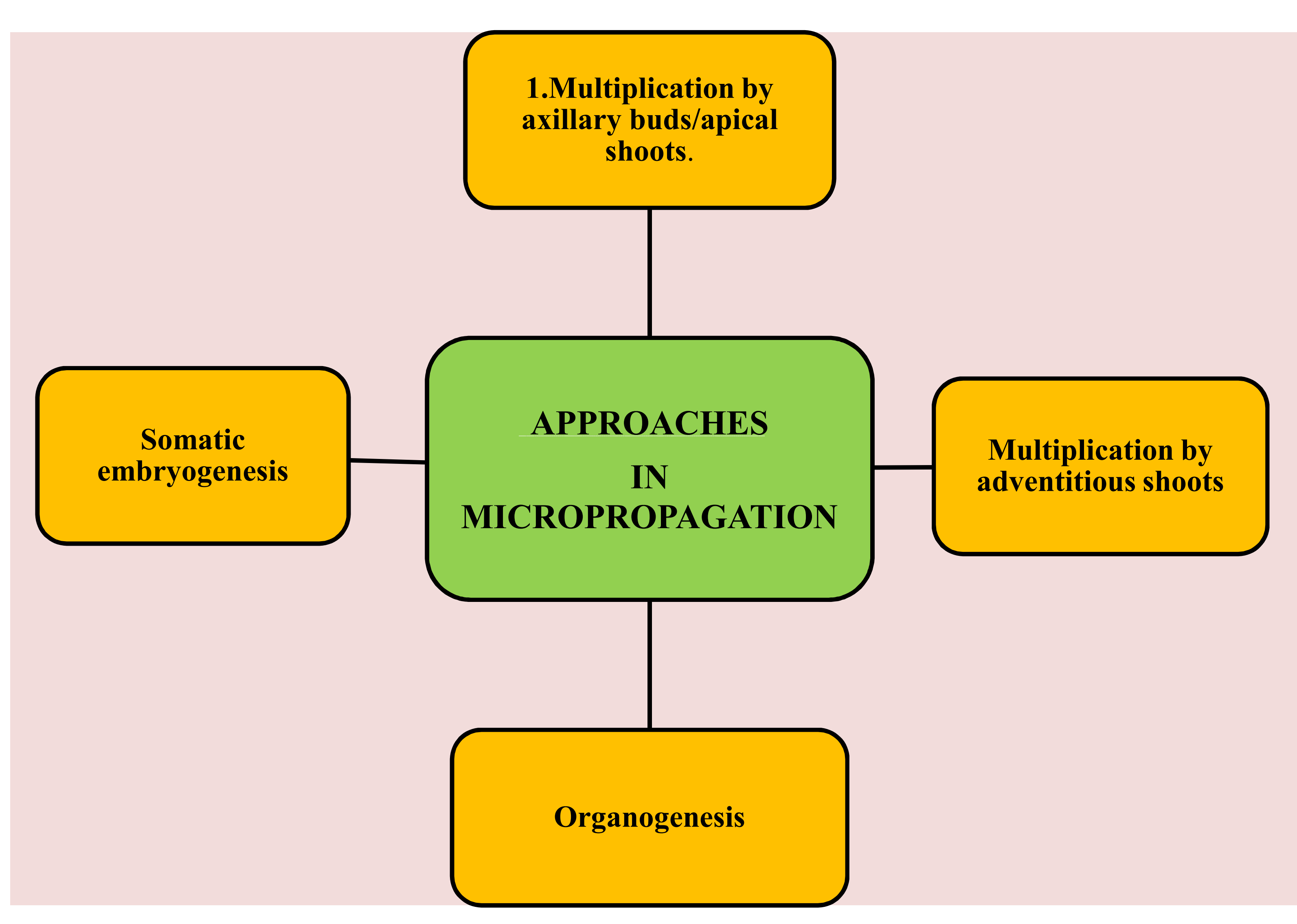
- In this stage, the major activity of micro propagation occurs in a defined culture medium.
- Stage 2 mainly involves multiplication of shoots or rapid embryo formation from the explant.

STAGES OF MICROPROPOGATION



https://microbenotes.com/micropropagation-stages-types-applications-advantages-limitations/

APPROACHES INVOLVED IN MICROPROPAGATION



1. **Multiplication by axillary buds/apical shoots**.

- Actively dividing meristems are present at the axillary buds and apical shoots (shoot tips).
- The axillary buds located in the axils of leaves are capable of developing into shoots.
- By means of induced in vitro multiplication in micro propagation, it is possible to develop plants from meristem and shoot tip cultures and from bud cultures.

https://www.slideshare.net/DrSureshSolleti/micropropagation-78526270

RAMA UNIVERSITY

Shoot tip cultures-

- Apical meristem is a dome of tissue located at the extreme tip of a shoot.
- The apical meristem along with the young leaf primordia constitutes the shoot apex.

