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PLANT BIOTECHNOLOGY

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I am happy at the efforts taken by the University in publishing this book not only in printed format, but also in PDF format in the Internet.

With warm regards

Dr. T. X. A. ANANTH
President – University Council

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CHAPTER I

Plant Cell Totipotency, Culture of plant cells, tissue and organs, scope historical review. Aseptic techniques: Culture media preparation and composition, methods ofsterilization: methods to overcome phenolic oxidation, inoculation, incubation and hardening.

Introduction:

An important aspect of all biotechnology processes is the culture of either the plants cells or animal cells or microorganisms (Fig. 1). The cells in culture can be used for recombinant DNA technology,genetic manipulations etc. Plant cell culture or the plant tissue culture is based on the unique property of the cell-totipotency. The concept of modern plant biotechnology originated in the year 1838, from the Cell theory of Schleiden and Schwann, which emphasises cell as the primary unit of all living things. In 1902, the concept of totipotency (The ability of a single cell to divide and produce all of the differentiated cells in an organism) was put forth by Galberlandt citing the production of somatic embryos from vegetative cells. From then on with leaping steps of technology growth Biotechnology hadstrodeahead.

Plant biotechnology has succeeded in development of transgenic plant which has now become the nodal point for many applications for plant biotechnology.

Fig. 1. Plants propagated in tissue culture media

TOTIPOTENCY

The inherent potentiality of a plant cell to give rise to a whole plant is described as cellular totipotency. This is a capacity which is retained even after a cell has undergone final differentiation in the plant body. In plants, even highly mature and differentiated cells retain the ability to regenerate to a meristematic state as long as they have an intact membrane system and a viable nucleus. This is contradicting to animals, where differentiation is generally irreversible. For a differentiated cell, to express its totipotency, it first undergoes dedifferentiation followed by redifferentiation. The phenomenon of a mature cell reverting to the meristematic state and forming undifferentiated callus tissue is termed ^differentiation'. the phenomenon of conversion of component cells of callus tissue to whole plant or plant organs is called as 'redifferentiation'

KEY WORDS USED IN IN VITRO CULTURES

Various terms are used to define the phenomenon in in vitro studies. For example differentiation, dedifferentiation, re-differentiation, regeneration and morphogenesis are terms with overlapping meanings.

Differentiation

The term differentiation is used in many different senses in biology. In broad sense, it is defined as the process by which meristematic

cells are converted into two or more types of cells, tissues or organs which are qualitatively different from each other.

De-differentiation

The term is used to denote the process of formation of unorganised tissues from the highly organized tissues.

Re-differentiation

The process of differentiation occurring in an undifferentiated tissue

Regeneration

It is defined as the structuring of any part, which has been removed or physiologically isolated from the organism. In other words, genesis of an entire plant from cultured explants directly or via callus indirectly is called regeneration.

Morphogenesis

Attainment of biological organization or form is termed as morphogenesis. Under in vitro conditions this can be achieved by two routes: de novo origin of organs, either shoots or roots from the cultured tissues precisely termed as organogenesis and de novo origin of embryos with distinct root and shoot poles on opposite ends from the somatic cells or cells cultured in vitro, otherwise called as somatic embryogenesis

Somatic Embryogenesis

An embryo is defined as a plant in its initial stage of development. Each embryo possesses two distinct poles, one to form root and the other shoot, and is the product of fusion of gametes. In some plant species, embryos are produced without the fusion of gametes and termed as asexual embryogenesis oradventitious embyrony.

In an intact plant this type of embryogenesis may occur in sporophytic tissues like integuments and nucellar tissues or from unfertilized gametic cells. Apart fromthe normal course of embryo formations viz., zygoticembryogenesis and adventitious embryony, instances of embryo formations from the tissues cultures in vitro

were reported.

Organogenesis

In plant tissue culture, organogenesis means genesis of organs like shoots, roots, leaves,flowers, etc. Using this concept, it has now become possible to achieve organogenesis in a large number of plant species by culturing explants, calli and cell suspension in a defined medium.

Historical Review of Plant tissue cultue

In 1902 Haberlandt demonstrated totipotency of plant cells by culturing isolated leaf cells in diluted Knop's solution for the first time, but his attempts failed due to inappropriate experimental conditions, inadequateencouraged subsequent attempts to regenerate whole plants from cultured cells. Mid 90s was markedby the development of new techniques and the improvement of plant tissue culture. These chniques were key for application of tissue culture to five broad areas namely, cell behaviour (cytology, nutition,metabolism,morphogenesis, embryogenesis and pathology), plant modification and improvement, pathogen-free plants and germplasm storage, clonal propogation, and product formation. Earlier attempts were made to culture isolated plant organs such as roots and shoot apices. Hanning in 1904 isolated embryos from crucifers and successfully grew on mineral salts and sugar solution.

The key steps for successful plant tissue culture include

Preparation of culture media

Selection of explants

Surface sterilization of explants

Inoculation of the explants

Incubation of the cultures

Successful regeneration of plants

Acclimatization of plants

Transfer of Tissue culture plants to field

1. Culture media:

Standardization of growth media is an essential criteria to be considered for plant tissue culture. The lack of availability of a nutrient solution that could support the growth of isolated plant cells and tissues hindered further progress for many years. The development of improved nutrient solutions, right choice of plant material, and appreciation of the importance of aseptic cultures, led to successful culturing of plant tissues. Molliard in France, Kotte in Germany, and Robbins in the United States, cultured fragments of embryos, and excised roots, on Knop's mineral solution (1 g potassium nitrate (KNO3), 1 g magnesium sulphate (MgSO4) 1 g potassium phosphate dibasic (K2HPO4), 3 g calcium nitrate (Ca(NO3)2 per litre). Some growth was observed on this minimal medium, but none of the cultures could be maintained for more than a few weeks. Philip Rodney White in the United States was the first to obtain indefinite growth of cultured plant tissues.

White in 1939 cultured tobacco tumor tissue from hybrid Nicotiana glauca and N. langsdorffii. The discovery of the naturally occurring auxin indole-3-aetic acid (IAA) by and its beneficial effects on plant growth led to its incorporation in plant nutrient media. Simon (1908) successfully regenerated callus, buds and roots from poplar tree in a medium containing IAA. Due to the dissatisfaction of Knop’s media, efforts were made by many individuals to develop nutrient solutions that could adequately support the growth of isolated plant tissues. White developed a new nutrient solution—the White's medium for algae, and the microelements that included glycine, nicotinic acid, thiamine and pyridoxine. It was the most widely used nutrient solution for plant tissue cultures until the 1960s.

Albert C. Hildebrandt at the University of Wisconsin performed elaborate studies to develop an ideal nutrient solution. This resulted in the development of solutions that contained greatly elevated

levels of mineral salts. Toshio Murashige, a graduate student in the laboratory of Folke Skoog, also at the University of Wisconsin, was culturing tobacco pith tissues. He found that the addition of an aqueous extract of tobacco leaves to White's medium resulted in greater than fourfold increase in growth. This was determined to be caused largely by the inorganic constituents of the leaf extract. Similar results were obtained when the ash of tobacco leaf extracts, or large amounts of ammonium, nitrate, phosphate and potassium salts were added to the White’s medium. These results led to the formulation of a new and completely defined nutrient solution, the Murashige and Skoog (1962) or MS medium (Table 1) , which also included chelated iron in order t;3o make it more stable and available during the life of the cultures, myo-inositol, and a mixture of four vitamins. It is the most widely used formulation for culture of plant tissues, and the publication describing the MS medium (Murashige and Skoog) remains one of the most highly cited publications in plant biology.

Toshio Murashige

Folke Skoog

The basic nutrient requirements of cultured plant cells are very similar to those of whole plants. Plant tissue and cell culture media are generally made up of some or all of the following components: macronutrients,micronutrients,vitamins, amino acids or other nitrogen supplements, sugar(s), other undefined organicsupplements, solidifying agents or support systems, and growth regulators.Several mediaformulations are commonly used for the majority of all cell and tissue culture work.

1 .2.1 Macronutrients

The macronutrients provide the six major elements-nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), and sulfur (S)-required for plant cell or tissue growth. The optimum concentration of each nutrient for achieving maximum growth rates varies considerably among species.Culture media should contain at least 25-60 mM of inorganic nitrogen for adequate plant cell growth. Plant cells may grow on nitrates alone, but considerably better results are obtained when the medium contains both a nitrate and ammonium nitrogen source. Certainspecies require ammonium or another source of reduced nitrogen for cell growth to occur. Nitrates areusually supplied inthe range of25-20 mM; typical ammonium concentrations range between 2 and 20 mM. However, ammonium concentrations in excess of 8 mM may be deleterious to cell growth of certain species. Cells can grow on a culture medium containing ammonium as the sole nitrogen source if one or more of the TCA cycle acids (e.g., citrate, succinate, or malate) are also included in the culture medium at concentrations of approximately 10 mM. When nitrate and ammonium sources of nitrogen are utilized together in the culture medium, the ammonium ions will be utilized together in the culture medium, the ammonium ions will be utilized more rapidly and

before the nitrate ions. Potassium is required for cell growth of most plant species. Most media contain K; in the nitrate or chloride form, at concentrations of 20-30 mM. The optimum concentrations of P, Mg, S, and Ca range from 1-3 mM when all other requirements for cell growth are satisfied. Higher concentrations of these nutrients may be required if deficiencies in other nutrients exist.

1 .2.2 Micronutrients

The essential micronutrients for plant cell and tissue growth include iron (Fe), manganese (Mn), zinc (Zn), boron (B), copper (Cu), and molybdenum (Mo). Chelated forms of iron and zinc are commonly used in preparing culture media. Iron may be the most critical of all the micronutrients. Iron citrate and tartrate may be used in culture media, but these compounds are difficult to dissolve and frequently precipitate after media are prepared. Murashige and Skoog used an ethylene diaminetetraacetic acid (EDTA)-iron chelate to bypass this problem. Cobalt (Co) and iodine (I) may also be added to certain media, but strict cell growthrequirements for these elements have not been established. Sodium (Na) and chlorine (Cl) are also used in some media but are not essential for cell growth. Copper and Cobalt are normally added to culture media at concentrations of 0.1 pM, Fe and Mo at 1 pM, I at 5 pM, Zn at 5-30 pM, Mn at 20-90 pM, and B at 25-100 pM.

1 .2.3 Carbon and Energy Source

The preferred carbohydrate in plant cell culture media is sucrose. Glucose and fructose may be substituted in some cases, glucose being as effective as sucrose and fructosebeing somewhat less effective. Other carbohydrates that have been tested include lactose, galactose, rafinose, maltose, and starch. Sucrose concentrations ofculture media normally range between 2 and 3 percent. Use of autoclaved fructose can be detrimental to cell growth. Carbohydrates must be supplied to the culture medium because few

plant cell lines have been isolated that are fullyautotropic, e.g., capable of supplying their owncarbohydrate needs byCO2 assimilation duringphotosynthesis.

Other vitamins such as biotin, folic acid, ascorbic acid, pantothenic acid, vitamin E (tocopherol), riboflavin, and p-aminobenzoic acid have been included in some cell culture media. The requirement for these vitamins by plant cell cultures is generally negligible, and they are notconsidered growth-limitingfactors. These vitamins are generally added to the culture medium only when the concentration of thiamin is below the desired level or when it is desirable to grow cells at very low population densities.

1.2.5. Amino Acids or Other Nitrogen Supplements

Although cultured cells are normally capable of synthesizing all of the required amino acids, theaddition of certain amino acids or amino acid mixtures may be used to further stimulate cell growth. The use of amino acids is particularly important for establishing cell cultures and protoplast cultures. Amino acids provide plant cells with an immediately available source of nitrogen, which generally can be taken up by the cells more rapidly than inorganic nitrogen. The most common sources of organic nitrogen used in culture media are amino acid mixtures (e.g., casein hydrolysate), L-glutamine, L-asparagine, and adenine. Casein hydrolysate is generally used at concentrations between 0.05 and 0.1 percent. When amino acids are added alone, care must be taken, as they can be inhibitory to cell growth. Examples of amino acids included in culture media to enhance cell growth are glycine at 2 mg/liter, glutamine up to 8 mM, asparagine at 100 mg/liter, L- arginine and cysteine at 10 mg/liter, and L-tyrosine at 100 mg/liter. Tyrosine has been used to stimulate morphogenesis in cell cultures but should only be used inan agar medium. Supplementation of the culture

medium with adenine sulfate can stimulate cell growth and greatly enhance shoot formation. Undefined Organic Supplements Addition of a wide variety of organic extracts to culture media often results in favorable tissue responses. Supplements that have been tested include protein hydrolysates, coconut milk, yeast extracts, malt extracts, ground banana, orange juice, and tomato juice. However, undefined organic supplements should only be used as a last resort, and only coconut milk and protein hydrolysates are used to any extent today.

Protein (casein) hydrolysates are generally added to culture mediaat a concentration of 0.05-0.1%, while coconut milk is commonly used at 5-20% (v/v). The addition ofactivated charcoal (AC) to culture media may have a beneficial effect. Theeffect of AC is generally attributed to one of three factors: absorption of inhibitory compounds, absorption of growth regulators from the culture medium, or darkening of the medium.

The inhibition of growth in the presence of AC is generally attributed to the absorption of phytohormones to AC.1-Napthaleneacetic acid (NAA), kinetin, 6-benzylaminopurine (BA), indole-3-acetic acid (IAA), and 6-y- Y-dimethylallylaminopurine (2iP) allbind to AC, with the latter two growth regulators binding quite rapidly. The stimulation of cell growth by AC is generally attributed to its ability to bind to toxic phenolic compounds produced during culture. Activatedcharcoal is generally acid- washed prior to addition to the culture medium at a concentration of 0.5-3.0 percent.

1.2.6 Solidifying Agents or Supports System

Agar is the most commonly used gelling agent for preparing semisolid and solid plant tissue culture media. Agar has several advantages over other gelling agents. First, when agar is mixed with

water, it forms a gel that melts at approximately 60°-100° C and solidifies at approximately 45°C; thus, agar gels are stable at all feasible incubation temperatures. Additionally, agar gels do not react with media constituents and are not digested by plant enzymes. The firmness of an agar gel is controlled by the concentration and brand of agar used in the culture medium and the pH of the medium. The agar concentrations commonly used in plant cell culture media range between 0.5 and 1.0%; these concentrations give a firm gel at the pH's typical of plant cell culture media. Another gelling agent commonly used for commercial as well as research purposes is Gelrite. This product is synthetic and Shouldbe used at 1.25-2.5 g/liter, resulting in a clear gel which aids in detecting contamination. Alternative methods of support have included use of perforated cellophane, filter paper bridges, filter paper wicks, polyurethane foam, and polyester fleece. Whether explants grow best on agar or on other supporting agents varies from one species of plant to the next.

1.2.7 Growth Regulators

Four broad classes of growth regulators are important in plant tissue culture; the auxins, cytokinins, gibberellins, and abscisic acid. Skoog and Miller were the first to report that the ration of auxin to cytokinin determined the type and extent of organogenesis in plant cell cultures. Both an auxin and cytokinin are usually added to culture media in order to obtain morphogenesis, although the ratio of hormones required for root and shoot induction is not universally the same. Considerable variability exists among genera, species, and even cultivars in the type and amount of auxin and cytokinin required for induction of morphogenesisThe auxin commonly used in plant tissue culture media are 1H-indole-3-acetic acid (IAA), 1H-indole-3-butyric acid (IBA), (2,4-dichlorophenoxy) acetic acid (2,4-D), and 1-napthaleneacetic acid (NAA). The only naturally occurring

auxin found in plant tissues is IAA. Other synthetic chlorophenoxyaceticacid (4-CPA, PCPA),(2,4,5-trichlorophenoxv)acetic acid (2,4,5T), 3,6-dichloro-2-methoxybenzoic acid(Dicamba), and 4-amino-3,5,6-trichloropicolinic acid (Picloram).

Auxins, abscisic acid, cytokinins, ethylene, and gibberellins are commonly recognized as the five main classes of naturally occurring plant hormones. Auxins, cytokinins, and auxin-cytokinin interactions are usually considered to be the most important for regulating growth and organized development in plant tissue and organ cultures, as these two classes of hormones are generally required. Other hormones abscisic acid, ethylene, g ibberell ins also play important regulatory roles in culture systems.

Auxins

Auxins (Fig. 2)exert a strong influence over processes such as cell growth expansion, cell wall acidification, initiation of cell division, and organization ofmeristems giving rise to either unorganized tissue (callus) or defined organs (generally roots) and promote vasculardifferentiation. Auxins play a key role in maintaining apical dominance, affecting abscission, promoting root formation, and tropistic curvatures, delaying leaf senescence, and fruit ripening. Auxins can occur naturally or can be synthetic. The most commonly detected natural auxin is indole-3-acetic acid (IAA), but depending on the species, age of the plant, season, and the conditions under which it has been growing, other naturalauxins have been identified such as 4-chloroindole-3-acetic acid, indole-3-acrylic acid,indole-3-butyric acid (IBA). Synthetically-prepared IAA and IBA are commonly used in plant culture media. They tend to be denatured in med'a and rapidly metabolized within plant tissues. These attributes can be useful when developmental phases in progress require less auxin.

Phenylacetic acid support callus growth and shoot formation in tobacco callus cultures. Commonly used synthetic auxins in tissue culture are 2,4-di- chlorophenoxyacetic acid (2,4-D; often used for callus induction and suspension cultures), and 1-naphthaleneacetic acid (NAA; when organogenesis is required).

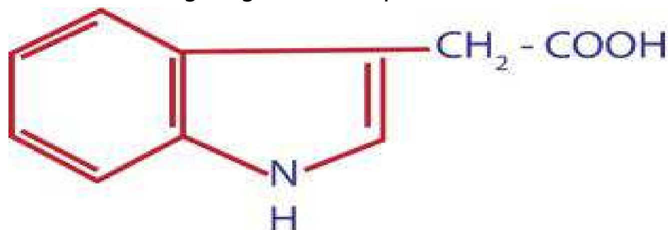


Fig. 2. Structure of Auxins

Cytokinins

Two major properties of cytokinins that are use(u[in culture are, stimulation of cell division and releaseof lateral bud dormancy. They can also induce adventilious bud formation(in cuttings and cultures). Cell division is regulated by the joint action of auxins andcytokinins, each of which influences different phases of cell cycle. The auxin and cytokinin levels in tissue culture media needto be carefully balanced and controlled. In intact plants, cytokininspromote lateral bud growth and leaf expansion, retard leaf senescence, promote chlorophyll synthesis, and enhance chloroplast development. The most commonlyused cytokinins in plant tissue culture are zeatin, 2-iP, dihydrozeatin, and zeatin riboside (Fig. 3). The most commonly used cytokinins are the substituted purines: kinetin and BA. Many aspects of cell growth, cell differentiation, and organogenesis in tissue and organ cultures have been found to be controlled by an interaction between cytokinins and auxins. The requisite concentration of each phytohormone varies greatly according to the kindof plant being cultured, the cultural conditions, and the form of

the phytohormone used. Although both auxin and cytokinin are usually required for growth and morphogenesis, auxin can inhibit cytokinin accumulation, whereas cytokinins can inhibit at least some of the actions of auxin.

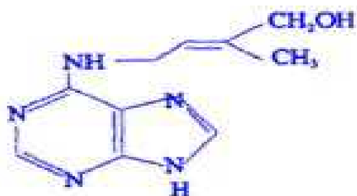


Fig. 3. Structure of cytokinins

Gibberellins

Gibberellins (GAs) (Fig. 4) can promote flowering (particularly in species that require long days and/or cold),that require long days and/or cold), cone initiation in some conifers, seed germination, and stem elongation (by increasing cell division and elongation). The ability to promote bolting and stem elongation can be exploited in vitro for the elongation of the shoots of woody species beforerooting. Some GA effects are caused by increases or decreases in the biosynthesis and activity of specific enzymes (e.g., increases in levels of aleurone hydrolytic enzymes). When GAs are added to plant tissue culture media, they oftendiminish or prevent the formation of roots, shoots, or somatic embryos. Some endogenousGAs are necessary for normal callus growth,and inhibition of GA biosynthesis influencedevelopment of cellsin liquid cultures. About 90 naturally occurring GAs are known. GAs has numerous interactions with other hormones. GA-induced a- amylase activity is antagonized by ABA. Ethylene blocks (or in rice, promotes) the ability of stems to respond to GAs. GA antagonizes the senescence promoting effects of ABA and ethylene in leaves and petals.

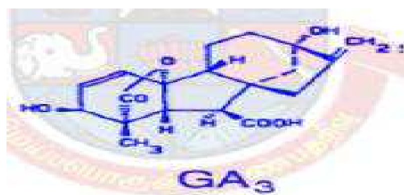


Fig. 4. Structure of GibbereMin

Abscisic acid

Abscisic acid (ABA) and other structurally related natural compounds with similar activity (Fig. 5) are most likely producedby the cleavage of xanthophyll. ABA is often regarded as being an inhibitor, as it maintains bud and seed dormancy, inhibits auxin-promoted cell wall acidification loosening, and slows cell elongation. ABA plays a key role in closing of stomatal apertures (reducingtranspiration), control of water and ion uptake by roots (in part by increasing hydraulic conductivity) and, with other phytohormones, promoting leaf abscission and senescence. ABA is important in seed maturation, as it induces the synthesis of storage proteins in developing seeds. It acts antagonistically to GAs in many systems. ABA, along with ethylene and jasmonic acid, aids in defense against insect wounding.With ethylene, ABA is intimately involved with plant responses to a wide range of environmental stresses. In tissue cultures, exogenously applied ABA can affect (generally positively at low concentrations, while high concentrations inhibit) callus growth and organogenesis (buds, roots, embryos). Some ABA is essential for the maturation and normal growth of somatic embryos. Manipulation of endogenous and/or exogenousABA levels increases the frequency of embryos reaching maturity. ABA increases freezing tolerance of axenically grown plants and cell cultures.

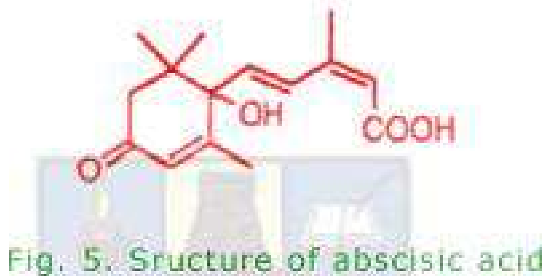


Fig. 5. Sructure of abscisic acid

Sterring Nutrient Media

Two methods (autoclaving and membrane filtration under positive pressure) are commonly used to sterilize culture media. Culture media, distilled water, and other stable mixtures can be autoclaved in glass containers that are sealed withcotton plugs, aluminum foil, or plastic closures. However, solutions that contain heat-labile components must be filter-sterilized. Generally, nutrient or plant tissue culture media are autoclaved at 1.05 kg/cm2 (15 psi) and 121°C. This hightemperature not only kills bacteria and fungi, but also their heat- resistant spores. Media can be sterilized in either an autoclave or pressure cooker with similar results. The time required for sterilization depends upon the volume of medium in the vessel. For small volumes of liquids (100 ml or less), the time required for autoclaving is 15-20 min, but for larger quantities (2-4liter), 30-40 min is required. The pressure should not exceed 20 psi, as higher pressures may lead to the decomposition of carbohydrates and other thermolabile components of a medium. There is evidence that medium exposed totemperatures in excess of 121 °C may not properly gel or may result in poor cell growth. The minimum times required for sterilization of different volumes of medium are listed below.

Since many proteins, vitamins, amino acids, plant extracts,

hormones, andcarbohydrates are thermolabile and may decompose during autoclaving, filter sterilization may be required. The porosity of the filter membraneshould be no larger than 0.2 microns (pm). Empty glassware that is to hold media must be sterilized in an autoclave before filter sterilization. Nutrient media that contain thermolabile components can be prepared in several steps. That is, a solution of the heat-stable components is sterilized in the usual way by autoclaving, and then cooled to 35 -50 °C under sterileconditions; in a separate operation, solutions of the thermolabile components are filter-sterilized. The sterilized solutions are then combined under aseptic conditions to give the complete media.

Aseptic conditions are necessary for maintenance of plant tissue cultures. All the containers, media, instruments and the explant should be strile.In order to avoid to contamination through air and external environment.All the experiments carried out inlaminar air-flow cabinet, which filters all the microorganisms.

Contamination of tissue culture may occur due to the fungal and bacterial microbes in the air in form of spores, pathogens on the surface of explants and through microbes from human body surface. Hence care should be taken for sterile conditions in all phases of tissue culture.
The contamination in tissue culture can be avoided with the following practices
Using sterile media, containers and instruments
Using sterile plant growth regulator stocks (by filter sterilization)
Aseptic working condition (Laminar air-flow)
Using sterile explants (isolated tissues) or surface sterilizing using chemical agents like mercuric chloride or sodium hypochlorite.

1.3.1. Autoclaving

This relies on the sterilization effect of super heated steam under pressure as in a domestic pressure cooker. The size of the equipment used can be as small as one litre or even as large as several thousand l i tres (Fig -6). Most instruments/nutrient media are sterilized with the use of an autoclave and the autoclave has a temperature range of media are sterilized with the use of an autoclave and the autoclave has atemperature range of 115- 1350 C. The standard conditions for autoclaving has a temperature of1210 C and a pressure of 15 psi (Pounds per square inch) for 15 minutes to achieve sterility. This figure is based on the conditions necessary to kill thermophilic microorganisms. The time taken for liquids to reach this temperature depends on their volume. It may also depend on the thickness of thevessel. The temperature of 121° C can only be achieved at 15 psi.

Fig.6. An autoclave

1.3.2 Filter sterilization

Some growth regulators like amino acids and vitamins are

heat labile and get destroyed on autoclaving with the rest of the nutrient medium. Therefore, it is sterilized by filtration through a sieve or a filtration assembly using filter membranes of 0.22 pm to 0.45pm size (Fig. 7).

Fig. 7. A filter sterilization unit

1.3.3 Irradiation

It can only be carried out under condition where UV radiation is available. Consequently, its use is restricted generally to purchased consumables like petridishes and pipettes. UV lights may be used to kill organisms in rooms or areas of work benches in which manipulation of cultures is carried out. It is however, dangerous and should not be turned on while any other work is in progress. UV light of some wavelengths can damage eyes and skin.

1 .3.4 Laminar Airflow Cabinet

Fig. 8. Working in a laminar flow hood

This is the primary equipment used for aseptic manipulation. This cabinet should be used for horizontal air-flow from the back to the front, and equipped with gas corks in the presence of gas burners (Fig. 8). Air is drawn in electric fans and passed through the coarse filter and then through the fine bacterial filter (HEPA). HEPA or High Efficiency Particulate Air Filter is an apparatus designed such that the air-flow through the working place flows in direct lines (i.e. laminar flow). Care is taken not to disturb this flow too much by vigorous movements. Before commencing any experiment it is desirable to clean the working surface with 70% alcohol. The air filters should be cleaned and changed periodically.

1 .3.5 TISSUE CULTURE LABORATORY

The tissue culture laboratory should be maintained in Golden standards to avoid contamination of the cultures. Installation of Hepa filters, or air filters over working areas would minimize the risk of contamination.

An enclosed entrance can precede the laboratory, laying of sticky

mats and removal of shoes will ensure complete aspectic conditions. The wash area, media preparation area, storage area and the transfer area should be outside the laboratory. It should accommodate the following areas:

Wash area and storage area:

The glassware washing area should be located near the sterilization and media preparation areas. When culture vessels are removed from the growth area, they are often autoclaved to kill contaminants or to soften semi-solid media.

The vessels can be easily moved to the washing area if the autoclave or pressure cooker is nearby. Locate the glassware storage area close to the wash area to expedite storage; these areas also need to be accessible to the media preparation area.Mobile drying racks can be stored nearby and lined with cheesecloth to prevent water dripping and loss of small objects. Locate ovens or hot air cabinets (75 C) close to the glassware washing and storage area. Dust-proof cabinets, low enough to allow easy access, can be used in the storage area.

Media preparation andsterilization area:

The water source and glassware storagearea should be convenient to the media preparation area. Benches, suitable forcomfortable working whilestanding (34 to 36m.) and deep enough (24 in.) to hold equipment listed below are essential. Their tops should be made with molded plasticlaminate surfaces that can tolerate frequent cleanings. The important equipment required in this area include Freezers, water distillation units, Balance, hot plate, stirrers, pH meter, autoclave, microwave oven etc.

INOCULATION, INCUBATION AND HARDENIN

1 .4 Inoculation

A piece of plant tissue (called an explant) is (Fig 9) (a) cut from the plant, (b) disinfested (removal of surface contaminants), and (c) placed on a medium. A medium typically contains mineral salts, sucrose, and a solidifying agent such as agar. The objective of this stage is to achieve an aseptic culture. An aseptic culture is one without contaminating bacteria or fungi.

Leaf Explant

Fig. 9. Different types of explants

Seed explants

Root Explant

Fig. 10. Process of inoculation of explants

Incubation

The aseptic culture is then incubated in a tissue culture room room where light, temperature and humidity are controlled. For some tissues, continuous darkness is needed and for some, light and dark cycles are required. The cultures are incubatedat 25°C in an aspectic condition in a sterile tissue culture room in racks under a controlled humidity condition. A light dark cycle of 16h light 8 hr darkness is provided. Light intensity of 4-10 X 103 lux for 16 hrs is given. The basic requirements needed for setting up a tissue culture room includes.

Isolation from foot traffic.
No contamination from adjacent rooms.
Thermostatically controlled heat.
Water and drains for a sink.
Adequate electrical service.
Provisions for a fan and intake blower for ventilation.
Good lighting.

Temperature, relative humidity, lighting units, and shelves need to be considered in the culture room. All of these environmental considerations will vary depending on the size of the growth room, its location, and the type of plants grown within it. Temperature is the primary concern in culture rooms; it affects decisions on lights, relative humidity, and shelving. Generally, temperatures are kept 76 +/-2 F. Heating can be accomplished by traditional heating systems supplemented with heat from light ballasts or space heaters. Cooling the room is usually obtained by installing heat pumps, air conditioners, or exhaustfans. Some plant cultures can be kept in complete darkness; however, most culture rooms are lighted at 1 klux (approximately 100ft-c) with some going up to 5 to 10 klux. The

plant species being micropropagated will determine the intensity used.

Automatic timers are needed to maintain desired photoperiods. Reflectors can be placed over bulbs to direct their light. Heat generated by the lights may cause condensation and temperature problems. Relative humidity (RH) is difficult to control inside growing vessels, but fluctuations in the culture room may have a deleterious effect.

Cultures can dry out if the room's RH is less than 50%; humidifiers can be used to correct this problem. If the RH becomes too high, a dehumidifier is recommended. Shelves can be built with wood. The wood for shelves should be exterior particleboard or plywood and should be painted white to reflect the room's light. Tempered glass is sometimes used for shelves to increase light penetration, but it is more prone to breaking. Air spaces, 2 to 4 in., between the lights and shelves will decrease bottom heat on upper shelves and condensation in culture vessels.

A room that is 8 ft high will accommodate 5 shelves, each 18 in. apart, when the bottom shelf is 4 in. off the floor. The top and bottom shelves may be difficult to work.

Fig. 11. Tissue culture room

During incubation, the cultures have to be subcultured at regular intervals to provide them with fresh nutrients, space for callus multiplication. The explants can be made to produce roots or shoots depending on the growth regulators or hormones provided to them in the medium. A growing explant can be induced to produce vegetative shoots by including a cytokinin in the medium. A cytokinin is a plant growth regulator that promotes shoot formation from growing plant cells. Roots can be induced by supplementing the media with high auxin and low cytokinin. Auxins are plant growth regulators that promote root formation.

1.6 Acclimatization Or Hardening

Transfering a tissue culture grown plant from the culture medium to soil is called acclimatization or hardening. During hardening the moisture should be maintained properly to avoid wilting. After transferring into pots or bags the plants have to be irrigated with water and nutrient solution. In order to avoid damage to the roots soft soil material like soilrite or vermiculite can be used. Before the plants are transferred to the field to grow in natural conditions, they

have to be maintained in pots with proper watering for 15-20 days in glass houses under controlled conditions of temperature and moisture.

