V- Explant

It is the piece of plant taken out and grown in closed vessel under sterile conditions in special nutrient media. It may be obtained from *in vivo* field-grown plant or plants growing under *in vitro* conditions. Potentially all living plant parts can be used as explant.

A- Different types of explants:

Plant segments used in tissue culture as explant are stem internode, root, leaf, flower, ovule, anther, cotyledon, epicotyl, hypocotyl, tubers, corm, ….. (details in lab). Such these explants form direct and indirect organs and embryos. Protoplast and thin cell layer can also be used as explant in some species while embryos (mature or immature) can be successfully used in cereals. Moreover, shoot tips and meristems may give successful results for shoot regeneration and multiplication.

B- Factors affecting explant's regeneration capacity

1- Origin

Regeneration capacity of explants shows a wide range among families, species and even within genotypes from the same species. Generally, explants from dicots regenerate more easily than those of monocots. Explants from some dicot families such as Solanaceae, Cruciferae, Gesneriaceae, Begoniaceae and Crassulaceae have a high regeneration capacity. In general, explants from herbaceous plants regenerate more easily than those of woody plants such as trees and shrubs.

2- Physiological stage of donor plant

Explants show an ability to express totipotency are the most suitable for tissue culture. Generally, vegetative segments of plants regenerate more easily *in vitro* than generative ones. Explants should be isolated from healthy plants with high cell division for successful response to tissue culture. On the other hand, regeneration capacity of mature tissues is quite low.

3- Explant age

Regeneration capacity of older plants is often low. As the organ using for explant source gets older, regeneration capacity decreases. The physiologically younger tissue is much more responsive *in vitro* and easier for surface sterilization.

4- Explant source

Plants grown under greenhouse conditions give rise to better results than the ones grown in field conditions. There are huge variations regarding tissue culture response in explants excised from plants grown in field condition depending on weather conditions during the year. However, the best results are obtained from explants excised from *in vitro* grown seedlings.

5- Season in which the explant is obtained

For example, buds or shoots taken during spring when shoots are in a flush state are more responsive than dormant buds. Additionally, contamination rates increase in summer.

6- Explant size and position in donor plant

It is so difficult to obtain a successful tissue culture response from small parts such as cells and meristems than from larger parts such as leaves and hypocotyls due to their limited nutrients and hormone reserves. Larger explants having a big amount of nutrition reserves such as tubers and bulbs can easily regenerate *in vitro* and are less dependent on nutrients and hormones in growth medium. In addition, *in vitro* growth of explants can be affected depending on the place from where they are excised due to the difference in endogenous level of growth regulators.

7- Plant quality

Healthy explants give better response compared with those derived from plants under nutritional or water stress or exhibiting disease symptoms.

8- Goal

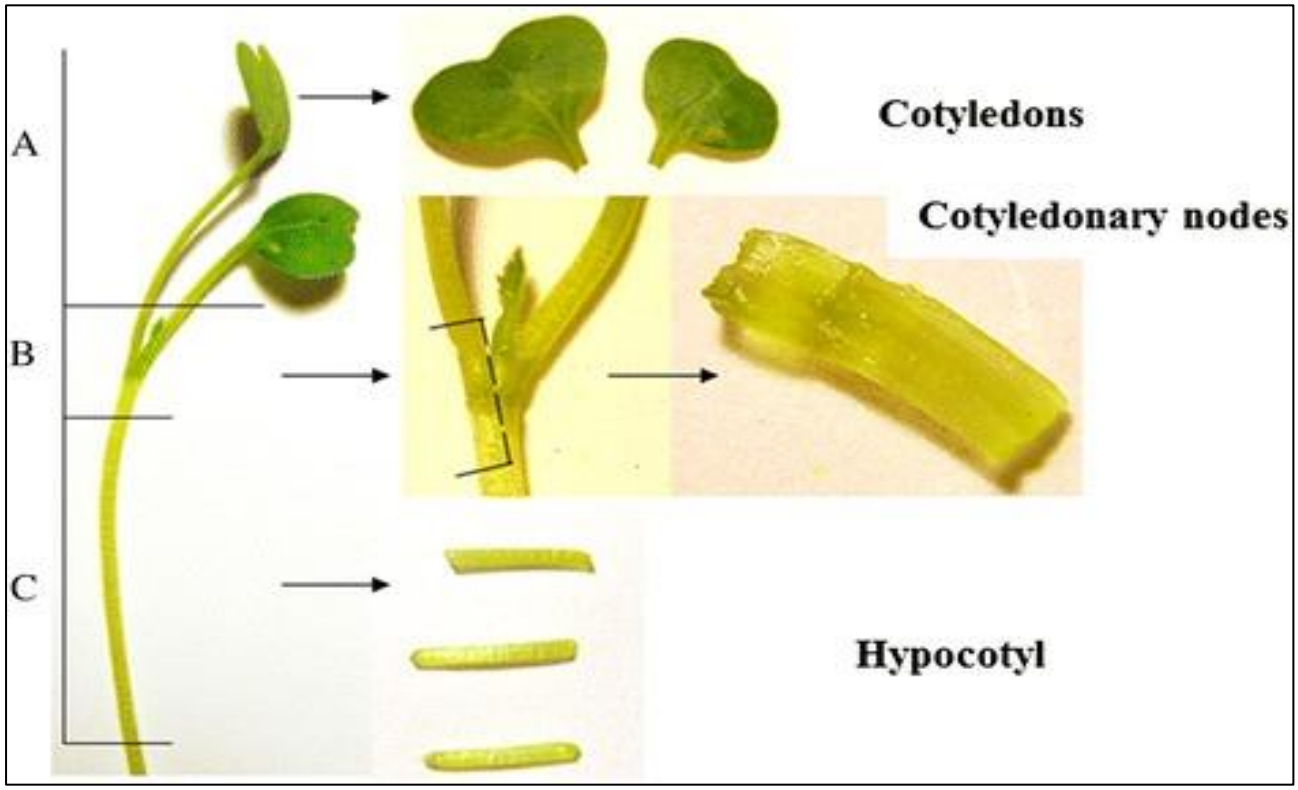
The choice of explant depends on what type of response required. For example, if clonal propagation is the goal lateral or terminal buds are used (to avoid **somaclonal variation**: genetic variations that may arise among regenerated plantlets) while leaf segments, hypocotyl sections and cotyledons are excellent explants for callus induction.

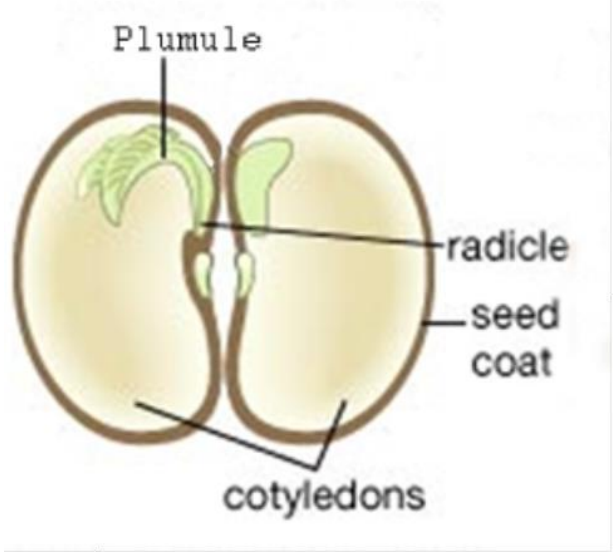
All of these parts, demonstrated in the next figures, can be used as a starting material for tissue cultures. In addition, protoplast can be used as a starting material for breeding and hybridization. A complicated procedure (involves enzymatic digestion of cell wall) is used to liberate protoplast.