RAMA UNIVERSITY
UTTAR PRADESH
KANPUR

- Meristem or shoot tip is isolated from a stem by a V- shaped cut.
- The size (frequently 0.2 to 0.5 mm) of the tip is critical for culture. In general, the larger the explant (shoot tip), the better are the chances for culture survival.
- For good results of micro propagation, explants should be taken from the actively growing shoot tips, and the ideal timing is at the end of the plants dormancy period.
- The most widely used media for meristem culture are MS medium andWhite's medium.

RAMA UNIVERSITY
UTTAR PRADESH
KANPUR

- In stage I, the culture of meristem is established. Addition of growth regulators namely cytokinins (kinetin, BA) and auxins (NAA or IBA) will support the growth and development.

- In stage II, shoot development along with axillary shoot proliferation occurs. High levels of cytokinins are required for this purpose.

- Stage III is associated with rooting of shoots and further growth of plantlet.

- Consequently, stage II medium and stage III medium should be different in composition. The optimal temperature for culture is in the range of 20-28°C (for majority 24-26°C). Lower light intensity is more appropriate for good micro propagation.

RAMA UNIVERSITY
UTTAR PRADESH
KANPUR

Bud Cultures

The plant buds possess quiescent or active meristems depending on the physiological state of the plant.

Two types of bud cultures are used:

- ✓ Single node culture
- ✓ Axillary bud culture.

RAMA UNIVERSITY
UTTAR PRADESH
KANPUR

Single node culture:

This is a natural method for vegetative propagation of plants both in vivo and in vitro conditions. A bud along with a piece of stem is isolated and cultured to develop into a plantlet. Closed buds are used to reduce the chances of infections. In single node culture, no cytokinin is added.

RAMA UNIVERSITY UTTAR PRADESH KANPUR

Axillary bud culture:

In this method, a shoot tip along with axillary bud is isolated. The cultures are carried out with high cytokinin concentration. As a result of this, apical dominance stops and axillary buds develop.

For a good axillary bud culture, the cytokinin/ auxin ratio is around 10: 1.This is however, variable and depends on the nature of the plant species and the developmental stage of the explant used. In general, juvenile explants require less cytokinin compared to adult explants. Sometimes, the presence of apical meristem may interfere with axillary shoot development. In such a case, it has to be removed.

RAMA UNIVERSITY
UTTAR PRADESH

2. Multiplication by adventitious shoots

- The stem and leaf structures that are naturally formed on plant tissues located in sites other than the normal leaf axil regions are regarded as adventitious shoots.
- There are many adventitious shoots which include stems, bulbs, tubers and rhizomes.
- The adventitious shoots are useful for in vivo and in vitro clonal propagation.
- The meristematic regions of adventitious shoots can be induced in a suitable medium to regenerate to plants.

RAMA UNIVERSITY UTTAR PRADESH KANPUR

3. ORGANOGENESIS

Definition:

“The formation of roots, shoots or flower buds from the cells in culture in manner similar to adventitious root or shoot formation in cuttings is called organogenesis”