Many tissue culture milestones have been achieved over the past 50 years. Micropropogation is one among them which is used for production of commercially viable plants and an alternative for vegetative propagation since the 1970s. Although many species are propagated using this technology there are still many more species that are either recalcitrantor cannot be cost-effectively propagated by tissue culture. Consequently, the commercialapplication of tissue culture propagation is restricted to mass propagating,high value, superior genotypes, and/or high health lines. Plant tissue culture relies on growing microbe-free plant material in a sterile environment, in conjunction with defi ned media containing nutrients, growth promoters, and a carbohydrate source. Typically plant tissue culture is carried out ona gelled medium within an enclosed clear or translucent culture vessel with limitedventilation, and placed under fluorescent lighting. To aid photosynthesis of tissue culture plants, an artificial lighting, sucrose is added to the medium as a carbohydrate source.

Major differences exist between the environments of plants growing intissue cultureand those in a greenhouse. These includedifferences in lighting, both quantityand quality; relativehumidity; nutrients and other growth promoters; the gaseouscomposition; and the medium substrate.In addition, the rooting procedurediffersmarkedly. In greenhouses a high-auxin quick dip is used forrooting cuttings. Excess auxin is flushed away in the free-draining, aerated potting medium.

Thiscontraststo in vitro rooting where a low auxin concentration is available over severalweeks in a poorly aerated, gelled medium. Therefore, it is not surprising that thetransfer of plantlets, whether

rooted or not, from the tissue culture environment tothe greenhouse causes tissue stress and is often associated with slow growth and significant plant losses. This period of plant stress often coincides with a changein plant ownership following the sale of the plants by the tissue culture laboratoryto the end, orintermediate, and user. Consequently, the tissue culture conditions thatthe plantlet has been grown in and the conditions the plants are to be transplantedinto are often poorly understood by the two parties.

Differences between thetwo environments and their effect onplants have been recognised in numerous studies that aim to understand the factors involved in the transition and establishment oftissue culture plantlets into a standard greenhouse environment and improve thesuccess rate.

WATER STRESS

The wilting of explants recently transferred from the in vitro to the ex vitro environmentis an early symptom of poor plant re-establishment. Even though free wateris available to these explants, incomplete leaf and stem development during theperiod in tissue culture contributes to the problems. Under standard tissuecultureconditions, where the relative humidity is usually greater than 95%, in vitro leavesmay not develop a waxy cuticle to the same extent as that found in ex vitro plants. Ventilation of the culture vessel, using loosely fi tting closures or vents, reduces therelative humidity in the vessel. A reduction in relative humidity leads to increasesin plant transpiration with associated development of functional stomata for controllingplant water loss. Besides increasing the rates of in vitro transpiration, reducing the relativehumidity can also increase nutrient uptake.

ROOT DEVELOPMENT

In vitro root development usually enhances transplanting success because functioningroots can create favourable plant waterbalance.Roots developed in vitroare believed to compensate for water loss caused by malfunctioning stomata.+Improved performance and increases in dry weight of these in vitro-rooted plants maybe due to extra nutrient uptake through the roots. The presence of roots in culture does not always improve transplant success. Inoculating plants at the time of transfer with mycorrhizal fungi can be beneficial for the survival and development of plantlets, leading to functional stomata and increased photosynthetic rates comparedto non-inoculated plantlets. When the growth retardant, paclobutrazol, is added to the rooting medium shootshave fewer and smaller leaves. This reduction in plant growth makesthem more wilt tolerant and increases the survival of plantlets following transfer tothe greenhouse.

AUTOTROPHIC GROWTH

The gaseous environment within the culture vessel can influence in vitro tissue growth since plant tissue generates and absorbs gases, altering the gas composition.The level of gaseous exchange from a tissue culture vessel will depend onhow well the vessel is sealed and whether it has vents. In addition, the type andamount of plant tissue in a culture vesselinfluences gas composition: ethylenemay be generated, such as in response to wounding during tissuemanipulations; carbon aioxide is a by-product of respiration; and during photosynthesis oxygen isgenerated and carbon dioxide absorbed.

In a closed vessel, plantlets are unable to achieve their photosyntheticcapacity as the carbon dioxideConcentrations are too low during most of the lightperiod.

Increasing light levels and improving the availability ofcarbon dioxide, either through venting or artificial enrichment, allows plant tissueto be grown photoautotrophically in a vessel. Photosynthesis is inhibited when sucroselevels increase from 3% to 6%, resulting in high levels of starch and sucrosein plantlets at the end of the in vitro period.

Although leaves formed in vitro may be photosynthetically competent, theseleaves are frequently replaced soon after transfer to the greenhouse by leaves withhigherphotosynthetic activity. Leaves formed duringthe acclimatisation period may still have a lower photosynthetic capacity thanleavesof greenhouse-grown plants. Increasing irradiance to around 300Mmol-m-2-s_1immediately after transfer enhances photosynthetic activity.The optimum nutrient levels in a photoautotrophic system may be very differentto those of a standard tissue culture system.

ALTERNATIVEPROPAGULES

Alternative propagules with little or no vegetative tissue present (e.g., bulbs ortubers) can be produced in culture with geophytes. As they sprout after transferto the greenhouse, the new shoots are more tolerant to greenhouse conditionsthansimilar shoots produced in culture. With mediaadjustments the vegetative growth senesces and tubers form over a 3- to 6-monthperiod. Besides the ease of acclimatisation, the compact and relatively robust formof microtubers makes them easy to handle and transport. They store well, allowingfor greater flexibility in time of planting. Inaddition, they can be quickly scatterplanted onto prepared greenhouse beds without the need for planting tubersupright. For rapid and even sprouting for in vitro bulbs and tubers, dormancybreaking needs to be synchronised. A period of cool storage is frequently used prior to planting out.

It is important to recognise and understand the differences between an in vitroand a greenhouse environment. By manipulating the in vitro environment, leavesthat have greater tolerance to water stress and are photosynthetically competentcan be developed as part of the acclimatisation process in preparing plantlets fortransferring out of culture. Roots formed in culture can be beneficial for enhancingearly growth following transfer from culture. The opt'mum growth rate of deflaskedplantlets frequently does not occur until new leaves and roots develop in the greenhouseenvironment. However, as species differ greatly in their requirements thereis no universal acclimatising protocol. However, for some cropsalternativepropagules (e.g., microtubers) are an option as they do not experience the acclimatization problems observed in tissue culture plantlets.

Green house

A greenhouse (also called a glasshouse) (Fig. 12) is abuilding or complex in which plants are grown in a controlled environment. After hardening, the plants can be acclimatized in the green houses. The structures and types of control systems that are used in the green houses are dictated by which of the environmental factors must be controlled, to what degree they must be controlled, and the cost of controlling them in relation to the value of the crop(s) being produced. Each environmental parameter that must be controlled increases facility and production cost. The objective is to design a controlled environment structure that allows for the control of those parameters that need to be controlled at the level of precision required. Doing more only adds to the cost of production. Common types of controlled environments include:

2-1 Hoop House

A true hoop house is generally only an arched structure or frame that provides cover, and thus some degree of light and temperature control, for crops. Hoop houses are generally used for over wintering plant materials or for starting hardy spring crops (broccoli, cabbage, ornamental perennials) early in the season. They typically do not have heating or cooling systems. Hoop houses may be covered with polyethylene film, shade fabric or may have no covering during warm seasons. Sometimes these structures are referred to as quonset houses.

However, one type of greenhouse design is the quonset-type structure. Therefore, the term quonset should be used only to refer to a type of greenhouse design.

2-2 Cold frames

Cold frames are similar to hoop houses and serve a similar purpose. The difference is that a cold frame may be partially set into the ground, is typically not as tall as a hoop house and may have a flat roof. Cold frames are generally used for overwintering plant materials or for starting hardy spring crops (i.e. broccoli, cabbage, ornamental perennials) early in the season. Cold frames may also be used to provide the necessary cold treatments to bulb crops. Cold frames have no heating or cooling systems.

2-3 Hot beds

Hot beds are similar to cold frames except that hot beds have some type of heat source and thus provide more control over temperature. The heat source may be hot water or steam from a boiler, electrical heating units, incandescent light bulbs or composting manures placed inside of the hot bed. Hot beds are most often used for starting plant materials in the early spring.

2-4 Shade houses

Shade houses (Sometimes referred to as Saran Houses) are structures that are covered with a fabric made of polypropylene, cotton, plastic or other material that is designed to partially exclude light. Some shading materials are aluminized so that light is actually reflected away from the structure. The cover material may beselected to block out varying amounts of light but typically shading materials excluding 20% to 60% are most common. These structures are generally used in subtropical (i.e. Florida) and tropical climates where reducing the light level and providing some measure of cooling (by shading) is desired. Shade Houses typically do not have heating or cooling systems. Shade houses are most often used in the production of cut flowers, foliage plants and nursery stock.

Coolers allow for plant materials to be held at low temperatures. Typically temperatures in the range of 35 - 50° F (2° - 10° C) are most common. In a few situations, temperatures below 32° F (0o C) may be required. Coolers are most often used for the storage of vegetables, fruits and cut flowers, holding nursery stock and providing a cold treatment (for vernalization or to break dormancy) to bulb crops.

2-6 Growth chambers

Growth chambers are computer- controlled enclosed units that potentially allow for very precise control of many or all of the environmental parameters previously discussed. Growth chambers are most often used for research purposes although they may be used in some propagation situations such as tissue culture. Growth chambers may be small reach-in chambers or large walk-in chambers.

2-7 Germination chambers

Germination chambers are similar to walk-in growth chambers except that they are primarily allow for the control of temperature, humidity and possibly light. They are often large walk-in rooms that are well insulated to minimize temperaturefluctuations, and they have some type of fog system used to maintain a high relative humidity. They are designed specifically to provide an optimal environment for seed germination.

Fig 12: Glass house for plant acclimatization

Methods to prevent Phenolic oxidation:

Phenolics are broadly distributed in the plant kingdom and are the most abundant secondary metabolites of plants. Phenolics are compounds which possess one or more aromatic rings with or more hydroxyl groups. The oxidation of the phenolics Phenolic exudation from explants used for tissue causes activation of oxidative enzymes like the polyphenol oxidase, phenyl alanine ammonia lyase and peroxidase, which caused browoning of the wounded region of the explants. This process of browning is called as phenolic oxidation, which results in inactivates the growth of tissues in culture. The

medium in which explants are grown become colored within an hour or two of subculture. This is a very common problem associated with tissue culture. Tissue browning is associated with accumulation of polyphenol oxidase and decrease of putrescinde, spermidind and spermine in browning callus, which inhibits callus growth, shoot differentiation and rooting of shoots. Browning can be caused by phenolic compounds at the chemcial level. The phenolic compounds activate polyphenoloxidase (PPO) of explants and change the metabolism of tissue cells and form brown quinine substance through its oxidtion.

Browning in tissue culture can be avoided by preteatment of explants with antioxidants, incorporation of antioxidants into the culture medium, incubation of culture in the dark and frequent subculture to fresh medium etc. Frequent transfer of explants to fresh medium two or three times at short intervals, is the simplest method to protect the explants from the detrimental effect of oxidative browning. During this period the cut end of the explant may become sealed up and the leaching phenolics stop. Keeping the culture initially in dark may also help to reduce the browning by preventing or reducing the activity of the enzymes concerned with both biosynthesis and oxidation of phenols.

CHAPTER II

Cell and organ differentiation; Clonal propagation or micropropagation (artificial seeds, virus free plants); Somaclonal variation; Overcoming crossing barriers (Pre fertilization and post fertilization barriersincluding in-vitroollination/fertilization andembryo rescue); Other uses of tissue culture (endosperm, nucellus culture, anther culture, ovule culture and bulbosum technique,germ plasmstorage including cryopreservation).

Introduction

Plant cell proliferation and organ differentiation is important for applying morden techniques in genetic transformation of plants. Although plants have remarkable capacity of totipotency, the behaviour of the explants in the -culture medium is unpredictable. The various terms predominantly used in plant tissue culture include differentiation, re-differentiation, dedifferentiation, morphogenesis and organogenesis. Differentiation is defined as a process by which meristematic cells are converted into two or more types of cells, tissuesor organs which are qualitatively different from each other. Dedifferentiation is the process of the process of formation of unorganized tissues from the highly organised tissues.The process of differentiation occurring in an undifferentiated tissue. Regeneration is is defined as the structuring of any part, which has been removed or physiologically isolated from the organism. Morphogenesis is attainment of biological organization or form is termed as morphogenesis.

Organogenesis

In plant tissue culture, organogenesis means genesis of organs like shoots, roots, leaves, flowers, etc. The earliest report on induction of

shoot organogenesis in vitro was by White (1939) using a tobacco hybrid; and the first observation of root formation were reported by Nobecourt (1939) using carrot callus. Till late 1950s, the basic regulatory mechanism underlying in organogenesis was not identified. Skoog and Miller (1957) were responsible to recognize the regulatory mechanism as a balance between auxin and cytokinin. As per their finding, arelatively high level of auxin to cytokinin favoured root formation and the reverse favoured shoot formation. Using this concept, it has now become possible to achieve organogenesis in a large number of plant species by culturing explants, calli and cell suspension in a defined medium. In organogenesis, the shoot or root may form first depending upon the nature of growth hormones in the basal medium. The genesis of shoot and root from the explants or calli is termed as caulogenesis (caulm = stem) and rhizogenesis (rhizo = root) respectively.Organogenesis or de novo regeneration is referred to the development of organized structures such as shoots, roots, flower buds, somatic embryos etc., from cultured cells or tissues. De novo organogenesis leading to complete plantlet regeneration is a multistage process consisting of at least three distinct stages.
Shoot bud formation,
Shoot development and multiplication
Rooting of developed shoots.

Caulogenesis is a type of organogenesis by which only adventitious shoot bud initiation takes place in the callus tissue. When organogenesis leads to root development, then it is known as rhizogenesis. Abnormal structures developed during organogenesis are called organoids. The localizedmeristematic cells on a callus which give rise to shoots and / or roots are termed as meristemoids. Meristemoids are characterized as an aggregation of meristem-like cells. These can occur directly on an explant or indirectly via callus.

Indirect organogenesis

Differentiation is influenced by the ratio of the exogenously supplied growth regulators auxin and cytokinin. In tobacco stem pith cultures a high ratio of auxin to cytokinin leads to initiation of roots whereas a low ratio leads to development of shoots.In general auxins (e.g. IAA (indoleacetic acid),NAA (a-naphthaleneacetic acid) and IBA indolebutyric acid)) will stimulate regeneration of roots, and cytokinins (e.g. BAP (6-benzylaminopurine) and kinetin) will promote regeneration of shoots or embryos (Fig.1).

Hence the auxin and cytokinins play an important role in organogenesis. Dedifferentiation and callus formation occur naturally in response to wounding. The wound responses involve auxin and cytokinins, which is the biological trigger for plant regeneration from somatic cells. However,sustained callus growth in vitro requires addition of one or more growth regulators.Prior to the chemical characterisation of IAA in 1934, attempts to obtain long-term callus cultures failed. With very few exceptions, auxin is essential for dedifferentiation and commonly 2,4-dichlorophenoxyacetic acid(2,4-D) is used to promote callus; cytokinin often enhances this process.

In tissues with a high endogenous level of auxin, culture of explants on a medium containing cytokinin as the only growthregulator may lead to development of shoots with very little callus. Not all living cells respond to auxin and this is particularly true of mature cells of grasses. Without dedifferentiation, it is not possible to move to the next stage of totipotent expression toplant regeneration.

Though auxin stimulates initial cell division in quiescent cells, continued presence of auxin can inhibit organised out-growth. This is a typical example of the sequential functions of a single hormone through a developmentalprogression.

In practical terms, cultures are usually transferred onto low or zero auxin media to permit or speed up shoot organogenesis.

Sometimes Removal' of auxin occurs when auxin in the medium is degraded either by the tissue itself or via chemical reactions such as photooxidation. Cytokinins promote outgrowth of shoots but are normally kept at very low concentration when root regeneration is wantedAuxins

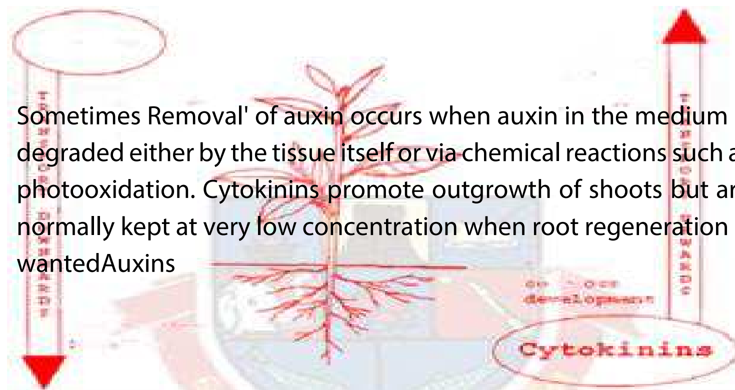


Fig. 1. Action of auxin and cytokinin

Direct organogenesis

Direct organogenesis bypasses the need for a callus phase. A good example is the formation of somatic embryos. Most evidence suggeststhat direct embryogenesis proceeds from cells which were already embryogenically competent while they were part of the original, differentiated tissue. These pre- embryogenic cells appear only to require favourable conditions (such as wounding or application of exogenous growth regulators) to allow release into cell division and expression of embryogenesis.

Such cells tend to be much more responsive than those involved in indirect organogenesis and do not seem to require the same auxin xpush' to initiate division; indeed, the cells may never have left the cell cycle and growth regulator application has some more subtle role.

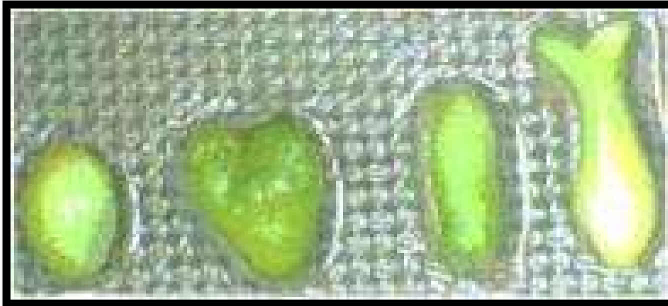
In Trifolium repens hypocotyl epidermis, BAP (a cytokinin) promotes reorientation of the plane of cell division, leading to initiation of a promeristemoid. An analogous response occurs in cotyledon explants of Abies amabilis where subepidermal cells develop intoshoots. In haploid embryos developed from Brassicanapus anther cultures, cytokininactually suppresses secondary embryoid

formation and instead promotes normal leafy shoots. This suggests a role for cytokinin in switching between shoot development and embryogenesis.

Although sucrose concentration and light modify the response, structures produced depend mainly on the auxin to cytokinin ratio. At 0.1:1, vegetative shoot buds form and at 100:1 roots are generated, but a 1:1 ratio promotes floral bud initiation. Incidentally, this remains a classic example

Somatic Embryogenesis

This is a method of direct organogenesis for development of plantlets for micropropagation or mass multiplication of specific plants. The cells, under a particular hormonal combination, change into the physiological state similar to zygotes (somatic zygotes) and follow an embryonic path of development to form somatic embryos (Fig. 2). These somatic embryos are similar to normal embryos (seed embryos) developed from zygotes formed by sexual fertilization. The somatic embryos can develop into a complete plant. Since somatic embryos can germinate into a complete plant, these can be used for the production of artificial seeds. Somatic embryos developed by tissue or cell cultures can be entrapped in certain inert polymers such as calcium alginate and used as artificial seeds. Since the production of artificial seed is amenable to mechanization and for bioreactors, it can be produced in large numbers.



Globular heal torpedo cotyledonary

Fig. 2. Stages of somatic embryos

Somatic embryos are structurally similar to zygotic embryosfound in seeds and possess many of their useful features, includingthe ability to grow into complete plants. 'However, somaticembryos differ in that they develop from somatic cells, instead ofzygotes (i.e., fusion product of male and female gametes) andthus; potentially can be used to produce duplicates of asinglegenotype. Since the natural seed develops as a result of a sexualprocess in cross-pollinating species, it is not genetically identical to one single parent. In contrast, somaticembryo develops from somatic cells (non-sexual) and does not involve sexualrecombination. This characteristic of somatic embryos allowsnot only clonal propagation but also specific and directed changesto be introduced into desirable elite individuals byinsertingisolated gene sequences into somatic cells. This bypassesgeneticrecombination and selection inherent in conventionalbreeding technology. If the productionefficency and convenience comparable to that of a true seed are achieved, somaticembryos can be potentially used as a clonal propagation system.

Advantages of Micro propogation:

From a very small tissue (explant) numerous plants can be developed, while inmethods only few plants can be generated.

In a conventional propagation method a large area is required for nursery space to maintaintissue culture container hundreds of plants can be maintained.The plants can be generated and maintained all round the year ignoring the seasonality of the plants.
The plants can be used for genetic transformation and can be used to develop transgenic plants with important agronomic traits.
Valuable germplasm can be conserved for long time without the need of fertilization and seed development.

EXPLANTS MICROPROPAGATION

Different kinds of explants were used in micropropagation. For example, incase of orchids, shoot tip (Anacamptis pyramidalis, Aranthera, Calanthe, Dendrobium), axillary bud (Aranda, Brassocattleya, Cattleya, Laelia), inflorescence segment (Aranda, Ascofinetia, Neostylis), lateral bud (Cattleya, Rhynocostylis gigantean), leaf base (Cattleya), leaf tip (Cattleya, Epidendrum), shoot tip (Cymbidium, Dendrobium,
Odontioda, Odontonia), nodal segment (Dendrobium), flower stalk segment (Dendrobium, Phalaenopsis) and root tips (Neottia, being Vanilla) are used in micropropagation.

METHODS OF MICROPROPOGATION IN PLANTS INOCULATION

Preparation of explants to be propagated is the first step of micropropagation. The stock (explants) should be free of bacterial, viral and fungal contaminations in order to get a healthy plant. After the plant material is chosen, the type of explant to be used have to be decided. The explants may be shoot tips, leaf, root, anthers, petals, pollen etc. Once the tissue type is selected, they have to be surface sterilised by repeated washing using bleach, ethanol,

mercuric chloride etc. Subsequently the explant has to be inoculated in a nutrient medium with sucrose as the energy source, micro and macro nutrients and plant growth regulators. For example cytokinins are used for developing branched shoots and auxins for root development.

Propogation:

Propogation is multiplying the shoot obtained in from the cultures by repeated subculturing. This is important to obtain multiple plants from a single explant. Through repeated cycles of this process, a single explant sample may be increased from one to hundreds or thousands of plants. Depending on the type of tissuegrown, multiplication can involve different methods and media.

If the plant material grown is callus tissue, it can be placed in a blender and cut into smaller pieces and recultured on the same type of culture medium to grow more callus tissue. If the tissue is grown as small plants called plantlets, hormones are often added that cause the plantlets to produce many small offshoots that can be removed and subcultured.

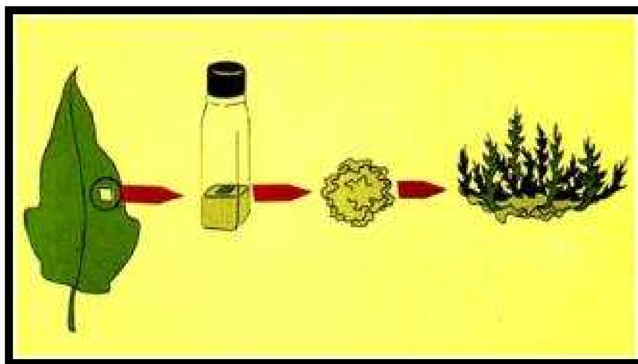


Fig. 3.Basics steps of Micropropogation

TYPE OF PROPOGATION

Through callusing:

The potentiality of plant cells to multiply indefinitely in cultures and theirtotipotent nature permit a very rapid multiplication of several plant types.

Differentiation of plants from cultured cells may occur via shoot – rootformation (organogenesis) orsomatic. Embryogenesis Somatic embryogenesis is most appealing from a commercial angle. A somatic embryogenesis system once established lends itself to better control than organogenesis.

Since somatic embryos are bipolar structures, with defined root and shoot meristems, the rooting stage required for microshoots gets eliminated. Above all, somatic embryos being small, uniform and bipolar are more amenable to automation at the multiplication stage and for field planting as synthetic seeds, offering cost advantages from labour savings, can also be stored through cold storage, cryopreservation or desiccation for prolonged periods. These characteristics make somatic embryogenesis potentially a less expensive and flexible system for micropropagation. The most serious objection against the use of callus cultures for shoot Multiplication is the genetic instability of the = r cells.

Adventitious budformation

Buds arising from any place other than leaf axil or the shoot apex are termed adventitious buds. The shoots differentiated from calli should also be treated as adventitious buds. In many crops, vegetative propagation through adventitious bud formation from root (blackberry, raspberry) and leaf (Begonia, Crassula) cuttings is standard horticultural practice. In such cases the rate of adventitious bud development can be considerably enhanced under culture conditions. For most bulbous plants (e.g. Lilley) adventitious bud

formation is the most important mode of multiplication and the best

Enhanced axillarybranching:

In cultures the rate of shoot multiplication by enhanced axillary branching can be substantially enhanced bygrowing shoots in a medium containing a suitable cytokinin at an appropriate concentration with or without auxin. Due to continuous availability ofcytokinin, the shoots formed by the bud, present on the explant, develops axillary buds which may grow directly into shoots. This process may be repeated several times and the initial explant transformed into a mass of branches.

PRETRANSPLANT

The ultimate success of commercial propagation depends on the ability to transfer plants out of culture on a large scale, at low cost and with high survival rates. The plants multiplied in vitro are exposed to a unique set of growth conditions (high levels of inorganic and organic nutrients, growth regulators, sucrose as carbon source, high humidity, low light, poor gaseous exchange) which may support rapid growth and multiplication but also induce structural and physiological abnormalities in the plants, rendering them unfit for survival under in vivo conditions. he two main deficiencies of in vitro grown plants are - poor control of water loss and heterotrophic mode of nutrition. Prior to the transplant of the tissue culture plants to field, they have to be acclimatized or hardened. Hardening is the process by which the plants are prepared for encountering the environmental conditions.

The plants are weaned from .deal conditions and are prepared to face the natural conditions. For example the plants are grown in a sterile condition in the test tubes during tissue culture phase. But, on exposure to the natural conditions, the plants are exposed to a number of microbes which include, pathogenic organisms and the

plants are prone to diseases. They have to accustom to the environment and develop strategies for defending themselves from the these harsh conditions. So prior to transplantation to field the plants are exposed to seminatural conditions. They are transferred to pots with loose soil like vermiculite or soilrite, so theat the roots are not damaged. A variety of potting mixtures such as peat, perlite, polystyrene beads, vermiculate, fine bark, coarse sand etc. or their mixtures in different combinations are used for transplantation. For initial 10-15 days, it is essential to maintain high humidity (90-100%) around the plants, to which they got adapted during culture. The humidity is gradually reduced to ambient level over a period of 2 -4 weeks Nutrition solution like Hoagland's solution should be supplemented.

SOMACLONAL VARIATION

Plants generally exhibit cytogenetic and genetic variations which help the plant breeders in crop improvement. When such variants arise through the cell and tissue culture process using any plant portion as an explant material, variations arising are termed as somaclonal variations . Variants obtained using callus cultures are referred as "Calliclones" while variants obtained using protoplast cultures are known as "Protoclones". Generally the term somaclonal variation is used for genetic variability present among all kinds of cell/plants obtained from cell cultures in vitro. Plants regenerated from tissue and cell cultures show heritable variation for both qualitative and quantitative traits. Several useful somaclonal variants have been obtained in large number of plant species such as, potato, sugarcane, banana, tomato etc

The basic cause of these variations may be attributed to changes in karyotype (chromosome number and structure), chromosome rearrangements, somatic crossing over, sister chromatid exchange, DNA amplification and deletion, transposable elements and DNA

methylation. Somaclonal variation can be characterized based on morphological,biochemical (isozymes) and DNA markers such as, Random Amplified Polymorphic DNA (RAPDs), Restriction Fragment Length Polymorphism (RFLPs) and Inter-Simple Sequence Repeats (ISSR).
The variations could also arise in tissue culture due to physiological changes induced by the culture conditions. Such variations are temporary and are caused by epigenetic changes. These are non-heritable variations and disappear when the culture conditions are removed.

There are different approaches (steps) to create somaclonal variations, which include:
Growth of callus or cell suspension cultures for several cycles. Regeneration of a large number of plants from such long term cultures.
Screening for desirable traits inthe regenerated plants and their progenies. Forexample, invitro selection to select agronomically desirable somaclones for tolerance to various biotic and abiotic stresses, herbicides, high salt concentration and extremes of temperature. Testing of selected variants in subsequent generations for desirable traits. Multiplication of stable variants to develop new breeding lines.

Applicationsof Somaclonal Variations

Variability generated at the genetic level proves to be a source of crop improvement which can be greatly beneficial to plant breeders. Distinctive mutations may sometimes give rise to elite characters in the regenerants which cannot be achieved by conventional methods of breeding.

Disease resistant genotypes of various plants can be attained. Resistance was first reported in sugarcane for eye spot disease (Heliminthosporium sacchari) and Fiji virus disease by regenerating plants from callus of susceptible clones.

Plants with characteristic resistance to abiotic stress (cold, draught, acidic or alkaline soil) can be obtained as somaclones.

Somatic genome exchange may give rise to regenerants where a part of alien genome can be introgressed thereby leading to germplasm widening.

Limitations of Somaclonal variations

Poor plant regeneration from long-term cultures of various cell lines.

Regeneration being limited to specific genotypes which may not be of much interest to breeders.

Some somaclones have undesirable features, such as aneuploidy, sterility etc.

Unpredictable variations that are often generated are of no use.

Variations attained may not always be stably integrated.

Variants attained may not always be novel. In majority of cases improved variants are not even selected for breeding programs.

OVERCOMING PRE- AND POSTFERTILIZATION BARRIER:

Interspecific and intergeneric crosses are made to introduce new genetic variation into cultivated plants. Inbreeding ornamental crops, interspecific hybridization is the mostimportant source of genetic variation. Many of the cultivars have originated from complex species crosses which have given rise to a broad range of shapes and colours to plants and flowers. The sexual barriers hindering interspecific hybridization are pre and post fertilization barrier many studies deal with methods for over coming pre fertilization barriers. once fertilization has occurred, hybridembryo growth may be restricted by post-fertilization barriers. Both embryo and endosperm have to develop an equilibrium for sharing nutrients

in an undisturbed developmental process Crossing barriers occur when crosses between plants of different species or genus (intra- or interspecific crosses).
These barriers are the result of incompatibility between the different plants. Sexual barriers preventing interspecific hybridization have been distinguished into pre- and postfertilization barriers. There are numerous techniques to overcome the barriers - bud pollination, stump pollination, use of mentor pollen and grafting of the style have been applied successfully to overcome prefertilization barriers. In vitro methods like ovary, ovary slice, ovule and embryo culture are used to overcome post-fertilization blocks which cause endosperm failure and embryo abortion. An integrated method of in vitro pollination and fertilization followed by embryo rescue has been applied in many crosses.

The sexual barriers hampering interspecific hybridization have been distinguished into preand postfertilization barriers (Stebbins 1958). Many studies deal with methods for erooming pre-fertilization barriers. Once fertilization has occurred, hybrid embryo growth may be restrictedbypost-fertilization
barriers. Both embryo and endosperm have to develop an equilibrium forsharing nutrients in an undisturbed developmental process. In general the first division of thezygote is delayed to favour the first division cycles of the endosperm cells. When the equilibrium inthe development of the zygote and endosperm is disturbed an abortion of the young embryo ordisintegration of endosperm follows. This abortion can take place in various stages ofdevelopment of the young seed. Depending on the stage of embryo abortion various in vitrotechniques can be applied to rescue the abortive embryo

PREFERTILIZATION BARRIERS

STIGMATAL AND STYLARBARRIERS

Use of mixed and mentor pollen

The use of mixed pollen i.e. mixture of compatible and incompatible pollen and mentor pollen, i.e. compatible pollen genetically inactivated by irradiation but still capable to germination, is reported to overcome inhibition in the style in many plant species,when used together with incompatible pollen

Influence of environmental conditions

The presence of the optimal level of receptivity of the stigma can vary from several hours (in mango) to more than one week (in lily). It determines the optimal time of pollination. A positive effect of high temperature in overcoming self incompatibiiity can be applied in breeding by heating the style or by pollinating at high temperatures. Style and ovary manipulations The gymnosperm can be manipulated to avoid self pollination. This has been demonstrated as early as 1945 by Blakeslee in Datura. Pollen tube growth inhibition in the style can be overcome using different pollination techniques. Removing the stigma and a part or whole of the style and pollinating the cut end. This is referred as stump pollination or ’cutstyle', ’intrastylar' or ’amputated- style' pollination. This results in arrest of pollen tube growth at different levels of the style. The pollen tube growth is arrested below the stigma (upper inhibition) or halfway in the style 3 to 4 days after pollination (lower-inhibition) results in medium sized pollen tubes. Following stump pollination, many pollen tubes of plants like lily, Lathyrus and Fritillaria grow normally into the ovary. In this way pollen escapes stylar and stigmatal barriers which can inhibit pollen tube growth.

