Lesson 3.2.1

TOTIPOTENCY CLONAL PROPAGATION

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Objective

In this chapter the concept of totipotency, expression of totipotency and its role in plant tissue culture are discussed.

3.2.1.1. Totipotency

1. Introduction

The term totipotency is coined by Morgan in 1901. The concept of totipotency is implicit in the statement of cell theory of Scheleiden & Schwan that each living cell of a multicellular organism would be capable of developing independently if provided with proper external conditions.

Totipotency is the genetic potential of a plant cell to produce the environment. So, totipotency is the cell characterstic in which the potential for forming all the cell types in the adult organism is retained.

The higher plant body is a complex, multicellular organization which is made up of a variety of tissue and cell types. All these cells have a common origin i.e. from the single cell of a newly formed zygote. The zygote divides repeatedly and produces the embryo with in the seed. Ultimately the embryo produces the entire plant during seed germination. Thus it is clear that the original single cell (zygote) conatins all the information that is required for the production of a complete plant. This information is stored in the chromosomal DNA of the nucleus. Exact copies of this information are duplicated during mitosis and then distributed in the daughter cells in each cell cycle. All the vegetative cells of the adult plant are derived by mitosis from the early embryo. So each of these cells must contain all the genetic information that is needed for the growth and development of an entire plant. This inherent potential of a cell is known as totipotency.

2. Expression of totipotency in culture

In tissue-culture stem, root or other plant parts are allowed to grow in culture medium containing mineral nutrients vitamins and hormones under sterile controlled conditions. The cells in culture will produce an unorganized proliferative mass of cells; which is known as callus tissue. The cells of this callus mass are totipotent. Thus a callus tissue can be able to regenerate back the normal plant, provided certain manipulations of the medium and the cultural environment. Thus the totipotency of the cell is manifested through the process of differentiation and the hormones play a main role in

this process. In 1957 F. Skoog and C.O. Miller proposed a hypothesis that organogenesis is controlled by a balance between cytokinin and auxin.

Skoog was able to demonstrate the role of kinetin in organogenesis in tobacco cultures. He found that when the ratio of kinetin to auxin was higher, only shoot developed. This is known as caulogenesis. When this ratio was lower only roots were formed. This is known as rhizogenesis. Later F.C. Steward etal, devised a method for growing carrot tissue, from the secondary phloem region of carrot root and culturing them in a moving liquid medium under aseptic conditions. The phloem tissue began to grow actively, in presence of coconut milk in the culture medium.

In moving liquid medium some single cells and small cellular aggregates were loosened from the surface of the growing tissue. When these isolated cells were grown separately it was found that some single cells developed somatic embryos (or) embryoids by a process that occurs in normal zygotic embryo. It is also observed in the same experiment that cells of some callus mass frequently differentiate into vascular elements like xylem and phloem without forming any plant organ (or) embryoids. This process is known as cytodifferentiation (or) histogenesis. Thus the totipotent cells may express themselves in different ways basing on the process of differntiation (i.e. may be embryo genesis, (or) organogesis (or) histogenesis).

Sometimes the totipotency of the cells is partially expressed (or) not expressed. This limitation on the capacity of a cell for development must have been imposed by the micro environment.

- In *in vitro* cultures the explant first forms the callus tissue in the callus inducing medium. This callus is maintained through some sub cultures. Then it is transferred to another regeneration medium which is favorable for the expression of totipotent cells on the regeneration medium, the totipotent cells of the callus tissue under go repeated cell divisions give rise to meristematic nodules (or) meristemoids. This may subsequently give rise to vascular differentiation (or) it may form a primordium which will give rise to a shoot (or) root. Some times the totipotent cells may produce embryoids through sequential stages of development. Such as globular stage, heart shaped stage and torpedo stage etc. It has been observed that the callus in some species can be able to grow on the standard maintenance medium which is devoid of growth hormones. Eg: *Nicotiana tabacum*. The cells of this habituated callus also remain

totipotent and are capable to regenerate a plant without any major manipulation.

The mode of expression of totipotency of plant cell in culture varies from plant to plant and also helps us to understand the process of differentiation. For example, a crown gall tumour cell has the capacity for unlimited growth independent of exogenous hormones. Thus they are totally lacking the organogenetic potential. So, such tissue have permanently lost the totipotentiality of the parent cell. In some plant species the crown gall bacterium induces a special type of tumour called teratomas. The cells of this tumour possess the capacity to differentiate shoot buds and leaves, when cultured for unlimited periods.

A callus mass in culture contains thousands of cells. But all the cells are not totipotent. If all the cells are totipotent and it is expressed at a time then equal no. of shoots (or) roots (or) embryoids will be regenerated from such totipotent cells. In the expts such results are not obtained. The reasons for the limited expression of totipotency vary from plant to plant. It has been observed that variation of chromosome number in the cells of callus tissue is one of the main factors for the limited expression of totipotency. This variation may be due to pre-existing variation in the somatic cells of the explant (i.e genetic) (or) it may be generated during tissue culture (i.e. epigenetic) changes in the chromosomal number will result in the formation of mixoploid callus tissue. But, within the mixoploid callus, organogenesis and embryogenesis mostly occurs in diploid cells. Therefore all the cells of the callus tissue are not able to express their totipotency. From the experimental observations it was proposed that an association of cells may be necessary to provide an appropriate environment for certain individuals to express their totipotency. It is also proposed that endogenous hormone level of a cell and exogenously supplied hormone makes a threshold level which will induce the totipotent cell to express in culture. The availability of hormones is not equal to all cells. There exists a gradient with regard to the hormones and nutrients within the callus tissue. This factor imposes a barrier to reach a threshold level of hormone equally in all cells for their expression of totipotency.

In culture, some cells are highly recalcitrant and in such cases totipotent cells don't respond to any morphogenetic stimuli. These cells cannot be easily differentiated. The other factors, like heterogeneity in the physical structure of the cells make a great difference in the degree of chemo differentiation of the cells will lead to an asynchronous situation due to which all cells are not able to

express their totipotency at a time. So, it seems apparently that all cells are not totipotent. Epidermal tissue and free uninucleate pollengrains, free cells from suspension cultures and protoplasts are more efficient systems for the synchronous expression of totipotency.

3. Importance of totipotency in plant science

The main objective in plant protoplast, cell and tissue culture is the reconstruction of plants. The totipotency of somatic cells has been exploited in the vegetative propagation of many economical, medicinal and agriculturally important plant species. So from fundamental to applied aspects of plant biology, cellular totipotency is highly important.

The success of the recent trends of plant tissue culture like genetic modification of plants, production of homozygous plants through haploid cell culture, somatic hybridization and mutation depends upon the expression of totipotency. Plant breeders, horticulturists, and commercial plant grociers are interested in plant tissue culture for the exploitation of totipotent cells in culture according to their desire. Totipotent cells within a bit of callus tissue can be stored in liquid nitrogen for a long period. Thus in the preservation of germplasm of endangered plant species, totipotency can be utilized. Thus, the success of different aims and objectives of plant tissue culture depends upon the expression of totipotency.

3.2.1.2. Clonal propagation

1. Introduction

Multiplication of genetically identical copies of a cultivar by asexual reproduction is called clonal propagation.

In nature plants are propagated by both sexual and asexual methods. Sexually propagated plants show a high amount of heterogeneity. Asexually propagated plants are genetically identical to the parent plant and thus permits the expression of the unique characters of the cultivars. Multiplication of genetically identical copies of a cultivar by asexual reproduction is called clonal propagation. A population derived from a single individual by asexual reproduction constitutes a clone. The most widely used *in vivo* methods of cloning, for agricultural crops include cutting of vegetative parts, layering, grafting and budding. These methods have been successfully applied for potato, apple, pear, ornamental bulbs, tuberous plants etc. But, this *in vivo* clonal propagation of plants is

often difficult, expensive and even unsuccessful in some plant sps. Clones are also generated through a process called apomixis (seed development without meiosis (or) fertilization). But this apomixes is restricted to only a few species. So, the horticulturists have adopted the methods of asexual or vegetative reproduction for clonally multiplying the selected cultivars. The advantages of vegetative reproduction over sexual reproduction are (1) Faster multiplication in contrast to seed propagation in plants with a long life-cycle. (2) There will not be the juvinile phase which is associated with seed-raised plants if we propagate them vegetatively directly from the adult material (3) Gene banks can be established by multiplying variants among clonally propagated plants.

Tissue culture methods offer an alternative means of plant vegetative propagation. Clonal propagation through tissue culture i.e. micropropagation can be achieved in a short time and space. Thus, it is possible to produce plants in large numbers from a single individual. Use of tissue culture for micropropagation was initiated by G. Morel in 1960. He considered this as the only commercially viable approach for orchid propagation. Since then several crop species have been micro propagated. Single cell and protoplast culture techniques also enable thousands of plants to be derived with in a short space and time. But, in practice, isolated cells / protoplasts, callus and cell suspension cultures may consist of abnormal and deviant idiotypes. So, their regenerate may generate novel types of genetic and epigenetic variations which are collectively called somaclonal variations. So, the most widely used methods of clonal propagation is based on the proliferation and induction of auxiliary (or) apical shoot meristems *invitro*. The major benefits of micropropagation are, (1) rapid multiplication of superior clones and maintenance of genetic uniformity. (2) Multiplication of disease free plants. (3) Multiplication of sexually derived sterile hybrids.

2. Technique of micropropagation

Invitro clonal propagation is a complicated process in which many stages are involved. There are 5 different stages that can be adopted for over all production technology of clones commercially. Stages I-III are followed under *invitro* conditions, and the state IV is accomplished in a green house environment. The adoption of all these stages simplifies the daily operation, accounting and product cost and it also allows greater ease in communication with other laboratories. Thus, a particular plant can be marketed (or) requested by specifying its stage.

Stage 0

This is an initial step of micropropagation in which stock plants used for culture initiation are grown for at least 3 months under carefully monitored conditions. Stock plants are grown at a relatively low humidity and watered either with irrigation tubes or by capillary sand beds (or) mats. During this stock plant preconditioning stage measures are also taken for the reduction of surface and systemic microbial contaminants.

Stage-I

Murashige defined this stage as the stage of initiation and establishment of aseptic cultures. The main steps involved are preparation of the explant followed by its establishment on a suitable culture medium. Cultures are initiated from explants of several organs but shoot tips and axillary buds are most often used for commercial micropropagation. Procedures to surface sterilize the explant and induce healthy growth in the culture medium for each species may be devised. It is also advisable to control microbial contamination with in the explant tissues if such efforts at stage-0 were not successful. Stage-I lasts 3 months to 2 years and it requires at least four passages of the subculture. Usually explants carrying a preformed vegetative bud are suitable for enhanced axillary branching. When the objective is to produce virus free plants from an infected individual, it becomes obligatory to use cultures derived from small shoot tips. If stock plants are tested virus free, the most suitable explants are nodal cutting. There are some disadvantages in using small sized explants for micropropagation because they have alow survival rate and show slow initial growth. Meristem tip cultures also result in the loss of certain horticultural traits exhibited by the presence of virus.

Eg: Clear vein character of Geranium CV. Crocodile.

Sub terminal (or) slightly older segments are desirable which can withstand the toxic effects of sterilization agents better than the terminal cutting. In some or ornamental plants terminal buds are used to initiate cultures since lateral buds fail to produce reculturable shoots. For rhizomatic plants runner tips are commonly used.

Stage-II

In this stage the micropropagation activity takes place using a defined culture medium that stimulates maximum proliferation of

regenerated shoots. Various approaches are followed for micropropagation. They are,

1. Multiplication through the growth and proliferation of meristems excised from apical and axillary shoots of the parent plant.

2. Induction and multiplication of adventitious meristems through process of organogenesis (or) somatic embryogenesis directly on explants.

3. Multiplication of callii derived from organs, tissues, cells (or) protoplast and their subsequent expression of either organogenesis (or) somatic embryogenesis in serial sub cultures. Shoots obtained from these calli can be further multiplied.

A passage or harvest cycle generally requires 4 weeks., shoots are harvested from the multiplying culture, which can be sold as stage – II product (or) carried on to stage III. Generally stage-II lasts 10-36 months with a large number of subcultures of similar age.

Stage-III

The shoots proliferated during state II are transferred to a rooting (storage) medium. Sometimes shoots are directly established in soil as microcuttings to develop roots. That possibility depends upon the particular species and at present, large no. of species cannot be handled in this way. So the shoots are generally rooted *invitro*. When the shoots are plantlets are prepared for soil, it may be necessary to evaluate several factors such as

1. Dividing the shoots and rooting them individually,

2. Hardening the shoots to increase their resistance to moisture stress and diseases,

3. Rendering plants capable of autotrophic development in contrast to the heterotrophic state induced by the culture and

4. Fulfilling the requirements of breaking dormancy, especially in bulb crops.

Stage III requires 1 – 6 weeks.

Stage-IV

Steps taken to ensure successful transfer of the plant lets of stage III from the aseptic environment of the laboratory to the environment of the green house comprise stage IV. Unrooted shoots of stage II are also acclimatized in suitable compost mixture (or) soil in pots under controlled condition of light, temperature and humidity in the greenhouse. In such cases stage III is skipped. Supplying the bottom heat acids to pots with plantlets or cuttings and maintenance of dense fine – particle fog system with in green house enhances the rooting process. Complete plant lets can also be established in artificial growing media such as soil less mixes, rock wood plugs or even sponges. It takes 4-16 weeks for the finished product to be ready for sale.

3. Different methods of clonal propagation

Clonal (or) vegetative propagation is achieved by the following processes, which are employed for the micropropagation of plant species: 1. Multiplication by axillary buds (2) Multiplication by adventitious shoots (3) Multiplication through callus culture.

1. Multiplication by axillary shoots

Axillary and apical shoots contain active meristems depending on the physiological state of the plant. Vascular plants which show indeterminate growth have subsidiary meristems in their leaf axils, which have the potential for growing into a shoot. But, only a limitea no. of axillary meristems have the capacity to develop invivo if the type of branching of a particular species displays apical dominance. Since the mechanism of apical dominance has been demonstrated to be under the control of various growth regulators, the proportion of these substance in the media can be manipulated in such away as to induce each meristem to regenerate a shoot in cultures. Shoot tips are cultured on a basal medium containing no growth regulators typically develop in to single seedling like shoots with strong apical dominance. When the shoots of the same explant material are grown on culture media containing cytokinin, axillary shoots develop precociously which proliferate to form clusters of secondary and tertiary shoots. These dusters can be further subdivided in to smaller clumps of shoots (or) separate shoots which, in turn will form similar clusters when subcultured on a fresh medium. This process continues indefinitely provided the basic nutrient formulations are adequate for normal growth. About 5-10 multiplication rates can be achieved on a regular 4-8 week micropropagation cycle which will lead to rapid

clonal propagation levels in the range of 0.1-3.0 x 10^6 with in one year.

In general the technique of proliferation by axillary shoots is applicable to any plant that produces regular axillary shoots and responds to cytokinins such as BAP, 2 ip and zeatin. Many forest and orchid tree species are suitable for invitro clonal propagation using axillary shoots.

Apical shoots of 1-5 mm are normally cultured on media containing mixture of auxin (.01-0.1 mg/lit) and cytokinin (.05-.5 mg/lit). The level of cytokinin is raised in subsequent subcultures to induce an acceptable rate of proliferation with out yellowing (or) distortion of shoots. If the presence of cytokinin in the medium inhibits root development, the cultured material is transferred to a rooting medium which contains no cytokinin (or) reduced levels of cytokinin. (Proliferation of axillary buds).

Fig-1

2. Multiplication by adventitious shoots

(Adventitious buds, bulbs, protocorms) Adventitious shoots are the stem and leaf structures that arise naturally on plant tissues located in extra axillary positions. The structures include stems, bulbs, corms, tubers, and rhizomes of these organs can be used as a cuttings in conventional propagation. Eg:- leave of Begonia and some ornamental plants produce shoots on a large scale. A similar type of adventitious shoot development can be induced in cultures by using a suitable explant from preconditioned plant material and appropriate levels of growth regulators in the medium. Bulbs and corms grow from meristems at the base of leaves and scales. These meristematic regions regenerate multiple shoots on a suitable culture medium. Levels of fidelity in propagated material of these speedes are quite high because the adventitious shoots arise only from single epidermal cell. Eg:- Adventitious shoots from stems of Tulipa. Compared to conventional propagation techniques the invitro micro propagation methods can produce 5-10 times more per annum. Continuous propagation by adventitious shoot proliferation from bulbs and corms can be achieved by cultivating two vertically split pieces of shoot bases. Clusters of shoots develop from around the abaxial surfaces of developing leaves and scales. Senescence and dormancy in such cultured materials can be prevented invitro by trimming of shoots within 2-3 nm of the basal plate. This approach has been found to ensure continuously productive cultures of Iris, Lilium and Tulipa

hybrids for indefinite periods. For bulbs with a strong apical dominance, it is necessary to destroy the main apex. This can be achieved by making two shallow vertical cuts at right angles to each other up to the level of the basal plate.

Clonal propagation by adventitious embryo formation is another useful approach followed or many plant species. Adventitious embryos can arise directly from a group of cells with in the original explants (or) from primary embryoids. Orchids produce a large no. of embryoids at the tip of leaves *in vivo.* The cultivars of citrus and mangifera shows polyembryony, where the embryos are formed from nuclear tissue. Adventitious embryoids and diploid embryos can be used as clonal material. Adventitious embryos obtained invitro by inducing embryogenesis on explants are also good materials for clonal propagation. Eg:- Leaf pieces of coffee trees from embryos directly when cultured on a based MS medium containing high levels of cytokinins.

3. Multiplication through callus culture: (Somatic embryogenesis)

Differentiation of plants from cultured cells via shoot-root formation (or) somatic embryogenesis, will be the fastest method of shoot multiplication and cloning of plant species. The cultures in which the celli are formed are having low preference as a means of micropropagation. The most serious draw back in the use of callus cultures for shoot multiplication is the genetic instability of their cells, due to which the initial plant regeneration capacity of the tissue may decline with the passage of time. But in case of some economically important crop species of cereals, forage, legumes, citrus, coffee, forest and tropical palm trees invitro propagation via organogenic (or) embryogenic calli is unavoidable. Plant regeneration from protoplasts also requires passage through at least one callus stage. The production of many thousands of plant lets from calli either derived from cell suspensions (or) isolated protoplasts constitutes unique cases of cloning viz. calli cones and protoclones. Such clones commonly exhibit somaclonal variations.

Genetically stable calli have also been derived from explants of various sps of Lilium, chrysanthemum and tomato. In these types of calli, slow-growing meristematic cells from which shoots and embryoids arise are derived from the peripheral layer of highly vacuolated inner cells. These meristematic layers comprise diploid cells expressing totipotency while the inner layers made up of mixoploid cells do not. These types of calli can be subdivided by random dissection or by placing in a homogenizer to produce many

thousands of propagules in a single operation. Each propagule may be used in mass propagation of multiple shoots.

4. Factors Affecting Shoot Multiplication

Clonal propagation of plants invitro on a large scale requires a quantitative approach. It is necessary to ascertain the number of propagaules which can be regenerated in a given amount of culture material over a given period of time (or) a single culture generation. Morphogenesis and proliferation rate of culture depend on the various factors influencing the relative incidence of organogenesis. This necessitates optmisation of conditions associated with invitro vegetative plant propagation.

1. Physiological Status of the Plant Material

Explants isolated from the more recently produced parts of a plant are more regenerative than those from older regions. The regenerative potential of tissue culture diminishes with each year of maturaton, particularly in perennial woody species. Generally, somatic embryogenesis is associated with cultures established from embryo explants than from the mature (or) non-embryonic tissues. Reports about season-linked regenerative capacity of tissue cultures have also appeared in the literature. Papaya tissue cultures can be established in hot summer months, where as flower-stem expants of Tulipa give rise to shoots only when excised during the dry storage phase (dormant). Once elongation of stems has commenced following dormancy, the regenerative potential of tulip explants is lost. Nodal explants to Dioscorea alata yams produce axillary shoot growth only when excised from do not plants growing under a 16 hr photoperiod. Exposure to shorter photoperiods gives rise to explants which either do not grow (or) show prolific callus development.

Familiarity with the donor plants natural propagation mechanism with reference to season and the growth stage is very much helpful in the determining the more suitable explant source. Typical examples of explants used in micropropagation of some important economic crops are given in the table.

2. Culture Media

The standard tissue culture media are more suitable for achieving stages I and II and micropropagation. Only stage III requires some modifications. The relative proportion of mineral components and two types of growth – regulating substances in the

culture medium largely govern the over all micropropagation performance of a culture system. In 1957, Skoog and Miller proposed a general concept which states that organ differentiation in plants is regulated by an interplay of auxin and cytokinin will work as a guide when developing a new medium for a new plant for micropropagation. For micropropagation relative concentration of NH_4^+ and K^+ in the medium influence the number and size of shoots produced from explants. These mineral components also influence the induction of somatic embryogenesis in cultures.

To induce adventitious root formation, after axillary shoot propagation, cytokinin is usually omitted and auxin is added. In some species root formation may occur on a medium with out hormones. Gibberellic acid and Abscisic acid and Abscisic acid in the medium are reported to inhibit root formation. Activated charcoal induces the formation of adventitious roots in some species. Its presence in the medium reduces the light supply to invitro regenerated shoots and helps to remove inhibition by the absorption of all inhibitors released in cultures.

3. Culture Environment

Though they are green in colour invitro regenerated shoots grow as heterotrophs as they derive their nourishment from the culture medium. Several studies have indicated that light, absorbed by photosynthetic pigments in cultured tissues, plays an important role in inducing morphogenesis in these tissues. In the excised leaf segments of lily it was observed that the bud induction is controlled by the photosynthetic system in its cells to a certain extent. The optimum light intensity found for shoot multiplication in most of the species is 100 lux. The quality of light also controls the organogenic differentiation and growth of shoots in cultures. Blue light induced bud formation in Tobacco shoots and the no. of shoots is doubled in the case of Letuce which are regenerated from callus cultures. There are several micropropagation systems in which morphogenesis is induced by red/far – red light treatments. The red light stimulates the induction of flower buds and far-red light stimulates root production.

Photoperiodic effects are dependent on the relative sensitivity of individual species. A diurnal illumination of 16 hr day and 8 hr night is generally found satisfactory for multiplication and proliferation of shoots. But, in case of cauliflower a 9 hr day light illumination is required. Temperature is also an important part of culture environment. In most of the micropropagating cultures, the

temperature is maintained constant around 25^{O}C. Some cultures require initial low temperature or morphogenic response.

Another factor of culture environment that effects the performance of cultures during micropropagation is the constitution of the gas phase with in the culture vessels. Ethylene, oxygen, carbon dioxide, ethanol and acetaldehyde are metabolically active gases with possible effects on morphogenesis and may promote unorganized growth of cells.

4. Genotype

Screening and selection of genotypes among segregating populations could be a fruitful approach in the improvement of micropropagation capabilities of plant species which are recalcitrant in tissue culture. Bingham et al. in 1975 followed this approach for clonal propagation of alfa alfa plants. By breeding different lines of Alfa alfa they screened the progeny for regeneration ability in cultures. Later it is revealed that the different genotypes of Nicotiana species differed in their regeneration capabilities in cultures. A similar response was observed in closely related genotypes of three hexaploid oat species. In case of grapes, different propagation coefficients were achieved from various cultivars, lines and hybrids. Cultivars with vigorous germination and branching capacity propagated rapidly, while those with low rates of germination and weak branching capability showed poor response in cultures. Genotypic effects of this nature are frequent and underscore the fact that a micro propagation system developed for one particular cultivar will not automatically be applicable to another cultiver. with in the same species.

5. Factors Effecting *Invitro* Rooting

Media having a low concentration of salts have proven satisfactory for rooting of shoots micropropagated at stage II. For species in which induction of shoot multiplication requires a full-strength MS medium, reduction of the salt concentration to half or one – quarter has been found satisfactory for rooting. Roots are induced in presence of a suitable auxin in the medium (NAA or IBA 0.1 – 1 mg/lit). The shoot of some plants may readily root on a hormone free medium. Riboflavin is reported to improve the quality of the root system in Eucalyptus ficifolia. Phloroglucinol has been found to promote rooting in a no. of rosaceous fruit trees.

Micropropagation of hard-to-root species can be achieved if their juvenile explants are induced to develop shoots by suckering,

coppicing or hormone treatment. In some plants shoot have been regenerated from adult materials but invitro multiplied shoots from roots only by passing them through a series of sub-cultures. The time required for invitro rooting may vary from 10-15 days. Plant with roots around 5 mm in length have been found convenient for handling during transplantation. Longer roots may break in this process and lead to high mortality of transplanted plants.

6. Acclimatisation of the Plant Transferred to Soil

Micropropagation on a large scale can be successful only when plants after transfer from culture to the soil show high survival rates and the cost involved in the process is low. Tissue culture plants generally show some structural and physiological abnormalities which include:

1. abnormal leaf morphology and anatomy.
2. poor photosynthetic efficiency.
3. marked decrease in epicuticular wax.
4. malfunctioning of stomata.

These characters along with heterotrophic mode of nutrition and poor mechanism for water loss control further render micropropagated plants vulnerable to transplantation shocks. So, the transfer of individual plant lets to a potting mix and their acclimatization under green house conditions require the application of various methods to harden the plants/shoots for transplantation.

Plants are transferred to the soil usually after the *invivo* rooting stage. The induction of *invivo* rooting of cultured shoots may be more economical and produces good quality roots. The lower parts of tissue culture plants (or) shoots should be washed thoroughly before their transfer to the potting mix. The transplanted plantlets (or) shoots are immediately irrigated with an inorganic nutrient solution and maintained under high humidity for the initial 10-15 days. This is required because plant lets are adopted to almost 90-100% humidity during culture. High humidity can be built up around the transplanted plants by covering them with clean transparent plastic bags having a small hole for air circulation. The size of the hole can be increased after two weeks in order to reduce humidity. This enables shoots to adopt well to green house conditions and to establish functional roots. Partial defoliation of plant lets and the application of anti transpirants in the initial stages of transplantation

improved the survival frequency due to reduction in water loss by plant lets. Nowadays most of the commercial laboratories have computerized hardening rooms with controlled conditions of light, temperature and humidity. Direct transplantation of cultured plants to the filed was tried incase of Rice and Tobacco. Transplants survived with high frequencies by applying a thin film composing 50% aqueous glycerol and grease or paraffin in an equal amount of diethyl ether on the surface of leaves with a brush before transplantation.

A major shock to the plants following transplantation is the change from a substrate rich in organic nutrients to a substrate providing mostly inorganic nutrients. The shoot system can be hardened by inducing autotrophism and development of surface wax on invitro formed leaves. Increase in the epicuticular wax deposition could be induced by exposing the cultures to $Cacl_2$ (or) covering the medium with a thinlayer of landing. These treatments helped in reducing humidity. $Cacl_2$ (or) lanolin are found to effect overall growth and development of plants. The relative humidity can also be reduced by opening the culture tubes inside a dessicator with $CaSO_4$ as the desiccant. This approach resulted in hardening of tissue culture plants invitro by the development of wax after seven days. These plants then acclimatized easily and had a higher survival rate (96%).

In case of some legumes a pre transplant stage is introduced which showed high survival rates. In this stage, plants are transferred to screw cap bottles containing sterilized quartz sand irrigated with an organic nutrient solution carrying an efficient strain of Rhizobium. Bottles are initially closed kept closed for two weeks and subsequently the caps are removed to maintain plants under controlled conditions of light and temperature ($25 \pm 2^{o}C$ and 18 Wm^{-2}) for another two weeks before their final transfer to the field.

Storage organs have been induced in cultured shoots of several species. These structures do not require hardening and they can be directly transplanted to the soil. The advantage of invitro tuberisation is that the additional step of rooting the shoots is eliminated. A wel-known example is the production of aerial tubers by the cultured shoots of potato under the influence of chloramequat. Other examples in which invitro tuberisation can be induced are Dioscorea bulbifera, D-alata and D. rotundata. Formation of bulblets is reported from Narcissus and Muscari species in the presence of activated charcoal. Formation of cormlets is reported from Gladiolus under high sucrose concentration. Transplantation of micropropagated plants of gladiolus has been a serious problem. Corms developed invitro can circumvent

this problem as they show high germination (75-80%) under field conditions.

fig-Acclimatization of plants to soil

7. Clonal Multiplication of Woody Species

Woody perennials comprise valuable crops such as fruit and not trees, plantation species, timber yielding and important trees of soil forestry. Due to the long term life cycle of these three crops. The improvement of woody perennials through application of conventional methods is very slow. The difficulty in rooting the cuttings from Elite tree species has complicated the process of their clonal propagation. Some perennial crops that can be propagated in tissue culture and the media used at various stages of their micropropagation, are listed in the table :

Considerable success has been achieved with respect to cloning of temperate fruit species and other trees through tissue culture. The response of forest trees like Populus, Eucalyptus and Tectonia, to micropropagation is highly positive. Tissue culture of nut trees also holds good. Recently success is achieved in tree tissue culture, Eg:- The controlled flowering of *invitro* propagated bamboo. Explants that have shown positive response in cultures for regeneration are mostly restricted to jullinile material. Clonal multiplication of woody

perennials *invitro* on a commercial scale requires enormous efforts directed towards establishing cultures enormous efforts directed towards establishing cultures from adult explants. Effective treatments to induce efficient rooting of *invitro* multiplied shoots and quality improvement of somatic embryos to achieve high efficiency conversion to plant lets must be found.

Shoot tips from the seed-lings of citrus, Jack fruit and black plums have already responded in culture. Immature embryo of Avocado have proved equally effective clonal materials in cultures. As the nucellar seedlings are naturally rejuvenated clones, they have been utilized as stock plants for micropropagation of several poly embryomic citrus cultivars and mango varieties. Mono embryonic species of citrus, varieties of mango and syzygium species regenerate by somatic embryogenesis using nucellar explants. Many tropical and subtropical fruit crops are reported to regenerate from somatic embryos induced on explants from mature trees.

The nature of the explant, its orientation in the culture medium and genotype influence the response of adult materials invitro. Shoot tips (or) nodal segments from tropical adult trees are reliable sources of explants. These explants can be excised from newly developed vegetative buds of jack fruit at the base of the main stem during the onset of flowering (or) during vigorous vegetative growth of giuava. In some cases hard pruning of the main stem stimulates the growth of lateral buds, thus providing enough meristematic material for clonal propogation. Conditioning of stock plants improves the culture response of shoot tips and nodal segments. Most citrus producing countries have established programmes for release of clonal material that has been indexed for freedom from Citrus tristeza virus after micro grafting of meristems on to seedling root stocks invitro. Root rot of Avocado caused by phytophthora cinnamoni, could be controlled if clonally propagated disease- resistant root-stocks were readily available.

Clonal multiplication of gymnosperms

Soft woods

Gymnosperms are the main source of soft wood. Clonal propagation of gymnosperms is desirable because of their long life span and open pollination. Vegetative multiplication by cuttings has drawbacks as cuttings lack the vigour of seedlings and their growth declines with increasing age of the ortet. The invitro techniques are

expected to overcome these problems and regenerate the plants at faster rate.

Tissue culture studies with gymnosperms were started by (1934) Gautheret in Pinus Pinaster and Abies alba. Later La Rue cultured the embryos of several Gymnosperms in to normal seedlings. In 1975 Sommer etal could regenerate the complete plant lets from *Pinus palustris*. Since then plantlets have been from about 35 gymnosperm species via somatic embryogenesis.

A large no. of adventitious buds can be induced on embryonal explants of gymnosperms. But the no. of plants finally established in the soil is extremely low because of losses during successive stages of development, rooting, transplantation etc. Only limited success has been achieved with micropropagation of plants from adult tissues. Micropropagation from older trees has been demonstrated in species such as Sequoia Sempervirens, Pinus, Pinaster, P. radiater.

Induction of somatic embryogenesis in the immature embryo cultures of picea abies and female gametophyte of Larix deciduas has been a major break through in the tissue culture of gymnospermous species yielding softwoods.

In case of pseudotsugu sps; Larix sps; Pinus taeda and Pinus strobes precotyledonary embryos were used for raising embryogenic callus.

In case of picea abies and pinus lambertiana somatic embryos were raised from the mature zygotic embryos. Well developed somatic embryos have been obtained from the protoplasts derived from embryogenic calli of Pinus taeda and Picea glauca.

8. Technical problems encountered in micropropagation

1. Contamination of cultures

During large-scale micropropagation of some plants slow growing microbial contaminants persist even after initial surface sterilization of explants. So, for the propagation of plants normally infected with latent (or) symptom less types of viruses (or) mycoplasmas, it is necessary to maintain stock plants of them which are disease-free.

2. Browning of the medium

During the micropropogation of woody perennials, the explants from adult tissues of these species often produce phenolic substances which turn the medium dark brown. Such a medium is toxic to tissues and inhibits their growth. Frequent transfer of explants to a fresh medium at short intervals could alleviate this problem in orchid bud cultures. Browning of the medium may also be prevented by dissecting explant tissues under the surface of liquids (or) by incorporating ascorbic acid (or) citric acid in the medium.

For teak tissue cultures, polyvinyl pyrrolidone has proved an effective amendment to the culture medium. Some of the phenolic compounds such as floroglucinol has been found essential for shoot multiplication and rooting in a number of rosaceous cultivars.

Eg: Einchona and pissardi plum.

9. Advantages of clonal propagation

1. The technique of invitro clonal propagation is a type of micropropagation, which is an alternative to conventional methods of vegetative propagation. It is applied with the objective of enhancing the rate of multiplication.

2. From a small, microscopic, piece of plant tissue, over a million plants can be grown through tissue culture with in 12 months. Such a higher rate of multiplication cannot be expected by any of the invivomethods of clonal propagation.

3. The advantage in propagation through tissue culture is that shoot multiplication has a short cycle (2-6 weaks) and each cycle results in logarithmic increase in the number of shoots.

4. Tissue culture give propagules such as minitubers or minicorms of plant multiplication thoughout the year irrespective of the season.

5. The small size of propagules and their ability to proliferate in a soil-free environment facilitates their storage on a large scale and also allows their large scale dissemination by suitable means of transport across the international boundaries.

6. Clonal propagation invitro appears to have a permanent advantage in the plant species in which there is serious problem with disease accurence.

7. In case of dioecious species, multiplication by cloning is important, when the plants of one sex are desired commercially. For example, male plants of asparagus officinalis are more valuable than the female plants of this species. In the seed-raised or chards, micropropagating female plants would save the losses suffered due to discarding a large no. of naturally arising male plants.

8. The major advantage of micropropagation is the minimum growing space required in commercial nurseries. This makes possible the propagation of clones on a commercial scale for large no. of horticultural species (Eucalyptus, banana, orchids, ferns, rhododendrons) in a single nursery.

Success of micropropagation is judged on the basis of rate of shoot multiplication and the success of transplantation to the field. A list of treeplants which have been micropropagated invitro is given in the table.1.

Teak is one of the most important timber trees of India and in neighbouring countries. The wood of teak is highly superior for its strength, durability and insect resistance. Similarly eucalyptus citridora is commercially valued for is wood and the essential oils of its leaves. The main component of oil is citronella. Suitable methods are not available for the vegetative propagation of such elite trees by cutting. Micropropagation of such plants have been developed by which about 500 plants of teak and 100,000 plants of Eucalyptus citroiodora can be obtained from a single bud in a year. This preliminary experiment clearly demonstrates the vast potentials of micropropagation technique for clonally multiplying Elite trees.

3.2.1.3. Meristem culture

Meristem culture involves the development of an already existing shoot meristem and subsequently the regeneration of adventitious roots from the developed shoots. In this chapter different aspects related to meristem culture and its applications are discussed.

1. Introduction

Cultivation of axillary (or) apical shoot meristems is known as meristem culture. In meristem culture generally shiny dome like structure measuring less than 0.1mm in length terminal portion of the shoot with one or two pairs of the young leaf primordial is cultured.

2. Principle

The excised shoot tips and meristem can be cultured aseptically solidified agar medium (or) an filter paper bridges dipped in to liquid medium, under appropriate conditions will grow into a small leafy shoot (or) multiple shoots. Alternately the meristem may form a small callus at its cut base on which a large no.of shoot primordial will develop. These shoot primordial will grow in to multiple shoots. These shoots can be propagated further by nodal cuttings. Each nodal segment contains one node. The axillary bud on each segment will growout in culture to form another shoot. In case of orchids, the excised stemtips in culture proliferate to form callus from which some organized jovinile structures known as protocorm develop. When the protocorms are separated and cultured on fresh medium, they develop in to normal plants.

Exogeneously supplied cytokinins in the nutrient medium plays a major role for the development of a leafy shoot (or) multiple shoots from the meristem. Addition of adenine sulfate in the nuctrient medium also induces the shoot tip multiplication in some cases. BAP is the most effective cytokinin commonly used in shoot tip culture. NAA is the most effective auxin used in shoot tip culture. Coconut milk and Gibberellic acid are also equally effective for the growth of shoot apices in some cases.

3. Explant

Shoot apical meristem lies in the `shoot tip beyond the youngest leaf primordium. It measures up to 100μm in diameter and 250 μm in length. A shoot tip of 100-500 μm would contain 1-3 leaf primordial in addition to the apical meristem. Generally, shoot tips of 1 μm are used when the object is virus elimination. Shoot-tip culture is widely used for rapid clonal propagation, for this prupose much larger (5-10mm) explants are used. So, most cases of meristem culture are shoot-tip cultures. Nodal explants of various sizes are also employed for rapid clonal propagation. Generally, explants taken from actively growing plants at the beginning of growing season are the most suitable.

