SEMESTER –IInd Semester

PAPER- I: TREE PROPAGATION & IMPROVEMENT

UNIT- I

Definition of Propagation

Plant propagation refers to the multiplication of an individual plant or group of plants, which have specific value to mankind. Perpetuation of plants is called propagation. It involves multiplication of one plant into several plants –development of new individuals. New plants or new individuals are required for establishing **new plantings / new gardens/ new orchards**.

Methods of propagation: Broadly grouped in to two.

A. **Sexual** and

B. A**sexual (Seed) Propagation or Vegetative Propagation**

Sexual Reproduction

It refers to multiplication of plants by seed. In sexual process male and female gametes are fused to produce seed. Meiosis division takes place in course of fusion and the chromosome numbers, as in parents is reduced to half, which after fertilization becomes normal. In sexual propagation during meiosis segregation, reassortment or rearrangement of characters takes place. So, the plants thus produced may or may not be similar to their parents and the propagated plants may also be different from each other**.** It is called as seed propagation; since the propagation is through seed and also sexual propagation because sexes are involved **Seed** is the result of fusion of male and female gametes. Seeds are fertilized ovules, containing embryos resulting from the union of a male and a female gamete during fertilization. The embryo in the seed gives rise to a new plant on germination. Plants that are produced from seeds are called **seedlings.**

Asexual propagation/ Vegetative Propagation

It is called with different names--Asexual propagation, Vegetative propagation, Clonal propagation. Asexual propagation is reproduction by means of vegetative parts of the plant such as roots, shoots, or leaves other than seed. In this propagation sexes are not involved–hence it is called **asexual propagation**. It involves the use of any part of the plant, other than seed i.e. vegetative parts –hence **vegetative propagation**. The vegetative organs of many plants have the capacity (ability) for regeneration, to produce new individuals. For instance: (a) stem pieces

(cuttings) produce root system (b) Root pieces (root cuttings) develop root system. (c) Leaves generate both roots and shoots.

Vegetative parts possess somatic cells. They divide (multiply) by mitosis –does not involve reduction in chromosomal number, but involves the duplication of chromosome structure -the same genetic constitution is seen in the resultant plants – no variation. Whatever the characters present in the parent –the same are carried in the new plants i.e. duplicated without any change – true to mother plant-variation is eliminated.

Object of Vegetative Propagation

For the past 30 years, interest in the propagation of native plants has been growing. Many desirable and ecologically important species, however, are difficult or very time consuming to propagate by seeds. Thus, nursery growers may want to investigate how to propagate a species of interest by vegetative propagation. This can be done by combining classic horticultural propagation techniques with an understanding of the ecological and reproductive characteristics of the species. By investigating how a species perpetuates under natural conditions, nursery growers may be able to vegetatively propagate the species and produce nursery stock in situations when there are constraints on using seed propagation.

Many native plants naturally propagate vegetatively (that is, without seeds or spores) as a method of ensuring reproduction. Vegetative propagation is commonly found with species that have short seed life, low seed viability, or complex or delayed seed dormancy strategies. Species that inhabit ecosystems with drastic weather patterns, short growing seasons, and endure fires and other disturbances often reproduce vegetatively. All new daughter plants that arise from vegetative propagation are genetically identical to the mother (donor) plant, and these resulting individuals are known as “clones”. Nursery managers can make use of a plant’s ability to regenerate vegetatively.

The following situations favor vegetative propagation over seed propagation:

Advantages or importance

- Disease-free nursery stock is required.
- Seed propagation is difficult, very time consuming, or few viable seeds are produced.
- Larger nursery stock is needed in a shorter period of time.

- An individual, unique plant needs to be propagated.
- There is a need to shorten time to flower for seed production.
- A uniform stock type is needed.
- Specific genotypes are desired.
- As there is no change in the genetic makeup of the plant propagated by this method, the fruit plants propagated vegetatively are true to type, and, as a result, it is possible to get uniformity in growth, yield and quality of fruit, which makes harvesting and marketing easy.
- Some fruits such as banana, pineapple and some guava varieties being seedless, the only way of further propagation is vegetative method.
- Vegetatively propagated fruit trees come into bearing earlier.
- Certain varieties of some fruit tree/plant are susceptible to certain diseases. By budding or grafting them on a resistant root stock, these varieties can be grown without pest or disease incidence.
- Hardiness to cold and other unfavourable conditions such as drought can be secured.
- Trees can considerably dwarfed by using proper root stocks.
- Methods like bridge grafting or buttressing can be used for healing of the wounds caused by rodents.
- By top working the inferior quality fruit trees can be converted into superior quality fruit trees.
- As a fancy, it is possible to grow 2-3 varieties on the same plant, e.g; one can get 3-4 varieties of roses on various branches of the stock plant.

Disadvantages/limitations

Some disadvantages of using vegetative propagation include:

- No, new variety can be evolved by means of the vegetative method of propagation.
- Greater production costs than seed propagation, usually because of increased labor.
- Reduced genetic diversity.
- Specialized propagation structures may be required, depending on the species or time of year.
- Vegetative propagation in many cases is more expensive than seed propagation.

- Vegetatively propagated plants are comparatively short lived. Lack of tap root system in vegetatively propagated plants results in poor anchorage in the soil. Consequently, such plants are easily uprooted in storms and or other such severe conditions.
- Vegetatively propagated plants are comparatively less hardy.
- Transmit viral diseases from plant to plant.

In general, vegetative propagation can be done with pieces of stems, leaves, roots, bulbs, corms, tubers, and rhizomes. Many factors, however, contribute to successful vegetative propagation of native plants. The type of vegetative material used, the time of year that material is collected, how it is handled and manipulated to induce rooting, and proper application of the correct environmental conditions all affect vegetative propagation. In addition, how plants are handled after rooting also plays an important role. Because vegetative propagation is more costly than growing seedlings, the production system must be efficient. A general rule of thumb is that at least 50 percent rooting must be obtained to produce cuttings economically. If rare species or individual plants are being propagated, however, costs may be less important. Consider these methods to reduce production costs:

- Develop a smooth production line, from the collection of material to the final product.
- Train nursery staff how to properly collect, process, plant, and grow material.
- Build a dibble for making holes in the rooting medium.
- Control waste caused by poor propagation or growing practices.
- Lift and harden cuttings properly to reduce mortality.
- Develop a good system for overwintering cuttings.
- Keep good records to improve your results and to document production costs.

Application of Vegetative Propagation in Forestry

Vegetative propagation of trees was originally used for the species like Poplar, *Eucalyptus* and *Cryptomeria* which reproduced vegetatively under natural condition. By the development of

other methods of vegetative propagation namely, cuttings and grafting it was made possible to multiply the selected superior trees for use in seed orchard and also to preserve germplasm in banks. Large-scale propagation from cuttings at costs similar to those of seedlings has been developed in many forest tree species.

Vegetative propagation has been used in forestry for production of quality planting stock. It has been used to develop fast and economical methods of raising superior planting stock in the intensive management of forests. These techniques become important in forestry owing to their potential in propagating superior trees to increase volume and quality of production. It has also been used for propagation of problem trees.

Vegetative propagation could be effectively used in species, which are not able to produce seeds frequently like bamboos. Propagation by vegetative means may be easy, more rapid and economical than by seed in many cases. In some tree species, germination may be poor or slow or there may exist complex dormancy problems or the seed may lose its viability very quickly like in recalcitrant species. Moreover, seedlings of many species grow slowly and take a long time to reach marketable size. In all these cases, use of vegetative multiplication is a more convenient method of propagation.

Since, most of the tree species are cross-pollinated and highly heterozygous, asexual clonal propagation helps in maintaining the genetic characteristics of each species. Because of this property, vegetative propagation methods can be used to develop clonal repository. Genotypes are preserved in such clonal banks. In genetic testing's, vegetative propagation has been used to evaluate genotypes and their interaction with the environment through clonal testing.

Clonal propagation will also help to conserve and multiply the valuable trees in a centralized area such as in a laboratory or green house for intensive study and breeding. Vegetative propagation has been used for maintenance of genetic gain. It helps to capture maximum genetic gains when used for regeneration in operational planting programme.

In addition to the above benefits, some forms of vegetative propagation can be used for production of disease free plants. In tree species that are often susceptible to some pests and diseases and while some may be partially or entirely resistant. Vegetative approaches like grafting will help to produce resistant clones.

Vegetative propagation can also be used to speed up the reproductive cycle for accelerated breeding and testing. Vegetatively propagated plants are precocious in bearing than seed propagated plants. Early induction of flowering thus induced will help to reduce the rotation of the tree species and also to increase the productivity.

Incorporation of Vegetative Propagation Techniques in Tree Breeding

Because of the various advantages of vegetative propagation in preserving the genetic characters of the propagated plants, it is used for the following uses in tree breeding programs.

1. Establishment of Clonal Seed Orchards

One of the objectives of a tree improvement program is to propagate the interim benefits of the program at the fastest rate to meet the propagules requirement of operational forestry through appropriate technologies. In advanced breeding programmes seed orchards are established for production of superior quality seeds. A seed orchard is a collection of selected clones or families established in one physical location and then managed to produce genetically improved seed for operational forestation.

Seed orchard are established either through seeds (seedling seed orchards) from open pollinated seed or clones (clonal seed orchard) by the use of grafts, cuttings, air-layered plants, tissue culture plantlets or other methods of vegetative propagation. Clonal seed orchard is generally preferred when vegetative propagation is possible and the seed orchard only serves as a production area for seeds or vegetative propagules. Clonal seed orchard is raised in species in which flowering can be initiated at an earlier age through vegetative propagation. A clonal seed orchard typically contains many clones.

2. Genetic Testing in a Breeding Programme

In conventional tree breeding programmes, the usual approach for testing candidates for the next generation involves progeny tests involving progenies by controlled crossings. The candidates must reach the flowering stage, which can take several years in trees. Vegetative propagation allows the candidates to be directly tested as clones, instead of indirectly, as in progeny tests.

Besides saving time, since it does not require trees to reach flowering competence, this strategy also increases the selection intensity, by allowing more candidates to be tested at the same cost.

Studies on this subject, with varying breeding parameters, have consistently concluded that clonal candidate testing is efficient.

3. Deployment of Genetically Superior Families through Bulk Propagation:

Large scale deployment of genetically superior families selected based on genetic tests is one of the common steps in tree improvement programmes. After controlled pollination of selected parents in the breeding program, families can be mass propagated without knowing the value of each progeny and without identifying the individual progeny. Quite often, it would be expensive to do controlled crosses to obtain large quantities of seed to meet requirement of operational forestry.

In such situations, vegetative propagation using the limited seeds produced through such controlled crosses is often resorted to. Each seed produced through such crossing would represent a different genotype and if 100 seeds are obtained from one cross we will end up with 100 genotypes. If 30 such crosses are made, the lot would contain 3000 genotypes. This mode of using vegetative propagation is called bulk propagation and provides the same genetic gain as if the material was propagated via seeds.

PROPAGATION TOOLS

The following tools are used in the propagation methods described above. For a full list of tools and equipment which can be used in propagation, the learner should consult the Learner Guide of Unit Standard 116205.

1. **Budding Knife** – A razor sharp knife used to make cuts on the seedlings and to cut off the bud-eye. The knife must always be sharp and in a good working condition to prevent tissue damage to the plant when cutting through it. If tissue damage occurs, the graft will most likely fail.
2. **Budding Tape** – Clear polyethylene strips, used to maximize contact between the bud and the rootstock until the union and the healing is complete. It also prevents drying and excess water from getting in and rotting the bud.
3. **Pruning Shears** – Bud-wood is cut using pruning shears. Pruning shears are also used where cuttings are used for propagation.

4. **Sharpening Stone** – All blades become blunt with use and require periodic sharpening. A sharpening stone, or wet stone, and honing oil are required.
5. **Sterilization Liquid** – Knives and shears must be periodically cleaned and sterilized properly with a solution of 10% bleach.

CUTTINGS

Segments of stem, leaf or root can be used for vegetative multiplication. Stem cuttings are induced to produce adventitious roots. Leaves are induced to produce roots as well as shoots, and the roots are induced to form shoots to get a complete plant. A cutting is the portion of a plant that is collected, treated, and planted to develop into a new intact plant complete with stems, leaves, and roots. Cuttings can be collected from mother plants in the wild, or special donor plants can be cultured in the nursery. Selection of mother plants, whether in the nursery or the wild, must be done carefully; it is just as important as the origin of seeds to ensure that nursery stock is well adapted to the out planting environment. Collection of cuttings should follow the same ethical guidelines as collection of seeds to establish proper genetic diversity and sustainability of wild populations. In addition, the ability of cuttings to root is often cloning specific, so it is important to record the origin of cuttings and subsequent rooting success.
Striking Cutting is the process of placing the cutting into soil or a rooting substrate. Often, propagators will say that cuttings have been "struck" to indicate that the cuttings have been placed in the rooting substrate.

PLANT PROPAGATION BY CUTTINGS

The process of propagation of plants by cuttings is known as cutting. A cutting is a part of a plant that will produce roots when put in soil media and eventually produce a new plant quite true to the parent plant.

A cutting may be a piece of stem, a leaf or part of a leaf, a piece of root, or root stock, or even a scale of bulb. **Classification of cuttings:** Cuttings are usually classified in to 3 groups according to the particular part of the plant used as cutting.

1. Stem cuttings
2. Root cuttings
3. Leaf cuttings

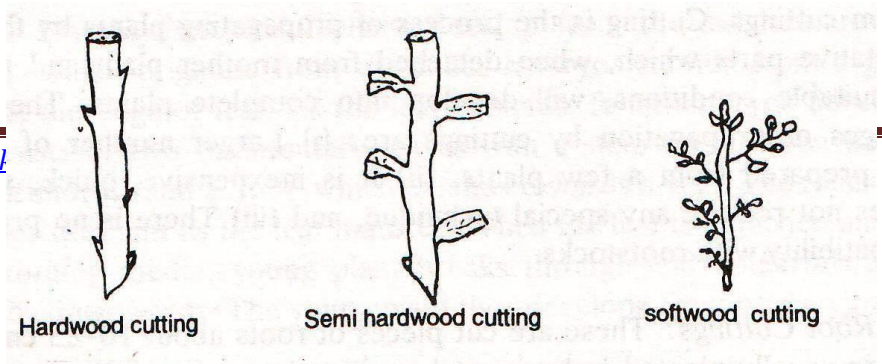
Stem cutting

The stem cutting is the most common and easy method for propagation of forest tree species. Tree species differ in their rooting ability and accordingly can be classified into

1. Very easy to root (*Salix sp., Morus alba, Bambusa vulgaris*)
2. Easy to root (*Ficus spp., Pongamia pinnata, Eucalyptus spp.*)
3. Moderately difficult to root (*Dalbergia latifolia, Grevillea spp., Pteridofolio*)
4. Difficult to root (*Tectona grandis, Emblica officinalis*)
5. Very difficult to root (*Hardwickia binnata, Albizia procera, Terminalia spp*.)

Shoot cuttings, also referred to as stem cuttings, are the most common type. A segment of stem branches can be rooted to produce full plant. The size of the cutting varies: it could be a few centimeters (*Eucalyptus, Casuarina*) to 1-2 meter poles (*Cassia, Siamea, Gliricidia, Sepium*). The presence of buds on the cutting is very essential for cutting propagation, because it supplies many factors responsible for rooting. Evenly the presence of leaves greatly affects rooting in the cuttings. Based on the presence or absence of leaves, the cuttings can be categorized as leafless cutting (Hardwood cuttings) and leafy cutting. These cuttings can be broadly placed into four categories depending on the time of year they are collected and based on the degree of maturity and lignification of wood used in making cuttings.

1. Hard wood stem cuttings
2. Semi hard wood stems cuttings
3. Soft wood stem cuttings Herbaceous stem cuttings



1. Hard wood stem cuttings: These cuttings are made from the past seasons growth or wood that has matured and lignified are known as hardwood cuttings. Hardwood cuttings are collected when plants are dormant, from late autumn through early spring. Hardwood cuttings, being leafless, do not dry up fast and thus can be rooted without much difficulty in an open environment.

Preparation and planting: Select a fully matured shoot with normal internodes from a healthy, vigorous plant growing in full sun light. Remove all the leaves without damaging the axillary buds. Give a slant cut just below the basal node of the selected shoot. Measure the required length (about 15 to 25cm and containing 3 to 4 buds) from the base of the shoot and give a horizontal cut 1 to 2.5cm above the top node. Repeat the procedure and prepare as many cuttings as possible from the shoot. In case of difficult to root species treat the prepared cuttings with recommended growth regulators to induce rooting. Make holes in the prepared bed or pot with the help of a stick or dibbler. Insert the cuttings in the hole such that at least two nodes are inside the soil. Take care of polarity while planting cuttings. After planting press the medium firmly around the cutting and water immediately.

Hard wood cuttings may be of three types: Straight or simple cutting, heel cutting and mallet cutting etc.

1. ***Straight or simple cutting:*** It is a branch segment that does not contain branches at its base or on any part of the older branch.
2. ***Heel cutting:*** *A* small chip of older wood is retained at the base of each cutting
3. **Mallet cutting:** A segment of older branches is present at the base of the cuttings and its appearance is somewhat like inverted- T.

		
Mallet cutting	*Heel Cutting*	*Straight Cutting*

2. Semi-hard wood stem cuttings: Semi hard wood cuttings are prepared from new shoots just after a flush of growth which is partially matured branches of woody trees.

Preparation and planting: Select partially matured shoots from a healthy and vigorous growing plant and take out the terminal 7 to 15cm portion by giving a horizontal cut just below a basal node. Remove all the leaves towards the base of the shoot and retain only the terminal leaves. If the retained leaves are very large, reduce their size by cutting the top half portion. This facilitates planting the cuttings closer and also minimizes the loss of water from cutting. Plant the cuttings in the same way as hard wood cuttings are planted.

3. Soft wood cuttings: Cuttings are prepared from the soft succulent new spring growth of species which are 4 to 6 months old and comparatively younger than the semi hardwood cuttings.

Preparation and planting: Select the soft succulent shoots from a healthy and vigorous growing plant, growing in full sun light and take out the terminal 7 to 15cm portion by giving a horizontal cut just below a basal node. Don‘t remove the leaves except for the part to be buried inside the rooting media. Soft wood cuttings should be kept in green house or in moist chamber where a high humidity can be maintained which keeps the tissues in turgid condition. Plant the cuttings in the same way as hard wood cuttings are planted.

4. Herbaceous stem cuttings: This type of cuttings is taken from soft, succulent herbaceous green house plants. Herbaceous cuttings are mostly used in floriculture (culture of ornamental flowering plants). This type of cutting roots fast, but is not used in forestry practice.

Preparation and planting: Select the succulent herbaceous shoots from a healthy and vigorous green house growing plant. Retain all the leaves. Give a basal cut below a basal node. Plant the cuttings in the same way as hard wood cuttings are planted.