Endosperm culture

In diploid plants, the endosperm is a triploid (i.e., having 3 sets of

chromosomes) tissue as a result of double fertilization, which is a unique process in higher plants. During the fertilization process, one of the male gametes fuses with the egg to form a zygote, which later forms the embryo; the other male gamete fuses with the central cell, which contains 2 haploid nuclei. This second fusion frequently results in a triploid structure, the endosperm. Hence, the endosperm is formed as a result of double fertilization and triple fusion (i.e., fusion between 3 different haploid nuclei, 1 from the paternal and 2 from the maternal side) and is present in all angiosperm families except Orchidaceae, Podostemaceae, and Trapaceae. The endosperm functions as a nutritive tissue, because growth and development of the embryo depends on the presence of the enaosperm. Moreover, the endosperm persists in some seeds (like cereals) as a reserve food. The endosperm represents about 60% of the world's food supply. When the endosperm fails to develop properly, abortion of the embryo results. Endosperm may be fully utilized by the developing embryo (non- endospermous), or it may persist in mature seeds (endospermous).

Callus tissue is induced from the endosperm explant in usual manner as with other explant. The endosperm tissue is homogenous in nature surrounded by a single peripheral layer of meristematic cells. These meristematic cells undergo repeated penclinal and anticlinal divisions resulting in increased girth of endosperm tissues and in turn producing callus with nodular structures on the surfaces or just below the outer most layer. Embryo cultures have been initiated from members of families ofEuphorbiaceae, Loranthatceae and Santalaceae. With respect to members of first two families the embryo has to be maintained intact along with endosperm in culture, to induce the callus from the endosperm. In these cases, immediately after the callus induction from the endosperm the embryo should be removed under aseptic conditions to avoid the

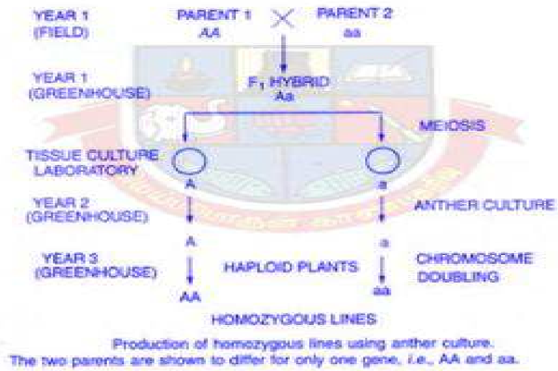
formation of embryo-endosperms callus mixtures. The embryo attached with the endosperm is that-duringgermination of the embryo, it releases gibberellin - like substances which turn in help in de novo synthesis of other enzymes responsible for the endosperm proliferation. These substances are otherwise called 'embryo factors'.

Application of endosperm culture

The cultured endosperm forms an excellent exper,mental system for physiological and morphogenetic studies. This system shows great promise in the study of metabolism and differentiation. The triploidy can be exploited in the crops viz. apple, banana, mulberry, sugarbeet, tea and watermelon where seeds are not of commercial importance. In some plants especially in clonally propagated ones triploids are superior to the diploids giving better pulp woods. Since these plants can be propagated vegetatively seed sterility is not a severe setback. In the case of conventional method of triploid production, crosses are made between auto tetraploids and diploids. Sometimes, these crosses may not be successful causing difficulty in triploid production.

Anther Culture

Anther culture is technique by which the developing anthers at a preciseand critical stage are excised .Aseptically from unopened flower bud and are cultured on a nutrientmedium where the microspore withinthe cultured anther develop into callus tissue or embryoids that give rise to haploid plantlets either through organogenesis orembryogenesis



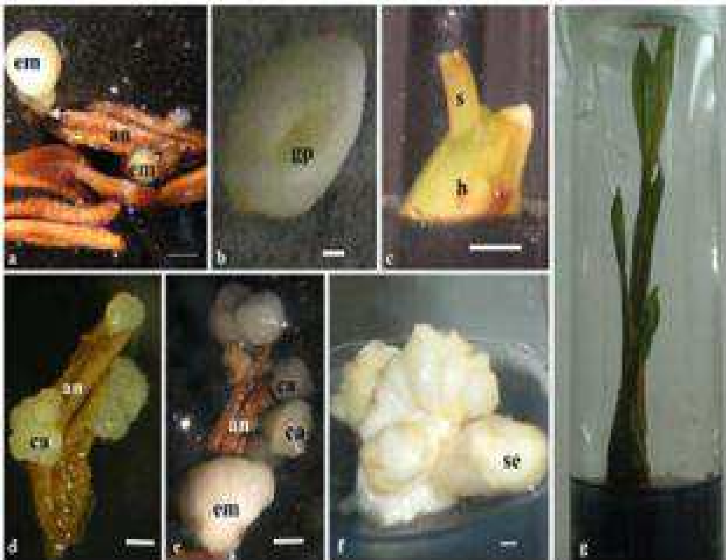


Fig. 4. Plant regeneration from anther culture in coconut

(Source: TNAU Agritech Portal)

Pollen Culture

Pollen or microspore culture is an in vitro technique by which the pollen grains, preferably at the uninucleated stage, are squeezed out aseptically from the intact anther and then cultured on nutrient medium where the microscope, without producing male gametes, develop into haploid embryoids or callus tissues that give rise to haploid plantlets by embryogenesis or organogenesis.

Chemical treatments

Application of growth regulators, such as auxins, cytokinins and gibberellins to the pedicel or the ovary at the time of or soon after pollination may improve fruit and seed set after interspecific pollination. Application of growth regulators to delay abscission of the style and show positive effects on the development of young fruits. In many crosses, application of growth substances promotes post-pollination development up to a stage when hybrid embryos can be excised and cultured. In interspecific crosses of Populus, treatment of the stigma with organic solvents such as hexane and ethyl acetate before pollination increases reported to be effective in overcoming prefertilization barriers. Also immunosuppressors such as amino-n-caproic acid, salicylic acid and acriflavin have been used to produce wide hybrids in many cereals and legumes. The female parent is treated with immunosuppressors before and/or after pollination for many days and the resulting embryo is cultured on a suitable medium.

TECHNIQUES FOR OVERCOMING POST-FERTILIZATION BARRIERS

There are numerous in vitro methods to overcome post-fertilization barriers in a number of plant species.

Embryo rescue

The term "embryo rescue" refers to a number of in v'tro techniques whose purpose is to promotethe development of an immature or weak embryo into a viable plant. Embryo rescue has beenwidely used for producing plants from hybridizations in which failure of endosperm to properlydevelop causes embryo abortion. In embryo rescue procedures, the artificial nutrientmedium servesas a substitute for the endosperm, thereby allowing the embryo to continue its development. Embryorescue techniques are among the oldest and most successful in vitro procedures.

One of the primary uses of embryo rescue has been to produce interspecific and intergenerichybrids. While interspecific incompatibility can occur for a Wide variety of reasons, one commoncause is embryo abortion. The production of small, shrunken seed following wide hybridization isindicative of a cross in which fertilization occurred but seed development was disrupted. Embryorescue procedures have been very successful in overcoming this barrier to wide hybridization in awide range of plant materials (Collins and Grosser, 1984). In addition, embryo rescue has beenused to recover maternal haploids that have developed as a result of chromosome eliminationfollowing interspecific hybridization.

Embryo rescue techniques also have been utilized to obtain progeny from intraspecific hybridizations that do not normally produce viable seed. For example, triploids have been recovered fromcrosses between diploid and tetraploid members of the same species, and progeny have beenobtained from crosses utilizingearly-ripening and "seedless," or stenospermacarpic, fruit genotypesas maternal parents. Embryo rescue techniques have also been used in situations in which embryoabortion is not a concern, such as for overcoming seed dormancy and studying seed developmentand germination.

Embryo culture techniques have many significant applications in

plant breeding, as well as basic studies in physiology and biochemistry. Immature embryo rescue and culture is a particularly attractive technique for recovering plants from sexual crosses where the majority of embryos cannot survive in vivo orbecome dormant for long periods of time. Overcoming embryo inviability is the most common reason for the application of embryo rescue techniques. Recently, fruit breeding programs have greatly increased the interest in exploiting interploid hybridization to combine desirable genetic traits of complementary parents at the triploid level for the purpose of developing improved seedless fruits. However, the success of this approach has only been reported in limited number of species due to various crossing barriers and embryo abortion at very early stages. Thus, immature embryo rescue provides an alternative means to recover triploid hybrids, which usually fail to completely develop in vivo.

Depending on the organ cultured, embryo rescue may be referred to as embryo, ovule, or ovaryculture. While the disinfestation and explant excision processes differ for these three techniques,many of the factors that contribute to the successful recovery of viable plants are similar. Thischapter will begin with a discussion of general factors that should be considered when utilizingembryo rescue and then turn to techniques specific to each type of embryo rescue procedure.

Factors affecting embryo culture:

Media

Murashige and Skoog (MS) and Gamborg's B-5 media are the most commonly used basal media for embryo rescue studies.Types and concentrations of media supplements required depend greatly on the stage of developmentof the embryo. There are two phases of embryo development:

The heterotrophic phase and the autotrophic phase.

In the heterotropic phase, theyoung embryo, which is often referred to as a proembryo, isdependent on the endosperm. Embryosinitiated at this stage require a complex medium. Amino acids, particularly glutamine and aspargine,are often added to the medium. Various vitamins may also be included. Natural extracts, such ascoconut milk and casein hydrolysate, have sometimes been used mstead of specific amino acids.Young embryos require a medium of high osmotic potential. Sucrose often serves both as a carbonsource and osmoticum. High osmotic concentration in the medium prevents precocious germinationand supports normal embryonic development. A sucrose concentration of 8- 25% is used.

The second stage of embryo development is the autotrophic phase, which usually begins in thelate heart-shaped embryo stage. At this time the embryo is capable ofsynthesizing substances required for its growth from salts and sugar. Germination will usually occur on a simpleinorganic medium, supplemented with 58 to 88 mM (2-3%) sucrose.

Growth regulators have been extensively used for Embryo resucue cultures. Lowconcentrationsof auxins have promoted normal growth, gibberellic acid has caused embryo enlargement and cytokinins have inhibited growth. Media requirements differ depending on stage of embryo development.Forcultures initiated using very young embryos, more than one media formulat:on may be needed.

Temperature and light

Temperature and light requirements vary among species. The growthrequirements of embryos often mimic those of their parents, with embryos of cool-season cropsrequiring lower temperatures than those of warm-season crops. Cultures are often incubated at

25to 30° C, although considerably lower temperatures are needed for some species. In species thatnormally exhibit seed dormancy, a cold treatment may be required.Cultures are usually initially cultured .n the dark to prevent precocious germination, but aremoved to a lighted environment to allow chlorophyll development after 1 to 2 weeks in the dark.

Time of culture

When attempting to rescueembryos of incompatible crosses, it is critical that the cultures beinitiated prior to embryo abortion. However, because it is more difficult to rear young embryosthan those that have reached the autotrophic phase of development, chances of success are maximized by allowing the embryo to develop in vivo as long as possible. Histological examinationscan be used to determine the time of endosperm failure and embryo abortion; however, theseevaluations can be very laborious.

Cultures are often initiated at variousintervals following pollination to maximize chances of recovering viable plants. Since an interaction between media andtime of culture is expected, it is important to test a range of media ranging from complex withhigh sucrose to simple with low sucrose at the various culture times

Embryo culture

The most commonly used embryo rescue procedure is embryo culture, in which embryos are excisedand placed directly onto culture medium. Fruit from controlled pollination of greenhouse- or fieldEmbryo culture can be applied successfully in crosses in which pollinated flowers can stayon the plant for a notable time, before natural abscission occurs. Sinceembryos are located in a sterile environment, disinfestation of the embryo itself is not required. Insome cases, the entire ovary is surface- sterilized. In other cases,

ovules are removed from the ovaryunder nonaseptic conditions and then disinfested. In either instance, a harsh disinfestation procedurecan usually be applied, since the embryo is protected by the surrounding tissue.

Careful excision of the embryo is critical to the success of embryo culture. A stereomicroscopeis usually required, and must be placed in the laminar flow hood in such a manner as not to restrictairflow. The best point of incision into the ovule differs among species. In some cases, embryoscan be extracted by cutting off the micropylar end of the ovule and then applying gentle pressureat the opposite end of the ovule. This results in the embryo being pushed out through the opening.It is crucial that the embryo be placed directly into culture after its excision so that it does notbecome dry. For heartshaped and younger embryos, the embryo should be excised with thesuspensor intact. Because of the extreme importance and frequent difficultyof excising embryos without causing damage, it may be helpful to develop and practice an excisiontechnique under nonaseptic conditions.

Embryo culture is sometimes preceded by ovule or ovary culture. One advantage of thistechnique (sometimes termed ovule-embryo or ovary-embryo culture) is that embryo excision isdelayed until the embryo becomes large enough to remove without damage. Also, the presence ofthe integument during the ovule or ovary culture phase has been found to reduce the possibility ofprecocious germination (Ramming, 1990). Once excised, the embryo may benefit from being indirect contact with the medium. Also, for those species affected by dormancy, removing the embryomay overcome any inhibitory effects imposed by the surrounding ovular tissues.

Nurse cultures have been used for rescuing embryos (Wiiliams et al., 1982). This techniqueinvolves inserting the embryo from an incompatible cross into endosperm removed from a relatedcompatible cross. For example, the embryo of an interspecific

hybrid may be inserted intoendosperm from an intraspecific cross involving one of the parental species. The embryo andendosperm are then placed into culture together

This method has been applied in a large number of crops Eg.,Allium, Lycopersicon and Solanum. To overcome the problems involved in isolating the young embryos from ovules and providing suitable conditions for their growth, embryo culture has been modified in some systems. The ovule is cut in half and the cut halves, or only the halves containing the embryo, are cultured in a liquid medium. Out of these half ovules germinating embryos emerge, which could be raised to plantlets on a solid medium.

Ovule culture

Embryos are difficult to excise when very young or from small-seeded species. To preventdamagingembryos during the excision process, they are sometimes cultured while still inside the ovule. Thistechnique is referred to as ovule culture or in ovolo embryo culture. As with embryo culture, ovariesare collected prior to the time at which embryo abortion is thought to occur. The ovary is surface- sterilized and the ovules removed and placed into culture. This step ranges from extremely easyto accomplish, for large-seeded species in which only a single ovule is present, to time-consumingand difficult, for small- seeded polyovulate species. Excision of the ovules may require the use ofa stereomicroscope

In those crops in which the fruit is aborted before embryo culture can be applied, ovule culture is an easy and fast method. This technique is applied in species of Alstroemeria, Lycopersicon, Nicotiana, Vitis etc. In Alstroemeria fertilization occurs 24 hours after pollination. Ovaries are harvested 2 days after pollination, the ovules are dissected and placed on MS medium containing 9 % sucrose. Six weeks later the ovules are transferred to an MS medium with 4-5 % sucrose. Germination of the ovules starts 1-2 weeks later. The ovules

are enlarged twice their size in the first two weeks due to proliferation of the inner and outer integuments. Plantlets can be developed after 42 days post inoculation from the cultured ovules. Depending on the genotypic combination of the interspecific crossing, the percentage of seedlings obtained from ovule culture varies from 0.5-22.5.

Ovary culture and ovary-slice culture

In ovary or pod culture, the entire ovary is placed into culture. Ovaries are collected and anyremaining flower parts removed. Disinfestation protocols must remove surface contaminants without damaging the ovary. The ovary is placed into culture so that the cut end of the pedicel is inthe medium. At the end of the experiment, seed are removed from the fruit that develop in culture (Fig. 5). A technique known as ovary-slice culture has been utilized for rescuing Tulipa interspecific hybrid embryos. Ovaries were cut transversely into sections and the basalcut end of the sections placed on the culture medium. In Tulipa, ovule culture and ovary-sliceculture produced similar germination rates;

However, the ovary-slice culture procedure was considered to be the superior of the two techniques because it was less time consuming. Ovary culture and ovary-slice culture has been applied in many species like Li Hum, Nerine and Tulipa etc. Ovaries are harvested 7-40 days after intrastylar pollination, surface sterilised, sliced into 2 mm thick disks and inoculated. Seed germination occurs 30-150 days post inoculation. By this method plantlets were obtained from very small embryos.

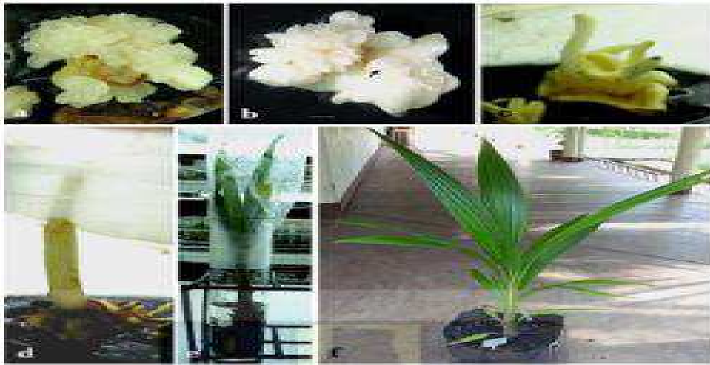


Fig. 5.Coconut plant regeneration from ov ovary culture (Source: TNAU Agritech rtapotal)

In vitro pollination/fertilization

In many instances, physical manipulation of the pollination/ fertilization process cancircumvent the genetic barriers of incompatibility and the physical barriers of anatomical problems such as pollen tube length versus style length. Incompatibility barriers are the genetically controlled male/female and self/nonself tubes penetrate into and discharge sperm within the female gametophyte, followed by processing of the sperm within that female gametophyte and the subsequent effecting of double syngamy between the two sperm nuclei and their respective mates: the egg nucleus and the fusion nucleus.It also is important to realize that ovule and seed are often used as synonyms, but this too is incorrect. An ovule is a sporophytic structure derived from the female parent within which the megagametophyte (female gametophyte) differentiates. When an ovule is mature,it contains a female gametophyte that is ready to receive a pollen tube and to befertilized (Fig.6). In contrast, a seed is a structure derived from a mature ovule, and within it the new sporophyte generation (embryo) develops following fertilization. When a seed is mature, it is

ready for dispersal, and it contains an embryo with or without endosperm. The significant difference is that when fertilization occurs, an ovule becomes a seed, and it is incorrect to persist in calling it an ovule. Thus, the term ovule culture is used correctly onlywhen fertilization has not occurred.

If fertilization has occurred, either naturally in vivo or experimentally in vitro, one must use the term seed culture when growing these structures under experimental conditions.

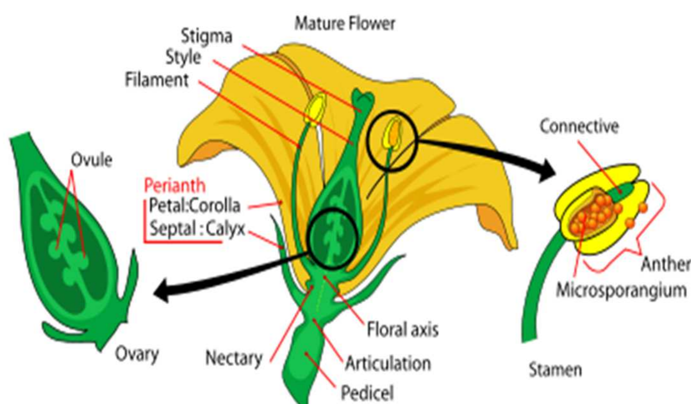


Fig.6: Diagram of a mature soyabean flower with petals removed illustrating male and female reproductive structure and growth of a pollen tube through the style into the ovary where it grows into an ovule. Sperm are thus conveyed from a pollen grain to a female gametophyte within which double fertilization occurs, b. In vitro pollination method in which a whole gynoecium (stigma, style and ovary) is placedinto culture and pollen is applied manually to its sigma. c. In vitro pollination method in which ovary wall is trimmed away to expose ovule and pollen is applied directly on ovules and placenta. d. In vitro pollination method in which ovules are excised, placed on an agar surface, and pollen is sprinkled nearby. (Tilton and Russel, 1984)

Methods for In vitro fertilization/pollination

The most widely used technique for in vitro fertilization is to culture unpollinated whole gynoecia (the female reproductive system, including stigma, style, and ovary, (Fig. 6a) and apply pollen to the stigmas when they become receptive (Fig. 6b).

This technique produces a good rate of success, and, as a general rule, the yield of viable seed in plants with numerous ovules per ovary often can be increased by culturing whole flowers rather than just isolated gynoecia. In soybeans, Glycine max (Leguminosae) and other plants with only a few ovules per ovary, however, high rates of fertilization and seed set can be obtained by pollinating and culturing isolated gynoecia.

Using this method, successful pollinations that result in fertilized soybean ovules can be detected soon after pollination by observing changes in the stigma and style.Pollination initiates a number of physiological and biochemical reactions throughout the gynoecium and, in the stigma and style, these reactions result inexternal changes that can be observed empirically. First, the pollen and stigma become brown and moribund; then the style withers.

The time required for these changes to occur varies with different taxa, but they usually are evidentwith in 48 hours In soybean we observe browing of the stigma in about 24 hours and, by about 36 hours, thethe styleusually has wilted. These changes in the stigma and style emulate natural changes that occur in vivo, but in our experience with soybeans, young fruits developing in vitro grow more slowly than do those growing in vivo.

Several variations of this basic in vitro pollination method are known, and some have been employed successfully to circumvent incompatibility barriers in a number of taxa. Examples of variations include: grafting the stigma and style of a compatible gynoecium

onto the ovary of an incompatible gynoecium; application of pollen to the cut surface of an ampu-tated style; and substitution of stigmal exudate, usually by removing exudate from the recipient stigma with alcohol and then depositing exudate from the donor stigma by direct physical contact and transfer between the two stigmas. A fourth and greatly different approach is to treat transmitting tissue chemically. For example one can colchicine to overcome problems caused byincompatibility.

The second technique used to achieve in vitro fertilization involves excising whole, unpollinated gynoecia and then trimming them to expose the ovules and placenta. These explants are put on solid culture medium and pollen is placed directly on the placenta or ovules (Fig. 6c). This method, known as intra ovarian/ placental pollination, is probably the most widely used technique for in vitro fertilization. It yields a good rate, usually 50-66%, of fertilization in soy-bean and has proven fruitful in a number of intraspecific crosses in several other taxa. It also has proven to be a successful technique to make interspecific and intergeneric wide crosses.

The third technique, pollinating ovules isolated from all other tissues, is the least effective (Fig. 6d). There is only one instance in which viable seeds were obtained from ovules that were excised, pollinated, fertilized, and the resulting seeds cultured in isolation from all other gynoecial tissue in Brassica oleraceae. This is the most important in wtropoNination/fertilization method to pursue and to make prac-tical because it offers the greatest degree of control over the process of fertilization on a one pollen tube/one ovule basis.

Germplasm Conservation in Plants

The whole set of genetic material of a species of plant is known as germplasm of the organism .It is based on the knowledge of germplasm that various breeding techniques of plants are developed. Hence the storage or preservation of germplasm is

important.

Conventionally seeds were used to store the germplasm. Conservation of plant genetic resources is necessary for food security and agrobiodiversity. Genetic diversity provides options to develop through selection and breeding of new and more productive crops, resistant to biological and environmental stresses. For more food, it will be necessary to make better use of a broader range of genetic diversity across the globe. Many plant species are now in danger of becoming extinct. More than fifteen million hectares of tropical forests are vanishing each year. Their preservation is essential for plantbreeding programs. Biodiversity provides a source of compounds to the medical, food and crop protection industries.Genetically uniformmodern varieties are being replacedwith highly diverse local cultivars and landraces of traditional agro-ecosystems. Deforestation, urbanization, pollution, habitat destruction, fragmentation and degradation, spread of invasive alien species, climate change, changing life styles, globalization, market economies, over-grazing andchanges in land-usepatternare contributing indirectly to the loss of diversity. These reductions are a threat for food security in the long term.

Genebanks were established in many countries for conservation of plants. Advances in biotechnology, especially in the area of in vitro culture techniques and molecular biology provide some important tools for improved conservation and management of plant genetic resources.

Conservation of plant genetic resources can be carried out either in the natural habitats (/» situ) or outside (ex situ). Ex situ conservation is generally used to safeguard populations, in danger of destruction, replacement or deterioration. An approach to ex situ conservation

includes methods like seed storage in seed banks, field gene banks, botanical gardens, DNA and pollen storage. Among these, seed storage is the most convenient method of long-term conservation for plant genetic resources. This involves desiccation of seeds to low moisture contents and storage at low temperatures. Seeds of some species, especially a large number of important tropical and sub-tropical tree species, are recalcitrant or intermediate, i.e. they cannot stand desiccation below a relatively high critical water content value (10-12% or 20% of fresh weight)and cold storage without losing viability.

Intermediate seeds can be stored by partial drying, although for shorter periods compared to orthodox seeds. Conservation of recalcitrant seeds under humid conditions can be carried out only for short periods, due to germination onset, fungal attack or viability loss. The second group of plants, which are not feasible for seed banking, are vegetatively propagated species. They are usually highly heterozygous and, in some cases do not produce seeds, such as banana, sweet potato, sugarcane, cassava, yam, potato and taro. Thesespecies are usually conserved in field gene banks. Field collections can lose germplasm (genetic erosion) because of pests, plaque attacks, diseases and adverse weather conditions and their maintenance is labor-intensive and expensive. In vitro culture is a feasible alternative for genetic conservation of plants where the seed banking is not possible. In vitro culture not only provides a method for clonal propagation and safe exchange of plant material but also used for medium-term germplasm conservation. Several in vitro techniques have been developed for storage of vegetatively propagated and recalcitrant seed producing specie.

In general, they fall under two categories: (1) slow growth procedures, where germplasm accessions are kept as sterile plant tissues or plantlets on nutrient media, which provide short- and medium storage options, and (2) cryopreservation, where plant

materials are stored in Liquid Nitrogen for long-term storage. Cryopreservation technique is based on the removal of all freezable water from tissues by physical or osmotic dehydration, followed by ultra-rapid freezing. Cryopreservation includes classical and new techniques. Classical cryopreservation techniques have been developed in the 70-80s. They comprise a cryoprotective treatment followed by slow freezing.

The mostcommon cryoprotective substances are dimethylsulfoxide (DMSO), polyethylene glycol (PEG), sucrose, sorbitol and mannitol. These substances have the osmotic actions; however some of them such as DMSO can enter to cells and protect cellular integrity during cryopreservation.
Classical cryopreservation methods are mainly used for freezing undifferentiated cultures such as cell suspensions and calluses. For freezing of differentiated tissues and organs such as seed, embryonic axes, shoot tips and zygotic and somatic embryos, new techniques include encapsulation-dehydration (ED), vitrification, encapsulation-vitrification, desiccation, pre-growth, pregrowth-desiccation and droplet freezing. Many of these techniques have been reported for conservation of plants germplasm. For successful cryopreservation, many factors such as source-plant status, starting materials, personnel, culture conditions, pretreatment conditions, cryopreservation methods, cryogenic facilities, regimes and post-thawing are involved.
Cryopreservation methods include both cryogenic (cryoprotectant and low temperature treatments) and non-cryoprotectant (pre- and post-storage culture) components. The success of a protocol depends on the tolerance and sensitivity of plant germplasm to the stresses. Most plant speciesneeds to be conserved at three broad levels; ecosystem level (in situ), genotype level (ex situ) and gene level (molecular). Cryopreservation and DNA storage may provide

longterm storage capabilities.Cryopreservation may besupplemented by DNA storage systems for long-term storage. DNA banks provide novel options for gene banks.

Germplasm Collection

Collecting involves gathering samples of a species from populations in the field or natural habitats for conservation and subsequent use. The unit of collection may be botanic seeds or vegetative propagules, depending on the breeding system of the species. Collecting may be easy in species producing small botanic seeds in abundance. However, it becomes problematic when seeds are unavailable or non-viabledue to: damage of plants by grazing or diseases; largeand fleshy seeds that are difficult to transport; or wheresamples are not likely to remain viable duringtransportation due to remoteness of the collecting sitefrom the genebank.

Advances in biotechnology provideuseful solutions for collecting such problem species. For example, in coconut (Cocos nucifera), where the major difficulty for standard seed collection is the large size of the seeds, in vitro techniques have been developed that allow collecting ofthe relatively small zygotic embryos in the field andtransporting them back in sterile conditions to the laboratory to inoculate and germinate them on a culture medium.In cacao (Theobroma cocoa), where collecting germplasm in the field is limited by the rapid deterioration of samples during transit as the seeds do not withstand desiccation. A simple in vitro method that involved collecting shoot nodal cuttings, followed by sterilization and inoculation of tissue into prepared culture vials containing semi-solidmedium.In vitro collecting methods were also developed for a range of other species including oil palm, forage grasses, banana, coffee, grape, Prunus and Citrus spp.

I Slow growth cultures

Slow growth of cultures involveslimiting the conditions of growth so that the culture does not grow and propagate in ordinary pace. This can be achieved by limiting the factors affecting the growth. This provides an attractive alternative to cryopreservation as the procedure is cost effective and simple comparatively.

Slow growth procedures allow clonal plant material to be held for 1-15 years under tissue culture conditions with periodic sub-culturing, depending on species. There are several methods by which slow growth can be maintained. In most cases, a low temperature often in combination with low light intensity or even darkness is used to limit growth. Temperatures in the range of 0-5°C are employed with cold tolerant species, but for tropical species which are generally sensitive to cold, temperatures between 15° and 20°C are used. It is also possible to limit growth by modifying the culture medium,mainly by reducing the sugar and/or mineral elements concentration and reduction of oxygen level available to cultures by covering explants with a layer of liquid medium or mineral oil.
Regeneration and successful propagation of geneticallystableseedling from culture are prerequisites for any In vitro conservation effort. Protocals for colnal multiplicationare wellestablished for several species. Generally, organized cultures such as shoots are used for slow growth storage since undifferentiated tissues such as callus are more vulnerable to somaclonal variation. Although slow growth procedures have been developed for a wide range of species, they are routinely used for conservation of genetic resources of only a few species including Musa spp., potato, sweet potato, cassava, yam, Allium spp. and temperate tree species. About 37,600 accessions are reportedly conserved by in vitro techniques in genebanks, worldwide (FAO, 1996)

Factors affecting slow growth cultures include

Temperature

The lowering of temperature beyond optimum level was found to affect the cultures by loweringthe growth pace.

I Nutrien t restriction:

The limiting of certain nutrient which is vital for growth and differentiation helped in achieving the slow growth culture.

Growth regulators:

In some case where temperature and nutrient control was not seen to be effective the culture is added with some growth regulators which regulate the growth of culture. The various growth regulators added include tri- idobenzoic acid (TIBA), chlormequat (CCC), abscisicacid(ABA).

Osmotic concentration:

Thelevel of osmotic concentration is another important method by which the slow growth of cultures can be achieved. The high levels of sucrose, mannitol or sorbitol were shown to reduce the growth of cultures.

Other factors: certain other factors such as oxygen concentration, culture vessel used for culturing, restricting the illumination received by cultures all affect the growth of cultures.

Cryopreservation

Cryopreservation involves storage of plant material at ultra-lowtemperatures in liquid nitrogen (-196°C). At this temperature, celldivision and metabolic activities remain suspended and the material can be stored without changes for long periods oftime.

Cryopreservation is the only available method for long-term conservation of vegetatively propagated plant germplasm. The choice of material includes cells, protoplasts, shoot apices, somatic embryos, seed or excised zygotic embryos.
Cryopreservation requires limited space, protects material fromcontamination, involves very littlemaintenance and is considered to be a cost-effective option.

The techniques for cryopreservation currently in use are quite varied and include the older classical techniques based on freeze-induced dehydration of cells as well as newer techniques based on vitrification. In classical techniques, tissues are cooled slowly at a controlled rate (usually 0.1-4°C/ min) down to about -40°C, followed by rapid immersion of samples in liquid nitrogen. Slow freezing is carried out using a programmable freezing apparatus. Cryoprotectants are added to the freezing mixtures tomaintain membraneintegrity and increase osmotic potential of the external medium. Classical cryopreservation procedures have been successfully applietoundifferentiated culture systems such as cell suspensions and calluses.