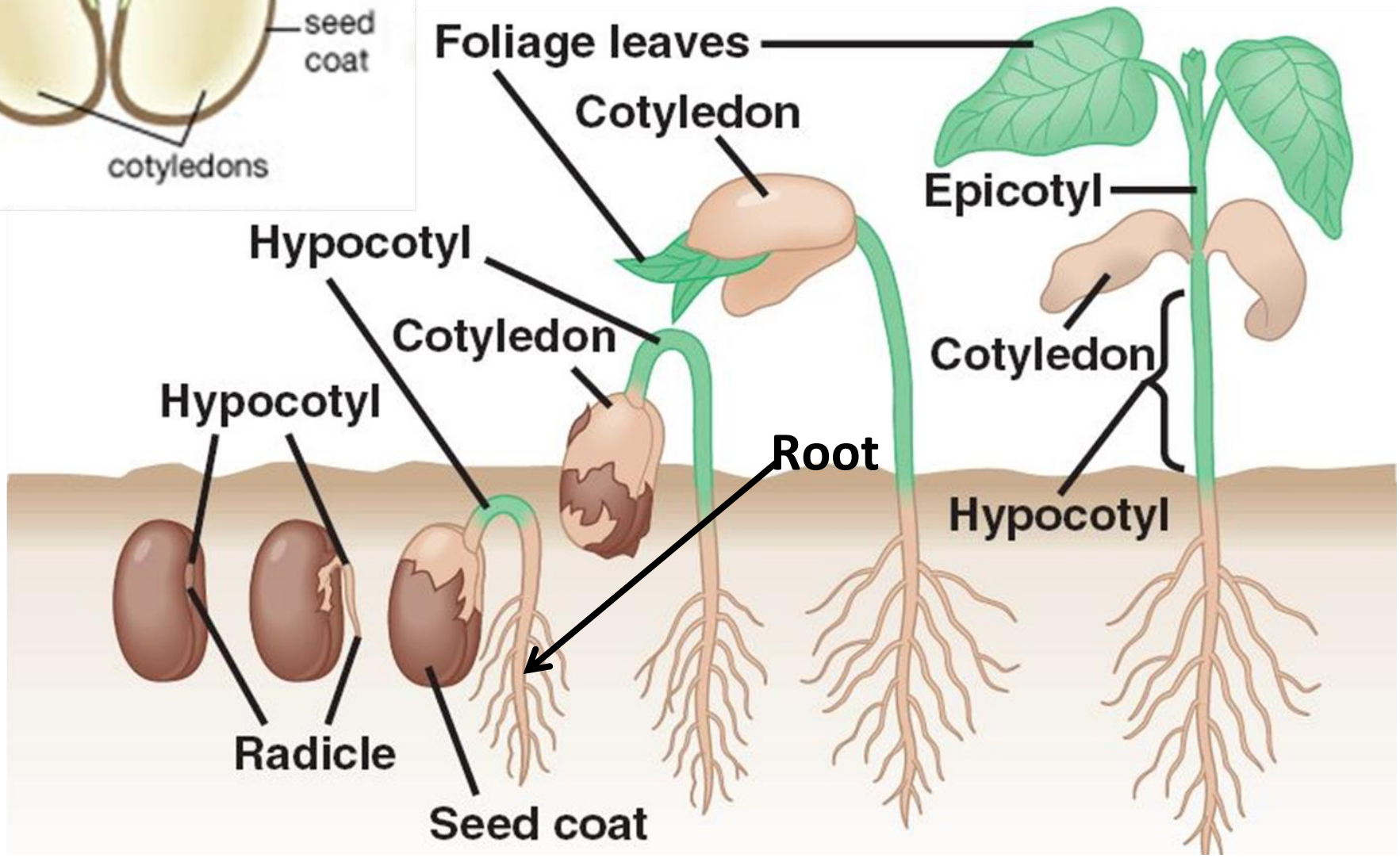
C- Surface sterilization of Explants

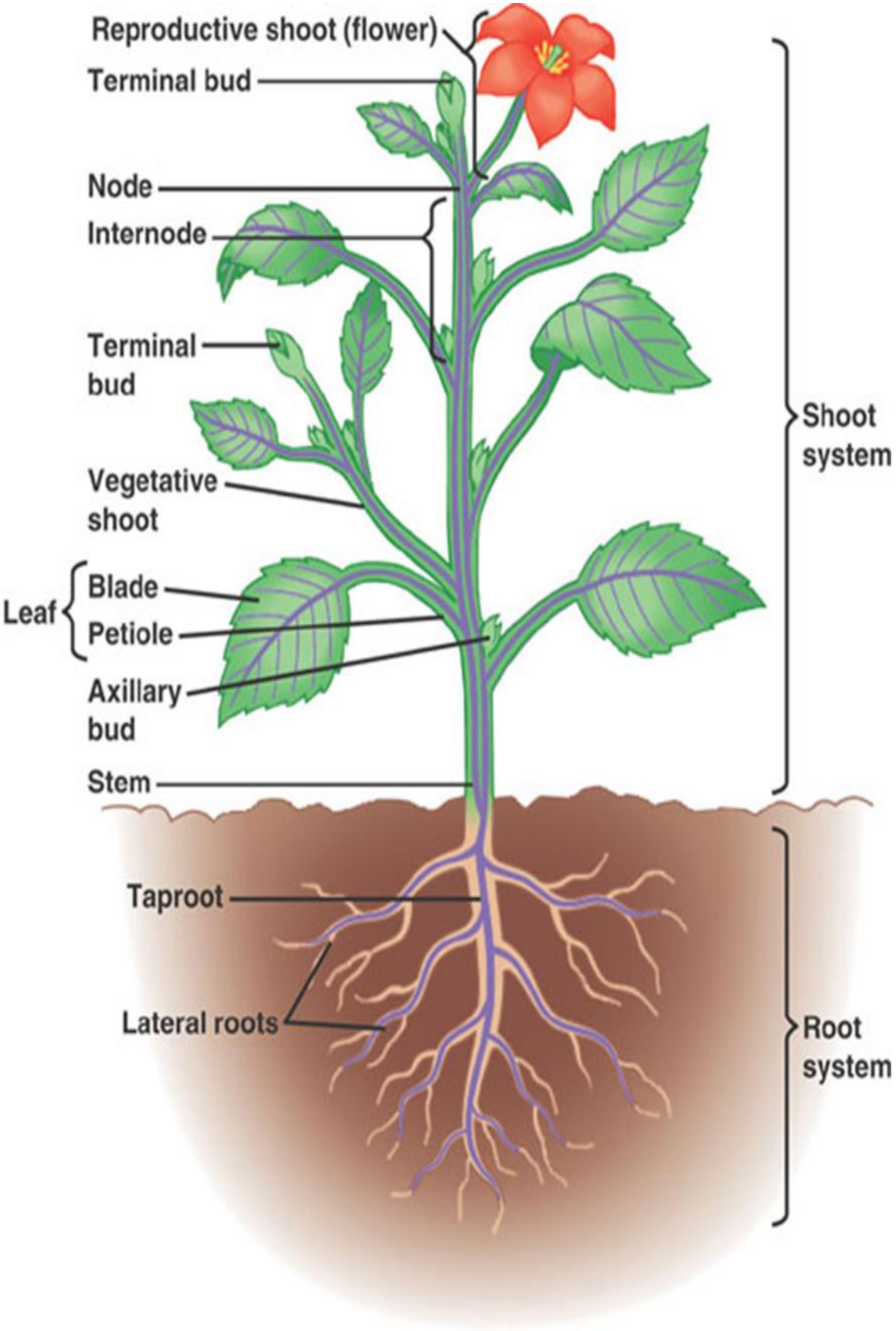
The explant is often the major source of contaminants (by microorganisms) that must be eliminated before placing on sterile media. Explants are first pre-washed in soapy water to remove soil particles and dust to enhance the contact of the disinfectant. Washed explants are then surface sterilized in disinfectant solution to which a wetting agent (eg: Tween-20) is added. Wetting agent acts as surfactant. After shaking for several minutes, explants are rinsed aseptically in sterile distilled water. Several disinfecting agents are used eg: commercial bleach (5.25% sodium hypochlorite), calcium hypochlorite, ethanol (70%), hydrogen peroxide, mercuric chloride and silver nitrate; however ethanol and sodium hypochlorite (NaOCl) has been most widely used.