4. Culture medium

MS medium has been found satisfactory for most of the plant species but for some species a much lower salt concentration may be adequate (or) even necessary since the high salt concentration of MS medium may be deleterious (or) even toxic. Eg: In blue berry, ¼ MS

salts are sufficient and the full MS is often toxic. Solid medium is most widely used for convenience but in some species, use of liquid medium is either necessary Eg: Cattleya (or) beneficial

Eg: Cephalotus.

Growth regulator requirement depends on the stage of culture process viz. 1. culture initiation (2) Shoot multiplication (3) rooting of shoots (4) Transfer of planlets to soil.

1. Culture initiation

Culture initiation consists of surface sterilization of explants and establishing them invitro. In this stage detection, elimination (or) control of contamination is broughtout. The growth of the explant may (or) may not occur. A growth regulator free based medium is used. In case of heavy contamination (or) endophytic contamination a suitable antibiotic (trimithoprim) or fungicide (Bavistin) may be added to the culture medium.

After 2-3 weeks, the cultures are transferred to a shoot multiplication medium, in which axillary branching is promoted. This medium contains a cytokinin (1-2 mg/lit) either alone or in combination with auxin (0.1 mg – 1mg/lit) chiefly depending on the plant species. BAP is the most commonly used cytokinin, but in some species viz. blue berry, garlic, rhododendrons etc. 2-ip is much more effective. NAA, IBA, and IAA are the commonly used auxins. 2,4-D is not used as it promotes callusfemation. Higher concentrations of cytokinin (> 2 mg /litre of BAP) induce adventitious buds retard shoot growth. If the shoot growth is retarded culture of shoots on basal medium (or) low cytokinin medium (or) GA_3 medium, promotes shootelongation before they can be rooted. So, the growth regulator combination should bedetermined to obtain optum shoot multiplication rates with minimum risk of adventitious shoot buds and without the need of shoot elongation step.

5. Culture environment

During culture initiation and shoot multiplication phases, the cultures are generally kept at a constant temperature of 25^{o}C and are illuminated with 1000 / Lux white light from fluorescent tubes. In some cases high light intensities (3000-10,000 / Lux) are showing beneficial effect on rooting and on plant survival after transfer to soil.

6. Browing of medium

in many species, phenolic leach in to the medium from the cut surface of explants. These phenolics turn darkbrown on oxidation and are detrimental to the cultures. This problem is very common in case of woody species, particularly when the explants are taken from mature trees. This problem can be overcome, in most species by any one of the following ways. But, in some species like mango control of phendics is the chief problem as the entire explant turns black and dies.

1. Frequent sub-culture (3-7 days) of explants on agarmedium may be sufficient to overcome this problem in many species.

2. A brief period of culture in the liquid medium may be sufficient to overcome this problem in many species. Eg: apple, rubus, eucalyptus etc. This may remove the phenols and other inhibitory substances from the explants.

3. In some cases antioxidants like ascorbic acid (50-100 mg/lit) cysteine HCl (100 mg/l) or citric acid (150 mg/lit) may be used to check the oxidation of polyphenols.

4. Adsorbents like activated charcoal (0.5 – 2 gm/lit) (or) polyvinyl pyrrollidone may be used to adsorb the polyphenols secreated in to the medium.

5. Culture in dark may be helpful since light enhances polyphenol oxidation.

7. Rooting of shoots

Generally, the rooting medium has low-salt concentration, which may be ½ or even ¼ of that of the MS medium and reduced sugar levels (1 gm/lit). Reduced salts is essential for rooting in some species like narcissus. In some species rooting occurs on growth regulator free medium.

Eg: straw-berry, narcissus. In most of the species 0.1 – 1 mg/lit NAA or IBA is required for rooting. In plants like citrus, a pulse treatment with an auxin (10 minuts with 100 mg /lit NAA (or) IBA) gives optimum rooting.

Shoots are usually rooted in an agar medium, but the recent trend is to root them directly in vermiculate (or) potting mix. The cutends of the shoots are treated with a suitable auxin solution (or)

powder mix and they are transplanted in to pots and kept under high relative humidity and low-light intensity. This saves the cost of production as rooting and soil transfer stages are combined and rooting medium is eliminated. Rooting takes about 10-15 days depending mainly on the species. Plant lets with 0.5 to 1 cm roots are usually transplanted in to pots since longer roots tend to get damaged.

8. Transfer of plant lets to soil

The rooted shoots are removed from the medium and the agar sticking to their roots is washed with tap water and they are transplanted in plastic cups containing a suitable potting mix. Initially the plants are kept in high humidity and low-light intensity. The plant lets are covered with plastic covers to maintain high humidity. The humidity is gradually decreased to the ambient level after 7-15 days and the light intensity is increased. The plants are finally exposed to the green house conditions.

9. Vitrification and morphological variations

During repeated cycles of invitro shoot multiplication a percentage of cultures show brittle, glassy, water soaked (or) almost translucent leaves. Such shoots exhibit a decline in the rate of growth and may ultimately die. This phenomenon is called vitrification. Vitrification may be represented by the symptoms not visible to the naked eye, such as poorly developed vascular bandles, abnormal wax quality, abnormal functioning of stomata etc. Vitrification is the cosequence of culture conditions. It may be overcome by the following ways: (1) increased agar levels (ca. 1%) (2) Bottom cooling of culture vessels (3) Addition of agar hydrolysates and (4) Use of growth retardants.

Morphological variants may arise during multiplication stage. Such variants are eliminated by visual selection in commercial ventures and the homogeneity is maintained in the plant lets. Many commential enterprises therefore prefer to multiply shoots for only four cycles from an explant, thereafter a fresh batch of cultures is initiated from field-tested plants.

10. Importance of meristem culture

1. This technique is used in germplasm storage of plants.
2. It is used in the micropropagation of agricultural and horticultural crops.

3. It is an effective method for the elimination of viruses and other pathogens from citrus and other plants.

4. The clones produced during meristem culture are used as scions which are grafted with etiolated seedlings.

3.2.1.4 Summary

Each somatic cell of the mature plant is derived by mitosis from the zygotic cell. So, each of these cells must also contain all the genetic information needed for growth and development of a whole organism. Therefore, totipotency is the genetic potential of a plant cell to produce the entireplant.

The expression of totipotency of different types of plant cell such as parenchyma, meristematic, leaf mesophyll, phloem, cambium cells and pollen grains can be demonstrated by means of plant tissue culture technique. Totipotency of the cells in culture is manifested through the process of differentiation and the hormones play a major role in this process than any other factors. F. Skoog and C.O. Muller of USA advanced an hypothesis that the regeneration of an organ from cultured tissue is controlled by a balanced ratio between auxin and cytokinin. A relatively high auxin –cytokinin ratio induces root formation and a low ratio of the same hormones favors shoot production.

Somatic tissues of carrot has shown evidence of embryo initiation, in single cells that are progenitors of bipolar embryos. Here, the balance of hormone levels led to the differentiation of embryo (or) embryoids by a process that occurs in normal zygotic embryos. It is also observed in some experiments, that the cells of some callus mass frequently differentiate in to vascular elements without forming any plant organs (or) embryoids. This process is known as histogenesis (or) cytodifferentiation. Therefore the totipotent cells may express themselves in different ways on the basis of differentiation process and manipulation.

The composition of the nutrient medium which is farowing the expression of totipotent cells is standardized by trial and error method. In callus culture all the cells are not totipotent. Variation of chromosomal number in the cells of callus tissue is one of the mainfactors that causes the limited expression of totipotency. Observation on organogenesis (or) embryogenesis in callus culture have led to propose that association of cells may be sometimes

required to provide the appropriate environment for certain individuals to express their totipotency.

The unavailability of threshold level of endogenous and exogenous hormone in all cells of callus tissue may impose a barrier for their expression of totipotency.

Besides these factors, heterogeneity in physical structure of the cell make a great difference in the degree of chemodifferentiation cells that make an asynchronous situation due to which all cells are not able to express their totipotency at a time.

From the fundamental to applied aspects of plantbiology, cellular totipotency is highly important. The success of different aims and objectives of plant tissue culture depends upon the expression of totipotency.

The progeny obtained by vegetative propagation (or) asexual reproduction of a single plant constitute a clone. The members of a single clone have the same genotype. Conventionally vegetative reproduction is achieved by cuttings, budding, grafting etc. Tissue culture also enables rapid clonal propagation of plants this is called micropropagation.

The main objective of clonal propagation is to produce progeny plants which are genetically identical to their parent plant. This is achieved by different processes, viz. (1) Proliferation of axillary buds (2) Induction of axillary buds, bulbs, protocorms etc. and (3) Somatic embryogenesis; which are employed for the micropropagation of different plant species.

In case of proliferation of axillary buds, cultures of either shoot tip (or) nodal explants are utilized which are grown on a medium supplemented with a cytokinin and auxin. The cytokinin stimulates the preexisting shoot buds present in the explant to develop in shoots. Each leaf on such shoots has an axillary bud. These axilary buds are also stimulated to develop into shoots. The individual shoots are then excised and subcultured on a on a fresh medium to initiate a new cycle of multiplication by axillary branching. In most of the plant species each explant would produce 5-6 shoots in 4-5 weeks and we can produce 5^{10}-6^{12} plants in one year from a single individual explant. Provided all the shoots produced could be cultured and there was 100% survival. This method is used in the propagation of straw-berry. In many plant species, rapid clonal propagation is achieved by inducing adventitious buds, bulbs, protocorms etc. protocorms are

produced from the excised shoot tips of orchids. Each protocorm divides to yield several protocorms. Each protocorm may be cut in to 4-6 pieces and subcultured, each piece again gives rise to several protocorms. When protocorms are left as such without chopping, each protocorm gives rise to a complete plant let on the same medium. This approach can yield over 10^6 orchid plants in one year from a single shoot tip. This technique is widely used in orchid industry for the micropropagation of both sympodial and monopodial orchids.

Micropropagation of Lily is done by inducing bulb formation on cultured pieces of bulb scales. A single bulb scale may yield about 100 bulblets. Each bulblet is separated and gives rise to a complete plant.

In some species adventitious shoot buds are used for micropropagation. A shoot bud arising any where other than a leaf axil (or) shoot tip is called adventitious shoot bud. Adventitious shoot buds are produced invivo in many plant species, Ex: Begonia leaves, Phlox roots etc. Adventitious shoot bud formation is exhibited by many plant species Eg: chrysanthemum, Hyacintuus, Ornitho galum etc.

Somatic embryos have been used for the micropropagation of several species. Eg: Oilpalm, datepalm, alfalfa, carrot, yellow poplar, Norway spruce etc.

Somatic embryos offer a very high rate of multiplication. An automated large scale micropropagation based on somatic embryos, bulblets, microtubers etc. has been devised. This system integrates a bioreactor with a bioprocessor for separation, isolation based size and subculturing of the propagules. An automated transplanting machine has also been developed which transplants up to 8000 plants / hr in to a soil mix.

Certain varieties of some ornamentals and fruit trees are chimeras such varieties must be propagated by axillary shoot-bud proliferation and somatic embryogenesis, in these cases adventitious bud (or) bulb regeneration must be avoided. In some ornamental varieties the desirable variegations are due to the presence of a virus. In such cases adventitious shoot bud / bulb (or) somatic embryo regeneration is the method of choice, while axillary bud proliferation must not be used since the latter may eliminate the virus.

The vast potentials of micropropagation techniques civil fulfil the requirements of reforestration and in future provide the timber, oil, fuel wood and the raw materials for paper industry.

Micropropagation has got some disadvantages such as browning of the medium. Several methods have been adopted currently to reduce this problem.

Meristem is a mass of undifferentiated parenchyma cells found at the extreme tip of the shoot and root systems. These cells have totipotency necessary to regenerate in to plant lets.

- These cells are allowed to grow in the culture medium for producing plantlets through the callus phase. The genetic composition of these plantlets is similar to that of their explant tissue (or) callus tissue.

- Shoot apical meristem lies in the shoot lip beyond the youngest leaf (or) first leaf primordium. Shoot tips of 1 μm are used when the objective is virus elimination. For clonal propagation shoot tips of 5-10 mm are used.

- When the objective is vegetative propagation, the size of the shoot tip used for culture is not important. But, when the objective is to free the stock from virus, it is essential that the apical meristem should be excised along with a minimum of the surrounding tissue.

- Generally, explants taken from actively growing plants at the beginning of growing season are the most suitable.

- MS medium has been found satisfactory for most of the plant species. But, for some species a much lower salt concentration may be adequate since high salt concentrations of MS may be deleterious (or) exen toxic.

- The growth regulator requirement depends on the stage of culture process. Viz. 1. culture initiation, (2) shoot multiplication (3) rooting of shoots (4) transfer of plant lets to soil.

- Culture initiation consists of surface sterilization of explants and establishing them invitro. In this stage

contamination is detected and controlled. Generally, a growth regulator free basal medium is used in this stage.

- After 2-3 weeks the culture is transferred to a shoot multiplication medium. It contains growth regulators and promotes axillary branching.

- During the culture initiation and shoot multiplication phases, the cultures are kept at constant temperature of 25^{o}C and are illuminated with about 1000 /ux white light from fluorescent tubes.

- In many species, phenolics leach in to the medium from the cut surfaces of explants. These phenolics turn dark brown on oxidation, so the medium also turns brown. This problem is common in case of woody species, which can overcome by frequent subculturing, by using antioxidants (or) activated charcoal (or) by culturing in dark.

- Rooting medium has low salt and reduced sugar levels generally auxins are used in rooting medium. Rooting takes about 10-15 days, depending on the species. Plantlet with 0.5 to 1 cm roots are transplanted in to pots

- Meristem culture is used in the production of virus free plants and in the micropropagation of agricultural and horti cultural crops.

3.2.1.4 Model Questions

1. What is totipotency ? Give a general account of the expression of totipotency of cells in culture.

2. Write short notes on –

   a. totipotency b. caulogenesis c. Rhizogenesis.

   d. Histogenesis e. Improtance of totipotency

3. What is clonal propagation? Describe the different methods of clonal propagation, mention the importance of clonal propagation?

4. What is meristem culture? Mention the significance of meristem culture.

3.2.1.5 Reference books

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Lesson 3.2.2
Meristem Culture

Objective

Meristem culture involves the development of an already existing shoot meristem and subsequently the regeneration of adventitious roots from the developed shoots. In this chapter different aspects related to meristem culture and its applications are discussed.

3.2.2.1. Introduction

Cultivation of axillary (or) apical shoot meristems is known as meristem culture. In meristem culture generally shiny dome like structure measuring less than 0.1mm in length terminal portion of the shoot with one or two pairs of the young leaf primordia is cultured.

3.2.2. 2. Principle

The excised shoot tips and meristem can be cultured aseptically on solidified agar medium (or) an filter paper bridges dipped in to liquid medium, under appropriate conditions will grow into a small leafy shoot (or) multiple shoots. Alternately the meristem may form a small callus at its cut base on which a large no. of shoot primordia will develop. These shoot primordia will grow in to multiple shoots. These shoots can be propagated further by nodal cuttings. Each nodal segment contains one node. The axillary bud on each segment will growout in culture to form another shoot. In case of orchids, the excised stemtips in culture proliferate to form callus from which some organized juvinile structures known as protocorms develop. When the protocorms are separated and cultured on fresh medium, they develop in to normal plants.

Exogeneously supplied cytokinins in the nutrient medium plays a major role for the development of a leafy shoot (or) multiple shoots from the meristem. Addition of adenine sulfate in the nutrient medium also induces the shoot tip multiplication in some cases. BAP is the most effective cytokinin commonly used in shoot tip culture is NAA. NAA is the most effective auxin used in shoot tip culture.

Coconut milk and Gibberellic acid are also equally effective for the growth of shoot apices in some cases.

1. Explant

Shoot apical meristem lies in the `shoot tip beyond the youngest leaf primordium. It measures up to 100μm in diameter and 250 μm in length. A shoot tip of 100-500 μm would contain 1-3 leaf primordial in addition to the apical meristem. Generally, shoot tips of 1 μm are used when the object is virus elimination. Shoot-tip culture is widely used for rapid clonal propagation, for this purpose much larger (5-10mm) explants are used. So, most cases of meristem culture are shoot-tip cultures. Nodal explants of various sizes are also employed for rapid clonal propagation. Generally, explants taken from actively growing plants at the beginning of growing season are more suitable.

2.Culture medium

MS medium has been found satisfactory for most of the plant species but for some species a much lower salt concentration may be adequate (or) even necessary. Since the high salt concentration of MS medium may be deleterious (or) even toxic. Eg: In blue berry, ¼ MS salts are sufficient and the full MS is often toxic. Solid medium is most widely used for convenience but in some species, use of liquid medium is either necessary.

Eg: Cephalotus.

Growth regulator requirement depends on the stage of culture process viz. 1. culture initiation (2) Shoot multiplication (3) rooting of shoots (4) Transfer of planlets to soil.

3.2.2.3 Culture initiation

Culture initiation consists of surface sterilization of explants and establishing them invitro. In this stage detection, elimination (or) control of contamination is broughtout. The growth of the explant may (or) may not occur. A growth regulator free based medium is used. In case of heavy contamination (or) endophytic contamination a suitable antibiotic (trimithoprim) or fungicide (Bavistin) may be added to the culture medium.

After 2-3 weeks, the cultures are transferred to a shoot multiplication medium, in which axillary branching is promoted. This medium contains a cytokinin (1-2 mg/lit) either alone or in combination with auxin (0.1 mg – 1mg/lit) chiefly depending on the plant species. BAP is the most commonly used cytokinin, but in some species viz. blue berry, garlic, rhododendrons etc. 2-ip is much more effective. NAA, IBA, and IAA are the commonly used auxins. 2,4-D is not used as it promotes callusing. Higher concentrations of cytokinin (> 2 mg /litre of BAP) induce adventitious buds retard shoot growth. If the shoot growth is retarded culture of shoots on basal medium (or) low cytokinin medium (or) GA_3 medium, promotes shootelongation before they can be rooted. So, the growth regulator combination should be determined to obtain optimum shoot multiplication rates with minimum risk of adventitious shoot buds and without the need of shoot alongation step.

3.2.2.4 Culture environment

During culture initiation and shoot multiplication phases, the cultures are generally kept at a constant temperature of 25^{o}C and are illuminated with 1000 / Lux white light from fluorescent tubes. In some cases high light intensities (3000-10,000 / Lux) are showing beneficial effect on rooting and on plant survival after transfer to soil.

1. Browning of medium

In many species, phenolics leach in to the medium from the cut surface of explants. These phenolics turn darkbrown on oxidation and are detrimental to the cultures. This problem is very common in case of woody species, particularly when the explants are taken from mature trees. This problem can be overcome, in most species by any one of the following ways. But, in some species like mango control of

phenolics is the chief problem as the entire explant turns black and dies.

1. Frequent sub-culture (3-7 days) of explants on agar medium may be sufficient to overcome this problem in many species.
2. A brief period of culture in the liquid medium may be sufficient to overcome this problem in many species. Eg: apple, rubus, eucalyptus etc. This may remove the phenols and other inhibitory substances from the explants.
3. In some cases antioxidants like ascorbic acid (50-100 mg/lit) cysteine HCl (100 mg/l) or citric acid (150 mg/lit) may be used to check the oxidation of polyphenols.
4. Adsorbents like activated charcoal (0.5 – 2 gm/lit) (or) polyvinylpyrrollidone may be used to adsorb the polyphenols secreated in to the medium.
5. Culture in dark may be helpful since light enhances polyphenol oxidation.

3.2.2.5 Rooting of shoots

Generally, the rooting medium has low-salt concentration, which may be ½ or even ¼ of that of the MS medium and reduced sugar levels (1 gm/lit). Reduced salts is essential for rooting in some species like narcissus. In some species rooting occurs on growth regulator free medium.

Eg: straw-berry, narcissus. In most of the species 0.1 – 1 mg/lit NAA or IBA is required for rooting. In plants like citrus, a pulse treatment

with an auxin (10 minutes with 100 mg /lit NAA (or) IBA) gives optimum rooting.

Shoots are usually rooted in an agar medium, but the recent trend is to root them directly in vermiculate (or) potting mix. The cutends of the shoots are treated with a suitable auxin solution (or) powder mix and they are transplanted in to pots and kept under high relative humidity and low-light intensity. This saves the cost of production as rooting and soil transfer stages are combined and rooting medium is eliminated. Rooting takes about 10-15 days depending mainly on the species. Plant lets with 0.5 to 1 cm roots are usually transplanted in to pots since longer roots tend to get damaged.

3.2.2.6 Transfer of plantlets to soil

The rooted shoots are removed from the medium and the agar sticking to their roots is washed with tapwater and they are transplanted in plastic cups containing a suitable potting mix. Initially the plants are kept in high humidity and low-light intensity. The plant lets are covered with plastic covers to maintain high humidity. The humidity is gradually decreased to the ambient level after 7-15 days and the light intensity is increased. The plants are finally exposed to the green house conditions.

Fig-1

3.2.2.7 Vitrification and morphological variations

During repeated cycles of invitro shoot multiplication a percentage of cultures show brittle, glassy, water soaked (or) almost translucent leaves. Such shoots exhibit a decline in the rate of growth and may ultimately die. This phenomenon is called vitrification. Vitrification may be represented by the symptoms not visible to the naked eye, such as poorly developed vascular bandles, abnormal wax quality, abnormal functioning of stomata etc. Vitrification is the cosequence of culture conditions. It may be overcome by the following ways: (1) increased agar levels (ca. 1%) (2) Bottom cooling of culture vessels (3) Addition of agar hydrolysates and (4) Use of growth retardants.

Morphological variants may arise during multiplication stage. Such variants are eliminated by visual selection in commercial ventures and the homogeneity is maintained in the plant lets. Many commential enterprises therefore prefer to multiply shoots for only four cycles from an explant, thereafter a fresh batch of cultures is initiated from field-tested plants.

3.2.2.8 Importance of meristem culture

1. This technique is used in germ plasm storage of plants.
2. It is used in the micro propagation of agricultural and horticultural crops.
3. It is an effective method for the elimination of viruses and other pathogens from citrus and other plants.
4. The clones produced during meristem culture are used as scions which are grafted with etiolated seedlings.

3.2.2.9 Summary

3.2.2.10 Model questions

1) What is meristem culture ? Mention the significance of meristem culture

3.2.2.11 Reference books

1. An introduction to plant tissue culture Dr.M.K. Razdan, Oxford & IBH publishing Co. Pvt., Ltd.

2. Plant cell and tissue culture, Narayana Swamy, Tata Mcgrow-hill.

3. An introduction to plant tissue culture, Dr.Kalyan Kumar De, New Central Book Agency.

4. Biotechnology, B.D.Singh, Kalyani Publishers.

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LESSON - 3.2.3

Production of Haploids

Objective

The main objective of this lesson is to bring the different processes and types involved in producing the haploid plants.

3.2.3.1

Introduction:

Introduction: In angiosperms, that haploid or gametophytic phase is extremely brief and is represented by pollen grains in anther and one or more cells in embryo sac of an ovule. A typical anther in cross section shows two anther lobes and each lobe possesses two pollen sacs. During microsporogenesis pollen mother cells (PMC) inside the pollen sacs form pollen tetrad by meiosis. In each pollen tetrad, four pollens are held together temporarily by their close wall. Each pollen possesses an unique genome where every gene is present as a single copy.

Exploitation of this unique genetic unit and the totipotency of the plant cell is the basis of anther a pollen culture for the production of haploid plants. On the other hand egg cell produced with in ovule is very difficult to separate from complex tissue integration. In culture, the anther swells and dehisces along its upper margin, length wise. This phenomenon helps to expose the pollen grains. Alternatively, huge amount of pollen grains can be isolated manually and can be culture aseptically very easily. Therefore, pollen is more suitable material than egg cell for the production of haploid.

The development & production of haploid plant *invitro* is very important for the study of fundamental and applied aspects of genetics in higher plants. Production of homozygous diploid by doubling the chromosome number of haploids *invitro* makes a pure line in a single step and such homozygous pure line is of great importance in plant breeding. Thus from the practical as well as fundamental point of view, anther and pollen culture open up a new avenue in the field of plant science.

3.2.3.2 History:

W. Tulecke (1953) was the first one to observe that mature pollen grains of Ginkgo biloba can be induced to proliferate in culture to form haploid callus. In 1964 S. Guha & S.C. Maheswari first reported the direct development of embryos from microspores of Datura innoxia by the culture of excised anther. In 1967 J.D. Bourgin & J.P. Nitsch obtained complete haploid plant lets from anther culture of Nicotiana tabacum.

3.2.3.3 Anther Culture & Pollen Culture:

Anther culture is a technique by which the developing anthers at a precise and critical stage are excised aseptically from unopened flower bud and are cultured on a nutrient medium where the microspores with in the cultured anther develop into callus tissue or embryoids that give rise to haploid plantlets either through organogenesis or embryogenesis.

Pollen or microspores culture is an invitro 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 microsproes, with out producing male gametes, develop into haploid embroids or callus tissue that give rise to haploid plant lets by embryogenesis or organogensis.

3.2.3.4 Development of Androgenic Haploids:

Fig.1

Androgenesis is the invitro development of haploid plants originating from totipotent pollen grains through a series of cell division and differentiation.

There are two modes of androgenesis:

(i) Direct androgenesis

(ii) Indirect androgenesis

(i) Direct androgenesis: In this type, microspore behaves life a zygote and undergoes change to form embryos which ultimately gives rise to a plant let.

(ii) Indirect androgenesis: In contrast to the direct androgenesis, the microspore instead of under going embryogenesis, divide, repeatedly to form a callus tissue which differentiates into haploid plant lets.

3.2.3.4.2 Principle of Anther & Pollen Culture:

The basic principle of anther and pollen culture is the production of haploid plants exploiting the totipotency of microspore and the occurrence of single set of chromosome (n) in microspore. In this process, the normal development and function of the pollen cell to become a male gamete is stopped and is diverted forcely to a new metabolic pathway for vegetative cell division. For this objective, microspores, either with in intact anther or in isolated state, are grown aseptically on the nutrient medium where the developing pollen grain will form the callus tissue or embryoids that ultimately give rise to haploid plant lets.

Infact, anther culture is in essence the pollen culture. The principle behind the anther culture is that without disturbing the natural habitat and environment of the enclosed anther. Pollen can be grown by culturing the intact anther. In culture condition, the diploid tissue of anther will remain living with out proliferation at the selective medium and at the same time, it will encourage the development of pollen by nursing and providing nutrient. The haploid embroyoids or the callus tissue can be seen as the anther dehisces in culture. But there is always the possibility that the diploid somatic cells of the anther will also respond to culture condition and so produce unwanted diploid callus or plant lets. In attempts to avoid this problem, free pollens isolated from the anther are grown in nutrient medium. The knowledge gained so far from anther and pollen culture has established that pollens at the uninucleate stage, just before the first mitosis, or during mitosis are most suitable for the induction of haploids.

Induction of haploids can be enhanced by keeping the anther or flower bud at low temperature. The low temperature has been ascribed to a number of factors such as dissolution of microtubules, alternation in the first mitosis or maintenance of higher ratio of viable. Pollen capable of embroygenesis. Cold treatment may also act to help the embryogenesis by repressing the gametophytic differentation or by lowering. The abscisic acid content of the anther which is considered to be inhibitory for the production of haploids.

The important aspect of anther or pollen culture is the nutrient medium. The nutritional requirements of the excised anther are much simpler then those of isolated microspores. In the isolated microspore, it is obvious that certain factors responsible for the induction of haploids. Which might have been provided through the medium. Rich medium may encourage the proliferation of the diploid tissue of anther wall and should be avoided. Incorporation of activated charcoal into the medium has stridulated the induction of androgenesis. The iron in the medium also plays a very important role for the indultion of haploids. Potato extract, coconut milk and growth regulators like a win and cytokinin are also used for anther and pollen culture due to their stimulatory effect on androgenesis.

In culture, pollen may divide mitotically or can follow the normal pathway of forming vegetative and generative nucleus. The generative nucleus remains quiescent and abort. The vegetative nucleus divides repeatedly, forming a multi nucleate pollen. The multi nucleate pollen undergoes segmentation which may lead to form either organised embryoid structure or callus tissue. Both types of development are utilised to form haploid plant lets.

The haploid plantlets are self-sterile due to presence of single set of chromosome which are not able to participate in meiotic segregation. By colchicine treatment haploids are made homoygous diploid or isogenic diploid which are fertile.

Haploids or homozygous diploid grown invitro are transferred to pot and grown to maturity in the glass house.

3.2.3.3.3 Protocol for Anther Culture:

fig 2.

Tobacco is the ideal material for anther culture. So the basic protocol described below should be applicable to anther culture in general with modification. The immature anthers containing uninucleate pollen at the time of first mitosis are the most suitable material for the induction of haploids. The steps of anther culture are given below:

1. Collect the flower buds of Nicotiana tabacum at the onset of flowering. Measure the length of each individual with a ruler. Select the bud of 17-22 mm in length when the length of the sepals equals that of the petals. Reject all flower buds which are beginning to open.
2. Transfer the selected flower buds to the laminar air flow. Each flower bud contains five anther and these are normally surface sterile in closed buds. The flower buds are surface sterilized by immersion in 70% ethanol for 10 seconds followed immediately by 10 minutes in 2% (v/v) Sodium hypochlorite. They are washed three times with sterile distilled water. Finally, transfer the buds to a sterile petridhish.
3. To remove the anthers, slit the side of the bud with with a sharp scalpel and remove them. With a pair of forceps, place the five anthers with the filaments to another petridish. The filament are cut gently. During excision of anthers, special care should be taken to ensure that they are not injured in any way. Damaged anthers should be discarded as they aften form callus tissue from the damaged parts other than the pollen.
4. Anthers are placed on agar solidifed basal MS or White or Nitsch and Nitsch medium.
5. The culture are kept initially in dark. After 3-4 weeks, the anthers normally andergo pollen embryogenesis and haploid plantlets appear

from the cultured anther. In some cases, anther may under go proliferation to form callus tissue which can be induced to differentiate into haploid plants.

6. At this stage, the cultures are incubated at 24-28^{o} C in a 14 hrs. daylaight regime at about 2,000 lux.
7. Approximately 50 mm tall plantlets are freed from agar by gently washing with running tap water and then transferred to small pot containing outoclaved potting compost. Cover each plantlet with a glass beaker to prevent desiccation and maintain in a well lit humid green-house. After one week, remove the glass beakers and transfer to larger pots when the plants will mature and finally flower.

3.2.3.3.4 Protocol for Pollen Culture:

Isolated pollen can be cultured by two methods –

Method 1:

This method is described here for the culture of isolated pollen of tobacco. This technique can be considered as the basic protocol for pollen culture and involves the following steps:

1. Selection of suitable unopened flower bud, sterilization excision of anther with out filament are the same as described previously in anther culture.
2. About 50 anthers are placed in small sterile beaker containing 20 ml of liquid basal medium (MS quality or Nitsch and Nitsch).
3. Anthers are then pressed against the side of the beaker with the sterile glass piston of a syringe to squeeze out the pollens.
4. The homogenized anthers are then filtered through a nylon sieve (pore diameter 40μ - 60 μ) to remove the anther tissue debris.
5. The filtrate pollen suspension is then centrifuged at low speed (500 – 800 revolution per minute) for 5- minutes. The supernatant containing fine debris is discorded and the pellet of pollen is

suspended in fresh liquid medium and washed twice by repeated centrifugation and resuspension in fresh liquid medium.

6. Pollens are mixed finally with measured volume of liquid basal medium so that is males the density of $10^3 – 10^4$ pollens/ml.
7. 2.5 ml of pollens suspension is pipetted off and is spread in 5 cm petridish. Pollens are best grown in liquid medium but, if necessary, that can be grown by plating in very soft agar added medium. Each dish is sealed with cellotape to avoid dehydration.
8. Petridishes are incubated at $27^o – 30^o$ C under low intensity of white cool light (500 lux, 16 hrs.)
9. Young embryoids can be observed after 30 days. The embryoids ultimately give rise to haploid plant lets.
10. Haploid plantlets are then incubated at $27^o – 50^o$C in a 16 hrs day light regime at about 2000 lux. Plantlets at maturity are transferred to soil as described in anther culture.

Method 2:

This method is known as nurse culture technique. Sharp et.at (1972) first introduced this method. The steps are given below:-

1. Selection of flower bud, sterilization, excision of anther, isolation of suitable pollen are the same as described previously.
2. In this method, the intact anthers are placed horizantally on the top of solid or semisolid basal medium with in a conical flask.
3. A small filter paper disc is placed over the intact anther and about 10 pollen grains in suspension are then placed on the filter paper disc. Hence the intact anthers re considered as the nurse tissue. A control set is also prepared in exactly. The same way except that the pollen grains on filter paper are directly kept on solid medium. Some times, callus tissue derived from any part of the plant is used as nurse tissue.
4. With this method, pollen grains in the control set did not grow at all. The pollen grains kept on nurse tissue grow and form a culture of

green parenchymatus tissue in two weeks. Such tissue ultimately form the haploid callus tissue.

3.2.3.3.5 Advantages of Pollen Culture Over Anther Culture:

fig 3.

It has been already proved that anther culture is an efficient way for the production of haploids from the microspores present with in the intact anther. In this process, there is always the possibility that somatic cells of the anther that are diploid, will also respond to the culture condition and so produce unwanted diploid calluses or plantlets. Sometimes, the development of microspore inside the anther may be interrupted due to growth inhibiting substances leaking out of the another wall in contact with nutrient medium. In attempts to avoid these problem, the culture of free pollen has been investigated. The culture of pollen offers the following additional advantages.

(i) Over crowding of pollen grain in anther is eliminated and isolated pollen grains are equally exposed to nutrient medium.

(ii) Unwanted growth of the anther wall and other associated tissue are eliminated.

(iii) The steps of androgenesis can be observed starting from single cell.

(iv) Various factor governing androgenesis can be better regulated.

(v) Pollen is ideal for uptake, transformation and mutagenic studies as pollens can be uniformly exposed to chemicals and physical mutagens.

(vi) Pollen may be directly transformed into an embryoid. So it is very suitable for understanding biochemistry and physiology of androgenesis.

(vii) Higher yields of haploid plants per anther could be expected in pollen culture than the anther culture.

3.2.3.5 Haploids from Unpollinated Ovaries:

Production of haploid individual by development of an unfertilised egg-cell as a result of deloged pollination is referred to as gynogenesis. Gynogenesis is found in the inter specific cross solanum tuberosum (2η = 4 x) X S. Phureja (2n = 2x) resulting in the production of a dihaploid (2n = 2n) potato. San Noeum (1976) was the first to demonstrate that gynogenesis, an essentially invivo phenomenon, can be induced under invitro conditons. She obtained gynogenic haploids using an ovary culture of Hordeum vulgore subsequently, gynogenic haploids have been obtained from unpollinated ovaries and ovules.

Induction of haploids from female gametophytes may not be inaccessible.

3.2.3.6 Homozygous Plants:

Fig .4

Haploid plants derived from either anther culture or pollen culture are sterile. These plants contain only one set of chromosomes. By doubling their chromosome number the plants can be made fertile and the resultant plants will be homozygous diploid plants can used as pure lines in breading programme.

3.3.3.5.1Colchicine treatment:

Haploids can be diplodized by a number of methods in which colchicine has been utilized widely as a spindle inhibitor to induce chromosome duplication and to produce polyploid plant. This method has been employed for obtaining homozygous diploid plants from haploid culture. The yound, plantlets while still enclosed with in anther are treated with 0.5% colchicine solution from 24-48 hrs. Treated plantlets are replanted in the medium after thorough washing. In case of mature haploid plantlets. 4% colchicine lanoline paste may be applied to the axil of the leaves.

Endomitosis:

It is known that haploid cells are unstable in culture and have a tendency to undergo endomitosis i.e chromosome duplication with out nuclear division. The property can be used for obtaining homozygous deploid plants. In this process, a small explant of stem from a haploid plant is cultured on auxin-cytokinin added medium where the segment forms the callus tissue. During callus growth diploid homozygous cells produced by endomitosis. Now large number of isogenic diploid plants can be obtained by organogenesis.

3.2.3.5.2 Fusion of Pollen Nuclies:

Homozygous diploid callus or embroyoids may form by the spontaneous fusion of two similar nuclei of the cultured pollen after first division. In Brassica the frequency of spontaneous nuclear fusion in microspore is high in culture.

3.2.3.6 Significance of Haploid Plants:

In a diploid cell the chromosomes exist in homologous pairs. The genes for specific characters are also formed in pairs which are known as allelic gene pairs. For an example. T gene (for tallness) is an allele of T gene (for dwarfness) and viceversa in heterozygous conditon. Each allele is location on one pair of homologous chromosome at a particular gene locus. Although each allele controls the same genetic trait (height of the plant). Yet, they, may control a contrasting phenotypic expression (tall / dwarf) of that trait. In heterozygous condition, the activity of only one of the alleles is expressed phenotypically, the allele is said to be dominant (suppose T gene). On the other hand, the activity of the other allele which is not expressed phenotypically until it is seperated from dominant allele, is said to be recessive (suppose T gene).

A chromosome contains a number of dominant and recessive genes whose allelic forms are present on the homologous parther chromosome at the same gene loci. In heterozygous diploid only dominant alleles are expressed phenotypically. Homdogous chromosme seperated during meiosis. Pollen grains receive only one set of homologous chromosomes.

