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APPLIED PLANT BIOTECHNOLOGY

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PREFACE

Plant tissue culture is a practice used to propagate plants under sterile conditions, often toproduce clones of a plant. Different techniques in plant tissue culture offer advantages overtraditional methods of propagation including the production of exact copies of plants even inabsence of seeds and low germinating seeds, production of genetically modified and disease free plants.

This handout entitled plant tissue culture course is a subject aimed at 3rd year Biotechnology and 1 st year Master genetic and amelioration of plant students. The content of this handout includes the program taught in the department of natural and life sciences at Ibn Khaldoun University in Tiaret. It is written in the form of a detailed course, with illustrative diagrams. It is presented in a very simple style which allows students to understand very quickly. The content of this handout is structured in different chapters, wich we devoted the important of tissue culture diffentspractises and we have included the description of the technic of transgenesis and modification of the genom.

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Introduction:

Plant tissue culture is broadly refers to the in vitro cultivation of plants, seeds and various parts of the plants. Plant cell/tissue culture, also relate to axenic, or sterile culture, is an important tool in both basic and applied studies as well as in commercial application. Although it's recommended a more restricted use of the term, plant tissue culture is generally used for the aseptic culture of cells, tissues, organs, and their components under defined physical and chemical conditions in vitro.

Or Plant tissue culture is an essential component of plant biotechnology the propagation of plants through "cloning" an asexual method of reproduction. A portion (explant) of a desired plant is cultured in vitro on a defined medium, which promotes rapid multiplication of cells. The new plants are removed from the culture and transferred to a standard potting medium.

A whole plant can be regenerated from a small tissue or plant cells in a suitable culture medium under controlled environment. The plantlets so produced are called tissue-culture raised plants. These plantlets are a true copy of the mother plant and show characteristics identical to the mother plant. Many plant species are presently being propagated through tissue culture successfully. This capacity of a single cell to grow into a complete plant is termed as Totipotency, which was first put forward by a German Botanist Haberlandt in 1902. Tissue culture is the propagation of plants wherein a part/tissue of the plant is placed in nutrient media that favors the production of shoots, roots following which they are hardened and transferred to soil. Quality planting material of economically important species can be produced in a large scale/desired quantity through tissue culture. Plant tissue culture can be initiated from almost any part of a plant however, for micropropagation or direct shoot regeneration, meristemetic tissue such as shoot tip is ideal. The physiological state of the plant does have an influence on its response to tissue culture. The mother plant must be healthy and free from obvious signs of disease or pest. The shoot tip explants being juvenile contain a higher proportion of actively dividing cells. It is important to use quality mother plant stock to initiatecultures. The cultural conditions required to initiate and sustain plant cells in culture, or to regenerate intact plants from cultured cells, are different for each plant species. Each variety or clone of a species often have a particular set of cultural requirements.

History

Perhaps the earliest step toward plant tissue culture was made by Henri-Louis Duhumel du Monceau in 1756, who, during his pioneering studies on wound-healing in plants, observed callus formation. Extensive microscopic studies led to the independent and almost simultaneous development of the cell theory by Schleiden (1838) and Schwann (1839).

Vöchting, 1878, work on callus formation and on the limits to divisibility of plant segments was perhaps the most important.

German botanist Haberlandt G. (1902) regarded as the Father of plant tissue culture, He also explained the concept of cell totipotency.

Gautheret, R. J. (1934) made preliminary attempts with liquid medium for cultivating plant issues but failed completely. Later he cultured the explants on medium solidified with agar, and got healthy calli from the explants. White, P.R. (1934) obtained indefinite survival of cultured tomato roots on sub culturing in liquid medium. White, P.R., Gautheret, R.J. and Nobecourt, P (1939) simultaneously announced the possibility of cultivating plant tissues for unlimited periods. Van Overbeek, J., Conklin, M.E. and Blackeslee, A.F (1941) established importance of coconut milk for growth and development of very young Datura embryos. White, P.R. and Braun, A.C (1942) initiated studies on crown gall and tumour formation in plants. Skoog, F (1944) started his work on organogenesis in tobacco callus. Guha, S. and Maheshwari, S.C (1964) cultured mature anthers of Datura innoxia to study the physiology of meiosis and accidentally noticed the development of embryoids from the anthers plated on basal medium supplemented with kinetin and coconut milk. Murashige, T (1974) developed the concept of developmental stages in cultures in vitro: Stage

I: Explant establishment; Stage

II: Multiplication of propagule and Stage

III: Rooting and hardening for planting into soil.

In1978, Melchers developed, somatic hybridization of tomato and potato resulting pomato

Larkin,1981- Introduction of the term somaclonal variation by.

Infection and transformation of leaf discs with *Agrobacterium tumefaciens*and regeneration of transformed plants by Horsch, 1985. Fraley, 1985, evelop the disarmed Ti-plasmid vector system for plant transformation.

I. Importance of Plant Tissue culture

Culture in Agriculture As an emerging technology, the plant tissue culture has a great impact on both agriculture and industry, through providing plants needed to meet the ever increasing world demand. It has made significant contributions to the advancement of agricultural sciences in recent times and today they constitute an indispensable tool in modern agriculture. Biotechnology has been introduced into agricultural practice at a rate without precedent. Tissue culture allows the production and propagation of genetically homogeneous, diseasefree plant material. Cell and tissue in vitro culture is a useful tool for the induction of somaclonal variation. Genetic variability induced by tissue culture could be used as a source of variability to obtain new stable genotypes. Interventions of biotechnological approaches for in vitro regeneration, mass micropropagation techniques and gene transfer studies in tree species have been encouraging. Genetic engineering can make possible a number of improved crop varieties with high yield potential and resistance against pests. Genetic transformation technology relies on the technical aspects of plant tissue culture and molecular biology for :

- Production of improved crop varieties
- Production of disease-free plants
- Genetic transformation
- Production of secondary metabolites
- Production of varieties tolerant to salinity, drought and heat stresses

Dedifferentiation and callus formation

The term "dedifferentiation" has many definitions: process by which mature or specialized cells lose their differentiated character and rejuvenate; a process in which tissues that have undergone cell differentiation can be made to reverse the process so as to become a primordial cell again; “involves a terminally differentiated cell reverting back to a less differentiated stage from within its own lineage; its distinguishing feature is the withdrawal from a given differentiated state into a stem cell -like state that confers pluripotentiality. The common in these definitions is that, contrary to differentiation, dedifferentiation increases the developmental potency of cells.

"Callus" is an actively-dividing non-organized mass of undifferentiated and differentiated cells often developing either from injury or in tissue culture in the presence of growth regulators. Explants from both mature and immature organs can be induced to form callus.

II. General technique of in vitro Culture:

General technique of plant cell, tissue and organ culture is almost the same with a little variation for different plant materials. There are certain basic steps for the regeneration of a complete plant from an explant cultured on the nutrient medium.

2.1. Selection and Sterilization of Explant: Suitable explant is selected and is then excised from the donor plant. Explant is then sterilized using disinfectants.

2.2. Preparation and Sterilization of Culture Medium: A suitable culture medium is prepared with special attention towards the objectives of culture and type of explant to be cultured. Prepared culture medium is transferred into sterilized vessels and then sterilized in autoclave.

2.3. Inoculation: Sterilized explant is inoculated on the culture medium under aseptic conditions.

2.4. Incubation: Cultures are then incubated in the culture room where appropriateconditions of light, temperature and humidity are provided for successful culturing.

2.5. Sub culturing: Cultured cells are transferred to a fresh nutrient medium to obtain the plantlets.

2.6. Transfer of Plantlets: After the hardening process (i.e., acclimatization of plantlet to the environment), the plantlets are transferred to green house or in pots.

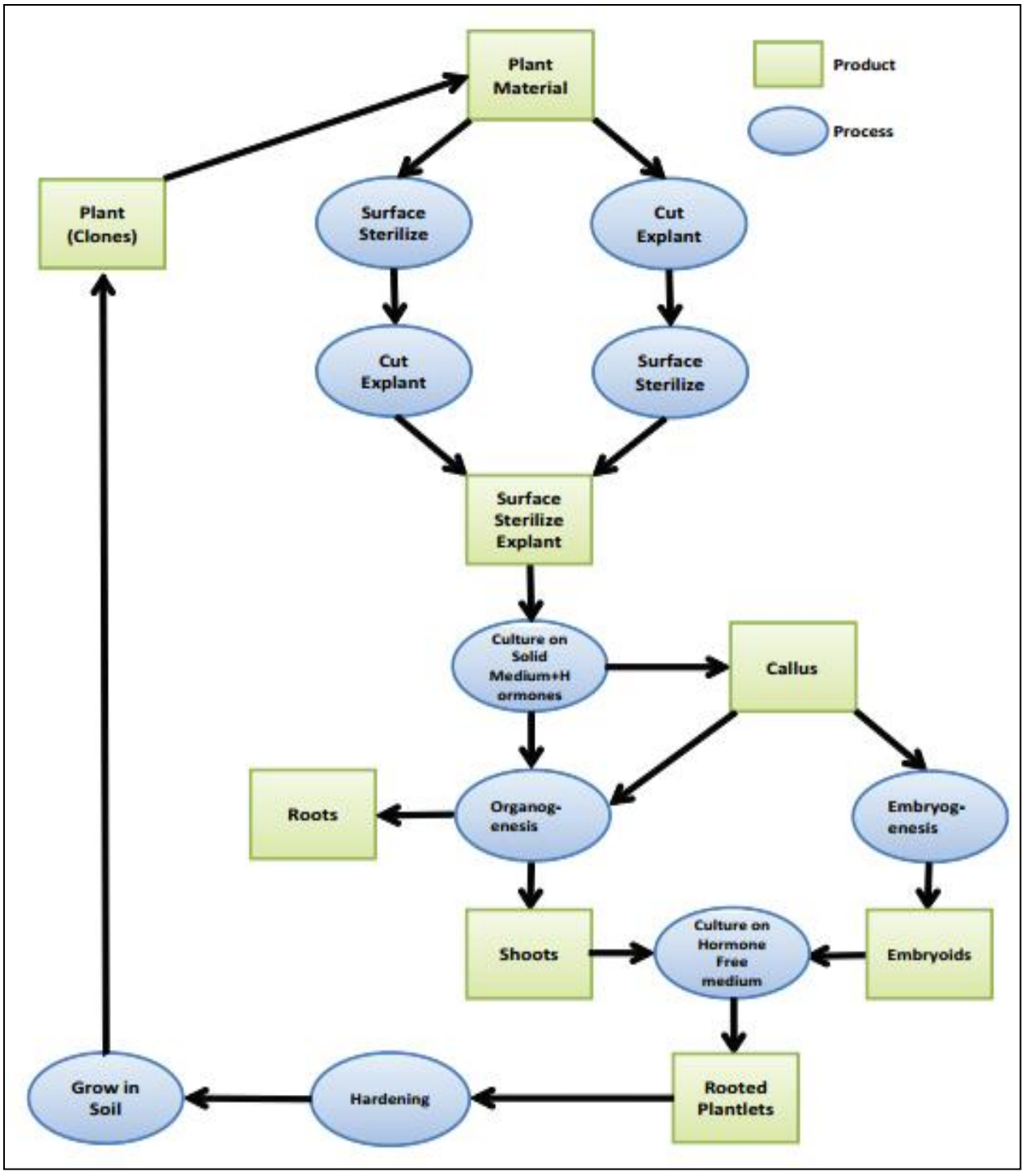

Figure 1. Flow chart summarizing tissue culture experiments.

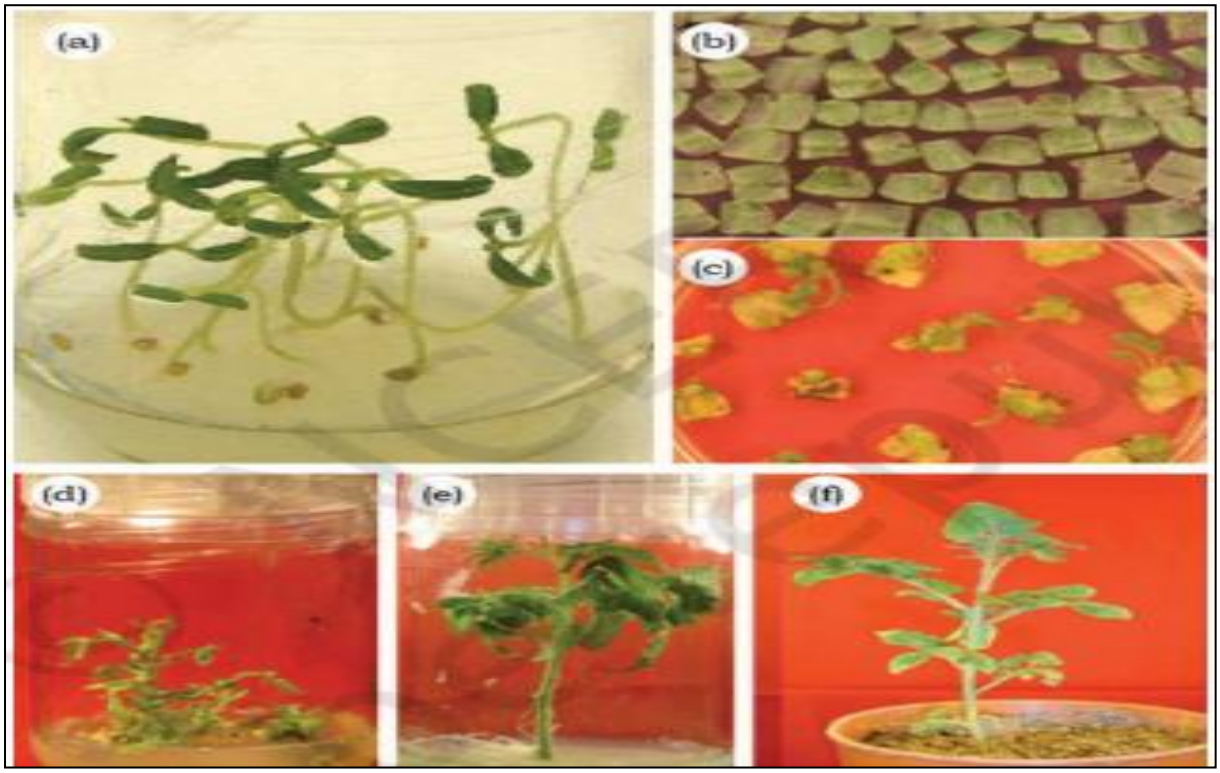

Figure 2.Various stages of regeneration tissue culture plant: (a) Germination of seeds on the culture medium in a culture bottle. (b) Explant preparation from germinated seedlings. (c) Regenerating callus (calli with shoot buds) from inoculated explants. (d) Regenerating shoots ready to be transferred to rooting media. (e) Rooting in the inoculated Shoots. (f) A plantlet transferred to the soil in pot.

III.Nutrient Media

A variety of nutrients and suitable environmental conditions are required for optimal growth and development of an explant. Depending upon the type of plant species, like monocot or dicot; domesticated or wild, etc., composition of the culture media varies. Even different tissues from the same plant may have different nutritional requirements for optimal growth. Therefore, success of in vitro culture of plants mainly depends upon the selection of the right composition of culture medium. Culture media used for in vitro plant cultures broadly contain the following components:

1. Inorganic components
2. Organic supplements
3. Carbon source
4. Gelling agents
5. Antibiotics
6. Plant growth hormones

3.1. Inorganic components

Many inorganic components are required in large amounts (milli molar concentrations) and are categorised as macronutrients. These include carbon (C), calcium (Ca), hydrogen (H), potassium (K), magnesium (Mg), nitrogen (N), oxygen (O), phosphorus (P) and sulphur (S). Several other essential inorganic components are required in small amounts (micro molar concentrations) and are categorised as micronutrients. These include boron (B), cobalt (Co), copper (Cu), iron (Fe), manganese (Mn), molybdenum (Mo) and zinc (Zn).