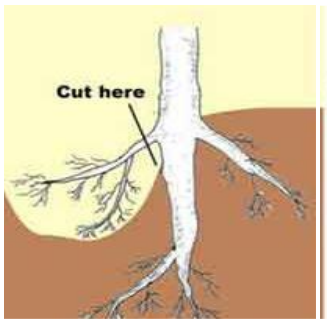
Leafy stem cutting

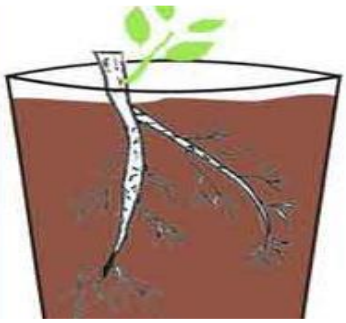
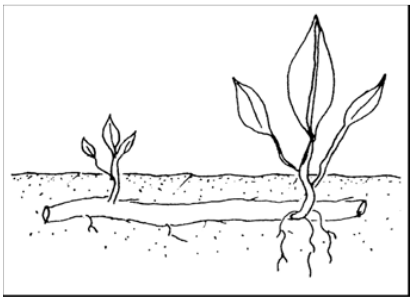
Leafy stem cutting, as the name indicates, are cutting that bear leaves or a part of the leaves. Usually one or two trimmed leaves are left with the cuttings. The presence of the leaves is advantageous to rooting, because they are the source of carbohydrates and auxins. However, presence of leaves makes cutting more prone to desiccation because of transpiration. The leafy cuttings thus require special propagating structures like mist chambers, polytunnels etc. to maintain high humidity conditions. Semi hardwood, softwood and herbaceous cutting are included in leafy cuttings.

Root Cuttings

Like stem and leaf parts, root segments are capable of regenerating into a full plant. The root cuttings are generally collected before the initiation of active growth period. At this stage, roots have sufficient stored food material, which can be utilized in the regeneration process. First adventitious shoots are produced from the root cutting, and then roots are formed from the base of the new shoots. These species having a natural tendency to propagate through root suckers can be multiplied via this method. In root cuttings it is preferable to maintain polarity of cuttings. The proximal end (the end facing main stem) of the cuttings should always face up.

Plants which give rise root suckers freely are propagated by root cuttings. Take root cuttings about 1 meter away from the tree trunk. These cuttings should be 20- 25 cm long and 1-2 cm thick. Place these cuttings horizontally into the soil about 10 cm deep until they shoot. This technique is useful for propagation of *Santalum album, Dalbergia sissoo, Populus alba, Albezia julinrissin, Azadiracta indica* etc.



Although not used as much as other types of cuttings root cuttings can be made by dividing roots into individual segments containing dormant shoot buds capable of developing into new plants. Root sections are collected from late autumn to early spring before new tissue emerges from buds. Root cuttings are planted horizontally in containers with the dormant leaf buds on the upper side. Some root cuttings are also planted in the containers vertically, but it is important to maintain the correct polarity. To ensure that root cuttings are planted correctly, cut the upper end of the cutting horizontally and cut the basal end diagonally. Root cuttings generally do not require a special rooting environment unless shoots are cut from the root piece and treated as a stem cutting.

Root cuttings, which are used in forestry propagation, should be taken from the young plant stock during the winter and spring months to ensure that they are saturated with stored foods. This time frame also prevents cutting during the time the parent plant is rapidly expanding shoot growth. Cutting during active expansion will take food stores away from the root system (Hartmann *et al.*, 1990). Another consideration when dealing with root cuttings is to make sure the polarity of the root is correct before planting. The portion of the root that was located nearest

the crown of the plant should be planted up. A uniform system of identification of the top and bottom of the root is a good idea to insure correct directional planting.

Leaf cuttings

Though not used extensively in forestry applications, this cutting method warrants a brief mentioning. This form of propagation utilizes the leaf to promote new plant growth. A root and shoot will form and develop, from the leaf cutting, into a new plant. The original leaf cutting does not remain as part of the new formed plant.

In leaf cuttings the plants are formed generally via two mechanisms: first, from pre-formed embryos present on the margin of leaves (*Bryophyllum* and ornamental plants) and second through induction of secondary meristem. Propagation by leaf cutting has limited utility. Only a small group of species can be multiplied by the method. In horticulture the method is utilized for some ornamental plants like *Saintpaulia* (African violet), *Gegonia rex, Bryophyllum pinnata* etc. Leaf bud cuttings are also a kind of leaf cutting- where leaf blade, petiole and a bud is the starting material and this structure is rooted and a full plant is produced (*Rhododendron, rubber*).

Leaf-bud cuttings

This type of cutting propagation also is not used extensively in forestry applications, but a brief description is warranted. The leaf-bud cutting includes the leaf itself, petiole, and a small piece of stem with the axially bud. This form of cutting propagation is useful when material is scare, because the same amount of stock will produce twice as many new plants as that of stem cuttings (Hartmann *et al.*, 1990).

Development of Roots in cuttings

Rooting in cutting is however, a complex physiological process. The roots that develop on the cuttings are adventitious in nature. These adventitious roots may develop either from a preformed root initial or from wound-induced initials or primordia. Root development through preformed root initials is quite simple. When cuttings from species having pre-formed root initials (*Populus, Salix, Willow, etc*.) are kept for rooting in rooting media under favourable conditions of high humidity. The root initials develop into adventitious roots. The presences of preformed root initials in stems of such species make them easy to root for cutting propagation.

Another way of root development is through wound-induced root initials. A cutting is a severed part of the plant, and thus the plant is wounded on being cut. When so called wounded cutting is kept alive under favourable conditions, it undergoes healing process. There is deposition of suberin at the cut end, followed by plugging of xylem vessels by gum. Next there is formation of undifferentiated mass of cells (callus) because of the repeated division of living cells just behind the cuts at the suberised end of the cutting. Some cambium cells near the vascular system give rise to root initials that develop into root primordia and later form the adventitious roots.

During the rooting process carbohydrates from the upper part of the cutting transported down and there starch is hydrolyzed into sugars, which is utilized by the dividing cells. Among phytohormones, auxins play an important root in root initiations. Auxins are needed during the early part of the root-initiation stage where differentiation of cells takes place with the formation of root initials. The development of root primordia and their elongation is not affected by the presence of auxins. Therefore physiologically there are two stages of root development in the cuttings, first, auxins requiring and another auxins not requiring. Besides carbohydrates and auxins there are many other factors that influence rooting. Cutting is a vague term unless specified which part is taken as a cutting.

FACTORS AFFECTING ROOTING IN CUTTINGS

Rooting phenomena is influenced by several plants (internal) and environmental (external) factors.

Plant factors

Physiological state of mother plant or cutting: the material used as a cutting must be juvenile. Mature cuttings do not have the capacity to form adventitious roots. Therefore juvenile material is necessary for cutting propagation. Perennial plants have two phases: juvenile and mature or an adult phase. The juvenile phase has some distinct characteristics. For example, in *Eucalyptus*, the juvenile leaves are broad and short and the stem is somewhat angular. In mature trees the leaves are narrow and long, and the branches are round or cylindrical. The adult branches have capacity to produce flowers. In a full grown tree, the lower parts of the tree or the lower branches are

more juvenile than the top portions. Therefore, the cuttings should be collected from the branches of the mother tree. Coppicing is a common method in forestry for getting juvenile cuttings. A full grown tree is cut close to the ground and the exposed area of the resulting stump is covers with some fungicide. After some time new shoots start coming out. These coppiced shoots after attaining some height can be cut and rooted. Another method of getting sufficient number of juvenile cuttings is to maintain trees in the form of hedges. The top portion is cut to get more number of side branches and shoots, which are again juvenile (*Pinus and Casuarina spp.*)

Effect of leaves and buds: leaves and buds supply auxin, carbohydrates and rooting co-factors, and thus are very essential for rooting. Without leaves, the cuttings can be rooted (hardwood cuttings), but without buds rooting of cutting ids not at all possible. Since movement of auxin is always downwards, it is extremely important that polarity of cutting is maintained. If cuttings are kept upside down, the rooting is not possible and cutting will not survive.

Position of cutting or bud on the mother tree (Topophysis): the variation in growth of the vegetatively propagated plants is due to the position of cutting or a bud on the mother tree. If a cutting is taken from a branch or shoot growing upright or an orthogeotropic shoot, the plant generated from it will produce upright shoots. Similarly, horizontally growing or plageotropic shoot or branch will produce plants with horizontally growing shoots. This is termed as Topophysis.

1. Propagation Media

No propagation method is going to work if the right media for growth is not used. In propagation, the air content of your media should be between 20 and 45 volume percent to promote root formation and growth. The volume percent in media should not drop below 15 volumes percent (Gislerod, 1983). This ensures adequate oxygen availability for the developing root systems. Increases in air within the media increases the oxygen diffusion rate (ODR). This increase is what will aid the root systems in acquiring the optimum amount of oxygen needed. The contents of media can vary for different regions and different species. Most mediums contain combinations of sand, peat, sphagnum moss, vermiculite, pearlite, compost, and

shredded bark/sawdust (Hartmann 1975). The following is a guideline to follow to help obtain good results from your media:

1. The media should be firm and dense enough to hold the cutting without movement during rooting. Excessive shrinkage, of media, after drying is not desirable.
2. The media should be able to hold moisture so that excessive watering is not needed.
3. Adequate existence of pores for the purpose of draining of excess water. This will permit sufficient aeration.
4. The media should not contain weeds, unwanted seeds, nematodes, and other noxious organisms.
5. Salinity levels should not be excessively high.
6. Media should be capable of being sterilized with steam.
7. The availability of nutrients for plant growth should be adequate

2. Hormones treatments

Hormones are organic compounds synthesized by plants that can regulate various processes of growth and development in a very low concentration. These hormones along with various other developmental events can also regulate rooting in plants and cuttings.

Auxins: these are the most important of all hormones involve in rooting. F.W. Went in 1926 first isolate auxin. Later this compound was identified as Indole -3-acetic acid (IAA). The auxins owing to their ability to induce adventitious roots are extensively used in cutting propagation. Synthetic auxins like Indole buteric acid(IBA), Indole propionic acid (IPA), and naphthalene acetic acid (NAA) have better effect than IAA in rooting of cuttings, because they are not destroyed by IAA oxidase (an enzyme involved in auxin degradation) present in plant tissues.

The concentration of auxin used in cutting propagation varies from species to species and also with the type of cutting. An optimum concentration of auxin is required, and a small excess of the hormone applied can inhibit development bud. Therefore one should be very careful while selecting the concentration of auxin for treating cuttings. For application auxin there are different

kinds of methods like the talcum powder based method, quick dip method, water based solutions (K-salt formulation).

Indole butyric acid (IBA) and naphthalene acetic acid (NAA) are two synthetic rooting chemicals that have been found to be reliable in the promotion of rooting in cuttings. IBA is widely applied in general use because it is non-toxic to most plants over a wide range and promotes root growth in a large number of plant species. Both of these chemicals are available in talc or in liquid formulations.

Method of application

1. ***Quick Dip Method:*** in this method, auxin is dissolved in alcohol (ethyl alcohol). The base the cutting is dipped for 1-5 sec. and after evaporation of alcohol the cutting is kept for rooting in a rooting medium. Usually high concentrations of auxins are used in this method.
2. ***Talcum powder:*** here the auxin is diluted with talcum powder and the base of cutting is put into the powder. The excess powder is removed by tapping and the cutting is kept for rooting in a rooting medium.
3. ***K- salt Formulations:*** such formulations are commercially available and are water soluble. For treatment the basal ends of cuttings are soaked in auxin solution for 4-12 hrs.

Cytokinins: Cytokinins are the hormones responsible for cell division. Normally they inhibit initiation of roots. However, at very low concentrations and in early stages of root initiation the Cytokinins may have beneficial effects. Cytokinins also have some role in rejuvenation and carbohydrate loading and both of these processes are helpful in the rooting phenomenon.

Gibberellins or Gibberelic Acid: these are the hormones responsible for cell elongation and are needed for the overall elongation of the stem internode. For rooting, GA has negative effect, which and probably is due to inhibition of cell division.

Ethylene: ethylene is involved in adventitious rooting in plants under waterlogging stress. However, in rooting of stem cuttings it can either stimulate or inhibit rooting process. Ethylene induced adventitious rooting occurs due to activation of auxin biosynthesis in tissue. It does not have a direct involvement in rooting.

Abscissic Acid (ABA): it is a growth inhibitor closely associated with various kinds of plant stress (drought, heat etc.). Abscissic acid (ABA) may have a positive effect, as it antagonizes gibberellic acids and Cytokinins. Both of them have inhibitory effect on rooting.

Rooting Co-factors: R. Bouillenne and F. Went during 1933-34 found that an extract from leaf, bud or cotyledon had stimulatory effect on root production in the cutting; they termed this biochemical factor Rhizocaline. Later during 1995, R. Bouillenne and M. Bouillenne- Walrand proposed that rhizocaline is a complex of auxins, some epecific enzymes located in root- farming tissues, and phenolic compound (Ortho- diphenols such as calechol and pyrogallol). The diphenols inhibit IAA oxidase enzyme and thus maintain higher level of auxins in the tissues. These rhizoclaines are considered important factors regarding rooting in the cuttings. Hess 1960s gave another term rooting co factors which are natural biochemical factors like oxygenated terpenoids, isochlorogenic acid and orthodiphenols, which along with auxin (not include in rooting co factors) promote rooting. Javavis, 1986 proposed thatinitially high auxins are needed for root induction, and further for development of roots auxin in high concentration is inhibitory. Therefore the phenolic compounds regulate the concentration of auxin by regulation of auxin oxidation. IAA oxidase enzyme was inhibited by ortho phenol and borate inhibits this inhibition of IAA oxidase by forming a complex with O-diphenol. Therefore when IAA oxidase is not inhibited, high auxin (IAA) concentration induces rooting and later auxin concentration is reduced as a result of its destruction by oxidase and root elongation which leads to further development.

3. Effects of cutting size on successful propagation

Very thin cuttings do not have the food reserves thicker cuttings have. This may lead to mortality before the cutting has a chance to root. The length of the cutting may vary depending on the species. Cutting sizes range from 2 inches to over 16 inches (Frampton and Hodges 1989, Foster 1990, Edson et al. 1991). Smalley and Dirr (1988) discovered that a two inch long cutting of red maple grew straighter than the larger cuttings, and the rates of growth after out planting was similar.

4. Shading

Shading of cuttings was once thought not to benefit the cuttings in their task of root development. It was believed that shading cuttings hindered the photosynthesis process, which would reduce the rooting activity. Photosensitize is responsible for the production of carbohydrates which will aid cuttings in the formation of roots. There are several reason associated with shading. It prevents leaf scorch, and aids in the prevention of excess buildup of carbohydrates, which will actually hinder the development of rooting systems (Stanley and Toogood 1981). An adequate amount of shading is around 20% from the beginning of spring to the end of fall.

5. Water quality and availability

The issue of water potential is extremely important with any type of cutting propagation. Since the cuttings have no root system, they cannot maintain turgor pressure in the absence of water, even for short periods of time. This makes the availability of water critical to cuttings survival. When the cutting is taken from the parent plant, the turgor pressure is broken. This pressure is similar to a rubber band, in that as water stress becomes higher, the force exerted from the top of the plant to get water from the roots increases. As water availability increases this pressure is reduced. Under normal conditions a plant can adapt, or adjust to situations of lower water availability through what is termed osmotic adjustment, in order to keep from wilting and dying. Osmotic adjustment occurs when the osmotic potential, at turgor loss, of a plant is lowered through the active accumulation of solutes in the cells (Turner and Jones 1980, Fan et al 1994). Cuttings do not have this luxury because they have no root system. Instead of adjusting, the cutting will wilt and die without constant water. As the root system develops, the plant will be able to take up water from the soil more efficiently.

6. Heat

Thermo-chemical reactions, which means the speed of plant activity is directly related to the temperature, it one of the most important aspects of successful propagation (Wells 1955). As temperature rises, the respiration of a plant will also raise. In common terms; higher temperatures, to some point, will generate higher activity levels in the plant. This section will examine the propagation programs in several countries. Other countries

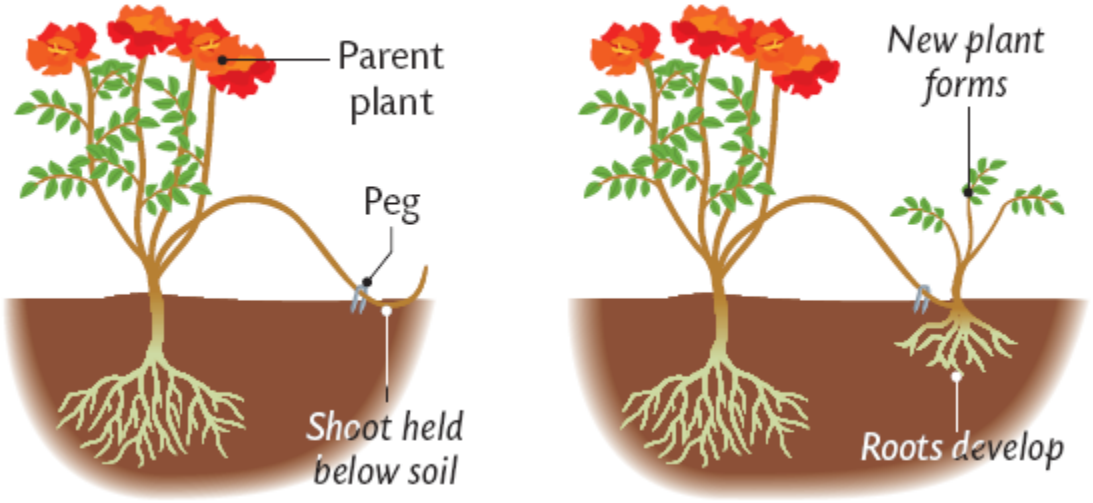
obviously have tree improvement programs, but these few countries comprise a large part of the active programs.

Vegetative Propagation by Layering

Layering is a technique by which adventitious roots are formed on a stem while still attached to the plant. Severed from the parent plant, the rooted stem becomes a new plant. This method of vegetative propagation, called layering, promotes a high success rate because it prevents the water stress and carbohydrate shortage that plague cuttings. Layering often occurs naturally without the assistance of a propagator. It is mostly used by nurseries with a long growing season and on those species that fail to root from stem or root cuttings. Layering is started when plants are dormant. Some plants layer themselves naturally, but sometimes plant propagators assist the process. Layering may be enhanced by wounding one side of the stem or by bending it very sharply. The rooting medium should always provide aeration and a constant supply of moisture.

Techniques of Layering Preparation

In layering, adventitious roots are initiated on the stem or branch of tree, which after induction of roots is detached and planted as a rooted plant. For induction of roots, a small part of the stem is selected and a ring of bark is removed. The area is kept moist by covering it with peat moss, soil, FYM or sphagnum mosses etc. and wrapping it with polythene covering tap to avoid moisture loss. After some time the roots are produced, and at this stage the branch can be cut and planted. The food material (Carbohydrates) and hormones (especially auxins) move downwards and accumulated at the place where bark has been removed. Consequently swelling at the upper part of the area takes place and from the swell end roots come out. The physiology is almost the same as that of rooting in stems cuttings. In layering, there is xylem connection, and hence there is adequate supply of nutrients and water to the branch and leaves, which contributes sufficient amount of carbohydrate production through photosynthesis. In addition, this help in better rooting in the layering. In contrast, if a cutting is detached from the mother plant and is kept for rooting, the plant utilizes stored food and if leaves are left the cutting may dry up fast because of excessive loss of water through transpiration



However, the root formation in layered stems completely depends upon continuous moisture supply, good aeration and moderate temperature around the rooting zone. Sometimes synthetic growth regulators like IBA, IAA etc, are also treated to layered stem to induce better rooting, as the auxins in layered stem is an important factor for rooting.

Advantages:

i. It is an easy method and does not require much care and arrangement like cutting.

ii. The mother plant supplies nutrient and other metabolites as it remains attached while rooting.

iii. By using a large branch a much larger plant can be obtained in the first instance.

iv. Some plants that cannot be satisfactorily started from cuttings can be propagated by layering.