As a result in pollen-derived haploid plants, all the recessive genes, along with dominant genes, will be expressed phenotypically as there is no masking of recessive genes will be doubled at their respective loci. So, even in diploid condition, all the recesive genes, along with dominant genes. Since the haploid plants are sterile, diploid fertile plant can be made by doubling the same existing chromosome. As a result, the dominant as well as recessive genes will be doubled at their respective loci. So, even in diploid conditon, all the recessive genes will be doubled at their respective loci. So, even in diploid condition, all the recessive genes will be expressed phenotypically. Such diploid plant is also called homozygous plant or isogenic diploid plant with homozygous diploid plant. One can identify the recessive characters which are not possible to identify in heterozygous condition.

Crossing over is an essential feature in the maiotic cycle by which random exchange of genetic material (genetic recomination) between two homologous chromatids takes place. The exchange is of great importance because if produces a new gene combination. As a result, four haploid nuclei, produced from a single diploid nucleus, differ from one another. Therefore, haploid plants, derived from four haploid pollens of a pollen tetrad, are significant because the plants will differ genotypically.

In haploid plants, each chromosome is represented only once and this is the reason there is no zygotene pairing in first meiotic division. Thus all the chromosomes appear as univalent. During Anaphase 1 each chromosome moves freely and form generally more than two groups. Gametes with less than the haploid number one generally not viable, therefore haploid plants are highly sterile.

3.2.3.7 Importance and Implication of Anther and Pollen Culture:

In vitro anther and pollen culture for the production of haploid and homozygous diploid have proved an important fool for fundamental and

applied plant biology. Haploids are very important for study of fundamental genetics in higher plants because all the recessive genes remain uncovered and the recessive. Genes remain uncovered and the recessive gene controlled traits are expressed phenotypically in regenerated plants. It is also valuable for mutation study. In addition haploids are very useful for plant breeding programmed.

For plant breeding Programmes:

The importance and implication of anther and pollen culture are given bellow:

Utility of Anther and Pollen Culture for Basic Research

1. Haploids derived from anther and pollen culture are useful in cytogenetic studies.
2. By comparing the heterozygous diploid with haploid or homozygous diploid population, recessive phenotypic characters can be identified very easity.
3. Critical genetic analysis of haploid population derived from individual microspore of pollen tetrad is very useful for the study of genetic recombination in higher plants.
4. The series of cell division and mode of differentation (embryogenesis or organagenesis), starting from single cell (microspore) and ending in whole organism, can be studied under microscope.
5. Double haploid, that are homozygous and fertile, are readily obtained, enabling the selection of desirable gene combition.
6. Culture of isolated pollen provides a novel experimental system for the study of factor controlling pollen embryogenesis of higher plants.
7. Study of meotic behaviour of haploids provides valuable clues to measure chromosome duplication with in a species and for understanding a phylogentic relationalship between species. It also provides information for the interpretation of chromosome homology.
8. Genetic analysis could be performed on haploid population to establish inheritance patterns.

9. Another application involves the use of haploids in the production of monosomics, nullisonics and other aneuploids. This approach has been used in tobacco for the isolation of nullisomics, trisomics and tetrasomics.

Use of Anther and Pollen Culture for Mutation Study:

One unique value of microspore culture lies in the study of somatic cell genetics. In such studies, mutant cell lines are specifically important. Several biochemical mutants have also been reported using haploid cells.

Normally, invivo, the majority of mutation are recessive and therefore, are not expressed in diploid cells in the presence of immutated dominant gene. Haploid callus cells have been employed to stdy the effect of various mutagens, both irradiation as well as chemical. A number of mutant cell lines have now successfully isolated and extensive work is being done to obtain cell lines that are resistant to environmental stresses, herbicides, phytopathotoxin, salts, drought, chilling, various drug, viruses and nematodes etc., salt resistant plans of Datura innoria from a cell line have been selected from haploids derived from anther culture.

In Ginkgo, arginine – requiring strains from pollen culture have been obtained. With the availability of the technique, a large number of biochemical mutants have been isolated in a number of plant species using haploid cell cellline, tissues and complete plants resistant to streptomycin, 5 - bromodeoxyuridine, methionine sulfoximine have been obtained. Nitrate reductase mutants have also been reported in Nicotiana tobacum. Recovery of aurotrops for pantothenate, adenine and nitrate reductase less variants have also been reported from Datura innoxia.

Subjecting young haploid plantlets while emerging from the anther to 1,500 – 3,000 pads of gaumma irradiation a high proportion of mutants have been isolated in Nicotiana tabacum. Alternatively, flower

buds of Nicotional tabacum are irradiated to X-ray and the excised anthers are subsequently cultured. By this process about 50% of haploid plant thus obtained are aberrant phenotypes. Differential radiosensitivity to ultraviolet and gamma-radiation and valine resistant mutants have also been regenerated from haploid cell culture using UV and gamma-irradiation.

3.2.3.8 Summary:

In angiospenns, the haploid a gametophyic phase is extremely brief and represented by microspore or pollen grain present inside the anther. By careful selection of developing anthers at a precise and critical stage, it is possible to establish cultures of a number of species that will give rise to haploid cells. Cultured anthers may give rise to a callus tissue which after subcultures can be regenerated into plantlets. Alter natively in a number of species, the developing pollen grain have been diverted from their normal pathway and have undergone to form haploid embryoids which utimately give rise to haploid plantlets. In 1904 S. Guba & S.C Maheswari first reported the direct development of embryoss from microspores of Datura innoxia by the culture of excised anther.

Androgenesis is the invitro development of haploid plants originating from totipotent pollen grains through aseries of cell division and differentiation.

There are two modes of androgenesis.

(i) direct androgenesis and

(ii) indirect androgenesis

The basic principle of anther culture is that without disturbing the natural habitat and environment of the enclosed anther, pollen can be grown by culturing the intact anther. But there is always the possibility that diploid somatic cells of the anther will also respond to culture condition and so produce unwanted diploid callus or plantlet.

In attempts to avoid this problem, free pollens isolated from the anther are grown innutrient medium. The knowledge gained. So far

from anther and pollen culture has established that pollens at the uninucleate stage just before the first mitosis or during mitosis are most suitable for the induction of haploids. Induction of haploids can be enhanced by keeping the anther or flower bud at low temperature.

In culture, pollen may divide mitotically to form either organised embroid or callus tissue. Both types of development are utilised to form haploid plantlets. The haploid plantlets are self sterile due to presence of single set of chromosome, which are not able to participated in meiotic segregation. By cochicine treatment haploids are made homozygous diploid or isogenic diploid which are fertile.

Haploids are very important for the study of fundamental genetics in higher plants, because all the recessive genes remain unmasked and the recessive gene controlled traits are expressed phenotypically in regenerated plants. It is also valuble for mutation study. In addition, haploids are very useful for plant breeding programmes.

3.2.3.9 Questions:

1. How haploid one produced in tissue culture mention the significance of haploids.
2. Describe the method & discuss the importance & implication of pollen culture.
3. Describe the method for the development of androgenic haploids from another & pollen culture. Discuss the importance of such plants.

3.2.3.10 Reference Books:

1. An introduction to plant tissue culture -
   Dr. M.K. Razdan, Oxford & IBH publishing Co-Pvt. Ltd.
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   Narayana Swamy, Tata Mcgrow-Hill
3. An introduction to Plant tissue Culture –
   Dr. Kalyan Kumar De, New Central Book Agency

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Lesson 3.2.4

Protoplast Isolation, Culture and Fusion

Objective

In this chapter we have clearly explained the different procedures of protoplasts isolation and fusion techniques

3.2.4.1 Introduction

Protoplast fusion is a physical phenomenon. During fusion, two or more protoplasts come incontact and adhere with one another and fuse either spontaneously in presence of fusion inducing chemicals.

Spontaneous fusion takes place among the similar parental protoplasts. It is strictly intraspecific fusion and gives rise to homokaryon. Spontaneous fusion has no practical use. Different steps and processes involved in protoplasts isolation were given below

1. Direct method

In this method the leaf segments are treated with macerozyme and cellulase simultaeneously. The enzyme mixture consists of 0.5% macerozyme 2% cellunate is 13% sorbitol or mammitol at pH 5.4. In this method the leaf segments are incubated over night with enzyme mixture at 25^{o}C and teased gently to liberate the protoplasts. The mixture is filtered through a fine wire gauze to remove leaf debris and then they are centrifuged at 100 g for 1 minute. The protoplasts form a pellet and the supernatant is removed. The process is repeated thrice and the protoplasts are washed with 13% sorbitol solution, which is later replaced by 20% sucrose solution and centrifuged at a speed of 200g for min. a pellet of clean protoplasts can be obtained.

2. Sequential method

This is a two step method. In the first step the cells are isolated using macerozyme (or) pectinase which dissolves the middlelamella. Then the cells are treated with cellulase, which dissolves the cell wall and the protoplast are released. In this method, the leaf segments are incubated with mixture A. (0.5% macerozyme + 0.3% potassium dexstran sulphate in 13% mannitol at pH 5.8). Then they are vacuum filtrated for 5 min., then transferred to a waterbath at 25^{o}C and it is subjected to slow shaking. After 15 mts, the enzyme mixture is replaced by fresh enzyme mixture a and the leaf segments are incubated for another hour. The mixture is filtered through nylon mesh, centrifuged at 100 g perminute and washed thrice with 13% mannitol to get a pure sample of isolated cells. These cells are then incubated with enzyme mixture B (2% cellulase in 13% solution of mannitol at pH 5.4) for 90 min at 30^{o}C. After incubation the mixture is centrifuged at 100 g for 1 min. the protoplasts form a pellet, which is cleaned thrice.

3.2.4.2 Purification of isolated protoplast

The isolated protoplasts are present in the media together with cell debris and broken cell organellae. So they are to be purified from this mixture. Two commonly used methods for purification of protoplast is described here.

a. Floatation

The protoplasts are lighter than the cell debris. So gradients may be used, which will allow the protoplasts to float and the cell debris to sediment. A concentrated solution of mannitol, sorbitol and sucrose may be used as a gradient and the crude protoplast suspension may be centrifuged in this gradient at 75g per minute. The protoplasts can be pipetted off from the top of the tube after centrifugation. This method causes less damage to the protoplasts.

b. Sedimentation and washing method

In this method, the crude protoplast suspension is centrifuged at low speed (50-100 g / 5 min). The intact protoplasts form a pellet and supernatant containing cell debris. The pellet is taken and it is resuspended in fresh culture medium and mannitol and rewashed. This process is repeated twice (or) thrice to get a clean protoplast preparation.

3.2.4.3 Viability and plating density of protoplasts

Viability of the protoplasts after isolation and during culture in liquid medium is very important. Cell wall formation, cell division, callus formation, etc. depend up on the viability protoplast.

The most frequently used staining methods for assessing protoplast viability are fluorescein diacetate (FDA), pheno safranine and calcofluor white (CFW).

1. 0.01% FDA is added to the protoplast culture. The chlorophyll from the broken protoplasts fluoresces red. The FDA accumulates with in the plasma membrane and the viable protoplasts fluoresce green (or) white. It should be examined within 5-15 minutes after the FDA treatment, after which FDA dissociates.

2. Phenosafranin (0.1%) detects dead protoplasts which will turn red and the viable protoplasts remain unstained even after 2 hours in the stain solution.

3. CFW (0.1%) detects on set of cell wall regeneration around the plasma membrane of viable protoplasts in the form of a ring of fluorescence.

Minimum plating density

A minimum plating density of protoplasts is required for growth to begin, because protoplasts enrich the culture medium due to cell leakage. The minimum plating density for Tobacco is $5x10^3$ to $1x10^5$ protoplasts / cm^3. However, there is a need to culture the individual protoplasts for their requirement in genetic engineering experiments. Different approaches are followed for this purpose.

1. Small value cultures

Small volume cultures of less than 1 cm^3 may be used to reduce the no. of protoplasts although minimum plating density is maintained. Hanging droplets and other similar techniques have been used to reduce the volume.

2. Conditioned media

It is the medium in which the plant cells have been already grown for about 48 hours and the cells are then filtered out. It can be used for growing isolated chloroplasts at lower densities.

3. Feeder layers

Feeder layers of protoplasts can be prepared by plating solid media with protoplasts followed by irradiation, which should inactivate but not kill these proplasts. Protoplasts at lower density (5-50 protoplasts / cm^3) can now be plated on this feeder layer.

3.2.4.4. Protoplast culture and regeneration of plants

For proplast culture Nagata and Takebe medium is used.

Several methods have been described for the culture of protoplasts, such as droplet culture, coculture, feeder layer, hanging droplet, bead culture, Bergmann's plating technique, multidrop array technique etc.

1. Drop let culture

Suspending protoplasts in liquid culture media are placed on petridishes in the form of droplets with the help of micropipette. This method enables the microscopic examination of protoplast development. In this method cultured protoplasts clump together at the centre of droplets.

2. Co culture

Sometimes fast growing protoplast is mixed in varying ratio with the less fast growing protoplast. The mixed protoplasts are plated in solid medium as described previously. The fast growing protoplast provides some growth factors which induces the growth and development of the desirable protoplasts. This is known as co culture technique.

3. Feeder layer technique

Fast growing protoplasts are some times made mitotically blocked by low doses of x-ray treatment. Such irradiated protoplasts are plated with agar medium. Up on this thin solidified layer of irradiated protoplast, desirable protoplasts are again plated at a low density with agar medium. So, there will be two agar layers containing irradiated protoplast in the lower layer and desirable protoplast in the upper layer. The lower irradiated protoplast is known as feeder layer which improves the growth and development of normal protoplasts even at lower density.

4. Hanging –drop method

Culture of protoplasts in an in an inverted liquid droplet (0.25-0.50µl) is known as hanging droplet method. Each droplet contains very small groups of protoplasts. A no. of droplets are placed on the inner surface of the lid of a petridish. Very thin layer of water is kept on the lower part of the petridish to make a humid condition inside the petridish as well as to prevent the dessication of the droplets. This technique facilitates to observe the development of protoplast under microscope. Protoplasts also gets better aeration as they go down to the hanging surface of the droplets.

5. Bead culture (or) immobilized culture

In this method the protoplast suspensions are mixed with several polymers like alginate, carrageenan, and melted standard difco agar. Small beads are made by dripping the mixture in to liquid medium. After that, beads in liquid medium are kept on a moving shaker. This entrapped protoplast culture has shown several advantages over static liquid culture (or) slowly moving liquid culture where they will suffer from mechanical breakage. This technique increases the mechanical stability, aeration and viability with biochemical activity.

Wall formation, cell division and callus formation

The viable protoplast in culture regenerates its own wall around them. After the formation of wall, the protoplast becomes a

regenerated cell. The protoplast remains naked for 10 minutes (or) a day. It depends up on the species. The onset of cell wall regeneration can be detected by staining with 0.1% calcofluor white (CFW) fluorescent stain. Cell wall regeneration is a prerequisite for nuclear and cell division. After the formation of cell wall, the walled cells expand and divide in to two cells. The protoplasts, Which are capable of dividing, undergo first division within 2-7 days and form multicellular colonies after 2-3 weeks. All the cells derived from the protoplasts do not divide and form the cell colonies. The percentage of cells which give rise to cell colonies is known as plating efficiency (or) colony forming efficiency. Several factors like genotype of the donorplant, culture medium, hormones and also some physical factors are important for the division of protoplast and callus formation. The small callus mass can be subcultured at regular intervals and can be subcultured at regular intervals and can be used for organogenesis.

For sub-culturing the plate containing dividing protoplasts are sliced in to several agar blocks. Each block is transferred to the surface of fresh medium. The plasmolyticum level in the culture medium is progressively reduced to zero by repeated subculturing.

Plant regeneration

The ultimate objective in protoplast culture is the reconstruction of plant from the single protoplast. The first step in regeneration of plants involves the transfer of callus to a medium capable of initiating differentiation which is species specific. It is noticed that plant regeneration occurs very easily in some plant species while others are recalcitrant. Plant regeneration from protoplast derived callus tissue have been reported mainly from solanaceous species. In recent years non-solanaceous sps. Like carrot, alfalfa, cassava, millet, clover, rape seed, asparagus, cabbage, citrus etc. are found to be capable of plant regeneration from protoplasts.

Properties of isolated protoplasts

1. Freshly isolated protoplasts in plasmolyticum solution are always spherical in shape.

2. The protoplasts are highly fragile in structure.

3. Newty released protoplasts show high pinocytic activity. This property helps to introduce foreign particles like DNA, cell organellae, virus, bacteria etc. in to the protoplasts.

4. Plant protoplast carry a negative surface charge. Depending on the species, this charge may vary from –10 to –30 mv.

5. Newly formed protoplast begins to synthesize a new cell-wall immediately after washing and enzyme removal. Deposition of cellulose microfibril around the plasma membrane can be detected in some plant sps. With in 10 minutes of culture after washing.

6. After the formation of cell wall, it enters the cell division and ultimately forms the callus tissue from which complete plant can be regenerated.

7. Freshly isolated protoplasts do not fuse and they repel each other because they carry similar charges. But in presence of some fusion inducing chemicals like poly Ethylene Glycol in the medium, the protoplasts isolated from distantly related plant sps can be induced to fuse. Such fusion may lead to form a somatic hybrid plant.

3.2.4.5. Protoplast fusion and somatic hybridization

Protoplast fusion (or) somatic hybridization is one of the most important uses of protoplast culture. This is particularly important for hybridization between species (or) genera, which cannot be made to cross by the conventional method of sexual hybridization. So, through this protoplast technology we can overcome the limitation of conventional sexual hybridization. In this technique protoplasts are isolated from any two genotypically different plants are fused to obtain hybrid protoplasts. The hybrid protoplast contains the cytoplasm from both the parental nuclei. So, the invitrofusion of plant protoplasts derived from the somatic cells of the same plant (or) from

two genetically different plants is called somatic hybridization. Sometimes the protoplasts from vegetative cell and gametic cell are fused and such fusion is called somato-gametic hybridization.

Fig-1

Techniques of protoplast fusion

1. Spontaneous fusion

During the isolation of protoplasts for culture, some of the protoplasts lying in close proximity, fuse and this phenomenon is called spontaneous fusion. Simply physical contact is sufficient to bring about the spontaneous fusion among the similar parental protoplasts. During the enzyme treatment for the isolation of protoplasts, it is found that protoplasts from adjoining cells fuse through their plasmodesmata to form a multinucleate protoplast. Electron microscopic studies have shown that as the cell walls are enzymatically degraded, the plasmodesmatal connection between the adjacent cells enlarge due to the removal of its constriction and the enlargement of pit fields. Gradually, the greater enlargement of plasmodesmata allow the entry of organellae in to neighbouring cells. Finally a complete coalescence of adjacent cell takes place. The spontaneous fusion is strictly intraspecific and gives rise to homokaryon. Spontaneous fusion can also be induced by bringing the protoplasts into intimate contact through micropipettes. There seems to be a correlation between the size of the leaf and the percentage of protoplasts undergoing spontaneous fusion. Protoplasts from young leaves are more likely to undergo this fusion.

Eg: Fusion of the microsporocyte protoplasts of Lolium longiflorum and Trillium kamtschaticum.

Spontaneous fusion is important in the studies of the nature and the function of plasmodesmata, the physiology and control of mitosis in multinucleated cells and nuclear fusion spontaneous fusion has some practical importance in chromosome doubling.

Induced fusion

Fusion of freely isolated protoplasts from different sources with the help of fusion inducing chemical agents is known as induced fusion. Normally, isolated protoplasts do not fuse with each other because the surface of the isolated protoplast carries negative charge (-10 mv to –30 mv) around the outer surface the plasma membrane and thus there is a strong tendency for the protoplasts to repel each other due to the same amount of charge possessed by them. So for the initiation of fusion, a fusion inducing chemical agent (or) a system which reduces the electronegativity of the isolated protoplasts and allow them to fuse with each other is necessary. This induced fusion is a highly important and a valuable technique because the protoplasts from widely different and sexually in compatible plants can be fused by this procedure. This technique has the ability to combine different genotypes beyond the limits imposed by sexual process. The main objectives of somatic hybridization are mainly based on induced protoplast fusion.

The isolated plant protoplasts can be induced to fuse by three ways. Viz.

1. Mechanical fusion
2. Chemical fusion
3. Electrofusion

1. Mechanical fusion

In this method, the isolated protoplasts are brought in to intimate physical contact mechanically under a microscope with the help of a micro manipulator and perfusion micropipette. This micropipette is partially blocked at its tip within 1 mm length by a sealed glassrod.

By doing so the protoplasts are retained and compressed by the flow of liquid. Occasional fusion of proto plasts has been observed by this technique.

2. Chemofusion

Many chemicals have been used to induce protoplast fusion. Sodium Nitrate, play ethylene glycol (PEG), calcium ions (Ca^{+2}), polyvinyl alcohol, etc. are the most commonly used chemical fusogens. Generally chemofusion techniques are followed in most of induced fusion experiments. Chemical fusogens cause the isolated protoplasts to adhere to one another and leads to tight agglutination followed by fusion of protoplast. The adhesion of isolated protoplasts takes place either due to the reduction of negative charges of protoplasts or due to attraction of protoplasts by electrostatic forces caused by chemical fusogens.

Different chemical fusion procedures have been proposed from time to time to improve the fusion frequency and reproducibility of the fused product. Each method has its own merits and demerits. Some chemofusion methods are described here.

a. Fusion induced by sodium (or) potassium nitrate

In this method, equal densities of protoplast from two different sources are mixed and then centrifuged at 100 g for 5 mts. To get a dense pellet. Then the pellet is resuspended in a solution of 5.5% sodium nitrate 10% sucrose. So the suspended protoplasts are kept in a waterbath at 35^{o}C for 30 mts. Fusion of protoplasts takesplace at the time of incubation. The pellet is again suspended in 0.1% sodium nitrate for 5-10 mts. The protoplasts are washed twice with liquid culture medium by repeated contrifugation. Finally they are plated in semisolid culture medium. By using this technique intra and inter specific fusions have been achieved. However, sodium nitrate is toxic to the cell at fusogenic concentration. The frequency of fusion is not very high in this method particularly when highly vacualated mesophyll chloroplasts were used. But it is useful for the protoplasts derived from meristematic cells. This method was successfully utilized for the fusion of protoplasts from shoot tips of Oat and maize seedlings.

b. Fusion induced by calcium ions at high pH

The method involves the centrifugation of protoplasts in a fusion inducing mixture at 37°C in a medium containing high concentration of Ca^{+2} ions at pH 10.5. Equal densities of protoplasts are taken in a centrifuge tube and the protoplasts are spun at 100g for 5 min. Then the pellet is suspended in a fusion inducing solution of 0.05m $CaCl_2$, $2H_2O$ in 0.4m mannitol at pH 10.5 and centrifuged for 30mts. at 50g, after which the tubes are placed in a water bath at 37°C for 40-50 min. This leads to the fusion of 20-50% of the protoplasts. This method is found to be superior to other methods in some plant sps. But the high pH may be toxic for some plant sps.

c. Polyethyleneglycol (PEG) treatment

Protoplast fusion has been successfully achieved in several crops by using polyethyleneglycol as a fusogen. This technique gives high frequency of fusion and involves low cytotoxicity. This technique can be used for the fusion of protoplasts from unrelated taxa. Eg: soybean – tobacco, soybean – maize and soybean – barley.

The agglutimation of protoplasts, during PEG – treatment, can be brought about by the following two methods. (1) When the protoplasts are available in sufficient quantities, 1 ml of culture medium with suspended protoplasts is added to 1 ml. of 56% solution of PEG and the tube is shaken for 5 sec, and the protoplasts are allowed to settle for 10 min, then they are washed with the growth medium and they were examined under the microscope to trace for successful agglutination and fusion.

2. If the protoplasts are available in low quantities, drop cultures can be used. The two types of protoplasts are mixed in equal quantities and 4-6 microdrops are placed in a small petriplate and allowed to settle for 5-10 min. at room temperature. Two to three microdrops of peg are added from the periphery in each petriplate which is incubated for 30 min at room temp. This leads to agglutination of

protoplasts. After PEG treatment, the protoplasts are gradually washed and during this process, most of the fusion is achieved. PEG is then replaced by the culture medium to allow the growth of fused protoplasts.

3. Electro fusion

In this method mild electrical stimulation is used to fuse protoplasts. Here the protoplasts are placed in to a small culture cell containing microelectrodes and a potential difference is applied. Then the protoplasts will line up between the electrodes in the form of a pearl chain. The aligned protoplasts are pushed, with a micromanipulator, at a gentle pace through a narrow gap between the two electrodes. When the two protoplasts that are to be fused are appropriately oriented opposite the electrodes and a short electric pulse of high voltage (100 kv/min) is applied, results in the electric breakdown of membrane and subsequent fusion of the protoplasts. The high voltage creates transient disturbances in the organization of plasma membrane which leads to the fusion of neighbouring protoplasts. The entire operation is carried out manually in a specially designed equipment, called electroporator, under a microscope. Many workers feel that this fusion technique is more desirable than the other techniques and highly effective for all most all the systems.

Fig-2

3.2.4.6 Mechanism of protoplast fusion

The mechanism of protoplast fusion is not fully known. Many explanations have been put forward to understand the mechanism of protoplast fusion. Some important explanations are,

1. When the proplasts are brought closer, changes will be induced in the electrostatic potential of the membrane which results in fusion.

2. The fusogens cause distrabance in the intramembranous proteins and glycoproteins of the protoplasts. This increases membrane fluidity, and when the protoplasts are closely adhered, creats a region where lipid molecules intermix allowing coalescence of adjacent membranes.

3. The protoplasts carry negative charge mainly due to intra membranous phosphate groups. The addition of Ca^{+2} ions reduces the zeta-potential of the plasma membrane and this leads to protoplast aggregation.

4. The highly alkaline solution used in chemofusion induces the intramembranous production of lysophospholipid which may be linked with membranous fusion.

5. the Ca^{+} ions link the –vely charged PEG and membrane surface. On elution of the PEG, the surface potentials are disturbed leading to intermembrane contact and subsequent fusion. In addition to this, the strong affinity of PEG for water may cause local dehydration of the membrane and increase in fluidity, thus inducing fusion.

6. PEG induces protoplast aggregation and α - tocopherol present as an impurity in commercial grade PEG, promotes membrane fusion.

1. Hybrid identification

Identification of the fused protoplasts is necessary to quantitate fusion frequency and to monitor the fusion products. The preliminary identification of fusion product is done under microscope. The microscopic identification is based on differences between the parental cells with respect to pigmentation, presence of chloroplast, nuclear staining, cytoplasmic markers etc. For example, the protoplasts of one species may be green and vacuolated, while those of the other species non vacuolated and nongreen. So, the protoplast fusion products between these two species can be easily identified. If both types of parental protoplasts look alike then the fusion product can be distinguished using nuclear staining technique. Sometimes a hybrid cellcontains two nuclei of the parental protoplasts. Such dikaryotic cells can be identified using aceto-caramine (or) aceto-orcein staining procedure. But the presence of two parental nuclei in the hybrid cell

i.e this heterodikaryotic condition, can be more precisely be distinguished using carbol-fuschin stains. This stain stains the two parental nuclei differently. For the identification of heterokaryons (or) fusion products non-toxic fluorochromes are also used.

Eg: Fluorescein Isothio Cyanate (FITC)

Rhodamine Isothiocyanate (RITC)

Rhodamine B.

The advantage of using fluorochromes for the identification of fusion products is that it doesnot depend up on the types of protoplasts being used. For example, the hetero karyons between FITC labeled suspension cell protoplast and unlabelled mesophyll protoplast exhibit an apple green fluorescene of FITC and red fluorescence from chlorophyll of the mesophyll partner.

2. Selection of hybrid cells

The protoplast suspension recovered after the treatment with a fusion inducing agent consists of the following cell types: unfused rpotoplasts of both the parental species, fusion products the same species (homokaryons), fusion products of both the species (heterokaryons). In somatic hybridization experiments only the heterokaryotic (or) hybrid protoplasts resulting from the fusion between the protoplasts of two parental species are of interest. They form only a small portion of the population (0.5%-10%). Therefore, an effective strategy has to be employed for identification and isolation of somatic hybrids. This step is called selection of hybrid cells.

A number of strategies have been used for the selection of hybrid protoplasts. (1) Some visual markers like pigmentation of the parental protoplasts may be used for the identification of hybrid under a microscope. These are then mechanically isolated and cultured. For example, the protoplasts at one species may be green and vacuolated (from mesophyll cells), while those of the other may be non-vacuolated and non-green (from cell cultures). When such features are not available, the protoplasts of two parental species may be separately labeled with different fluorescent agents.

Some procedures have been devised for selecting the hybrid cells. These procedures exploit some properties of parental species which are not expressed in the hybrid cells due to complementation between their genetic systems. The examples for such properties are sensitivity to culture medium constituents, antimetabolites, temperature etc., inability to produce essential biochemicals, chlorophyll (or) some other pigmentation.

These selection procedures can be exemplified as follows:

1. Use antibiotics

In case of petunia, the protoplasts of Petunia hybrida form calli on the MS medium and the protoplasts of Petunia parodii produce only small cell colonies. Actinomycin-D (1 μg/ml) inhibits cell division of P.hybrida protoplasts, but it has no effect on the protoplasts of P. parodii. Thus protoplasts of both these Petunia species fail to produce macroscopic colonies on MS medium supplemented with 1 μg/ml actinomycin d. However, their hybrid cells divide normally on this medium to produce macroscopic colonies. This selection strategy exploits those natural properties of the two parental species which show complementation in the hybrid cells and permits their selection.

In case of Nicotiana Sylvestris Kanamycin resistant cell line has been used as a genetic marker to identify the fusion products between N-Sylvestnis and N.Kinghtiana. Streptomycin resistant mutant of N.tobalcum are also used to recover the interspecific hybrids with N.Sylvestris cycloheximide resistant cell line of Daucus carota can be used as marker for the fusion with albino cell line of D. carota.

2. Use of auxotrophic mutant

Nutritional (or) auxotrophic mutant is the most suitable material because the hybrid can be selected at the cellular level and

plant regeneration is not an essential part of this selection procedure. Auxotrophic mutants have been successfully used to isolate hybrid protoplast in spherocarpus donnelii. Hybrids obtained by the fusion of protoplasts from nicotinic acid and glucose requiring mutants are selected on minimal media. The regenerated hybrid plants are identified on the basis of morphology and karyotype. Nutritional mutants.

3. Use of metabolic mutants

Many nitrate reductase deficient mutants have been obtained from mutagenized haploid cells of Nicotiana tobaccum cultured on medium containing chlorate and with amino acids as the nitrogen source. Cells with nitrate reductase convert chlorate to chlorite which is cytotoxic. The isolated mutants are unable to grow on nitrate containing medium and lack nitrate reductase and other molybdenum – protein containing enzymes. Such mutants may be suitable for hybrid selection.

Chlorophyll deficient mutants have been selected from haploid cells of Datura innoxia after irradiation.

Metabolic mutants are reported in Arabidopsis and a proline requiring mutant is reported. In case of Nicotiana Plumbagenifolia, Threonine deaminase and nitrate reductase deficient mutants have been obtained from haploid plants.

4. Isoenzyme analysis

Isoenzymes are multiple molecular forms of an enzyme with similar (or) identical substrate specificity occurring with in the same organism. Isoenzymes are multiple molecular forms of an enzyme with similar (or identical substrate specificity occurring within the

same organism. Isoenzyme analysis can be used to verify hybridity. Isoenzymes of different constitutive enzymes exhibit the unique banding pattern (or) zymograms in polyacrylamide gel electrophoresis. The number of bands and Rf value of isoenzyme are constant and specific for each parental plant species. The summation (or) intermediate banding pattern of isoenzyme may be found in the hybrid callus tissue. This analysis helps to select hybrid cells.

5. Use of herbicides

Plants prossess differences in their capacity to metabolize herbicides. This property is utilized for selection. For example rice plants are resistant to propanil (3,4-dichloropropionalide). This resistance is based on the ability of rice cells to metabolize propanil.

6. Chromosome analysis of hybrid

Chromosome preparation from actively growing small cell colonies derived from the protoplasts indicates that their karyotype is showing hybrid characterstics. A more general and widely applicable strategy, is to culture the entire protoplast population without applying any selection for hybrid cells. All the types of protoplasts form calli the hybrid calli can be identified on the basis of callus morphology, chromosome constitution, protein and enzyme banding patterns etc. In some cases, the identification may be delayed till plants are regenerated. In such case it is desirable to culture the protoplasts in very low densities since the neighbouring colonies may get fused at higher densities. So, they should be cultured in micro drops, each drop containing a single cell. Many workers favour this approach since it doesnot depend on the presence of markers, especially for those species where it is difficult to find the markers.

7. Hybrid isolation

Several selection methods described above are not applicable for the selection of all types of fusion products at the culture level. Various mutant cell lines are used in some selection methods. But those methods are limited by the non-availability of the mutant cell lines in plants. In some cases it is observed that in the fusion product, chromosome elimination may occur. Therefore, the use of mutant (or) genetic complementation may tail in the selection of hybrid produced from widely related sexually incompatible genera. To overcome the limitations of selection methods, recently the fusion products are isolated physically before culturing them. The hybrid isolation methods are:

1. Micropipette technique

In this technique, heterokaryons are isolated from the fusogen treated protoplast suspension, under a microscope using micropipette. Very few hetrokaryons will obtained after a lot of time and effort.

2. Density gradient fractionation of protoplast suspension after fusion

The protoplast suspension after fusion is placed on the top of a density gradients. The gradients are centrifuged at 20^{o}C for 5 mts. at 50-100g. The fused protoplasts will form a band in the intermediate density position. These heterokaryons can be carefully pipetted off using Pasteur pipettes and are examined under a microscope to determine the number. Finally they are washed with liquid culture medium before plating.

3.2.4.7. Post fusion events

After membrane fusion, the cytoplasm and organellae of both the parental protoplasts are intermixed with each other and form a heteroplasmic cytoplasm. This offers an opertunity of obtaining heterozygosity of extrachromosomal material. This fusion differs from a zygote in that there is no strict maternal inheritance of cytoplasmic organellae. In fused protoplasts dikaryotic condition is established. Here one nucleus of each species occurs. Two types of dikaryotic conditions may be observed. The fused protoplasts may be homokaryons (or) heterokaryons. Homokaryons are formed from the fusion of similar parental protoplasts. Heterokaryons are formed by the fusion of dissimilar parental protoplasts. Thus the protoplast population in culture is composed of a mixture of unfused protoplasts and fused homokaryons and heterokaryon. Sometimes more than 2 protoplasts may involve in fusion and produces multinucleate cells which are incapable of undergoing mitosis and further development.

A Hetero Karyon produce either a hybrid (or) a Cybrid. If only nuclear fusion occurs it results in the formation of a hybrid. Some times the nuclei of the heterodikaryotic cell do not fuse and one nucleus of any one of the parents may be eliminated in the subsequent developmental stages. Thus a cybrid cell is produced. It shows the nuclear genome of any one of the parents and the cytoplasm of both the parents.

The hybrid (or) cybrid protoplasts regenerate a wall around them and enter the mitotic cycle. It undergoes mitotic divisions and forms callus tissue.

1. Regeneration of hybrid plants

Complete hybrid (or) cybrid plants can be regenerated from the callus tissue. But, plant regeneration has been achieved successfully in a small number of species and is mainly confined to some interspecific sexually compatible species. In sexually incompatible

species and in some sexually compatible sps., the regeneration is very limited. This phenomenon is called "somatic incompatibility". The hybrid plants must be atleast partially fertile, in adition to having some useful property, for using them breeding schemes.

2. Somatic hybrids and cybrids

Somatic hybridization produces either symmetric (or) asymmetric hybrids.

a. Symmetric hybrids

Some somatic hybrid plants retain the full (or) nearly full somatic complements of the two parental species. These are called symmetric hybrids. These hybrids provide unique opportunities for synthesizing new species. Some of the symmetric hybrids between distantly related sexually incompatible species are sterile, they cannot be used in breeding programmes. This problem can be overcome by producing 3n somatic hybrids by fusing somatic (2n) cells of one species with haploid (n) cells of the other species such 3n plants are expected to be partially fertile. These somatic hybrids can now be used in breeding programmes for limited gene / chromosome introgression from the species contributing the haploid protoplast.

The fusion of somatic hybrid protoplast with that from one of its parents is called "somatic back hybridization". This is an approach to the improvement of useless somatic hybrids. For example, non-flowering somatic hybrid Daucus carota + Aegopodium podagraria. Here the protoplasts from the hybrid are fused with those of one of its parental species. When the protoplasts from the above hybrid are fused with carrot protoplasts, the resulting somatic hybrid produced flowers. Such hybrids can now be used in breeding programmes with the aim of gene / chromosome introgression.

Some of the symmetric hybrids express useful genes ad are fertile. These can be used in breeding programmes. For example, the protoplasts of Brassica napus (genomes AACC) were fused with a phomalingam resistant cultivar of Brassica nigra (genome BB). (P. lingam, the causal organism of black by disease the somatic hybrids thus obtained had the genomic constitution AABBCC and were resistant to P. Lingam. These hybrids are being used in the conventional breeding programmes to develop P.lingam resistant cultivars of rapeseed. In case of Tomato, somatic hybrids produced with some of its wild relatives are being exploited in conventional breeding. Similarly in case of potato somatic hybrids are produced with some of its wild, relatives viz. Solanum brevidens, S. phureja and Lycopersicon pennellii. The resulting somatic hybrids are resistant to potato leaf roll virus, potato virus-Y, and erwinia soft rot and have other useful traits which are being used in breeding programmes.

An example of useful symmetric hybrids is provided by Datura hybrids, D.innoxia + D. discolor and D. innoxia + D. stramonium. These somatic hybrids show heterosis for alkaloid content, which is 20-25% higher than those in the parent species. So, they can be used in breeding programmes for developing a Datura species for industrial production of scopolamine. All the examples of symmetric hybrids cited above involve the species which donot produce sexual hybrids. Somatic hybrids must be atleast partially fertile for using them in breeding programmes. Generally somatic hybrids would be used for the transfer of useful genes,

Eg: disease resistance, resistance to abiotic stresses etc., from wild species in to cultivated species.