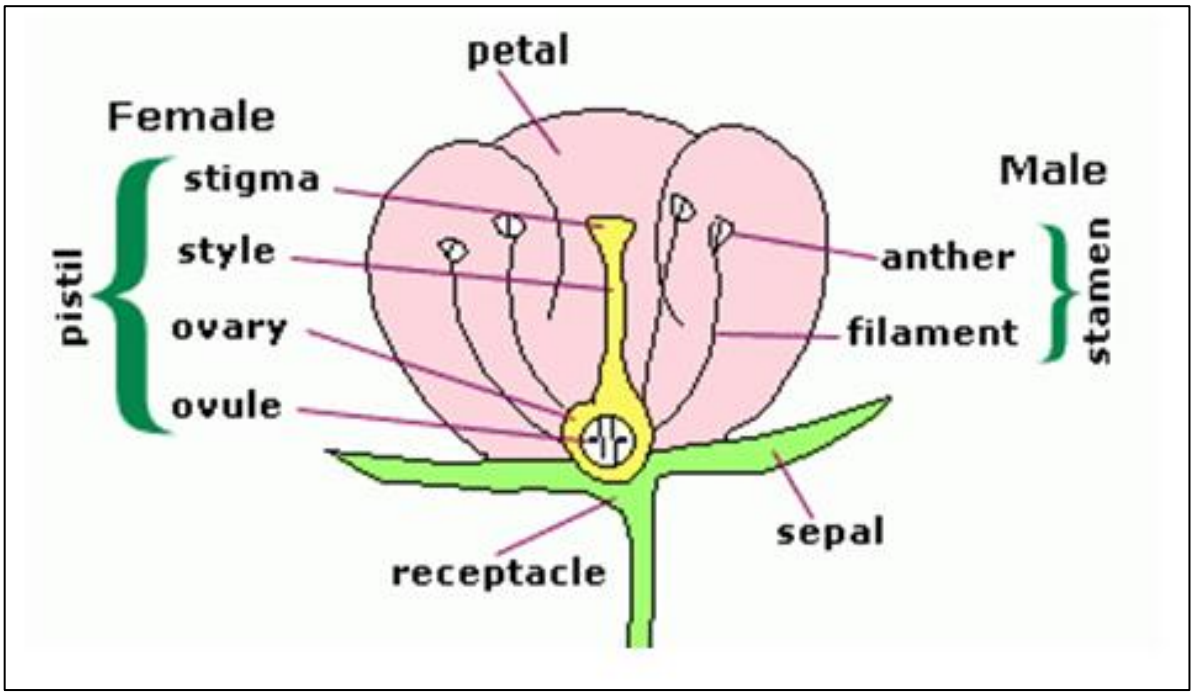
Disinfectants also have lethal effects on tissues of explant; consequently, trial and error or literature reference determines the concentration and exposure time to design the treatment that eliminates contaminants with minimum damage for explant tissues. Usually two disinfectants are used in sequential manner for better results. In case of internal contamination, explants from rapidly growing tips that are usually least likely to harbor internal contaminants. The use of antibiotics and fungicides in media is usually not successful.

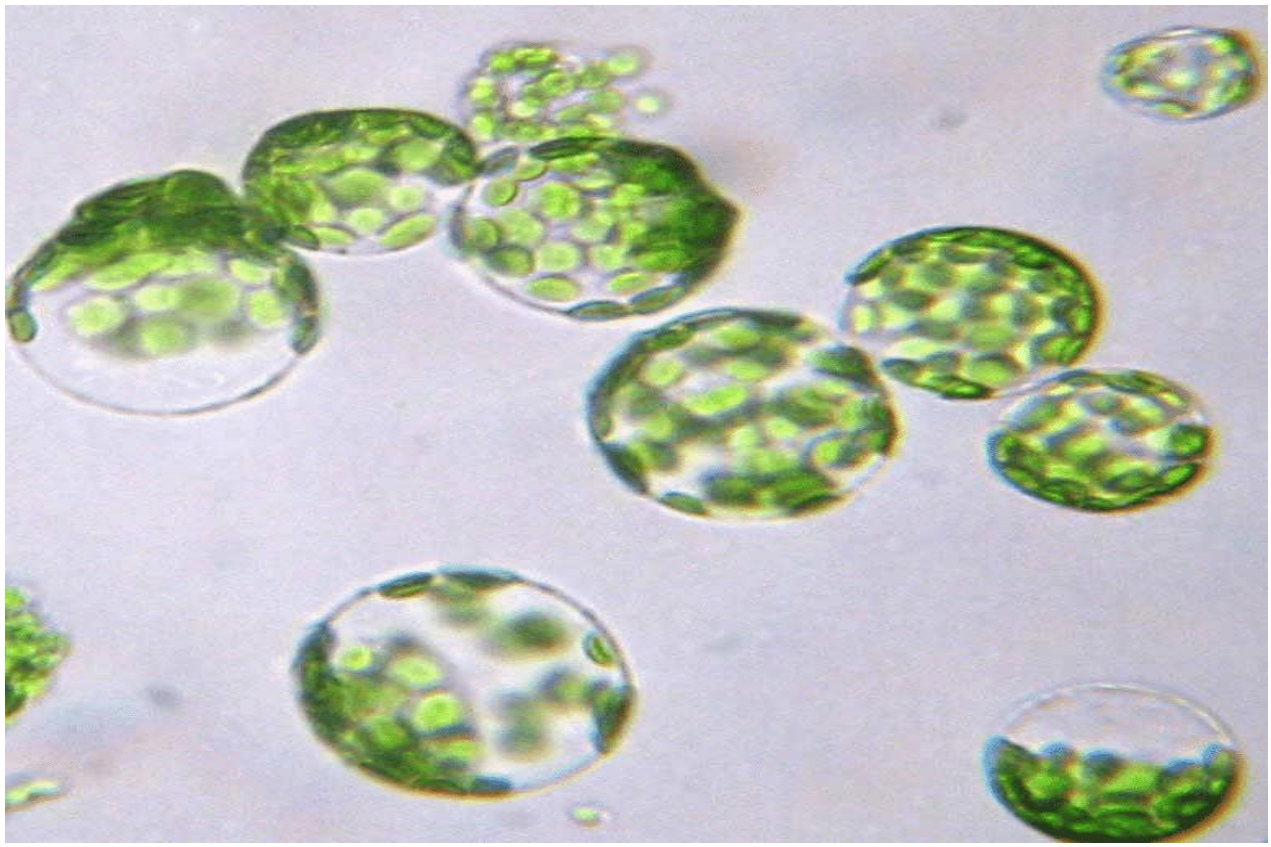



Plumule
radicle
seed coat
cotyledons


Foliage leaves
Cotyledon
Epicotyl
Hypocotyl
Cotyledon
Cotyledon
Hypocotyl
Root
Hypocotyl
Radicle
Seed coat