Disadvantages:

i. It is a costlier method.

ii. It is a slow process

iii. Limited number of plants can be propagated

iv. Layered plants are generally shallow rooted

v. Interference with cultivation

vi. Require more individual attention

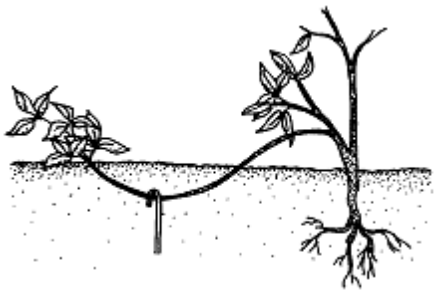
vii. The beneficial effect of root stock cannot be exploited

Types of Layering

Four types of layering can be used by propagators:

1. **Simple layering:** It is used on species that produce many shoots annually. In this method, a branch is bent to the ground and some portion of it is covered by soil leaving the terminal end of the branch exposed. Root initiation takes place at the bent and buried portion. After allowing sufficient time for root formation, the rooted stem is separated from the mother plant. To increase the removal of root bark, girdling, wiring etc. can be done to the buried part. All these treatments favour accumulation of carbohydrates by blocking downward the transport of food material.

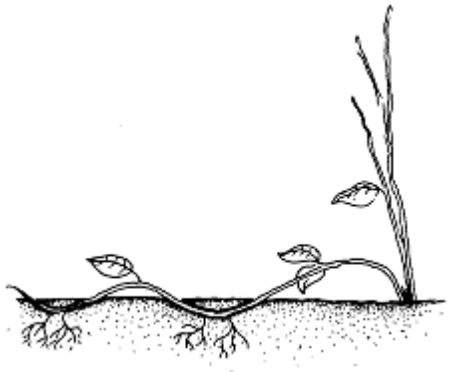
Procedure: Select a healthy, flexible and sufficiently long (50 to 60cm) branch towards the base of the plant. The selected branch should be closer to the ground. At a distance of about 15 to 30 cm back from the tip give a sharp, slanting inward and upward cut 1.5 to 2.5cm below a node and insert a small wood splinter. Bend the shoot gently to the ground so that the treated part can conveniently be inserted into the soil. Cover the treated region with soil. Peg down the shoot or keep a stone of brick on the covered soil to keep the layered shoot in place. Drive a vertical stake into the soil by the side of the layered branch and tie the terminal portion of the branch to keep it upright. Water the layered portion regularly so as to keep it moist all through till root initiation take place. After sufficient root formation separate the layer by cutting just below the rooted zone.



Simple layering

2. **Serpentine or Compound Layering:** Serpentine or Compound layering is essentially the same as the simple layering except that the branch is alternatively covered and exposed along its length. The branch for compound layering must be long and flexible so that it can be layered at different places along its length. It means the long flexible branch is buried at more than one point, giving it the appearance of a snake. In this, more than one plants are obtained from a single branch.

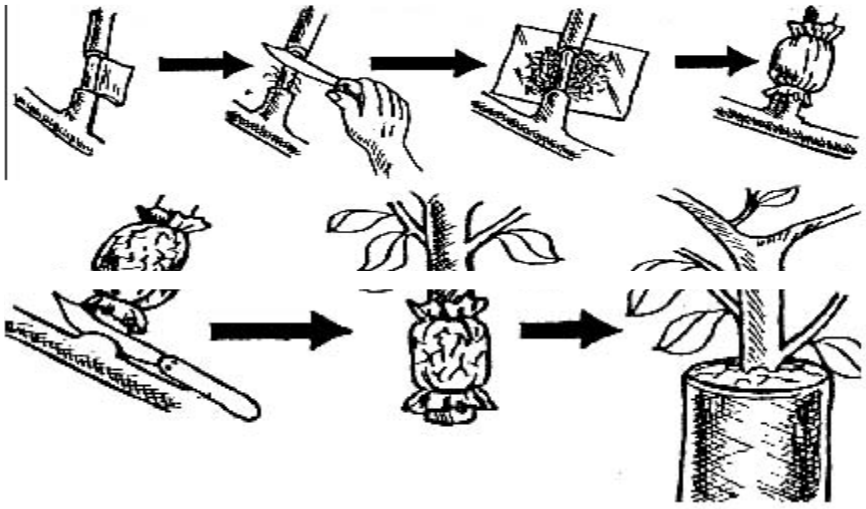
Procedure: Select a healthy, flexible and sufficiently long (100 to 250 cm) basal branch that is close to the ground. Give a sharp slanting, inward and upward cut 1.5 to 2.5 cm below a node at 30cm interval starting from the tip leaving 3 to 3 buds in between two such cuts. Bend the shoot gently to the ground, and insert and cover the cut portions with the soil exposing the uncut portions. The remaining steps are same as in simple layering.



Serpentine or Compound Layering

3. **Air Layering:** This type of layering is more useful in forestry than other methods**.** Inthis mothod the bark is completely removed from a small area of the branch, and the exposed area is covered with moist moss and again covered with polythene. After some days, the roots develop and at this stage the branch can be removed and planted as a separate plant. The exposed area of the branch can be treated with auxin for better results. In air layering roots form on an aerial shoot. The rooting medium is tied to the shoot for getting root initiation. Sphagnum moss is the best rooting medium for air layering as it holds large quantities of water till root initiation and through the root initiation and through the root development.

Procedure: Select a healthy branch of previous season‘s growth. At a point 15 to 30 cm back from the tip of the shoot make a girdle just below a node by completely removing a strip of bark 2 to 3.5 cm wide all around the shot. Scrape the exposed surface lightly to remove traces a phloem or cambium to retard healing. In difficult-to-root species treat the girdled portion with the recommended growth regulator to induce better rooting. Cover the girdled portion with moist propagating medium. Sphagnum mass saw dust, vermiculite. Tie the medium around the girdled portion using a polyethylene sheet. Tying should be perfect so that no water can enter the treated part. After observing the fully developed roots through the transparent polyethylene sheet, separate the root zone and transplant the layer appropriately.

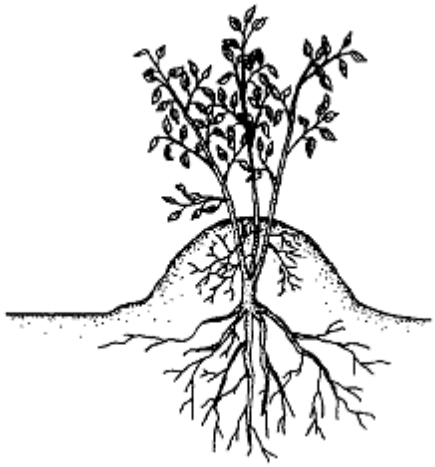


Stages of preparing an Air layer- piece of bark removed, scraping the exposed wood, wrapping with moist rooting medium, tying, separating the branch from the mother plant, separated air layer, planted air layer

4. **Mound (stool) Layering**: It is also known as stool layering. In this method, the stem is cut close to the ground during the dormant season and soil is heaped around the base of the newly developing shoots. After some time, new shoots start coming out of the stem and when they attain certain height the base of the shoots is covered by soil or a rooting medium (saw-dust,

peat, sand etc.) and left for some time. After induction of the roots the shoots are removed and planted as individual rooted plant. The base of the shoots may be girdled, wired or may be treated with auxin to induce better rooting. Mound layering is practiced in trees like *Santalum album* and *Pterocarpus santalinus.*

Procedure: Select the plant to be mound layered or plant a rooted layer in a trench and allow it to grow for a year. Cut back the plant to 2.5 cm from the ground level just before growth begins. Allow the new shoots to develop. When these shoots have grown 7 to 15 cm tall, girdle them at the base and treat the girdle portion with the recommended growth regulator and draw up the loose soil round each shoot to half its height. When these shoots have are 20 to 25 cm tall add soil again to half their height. Add soil again when the shoots grow to a height of about 35 to 45 cm. Water the heaped soil regularly and allow sufficient time for the initiation of roots. A depression can be made in the centre of the heap to hold water. After sufficient root formation, remove the heaped soil and cut the rooted shoots individually to their base. Transplant the rooted shots in pots or suitable containers.

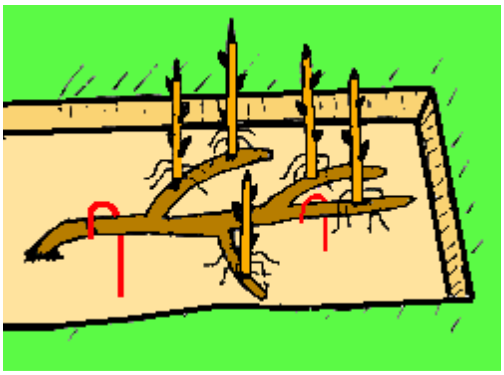


Mound or stool layering

5. **Trench Layering:** Trench layering consists of growing a plant or a branch of a plant in a horizontal position in the base of a trench and filling in soil around the new shoots as they

develop, so that the shoot bases are etiolated. Roots develop from the base of these new shoots. Etiolated roots develop from the base of these new shoots. Trench layering is used primarily for woody species difficult to propagate by mound layering. Trench layering is used primarily for woody species difficult to propagate by mound layering.

Procedure: Dig small trenches of about 25-30cm deep and in about 1 m wide rows. Plant rooted layers or one year old nursery – budded or grafted plants in the trenches in rows at an angle of 30^{O} to 45^{O} and 50 to 10 cm apart within the row. The rows should be1.2 to 1.5 m apart. Just before growth begins, lay the plant or a branch flat on the bottom of the trench. Plants must be kept completely flat with wooden pegs or wire fasteners**.** Cut back the shoots slightly and remove the weak branches. Add roots medium (sand or sawdust or peat moss) or their mixture at intervals to produce etiolating on 5 to 10 cm of the base of the developing shoots. Apply first 2.5 to 5cm layer before buds swell and repeat as shoots emerge and expand. At the end of the season, remove the medium and cut off the rooted shoots close to the parent plant. Transplant the rooted shoots in pots or suitable containers.



Trench Layering

Grafting

Grafting is the process of attaching a shoot (or less frequently a bud) from the individual you want to clone to the root system and lower stem, collectively called the **root stock**, of another tree. It produces a genetic mosaic, where most of the stem and crown of a tree or shrub are one genotype, and the root system and lower stem are another. This can come in handy for combining different roots and shoots with desirable properties. It is the only available method of cloning older trees of many species.

Typically a dormant **scion** (shoot cutting with terminal bud) is harvested from the upper crown of the tree to be cloned (the ramet) in mid to late winter, then attached to the top of the cut stem of a seedling of approximately the same diameter. It is important that the xylem, phloem and cambium of the rootstock and scion are in contact. Initially, wound callus is formed by both the rootstock and scion. The two then grow together and develop continuous vascular tissue. Root stock and scion need to be genetically compatible; otherwise a proper union will not develop and the tree will eventually die. Breeding programs have produced lines of rootstock that have high levels of compatability for other genotypes for some species such as Douglas-fir (*Pseudotsuga menziesii*).

Grafting is commonly used to establish genetically superior trees in seed orchards (mature scion material is used so graft will flower sooner) and to propagate unique trees for horticultural purposes (e.g., weeping varieties, infertile flowering cherries).

Vegetative Propagation by Grafting

Two types of rootstock can be used for grafting: the cultivar and the seedling rootstock. The cultivar rootstock is produced by vegetative methods, generally by layering and cuttings. Seedling rootstocks grow from seed. One of the best examples for cultivar rootstock is the apple and for the seedling rootstock, the mango.

During the selection of the scion wood we have to consider some important aspects:

1. The scion wood must carry healthy buds that will grow into leafy shoot
2. It should come from a tree which is free from any pests or diseases
3. The plant should have the required characteristics. This includes, that we take scions from plants, which is already bearing fruits.
4. Buds, which already begin to grow, are useless for grafting. The grafting process will fail.
5. The one year old wood is the best for grafting. These shoots grew during the previous year. Water sprouts from up in the tree make straight scions.

There are some rules, which must be taken consideration for any grafting method to be successful:

1. Two incompatible plants cannot be grafted
2. The cambium layers of the rootstock and the scion must touch
3. The scion must be the right way up when you graft it
4. You can grafting in any time of the year, but the best time for deciduous plants is, when the plant drops its leaves and is dormant
5. Cool, cloudy day without wind prevents the graft from drying out, therefore this type of weather is the best for grafting
6. The care activities are very important until the rootstock and scion are properly joined

These grafting processes use a sharp knife, tape and grafting wax. Grafting wax seals the join wound and avoids water loss and disease infection. Therefore, its use is strongly recommended. If grafting wax is not available, we can use petroleum jelly (Vaseline) or we can produce homemade grafting wax. There are two ways to make grafting wax:

5 Kg resin
1 Kg bee wax
½ Kg siliceous earth (Fuller's earth)
Or

2½ Kg paraffin wax
1½ Kg siliceous earth
½ Kg Zinc oxide

In some cases, a section of trunk is introduced between the rootstock and scion. It is called inter-stem or inter-stock. This is done in order to have a desired effect or characteristic on the tree. These effects can be disease resistance, winter resistance or effects on the size of the tree. It is also used to join two incompatible cultivars together.

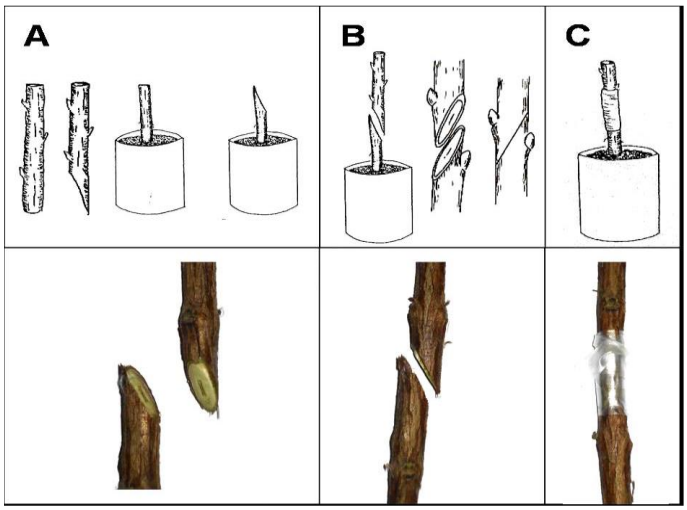
Whip Grafting

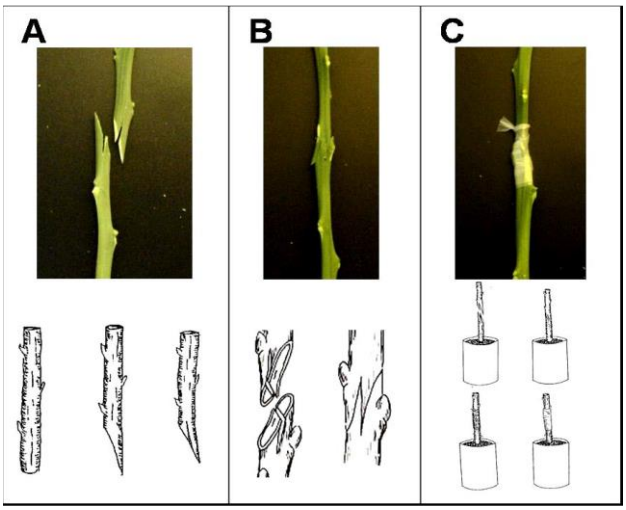
The whip graft is useful for plants that unite easily. This method is useful for apples, mangos and pears. It can be used to graft root, stem or top graft. The diameter of the scion and rootstock should be the same, from the size of a pencil to 10-15 mm.

Simple Whip Grafting: This type of grafting practice includes the process of a simple sloping cut on both the scion and the rootstock. The two parts should over lap each other perfectly. In any case, one rule must be followed: The wider the scion and root stock, the longer the cut surface.

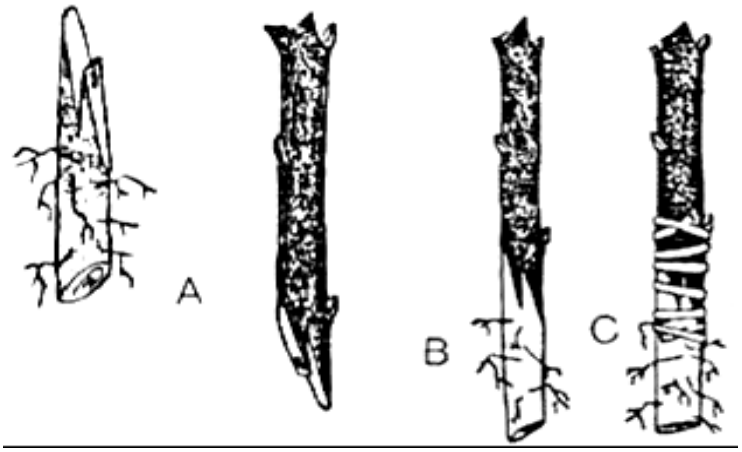
English (Tongue) Whip Grafting: This method is more common in practice, especially in the case of pear and apple trees. It is used to graft thin stems. It may be used on roots, stems or tops. The scion should have two or three buds with the graft made below the bottom bud. The first cut is a 2-5 cm sloping cut at the bottom of the scion. The second cut is made with a distance 1/3cm from the tip of the first cut. The same process is repeated on the rootstock. In apple propagation, the tongue grafting is mainly used to graft on M4 (semi-dwarfing – 5m height) and M9 (very dwarfing – 3m height) rootstock.

Simple Whip Grafting **Tongue Whip Grafting**





Root Tongue Grafting: Root grafting is used for propagation on a rootstock seedling, however the rootstock cannot belong to the dwarf category. This is due to the fact that it causes the rooting of the scion. We use a piece of 8-10 cm long root and a little bit longer scion. Root grafting is done when the apple rootstock and scion are dormant. This method is normally not used for pears.