However, incase of differentiated structures, they have been employed for storage of apices or embryonic axes of only cold-tolerant species, and their utilization for tropical species has been limited.Verification- based producers involve removal of most or all freezable water by physical orosmotic dehydration of explants, followed by ultra-rapid freezing which results in vitrification of intracellular solutes, i.e. formation of an amorphous glassy structure without occurrence of ice crystals which are detrimental to cellular structural integrity. These techniques are more appropriate for complex organs like embryos and shoot apices; they are also less complex and do not require a programmable freezer, hence are

suited for use in any laboratory with basic facilities for tissue culture. There are seven vitrification-based procedures in use for cryopreservation: (1) encapsulation-dehydration, (2) vitrification, (3) encapsulation-vitrification, (4) desiccation, (5) pregrowth, (6) pregrowth-desiccation, and (7) droplet freezing.

With the advent of these new cryogenic procedures, especially vitrification, encapsulation-vitrification andencapsulation-dehydration, the number of species cryopreserved has increased significantly in recent years. In general, cryopreservation is well established for vegetatively propagated species. However, it is much less advanced for recalcitrant seed species due to some of their characteristics, including their very high sensitivity to desiccation, structural complexity and heterogeneity in terms of developmental stage and water content at maturity. The new cryopreservation techniques have been successfully applied for more than 80 species and they are under development or vigorous testing for several other species. However, examples of their routine use for long-term conservation are still limited only to oil palm and potato. As research carried out by various teams worldwide is progressively improving our understanding of mechanisms involved incryopreservation, it is expected that the utilization of cryopreservation in genetic resources conservation will increase steadily in the coming years

STEPS INVOLVED IN CRYOPROTECTION

Freezing: The procedure of freezing may be conducted slowly, rapidly orinitial freezing by dropping temperature slowly and followed by a rapid decrease in temperature. In order that the plants are not affected by the sudden decrease in temperature, treatment of cells with plant vitrification solution helps cells and tissue to overcome the harsh temperature. The medium was added with cryoprotectant like DMSO, glycerol, and proline to the culture

medium to protect cells from injury. The addition of cryoprotectant protects the cell by prevention of large crystals inside cell, protect from water loss from cell. The frozen cellsare stored in a refrigerator containing liquid nitrogen. The temperature of such refrigerator is maintained at or below -130 degree C. Organised tissues like shoot tips, somatic and zygotic embryos are usually chosen for storage. Alternatively cells can be immobilised in sodium alginate and then cryopreserved.

Thawing: Thawing of cultures is done in a rapid process. The freeze preserved culture is dipped in a water bath containing water at about 37-40 degree C for about 90 seconds. This process in done rapidly so that no ice crystals are formed. The thawed culture is washed several times to remove cryoprotectant. In the recent times, the cryprotectant is removed by diluting. This is done by fixing the culture along with a cryoprotectant onto a disk and is kept on a suitable medium. This disk is frequently transferred into a fresh medium. This frequent transfer dilutes out the cryoprotectant.

Reculture: The culture which is freeze preserved need to be thawed and cultured to bring it back to normal life. The optimum conditions of freeze preserved plants have to be determined fordeveloping a successful reculture. Aftercryopreservation, some plants tend to show special requirement for growth which was not necessary under normal propagation of the corresponding plants. For eg: tomato shoot tips when cryopreserved, thawed and recultured, the culture required some levels of abscisic acid in their medium in order to initiate and develop shoot tip from callus formed.It is found that mostly meristematic cells survive cryopreservation than other cells. In plants where the germplasm cannot be stored in seeds or other parts the cryopreservtaion provides a good option of storage and future usage.

ARTIFICIAL SEEDS

Another mechanism of germplasm conservation is by desiccating embryos and storing it as artificial seeds (Fig. 7). This has proved to be an effective mechanism, but was possible only with somatic embryos and in certain cases by shoot tips. The process of germplasm conservations offers several advantages like cost effective, availability of germplasm of specific plants to propagate, small storage space, and longer terms of storage. It also includes risks such as cell damage by cryopreservation, high technology involved etc.Artificial seeds make a promising technique for propagation of transgenic plants, non-seedproducing plants, polyploids with elite traits and plant lines with problems in seed propagation. Being clonal in nature the technique cuts short laborious selection procedure of the conventional recombination breeding and can bring the advancements of biotechnology to the doorsteps of thefarmer in a cost- effective manner.Artificial seed technology involves the production of tissue culture derived somatic embryos encased in a protective coating. Artificial seeds have also been often referred to as synthetic seeds.

Basic Requirement for Production of ArtificialSeeds

Recently, production of synthetic seeds by encapsulating somatic embryos has been reported in fewspecies. One prerequisite for the application of synthetic seed technology in micropropagation is the production of high-quality, vigorous somatic embryos that can produce plants with frequencies comparableto natural seeds. Inability to recover such embryos is often a major limitation in the development of synthetic seeds.Synthetic seed technology requires the inexpensive productionoflarge numbers of high quality somatic embryos with synchronousmaturation. The overall quality of the somatic embryos iscritical for achieving high conversion frequencies.

Encapsulationand coating systems, though important for delivery of somatic embryos, are not the limiting factors for development ofsyn thetic seeds.At present, the characteristic lack of developmental synchronyin embryogenic systems stymies multi-step procedures for guidingsomatic embryos through maturation. The lack of synchronyof somatic embryos is, arguably, the single most importanthurdle to be overcome before advances leading to widespreadcommercialization of synthetic seeds can occur. Synchronizedembryoid development is required for the efficientproduction of synthetic seeds.Gelling agents for seed encapsulation
Several gels like agar, alginate, pol yco 2133 (Bordon Co.), carboxy methyl cellulose, carrageenan, gelrite (Kelko. Co.), guargum,sodium pee tate, tragacanth gum, etc. were tested for syntheticseed production, out of which alginate encapsulation was foundto be more suitable and practicable for synthetic seed production.Alginate hydrogel is frequently selected as a matrix forsynthetic seed because of its moderate viscosity andlowspinnability of solution, low toxicity for somatic embryos andquick gellation, low cost and bio-compatibility characteristics.The use of agar as gel matrix was deliberately avoided as it is considered inferior to alginate with respect to long term storage.Alginate was chosen because it enhances capsule formation andalso the rigidity of alginate beads provides better protection(than agar) to the encased somatic embryos against mechanicalinjury.

Artificial Endosperm

Somatic embryos lack seed coat (testa) and endosperm that provide protection and nutrition for zygotic embryos in developing seeds. To augment these deficiencies, addition of nutrients and growth regulators to the encapsulation matrix is desired, which serves as an

artificial endosperm. Addition of nutrients and growth regulators to the encapsulation matrix results in increase in efficiency of germination and viability of encapsulated somatic embryos. These synthetic seeds can be stored for a longer period of time even up to 6 months wrthout losing viability, especially when stored at 4°C.

Addition of Adjuvants to the Matrix

In addition to preventing the embryo from desiccation and mechanicalinjury, a number of useful materials such as nutrients, fungicides, pesticides, antibiotics andmicroorganisms (eg. rhizobia) may be incorporated into the encapsulation matrix. Incorporation of activated charcoal improves the conversion and vigour of the encapsulated somatic embryos. It has been suggested that charcoal breaks up the alginate and thusincreases respiration of somatic embryos (which otherwise lose vigour within a short period of storage). In addition, charcoal retains nutrients within the hydrogel capsule and slowly releases them to the growing embryo.

Fig. 7 Cactus artificial seeds (Source: University of Malaya Website)

Application of synthetic seeds

The artificial seeds can be used for specific purposes, not ably multiplication of non-seed producing plants, ornamental hybrids (currently propagated by cuttings) or the propagation of polyploid plants with elite traits. The artificial seed system can also be employed in the propagation of male or female steri lepla nts for hybrid seed production. Cryo-preserved artificial seeds may also be used for germplasm preservation, particularly in recalcitrant species (such as mango, cocoa and coconut), as these seeds will not undergo desiccation. Furthermore, transgenic plants, which require separate growth facilities tomaintain original genotypes may also be preserved using somatic embryos. Somatic embryogenesis is a potential tool in the genetic engineering of plants.

Potentially, a single gene can be inserted into a somatic cell. In plants that are regenerated by somatic embryos from a single transgenic cell, the progeny willnot be chimeric. Multiplication of elite plants selected in plant breeding programs via somatic embryos avoids the genetic recombination, and therefore does not warrant continued selection inherent in conventional plant breeding, saving considerable amount of time and other resources. Artificial seeds produced in tissue culture are free of pathogens. Thus, another advantage is the transport of pathogen free propagules across the international borders avoiding bulk transportation of plants, quarantine and spread of diseases.

CHAPTER III

Isolation of protoplasts; Purification of protoplasts; Visibility and plating density of protoplasts; Protoplast culture and regeneration of plants; Protoplast fusion and somatic hybridization (techniques of fusion, selection of fused protoplasts, chromosome status of fused protoplasts, uses of somatic hybrids); Cytoplasmic hybrids or Cybrids; Genetic modification of protoplasts.

Indroduction

A fundamental difference between plant and animal cells is that plant cells are totipotent. Plant cell wall is vital for structure and function, it hinders in procedures like direct DNA transfer to individual cells and production of somatic hybrids by cell fusion. The plant cell wall can be removed without affecting cell viability. Plant cells devoid of cell wall are termed as protoplasts. The plant protoplasts can exist as separate cells without cytoplasmic continuity with neighbouring cells.

Protoplast culture

Plant protoplasts were first isolated from onion bulb scales by slicing onion with a fine knife in a plasmolyzing solution and releasing the protoplast from the cell wall by Klercker in 1892. Plant protoplast can be isolated by incubating plant tissues in a concentrated cellulase solution. Macerozyme is a cell wall degrading enzymes commonly used for protoplast isolation.

Fig. 1. Scheme of protoplast culture Source: Pl plant- Tissue-

Culture / Introduction- And-Hi history – Of - Plant- Tissue-Culture.aspx

ISOLATION OF PLANT PROTOPLAST

Klercker (1892) was the first to isolate protoplasts from plasmolysed cell of Stratiates aloides. Later protoplast isolation from tissues of onion bulbs was accomplished. Scales immersed in 1.0 M sucrose until the protoplast shrunk away from their enclosing wall and then the plasmolysed tissue was cut with a sharp knife at such a thickness that only the cell walls were cut without damaging the protoplasts in strips. The protoplasts were released by osmotic swelling when these strips of the tissue are placed in a low concentration sucrose solution.

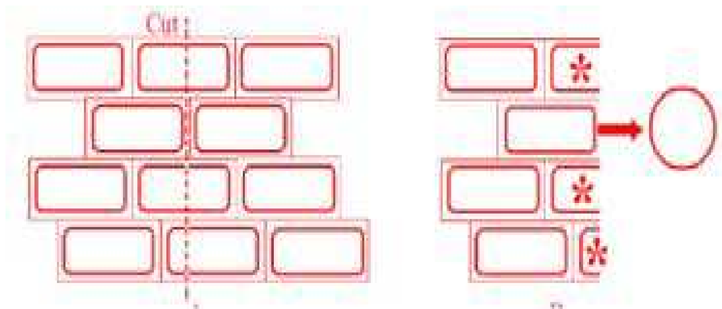


Fig. 2.Protoplast isolation by mechanical cutting though the cells without damaging the protoplasts in strips.

Plant protoplasts can be produced by two procedures

Mechanically slicing or chopping of plant tissues: To isolate protoplasts by mechanical slicing, numerous cuts are made through plant tissue. This allows some of the protoplasts to slip out of tangentially sectioned cells. This procedure produced protoplasts rapidly, but the yields are low. It is best for isolation of protoplasts from thick tissues like potato tubers or apple fruit.

Enzymatically digesting the cell wall using various hydrolytic enzymes: Protoplast isolation by enzymatic cell wall digestion using enzymes like cellulase, hemicellulose and pectinase, which are extracted from various sources including fungi, snail gut and termite gut. Digestion by a combination of these enzymes is generally conducted at a pH of 5.5-5.8 over a period of 3-16 h. Subsequently the protoplasts can be collected and purified by centrifugation to separate broken and damaged cells from intact protoplasts.

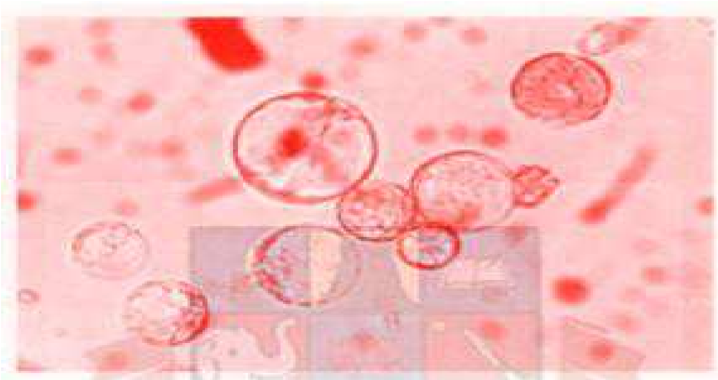


Fig. 3. Freshly Isolated protoplast from olaSolaum phureja (Compton *et al.* 1996)

Purification of protoplast

The successful culture of protoplasts requires a pure population of intact and viable protoplast at a high yield. So the protoplasts require to be purified by removing the undigested material, damaged protoplast and enzymes.

Removal of Debris and enzymes

Debris can be removed from the protoplast suspension by filtering the preparation through a steel or nylon mesh of 100 p pore size. Enzyme is removed by centrifuging the protoplast suspension at 600 rpm for 5 minutes. The protoplasts settle to the bottom of the centrifuge tube, while the supernatant is removed with the help of a

pipette. The protoplasts are then resuspended in a washing medium containing an osmoticum only or osmoticum withnutrient medium or hydrated calcium chloride. The suspension is centrifuged again to setle the protoplasts and the washing medium decanted. Traces of enzyme are removed by washing the protoplasts twice or thrice with the medium

Removal of BrokenProtoplasts

Intact protoplasts are sparated from the broken debris by suspending the protoplast preparation 20-40% sucrose solution and centrifugation at 350 rpm for three minutes. Intact protoplast collect at the top of the sucrose solution ane are carefully removed with a pipette. Ficoll or density buffer can be used for purification of protoplast. In this method 0.5-3.0 volumes of crude protoplast preparation after filtration through sterile muslin cloth is layered on lympoprep in the centrifuge tube and spun at 50-200 g for about 10 minutes. The protoplast collect as a ring between the enzyme solution and lymphoprep and debris settle to the bottom. The protoplasts are removed fromt the interphase with a pasture pipette

Fig. 4.Protoplast purification. A. Purified protoplast ring. B. Purified protoplast population

Nutritional requirements for protoplast culture

Nutritional requirement for the growth of protoplasts differ from the nutritional requirement for tissues and cell culture. Protoplast leak in cultures as they are devoid of cell walls, and moreover, they have a greater surface are for the diffusion of metabolites than the tissues. So the concentration of different metabolites in protoplast is less than that in the tissues and cells. To compensate for these loses from protoplast the number of metabolites provided in their culture media is more.

The necessity to develop protoplast- to-plant systems, particularly for economically important species, has demanded a major investment of resources. Typically, isolated protoplast commences cell wall regeneration within a short time (often minutes) followingintroduction into culture. However, they require osmotic protection until their new primary walls can counteract the turgor pressure exerted by the cytoplasm. In some cases, gradual reduction of the osmotic pressure by diluting the culture medium with a solution of similar composition, but of reduced osmotic pressure, is essential for sustaining mitotic division, leading to the formation of daughter cells and tissues.

Protoplasts from different species and from different tissues of the same species have different nutritional requirements, which has to be standardized. The major media are based on the MSand B5 formulations, with additionof an osmoticum, usually anonmetabolisable sugar alcohol, such as mannitol, or sorbitol. A complex, undefinedmedium containing coconut milk can also so be used for the culture ofprotoplasts at very low densities.

The major growth regulators, auxins and cytokinins, are normally essential forsustained protoplast growth, although exceptions exist where only auxin is required, as incarrot and A. thaliana.In contrast, auxins and cytokinins aredetrimental to growth in citrus.The growth

requirements of protoplastsoften change during culture, necessitating modification of medium composition, typicallyinvolving a reduction of the auxin concentration. Phenylureaderivatives, such as N- (2-chloro-4-pyridyl)-NV-phenylurea, and brassinosteroids, which are similar structurally to animal steroidal hormones, canpromotedivision of protoplast-derived cells. Cyclophilinimmunophilins may play a role in actively growing protoplast-derived cellsand intact plants, particularly during early flower development. Sucrose and glucose are the regular choices of carbon sources in most media, although maltose promotes shootregeneration for protoplast- derived cells of cereals

Procuedue for culture of isolated protoplasts

Protoplast culture technique is generally based on liquid or semi-solid media, or their combination. Protoplast are dispensed into petri dishes and it is the easiest and simples method as the medium can be easily replaced to gradually reduce its osmolarity,therebymaintaining protoplast growth. Suspension culture by droplets of suspension of ca. 100-150 pl involume are useful when limited numbers of protoplasts are available. Semi solid media can be used as the isolated protoplasts can withstand the rigours of being embedded in semi-solid media.

Protoplastsremain separated in the semi-solid medium, with the latter supporting wall regeneration andpromoting mitotic division. Semi-solid media containing suspended protoplasts can bedispensed as a layer or droplets, with the latter usually up to 250 pi in volume in Petridishes. Dissecting the layer of medium into sectors, with subsequent bathing of the sectorsor droplets in liquid medium of the same composition, promotes protoplast growth.Stepwise reduction of the osmotic pressure is readily achieved by changing the bathingmedium.

Alginate has also been used to semi- solidify media, with gelling

being induced byexposure to calcium ions. Crucially, protoplast-derived colonies may be released bydepolymerising the alginate by treatment with sodium citrate to remove the calcium ions.Suspending protoplasts in a thin layer of liquid over semi-solid medium stimulates cell colony formation, particularly when a filter paper is included at the liquid/semisolid interface. The filter paper can be replaced with abacterial membrane filter (pore size 0.2 Am) to produce a similar effect. Nylon mesh has also been used as a support for protoplasts in bothliquid and semi-solid culture systems.
Removal of the filter paper, bacterial filter, cellophane, or nylon mesh facilitatestransfer of protoplast- derived cells to new medium.

SOURCES OF PROTOPLAT

Leaves:

The leaf is the most convenient and popular source of plant protoplast because it allows isolation of a large number of relatively uniform cells. Protoplasts isolation from leaves involve five basic stages: a) Sterilization of leaves, b) removal of epidermal cell layer c) Pre-enzyme treatment d) incubation in enzyme and e) isolation by filtration and centrifugation.In case of monocots, leaf material is cut into small places (1mm2) and then combined with vacuum infiltration. This procedure allows adequate infiltration of enzymes into leaf cells. As soon as the vacuum is removed the leaf piece will sink and eventually release the mesophyll protoplasts (Fig. 5).

Callus Culture:

Young actively growing callus is subcultured and used after two weeks for protoplasts isolation.

Cell Suspension Culture:

A high - density cell suspension is centrifuged. After removing the supernant, cell are incubated in enzyme mixture (cellulose +

pectinase) in a culture flask placed on a platform shaker for 6 hrs to overnight depending on to the concentration of enzymes. A lower concentration cf enzymes helps to prevent the formation of aggregates in the cell suspenion in order to obtain better yield.

Preconditioned PlantMaterials:

Mesophyll protoplast of some crop plants have a low morphogenetic response. This is because of the fact that thephysiological state of growth of a donor plant under natural condition largely affect the regeneration potential of protoplasts in this system. On the contrary, tissue cultured regenerated plants are maintained under uniform physiological conditions and therefore provides materials preconditioned for protoplasts isolaton, and regeneration. This approach is particularly essential for regeneration of potato protoplasts.

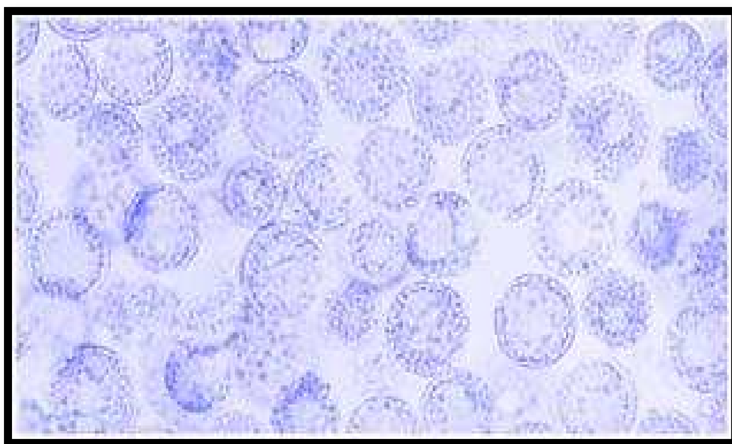


Fig. 5 Protoplast isolated from leaf mesophyll of TriTriticum aestivum.

FACTORS AFFECTING PROTOPLAST CULTURE

Successful growth and regeneration of protoplasts is dependent on a number of factors ranging from the status of the donor plant to the culture conditions.

Plant Species and Varieties

It is well documented fact that even very small genetic difference leads to varying protoplast responses to culture conditions.

Plant Age and Organ

A second important factor for the successful culture is the age of the donor plant and developmental stage of the donor part used for isolation of the protoplasts. The stages with respect to their responses are germinating embryos, followed by seedling of one week, planlets, leaves, juvenile tissue from mature plant and mature tissue. Though having the same genetic information protoplasts isolated from these parts behave differently in culture conditions. Plant regeneration has been most successful from leaf protoplasts of herbaceous species.

Improved techniques for protoplast culture

Electrical stimulation of protoplasts

Some cells of plants and animalsdetect voltage gradients and current densities as lowas 0.5 µV m-1 and 5 nA cm-2respectively, with weak electricalcurrents of prolongeddurationstimulating growth, healing of wounds and bones, and organ regeneration

In animals. Electro stimulation of protoplasts of *Medicago sativa*, resulted in development of protoplasts directly intosomatic embryos, prior to plant regeneration. Electrical currents not only alter cell polarity, but also affect auxin transport.

Electrical effects were influencedby several variables, including the voltage and its duration, and protoplast size. Electrotreated protoplasts of Prunus, Pyrus, and Solanumentered mitosis within 5 days of culture compared to untreated protoplasts, which hadlagperiods of 15, 9, and 1 days, respectively. This simple

technology cans beapplied to protoplasts from embryogenic cell suspensions recalcitrant species. Experiments withprotoplasts and cultured mammalian cells confirmed that electricalpulses stimulate DNA synthesis, which, in turn, is reflected in the earlier onset of mitosisin protoplast-derived cells. A key feature of electrostimulated protoplasts is thatenhancement of division was sustained to the callus stage. Shoot regeneration is also stimulated from callusfollowing electrostimulation. The electro stimulated plant regenerate more vigorously than controls.

Supplementation ofculture media withsurfactants, antibiotics, and polyamines

Supplementation of the culture medium with nonionicsurfactants is a novel approach for enhancing the mitotic division of plant protoplast-derived cells. PluronicR F-68, apolyoxyethylene-polyoxypropylene copolymer, is used extensively asa nontoxic, low-cost cell- protecting agent during theculture of both animal and plant cells. For example, in experiments with plant protoplasts, supplementationof culture medium with 0.1% (wt/ vol) PluronicR F-68 increased the plating efficiency ofprotoplasts of Solanum dulcamara by 26% over control. PluronicR F-68 exerts astimulatory effect on cell growth and differentiation by promoting the uptake of nutrients, growth regulators, and oxygen.

Some antibiotics stimulate the division of protoplast-derived cells. For example, the cephalosporin antibiotic, cefotaxime, promotes mitotic division and cell colonyformation of protoplasts isolated from seedling leaves of the woody plant passionfruit (Pass/77or3 edulis) when added to the culturemedium at 250 pg ml"1. Polyamines influence plant cell morphogenesis by regulating DNA replication, transcription, translation, cell division, and differentiation, and are regarded as a newclass of plant growth regulators and a reserve of carbon and nitrogen in cultured tissues.

Thus, polyamines stimulate DNA synthesis and mitotic activity inoat protoplasts, with arginine also stimulating division in protoplasts of almond.

Manipulation ofrespiratory gases during protoplast culture

The gaseous environment plays a fundamentalcomplementary role in growth and differentiation of protoplsts. Importantly, medium, headspace, andplant tissues interact in complex ways that have often been overlooked. The photosyntheticcapability of some tissues and their biomass relative to the capacity of the vesselsand the culture room environment contribute to the composition of the gases within culturevessels. There is a need to manipulate headspace gases to ensure that oxygen and carbondioxide do not deviate dramatically from their normal atmospheric concentrations of 21%and 0.03% (vol/vol), respectively and thatethylene does not accumulate. Adjusting the protoplast density, depth of themedium, and oxygen concentration in the headspace results in a successful protoplast culture. The concentration of the oxygen in the head space can be manually maintained.

Culture of protoplasts in small volumes of liquid mediumon the surface of filters increases gaseous exchange, often with nurse cells in anunderlying semisolid layer. Another simple system involves the insertion of glass rods,each approximately 6 mm in diameter and 8 mm in length, into a layer of semi- solidculture medium. Protoplasts plated in liquid medium over the semi-solid phase aggregates in the menisci around the glass rods and at the sides of the dishes. Protoplasts in these regions are stimulated to divide, probably because of improved aeration at the menisci.

Gassing of protoplast cultures

Oxygenenrichment of the headspace, by placing culture vessels in screw-capped glass jars with inlet and outlet valves, prior to gassing

of the jars with oxygen and sealing of the valvesenhances the protoplast division in culture. Following initial enrichment, the oxygen concentration inthe headspace is believed to decline gradually, as slow gaseous exchange is possiblethrough the seals of the vessels. The shoot regeneration frequency from protoplast-derivedtissues increases byexposure of protoplasts to an oxygen-enriched atmosphere, indicating a long-term effect.

Uses of Plant protoplasts

Plant protoplasts are used for crop improvement by breeders. Protoplasts are used for sexual hybridization to improve agronomic traits in crop plats. This technique can be used for closely related species or with wild varieties. Sexual hybrids between distantly related species are also possible for introduction of single gene traits. Intrasepecific and interspecific incompatibility barriers limit the use of sexual hybridization for crop improvement. Protoplast fusion can be usedful to develop plants with improved agronomic and horticultural characteristics like insects resistance etc

By isolating protoplast and regenerating plants from them, plants with improvedcharacteristic have beenretrieved by somaclonal variation Parasexual or somatic hybrids can be obtained by fusing protoplasts of unrelated or distantly related speciesImproved plants obtained by protoplast manipulations can be used in breeding programes to develop new cultivars.

Regenerating plants from protoplasts:

Leaf mesophyll tissue is commonly used as a source of plant protoplast. The cells within the leaf mesophyll are lossely packed with allows penetration of digestive enzymes that facilitate protoplast release.Typically 10 to 5 10 protoplasts can be obtained per gram of leaf tissue. Generally, the youngest, fully expanded

leaves from young plants or seedlings are used.
Preconditioning of the plants by placing them in darkness for 24-72 h before protoplast isolation or givingthem a cold treatment (4-100 C) improves protoplast yield. In vitro plantlets are often preferred as a source of protoplasts as they confer aseptic conditions. While using greenhouse or garden grown plants surface sterilization has to be done to the tissues prior to protoplast isolation. In vitro sources of protoplasts include callus, cotyledons, hypocotyls, embryogenic suspension cultures, leaves, shoots or somatic embryos.
Before protoplast isolation, plant tissues are cut into small pieces and flated in an isotonic solution at 20-250 C for 1-24 h. During this step water moves out of the cells, which causes their contents to shrink and draw away from the cell wall (plasmolysis). This step is done atlow temperatures (4-10° C). The pieces are incubated in digestive enzymes for removal of the cell walls and middle lamellae. Incubation is done in darkness in a shaker for 4-18h at 25-30° C. Subsequently the digest is gently swirled to release protoplasts and filtered to separate protoplast from large debris. The protoplast suspension is centrifuged at low speed (50 x g) for about 10 min. Protoplasts and debris is collected in the pellet. The supernatant is discarded ad protoplasts resuspended in a high sucrose medium, which is overlaid with 1 ml of rinse medium containing mannitol. During centrifugation, viable protoplast collects at the interface of the two media while debris concentrates in the pellet. Protoplasts are removed from the interface with a Pasteur pipet and washed two or three times before transfer to culture medium.

Seeding density

Protoplasts density is very crucial as it influences the plating efficiency and better surviving of protoplasts.When plated at higher density protoplasts compete with one another, while at lower density losses of metabolits from protoplast is more. The later

situation of protoplast leakiness can be circumvented by addition of required matabolites to make medium isotonic.

After the final wash, protoplasts are suspended in a small volume (about 1 ml) of liquid culture medium. A sample is removed and transferred to a hemocytometer to determine protoplast density. Enough culture medium should be added to adjustthe protoplast density to 104-106 cells per millilitre. Protoplasts may be cultured in a thin layer of liquid medium, embedded in agarose, in liquid medium on top of solid medium or in agarose droplets suspended in liquid. Liquid medium is used to facilitate adjustment of cell density and osmotic pressure. The final (overall) density of protoplasts in the culture medium (plating density) is crucial for maximising wall regeneration and concomitant daughter cell formation. Generally, the optimum plating density is in therange 5 X 104-1 X 106 protoplasts ml-1

.

An excessively high plating density rapidly depletes nutrients, andprotoplast-derived cells can fail to undergo sustained division. Cells stimulate mitotic division of adjacent cells by releasing growth factors, including amino acids, into the surrounding medium, aprocess commonly known as 'medium conditioning' or 'nurse' culture. Consequently, protoplasts fail to undergo sustained division when cultured below a minimum inoculumdensity threshold. Medium preconditioned by supporting the growth of actively dividingcells for a limited period is valuable in stimulating growth of isolated protoplasts.Similarly, activelydividing cells can promote or ^urse' the growth of recently isolatedprotoplasts. Nurse cells can be from the same or different species. Protoplasts/spheroplasts or cells that have been X-irradiated to inhibit division can also exert a similar nurse effect.

Viability:

Protoplast viability is estimated by staining a sample with fluorescein diacetate (FDA) stain. The stain is mixed with protoplast and fluorescence observed under UV light. Viable protoplasts actively absorb the stain and exhibit a green fluorescence, whereas nonviable protoplasts don not. Although nonviable mesophyll protoplasts fluoresence red because of the auto florescence of chlorophyll, they are not metabolically active. Evans blue can also be used as an alternate staining agent.

Freshly isolated protoplasts are spherical because they are unrestricted by a cell wall. Under suitable conditions, viableprotoplasts regenerate a new cell wall within 48-96 h after isolation. Presence of the cell wall can be determined by a change in shape from spherical to ovoid or by staining cell with Calcofluor white, which detects the presence of cellulosic wall materials. Stained cells or protoplasts with remnant cell wall material or protoplast that have resynthesized a new cell wall fluoresce bluish white under UV light. Protoplast without a wall do not fluoresce. Protoplasts that fail to regenerate a wall generally will not divide and eventually die (Fig. 6).

Not all healthy protoplasts divide. Therefore, the plating efficiency (PE = number of div.ding protoplasts/ total number of protoplasts) is used to estimate cell vigor. PE is usually calculated 1 week after protoplast isolation and varies from 0.1 - 80%. The first cell division often occurs within 14-21 days and macroscopic calli within 4 weeks. Plant regeneration occurs by shoot regeneration of somaticembryogenesis. The first shoots or embryos may be seen as early as 1 month after protoplast isolation but may require 6 months to 1 year.