3.2. Organic components

Amino acids and vitamins are common organic supplements that are used in culture media. Amino acids serve as nitrogen source and commonly used amino acids are arginine, asparagine, glycine or proline. Vitamins added in the culture medium are thiamine (Vitamin B1), nicotinic acid (Vitamin B3) and myoinositol.

3.3. Carbon source

For in vitro cultures, sucrose is considered as the best carbon source and is used at concentration of about 2–5 percent. Other sources for carbon are glucose, fructose and mannose.

Further, pH of the nutrient media is usually adjusted to about 5.8 to 6.0. Increase in the pH increases the hardness of the medium and decrease in pH leads to poor solidification of the media. pH of the nutrient media also affects the uptake of the nutrients by plant cells and solubility of the media salts.

3.4. Gelling agent is required for solid culture media. Agar is the most commonly used gelling agent and is ideal for routine applications.

3.5. Antibiotics can be used to suppress the bacteria and antifungal agents for mould infections in cultures. The recipe of plant tissue culture media is usually directed by plant species, however (MS) / media is the most commonly used media composition in plant tissue culture..

Table 01. Composition of commonly used Plant tissue culture media (White's and MS Media (1962)

Components	White's	Murashinge and Skoog (MS)
	Amount (mg l^{-1})	
Macronutrients		
$MgSO_4 \cdot 7H_2O$	750	370
KH_2PO_4	-	170
$NaH_2PO_4 \cdot H_2O$	19	-
KNO_3	80	1900
NH_4NO_3	-	1650
$CaCl_2 \cdot 2H_2O$	-	440
Micronutrients		
H_3BO_3	1.5	6.2
$MnSO_4 \cdot 4H_2O$	5	22.3
$ZnSO_4 \cdot 7H_2O$	3	8.6
$Na_2MoO_4 \cdot 2H_2O$	-	0.025
$CuSO_4 \cdot 5H_2O$	0.01	0.025
$CoCl_2 \cdot 6H_2O$	-	0.025
KI	0.75	0.83
$FeSO_4 \cdot 7H_2O$	-	27.8
$Na_2EDTA \cdot 2H_2O$	-	37.3
Sucrose (g)	20	30
Organic supplements		
Vitamins		
Thiamine HCl	0.01	0.5
Pyridoxine (HCl)	0.01	0.5
Nicotinic acid	0.05	0.5
Myoinositol	-	100
Others		
Glycine	3	2
pH	5.8	5.8

3.6. Plant growth regulators in tissue culture

Hormones are organic compounds which are synthesized in the tissues of plants. They are requied in only very low concentrations to influence plant growth and development. Many synthetic organic molecules have been.found which have biological activity similar to the hormones. As a group the synthetic compounds and the naturally occurring hormones are known as growth regulators.

Tissue culture is the manipulation of plant growth under carefully controlled conditions and the auxins and cytokinins are of particular significance.Most explants produce some (endogenous) auxin and cytokinin. In tissue culture additional (exogenous) growth regulators are appbed to achievegrowth effect. As a general guide either auxin or cytokimn or both auxin and cytokinin are added to the culture to achieve the desired growth response.

4.6.1. Some practical aspects of the use of growth regulators.

Growth regulators for use in media are stored in the dark in the refrigerator as stock solutions. Small volumes (say 50 mL) of concentrated solutions containing 1 mg mL^{-1} of the growth regulator may be stored for some time in this manner. The stability of growth regulators varies; kinetin and IAA are unstable in the light so are usually stored in dark bottles. Also, IAA loses its activity in aqueous solutions so stock solutions of IAA should not be kept for long periods. De Fossard (1976) provides very useful details on preparation of stock solutions. In general auxins should be dissolved in a small volume of 95% alcohol before the correct volume is obtained by the addition of water. Cytokinins are dissolved in a small volume of 1 N hydrochloric acid and then water is added to achieve the final volume. Appendix 1 provides detailed information on a wide range of plant growth regulators in use in plant tissue culture.

Auxins

This group includes the naturally occurring IAA and the synthetic auxins rnA, NAA and 2, 4-D. The auxins are added to the nutrient medium in concentrations within the range of 0.01-10 mg L^{-1}. The synthetic auxins are relatively more active than IAA and have the added advantage that they are not degraded by enzymes present in the plant tissues. Auxins have a number of different roles in plant growth and development. They stimulate cell elongation and growth, cell division particularly in callus formation and the formation of adventitious roots. Auxins also inhibit axillary bud development and the formauon of somatic embryos from callus cultures.

Cytokinins

These include the naturally occurring 2iP and ~e.atin and the syntheticcytokinins and kinetin (see Figure 3). The synthetic cytokimns have very high bio!oglcal activity and are not expensive so have wide app~ication in tissue culture. Cytokimns cause tissues to swell, induce the development of axillary buds and the adventmous buds and promote cell division. Their role in inducing axillary bud development through decreasing apicaldommanceis veryimportant in tissue culture. Cytokinins are heat stable and may beadded to the medium pnor to autoclaving.

Gibberellic Acid

This group includes about sixty compounds of which GA3 (see Figure 3) is mostwidespre.ad In plants. Gibberelhc acid is not frequently used in tissue culture work. It isheat labIle and cannot be autoclaved and thus must be added to the medium afterautoclaving using milliporefitration (filter sterilization). Thegeneral role of gibberellicacid is to promote seed germination and toinduce elongation of the internode. It has beenused In culture media to facIlItate elongation of very small shoots and to stimulate embryoformation from callus (Figure 3).

Abscisic Acid

Abscisic acid (ABA see Figure 3) is found widely distributed in plant tissues and isregarded as being a growth inhibitor. It is not often used in tissue culture but it hasspecific applications such as stimulating embryoid development from callus. These roles for ABA may be related to its ability to modify cytokininsynthesis and as a gibberellin antagonist.

Ethylene

Ethylene is a gas of simple structure (Figure 3). It is produced by plants and in closedculture vessels the accumulation of ethylene is believed to result in growth. Ethylene is also produced by some plastic containers andflaming also produces some ethylene. It appears to interact with other growth regulatorsand is implicated in the senescence of plant tissues. Ethylene is produced in response towaterlogging, a condition analogous to *in vitro* culture. At higher concentrations ethyleneisknown to induce vitrification.

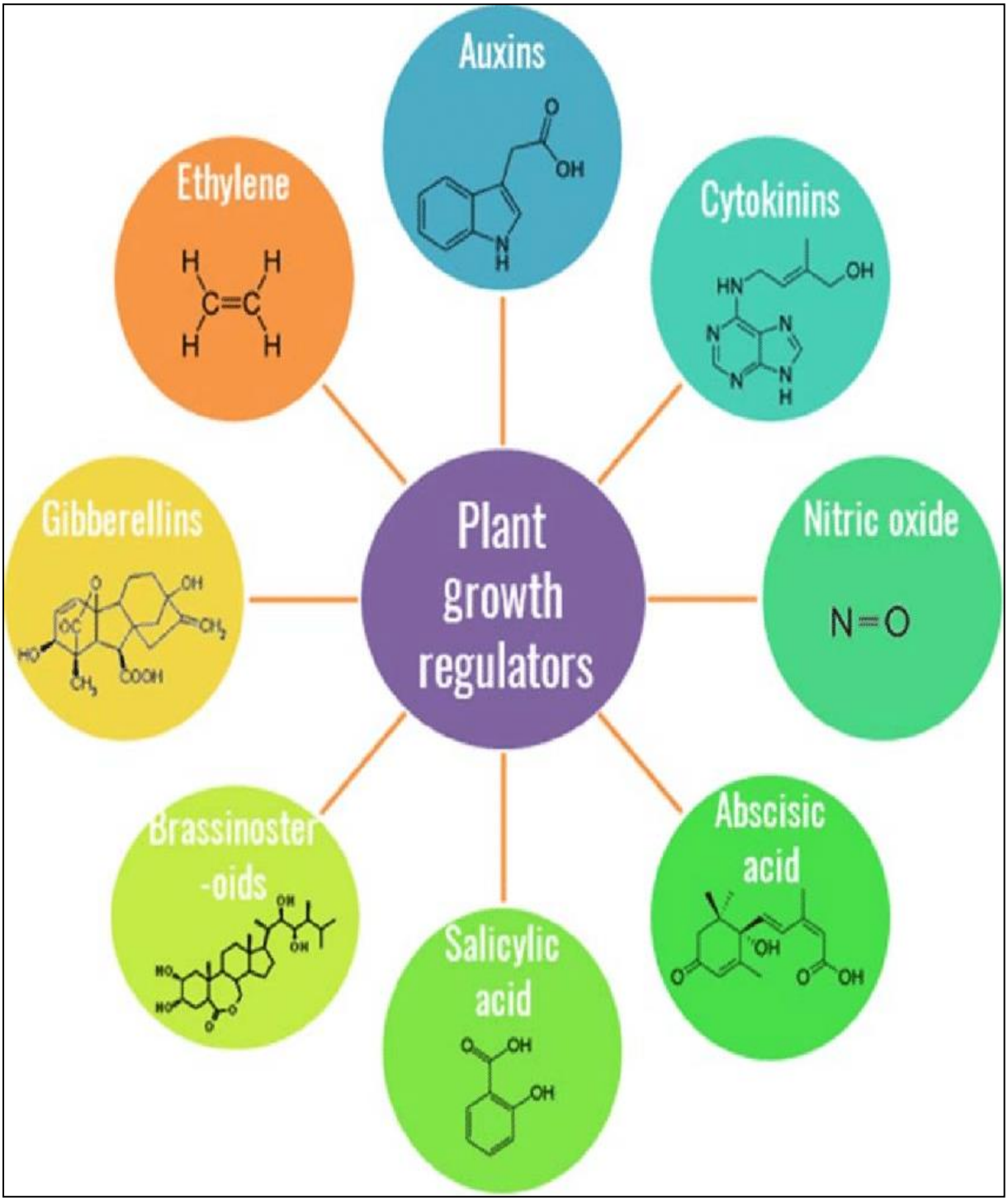

Figure 3. Plant regulator structure

3.6.2. Other Substances

Adenine

Adenine is frequently added to culture media and has been shown to have a beneficialeffect on shoot production. While the mode of action of adenine is unknown it is likelythat it exerts its effects through allowing greater synthesis of natural cytokinins for whichit is a precursor.

Activated carbon

Is used at concentrations from 0.2 to 3% in culture media. Activated carbon has thecapacity to remove substances from the medium by adsorption, thus makipg thesesubstances unavailable to the tissues. It has been shown that actIvated carbon will adsorbgrowth regulators from the medium as well as othersubstances such as polyphenols which are toxic to cell growth. This suggests that thegrowth regulator role proposed for activated carbon is related to lts absorptive propertiesrather than any direct effect. Activated carbon is often used In root InductIon media whereits role may be related to reducing light to enhance root growth as well as absorptlon ofgrowth regulators (see Appendix1for more details).

3.6.3.Hormonale balance

Auxins and cytokininare most commonly used in plant tissue culture. Ratio of their concentrations determines the type of organs produced from the cultured cell or tissues. For example, higher concentration of cytokinin results in shoot regeneration in general. Further, different plant tissues need different amount of hormones for their growth and therefore, depending upon the type of explant, their concentration may vary in the nutrient media.

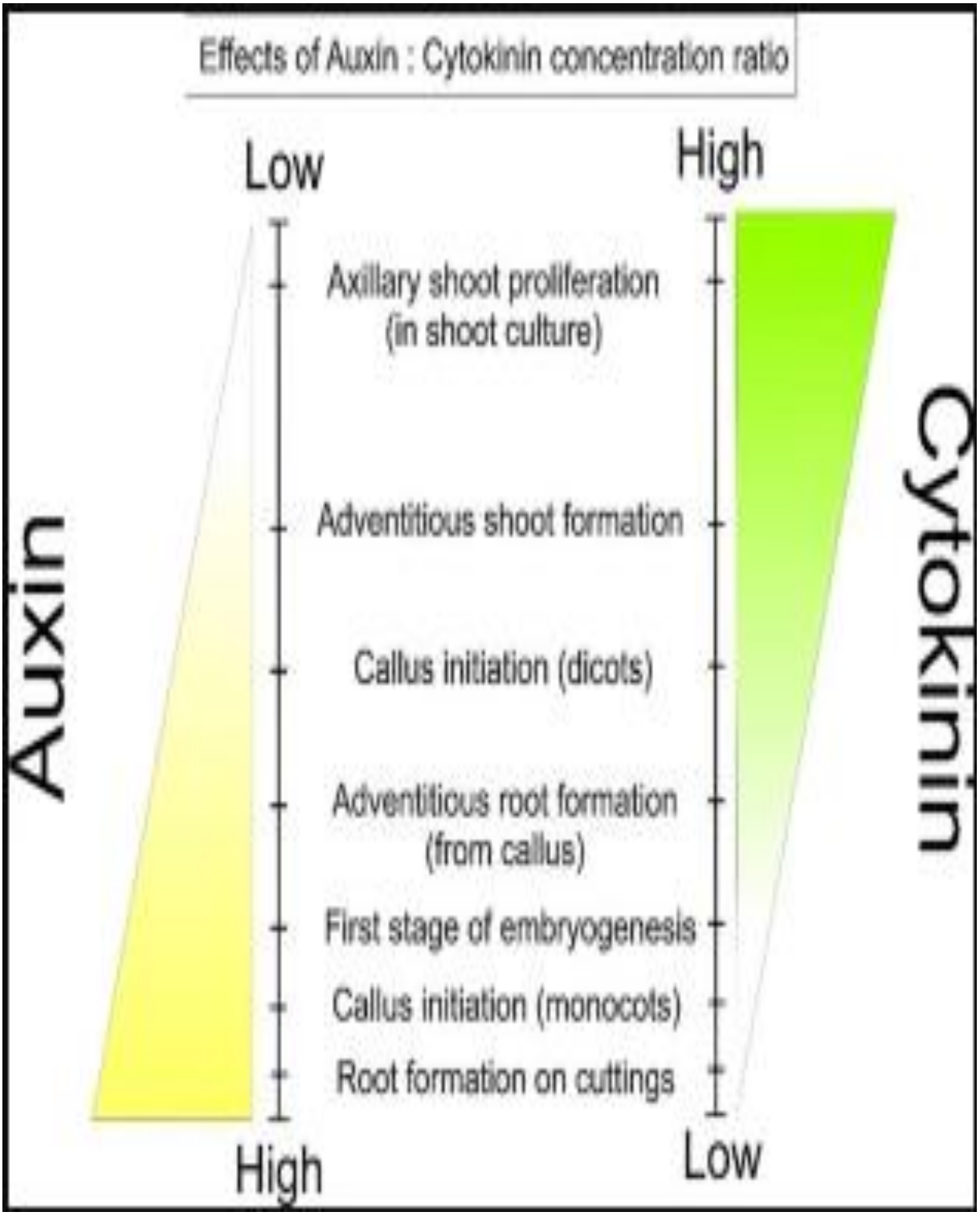

Figure 4. The effects of different ratio of the two hormones; auxin and cytokinins

IV.Applications of Plant Cell and Tissue Culture

4.1. Commercial production of secondary metabolites

The compounds/ biochemical are which are not directly involved in primary metabolic processes like respiration, photosynthesis etc are secondary metabolites. These include a variety of compounds like alkaloids, terpenoids, etc with various biological activities like antimicrobial, antibiotic, insecticidal, valuable pharmacological and pharmaceutical activities. Therefore, micropropagation allows their commercial scale production from cell cultures viz. shikonin derivatives used in dyes, pharmaceuticals are produced from cell cultures of Lithospermumerythrorhizon. Also, cultured cells of many plant species produce novel biochemical's which have otherwise not been detected in whole plants.