Reproductive shoot (flower)
Terminal bud
Node
Internode
Terminal bud
Vegetative shoot
Leaf
Blade
Petiole
Axillary bud
Stem
Shoot system
Taproot
Lateral roots
Root system


petal
Female
stigma
style
ovary
ovule
pistil
Male
anther
filament
stamen
sepal
receptacle



Protoplast

IV- Physical growth conditions

Physical growth conditions *in vitro*, especially light, temperature, humidity and oxygen, have a profound effect on growth and development of plant:

- Different plant species differ in their light requirements for the same process. Wave length, light intensity and **photoperiod** (the length of time a plant receives light in 24 hours) must be considered when determining light regime applied during incubation. Interaction between light and different growth regulators is recorded by several authors. The most common light intensity is 60 – 70 μmol m^{-2} s^{-1} with 16 h or 24 h photoperiod.
- Incubation temperature also varies among different species and with purposes. Constant temperature is generally used during incubation for certain purpose. Usually, the temperature of a growth room is kept constant at 24-28°C (average), lower temperature (18°C) for bulbous species, or higher temperature (28°C) for tropical species. However, sometimes altering temperature may be required (26°C day/15°C night), especially in seed germination of some species. A heat shock is applied at the beginning of *in vitro* germination of some species. The temperature within the closed vessels is 3-4°C higher than the growth room due to irradiance.
- A growth room with high **humidity** increases the chances of contamination. Humidity is usually very high in the closed vessels (90-100%).
- Plant tissue cultures employ sealed containers that limit gas exchange. In very tightly closed vessels, especially when **subculturing** (transfer to fresh medium) is performed with long interval, tissues may suffer anoxia due to oxygen consumption. These conditions also encourage accumulation of carbon dioxide, ethylene and water vapor (increases relative humidity). In addition, **oxygen availability** is important for *in vitro* root formation and it can be provided through liquid medium more than solid.

Tissue culture Types

Tissue culture types can be classified either by the explant used or/and by their purposes. We will point out on Seed cultures, Bud cultures (Micropropagation), Meristem cultures (virus free), Organogenesis and embryogenesis cultures, Embryo cultures, Callus and Cell Suspension cultures, Protoplast cultures and Haploid cultures.

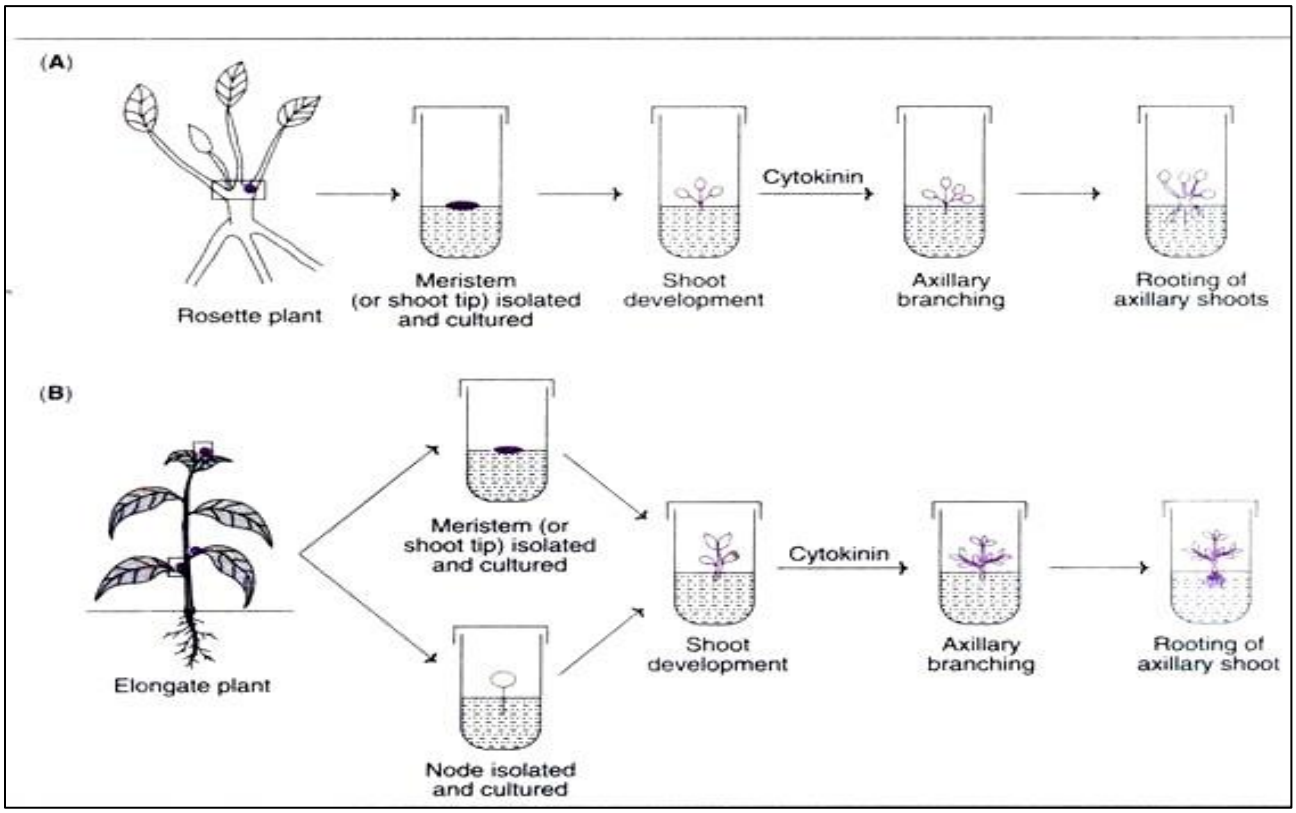
1. Seed Culture

The seed (or zygotic seed) is the vehicle that connects one generation to another in much of the plant kingdom. By means of seed, plants are able to transmit their genetic constitution in generations and therefore seeds are the most appropriate means of propagation, storage and dispersal. Seeds may be cultured *in vitro* to generate seedlings or plants. It is the best method for raising the sterile seedling. The seed culture is done to get the different kinds of explants from aseptically grown plants which help in better maintenance of aseptic tissue.

2. Micropropagation/Bud culture

Micropropagation is defined as the true-to-type propagation of selected genotypes using *in vitro* culture techniques. It achieved through pre-formed meristems by apical/axillary bud culture (isolated bud) [shoot culture (one bud in case of elongated plant) or (numerous buds in case of rosette plant) and node culture (one bud/node)] and development after de novo formation of adventitious shoots through organogenesis or somatic embryogenesis (item no.