But some somatic hybrids, for example Datura hybrids, show sufficient promise for developing a novel crop species.

2. Asymmetric hybrids

Many somatic hybrids exhibit the full somatic complement of one parental species, while all (or) nearly all chromosomes of the other parental species are lost during the preceding mitotic divisions. Such, hybrids are referred to as asymmetric hybrids. Such hybrids are likely to show a limited introgression of chromosome segments from the eliminated genome due to due to drastically enhanced chromosomal aberrations and mitotic crossing over invitro. Asymmetric hybrids can be obtained even from those combinations which normally produce symmetric hybrids by the following approach: protoplasts of one of the parental species are irradiated with a suitable dose of x-rays (or) gammarays to induce extensive chromosome breakage. In such conditions, chromosome segment introgression may be markedly enhanced. The asymmetric hybrids are essentially cytoplasmic hybrids (or) cybrids except for the introgressed genes.

Asymmetric hybrids are more promising for practical application as they can provide an opportunity for the introgression of few desirable genes from one fusion partner in to the other. Many examples of such gene transfers have been reported. Viz. r DNA genes, genes for isozymes, nitrate reductase genes etc. For example, protoplasts of a nitrate reductase mutant of Nicotiana plumbaginifolia were fused with irradiated protoplasts of Atropa belladoma and the somatic hybrids were selected on nitrate medium. The hybrids showed 4 or 6 genomes of N. plumbagenifolia plus 6-26 chroosomes of A. belladonna. Many of these asymmetric hybrids were self-fertile. After two back crosses with N. Plumbagenifolia plants having diploid complement of N. Plumbagenifolia and 1-2 A. belladonna chromosomes were recovered. They contained the nitrate reductase gene and r-DNA repeats of A. belladonna, many clones also showed one or more isozymes of A. belladonna. The degree of chromosome elimination doesnot appear to depend on the irradiation dose. It seems to depend mainly on the species combination used in the fusion. Asymmetric hybrids seem to possess multiple genomes of non-irradiated parent. In contrast, symmetric hybrids involving the same two parents (protoplasts are not irradiated) usually have only two copies each of their genomes. Thus the feasibility of asymmetric hybrid production and the gene transfer from the irradiated parent have been demonstrated.

3. Cybrids (or) cytoplasmic hybrids

Cybrids are the cells (or) plants containing the nucleus of one species but the cytoplasm from both the parental species cybrids may be produced by the following ways in the proplast fusion experiments. Viz. 1. fusion of a normal protoplast of one species with an enucleate protoplast of the other species. 2. elimination of the nucleus of one species from a normal heterokaryon 3. gradual elimination of chromosomes of one species from a hybrid cell during subsequent mitotic divisions cybrids may be produced in high frequency by irradiating the protoplasts of one species (with x-rays (or) gamma rays) prior to fusion in order to inactivate their nuclei (or) by preparing enucleate protoplasts (cytoplasts) of one species and fusing them with normal protoplasts of the other species.

The objective of cybrid production is to combine the cytoplasmic genes of one species with nuclear and cytoplasmic genes of another species. But the mitotic segregation of plasmagenes, as evidenced by the distribution of chloroplasts, leads to the recovery of plants having plasmagenes of one (or) the other species only. Only a small portion of the plants remain `cybrid' which would further segregate in to the two parental types.

This provides any unique opportunities:

1. Transfer of plasmagenes of one species into the nuclear background of another species in a single generation.

2. This transfer is possible even in sexually incompatible combinations.

3. Recovery of recombinants between the parental mitochondrial (or) chloroplast DNAs (genomes) and production of a wide variety of combinations of the parental and recombinant chloroplasts with the parental (or) recombinant chloroplasts with the parental (or) recombinant mitochondria.

4. When eybrids are produced by irradiating the protoplasts of one species prior to fusion, they provide additional opportunity for the recovery of chromosome segment introgressions from the lost genome, in combination with variations in the plasmon. The cybrid approach has been used for the transfer of cytoplasmic made sterility from Nicotiana tabacum to N. sylvestris and from retunia hybrids to p.axillaries.

5. The mitochondria from one parental species may be combined with the chloroplasts of the other parental species.

Cybrids contain nucleus from one species and cytoplasm from both the species involved in fusion. This approach has been used to transfer cytoplasmic male sterility and triazine resistance in tobacco, cabbage, rice, potato and petunia.

Cybrids provide opportunity for combining mitochondria of one species with the chloroplasts of another species and generating recombinant organellae. This may be an important objective in case of several alloplasmic lines in which one organellae confers desirable features, while the other has deleterious effects. An alloplasmic line has the nucleus of one species and cytoplasm from a different species. For example, Brassica napus alloplasmic lines produced sexually by repeated back crossing carry radish cytoplasm and Ogura cytoplasm are male sterile due to the mitochondrial genome but show chlorophyll deficiency under low temperature due to chloroplast genome. Cybrids were produced between CMS (ogura cytoplasm) B. napus and male fertile B. napus. Some of the cybrid regenerants were male sterile but did not show chlorophyll deficiency under low temperature because,

these cybrids contained radish mitochondria and B. napus chloroplasts.

3.2.4.8. Advantages and limitations of protoplast culture

Advantages

1. Symmetric hybrids can be produced between species which cannot be produced between species which cannot be hybridized sexually. These hybrids can be used in breeding experiments for the transfer of useful genes to crops (or) may be used as new crop species.

2. Hybrids can be produced even between the strains which are completely sterile.

   Eg: Monoploids

3. Cytoplasm transfer can be effected in one year, while back crossing may take 5-6 years. Back-crossing is not possible between some species. In such cases cytoplasm transfers can be made using this approach.

4. Mitochondria of one species can be combined with the chloroplasts of another species. This may be very important in somecases. This cannot be achieved by sexual means exen between easily crossable species.

5. Recombinant organellae genomes, especially of mitochondria are generated in somatic hybrids and cybrids. Some of these recombinant genomes may possess useful features.

Limitations

1. The techniques for the isolation, fusion and culture are not available for many important crop species like many cereals and pulses.

2. In many cases, chromosome elimination occurs from somatic hybrids leading to asymmetric hybrids. Such hybrids may be useful, but there is no control on chromosome elimination.

3. Many somatic hybrids show genetic instability which may be an inherent feature of some species combinations.

4. Many somatic hybrids donot regenerate or give rise to sterile regenerants. Such hybrids are useful for crop improvement. All interfamily somatic hybrids are genetically unstable and morphologically abnormal, while intergeneric and intertribal hybrids are genetically stable but produce abnormal and sterile plants.

5. Conventionally, cytoplasm transfer from one strain into anther is achieved by back crossmethod which requires 6-7 years. This can be done with in one year by using proplast fusion technique.

6. There is no standardized method which is applicable for all material, for the identification, selection and isolation of protoplasts at the culture level.

3.2.4.9. Summary

Protoplast can be isolated from almost every plant species and could be fused with the protoplast of a different species and a hybrid protoplast can be obtained.

This hybrid prtoplast regenerates a cell wall and its two different nuclei fuse together during celldivision and a hybrid cell will be produced. A plant could be regenerated from this cell, it would be a hybrid between the two species involved. This technique is referred as protoplast fusion (or) somatic hybridization.

Isolated protoplasts from different sources generally donot fuse with eachother because they carry negative charges outside and thus repel one another due to their same charges. So a fusion inducing chemical (or) agent is needed to reduce the electronegativity and allow them to fuse with eachother. Sodium nitrate, polyethylene glycol (PEG), calcium ions, polyvinyl alcohol etc. are the most commonly used protoplast fusion inducing chemicals which are commonly known as chemical fusogens. Mild electrical stimulation can also be used to fuse protoplasts. This technique is known as electrofusion.

The exact mechanism of protoplast fusion is not fully known. The possible explanation of PEG induced fusion is that the high molecular bridge connecting the protoplasts and Ca^{+2} ions link the negatively charged PEG and the membrane surface leading to intermembrane contact and subsequent fusion. In addition to this, the strong affinity of PEG for water may cause local dehydration of the membrane and increases fluidity, thus inducing fusion.

Identification of fusion product is essential to estimate the fusion frequency and to monitor the fusion products. The microscopic identification of fusion product is based on differences between the parental cell with respect to pigmentation, presence of chloroplast, nuclear staining, cytoplasmic marker etc. Non-toxic fluorochromes

such as fluorescein isothiocyanate or rhodamine isothiocyanate are often used for the identification of heterokaryon (or) fusion products.

Some types of selection techniques are also required at the level of culture to recover hybrid cells and its callus tissue following fusion. Many selection procedures such as auxin autotrophy, use of genetic complemtnation, use of uncommon aminoacids, use of cell resistance to aminoacid analogues, use of phytotoxin, use of antibiotics, use of auxotrophic mutants, use of metabolic mutants, isoenzyme analysis, use of herbicides, chromosome analysis have been employed to confirm the hybridity. Micropipette technique and density gradient fractionation have also been employed to isolate only the fusion products from the unfused parental protoplast population.

After the fusion of membranes, the cytoplasm and its organellae of both parental protoplasts are inter mixed and dikaryotic condition is established. The fusion of nuclei in a binucleate hetero karyon results in the formation of a true hybrid protoplast.

The fusion of two protoplasts from the same culture results in a homokaryon. Frequently the genetic information is lost from one of the two nuclei. If one nucleus completely disappears, the cytoplasm of the two parental protoplasts are get hybridized and the fusion product is known as cybrid. Certain genetic factors are carried in the cytoplasmic inheritance system instead of in the nucleargenes. Cybrids are useful in plant breeding programmes.

Protoplast fusion and somatic hybridization have opened up a new avenue in plant science. Protoplast fusion provides a method of combining the different genomes of different genera and species, with the potential of overcoming sexual incompatibility barrier between plants. The studies of fusion product can give information about compatibility (or) incompatibility of the nuclei (or) cytoplasm. By protoplast fusion, it is possible to transfer some useful genes such as disease resistance, nitrogen fixation, rapid growth rate, protein quality, frost resistance drought resistance etc. This provides an opportunity for the production of new genetic variation and there by widen the genetic base for plant breeding. In the case of vegetatively

propagating plants the genetic variation can be induced through protoplast fusion. Intergeneric crosses between widely related plants are not possible due to sexually incompatibility. In such cases protoplast fusion technique is used.

3.2.4.10 Model Questions

1. What is somatic hybridization ? what are the advantages of protoplast fusion over traditional methods of sexual hybrization?
2. Discuss the different types of protoplast fusion ? What chemical compounds have been used as fusogen agents ?
3. Describe the procedures of chemical fusion. State the possible mechanisms of protoplast fusion ?
4. What types of procedures can be used for the identification, selection and isolation of hybrid cells ?
5. What is cybrid ? How does this phenomenon occur ? Does it have any significance in the breeding of plants ?
6. Write short notes on –
    a. Protoplast fusion / cell fusion
    b. Somatic hybridization.
    c. Cybrid
    d. Fusion agents

3.2.4.11 Reference books

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Lesson 3.3.1

Biology of cultured cells

Objective:

Objective:

Animal cells behaves in a different way than plant cells in the culture. This chapter includes the growth, behavior and requirements of animal cells in culture. Constituents of serum and serum free media were also explained.

3.3.1.1 Introduction

Animal cells are more difficult to culture than microorganisms because they require many more nutrients and typically grow only when attached to specially coated surfaces. Despite these difficulties, various types of animal cells, including both undifferentiated and differentiated ones, can be cultured successfully.

3.3.1.2 Rich Media Are Required for Culture of Animal Cells

Nine amino acids, referred to as the *essential amino acids*, cannot be synthesized by adult vertebrate animals and thus must be obtained from their diet. Animal cells grown in culture also must be supplied with these nine amino acids, namely, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. In addition, most cultured cells require cysteine, glutamine, and tyrosine. In the intact animal, these three amino acids are synthesized by specialized cells; for example, liver cells make tyrosine from phenylalanine, and both liver and kidney cells can make glutamine. Animal cells both within the organism and in culture can synthesize the 8 remaining amino acids; thus these amino acids need not be present in the diet or culture medium. The other essential components of a medium for culturing animal cells are vitamins, which the cells cannot make at all or in adequate amounts; various salts; glucose; and serum, the noncellular part of the blood.

Serum, a mixture of hundreds of proteins, contains various factors needed for proliferation of cells in culture. For example, it contains insulin, a hormone required for growth of many cultured vertebrate cells, and transferrin, an iron-transporting protein essential for incorporation of iron by cells in culture. Although many animal cells can grow in a serum-containing medium, such as Eagle's medium, certain cell types require specific protein growth factors that are not present in serum. For instance, precursors of red blood cells require the hormone erythropoietin, and T lymphocytes of the immune system require interleukin 2 (IL-2). These factors bind to receptor proteins that span the plasma membrane, signaling the cells to increase in size and mass and undergo cell division. A few mammalian cell types can be grown in a completely defined, serum-free medium supplemented with trace minerals, specific protein growth factors, and other components.

3.3.1.3 Most Cultured Animal Cells Grow Only on Special Solid Surfaces

Within the tissues of intact animals, most cells tightly contact and interact specifically with other cells via various

cellular junctions. The cells also contact the extracellular matrix, a complex network of secreted proteins and carbohydrates that fill the spaces between cells. The matrix, whose constituents are secreted by cells themselves, helps to bind the cells in tissues together; it also provides a lattice through which cells can move, particularly during the early stages of animal differentiation.

The extracellular matrices in various animal tissues consist of several common components: fibrous collagen proteins; hyaluronan (or hyaluronic acid), a large mucopolysaccharide; and covalently linked polysaccharides, proteins in the form of proteoglycans (mostly carbohydrate) and glycoproteins (mostly protein). However, the exact composition of the matrix in different tissues varies, reflecting the specialized function of a tissue. In connective tissue, for example, the major protein of the extracellular matrix is a type of collagen that forms insoluble fibers with a very high tensile strength. Fibroblasts, the principal cell type in connective tissue, secrete this type of collagen as well as the other matrix components. Receptor proteins in the plasma membrane of cells bind various matrix elements, imparting strength and rigidity to tissues. The tendency of animal cells *in vivo* to interact with one another and with the surrounding extracellular matrix is mimicked in their growth in culture. Unlike bacterial and yeast cells, which can be grown in suspension, most cultured animal cells require a surface to grow on. Many types of cells can adhere to and grow on glass, or on specially treated plastics with negatively charged groups on the surface (e.g., SO_3^{2-}). The cultured cells secrete collagens and other matrix components; these bind to the culture surface and function as a bridge between it and the cells. Cells cultured from single cells on a glass or a plastic dish form visible colonies in 10 – 14 days. Some tumor cells can be grown in suspension, a considerable experimental advantage because equivalent samples are easier to obtain from suspension cultures than from colonies grown in a dish.

Primary Cell Cultures Are useful, but Have a Finite Life Span Normal animal tissues (e.g., skin, kidney, liver) or whole embryos commonly are used to establish *primary cell cultures.* To prepare tissue cells for culture (or to remove adherent cells from a culture dish for biochemical studies), trypsin or another

protease is used to destroy the proteins in the junctions that normally interconnect cells. For many years, most cell types were difficult, if not impossible, to culture. But the identification and preparation of various protein growth factors that stimulate the replication of specific cell types, as well as other recent modifications in culture methods, now permit experimenters to grow various types of specialized cells.

Many studies with vertebrate cells, however, still are performed with those few cell types that grow most readily in culture. These are not cells of a defined type; rather, they represent whatever grows when a tissue or an embryo is placed in culture. The cell type that usually predominates in such cultures is called a fibroblast because it secretes the types of proteins associated with fibroblasts in fibrous connective tissue of animals. Cultured fibroblasts have the morphology of tissue fibroblasts, but they retain the ability to differentiate into other cell types; thus they are not as differentiated as tissue fibroblasts.

Some studies are conducted with primary cultures of epithelial cells. In general, external and internal surfaces of tissues and organs are covered by a layer of epithelial cells called an epithelium. These highly differentiated cells are said to be *polarized* because the plasma membrane is organized into at least two discrete regions. For example, the epithelial cells that line the intestine form a simple columnar epithelium. That portion of the plasma membrane facing the lumen of the intestine, the *apical* surface, is specialized for absorption; the rest of the plasma membrane, the *basolateral* surface, mediates transport of nutrients from the cell to the blood and forms junctions with adjacent cells and the underlying extra cellular matrix called the basal lamina. Certain cells cultured from blood, spleen, or bone marrow adhere poorly, if at all, to a culture dish but nonetheless grow well. In the body, such nonadherent cells are held in suspension (in the blood), or they are loosely adherent (in the bone marrow and spleen). Because these cells often come from immature stages in the development of differentiated blood cells, they are very useful for studying normal blood cell differentiation and the abnormal development of leukemias.

When cells are removed from an embryo or an adult animal, most of the adherent ones grow continuously in culture for only a limited time before they spontaneously cease growing. Such a culture eventually dies out after many cell doublings, even if it is provided with fresh supplies of all the known nutrients that cells need to grow, including serum. For instance, when human fetal cells are explanted into cell culture, the majority of cells die within a relatively short time; "fibroblasts," although also destined to die, proliferate for a while and soon become the predominant cell type. They divide about 50 times before they cease growth. Starting with 10^6 cells, 50 doublings can produce $10^6 \times 2^{50}$, or more than 10^{20} cells, which is equivalent to the weight of about 10^5 people. Thus, even though its lifetime is limited, a single culture, if carefully maintained, can be studied through many generations. Such a lineage of cells originating from one initial primary culture is called a cell strain.

Transformed Cells Can Grow Indefinitely in Culture

To be able to clone individual cells, modify cell behavior, or select mutants, biologists often want to maintain cell cultures for many more than 100 doublings. This is possible with cells derived from some tumors and with rare cells that arise spontaneously because they have undergone genetic changes that endow them with the ability to grow indefinitely. The genetic changes that allow these cells to grow indefinitely are collectively called *oncogenic* transformation, and the cells are said to be *oncogenically transformed,* or simply *transformed.* A culture of cells with an indefinite life span is considered immortal; such a culture is called a cell line to distinguish it from an impermanent cell *strain.*

The ability of cultured cells to grow indefinitely or their tendency to be transformed varies depending on the animal species from which the cells originate. Normal chicken cells rarely are transformed and die out after only a few doublings; even tumor cells from chickens almost never exhibit immortality. Among human cells, only tumor cells grow indefinitely. The HeLa cell, the first human cell type to be grown in culture, was originally obtained in 1952 from a malignant tumor (carcinoma)

of the uterine cervix. This cell line has been invaluable for research on human cells.

In contrast to human and chicken cells, cultures of embryonic adherent cells from rodents routinely give rise to cell lines. When adherent rodent cells are first explanted, they grow well, but after a number of serial replatings they lose growth potential and the culture goes into crisis. During this period most of the cells die, but often a rapidly dividing variant cell arises spontaneously and takes over the culture. A cell line derived from such a variant will grow forever if it is provided with the necessary nutrients. Cells in spontaneously established rodent cell lines and in cell lines derived from tumors often have abnormal chromosomes. In addition, their chromosome number usually is greater than that of the normal cell from which they arose, and it continually expands and contracts in culture. Such cells are said to be *aneuploid* (i.e., have an inappropriate number of chromosomes) and are obviously mutants.

Although most cell lines are undifferentiated, some can carry out many of the functions characteristic of the normal differentiated cells from which they are derived. One example is certain hepatoma cell lines (e.g., HepG2) that synthesize most of the serum proteins made by normal hepatocytes (the major cell type in the liver) from which they are derived. These highly differentiated hepatoma cells are often studied as models of normal hepatocytes. Cultured *myoblasts* (muscle precursor cells) are another example of transformed cells that continue to perform many functions of a specialized, differentiated cell. When grown in culture, transformed myoblasts can be induced to fuse to form myotubes. These resemble differentiated multinucleated muscle cells and synthesize many of, if not all, the specialized proteins associated with contraction. Certain lines of epithelial cells also have been cultured successfully. One such line, Madin-Darby canine kidney (MDCK) cells, forms a continuous sheet of polarized epithelial cells of one cell thick that exhibits many of the properties of the normal canine kidney epithelium from which it was derived. This type of preparation has proved valuble as a model for studying the functions of epithelial cells.

Fusion of cultured Animal cells can yield interspecific hybrids useful in somatic-cell genetics cultured animal cells infrequently undergo cell fusion spontaneously. The fusion rate, however, increases greatly in the presence of certain viruses that have a lipoprotein envelope similar to the plasma membrane of animal cells. A mutant viral glycoprotein in the envelope promotes cell fusion. Cell fusion also is promoted by polyethylene glycol, which causes the plasma membranes of adjacent cells to adhere to each other and to fuse As most fused animal cells undergo cell division, the nuclei eventually fuse, producing viable cells with a single nucleus that contains chromosomes from both "parents." The fusion of two cells that are genetically different yields a hybrid cell called a heterokaryon.

Because some **somatic cells** from animals can be cultured from single cells in a well-defined medium, it is possible to select for genetically distinct cultured animal cells, just as is done with bacterial and yeast cells. Moreover, during mitosis the chromosomes in an animal cell are large and highly visible after staining, making it easy to distinguish individual chromosomes. Genetic studies of cultured animal cells are called *somatic-cell genetics* to distinguish them from *classical genetics,* which deals with whole organisms derived from **germ cells** (sperm and eggs).

Cultured cells from different mammals can be fused to produce interspecific hybrids, which have been widely used in somatic-cell genetics. For instance, hybrids can be prepared from human cells and mutant mouse cells that lack an enzyme required for synthesis of a particular essential metabolite. As the human-mouse hybrid cells grow and divide, they gradually lose human chromosomes in random order, but retain the mouse chromosomes. In a medium that can support growth of both the human cells and mutant mouse cells, the hybrids eventually lose all human chromosomes. However, in a medium lacking the essential metabolite that the mouse cells cannot produce, the one human chromosome that contains the gene encoding the needed enzyme will be retained, because any hybrid cells that lose it following mitosis will die. All other human chromosomes eventually are lost.

By using different mutant mouse cells and media in which they cannot grow, researchers have prepared various panels of hybrid cell lines. Each cell line in a panel contains either a single human chromosome or a small number of human chromosomes, and a full set of mouse chromosomes. Because each chromosome can be identified visually under a light microscope, such hybrid cells provide a means for assigning, or "mapping," individual genes to specific chromosomes. For example, suppose a hybrid cell line is shown microscopically to contain a particular human chromosome. That hybrid cell line can then be tested biochemically for the presence of various human enzymes, exposed to specific antibodies to detect human surface antigens, or subjected to DNA hybridization and cloning techniques to locate particular human DNA sequences. The genes encoding a human protein or containing a human DNA sequence detected in such tests must be located on the particular human chromosome carried by the cell line being tested. Panels of hybrids between normal mouse and mutant hamster cells also have been established; in these hybrid cells, the majority of mouse chromosomes are lost, allowing mouse genes to be mapped to specific mouse chromosomes. Hybrid cells often are selected on HAT Medium

One metabolic pathway has been particularly useful in cell-fusion experiments. Most animal cells can synthesize the purine and pyrimidine nucleotides de novo from simpler carbon and nitrogen compounds, rather than from already formed purines and pyrimidines. The folic acid antagonists amethopterin and aminopterin interfere with the donation of methyl and formyl groups by tetrahydrofolic acid in the early stages of de novo synthesis of glycine, purine nucleoside monophosphates, and thymidine monophosphate. These drugs are called *antifolates,* since they block reactions involving tetrahydrofolate, an active form of folic acid. Many cells, however, contain enzymes that can synthesize the necessary nucleotides from purine bases and thymidine if they are provided in the medium; these *salvage pathways* bypass the metabolic blocks imposed by antifolates. A number of mutant cell lines lacking the enzyme needed to catalyze one of the steps in a salvage pathway have been isolated. For example, cell lines lacking thymidine kinase (TK) can be selected because such cells are resistant to the otherwise

toxic thymidine analog 5-bromodeoxyuridine. Cells containing TK convert 5-bromodeoxyuridine into 5-bromodeoxyuridine monophosphate. This nucleoside mono- phosphate is then converted into a nucleoside triphosphate by other enzymes and is incorporated by DNA polymerase into DNA, where it exerts its toxic effects. This pathway is blocked in cells with a *TK* mutation that prevents production of functional TK enzyme. Hence, TK^- mutants are resistant to the toxic effects of 5-bromodeoxyuridine. Similarly, cells lacking the HGPRT enzyme have been selected because they are resistant to the otherwise toxic guanine analog 6-thioguanine. As we will see next, $HGPRT^-$ cells and TK^- cells are useful partners in cell fusions with one another or with cells that have salvage-pathway enzymes but that are differentiated and cannot grow in culture by themselves.

The medium most often used to select hybrid cells is called *HAT medium,* because it contains hypoxanthine (a purine), aminopterin, and thymidine. Normal cells can grow in HAT medium because even though aminopterin blocks de novo synthesis of purines and TMP, the thymidine in the media is transported into the cell and converted to TMP by TK and the hypoxanthine is transported and converted into usable purines by HGPRT. On the other hand, neither TK^- nor $HGPRT^-$ cells can grow in HAT medium because each lacks an enzyme of the salvage pathway. However, hybrids formed by fusion of these two mutants will carry a normal *TK* gene from the $HGPRT^-$ parent and a normal *HGPRT* gene from the TK^-parent. The hybrids thus will produce both functional salvage-pathway enzymes and grow on HAT medium. Likewise, hybrids formed by fusion of mutant cells and normal cells can grow in HAT medium.

Hybridomas Are Used to Produce Monoclonal Antibodies

Each normal B **lymphocyte** in an animal is capable of producing a single type of antibody directed against a specific determinant, or epitope, on an antigen molecule. If an animal is injected with an antigen, B lymphocytes that make antibody recognizing the antigen are stimulated to grow and proliferate. Each antigen-activated B lymphocyte forms a clone of cells in the spleen or lymph nodes, with each cell of the clone producing identical antibody, termed monoclonal antibody. Because most

natural antigens contain multiple epitopes, exposure of an animal to an antigen usually stimulates formation of several different B-lymphocyte clones, each producing a different antibody; a mixture of antibodies that recognize different epitopes on the same antigen is said to be *polyclonal.*

For many types of studies involving antibodies, monoclonal antibody is preferable to polyclonal antibody. However, biochemical purification of monoclonal antibody from serum is not feasible, in part because the concentration of any given antibody is quite low. For this reason, researchers looked to culture techniques in order to obtain usable quantities of monoclonal antibody. Because primary cultures of normal B lymphocytes do not grow indefinitely, such cultures have limited usefulness for production of monoclonal antibody. This limitation can be avoided by fusing normal B lymphocytes with oncogenically transformed lymphocytes called *myeloma cells,* which are immortal.

Fusion of a myeloma cell with a normal antibody-producing cell from a rat or mouse spleen yields a hybrid that proliferates into a clone called a hybridoma. Like myeloma cells, hybridoma cells are immortal. Each hybridoma produces the monoclonal antibody encoded by its B-lymphocyte partner. Many different myeloma cell lines from mice and rats have been established; from these, $HGPRT^-$ lines have been selected based on their resistance to 6-thioguanine as described above. If such mutant myeloma cells are fused with normal B lymphocytes, any fused cells that result can grow in HAT medium, but the parental cells cannot. Each selected hybridoma then is tested for production of the desired antibody; any clone producing that antibody then is grown in large cultures, from which a substantial quantity of pure monoclonal antibody can be obtained.

Such pure antibodies are very valuable research reagents. For example, a monoclonal antibody that interacts with protein X can be used to label, and thus locate, protein X in specific cells of an organ or in specific cell fractions. Once identified, even very scarce proteins can be isolated by affinity chromatography in columns to which the monoclonal antibody is bound (see Figure 3-43c). Monoclonal antibodies also have become important

diagnostic and therapeutic tools in medicine. Monoclonal antibodies that bind to and inactivate toxic proteins (toxins) secreted by bacterial pathogens are used to treat diseases caused by these pathogens. Other monoclonal antibodies are specific for cell-surface proteins expressed by certain types of tumor cells; chemical complexes of such monoclonal antibodies with toxic drugs are being developed for cancer chemotherapy.

3.3.1.4 SUMMARY

- Growth of vertebrate cells in culture requires rich media containing essential amino acids, vitamins, and peptide or protein growth factors, frequently provided by serum. Most cultured vertebrate cells will grow only when attached to a negatively charged substratum that mimics the extra cellular matrix in animal tissues.

- Primary cells, which are derived directly from animal tissue, have limited growth potential in culture and may give rise to a cell strain.

- Transformed cells, which are derived from animal tumors or arise spontaneously from primary rodent cells, grow indefinitely in culture. They usually have an unstable, aneuploid complement of chromosomes, including abnormal chromosomes. Transformed cells derived from a single parental cell are called *cell lines.*

- Cultured cells can be induced to fuse into heterokaryons (hybrids) by treatment with certain viruses or polyethylene glycol. Heterokaryons between cells of different species tend to lose the chromosomes of one species as they divide.

- Panels of hybrid lines prepared from mutant mouse cells and normal human cells, each containing different human chromosomes, can be used to map the gene encoding a specific human protein to a specific human chromosome.

- Fusion of an $HGPRT^-$ myeloma cell and a single B lymphocyte yields a hybrid cell that can grow on HAT

medium and proliferate indefinitely, forming a clone called a *hybridoma.* Since each individual B lymphocyte produces antibodies specific for one antigenic determinant (epitope), a hybridoma produces only the monoclonal antibody synthesized by its original B-lymphocyte parental cell.

3.3.1.5 Model Questions

1)Explain the different conditions required for cell culture.

2) Explain the biology of cultured cells?

3.3.1.6 Reference books

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Lesson 3.3.2

Animal Cell Culture Media And Sources Of Material For Cell culture

Objective :

Objective

Growing of animal cells *invitro* is entirely different from culturing plant cells One should well known about the constituents of media, their properties and their role in animal cell growth. The present chapter includes different media components and their role in animal cell culture.

3.3.2.1 Introduction

With our present knowledge of metabolism it is obvious that there will never be a universal medium for all cell types. Thus, The requirements for plant cells and animal cells have many things in common but are ,on the hole ,quite different .Among animals it is not surprising that the requirement for insect tissues should be different from those for mammalian cells. And although most media for mammalian cells seem to be equally effective for different cell types.

3.3.2.2.Culture media

Even after several years of extensive research. media obtained from natural sources are still widely used for many purposes especially for growing tissue and cells, freshly isolated from the organism. However, they suffer from disadvantages like, their composition is unknown, variable time to time, virtually impossible to reproduce the same conditions exactly from one experiment to the other. To solve this problem, attempts were made to develop a media composed entirely of defined contents by two methods, analytical and synthetic approach.

In analytical method, attempts were made to analyze the medium totally and to know the essential components, but this has not given encouraging results because of the time constraint. In the synthetic method, the metabolites shown to be essential or valuable for the survival and development of the intact organism were combined to form as media, which is being tested for its ability to keep cells alive. However, it is apparent that both the methods of approach were dependent mutually.

Most media in general consists of mixture of substances, which were proved essential, generally beneficial and harmless.

To grow cells *invitro*, culture conditions must mimic in vivo conditions with respect to temperature, oxygen and CO_2 concentration, pH, Osmolality and nutrition. Most basal cell culture media can't, by themselves support the growth of the cells and it is common practice to supplement cell culture media with animal sera. Growing cells in serum free media has many advantages but the ideal general purpose of serum free media has not yet been developed. The main function of cell culture media are to maintain the pH and osmolality essential for cell viability and to provide the nutrients and energy needed for cell growth and multiplication. The temperature, oxygen and CO_2 concentration of the cell cultures must also be controlled.

A complete cell culture medium can be considered to be composed of two distinct parts : (1)A basal medium that satisfies all cellular requirements for nutrients (2) A set of components that satisfy other types of cellular requirements and permit growth of cells in the basal medium.

3.3.2.3 Basal media

The culture media is by far the most important single factor in culturing animal cells. The function of this medium is to provide an environment for survival and also to provide substances required by the cells which they can't synthesize directly. The composition of early tissue culture media was based on biological fluids such as plasma lymph and serum and tissue extracts especially of embryonic origin. Basal tissue culture media were developed only to include nominal components which were essential for growth.

Constituents basal medium

The common constituents of basal medium may be considered as follows

Balanced salt solution

Balanced salt solution (BSSs) have been used since the early attempts at cell culture *invitro*. A BSS is composed of a combination of inorganic salts that maintain physiological pH and osmotic

pressure .In addition to these effects the inorganic ions used have other important physiological roles including the maintenance of membrane potential and as cofactors in enzyme reactions and in cell attachment. The inorganic ions employed are chiefly Na+, K+, Mg^{+2}, Ca^{+2}, Ci-, SO4-$^{-2}$, PO4-$^{-3}$ and HCO^{3-}. When necessary osmolality may be adjusted by modifying the concentration of NaCl.Most BSSs do not contain the nutrients required by cell for long term maintenance and growth although glucose may be included. The four main categories of BSS are

1.Earles balanced salt solution (EBSS)

2.Dulbeccos phosphate –buffered saline (DPBS)

3.Hanks balanced salt solution (HBSS)

4.Eagles spinner salt solution (ESSS)

HBSS and DPBS are intended for use equilibrated with air while EBSS and ESSS requires equilibration with gas phase containing 5% CO_2 inorder to maintain correct pH

Buffering Systems

Culture media need to be buffered to compensate for evolution of CO_2 and production of lactic acid from the metabolism of glucose Media have traditionally been buffered with a bicarbonate buffer, often at a final concentration of 24mm. Bicarbonate forms buffer system with dissolved CO_2 produced by growing cells. However when cells are growing at a low cell density or in a lag phase, insufficient CO_2 may be produced to maintain the required optimal pH For this reason, these cultures need to be maintained at atmosphere of 5-10% CO_2. Bicarbonate is both cheep and non toxic to the cells but its Pka (6.1)results in sub optimal buffering in the physiological range some media are designed to contain low HCO3-but high PO_4^{-3} concentration and there fore do not require incubation in a CO_2 enriched atmosphere. Sodium –beta –glycerophosphte is also used as a buffer in some formulations.

For more effective buffering, with out the need for elevated levels CO_2 a range of organic buffers can be employed. The mostly used of this is Hepes (N-2- hydroxy ethyl piperazine –N1-2 ethane sulphonic acid)Hepes is very effective buffer in the pH range 7.2-7.6 and is most resistant to rapid pH changes than bicarbonate. Some media are buffered with both bicarbonate and Hepes. However Hepes is both expensive and toxic to the cells at concentrations above 100mm.

Energy sources

Carbohydrates are major source energy for cultured cells. Glucose is the most frequently used sugar. Other sugars, e.g. maltose, sucrose fructose, galactose and mannose may also be included. Glutamine can also supply a major proportion of the required energy in some cells .

Amino acids

Most animal cells have a requirement for essential amino acids i.e. those which are not synthesized by the body together with cysteine and tyrosine in addition although individual requirements for amino acids vary from one cell to another Other non essential requirements are often added as well, to compensate either a particular cell type s incapacity to make them or they made, but lost by leakage in to the medium The concentration amino acids limits the maximum cell concentration attainable, and the balance may influence cell survival and growth rate. Glutamine is required by most cells although some cells utilize glutamate, some evidences says that glutamine is also used by cultured cells as a source of energy and carbon.

Vitamins

Several vitamins of B group are necessary for cell growth and multiplication. Many vitamins are precursors for cofactors The vitamins most commonly added to basal media are Para –amino benzoic acid, biotin, choline, folic acid, nicotinic acid, pantothenic acid, pyridoxal, riboflavin, thiamine and inositol. The importance of other water soluble vitamins is less clear. Vitamin B12 has been

reported to be essential for some cells and is included in F12 medium Few data are available on the role of fat soluble vitamins in medium.

Hormones and growth factors

Hormones and growth factors exhibit a variety of different effects on cells. These are included in some media (especially serum free media) at relatively low concentrations .Insulin and hydro cortisone are main examples but growth factors like NGF (nerve growth factors) and EGF (epidermal growth factor)has also been used as well as certain interleukins, colony stimulating factors, and fibroblast growth factors (FGFs)…etc

Proteins and peptides

Although an absolute requirements for protein and peptides by cells in culture has not been established, relatively few media have been established in which the cells grow rapidly in the total absence of proteins or polypeptides. Common examples of protein supplements used are fetuin, alpha globulins fibronectin, albumin and transferrin.

Fatty acids and lipids

As with proteins and peptides, there is no consensus regarding an essential role for lipids in cell culture. However, fatty acids and lipids are important components of several serum free media.

Accessory factors

Amongst these are the trace elements, especially iron, copper, zinc, and selenium. A variety of other compounds, including nucleosides and intermediates of tricarboxylic acid cycle may also be used.

Antibiotics

Antibiotics were originally introduced in to the culture media to reduce the frequency of contamination. However, the use of laminar hoods, coupled with strict aseptic technique, makes antibiotics unnecessary. Indeed, antibiotics have number of significant disadvantages ,

1.They encourage development of antibiotic development resistant organisms

2.They hide the presence of low level ,cryptic contaminations that can become fully operative if the antibiotics were removed, the culture conditions change or resistant strains developed.