4.2. Production of synthetic seeds

Synthetic seed is a bead of gel containing somatic embryo or shoot bud with growth regulator, nutrients, fungicides, pesticides etc needed for development of complete plantlet. These are better propagule as don't need hardening and can be sown directly in field.

4.3. Raising somaclonal variant:

The genetic variability occurring in somatic cells, plants produced in vitro by tissue culture are referred to as somaclonal. When these variations involve traits of economic importance, these are raised and maintained by micropropagation.

4.4. Production of disease free plant:

Most of the horticultural fruit and ornamental crops are infected by fungal, viral, bacterial diseases. Micropropagation provides a rapid method for production of pathogen free plants. In case of viral diseases especially, the apical meristems of infected plants are free or carry very low concentrations of viruses. Thus culturing meristem tips provides disease free plants.

4.5. Micropropagation Methods:

The ability of mature cell to dedifferentiate into callus tissue and the technique of cloning isolated single cell in vitro discussed earlier in this chapter have demonstrated that the somatic cells can differentiate to a whole plant under particular conditions. This potential of cell to divide and develop into multicellular plant is termed as cellular totipotency. To express totipotency, after dedifferentiation, the cell has to undergo redifferentiation or regeneration which is the ability of dedifferentiated cell to form plant or plant organs.

4.6. Germplasm conservation

Germplasm refers to the sum total of all the genes present in a crop and its related species. The conservation of germplasm involves the preservation of the genetic diversity of a particular plant or genetic stock for its use at any time in future. It is important to conserve the

endangered plants or else some of the valuable genetic traits present in the existing and primitive plants will be lost. The germplasm is preserved by following two ways.

(a) In-situ conservationThe germplasm is conserved in natural environment by establishing biosphere reserves such as national parks, sanctuaries. This is used in the preservation of land plants in a near natural habitat along with the several wild types.

(b) Ex-situ conservationThis method is used for the preservation of germplasm obtained from cultivated and wild plant materials. The genetic material in the form of seeds or in vitro cultures are preserved and stored as gene banks for long term use. Cryopreservation plays a vital role in the long-term in vitro conservation of essential biological material and genetic resources. It involves the storage of in vitro cells or tissues in liquid nitrogen that results in cryo-injury on the exposure of tissues to physical and chemical stresses.

4.7. Genetic transformation

Genetic transformation is the most recent aspect of plant cell and tissue culture that provides the mean of transfer of genes with desirable trait into host plants and recovery of transgenic plants. The technique has a great potential of genetic improvement of various crop plants by integrating in plant biotechnology and breeding programmes. It has a promising role for the introduction of agronomically important traits such as increased yield, better quality and enhanced resistance to pests and diseases.

4.8. Protoplast technology Somatic hybridization is an important tool of plant breeding and crop improvement by the production of interspecific and intergeneric hybrids. The technique involves the fusion of protoplasts of two different genomes followed by the selection of desired somatic hybrid cells and regeneration of hybrid plants. Protoplast fusion provides an efficient mean of gene transfer with desired trait from one species to another and has an increasing impact on crop improvement. Somatic hybrids were produced by fusion of protoplasts from rice and ditch reed using electrofusion treatment for salt tolerance.

4.9. Haploid production

The tissue culture techniques enable to produce homozygous plants in relatively short time period through the protoplast, anther and microspore cultures instead of conventional breeding .

4.10. Production of transgenic plants :

With beneficial traits such as herbicide resistance, stress resistance, insect resistance, etc

• Herbicide resistance Weeds are unwanted plants which decrease the crop yields and by competing with crop plants for light, water and nutrients. Several biotechnological strategies for weed control are being used. The biological manipulations using genetic engineering to

develop herbicide resistant plants are: (a) over-expression of the target protein by integrating multiple copies of the gene or by using a strong promoter., (b) enhancing the plant detoxification system which helps in reducing the effect of herbicide., (c) detoxifying the herbicide by using a foreign gene., and (d) modification of the target protein by mutation.e.g. Glyphosate resistance

• Stress resistance Resistance to abiotic stresses includes stress induced by herbicides, temperature (heat, chilling, and freezing), drought, salinity, ozone and intense light. These environmental stresses result in the destruction, deterioration of crop plants which leads to low crop productivity. Several strategies have been used and developed to build resistance in the plants against these stresses.

• Insect resistance

A variety of insects, mites and nematodes significantly reduce the yield and quality of the crop plants. The conventional method is to use synthetic pesticides, which also have severe effects on human health and environment. The transgenic technology uses an innovative and ecofriendly method to improve pest control management.

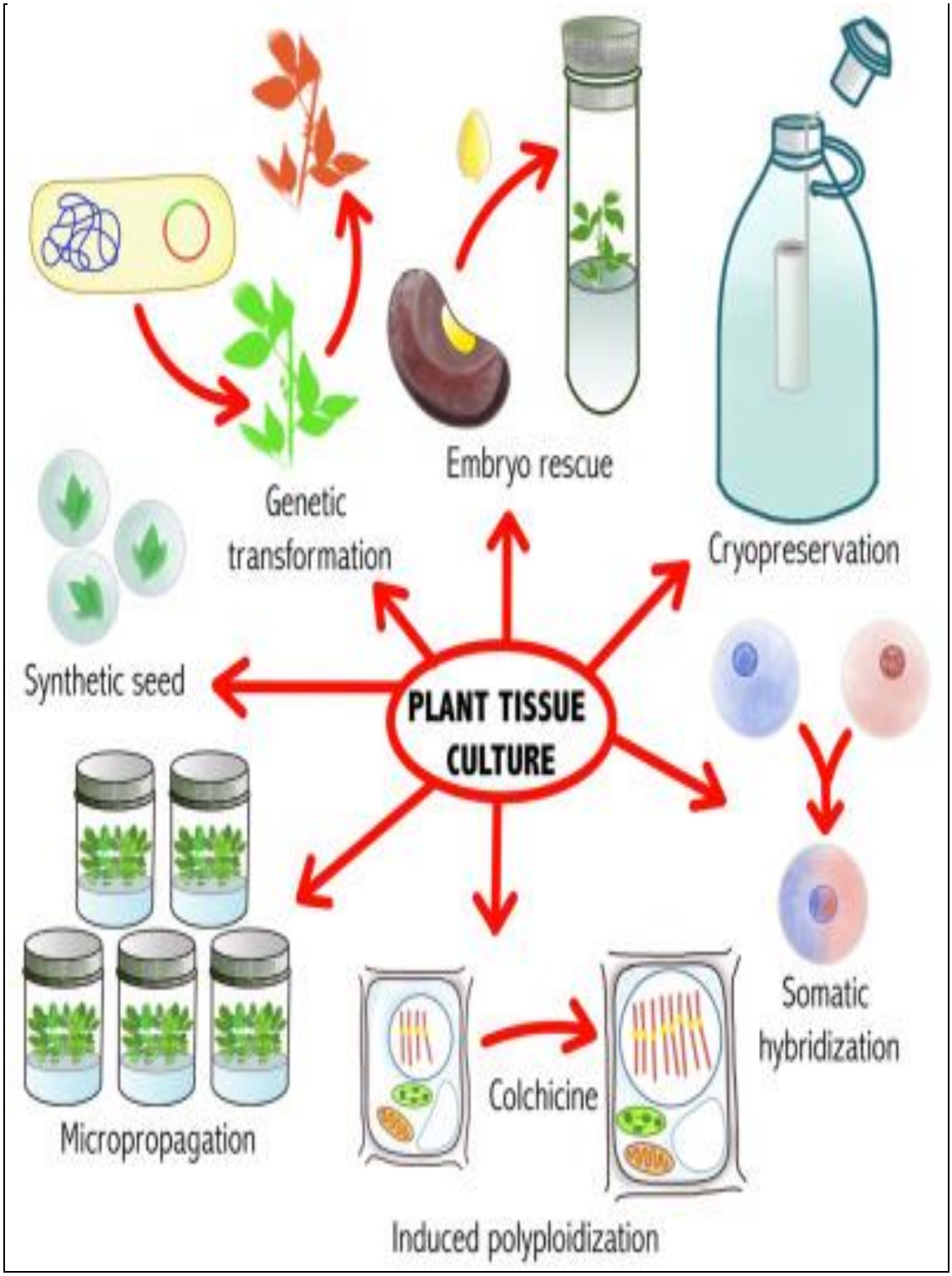

Figure 5. Salient applications of plant tissue culture.

V. Micropropagation

It is a tissue culture technique that is used to multiply plants without sexual reproduction or seed formation. Each of the new plant is genetically identical to its parent and can be called clone. Traditionally, it is done by using cuttings, budding, grafting, corms, tubers or other vegetative propagules. However, these traditional procedures are laborious, dependent on environmental conditions and the success rate is also low. Micropropagation can be used to address the above mentioned problems. It results in rapid multiplication of plants within a short period of time in a small space. Since it is performed under controlled environmental conditions, micropropagation is not season dependent. This method is useful for the multiplication of non-fertile plants, rare plants, endangered plants or other plants for which the character of choice cannot be maintained by sexual reproduction (elite plants). Micropropagation has been employed successfully in agriculture, horticulture and forestry like potato, banana, carnation, chrysanthemum, etc. Banana tissue culture technology is very popular for the supply of disease-free quality planting materials and has been proven to be profitable to the farmers. Banana is one of the major tissue culture-raised crops in India. More than 400 million plantlets are regenerated annually through tissue culture.

Explants used in micropropagation Different kinds of explants have been used in micropropagation. In orchids, shoot tip (Anacamptispyramidalis, Aranthera, Calanthe, Dendrobium, Cymbidium, Odontioda), axillary bud (Aranda, Brassocattleya, Cattleya, Laelia), inflorescence segment (Aranda, Ascofinetia, Neostylis, Vascostylis), lateral bud (Cattleya, Rhynocostylis gigantean), leaf base (Cattleya), leaf tip (Cattleya, Epidendrum), nodal segment (Dendrobium), flower stalk segment (Dendrobium, Phalaenopsis) and root tips (Neottia, Vanilla) are being used in micropropagation. Various steps involved in the process of micropropagation have been listed below: Stage 0: Preparation of donor plant Suitable plant tissue or explant is taken from the plant growing under natural conditions and cultured under in vitro conditions. Stage I: Initiation stage In this stage, initiation and establishment of a culture in a suitable medium is done. The explant is surface sterilized and transferred into a nutrient medium. The surface sterilization removes contaminants with minimal damage to plant cells. The most commonly used disinfectants are sodium hypochlorite, calcium hypochlorite, ethanol and mercuric chloride. Thereafter, the cultures are incubated in a growth chamber either under light or dark conditions. Stage II: Multiplication stage This stage mainly involves the multiplication of shoots or formation of embryo from the explant. The cultures are maintained in growth chamber at of 20– 24° C and light intensity of 2000- to 4000-lux. During this stage, the number of propagules is increased. This is done by repeated subcultures

until the desired number of plantlets is obtained. Stage III: Rooting stage This stage involves the transfer of shoots to a medium that help in the formation of roots. The rooting is induced by addition of plant growth regulators such as auxins in the media. Stage IV: Acclimatization Stage The plants raised under in vitro conditions are weaned and hardened. Hardening is done by transferring the plant from conditions of high to low humidity and from low light intensity to high light intensity. In this stage plantlets are established in the soil. The plantlets of stage III from the laboratory are transferred to the greenhouse. In case of some plant species, shoots are directly planted in pots or suitable compost mix.

Advantages

• Micropropagation has a number of advantages over traditional plant propagationtechniques:

• The main advantage of micropropagation is the production of many plants that are clones of each other.

• used to produce disease-free plants.

• It can have an extraordinarily high fecundity rate, producing thousands ofpropagules while conventional techniques might only produce a fraction of thisnumber.

• regenerating genetically modified cells or cells after protoplast fusion.

• It is useful in multiplying plants which produce seeds in uneconomical amounts,or when plants are sterile and do not produce viable seeds or when seed cannot bestored (see recalcitrant seeds).

• Some plants with very small seeds, including most orchids, are most reliablygrown from seed in sterile culture.

• A greater number of plants can be produced per square meter and the propagulescan be stored longer and in a smaller area.

Disadvantages of micropropagation

• Expensive laboratory equipments are required.

• Plants are not autotrophic during culture conditions.

•The plants are poorly adapted to field conditions and need to be acclimatized before transferring to field.

• There is a risk of genetic changes.

• Mass propagation is not possible for all crops. For e.g. plants such as cowpea is highly recalcitrant, and attempts to regenerate it using in vitro-cultured explants have not been very successful.

• Adult woody plants cannot be easily regenerated via this technique.

• The induction of roots is not easily achieved in many species.

• Each explant has different in vitro growth rates and maturation, hence uniform growth of plants cannot be obtained from tissue culture in the case of many flowering plants.

5.1. Meristem Tip Culture

The active growing point of the plant shoot is the rieristem. This is a small organ composed of rapidly dividing (meristematic) cells. For propagating potato shoot cultures, it is the ideal starting material as it has two favorable characteristics.

- The isolated meristem develops in culture in a genetically stable form. This is not the case, for example, in disorganized callus cultures that show major genetic irregularities. - The isolation of meristems reduces the level of viral infection in the tissue and, under appropriate conditions, can be used for complete pathogen eradication. The dome of a shoot apical meristem contains the truly meristematic cells and is surrounded by leaf primordia and primary leaves. Since the more differentiated vascular tissues occur away from the meristem (towards to older tissues of the stem), the vascular elements of the leaf primordia are still very incipient, and these elements have not yet made contact with the main strand of the vascular system in the stem. Therefore, virus particles, which may be present in the vascular system, can reach the meristemati6 region of the apex only through movement from cell to cell. This is one of the main reasons why in a virus infected plant, virus concentration decreases acropetally toward the meristem of the apical as well as the axillary buds.

In many areas of a plant there are small regions where cell division is nuch more rapid than in others. These areas of rapid cell division are referred to as meristematic regions or simply meristems. Any of the several meristems could be used for culturing but the most practical for dahlias seems to be the one near the tip of the growing shoots. This meristem is not extreme tip of the growing shoot but is located a few rows of cells behind or back of the tip. The meristem is exposed by removing as many leaves as practicable from the growing shoot. The growing tip then appears rounded, shiny and semi-transparent. When the visible leaves have been removed and the shoot tip placed under a fairly strong microscope several protuberances may be seen about the rounded tip. These are leaves in the process of being formed (an example of cell differentiation) and are called primordial leaves. As many of these are removed as possible without damaging the rounded shoot tip:

This tip, very tiny (smaller than a period) , is then cut from the shoot and placed in a culture tube containing the nutrient solution. The culture tubes are then capped and placed in an environment with controlled heat and artificial light to facilitate their development into a plant. All this must be done under extremely sanitary conditions to prevent contamination of the cuttings or tube by fungus spores.