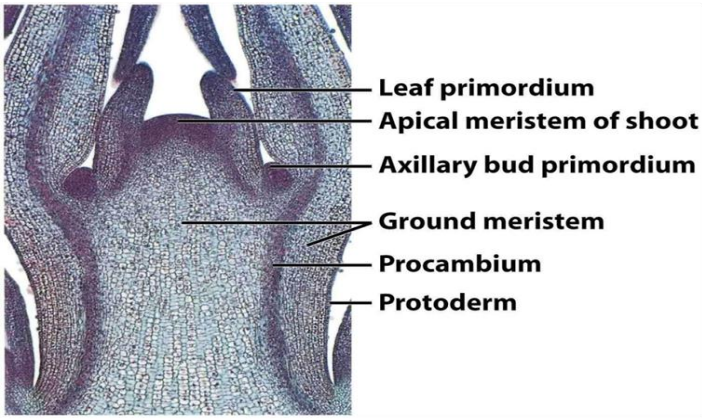
4). Buds contain quiescent or active meristems in the leaf axils or shoot tip, which are capable of growing into a shoot. In single node culture, each node of the stem is cut and allowed to grow on a nutrient media to develop the shoot tip from the leaf axil, which ultimately develops into new plantlet. In axillary bud method, the axillary buds are isolated from the leaf axils and develop into shoot tip under little high cytokinin concentration. The most frequently used micropropagation method for commercial production utilizes shoot and node cultures that provide easy genetically stable method.

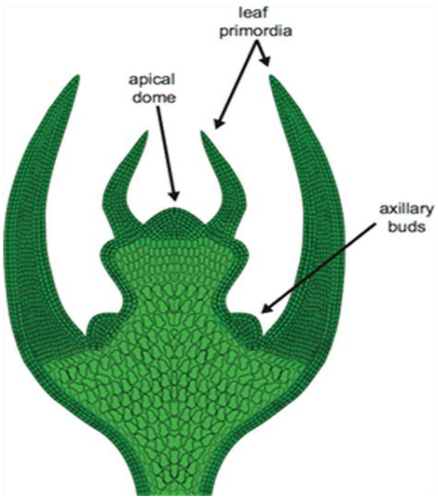


Shoot apical bud stucture

Shoot apical buds are located at the apices of the main and lateral branches of plants. Cells derived from them subsequently undergo differentiation to form the mature tissues of the plant body. Because of their highly organized structure, apical buds tend to be genetically stable. Shoot apical bud consists of a layered dome of actively dividing meristematic cells located at the extreme tip of shoot (0.1-0.2 mm diameter and 0.2-0.3 mm length) in addition to leaf primordia and their buds. It has no connection to xylem and phloem. Below the apical meristem, localized areas of cell division and elongation represent sites of newly developing

leaf primordia. Lateral buds each containing meristemic cells develop within the axils of the subtending leaves. In intact plants, outgoing of the lateral buds is usually inhibited by apical dominance of terminal shoot tip. Shoot apical bud culture is started mainly with meristematic dome plus one or two leaf primordial.





L.S. in shoot apex

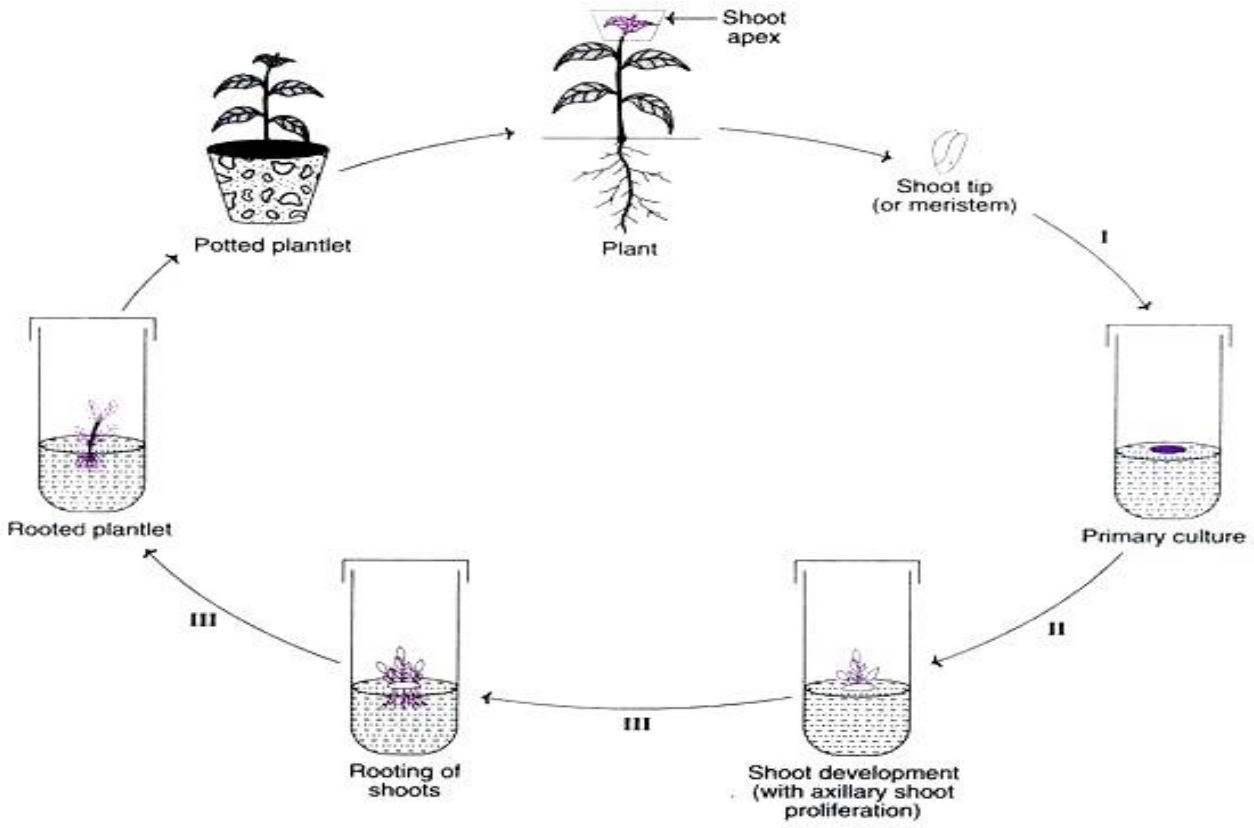


*Fig. 47.2 : A diagrammatic representation of shoot tip (or meristem) culture in micropropagation (**Note : I, II and III** represent stages in micropropagation)*

Axillary, Shoot and node cultures

Both methods depend on the stimulation of axillary shoot growth from lateral buds following disruption of apical dominance of shoot apex. Those cultures refer to *in vitro* propagation by repeated enhanced formation of axillary shoots from shoot tips (apical bud) or nodal cutting or lateral (axillary) buds. In turn, those produced axillary shoots are either subdivided into shoot tips and nodal segments that serve as secondary explants for further proliferation or serve as microcuttings for rooting. Node culture is a simplified form of shoot culture where shoot is cut into nodal segments (Single elongated unbranched shoot consists of one/several nodes).