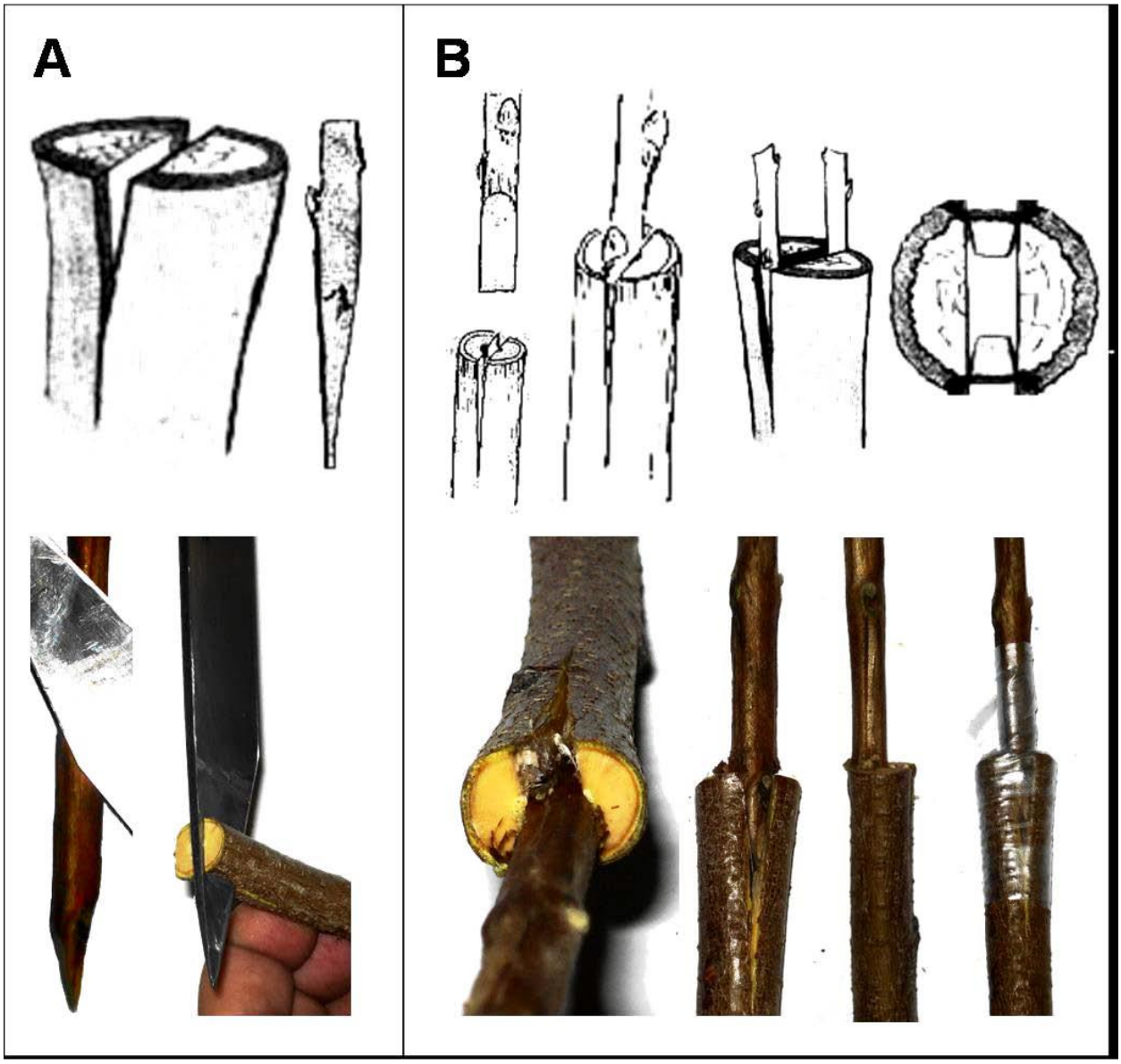
Cleft Grafting

This method has been practiced throughout the history of horticulture and is one of the oldest fruit propagation techniques. It is suited for apple and pears, but, in tropical areas, in can also be used for propagation of mango and avocado trees. Citrus and guava trees also use this method. In the case of top and side work, the scaffold limb is usually wider than the scion. In the case of tree propagation, both parts, the rootstock and scion, should be the same size.

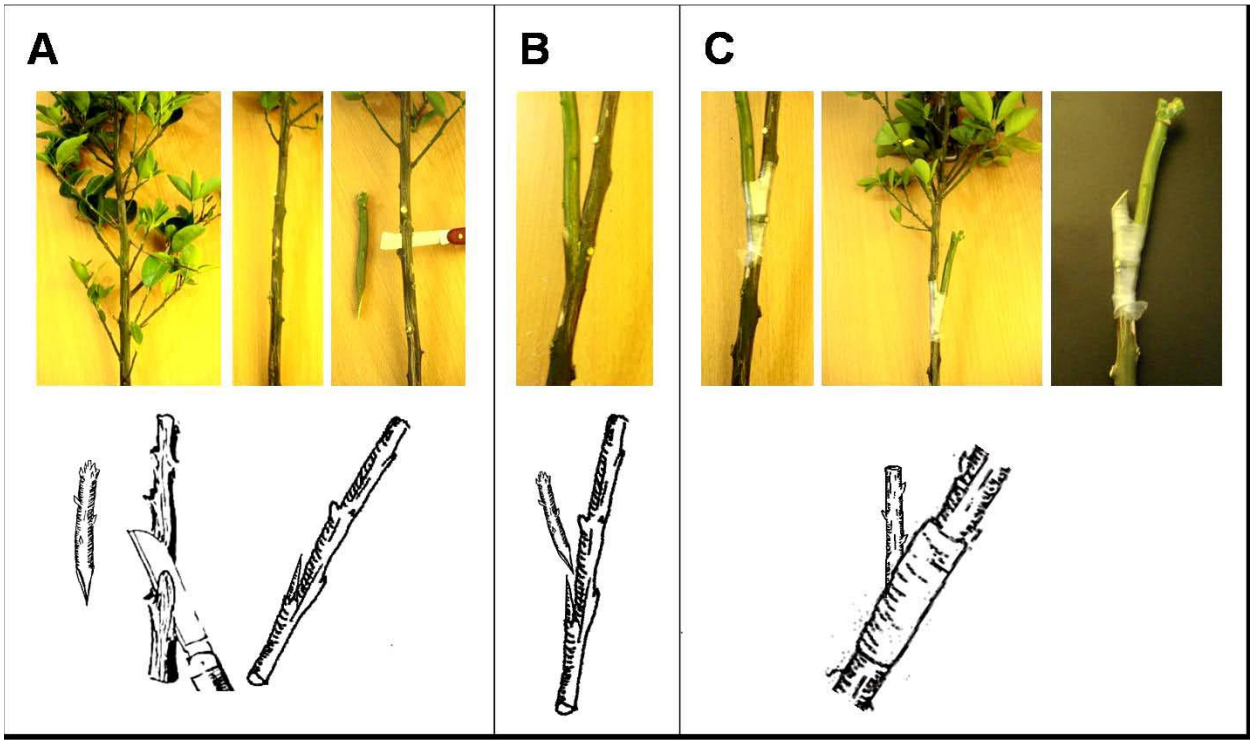
Top Cleft Grafting: For this method, the scaffold limb of the stock should be 4-6 cm wide. It should be straight and growing vertically. It should be free from spurs, knots and cankers. The limb must be cut where the amputation point is keeping the limb from splitting or the bark from peeling. After that, the stock must be split across the center to a depth of about 15 cm. Next, cut the scion 20 cm long and make two sloping cuts about 4-5 cm long. The wedge of the knife should be driven into the center of the split that was held apart and subsequently joined with the scion and the stock in a way that the two cambia will be in contact with each other. Usually, we use two dormant scions for the two opposite sides of the split.

Side Cleft Grafting: The processes are similar to that of the top grafting method, however, grafting is done on the upward side of the limb. Additionally, the limbs are not amputated until the grafting scion begins to shoot. The stocks are then split with a diagonal cut about 3-4 cm long and 1/2 cm deep and are joined with the scion at the stock. The next step is to tie and wax the graft. The scion must be covered with a small, thin plastic bag that contains a piece of paper to prevent the scion from drying up. This bag can be removed after 7-10 days. When the scion begins shooting, cut the limb closest to the grafting point.

Top Cleft Grafting



Side Cleft Grafting



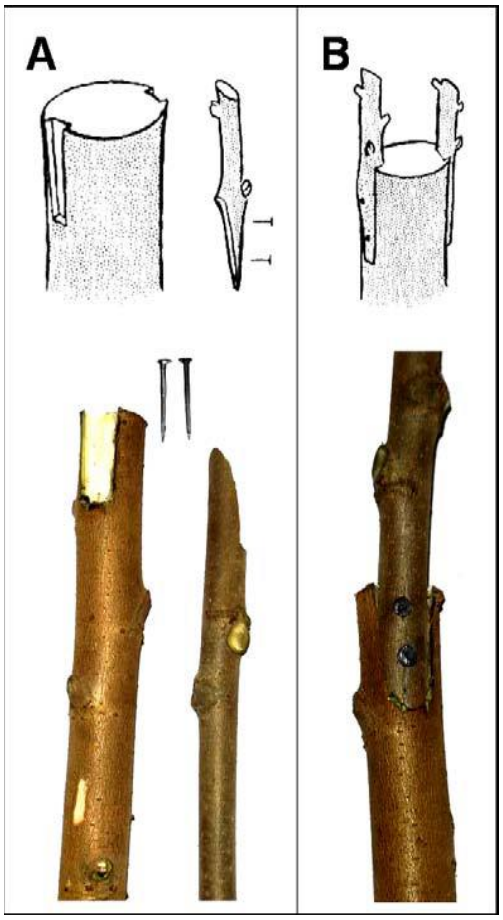
Bark Grafting

Bark grafting is used when the stock is too large for whip grafting. It is one of the most difficult grafting techniques. Perfect application of this method requires much practice and experience. The use of this technique is common for pear, apple and different nuts grafting.

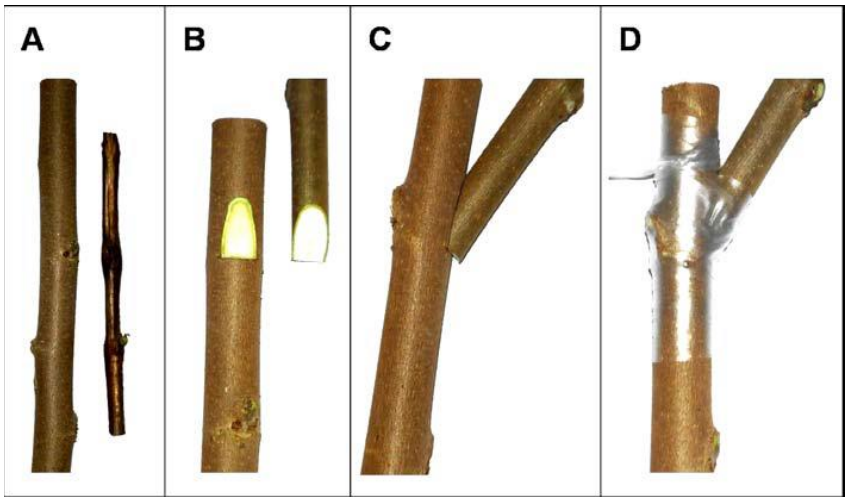
Top Bark Grafting: In this method the dormant scion should be used. The stock should be grafted when the bark begins to slip. The first step is to cut squarely across trunk (4-6 cm diameter). After the scion is cut across, a slopping cut of about 4-6 cm is made, which is done above the top bud (7-8 mm). The scion must be joined at the surface against the side of the stock. Finally, fix the scion with 2 nails and wax it once this is complete. Keep the trunk below the joining point so that it can be free from sprouts and shoots.

Side Bark Grafting: This technique is very similar to the top bark grafting. The square cut is 2-3 cm long and 8-10 cm wide. The joining part is then tied. Nails are not used and the branch of the stock above the joining point should be cut off after the scion begins shooting. This method is most commonly used during the growing season.

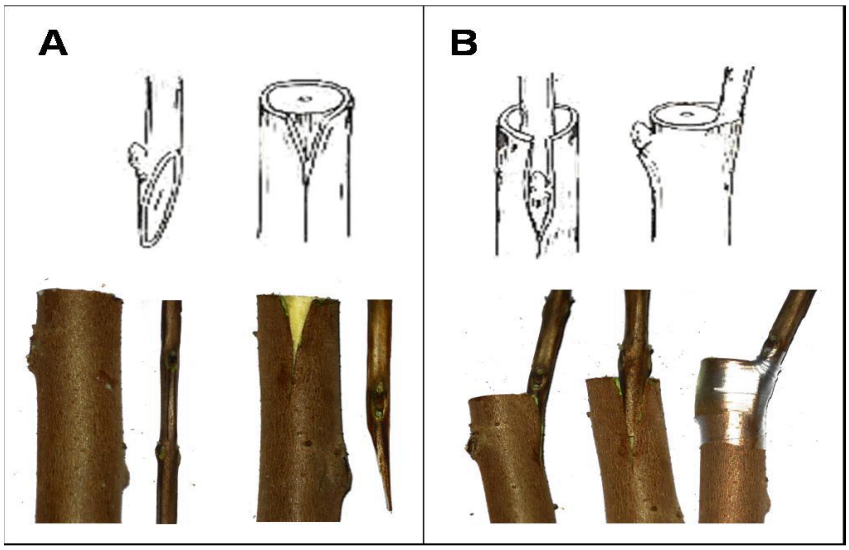
Top Bark Grafting



Side Bark Grafting



Slipping Bark Grafting

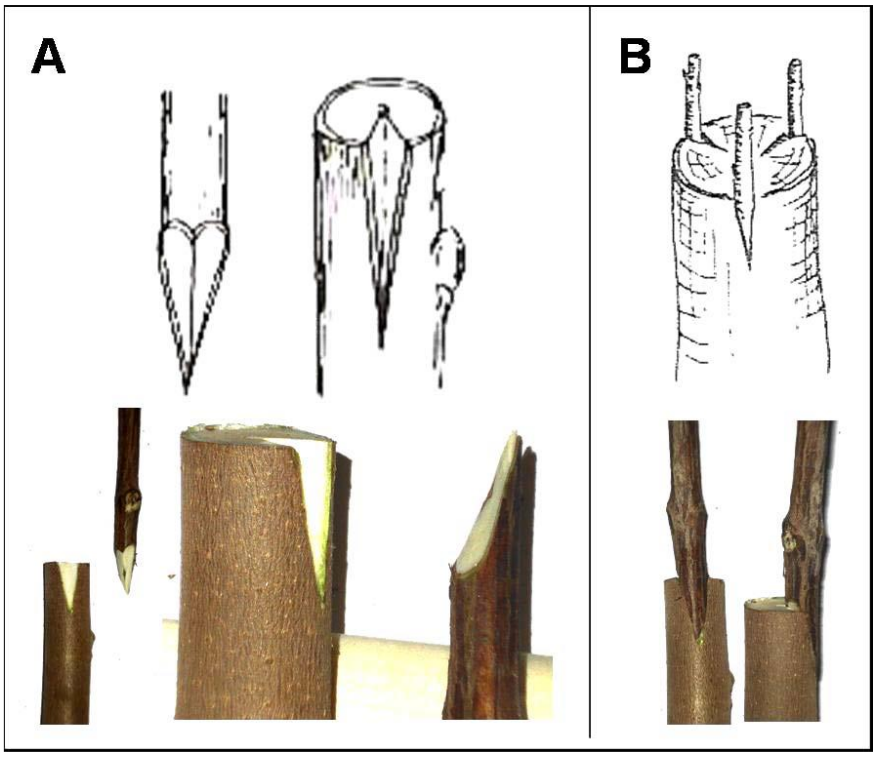


Slipping Bark Grafting: The technique has many similarities with the budding methods and can be applied during the same period, when the bark peels easily off the stock. The first step is to cut back the stock. Next, make a 15-20 cm long cut on the bark from the point where the stock was cut back. The cut is similar to the cut used for T-budding; however, only cut the bark for slipping bark grafting. The scion preparation is easy. First, make a sloping cut on the stick. A slight twist with the grafting knife may open the two flaps of bark. After that, the scion should be inserted under the two flaps of bark by pushing it downward. Finally the incision should be closed with budding tape, which should be wrapped tightly around the stem.

Wedge Grafting

This method is one of the most difficult propagation techniques. To perform a good quality wedge grafting requires a high level of skill and a great degree of experience. This method is to be used for working on the tops of trees. For small trees, graft into the trunk; while for large trees, graft into the main branches. The stock may be much wider than the scion. The method may be used during dormant stage. The scion, like in other cases, should contain a minimum of 3 buds and its length should be approximately 20- 25 cm. Now, cut the base of the scion to a long wedge that is sloping both downward and inward. Use a thin-bladed saw to make a cut (or various cuts according to the size of the stock) to approximately the center of the stub. Wide the cuts with a round-bladed grafting knife to fit the cuts on the scion. Place the scion into the cut. If the scion matches the cut in the stub, they are held by being tapped in place. Be sure, that the cambium of both, the scion and stock, is in contact to each other. Finally, cover the graft union and the end of the scion with grafting wax.

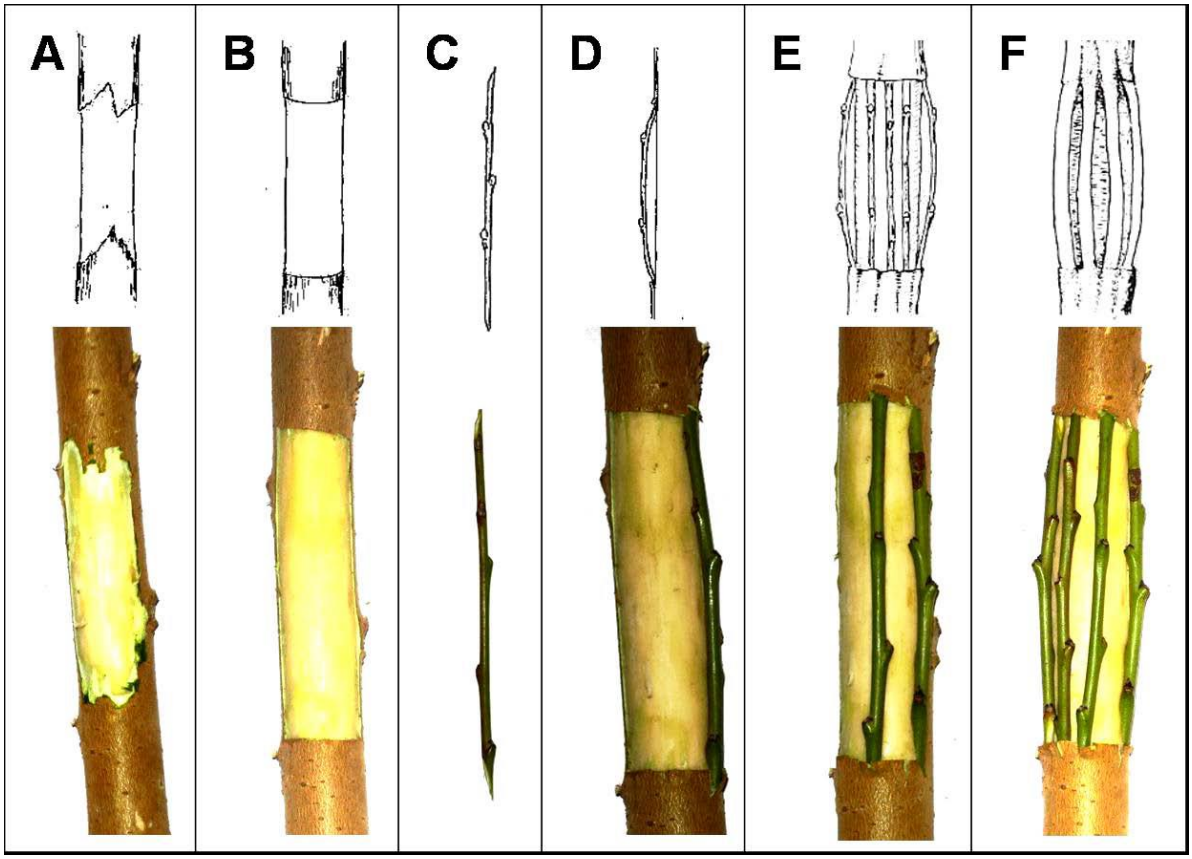
Wedge Grafting



Bridge Grafting

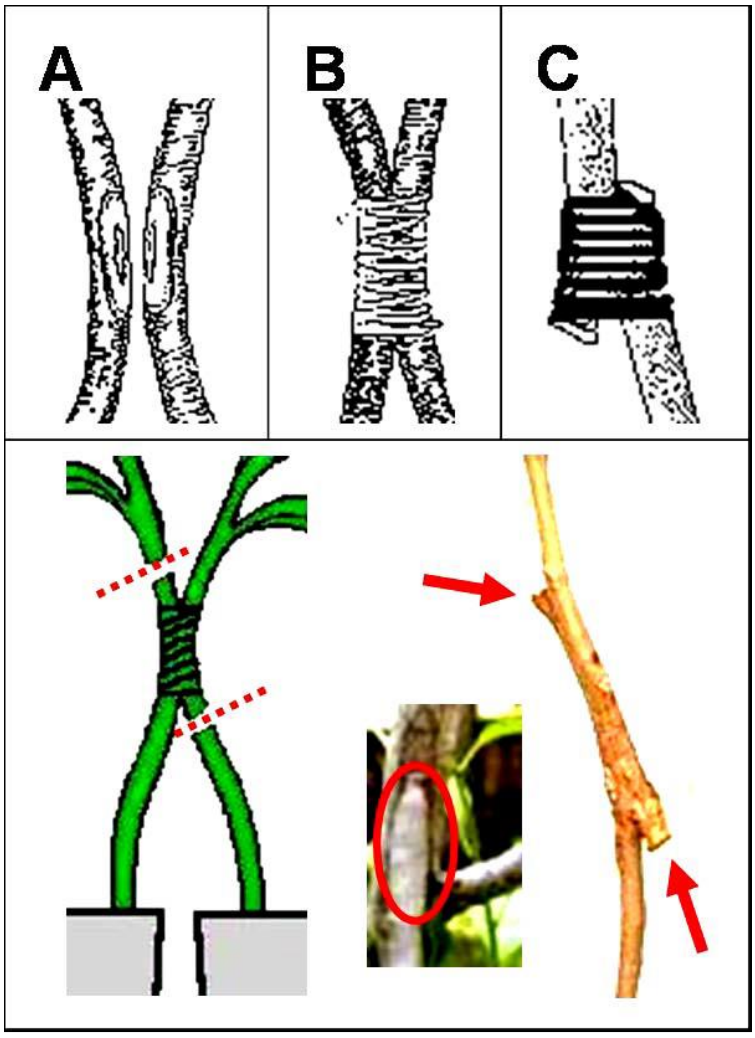
This technique is mainly used to repair damaged trees or branches and not for propagation. Like in the case of the slipping grafting, bridge grafting requires grafting under the bark. Where bark of the branch or trunk is damaged, first clean up the surface and cut a wedge in the bark horizontally. Next, prepare 3-6 scions according the size of the damaged area and graft both ends of each of the scions under the bark.