5.1.1 Mersitem culture - Why meristems are virus free?

Failure to invade meristem is due to:

Lack of a vascular system. Spreading cell to cell via plasmodesmata which are too small to allow the passage of virus particles

High metabolic activity: active mitosis – the synthesis of RNA for viral multiplication may suppressed. Active metabolic process which is not suitable for virus multiplication

High auxin concentration in meristematic cells in hibit virus multiplication : interfere nucleic acid metabolism

Competition for nutrients enzymes for virus replication

5.2. Somatic embryogenesis

Is the process where in somatic cells differentiate into somatic embryos.

It is not a naturally occurring process, an artificial one wherein an embryo or plant is obtained from one somatic cell. Somatic embryos take form from the cells of the plants, which usually do not take part in embryo development. Neither a seed coat nor endosperm is formed around the somatic embryo.

In the process, one cell or a cluster of cells initiates the developmental route, which results inreproducible regeneration of non-zygotic embryos, which can germinate for the formation of an entire plant. The cells which are derived from potential source tissues are subject to a culture medium for the formation of an undifferentiated cluster of cells referred to as the callus. In the tissue culture medium, the plant growth regulators can be formed for the induction of the formation of calluses and hence modified to induce the embryos for the formation of calluses.

5.2.1. Process of Somatic Embryogenesis

The somatic embryogenesis procedure is a three-step procedure, which causes the induction of embryogenesis, development of the embryo and its maturation.

The principle of somatic embryogenesis finds its basis on the topic of totipotency of the plant cells; it illustrates two facets of plant embryogenesis:

- The process of fertilization can be replaced by an endogenous mechanism.
- The other types of cells of the plant, apart from the fertilized egg cells, can retrieve the capacity to form an embryo.

Since the process of somatic embryogenesis does not entail the procedure of fertilization, it promotes the large scale propagation of plants at a faster rate. In addition, it also assists in the genetic transformation of plants, serving as a promising resource for the cryo-storage of the embryo and germplasm.

5.2.2. Somatic embryogenesis induction

Cells are reactivated to differentiate and develop embryos, which occur through two processes:

Direct somatic embryogenesis and indirect somatic embryogenesis.

5.2.3. Direct somatic embryogenesis

It involves the development of the embryos in a direct way from the cells of the explants, such as the cells of the immature embryos. Here, there is no intermediary stage (like the formation of the callus). The explants of the somatic embryogenesis are seen to entail PEDCs (pre-embryogenic determined cells).

5.2.4. Indirect somaticembryogenesis

It includes the formation of somatic embryos by reiterating numerous cycles of cell divisions. It includes intermediary steps of growth of the callus, and hence the process includes multiplesteps. The cells which do not carry the pre-embryogenic determined cells are caused todifferentiate for the formation of the embryo by revealing different treatments. The cells modify into IEDs (induced embryogenic pre-determined cells).

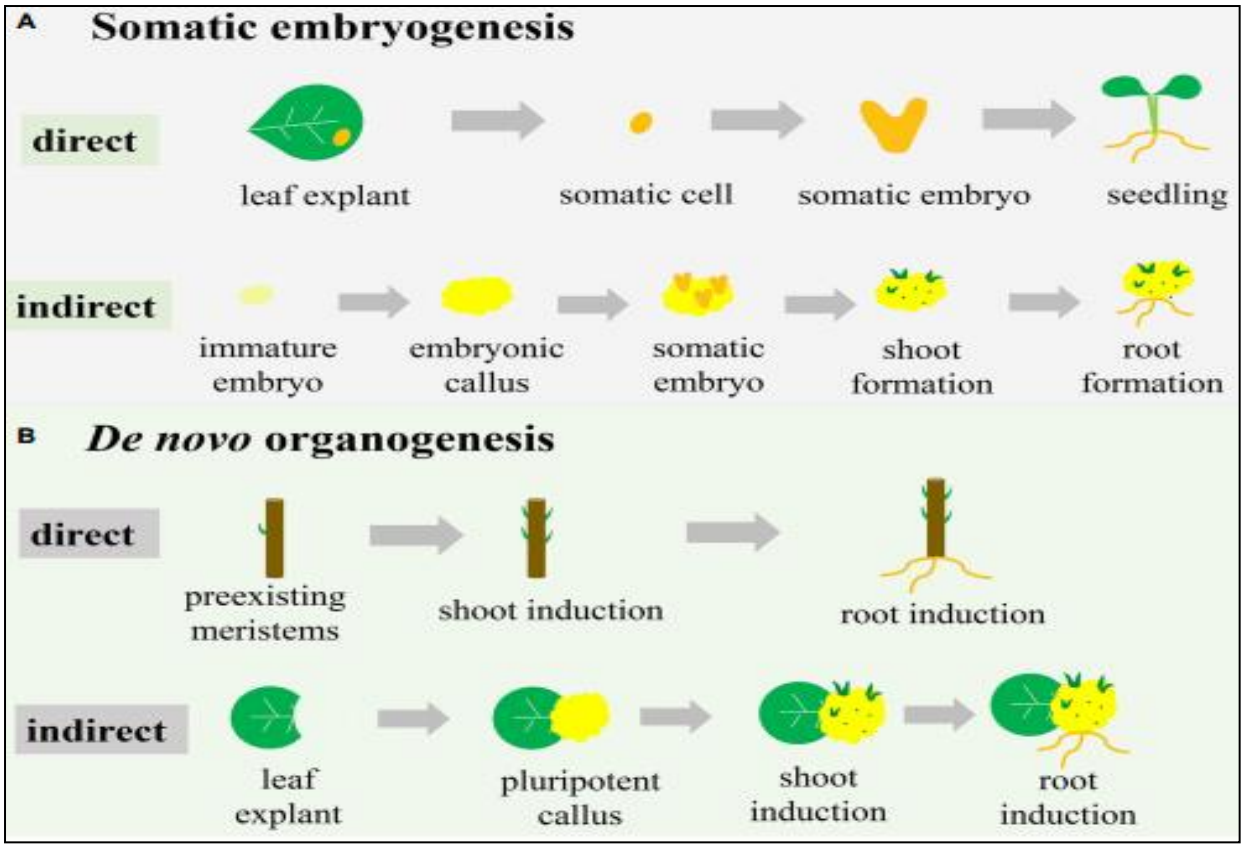

Figure 6. Different pathways of plant regeneration. (A) Somatic embryogenesis. In the direct pathway, the somatic cell originated from explants (e.g., a leaf) is induced to form the somatic embryo, which subsequently drives the development of the whole plant. In the indirect pathway, the explant (e.g., an immature embryo) is induced to initiate the embryonic callus, on which somatic embryos are formed to subsequently develop shoots and roots. (B) De novo organogenesis. In the direct pathway, shoots and roots are induced directly on the stem with pre-existing meristems. In the indirect pathway, pluripotent callus is produced around the wound in a leaf explant, with formation of shoots and roots subsequently induced.

5.2.5. Procedure:

1. Wash seeds by submerging in water with a few drops of detergent in a beaker and shakeby hand, or wrap seeds in two layers of cheese cloth/muslin cloth/nylon pouch and then washwith water.
2. Submerge the seeds in 70% alcohol for 30-60s. Decant the alcohol.
3. Transfer the seeds to a flask or beaker containing 20-40% commercial sodiumhypochlorite for 15-20 min. Rinse4xwithsteriledistilled water.
4. Place 2-3 seeds per culture vessel on the surface of MS agar medium.
5. Incubate the cultures at 25°C under 16 h photoperiod with -1000 lux light intensity for 1-2weeks.

6. Collect the germinated seedlings when the cotyledons are fully expanded. Place eachseedling on a sterile petri dish and excise the hypocotyl from each seedling and cut themtransversely into two parts.

7. Place the hypocotyl sections on the following medium: MS + 1-2 mg/l 2,4-D.

8. Incubate the cultures in dark at 25°C for 4-8 weeks.

9. Maintain the callus by subculturing small pieces on fresh medium every 3- 4 weeks. Callus will contain pro- embryo initial cells as well as minute microscopic embryos in the earlystages of development.

10. Place 0.5 to 1 cm 2 callus pieces on MS agar medium without growth regulators and ncubate the cultures at 25°C under the 16h photoperiod with ~1000 lux light intensity.

Within 2-3 weeks of cultures will exhibit embryos and green plantlets.

11. Tease out individual or group of plantlets from the callus mass and transfer on halfstrength MS medium under 16h photoperiod with high light intensity of ~5 lux. Within 4-5weeks the cultures will resemble seedling carrots.

12. Transfer the plantlets to small pots containing sterile peat moss and vermiculite in a 1:1ratio. Enclose the plantlets with plastic containers to maintain high humidity.

13. Transfer the plants to soil and follow the procedure of plant establishment and hardening.

5.2.6.Growth Regulators and Other Factors for Somatic Embryogenesis

In most species an auxin (generally 2,4-D, at 0.5-5 mg/l) is essential for somaticembryogenesis. The auxin causes dedifferentiation of a proportion of cells of the explant, which begin to divide. Eg In carrot, these small, compact cells divide asymmetrically, and their daughter cells stick together to produce cell masses called proembryogenic masses or embryogenic clumps (ECs). In the presence of auxin, the ECs grow and break up into smaller cell masses, which again produce ECs. But when the auxin is either removed or reduced (0.01-0.1 mg/l) and cell density is lowered, each EC gives rise to few to several SEs; each SE is believed to develop from a single superficial cell. The ability to regenerate SEs, i.e., totipotency, is acquired by cells during dedifferentiation in response to high auxin treatment but the mechanism is not well known. Some glycoprotein's produced by totipotent cellmasses are secreted into the medium; when these proteins are added into the culture medium they speed up the process of acquisition of totipotency. A class of proteins, called arabinogalactan proteins, induces SE regeneration in undifferentiated Eg In carrot cells, indicating their role in the process. Auxins promote hypermethylation of DNA whichmay have a role in totipotency acquisition. In many species like Eg*Embeliaribes*, In carrot,coffee; alfalfa etc., somatic embryogenesis is a two step process:

(i) SE induction on high auxin (up to 40-60 mg/l, 2, 4 D).

(ii) SE development on a low auxin or OR-free medium.

In the SE induction phase, explant cells dedifferentiate, became totipotent and, in manyspecies, form embryogenic clumps (ECs). Cells can be maintained in embryogenic stage on the induction medium for prolonged periods (over 10 years in carrot). When ECs are transferred from induction medium to an appropriate medium, SE differentiation proceeds from globular, heartshaped, torpedo to cotyledonary stages; this is called SE development phase. Clearly in species like Eg In carrot, etc., OR requirements for the two phases are drastically different.

In most cases, SEs begin to germinate immediately after the cotyeledonary stage; thisis called SE conversion. But often the plantlets so obtained are rather weak. It is, therefore,desirable to subject SEs to a maturation phase, following their development; in this phase theSEs usually do not grow but undergo biochemical changes to become more sturdy and hardy.SE maturation is achieved by culturing than on a high sucrose (up to 6% or even 40%) medium or in presence of a suitable concentration (0.2-0.4 mg/l) of ABA, or by subjecting them to partial desiccation. In most species, SE maturation improves their conversion, often by several-fold. In some species, e.g., embeliaribes etc., SE induction and development may take place on the same high auxin medium, although the frequency of mature embryos is rather low. In some species, SEs are produced in response to a cytokinin, e.g., BAP induces SEs in hypocotyls of young zygotic embryos of EgInTrifolium sp., pea, etc. But SEs are produced on immature cotyledons of these explants when 2,4-D is used in the medium. It seems that cytokinins are effective in SE regeneration from embryogenic cells of young zygotic embryos, while auxins are effective on differentiated cells of both embryos and somatic tissues. Many workers have used combinations of auxins and cytokinins for SEregeneration in different species, but the role of cytokinin in these systems is not known.

6. Modification of plant's genome

6.1. Haploid production through androgenesis and gynogenesis

- **Methods based on in vitro androgenesis**

The higher plants are normally diploid, with two sets of chromosomes in their somatic cells. Haploids (with one set of chromosomes) arise in nature by parthenogenesis due to malfunction in the normal sexual process. Haploids are sterile having only a single set of chromosomes and can be converted into homozygous diploids by spontaneous or induced chromosome doubling. Haploid plants are of great significance as they produce homozygous lines (homozygous plants). The production of haploids was first discovered in 1921 by Bergner in *Datura stramonium*. The development of haploid embryos and plantlets from microspores of *Datura innoxia* by the cultures of excised anthers was first reported by Guha and Maheshwari. To date androgenic haploids of over 200 species including many major crop plants (cereals, mustards, potato, and tomato) have been developed. For the culture, anthers at the late uninucleate stage of microspore development are excised from surface-sterilized buds and cultured on a nutrient medium. A low temperature (4-5° C) treatment is given for initial 2-3 days to enhance the androgenic response. However, in some cases like Brassica, a higher temperature (30-35° C) is required. The microspores undergo repeated divisions to form multicellular structures. Such structures directly develop into an embryo or form a callus from which plants are regenerated via organogenesis or embryogenesis. A summary of the standard protocol/procedure employed in raising the androgenic haploids is outlined in Fig 7.

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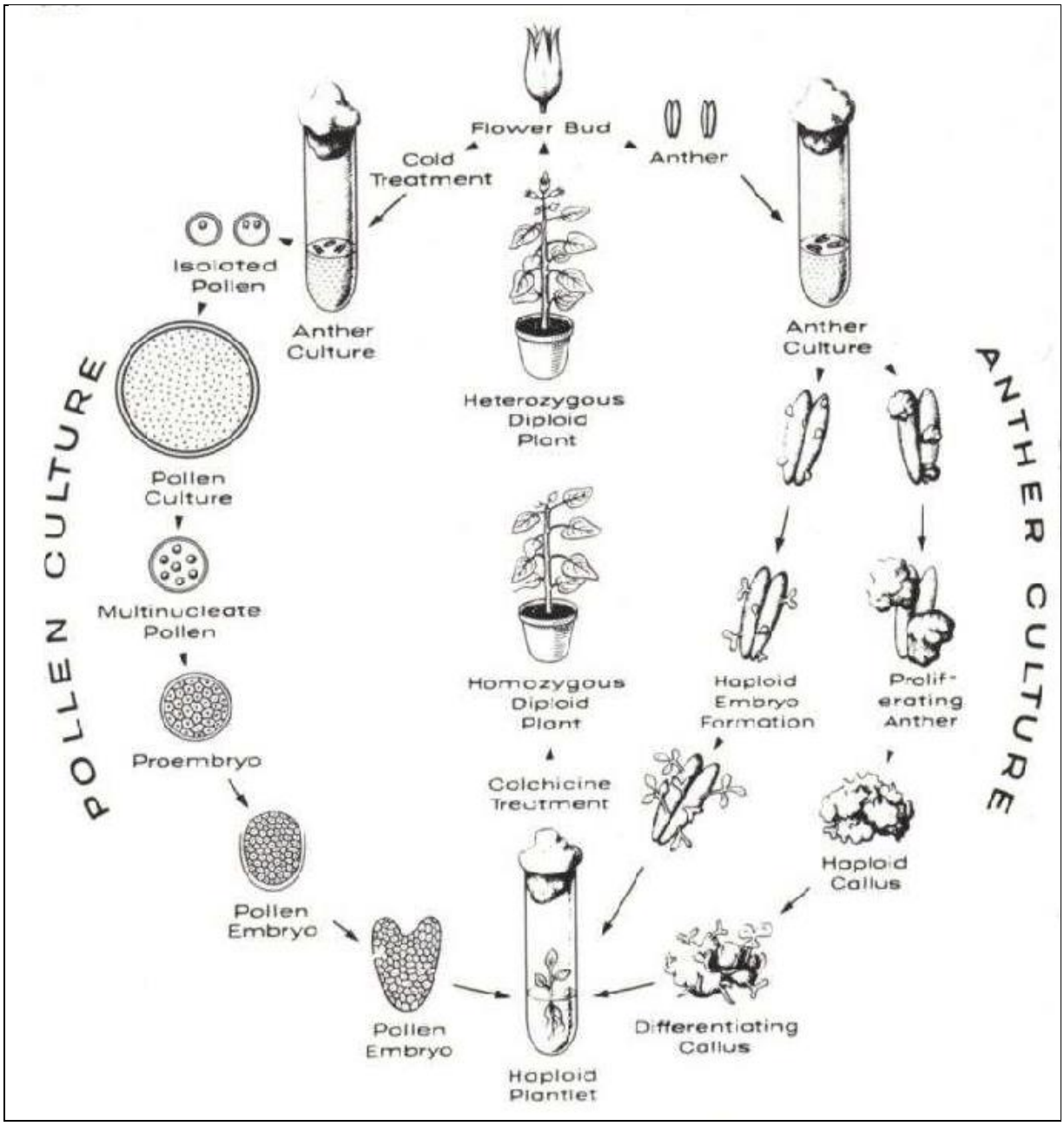

Figure 7. Diagrammatic representation of the production of plants by anther and pollen culture.