Micropropagation stages

Five stages can be recognized in the previous micropropagation method:

Stage 0: Donor plant selection and preparation,

Stage 1: Establishment of aseptic culture,

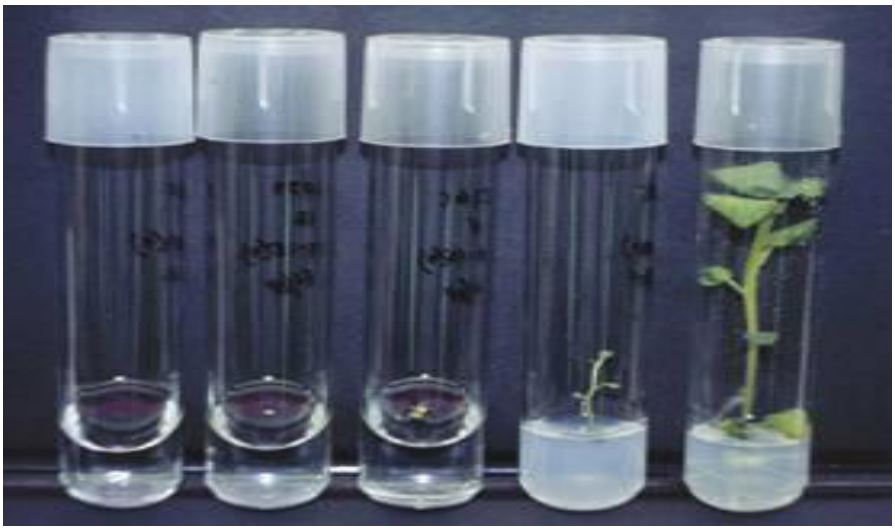
Stage 2: Proliferation of axillary shoots,

Stage 3: Pretransplant (Rooting) and

Stage 4: Transfer to natural environment.

Donor plants must be pathogen free, grown under clean and relatively low humidity to reduce risk of contamination. The response on culture media can be enhanced through spraying donor plants with cytokinin or gibberellin. Initiation step (stage 1) is affected with explant size, position, and time of explantation. It frequently requires cytokinins and auxins to enhance explant survival and shoot development. However, type and concentration of these hormones is genotype dependent. The next stage (stage 2) is characterized by repeated enhanced formation of axillary shoots from shoot tips or lateral buds cultured on a cytokinin rich medium to disrupt apical dominance of the shoot tip. A subculture interval of 4 weeks is used in this stage. Increase in number of subcultures is associated with appearance of off-types (somaclonal variation) to an unacceptable level. Rooting stage (stage 3) involves preparation of stage 2 shoots for successful transfer to soil, during which elongation of shoots and rooting occurs. It is achieved on hormone-free basal medium or auxin-containing one. The ultimate success of micropropagation depends on the ability to reestablish vigorously growing plants from *in vitro* to greenhouse conditions (stage 4). However, micropropagated

plants are difficult to transplant due to heterotrophic mode of *in vitro* plants and poor control of water loss.



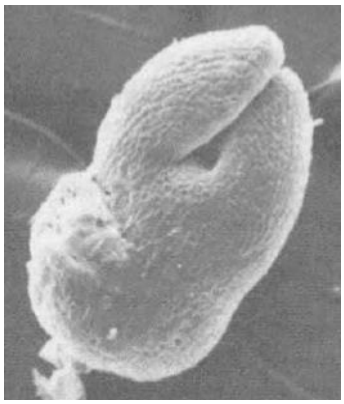
Stages of meristems culture

3. Meristem culture/Virus free

Culture of the apical meristematic dome alone is termed meristem culture. It is rarely used because of its low survival rate.

Virus free plants

Plant meristem culture is a unique technique to free away various pathogens including viruses, viroides, mycoplasma, bacteria and fungi. Meristems are frequently devoid of systemic pathogen due to the absence of differentiated conducting tissues (xylem and phloem).



Meristem dome

Different researchers have shown that the rate of obtaining a virus-free regenerated plant is inversely related to the size of the isolated meristem. However, the capacity of the meristem (meristem tip culture is usually used) to regenerate into a full plant is also directly related to the size of the explant. Hence, a balance between successful virus elimination and the probability for plant development is needed. This method is more successful in case of herbaceous plants than woody plants. In case of woody plants, the success is obtained when the explant is taken after the dormancy period is over. After the shoot tip proliferation, the rooting is done and then the rooted plantlet is potted.

4. Organogenesis and Embryogenesis

Medium and growth conditions are manipulated to obtain a complete plant from explant through either organogenesis or embryogenesis; both of them may be direct or indirect following callus (a mass of unorganized cells) phase.

Organogenesis is the ability of plant tissue to form various organs *de novo* on non-meristematic explant. It describes many processes including regenerating shoots or roots on explant, callus or even *in vivo* root formation on stem cuttings. Shoots are regenerated first then transferred to rooting medium. If root is formed first, shooting is usually not attained. Organogenesis is divided into **three** phases: dedifferentiation, induction and differentiation.

- Dedifferentiation involves the reversion to a less committed, more flexible developmental state that may or may not give rise to callus tissue. At the end of this phase, cells acquire a state of competence which is defined by the ability to respond to organogenic stimuli.
- The induction phase is characterized with a cell or group of cells become fully committed to the production of shoot or root.
- At differentiation phase, morphological differentiation and development of the nascent organ begins.

It is worth to mention that cytological studies showed that regenerated organs are multicellular in origin.

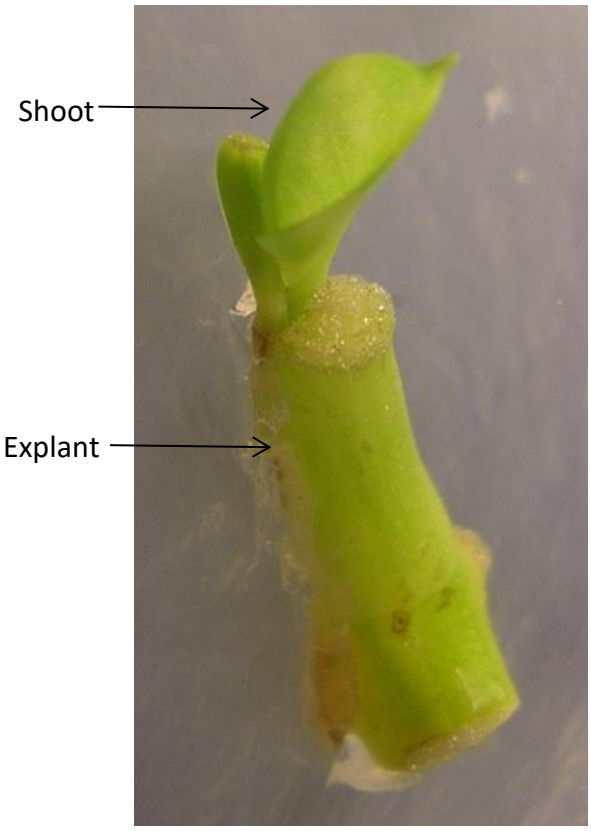
Embryogenesis:

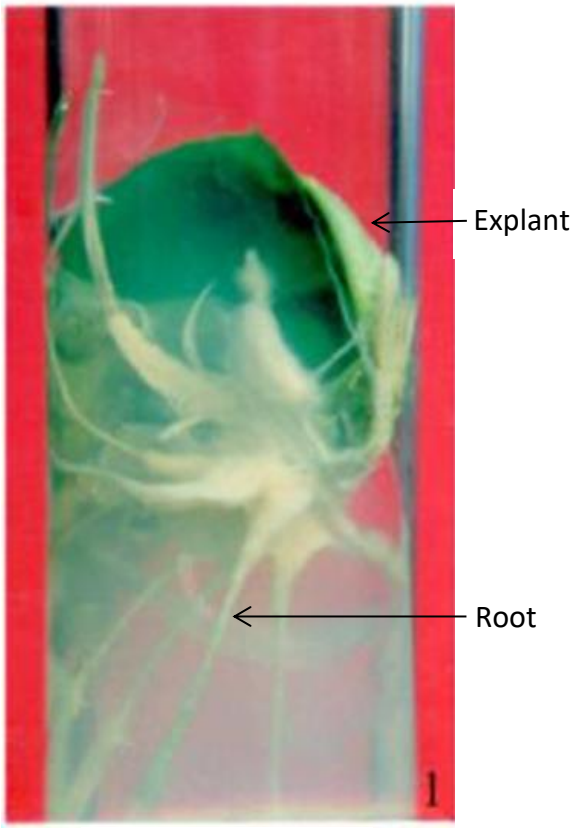
Embryo is a morphologically distinct entity that functions as an intermediate stage in the transition between gametophytic to sporophytic life cycle. In higher plants, embryos develop within seeds as a result of gametic fusion and are called zygotic embryos. In tissue cultures, somatic embryos (non-zygotic embryos) resulted without gametic fusion. In contrast to organogenesis, any somatic embryo is unicellular in origin. Somatic embryogenesis involves 3 phases: initiation of embryogenic cell, embryo development and embryo maturation phases.