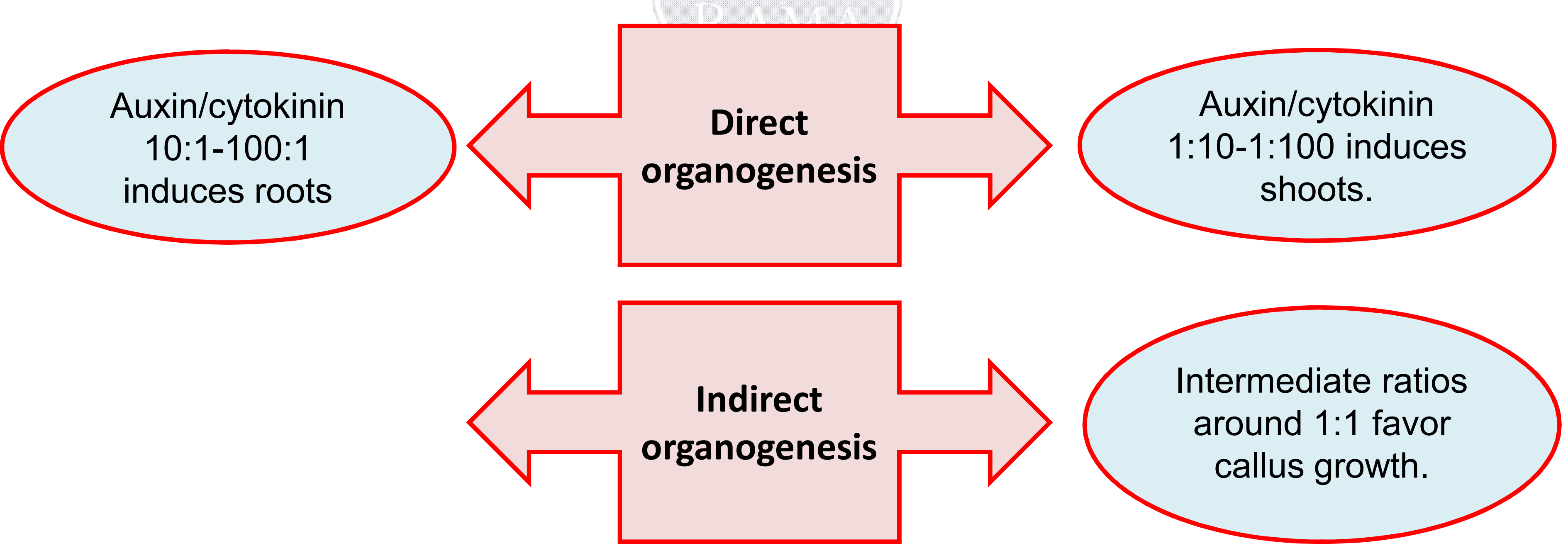
- ✓ Organogenesis starts in the callus in response to the stimulation given by the chemicals in the medium.
- ✓ Organogenesis takes place in two stages, namely caulogenesis or shoot initiation and rhizogenesis or root initiation.
- ✓ Both types of organogenesis are controlled by the hormones present in the medium. generally a high auxin:cytokinin ratio induce shoot formation.
- ✓ Organogenesis starts with the development of a group of meristematic cells called meristemoids, which initiate the formation of a primordium.
- ✓ Depending on the factors within the system, this primordium develops into shoot, root or embryoid.

RAMA UNIVERSITY UTTAR PRADESH KANPUR

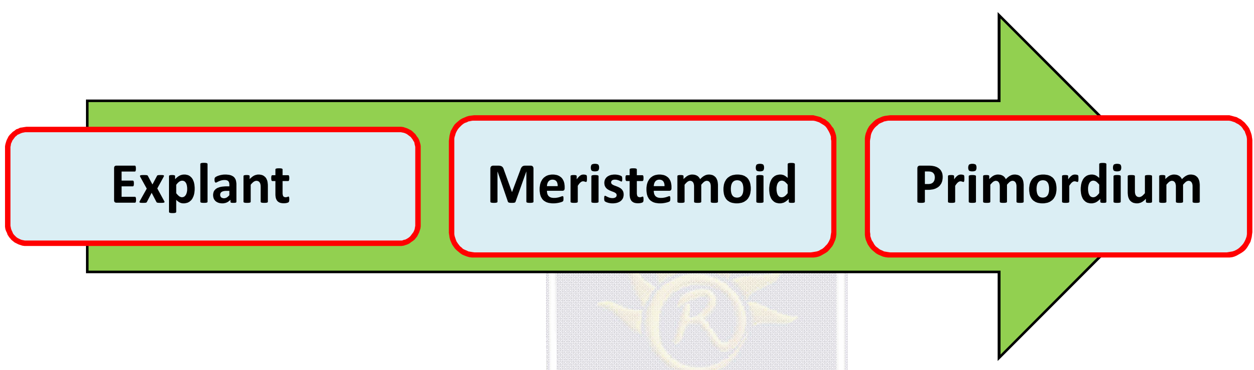
Two types of organogenesis

1. Direct organogenesis
2. Indirect organogenesis

This two types depend on hormonal combination of the culture media.



Direct organogenesis



- In many plants, subculturing of callus results in undesired variations of clones (somaclonal variations).
- To avoid this, direct regeneration of the explants into plantlets can be tried.
- This has been achieved in many plant species by altering the hormonal combination of the culture media.

RAMA UNIVERSITY UTTAR PRADESH KANPUR

Direct organogenesis from different explants.