- **Methods based on in vitro gynogenesis**

Gynogenesis would exploit the ability of egg cells to develop in the embryo sac as a haploid zygote without fertilization. This alternative to the normaldevelopment of the megagametophyte was first described in vitro in 1976 by San et al.. It would therefore be a form of female haploid parthenogenesis (from the Greek words parthenos, meaning "virgin" and genesis, meaning "origin". In some species, the gynogenic embryo is believed to originate from antipodal or synergid cells, but in the vast majority of cases the gynogenic embryo is derived from the egg cell. Gynogenic embryos are mostly haploid, which implies that in order

to obtain the desired double haploid, the application of additional treatments for chromosome duplication should be considered in nearly all cases. As in the case of androgenesis, colchicine is the most effective and therefore the most widely used antimitotic The success of gynogenesis induction is influenced by many different factors, including the developmental stage of the embryo sac and the in vitro culture conditions. However, the genotype is the most important, even more than for microspore embryogenesis. In fact, this is the most limiting factor for the practical application of this technique, since there are very few responsive genotypes, much less than those that respond to microspore embryogenesis. Other limitations include a low efficiency, much lower than microspore embryogenesis (there are much less egg cells than microspores in a flower), a very low rate of spontaneous duplication of the genome, and low levels of embryo regeneration, perhaps due to the instability of haploid genomes, prone to chromosomal alterations. All these limitations make in vitro gynogenesis-based approaches a secondary alternative, used in a reduced range of species where other in vitro approaches (microspore embryogenesis) have proven ineffective

6.1.2. Applications of Haploids

i)Using this technique, homozygous plants can be obtained in a relatively short time as compared to the conventional breeding methods. The doubling of chromosomes is achieved by colchicinestreatment which restores the fertility of plants. The double haploids show the potential to become pure breeding new cultivars.

ii) Haploids help to detect recessive mutations which are normally not expressed in heterozygous diploids.

iii) This technique also saves the plant breeders from the laborious and lengthy procedure of inbreeding.

iv) In vitroandrogenesis allows the breeders to detect gametoclonal variation (variations occur due to recombination and segregation during meiosis).

v) Anther culture has also contributed to the production of super males (male populations with desirable features associated with male plants).

vi) Since the haploids show chromosomal instability, they can be used for the introduction of alien chromosomes or genes in breeding programs. Genetically transformed haploids have shown promising results by exhibiting resistance to various biotic and abiotic stresses.

6.1.3. Diploidization of haploid plants

Haploids plants are sterile as these plants contain only one set of chromosomes. Bydoubling their chromosomes number, the plants can be made fertile and resultantplants will be homozygous diploid or isogenic diploid. The fertile homozygous diploidplants are more important than the sterile haploid plants and can be used as pure linesin breeding programme. Haploids plants can be diplodized by following methods:

i) Colchicine Treatment: Colchicine has been utilised widely as spindle inhibitor toinduce chromosome duplication and to produce polyploid plants. The youngplantlets are treated with 0.5% colchicine solution for 24-48 hrs. Treated plantletsafter thorough washing.

ii) Endomitosis: Haploids cells are unstable in culture and have tendency to undergoendomitosis. i.e chromosome duplication without nuclear division. This propertycan be used for obtaining homozygous diploid plants.

iii) Fusion of Pollen Nuclei: Homozygous diploid callus or embryoids may arise fromthe spontaneous fusion of two similar nuclei of the cultured pollen after first division.In Brassica, the frequency of spontaneous nuclear fusion in microspore is high inculture.

6.2. Somatic hybridization

Somatic hybridization is a technique which allows the manipulation of cellular genomes by protoplast fusion. Its major contribution to plant breeding is in overcoming common crossing barriers among plant species and in organelle genetics and breeding. Several reviews on different aspects of somatic hybridization were published recently. Several steps are involved in the somatic hybridization process such as (1) the source of protoplasts, (2) the isolation of protoplasts, (3) plating of protoplasts, (4) regeneration of plants, (5) fusion of protoplasts, (6) selection procedures, and (7) identification and characterization of the somatic hybrid plants.

6.2.1. Importance of Protoplast Isolation And Culture

The isolation, culture and fusion of protoplast are one of the most fascinating fieldsof research. The techniques are important for the following reasons:- – To develop novel hybrid plant through protoplast fusion, genetic engineering would continued to be an exciting area of research in modern plant biotechnology. This technology holds great promises to synthesise a plant of desired characteristics. – This helps incrop improvement by somatic hybridisation and cell modification. – The protoplast in culture can be regenerated intoan entire plant. – Itprovides a tool for isolating protoplasts and exploring the possibilities of genetic engineering. – The technique in future will be one of the most frequently used research tools for tissue culturists, physiologists, pathologists molecular biologists, cytogenetics and biotechnologists

6.2.2. Methodologies for Protoplast Isolation and Regeneration

Plant protoplasts were first isolated enzymatically, by Cocking in 1960 from tomato root cells. Subsequent physiological studies over the intervening years have provided efficient methods for the release and maintenance of abundant quantities of viable plant protoplasts from a myriad of plant species, cell, tissue, and organ types. Currently, isolated protoplasts playa key role in elucidating our understanding of cell biology, structure, function, and in emerging studies on gene transfer and manipulation. Protoplast research is regarded as laborintensive, and requires meticulous attention and timely observations during the course of experiments that are conducted primarily through an empirical approach. This research field is, however, progressively moving toward more objective evaluations and statistical analysis of experiments. For example, most workers now routinely use Calcofluor and trypan blue, FDA or similar compounds to determine cell wall regeneration and viability respectively. Nevertheless, it still remains a common practice to publish only the in vitro protocol which succeeded, without reference or comparison to control or other variable treatments. The growing use of experimental designs and concomitant quantitative data collection and analysis should lead to a better interpretation of the role that various genotypes, media, and environmental factors play in determining cellular growth patterns. Recent review articles provide a thorough insight into the overall progress made in methodologies to isolate and culture plant protoplasts and regenerate and genetically characterize the plants derived from them. In the following sections the focal point is on more recent technological changes which have been implemented by researchers in this field to attain efficient protoplast culture schemes and the regeneration of plants.

6.2.3. Source of Protoplasts Preliminary tissue culture studies using leaf tissues and derived callus cultures of the species to be studied are often valuable to protoplast work; especially in providing information on the conditions required to obtain shoot regeneration. For example, some studies included such experiments on the regeneration of callus cultures of Trigonella, and showed an association between a morphogenic cell suspension culture and the ability of derived protoplasts to regenerate plants of barley. In the former study, the protoplast-derived callus required a different medium formulation for shoot regeneration than did the leaf explant callus. However, the tissue culture experiments were beneficial because the medium which induced callus from leaf explants also was used to initiate and maintain the cell suspension cultures from which protoplasts were isolated. The common approach taken in devising a regenerating protoplast scheme for a plant species, particularly dicots, has been to use leaf tissues as the initial cell source. In general, leaf cells are available in quantity and are readily

digested by the use of commercial enzyme(s) treatment to release ample protoplasts. Another reason for using leaf material initially is that the regeneration capacity may be optimized by a shorter plant cell to intact plant cycle compared to suspension cultures. More recently, however, in dicots seedling tissues such as soybean cotyledon, browallia hypocotyl and root, or leaf-derived callus of apple, as examples, are becoming more popular. Still another advantage of mesophyll- or seedling-derived protoplasts is the stable chromosome number, which may be upset in suspension culture protoplasts.

6.2.4. Protoplast fusion

Protoplast fusion has opened up a novel approach to raising new hybrids. This technique of hybrid production through the fusion of body cells, bypassing sex altogether, is called somatic hybridization. Somatic cell fusion, thus, offers new ground to achieve novel genetic changes in plants.

During enzymatic degradation of cell walls some of the adjacent protoplasts fuse together forming homokaryons (also referred to as homokaryocytes, each with two to several nuclei.

This type of protoplast fusion, called 'spontaneous fusion', has been ascribed to the expansion and subsequent coalescence of the plasmodesmatal connections between the cells. The occurrence of multinucleate fusion bodies is more frequent when protoplasts are prepared from actively dividing cultured cells.

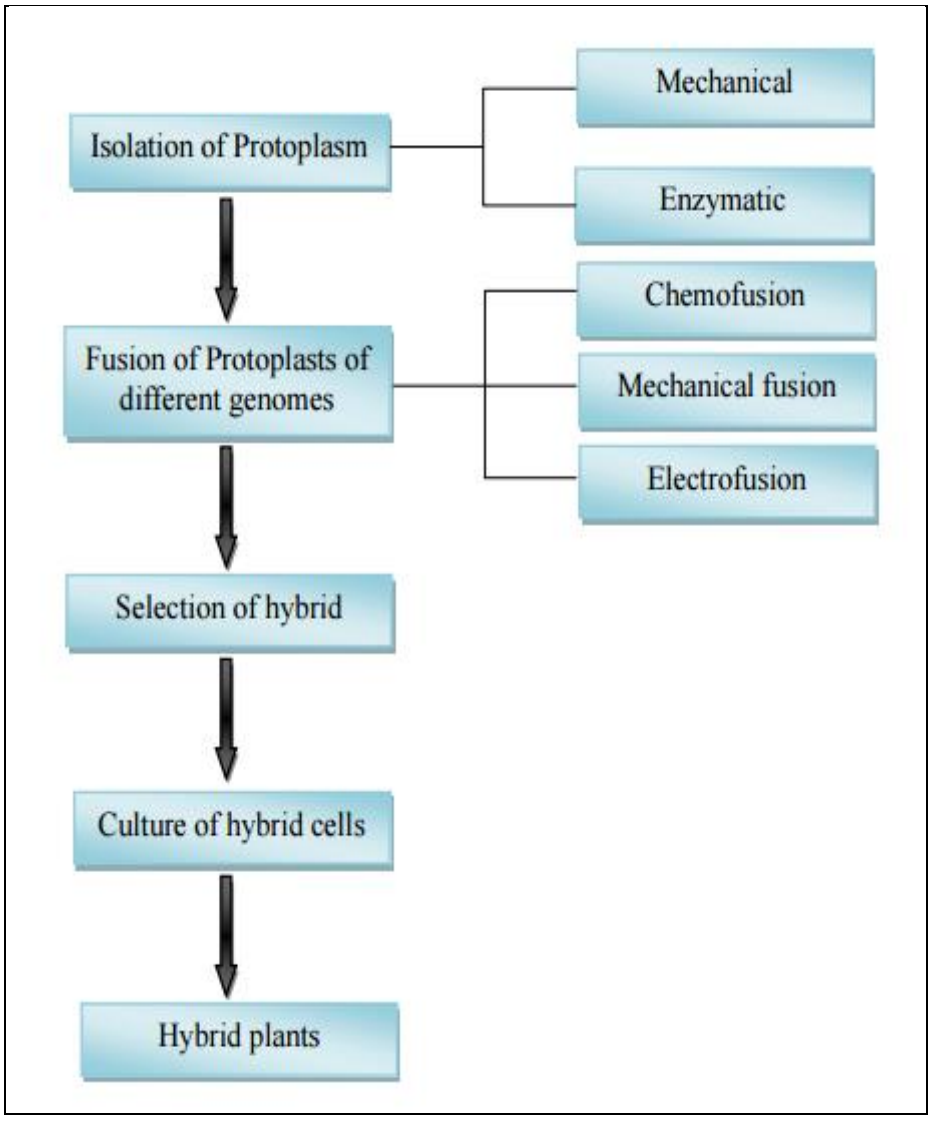

Figure 8. Schematic representation of production of hybrid plant via protoplast fusion

6.2.5. Chemical fusion

(i) NaNO treatment:

As early as 1909, Kuster demonstrated that a hypotonic solution of NaNO3 induces the fusion of sub-protoplasts within a plasmolysed epidermal cell.

(ii) High pH and high Ca^{+2} treatments:

In 1973 Keller and Melchers reported that mesophyll protoplasts of two lines of tobacco could be readily fused by treating them in a highly alkaline (pH 10.5) solution of high Ca^{+2} ions (50 mM CaC12.2H20) at 37~ for about 30 min.

(iii)Polyethylene glycol (PEG) treatment:

PEG has achieved widespread acceptance as a fusogen of plant protoplasts because of the reproducible high frequency heterokaryon formation and comparatively low cytotoxicity to

most cell types. Another merit of PEG-induced fusion, over the other two methods of chemical fusion of protoplasts, is the formation of a high proportion of binucleateheterokaryons.

Briefly, the freshly isolated protoplasts from the two selected parents are mixed in appropriate proportions and treated with 15-45% PEG (1500-6000 MW) solution for 15-30 min followed by gradual washing of the protoplasts with the culture medium. Kao et al. (1974) observed that eluting PEG with a highly alkaline solution (pH 9-10) containing a high 376 Ca 2+ ion concentration (50 mM CaC12.2H20) led to a higher frequency of fusion than washing with the culture medium (see also Kao and Wetter, 1977). This method, which is essentially a combination of the original PEG method described by Kao and Michayluk (1974) and the high pH high Ca 2§ ions method of Keller and Melchers (1973), is currently the most widely used method for plant protoplast fusion.

6.2.6. Several factors affect protoplast fusion by PEG:

(a) PEG of molecular weight (MW) higher than 1000 induces tight adhesion and high frequency fusion of protoplasts. Generally, PEG of MW 1500-6000 has been used at concentrations ranging from 15 to 45%.

(b) PEG-induced fusion is enhanced by enriching the PEG solution with Ca^{+2} ions.

(c) The dilution of PEG should be gradual. Drastic elution would result in the formation of very few heterokaryons.

(d) Prolonged incubation in PEG solution reduces heterokaryon formation.

(e) Protoplasts from young leaves and fast growing calli give better fusion.