Initiation of embryogenic cell is a dramatic change in the fate of **non-embryogenic cell (in explant or callus)** including dedifferentiation followed by redetermination toward an embryogenic cell type. Some genetic mechanisms for somatic embryogenesis can be related to mechanisms of zygotic embryo formation. Immature zygotic embryo can be used as explant; in such case, we need only to propagate these embryogenic cells *in vitro*.

Embryo development is the observable transition from non-embryogenic to embryogenic cell; it occurs when the cell undergoes an unequal division, resulting in a larger vacuolated cell and a smaller, densely cytoplasmic (embryogenic) cell. Embryogenic cell divides to form somatic embryo. Zygotic and somatic embryos share the same gross pattern of development (globular, heart, torpedo and cotyledonary stages).

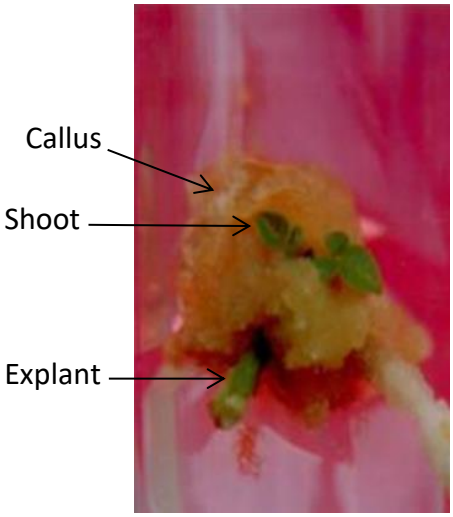
Embryo maturation is the terminal event of embryogenesis; it is characterized, in zygotic embryo, by attainment of mature embryo morphology, accumulation of storage food materials, reduction in water content and often gradual cessation of metabolism. Complete maturation is not absolutely necessary to obtain plants from somatic embryos. It is required to achieve high rate of plant recovery. High sucrose concentration, ABA and polyethylene glycol are shown to enhance maturation of somatic embryos. In some rare cases, somatic embryo enters resting stage like that observed for zygotic embryos.