a) Shoot induction from shoot tip explant on a medium,

b) Shoot induction from hypocotyls explant on a medium,

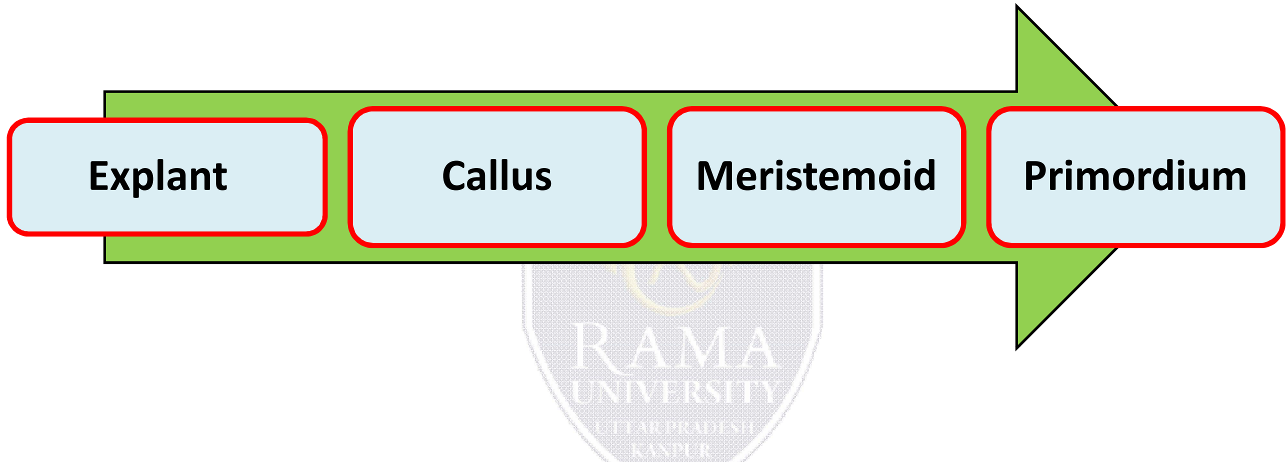
c) Shoot induction from cotyledon explant on a medium,

d) Regenerated shoot showing rhizogenesis and

e) Hardened plantlet regenerated from different explants

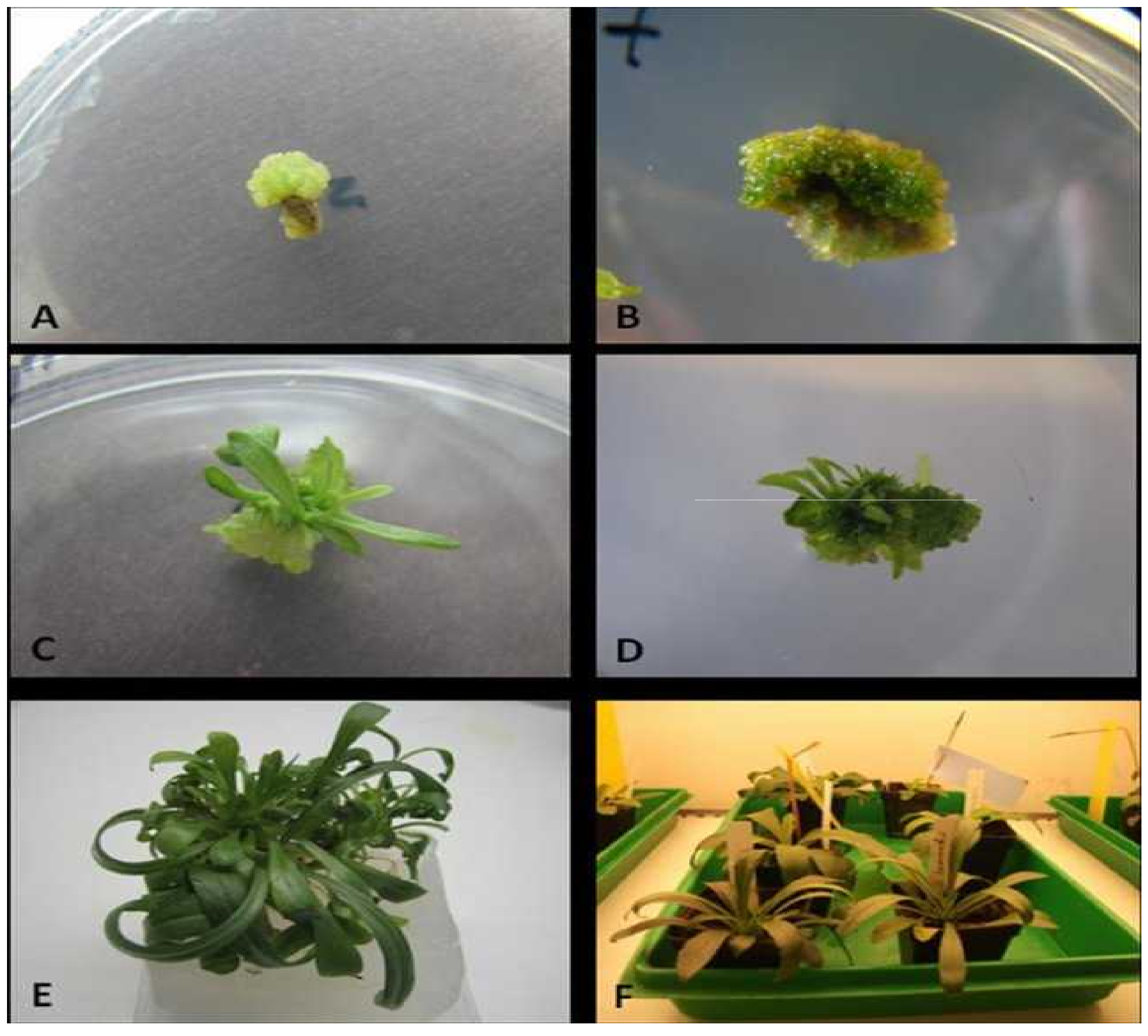
https://www.researchgate.net/publication/267630716_Influence_of_different_cytokinins_on_direct_shoot_regeneration_from_the_different_explants_of_Carthamus_tinctorius_L_Var_Annigeri-2_A_high_oil-yielding_variety/figures?lo=1