(f) Excessive dilution of the enzyme solution leads to poor fusion, probably because of rapid wall synthesis by the protoplasts.

(g) The types of enzymes and their concentrations used for protoplast isolation is another factor influencing protoplast fusion.

Mechanism of fusion. Protoplast fusion consists of three main phases: (a) agglutination, during which the plasma membrane of two or more protoplasts are brought into close proximity, (b) membrane fusion at small localized regions of close adhesion resulting in the formation of cytoplasmic continuities or bridges between protoplasts and, (c) rounding-off of the fused protoplast due to the expansion of the cytoplasmic bridges forming spherical hetero- or homokaryons. Protoplast adhesion, which is temperature independent, can be induced by a variety of treatments but this does not necessarily lead to membrane fusion.

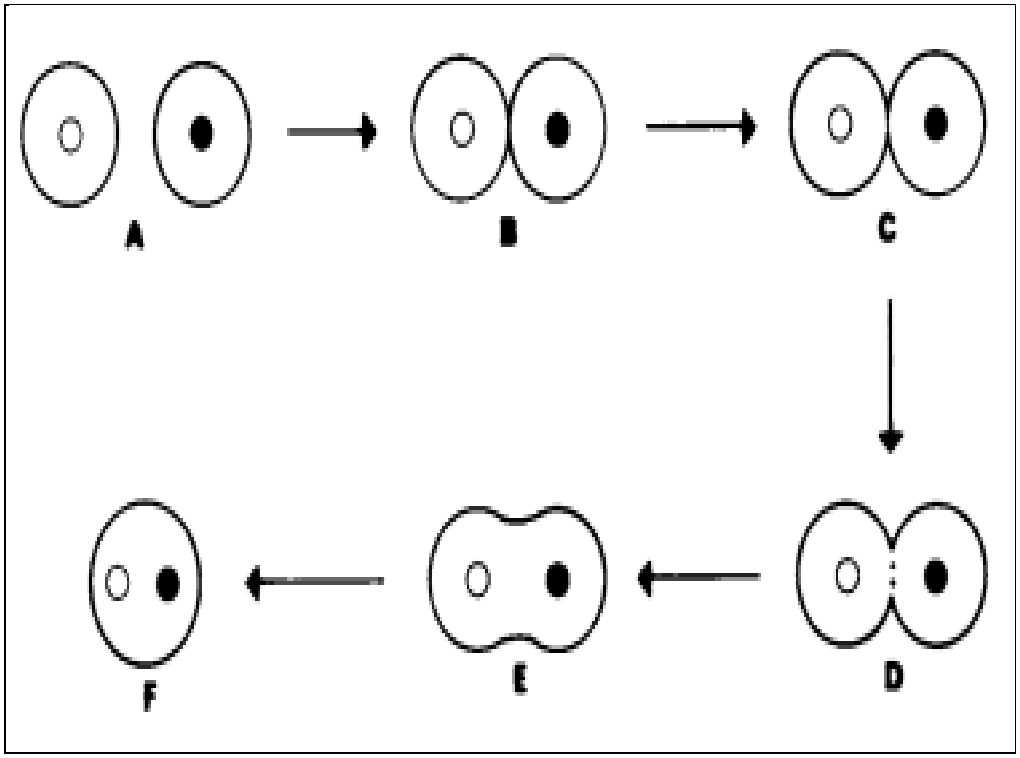

Figure 9. Diagrams showing the sequential stages in protoplast fusion. (A) Two separate protoplasts. (B) Agglutination of two protoplasts. (C,D) Membrane fusion at localized sites. (E,F) Formation of a spherical heterokaryon.

6.2.7. Electrofusion

Chemical fusion of plant protoplasts has many disadvantages: (1) The fusogens are toxic to some cell systems. The destruction of mitochondria following PEG treatment at fusogenic level. (2) It produces random, multiple cell aggregates. (3) The fusogenmust be removed before culture. In contrast, electrofusion (Fig. 10), is rapid (usually complete within 15 min), simple, synchronous, and more easily controlled.

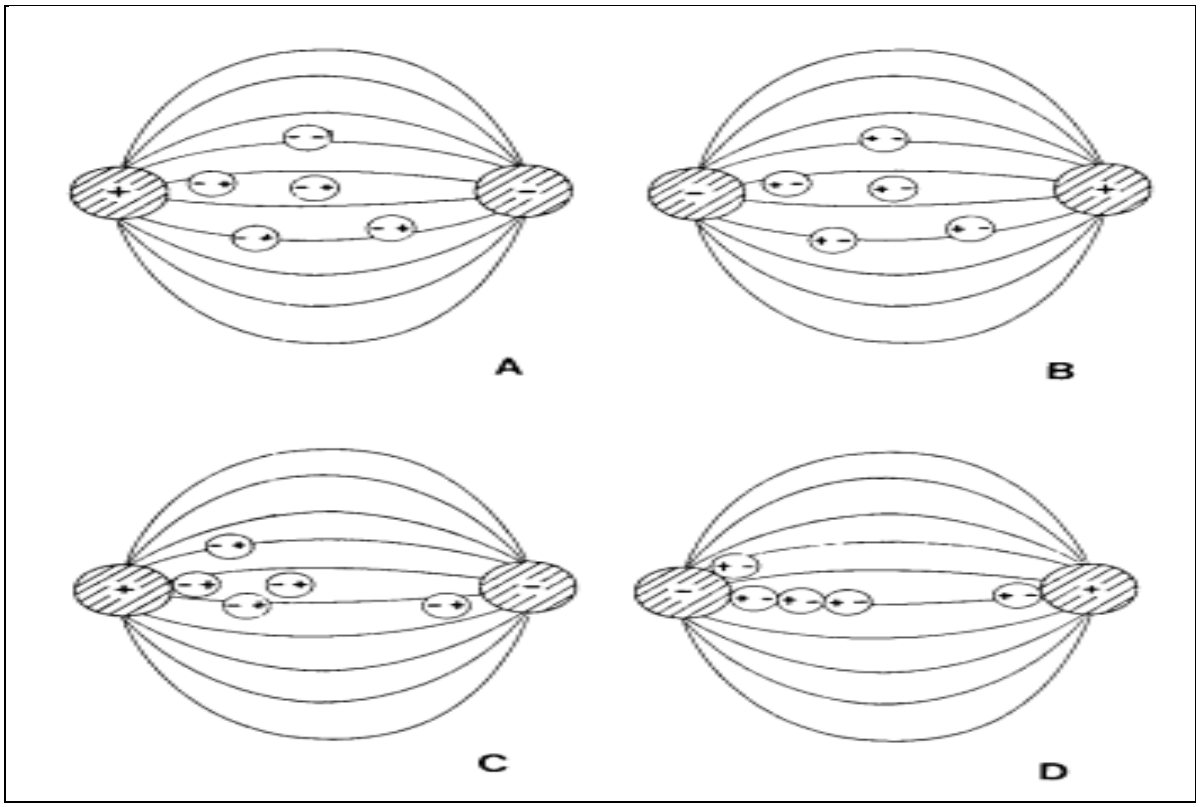

Figure 10. Dielectropole collection of protoplasts (blank circles) in a non-homogeneous AC field. (A,B) The electric field-induced transient dipole in the protoplast membrane (represented by + and -). (C,D) Due to non-homogeneous electric field, the protoplasts move in the direction of higher field strength and attach to the nearest surface of the electrode (hatched circles). Since the bathing medium is of relatively low conductivity (in comparison to the cells) the protoplasts attached to the electrode surface act as local high field-strength region and attract other protoplasts. This leads to the formation of pearl-chain of protoplasts.Electrofusion is more suitable for the fusion of mesophyll protoplasts than root or callus protoplasts. The presence of large vacuole or amyloplasts is detrimental for the protoplasts during the fu-sion process. Despite the several advantages of the electrofusion method over PEG-induced fusion, the latter continues to be more popular probably because of high technical accuracy and, to some extent, high initial investment associated with the former.

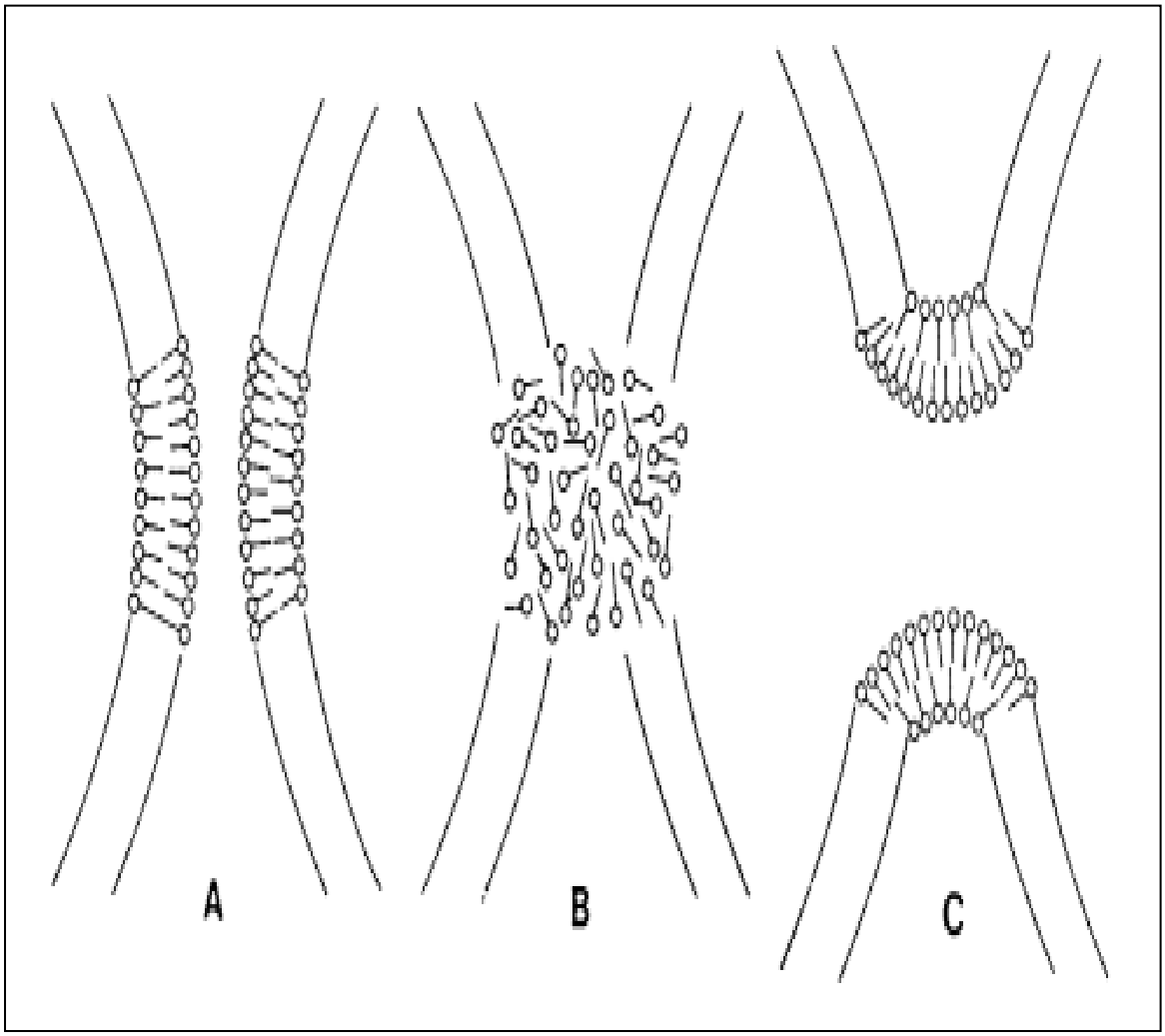

Figure 11. Model of the molecular events occurring during electrofusion. (A) Membranes of neighbouring protoplasts are brought together and held in intimate contact by the process of dielectrophoresis. (B) Application of a single, high strength DC pulse leads to a breakdown of the plasma membrane at the poles of the cells. (C) Reannealing of membranes following fusion pulse. If the two neighbouring protoplasts were in close proximity at the time of local membrane disruption, then lipids reassemble into a single bilayer, fusing the protoplasts

6.3. Embryo Rescue

Studies utilizing immature zygotic embryos help researchers gain greater insight into embryo development and seed maturation. Embryo culture of immature orchid embryos has become important in obtaining rare hybrids. Embryo rescue has proven itself as a valuable tool for plant breeders to obtain hybrids from crosses that would otherwise abort on the plant. There are numerous reports in the literature establishing the effectiveness of this technique in plant improvement programs. Immature heart stage and early cotyledonary stage embryos obtained from crossing Salix and Populus species were cultured on a half-strength Murashige and Skoog (1962) medium with 3% sucrose into plants. Plant material was confirmed to be hybrid using scanning electron microscopy, flow cytometry, and random amplification of polymorphic DNA screening. Interspecific hybrids of cotton have been obtained by embryo rescue. Some research obtained interploid hybrids from St. Augustine grass by embryo culture. Unique interspecific crosses in azalea, where there are both prezygotic and postzygotic barriers among interspecific crosses, were rescued by embryo culture. Embryo rescue techniques were used to circumvent lengthy seed to seed cycle and fungal seed-borne diseases in globe artichoke breeding.

6.3.1.Applications

• Breeding of incompatible interspecific and intergeneric species.

• Embryo culture is also used in crosses between diploids and tetraploids.

• To overcome seed dormancy and for shortening the breeding cycle of deciduous trees

• To determine seed viability.

• Recovery of maternal haploids that develop as a result of chromosome elimination following interspecific hybridization.

• Used in studies on the physiology of seed germination and development.

• Embryos are excellent materials for in vitro clonal propagation. This is especially true for conifers and members of Gramineae family of rare plants.

• Germination of seeds of obligatory parasites without the host is impossible in vivo, but is achievable with embryo culture.

• To study morphogenesis and nutritional requirements.

6.4. Transgenesis

Transgenic plants are the ones, whose DNA is modified using genetic engineering techniques. The aim is to introduce a new trait to the plant which does not occur naturally in the species. A transgenic plant contains a gene or genes that have been artificially inserted. The inserted gene sequence is known as the transgene, it may come from an unrelated plant or from a completely different species. The purpose of inserting a combination of genes in a plant, is to make it as useful and productive as possible. This process provides advantages like improving shelf life, higher yield, improved quality, pest resistance, tolerant to heat, cold and drought resistance, against a variety of biotic and abiotic stresses. Transgenic plants can also be produced in such a way that they express foreign proteins with industrial and pharmaceutical value

6.5. Methodologies for the development of transgenic plants

The transfer of exogenous DNA to higher plants can be accomplished by various methods. Here, only the classical and widely used methodologies for the development of transgenic plants will be addressed. Variations to the methods discussed in this chapter as well as other methods for the development of transgenic plants can be found in the literature.

Agrobacterium tumefaciens is an aerobic, Gram-negative bacterial species that belongs to the Rhizobiaceae family and is found in soil (Zambryski, 1988). It is a pathogen that infects a wide range of host plants (over 600 species). Agrobacterium tumefaciens causes crown gall, a tumor-forming disease, in infected plants, and it has the ability to transfer part of its genetic material into the host plant, altering the gene expression of the host for its own benefit.