3.They may hide mycoplasma infections

4.They have antimetabolic effects that can cross react with mammalian cells

5. They encourage poor aseptic techniques

For all these reasons, it is strongly recommended that routine cultures can be performed in the absence of antibiotics and their use be restricted to primary cultures or large scale labor- intensive experiments with a high cost of consumables. If the conditions demand the use of antibiotics, then they should be removed as soon

as possible, or if they are used in long term, parallel culture should be maintained free of antibiotics. A number of antibiotics used in tissue culture are moderately effective in controlling bacterial infections. However, a significant number of bacterial strains are resistant to anti biotic, either naturally poor by selection, so the control that they provide is never absolute. Fungal and yeast contaminations are particularly hard to control with antibiotics, they may be held in check about seldom eliminated when antibiotics are to be used in cell cultures, the key factors governing their choice are

1.Absence of cytotoxicity

2. Broad anti-microbial spectrums

3.Acceptable cost

4.Minimum tendency to induce formation of resistant microorganisms

3.3.2.4 Serum media

Historically the first tissue culture experiments were performed using animal body fluids such as lymph to support cell growth. When Eagle and others, in the late 1950s, produced basal media containing amino acids, carbohydrates, vitamins and minerals, it became apparent that supplementation of medium with body fluid was still needed to provide un identified, but essential, factors needed for efficient cell growth Supplementation of basal medium up to 20%of animal serum became widely used. Serum contains growth factors, which promotes cell proliferation, and adhesion factors and anti trypsin activity, which promotes cell attachments. Serum is also a source of minerals, lipids, hormones, many of which may bound to proteins. The sera used in most tissue culture laboratory are calf (bovine), fetal bovine, horse and human serum. Calf(CF) and fetal bovine serum(FBS) are the most widely used, the later for more demanding cell lines and for cloning. Human serum is some times used in conjunction with some human cell lines, but it needs to be screened for viruses, such as HIV and hepatitis horse serum is preferred to calf serum by some workers, as it can be obtained from a

closed herd and is often consistent from batch to batch Horse serum is also be less likely to metabolize poly amines due to lower levels of poly amine oxidase, Polyamines are mitogenic for more cells.

3.3.2.5 Advantages of serum

1. Serum represents a cocktail of most of the factors required for cell proliferation and maintenance

2. Serum is an all most universal growth supplement which is effective with most cells. Using serum supplemented medium there fore reduces the need to spend time developing a specific, optimized medium formulation for every cell type under investigation.

3. Serum buffers the cell culture system against a variety of perturbations and toxic effects such as pH change, or presence of heavy metal ions, photolytic action or end toxin.

The use of serum also imposes a number of difficulties which impacts on the safety, reproducibility, and cost of bio pharmaceutical as produced in animal cells .these difficulties can be minimized by careful selection and validation serum sources although almost all new manufacturing processing's using animal cells are designed for serum- free media in order to avoid these difficulties, many existing processes still use FBS supplemented medium. This situation is unlikely to change fundamentally in near future since regulatory constraints generally make it impractical and uneconomic to alter existing processes.

Despite its high cost FBS remains the most frequently used serum for medium supplementation. Several different types of serum have been proposed as cheaper alternative to FBS Calf serum is quite widely used industrially and as available as either new born serum (which has high level of biotin) or as mature calf serum. New born calf gama globulins are as a result of the colostrums immediately after birth. Adult bovine serum is used occasionally but is not usually as effective as FBS or calf serum. Horse serum is also used particularly with some human cell lines the use of human serum has been

proposed for some fastidious human cell lines, but it is not clearly established that clearly that human serum better in general than FBS.

Constituents of Serum

Serum is an effective growth promoting supplement for practically all types of cells because of its complexity and multiplicity of growth promoting, cell protection, and nutritional factors that it contains .These can be divided in to specific polypeptides which stimulates cell growth (growth factors), carrier proteins, cell protective agents, cell attachment factors, and nutrients (some of which may be small molecules which are attached to carrier proteins). Some serum macro molecules can fill more than one of these roles.

Growth factors

Polypeptide growth factors are of particular importance in serum .These 5-30 kD proteins act via specific cell surface receptors as signals which stimulates cell proliferation or differentiation. In some cases ,the presence of certain growth factors may not be stimulatory as such but may still be essential since deprivation of the factor initiates a pre programmed auto destructive sequence of events (apoptosis) which results in death of cells even though they may be fully provided with nutrients and be maintained under optimal culture conditions.

Different cell types have different growth factor requirements and same growth factors may stimulate or inhibit depending up on cell type and the growth factor concentration. Different types of serum may contain different absolute and relative levels of different growth factors. This is one of the main reason why growth testing of serum batches is necessary to ensure satisfactory performance with the specific cell line of interest.

Albumin

Albumin is the majority protein components of serum and exerts several effects which contribute to the growth and maintenance of cell lines in culture it functions as a carrier protein for a range of small molecules, particularly lipids. Transport of fatty acids is an important function of albumin since these are essential for cells and but are toxic in the unbound form and are also very poorly soluble in water. Steroids and fat soluble vitamins may also bound to albumin. Albumin also has specific binding site for thyroxin and metal

ions such as Ni+2 and Cu+2 .There is evidence that albumin also bind some other metal ions and also carry other, undefined components which supports cells in culture.

3.3.2.6 Disadvantages of serum

1.Physiological variability :

The major constituents of serum such as albumin and transferrin are known, but serum also contains a wide range of minor components that may have considerable effect on cell growth .These components include nutrients (amino acids, nucleosides, sugars etc), peptide growth factors, hormones, minerals, and lipids, the concentration and action of which have not been fully determined.

2. Shelf life and consistency :

Serum varies from batch to batch, and at best a batch will last one year, perhaps deteriorating during that time. It must then be replaced with another batch that may be selected as similar but will never be identical, to the first batch.

3. Quality control:

Changing serum batches require extensive testing to ensure that replacement is as close as possible to the previous batch. This can involve several tests (for growth plating efficiency, and special functions)and a number of different cell lines.

4. Specificity:

If more than one cell type is used each type may require different batch of serum so that several batches must be held on reserve simultaneously. Coculturing different cell types will present an even greater problem.

5. Availability:

Periodically, the supply of serum is restricted due to drought in the cattle rearing areas, the spread of disease among the cattle's, or economic or political reasons. This can create problems at any time, restricting the amount of serum available and the number of batches to choose from, but can be particularly acute at times of high demand To day demand is increasing and it will probably exceed supply unless the majority of commercial users are able to adopt serum-free media. While an average research laboratory may reserve 100-200 liters of

serum per year, a commercial laboratory can use that amount or more in a weak.

6. Down stream processing:

To any one interested in recovering cell products, the presence of serum creates a major problem to purification and even limit the pharmaceutical acceptance of the product..

7. Contamination :

Serum is frequently contaminated with viruses, which may be harmless to cell culture, but represents an un known additional factor out side the operators control. Fortunately improvement in serum sterilization techniques have virtually eliminated the risk of mycoplasma infection from sera from most reputable suppliers, but this cants be guaranteed for viral contamination, in spite of claims that some filters claim removal of viruses. Because of the risk of the spreading of bovine spongiform encephalitis among cattle's, cell cultures and serum supplied to united states and Australia require information about the country of origin and the batch number of the serum. Serum derived from cattle in Newzealand probably has the lowest endogenous viral contamination, as many of the found in European and North American cattle are not found in New Zealand.

8. Cost :

Cost is often cited as disadvantage of serum supplementation.C3ertainly serum constitutes the major part of the cost of the bottle of the medium (more than 10 times the cost of chemical constituents) but it is replaced by definite constituents, the cost these may as high as that of the serum. However, as the demand for such items as transferrin, selenium, insulin, etc. rises the cost is likely to come down with increasing market size, and serum free media will become relatively cheaper. The availability of recombinant growth factors, coupled with market demand, particularly in the form of growth factors in the form of pharmaceuticals, may help to reduce their intrinsic cost.

9. Growth inhibitors :

As well as its growth promoting activity, serum contains growth inhibiting activity, and although stimulation usually predominates, the net effect of the serum is unpredictable combination of both inhibition and stimulation of growth. While substances such as PDGF

may be mitogenic to fibroblasts, other constituents of serum can be cytotstatic. Hydrocortisone, present at around 10-8 M in fetal serum is cytostatic to many cell types such as glia and lung epithelium, at high level densities (though it may be mitogenic at low cell densities), and TGF-beta, released from platelets, is cytostatic to many epithelial cells.

10. Standardisation:

Standardization of experimental and production protocols is difficult, both at different times and among different laboratories, due to batch to batch variations.

3.3.2.7 Replacement of serum in medium

Much of the present requirements for cells in culture system comes from Eagles work on the fundamental requirements for growing mammalian cells. Based on this information many attempts have been made to replace serum in part or in full by serum –derived factors or by completely synthetic media. One approach is to reduce the serum requirement by supplementing the culture medium with processed serum products. Controlled processed serum replacement (CPSR)are prepared by processes that yield defined products with much higher batch to batch consistency than serum CPSR products are derived from bovine plasma, and have lower protein and endotoxin levels than serum. Natural serum can also be replaced by supplemented or fortified serum. Serum may be fortified with mitogens, growth factors, hormones, proteins and other protein stabilizers, and trace elements. Such fortified serum can be used at a much lower concentration than normal serum.

3.3.2.8 Serum-free media

For the disadvantages discussed in serum media when ever possible it is desirable to culture cells in serum free medium. A properly designed serum-free medium :

1. Is reproducible

2. Is not reliant on the economics of the world cattle market

3. Simplifies down stream purification

4. Has no unknown factors e.g. viruses or growth inhibitors

A number of cell types have been grown successfully in serum-free media, usually in a medium specifically developed for one cell line. The requirements of cell lines differ greatly and success of a serum-free medium formulation with one cell line does not guarantees with other, even with closely similar cell lines. A great deal of effort has gone into developing serum free media, but until recently, success has been limited. However, with the identification of essential growth factors and nutrients required by different cells, several very effective serum-free media have been formulated. A variety of tissue sources and species are represented clearly there are unique combinations of growth factors and hormones that promote optimal proliferation of specific cell types. The most consistent requirement appears to be for the polypeptide hormone, insulin and iron transport protein transferrin. Other supplements includes polypeptides and steroid growth hormones, polypeptide growth factors, trace elements reducing agents, diamines, vitamins and albumin complexed with unsaturated fatty acids. An important consideration for some application is that animal derived supplements or proteins can pose contamination risks similar to those of serum.

Several commercially produced ,ready to use serum-free media are now available which have been designed for a particular cell types .It should however be remembered that different strains of the same cell type may have different medium requirements now, and that fine tuning of these commercial media may be necessary to optimum results with specific individual strain or construct.

3.3.2.9 Design of serum-free media

A defined serum free media is one in which a group of components are formulated together to optimize performance of single cell type .Each component included of known purity and is present at a known concentration. Several important factors must be considered to achieve this goal. Amongst these the origin of cell lines, i.e. species and tissue, the compatibility of media components and their interaction., and the specific application for which the cell line being cultured, e.g. production of biomass or generation of products. The two approaches generally followed in designing a free media are :

a) Reduced serum: In this approach the concentration of serum in the basal medium is progressively reduced while other components, e.g. growth factors and hormones are added to identify the factors capable of restoring the growth to the level obtained in the presence of serum. This process can be very lengthy because at each change, growth

assays using the serum –supplemented control, and repeat the verification assays need to be done.

b) Basal medium: A different approach i9s to add components (singly or in combination)to a basal medium in a step wise manner until a medium is progressively built up to give a similar or equal at cell growth to the serum supplemented medium.

Both of these approaches, the following critical factors need to be considered in designing an efficient serum-free medium.

Basal medium: The selection of basal medium is extremely important in terms of energy sources, buffers, and inorganic ions. Generally the starting basal medium formulation is chosen on the basis of the known preference of the required cell line.

Lipids:

These include ethonolamine,phosphoethanolamine,sterols, fatty acids and phospho lipid. In serum supplemented media, they are usually carried on macro molecules, principally proteins. In serum free media fatty acids are usually provided in a bound form (either to albumin or to the other serum proteins) or in the form of phospholipids enclosed vesicles (liposomes). If serum albumin is used directly as a lipid source, it should be noted that the endogenous lipid content of may be dependent on the methods used for its [purification; the solvent precipitation frequently used may results in substantial stripping of lipid protein. It should also be noted that pasteurized human albumin will have been stabilized with octanoic acid or other hydro phobic stabilizing agents prior to heating and that it may be important to replace these with more physiologically relevant lipids before use in cell culture. Resent developments have permitted the use of totally synthetic hydrophilic carriers such as cyclodextrins for the trans port.

Buffering :

Buffers maintain a proper environment for the metabolism,growth,and functioning, of cells. major ions (Na+, K+, HCO^{3-} and HPO_4^{-2}) are usually regarded as the principle components, in pH control, along along with H+ and OH-, which enters into ionic balance. Other components, including amino acids, if present in high concentrations, can contribute to the buffering power of a medium. Besides bicarbonate, zwitter ionic buffers like Hepes, Bes and Tes may be used in systems in may be used in which the strict control of gas

phase in\s not required. However, careful consideration must be given to the concentration of these buffers which can be toxic to the cells. Some of these buffer chelate biologically important cautions. A useful buffer for the use in the presence of low or no bicarbonate is sodium glycerophosphate.

Trace elements :

The major ions, i.e. Na+,K+,Ca+,Mg+2,Cl-,HPO4-and HCO3-, are principally involved in maintaining electrolytes balance and contributing to osmotic equilibrium, of the system. Trace elements are also included in many serum free -media, because of their beneficial effects. Inter relation ships exists between Fe+2,Zn+2,and Cu+2ions which are needed for many cells. Most serum free media also include, Co+2 and SeO3-2. Cells derived from heart and kidney tissue have high requirement for K+ whilst Ca+2, is require for control of mitosis and the Ca+2/Mg+2 ratio is important in controlling cell proliferation and transformation. Selenium is proving to be important for many cell types. Other trace elements include Sn, V, Al, and As. Iron is frequently added as a transferrin but can also be added in other forms such as ferric citrate, ferrous nitrate, or ferrous sulphate. Mechanical stabilizers and adhesion factors : For optimal growth suspension culture require protection from shear due to agitation (air bubbles, stirrer, shaker). Shear damage can be reduced by increasing the viscosity of the medium.Carboxy methyl cellulose and polyvinylpyrrolidone have been used for this purpose. The most widely used shear protectant is Pluronic F-68. This is a non ionic block copolymer with an average molecular weight of 8400Da, consisting of a central block of polypropylene (20% by weight) and blocks of polyoxyethylene at both ends. Pluronic F-68 has been demonstrated to have a significant effect in protecting animals grown in suspension in sparged or stirred bioreactors. The protective effect is thought to be exerted through the formation of an interfacial structure of adsorbed molecules on the cell surface. It is thought that the hydro phobic portion of molecule interacts with the cell membrane, while poly oxymethylene oxygen may form hydrogen bonds with water molecules to generate a hydrogen sheath, which provides the protection from laminar share stress and cell bubble interactions .

Cell attachment and growth of anchorage dependent cells can be improved by pretreatment of the substrate in a variety of ways. the substrate can be treated with adhesive glycol proteins such as fibronectin ,laminin ,chondroitin.epibolin,or serum spreading factor.

3.3.2.10 Difficulties that may be encountered with serum free media

When cells are grown in serum free- conditions, they no longer benefit from the multiple protective and nutritional effects that serum provides. The robustness of the process in serum free medium depends on attention on the following points;

1) Cells appear more fastidious in the absence of serum: design of dedicated medium for each cell type is usually necessary for optimal results.

2) Cultured conditions become more critical in serum free medium: better control of key processes parameters (PH,oxygenation etc) is there fore necessary.

3) Serum free media has a reduced capacity to inactivate or to absorb the toxic materials (e.g. heavy metals, endotoxin, etc.) Greater attention to the purity of components and depyrogenation is required .Antibiotics may exhibit cytotoxicity in serum free medium

4) Specific shere protective agents may need to be added.

5)A significant adaptation may need to be required before cells are fully weaned to serum-free medium .This makes the design and testing of serum free medium a long and labor intensive process.

3.3.2.11 SOURCES OF MATERIAL FOR CELL CULTURE

The expression of specialized functions in a culture is controlled by nutritional constitution of the medium, the presence of hormones and other induce & repressor substances and the interaction of the cells with the substrate and other cells. There are many reviews of culture techniques for specific cell types.

The development of techniques for cell line immortalization has meant that it has been possible to generate continuous cell lines from a number of finite lines

Untransformed tissue. In many cases, the differentiated properties are lost, but by using a switchable promoter (eg. Temperature sensitivity) it may prove possible to recover the differentiated phenotype. The development of transgenic mouse carrying the large 'T' gene of SV 40 has opened up a wide range of possibilities because cells cultured from these animals are already immortalized, but still retain some differentiated functions. Many specialized cells (eg. Epidernal keratinocytes, melanocytes endothelial cells smooth muscle cells, dismal fibroblasts and mammary epithelium) are commercially available. The cost is naturally very high, but as demand increases, the cost may fall. Skin cultures are also available for cytotoxicity and inflammation research, such as Episkin and Epiderm and from advanced tissue sciences. These products are prepared in filter wells by combining keratinocytes with dermal fibroblast and collagen supported by a nylon net in a so called " skin equivalent" other tissues in particular, the Cornea – has also been prepared in a similar way for toxicity studies.

A number of specialized procedures have now been devised and some representative examples have been contributed by experts in many areas.

Epithelial Cells:

Epithelial cells are responsible for the recognised functions of many organs (eg. Controlled absorption in the Kidney and gut secretion in the liver and pancreas, gas exchange in the lung, barrier protection in skin). Epithelial cells are also of interest as models of differentiation and stem cell kinetics (eg. Epidermal keratinocytes) and are among the principle tissues in which common cancers arise. Consequently, the culture of barrios epithelial cells has been a focus of attention for many years. The

major problem in the culture of pure epithelium has been the overgrowth of culture by stromal cells, such as connective tissue, fibroblasts and vascular endothelium. Most of the variations in the technique are aimed at preventing such overgrowth by nutritional manipulation of the medium or alternations in the culture substrate, that promote the growth of the undifferentiated epidermis and preferably the stem cells subsequent modifications may then be employed to enhance epithelial differentiation.

Factors contained in serum – many of them derived from platelets have a strong mitogenic effect on fibroblasts and tend to inhibit epithelia proliferation by inducing terminal differentiation. Consequently, one of the most significant events in the isolation and propagation of specialized cell cultures has been the development of selective, serum free media, supplemented with specific growth factors as appropriate . The isolation of epithelial cells from donor tissue is best performed with collagenase which disperses the stroma but leaves the epithelial cells in small clusters, favoring their subsequent survival.

Epidermis:

Because of the progress in basic cell structure, technology and in our understanding of the culture requirements of the various epithelial tissues, keratinocytes of most stratified epithelia can now be grown and studied in cell culture. Mostly the squamous epithelia of the skin and their isolated epithelial cells, the keratinocytes, have been used to study their physiology and pathology *in vitro*. In addition, cells of the oral mucous, as well as the skin appendages such as hair follicles, have been isolated and cultured under various conditions, and reconstructed tissues have been formed in culture as well as in transplants.

In order to avoid overgrowth by mesenchymal cells, the epithelial compartment has to be separated from the connective tissue and disposed into single cells, which are then cultured on different substrata, by using different media formulations. As a practical assessment for mesenchymal cells contamination cells should be plated at clonal densities (1-5 x 10^2 cells / cm^2) on feeder cells, and cloned morphology should be identified at low magnification following fixation and Hemalium and Eosin (HE) staining of 1- 14 – days cultures.

A more specific and highly sensitive method to identify contaminating fibroblast is the analysis of expression of keratinocyte growth factor (LGF) by RT – PCR. Since, this factor is produced in fibroblast and not in keratinocytes, it represents a selective marker. More over, KGF expression is enhanced by co-cultured keratinocytes so that a minority of contaminating fibroblasts will be detected by this assay.

Variations:

Keratinocytes can be obtained from the outer root sheaths (ORS) of plucked scalp hair follicles by dissociating cells from the dissected follicle. Up to 5 x 10^3 cells can be obtained from three hair follicles and plated on fibroblast feeder cells, resulting in about 1 x 10^6 cells with 15 d. Like intermolecular keratinocytes, ORS cells can be sub cultured on feeder layer dishes and cryopreserved. The ORS derived kerationocytes are similar in culture to intermolecular cells, form a rectangular reoepidernis when transplanted onto nude nice, and are used clinically to cover chronic wounds.

Keratinocytes can also be grown at clonal density to study colonel cell populations and their different proliferation potentials. For this purpose keratinocytes are co-cultured with X – irradiated

3T3 cells, at reduced Ca^{+2} concentration with fibroblasts conditioned medium or in defined serum free medium.

In order to provide more in vivo like conditions and to study the regulation of skin physiology by epithelial – mesenchymal interactions, organotypic co-culture systems have been developed by seeding the cells on collagen gels. Under these improved growth conditions, keratinocytes express many aspects of growth and differentiation of epidermis *in vivo*, including ultra structural features and a complete basement membrane, features that are absent or less pronounced in submerged cultures on plastic, such organatypic cultures can now also be established and maintained for three weeks in defined SKDM medium, allowing molecular interactions between epithelial cells and mesenchymal cells to be analyzed without disturbing influences of serum or other undefined tissue extracts.

Optimal growth and differentiation of isolated keratinocytes are obtained under *in vivo* conditions when the cells are transplanted as intact cultures or in suspension onto nude mice. This leads to an almost complete expression of cells, differentiation characteristics of normal epidermis, and all biochemical and with a structural features of a completely keratinized epithelium, including the formation of basement membrane. Combined cultures of epidermis and stream, mounted on ness fibers are now commercially available and have been suggested as models for irritation and inflammation research.

CORNEA:

There have been a number of attempts to replace the rabbits eye test by using cultured corneal epithelium, so there has been considerable interest in culturing cornea. The following protocol

for the culture of normal human corneal cells in a serum free medium was provided by Carolyn cann.

Phenotypic Development in Vitro:

Both primary cultures and HCE lines retain phenotypic characters of corneal epithelium in situ. They continue to synthesize collagenase and express EGF repots and specific cytokeratins , although the level of expression under current culture conditions are less than observed *insitu.* When corneal epithelium is cultured upon collagen membranes at air- liquid interfaces, its morphology and barrier function are fairly well preserved stratified membranes develop that are able to inhibit the diffusion of Na-fluorescein. Air-liquid interface cultures survive for 2 weeks *in vitro* and have been used to investigate injury and repair mechanisms.

Human corneal epithelial cells propagated *in vitro* may provide a suitable model for exploring basic cell biological mechanism as well as toxicological phenomena. Although the primary cultures are adequate *in vitro* studies, HCE lines with an excellent extended life span provide a retable source of material that can be shared among laboratories.

BREAST:

Milk and reduction mannoplasty of suitable sources of normal ductile epithelium from the breast, the formal gives purer cultures of epithelial cells. The disaggregation in collagenase is preferred for primary disaggregation , growth on confluent feeder layers of fetal human intestine represses stream contamination of both normal and malignant tissues, and optimization of the medium enables serial passage and cloning of the epithelial cells. Cultivation in collagen gel allows three dimensional structures to form that correlate well with the histology of the original donor tissue.

As with epidermis, cholera toxin and EGF stimulate the growth of epithelia cells from normal breast tissue *in vitro.*

The hormonal picture is more complex. Many epithelial cells survive better with insulin, (~1 x10 1μ /ml) and hydrocortisone (~1 x 10 $^{-8}$M), added to the culture. The differentiation acinar breast epithelium in organ culture requires hydrocortisone, insulin, prolactin, estrogen, progesterone and growth harmone can also been shown to be required in cell culture.

Variations:

It is convenient to use the macrophages that are already present in the milk as feeders, but they are gradually lost as the epithelial colonies expand. However, macrophages can be removed by adsorption to glass, and in that case other feeders must be added. Irradiated or mitomycin 3T6 cells show the best growth promoting activity. Analogues of c AMP can be used to replace the chlorine toxin, although this is not possible with macrophage feeders, which are killed by the analogues.

Uses and applications of milk epithelial cell culture:

Milk cultures provide cells from the fully functional gland & allow the definition of phenotypes by in immunological markers. Milk cells have been successfully transferred by SV 40 virus and provide an important source of normal cells for comparison with breast cancer cells.

Gastro Intestinal Tract:

The culture of normal epithelium from the gut lining has not been extensively reported, although there are numerous reports in the literature of continuous lines from human colon carcinoma. Cell cultures from fetal human intestine, extensive use has been made of the rat intestinal cell line IEC-6.

LIVER:

Although cultures from adult liver do not express all the properties of liver parenchyma, there is little doubt that the correct lineage of cells may be cultured. So far attempts generating proliferating cell lines have not been particularly successful, but functional hepatocytes can be cultured under correct conditions.

Some of the most useful continuos liver cell lines were derived from Reuber H 35 and Morris minimal deviation hepatomas of the rat. Inducing tyrosine amino transferase in the cell lines with dexamethasome proved to be valuable model for studying the regulation of enzyme adaptation in mammalian cells. Cell lines such as Hep-C_2 have also been generated from human hepatica and retain some of these metabolising properties of normal liver.

PANCREAS:

Both acinar and islet cells have been grown from pancreatic tissue, and lines have been established from the immortomouse". The conversion of adenoma to carcinoma has generated particular interest.

KIDNEY:

The kidney is a structurally complex organ in which the system of reforms and collecting ducts is made up of numerous functionally & phenotypically distinct segments. This segmental heterogeneity is compounded by a cultural diversity that has yet to be fully characterized. Some tubular segments posses several morphologically distinct cell types. In addition evidence point to rapid adaptive changes in cell ultra structure that may correlate with changes in cell function. The structural and cellular heterogeneity presents a challenge to cell cultures who is interested in isolating pure and highly enriched cell populations. The difficulty of the problem is further compounded in studies of

human kidney. With which form and access to the specimen may make some manipulations, such as vascular perfusion, difficult or impossible.

Several approaches have been used successfully to culture the cells of specific tubular segments. Density gradient methods are now commonly employed to isolate enriched populations of enzyme digested tubules segments and are particularly effective in establishing proximal tubule cell cultures from experimental animals. Specific nephron or collecting duct segments can also be isolated by microdissection and then explanted to the culture substrate. This method, developed with the use of experimental animals has been applied to the culture of human kidneys and cyst wall epithelium of polycystic kidneys. These elegant methods hold considerable promise for the study of specific kidney cell types in health and disease, but yet have been applied almost exclusively to studies on experimental animals.

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PROSTATE:

A number of reports have used cells cultured from prostate tissue with a particular interest in regulating cell proliferation and differentiation by paracrine growth factors Variation: The foregoing procedure can be applied to the culture of human prostate epithelial cells with modifications described in chaproniere .

The purified growth factor, prostatropin, is identical to heparin binding fibroblast growth factor type 2, previously called acid FGF-1 or HBGF-I. Molecular characterization of new members of the FGF – ligand family as well as the FGF – receptor family in prostate cells, has revealed that prostate epithelial cells express a specific splice variant of one of the four FGF receptor genes. The specific receptor recongnises FGF –1 and stromal cell – derived FGF – 7

also called (Keratinocyte growth factor) but not FGF – 2, previously called b FGF.

MESENCHYMAL CELLS:

Included under this are those which are derived from the embryonic mesoderm, but exclude the hematopoietic system. Mesenchymal cells include the structural and vascular cells.

CONNECTIVE TISSUE:

Connective tissue cells are generally regarded as the weeds of tissue culturists garden. They survive most of the enzymatic and mechanical explanation techniques and may be cultured is many simple media.

Although cells loosely called fibroblasts have been isolated from many different tissues and assumed to connective tissue cells, the precise identity of cells in this class remains some what obscure. Fibroblasts, synthesis of Type – I in relatively large amounts is characteristic of connective tissue. However, $3T_3$ cells can also be induced to differentiate into adipose cells. It is possible that cells will transfer from one lineage to another under certain conditions, but such transdifferentiation has rarely been confirmed. It is more likely that mouse embryo fibroblast cells lines are primitive mesodernal cells that may be induced to differentiate in more than one direction.

Human, hamster, and the chick fibroblasts are more physiologically distinct from mouse fibroblast, as they assume a spindle shaped morphology at confluence producing characteristic parallel assays to cells distinct from the pavement like appearance of mouse fibroblasts. The spindle shaped cells may represent a more highly committed precursor and may be more correctly termed as fibroblasts. NIH 3T3 cells may become spindle shaped if allowed to remain at high cell density. It has also been suggested

that fibroblastic cell lines may be of vascular parasites, connective tissue like cells in blood vessels.

Clearly cell lines loosely termed fibroblastic can be cultivated from embryonic and adult tissues but these lines should not be regarded as identical or classed as fibroblasts without confirmation by appropriate markers. Collagen, typy1 is one such marker.

ADIPOSE TISSUE:

While it may be possible to prepare cultures from mature fat cells, differentiation may be induced is cultures of messnchymal cells by maintaining the cells at high density for several days. An adipogenic factor in serum is found to be responsible for induction primary culture of fat cells may be prepared from rat epididymus by collegians digestion.

Adipose cells are terminally differentiated, specialized cells whose primary physiological role has classically been described as an energy reservoirs for the body. Adipocytes are a storage form of triglycerides in times of energy excess and a source of energy in the form of free fatly acids released by lipolysis during times of energy need. Recently the important role of adipocytes as active regulators of Carbohydrate and lipid metabolism has received increased attention. It is likely that specific abnormalities in adipose tissue can contribute directly to the pathogenesis of common diseases such as diabetes, hypertension and obesity.

MUSELE:

Cardiac muscle can be isolated using the cold-tyrosine method and will show concentration after about 3 days is culture. Human cardiac myocytes have also been grown smooth mussel cells have been grown from vascular tissue and cocultured with vesicular endothelium.

CARTICLAGE:

The methods for culturing cells from articulator cartilage was contributed by Francois Lemave.

Chondrocytes are highly specialized cells of mesodermal origin that are responsible for synthesis, maintenance and degradation of the cartilage matrix. A great deal of research in the field of rheumatology has been focused on understanding the mechanism that induce metabolic changes in articulate chondrocytes during ostroarthritis and rheumatoid arthritis. Articular chondrocytes in culture are a very useful tool, but, cultured in a minelayer, they rapidly divide, become fibroblastic and lose their biochemical characteristics. As early as the first passage, there is a gradual shift from the synthesis of type –II collagen to types – I &III colognes and from aggrecan to low molecular weight proteoglycans. In parallel with the phenotypic modulation, the metabolic response to interleukin – I - β is quantitatively estimated by subculture.

ANALYSIS OF CHONDROCYTE PHENOTYPE:

Collagen: Type – II colognes represents 85-90% of the total collagen synthesized by chondrocytes in cartilage or primary culture. Type – II collagen is highly specific to chondrocytes and is considered as their main differentiation marker. The expression of Type – II collagen can be analyzed by indirect immunofluorescence using specific antibodies. These must be very carefully checked, however, to make sure that there is no cross reactivity with type – I collagen, which commonly occurs. Polyclonal antibodies against type-II collagen can be purchased from southern Biotechnology Association, Inc. The immunocytochemical study of collagen expression in chondrocytes cultured in alginate beads necessitates sectioning the beads.

For more precise analysis of the collagen phenotype SDS-PAGE and ttwo dimensional CNBr, peptide maps of purified collagen after (^{3}H) proline incorporation have to be performed.

Concerning major collegens, SDS-PAGE analysis can reveal the presence of type-III collagen and of the α-2 chain of collagen-1. However, it can't separate the α -1 chain of type –1 & type – II, which is why two dimensional CNBr peptide mapping is necessary to asserts type – II collagen expression.

The expressions of these genetically distinct collegians can also be analysed at the transcriptional level with conventional transfer and hybridization techniques because of sequence homology between different collagen genes, hybridization and washing conditions must be tested and needed to be stringent enough to avoid cross hybridization.

Proteoglycans: Aleian blue staining at acidic P^{H} is widely used as indicator of the presence of sulfated aggrecan. As with immunocytochemical studies this staining necessities sectioning the beads. A more precise analysis of the different types of proteoglycans synthesized can be performed after $^{35}SO_4$ incorporation. CDNA probes for aggrecan and link protein allow an analysis at transcriptional level of the expression of these specific proteins.

BONE:

Although bone is mechanically difficult to handle, thin slices treated with EDTA and digested in collagenase give rise to cultures of osteoblasts that have some functional characteristics of tissue. Antiserum against collagen has been used to prevent fibroblastic overgrowth without inhibiting the osteoblasts. Propagated lines

have been auctioned from osteosarcoma, but not from normal osteoblasts.

ENDOTHELIUM:

Endothelium has been successfully cultured by collagenase perfusion of bovine aorta and human umbilical vein, trypsinization of white matter from rat cerebral cortex, and microdissection of adrenal cortex.

Endothelium can be characterized by the presence of factor VIII antigen and type – IV collagen, Wisbel – Pelade bodies, and , sometimes, the formation of right junctions, although the last feature is not always demonstrated readily in culture.

Endothelial cultures are good models for contact inhibition and density limitation of growth, as cell proliferation is strongly inhibited after conference is reached.

Much interest has been generated in endothelial cell culture because of the potential involvement of endothelial cells in vascular diseases, the repair of blood vessels, and angiogenesis in cancer. Growth factors, including angiogenesis factor derived from walker 256 cells in vitro, play an important part in maintaining cell proliferation and survival so that, secondary structures can be forced.

NEURO ECTODERMAL CELLS:

Neurons: nerve cells appear to be more fastidious is their choice of substrate than most other cells. They will not survive well on untreated glass or plastic, but will demonstrate neurites outgrowth in collagen and ploy.D-lysine. Neurite outgrowth is encouraged by ploy peptide nerve growth factor and factors, secreted by glial cells, that are immunologically distinct from NGF. Cell proliferation has not been found in cultures of most neurons, even with cells from embryonic stages in which mitosis was apparent in vivo. However,

recent studies with embryonic stem cells have shown that some neurons can be made to proliferate *in vitro* and recognize in vivo.

GLIAL CELLS:

Glial cells have been cultured from avian, rodent, and human brains. Human adult normal brain lines express glial fibrulary acidic protein, highly affinity gamma amino butyric acid and glutamate uptake and glutamate synthetase activity. Oligo dendrocytes do not readily survive sub-culture, but schwann cells from optic nerve have been sub cultured using cholera toxin as a mitogen. Cultures of human glyoma can also be prepared by mechanical disaggregation, typsinization or collagenase digestion. The right temporal lobe from human maels appears to be marginally better than other regions of the brain, but most regions have a good chance of success.

A number of glyomas have been cultured from rodents among which the C-6 deserves special mention . This cell line expresses the astrocytic marker- glial fibrellary acidic protein – in up to 98% of cells, but still caries the enzyme glycerol phosphate dehydrogenate and 2'3' cyclic nucleotide phosphorylase, both of which are oligodendrocytic markers, the lines appears to be an interesting example of a precursor cell tumor that can mature along two district phenotype routes simultaneously.

ENDOCRINE CELLS:

The problems of culturing endocrine cells are accentuated because a relative number of secondary cells may be quite small. Sato and colleagues cultured functional adrenal and pituitary cells from rat tumors by mechanical disaggregation of the tumor and regular monolayer of the culture. The functional integrity of cells was retained by intermittent passage of cells as tumors in rats.

These line are now fully adapted to culture and can be maintained without animal passage.

Fibroblasts have been reduced in cultures of pancreatic islets cells by treatment with ethyl mercury thiosalicylate and also been purified by density gradient centrifugation and by centrifugal elutriations.

Pituitary cells produce pituitary Hormone for several sub cultures have been isolated from the mouse. Although normal human pituitary cells do not survive well and pituitary cells gradually loose the capacity to synthesize hormones, 3 – dimensional cultures of pituitary adenomas continue to secrete prolactin.

HEMATOPOIETIC CELLS:

Hematopoietic cells have been grown in colony forming assays, in long term cultures from bone narrow, and as continuos cell lines. Under the control of specific growth factors, hematopoetic stem cells or can be recloned from agar cultures. MPF - 1α appears to be the main factor that keeps the stem cells in the regenerative compartment. However, most suspension colonises, which contain cells of only one lineage, survive only as primary cultures that lose their repopulating efficiency and hence cannot be cultured, but under the appropriate conditions, other lineages can be produced.

A number of myeloid cell lines have been developed from Maurine leukemias and like some of the human lymphoblastoid lines, have been shown to make globulin chains and in some cases, complete $\propto$ and γ globulin's. Some of the lines can be grown in serum-free medium. T – cells lines requires T- Cell growth factors and B- cell growth factors have also been described.

The first human lymphoblastoid cell lines were derived by culturing peripheral lymphocytes from blood at very high cell densities, usually in deep cultures.

Rossi and Friend demonstrated that a mouse RNA virus could cause splenomegaly and erythroblastosis in infected mice. Cell cultures taken from minced spleens of these animals could, in some cases, give rise to continuous cell lines of erythrolukemia cells. All of these lines are transformed by what is now recognised as a complex defective and helper viruses derived from Moloney sarcoma virus.

Macrophages may be isolated from many tissues collecting the cells that attach during enzymatic aggregation. The yield is rather low, however, a number of techniques have been developed to obtain large number of macrophages. If necessary, macrophages may be identified by their ability to attach to the culture substrate in the presence of protease's. Macrophages can be subcultured only with difficulty, because of their sensitivity to tyrosine.

GONADA:

Ovarian granular cells can be maintained and are apparently functional is primary culture, but specific functions are lost on subculture. A cell line started from Chinese hampster ovary has been in culture for many years, but its lineage still has not been identified. Although epithelioid at some stages of growth, it undergoes a fibroblastic like modification when it is cultured in dibutyrye cAMP.