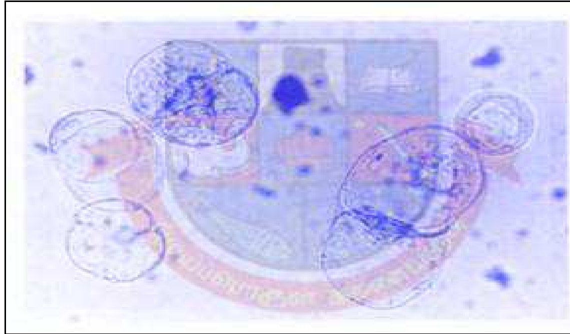


Fig. 6. Mitotic division of isolated protoplast (Compton *et al.* 1996)

SOMATIC HYBRIDIZATION

The Somatic cell hybridization/ parasexual hybridization orProtoplast fusion offers an alternative method for obtaining distant hybrids with desirable traits significantly between species or genera, which cannot be made to cross by conventional method of sexual hybridization. Plant cells from which the cell wall has been enzymatically or mechanically removed are called protoplasts. Theoretically, protoplasts are totipotent, meaning that after their isolation and subsequent culture they have the capability to dedifferentiate, re-start the cell cycle, go through repeated mitotic divisions and then proliferate or regenerate into various organs. Fusion of protoplasts from different species can therefore be a practical breeding tool and circumvents sexual hybridization related prezygotic or postzygotic barriers. Somatic hybridization differs from other techniques in many respects. When comparing somatic hybridization to transgenic approaches, somatic hybridization enables broadening of the germplasm base, allows the transfer of uncloned multiple genes and generates products that are not subjected to the same legal regulations as transgenic lines. Also, it transfers both mono- and polygenic traits. The first protoplast fusion

was described more than a century ago by Kuster in 1909. Tobacco was the first crop in which successful interspecific somatic hybridization was reported by Carlson in 1972. Since then, improvements and somatic hybrids have been made in many species and complete plant regeneration was accomplished. Somatic hybridization broadly involves in vitro fusion of isolated protoplasts to form a hybrid cell and its subsequent development to form a hybrid plant. The process involves: a) fusion of protoplasts, (b) Selection of hybrid cells, (c) identification of hybrid plants. During the last two decades, a variety of treatments have been used to bring about the fusion of plant protoplasts. Protoplast fusion can be achieved by spontaneous, mechanical, or induced fusion methods.. These treatments include the use of fusogens like NaNO3, high pH with high Ca2 + + ion concentration, use of polyethylene glycol (PEG), and electrofusion. These inducing agents used in protoplast fusion are called 'fusogen'.PEG treatment is the most widely used method for protoplast fusion as it has certain advantages over others. These are: (a) it results in a reproducible high-frequency of heterokaryon formation, (b) The PEG fusion is non specific and therefore can be used for a wide range of plants, (c) It has low toxicity to the cell and (d) The formation of binucleate heterokaryons is low.

Protoplast fusion can be either symmetric or asymmetric depending on the nature of genetic contribution of the fusion partners. A somatic breeding protocol can typically be subdivided into the following steps: isolation, fragmentation (in case of asymmetric hybridization), fusion, regeneration and selection. In symmetric fusion the complete genomes of both parent protoplasts are fused. However, when two complete genomes fuse, a phenomenon called "gene conflict" may arise, because certain chromosomes repel one another. Moreover, the technique introduces a significant amount of

unwanted genetic material. These limitations result in abnormal growth, regeneration of hybrids with low fertil-ity, non-rooted shoots, slow hybrid growth, and recalcitrant calli or microcalli (Fig.7).

In asymmetric fusion, after genome fragmentation only a limited amount of one genome s transferred to thefusion product. Cytoplasmic genomes can also be recombined with nuclear genomes for applications such as cytoplasmic male sterility (CMS) intro- duction. The technique is being applied to circumvent the above mentioned barriers in symmetric fusion. For example, symmetric hybrids betweenBrassica napus and Lesquerella fendleri are self-sterile, whereas asymmetric hybrids between the same fusion parents are self- fertile. Similarly, symmetric fusion between Orychophragmus violaceus and B. napus yields sterile hybrids, whereas asymmetric hybrids are fertile and can set seeds. By introgressing fewer genes than after sexual crossing or symmetric somatic fusion, the number of backcrosses can also be significantly reduced. Genome fragmentation of the donor parent encourages the elimi- nation of much of its redundant genetic material in the somatic hybrid. Moreover, in asymmetric fusions, most karyotype instability causing donor genes are eliminated during the first post-fusion mitoses, as opposed to symmetrical fusions after which eliminations can occur up to the first sexually derived generation.

In other words, not only does asymmetric fusion introduce fewer genes in a recipient genome after fragmenting the donor genome, but elimination of disadvantageous genes or chromosomes also proceeds quicker. Nevertheless, chromosomes and chromosome fragments can still be lost during meiosis due to rearrangements.

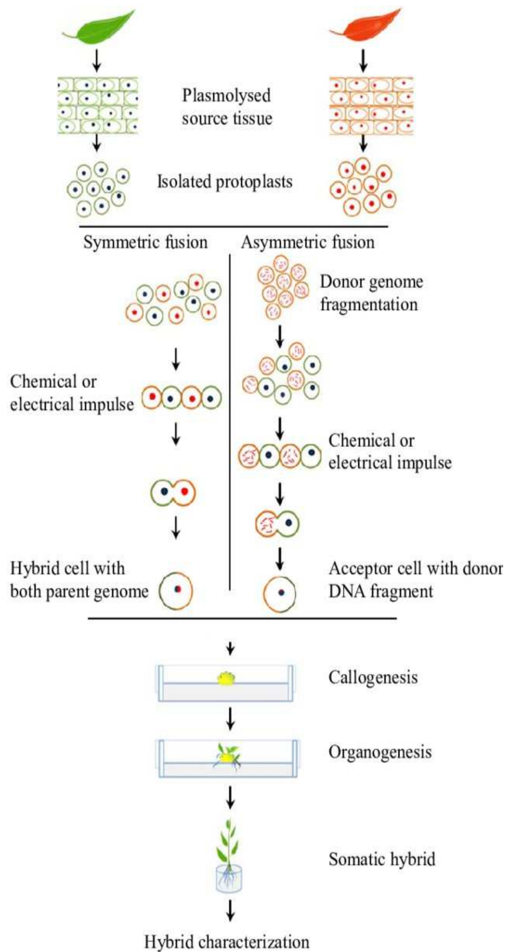


Fig. 7.Schematic representation of symmetric and asymmetric somatic hybridization techniques.(Shankar et al. 2013)

MECHANISMOF FUSION

The fusion of protoplasts takes place in three phases- agglutination, plasma membrane fusion and formation of heterokaryons. When the two protoplasts come in close contact with each other, they adhere to each other. This agglutination can be induced by PEG, high pH and highCa2+. The protoplast membranes get fused at localized sites at the point of adhesion. This leads to the formation of cytoplasmic bridges betweenprotoplasts. High pH and high Ca2+ ions neutralize the surface charges on the protoplasts which allowscloser contact and membrane fusion between agglutinated protoplasts. The fused protoplasts become round as a result of cytoplasmic bridges which leads to the formation of spherical homokaryon orheterokaryon.

High calcium and high pH induced protoplast fusion

Physical contact of two protoplasts is essential for their fusion. However, protoplast does not fuse easily due to two main reasons: i. they have a net negative charge on their membrane surfaces and force of repulsion works between protoplasts, and ii. It is difficult to remove water from hydrophilic sufaces of protoplasts which also create a repulsive force between two protoplast. It was observed that positively charged ions reduce the negative charges of membranes reducing the repulsive force considerably. Calcium ions are suitable for such purposes and methods havebeen standardised for protoplast fusion using calcium ions in a high pH solution.

PEG Induced fusion

Polyethylene glycol (PEG) induced protoplast fusion was developed by Kao and Michayluk in 1974. PEG molecules have polarity like membrane phospholipid molecules and get attached with membrane proteins. When the attached PEG between two protoplast is removes it results in breakdown of membranes at the contact points causing protoplasts to fuse with subsequent rejoining of plasmamembranes of the adjacent protoplasts.

Electric field induced fusion

In this method, developed by U. Zimmerman, protoplasts are placed in an electric field and are exposed to high intensity electric pulse for a short duration (nano-micro second). This exposure to electric field

reversibly increases permeability of cell membrane. Local electrical charge breakdown occurs in the plasma membranes resulting in fusion of adjacent protoplast. The original properties of membrane are restores within micro second to minutes depending on the experimental conditions and membrane properties (Fig. 8).

Steps involved inelectrophoresis are

Dielectrophoresis
Mutual dielectrophorasis
Membrane contact
Electric break down of membranes
Protoplast fusion

Dielectrophoresis:
Closemembrane contact of two protoplasts is one of the prerequisite for protoplast fusion. This step is achieved by dielectrophoresis, which is concerned with motion of neutral particle in a non uniform electric field. Neutral particles become polarized in the presence of electric field with negative side toward anode and positive charge of the same magnitude on the opposite side, i.e., toward cathode. In uniform electric field the field strength is equal on both the sides. And in such field these induced bipolar particles will no move. But under non uniform field a net force acting upon the particle results in linear motion towards the region of highest field intensity.

Mutual Dielectrophoresis:
Oneprotoplast approaching another polarized protoplast during the movement towards the region of highest field intensity, will encounter an enhancement of local field divergence and will tend to move toward the protplast since the field strength is higher near that cell. As a result protoplasts in uniform field form chain like aggregates (pearl chain) with point to point membrane contact.

Electric Break Down:

Reversible electric break down in the zone of membrane contact is the primary process responsible for the initiation of fusion. Phospholipids are arranged in a planer bMayer into which peripheral or integral structural carrier protein are embedded in a mosaic like fashion. The lateral fluidity of phospholipids is very high, while the movement vertical to the membrane surface is severely limited. A flip-flop movement of lipids in this direction is very difficult. The reversible electrical break downof protoplast membranes lead to perturbation of membrane structure, which permits an exchange of materials between the protoplast and its environments. The electric field affects the protoplast adhering to each other in the orientation to the field direction. Electrical break down occurs at the poses of cells and zone of contact between two

SELECTION OF HYBRID CELLS

Successful somatic hybridization required the induction of protoplast fusion resulting in the formation of heterokaryocytes and somatic hybrid, their survival, selection, and recovery of hybrids, subsequent

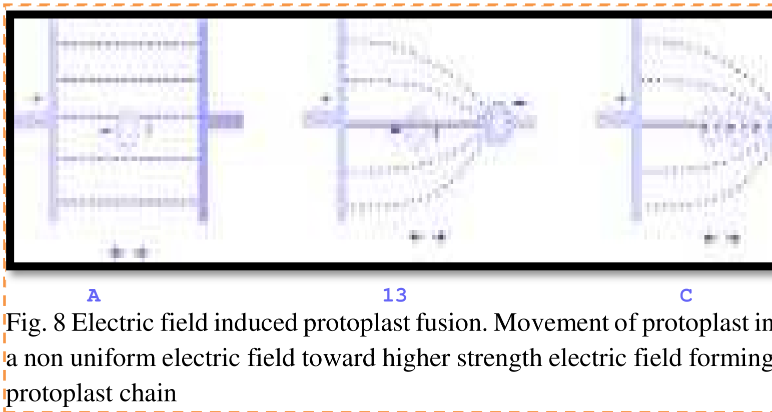


Fig. 8 Electric field induced protoplast fusion. Movement of protoplast in a non uniform electric field toward higher strength electric field forming protoplast chain

division of fusion products and morphogenesis giving rice to hybrid plants. The methods used for the selection of hybrid cells are biochemical, visualand cytometric methodsusing fluorescent dyes. The biochemical methods for selection of hybrid cells are based on the use

of biochemical compounds in the medium. The drug sensitivity method is useful for the selection hybrids of two plants species, if one of them is sensitive to a drug. Another method, auxotrophic mutant selection method involves the auxotrophs which are mutants that cannot grow on a minimal medium. Therefore specific compounds are added in the medium. The selection of auxotropic mutants is possible only if the hybrid cells can grow on a minimal medium. The visual method involves the identification of heterokaryons under the light microscope. In some of the somatic hybridizations, the chloroplast deficient protoplast of one plant species is fused with the green protoplast of another plant species. The heterokaryons obtained are bigger and green in colour while the parental protoplasts are either small or colourless. The cytometric method uses flow cytometry and flourescent-activated cell sorting techniques for the analysis of plant protoplasts.
The methods used for selection of somatic hybrids can be classified as follows
Selection of somatic hybrids by culturing them on such a medium on which only somatic hybrid protoplast can grow Complementary selection of somatic hybrids on specificculture medium Mechanical isolation by visual means and knowledge of identification of somatic hybridsmorphology of the plant after regeneration

APPLICATION OF SOMATIC HYBRIDIZATION

Creation of hybrids with disease resistance Many disease resistance genes (e.g. tobacco mosaic virus, potato virus X, club rot disease) could be successfully transferred from one species to another. E.g resistance has been introducedin tomato against diseases such as TMV, spotted wilt virus and insect pests.

Environmental tolerance

Using somatic hybridization the genes conferring tolerance for cold, frost and salt were introduced in e.g. in tomato.

Cytoplasmic male sterility

Using cybridization method, it was possible to transfer cytoplasmic

male sterility.

Quality characters

Somatichybrids with selectivecharacteristics have been developed e.g. the production of high nicotine content.

Somatic hybridization for genetic recombination in asexual or sterile plants Somatic cell fusion appears to be the only approach through which two different parental genomes can be recombined among plants that cannot reproduce sexually. Further, protoplasts of sexually sterile (haploid, tripled and aneuploid) plants can be fused to produce fertile diploids and polyploids. There are several reports describing the amphidiploid and hexaploid plants produced from fusion of haploid protoplasts of tobacco. Protoplasts isolated from dihaploid potato clones have been fused with isolated protoplasts of Solanum brevidens to produce hybrids of practical breeding value. Haploid protoplasts from an anther-derived callus of rice cultivars, upon fusion also produce fertile diploid and triploid hybrids.

Somatic hybridizations to overcome sexualincompatibility

In plant breeding programmes, sexual crossings at interspecific or intergeneric levels often fail to produce hybrids due to incompatibility barriers. The bottlenecks in sexual hybridisation may therefore, be overcome by somatic cell fusion. In some cases somatic hybrids between two incompatible plants have also found application in industry or agriculture.

Nicotiana repanda, N. nesophila and N. stockonii are resistant to a number of diseases but are not sexually crossable with tobacco (/V. tabacum). However, fertile hybrids have been reported in combination N. tabacum (+)N. nesophila and N. tabacum (+) N. stocktonii by protoplast fusion. Somatic hybridisation of dihaploid and tetraploid potato protoplasts with isolated protoplasts of Solanum brevidens, S. phureja and S. pennelii resulted in the synthesis of fertile, partially amphieuploid plants possessing important agricultural traits, e.g., resistance to potato leaf virus, potato virus Y and Erwinia soft rot. Using this approach, tomato (Lycopersicon esculentum) hybridised

somatically with a number of wild species has resulted in the synthesis of hybrids which are fertile and used in breeding programmes. Interspecific somatic hybridisation involving species that are sexually incompatible with egg-plant (Solarium melongena) has also resulted in the production of amphidiploids with traits resistant to verticillium wilt.

Somatic hybridization to generate novel plants

A number of unique plants have been generated throughsomatichybridisation by protoplast fusion. A number of improvements have been done in fusion technology like electrofusion. Electrofusion is preferred as it aids in maintenance of protoplast viability and reduction in membrane damage, general protoplast, distortion/disruption, and organelle fusion. A number of novel plants with improved agronomic traits have been obtained by somatic hybridization.
In 1972, Carlson and his associates produced the first inter-specific somatic hybrid between Nicotiana glauca and N. langsdorffii. In 1978, Melchers and his co-workers developed the first inter-genetic somatic hybrids between Solanum tuberosum (potato) and Lycopersicon esculentum (tomato). The hybrids are known as 'Pomatoes or Topatoes' Considerable basic and applied research has focused on Citrus because of its global commercial importance. Vitrus somatic hybrids are easier to obtain as interspecific and intergeneric somatic hybrids can be produced, with wild relatives of cultivated citrus representing an untapped gene pool reservoir to generate novel somatic hybrid and cybrid plants. Electrofusion was successful in obtaining cybrids of protoplasts of Valencia sweet orange (Citrus sinensis) and iodoacetate-treatedprotoplasts of Murcott tangor. Cell suspensions of Bonnaza navel orange (C. sinensis) with mesophyll protoplasts fromseedless Red Blush grapefruit (C. paradisi) were electrofused to generate tetraploid and diploid plants. Some of the tetraploids exhibited precocious flowering. These somatichybrids are excellent pollen parents for improving cultivars bygenerating triploid seedless hybrids.

Much attention has focused, over several years, on somatic

hybridisation of Brassicaceae has been focussed for severalyears and somatic hybrids between Brassica napus and B.rapa has been successfully accomplished. In the hybrids, the parental material influenced the characteristics of theresultant somatic hybrid plants.Somatic hybrids have potential in bioremediation and environmental clean-up. Electrofused protoplasts of the zinc accumulator Thlaspi caerulescens withthose of B. napusresulted in hybrid cells that were zinc tolerant and failed to adhere to the walls of culture vessel.

Regenerated hybridplantsaccumulated zinc and cadmium to concentrations normally toxic to B. napus,showing that the transfer of the trait for metal accumulation in plants is achievable byprotoplast fusion.

Somatic hybridization for cytoplasmic male sterility Methods were also developed to substitute the nucleus of one species into the cytoplasm of another species, whose mitochondria were inactivated. This type of substitution in some cases, led to generation of cytoplasmic male sterility. For this purpose, the two types of protoplasts, used for the production of somatic hybrids, were treated differently, as follows: mesophyll protoplasts of tomato (Lycopersicon esculentum) were treated with iodoacetamide (IOA) to inactivate mitochondria.
Mesophyll protoplast of Solanum acaule (or tuberosum) were irradiated with g or x-rays to inactivate nuclei

The protoplasts were mixed in 1:1 ratio and induced to fuse using Ca^{2+} and PEG, leading to the production of heterologous or alloplasmic hybrids. Among the fusion products, some hybrid tomato plants were indistinguishable from the original cultivars, with respect to morphology, physiology and chromosome number (2n = 24), but exhibited various degrees of male sterility. In five tomato cultivars, male sterility induced >n this manner was inherited maternally over several generations. Therefore, it was obviously cytoplasmic male sterility. The mitochondrial DNA of these CMS hybrids did not resemble mtDNA of either parent, and was instead recombinant type, representing a hybrid

mitochondrial genome. Therefore, protoplast fusion can be effectively used for production of CMS lines and has the following advantages:
Only one step is required;
The nuclear genotype of the cultivar remains unaffected,
There are prospects that 100% of the progenies of somatic hybrids will be CMS. The restorer lines for these CMS lines have also been shown to be available in tomato, so that hybrid seed can be produced without manual emasculation.

CHROMOSOME NUMBER IN SOMATIC HYBRIDS

The chromosome number in the somatic hybrids is generally more than the total number of both of the parental protoplasts. If the chromosome number in the hybrid is the sum of the chromosomes of the two parental protoplasts, the hybrid is said to be symmetric hybrid. Asymmetric hybrids have abnormal or wide variations in the chromosome number than the exact total of two species.

LIMITATIONS OF SOMATIC HYBRIDIZATION

Somatic hybridization does not always produce plants that give fertile and visible seeds.
There is genitc instability associated with protoplast culture. There are limitations in the selection methods of hybrids, as many of them are not Somatic hybridization between two diploids results efficient.in the formation of an amphidiploid which is not favourable therefore haploid protoplasts are recommended in somatic hybridization. It is not certain that a specific character will get expressed in somatic hybridization. Regenerated plants obtainedfrom somatic hybridization are often variable due to somaclonal variations, chromosomal elimination orgenelle segregation etc.

Protoplast fusion between different species/genus iseasy, but the production of viable somatic hybrids is not always possible.

CYBRIDS

The cytoplasmic hybrids where the nucleus is derived from only one parent and the cytoplasm is derived from both the parents are referred

to as cybrids. The process of formation of cybrids is called cybridization. During the process of cybridization and heterokaryon formation, the nuclei are stimulated to segregate so that one protoplast contributes to the cytoplasm whilethe other contributes nucleus alone. The irradiation with gamma rays and X-rays and use of metabolic inhibitors makes the protoplasts inactive and non-dividing. Some of the genetic traits in certain plants are cytoplasmically controlled. This includes certain types of male sterility, resistance to certain antibiotics and herbicides.

Therefore cybrids are important for the transfer of cytoplasmic male sterility (CMS), antibiotic and herbicide resistance in agriculturally useful plants. Cybrids of Brassica raphanus that contain nucleus of B. napus, chloroplasts of atrazinc resistant B. capestris andmale sterility from Raphanus sativas have been developed.

Genetic transformation of protoplasts

Early work on plant transformation suggested that monocotyledonous species were recalcitrant to infection by Agrobacterium. This led to exploration of other avenues of transformation for such species, and plant protoplasts became an obvious target, building on experience gained from animal cell systems. Evidence was obtained quite early on that DNA could be introduced into plant protoplasts via polyethylene glycol (PEG) fusion, electroporation, and microinjection. Although a range of species has now been transformed in this way, a major disadvantage of methods utilizing protoplasts is that the regeneration of plants from protoplast cultures can be a complex and time-consuming process. As a result, protoplast transformation has been largely superceded by alternative faster and more efficient regeneration systems. However, protoplast systemsstill have applications for research purposes such as analysis of gene and gene construct function and plastid transformation. In studies where regeneration is not required, protoplast electroporation can be auseful tool in transient expression assays;electroporation has been found to result in single copy plasmid insertions rather than the multiple plasmid copies encountered following particle bombardment.Genetictransformation of protoplasts has been accomplished by co-cultivating protoplasts wit

Agrobacterium tumefaciens or by direct DIM A transfer using polycationic chemicals, electroporation, liposomes,microinjection or sonication. Transformation of plant protoplasts can be done using polycationic chemicals and/ or electroporation.

Agrobacterium-mediated transformation

A.tumefaciens, can be used for transforming protoplasts. For Agrobacterium -mediated gene transfer protoplasts are isolated and cultured for 2-3 days prior to co-cultivation withAgrobacterium cells. Protoplasts are mixed with Agrobacterium ata rate of 10^-106 bacterial cells per ml and incubated together for 24-30 h. After co-cultivation, antibiotics that inhibit growth of the bacterium are added to the culture medium. Treated cells are allowed to grow to the multicellular stage befor theaddition of sselective antibiotics (kanamycin, hygromycin etc.) that inhibit growth of untransformed cells.Transformed cells grown on selective medium are able to regenerate plants with introduced gene.

Polycationic chemical-mediated transformation

The most commonly used polycationic chemical for direct DNA transformation is PEG. Transformation frequencies vary from 0.8 X 10-6 to 6 X 10-3 depending on the plant species. To transform plant cells using PEG, protoplasts are isolated andadjusted to the proper cell density before adding transforming DNA and PEG. The mixture is incubated for 10-30 min before dilution with culture medium. PEG interacts with negatively charged DNA to forma positively charged complex that binds to the anionic plsmalemma. Absorption of DNA into cells occurs through endocytosis or active uptake

Electroporation-mediatedtransformation

DNA uptake is induced by an electric pulse. The DNA uptake occurs through pores in the cell membrane in response to the electric pulse. The transforming DNA is mixed with plant protoplasts prior to one or more electric pulses of 200-1000 V/ cm. Short pulses are typically less lethal than long pulses. Transformation efficiency is lower for electroporation than PEG. Stable transformation using electroporation

with or without PEG can be achieved in many plant species. It is fast and efficient means of delivering DNA to plant cells and protoplasts and I not limited by the size or structure of transforming DNA and does not have a limited host range.

Sonication-, liposome- and microinjection-mediated transformation

Sonication is a method of protoplast transformation which uses sonic pulses for delivery of DNA. During sonication, protoplasts are mixed with DNA and given sonic pulses (500-900 msec at 0.65-1.6 V/ cm) that disrupt the cell membrane and allow DNA to enter cells through pores created in response to electric pulse. Liposomes are hydrogenated phospholipids in which DNA or RNA is encapsulated and delivered directly to plant cells throughendocytosis or active uptake. Microinjection uses a fine capillary needle to deliver DNA, chromosomes or nuclei directly into plant cells. Plants with improved characteristics have been obtained through somaclonal variations and somatic hybridization. Direct gene transfer utilizingprotoplasts can be used to insert beneficial genes into crops such as cereals and grasses, which are not amenable toAgrobacterium-mediated transformation. These methods can be used to create plants with improved agronomic andhorticultural characteristics as well as increased insect and disease resistance.

CHAPTER IV

Target cell for crop improvement; vectors for gene transfer (based on Ti and Ri plasmids; co integrate, intermediate and helper plasmids; binary vectors; viruses as vectors); gene transfer techniques using Agrobacterium', selectable and scorable markers (genes); Agroinfection and gene transfer; Physical delivery methods.

Introduction

A plant in which a gene has been transferred through genetic engineering is called a transgenic plant. Transformation is the genetic alteration of a cell resulting from the introduction, uptake and expression of foreign genetic material (DNA or RNA). The foreign DNA can be delivered into the plants through Agrobacterium vectors. The Ti plasmid or the Ri plasmid vectors can be designed to deliver the T-DNA along with the GOI to genetically modify a plant.

Agrobacteriunri is a gram negative, rod shaped soil bacteria which belongs to the family rhizobeaceae which has the capacity of transferring or delivering its DNA to a plant cell and hence it is referred to as 'natural genetic engineerThis natural ability of Agrobacterium is utilized to transfer our "gene of interest" into the plant. The Agrobacterium strain A. tumefaciens harbours the Ti plasmid and the A. rhizogenes harbours the Ri plasmid. Agrobacterium can transfer a part of its DNA from the Ti plasmid or Ri plasmid to the plant cell, where the DNA gets integrated into the plant genome and express the gene product. In nature the piece of DNA transferred into the plant, produce plant growth factors, sugar and amino acid derivatives called opines. Agrobacterium tumefaciens causes crown gall disease (Fig. 1). Expression of T-DNA genes from Ti plasmids leads to production of phytohormones, auxin and cytokinin the induce proliferation of transformed cells to form undifferentiated tumors or "crown galls". Agrobacterium rhizogenes causes hairy root disease (Fig. 1b). Expression of rot A, B, C genes from T-DNA the Ri plasmid produces "hairy roots".

Agrobacterium tumefaciens

A. tumefaciens remains a majormethod of choice for transforming plant cells. A. tumefaciens initiallywas used for transformingdicotyledonous plants. Themonocotyledonousspecies, including important crop plants such as maize, rice, wheat, and barley werenot asreadily amenable to

Fig.1 Crown gall disease.b.Hairy root disease

transformationun like dicotyledonous plants such astobaccoand petunia, as dicots are thenatural hosts for the bacterium. Butlater it was proved that monocots, such as rice, could be successfullytransformed by the bacterium.

Many different techniques have beenused to improve the efficiency oftransformation with Agrobacterium.Methods such as sonication ofembryogenic suspension cultures, wounding by bombardment prior to inoculation, or bombardment with dried Agrobacterium are being used to improve the efficiency of Agrobacterium mediated plant transformation. In plantatransformation with Agrobacterium is a successful method for transforming small plants like Arabidopsis thalianawhich saves time. Agrobacterium- mediated
DNA transfer is preferred over other methods as it results in delivery of

fewer copies of T-DNA in a precise manner.

Agrobacterium rhizogenes

a. rhizogenes induces the formation of "hairy roots" in dicotyledonous plants by incorporatingT-DNA from the Ri plasmid into host plant nuclear DNA. Plants transformed withA.rhizogenesthe'w progeny carrying the Ri plasmid are morphologically abnormal, ie, they show hairy root formation and this is considered as a disadvantage and hence A. tumefaciens is preferred as a routine method of producing transgenic plants. But hairy root has an important alternative application. The plant hairy rootscan be used for synthesizing secondary metabolits as plant roots in their natural state are able to synthesize extraordinary diversity ofsecondary metabolites (Fig. 2). The hairy roots are advantageous for secondary metabolite synthesis because, they show rapid growth and genetic stability and can produce high-level of secondary metabolites. They can be considered as bioreactors forlarge scale production ofsecondary metabolites.

Fig. 2. Hairy Root culcultures

Agrobacterium vectors

Agrobacterium transfers T-DNA, a small region of a resident Ti-plasmid or root-inducing plasmid (Ri - plasmid), to a number of plants and animal cells. The Ti plasmids are 200 to 800 kbp in size. T-DNA region of the native Ti and Ri plasmids are 10-30 kbps in size. The processing of the T-DNAfrom the Ti plasmid and its subsequent export from the

bacterium to the plant cell result in large part from the activityof virulence (vir) genes carried by the Ti plasmid (Fig. 3). The virulence proteins are coded by the vir genes (virA to virG) present on the Ti plasmid. There are various vir proteins which help in the different stages of T-DNA transfer. Vir region on the Ti/Ri plasmid is a collection of genes whose collective function is to excise the T-DNA region of the plasmid and promote its transfer and integrate into the plant genome. The vir genes are induced by signals produced by plants following wounding. Phenolic compounds such as acetosyringone, activate the virA gene. VirA phosphorylates the VirG gene. The activated VirG which is a transcription activator, activates the remaining Vir gene operons. The virD proteins make single-stranded cuts at the left and right borders of the T-DNA. VirE acts as a ssDNA binding protein, protecting the single stranded T-DNA in the plant cell. These vir genes are necessary for transfer of the T-DNA. Vir genes can act in trans, ie they can transfer a T-DNA which is not present in their own plasmid.

Agrobacterium plasmids (Ti ad Ri plasmids) are modified so that they can be used for delivering our gene of interest (GOI) into the plants. AnyDNA fragment placed between border sequences can be transferred into the plants efficiently. Originally the T- DNA harbours tumour inducing genes or the root hair inducing genes. To utilize the T-DNA vectors for plant transformation, the oncogenes are deleted from the plasmids and the GOI, plant selectable markers and reporter genes are cloned in the T- DNA region between the border sequences. These modified plasmids can efficiently deliver the GOI to be delivered into the plants. These modified plasmids are called plant transformation vectors. The essential features of plant transformation vectors are:

The transferred DNA (T-DNA)

vir genes. Approximately35 vir genes map outside the T- DNA (transferred DNA) region and encode products required for excision, transfer, and integration of T-DNA into a plant genome, vir genes act in trans, meaning they do not need to be physically attached to the T-DNA to cause integration into the plant genome.

T-DNA border sequences.

These are sequences of 25 bp imperfect repeats that flank the T-DNA and are required for its transfer. Border sequences encompass the recognition sites for a site-specific endonuclease, which is encoded by the vir D operon, part of the vir genes. The endonuclease cleaves the lower DNA strand of the T-DNA marking the starting point of the transfer.

c/s- regulatory regions. These include the right and left T-DNA borders, which are physically attached to the genes to betransferred into the plantgenome. Other c/s -regulatory regions include promoters and terminators that flank thetransgenes and regulate their expression. Commonly usedpromoters and terminators are the nopaline synthesis gene (NOS) and Cauliflower Mosaic Virus (CaMV) 35S promoter.

Selectable marker genes. Inplants, they allow the identification and selection of cells with the gene of interest incorporated in their genome. Bacterial selectable markers permit the identification of bacteria transformed with a vector carrying the marker. Examples of plant and bacterial selectable markers are hygromycin phosphotransferase and kanamycin, respectively.

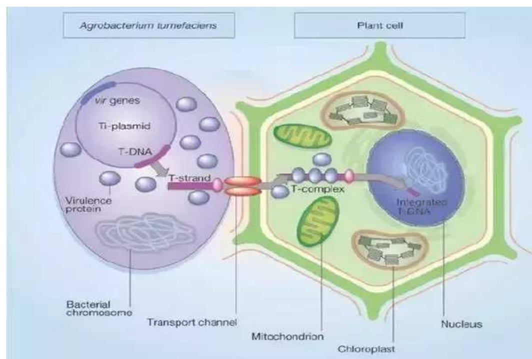
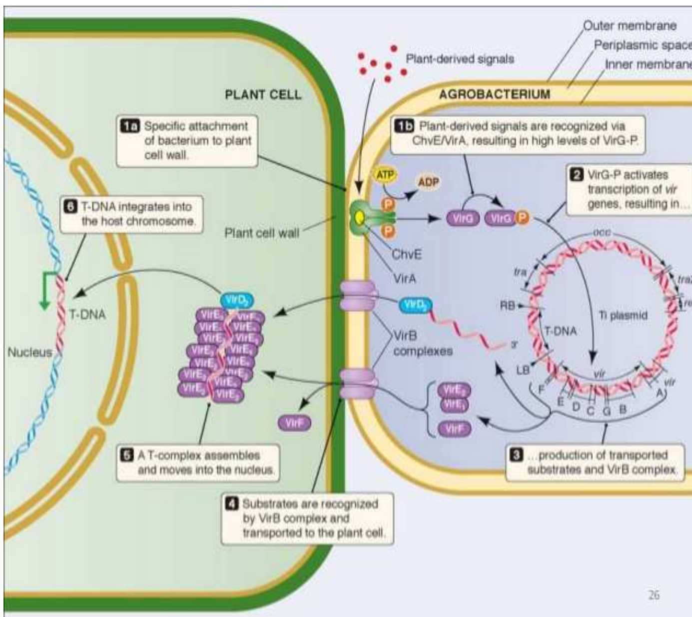


Fig. 3.Agrobacterium T-DNA transfer

Virulent strains of A. tumefaciens andA.rhizogenes contain a large megaplasmid (more than 200 kb) which plays a key role in tumour

induction and for this reason it was named Ti plasmid, or Ri in the case of A. rhizogenes. The T-DNA fragment is flanked by 25-bp direct repeats, which act as a c/s element signal for the transfer apparatus. The transfer is mediated by the cooperative action of proteins encoded by genes determined in the Ti plasmid virulence region (vir genes) and in the bacterial chromosome. The 30 kb virulence (vir) region is a regulon organised in six operons thatare essential for the T-DNA transfer (virA, virB, virD, and virG) or for the increasing of transfer efficiency (virC andvirE). Differentchromosomal-determined genetic elements have shown their functional role in the attachment of A. tumefaciens to the plant cell and bacterial colonisation.