Indirect organogenesis



RAMA UNIVERSITY UTTAR PRADESH KANPUR

- ✓ In indirect organogenesis, callus is first produced from the explant.
- ✓ Organs can then be produced from the callus tissue or from a cell suspension produced from that callus.



Steps of indirect organogenesis in *D. lamarckii*.

A. Hypocotyl explants producing organogenic callus on medium containing 5.0 mg/BA and 1.0 mg/L NAA

B. organogenic callus on medium containing 0.5 mg/L BA and GA 3

C and D. Indirect shoot formation after a 6 weeks of cultivation

E. Regenerants after rooting stage

F. Regenerants being hardened off under growth chamber conditions.

https://www.researchgate.net/publication/267802786_IN_VITRO_PROPAGATION_AND_CARDIAC_GLYCOSIDE_PRODUCTION_IN_ENDEMIC_DIGITALIS_L_SPECIES_OF_ANATOLIA

4. Somatic Embryogenesis

- Embryo is defined as the earliest recognisable multicellular stage of an individual that occurs before the development of characteristic organs of the given species.
- Production of embryo like structure from callus is known as embryogenesis. In higher plants such embroys usually arise from zygote formation and are termed as zygotic embryos.
- Various types of cells and tissues can be used as source of embryogenic cells. It may be microspores (1n), zygote (2n), somatic cells (2n) or somatic hybrids (4n).
- Embryogenesis can be initiated in an explant only from the more juvenile or meristematic tissues.
- Immature zygotic embryos, cotyledons and hypocotyl dissected from ungerminated seeds are common explants.
- Isolated somatic cells can develop into embroys.
- Embryo development occurs through an organised sequence of cell division, enlargement and differentiation.
- The final stages of development towards maturation are distinguished by overall enlargement and matured embryo morphology.

RAMA UNIVERSITY UTTAR PRADESH KANPUR

Somatic embryogenesis involves three distinct steps which are absent in organogenesis

1.Induction

- It is the initiative phase where cells of callus are induced to divide and differentiate into groups of meristematic cells called embryogenic clumps (ECs).
- These ECs develop into initial stages of somatic embryo i.e. globular stage.

2.Maturation

- In this phase somatic embryos develop into mature embryos by differentiating from globular to heart shaped and the mature embryo here undergoes biochemical changes to acquire hardness.

3.Conversion

- Embryos germinate to produce seedlings.

RAMA UNIVERSITY

How Somatic Embryos produced?

- ✓ In somatic embryogenesis, embryo-like structures, which can develop into whole plants in a way analogous to zygotic embryos, are formed from somatic tissues.
- ✓ These somatic embryos (SE) can be produced either directly or indirectly.
- ✓ Two ways of somatic embryogenesis:

    1. Direct embryogenesis
    2. Indirect embryogenesis

RAMA UNIVERSITY KANPUR

1. Direct embryogenesis

In direct somatic embryogenesis, cells of explant undergo direct embryogenesis from proembryonic determined cells in absence of callus proliferation.

2. Indirect embryogenesis

In indirect somatic embryogenesis, cells of explant first undergo callus proliferation and embryoids develop within the callus tissue from induced embryonic cells.

RAMA UNIVERSITY
UTTAR PRADESH
KANPUR

Importance of Somatic Embryogenesis

- ✓ The mass production of adventitious embryos in cell culture is still regarded by many as the ideal propagation system.
- ✓ The adventitious embryo is a bipolar structure that develops directly into a complete plantlet and there is no need for a separate rooting phase as with shoot culture.
- ✓ Somatic embryo has no food reserves, but suitable nutrients could be packaged by coating or encapsulation to form some kind of artificial seeds. Such artificial seeds produce the plantlets directly into the field.
- ✓ Unlike organogenesis, somatic embryos may arise from single cells and so it is of special significance in mutagenic studies.
- ✓ Plants derived from asexual embryos may in some cases be free of viral and other pathogens. So it is an alternative approach for the production of disease-free plants.