Bridge Grafting



Approach Grafting

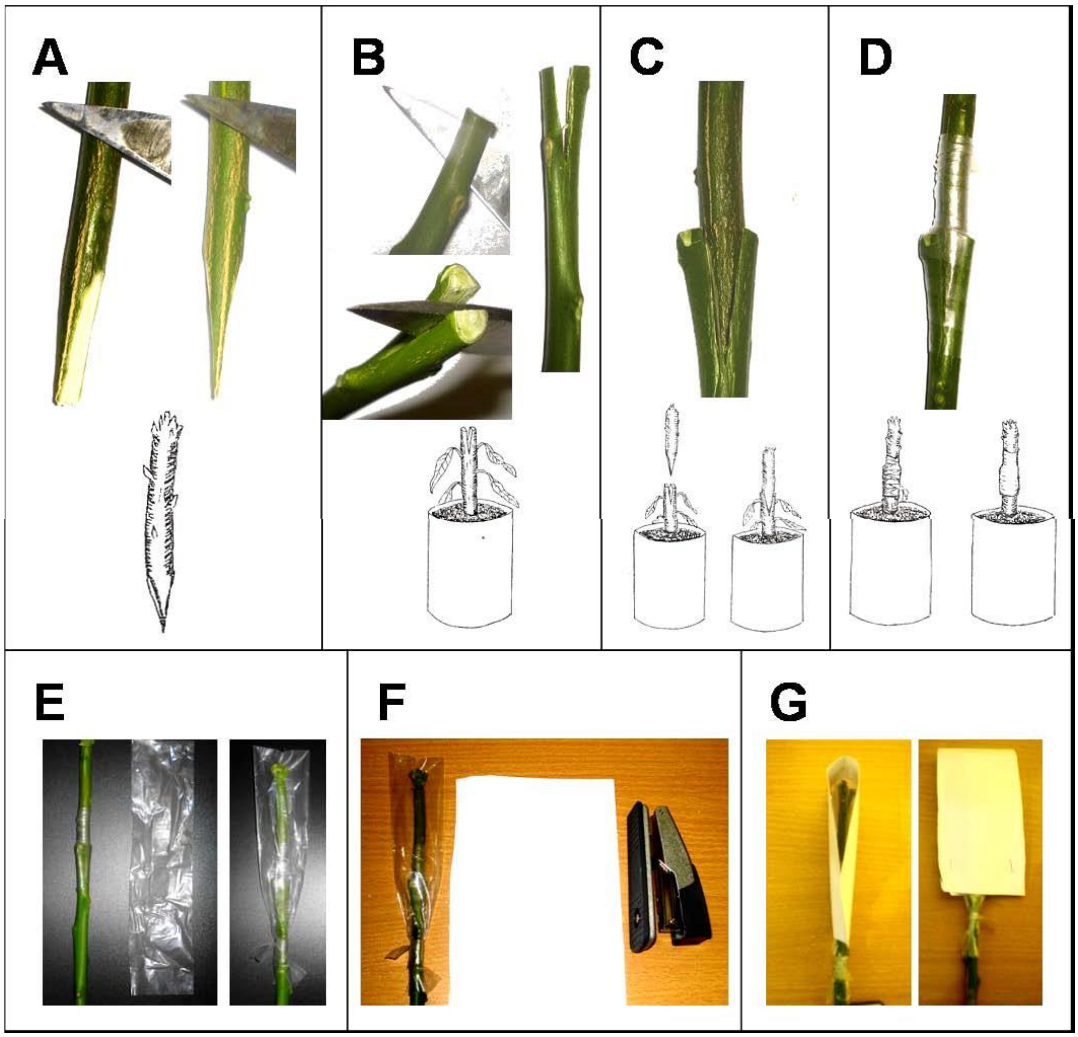
Some trees are very difficult to graft such as mango and macadamia. In these cases we can use the approach grafting method. The main difference between these techniques and other methods are that the scion is attached to its root system during the grafting process. Take two plants. One will be the rootstock and the other the scion. Make the same cut on both stems at the same height. Hold the two stems together and tie them with tape. When the grafts have joined, remove the top of the rootstock plant with a cut above the joining point and remove the bottom of the scion plant with a cut below the joining point.



Green Grafting

In some cases such as grafting gooseberry, it is very difficult to graft wooded plant parts. Therefore the two options to use are top grafting, when the stock was cut back or side grafting. Both cases use cleft or whip grafting methods. Top cleft grafting to produce a fruit tree requires a cut in the scion 20 cm long and also requires the removal of all leaves. Be sure that the top bud is well developed and healthy. Make two sloping cuts 2-5 cm long. Cut the top of the rootstock 20-40 cm above the surface. Make one straight cut across the center, the same length as the cut on the scion. Firmly join the two parts. Finally, tie and wax the graft.

Green Cleft Grafting



Taking

Care of Grafted and Budded Plants

1. During the grafting process, be sure to clean and sharpen budding and grafting knives that have been used. The ready graft should be fixed with tape and the wounded surface should be covered with grafting wax in order to keep the scion or bud from losing water and drying up.
2. Five days after grafting we need to check the graft and re-wax it if the wax has cracked.
3. Any shoots which grow below the graft on the rootstock should be removed, because they compete with the shoots of the scion.
4. Recently grafted trees need a lot of water distributed on a regular basis.
5. In the first year after grafting, avoid the application of any fertilizer, manure or compost, because the tree will begin to grow fast prematurely, which will not allow the graft to heal properly.
6. Budded plants should be kept under cool conditions until the graft has joined in order to keep the bud from growing prematurely.
7. As soon as the growing season starts, cut off the rootstock above the plant with a sloping cut. This will help the bud to begin growing.
8. Rub off the buds on the rootstock below the grafted bud as these other buds will only provide unnecessary competition with the grafted bud. This activity should be done on a regular basis until the rootstock buds stop appearing.

Vegetative Propagation by Budding or Bud Grafting

The method of budding is the most common technique for plant propagation in commercial nurseries. First, one must graft a single bud attached to the stem of the rootstock. The stem or branch may not be thicker than 2 cm diameter. Therefore, this method is only applicable for young rootstock plants or smaller branches of large plants.

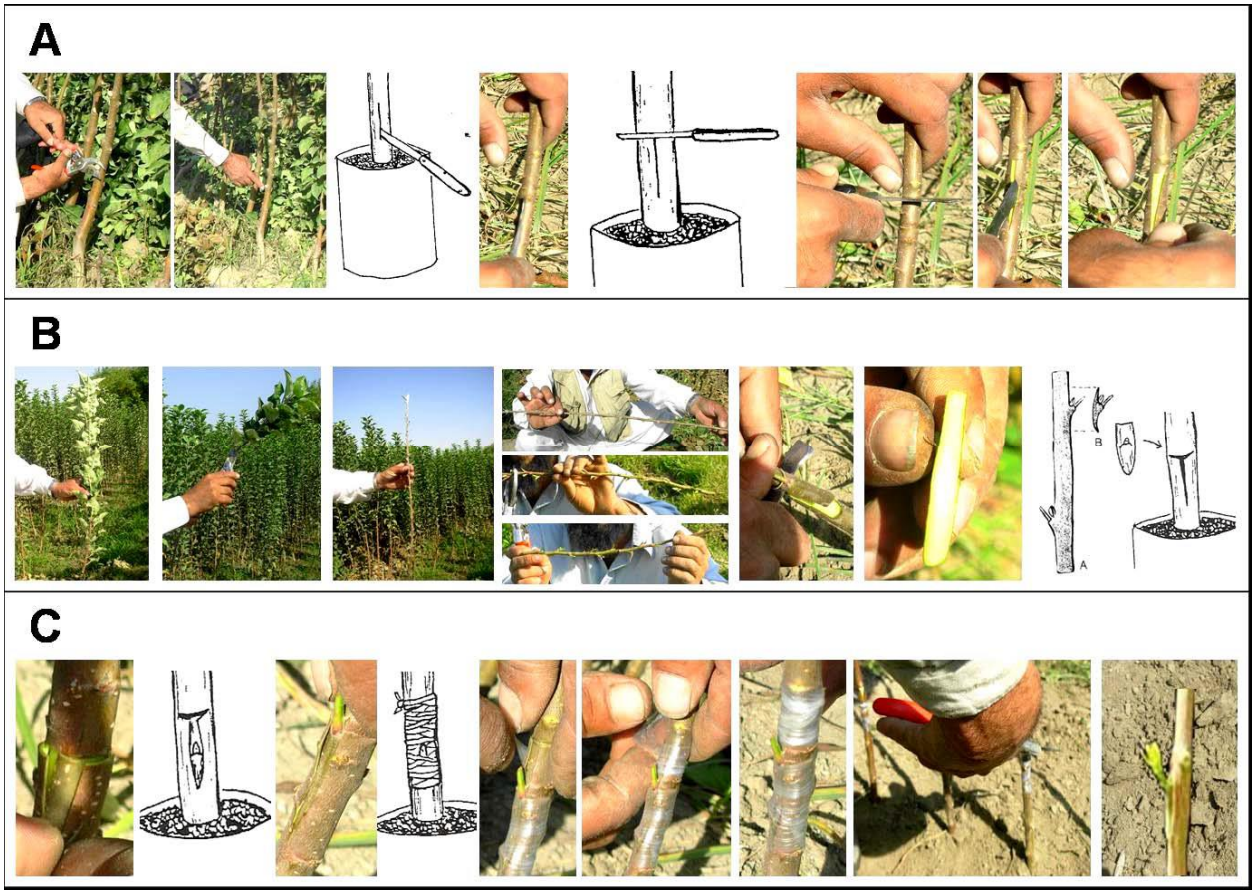
For best results, use bud wood or bud sticks which are of a vigorous current season growth. Remove the top and bottom part of the branch, because the tip buds are too immature and the bottom buds may be a cluster of buds or they are too weak to use for budding. The length of the stick is approximately 30 cm. Remove the leaves leaving a 1- 1.5 cm long of leaf petiole on the stem.

The time for budding comes when the bark peels easily on the stock. Irrigation a few days before budding helps to slip the bark. One should bud graft into the root neck, or into a higher part of the plant. Normally, budding should be done about 15-20 cm above the root neck avoiding the possibility that the scion will root into the soil.

There are two periods of time to use the method of bud grafting. One option is to implement it in the beginning of the growing season. Bud grafting during this time comes with the risk that the new shoot will not be sufficiently strong and matured to survive freezing conditions during the

winter. The other and most common option is budding in the beginning of the dormant stage. This means that the bud will remain dormant until the following spring. Just as growth begins, all top growth is cut off with a sloping cut 5-7 mm above the bud. All growth except the inserted bud must be removed on a weekly basis.

The bud preparation starts about 1 cm below the bud with a slicing cut under and about 2 cm beyond the bud (5-7 mm above the bud). The dept of the cut is such that only a very small amount of wood appears directly under the bud. This wood underneath the bud need not be removed.



T-budding

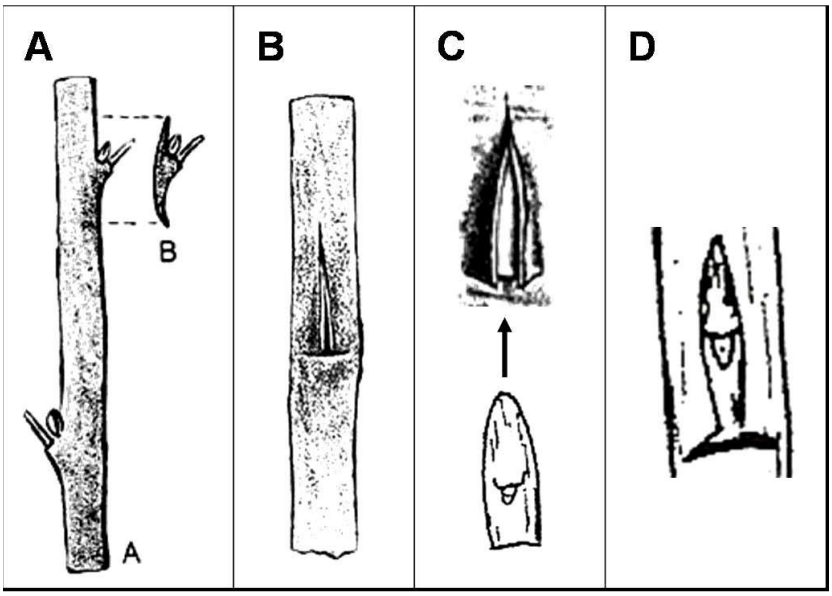
T-budding

The “T” cut on the stock is done about 20-25 cm above the surface with a 2 cm long vertical cut and a 7-8 mm long horizontal cut on the stock. A slight twist with the budding knife may open the two flaps of bark. After that, the bud should be inserted under the two flaps of bark by pushing downward. If part of the bud remains above the horizontal cut, it must be cut off. This will allow the flaps to be closed tightly. Finally, the incision should be closed with budding tape, which should be wrapped tightly around the stem. Tying must start at the bottom or the top end of the incision. After 3-4 weeks, the tape should be removed (if it did not already fall off). At this time, the shield of the bud and the petiole may indicate the condition of the bud. If the shield is shriveled and the petiole does not fall off at the touch, the bud is possibly dead and the budding process should be repeated.

The inverted T-budding technique is exactly same as the normal T-budding method with the exception that the horizontal cut is made on the bottom end of the incision. In this case, the bud is cut from the bud stick by starting above the bud and exiting below it.

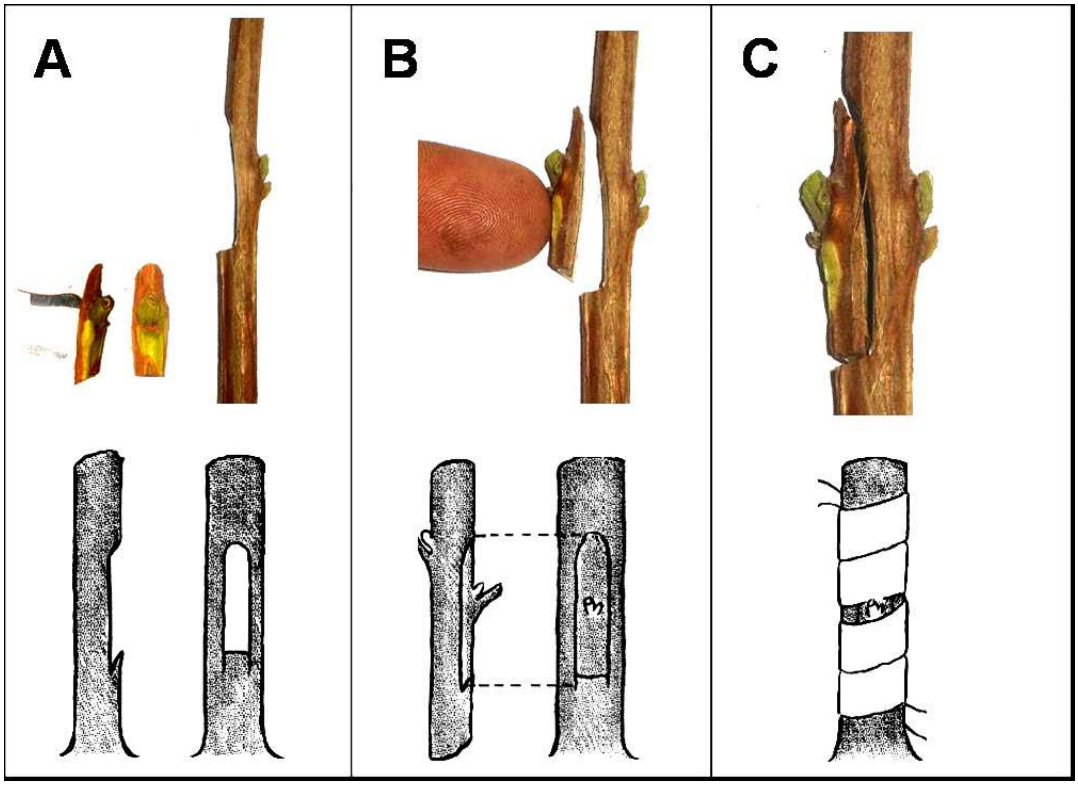
Currently most fruit trees are propagated with the T-budding method. However, the use of inverted T-budding technique, it is much more effective due to the downward flow of hormones that are intercepted below the bud. Therefore, the union will be stronger and the healing process will be faster (as opposed to the normal T-budding method).

Inverted T-budding



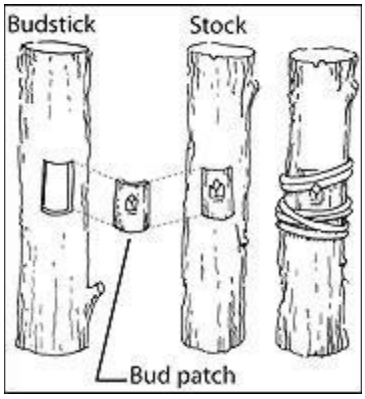
Chip-budding

Chip-budding does not use the protective bark flaps as T-budding does, but it also does not use slipping bark. The first step is to make a cut about 2-2.5 cm long with a depth of ¼ to 1/5 the diameter of the stock. With a horizontal cut made on the bottom, the cutting can be removed. The bud can also be cut off if necessary. The bud stick and stock must be the same diameter. The stock and scion must be placed together in such a way that allows the cambia of the bud and stock to match together as much as possible. Desiccation is a high risk when we use this method, therefore, the wound should be wrapped tightly with grafting tape.