Molecular mechanism of gene transfer byAgrobacterium (Fig.4)

Theloci chvA and chvB, involved in the synthesis and excretion of the p-1,2 glucan; the chvE required for the sugar enhancement of vir genes induction and bacterial chemotaxis; the cel locus, responsible for the synthesis of cellulose fibrils; thepscA (exoC) locus, playing its role in the synthesis of both cyclic glucan and acid succinoglycan; and the att

locus, which is involved in the cell surface proteins.
There are three important facts for the practical use of this process in plants transformation. Firstly, the tumour formation is a transformation process of plant cells resulted from transfer and integration of T-DNA and the subsequent expression of T-DNA genes. Secondly, the T-DNA genes are transcribed only in plant cells and do not play any role during the transfer process. Thirdly, any foreign DNA placed between the T-DNA borders can be transferred to plant cell, no matter where it comes from. These well-established facts, allowed the construction of the first vector and bacterial strain systems for plant transformation.
Steps inAgrobacterium T-DNA transfer
The steps in Agrobacterium T- DNA transfer are
Bacterial colonization
Induction of bacterial virulence
Generation of T-DNA transfer complex
T-DNA transfer
Integration of T-DNA into the plant genome

Bacterial colonization

Bacterial colonisation is an essential and the earliest step in tumour induction and it takes place when Atumefaciens is attached to the plant cell surface. The polysaccharides of the A.tumefaciens{ l ipo polysaccharides (LPS), and capsularpolysacha rides (K-antigens)) play an important role in the colonising process. The LPS are integral part of outer membrane and capsular polysaccharides (K- antigens), lacking of lipid anchor, have strong anionic nature. There are evidences that capsular polysaccharides may play specific role during the interaction with the host plant.
The chromosomal 20kb att locus contains the genes required for successful bacterium attachment to the plant cell. This locus has been extensively studied. Insertions in the left 10 kb side of this region produce avirulent mutants that could restore its attachment capacity if the culture medium is previously conditioned by the incubation of wild-type virulent bacterium with plant cells. The genes, placed at att left side, are involved in molecular signalling events, while the right side genes are likely to be responsible for the synthesis of fundamental

components.

Induction of bacterial virulence system

The T-DNA transfer is mediated by products encoded by the 30-40 kb vir region of the Ti plasmid. This region is composed by at least six essential operons {vir A, vir B, vir C, vir D, vir E, virG) and two non-essential (virF, virH). The number of genes per operon differs, virA, virG and virF have only one gene; virE, virC, virH have two genes while virD and virB havefour and eleven genes respectively. Theonlyconstitutive expressed operons are virA and virG, coding for a two-component (VirA-VirG) system activating thetranscription of theother vir genes.

VirA is a transmembrane protein that detects signal molecules, released from wounded plants and it acts as an antenna to sense the host. The signals for VirA activation include acidic pH, phenolic compounds, such as acetosyringone, and certain class of monosaccharides which acts sinergically with phelolic compounds. VirA protein can be structurally defined into three domains: the periplasmic or input domain and two transmembrane domains (TM1 and TM2). The TM1 and TM2 domains act as a transmitter (signaling) and receiver (sensor). Monosaccharide detection by VirA is important amplification system to respond to low levels of phenolic compounds. The induction of this system is only possible through the periplasmic sugar (glucose/ galactose) binding protein ChvE, which interacts with VirA. Activated VirA has the capacity to transfer its phosphate to a conserved aspartate residue of the cytoplasmic DNA binding protein VirG. VirG functions as transcriptional factor regulating the expression of vir genes when it is phosphorilated by VirA. The C-terminal region is responsible for the DNA binding activity, while the N-terminal is the phosphorilation domain and shows homology with the VirA receiver (sensor) domain.

The activation of vir system also depends on external factors like temperature and pH. At temperatures greater than 32°C the vir genes are not expressed because of a conformational change in the folding of VirA induce the inactivation of its properties.

Generation of T-DNAtransfer complex

The activation of vir genes carries out the generation of single-stranded

(ss) molecules representing the copy of the bottom T-DNA strand. Any DNA placed between T-DNA borders will be transferred to the plant cell, as single strand DNA, and integrated into plant genome. These are the only cis acting elements of the T-DNA transfer system. The proteins VirD1 andVirD2 play the key role in this step are, recognising the T-DNA border sequences and nicking (endonuclease activity) the bottom strand at each border. The nick sites are assumed as the initiation and termination sites for T-strand recovery. After endonucleotidic cleavage VirD2 remains covalently attached to the 5'-end of the ss-T-strand. This association prevents the exonucleolitic attack to the 5'- end of the ss-T-strand and distinguishes the 5'-end as the leading end of the T-DNA transfer complex. VirD1 interacts with the region where the ss-T- strand will be originated.

The T-strand synthesis is in 5' to 3' direction and it is initiated at the right border and that the termination process take place even when the left border is mutated or completely absent. The presence of an enhancer or "overdrive" sequence next to the right border, which specifically recognised by VirCl protein, makes the difference between both T-DNA borders.

Translocation of T-DNA- complex

The transferring vehicle to the plant nucleus is a ssT-DNA- protein complex. According to the most accepted model, the ssT-DNA-VirD2 complex is coated by the 69 kDa VirE2 protein, a single strand DNA binding protein. This co-operative association prevents the attack of nucleases and, in addition, extends the ssT-DNA strand reducing the complex diameter to approximately 2 nm, making easier the translocation through membrane channels. VirE2 contains two plant nuclear location signals (NLS) and VirD2 one. These two proteins play important role once the complex is in the plant cell mediating the complex uptake to the nucleus.VirEl is essential for the export of VirE2 to the plant cell.

VirB are proteins that present the hydrophathy characteristics similar others membrane- associated proteins. The majority of VirB proteins are assembled as a membrane-spann ng protein channel involved

bothmembranes. VirB4 and VirBll are hydrophilic ATPases necessary for active DNA transfer. Vir B11 lacks continuous sequence of hydrophobic residues, motivating of periplasmic domains. The VirB7-VirB9 heterodimer isassumed to stabilises other Vir proteins during assembly of functional transmembrane channel.

Some of the initial steps of biogenesis of ssT-DNA complex apparatusis as follow. Firstly, VirB7 and VirB9 monomers are exported to the membrane and processed. They interact each other to form covalently cross- linked homo- andheterodimmers. Subsequently the VirB7-VirB9 heterodimmer is sorted to the outer membrane. The next steps involve the interaction with the other Vir proteins for assembling the transfer channel with the contribution of the transglycosidase. Other two accessories via operons present in the octopine Ti-plasmid are virF and virH

The virF operon encoding for a 23-kDa protein that functions once the T-DNA complex is inside the plant cells via the conjugal channel or mdependently. The role of VirF is seems to be related with the nuclear targeting of the ssT-DNA complex.

The virH operon consists in two genes that code for VirHl and VirH2 proteins. These Vir proteins are not essential but enhance the transfer efficiency, detoxifying certain plant compounds that can affect the growth of bacterial. They play a role in the host range specificity of bacterial strain for different plant species.

Integration of T-DNA into plant genome

Inside the plant cell, the ssT- DNA complex is targeted to the nucleus crossing the nuclear membrane. Two Vir proteins have been found to be important in this step: VirD2 and VirE2, which are the most important; and probably VirF, which has minor contribution to thisprocess. The two NLS of VirE2 have been considered important for the continuous nuclear import of ss-T-DNA complex, probably by keeping both sides of nuclear pore simultaneously open. The nuclear import is probably also mediated by specific NLS-binding proteins,

which are present in plant cytoplasm.

The final step of T-DNA transfer is its integration into plant genome. It is considered that the integration occurs by illegitimate recombination. According to this model, paring of a few bases provides just a minimum specificity for the recombination process by positioning VirD2 for the ligation. The 3'-end or adjacent sequences of T-DNA find some low homologies with plant DNA resulting in the first contact (synapses) between the T-strand and plant DNA forms a gap in 3'-5' strand of plant DNA. Displaced plant DNA is subsequently cut at the 3'-end position of the gap by endonucleases, and the first nucleotide of the 5' attached to VirD2 pairs with a nucleotide in the top (5'-3') plant DNA strand. The 3' overhanging part of T-DNA together with displaced plant DNA are digested away, either by endonucleases or by 3'-5' exonucleases. The, the 5′ attached to VirD2 end and other 3'-end of T-strand (paired with plant DNA during since the first step of integration process) joins the nicks in the bottom plant DNA strand. Once the introduction of T-strand in the 3'-5' strand of the plant DNA is completed, a torsion followed by a nick into opposite plant DNA strand is produced. This situation activates the repair mechanism of the plant cell and the complementary strand is synthesised using the early inserted T-DNA strand as a template.

VirD2 has an active role in the precise integration on T-strand in the plant chromosome. The release of VirD2 protein may provide the energy containing in its phosphodiester bond, at the Tyr29 residue, with the first nucleotide of T-strand, providing the 5'-end of the T-strand for ligation to the plant DNA. This phosphodiester bound can serve as electrophilic substrate for nucleophilic 3'-OH from nicked plant DNA.

Plant transformationmediated by Agrobacterium tumefaciens

The first plant transformed by Agrobacterium tumefaciens was tobacco in 1983(Herrera-Estrella, 1983). Since that crucial moment in the development of plant science, a great progress in understanding theAgrobacterium-med\ated gene transfer to plant cells has been archived. However, Agrobacterium tumefaciens naturally infects only dicotyledonous plants and many economically important plants, including the cereals, remained accessible for genetic

manipulationduring long time.

The monocots have been considered to be outside the Agrobacterium host range and other gene-transfer methods were developed for these plants. To develop this methodologies for a monocot plant it is important to take in consideration the critical aspects in the Agrobacterium tumefaciens-plant interaction, the cellular and tissue culture methodologies developed for that species. The su;table genetic materials (bacterial strains, binary vectors, reporter and marker genes, promoters) and molecular biology techniques available in the laboratory, are necessary for selection of DNA to be introduce. This DNA must be expressible in plant making possible the identification of transformed plants in selectable medium and using molecular biology techniques test and characterise the transformation events.
Transformation is currently used for genetic manipulation of more than 120 species of at least 35 families, including the most major economic crops, vegetables, ornamental, medicinal, fruit, tree and pasture plants, using Agrobacterium-mediated or direct transformation methods. The idea, that some species cannot accept the integration of foreign DNA in its genome and lack the capacity to be transformed is unacceptable under the increasing number species that have been transformedThe optimisation of Agrobacterium tumefaciens-plant interaction is probably the most important aspect to be considered. It includes the integrity of bacterial strain its correct manipulation as warranty of the virulence machine integrity and the study of reaction in wounded plant tissue, which may develop necrotic process in the wounded tissue or affect the interaction and release compounds inducers or repressors of Agrobacterium virulence system. The type of explant is also important fact and it must be suitable for regeneration allowing the recovering of whole transgenic plants. The establishment of method for efficientregeneration for one species is crucial transformation

PURPOSES OF PLANT TRANSFORMATION

A system to study Plant Physiology and plant functional genomics .The capacity to introduce and express (or inactivate) specific genes in plantsprovides a powerful new experimental tool, allowing direct testing of somehypotheses in plant physiology and functional genomics that have been exceedingly difficult to resolveusing other. Excitingmoleculargenetic analysis of cellular signals controlling sexualreproduction andplant-microbe interactions; the roles of specific enzymes in metabolicprocesses determining partitioning of photosynthates, and thus harvestable yield; and the roles of specific enzymes and hormones in plantdevelopmental processes, including those affecting quality and storage life of marketedplant product.

Practical Tool for PlantImprovement

Much of the support for plant transformation research (and more broadly for plant molecular biology) has been provided because of expectations that thisapproach could: (a) generate plants with useful phenotypes unachievable byconventional plant breeding, (b) correct faults in cultivars more efficiently thanconventional breeding, or (c) allow the commercial value of improved plantlines to be captured by those investing in the research more fully than ispossible under intellectual property laws governing conventionally bred plants.The first of these expectations has been met, with production of the firstcommercial plant lines expressing foreign gene conferring resistance to viruses,insects, herbicides, or post-harvest deterioration,andaccumulation of usefully modified storage products, including several cases where there was no source of the desired trait in the gene pool forconventional breeding. The future prospects in this respect are also exciting, with preliminary indications that novel genes can be introduced to generateplant lines useful for production of materials ranging from pharmaceuticalsto biodegradable plastics.The extent to which the other practical or commercialexpectations ofplanttransformation can be met depends on the efficiency and predictability ofproduction of lines with the desired phenotype, and without undesired sideeffects of the transformation process.

BIOLOGICALREQUIREMENTS FOR TRANSFORMATION

The essential requirements in a gene transfer system for production of transgenic plants are: (a) availability of a target tissue including cells competent for plant regeneration, (b) a method to introduce DNA into those regenerable cells, and (c) a procedure to select and regeneratetransformed plants at a satisfactory frequency.One of the simplest available plant transformation systems is the floral dip method, which involves infiltrationof Agrobacterium cells into Arabidopsis plants before flowering, anddirect selection for rare transformants in the resulting seedlingpopulations.But this technique can be applied to few plants as it needs small plant size, rapid generation time, and high seed yieldper plant are prerequisites for this method. These features are not shared byany economically important plant species. Floral dip method of transformation, involves inoculation of the developingfloral parts with Agrobacterium which results in transformed gametes that develop into transgenic plants. This high throughput technique can be widely used for transforming large population of plants. Therefore, thetotipotency of some somatic plant cells underlies most plant transformationsystems.

The efficiency with which such cells can be prepared as targets for transformation is today the limiting factor in achievement of transformation inrecalcitrant plant species.Using either Agrobacterium or particle bombardment, it is now possible to introduce DNA into virtually any regenerable plant celltype. Only a smallproportion of target cells typically receive the DNA during these treatments, and only a small proportion of these cells survive the treatment and stablyintegrate introduced DNA. It is therefore generally essential to efficientlydetect or select for transformed cells among a large excess of untransformed cells, and to establish regenerationconditions allowing recoveryof intact plants derived from single transformed cells.

ESSENTIAL REQUIREMENTSOF TRANSFORMATION SYSTEMS

The desired characteristics needed for a transformation protocol for crop improvement include:

Ready availability of the target tissue. The resources required to

maintain acontinuous supply of explants such as immature embryos at the correct developmental stage for transformation canbesubstantial.
Applicability to a range of cultivars. Genotype-specific techniques are of lower value because of the added complexity of extended breeding worktomove desired genes into preferred cultivars.
High efficiency, economy, andreproducibility, to readily produce manyindependent

Transformants for testing
Safety to operators, avoiding procedures, or substances requiringcumbersomeprecautions to avoid a high hazard to operators (e.g. potential carcinogenicityofsilicone carbidewhiskers).
Technical simplicity, involving a minimum of demanding or inherentlyvariablemanipulations, such as protoplast production and regeneration.
Frequent cotransformation with multiple genes, so that a high proportion ofplant lines selected for marker gene expression will also incorporatecotransformed useful genes.
Unequivocal selection or efficient screening to recover transgenic plantsfrom transformed cells.
Minimum time in tissue culture,to reduce associated costs and avoid undesired somaclonalvariation.
Stable, uniform (nonchimeric) transformants for vegetatively propagatedspecies, or fertile germline transformants for sexually propagated species
Capacity to introduce defined DNA sequences without accompanying vectorsequences not required for integration or expression of the introduced genes.
Capacity to remove reporter genes or other sequences not required followingselection of transformed lines.
Simple integration patterns and low copy number of introduced genes, tominimize the probability of undesired gene disruption at insertion sites, ormulticopy associated transgene silencing.
Stable expression of introduced genes in the pattern expectedfrom thechosen gene control sequences, rather than patterns associated with thestate of the cells at the time of transformation, or the chance

site ofintegration. Optionally applicable to transformation of organelle genomes. Absence of valid patent claims on products.

VECTORS FOR INDIRECT GENE TRANSFER

Among the various vectors used in plant transformation, the Ti plasmid of Agrobacterium tumefaciens has been widely used. This bacteria is known as "natural genetic engineer" of plants because these bacteria have natural ability to transfer T-DNA of their plasmids into plant genome upon infection of cells at the wound site and cause an unorganized growth of a cell mass known as crown gall. Ti plasmids are used as gene vectors for delivering useful foreign genes into target plant cells and tissues.

However, Ti plasmids are very large and T-DNA regions do not generally contain unique restriction endonuclease sites notfound elsewhere on the Ti plasmid. Therefore, one cannot simply clone a gene of interest into the T-region. Scientiststherefore developed a number of strategies to introduce foreign genes into the T-DNA. These strategies involved two different approaches: cloning the gene, by indirect means, into the Ti plasmid such that the new gene was in cis with thevirulence genes on the same plasmid (cointegrate vectors), or cloning the gene into a T-region that was on a separate replicon from the virgenes (T-DNA binary vectors).

The Two basic types of vectors used to transform a wide range of plants via Agrobacterium

Cointegrate vectors

Binary vectorsCointegrate vectors

These vectors result fromthe recombination of a small vector plasmid, for example an E.coli vector, and a Ti plasmid harbored in A. tumefaciens (Fig.5). The oncogenes are removed from the T- DNA, and the GOI is placed by homologous recombination.Forconstruction of cointegrate vectors, the GOI is cloned a E. coli plasmid which has a common region (Antibiotic resistance gene) with the Ti plasmid, to facilitate homologous recombination. This is called as an intermediate vector. The plasmid from E. coli is transferred to Agrobacterium by conjugation or mobilization. Recombination takes place through a

homologous region present in both of the plasmids.The GOI is placed in the Ti plasmid by double homologous recombination. The coitegrate vectors harbour the T- DNA and the vir genes in the same Ti plasmid, and hence vir proteins function in c/s. The cointegrate vectors are modified resident Ti plasmids and hence reside as single copy per Agrobacterium cell.

In order to introduce a foreign DNA in the cointegrate vectors, the ColE1 replicon such as pBR322 is first introduced into the T-region of a Ti plasmid. DNA to be integrated into this T-region is cloned into a separatepBR322-derived moleculecontaining a second antibiotic resistance marker.
This plasmidis introduced into the altered Agrobacterium strain, and the resulting strain is selected for resistance to the secondantibiotic. Because ColEl replicons cannot function in Agrobacterium, the pBR322- based plasmidwould have to cointegrate into the pBR322 segment of the altered T-region for the stable expression of the plasmid-encodedresistance gene.

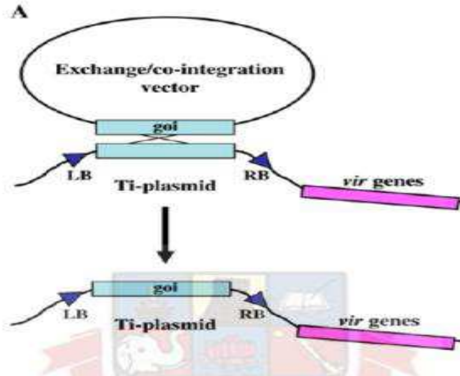


Fig. 5. Cointegrate Vector system (Lee and GeGelvin 2008,

Binary vectors

The construction of cointegrate vectors to introduce GOI into the T-DNA involves cumbersome genetic manipulations. This was because Ti/ Ri-plasmids are very large, low copy numberin Agrobacterium, difficult to isolate and manipulate in vitro, and do not replicate in Escherichia

coli, thefavored host for genetic manipulation. T-DNA regions from wild-type Ti-plasmids are generally large and donot contain unique restriction endonuclease sites suitable for cloning a GOI.

The break through discovery that vir genes can act in trans, helped in development of the next generation of plant transformation of binary vectors, the binary vectors. The vir and
T-DNA regions of Ti-plasmids could be split onto twoseparate replicons.As long as both of these repliconsare located within the same Agrobacterium cell, proteinsencoded by vir genes could act upon T-DNA intrans to mediate its processing and export to the plant.Systems in which T-DNA and vir genes are located onseparate replicons were eventually termed T-DNAbinary systems (Fig. 6). T-DNA is located on thebinary vector (the non-T-DNA region of this vectorcontaining orig i n [s] of replication that could functionboth in E. coli and in Agrobacterium tumefaciens, andantibiotic-resistance genes used to select for the presence of the binary vector in bacteria, became known asvector backbonesequences). The repliconcontainingthe virgenes is known as the vir helper. Strainsharboring this replicon and a T-DNA are considereddisarmed if they do not contain oncogenes that couldbe transferred to a plant.

A binary vector is a standard tool in the transformation of higher plants mediated by Agrobacterium tumefaciens. K T-DNA binary system consists of two plasmids, a binary vector and a helper plasmid. As a pair of plasmids are used for plant transformation, they are called as binary system. These two vectors are modified Ti plasmid. The binary plasmid is a shuttle vector as it can replicate in both E.coli and Agrobacterium and it harbours the T- DNA region. It is composed of the borders of T-DNA, multiple cloning sites, replication functions for Escherichia coli and A. tumefaciens, selectable marker genes, reporter genes, and other accessory elements that can improve the efficiency of and/or give further capability to the system. The GOI is cloned in the binary vector in E. coli. And later, the binary vector is mobilized into the Agrobacterium strain. They lack the vir genes. The vir genes are present on the second plasmid, called as the vir helper plasmid. The plasmid containing the T-DNA does notcontain the vir genes and hence

considered as disarmed. A super-binary vector carries additional virulence genes from a Ti plasmid, and exhibits very high frequency of transformation, which is valuable for recalcitrant plants such as cereals. A large number of binary vectors are available commercially and can be used for cloning fragments as large as 15 kb.

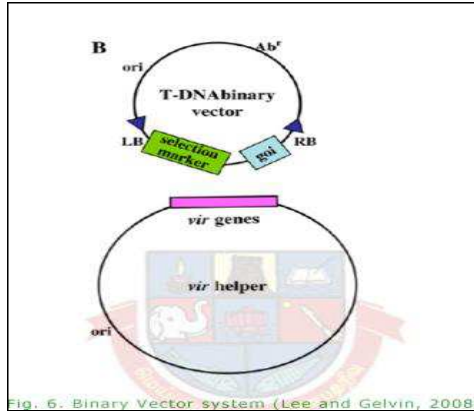


Fig. 6. Binary Vector system (Lee and Gelvin, 2008)

To transform plants, leaf discs (in case of dicots) or embryogenic callus (in case of monocots) are collected and infectedwith Agrobacterium carrying recombinant disarmed Ti-plasmidvector. The infected tissue is then cultured (co-cultivation) on shoot regeneration medium for 2-3 days during which time the transfer of T- DNA along with foreign genes takes place. After this, the transformed tissues (leaf discs/calli) are transferred onto selection cum plant regeneration medium supplemented with usually lethal concentration of an antibiotic to selectively eliminate non-transformed tissues. After 3-5 weeks, the regenerated shoots (from leaf discs) are transferred to root- inducing medium, and after another 3-4 weeks, complete plants are transferred to soil following the hardening (acclimatization) of regenerated plants. The molecular techniques like PCR and southern hybridization are used to detect the presence of foreign genes in the transgenic plants.

PLANT VIRAL VECTORS

Plant viral vectors delivered by Agrobacter um are the basis of several manufacturing processes that are currently in use for producing a wide range of proteins for multiple applications, including vaccine antigens,

antibodies, protein nanoparticles such as virus-like particles (VLPs), and other protein and protein-RNA scaffolds. Viral vectors delivered by agrobacterial T-
DNA transfer (magnifection) have also become important tools in research. The strategy relies on Agrobacterium as a vector to deliver DNA copies of one or more viral RNA/DNA replicons; the bacteria are delivered into leaves by vacuum infiltration, and the v>ral machinery takes over from the point of T-DNA transfer to the plant cell nucleus, driving massive RNA and protein production and, if required, cell-to-cell spread of the replicons. Among the most often used viral backbones are those of the RNA viruses Tobacco mosaic virus (TMV), Potato virus X (PVX) and Cowpea mosaic virus (CPMV), and the DNA geminivirusBean yellow dwarf virus. Prototypes of industrial processes that provide for high yield, rapid scale up and fast manufacturing cycles have been designed, and several GMP-compliant and GMP-certified manufacturing facilities are in place. These efforts have been successful as evidenced by the fact that several antibodies and vaccine antigens produced by magnifection are currently in clinical development.
The development of plant virus gene vectors for expression of foreign genesin plants provides attractive biotechnological tools to complement conventionalbreeding and transgenic methodology. The benefits of virus-based transient RNAand DNA replicons versus transgenic gene expression include rapid and convenient engineering. This results in high levels of expression of the foreign gene when compared to Agrobacterium T-DNA vectors (Fig.7). These characteristics are especially advantageous when very highlevels of gene expression are desired within a short time, although instability ofthe foreign gene in the viral genome can present some problems. The strategiesthat have been tested for foreign gene expression in various virus-based vectorsinclude gene replacement, gene insertion, epitope presentation, use of virus controlled geneexpression cassettes, andcomplementation

Autonomously replicating viruses offer numerous advantages for use as vehicles for transient expression of foreign genes, including their characteristic high levels of multiplication and concomitant levels of transient gene expression. Because of the nature of virusinfections, the

maximum level of foreign gene expression from viral genomes occurs within a short time- period, usually within one or two weeks after inoculation. Another practical advantage is that many plant viruses are easily transmissible and could potentially be used commercially for rapid mechanical inoculation of large acreages of crop plants. Also, once a suitable transient gene vector is identified, the expression of foreign genes can be tested rapidly in a variety of different plants that are hosts for the virus. This property is also especially useful for preliminary investigations of genes that induce measurablephenotypic effects. Therefore, this strategy has the potential to expedite initial screening procedures for selection by testing the possible effects of mutated genes before implementing more time and resource consuming transgenic approaches for genetic analyses or breeding.

SELECTION OF TRANSGENIC PLANTS

For transformation systems that generate substantial numbers of nonchimericprimarytransformants, genes conferring resistance to a selective chemicalagent, genes conferring a phenotype allowing visual or physical screening, or even

PCR screening to identify plants containing transferredgenes can all be used to recover transformants. Transformationsystems that generate chimeric primary transformants including transformedgermline cells, as intermediates in the production of homogeneously transformed(Rl) progeny plants, generally require screening rather than lethalselection to reveal primary transformants. Screening approaches are expensive unless the transformation efficiency ishigh, and generally impractical if the proportion of transformants among regeneratedlines is below10-2 to 10-3. Under these circumstances, antibiotic selection may allow a higher proportion of transformed cells to multiply and regenerate, in addition to facilitatingthe recognition of transformants.

Simple, and robust selection regimens now exist fortransgenic plants, requiring littleexperimentation with the timing and concentrationof selective agents to match the target tissue and gene transfer system.However, it is still important to consider the physiology of antibiotic actionand resistance mechanisms when choosing or modifying selection protocols.

Attention is increasingly being directed to introduction of multipleagronomically useful genes into plant lines, without having to pyramidselectable genes in the process. These selectable markers are not necessary once the primary transformants are identified and screened. They become a metabolic burden to the plants and an environmental hazard, as most of the plant selection markers are antibiotic resistance genes or herbicide resistance genes. A number of strategies have been developed to eliminate the markers form the transgenic plants once the transformants are selected, and to obtain marker-free plants.

PLANT SELECTABLE MARKERS

After transfer and integration of foreign DNA into plant cells it is important to select the transformed cells or the cells which has received the foreign DNA. Plant Selectable markers are used for recovery and recognition of transformed cells from among the untransformed plants. There are a number of plant selectable markers. A few like neomycin phosphotransferase gene which gives resistance to the antibiotic kanamycin and the hygromycin phosphotransferase genewhich gives resistance to theantibiotic hygromycin are widely used for selection of transformed plants. The widely used SM genes include the antibiotic resistance genes and herbicide resistance genes. Among the antibiotic resistance genes, the genes encoding neomycin phosphotransferase-II {nptII) and hygromycin phosphotransferase(hph) which confer resistance to the antibiotics kanamycin andhygromycin, respectively, areextensively used as SM in the transformation of many crop plants, both monocots and dicots. The herbicide resistance gene encoding phosphinothricin acetyltransferase conferring resistancephosphinothricin is used in efficient selection of transgenics in a number of crops. The SM genes can be classified as positive, negative, conditional and non-conditional selection systems.

The positive-selection system consists of a gene encoding a protein (enzyme) that promotes the growth and differentiation of transformed cells. The conditional-positive selection systems require a substrate that does not promote growth of the untransformed cells. The SM genes

convert the substrate into molecules that provide the transformed cells a growth advantage. Xylose isomeraseand phosphomannose isomerase convert the non-toxic inert substrates, xylose andmannose-6-phosphate intoD-xylulose and frusctose-6-phosphate, respectively, so that they can be utilized as carbon sources by plants. The non-conditional-positive SM genes do not require external substrates, yet promote the selective growth and differentiation of transformed tissues, which are different from the normal untransformed tissues. The ipt gene that codes for isopentyl transferase induces a shooty phenotype in the transformed tissues on a hormone-free medium. The rol genes of Agrobacterium rhizogenes induce a hairy root phenotype in the transformed tissues. The abnormal morphology of the transformed tissues necessitates the use of inducible promoters for these markers.

A negative SM gene causes death of the transformed cells. When the action of the toxic gene requires a substrate to express toxicity, the system is a conditional-negative selection system. Conditionalnegative SM genes are frequently used for plant transformation. They convert non-toxic agents in the medium to toxic agents which cause the death of the transformed tissues.

They include the codA gene, the bacterial cytochrome P450 mono-oxygenase gene, the bacterial haloalkane dehalogenase gene and theArabidopsisalcoholdehydrogenase gene. When the selection is substrate-independent, it is a non-conditional-selection system. They include genes encoding diphtheria toxin a subunit and ribosome inactivating proteins.Neomycin phosphotransferase H (NPTII): It is the most widely used selectable marker for plant transformations.

The enzyme is encoded by the nptII (or neo) gene, derived from the transposon Tn5, and inactivates by phosphorylation a number of aminoglycoside antibiotics such as kanamycin, neomycin, geneticin and paromomycin. Kanamycin is mostly used as the selective agent, normally in concentrations ranging from 50 to 500 mg/l.

Hygromycinphosphotransferase

Hygromycin B is an aminocyclitol antibiotic that interferes with protein synthesis. The hygromycin phosphotransferase gene (hpt) is derived from E. coli. The bacterial hpt-coding sequence was modified for expression in plant cells by Waldron et al. in 1985 and has since found wide application as a resistance gene for plant transformation.. Hygromycin B is usually more toxic than kanamycin and kills sensitive cells more quickly. Selection in vitro is applied at concentrations rang ing from 20 mg/ l for Arabidopsis thaliana to 200 mg/l for tall fescue.

Bleomycin and phleomycin resistance

Plant cells are sensitive to the glycopeptide antibiotics Neomycin and phleomycin, which produce single-stranded and double-stranded breaks in DNA. Two genes encoding Neomycin-binding proteins are usedto construct dominant Neomycin or phleomycin resistance markers for plant cell transformation: a bleomycin resistance gene from Tn5 and one derived from the actinomycete Streptoalloteichus hindustanus.