Agrobacterium will recognize and contact the plant cell. This process of recognition and contact is mediated by signaling molecules such as low molecular weight phenolic compounds, amino acids, and sugars released by the injured root. After contact is established, the signaling molecules promote the activation of virulence genes (vir genes) located in the Vir region of the tumor-inducing (Ti) plasmid in A. tumefaciens (Figure 12). There are six groups of genes in the vir region that are either essential (virA, virB, virC, and virD) or function to increase the efficiency of the host cell's transformation (virE and virG). Additionally, other virulence genes located on the bacterial chromosome are involved in the early recognition and contact stages of the infection process The proteins encoded by vir genes are essential for the transfer of the T-DNA (transferred DNA) region of the A. tumefaciensTi plasmid to the nucleus of the host plant cell. The T-DNA region is flanked by left and right border segments, which are repeated sequences of approximately 25 base pairs. The left and right borders are critical for the recognition of the transfer region that must be

released. A single strand of the T-DNA complex is transferred, along with the vir genes, to the plant cell nucleus, where it is inserted and expressed. It is believed that its integration into the genome is random, but there are indications that integration occurs specifically in transcriptionally active regions. Regulatory elements present in the T-DNA of A. tumefaciens (a prokaryote) allow for transcription of the genes present in this region using the plant eukaryotic transcription system. In addition, the T-DNA has genes encoding enzymes that are involved in the synthesis of opines (modified amino acids or carbohydrates). A. tumefaciens infections allow portions of a prokaryotic genome (the T-DNA region of Ti plasmid) to be incorporated into the DNA of a eukaryotic organism, a natural property unique to this bacteria. Understanding this natural process of transferring genetic elements between different organisms was essential to obtain transgenic plants.

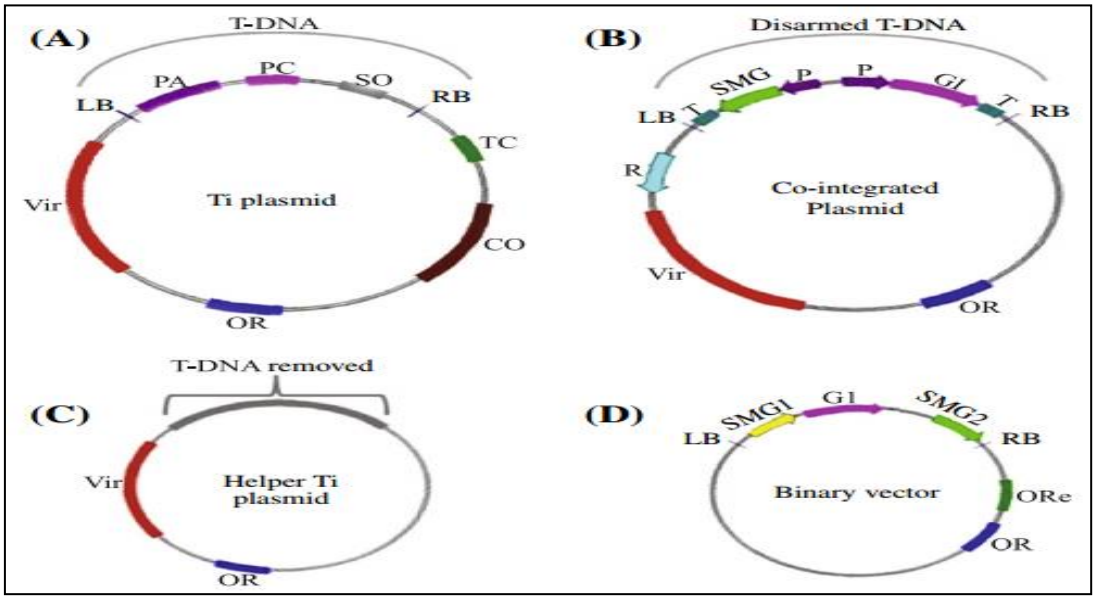

Figure 12 : A schematic representation of the Ti plasmid of Agrobacterium tumefaciens and its modifications for plant transformation. (A) The A. tumefaciens native Ti plasmid. The oncogenes responsible for the production of cytokinin (PC) and auxin (PA) as well as the gene responsible for opine synthesis (SO) are found in the transferred DNA (T-DNA) region. The T-DNA region is flanked by the Right Border (RB) and Left Border (LB) regions, which are both essential for the T-DNA transfer process. In addition, the Ti plasmid has a virulence region (Vir), a region responsible for conjugative transfer (TC), a region responsible for the expression of genes involved in opine catabolism (CO) and the origin of replication for A. tumefaciens (OR). (B) To disarm the Ti plasmid, the PC, PA, SO, TC, and CO regions of the T-DNA are removed, and an exogenous T-DNA section is inserted. The exogenous T-DNA introduces the exogenous gene of interest (GI), the selection marker gene (SMG), and the promoter (P) and terminator (T) regions. Additionally, an antibiotic resistance gene (R) is inserted to allow for selection of the transformed bacteria. (C) The Ti plasmid with excised T-DNA is used as a helper in the T-DNA binary vector system. (D) A binary vector has the GI as well as the SMGs for both A. tumefaciens (SMG2) and plants (SMG1). It also has an origin of replication for E. coli (Ore). A. tumefaciens bacteria carrying a helper Ti plasmid containing the binary vector can be used for plant transformation

6.5.1. Direct Methods

The introduction of genes into the nucleus of target cells by direct methods is dependent on making physical and chemical changes to the plant's cell walls and membranes. The exogenous gene to be introduced into the cell nucleus is inserted into a vector. The vector is then adhered to a metallic microparticle for its introduction to and transformation of the recipient cell. Theoretically, direct methods may be used to transfer DNA to virtually all plant species.

6.5.2. Particle bombardment (Biolistics)

For exogenous DNA to enter the nucleus of a host cell, it must overcome the cell wall and the plasma membrane. Particle bombardment is the acceleration of inert metal microparticles that are carrying DNA into the target cell. Of course, these microparticles must overcome the cell's barriers without causing cell death.

Initially, the exogenous DNA must be incorporated into bacterial plasmids.

In this step, carrier DNA (usually salmon sperm DNA) can also be added to increase the transformation efficiency. This complex of vector, carrier DNA, and metal microparticlesmust be accelerated to reach the inside of the recipient plant cell nucleus. Most of the systems used for accelerating the metal microparticlesare based on generating great power to displace a membrane that contains the DNA-coated microparticles.

High-density microparticles, such as gold and tungsten, are commonly used in the transformation process.

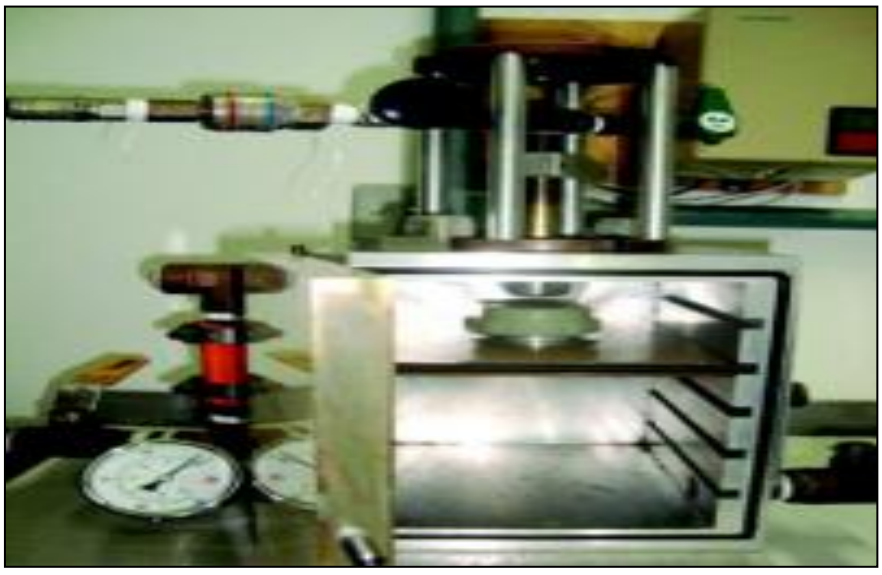

Figure 13.A microparticle accelerator chamber, into which the plant material is placed for the biolistic transformation process.Upon the microparticle's entry into a cell, the attached exogenous DNA dissociates by interaction with cellular fluids and is randomly integrated into the recipient cell genome.

6.6.3. Protoplast Electroporation

Protoplast electroporation is used to introduce exogenous macromolecules such as DNA to plant cells by reversibly changing the permeability of the plasma membrane. Protoplasts are plant cells that have been stripped of their cell walls through the action of pectinases and cellulases. As described for the other plant transformation techniques, it is necessary to obtain a vector containing the exogenous DNA of interest and a selection gene. To introduce the exogenous DNA into the recipient cell interior, purified protoplasts are mixed with the vectors and the carrier DNA; this mixture is then exposed to short pulses of a continuous current with high voltage. Pores form temporarily in the plasma membrane, allowing the vector carrying the exogenous gene of interest to enter.

As an alternative to electroporation, polyethylene glycol (PEG) associated with calcium and magnesium at an alkaline pH can be used to promote the binding of exogenous DNA to protoplasts. In this method, DNA adheres to the cell's surface and is absorbed by endocytosis. Treatment with PEG can also affect the regeneration of plants from protoplasts, increasing the difficulty of obtaining transformed plants.

6.4.4. Laboratory steps for the development of transgenic plants

Several specific protocols for plant transformation have been described in the literature. This section will only address the general laboratory steps needed to obtain transgenic plants:

(1) the isolation and cloning of the gene of interest;

(2) the preparation of the plant material (fragments of leaves, embryos, protoplasts, and others);

(3) the DNA transfer (gene transformation); and

(4) the regeneration of a plant from the transformed cell.

Isolation and Cloning of the Gene of Interest In this step, the gene of interest is isolated from the total DNA of the donor organism and cloned into a vector to transform E. coli competent cells. The gene of interest can be isolated by constructing a genomic library and using appropriate probes to recognize the gene, as well as by amplifying the gene using the specific primers for the coding region. The isolated gene of interest is used for vector construction. In addition to the transgene, the vector must contain a selection marker gene to track the successfully transformed plant cells. In the case of genetic transformation by A. tumefaciens, the gene of interest should be either transferred to a binary vector or co-integrated into the bacteria's Ti plasmid.

6.4.4.1.Preparation of the Plant Material

To produce protoplasts, leaf fragments must be kept in an enzyme solution with the cut surface in contact with the solution. Leaf fragments that have been macerated by enzymatic action are strained, and the recovered solution contains the protoplasts. This solution is subjected to successive centrifugations, and the protoplasts are then resuspended in a specific buffer. Subsequently, the protoplasts are counted in a Neubauer chamber. After being counted, the protoplasts may be used for electroporation or for treatment with PEG.

When using A. tumefaciens for genetic transformation, incisions must be made in the explants to facilitate the process of infection and the transfer of exogenous DNA.

6.4.4.2. DNA Transfer

In the transformation method mediated by A. tumefaciens, its co cultivation with the explants is carried out after the pre culture step. First, the culture of A. tumefaciens with the plasmid containing the gene of interest is incubated at 28°C in a liquid medium with specific antibiotics, and the cultures are then subjected to centrifugation.

6.4.4.3. Regenerating Plants from the Transformed Cells

Undifferentiated cells are totipotent, able to differentiate into any type of plant tissue. When explants or protoplasts are placed in contact with a selective regeneration medium, calli (undifferentiated cells) are formed. After this regeneration step, the calliare transferred to an elongation medium for approximately 30days. The differentiated and elongated shoots are sectioned and transferred to the rooting medium. Rooted plants with developed shoots are then transferred to a substrate in the greenhouse. Once the expression of the transgene is confirmed, the hemizygous transgenic plant (called T0 or R0) is self-fertilized to obtain homozygous transgenic progeny.

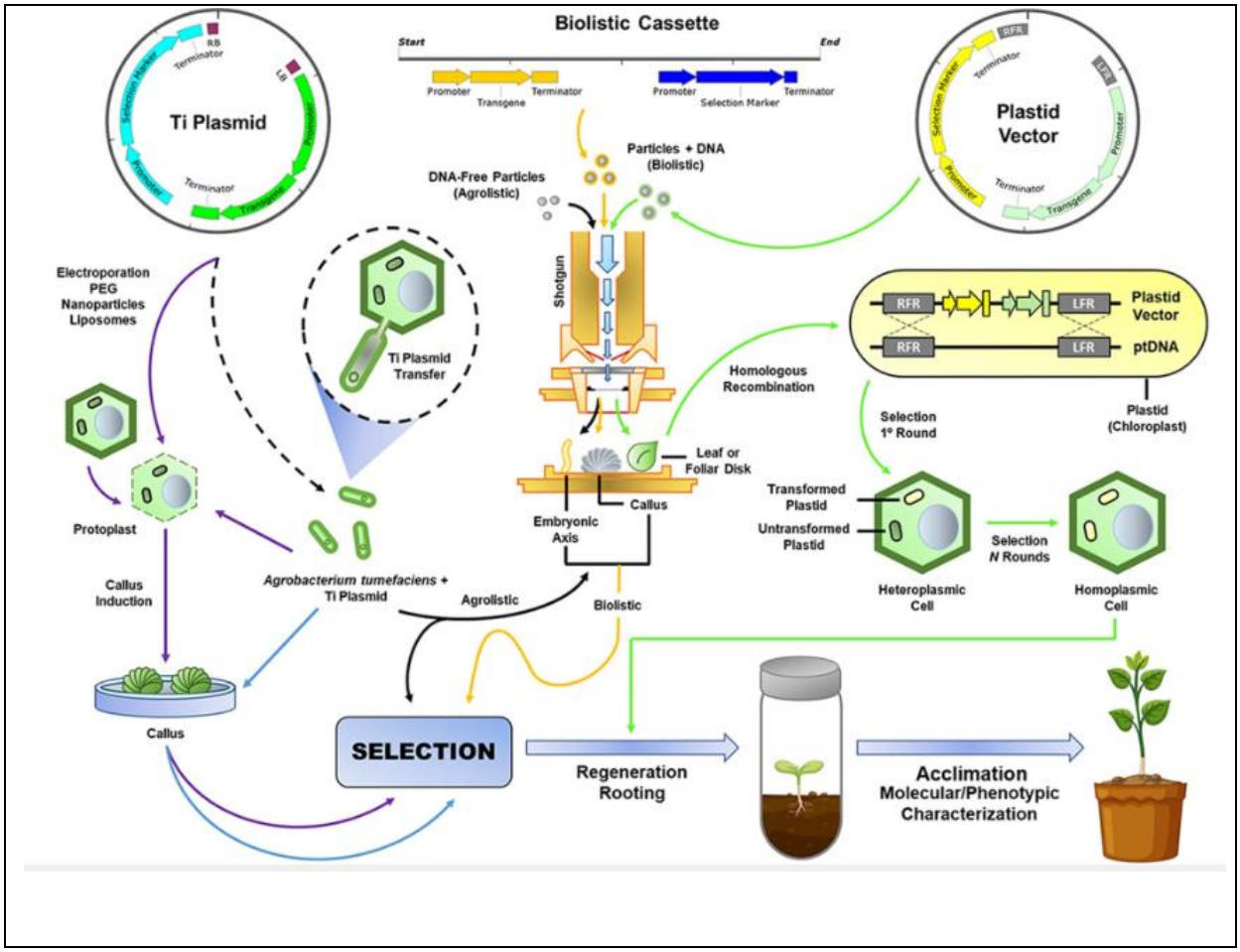

Figure 14. Plant genetic transformation approaches.