Patch Budding: In this method a regular patch of bark is completely removed from the stock plant and is replaced with a patch of bark of the same size containing a bud from the desired mother plant. For this method to be successful the bark of the stock and bud stick should be easily slipping .The diameter of the stock and bud stick should be preferably by about the same (1.5 to 2.75cm) E.g., Ber, Citrus, Cocoa and rubber.

Procedure: On the selected stock plant at the desired place (10-15cm above the ground level) give two transverse parallel cuts through the bark and about 1-1.5 cm long or 1/3rd the distance around the stock. The distance between the cuts may be 2-3 64
cm.Join the two transverse cuts at their ends by two vertical cuts. Remove the patch of bark and keep it in place again until the bark patch with the bud from the selected mother plant is ready. On the bud stick give two transverse cuts-one above and one below the bud-and two vertical cuts on each side of the bud. The dimensions of the transverse and vertical should correspond to those given on the stock. Remove the bark patch with bud by sliding side ways. Cuts with bud by sliding side ways. Insert the bud patch immediately on the stock in such a way that the horizontal cuts of the bark patch and those on the stock plant match together perfectly. Wrap the inserted bud patch with polythene strip covering all the cut surfaces but exposing the bud properly.



Ring budding: The bud is prepared by taking a ring of a bark, 3cm long with the bud in the centre. In the root stock, two transverse cut 1.5cm apart are made and these are connected with a vertical cut and a ring of bark is removed. The prepared scion bud with the ring of bark is fitted in the exposed portion of the rootstock and tied.

UNIT- IV

Biotechnology: Definitions, Concepts and Importance

Introduction

The term Biotec hnology was coined by karl Ereky a Hungarian engineer in 1919. This term is derived from a fusion of Biology and Technology Biotechnology is not a pure science but an integrated affect of these two areas, the root of which lies in biological sciences It is truly multidisciplinary in nature and it encompasses several disciplines of basic sciences and engineering

The science disciplines from which biotechnology draws heavily are Microbiology, Biochemistry, Chemistry, Genetics, Molecular biology, Immunology and Physiology On engineering side it leans heavily on processes chemical and biochemical engineering since large multiplication of microorganisms and cells their down stream processing etc. are based on them. It is a fast growing science and it has been defined in different ways by different group of workers.

Definition

Biotechnology is the application of scientific and engineering principles to the processing of materials by biological agents to provide goods and services. This was given by OECD – the organization for economic cooperation and development in 1981.

Although the term was recent origin the discipline itself is very old. Man began employing microorganisms as early as in 5000 B.C for making wine vinegar curd etc.

All these processes which are based on the natural capabilities of microorganisms are commonly considered as old biotechnology. The development of recombinant technology allowed to modify microorganisms and other organisms to create in them highly valuable, novel and naturally non-existing capabilities. Eg:- The human gene producing Insulin has been transferred and expressed in bacterium like *E.coli* and it is being used in management of Diabetes. Crop varieties and animal breeds with entirely new and highly useful traits are being created with the help of recombinant DNA technology.

These and many other similar examples constitutes modern the new Biotechnology in India.

In 1982 Government of India set up an official agency National Biotechnology Board (NBTB) which started functioning under the Department of Science and Technology (DST).
In 1986 NBTB was replaced by a full fieldged department, the Department of Biotechnology (DBT) in the ministry of sciences. Technology for planning, promotion and coordination of various biotechnological programmes.

More over on the proposal of United Nations Organization (UNO) the International Center of Genetic Engineering and Biotechnology (ICGEB) was established to help the developing countries. ICGEB has its two centers one in New Delhi and the other in Trieste (Italy).

The New Delhi center of ICGEB is functioning proper way since in 1988.
The other central organizations for Biotechnology research in India are

IARI : Indian Agricultural Research Institute, New Delhi
JNU : Jawaharlal Nehru University, New Delhi
IVRI : Indian Veterinary Research Institute, Izatnagar
CFTRI : Central Food Technology Research Institute, Mysore
NDRI : National Dairy Research Institute - Karnal - Haryana
MRC : Malaria Research Center – New Delhi
RRL : Regional Research Laboratory – Jammu
CDRI : Central Drug Research Institute – Lucknow
CIMAP : Central Institute of Medicine and Aromatic plants - Lucknow and Hyderabad
IIT : Indian Institute of Technology – Kanpur, New Delhi
IISC : Indian Institute of sciences – Bangalore
IMTECH : Institute of Microbial Technology – Chendiger
NIM/NII : National Institute of Immunology – New Delhi
NCL : National Chemical Laboratory – Pune
CCMB : Center for Cellular and Molecular Biology – Hyderabad
CDFD : Center for DNA Finger Printing and Diagnostics – Hyderabad
CPMB : Center for Plant Molecular Biology – 7' centers
BARC : Baba Atomic Research Center – Mumbai

History of plant tissue culture

The term 'plant tissue culture culture' broadly refers to the *in vitro* cultivation of plants, seeds, plant parts on nutrient media under aseptic conditions.

During the 1800s, the cell theory (Schleiden and Schwann) which states that the cell is the basic structural unit of all living organisms, was very quick to gain acceptance. However, the second portion of the cell theory states that these structural units are distinct and potentially totipotent physiological and developmental units, failed to gain universal acceptance.

In 1902, Gottlieb Haberlandt, a German plant physiologist, attempted to cultivate plant tissue culture cell *in vitro.* He is regarded as the father of plant tissue culture. Totipotency is the ability of plant cell to perform all functions of development, which are characterstic of zygote i.e its ability to develop into a complete plant. In 1902, Haberlandt attempted culture of isolated single

palisade cells from leaves in knop's salt solution enriched with sucrose. The cells remaine d alive for up to one month, increased in size, accumulated starch but failed to devide. Demonstration of totipotency led to the development of techniques for cultivation of plant cells under defined conditions. The first embryo culture, although crude, was done by Hanning in 1904.

In 1925 Laibach recovered hybrid progeny from an inter specific cross in Linum.

In 1964 Maheshwari and Guha were first produced haploid plants from pollen grains, by culturing anthers of Datura.

In 1960, cocking isolated protoplast for culturing.

In 1972 Carlson et al produced first somatic hybrid plants by fusing the protoplasts of *N. glauca x N. langsdorfli*

Plant cell and tissue culture
Steps in general tissue culture techniques – merits and limitations –

Plant tissue culture is the aseptic method of growing cells and organs such as meristems, leaves, roots etc either in solid or liquid medium under controlled condition. In this technique small pieces of viable tissues called ex-plant are isolated from parent plants and grown in a defined nutritional medium and maintained in controlled environment for prolonged period under aseptic condition.

The general technique of plant tissue culture involves four main stages. They are Initiation of culture Multiplication (or) sub culture Development and differentiation Hardening

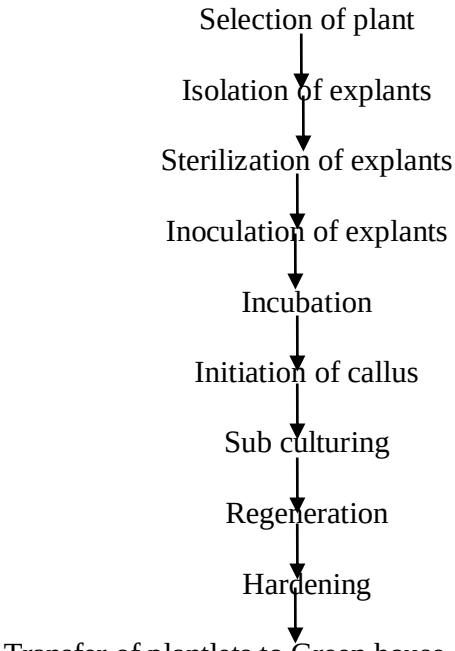
1. **Intiation of culture:** The most important factor in tissue culture technique is the maintenance of aseptic condition. For this purpose the culture medium generally, a GR-free medium is used Immediately after preparation the culture vessel has to be plugged and autoclaved at 121oC 15 psi (pounds per sq. inch) for an about 15-20min. The plant material has to be surface sterilized with a suitable sterilent. The transfer area should also maintain free of micro organisms. Strict precautions are to be taken to prevent the entry of micro organisms.

   The plug of a culture vessel is removed carefully to transfer plant material to the nutrient medium during sub culturing. After inoculation the cultures are incubated in culture room under controlled condition at 25 + 12oC temperature and 1000 lux light intensity generated by florescent tube and at a constant photoperiod regulated by automatic timers.

2. **Multiplication / Subculture:** After 2-3 weeks the explants show visible growth by forming either callus (or) differentiated organs like shoots, roots (or) complete plantlets, depending upon the composition of the medium. Periodically sub-culturing of callus (or) organs (or) plantlets to the fresh medium is done to multiply the callus (or) organs (or) to obtain large number of plantlets from the callus.

3. **Development and Differentiation / organogenesis:** The concentration of phytoharmones in the medium are altered to induce differentiation in callus. A high cytokinins to auxin ratio induces shoot formation (caulogenesis) (basal medium + low cytokinins / GA_3 medium is used before they can be rooted. Higher concentration (>2 mg/l BAP) of cytokinins induce adventitious shoot buds and retard shoot growth. Very high auxins to cytokinin ratio induces root formation (Rhizogenesis). The development of organ structures like shoot, roots etc. from the cultured cells (or) tissues is known as organogenesis. Alternatively media composition can also be altered to induce the development of somatic embryos and the process is known as somatic embryogenesis. Further, an entire plantlet can be induced to grow on culture media by manipulating the phytoharmone balance correctly and the process is called Regeneration. The regeneration may be either direct or callus mediated. The *in vitro* induced shoots must be transferred to the culture media that supports root induction.

Steps in plant tissue culture technique



4. **Hardening:** The *in vitro* cultured rooted pla nts are first subjected to acclimatization before transferring to the field. The gradual acclimatization of *in vitro* grown plant to *in vivo* conditions is called hardening. The plantlet is taken out from the rooting medium and is washed thoroughly to remove entire agar from the surface of plantlet as agar may attract

microbes to grow and destroy the plantlets. The plantlet is now kept in a low minimal salt medium for 24-48hrs and transferred to a pot that contains autoclaved sterilized mixture of clay soil, coarse sand and leaf moulds in 1 : 1 : 1 ratio proportion. The pot containing plantlet is covered generally with the transparent polythene cover having holes for aeration to maintain the humidity. The plantlets are maintained for about 15-30 days in this condition. The plantlets are then transferred to the soil and are ready for transfer either to the green house or main field.

Applications of plant tissue culture in crop improvement

1. Micro propagation helps in mass multiplication of plants which are difficult to propagate through conventional methods.
2. Some perennial crop plants like ornamental and fruit crops can not be propagated through seeds. The vegetative propagation like grafting, budding are tedious and time consuming. In such crops micro propagation helps in rapid multiplication.
3. Rapid multiplication of rare and elite genotypes such as Aromatic and Medicinal plants. Isolation of *in vitro* mutants for a large number of desirable character Eg:- Isolation of biochemical mutants and mutants resistant to biotic (pest and disease) abiotic (salt and drought, cold, herbicide etc) stresses through the use of somaclonal variation.
4. Screening of large number of cells in small space.
5. Cross pollinated crops like cordamum, Eucalyptus, coconut, oil palm do not give true to type plants, when multiplied through seed. Development of genetically uniform plants in cross pollinated crops is possible through tissue culture.
6. In case of certain horticultural crops orchids etc seed will not germinate under natural conditions, such seed can be made to germinate *in vitro* by providing suitable environment.
7. Induction of flowering in some trees that do not flower or delay in flowering. Eg:- Bamboo flowers only once in its life time of 50 years.
8. Virus free plants can be produced through meristem culture.
9. Large amount of germplasm can be stored within a small space and lesser cost for prolonged periods under *in vitro* condition at low temperature. The preservation of cells tissues, organs in liquid Nitrogen at – 196^{o}C is called cryopreservation.
10. Production of secondary metabolites. Eg:- Caffine from *coffea arabica*, Nicotine from *Nicotiana rustica.*
11. Plant tissue culture can also be used for studying the biochemical pathways and gene regulation.
12. Anther and pollen culture can be used for production of halploids and by doubling the chromosome number of haploids using cholchicine homogygous diploids can be produced. They are called dihaploids.
13. In case of certain fruit crops and vegetative propagated plants where seed is not of much economic impor tant, triploids can be produced through endosperm culture.
14. Inter specific and inter generic hybrids can be produced through embryo rescue technique which is not possible through conventional method. In such crosses *in vitro* fertilization

helps to overcome pre-fertilization barrier while the embryo rescue technique helps to over come post fertilization barrier.

15. Somatic hybrids and cybrids can be produced through protoplast fusion (or) somatic hybridization.
16. Ovary culture is helpful to know the physiology of fruit development.
17. Development of transgenic plants.

Advantages of tissue culture

- Rapid multiplication within a limited space
- It is not time bound and not season bound
- Free from pests and diseases

Limitations (or) Disadvantages

Laborious, costly, special risk is required.

Nutritional requirements of Tissue culture

The isolated plant tissues are grown on a suitable artificially prepared nutrient medium called culture medium. The medium is substrate for plant growth and it refers to the mixture of certain chemical compounds of form a nutrient rich gel (or) liquid for growing cultures, whether cells, organs (or) plantlets. The culture media has to supply all the essential mineral ions required for *in vitro* growth and morphogenesis of plant tissue.The major constituents of most plant tissue culture methods are :

1. Inorganic nutrients : micro and macro
2. Carbon source
3. Organic supplements
4. Growth regulators
5. Solidifying agents

1. **Inorganic Nutrients:** A variety of mineral elements (salts) supply the macro & micro nutrients required in the life of plant. Elements required in concentration 0.5 ml / lit. are referred to as macro nutrients and those required in less than 0.5 ml / lit. concentration are considered as micro nutrients

   A. Macronutrients: They include six major elements N.P.K.Ca Mg & S present as salts in the media which are essential for plant cell and tissue growth Nitrogen is the element which is required in greatest amount. It is most commonly supplied as a mixture of Nitrate lons (KNO_3) Ammonium ions (NH_4NO_3) Phosphorous is usually supplied as phosphate ion of Ammonium, sodium and Potassium salts. Other major elements Ca.Mg,S, are also required to be incorporated in the medium.

   B. Micro nutrients: These are Mn.Zn. B, Cu. Mo. Fe. Co. I.

Iron is generally added as a chela te with with EDTA. (Ethylene Diamino Tetra Acetic acid) In this form iron is gradually released and utilized by living cells and remains available up to a PH of 8.

Some of the elements are important for plant nutrition and their physiological function.

Element Function

Nitrogen (N2): Component of proteins nucleic acids some co-enzymes
Phosphrous (P): Component of nucleic acids energy transfer component of intermediate in respiration and photosynthesis
Potassium (K): Regulates osmatic potential principal in organic cation
Calcium (Ca): Cell wall synthesis. Membrane function cell signally
Magnesium (Mg): Enzyme co-factor component of chlorophyl
Sulphur (S): Component of some amino acids (Methionine cysteine) some co-factors
Chlorin (Cl): Required for photosynthesis
Iron (Fe): Electron transfer as a component of cytochromes
Managanese (Mn): Enzyme co-factor
Cobalt (Co): Component of some vitamins
Copper (Cu): Enzyme co-factor electron transfer reaction
Zinc (Zn): Enzyme co-factor chlorophyll biosynthesis
Molybdenum (Mo): Enzyme co-factor component of nitrate reductase.

Preparation of Nutrient Medium

(Preparation of composition of Murashige and Skoog medium)

The nutrients required for optimal growth of plant organ tissue and protoplast *in vitro* generally vary from species to species eve n tissues from different parts of a plant may have different requirements for satisfactory growth

Carbon source: Plants cells and tissues in culture medium lack autotrophic ability and therefore need external carbon for energy. The most preferred carbon energy source in plant tissue culture is sucrose. It is generally used at a conc of 2-5% while autoclaving the medium sucrose is converted to Glucose and Fructose. In the process first Glucose is used and then Fructose, Glucose supports good growth while fructose less efficient. Maltose Galactose then lactose are mannose and the other sources of carbon. Most media contain myoenosital at a concentration of approximately Ca 100 mg l-1 which improves cell growth.

Organic supplements

Vitamins: Plants synthesize vitamins endogenously and these are used as catalysts in various metabolic processes. When plant cells and tissues are grown in *in vitro* some essential vitamins are synthesized but only in suboptimal quantities. Hence it is necessary to supplement the medium with required vitamins and amino acids to get best growth of tissue. The most commonly used vitamins is thiamine (vitamin B) the other vitamin which improve growth of

cultured plants are Nicotinic acid, Panthothenic acid Pyridoxin (B6) Folic acid A mynobenzoic acid. (ABA)

Amino acids: Cultured tissues are normally capable of synthesing Amino acids necessary for various metabolic processes. In spite of this the addition of Amino acid to the media is important for stimulating cell growth in protoplast cultures and for establishing cell culture. Among the amino acids glycine is most commonly used Amino acids Glutamine Aspargine, Arginine Cystine are the other common sources of organic Nitrogen used in culture media.

Other organic supplements: These include organic extracts Eg:- Protein (casein) hydrolysate, coconut milk, yeast & malt extract, ground banana, orange juice, Tomato juice, Activated charcoal. The addition of activated charcoal to culture media stimulates growth and differentiation in orchids, Carrot and Tomato Activated charcoal adsorbs inhibitory compounds & darkening of medium occurs. It also helps in to reduce toxicity by removing toxic compounds Eg:- Phenols produced during the culture permits un hindered cell growth

Antibiotics: Some pla nt cells have systematic infection of micro organisms. To prevent the growth of these microbes it is essential to enrich the media with antibiotics Eg:- Streptomycin or Kanamycin at low concentration effectively controls systemic infection and do inhibit the growth of cell cultures

Growth regulators: These include auxins, cytokinins, gibberillins, ABA. The growth differentiation and organogenesis of tissue occurs only on the addition of one (or) more of these hormones to the medium.

Auxins: Auxins have the property of cell division, cell elongation, elongation of stem, internodes, tropism, Apical dominance abscission and rooting commonly used auxins are

IAA (Indole 3-Acetic Acid)
IBA (Indole 3-Butyric Acid)
2,4-D (Dichloro Phenoxy Acetic Acid)
NAA (Naphthylene Acitic Acid)
NOA (Naphthoxy Acitic Acid)
The 2,4-D is used for callus induction where as the other auxins are used for root induction.

Cytokinins: Cytokinins are adenine derivaties which are mainly concerned with cell division modification of apical dominance, and shoot differentiation in tissue culture. Cytokinins have been shown to activate RNA synthesis and to stimulate protein and the enzymatic activity in certain tissues commonly used Cytokinins are

BAP (6-Benzylamino purine)
BA (Benzy adenine)
2ip (Isopentyl adenine)
Kinetine (6 – furfur aminopurine)
Zeatin (4 – hydroxy 3 methyl trans 2 butinyl aminopurine)

Gibberillins and Abscisic acid: GA3 is most common gibberillin used in tissue culture. It promotes the growth of the cell culture at low density. Enhances callus growth and simulates the elongation of dwarf or stunted plantlets formation from adventive embryos formed in culture. ABA in culture medium either stimulates or inhibits culture growth depending on specie s. It is most commonly used in plant tissue culture to promote distinct developmental pathways such as somatic embryogenesis.