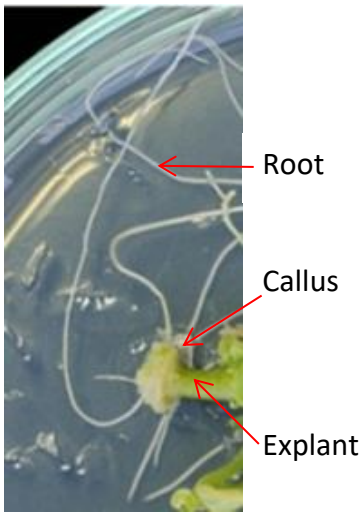




Shoot formation **Root formation**

Direct Organogenesis



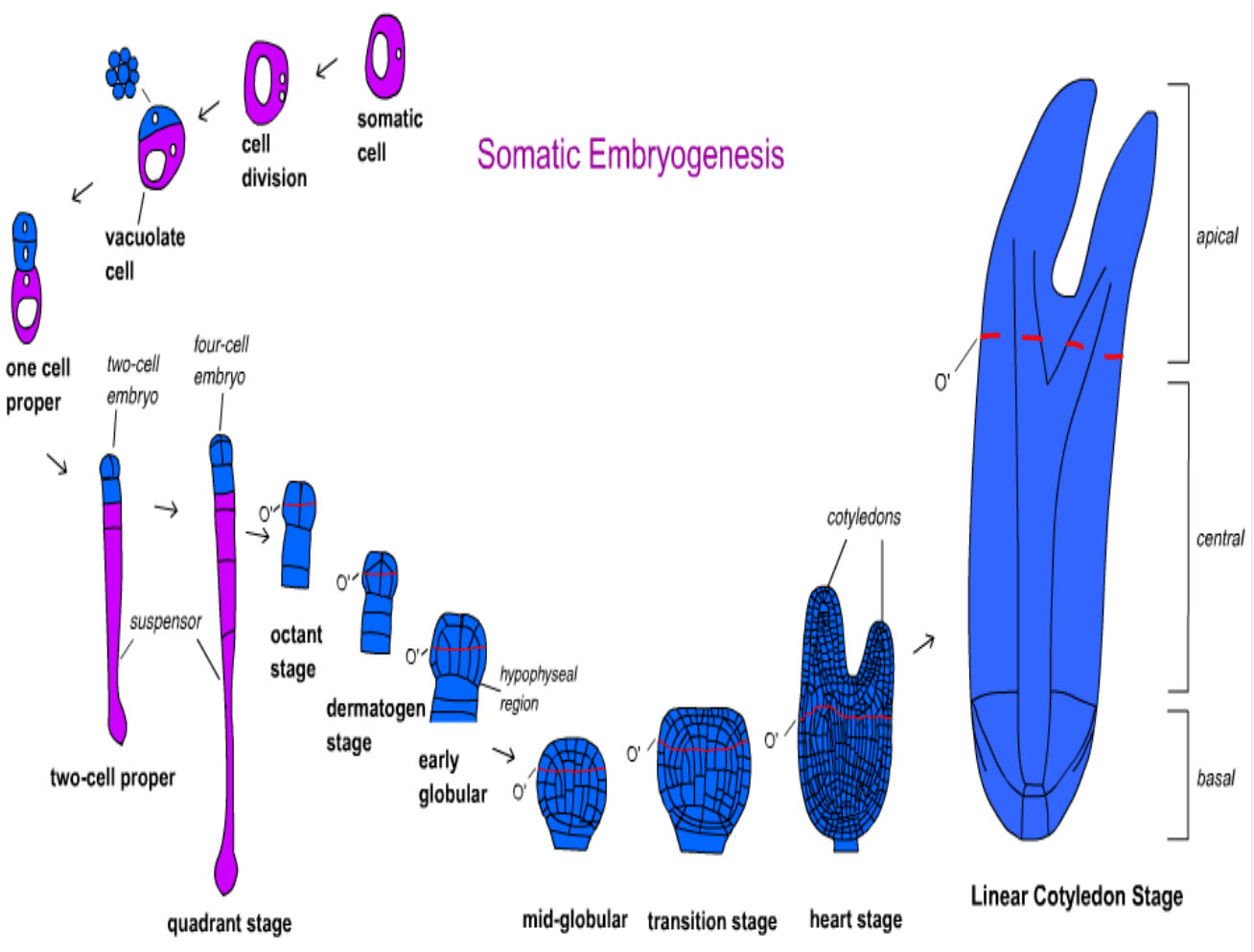


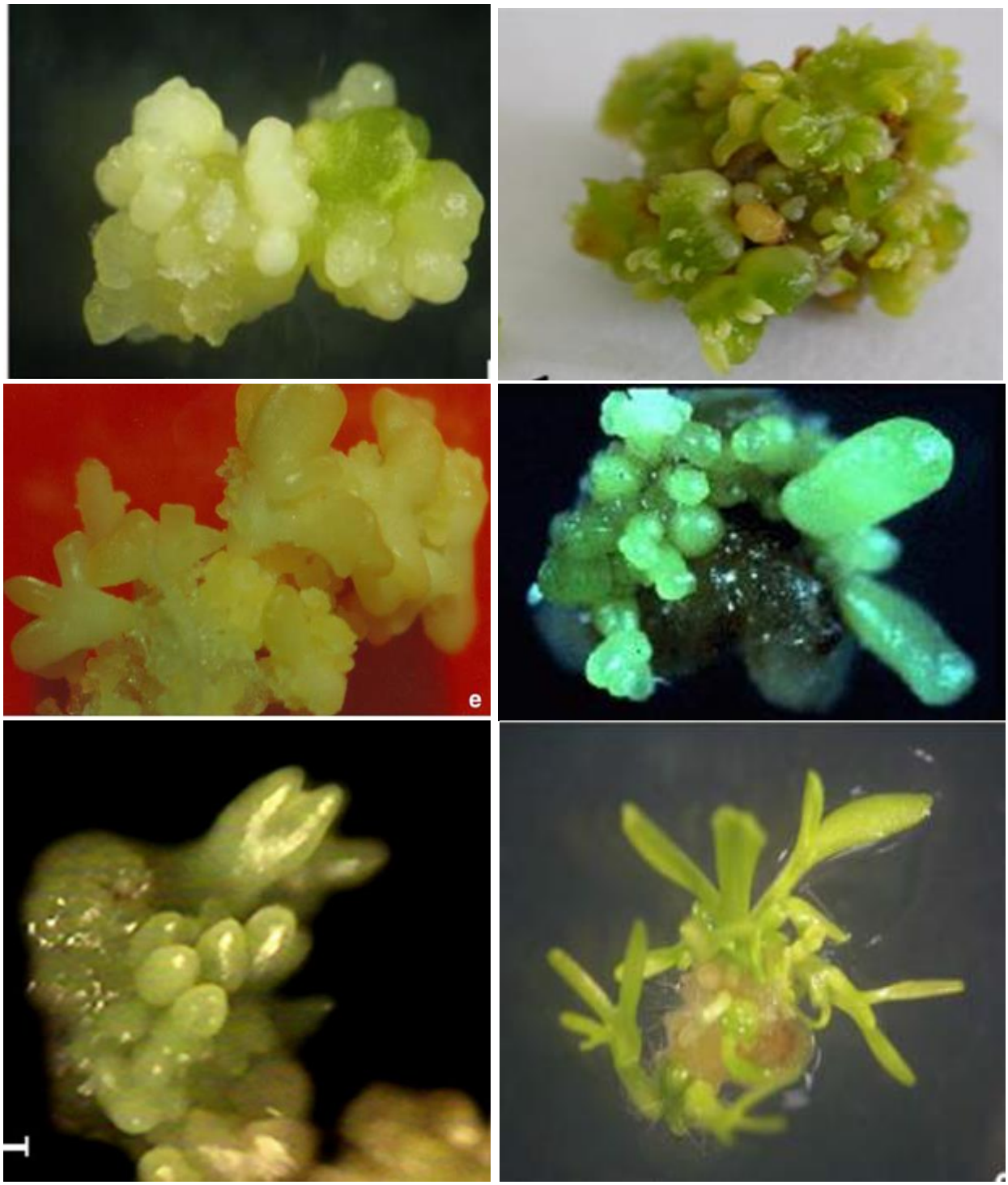
Shoot formation **Root formation**

Indirect Organogenesis

Globular **Heart** **torpedo** **cotyledonary**

Stages of embryogenesis

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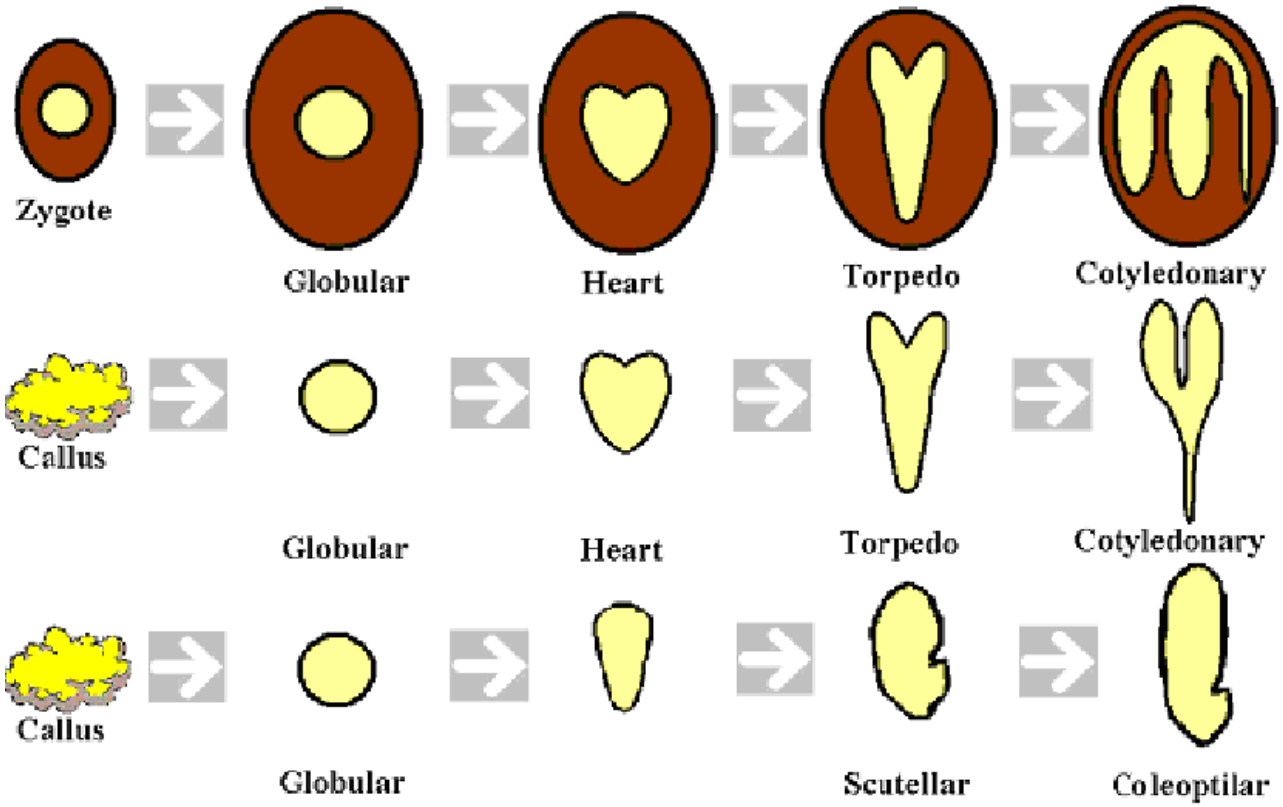

zygotic and somatic embryogenesis

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