RAMA UNIVERSITY UTTAR PRADESH KANPUR

ADVANTAGES OF MICROPROPOGATION

1. Clonal mass propagation - extremely large numbers of plants can be produced. Rather than getting 10000 plants per year from an initial cutting in vegetative propagation, one can obtain more than 1,000,000 plants per year from one initial explant through micropropagation.

2. Culture is initialized from small parts of plants – so no need of much space: from 1 m2 space in culture room, 20000 - 100000 plants can be produced per year.

3. Production of disease and virus free plantlets. This leads to simplification of international 8 exchange of plants

RAMA UNIVERSITY UTTAR PRADESH

4. Micropropagation enables growers to increase the production of plants that normally propagate very slowly such as Narcissus and other bulbous crops.

5. Introduction of disease free new cultivars is possible through micropropagation

6. Vegetative propagation of sterile hybrids can be used as parent plants for seed production. Eg. Cabbage

7. One of the rapid methods for cloning of disease free trees.

8. In vitro cultures can be stored for long time through cryopreservation.

9. Breeding cycle can be shortened.

RAMA UNIVERSITY
UTTAR PRADESH
KANPUR

DISADVANTAGES OF MICROPROPOGATION

1. Expensive laboratory equipment and service
2. No possibility of using mechanization
3. Plants are not autotrophic
4. Poor Acclimatization to the field is a common problem
5. Risk of genetic changes if 'de novo' regeneration is used
6. Mass propagation cannot be done with all crops to date. In cereals much less success is achieved
7. Regeneration is often not possible, especially with adult woody plant material.
8. More problems in inducing rooting

RAMA UNIVERSITY UTTAR PRADESH

APPLICATIONS OF MICROPROPOGATION

1. Clonal mass propagation

The important point here is that extremely large numbers of plants can be produced. Rather than getting 10000 plants per year from an initial cutting, one can obtain upwards of 1,000,000 plants per year from one initial explant.

2. Difficult or slow to propagate plants

Micropropagation enables growers to increase the production of plants that normally propagate very slowly such as narcissus and other bulbous crops.

3. Introduction of new cultivars

For example: Dutch iris. Get 5 daughter bulbs annually. Takes 10 years for commercial quantities of new cultivars to be built up. Can get 100-1000 bulbs per stem section.

RAMA
UTTAR PRADESH
KANPUR

4. Vegetative propagation of sterile hybrids

Used as parent plants for seed production. Eg. cabbage.

5. Pathology - Eliminate viruses, bacteria, fungi

Use heat treatment and meristem culture. Used routinely for potatoes, carnation, mum, geranium, garlic, gypsophila

6. Storage of germplasm

Generally the only successful method to date is keeping them in refrigerator. Slows down, but does not eliminate, alterations in genotype.

RAMA UNIVERSITY KANPUR

Regeneration

- The process whereby a part of a plant can be turned into a whole new plant.
- Regeneration is possible because plant cells can be made totipotent using hormones.
- Differentiated tissue: stems, leaves, roots, etc.
- Undifferentiated (embryonic) cells are totipotent: can become a whole new plant by differentiating into a whole new plant.

RAMA UNIVERSITY KANPUR

Steps involved in Tissue Regeneration

1) **Sterilization:** Tissue must be sterile- completely free of any microoganisms; done using aseptic technique
2) **Differentiation:** Starting tissue is called an ex-plant., differentiated cells (these cells have developed to be part of specialized tissue (root, leaf, stem, ovary, cotyledon, etc.).
3) **Transfer of explants** are plated on a sterile petridish containing hormones and nutrients that promote the explant cells to develop into callus.
4) **Callus development:** a mass of undifferentiated cells developed into seedlings.
5) **Transfer of callus cells** to petridishes: Individual cells (or clumps of cells) of the callus are transferred aseptically to a different petri dish containing sterile medium that encourages the undifferentiated callus cells to become shoots and roots.

CONTINOUS CULTURE

- In continuous culture system, the old liquid medium is replaced continuously by the fresh liquid medium to stabilize the physiological states of the growing cells.
- In this system, nutrient depletion does not occur due to the continuous flow of nutrients and the cells always remain in the steady growth phase.
- Cell proliferation takes place under constant condition.