Solidfying agent: Gelling and solidifying agents are commonly used for preparing semisolid or solid tissue culture media. Agar (polysaccharide obtained from marine sea weeds) is used to solidify the medium. Normally 0.5-1% Agar is used in the medium to form a firm gel at the pH typical of plant tissue culture media. Use of high concentration of agar makes the medium hard and prevents the diffusion of nutrients into tissues.

pH: Plant cells and tissues require optimum pH for growth and development in cultures. The pH effects the uptake of ions, hence it must be adjusted below 5-6.0 by adding 0.1N NaOH (or) HCLusually the pH higher than six results in a fairly hard medium where as pH below five does not allow satisfactory solidification of medium.

Preparation of Nutrient Media

The nutritional requirement for optimum growth of plant organ, tissue and protoplast *in vitro* generally vary from species to species even tissues from different parts of plant may have different requirement for satisfactory growth. Therefore no single media as such can be suggested as being entirely satisfactory for all types of *in vitro* culture. In order to formulate a suitable medium for a new system a well known basal medium such as Ms (Murashigel and skoog) B5 (gamborg et al) etc.

The composition of MS media is given below

Macro salts Concentration

NA_4NO_3 ……. 1.65 g
KNO_3 ……. 1.90 g
$CaCl_2$ 2 H_2O ……. 0.44 g
$MgSO_4$ 7H_2O ……. 0.37 g
KH_2PO_4 ……. 0.17 g

Micro salts

$FeSO_4$ 7H_2O ……. 27.80 mg
Na_2EDTA 2H $_2O$ ……. 33.60 mg
Kl ……. 0.83 mg
K_3BO_4 ……. 6.20 mg
$MnSO_4$ 4H_2O ……. 22.30 mg
$ZnSO_4$ 7H_2O ……. 8.60 mg
Na_2MoO_4 2H_2O ……. 0.25 mg
$CuSO_4$ 5 H_2O ……. 0.025 mg
$CoCl_2$ 6H_2O ……. 0.025 mg

Organic supplements

Myoinositol …… 100.00 mg
Nicotinic acid …… 0.05 mg
Pyridoxine HCl …… 0.05 mg
Thiamine HCl …… 0.05 mg
Glycine …… 0.20 mg
Sucrose …… 20.00 mg
Growth regulators As per need
Gelling agent (only for salid medium)
Agar …… (0.5-1%) 6-8 g/lig.
pH …… 5.8

By making minor quantitative and qualitative changes a new media can develop to accommodate the specific requirements of the desired plant material

Preparation of the medium

The most suitable method for preparing media now -a-days is to use commercially available dry powdered media. These media contains all the required nutrients. The powder is dissolved in distilled water generally 10% less than final volume of medium and after adding sugar, agar and other desired supplements. The final volume is made up with distilled H2o. The pH is adjusted and media is autoclaved, Another method of preparing media is to prepare concentrated stock solutions by dissolving required quantities of chemicals of high purity in distilled water. Separate stock solution are prepared for different media components

1. Major salts
2. Minor salts
3. Iron
4. Organic nutrients except sucrose

For each growth regulator a separate stock solutions is prepared. All the stock solutions are stored in proper glass or plastic conta iners at low temperature in refrigerators. Stock solution of Iron is stored in amber coloured bottles. Substances which are unstable in frozen state must be freshly added to the final mixture of stock solution at the time of medium preparation, Contaminated (or) precipitated stock solution should not be used.

The following sequential steps are followed for preparation of media

1. Appropriate quantity of Agar and sucrose is dissolved in distilled water.
2. Required quantities of stock solution, heat stable growth harmones (or) other substances are added by continuous stirring.
3. Additional quantity of distilled water is added to make final volume of the medium.
4. While stirring the pH of the medium is adjusted by using 0.1 NaoH (or) HCL.
5. If a gelling agent is used heat t he solution until it is clear.
6. Medium is dispensed into the culture tubes, flasks, (or) any other containers.

7. The culture vessels are either plugged with non-absorbant cotton wool rapped in cheese cloth or closed with plastic caps.
8. Culture vessels are sterilized in autoclave at 121oC 15Psi (1.06kg / cm2)for about 15-20 min.
9. Heat labile constituents are added to the autoclaved medium after cooling to 30-40oC under a Laminar airflow cabinet.
10. Culture medium is allowed to cool at room temperature and used or stored at 4oC (1or2 days)

Sterilization in plant tissue culture, growth room chambers and instruments

The media used for plant tissue culture contain sugar as a carbon source there by attracting a variety of micro organisms including bacteria and fungi. These organisms grow much faster than the cultured tissues and produce metabolic substances which are toxic to plant tissues. There are a number of sources through which the media may get contaminated which include culture vessels, instruments, media, explants, transfer area and culture room. Therefore sterilization is absolutely essential to provide and maintain a completely aseptic environment during *in vitro* cultivation of plant cells (or) organs. Sterilization is a procedure used for elimination of micro organisms and maintaining aseptic (or) sterile conditions for successful culture of plant tissues (or) organs.

The different techniques used for sterilization of plant tissue culture growth room chambers and instruments are

1. Dry sterilization
2. Wet heat / autoclaving / steam sterilization
3. Ultra filtration (or) Filter sterilization
4. Ultra violet sterilization
5. Flame sterilization
6. Surface sterilization (or) chemical sterilization
7. Wiping with 70% alcohol

Dry heat sterilization : Empty glass ware (culture vessels, petriplates etc) certain plastic ware (Teflon, FFp), Metallic instruments (scalpels, foreceps, needles etc) aluminium foils, paper products can be sterilized by exposure to hot dry air at 160o- 180oC for 2-4hr in hot air oven. All items should be properly sealed before sterilization.

Wet heat sterilization (or) autoclaving steam sterilization: It is a method of sterilization with water vapour under high pressure to kill all microbes by exposing to the super heated steam of an autoclave. Normally the tissue culture media in glass containers sealed with cotton plugs (or) Aluminium, foils, plastic caps are autoclaved with a pressure of 15psi at 121oC for 15-20 minutes. From the time the medium reaches the required temperature some types of plastic glassware can also be repeatedly sterilized by autoclaving (Good sterilization relies on time, pressure, temperature and volume of the object to be sterilized). The advantages of an autoclave are speed, simplicit y and distruction of viruses, while disadvantages are change in pH by 0.3 – 0.5 units.

Ultra filteration / Filter sterilization : Vitamins, amino acids, plant extracts, harmones, growth Regulators are thermolabile and get destroyed during autoclaving. Such chemicals are filter sterilised by passing through a bacterial proof membrane filter under positive pressure. A millipore (or) seitiz filter with a pore size of not more than O,2µ is generally used in filter sterilization. This procedure has to be carried out only in aseptic working space created by laminar air flow cabinet. Filter sterilised thermolabile compounds are added to an autoclaved media after cooling at about 40^{o}C temperature.

Laminar air flow cabinets are used to create an aseptic working space blowing filter sterilized air through an enclosed space. The air is first filtered through a coarse free filter to remove large particles. It is then passed through HEPA filters which filters out all particles larger than 0.3 lm. This sterilized air blows through the working area in a cabinet at a constant speed of 1.8km/hr^{-1}. These filters not only eliminate dust and other particles but also fungal and bacterial spores. Thus an aseptic environment is maintained at the time of tissue inoculation.

Ultra violet sterilization: UV light sterilizes the interior portion of the inoculation chamber and eliminates atmospheric contamination. Materials like nutrient media, disposable plastic ware used for tissue culture and other similar materials are sterilized using UV rays to remove the contaminates.

Flame sterilization: Metalic instruments like foreceps, scalpels, needle, spatula are sterilised by dipping in 95%. ethanal followed by flaming and cooling. This technique is called flame sterilization. Autoclaving of metalic instrument is generally avoided as they rust and become blunt. These instruments are repeatedly sterilized during their use and time of inoculation to avoid contamination. The mouths of culture vessels are need to be expose to flame prior to inoculation (or) sub culture

Chemical sterilization / Surface: The explant before its transfer to the nutrient medium contain in the culture vessels is treated with an appropriate sterilizing agent to inactivate the microbes present on their surfaces. This is known as surface sterilization. The most commonly used sterilization for surface disinfection are Mercuric chloride 0.1% for 3-10min, Calcium hypochlorite 5% for 20 min, Sodium hypochlorite 0.5-1% for 15 min, Bromine water 1% for 2-10min, Chloramines 10-20% for 20-30min, and Other H_2O_2 $AgNo_3$ Antibiotic etc. are also used. The plant material to be sterilized is dipped in sterilant solution for prescribed period and then the explants is taken out and washed with sterile distilled water for 2-3 times thoroughly so as to remove all the traces of sterilant adhere to the plant material before its transfer to nutrient media.

Wiping with 70% ethanol : The surfaces that cannot be sterilized by other techniques example plot form of laminar air flow cabinet, hands of operator etc are sterilized by wiping them thoroughly with 70% alcohol and the alcohol is allowed to dry.

Totipotency, Growth and differentiation in cultures

Explants: A plant organ (or) an exised part used to initiate Tissue culture

Growth: An increase in size (vol/wt/length) due to cell division and subsequent enlargement

Differentiation: The development of cells / tissues with the specific function and / or the regeneration of organs / organ like structires / proembryos. The phenomenon of mature cells reverting to a meristematic state and forming undifferentiated callus tissue is termed as 'De differentiation'

Callus: The ability of component cells of the callus to differentiate into a whole plant or a plant organ is termed as Re-differentiation. Callus may be defined as an unorganized mass of loosely arranged parenchymatous tissue which develop from parent cells due to proliferation of cells

Cellular Totipotency: The capacity of a plant cell to give rise to a whole plant is known as cellular Totipotency. Generally a callus phase is involved before the cells can undergo redifferentiation leading to regeneration of a whole plant. The dedifferentiation cells can rarely give rise to whole plant directly without an intermediate callus phase (Direct regeneration) Growth and differentiation although proceed together they are independent

Differentiation may be categorized into 2 groups
A. Structural B. Physiological

Structural differentiation: It is further distinguished into external and Internal differentiation.

- **a) External:** Most common example is root and shoot differentiation another familiar example of is vegetative and reproductive phases of life cycle. Further differentiation in reproductive organs results in male and female organs
- **b) Internal:** This includes differentiation of various types of cells and tissues. Differentied cells mostly occur into groups forming different type of tissues Eg:- Vascular tissues
- **c) Physiological:** The variations in the structure between root and shoot are the expressions of fundamental physiological differences

Cyto differentiation: In both plants and animals specialized cells perform different functions. This specialization is known as cytodifferentiation.The cells in a callus are parenchymatous in nature. The differentiation of these cells into a variety of cells is required during re-differentiation of the cells into whole plants. This re-differentiation of cells is known as cyto-differentiation. Eg:- Vascular tissue differentiation (Xylem and phloem).

Organogenic differentiation: For the regeneration of whole plant from cell (or) callus tissue cyto differentiation is not enough and there should be differentiation leading to shoot bud and embryo formation. This may occur either through organogenesis (or) somatic embryogenesis. Organogenesis refers to the process by which the explants, tissues (or) cells can be induced to form root and the (or) shoot and even whole plants. In other words formation of organs is called organogenesis this may be

categarised into 2 groups. Rhizogenesis. The process of root formation Caulogenesis. The process of shoot initiation

Somatic embroyogenesis: Development of embryos from somatic cells in culture whose structure is similar to zygotic embryos found in seeds and with analogous embryonic organs such as cotyledons (or) cotyledony leaves.

Factors affecting cyto-differentiation

1) Phytoharmones:- Auxins at low concentration stimulates xylogenesis. Cytokinins and Gibberilins also stimulates tracheary element differentiation. When auxin and kinetin are used together they have a synergistic effect

2) Sugar:- Sucrose plays an important role in vascular tissue differentiation. Its concentration directly effects the relative amounts of xylem and phloem formed in the callus in the presence of low concentrations of auxin, 1% sucrose induces little xylem formation. Better xylem differentiation with little (or) no phloem was observed when sucrose level was increased to 2% both xylem and phloem are differentiated at 2.5 to 3.5% sucrose concentration. With an increase in sucrose concentration (4%) phloem was formed with little (or) no xylem.

3) Nitrogen:- Presence of Ammonia and Nitrate in the medium directly effects the differentiation of tracheary elements

4) Physical factors :- The effect of light on vascular tissue differentiation varies between cultured tissues. Temparature also effects cytodifferention. The temparature within 17-31$_{o}$C promotes vascular differention besides light and temparature other physical factors such as pH of medium greatly effects cytodifferention

Callus growth pattern

Growth of callus is measured in terms of increasc in fresh weight, dry weight (or) cell nember A generalized growth pattern takes the form of a sigmoid curve

Three district phases can be observed during growth of the callus

Lag phase: a period of little (or) no cell division (Biomass remain unchanged) **Cell division followed by linear phase (logphase)**:- a period of cell division and expansion rate of division. **Stationary phase (or) regeneration phase**:- As the nutrient supply of medium depleats, a gradual cessation of cell division occurs. This phase is associated with the initiation of structural organization of the cell which increases the production of secondary metabolites.

Types of cultures – callus and suspension cultures

Types of Cultures

1) Callus culture:- callus culture may be derived from a wide variety of plant organs roots, shoots, leaves (or) specific cell types. Eg:- Endosperm, pollen. Thus when any tissue (or) cell cultured on an agar gel medium forms an unorganized growing and dividing mass of cells called callus culture.

In culture, this proliferation can be maintained more (or) less indefinitely by subculturing at every 4-6 weeks, in view of cell growth, nutrient depletion and medium drying. Callus cultures are easy to maintain and most wide ly used in Biotechnology. Manipulation of auxin to cytokinin ratio in medium can lead to development of shoots or somatic embryos from which whole plants can be produced subsequently. Callus culture can be used to initiate cell suspensions which are used in a variety of ways in plant transformation studies.

Callus cultures broadly speaking fall into one of the two categories.
1) compact 2) friable callus
In compact callus the cells are densly aggregated. Where as in friable callus the cells are only loosly associated with each other and callus becomes soft and break a part easily. It provides inoculum to form cell suspension culture.

Suspension culture

When friable callus is placed into a liquid medium (usually the same composition as the solid medium used for callus culture) and then agitated single cells and / or small clumps of few to many cells are produced in the medium is called suspension culture Liquid cultures may be constantly agitated generally by a gyratory shaker of 100-250 rpm to facilitate aeration and dissociation of cell clumps into small pieces. Suspension cultures grow much faster than callus cultures, need to be sub-cultured at every week, allow a more accurate determination of the nutritional requirement of cells and even somatic embryos.

The suspension culture broadly grouped as 1) Batch culture 2) Continuous culture
1) Batch culture
A batch culture is a cell suspension culture grown in a fixed volume of nutrient culture medium. Cell suspension increases in biomass by cell division and cell growth until a factor in the culture environment (nutrient or oxygen availability) becomes limiting and the growth ceases. The cells in culture exhibit the following five phases of a growth cycle.
i. Lag phase, where cells prepare to divide
ii. Exponential phase, where the rate of cell division is highest.
iii. Linear phase, where cell division shows but the rate of cells expansion increases.
iv. Deceleration phase, where the rates of cell division and elongation decreases.
v. Stationary phase, where the number and size of cells remain constant.

When cells are subcultured into fresh medium there is a lag phase. It is the initial period of a batch culture when no cell division is apparent. It may also be used with reference to the synthesis of a specific metabolite or the rate of a physiological activity. Then follows a period of cell division (exponential phase). It is a finite period of time early in a batch culture during which the rate of increase of biomass per unit of biomass concentration (specific growth rate) is constant and measurable. Biomass is usually referred to in terms of the number of cells per ml of culture. After 3 to 4 cell generations the growth declines. Finally, the cell population reaches a stationary phase during which cell dry weight declines. It is the terminal phase of batch culture growth cycle where no net synthesis of biomass or increase in cell number is apparent.

In batch culture, the same medium and all the cells produced are retained in the culture vessel (Eg. culture flask 100-250 ml). the cell number or biomass of a batch culture exhibits a typical sigmoidal curve. Batch cultures are maintained by sub-culturing and are used for initiation of cull suspensions.

2) Continuous culture:-

These cultures are maintained in a steady state for a long period by draining out the used (or) spent medium and adding the fresh medium. such subculture systems are either closed (or) open type.

1) Closed:-

The cells separated from used medium taken out for replacement and added back to the suspension culture. So that the cell biomass keeps on increasing

2) Open:-

Both cells and the used medium are takenout from open continuously cultures and replaced by equal volume of fresh medium. The replacement volume is so adjusted that cultures remain at sub-maximal growth indefinitely.

What is Cryopreservation?

Cryopreservation is the method of keeping the live cells, tissues and other biological samples in a deep freeze at subzero temperatures for the storage or preservation. The sample is commonly kept at −196°C.

At such low temperatures, all the biological activities of the cells stop and the cell dies. Cryopreservation helps the cells to survive freezing and thawing.

The ice formation inside the cells can break the cell membrane. This can be prevented by regulating the freezing rate and carefully choosing the freezing medium.

Cryopreservation Process

In this process, biological materials including cells, oocytes, spermatozoa, tissues, ovarian tissues, pre-implantation embryos, organs, etc. are kept in extremely cold temperatures without affecting the cell's viability.

Dry Ice and liquid nitrogen are generally used in this method.

Cryopreservation Steps

The complete procedure steps involved in preserving the obtained biological samples are as follows:

1. **Harvesting or Selection of material**– Few important criteria should be followed while selecting the biological materials such as – volume, density, pH, morphology, and without any damage.
2. **Addition of cryo-protectant** – Cryoprotective agents such as glycerol, FBS, salts, sugars, and glycols are added to the samples as it reduces the freezing point of the medium and also allow slower cooling rate, which reduces the risk of crystallization.
3. **Freezing** – Different methods of freezing are applied in this method of cryopreservation to protect cells from damage and cell death by their exposure to the warm solutions of cryoprotective agents.
4. **Storage in liquid nitrogen**– The cryopreserved samples are stored in extreme cold or -80°C in a freezer for at least 5 to 24 hours before transferring it to the storage vessels.
5. **Thawing-** The process of warming the biological samples in order to control the rate of cooling and prevent the cell damage caused by the crystallization.

Cryopreservation of Embryos

During the infertility treatment, hormones are used to stimulate the development of eggs. The eggs are then taken out and fertilized in the lab. More embryos can be created and transplanted to the woman's uterus. These embryos can be cryopreserved and can be used at some later date. By

this, the female can get an additional transfer of embryo in future, without spending on another IVF cycle.

Oocyte Cryopreservation

In the vitrification method, the eggs freeze rapidly so that there is less time available for the formation of ice crystals. New cryoprotectants are used with a high concentration of products with anti-freeze property.

The oocyte is first placed in a bath containing a low concentration of anti-freeze like cryoprotectant. Some sucrose is added to help draw some water out of the egg. The egg is then shifted to high concentration anti-freeze cryoprotectant for very few seconds and then immediately transferred to liquid nitrogen. When the egg is thawed and used for the transplantation into the woman.

Cryopreservation of Sperm

The semen sample is mixed with a solution, which provides protection during freezing and thawing. Followed by transfer to plastic vials, which are then kept in liquid nitrogen for freezing.

This process ensures the chances of conception in future. The sperm can also be deposited, froze and stored in cryobanks for less than a year. These sperms can later be used for certain infertility treatment procedures.