Conclusion

Plant tissue culture represents the most promising areas of application at present time and giving an out look into the future. The areas ranges from micropropagation of ornamental and forest trees, production of pharmaceutically interesting compounds, and plant breeding for improved nutritional value of staple crop plants. The rapid production of high quality, disease free and uniform planting stock is only possible through micro-propagation. New opportunities has been created for producers, farmers and nursery owners for high quality planting materials of fruits, ornamentals, forest tree species and vegetables. Plant production can be carried out throughout the year irrespective of season and weather. The in vitro culture has a unique role in sustainable and competitive agriculture and forestry and has been successfully applied in plant breeding for rapid introduction of improved plants. Plant tissue culture has become an integral part of plant breeding. It can also be used for the production of plants as a source of edible vaccines. There are many useful plant-derived substances which can be produced in tissue cultures.Plant cell culture has made great advances. Perhaps the most significant role that plant cell culture has to play in the future will be in its association with transgenic plants. It has a great role to play in agricultural development and productivity.

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Commonly used terms in tissue culture

Tissue Culture – The maintenance or growth of tissues *in vitro* in a way that allows for dedifferentiation, differentiation and preservation of their architecture or function or both.

In vitro – Growing cells on a defined medium under sterile conditions.

In vitro Propagation – Propagation of plants in a controlled environment using culture vessels on a defined medium under sterile conditions.

Clonal Propagation – Asexual reproduction of plants that are considered to be physiologically and/or genetically uniform and to have originated from a single individual or explant / single cell.

Micropropagation – Clonal propagation of plants from small explants.

TotipotencyTotipotency is the ability of plant cell to perform all the functions of development, which are characteristics of zygote, i.e., ability to develop into a complete plant. Morgan (1901) coined the term "Totipotency" to denote this capacity of cell to develop into an organism by regeneration.

Adventitious means in an unusual anatomical position. Hence, the term 'adventitious shoots' mean the shoots are grown from the place where they are not formed normally. The explants used for adventitious shoot induction include internodes, leaf blades, cotyledons, root tips, bulb, corms, tubers, rhizomes and so on.

Anther Culture– The *in vitro* culture of anthers containing microspores on a defined medium.

HaploidPlants – The microspores in the anthers may form haploid callus or develop directly into haploid plants.

Differentiation – Development of organization within a tissue to the formation of an organ, shoot or somatic embryo.

Embryogenesis - The process of embryo-like structure initiation and development.

Embryoid– Mass of cells that resembles an embryo (embryo-like structures). Somatic embryoids / Haploid embryoids

Embryo culture – *In vitro* development or maintenance of isolated mature or immature embryos.

Embryo-rescue – Embryo culture to facilitate recovery of progeny from wild crossing between different species (inter-specific cross).

Meristem Culture –*In vitro* culture of apical meristem (dome-like structure), excised from shoot apex.

Morphogenesis – The evolution of a structure from an undifferentiated to a differentiated state. The growth and development of differentiated structures.

Organogenesis– A process of differentiation by which plant organs are formed *de nova* or from preexisting structures of precursor cells.

Plant regeneration – The process of recovering plantlets from *in vitro* cultures through organogenesis or embryogenesis.

Somoclonal variation – Phenotypic variation, either genetic or epigenetic in origin.

Suspension culture – Cells in liquid culture.

Plant Protoplast – A plant cell from which the entire cell wall has been removed.

Protoplast fusion– Technique in which protoplasts are fused into a single cell. (To overcome compatibility barriers)

Auxins – A broad class of heterocyclic ringed compounds. Plant growth regulators – IAA, NAA, 2,4-D

Cytokinins – A broad class of substituted adenine derivatives. Plant growth regulators – Kinetin, BA, 2iP

Explant – Tissue taken from its original site and transferred to an artificial medium for growth or maintenance.

Adventitious: development of organs such as buds, leaves, roots, shoots and somatic embryos from shoot and root tissues and callus.

Agar: Natural gelling agent made from algae Aseptic technique: procedures used to prevent the introduction of microorganisms such as fungi, bacteria, viruses and phytoplasmas into cell, tissue and organ cultures, and cross contamination of cultures.

Autoclave: A machine capable of sterilizing by steam under pressure

Axenic culture: a culture without foreign or undesired life forms but may include the deliberate co-culture with different types of cells, tissues or organisms.

Callus: an unorganized mass of differentiated plant cells.

Cell culture: culture of cells or their maintenance in vitro including the culture of single cells.

Chemically defined medium: a nutritive solution or substrate for culturing cells in which each component is specified.

Clonal propagation: asexual multiplication of plants from a single individual or explant.

Clones: a group of plants propagated from vegetative parts, which have been derived by repeated propagation from a single individual. Clones are considered to be genetically uniform. Contamination: infected by unwanted microorganisms in controlled environment

Cryopreservation: ultra-low temperature storage of cells, tissues, embryos and seeds.
Culture: A plant growing in vitro in a sterile environment

Embryo culture: In vitro culture of isolated mature or immature embryos. Explant: an excised piece or part of a plant used to initiate a tissue culture.

Ex vitro: Organisms removed from tissue culture and transplanted; generally plants to soil or potting mixture. Hormone: Generally naturally occurring chemicals that strongly affect plant growth

In Vitro: To be grown in glass

In Vivo: To be grown naturally

Laminar Flow Hood: An enclosed work area where the air is cleaned using HEPA filters Medium: a solid or liquid nutritive solution used for culturing cells

APPENDIX 1

Plant Growth Regulators In Vitro

The following are lists of Auxins, Cytokinins and other plant growth regulators used in tissue culture.

Auxins

P-Chlorophenoxyacetic Acid (4-CPA; CPA)

autoclavable working conc.:5.0-50.0 µM

2,4-Dichlorophenoxy Acetic Acid (2,4-D)

Autoclavable working conc.:0.05-25.0 µM

Indole-3-Acetic Acid (IAA)

some loss of activity may occur during autoclaving, compensate by increasing the component concentration, for critical experimentation should be filter sterilized.

light sensitive working conc.:5.0-15.0 µM

Indole-3-Acetyl-L-Alanine (IAA-L-Alanine)

filter sterilization only working conc.:0.05-25.0 µM

Indole-3-Acetyl-L-Aspartic Acid (IAA-L-Aspartic Acid)

filter sterilization only working conc.:0.05-25.0 µM

Indole-3-Acetylgl Ycine (IAA-Glycine)

filter sterilization only working conc.:0.05-25.0 µM

Indole-3-Acetyl-L-Phenylalanine (IAA-L-Phenylalanine)

filter sterilization only working conc.:0.05-25.0 µm

Indole-3-Butyric Acid = 4-[3-Indolyl]Butyric Acid (IBA)

autoclavable, some loss of activity may occur during autoclaving, compensate by increasing the component concentration. slightly light sensitive working conc.:0.5-50.0 µM

I-Naphtiialeneacetic Acid (NAA)

Autoclavable working conc.:0.5-50.0 µM

NaphthoxyaceticAcid (NOA)

Autoclavable working conc.:0.05-50.0 µM Auxins can be dissolved in a few drops of concentrated alcohol. Add distilled water to make up to the required volume.

CYTOKININS

Adenine

Autoclavable working

conc.:250-1250 µM

Adenine HemisulphaTe

Autoclavable

working conc.:250-1250 µM

6-Benzylaminopurine = N6_Benzyladenine (BAP Or Ba)

Autoclavable

working conc.:O.5-25 µM

6-Benzylaminopurine Ridoside = N6-Benzyladenosine

filter sterilization

working conc.:O.05-25 µM

N -Benzyl-9-(2-Tetrahydropyran YL)Adenlne = 6-Benzylamino-9-[2-Tetrahydropyranyl]9h Purine (Bpa)

some loss of activity may occur during autoclaving

working conc.:O.05-25 µm

6-(y;yDimethylallYlamino)-Purine = N6_[2-Isopentenyl]Adenine (2IP)

some loss of activity may occur during autoclaving

working conc.:5.0-150 µM

6-(y,yDimethylallylamino)-Purine Riboside = N6y,y DimethylallYladenosine = N6_[2-Isopentenyl] Adenosine

filter sterilization

working conc.:5.0-150 µM

Kinetine = 6-Furfur Ylaminopurine

Autoclavable

working conc.:0.5-25 µM

Kinetine Riboside = 6-Furfurylaminopurine Riboside = N6-Furfur Yladenosine

filter sterilization working conc.:O.05-25 µM

Zeatin = 6-[4-Hydroxy-3-Methyl-But-2-Enylamino] Purine Trans And mixed isomers some loss of activity may occur during autoclaving working conc.:0.05-25 µM

T-Zea Tin Riboside

filter sterilization

working conc.:0.05-25 µM

Cytokininscan be dissolved in a few drops of IN HCl. Add distilled water to make up tothe required volume.

Other plant growth regulators used in tissue **culture**

Abscisic Acid

Some loss of activity may occur during autoclaving

working conc.:0.5-50 µM

Trans-Cinnamic Acid = β-PhenylacrylicAcid = 3-Phenyl PropenoicAcid

some loss of activity may occur during autoclaving working conc.:0.5-50 µM

1,3-Diphenylurea (Carbanilide)

filter sterilization

working conc.:0.5-50 µM

Gibberellic Acid (GA3)

Some loss of activity may occur during autoclaving

working conc.:0.05-25 µM

3-0xo-2-(2'-Pentenyl)-Cyclopen-Taneacetic Acid (Jasmonic Acid)

Autoclavable

working conc.:0.05-15 µm

N-Phenyl-N'-(2-Chloro-4-Pyridyle) Urea = (4PU-30)

some loss of activity may occur during autoclaving

working conc.:0.1-25 µm

N-Phenyl-N'1,2,3-Tiiidiazol-5-Yl Urea = TDZ

some loss of activity may occur during autoclaving

working conc.:0.1-25 µM

Phloroglucinol = 1,3,5-TrIhiydroxybenzene

some loss of activity may occur during autoclaving

working conc.:5.0-50 µM

Succinic Acid 2,2-Dimetiiyl Hydrazide (Daminozide)

some loss of activity may occur during autoclaving

working conc.:5.0-50 µM

Prepare stock solutions of 1000 µM for all the chemicals. Add appropriate quantity of the required stock solution to the medium to obtain the desired concentration. Use the following equation to work out the concentrations in the medium:

Cs x V s = em x V m

C = concentration

V = volume

S = stock

M=medium

APPENDIX 2.

PROTOCOLS FOR FUSING PLANT PROTOPLASTS

1. High pH and high Ca^{+2} fusion (after Keller and Melchers, 1973; Melchers and Labib, 1974)

(a) Mix freshly isolated protoplasts of the selected parents in a ratio of 1:1 with a final density of ca. 2.5 x 10 ~ protoplasts m1-1. (b) Pellet the protoplasts by centrifuging at 50 x g for 3-5 min. (c)Remove the supernatant and add 2 ml of the fusion mixture, containing 50 mM glycine-NaOH buffer, 50 mM $CaC1_2.2H_20$, and 400 mM mannitol (pH 10.5). (d) Pellet the protoplasts by centrifuging at 50 x g for 3-5 min. (e) Place the centrifuge tube in a water bath at 37°C for 10-30 min. (f) Replace the fusion mixture by washing medium (600 mM mannitol, 50 mM $CaC1_2.2H_20$) and leave for 30 min. (g)Wash twicewith the washing medium. (h) Suspend the protoplasts in culture medium and culture as small drops.

2. PEG-induced fusion (after Kao, 1976)

(a)Mix freshly isolated protoplasts (while still in the enzyme solution) of the two desired parents in a ratio of 1:1. Pass the suspension through a 62µm pore size filter and collect the filtrate in a centrifuge tube. Seal the mouth of the tube with a screw cap.

(b)Centrifuge the filtrate at 50 x g for 6 min to sediment the protoplasts.

(c)Remove the supernatant with a Pasteur pipette.

(d)Wash the protoplasts with 10 ml of solution I (500 mM glucose, 0.7 mM KH_2PO_4-H_20 and 3.5 mM CaC12.2H20, pH 5.5).

(e)Resuspend the washed protoplasts in solution I to make a suspension with 4-5% (v/v) protoplasts $m1^{-1}$.

(f)Put a 2-3 ml drop of Silicon 200 fluid (100 cs) in a 60x15 mm petri dish.

(g)Place a 22 x 22 cm coverslip on the drop.

(h)Pipette ca. 150µl of the protoplast suspension onto the coverslip with a Pasteur pipette.

Student Activity

Tissue culture uses a small piece of tissue from a mother plant to grow many new copies of the original plant

1-What is the term used to refer to this small piece of tissue?

2-What are some of the plants that we might use for tissue culture?

3-Why is tissue culture used for propagation of some plants rather than just planting seeds?

4-What is a sterile environment?

5-Why is a sterile environment important in tissue culture?

6-How did you or your teacher sterilize the instruments that were used in this tissue culture activity?

7-Could we sterilize the plant tissue in the same manner? Why or why not?

8-What happens if you open your sterile plant container when it is not inside a sterile environment?

Answers to Student Activity

1-Explant

2-cauliflower, african violet, rose, carnation or almost any other plants students might mention

3-We use tissue culture when:

a) we want to have many plants that are genetically identical,

b) we only have a very small amount of tissue or small number of plants but we need to increase them,

c) we have genetically transformed single plant cells that we need to develop into mature plants

4-An environment that has been treated so that is free of all bacteria, viruses and fungi.

5-The medium used for tissue culture provides a good food source for the rapid growth of many types of cells. Bacteria, viruses and fungi will grow more quickly in this environment than the plant cells that we are trying to culture. Thus the cultures will rapidly become contaminated with excessive growth of contaminants in a non-sterile environment.

6-They were wrapped in foil, sealed and then sterilized in a pressure cooker or oven.

7-No. If we tried to sterilize plant materials using high heat we would kill the plant.

8-Bacteria, viruses or fungi from the air in a non-sterile environment could fall into our culture container and begin to grow, contaminating our cultured tissue explant.

EXERCISES

What is plant tissue culture?

2. Describe the various components of plant tissue culture media.

3. What are the general steps of plant tissue culture?

4. Describe various applications of plant tissue culture.

5. How are somatic hybrids developed? 6. What are somaclonal variations?

7. Define explant and list five most commonly used explants for plant tissue culture.

8. Describe somatic embryogenesis and their application for the development of synthetic seeds.

9. Describe briefly the role of pH in nutrient media.

10. Describe the method of somatic hybridization and its advantages.

11. What are somaclonal variations and discuss their role for improving crops.

Multiple Choice Questions

12. Which of the following tissues can be used as explant for regenerating complete plant through tissue culture?

(a) Shoot apical meristem

(b) Embryo

(c) Leaf segments

(d) All of the above

13. Which of the following explants are suitable for the production of virus free plants?

(a) Leaf segments

(b) Seeds

(c) Apical meristem

(d) Stem cuttings

14. The process of combining the nuclear genomes of one parent with the cytoplasmic genome of the other parent is called as:

(a) Cybridization

(b) Micropropagation

(c) Regeneration

(d) None of them

15. Which of the following components is not essential for Murashige and Skoog media?

(a) Inorganicnutrients

(b) Carbon source

(c) Antibiotics

(d) Organic Nutrients

16. Decrease in the pH of the media may result in:

(a) Increase in hardiness of the solidified medium.

(b) May interfere with the solubility of media salts.

(c) Interfere with solidification of the medium and results in poor solidification.

(d) All of the above.