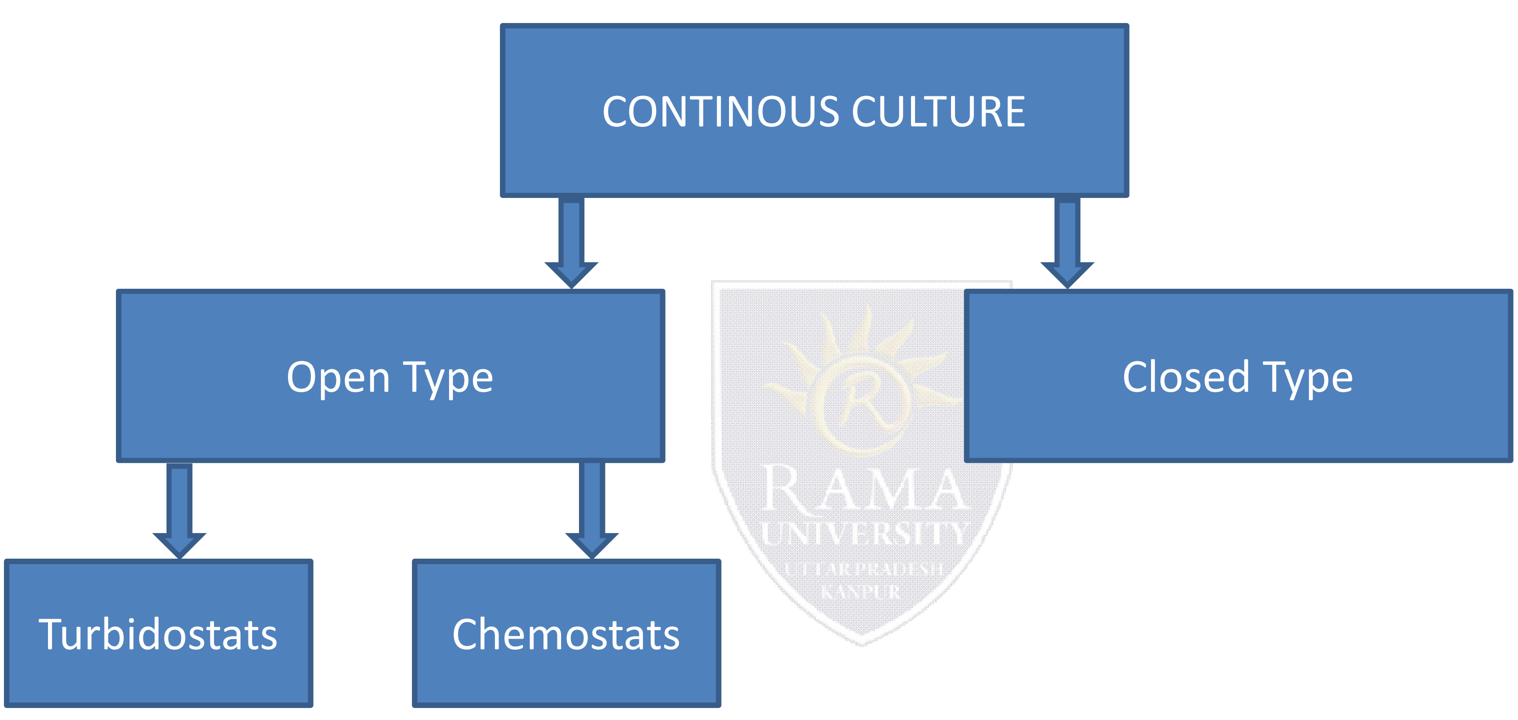
- This system allows
  - ✓ **Establishment** of steady states of growth and metabolism.
  - ✓ **Study** of the changes which occur in transitions from one steady state to another.
  - ✓ **Identification** of the controlling factors.

- Such culture systems are of **two types:**

  1. Open type

  2. Closed type

RAMA UNIVERSITY KANPUR



RAMA
UNIVERSITY
UTTAR PRADESH
KANPUR

1. **Open type**

- In open type, both the cells and used medium are replaced with fresh medium thus maintaining culture at constant and submaximal growth rate.

- Open continuous cell suspension culture is of two types :

    1. Chemostat
    2. Turbidostat

RAMA UNIVERSITY
UTTAR PRADESH
KANPUR

Chemostat

- ✓ In this system, culture vessels are usually cylindrical or circular in shape and possess inlet and outlet pores for aeration and the introduction and removal of cells and medium.
- ✓ Such a system is maintained in steady state.
- ✓ Thus in steady state condition the density, growth rate, chemical composition and metabolic activity of the cells all remain constant.
- ✓ Such continuous cultures are ideal for studying growth kinetics and the regulation of metabolic activity in higher plants.

UNIVERSITY
UTTAR PRADESH

Turbidostat

- ✓ A turbidostat is a continuous culturing method where the turbidity of the culture is held constant by manipulating the rate at which medium is fed.
- ✓ In this system, the cells are allowed to grow upto a certain turbidity, when the predetermined volume of culture is replaced by fresh culture.
- ✓ The turbidity is measured by the changes of optical density of medium.
- ✓ An automatic monitoring unit is connected with the culture vessel and such unit adjusts the medium flow in such a way, as to maintain the optical density or pH at chosen, present level.

RAMA UNIVERSITY
UTTAR PRADESH

2. Closed type

In closed type, the used medium is replaced with the fresh medium, hence, the cells from used medium are mechanically retrieved and added back to the culture and thus, the cell biomass keeps increasing.