Cellular fractions from testes have been separated by velocity sedimentation at unit gravity, but no prolonged culture of fractions has been reported. The TM_4 is an epithelial line from mouse testis although its differentiated features have not been reported sertoli cells have also been cultured from testis.

GREM CELLS:

These have been many reports of germ cell cultures, but the cultures are derived from early embryos rather than the gonads. When cells from an embryo are implanted into an adult (eg. under the Kidney capsule) they give rise to tumors known as Teratomas, or embryonic carcinomas. These kinds of Tumours also arise spontaneously when group of embryonic cells or single cells are carried over into the adult, often at an inappropriate site. Artificially derived Teratomas have been used extensively to study differentiation. Teratoma cells on feeder layer of, for eg: mouse fibroblast, proliferate, but not differentiate, whereas, when the cells are grown on gelatin without a feeder layer or in non-adherent plastic dishes, nodules from that eventually differentiate.

3.3.2.12 Summary

The medium is by far the most important in culturing cells and tissues. The function of this medium is to provide the physical conditions of pH, osmotic pressure ..etc for their survival and the complicated chemical substances by the tissue which can't be synthesized by the tissue itself. The serum and serum free media includes these substances in required concentrations for cell culture. The serum itself is having potential nutrients in it as it is a cocktail of most of the factors required for cell proliferation. As the availability and Shelf life are the major problems with the with serum now people are trying to adapt serum free media**.**The most important thing in cell culture is the selection of source for cell culture.generally cells from embryonic tissues are generally have good capability to grow faster than adult tissue.Selection varies from type of experiment to be performed.

3.3.2.13 Questions

1. Write about the different components of serum media.

2. Discuss the advantages and disadvantages of serum free media.

3.3.2.14 Reference books

1) Cell and tissue culture by John Paul,fifthe edition

2) Basic cell culture techniques by Ian R . Freshney.

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3.3.3

MAINTENANCE OF THE CULTURE and CELL LINES

Objective : the main objective of this lesson is to bring the information regarding the maintenance of cell lines during culture

3.3.3.1 Introduction:

The first subculture represent an important transition for the culture. The need to subculture implies that the primary culture has increased to occupy all of the available substrate. Hence cell proliferation has become an important feature. While the primary culture may have a variable growth fraction depending on the type of cells present in culture, after the first subculture the growth fraction in usually high. (80% or more).

From a very heterogeneous primary culture, containing may of the cell types present in the original tissue, a more homogeneous cell line emerges. In addition to its biological significance, this has also considerable practical importance as the culture can now be propagated, characterized, and stored, and the potential increase in cell number and

uniformity of the cells opens up a much wider range of experimental possibilities

Once a primary culture is subcultured) or " Passaged" or "transferred"), it becomes known as a "cell line". This term implies the presence of several cell line ages either of similar or distinct phenotypes. If one cell lineage is selected, by cloning by physical cell separation, or by any other selection technique, to have certain specific properties which have been identified in the bulk of the cells in the culture, this becomes known as "cell strain". Some commonly used cell lines and cell strains are listed in table 10.2. If a cell line transforms in vitro this gives rise to a continuous cell line and if selected or cloned and characterised it is known as continuous cell strain. The relative advantage and disadvantages of finite cell lines or continuous cell lines are listed in table

Table-1

Advantages	Disadvantages
Propagation More Cells Cloning Homogeneity Characterisation of replicate samples Frozen storage eventually	Selection, overgrowth Loss of differentiated properties (may be indictable) Genetic instability Trauma of desegregation Enzymatic and mechanical damage.

3.3.3.2 Nomenclature:

The first subculture gives rise to a "secondary" culture, the secondary to a "tertiary" and so on, although in practice this nomenclature is seldom used beyond tertiary. Since the importance of culture life time was highlighted by Hay flick and others with diploid fibroblast (Hayflick and Moorhead, 1961), where each subculture divided

the culture is half ("split ratio"- 1:2), passage number has often been confused with "generation number". Cell lines with limited culture life spans ("finite" cell lines) behave in a fairly reproducible fashion. They will grow through a limited number of cell generations, usually between 20 & 80 population doublings, before extinction. The actual number depends on strain differences and culture conditions but is consistent for one cell line grown under the same conditions. It is, therefore, important that reference to a cell line should express the approximate generation number or number of doublings since explanation, "approximate" because the number of generations which have elapsed in the primary culture is difficult to assess.

The cell line should also be given a code or designation (eg. NHB, normal human brain), a cell strain or cell line number (if several cell lines were derived from the same source), NHB1, NHB2, etc., and if cloned a clone number, NHB 2-1, NHB2-2, etc, together with the number of population doublings, this becomes, for example, NHB2/2 and will increase by one for a split ration 1: 2 (NHB 2/2, NHB 2/3, etc); by two for a split ratio of 1:4 9NHB 2/2, NHB 2/4, etc.,), and so on. For publication each cell line should be prefixed with a code designating the laboratory in which it was derived. Eg. WI Wistar Institute (Feder off, 1975).

3.3.3.3 Routine maintenance:

Once a culture is initiated, whether it be primary culture or a subculture of a cell line, it will need a periodic medium change ("feeding") followed eventually by subculture if the cells are proliferating. In non-proliferating cultures, the medium will still need to be changed periodically as the cells will still metabolize, and some constituents of the medium will become exhausted or will degrade spontaneously. Intervals between medium changes and between subcultures very from one cell line to another depending on the rate of growth and metabolism; rapidly

grooving cell lines such as Hela are usually sub cultured once per week and the medium changed 4 d later. Mare slowly grooving cell line may only require to be sub cultured every 2,3 or even 4 WK, and the medium changed weekly between subcultures.

Table-2 Characters of finite and continuous cell lines

Character	Finite	Continuous
Ploidy	Diploid	Heteroploid
Transformation	Euploid	Aneuploid
Tumorigenicity	Normal	Transformed
Anchorage dependence	Non-tumorigenic	Tumorigenic
Contact inhibition	Yes	No
Density limitation of growth	Yes	No
	Yes	No (or less so)
Mode of growth	Monolayer	Monolayer or Suspension
Maintenance	Cyclic	Steady state possible
Serum requirement (in simple media)	High	Low
Cloning efficiency	Low	High
Markers	May be tissue specific	Chromosomal, enzymic
Special functions (e.g. Veins susceptibility differentiation)	May be retained	Often lost
Growth rate	Slow (24-96 hr.doubling time)	Rapid (12024 hr. doubling time)
Yield	Low (<10^6 cells/ml, 10^5 cells /cm^2)	High (> 10^6 cells/ml, >10^5 cells/cm^2)
Control features	Generation number *invivo* markers	Strain characteristics

TABLE- 3 Cell Dissociation for transfer or Counting – Procedures of gradually increasing severity.

1. Shake – off	Mitotic or other loosely adherent cells
2. Trypsin* PBS (0.01-05% as required, Usually 0.25%, 5-15 min)	Most continuous cell lines
3. Prewash with PBS or CMF, then 0.25% Trypsin* in PBS or saline-	Some strongly adherent continuous cell lines and many cell lines at early passage stages.

citrate. 4. Prewash with mM EDTA in PBS or CMF then 0.25% trypsin* in citrate	Some strongly adherent early passage cell lines. Epithelial cells, although some may be sensitive to EDTA.
5. Prewash with IMH EDTA, then EDTA 2nd rise, and leave on, 1ml/5cm	Strongly adherent cells, particular epithelial and some tumor cells (note: EDTA can be toxic to some cells)
6. EDTA prewash, then 0.25% trypsin* with IMH EDTA	Thick cultures, multi layers, particularly collagen-producing dense cultures.
7. IMH EDTA prewash, then 0.25% trypsin* and collagemase* 200 units/ml PBS or saline –litrate or EDTA/PBS 8. Scraping	All cultures, but may cause mechanical damage and usually will not give a single cell suspension.
9. Add dispase (0.1-1.0 mg/ml) or peonas (0.1 – 1.0 mg/ml) to medium and encubate till cells detach.	Will dislodge most cells, but requires centrifugation step to remove enzyme not inactivated by serum. May be harmful to some cells.

- Digestive enzymes are available (Difco, Worthington, bochringes, Mannheim, Sigma) in varying degrees of purity. Crude preparations, eg. Difco Trypsin 1: 250 or Worthington CLS grade collagenase, contain other protease's which may be helpful in dissociating some cells but may be tonic to others. Start with a crude preparation and progress to purer grades if necessary. Purer grades are often used at a lower concentration (mg/ml) as their specific activities (enzyme units /g) are higher. Purified trypsin at 4°C. has been recommended for cells grown in low serum concentration's or in the absence of serum (MC Keetan, 1977), and will generally be found to be more consistent. Batch testing and reservation, as for serum, may be necessary for some applications.

Replacement of Medium:

Four factors indicate the need for the replacement of culture medium.

A Drop in P^H: The rate of fall and absolute level should be considered. Most cells will stop growing as the P^H falls from P^H 7.0 to P^H 6.5 and will start to lose viability between P^H 6.5 & P^H 6.0, so if the medium goes from red through orange to yellow, the medium should be changed. Try to estimate the rate of fall; a culture which falls at 0.1 P^H units in 1 d will not come to harm if left a day or two longer before feeding, but a culture that falls 0.4 P^H units in 1d will need to be fed 24-48 hr later and cannot be left over a weekend.

Cell concentration: Cultures at high cell concentrations will use up the medium fasten than at a low concentration. This is usually evident in the P^H change but not always.

Cell Type: Normal cells (eg: diploid fibroblast) will usually stop dividing at a high cell density (density limitation of growth) due to growth factor depletion and other factors. The cells block in the GL phase of the cell cycle and deteriorate very little even if left for 2-3wk. Transformed cells, continuous cell lines, and some embryonic cells, however, will deteriorate rapidly at high cell densities unless the medium is changed daily or they are subcultured.

Figure – 10.1 Page no: 130, Material no: CBT

Figure – 10.2 Page no: 131, Material no: CBT

Figure – 10. (graphs) Page no: 131, Material no: CBT

Cell Morphology: when checking a culture for routine maintenance, be alert to sings of morphological deterioration: granularity around the nucleus, cytoplasmic vacillation, and rounding up of the cells with detachment from the substrate (Fig 10.1)

This may imply that the culture required a medium change, or may indicate amore serious problem, eg: inadequate as toxic medium or serum, microbiological contamination on senescence of the cell line.

During routine maintenance, the medium change or subculture frequency should prevent such deterioration.

Volume, Depth and Surface Area:

This usual ration of medium volume to surface area is 0.2 – 0.5 ml/cm^2. The upper limit is set by gaseous diffusion through the liquid layer and the optimum will depend on the oxygen requirement of the cells. Cells with a high oxygen requirement will be better in shallow medium (2mm) and those with a low requirement may do better in deep medium (5mm). If the depth is greater than 5mm, then gaseous diffusion may be come limiting. With monolayer cultures this can become limit be overcome by rolling the bottle on perfusing the culture with medium and arranging for gas exchange in an intermediate reservation. When the depth of suspension culture is increased, it should be stirred with a bar magnet. To prevent frothing, the depth of stirrer cultures must be a minimum of 5cm. For intermediate depths of medium between 5mm and 5cm use a roller bottle (see table 7.1).

Holding Medium:

A holding medium may be used where stimulation of mitosis. Which usually accompanies a medium change, even at high cell densities, is undesirable. Holding media are usually regular media with the serum concentration reduced to or 2% an eliminated completely. This will not stimulate mitosis in most untransformed cells unless a special serum-free formulation is used. Transformed cell lines are unsuitable for this procedure as they may either continue to divide successfully on the culture may deteriorate as transformed cells do not block n a, regulated fashion in G_1.

Holding medium is also used to maintain cell lines with finite life span without up the limited number of cell generations available to them. Reduction of serum and cessation of cell proliferation also promotes expression of the differentiated phenotype in some cells (Schousboe et al

1979; Maltese and volpe, 1979). Medium used for the collection of biopsy samples can also be referred to as " holding medium".

Subculture:

The growth of cells in culture usually follows the pattern depicted in figure 10.3. A lag following seeding is followed by a period of exponential growth (10g phase). When all the available substrate is occupied, or when the cell concentration exceeds the capacity of the medium, growth ceases or is greatly reduced, then either the frequency of medium changing must increase an the culture must be divided. The usual practice in sub culturing an adherent cell line involves removal of the medium and dissociation of the cells in the monolayer with trypsin, although some loosely adherent cells (eg. Hela-S_3) may be subcultured by shaking the bottle and collecting the cells in the medium, and diluting as appropriate in fresh medium in new bottles exceptionally, some cell monlayer cannot be dissociated in trypsin and require the action of alternative protease's such as propose, dispose or collagenase (Table 10.1) Lfoley and aftonomos, 1973). The attachment at cells to each other and to the culture substrate is mediated by cell surface glycoproteins and Ca^{+2} ions. Other protein derived from the cells and from the serum, become associated with the cell surface and the surface of the substrate and facilitate cell adhesion.

For finite cell lines, it is convenient to reduce the cell concentration at subculture by two – four eight or 16 – fold, making the calculation of the number of population doublings easier (2=1, 4=2, 8=3, 16=4), eg; a culture divided eightfold will require three doublings to achieve the same cell density. With continuous cell lines, where generation number is not usually recorded, the cell concentration is more conveniently reduced to a round figure, eg; 5 x 10^4 cells/ml. In both cases, the cell number should be recorded so that growth rate can be estimated at each subculture and consistency monitored.

Propagation in Suspension:

The preceding instructions refer to subculture of monolayers, as most primary cultures an continuous lines grow in this way. Cells which grow continuously in suspension, either because they are nonadhesive (eg; many leukemia's and marine ascities tumors) or because they have been kept in suspension mechanically or selected, may be subcultured like micro organisms. Trypsin treatment is not required and the whole process is quicker and less traumatic for the cells. Medium replacement is not usually carried out with suspension cultures as this would require centrifugation of the cells. Routine maintenance is, therefore, reduced to one of two alternative procedures, i.e. subculture by dilution, or increase of the volume with out subculture.

Suspension cultures have a number of advantages the production and harvesting of large quantities of cells may be achieved with out increasing the surface area of the substrate. Further nose, if dilution of the culture is continuous and the cell concentration kept constant, a steady state can be achieved; this is not readily achieved in monolayer culture. Maintenance of monolayer cultures is essentially cyclic with the results that growth rate and metabolism varies depending on the phase of the growth cycle.

Monolayers are convenient for cytological and immunological observation, cloning, mitotic, "shake off" (for cell synchronization of chromosome preparation) and insite extractions with out centrifugation.

Table 4

	Monolayer	Suspension
Maintenance	Cyclic pattern of propagation require dissociation dependent on availability of substrate	Can be maintained at "Steady State" simple dilution at passage dependent on medium volume only (with adequate gas exchange)
Results of differences in Geometry	Cell Interaction: Metabolic cooperation, junctional communication	Homogenous suspension Cell density limited by nutrient and

Sampling and Analysis	contact inhibition of movement and membrane activity, density, limitation of growth. Diffusion boundary of effects Establishment of polarity differentiation cell shape and cytoskeleton spreading, motility, over lapping, under lapping	hormonal concentration of the medium only shearing effects in stirred cultures may damage some cells.
Which Cells?	Good cytological preparation Chromosomes, Immunofluoroscence histo chemistry enrichment of mituses by "Shake-off" serial extractions insite possible with out centrifugation	Bulk production of cells. Ease of harvesting (no trypsin ration required)
	Most cell types except some hemopoietic cells and ascites tumors	Transformed cells and eymphoblastoid cell lines

3.3.3.4 Slow Cell Growth:

Even in the best - run laboratories problems may arise in routine cell maintenance. Some may be attributed to microbiological contamination, but often the cause lies in one or more alterations in culture conditions. The following check list may help to track these down:

1. Any change in procedure or equipment?
2. Medium:
   a) Medium adequate/ - check against other media
   b) Frequency of changing correct?
   c) P^H : check that it is with in 7.0 – 7.4 during culture
   d) Osmolality: check in osmometer
   e) Component missed out: make up fresh batch
   f) New batch of stock medium which is faulty?

g) Is BSS – based, is BSS satisfactory? (check with other users)
h) If water – based, is water satisfactory (check with other users, or against fresh IX medium, bought in).
i) Check still – deionizer – conductivity, contamination – glass boils – residue.
j) Storage vessel, for algal on fungal contamination chemical traces in plastic
k) HCO_3
l) Antibiotics

3. Serum:
   a) New batch? Check suppliers quality control
   b) Check concentration 700 low or too high?
   c) Reconfirm lack of toxicity, growth promotion and plating efficiency
4. Glassware of plastics:
   a) If new, check against previous stock.
   b) Wash-up other cells showing symptoms?
   c) Trace contamination of glass? Check growth an plastic
5. Cells (if other people's cells are all right):
   a) Contamination
      i) Bacterial, fungal-grow up with out antibiotics
      ii) Mycoplasma
         a) Stain culture with Hoechst 33258
         b) Check for cytoplasmic DNA incorporation of radioactive thymidine) by to
            Radiography
         c) Get commercial test done (eg. Flow laboratories or Microbiological
            Associates)
      iii) Viral – difficult to detect – try E M or fluorescent antibody.
   b) Seeding density too low at transfer
   c) Transferred too frequently

d) Allowed to remain for too long in plateau before transfer.

6. Subculture routine:
   a) Change in batch of trypsin or other dissociation agent.
   b) Severity of dissociation – too long, agent too concentrated or too high specific activity
   c) Pipetting during dissociation too vigorous
   d) Sensitive to EDTA used)
7. Hot room and incubators : Check temperature and stability
   a) Faculty thermostats
   b) Access too frequent
   c) Humidity of humid CO_2 incubators
   d) CO_2 concentration (check P^H insites)

At subculture a fragile or slowly growing line should be split 1:2, and a vigorous, rapidly growing line, 1:8 or 1:16. Once a cell line becomes contnuous usually taken as beyond 150 or 200 generations) the generation number is disregarded and the culture should simply be cut back to between 10^4 and 10^5 cells/ml. The split ratio or dilution is also chosen to establish a convenient subculture interval (perhaps 1 or 2 wk) and to ensure that the cells (1) are not diluted below that concentration which permits them to reenter the growth cycle with a lag of 24 hr or less and 21 do not enter plateau before the next subculture .

3.3.3.5 Summary

The first subculture represent an important transition for the culture. The need to subculture implies that the primary culture has increased to occupy all of the available substrate. The proliferative capacity is high during the fist sub culture. During sub culturing care should be taken during different steps like trypsinisation, and inoculation to avoid the contamination. If any slow growth is observed one should concentrate on any changes in procedures medium and equipment used.

3.3.3. 6 Model Questions

1)Explain the routine maintenance of cell lines

2)Give precautions that should be taken to reduce the cell growth

3.3.3.7 Reference books

1) Cell and tissue culture by John Paul,fifthe edition

2) Basic cell culture techniques by Ian R . Freshney.

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Lesson 3.3.4

Techniques of Animal Tissue and Cell Culture

Objective :

For practicing the animal cell culture one should have clear idea of different techniques of cell culture. The present chapter includes different culture techniques in culturing tissues, organs and embryos etc.

3.3.4.1 Introduction

For the development of primary cultures and cell lines, a variety of tissues and disaggregation methods are used to give good yield of separate cells. It is, therefore, obvious that for any exercise involving animal cell and tissue culture, tissue needs to be obtained under aseptic and sterile conditions, although it may or may not be cultured, depending upon whether or not it is immediately subjected to disaggregation to give an yield of cells. However, even for obtaining cell cultures, often the tissue is cultured using **'primary explantation technique'** developed by Harrison (1907), Carrel (1912) and others. This technique is used for cultivation of pieces of fresh tissue derived from the organism, and this was almost the exclusive technique used for animal tissue culture till about 1945. Different forms of primary explantation techniques are still widely used and will continue to be used for a very long time. These techniques differ only in the type of vessels (flasks, test tubes, etc.) used for growing the tissue, but are uniform in principle. The primary explantation technique is also used for embryo and organ culture, but are variously modified to become specialized techniques.

3.3.3.2 TISSUE CULTURE TECHNIQUES

The different explantation techniques are classified into the following: (i) **slide cultures**, (ii) **carrel flask cultures** and (iii) **roller test tube cultures**. These three techniques of tissue culture and the techniques of organ culture (including tissue engineering) will be discussed in this chapter.

1)Slide or coverslip cultures

In this technique, slides or cover slips are prepared by placing a fragment of tissue (**explantation**) onto a coverslip, which is subsequently inverted over the cavity of a depression slide. This is the oldest method of tissue culture and is still quite widely sued. This has a number of advantages and disadvantages listed in Table. From this Table, it is obvious that the application of slide culture is limited but it may be very useful for morphological studies through the use of time-lapse cinemicrographic investigations. There are several general methods for preparation of slide culture; three of these methods will be briefly described in this section.

Table 1 : Advantages and disadvantages of slide and coverslip cultures of tissue.

S.No.	ADVANTAGES	DISADVANTAGES
1	It is simple and relatively inexpensive.	Supply of oxygen and nutrients is rapidly exhausted, so that the medium quickly becomes acidic and requires transfer of rapidly growing tissue.
2	Cells in living state are spread out in a manner suitable for microscopy and photography.	Sterility can not be maintained for a long period.
3	Cells grow directly on coverslip and can be fixed and stained to make permanent slides.	Only very small amounts of tissue can be cultured.

2)Single coverslip with plasma clot.

This technique developed by Harrison (1907) has been most commonly used during the last more than fifty years and includes the following steps

(i) Prepare medium in two parts, one containing 50% plasma in BSS (Balanced Salt Solution) and the other containing 50% embryo extract in serum; (ii) under sterile and aseptic conditions, using a capillary pipette, place one drop of plasma containing solution in the centre of each of one, or more coverslips (22mm); (iii) transfer a fragment (one or two pieces) of tissue (called explant) to this drop without crushing the tissue; (iv) add the embryo extract containing solution and mix thoroughly before clotting starts and then locate the explant; (v) place

two small spots of petroleum jelly (using a glass rod) near the concavity of a depression slide and invert this slide over the coverslip; apply gentle pressue, so that jelly sticks to coverslip; (vi) allow culture medium to clot; (vii) turn over the slide and seal the margins of coverslip with paraffin (viii) label and incubate at 37^{O}C.

3)Double coverslip with plasma clot

This technique was developed by Maximov and resembles the single coverslip method described above. It includes the following steps: (i) A small drop of BSS is placed on a large coverslip (40mm); (ii) a square or round coverslip (22mm) is placed over BSS in the centre of large coverslip. These two steps are then followed by the steps listed above for single coverslip method. A large depression slide is used and the entire preparation is attached to it by petroleum jelly and wax in such a way that the small coverslip is not in contact with the slide at any point

3)Single coverslip with liquid medium (lying and hanging drop cultures)

Following steps are involved in this method: (i) prepared explants are placed in culture medium in a watch glass; (ii) the explants are drawn into the tip of a capillary pipette, and one explant is deposited in the centre of each coverslip; (iii) the liquid medium can be spread out in a very thin circular film with the explant protruding above the surface; (iv) a depression slide with petroleum jelly is applied immediately and preparation turned over with a quick flip to prevent the fluid from running into the crevice between the slide and the coverslip; (v) the coverslip is sealed and the slide incubated at 37^{O}C, upright or inverted; the tissue grows on the coverglass.

After-care of slide cultures

Single coverslip cultures are very useful for short term studies but they are difficult to handle subsequently except by a process of transfer. Therefore, double cover slip method is recommended, whenever it is desirable to leave the explant in its original location to obtain long term tissue cultures. This would require washing, feeding, patching and transfer of cultures.

(a) Washing and feeding double coverslip cultures

Washing and feeding involves the following steps: (i) remove seal using razor blade and remove large coverslip with small one still attached and flip it over, so that culture is uppermost in orientation; (ii) detach small coverslip from the large coverslip using needle and forceps and transfer it to a Columbia staining dish (watch glass or Petri dish may also be used) containing balanced salt solution (BSS); (iii) remove small cover slip treated as above (one at a time) and place it on a large coverslip with culture up; while removing small coverslip from dish with BSS, the mount of BSS brought with it may be controlled by the rate of removal (too much liquid will interfere in feeding operation and too little will allow air bubbles); (iv) feed the culture by adding a drop of feeding solution (e.g. BSS : serum : 50% embryo extract = 1:1:1) to the small coverslip; (v) attach a clean depression slide, using petroleum jelly, as done earlier.

(b) Patching the plasma clot in slide cultures

If there is evidence of liquefaction, plasma clot should be patched as followed: (i) wash small coverslip with culture as above; (ii) in a watch glass take two drops of a mixture of plasma and BSS and to this add two drops of a mixture of serum and embryo extract; mix this quickly and place a drop on each washed coverslip having a culture; (iii) a clean depression slide with petroleum jelly is then attached, as usual.

(c) Transfer of slide culture

The coverslip culture may need to be transferred using the following steps; (i) remove and wash coverslip with culture, and using a Bard-Parker knife, cut through the outgrowth; (ii) the square tissue may be cut into two or four pieces, each transferred to new coverslip and treated as a new explant.

4)Flask cultures

The main use of flask cultures is in the establishment of a strain from fresh explants of tissue. A good Carrel flask has excellent optical properties for microscopic examination, even though polystyrene culture flasks can also be used, provided they have a wide neck for handling the explants. The flask technique has the following advantages: (i) tissue can be maintained in the same flask for months or even years; (ii) large number of cultures can be easily prepared and large amount of tissue can be grown with large amount of medium.

There are two types of flask techniques: (i) **`thick clot cultures'**, which allow rapid growth suitable for short term cultures and (ii) **`thin clot cultures'**, which can be maintained for a considerable period of time.

Preparation of flask cultures

Following steps are involved in the preparation of flask cultures (i) place upto six D3.5 **Carrel Flasks** in a rack with their necks flamed and pointing to the right; (ii) place a drop of plasma on the floor of flask and spread this plasma out in a circle; (iii) with the help of spatula, transfer the desired number of explants to the plasma and allow clotting to occur; (iv) after the plasma clots and explants fixed in position, and extra medium; for thick clots 1.2ml of dilute plasma and for thin clots 1.2ml of dilute serum is added instead of plasma; the whole thing is left for clotting; (v) the flasks are gassed with gas phase (5% CO_2 in air).

Renewal of medium

The medium may be periodically replaced in flask cultures as follows: (i) old fluid is drawn off by means of a pipette; (ii) 1.2ml of fluid medium is added as replacement; (iii) the flask is gassed as above.

Transfer of Culture

The culture grown in a flask culture need to be removed and cut into pieces, when there is a need for transfer. These pieces are then used for replantation as usual.

5)Test tube cultures

Test tubes are cheap and convenient vessels for tissue culture and can be used for preparing a large number of cultures, which can be placed in stationary racks or roller drums. However, this technique has disadvantages like poor optical property for microscopy, difficulty

in quantitation due to the curvature and high risk of contamination. Cultures on plasma clots in test tube are prepared just like those in flasks, but tissues may be grown on the wall of test tube without a plasma clot. Test tubes may also be used for developing suspension cultures. The feeding, patching and transfer of culture is done as in other primary explantation techniques.

3.3.4.3 Organ Culture

Organ culture usually implies culturing pieces of an organ (not necessarily whole organ) *invitro*, and its objective is to maintain the architecture of the tissue and direct it towards normal development such as occurs *invivo*. In order to achieve this aim, it is essential that the tissue should never be disrupted or damaged and this requires careful handling. In view of this, organ culture techniques generally demand more careful manipulation than tissue culture techniques discussed earlier in this chapter. Media used for growing organ cultures are generally the same as those used for tissue culture. The techniques of organ culture can be divided into (i) those employing a solid medium and (ii) those employing liquid medium.

1)Culture of embryonic organs

It is easier to culture embryonic organs than to culture organs from adult animals and the techniques used in two cases. Embryo organs can be cultured, using any of the following three techniques.

2)Organ cultures on plasma clots

The organ cultures can be prepared on plasma clots using the following steps: (i) prepare a plasma clots by mixing 15 drops of plasma with five drops of embryo extract in a watch glass; (ii) place the watch glass on a pad of cotton wool in a Petri dish; cotton wool is kept moist to prevent excessive evaporation from the dish; (iii) place a small, carefully dissected piece of tissue on top of the plasma clot in the watch glass. The technique has now been modified, and a raft of lens paper or rayon net is used on which the tissue is placed. Transfer of the tissue can then be achieved by moving the raft easily. This also facilitates feeding of the culture, so that the net may be removed. Excess fluid is removed and the net with the tissue placed again on fresh pool of medium.

3)Organ cultures on agar

Media solidified with agar are also used for organ culture and these media consist of 7 parts 1% agar in BSS, 3 parts chick embryo extract and 3 parts horse serum. In some other studies, denied media with or without serum are used with agar. The medium with agar has the advantage that the support for organ culture does not liquefy and no additional mechanical support is required. Embryonic organs generally grow well on this medium, but tumours from adults will not survive.

4)Organ cultures on fluid media

Organ cultures also make use of liquid media. In this method, often a raft made of lens paper, or cellulose acetate net or perforated metal gauze is used.

5)Culture of adult organs

The culture of organs or parts from adult animal is more difficult due to their greater requirement of O_2. A variety of adult organs (e.g. liver) have been cultured using special media (e.g. T8; see Paul, 1975) with a special apparatus (Towell's Type II culture chamber). Since serum was found to be toxic, serum free media were used, and the special apparatus permitted the use of 95% O_2.

3.3.4.4 WHOLE EMBRYO CULTURE

Culture of chick embryo

The effect of metabolic inhibitors on embryonic development was studied by Spratt (1956, 1957), using embryo cultures. In this technique, 40-hours old embryos were used and the embryo development could be followed for another 24-48 hours *invitro* before the embryo dies. This involves the following steps: (i) prepare a suitable defined medium or a synthetic medium (consult a manual), and 1ml aliquots of the medium are added to sterile watch glasses, placed on moist absorbent cotton wool pads in Petri dishes (as for organ culture); (ii) incubate hen's eggs at $38^{O}C$ for 40-42 hours to

provide about a dozen embryos; (iii) the shell is wiped with alcohol and broken into a sterile evaporating dish containing 50ml chick saline or BSS; (IV) a circular cut is made (using scissors) into the vitelline membrane around the blastoderm and the latter is transferred to a Petri dish containing BSS; (v) the adherent vitelline membrane is removed with the aid of forceps and the embryo is examined under the microscope to determine the stage of development; (vi) the blastoderm is transferred to the top of the medium in the watch glass prepared in (i) above; (vii) the blastoderm is spread on agar gel (ventral side down) and the excess BSS is removed; (viii) culture is incubated at 37.5^{O}C.

Culture of mammalian embryos or ova

The mammalian embryos (rabbit or mouse), as young as 2-8 celled fertilized ova, have been cultured. It was shown in these cases that considerable development can really take place in vitro, and mouse embryos were shown to develop upto blastocysts. Such cultured embryos or ova can be reimplanted and give rise to healthy animals. The media used for this purpose varied from 100% serum, through the common types of plasma-embryo extract media to simple Krebs-Ringer solutions supplemented with 1% of thin egg white or bovine albumin. The embryo can be cultured either in the pre-implantation stage or at the post-implantation stage. In the latter case, special care is needed to remove the embryo in an undamaged form.

3.3.4.5 Cell culture techniques

Cell culture can be performed in two ways.They are monolayer culture and suspension culture

1) Monolayer cultures

Preparation of monolayer includes different steps like isolation of tissue dissagregation and culturing in flasks

a)Tissue isolation

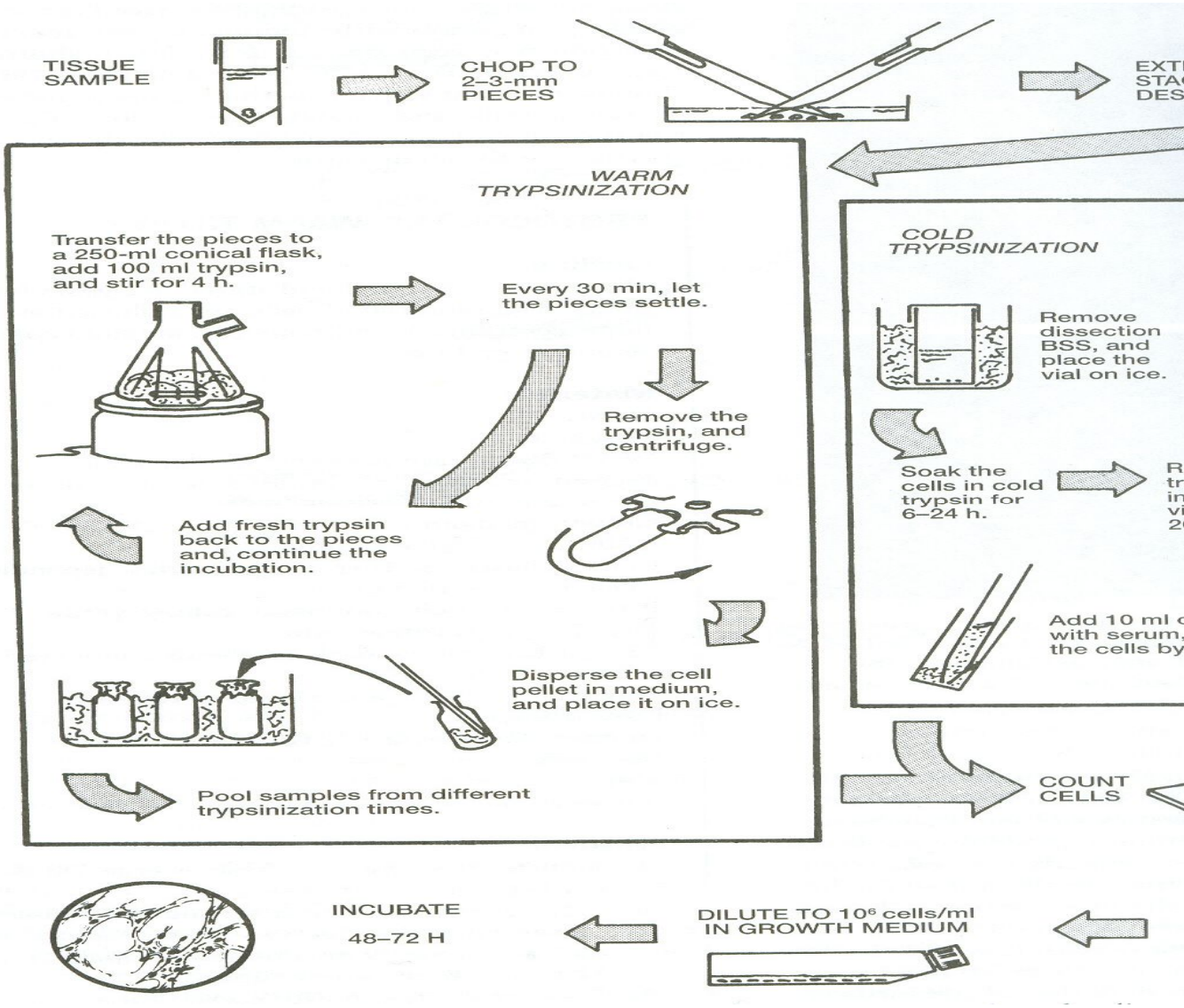
The tissue is isolated from the patient or from any other sources after getting the concerts from he patient , surgeon and from he respective people. The tissue must be chopped in with fine knife taking care should be taken to avoid the maximum damage tissue . once isolated tissue should be transferred to medium and should be sent to the laboratory with in least possible time to get the maximum number of viable cells

While isolating cells one should consider the following

- Fat or necrotic tissue should be removed. Tissue should be finely chopped with sharp instrument (to minimize the damage) Higher cell concentration is needed for primary isolation

- Nutrient rich medium should be used to transfer the isolated tissue

b)Disaggregation: The tissue is diaggregated by means of mechanical disruption or enzymatic disaggregation into individual cells.Mechanical disruption is generally carried out by perfusion method.But most commonly used technique is enzymatic disaggregation where the tissue is treated with different types of enzymes and their combinations at different temperatuires and time intervals,so that he proteolytic connections present between cell can be lysed by these proteolytic enzymes.Moost commonly used enzymes are trypsin, collagenase,pronase and dispase .

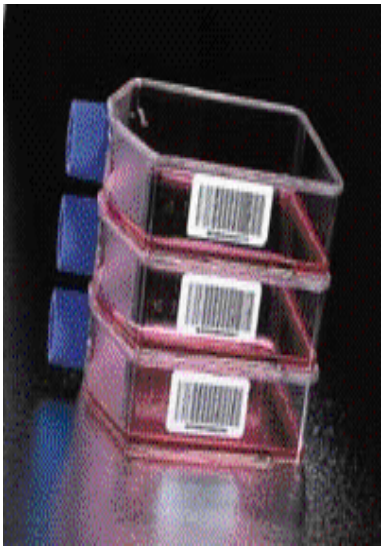


c)Culturing in Flasks

The disaggregated cells at the concentration of 10^{6} cells /ml is inoculated into different flasks with the suitable medium.The flasks were generally incubated in CO2 incubator for 3-4 days .During the

incubation the cels first attches to surface of the substrate (surface of the flsks) and proliferate to form monolayers.

Fig-Culturing invessels



Properties of monolayer

a)Confluency:

A culture population which occupies all a vailable growth surface. In practice it is not wise to maintain cells beyond the point of confluency.