Streptomycin and spectinomycin resistance

Streptomycin and spectinomycin resistance markers differ from the above described markers in that they allow differentiation by color rather than by killing. Under appropriate conditions sensitive plant cells bleach but do not die, whereas resistant cells remain green. Two dominant resistance genes used for plant transformation work are the streptomycin phosphotransferase (SPT) gene from Tn5 that provides resistance to streptomycin and the aminoglycoside-3'''-adanylyltransferase gene (aadA) conferring resistance to both streptomycin and spectinomycin. AadA gene is also used as a marker for plastid transformation

Phosphinothricinacetyltransferase

Phosphinothricin acetyltransferase is encoded by the bar gene from Streptomyces hygroscopicus or by the pat gene from S.viridiochromogenes which confer resistance to the herbicide compound phosphinothricin. Phosphinothricin (PPT), as a glutamate

analogue, is an irreversible inhibitor of glutamate synthetase, a plant enzyme involved in ammonia assimilation.

Phosphinothricin derivatives with phytotoxic activity, for example bialaphos a tripeptide with a phosphinothric; n residue produced by S. hygroscopicus and Basra (Hoechst, Germany) - a synthetic phosphinothricin (glufosinateammonium-GLA), are nonselective herbicides used in agriculture and can both be used in selection schemes. PAT can be used as a selectable marker in mono- and dicotyledonous plant species. In cereal transformation, its use is more widespread than the other selectable markers. Selection can be applied in the medium in vitro as well as by spraying whole plants either in vitro or in the field or green-house.

Green fluorescent protein (GFP)

Green fluorescent protein (GFP) is responsible for the green bioluminescence from the jellyfish Aquorea victoria. Aequoreabioluminescence is activated when Ca 2+ binds to aquorin, which oxidizes bound coelenterazine transferring the released energy to GFP. The Aequorea GFP has two excitation peaks at 395 nm and 470 nm, and the emission spectrum in the green (509 nm). In addition, the GFP is a stable protein. The GFP has a number of advantages. First, its detection, given a sufficient level of expression, is easy as it can be detected by irradiation using standard long-wave UV light sources. Introduction of a substrate is not required, unlike other commonly used reporter genes, such as p, 3- galactosidase, firefly luciferase, CAT or GUS. Third, its relatively small molecularsize (27 kDa) and monomeric nature makes protein fusion manageable. Forth, amino acid replacements in the polypeptide yield different color and brighter forms.

Uses of GFP in biological sciences

Identification of transformed cells (e.g. byfluorescence- activated cell sorting or fluorescence microscopy) Studies of gene ex pression in vttroLabeling and localization of fusion proteins.

Labeling of unicellular organisms

Labeling of specific cells in multicellular organisms (e.g. to study cell lineages); Identification of organisms released to the environment Several modifications of the gfp gene were introduced in order to improve it for specific uses: codon changes for optimal expression in particular organisms due to different codon usage, addition of peptide signals for organellar targeting, brighter green fluorescence GFP mutant to enhance sensitivity in gene expression studies.

PHYSICAL METHODS OFTRANSFORMATION

In the direct gene transfer methods, the foreign gene of interest is delivered into the host plant cell without the help of a vector. The methods used for direct gene transfer in plants are:

Chemical mediated gene transfer chemicals like polyethylene glycol (PEG) and dextran sulphate induce DNA uptake into plant protoplasts.Calcium phosphate is also used to transfer DNA into cultured cells. Microinjection where the DNA is directly injected into plant protoplasts or cells (specifically into the nucleus or cytoplasm) using fine tipped (0.5 - 1.0 micrometerdiameter) glass needle or micropipette. This method of gene transfer is used to introduce DNA into large cells such as oocytes, eggs, and the cells of early embryo. Direct injection of DNA into single plant cellsor multicellular tissues has the potential to be spedesand variety independent.

Tissue Electroporation

Electroporation is a technique that uses an electrical pulse to render cell walls or protoplast membranes permeable, so that DNA can be taken up into the cells. A high-voltage electrical pulse of short duration causes the formation of temporary pores, which allow cells to take up plasmid DNA; this may lead to stable or transient DNA expression. The method was originally applied to protoplasts, but has been found applicable to cells and even tissues. This method has been used successfully with immature zygotic embryos and embryogenic callus to produce transgenic maize and with intact nodal meristems to produce transgenic legumes.

Particle gun/Particlebombardment - In this method, the foreign DNA

containing the genes to be transferred is coated onto the surface of minute gold or tungsten particles (1-3 micrometers) and bombarded onto the target tissue or cells using a particle gun (also called as gene gun/shot gun/microprojectile gun) (Fig. 8).The microprojectile bombardment method was initially named as biolistics by its inventor Sanford (1983). Two types of plant tissue are commonly used for particle bombardment- Primary explants and the proliferating embryonic tissues. The DNA of choice is precipitated onto microscopic particles, which are then delivered into plant cells: as suspension cultures, tissues in culture, wholeplant parts, etc. The main advantage of this system is that it is species independent and avoids the complex interaction between bacterium and plant tissue, with the result that the DNA to be introduced does not need to contain the sequences necessary for T-DNA transfer. Particle bombardment is thus a simple physical process compared with Agrobacterium-mediated DNAtransfer, where transformation occurs by way of a complex process that is still not thoroughly understood. Transfer of DNA into plants by particle bombardment has become a major method of choice for the production of transgenic plants.

It has been used successfully in a wide range of species and has been used in transforming species that are not readily amenable to transformation with Agrobacterium or that are recalcitrant to otherdirect transfer methods. Particle gun delivery of DNA has been shown to result in integration of rearranged and/or truncated DNA sequences, as well as multiple copies of T-DNA. Usually, DNA is coated onto gold or tungsten particles for delivery, but biological projectiles such as bacteria (Escherichia coli), yeast, and phage have been complexed with tungsten and used as particles for bombardment.

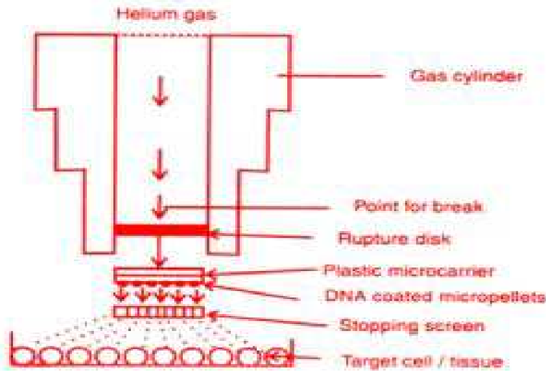


Fig. 8. Gene Gun

Liposome mediated gene transfer or Lipofection

Liposomes are circular lipid molecules with an aqueous interior that can carry nucleic acids. Liposomes encapsulate the DNA fragments and then adher to the cell membranes and fuse with them to transfer DNA fragments. Thus, the

DNA enters the cell and then to the nucleus. Lipofection is a very efficient technique used to transfer genes in bacterial, animal and plant cells.

Silicon Carbide Whiskers

Silicon carbide whiskers have been used to introduce DNA into plant cells. This method is limited, however, to fine suspension cultures of cells that can be readily penetrated by the whiskers, and thus has the associated problems of regeneration into whole plants. Another disadvantage is that silicon fibers have similar properties to asbestos fibers and care must be taken when working with them. While not widely used, the technique is a useful alternative to particle bombardment if the latter is not appropriate for any reason.

AGROIN FILTRATION

Agroinfiltration is a method of transformation to induce transient expression of genes in a plant or to produce a desired protein. In the methoda suspension of Agrobacterium tumefaciens is injected into a plantleaf, where it transfers the desired gene to plant cells. The benefit

of agroinfiltration when compared to traditional plant transformation is speed and convenience.

First step of the protocol is to introduce a gene of interest to a strain of Agrobacterium. Subsequently the strain is grown in a liquid cultureand the resulting bacteria are washed and suspended into a suitable buffer solution. This solution is then placed in a syringe (without a needle). The tip of the syringe is pressed against the underside of a leaf whilesimultaneously applying gentle counterpressure to the other side of the leaf.
The Agrobacterium solution is then injected into the airspaces inside the leaf through stomata, or sometimes through a tiny incision made to the underside of the leaf (Fig.10).Vacuum infiltration is another way to penetrate Agrobacterium deep into plant tissue. In this procedure, leaf disks, leaves, or whole plants are submerged in a beaker containing the solution, and the beaker is placed in a vacuum chamber. The vacuum is then applied, forcing air out of the stomata. When the vacuum is released, the pressure difference forces solution through the stomata and into the mesophyll. Once inside the leaf the Agrobacterium remains in the intercellular space and transfers the gene of interest in high copy numbers into the plant cells. The gene is then transiently expressed (no selection for stable integration is performed). The plant can be monitored for a possible effect in the phenotype, subjected to experimental conditions or harvested and used for purification of the protein of interest. Many plant species can be processed using this method, but the most common ones are Nicotiana benthamiana and Nicotiana tabacum.

Fig. 10. Agroinfiltration of pla plant leaves

OTHER APPLICATIONS OF PLANT TRANSFORMATION

Lower PlantsUnicellular algae likeChlamydomonas reinhardtii are also amenable for transformation with Agrobacterium. The advantage of transforming microalgae in particular is that they complete their cell cycle in a few hours, thereby rendering genetic selection and strain screening relatively quick and easy. A transformation system has been developed for the unicellular species Chlorella kessleri, which has been widely utilized for investigations of cellular processes, and C reinhardtii has been the subject of studies involving nuclear transformation , chloroplast transformation, and mitochondrial transformation . Other specific examples include the diatom, Phaedactylum tricornutum, and the multicellular alga, Volvox carteri. Bryophyts are also useful modelsfor studying the biology of land plants owing to their morphological simplicity, ease of cultureand short life cycle. Genetic transformation has been successful for species such as Physcomitrella patens and Ceratodon purpureus.

Plastids

DNA can also be transferred by Agrobacterium to plant chloroplasts. Evidence for this was firstobtained when a Ti plasmid vector was utilized that contained a marker gene capable of expression in chloroplasts. Particle bombardment and polyethylene glycol treatment

can be employed to produce stable chloroplast DNA transformants. Chloroplast transformation is advantageous over nuclear transformation and has been proposed as a possible solution to some of the problems encountered with nuclear transgenes, such as unstable or variable expression. For example, gene integration into the plastome occurs via homologous recombination so that there are no position effects, as occur following random insertion of transgenes in nuclear transformation; also, gene silencing has not been found to occur in plastids and therefore transgene expression is stable in progeny. High levels of protein expression and accumulation of protein can be achieved by plastid transformation. In addition, there is the potential for greater containment of transgenes, because plastids are not transmitted through the pollen in engineered crop plants and thus transgene spread via the pollen can be minimized. Protoplast Transformation Early work on plant transformation suggested that monocotyledonous species were recalcitrantto infection by Agrobacterium. An alternate strategy to overcome recalcitrance is protoplast transformation. DNA can be introduced into plant protoplasts via polyethylene glycol (PEG) fusion, electroporation, and microinjection. A number of species has been transformed in this way, a major disadvantage of methods utilizing protoplasts is that the regenerationof plants from protoplast cultures canbe a complex and time-consuming process. However, protoplast systems still have applications for research purposes such as analysis of gene and gene construct function and plastid transformation. Protoplast electroporation can be a useful tool in transient expression assays; electroporation has been found to result in single copy plasmid insertions rather than the multiple plasmid copies encountered following particle bombardment.

Conclusions

Growing interest on biotechnological research demands the development of novel strategies to manipulate and incorporatespecific genetic sequences into plants to improve theircharacteristics in agreement with the society needsin an easy, safe, trusty and reproducible form. Genetic plant transformation whether performed by

physical or othermethods, currently faces major challenges. Random integration of the transgenes continues to be a major issue; howevermethods to overcome this have been developed, such as the ones that utilize Zinc- finger nucleases which canbe used to generate high-frequency homologous recombination to modify specific plant genes. Transgene silencingis also a major challenge and to address it, several virus-derived proteins have been employed. To suppress specific transgenes, methods such as antisense and RNAi have been developed.The RNAi method is more powerful and its ability to suppress, or silence, expression of specific genes has made it amajor new tool for functional genomics and genetic engineering of many organisms. However, little is known aboutefficiency and stability of RNAi- induced gene suppression in the diversity of organisms where it has been applied.So far, most of the methods employed have relied on the use of Agrobacterium, but due to the limitations described above, direct, physical methods represent an interesting alternative to overcome some of these obstacles. They mayseem the method of choice if one wishes to exclude vector sequences and for species recalcitrant to Agrobacterium transformation.

Nevertheless, for aproperimplementation, it is important to understand the physics behind many ofthese methods to make a better use of the technique and eventually to enhance penetration of the cellular wall andintegration of the transgene. Some techniques have been successfully established for few plant types, but there is still alot of research to be done in order to effectively exploit them in a wide variety of species and to increase the efficiencyand reproducibility of the genetic transformations.

UNIT V

Transgenic plants for crop improvement (dicots and monocots including maize, rice, wheat, oats, etc.; resistance to herbicide, insecticide, virus and other diseases; transgenic plants for molecular farming; transgenic plants to regulated gene expression, Chloroplast and Mitochondrion engineering.

Introduction

The stable introduction of foreign genes into plants represents one of the most significant developments in a continuum of advances inagricultural technology that includes modern plant breeding, hybrid seed production, farm mechanization, and the use of agrichemicals to provide nutrients and control pests. The first- generation applications of genetic engineering to crop agriculture are targeted at issues that are currently being addressed by traditional breeding and agrichemical discovery efforts: (i) improved productionefficiency, (ii) increased market focus, and (iii) enhanced environmental conservation. Genetic engineering methods complement plant breeding efforts by increasing the diversity of genes and germplasm available for incorporation into cropsand by shortening the time required for the production of new varieties and hybrids. Genetic engineering of plants also offers exciting opportunities for the agrichemical, food processing, specialty chemical and pharmaceutical industries to develop new products and manufacturing processes. The first transgenic plants expressing engineered foreign genes were tobacco plants produced by the use of Agrobacterium tumefaciens

FIG. 1.Applications of plant transformations.

What are genetically-modified foods?

The term GM foods or GMOs (genetically-modified organisms) is used to refer to crop plants created for human or animal consumption using the latest molecular biology techniques. These plants have been modified in the laboratory to enhance desired traits such as increased resistance to herbicides or improved nutritional content. The enhancement of desired traits has traditionally been undertaken through breeding, but conventional plant breeding methods can be very time consuming and are often not very accurate. Genetic engineering, on the other hand, can create plants with the exact desired trait very rapidly and with great accuracy.Not only can genes be transferred from one plant to another, butgenes from non-plant organisms also can be used. The best known example of this is the useof B.t. genes in corn and other crops. B.t. (Bacillus thuringiensis) is a bacterium which produces proteins thatare lethal to insects - the plant produces its own pesticides against insects.

Advantages of GM foods

Ensuring an adequate food supply for the population is going to be a major challenge in the years to come. GM foods promise to meet this need in a number of ways:

Pest resistance: The consumers do not wish to eat food that has been treated with pesticides because of potential health hazards, and agricultural wastes from the use of pesticides and fertilizers can poison

the water and can harm the environment.

Herbicide tolerance: Weeds can be removed by physical means such as tilling, but farmers often spray different herbicides (weed-killer) to destroy weeds.

Disease resistance: There are many viruses, fungi and bacteria that cause plant diseases. Plant biologists are working to create plants with genetically-engineered resistance to these diseases.

Cold tolerance: Frost can destroy sensitive seedlings. With the antifreeze gene, plants areable to tolerate cold temperatures that would normally kill unmodified seedlings.

Drought tolerance: As the world population grows and more land is used for housinginstead of food production, farmers will need to grow crops in locations previously unsuited for plant cultivation.

Nutrition: Malnutrition is common in third world countries where people rely on a single crop such as rice. Rice does not contain adequate amounts of all necessary nutrients to prevent malnutrition. Rice could be genetically engineered to contain additional vitamins and minerals.

Disadvantages of GM foods

Most concerns about GM foods fall into three categories: environmental hazards, humanhealth risks and economic concerns:

Environmental hazards

Unintended harm to other organisms

Reduced effectiveness of pesticides

Gene transfer to non-target species

Allergies

Unknown effects on human health

Seed patenting

Insect resistant transgenic plants

Yield loss in crop plants is 37% of the agricultural production world-wide, with 13% due to insects. Plants can be engineered for insect resistance by by insertion and expression of insecticidal proteins. Cry genes of Bacillus thuringiensis coding for the delta endotoxins is the most vividly used gene for genetic engineering insect resistance in

plants.The insecticidal secondary metabolites coded by the plants can be broadly classified as i) non-protein antimetabolites like alkaloides, nonprotein aminoacids, terpenoids, rotenoids (isoflavanoids), tannins, polysaccharides, glucosinolates and cyanogenic glycosides and (ii) protein antimetabolites like proteinase inhibitors, a-amylase inhibitors, lectins and arcelins.

Cry genes from Bacillus thuringiensis

Cry(Bt) genes (from bacterium Bacillus thuringiensis) were the first transgenes to be used for generating insect resistant genetically modified plants. Plants harbouring the Bt gene are the only insect resistant transgenic crops grown commercially. B. thuringiensis (Bt) is a gram-positive bacterium producing highly insecticidal protein crystals ,5-endotoxin insecticidal proteins also called Bt toxins during sporulation. These toxins are active against a wide range of agriculturally and medically important pests with a high degree of specificity. Delta endotoxins are pore forming toxins that may induce cell death by forming ionic pores in the membrane of midgut epithelial cells in the target insect by triggering the activation of a cascade signaling pathway after toxin interaction with a specific receptor in the gut membrane.

The mode of action of Bt toxins is complex, involves multiple steps and sequential binding to receptors and is still incompletely understood. The ingested toxin is activated by insect gut proteases, interacts with the primary receptor and then undergoes further proteolytic processing. The toxin then binds to a second receptor resulting in toxin oligomerization and insertion into the membrane forming pores that cause osmotic shock, bursting of the midgut cells and insect death . Differen cry genes isolated from aifferent strains of B. thuringiensis have been used in a wide variety of crops for resistance against a broad spectrum of insects (Table. 1)

Table 1 : Toxicity of *B. thuringenesis* (Bt) toxins against aphids.

Toxin	Toxicity	Specificity
Cry2, Cry3A, Cry4	Some	Potato aphid, *Macrosiphum euphorbiae*

Toxin	Toxicity	Specificity
Cry4Aa Cry11Aa Cry3A,	LC50: 70-100 µg/mL 100% mortality at 500 µg/mL 60% mortality at 500 µg/mL	Pea aphid, *Acyrthosiphon pisum*
Vip1Ae-Vip2Ae	LC50: 0.576 µg/mL	Cotton aphid, *Aphis gossypii*

Transgenic maize expressing the cry gene from B. thuringiensis ssp. Kurstaki engineered forresistance agains the European corn borer was the first BT crop to be approved by the European Union (EU) for Food and Feed.

Bt cotton is the second most important transgenic insect resistant crop. Several insect resistant cotton events, which confer resistance to lepidopteran pests of cotton, have been approved in the EU so far.

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Genes from other Microbes

Vip genes, are insect resistant genes isolated from Bacillusspecies (B. thuringiensis and B. cereus). The products of the vipgenes (Vip proteins) are expressed in the vegetative stage of growth starting at midlog phase as well as during sporulation. More than 50 Vip proteins have been identified so far. Vip proteins causes swelling and disruption of the midgut epithelial cells by osmotic lysis in the target insects. One group of Vip toxins consists of binary toxins that are made of two components, Vip1 and Vip2. The combination of Vip1 and Vip2 is highly insecticidal to an agriculturally important insect, the western corn rootworm (Diabrotica virgifera), but does not show any insecticidal activity to lepidopteran insects. The other group consists of Vip3 toxins, which share no sequence similarity to Vipl or Vip2. The first-identified Vip3 toxin, Vip3Aal, is highly insecticidal to several major lepidopteran pests of maize and cotton. According to the Agricultural Biotechnology Annual Report for Australia (Crothers, 2006), GM VIP cotton has the licence for limited and controlled release.

Cholesterol oxidase

A cholesterol oxidase isolated from the culture filtrate of Streptomyces has been found to disrupt the gut epithelium of boll weevil upon ingestion. Tobacco (Nicotiana tabacum L.) plants transformed with the cholesterol oxidase choM gene caused death of the boll weevil larvae by disrupting its midgut. Another set of proteins encoded by the

bacterium Photorhabdusluminescens, which lives in the gut of entomophagous nematodes. In insects infected with the nematode, the bacteria are released into the insect hemocoel; the insect dies andthe nematodes and bacteria replicate in the cadaver.The toxin consists of a series of four native complexes encoded by toxin complex loci tca, tcb, tcc and tcd. Both tca and tcdencode complexes with high toxicity to tobacco hornworm (Manduca sexta) and therefore they represent potential alternatives to Bt for transgenic deployment. TcdA gene encoded by Photorhabdus luminescens was engineered into Arabidopsis to generatetransgenic Arabidopsis resistant to southern corn rootworm (Diabroticaundecimpunctata howardi).Resistance genes from insects or sources other than plantsAlthough insects producechitinases during molting Insects feeding on constitutively expressing the Insect Chitinases have been found to be affected due to inappropriately timed exposure to chitinase. Transgenic tobacco plants expressing insect (Manduca sexta) chitinasetransgene have enhancedresistance to budworm (Heliothis virescens) larvae. Protein inhibitors of proteases are compounds which are present inmultiple forms in numerous tissues of animals and plants as well as in microorganisms. Overexpression of protease inhibitors (PIs) can protect plants against some insect species. Transgenic tobacco expressing the insect encoded anti-tryps:n, anti-chymotrypsin and anti-elastase protease inhibitor genes from Manduca sexta showed enhanced resistance to potato whitefly type B, Bemisia tabaci. Insect neuropeptides are neuro transmitters that are able to interfere with a variety of physiological processes in insectscan be used to engineer insect resistance in plants. They are active at very low concentrations and are non-toxic to vertebrates, including humans.

Orally administered peptides may penetrate the insect gut and that undegraded fractions target haemocoelic cells and enter the haemolymph intact. Proctolin is the first isolated and characterised insectneuropeptide. It is only 5 amino acids long and it functions as a visceral and a skeletal neuromuscular transmitter and is a potent insecticidal protein. Avidin is a glycoprotein from chicken (Gallus gallus) egg white that binds its ligand, biotin, with very high affinity. Biotin is a coenzyme required for all forms of life, so feeding avidin to

many insects causes a biotin deficiency that leads to a stunted growth and mortality. Avidin is toxic to seven species of stored-product beetles (Coleoptera) and moths (Lepidoptera). Avidin is also toxic to housefly (Musa domestica) (Levinson and Bergmann, 1959) and olive fruit fly (Dacus oleae). Hence this can be used as a candidate gene for insect resistance in transgenic plants. Avidin expressed in transgenic tobacco and apple conferred a high level of insect resistance to potato tuber moth, Phthorimaea operculella (Zeller) and lightbrown apple moth, Epiphyas postvittana.

RESISTANCE GENES FROM HIGHER PLANTS

These include

Proteinase inhibitors

a-amylase

Lectin

Proteinase inhibitors

Proteinase inhibiotors (PIs) are ubiquitous proteins widely distributed in multiple forms in several tissues ofanimals, plants and in microorganisms. Insectproteinases are essential digestive enzymes that catalyse the release of amino acids from dietary protein to provide the nutrients required for larval growth and development. Whereas serine proteinases (trypsin-, chymotrypsin- and elastase like proteinases) are predominant in lepidopteran midgut,midguts of coleopteran species are rich in cysteine and aspartic proteinases. The mode of PI action on insects is still under debate, and it remains to ascertain whether PIs' deleterious effects stem from an antidigestive effect through proteolysis inhibition. PIs can also affect the water balance, moulting and enzyme regulation of the insects. Consequences are reduced growth and development of insects but also death. PIs reduces proteolytic enzyme activity in vitro in a number of insect species. Tobacco plants transformed with trypsin inhibitor gene (CpTI) fromVigna unguiculatashowedsignificant resistance to Heliothis virescens.

a-amylase inhibitors

Protein a-amylase inhibitors are widespread and have been isolated from a variety of plant species and microorganisms. Thephysiological role of a- amylase inhibitors in plants is uncertain, they act as protein reserve in seeds. They affect the insects by interfering with their nutrient utilization. Their mode of action is similar to the proteinase inhibitors. Bytransforming pea plants with the a-amylase inhibitors, transgenic seeds resistant to bruchid beetles (Callosobruchus maculatus and C. chinensis), pea weevil (Bruchus pisorum) were obtainted.

Lectins

Lectins are carbohydratebinding proteins that bind glycans of glycoproteins, g lyco lipids, or polysaccharides with high affinity. Although many roles have been proposed for plant lectins, the most likely function for vacuolar lectins is plant defence. In insect, toxic effects is mediated through binding of the lectins to the midgut epithelial cells with consequent disruption of the cell function. The bound lectins may inhibit nutrient absorption or disrupt midgut cells by stimulating endocytosis of the lectins, and possibly other toxic metabolites present in the midgut.Lectins can be used to control sap-sucking insects belonging to the order Homoptera, which includes some of the mostdevastating pests worldwide. Crop damage caused by these insects is not only due to feeding, but also to their role as vectors of plant viruses. Transgenic plants expressing Galanthus nivalis lectins, (GNA) (tobacco, potato and wheat) caused significantly reducedparthenogenetic fecundity, but only marginal or none decrease in aphid survival. Lectins from species other than Galanthus nivalis also possess insecticidal activity. Transgenic expression of pea lectin in oilseed raperesulted in significant reduction in pollen beetle larval weight, and small but significant reduction of larval survival.

HERBICIDE RESISTANCE

Herbicides are synthetic compounds that are commonly applied to eliminate weeds. These herbicides should not act on the crop plants and should be phytotoxic to major weeds. Hence there is an ever

increasing demand for herbicides which are effective, environmentally safe and rapidly biodegraded. Many herbicides exert their effects by inactivating target proteins' essential for vital functions such as photosynthetic or other biosynthetic pathways unique plants. As all the plants share these processes, the herbicides are not specific to weeds alone and they can target the crop plants also. Selective herbicides which do not affect the crop plants mainly as a consequence of a differential uptake or metabolism of the herbicide or by a precise localized application of the herbicide can be used. But an alternative strategy is to confer resistance to crops against broad-spectrum herbicide by genetic engineering. There are two approaches for engineering herbicide resistance in plants. 1. The modification of the enzyme or the target for herbicidal action in the plants to render it insensitive to the herbicide or by inducing the overproduction of the unmodified target protein, thus permitting normal metabolism to occur in spite of the presence of the herbicide. 2. The introduction of a enzyme or enzyme system to degrade and/or detoxify the herbicide in the plant before it can act. Herbicide resistance can be genetically engineered into plants with the above said starategies.

Modification of the target of herbicide action

This approach depends on the identification of molecular andbiochemical mode of action of the herbicide and to modify it to become resistant to the herbicide.

Glyphosate tolerance

Glyphosate is a widely used herbicide and it inhibits the enzyme5-e no l pyruvylshiki mate-3-phosphate synthase (EPSP), which is essential for biosynthesis of aromatic amino acids in bacteria and plants. A gene (aroA)coding for a glyphosate-tolerant form of EPSP has been isolated from bacterium Salmonellatyphimurium, has been successfully used for conferringGlyphosate resistance in transgenic plants expressing this gene. Transgenic tobacco and tomato plants over-expressing the aroA gene was found to show varied levels of resistance to the herbicide glyphosate

Sulphonylureas andimidazolines

The sulphonylurea and imidazoline herbicides inhibit the acetolactate synthase (ALS) from bacteria, yeast and plants and it is essential for biosynthesis of branched-chain amino acids leucine, isoleucine and valine. Dominant mutation in the ALS genes have been found to conferresistance to Sulphonylurea. Tobacco plants and Arabidopsis plantsresistant to Sulphonylureas by culturing them on the tissue culture media and selecting the resistant plants. A mutant allele crsl has been isolated from Arabidopsis thaliana. Transgenic expression of crsl in tobacco was found to confer resistance to sulphonyl urea. Imidazoline resistance have been achieved by selecting plants with mutation in the ALS enzyme. Plants with homozygous mutation showed 300 times imidazoline resistance when compared to normal plants.

Phosphinothricin resistance

Phosphinothricin (PPT) is an analogue of glutamate and it inhibits glutamine synthesis in bacteria and plants. Glutamine synthase is involved in primary assimilation of ammonia produced by nitrate reduction of nitrogen fixation in roots as well as the ressimilation of ammonia released by photorespiration in plants. In higher plants PPT causes rapid accumulation of ammonia and Inhibits photosynthesis, which leads to death of the plant cell. Expression of the Glutaminesynthase under the control of CaMV 35 S cassette was found to confer resistance to PPT in transgenic tobacco plants.Transgenic plants for fungal and viral resistance

FUNGAL RESISTANCE

Fungal disease has been one of the principal causes of crop losses ever since humans started to cultivate plants. Fungal diseases have been the principal causes of crop losses ever since humans started cultivating plants. About 70% of crop diseases are caused by fungi. Fungal diseases can be managed by crop husbandry techniques like breeding fungus-resistant cultivars of crops and application of agrochemicals. Chemical (fungicides) management is an effective disease management but the growing concern about the environment, and insistence on cost effective methods encourage the development of cultivars which

require lesser chemicals. Breeding practices are mostly deployed for yield and quality enrichment, rather than pest and disease resistance (Van Den Elzen et al., 1993). The time taken for crosses and backcrosses and subsequent selection of progeny for the presence of resistance traits makes plant breeding difficult to obtain resistant cultivars. Genetic engineering accelerates the process of obtaining resistant cultivars.

Genetic engineering approaches for fungal resistance have deployed many genes of the pathogens as well as the plant. Genes involved in plant resistance reaction, from the first step of pathogen recognition till the PR-gene expression have been used in plant genetic engineering (Cornelissen and Melchers, 1993; De Boer, 2003; Grover and Gowthaman, 2003; Honee, 1999; Melchers and Stuvier, 2000; Punja, 2001; Punja, 2006; Shah, 1997). Genetic engineering for fungal resistance broadly falls into two categories. The first approach for achieving fungal resistance relies on the activation of the whole array of defense response and the second approach involves constitutive overexpression of antifungal genes from plants (PR- proteins) and other organisms (antimicrobial peptides). Fungal disease resistant transgenic plants can be classified into nine categories based on the basis of action of the transgenes they possess

Expression of PR-protein genes

Proteins that target structural components of fungal pathogens

b)Antimicrobial peptides

C)Lipid transfer proteins

d) Combined expression of PR- protein genes

Expression of ribosome inactivating proteins

Expression of polygalacturonase- inhibiting proteins

Expression of genes that encode phytoalexm
biosynthesis

Expression of defense-related genes

Expression of antifungal proteins of microorganisms

Expression of recombinant antibodies

Expression of antimicrobial peptides from plant and
nonplant sources

Expression of detoxifiers of fungal pathogen
Expression of PR-protein genes
Proteins that target structural components of fungal pathogens

The fungal cell wall is an essential organelle that accounts for 15-30% of the cellular dry weight. As chitin and p-1,3-glucan are the major components of the fungal cell wall, expressionof PR-proteins with chitinase or p-1,3-glucanase activities has been found to be very effective. They include the chitinase (PR-3, 4, 8, and 11) and glucanase (PR-2). They play a dual role in defense: first they inhibit the fungal growth by cell wall dissolution and secondly they release pathogen-borne elicitors that may induce defense-related responses in the host plant. Chitinase and p-1,3-glucanase synergistically degrade fungal cell wall at the hyphal tips. As chitinase andp-1,3-glucanase efficiently inhibit fungal pathogens, they are used in a majority of the transgenic plants engineered for fungal resistance. Thaumatin-like proteins (TLPs) are PR-5 proteins which act by hydrolyzing the p-l,3-glucan of the fungal cell wall (Grenier et al., 1999) and also permeabilize the plasma membrane causing leakage of the cellular contents. Oxalic acid facilitates penetration of fungal pathogens by acidification to accelerate cell wall degradation.Oxalateoxidase (PR-15) protein degrades oxalic acid to carbon dioxide and hydrogen peroxide and considered to have a role in defense responses of plants. Transgenic over expression of Oxalate oxidase provides resistance to a number of fungi.