RAMA UNIVERSITY UTTAR PRADESH KANPUR

Plant cell Immobilization

- It is defined as a technique, which confines to a catalytically active enzyme (or to a cell) with in a reactors system and prevents its entry into the mobile phase, which carries the substrate and product.

- It involves the entrapment of cells within a gel or passive adsorption on solid support materials, thus creating a situation for cell to imitate membership on a tissue of a whole plant.

RAMA UNIVERSITY KANPUR

- It is a controlled process of agglutination, formation and adhesion on a matrix under controlled condition.
- It is a process in which highly reactive and sensitive enzyme is embedded in a matrix so that only substance and product can pass through matrix.
- Cells cease to grow & accumulate metabolites.
- In immobilization technique the plant cells are entrapped in different polymerize matrices. E.g. alginate, agar, agarose etc. and converted into hetrogeneous catalyst.

RAMA UNIVERSITY KANPUR

TYPES OF IMMOBILIZATION

1. **Adsorption**

   Direct intracellular binding due to natural affinity (adhesion or agglutination)

2. **Covalent linkage**

   Covalent coupling on otherwise inert matrices.

3. **Cross linking**

   Intracellular connection via bi or poly functional reagent

4. **Embedding**

   Mixing with suitable materials by changing their consistency with temperature.

5. **Entrapment**

   Physical retention within the frame work of diverse pore size and permeability (Micro encapsulation)

RAMA UNIVERSITY UTTAR PRADESH KANPUR

Mechanism/methods of immobilization system

1. Entrapment

- ✓ Gel entrapment by polymerization with polymers like polyacrylamide e.g. vinca
- ✓ Gel entrapment by ionic network formation: Entrapment of cell in calcium alginate
- ✓ Gel entrapment by formation by precipitation of some natural and synthetic polymer by changing one or more parameters such as pH, temperature, salinity etc.
- ✓ Entrapment in performed structures like hollow fibre reactors e.g. Polyurethane foam.

RAMA UNIVERSITY

2. Surface immobilization with help of nylon, cellulose etc.

3. Immobilization by embedding in agar, agarose etc.

Applications of plant cell immobilization:

- ✓ Biotransformation
- ✓ Rapid biosynthesis of secondary metabolites
- ✓ Synthesis from precursor
- ✓ The biosynthesis of secondary metabolites with increased yield over extended period of time.

Examples: Immobilization of various plant like Capsicum, Coffee, Vinca, Mentha, Tobacco etc.

RAMA UNIVERSITY UTTAR PRADESH KANPUR

ARTIFICIAL SEEDS

- Synthetic seeds are defined as **artificially encapsulated** somatic embryos, shoot buds, cell aggregates or any other tissue that can be used for sowing a seed and that possess the ability to convert into a plant under in vitro or ex vitro conditions and that retain this potential also after storage.

RAMA
UNIVERSITY
UTTAR PRADESH
KANPUR

- Various plant species which are reported for artificial seed production are Carrot, Alfa alfa (somatic embryos), Banana, Cardamom (Shoot buds or shoot tips), Ecalyptus (Axillary buds) etc.

- They are classified as Desiccated and Hydrated. **These two are again classified into encapsulated and uncoated.**

- The **desiccated synthetic seeds** are produced from somatic embryos either naked or encapsulated in polyoxy ethylene glycol followed by their desiccation. E.g. Wheat, Soyabean (Uncoated), Carrot (Encapsulated)

- **Hydrated synthetic seeds** are produced in those plant species where somatic embryos are recalcitrant and sensitive to desiccation. Hydrated seeds are produced by encapsulating the somatic embryos or somatic propagules in hydrogel capsules. E.g. Carrot, tomato (Uncoated), Mango, Alfa alfa (Encapsulated)

UNIVERSITY
UTTAR PRADESH
KANPUR

Advantages of synthetic seeds

1. Stored up to a year without loss of viability

2. Easy to handle, and useful as units of delivery

3. Directly sown in the soil like natural seeds and do not need hardening in green house.

RAMA UNIVERSITY
UTTAR PRADESH
KANPUR

Applications of synthetic seeds

1. Micropropagation through artificial seeds.
2. For development of plants for breeding purpose
3. Propagation of variety of crop plants especially crops for which true seeds are not used or not readily available for multiplication or the true seeds are expensive. Hybrid plants may vegetatively propagated plants which are prone to infections e.g. Garlic, potato, tomato, hybrid rice etc.
4. Transplanting improved or selected material in forestry to reduce the cost of breeding e.g. European larch, white spruce etc.
5. Germplasm conservation of endangered species through cryopreservation of synseeds in near future.

RAMA UNIVERSITY