The most commonly studied, well characterized cell lines include

Human Diploid, fibroblastic cell line, WI-38 Human cervical adenocarcinoma epitheloid, HeLa Mouse embryonic, fibroblastic, 3T3.

b)Ideal surfaces for monolayer are:

Non-toxic, biologically inert and optically transparent

Advantages of Monolayer Culture

Advantages

Easy to change medium and wash cells before adding fresh medium

- Expression is better in attachment cells
- Flexible and can be used for all cell type

Disadvantages

- Scale-up is difficult and expensive
- Require more space than suspension cultures
- Can not aliquot a sample to monitor cell growth
- Oxygen and pH measurements is not easy

2)Suspension cultures:

Cells which are not dependent on surface for their growth are generally cultured in suspensions.the cells inoculated are maintained in suspension either by means of shaking or rotation. Large scale culture can be grown in fermentors**.**

Advantages of suspension culture

.Does not require any support

- No contact inhibition
- Cells will be generally oval in shape
- Easy to scale up
- Most of the cells wont grow in this fashion

3.3.4.6 tissue Engineering

As discussed in the last chapter and earlier in this chapter, a variety of animal tissues can now be cultured. The number of types of these human tissues is more than 200. During the last decade, the tissue culture work in animals demonstrated that virtually any human tissue or organ can be grown in culture . This has become possible when it was found that the ability of cultured cells to undergo differentiation can be restored. These developments led to the

emergence of a new science called `tissue engineering'. Some of the goals of tissue engineering are listed in Table given below `Skin' was the first organ, which could be cultured in artificial media and could be used successfully for transplantation, after serious skin burns. Eugene Bell, who is the pioneer of artificial skin development, feels that eventually a tissue engineer will be able to reconstitute body parts for (i) use in grafting, and to be used as (ii) better models for drug delivery and action.

Table 2 Some of the technological goals of tissue engineering.

1	Growth of cells in three-dimensional systems
2	Delivery systems for protein therapeutics
3	Cell cultivation methods for culturing `recalcitrant cells'
4	Transgenic protein expression in transplantable cells
5	Vehicles for delivering transplantable cells
6	Avoiding immunogenicity in transplantation systems
7	Development of markers for tracking transplanted cells
8	Developing *invivo* and ex vivo biosensors for monitoring cell behaviour during tissue production

It is believed by tissue engineers like Rebort (Bob) Nerem of USA, that after the success of artificial skin, the next step is the growth of functional artificial organs like pancreas and liver damaged due to a disease. Although the clinical application of these other artificial organs is remote, the potential of growing and using these artificial organs does exist.

A `tissue engineering conference' was held in 1990 at Keystone, Colorado (USA), where possibility of replacing `Cartilage' in stress bearing joints was found to be a reality. Donald Ingber of USA works

on developing `extra cellular matrices (ECMs)' that will allow tissue growth and differentiation in vitro, as a means to organ replacement. However, tissue engineering would need a knowledge of physics and chemistry along with the knowledge about cell and tissue culture. For example, one will have to know, how the cellular environment affects three dimensional structure of tissue grown in culture. One will also have to know the fine structure of carbohydrate aggregates surrounding the cells. Detailed knowledge on these aspects will allow the induction of stem cells to differentiate in required manner. It is also believed that stem cells exist for all tissue types, and if these can be recovered, any human tissue can be induced to reform.

Artificial skin

There are three biotech companies in USA, which are developing artificial skin to the stage of clinical trials (i) ATS (Advanced Tissue Science), located at La Jolla, California which is now merged with Neomorphics (Lexington, MA), (ii) Biosurface Technology (BTI, Cambridge, MA) and (iii) Organogenesis. These three companies used different technological approaches, which are really the basis of all future tissue engineering.

The real breakthrough in culturing artificial skin was made in 1970s, at Harvard. It was demonstrated that when an irradiated 3T3 fibroblast cell line is grown with skin cells (keratinocytes, which make 90% of the skin epidermis), 3T3 cells stimulated growth of skin cells and allowed their differentiation into epidermis. 3T3 cells are believed to secrete factors which help in growth and differentiation. This technique was used by BTI, where postage stamp sized skin biopsies are taken and expanded 10,000 times within three to four weeks. This gives only the cultured epidermis, which when applied to burnt area, helps regeneration of dermis underneath. Since in severely burned patients, skin biopsies may not be available from the patients, following two approaches were used to overcome the problem: (i) Skin from cadavers (dead bodies) are taken to cover and protect against infection and desiccation, until their own skin is grown. (ii) Neonatal foreskin (loose skin from the tip of penis of newly born child) is cultured, which creates artificial dermis layer. This leads to allogeneic grafts stimulating rapid healing. New born skin cells are used, since they grow relatively more vigorously. ATS company uses a synthetic polymer, PGA, for growing artificial skin from neonatal foreskin. The use of synthetic polymers allows cells to grow into skin without scars,

so that it is believed that this technology will be the basis for future organ development.

A further advancement was made by the company `Organogenesis' which claims to have created a `living skin equivalent (LSE)' comprising of both a lower dermis layer and an upper epithelial layer. Collagen matrix is used as a support for the growth of LSE. This LSE when used for grafting may be replaced by host cells eventually, but is not rejected.

3.3.4.7 Summary :

Since 18th century several technique were used by different people for culturing animal tissues, organ as cells of animals for different purposes. For culturing tissues coverslip culture and flask cultures techniques were generally used. Organs are generally cultured of plasma clots, agar clots and some times on third media we can also culture entire embryos on suitable media. Cells can be cultured either by monolayer culture or by suspension cultures.

3.3.4.8 Model Questions :

1. Explain different tissue culture techniques?
2. Write about embryo culture?

3.3.4.9 References

1. Animal cell culture techniques by Ian Freshney.
2. Animal Cell culture by Davidson.

(Author-Jaganmohan Rao)

Lesson 3.4.1

Microcarrier Culture

Objective

If an anchorage-dependent cell line needs to be grown in suspension it becomes necessary to use a carrier system such as microcarriers. This chapter deals with development ,technique and applications of microcarriers.

3.4.1.1 Introduction:

Cell culture techniques have become vital to the study of animal cell structure, function and differentiation and for the production of many important biological materials such

as vaccines, enzymes, hormones, antibodies, interferon's and nucleic acids. Microcarrier culture introduces new possibilities and for the first time makes possible the practical high yield culture of anchorage-dependent cells. In microcarrier culture cells grow as monolayers on the surface of small spheres which are usually suspended in culture medium by gentle stirring. By using microcarriers in simple suspension culture systems it is possible to achieve yields of several million cells per milliliter.

3.4.1.2 Development of microcarriers:

With the development of microcarriers in 1967 by van Wezel the production of biologicals from anchorage dependent cells is a dream come true. van Wezel made microcarriers composed of cross-linked dextran beads charged with tertiary amine groups (DEAE) having an exchange capacity of 3.5 milli equivalent / gm dry weight. He demonstrated the growth of primary cells and cells from a human diploid cell strain on them as well as the propagation of poliomyelitis virus in the cells grown on microcarriers.

These microcarriers faced the problem of cell attachment due to large charge densities on their surfaces (van Wezel 1973; 1977). It was observed that above a bead concentration of 1 gm/liter, there were increased innoculum losses, long lag periods and diminished capacity for cell growth. Complete cell death was seen at bead concentrations of more than 2 gm/liter. To overcome this problem, many approaches were tried like coating the beads with serum proteins, nitrocellulose or carboxymethyl cellulose.

It was Levine *et al* 1979 who developed microcarriers of **reduced ion exchange capacity** (1.5 meq./gm dry weight). These could be used at concentrations as high as 6

gms/liter with no deleterious effect on cell growth. These low charged beads could be used to cultivate primary, diploid as well as established cell lines. Subsequently a lot of work was done to improve the quality of the beads. Different types of microcarriers were developed and patented. Henderson T. M has obtained a patent (US patent no. 4448884) for glass microcarriers. These have an outer layer of glass below which a layer of magnetic material has been added so that the microcarriers may be separated from the culture medium under the influence of a magnetic field. Cross-linked dextran has been found to be the best material for making microcarriers. Also, microcarriers were derived such that the surface charge was limited to the surface layers only. In such microcarriers the absorption of proteins like IgG or albumin present in the serum was low. Collagen has also been used for producing microcarriers. These beads have the advantage that they can be digested by enzymes, thereby facilitating harvesting (Gebb *et al* 1984).

3.4.1.3 Advantages of microcarrier cultures:

This system has the following advantages over other methods of large-scale cultivation:

- High surface area to volume ratio can be achieved which, can be varied by changing the microcarrier concentration. This leads to high cell densities per unit volume with a potential for obtaining highly concentrated cell products.
- Cell propagation can be carried out in a single high productivity vessel instead of using many low productivity units, thus achieving a better utilization and a considerable saving of medium.
- Since the microcarrier culture is well mixed, it is easy to monitor and control different environmental conditions such as pH, pO_2, pCO_2 etc.

- Cell sampling is easy.
- Since the beads settle down easily, cell harvesting and downstream processing of products is easy.
- Microcarrier cultures can be relatively easily scaled up using conventional equipment like fermenters that have been suitably modified (Spier and Horaud 1985).

Because of the many advantages of the technique itself, it has gained great popularity. Thus, a large variety of microcarriers are available in the market.

Commercially available microcarriers:

Due to the growing need for large quantities of anchorage-dependent cells, a variety of microcarriers are now available in the market. A list of some of the commercially available microcarriers, together with the names of their manufacturers is given in Table 2.

Table 2 : Commercially available microcarriers

Type	Trade Name	Company ?	Country	Composition
1. Dextran	Cytodex-1	Pharmacia ?	Sweden	DEAE - dextran
	Cytodex-2	Pharmacia	Sweden	Quaternary amine coated dextran
	Superbeads?	Flow Labs	USA ?	DEAE - dextran

	Microdex ?	Dextran products	Canada	DEAE dextran
	Dormacell	Pfeifer and Langer	Germany	DEAE-dimers-dextran
2. Plastic ?	Biosilon	Nunc ?	Denmark	Polystyrene - charged
	Biocarriers	Biorad	USA	Polyacrylamide/ DMAP
	Cytospheres	Lux	USA	Polystyrene charged
3. Gelatin	Cytodex - 3	Pharmacia	Sweden	Gelatin - coated dextran
	Gelibeads	KC Biologicals/ Hazelton Labs	USA?	Gelatin
4. Glass	Bioglas	Solohil Engs	USA	Glass- coated plastic
	Ventreglas	Ventrea	USA	Glass- coated plastic
5. Cellulose	DE-52/53	Whatman	UK	DEAE - Cellulose.

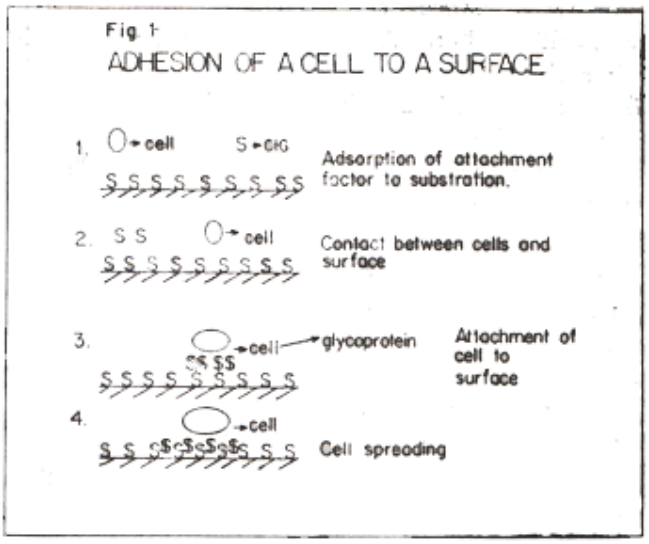
The availability of a wide range of microcarrier products in the market makes it necessary to define the exact requirements of a good microcarrier.

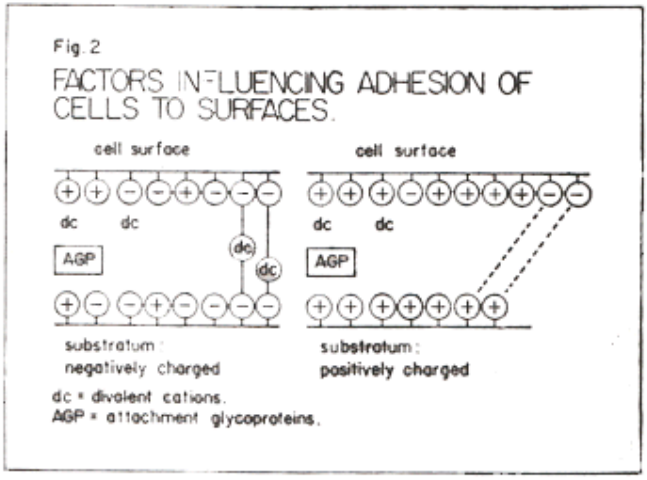
3.4.1.4 Requirements for an optimum microcarrier:

- The surface properties of the beads should be such that cells can adhere and proliferate rapidly. In other words, the contour should be even.
- The density of the beads should be slightly more than that of the culture medium, so as to facilitate easy separation. Conventional culture media are aqueous in nature and have densities ranging from 1.03-1.09 g/cc. However the density should not exceed a certain limit the optimum range being 1.03-1.045 gms/ml. Gentle stirring, which will not harm the shear-sensitive cells, should be sufficient to keep them in suspension. If the beads settle down cell growth will be prevented.
- The size-distribution of the beads should be narrow so that an even suspension of all microcarriers is achieved and cells attain confluency at approximately the same time.
- The optical properties should enable easy microscopic observation.
- They should be non-toxic not only for the survival and good growth of the cells but also for cell culture products that are used for veterinary or clinical purposes.
- The matrix of the beads should be such that collisions, which occur during stirring of the culture, do not cause fragmentation of the beads.
- Ideally, the beads should be such that they can be reused (Levine *et al* 1979).

3.4.1.5 Choice of microcarriers:

The choice of the microcarrier depends primarily on the objective of the culture. For instance, in the case of extracellular viruses and cell products obtained from established cell lines, dextran microcarriers prove to be the best. If the cells need to be trypsinised or detached then glass or gelatin beads are preferred. When a particular type of cell tends to have a low plating efficiency, it is important to use that microcarrier which enables attachment of the maximum number of cells. The choice of the microcarrier also depends on the morphology of the cells. For instance, when culturing cells with fibroblast-like morphology, microcarriers with a layer of surface charge on the periphery of the bead tends to show better attachment of cells as compared with others. For cells with an epithelial-like morphology, the attachment is better on beads coated with a protein like gelatin (Levine *et al* 1979, Spier and Horaud 1985). Macrocarriers can be used when cells are extremely shear sensitive.





3.4.1.6 Adhesion of cells to microcarrier surface:

The surface on which the cells are grown as well as the cells at physiological pH have either a net negative or a positive charge. The charge density on these surfaces rather than their polarity is responsible for attachment and spreading of cells. It is also known that cell

adhesion is mediated by specific cell surface receptors.

Maroudas's theory of attachment states that cell surface contact is bridged by amphoteric proteins (Figure 1). According to him, the prerequisite for attachment of cells onto any surface is the adsorption of protein factors to culture surface (Mukhopadhay *et al* 1993) after which the contact between cells and surface can take place. The cells attach onto the substratum before which they produce their own glycoproteins and matrix proteins and then spread out (Figure 2). It is this matrix that adheres to the charged surface and the cells then bind to the matrix via specific receptors. Therefore many a times it has been observed that surfaces that have been conditioned by previous cell growth often provide a better surface for attachment.

Three major classes of transmembrane proteins have been shown to be involved in cell-cell and cell-substrate adhesion viz.

1. Cell-cell adhesion molecules (CAMS) which are calcium independent and Cadherins which are calcium dependent

2. Integrins which are receptors for matrix molecules such as fibronectin, laminin and collagen which bind to them with the help of a arginine-glycine-aspartic acid sequence known as the RGD motif

3. Transmembrane proteoglycans which interact with matrix molecules like collagen but not with the help of the RGD motif (Freshney 1994).

Cell adhesion also depends on a functional contractile system. It involves multiple contacts with the surface. Next numerous filopodia are formed. They fit into a lattice structure formed by the glycoproteins on the substratum. This is followed by cytoplasmic webbing and flattening of

the cell mass. Thus the cells get attached onto the substratum (Hirtenstein *et al* 1980).

Once the cells have attached onto the microcarriers, they grow using the nutrients provided in their culture medium. The complexities of this culture system are mainly due to the large number of parameters affecting cell yield. Medium composition now assumes importance since it contains the carbon and nitrogen source, the energy source, growth factors and dissolved oxygen and other gases. Besides nutrient limitation, growth of cells is also affected by the accumulation of toxic metabolites. Other important considerations are environmental factors like pH and temperature and shear sensitivity of the cells, especially in case of microcarrier cultures employing spinner bottles and fermentors. (See figures 3 and 4).

3.4.1.7 Applications of microcarrier cultures:

A wide range of cells have been cultured on microcarriers. For instance, cells from invertebrates, from fish, birds and cells of mammalian origin have been cultivated on microcarriers. Transformed and normal cell lines, fibroblastic and epithelial cells and even genetically engineered cells (Schmid *et al* 1992) have been cultivated on microcarriers for various biologicals such as for the production of immunologicals like interferons, interleukins, growth factors etc. Cells cultured on microcarriers also serve as hosts for a variety of viruses that are used as vaccines like foot and mouth disease or rabies.

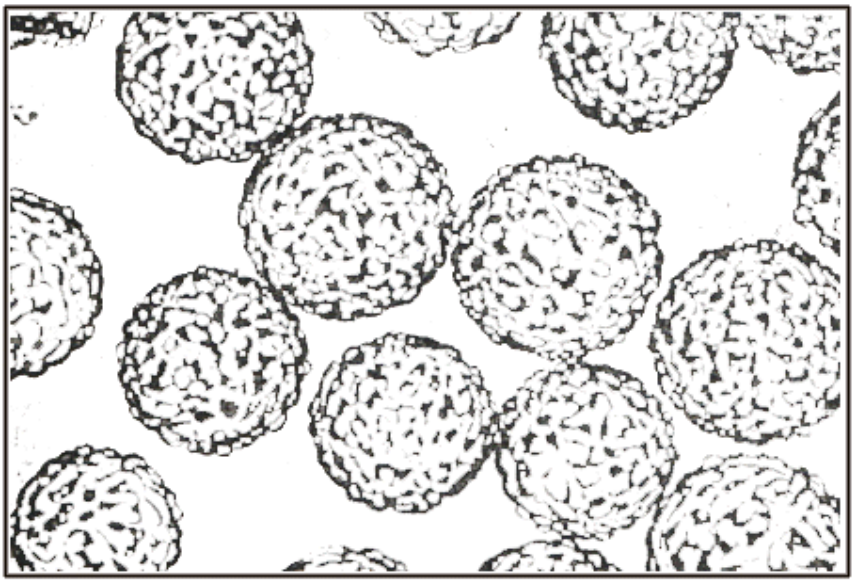


Figure3: Cells growing on microcarriers at confluency

Microcarrier cultures have found a wide number of applications other than mass cultivation as well. Cells growing on microcarriers serve as an excellent tool for studying different aspects of cell biology such as cell-to-cell or cell-to-substratum interactions. Cell differentiation and maturation, metabolic studies have also been carried using microcarriers (Tang et al 1994). Such cells can also be used for electron microscopic examinations or for the isolation of cell organelles such as the cell membrane. Also, this system is essentially a three-dimensional system and serves as a good 3-D model (Jessup *et al* 1997). Similarly, co-cultivation of cells can be done using this system (Johns *et al* 1995).

Microcarriers have also been used for the depletion of macrophages from a population of spleen cells. DEAE-dextran microcarriers can potentiate stimulation of lymphocytes by concanavalin A (con A). Microcarrier beads confluent with allogenic tumour cells can be injected in mice to increase humoral and cell-mediated immunity. Plant

protoplasts can be immobilised on DEAE-dextran microcarriers (Maroudas 1977).

Due to the large surface area to volume ratio provided by microcarriers, they are now successfully being used for a variety of biologicals on a laboratory as well as an industrial scale of even 4000 liters (Meigner *et al* 1980; Griffiths 1992; Montagnon *et al* 1984; van Wezel *et al* 1978; 1984; Montagnon 1989).

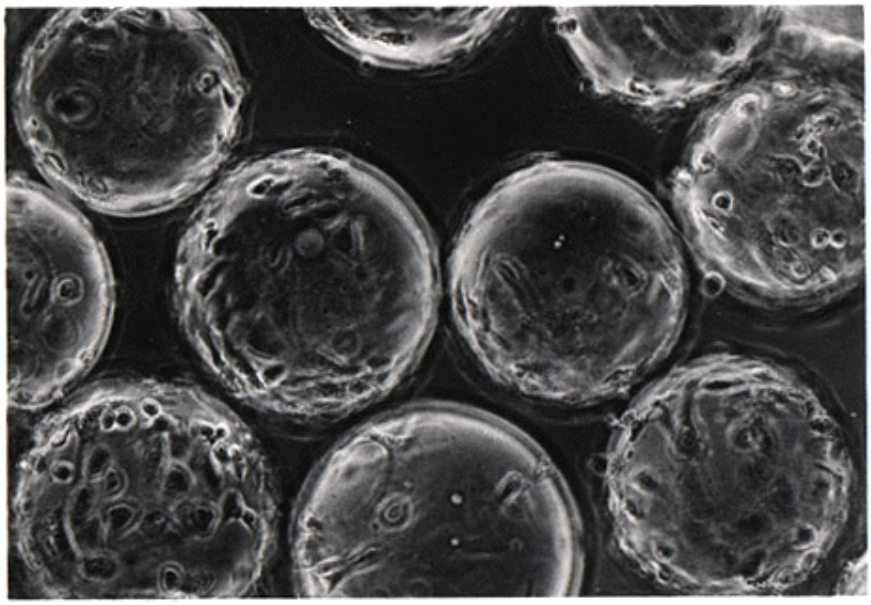


Figure 4: Vero cells cultured on Cytodex microcarriers

The following table shows some of the different products obtained from cells growing on microcarriers.

Table 3: Products obtained from cells growing on microcarriers

Products (Cells/Cellular products) / Reference

Interferon	Giard *et al* 1979Vascular
endothelial cells	Davies 1981 Bing *et al* 1991
Primary and established cell lines	Reuveny *et al* 1982
Pancreatic islet cells	Bone *et al* 1982
Proteolytic enzymes	Varani *et al* 1986
Arachidonic acid	Varani *et al* 1986
Tissue plasminogen activator	Nilsson *et al* 1988
Growth inhibitor	Spier & Fowler 1985
Nerve growth promotor	Norrgren et al 1983
Kallikrien	Kumar et al 1999

VIRAL PRODUCTS

Sindbis	Giard *et al* 1977
VSV	Giard *et al* 1977
Oncorna	Manousos *et al* 1980
Herpes simplex	Griffiths *et al* 1982
Hepatitis A	Widell *et al* 1984
Channel cat fish virus	Buck *et al* 1985
RSV	Hayle 1986

Corona virus	Talbot *et al* 1989
FMDV	van Wezel 1977, Meigner 1979
Rabies	van Wezel *et al*1978,1979, Montagnon 1989
Polio	Montagnon *et al* 1984
Reo virus	Berry *et al* 1999
Measles and mumps??	Siderenko et al 1989

In conclusion, it can be said that microcarrier technology is a wonderful tool for the industry since the technology can be scaled up easily to any extent and can be easily optimised.

3.4.1.8 Model Questions

1) What are microcarriers ,explain the technique of microcarrier culture

2) Discuss the applications of microcarrier culture

3.4.1.9 Reference books

1. Animal cell culture techniques by Ian Freshney.

2. Animal Cell culture by Jhon Master.

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Lesson 3.4.2

Cell Synchronization , cell growth and Cell transformation

Objective

Objective

This chapter deals with the different methods of cell syncronisation, cell growth and cell trans formation.

3.4.2.1 Introduction

In order to study many of the control mechanisms involved in cell cycle regulation, it is critical to synchronize the cells so that the distinct phases of the cycle are being studied Synchronizing cells allows the precise study of regulatory mechanisms which determine cell cycle regulation at the level of gene expression and proteinphosphorylation, facilitating drug discovery.

The term **cell growth** is used in two different ways in biology. When used in the context of reproduction of living cells the phrase "cell growth" is short hand for the idea of "growth in cell numbers by means of cell reproduction

3.4.2.2 Cell Synchronization

To synchronize cells, several methods are used. Commonly, researchers induce cells to quiescence by serum starvation before releasing them from this state, or treat cells with chemical inhibitors which arrest cells in distinct phases of the cycle. The following is a brief summary of these methods.

A. Serum starvation

In order to ensure that cells progress through the cell cycle in a synchronous manner, it is common to grow cells in serum-free media.

This deprives the cells of the nutrients required to proliferate and forces the cells into quiescence (G0).Depending on the doubling time for a specific cell population, cells may be maintained in serum-free media from 12-48 hours. In order to synchronize cells in thisway, it is important to ensure that cells are not at confluence when the serum is removed. The cells can be released from quiescence by adding back serum or by treating with specific growth factors. The major disadvantage of this method is that cells with high doubling times may require longer periods without media to enter quiescence. This has the disadvantage of stressing the cells, which may induce apoptosis or alter cellular responses. One way to circumvent this problem is to slowly decrease the serum content of the media, to acclimatize the cells to this serum free environment. For example, cells that are normally maintained in 10% fetal calf serum are maintained in 5% serum for 24 hours. This amount of serum is reduced to 1% for a further 24 hours before finally moving cells into serum-free conditions.

B. Use of chemical inhibitors to synchronize cells

Several chemicals can be added to proliferating cells to arrest them in certain stage of the cell cycle. The inhibitor can then be removed from the media and the cells can progress through the cell cycle in a synchronous manner. There are many inhibitors available that can be used to arrest cells in various stages of the cell cycle;however, since the major aim is to release the cells in order to synchronize them, itis important that reversible inhibitors are used. Two commonly used drugs are (1)

Aphidocholin that arrests cells in G1/S phase by inhibiting DNA polymerase and (2) nocozadole which promotes tubulin depolymerization that blocks mitosis,thereby arresting the cells in G2/M.

3.4.2.3 Method for detecting the synchronization of cells by flow cytometry.

Two methods are commonly used to detect synchronization by flow cytometry: (1) measuring DNA content of cells using DNA binding dyes such as propidium iodide; or (2) studying antigen expression patterns to determine cell position within the cell

cycle.

Propidium iodide staining

1. Wash cells of interest twice in 5 ml PBS without Mg2+ and Ca2+.

2. Fix cells in 25 ml ice-cold Fixation Buffer. Cells should be fixed in Fixation Buffer at -20°C for at least 2 hours prior to staining and can be stored at -20°Cin this buffer for up to 1 month prior to use.

3. Just prior to staining, remove fixation buffer by spinning at 300 x g for 10 minutes and wash cells twice in 25 ml PBS.

4. Resuspend in 0.5 ml Propidium Iodide Buffer to stain DNA.

5. Incubate cells at 37°C for 20 minutes in the dark prior to flow cytometry analysis.

3.4.2.4 Cell growth

The term **cell growth** is used in two different ways in biology. When used in the context of reproduction of living cells the phrase "cell growth" is short hand for the idea of "growth in cell numbers by means of cell reproduction." During cell reproduction one cell (the

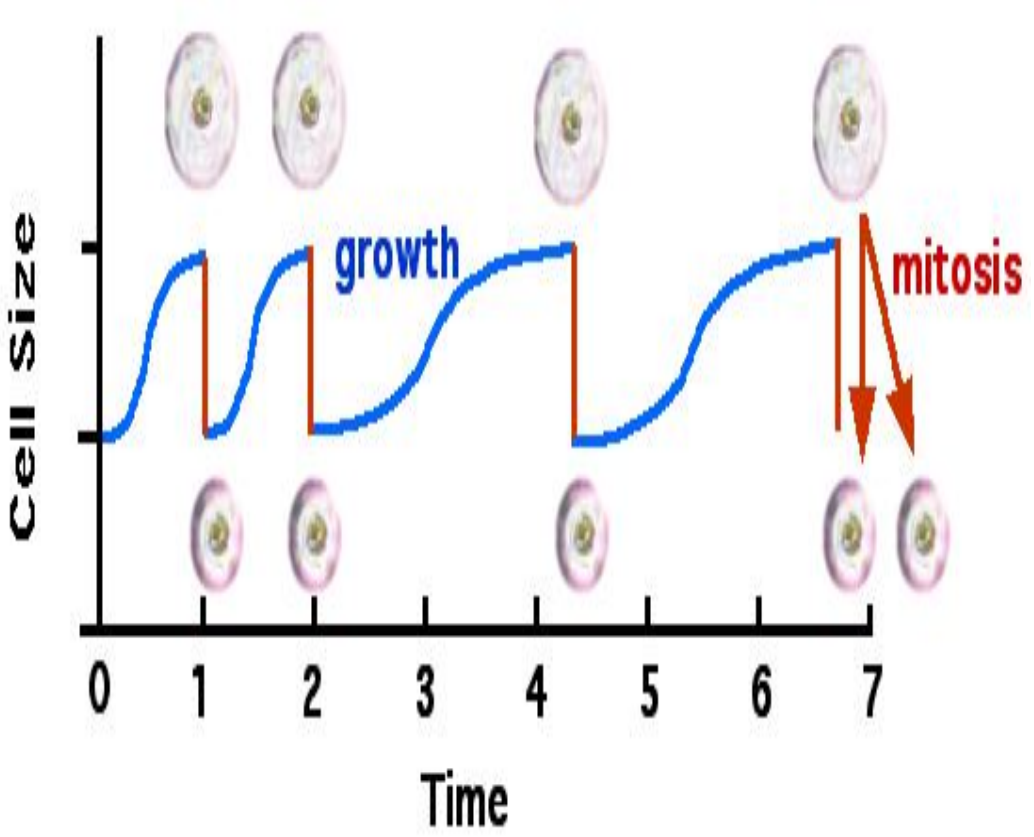
"parental" cell) divides to produce daughter cells. In other contexts, "cell growth" refers to increases in cell size.

3.4.2.5 Cell size

Many cells never have a large increase in size after they are first formed from a parental cell. Typical stem cells reproduce, double in size, then reproduce again. Most Cytosolic contents such as the endomembrane system and the cytoplasm easily scale to larger sizes in larger cells. If a cell becomes too large, the normal cellular amount of DNA may not be adequate to keep the cell supplied with RNA. Large cells often replicate their chromosomes to an abnormally high copy number or become multinucleated. Large cells that are primarily for nutrient storage can have a smooth surface membrane, but metabolically active large cells often have some sort of folding of the cell surface membrane in order to increase the surface area available for transport functions.

Yeast cell size regulation

The relationship between cell size and cell division has been extensively studied in yeast. For some cells, there is a mechanism by which cell division is not initiated until a cell has reached a certain size. If the nutrient supply is restricted (after time t = 2 in the diagram, below) and the rate of increase in cell size is slowed, the time period between cell divisions is increased. Yeast cell size mutants were isolated that begin cell division before reaching the normal size (*wee* mutants). The Wee1 protein is a tyrosine kinase. It normally phosphorylates the Cdc2 cell cycle regulatory protein on a tyrosine residue. This covalent modification of the molecular structure of Cdc2 inhibits the enzymatic activity of Cdc2 and prevents cell division. In Wee1 mutants, there is less Wee1 activity and Cdc2 becomes active in smaller cells, causing cell division before the yeast cells reach their normal size. Cell division may be regulated in part by dilution of Wee1 protein in cells as they grow larger.



Cell size regulation in mammals

The protein mTOR is a serine/threonine kinase that regulates translation and cell division. Nutrient availability influences mTOR so that when cells are not able to grow to normal size they will not undergo cell division. The details of the molecular mechanisms of mammalian cell size control are currently being investigated.

The size of post-mitotic neurons depends on the size of the cell body, axon and dendrites. In vertebrates, neuron size is often a reflection of the number of synaptic contacts onto the neuron or from a neuron onto other cells. For example, the size of motoneurons usually reflects the size of the motor unit that is controlled by the motoneuron. Invertebrates often have giant neurons and axons that provide special functions such as rapid action potential propagation. Mammals also use this trick for increasing the speed of signals in the nervous system, but they can also use myelin to accomplish this.

Other experimental systems for the study of cell size regulation

One common means to produce very large cells is by cell fusion to form syncytia. For example, very long (several inches) skeletal muscle cells are formed by fusion of thousands of myocytes. Genetic studies of the fruit fly *Drosophila* have revealed several genes that are required for the formation of multinucleated muscle cells by fusion of myocytes. Some of the key proteins are important for cell adhesion between myocytes and some are involved in adhesion-dependent cell-to-cell signaling that allows for a cascade of cell fusion events.

Oocytes can be unusually large cells in species for which embryonic development takes place away from the mother's body. Their large size can be achieved either by pumping in cytosolic components from adjacent cells through cytoplasmic bridges (*Drosophila*) or by internalization of nutrient storage granules (yolk granules) by endocytosis (frogs).

Increases in the size of plant cells is complicated by the fact that almost all plant cells are inside of a solid cell wall. Under the influence of certain plant hormones the cell wall can be remodeled, allowing for increases in cell size that are important for the growth of some plant tissues.

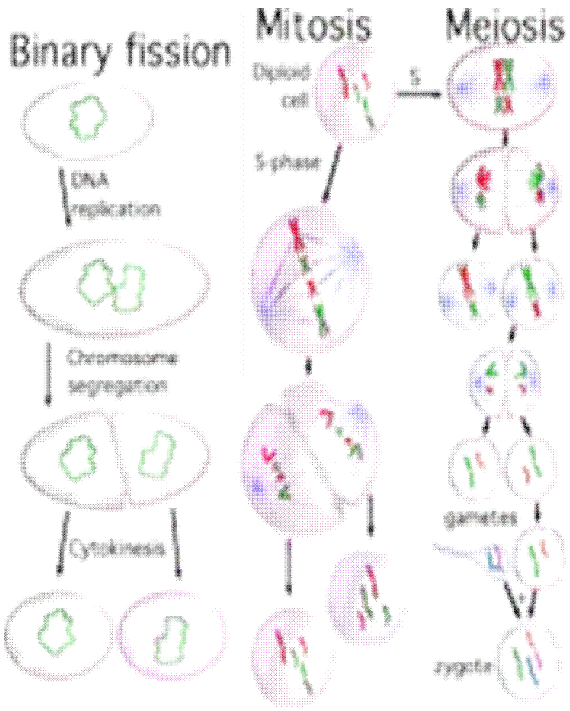
3.4.2.5 Cell reproduction

The process of cell reproduction has three major parts. The first part of cell reproduction involves the replication of the parental cell's DNA. The second major issue is the separation of the duplicated DNA into two equally sized groups of chromosomess. The third major aspect of cell reproduction is the physical division of entire cells, usually called cytokinesis.

Cell reproduction is more complex in eukaryotes than in other organisms. Non-eukaryotic cells such as bacterial cells reproduce by binary fission, a process that includes DNA replication, chromosome segregation, and cytokinesis. Eukaryotic cell reproduction either involves mitosis or a more complex process called meiosis. Mitosis and meiosis are sometimes called the two "nuclear division" processes. Binary fission is similar to eukaryotic cell reproduction that involves mitosis. Both lead to the production of two daughter cells with the same number of chromosomes as the parental cell. Meiosis is used for a special cell reproduction process of diploid organisms. It produces

four special daughter cells (gametes) which have half the normal cellular amount of DNA. A male and a female gamete can then combine to produce a zygote, a cell which again has the normal amount of chromosomes.

For details see the individual articles on DNA replication, binary fission, mitosis, meiosis, and cytokinesis. The rest of this article is a comparison of the main features of the three types of cell reproduction that either involve binary fission, mitosis, or meiosis. The diagram below depicts the similarities and differences of these three types of cell reproduction.



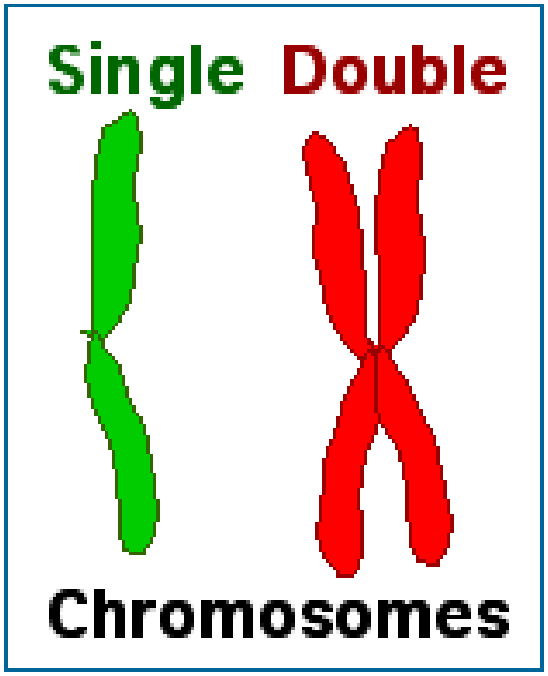
Cell growth

Comparison of the three types of cell reproduction

The DNA content of a cell is duplicated at the start of the cell reproduction process. Prior to DNA replication, the DNA content of a cell can be represented as the amount **Z** (the cell has Z chromosomes). After the DNA replication process, the amount of DNA in the cell is **2Z**

(multiplication: 2 x Z = 2Z). During Binary fission and mitosis the duplicated DNA content of the reproducing parental cell is separated into two equal halves that are destined to end up in the two daughter cells. The final part of the cell reproduction process is cell division, when daughter cells physically split apart from a parental cell. During meiosis, there are two cell division steps that together produce the four daughter cells.