Benefits of Cryopreservation

There are many benefits of cryopreservation technique. These include:

- Fertility treatments.
- Minimal space and labour required.
- Safety from genetic contamination.
- Safeguards genetic integrity of valuable stains.
- Safeguards the germplasm of endangered species.
- Biological samples can be preserved for a longer period of time.
- Protects the samples from disease and microbial contamination.
- Prevents genetic drift by cryopreservation of gametes, embryos, etc.

Applications of Cryopreservation

Cryopreservation is a long-term storage technique, which is mainly used for preserving and maintaining viability of the biological samples for a longer duration.

This method of preservation is widely used in different sectors including cryosurgery, molecular biology, ecology, food science, plant physiology, and in many medical applications. Other applications of cryopreservation process are:

1. Seed Bank.
2. Gene Bank.
3. Blood transfusion.
4. In vitro fertilization.
5. Organ transplantation.
6. Artificial insemination.
7. Storage of rare germplasm.
8. Freezing of cell cultures.
9. Conservation of endangered plant species.

Biodiversity conservation.

Introduction

Biotechnology is essentially the product of interaction between the science of biology and technology. The products of biotechnology are going to have a major impact on the quality of human life, productivity, trade and economics in the world. Already biotechnology is being used in the areas of diagnosis, prevention and cure of diseases, in the production of new and cheaper biochemical products e.g. pharmaceutical drugs, in enhanced production of new food resources, in environmental protection and energy conservation.

Biotechnology is as old as human civilization and has been an integral part of the human life. Many scientists use the term **old or traditional biotechnology** to the natural processes that have been used since many centuries to produce beer, wine, curd, cheese and many other foods. The new or modern biotechnology includes all the genetic manipulations, cell fusion techniques and the improvements made in the old biotechnological processes.

The term biotechnology was introduced in 1917 by Karl Ereky, a Hungarian Engineer. He used the sugar beets as the source of food for large scale production of pigs. Ereky defined biotechnology as "all lines of work by which products are produced from raw materials with the aid of living things". The multidisciplinary character of biotechnology makes it rather difficult to define biotechnology. However there are several definitions available.

Biotechnology is "the integrated use of biochemistry, microbiology, and engineering sciences in order to achieve technological (industrial) application of the capabilities of microorganisms, cultured tissue cells and parts thereof".(European Federation of Biotechnology)

Biotechnology is "the application of biological organisms, system or processes to manufacturing and service industries". (British Biotechnologists)

Biotechnology is "a technology using biological phenomena for copying and manufacturing various kinds of useful substances".(Japanese Biotechnologists)

Biotechnology is defined as "the controlled use of biological agents, such as microorganisms or cellular components, for beneficial use". (US National Science Foundation).

Biotechnology is the application of scientific and engineering principles to the processing of materials by biological agents to provide goods and service. (The Organization for the Economic Cooperation and Development (OECD), 1981)

The application of biochemistry, biology, microbiology and chemical engineering to industrial process and products and on environment.(International Union of Pure and Applied Chemistry (IUPAC), 1981).

A new definition after combining all aspects of biotechnology/genetic engineering was given by

Smith in 1996- The formation of new combinations of heritable material by the insertion of nucleic acid molecules produced by whatever means outside the cell, into any virus, bacterial plasmid or other vector system so as to allow their incorporation into a host organism in which they do not naturally occur but in which they are capable of continued propagation.

Plant Biotechnology

An important aspect of all biotechnology processes is the culture of either the plant cells or animal cells or microorganisms. The cells in culture can be used for recombinant DNA technology, genetic manipulations etc.

Plant cell culture is based on the unique property of the cell-totipotency. CELL-TOTIPOTENCY is the ability of the plant cell to regenerate into whole plant. This property of the plant cells has been exploited to regenerate plant cells under the laboratory conditions using artificial nutrient mediums. With the advances made in genetic engineering, it became possible to introduce foreign genes into cell and tissue culture systems. This led to the development of Genetically Modified (GM) or Transgenetic Crops which had improved traits and characteristics.

History of cell culture

In the early 19th century, Schleiden and Schwann proposed the concept of the 'cell theory'. In 1902, Gottlieb Haberlandt, the german botanist and regarded as the father of plant tissue culture, first attempted to cultivate the mechanically isolated plant leaf cells on a simple nutrient medium. He did not succeed in achieving the growth and differentiation of the cultured cells, however, he predicted the concept of growth hormones, the use of embryo sac fluids, the cultivation of artificial embryos from somatic cells, etc.

During the period 1902 - 1930, attempts were made to culture the isolated plant organs such as roots and shoot apices (organ culture). Hanning (1904) isolated embryos of some crucifers and successfully grew on mineral salts and sugar solutions. Simon (1908) successfully regenerated a bulky callus, buds, roots from a poplar tree on the surface of medium containing IAA which proliferated cell division. Gautheret, White and Nobecourt (1934-1940) largely contributed to the developments made in plant tissue culture. White (1939) cultured tobacco tumour tissue from the hybrid Nicotiana glauca, and N. Langsdorffii.

The period of 1940 - 1970s saw the development of suitable nutrient media to culture plant tissues, embryos, anthers, pollen, cells and protoplasts, and the regeneration of complete plants (in vitro morphogenesis) from cultured tissues and cells. In 1941, van Overbek and coworkers used coconut milk (embryo sac fluid) for embryo development and callus formation in Datura. Steward and Reinert (1959) first discovered somatic embryo production in vitro.

Maheswari and Guha (1964) developed the anther culture for the production of haplid plants.

Skoog and Miller (1957) advanced the hypothesis of organogenesis in cultured callus by varying the ratio of auxin and cytokinin in the growth medium. Muir (1953) developed a successful technique for the culture of single isolated cells wich is commonly known as paper-raft nurse technique (placing a single cell on filter paper kept on an actively growing nurse tissue). In 1952, the Pfizer Inc., New York (U.S.A) got the US patent and started producing industrially the secondary metabolites of plants. The first commercial production of a natural product shikonin by cell suspension culture was obtained.

In 1980s using Genetic engineering, for the first time, it was possible to introduce foreign genes into cell and tissue culture systems to develop plants with improved characteristics (transgenic crops) which may contribute to the path towards the second green revolution.

Plant Cell and Tissue Culture Techniques

The whole plants can be regenerated virtually from any plant part (referred to as explants) or cells. Plant tissue culture techniques involve the following steps:

1. Preparation and selection of suitable nutrient media.
2. Selection of explants such as shoot tip.
3. Surface sterilization of the explants by disinfectants e.g. sodium or calcium hypochorite solution 0.3- 0.6%) followed by washing the explants with sterile distilled water.
4. Inoculation or Transfer of the explants onto the suitable nutrient medium (sterilized by autoclaving) in culture vessels under sterile conditions (using laminar flow hood).
5. Incubation or Growing the cultures in the growth chamber or plant tissue culture room at optimum physical conditions of light (16 hours of photoperiod), diurnal illumination, temperature (25+/- 20C and relative humidity (50%-60%).
6. Regeneration of plants from cultured plant tissues.
7. Hardening: it is the gradual exposure of plantlets for acclimatization to environmental conditions.
8. Transfer of plants to the field conditions following the acclimatization/ hardening of the regenerated plants.

Nutrient Media

The intact plants can make their own food but the ***in vitro*** culture of plant parts or cells requires a variety of nutrients and suitable physical conditions for their growth. The composition of plant tissue culture medium depends upon the type of plant tissues or cells that are used for culture. No single medium can be used for all types of plants and organs, so the composition of the culture medium for each plant material has to be worked out.

A typical nutrient medium consists of the following components:

A. **Inorganic nutrients** (both micro- and macro-elements - C, H, O, N, P, S, Ca, K, Mg, Fe, Mn, Cu, Zn, B, Mb), The six elements namely nitrogen, phosphorus, potassium, calcium, magnesium and sulfur are the essential macronutrients for tissue culture. The ideal concentration of nitrogen, and potassium is around 25 mmol l-1 while for calcium, phosphorus, sulfur and magnesium, it is in the range of 1-3 mmol l-1. Among the micronutrients, iron requirement is very critical. Chelated forma of iron and copper are commonly used in culture media.

B. **A carbon source and energy source** (usually sucrose))- Plant cells and tissues in the culture mediumare heterotrophic and therefore depend on the external carbon for energy. Among the various energy sources, sucrose is the most preferred. During the sterilization of the medium, sucrose gets hydrolysed to glucose and fructose and the plant cells utilize first the glucose and than the fructose. The other carbohydrates such as lactose, maltose, galactose etc have been used in culture media but with limited success.

C. **Organic supplements vitamins** (e.g. nicotinic acid, thiamine, pyridoxine and myo-inositol), amino acids (e.g. arginine) The plant cells in culture are able to synthesize vitamins just like natural plants, but in suboptimal quantities which does not support proper growth of cells in culture. Therefore the medium is supplemented with vitamins to achieve good growth of cells. Similarly amino acids are added to the cell cultures to stimulate the cell growth and estabilish the cell lines. Organic acids especially the intermediates of krebs cycle e.g. citrate, malate, succinate, pyruvate also enhances the growth of plant cells. Sometimes antibiotics (e.g. streptomycin, kanamycin) are also added to the medium to prevent the growth of the microorganisms.

D. **Growth regulators** (e.g. auxins, cytokinins and gibberellins Plant hormones play an important role in growth and differentiation of cultured cells and tissues. The growth hormones included in culture media involve: auxins, cytokinins, and gibberellins. The auxins facilitate the cell division and root differentiation. The cytokinins induce cell division and differentiation and the gibberellins is mainly used to induce plantlet formation from adventive embryos formed in culture. Auxins induce cell division, cell elongation, and formation of callus in cultures. 2,4- dichlorophenoxy acetic acid is one of the most commonly added auxins in plant cell cultures. Cytokinins, promotes RNA synthesis and stimulate protein and enzyme activities in tissues. Kinetin and benzyl-aminopurine are the most frequently used cytokinins in plant cell cultures. The ratio of auxins and cytokinins play an important role in the morphogenesis of culture systems. When the ratio of auxins to cytokinins is high, embryogenesis, callus initiation, and root initiation occur. For axillary and shoot proliferation, the ratio of auxins to cytokinins is kept low.

   Among the gibberellins, gibberellin A3 (GA3) is the most commonly used for tissue culture. GA3 enhances callus growth and induces dwarf plantlets to elongate.

E. **Solidifying agents like agar**: Generally a gelling agent agar (a polysaccharide obtained from red algae, ***Gelidium amansil***) is added to the liquid medium for its solidification. . The agar obtained from seaweeds provides solid surface for the growth of cells because in the liquid medium, the tissue will be submerged and die due to lack of oxygen. Cells

are grown in suspension medium with out agar but such cultures are aerated regularly either by bubbling sterile air or by gentle agitation. Some other less frequently used solidifying agents are biogel (polyacrlyamide pellets), phytagel, gelrite, and purified agarose.

F. **Other compounds** like casein hydrolysate, coconut milk, malt extract, yeast extract, ***tomato***juice, etc. may be added for specific purposes.
G. **pH:** An optimum pH (usually 5.7) is also very important. At pH higher than 7.0 and lower than 4.5, the plant cells stop growing in cultures. The most extensively used nutrient medium is MS medium (developed by Murashige and Skoog in 1962).

Major Types of Media

1. **White's medium** - is one of the earliest plant tissue culture media.
2. **MS medium** - formulated by Murashige and Skoog (MS) is most widely used for many types of culture systems**.**
3. **B5 medium** - developed by Gamborg for cell suspension and callus cultures and at present it's modified form used for protoplast culture.
4. **N6 medium** - formulated by Chu and used for cereal anther culture.
5. Nitsch's medium developed by Nitsch and Nitsch and used for anther culture

Preparation of Media

The methodology for media preparation involves preparation of stock solutions (in the range of 10x to 100x concentrations) of highly purified chemicals and demineralized water. The stock solutions are stored in glass or plastic containers and frozen till further requirement. Now a days, plant tissue culture media are commercially prepared, and are available in the market as dry powders. The culture media is usually sterilized in an autoclave at 1210C and 15 psi for 20 minutes. Hormones and other heat sensitive organic compounds are filter sterilized and added to the autoclaved medium.

Maintenance of Aseptic Environment

It is very important to maintain aseptic environment during the in vitro culture of plant cells and tissues. Following are some of the methods adopted for sterilization:

A. Sterilization of Glassware- The glassware can be sterilized in a hot air oven at 160-1800C for 2-4 hours.
B. Sterilization of instruments- The metallic instruments are incinerated by dipping them in 75% ethanol followed by flaming and cooling.
C. Sterilization of nutrient media- The culture media are transferred into glass container, plugged with cotton or sealed with plastic closures and sterilized by autoclaving at 15 psi for 30 min. The autoclaving denatures the vitamins, plant extracts, amino acids and

hormones therefore the solution of these compounds are sterilized by using Millipore filter paper with pore size of 0.2 micrometer diameter.

D. Sterilization of plant materials- The surface of the plant material is made sterile by using disinfectants e.g. sodium hypochlorite, hydrogen peroxide, mercuric chloride, or ethanol. The transfer of sterile plant material on to the nutrient medium is done under the cabinet of laminar airflow.
E. Sterilization of Culture room and transfer area- the floor and walls of the culture room should be washed with detergent followed by 2% sodium hypochlorite or 95% ethanol. The sterilization can also be done by exposure to UV light. The cabinet of laminar air flow is sterilized by exposing to UV light for 30 min. and 95% ethanol 15 minutes before starting the work.

Types of Culture

Organ Culture

It deals with the culture of the isolated organs (roots) under laboratory conditions ***(in vitro)***. Different names are given depending upon the organ used for the culture. For instance the culture of roots, endosperm, ovary, and ovule are called as root culture, endosperm culture, ovary culture etc. It was Skoog (1944), who for the first time suggested that the organogenesis could be chemically controlled. Skoog and Miller (1957) also demonstrated that a high ratio of auxin: cytokinin stimulated the formation of root in tobacco callus, but a low ratio of the same induced shoot information.

Explants culture

The culture of plant parts (explants) is known as explant culture. The explants can be any part of the plant e.g. the piece of stem, leaf, hypocotyl, etc. The explant cultures are generally used to induce callus or plant regeneration.

Callus culture

Callus refers to an unorganized mass of cells generally parenchymatous in nature. The unique feature of callus is that the abnormal growth has biological potential to develop normal root, shoots, and embryoids, ultimately forming a plant. Naturally, the callus is formed due to the infection of microorganisms from wounds due to stimulation by endogenous growth hormones, the auxins and cytokinins. However, it has been possible to artificially develop callus by using tissue culture techniques. Auxins are added to culture medium for callus induction but the nature and quantity of auxin added, depends on the nature and source of explant and its genotype besides other factors. Callus cultures can be maintained for prolonged periods by repeated sub-culturing. Callus cultures are used for a) plant regeneration, b) preparation of single cell suspensions and protoplasts, and, c) genetic transformation studies.

Factors affecting Callus Culture

1. The source and the genotype of the explants.
2. Composition of the medium (most commonly used-MS medium).
3. Temperature (22-28^{0}C suitable for callus formation).
4. Growth regulators e.g. auxins, cytokinins alone or combination of these.
5. Age of the plant.
6. Location of the explants.
7. Physiology and growth condition of the plant

Cell suspension cultures

Cell suspension is prepared by transferring a fragment of callus to the liquid medium and agitating them aseptically to make the cells free. Single cells can be isolated from either callus or any other part of the plant and cultured in liquid medium using both mechanical and enzymatic methods. Mechanical methods involve grinding of the tissue to a fine suspension in a buffered medium followed by filtration and centrifugation to get rid of cell debris. The enzymatic method uses the enzymes (pectinase or macerozyme) to dissolve the middle lammela between the cells. After the isolation of the cells, they are cultured by batch cultures or continuous cultures.As the medium is liquid in nature, the pieces of callus remain submerged which creates anaerobic conditions. To overcome this problem, the suspension cultures are agitated by a rotary shaker which disperses the cells and expose them to air.

The advantages of cell suspension cultures over the callus culture:

1. The suspension can be pipetted.
2. They are less heterogeneous and cell differentiation is less pronounced.
3. They can be cultured in volumes upto 1,500 litres.
4. They can be subjected to more stringent environmental controls.
5. The manipulations for the production of natural products by feeding precursors, is possible.

Batch cultures are initiated as single cells in 100- 250 ml flasks and are propagated by transferring regularly small aliquots of suspension to a fresh medium. Continuous cultures are maintained in a steady state for long period by draining out the used medium and adding fresh medium.
The cell suspension cultures can be used for a) induction of somatic embryos/shoots, b) ***in vitro***mutagenesis and mutant selection, c) genetic transformation, d) production of secondary metabolites.

Mass cell culture

Plant cells are cultured in specially designed 'plant bioreactors' which essentially do not have a stirrer as plant cells are shear sensitive. In place of stirrer, gas is gently bubbled which provides stirring as well as meet the demand of a higher oxygen supply.

Protoplast culture

Protoplasts are plant cells without cell wall and can be isolated by using enzymes like cellulases, pectinases) from leaf, seedling, calli, pollen grains, embryo sacs etc.The protoplasts regenerate cell wall, undergo cell division, and form callus. The callus can also be subcultured. Some of the examples of plant species that have been regenerated from protoplasts are--- Cucumis sativus, Capsicum annum, Ipomoea batata, Glycine max, Chrysanthemum sp. These cultures are used for a) various biochemical and metabolic studies, b) fusion of two somatic cells to create somatic hybrids, c) fusion of enucleated and nucleated protoplasts to create Cybrids (cytoplasmic hybrids) and d) genetic manipulation. e) drug sensitivity.

Applications of Cell and Tissue Culture

Micropropagation /Clonal Propagation

Clonal propagation refers to the process of asexual reproduction by multiplication of genetically identical copies of individual plants. The vegetative propagation of plants is labour-intensive, low in productivity and seasonal. The tissue culture methods of plant propagation, known as 'micropropagation' utilizes the culture of apical shoots, axillary buds and meristems on suitable nutrient medium.The regeneration of plantlets in cultured tissue was described by Murashige in 1974. Fossard (1987) gave a detailed account of stages of micropropagation.

The micropropagation is rapid and has been adopted for commercialization of important plants such as banana, apple, pears, strawberry, cardamom, many ornamentals (e.g. Orchids) and other plants.The micropropagation techniques are preferred over the conventional asexual propagation methods because of the following reasons: (a) In the micropropagation method, only a small amount of tissue is required to regenerate millions of clonal plants in a year., (b) micropropagation is also used as a method to develop resistance in many species., (c) in vitro stock can be quickly proliferated as it is season independent,. (d) long term storage of valuable germplasm possible. The steps in micropropagation method are: a) Initiation of culture - from an explant like shoot tip on a suitable nutrient medium, b) multiple shoots formation from the cultured explant, c) rooting of ***in vitro*** developed shoots and, d) transplantation - transplantation to the field following acclimatization.

The factors that affect micropropagation are: (a) genotype and the physiological status of the plant e.g. plants with vigorous germination are more suitable for micropropagation., (b) the culture medium and the culture environment like light, temperature etc. For example an illumination of 16 hours a day and 8 hours night is satisfactory for shoot proliferation and a temperature of 250C is optimal for the growth.

The benefits of micropropagation this method are:

A. Rapid multiplication of superior clones can be carried out through out the year, irrespective of seasonal variations.
B. Multiplication of disease free plants
C. Multiplication of sexually derived sterile hybrids
D. It is a cost effective process as it requires minimum growing space.