Antimicrobial peptides

These include the antifungal proteins, defensins (PR-12) and thionins (PR-13). They constitute a family of low molecular weight (~5 kDa) basic peptides rich in basic and sulfur-containing amino acid residues. The thionins are low molecular weight proteins and their antifungal activity is based on their capacity to form pores in cell membrane resulting in membrane disruption and cell death. Thionins have been successfully used to obtain transgenic plants with resistance tophytopathogenic fungi.Defensins are structural and functional homologues of insect and mammalian proteins that play a role in host defense. In

Arabidopsis,there are 13 putative defensin genes encoding 11 different plant defensins. The defensins cause hyperbranching of the fungal hyphae, which is associated with their antifungal activity. They form multimeric ion- permeable channels in a voltage-dependent manner within the microbial membranes by effectively inserting themselves into the cell membrane. They bind to fungal membranes and cause fungal hyphae destruction. Theseantifungal proteins can be used to engineer fungal resistance in plants.

Lipid transfer proteins (PR-14)

They are antimicrobial peptides and belong to the PR-14 family. Thenonspecific-lipid transfer proteins (nsLTPs) help in the cytoplasmic transfer of lipids from the endoplasmic reticulum to organelle membranes such as mictochondria which cannot synthesize specific lipid molecules. They are also involved in the secretion of extracellular lipophillic materials like wax and cutin. The plant nsLTPs are ubiquitous and form a single family of similar 90-95 aa polypeptides. The lipid transfer protein (LTPs) expression is upregulated during infection. Fungal resistance in plants can be successfully engineered in plants by overexpressing the nsLTPs.

Combined expression of PR-proteins

Hallmark response of plants to pathogen attack is activation of a battery of PR- protein genes, suggesting that different mechanisms may have complementary roles in expression of resistance. Diseaseresistance is a complex trait and is controlled by expression of a number of genes. In a natural disease resistance response, many PR-protein genes function in a concerted manner to prevent the spread of pathogens combined overexpression of PR-proteins has been sought to be an effective method to control fungal diseases. Combination of genes with synergistic effect on the pathogen is an effective method to control the disease. PR-proteins with different modes of action would ensure durable and enhanced broad-spectrum resistance.

Expression of ribosomeinactivating proteins

The ribosome-inactivatingproteins (RIPs) exhibit antifungal activity. They inhibit protein synthesis by depurmating a specific residue near the 3' end of 26-28S rRNA. Although they are present in seeds of many plants, they do not affect thehost protein synthesis but act selectively on foreign ribosomes. RIPs used under the control of a wound-inducible promoter in tobacco, were effective against Alternaria alternata and Botrytis cinerea. PAPII, an RIP from pokeweed (Phytolaccaamericana) offered broad- spectrum resistance to viral as well as fungal pathogens in tobacco

Expression ofpolygalacturonase- inhibiting proteins (PGIP)

Endopolygalacturonases (PGs) produced by fungal pathogens play an important role in fungal pathogenesis in the early stages of infection. PGs cause the release of oligogalacturonides (OGs) from the plant cell wall. The OGs elicit a variety of defense responses including accumulation of phytoalexins, glucanase and chitinase. The polygalacturonase-inhibiting proteins (PGIPs) are extracellular plant glycoptoteins capable of inhibiting fungal PGs. They play the role of R genes to mount a defense response in plants. The PGIPs are localized in the cell wall of many plants and have a great potential for limiting fungal colonization by acting as both inhibitor and regulator of PG activity. The PGIPs directly antagonize the PGs.Overexpression of PGIPs has been used as a strategy to control fungal diseases in plants.

Expression of genes that encode phytoalexinSynthesis

Phytoalexins are antimicrobial, low molecular weight secondary metabolites which accumulate following an incompatible interaction between a resistant plant and the pathogen. Synthesis of phytoalexins is one of the active defense mechanisms in plants. Theexpression of phytoalexinbiosynthesis genes in a hetrologous host increases fungal resistance, since host- specific pathogens have not experienced selection for detoxifying or metabolizing the novel antifungal compounds. Stilbenes, pinosylvins and resveratrol are classes of Phyto alexins and the genes encoding their biosynthetic pathway were deployed for engineering fungal disease resistance.

Expression of defense- related genes

Cloning and characterization of R genes have lead to the use of these genes for engineering disease resistance in transgenic plants. Expression of a single R gene with proven durable resistance is a good strategy for engineering broad-spectrum disease resistance. Successful transfer of a functional R gene between closely related species through genetic transformation has paved the way for deploying the R gene for engineered resistance in transgenic crops. R gene transfer from model species to crops or between distantly related crops is hampered because of 'restricted taxonomic functionality'. For example, the R gene, Bs2 from tomato can function as a transgene in the members of the Solanaceae family but not in Arabidopsis. The Rpgl gene from barley, the tomato Cf9 gene and tomato Vel and Ve2 genes provided enhanced resistance against stem rust in barley, Leptophaeria in canola and Verticillium wilt in potato. The drawback of using the R genes in disease resistance is that they cause phenotypic abnormalities like stunted growth in the transgenic plants.

The pathogen elicitors can be manipulated to engineer broad host range resistance in plants. It is based on eliciting HR in plants containing the R gene with pathogen-inducible expression of the cognate Avr gene. Although the elicitors vary widely in their chemical composition and mechanisms by which plant perceive them, they trigger a common cascade of signaling pathways that coordinate the overall defense response of plants. Controlled expression of these genes may result in a broad-spectrum diseaseresistance in transgenic plants.The expression of cryptogein, a candidate elicitor ofPhytophthora cryptogea, under a defense-related gene promoter leads to resistance of transgenic tobacco to Thielaviopsis basicola, Erysiphe cichoracearum, and B. cinerea. An elicitor from Fusarium solani encodes a DNase. The expression of this elicitor under a pathogen- induced promoter in tobacco, enhanced resistance to A. alternata.

Expression of defense-inducing genes in plants through genetic engineering is a prospective strategy for developing fungal resistance. Disease resistancecan be engineered by constitutive expression of

transcription factors which upregulate the defense-related genes. The constitutive expression of ETHYLENE- R ESPONSE- FACTOR1 geneinArabidopsis conferred resistance to several necrotrophic fungi. The ethylene-responsiveelement-binding proteins bind to the promoters of PR-genes and induce their expression. Tsi, coding for an ethylene- responsive element-bindingprotein in tobacco, when expressed in hot pepper resultein enhanced resistance to Phytophthora capsid.

Expression of transgenes that lead to the production of signaling molecules like SA enhances PR-protein production in plants and results in fungal resistance. Bacterio-opsin from Halobacteria halobium functions as a proton pump to induce programmed cell death resulting in HR and broad-spectrum pathogen resistance. The fungal endo-polygalacturonase hydrolyzes thehomogalacturonan of the plant cell wall for effectiveoligogalacturonides (OGs) that are perceived by the plant cell as host-associated molecular patterns and induce a defense response. Transgenic expression of endo-polygalacturonase in tobacco and Arabidopsis confers resistance to Botrytis cinerea.

Antifungal proteins of microorganisms

Antifungal proteins from microorganisms have been deployed to enhance disease resistance in plants. An antifungal protein (AFP) from Aspergillus giganteus has been found to inhibit the growth of a number of economically
important fungal pathogens. Constitutive expression of AFP in the rice plants has been shown to enhance resistance to M. grisea. Expression of bacterial chitinase, viral chitinase and chitinase from Trichoderma species is a prospective strategy for engineering fungal resistance. The fungal pathogen, Ustilago maydis of Zea mays harbours a double-stranded RNA virus that encodes anti fungal proteinaceous killer toxins, KP1, KP4 and KP6. These proteins have been assessed to enhance resistance against the smut fungi infecting wheat. Expression of KP4 and KP6 as secretory proteins in transgenic tobacco plants can render them resistant to fungal pathogens.

Expression of recombinant antibodies

Recombinant antibody (rABs) engineering is a novel approach to generate pathogen-resistant transgenic plants. Crop resistance can be engineered by the expression of pathogen- specific antibodies, antibody fragments or antibody fusion proteins. The advantages of this approach are that the rABs can specifically target the pathogen molecule and a targeted defense is more advantageous than non-specific approaches.

The expression of plant antibodies 'plantibodies' against pathogen virulence products is a potential strategy for engineering disease resistance. The antibodies can bind to and inactivate enzymes, toxins or other pathogen factors. In vitro application of such antibodies abrogated disease development in plants. A recombinant gene representing a chicken-derived single-chain antibody specific to an antigen displayed on the Fusarium cell surface fused to an AFP was expressed in transgenic Arabidopsis plants. As the antifungal protein was directly targeted to the fungal cell wall, the antibody fusion proteins provided effective protection against Fusarium oxysporum.

Expression of antimicrobial peptides (AMPs) from plant and non-plant sources

Antimicrobial peptides (AMPs), mediators of innate defenses, in all species of life, have been exploited to genetically engineer disease-resistant crop plants. The AMPs are small cationic and amphipathic peptides. They represent a diverse family ofnaturally occurring defensive molecules found in invertebrates, plants and vertebrate animals. Over 500 AMPs have been reported, which include small (less than 50 aa) polycationic peptides with excess lysine and arginine residues and membrane active peptides.

Synthetic AMPs are more efficient in their antifungal activity as they are more target-specific and have increased efficacy at lower concentrations. In plants, AMPs are produced either constitutively or can be induced at the site of the challenge by a microbial pathogen. Apart fromthe AMPs that are classified as PR-proteins, the other classes of antimicrobial peptides include, p- barellin from Macadamia integrifolia, snakin from Solanum tuberosum, vicilin

fragment from Macadamia integrifolia, chitinbinding peptide from Amaranthus caudatus, Knottin-like protein from Mirabiiis jalapa, cyclotide from Oldenlandia affinis, impatiens from Imaptiens balsamina, cicerin and arietin from chickpea and angularin from red beans. AMPs have been used to engineer specific or broa-spectrum disease resistance in many plants

Expression of detoxifiers of fungal toxins

Many pathogens produce phytotoxins that cause diseases in plants. The toxin resistance in plants is based on the ability of plants to detoxify the pathogenic toxins. Degradation of these toxins by enzymes expressed in transgenic plants provides an opportunity to enhance disease resistance. NADPH-dependent HC-toxin reductase (HCTR), encoded by the Hm1 gene in maize, inactivates HC-toxin produced by the fungus. Overexpression of rice YK1, a homologue to the maize Hm1 gene, conferred resistance to rice blast disease caused by M. grisea. The fungal toxin eutypine from the fungus Eutypa lata acts as an important virulence factor involved in symptomdevelopment of Eutypa dieback disease. Eutypine is converted into its non-toxic form (eutypinol) by a NADPH- dependent aldehyde reductase coded by Vr-ERE.Transgenic grapevine plants expressing the Vigna radiataVr-ERE exhibited enhanced resistance towards Eutypa lata. A fungal toxin like cercosporin in the presence of light, reacts with oxygen, causing oxidative stress in plants. Deoxynivalenol (DON), the trichothecene mycotoxin produced by Fusarium species, is known to be a fungal pathogenicity factor and a potent inhibitor of protein synthesis in eukaryotes. Expression of the fungal tricotheceneacetyltranferase gene that degrades DON resulted in reduced growth of Fusarium graminearum in wheat.

Virus Resistance

Viral disease cause devastating yield loss in agricultural crops. Viruses are intracellular parasites, which exploit the host cellular transcriptional and translational machineries for its survival. Life cycle of plant virus includes the following steps (Fig. 4). Plant viruses enter a host plant cell, by penetrating the cell wall, following abrasive mechanical damage, or

via fungi, insects, mites, or nematodes that penetrate the plant cell wall during infection or feeding. The virus particle disassemble exposing the viral DNA or RNA. If the virus possesses mRNA as genetic material, translation will begin to produce the virus-specific proteins required for replication. DNA viruses generally enter the nucleus and utilize host enzymes to produce mRNAs suitable for translation. Each stage of the infection cycle can be inhibited, i.e. at uncoating, translation, replication, and/or movement. This can be achieved by introducing a portion of the viral genome with or without expression of the encoded protein by genetic engineering strategies. This is termed as pathogen derived resistance.

Plant viruses are intracellular, molecular obligate parasites and cause significanteconomic losses worldwide. Traditional approaches for managing plantvirus diseases include avoiding virus-infected material, chemicalcontrol ofarthropod vectors and, when available, use of virus-resistance in cultivatedcrops. However, all of these are labor intensive, and chemical control of insectvectors is becoming more expensive with potential undesirable side effects, including environmental hazards and the generation of insecticide resistance invectorpopulations and those of other insect pests.The inoculation of mild virus strains on plants showed protection of the plants against more severe strains, which showed a immune response like process in the plants. This became the basis of developing strategies for virus resistance by transgenic approaches.

There are mainly two approaches for developing genetically engineered resistance depending on the source of the genes used. The genes can be either from the pathogenicvirus itself or from any other source. The former approach is based on the concept of pathogen-derived resistance (PDR). For PDR, a part, or a complete viral gene is introduced into the plant, which, subsequently, interferes with one or more essential steps in the life cycle of the virus. This was first illustrated in tobacco by the group of Roger Beachy, who introduced the coat protein (CP) of tobacco mosaic virus (TMV) into tobaccoand observed TMV resistance in the transgenic plants.The concept of PDR has generated lot of interest andtoday there are several host-virus systems in which it hasbeen fully established. Nonpathogen-derived resistance,

on the other hand, is based on utilizing host resistancegenes and other genes responsible for adaptive host processes,elicited in response to pathogen attack, to obtaintransgenics resistant to the virus. The use of non-PDRtype of resistance, even though reported much less in theliterature incomparison to PDR-basedapproaches, holdsa better promise to achieve durable resistance.

In a number of crops, transgenics resistant to an infectivevirus have been developed by introducing a sequence ofthe viral genome in the target crop by genetic transformation.
Virus-resistant transgenics have been developedin many crops by introducing either viral CP orreplicase gene encoding sequences.Expression of the coat-protein of tobacco mosaic virus conferred resistance to TMV in transgenic tobacco. Geminiviral Rep proteins have been widely exploited to generate resistance. The Rep gene of African cassava mosaic virus (ACMV) inhibited virus replication in protoplasts and induced virus resistance in plants. A protein-mediated resistance was described with a truncated Tomato yellow leaf curl Sardinia virus (TYLCSV) Rep protein (210 amino acids), that strongly inhibited virus replication in protoplasts and induced resistance when expressed at high levels. Movement proteins (MPs) are encoded by plant viruses and enable infections to spread between adjacent cells (local spread) as well as systemically. Intercellular spread involves plasmodesmata, the channels that traverse plant cell walls and provide symplastic continuity between cells and tissues. Virus resistance in plants can be engineered by expression of a dysfunctional movement protein. Resistance conferred by transgenic expression of a dysfunctional MP is likely due to competition for plasmodesmatal binding sites between the mutant MP and the wild- type MP of the inoculated virus.

Pathogen derived resistance

Coat-protein mediated resistance (CPMR)

The use of viral CP as a transgene for producing virus resistant plants is one of the most spectacular successesachieved in plant biotechnology.CPMR refers to the resistance to virus infection caused

by expression of a coat protein (CP) gene in transgenic plants. The expression of a CP gene confers resistance to the virus from which the CP gene was derived. Resistance is associated to the expression of the CP gene, and is stably inherited to subsequent generations.

The CP strategy is the expression by the plant of the viral CP gene integrated into the plant genome. Construction of the chimaeric gene should include the selection of anappropriatetranscriptional promoter to cause the expression of the CP gene at sufficient levels to produce disease resistance. Several different transcriptional promoters have been used and the promoter that has proven most effective is the CaMV 35s promoter leads to high levels of mRNA and protein in most of the plants in which been tested.

Numerous crops have been transformed to express viral CP and have beenreported to show high levels of resistance in comparison to untransformed plants. CP- expressing plants indicate that resistance is primarily to virus particles, because of a likely interference in the initial events during the infection process. CP mediated resistance to TMV requires that the CP produced from thetransgeneis capable of subunit-subunit interactions but not necessarily capable of forming virus particles. CP apparently interferes with the disassembly of TMV, thereby preventing infection; furthermore, there is a direct correlation between the amount of CP and the level of resistance.

Replicase (Rep) mediated resistance:

Replicase (Rep) protein- mediated resistance against a virus in transgenic plants was first shown in tobacco against TMV in plants containing the 54 kDa putative Rep gene. Similar resistances have been developed for several other viruses namely pea early browning virus PVY22 and CMV23. Gene constructs of Rep genes that have been used forresistance include full-length, truncated or mutated genes. The resistance responses have been shown not to require protein synthesis and to be mediated at the RNA level. This type ofresistance remains confined only to a narrow spectrum ofviruses, the spectrum being narrower than that of CPMR.To make the resistance broad-based, it may be necessaryto pyramid such genes from several dissimilar virussourcesinto the test plant genome.

Movement protein

Movement proteins (MP) are essential for cell-to- cellmovement of plant viruses. These proteins have beenshown to modify the gating function of plasmodesmata, there by allowing the virus particles or their nudeoprotein derivatives to spread to adjacent cells. This phenomenonwas first used to engineer resistance against TMV intobacco by producing modified MP which are partiallyactive as a transgene. The conferred resistance is believed to be based on the competition between wild type virus encoded MP and the preformed dysfunctional MP to bind to theplasmodesmatal sites. The MP mediated is effective against distantly related orunrelated viruses, forexample resistance against TMVcould be achieved in tobacco using the MP derived frombrome mosaic virus, suggesting functionalconservation of this protein among several viruses.

Defective-interferingviral nucleic acids

In several viruses, truncated genomic components are often detectable in infected tissues, which interfere withthe replication of the genomic components. These speciesof DNA are also called defective interfering (DI) DNAand expression ofdelayed disease symptoms and recovery,coupled with increased resistance upon repeatedinoculation have been observed in plants engineered withDI DNA. For example, incorporation of subgenomicDNA B that interferes with thereplication of full length genomsc DNA A and B confers resistance to ACMV inN. benthamiana.

NON-PATHOGEN DERIVED RESISTANCE A .Post-transcriptional gene silencingPost-transcriptional silencing (PTGS) is a specificRNA degradation mechanism of any organism that takescare of aberrant, unwanted excess or foreign RNA intracellularlyin a homology-dependent manner. It is prevalentin various forms of life, namely plant, fungus and invertebrate animals. This activity could be present constitutivelyto help normal development or induced inresponse to cellular defense against pathogens.Inthismechanism, the elicitor double-stranded RNA (ds RNA),commonly produced during viral infection, is degraded to21-25nucleotides, termed as small interfering

RNA(siRNA), with the help of a variety of factors that havealready been or are being identified. This degradation process, initiating from aconcernedcell having the elicitor RNA, spreads later withinthe entire organism in a systemic fashion. This process isgenerally regarded to have evolved as a plantdefensemechanism against invading viruses containing eitherRNA or DNA genomes.

When the trigger for this silencing mechanism is delivered into the plant in the form of double-stranded RNA constructs, with sequences homologous to an invading virus, the silencing mechanism is initiated, which inhibits the viral replication inside the cell.
b. Plant diseaseresistance genes

A number of disease resistance genes (R) have been reported against viruses of crop plants. (They encode products which respond to viral signals (avirulence (avr) gene products) culminating in a number of resistance responses in the plant. Many of the corresponding viral avr genes have also been identified. Some of the R genes have been shown to complement the disease susceptibility phenotype in the correspondingcultivars when used as transgenes, furnishinga direct proof of their action. R genes inplants are defined by the classical geneforgenehypothesis, which states that for every incompatiblehost pathogen interaction, there exist matching Rgenes in the host and avr genes in the pathogen. Resistance reaction against pathogen results generally bydirect interaction between the products of R and avrgenes.

This interaction, in many cases, results in a resistancereaction, known as hypersensitive reaction (HR),which can be defined asa specific response of a hosttowards a pathogen. HR results in localized cell death,appearing as necrotic lesions at the site of pathogenentry. HR results in the arrest of pathogen spread, the re byeffective ly restricting it to the dead cells.All known R genes encode products having two basicfunctions: to act as sensors for the corresponding avr factors/ elicitors and to initiate signalling cascades for theexpression of defence-related genes. A number of conserved across several R geneproducts. These include leucine-rich repeat (LRR), nudeotide-binding site (NBS),

serine-threonine kinase, leucine zipper, toll- interleukin region (TIR), etc. Thesestructural features are believed to have important roles toplay in the execution of the above functions.

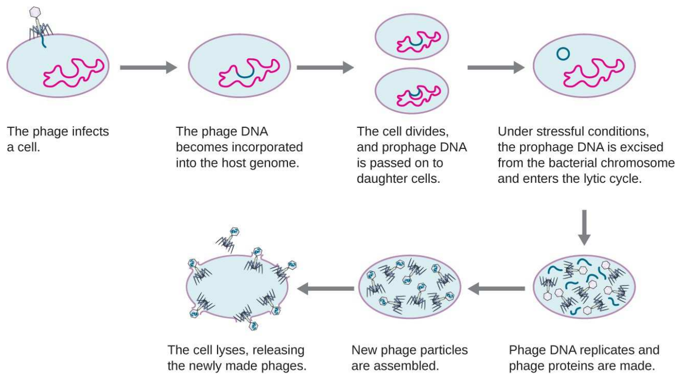


Fig. 4. Schematic representation of viral life

MOLEUCLAR FARMING

Molecular farming is the production of recombinant pharmaceuticals outside their natural source. The expression of recombinant insulin in bacteria is a classical example for molecular farming. Production of pharmaceutically important ancommercially valuable proteins in plants is molecular farming. The use of plants for medicinal purposes dates back thousands of years but genetic engineering of plants to produce desired biopharmaceuticals is much more recent. As the demand for biopharmaceuticals is expected to increase, it would be wise to ensure that they will be available in significantly larger amounts, on a cost-effective basis.

Currently, the cost of biopharmaceuticals limits their availability. Plant-derived biopharmaceuticals are cheap to produce and store, easy to scale up for mass production, and safer than those derived from animals. It is a safe and inexpensive means for the mass production of recombinant pharmaceutical proteins. Large scale of proteins can be expressed in plants because the expressed proteins are functional and almost indistinguishable from their original counterparts. Therapeutic proteins produced by plants include interleukins, recombinant

antibodies, plasma proteins, cytokines and growth factors. Molecular farming in plants has the potential to provide virtually unlimited quantities of recombinant proteins for use as diagnostic and therapeutictools in health care and the life sciences.

The potential of using plants as a production system for recombinant pharmaceuticals wasestablishedbetween 1986 and 1990 with the successful expression of a human growth hormone fusion protein, an interferonand human serum albumin. A crucial advance came with the successful expression of functional antibodies in plants in 1989 and 1990. This was a significant breakthrough for it showed that plants had the potential to produce complex mammalian proteins of medical importance. By analogy to the production of insulin in bacteria, the production of antibodies in plants had the potential to make largeamounts of safe, inexpensive antibodies available. transgenic tobacco, plants have proven to be versatile production systems for many forms of antibodies. These include full-sized IgG and IgA, chimeric IgG and IgA, secretory IgG and IgA, single-chain Fv fragments (scFv), Fab fragments and heavy-chain variable domains.

To date, only four antibodies have been made in plants that are potentially useful as human therapeutics. Only one of these has been tested in humans: a chimeric secretory IgG-IgA antibody against a surface antigen of Streptococcus mutans, the primary causal agent of tooth decay. This tobacco produced antibody was applied topically toteeth. The second antibody, a humanized anti-herpes-simplex virus (HSV) antibody made in soybean, was effective in the prevention of vaginal HSV-2 transmission in a mouse mode. A third antibody, against carcinoembryonic antigen (CEA), has recently been expressed in rice and wheat. CEA, a cell-surface glycoprotein, is one of the best-characterized tumor-associated antigens.
Antibodies against CEA are used for in vivo tumor imaging, as well as in antibody-based cancer therapy. This same antibody has been expressed in a rice cell culture. The fourth antibody is an example of both a novel use of plant-produced antibodies and an alternative production system. A plant virus vector has beenused to produce a tumor-specific vaccine

transientlyin tobacco for the treatment of lymphoma. There are no plantibodies yet in commercialproduction.

EDIBLE VACCINES

Oral delivery of vaccines is an attractive alternative to injection, largely for reasons of low costand easy administration. The chances of acquiringmucosal immunity against infectious agents thatenter the body across a mucosal surface are alsoincreased with oral vaccines.
However, a majorconcern with oral vaccines is the degradation ofprotein components in the stomach and gut beforethey can elicit an immune response. Plant tissues can be used to deliver edible vaccines without degradation.

Plant-derivedbiopharmaceuticals andhuman proteins

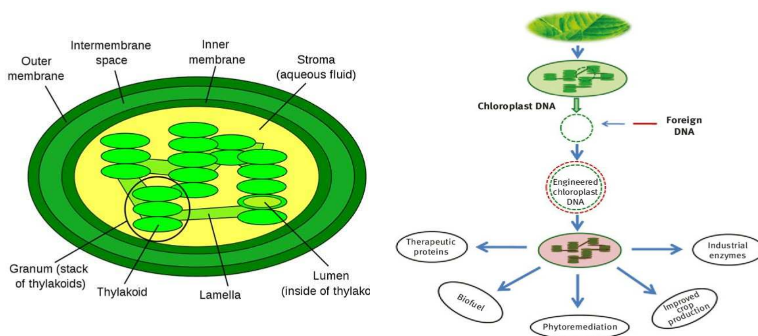
Generally, levels of pharmaceutical proteins produced in transgenic plants have been less than the 1% of total soluble protein that is needed for commercial feasibility if the protein must be purified34. Plant derived recombinant hepatitis-B surface antigen induced only a low level serum antibody response in a small human study, probably reflecting the low level of expression (1-5 ng g-1 fresh weight) in transgenic lettuce. In spite of recent improvements in expression levels in potato with a view to clinical trials31, expression levels should be increased further for practical purposes. Also, even though Norwalk virus capsid protein expressed in potatoes caused oral immunization when consumed as food, expressionlevels are too low for large-scale oral administration (0.37% of total soluble protein). Expression of genes encoding other.

Chloroplast engineering

Plant cells have three genomes: chloroplast, mitochondrial and nuclear genome. The plastid genome of photosynthetically active seed plants is a small circularly mapping genome of 120-220 kb, encoding 120-130 genes. It can be engineered by genetictransformation. Chloroplasttransformation is an environmentally friendly approach to plant genetic engineering that minimizes outcrossing of transgenes to related weeds or crops and reduces the potential toxicity of transgenic

pollen to non-target insects. Number of plastid genome is very high per cell, transformation of chloroplasts permits the introduction of thousands of copies of foreign genes per plant cell, and generates extraordinarily high levels of foreign protein. Unlike nuclear transformation, where the foreign DNA is introduced randomly by illegitimate integration, chloroplast engineering can be accomplished by homologousrecombination.
Chloroplasttransformation vectors use two targeting sequences that flank the foreign genes and insert them, through homologous recombination, at a precise, predetermined location in the organelle genome.Chloroplast engineering encompasses the following steps (Fig. 5)

Construction of a chloroplast transformation vector with the gene of interest flanked by regulatory sequences at the 5' and the 3' termini.



Placing homologous sequences flanking the Gene of Interest tofecilitate homologousrecombination.

Gene delivery by particlebombardment

Antibiotic selection with spectinomycin or streptomycin

Selecting the homoplastic transformants

Sexual reproduction of the transplastomic plants to get To plants with maternal inheritance of the transgenic trait, which does not segregate

`Right from the first chloroplast engineering of the seed plant tobacco two decades before, a number of important traits have been engineered by chloroplastengineering in plants. The crops and the traits

Mitochondrial transformation

Mitrochondrial transformations have been successfully done in yeast and C. reinhardtii. Transformation of plant mitochondria is easier when compared to animal systems. The advantage offered by plant mitochondrial systems are as follows.

Maternal inheritance
No pleiotropic effects
Absence of gene silencing
Multigene engineering
No position effect

No degradation of transgene RNA at post transcriptional levelTransformation of mitochondrial DNA can be achieved by protoplast fusion, Agrobacterium-mediated transformation, particlebombardment and microinjection. Transgenic plants pose the threat of gene transfer to related species through pollen. Mitochondrial transformation is an alternate method for developingenvironmentally safe transgenic plants. Mitochondria has a unique transcription and translation system and due to its maternal inheritance, shows better transgene containment with high levels of expression. Many of the economically important traits like yield, disease resistance, male sterility, heterosis, temperature and drought tolerance are controlled by interaction between the organellar and nuclear genomes. In order to understand the control of these traits approaches involving genetic transformation of the organellar genomes become important.

References:

A. Slater, N. Scott, and M. Fowler. 2004. Plant biotechnology—The genetic manipulation of plants. Oxford University Press, Oxford, pp. 346.

Alternatives and biosafety. J. Biotechnol. 107:193-232.

Birch.R. G.1997. Plant transformation: problems and strategies for practical application. Annual Review of Plant Physiology and PlantMolecular Bio logy. 48:297-326.

C. Neal Stewart Jr. 2007. Plant Biotechnology and Genetics: Principles, Techniques, and Applications. John Wiley & Sons, Inc.

Compton, M. E. , J. A. Saunders and R. E. Veilleux. 1996. Use of protoplast for plant -mprovement. Plant tissue culture concepts and laboratory practices.Ed.R. N. Trigiano and D. J. Gray.CRC Press.

Davey, M. R., P. Anthony, J. B. Power, and K. C. Lowe.2005. Plant protoplasts: status andbiotechnological perspectives.Biotechnology Advances. 23: 131-171.

Gamburg OL and Philips GC. 1995. Plant Cell, Tissue and Organ Culture fundamental Methods. Springer.

Gaspar, T., C. Kevers, C. Penel, H. Greppin, D.M. Reid, and T. A. Thorpe. 1996. LPlant hormones and plant growth regulators in plant tissue culture. Invitro Cellular and Developmental Biology. 32: 272-289.

Hans-Walter Heldt, Birgit Piechulla, 2010. Plant Biochemistry. Academic Press, Elsevier, pp.656.

Herrera-Estrella L. 1983. Transfer and expression of foreign genes in plants.PhD thesis.Laboratory of Genetics, Gent University, Belgium.

K. N. Kao, F. Constabel, M. R. Michayluk and O. L. Gamborg.1974. Plant Protoplast Fusion and Growth of Intergeneric Hybrid Cells.Planta. 120: 215-227.

Klein, T. M., E.D. Wolf, R. Wu, and J. C. Sanford. 1987. High-velocity microprojectiles for delivering nucleic acids into living cells. Nature.327: 70-73.

Lee, L-Y., and S. B. Gelvin. 2008. T-DNA Binary Vectors and Systems. Plant Physiology. 146: 325-332.

Me Cullen, C. A., A. N. Birins. 2006. Agrobacterium tumefaciens and Plant Cell Interactions and activities required for interkingdommacromolecular transfer. Annual Review of Cell and Developmental Biology.22:101-27

Miki, B., and S. McHugh. 2004. Selectable marker genes in transgenic plants: applications,

Murashige, T., and F. Skoog. 1962. Revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant 15:473-497.

Newell. C. A. 2000. Plant transformation Technology:Applications and Development. Molecular Biotechnology. 16: 53-63.

R. Keshavachandran, K.V. Peter. 2008. Plant Biotechnology: Methods in Tissue Culture and Gene Transfer. Orient Blackswan, pp. 312.

S. Carlson, H. H. Smith and R. D. Dearing. 1972. "Parasexual Interspecific Plant Hybridization," Proceedings of the National Academy of Sciences USA. 69: 2292-2294.

Scholthof, H. B. and K-B. G. Scholthof. 1996. Plant virus gene vectors for transient expression of foreign proteins in plants. Annual Review of Phytopathology. 34: 299-323

Seelye, J. F., G. K. Burge, and Ed R. Morgan.2003.Acclimatizing Tissue Culture Plants: Reducing the Shock. Combined Proceedings International Plant Propagators' Society, 86 :85-90.

Shankar, L. P., E. Tom, D. Dieter, Van B. Erik, and Van H. Johan.2013. Asymmetric Somatic Plant Hybridization: Status andApplications. American Journal of Plant Sciences.4: 1-10.

Skoog F, C. O.Miller 1957.chemical regulation of growth and organ formation in plant tissues cultured in vitro. Symp. Soc. Exp. Biol.11:118-131.

Tilton V. R. and S. H. Russell.1984.Applications of in vitro Pollination/F-'erti lization Technology.BioScience. 34: 239-242.

Vasil.I.K. 2008.A history of plant biotechnology: from the Cell Theory of Schleiden and Schwann to biotech crops. Plant CellReports 27:1423-1440

Vasil.I.K.2008.A short history of plant biotechnology. Phytochemistry Reviews. 7:387-394

White, P. R. 1939. Potentially unlimited growth of excised plant callus in an artificial nutrient.American Journal of Botany. 26:59-64