After the completion of binary fission or cell reproduction involving mitosis, each daughter cell has the same amount of DNA (**Z**) as what the parental cell had before it replicated its DNA. These two types of cell reproduction produced two daughter cells that have the same number of chromosomes as the parental cell. After meiotic cell reproduction the four daughter cells have half the number of chromosomes that the parental cell originally had. This is the haploid amount of DNA, often symbolized as **N**. Meiosis is used by diploid organisms to produce haploid gametes. In a diploid organism such as the human organism, most cells of the body have the haploid amount of DNA, **2N**. Using this notation for counting chromosomes we say that human somatic cells have 46 chromosomes (2N = 46) while human sperm and eggs have 23 chromosomes (N = 23). Humans have 23 distinct types of chromosomes, the 22 autosomes and the special category of sex chromosomes. There are two distinct sex chromosomes, the X chromosome and the Y chromosome. A diploid human cell has 23 chromosomes from that person's father and 23 from the mother. That is, your body has two copies of human chromosome number 2, one from each of your parents.



Immediately after DNA replication a human cell will have 46 "double chromosomes". In each double chromosome there are two copies of that chromosome's DNA molecule. During mitosis the double chromosomes are split to produce 92 "single chromosomes", half of which go into each daughter cell. During meiosis, there are two chromosome separation steps which assure that each of the four daughter cells gets one copy of each of the 23 types of chromosome.

3.4.2.6 CELL TRANSFORMATION

One of the main driving forces in the development of animal cell and tissue culture techniques has been the conviction that it could provide the means for coming to grips with the problem of cancer. It was mentioned that in an experiment by Beebe and Ewing on the culture of canine lymphosarcoma cells preceded Harrison's

experiments. From the to the present day one can follow an unbroken trail of pioneers, such as Carrel, Warburg, Earle, Rous, Gey, Murray. However, a systematic full-scale attack on the cancer problem has only been mounted since the early'1960s. The breakthrough was the development of quantitative assay procedures for tumour viruses, stimulated by the exploitation of cell culture methods by virologists.

Tumour viruses

Since Rous demonstrated the infective nature of the fowl sarcoma bearing his name, many other infective cancer-producing agents have been recognised.

Some years ago, Carrel demonstrated that the Rous sarcoma virus could be propagated in chick embryo tissue cultures and subsequently intermittent reports of the same nature appeared from Halberstaedter and Doljanski in 1939 and from Manaker and Group in 1956. This particular problem has been reinvestigated by Rubin and Temin (1958, 1959) using Dulbecco's method for plating viruses on monolayers of trypsinised chick embryonic tissue. Proliferative or degenerative foci indicate loci of virus infection and in this way the virus can be studied quantitatively.

Much of the present interest in this problem has stemmed from the observations of Stewart and Eddy that the polyoma virus of mice can be propagated serially in cultures of mouse cells, in which it produces typical necrotic foci. This virus is easy to dandle and is remarkable for extraordinary variety of tumours it can produce in the mouse.

It is surprising perhaps that viruses which cause tumours in the animal should cause rapid death in vitro. This may not be a general property of tumour viruses, however, although it is obviously a feature which makes recognition of the presence of an infective agent

easier. By inoculating cell populations during cloning it has now been recognised that tumour viruses, such as the polyoma virus, can cause cellular transformation without causing cell death. At the time of writing transformation of baby hamster kidney cells by polyoma virus has been most thoroughly studied. Transformation of human cell cultures by the simian virus SV40 has also been investigated. Suggestive evidence concerning other viruses of this kind are the report by Coman of passage of the Shope papilloma virus through cultures of rabbit skin and also the reports by Lasfargues of growth of the Bittner agent in cultures of mouse mammary tissue.

Tumour viruses are divided into the DNA viruses, which include polyoma, SV40, papilloma, adeno and herpes viruses, and RNA viruses (oncornaviruses) which include Rous sarcoma virus (RSV) murine leukaemia virus (MLV), murine sarcoma virus (MSV) and the Rauscher and Friend viruses. In general the DNA viruses are smaller than RNA viruses. They are associated with some naturally occurring tumours such as papillomas. Burkitt's lymphoma and Marek's disease of chickens. RNA viruses are associated with naturally occurring sarcomas and leukaemias in rodents and fowls.

Many tumour viruses are ' defective' in non-permissive cell lines, that is they are unable to go through a complete reproductive cycle in them. However, they can transform non-permissive cells. An example is the transformation of baby hamster cells by the polyoma virus. After infection the cells become heritably altere (Plate 23) but virus cannot be recovered from them (although evidence can be obtained that viral DNA is not present in the DNA in the cell's chromosomes). The same virus, in 'permissive cells' which, in this instance, are mouse fibroblasts, is capable of complete reproduction; the cells are killed and new virus appear in the medium. In the 'non-

permissive' host some factor needs for viral replication is missing; in the 'permissive' host this provided either by the host cell itself or by another virus (a'helper virus) which coincidentally infects the cell. The situation is not always as black and white as this; man tumour viruses are produced by cells transformed by them.

The tissue culture assays for tumour viruses are based on transformation or cytocidal assays. A cytocidal assay for polyoma virus was developed originally by Stewart and Eddy using the plaque assay technique for infectious viruses. Transformation assays for tumour virus are performed in the same way except that instead of noting plaques as foci of killed cells they are recognised as foci of transformed cells usually by virtue of altered morphology-changes of cells shape, piling up of cells in a contact-inhibited culture or, in some cases formation of giant cells. An example is the assay commonly used for RSV (Rous sarcoma virus).

Temin-Rubin assay for RSV (1958)

1. Primary chick embryo carcass cultures are prepared .
2. After 3-5 days incubation secondary cultures are made by trypsinising primary cultures and 2-3 x 10^5 inoculated into each of a number of 50 mm.Petri dishes in 5 ml. medium.
3. The following day the medium is removed and replaced with 0.1 to 0.8 ml. virus in BSS or medium. Adsorption is permitted for 10-60 minutes and 5 ml. of medium containing 0.6 per cent. agar are added (Double strength agar in water is melted and cooled to 45^o in a water bath. Double strength medium is heated to 45^o in the same water bath. The two are mixed aseptically immediately before use.)
4. The culture is incubated for three days, fed then with 2 ml. of agar-containing medium added to the original medium, and two to four

days later examined for foci. Identification of foci can be facilitated by staining the cells, either in vivo by adding 1/20,000 phenol red for two hours or, after fixation, by Giemsa staining.

Many strains of RSV give rise to transformed foci which release virus. The object of adding agar to the medium is to prevent secondary foci appearing as a result of diffusion of virus from primary foci.

The number of foci formed in an assay of this kind is proportional to the amount of virus added and is measured in plaque forming units (PFU). In the case of RSV one PFU may correspond to one virus particle but this ration varies with the virus and the virus preparation.

Similar assays are used to titrate SV40 virus in 3T3 cells and polyoma virus in baby hamster kidney cells. In the latter assay the BHK21 cell line is commonly used.

Transformed Colonies

Foci of transformed cells within a normal monolayer are sometimes difficult to recognise although individual colonies of normal or transformed cells can be easily distinguished. Morevoer, sometimes it is desirable to be able to grow colonies from cells as soon as possible after treatment with virus. An alternative strategy is, therefore, to adsorb virus to cells in suspension and plate these out as for cell cloning by the dilution method. As an example the formation of transformed colonies of baby hamster kidney cells by polyoma virus is described.

1. Aliquots of freshly-trypsinised baby hamster kidney cells are suspended at a concentration of 3-4 x 10^6 in 1 ml. dilutons of virus

in medium and allowed to adsorb for 30-60 minutes at 37^{o} with periodic agitation.

2. The cells are recounted and on the basis of this count (0 mm. Petri dishes are inoculated with 10^3 to 5 x 10^5 cells .
3. After seven to nine days incubation in the CO_2 incubator the plates are examined with an inverted microscope. The untransformed colonies of cells showing marked parallel alignment can be clearly distinguished from transformed colonies, in which the cells show no parallel alignment and pile up to form much thicker layers. The colonies may be picked or stained with Giemsa or crystal violet.

Agar transformation assay

A selective technique for transformed cells would clearly be valuable in studies of this kind and the demonstration that transformed but not untransformed cells will grow in sloppy agar made such an assay possible. In the assay of Mcpherson and Montagnier (1964) 60 mm petri dishes are prepared by forming a base layer of 0.5 per cent Difco Bactoagar in medium (7ml.). For the assay this is then overlaid with 1.5 ml. of medium containing 0.3 per cent agar with 10^3 to 5 x 10^5 cells.

Virus is adsorbed to cells, as described above, before plating. Uninfected cells yield no colonies but colonies derived from transformed cells form easily recognisable spheres of cells which can, if required, be picked with a Pasteur pipette.

Transformation by other Carcinogens

Using transformation assays similar to those developed for assay of viral transformation, induction of transformed colonies by radiation and by chemical carcinogens can be demonstrated. Among other systems, primary cultures of Syrian hamster embryonic carcass

cells have been used by Berwald and Sachs (1963) and a line of prostate cells from C3H mice by Chen and Heidelberger (1969).

Cells are inoculated at cloning densities, e.g. 1,000 cells per 35 mm petri dish, on irradiated feeding layers, if desired. Hydrocarbon carcinogens are dissolved in dimethylsulphoxide (DMSO) and added to the medium so that the DMSO content is 0.5 per cent. or less. Control cultures are treated with DMSO alone at the same concentration. Serial dilution of carcinogens may be made before adding to medium. Medium is routinely changed twice weekly. After one week the carcinogen-containing medium is replaced with normal medium. Some cultures are removed and stained two days later to give an estimate of overall plating efficiency. The remaining cultures are examined after a further two to three weeks for transformed colonies.

Assay of cytotoxicity

Cytotoxicity assays have been used for two main purposes in cancer research, estimating cytostatic or cytotoxic activity or chemotherapeutic agents and measuring lymphocyte-mediated immunity.

These tests can be carried out by measuring inhibition of colony formation or by directly observing cytotxicity. In both types of test Petri dishes can be used. For colony formation the dishes are inoculated with sufficient cells to give 50-100 colonies in controls.

For cytotoxicity micro titration plates are more convenient. . A detailed protocol for assaying lymphocyte mediated immunity, following Hellstrom (1967) and Takasagi and Klein (1970) is as follows.

Microtitration plate assay for lymphocyte mediated cellular immunity

1. Trypsinise target cells gently by the technique and suspend the cells in a small volume of serum containing medium. Count the cells.
2. Dilute with medium and dispense 40-200 cells in 0.2m.. medium to each well. Incubate overnight.
3. Remove medium.
4. Add 0.2 ml / well of a suspension of the lymphocytes to be tested (at about 2 x 10^6/ ml. medium if possible, but down to 2 x 10^5 / ml. can be used if necessary) in serum-free medium.
5. Incubate 45 minutes on a rocker.
6. To each well add 0.05 ml. 50 per cent, inactivated calf serum in medium.
7. Incubate two days at 37^o in CO_2 incubator.
8. Remove medium, wash several times with PBS, stain with crystal violet and examine.

Tumour virus production in cell cultures

In some instances viruses cause transformation but do not give rise to productive infections (for example, polyoma virus in BEK21 cells). Conversely, for virus production the first requirement is a productive virus infection. Many tumours associated with oncorna viruses. (RNA tumour viruses) are productively infected, for example, the Rous sarcoms, murine leukaemias and many marine sarcomas. Virus can be recovered from the medium in which these tumours are grown. However, many viruses of this group are unusable and undergo rapid degradation. To obtain good yields of intact virus the culture has, therefore, to be 'milked', that is, medium is removed and replaced frequently, for example every 12 to 24 hours. Many tumours

produced by DNA viruses, such as Burkitt's lymphoma, also yield virus (in this case the EBV virus).

Culture of tumour cells

Many investigations in cancer research start with attempts to culture tumour cells – to provide a source of virus, an object for the study of tumour cell metabolism or material to study sensitivity to anticancer agents.

The ease of difficulty of initiating cultures from tumours depends largely on the amount of connective tissue present and on whether the tumour has specific nutritional requirements.

Many experimental animal tumours are easy to culture by standard techniques. Generally speaking, in setting up primary cultures; however, a rich medium, with a total amino acid supplement, should be usued and the tissue should be handled gently.

Some human tumours, especially cellular tumours and ascites tumours (such as some ovarian tumours) are relatively easy to establish. Tumours with a high connective tissue content present more difficulty but, following dissociation with collagenase and trypsin or pronase, cultures can be obtained from 60-80 per cent. The problem then is to determine whether the culture consists of tumour cells or normal cells from connective tissue or other elements. If the cells have characteristic features it may be possible to have reasonable confidence about this but often it is difficult to be certain and no general method has yet been developed for distinguishing between the two.

3.4.2.6 Summary

Cell synchrony may be obtained by treating the cells at different stages of their growth by serum starvation or by chemical treatment.The growth of the cells in the cultutre can be estimated either by measuring the cell size or by conting the cll number . The latter is generally preferable.Transformation of cells is generally

carried out by means chemicals or by means of viruses like polyoma virus.

3.4.2.7 Model questions

1) Explain different methods for cell synchronization

2)Write a short note on cell trans formation

3.4.2.8 References

1. Animal cell culture techniques by Ian Freshney.
2. Animal Cell culture by Jhon Master.

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Lesson 3.4.3

In Vitro Fertilization and Embryo transfer(IVF-ET)

Objective

This chapter deals with the invitro fertilization ,and oits applications and potential risks involved in it.

3.4.3.1 Introduction

Invitro Fertilization Pre-Embryo Transfer (IVF-ET) is a fertility procedure which first succeeded as recently as 1978 by Dr. Edwards (an embryologist) and Dr. Steptoe (a gynecologist) in England. Since then the technology has been further refined and developed by physicians and embryologists, with over 20,000 babies born worldwide.

The possibility of a continuing pregnancy being achieved by IVF has improved from practically nil to one chance in 4 to 6 at IVF centers worldwide.

The possibility of a pregnancy being achieved for any one patient cannot be predicted, as it depends on many variables - such as age and the reproductive health of both the wife and the husband. Although the chance of success varies from case to case, a thorough evaluation is required to predict the probability of pregnancy in any given situation

Definition

In vitro fertilization (IVF) is a procedure in which eggs (ova) from a woman's ovary are retrived. They are fertilized with sperm in a laboratory procedure, and then the fertilized egg (embryo) is returned to the woman's uterus.

3.4.3.2 Steps involved in *in vitro* Fertilization

Step One: Ovarian Stimulation and Monitoring

(Controlled Ovarian Stimulation or Superovulation) Prior to the actual egg retrieval process, the woman undergoes a stimulation period where her ovaries are primed to produce the maximum number of viable eggs. During this time, the woman receives medications so those eggs are not released prematurely (prior to the time of egg retreival). The medications that are used to prevent premature egg release (i.e. ovulation) are called GnRH agonists. The most common medication in this class is known as Lupron®. Lupron may be initiated at different times depending on the protocol being used. Most commonly, it is begun one week before the anticipated

menses of the upcoming treatment cycle. Lupron prevents premature ovulation, which would result in cancellation of the cycle. About 20% of cycles are cancelled when Lupron is not used.

On a specified day, the woman begins injections of gonadotrophins, according to a schedule that is provided by the clinic. Gonadotropins (known as hMG or FSH) are used to stimulate the ovaries to develop as many ovarian follicles as possible. Follicles are fluid-filled sacs which contain developing eggs. The purpose of these medications is to induce the woman's ovaries to produce more than one egg. The greater the number of eggs that are produced, the greater the likelihood that fertilization of one or more of them will occur and eventually result in pregnancy.

The timing, dosage, and administration of the hormonal medications are critical to the success of the cycle. Excessive amounts of these medications may result in hyperstimulation of the ovaries (known as the ovarian hyperstimulation syndrome or OHSS), whereas insufficient quantities of the drugs may result in an unsuccessful cycle leading to cancellation. Therefore, careful physician monitoring is necessary to adjust dosages and ensure an optimal stimulation period. Typically, the physician will monitor the patient with vaginal or abdominal ultrasounds and blood tests. The ultrasound scans provide an actual image of the ovaries and aid in the assessment of follicular growth. These ultrasounds are begun around the eighth day of the cycle. The physician will check the growth, number, size of each follicle, and any signs of difficulty that require alterations in treatment. Blood tests are typically administered every other day in order to monitor levels of the estrogen hormone called estradiol. The estradiol level helps the doctor determine the optimal timing for administering HCG (human chorionic gonadotropin). HCG is a medication that is used to stimulate the final maturation

of the follicles. Egg retrieval typically follows within approximately 36 hours following HCG administration.

Step Two: Egg Retrieval

Once the follicle has ruptured, the physician attempts to remove as many eggs as possible. The two methods used to retrieve eggs are laproscopy and ultrasound-guided aspiration. In laproscopy, a surgical procedure requiring general anesthesia, the physician uses a surgical instrument called the laproscope inserted into the ovaries. The laparoscope contains an aspiration system that uses light suction to retrieve the egg from the follicle. The result of each attempt to retrieve the egg from the follicle is instantly examined under a powerful microscope. If the egg was not retrieved, fine adjustments are made for subsequent attempts until all the mature follicles have been aspirated.

In almost all cases, egg retrieval is accomplished non-surgically using a vaginal ultrasound guided aspiration, where a probe is used to guide a needle into the ovaries. The procedure does not require general anesthesia. Intravenous sedation is adequate and the recovery from both the procedure itself and the sedation is relatively easy. The ultrasound image allows more accurate aspiration attempts because the physician can guide the needle into each follicle in order to withdraw the eggs.

After recovering the eggs, they are transferred to a sterile container to await fertilization in the laboratory. Not all of the eggs retrieved will be used in the current IVF cycle. Unhealthy eggs and any eggs that fail to fertilize are not used. Any successfully fertilized eggs that are not needed for transfer in this first IVF cycle may be cryopreserved (frozen) for use in a later cycle.

Step Three: Fertilization

A semen sample is collected from the male partner approximately two hours before the female partner's eggs are retrieved. These sperm are then processed (a procedure called sperm washing), using various laboratory techniques. Sperm processing helps to select the strongest, healthiest, and most active sperm in the semen sample.

The mature, healthy eggs are then placed together in the laboratory with the selected sperm. They are incubated at a temperature identical to that of the woman's body. After approximately 48 hours, the eggs that have successfully fertilized and are growing normally, are called embryos. The embryos are then ready for the next step - Transfer into the womb. One of the major benefits of IVF is the ability to know at this point if the male's sperm have actually fertilized the eggs. In some cases of male infertility, fertilization fails to take place. Adjustments can be made in the treatment of the male partner, as well as changes in the semen processing or conditions to encourage fertilization in future IVF attempts.

Step four: Embryo Transfer

Embryo transfer is not a complicated procedure and can be performed without anesthesia. The embryos are placed in a catheter, which is a tubular instrument used to transport the embryos from the laboratory container to the womb. The physician inserts the catheter through the female partner's vagina and cervix and then into the uterus. Normal implantation and maturation of the embryo is anticipated to achieve pregnancy. Each physician who performs IVF together with the couple undergoing the procedure will determine the number of embryos for transfer. Most ART Centers have determined the optimal number of embryos to

achieve successful pregnancy. If there are any remaining embryos that are not needed for transfer in this cycle, cryopreservation may be an option to save the embryos for other IVF procedures in future cycles.

Cryopreservation is the process of freezing embryos to preserve them over time. Frozen embryos have been successfully thawed and used in later cycles that resulted in pregnancy. About two thirds of embryos will survive the process of freezing and thawing. The probability of pregnancy may be slightly improved using the frozen embryos since the embryo transfer can be performed during the female's normal ovulatory cycle (rather than a "stimulated" cycle). An added benefit is the lower cost since ovarian stimulation, egg retrieval, and fertilization procedures do not need to be repeated with the subsequent cycle.

Preparation

Once a woman is determined to be a good candidate for in vitro fertilization, she will generally be given "fertility drugs" to stimulate ovulation and the development of multiple eggs. These drugs may include gonadotropin releasing hormone agonists (GnRHa), Pergonal, Clomid, or human chorionic gonadotropin (hcg). The maturation of the eggs is then monitored with ultrasound tests and frequent blood tests. If enough eggs mature, the physician will perform the procedure to remove them. The woman may be given a sedative prior to the procedure. A local anesthetic agent may also be used to reduce discomfort during the procedure.

Aftercare

After the IVF procedure is performed the woman can resume normal activities. A pregnancy test can be done approximately 12-14 days later to determine if the procedure was successful.

Precautions

The screening procedures and treatments for infertility can become a long, expensive, and sometimes, disappointing process. Each IVF attempt takes at least an entire menstrual cycle and can cost $5000-$10,000, which may or may not be covered by health insurance. The anxiety of dealing with infertility can challenge both individuals and their relationship. The added stress and expense of multiple clinic visits, testing, treatments, and surgical procedures can become overwhelming. Couples may want to receive counseling and support through the process.

Normal results

Success rates vary widely between clinics and between physicians performing the procedure. A couple has about a 10% chance of becoming pregnant each time the procedure is performed. Therefore, the procedure may have to be repeated more than once to achieve pregnancy.

Abnormal results

An ectopic or multiple pregnancy may abort spontaneously or may require termination if the health of the mother is at risk.

Terms:

Fallopian tubes

In a woman's reproductive system, a pair of narrow tubes that carry the egg from the ovary to the uterus.

GIFT

Stands for gamete intrafallopian tube transfer. This is a

process where eggs are taken from a woman's ovaries, mixed with sperm, and then deposited into the woman's fallopian tube.

ICSI

Stands for intracytoplasmic sperm injection. This process is used to inject a single sperm into each egg before the fertilized eggs are put back into the woman's body. The procedure may be used if the male has a low sperm count.

ZIFT

Stands for zygote intrafallopian tube transfer. In this process of in vitro fertilization, the eggs are fertilized in a laboratory dish and then placed in the woman's fallopian tube.

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3.4.3.3 Risks of Therapy

Superovulation Stimulates Egg Development

The controlled "superovulation" techniques used in IVF are designed to stimulate the ovaries to produce several eggs (oocytes) rather than the usual single egg as in a natural cycle. Multiple eggs increase the potential availability of multiple embryos (fertilized eggs) for transfer and ultimately increase the probability of conception.

The medications required to boost egg production may include, but are not limited to the following: Lupron (gonadotropin releasing hormone-agonist), Antagon or Cetrotide (gonadotropin releasing hormone-antagonist), Follistim, Bravelle or Gonal-F (FSH, follicle stimulating hormone), Repronex (combination of FSH and LH,

luteinizing hormone), and Pregnyl or Novarel (hCG, human chorionic gonadotropin). Each is administered by injection only. Most medications are given subcutaneously (beneath the skin), though some are intramuscular injections (into the muscle). Risks associated with injectable fertility medications may include but are not limited to, tenderness, infection, hematoma, and swelling or bruising at the injection site.

Risks associated with the medications may include, but are not limited to, allergic reactions, hyperstimulation of the ovaries (mild, moderate or severe), failure of the ovaries to respond and cancellation of the treatment cycle.

There are situations that can occur during a stimulation that may necessitate canceling your IVF cycle and stopping treatment for a period of time. This occurs because the ovaries produce either too many or too few eggs in response to drug stimulation protocol. Although we realize that this can be a big disappointment, at times it is necessary to discontinue the use of the medications to avoid the possibility of complications and to afford you the best chance of future success. If canceling the cycle becomes necessary, you will be told to stop your injections. No HCG injection will be given and no egg retrieval will occur. You will be asked to schedule an appointment with your physician to make decisions regarding future treatment cycles.

When ovulation induction medications are used in fertility therapy, the ovaries are coaxed to produce more than one egg to the point of maturity. Consequently, hormone levels of estrogen and progesterone reach much higher than normal values. When the estrogen level becomes mildly to moderately elevated, side effects that may be experienced include, but are not limited to, fluid retention with slight transient weight gain, nausea, diarrhoea, pelvic discomfort

due to enlarged cystic ovaries, breast tenderness, mood swings, headache and fatigue.

Ovarian Hyperstimulation Syndrome (OHSS)

If the estrogen level rises excessively and HCG is administered to trigger final maturation of the eggs, the following more serious complications may result:

1. Excessive fluid retention with fluid in the abdomen and/or chest cavity.
2. Thrombosis of arteries and/or veins (formation of blood clots) which may lead to stroke, embolus, or potentially fatal complications.
3. Abnormally enlarged ovaries, which have the possibility of rupturing or twisting (a surgical emergency)

Any of the three problems listed above may require prolonged hospitalization.

Given the potential for such severe complications, it is important that we carefully monitor your response to these medications. This monitoring also allows your physician to determine when the eggs are ready for the next stage, oocyte (egg) retrieval. Monitoring includes frequent blood drawing for estradiol (estrogen) and possibly progesterone, LH and FSH levels. These blood tests will take place over approximately a twelve-day period. Risks associated with blood drawing may include, but are not limited to:

1. Pain at the site of needle stick
2. Tenderness or infection of the skin
3. Bruising or scarring of the site of blood draw

4. Development of a blood clot in the vein (thrombosis, thrombophlebitis)

The second portion of the monitoring phase in IVF involves the use of intravaginal ultrasound to track follicular growth. The eggs develop inside fluid-filled cysts of the ovaries called follicles, which enlarge as the eggs mature. Ultrasound studies usually begin after an estrogen response has been measured and continue on a frequent basis until oocyte (egg) retrieval. The ultrasound studies are performed using a vaginal probe. Vaginal sonograms carry no appreciable risk but may cause slight discomfort, particularly as you near the point of ovulation.

Ovarian stimulation with the fertility medications causes multiple follicles to develop. This is desirable in IVF because as the number of eggs increases, the chance for success increases. Multiple embryos can also increase the risk of multiple pregnancy. Approximately 20-25% of pregnancies with IVF will be multiple. Most of these will be twins, but triplets, quadruplets or even greater multiple pregnancies can occur. A procedure called "selective reduction of pregnancy" has been performed in several medical centers across the country in selected cases of triplets or more. Selective reduction is not offered on site or by GRS staff. More information on this procedure and recommended centers is available on request.

Retrieving the Oocytes (egg retrieval)

For IVF, collection of eggs is usually performed under transvaginal ultrasound guidance. To accomplish this, a needle is inserted (under IV sedation) through the vaginal wall into the ovaries using ultrasound to locate each follicle. The follicular fluid is drawn up into a test tube to obtain the eggs. Although patients are given pain medications intravenously and are carefully monitored by an

anesthesiology staff, some women may experience some discomfort during the procedure. Generally, the oocyte (egg) retrieval takes 20-30 minutes. Patients are usually discharged home within hours after the retrieval. Risks of oocyte (egg) retrieval may include, but are not limited to, the following:

1. Potential reactions from the drugs and procedures used in the administration of anesthesia.

2. Risks associated with the passage of the needle through the vagina into the ovaries (including infection, bleeding, inadvertent damage to adjacent structures including, but not limited to, the bowel, bladder, blood vessels, ureter, uterus or ovary(ies), and adhesion formation (internal scarring) following the procedure. Although uncommon, significant bleeding or damage to the bowel may occur, and surgery may be required to repair such damage; this is a very uncommon event. Rarely, infection may become severe enough to require hysterectomy and/or removal of one or both ovaries.

Collecting and Preparing the Sperm

A semen sample will be obtained from the partner by masturbation on the day of the oocyte (egg) retrieval. This is usually obtained while the retrieval is being performed. Abstinence from ejaculation for two to five days prior to providing this semen specimen is recommended. After the specimen is produced, the sperm will be prepared for inseminating the collected eggs in our laboratory. Because this can be a stressful time period for men, the man/partner may be unable to produce a specimen when needed. Men who feel that they may have difficulty producing a semen specimen have the opportunity to have their specimens frozen by our laboratory ahead of time for

use in this situation. Testicular biopsy can also be performed as a method to extract sperm for IVF.

Insemination of Eggs and Embryo Culture

Following egg retrieval, the follicular fluid is immediately transferred to the adjacent laboratory for identification of eggs, evaluation, and preparation for insemination. In the process of collecting the follicular fluid, it is possible that a large number of eggs may be retrieved. It is strongly recommended that all of these eggs be inseminated to maximize the number of embryos available for subsequent transfer. Any objection(s) to this policy should be stated in writing and attached to the IVF-ET consent form with the understanding that pregnancy success may be reduced. Otherwise, the prepared sperm will be added to each egg and they will be allowed to incubate overnight under controlled laboratory conditions. The next day, each egg is evaluated for evidence of fertilization. However, it is possible that no eggs are fertilized. If this happens, the laboratory staff will re-inseminate the eggs or perform intracytoplasmic sperm injection (ICSI) in hopes of obtaining embryos for transfer. If fertilization still does not occur, the eggs will be discarded and the remainder of the procedure will be cancelled. In the case of severe male factor, the couple may be asked to consider the option of using anonymous donor sperm (obtained through a licensed sperm bank for use as a "backup" or secondary sperm source) if it is not possible to obtain sufficient sperm from the partner at the time of fertilization.

The eggs that have fertilized will be allowed to develop for two or more additional days under controlled laboratory conditions before they are placed inside the woman's uterus. Depending upon the couple's wishes, some fertilized eggs/ embryos may be frozen and stored for future use.

After the embryos are transferred to the womb, the woman will continue progesterone supplementation that begins on the evening of your egg retrieval procedure. Progesterone can be taken as a combination of oral troches and rectal/vaginal suppositories or by injections. Administration of these medications after egg collection has been shown to create a more favorable uterine environment for the embryos, which therefore increases pregnancy rates. Side effects of progesterone may include, but are not limited to the following:

1. Vaginal dryness;
2. Bloating, breast tenderness;
3. Depression, mood swings;
4. Delay of menses.

Synthetic progesterone-like medications have been associated with certain birth defects. By using only natural progesterone, the risk of drug-induced birth defects is significantly reduced. It is important to note, however, that birth defects occur in approximately 3% of spontaneously-conceived pregnancies in the USA. Therefore, use of natural progesterone does not guarantee a child without a birth defect.

Transferring Embryos to the Uterus

Embryos are transferred on either, day three or day five of development. The embryologists at GRS are highly-skilled in identifying "healthy" embryos and in some cases will recommend that a patient extend embryo development to day five, known as the blastocyst stage. Blastocyst transfer has become quite common in IVF cycles as it can increase chances for success while decreasing the likelihood of multiples. Your physician will work closely with the

embryologists to determine if a day three or day five transfer would be ideal for your cycle.

Embryos are transferred to the uterus through a small tube (catheter). This procedure is much like a pap smear and does not require any anesthesia and is usually painless. The embryos are placed in a small amount of fluid inside the catheter, which is passed through the cervix at the time of a speculum examination. The embryos are placed in a manner so they reach the top part of the uterus. The number of embryos transferred depends on individual circumstances of the couple, and this decision will be made collectively by you, your physicians and the embryologist. Typically, two to four embryos are be transferred in one treatment cycle.

Embryo transfer can cause mild cramping. Although unlikely, during the embryo transfer the embryo(s) may be displaced through the cervix (causing loss of embryos) or into the fallopian tubes (causing possible tubal pregnancy). There is a small risk of bleeding or infection as a result of the transfer procedure.

After transfer, the woman may get dressed and leave after a brief recovery period. A pregnancy test will be done twelve to fourteen days after the transfer, regardless of the occurance of any uterine bleeding.

The transfer of several embryos increases the probability of success. A multiple embryo transfer also increases the risk of a multiple pregnancy. Any multiple pregnancy carries an increased risk of miscarriage(s), premature labor and premature birth as well as an increased financial and emotional cost. Pregnancy-induced high blood pressure and diabetes are more common in women pregnant with more than one fetus. Prolonged hospitalization may be necessary for these pregnant women and for the mother and babies after delivery. Tubal (ectopic) pregnancy is also possible,

and a combination of normal pregnancy and ectopic pregnancy may occur. A tubal pregnancy is a condition that may require laparoscopy or major surgery for treatment. Like spontaneous (natural) conceptions, pregnancies that arise through IVF may result in miscarriage. In the event of a miscarriage, a dilatation and curettage (D&C) may be necessary.

Couples going through therapy must choose and formalize their choice in the appropriate GRS consent form by indicating one of the following options for handling of any remaining embryos:

1. Freezing (cryopreservation) of remaining embryos for use by the couple in future treatment cycles

2. Anonymously donating the embryos for use by another infertile couple(s), if the donating couple and the donated embryos meet the screening criteria (You will not receive any money for this donation, nor will GRS "sell" them. GRS reserves the right to cryopreserve (freeze) any donated embryos as well as the right to discard any donated embryos if a suitable woman cannot be identified to receive the embryos)

3. Allowing the embryos to develop in the laboratory until they perish, at which time they would be disposed of in a manner consistent with professional ethical standards and applicable legal requirements (This usually occurs within six to eight days after egg collection)

Other Issues:

Any assisted reproduction process or technique can be psycologically stressful. Significant anxiety and disappointment may occur. We encourage you to consider short-term supportive counseling during this time and we

are happy to provide you with a list of psychiatrists, psycologists, counselors and social workers who may help you through this difficult time.

A substantial time commitment is required by both partners to complete an entire course of IVF therapy. It will be necessary for couples to adjust their schedules to undergo the required testing and therapies associated with IVF-ET. It is the responsibility of the woman to report to our office as scheduled for repeated ultrasound examinations and blood tests over several days or weeks before and after the expected time of egg collection. It is the responsibility of the man to be available at the time identified by the physician to provide sperm.

Theoretical Concerns & Potential for Success:

Unfortunately, neither conception nor a successful outcome of pregnancy is guaranteed by the IVF-ET procedure. There are many reasons why pregnancy may not occur with the IVF-ET procedure. In fact, there are complex and largely unknown factors that limit pregnancy rates following assisted reproductive techniques. Some of the known reasons for failure may include, but are not limited to:

1. There may be a failure to recover an egg because:
   - follicles that contain mature eggs may not develop in the treatment cycle

- ovulation has occurred before time of egg recovery
- one or more eggs cannot be recovered
- pre-existing pelvic scarring and/or technical difficulties prevent safe egg recovery

2. The eggs that are recovered may not be normal.

3. There may be insufficient semen to attempt fertilization of the recovered eggs because the man is unable to

produce a semen specimen, because the specimen contains an insufficient number of sperm to attempt fertilization, because the laboratory is unable to adequately process the specimen provided, or because the option to use a donor sperm as a "backup" was declined.

4. Fertilization of the eggs to form embryos may fail even when the egg(s) and sperm are normal.

5. The embryos may not develop normally or may not develop at all. Embryos that display any abnormal development will not be transferred.

6. Embryo transfer into the uterus may be difficult/impossible, or implantation(s) may not occur after transfer, or the embryo(s) may not grow or develop normally after implantation.

7. Any step in the IVF-ET process may be complicated by unforeseen events, such as hazardous or catastrophic weather, equipment failure, laboratory conditions, infection, human error and the like.

In the event the couple should die before embryo transfer, the embryo(s) will be discarded unless other provisions are made in writing.

When pregnancy occurs following IVF, it will typically be a normal pregnancy. However, there is always a risk of abnormal pregnancy, miscarriage, blighted ovum, ectopic pregnancy or premature delivery. This is because the process of IVF-ET does not protect against such normal occurrences. Congenital abnormalities, genetic abnormalities, mental retardation or other birth defects which occur in approximately 3% of spontaneously-conceived pregnancies may still occur in children born following assisted reproductive techniques. A large review of a subset of children born following assisted reproductive

procedures found the incidence of developmental anomalies similar to a control group of children spontaneously conceived. Women with multiple pregnancies have a much higher risk of complicated pregnancies, which may include the following: Toxemia, pre-eclampsia, miscarriage, premature labor and delivery, stillbirth, birth defects, and other complications.

3.4.3.4 Alternatives to IVF-ET:

Depending upon the individual and unique cause(s) of infertility for each couple, the chance of conception through alternative means, including intrauterine insemination (IUI) and medicinal therapy, other than IVF-ET may or may not exist. Possible success rates of these alternatives may vary depending upon the type and severity of the cause of the infertility. For some couples, it may even be possible to conceive spontaneously without a physician's help. You should discuss these alternative treatment methods with your physician before you proceed with IVF-ET therapy.

3.4.3.5 Summary

In vitro fertilization (IVF) is a procedure in which eggs (ova) from a woman's ovary are removed. They are fertilized with sperm in a laboratory procedure, and then the fertilized egg (embryo) is returned to the woman's uterus. In the Invitro fertilization the females are super ovulated by giving injections of gonodotrophin hormones.The eggs from the females are collected (egg retrival).At the same time a semen sample is collected from the male partner,processed and placed together with the healthy eggs and allowed to fertilize to produce embroyes which are then transferred in to the womb

3.4.3.6 Model Questions

1)Explain the steps involved in *invitro* fertilization and embryo transfer

2)Discuss the merits and demerits of *invitro* fertilization

3.4.3.7 References

1. Animal cell culture techniques by Ian Freshney.
2. Animal Cell culture by David son.

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Lesson 3.4.4

Stem cell culture and applications

objective

This chapter deals with the stem cell culture and their applications in different biological fields.

3.4.4.1 Introduction

. A stem cell is a special kind of cell that has a unique capacity to renew itself and to give rise to specialized cell types. Although most cells of the body, such as heart cells or skin cells, are committed to conduct a specific function, a stem cell is

uncommitted and remains uncommitted, until it receives a signal to develop into a specialized cell. Their proliferative capacity combined with the ability to become specialized makes stem cells unique. Researchers have for years looked for ways to use stem cells to replace cells and tissues that are damaged or diseased. Recently, stem cells have received much attention. What is "new" and what has brought stem cell biology to the forefront of science and public policy?

Scientists interested in human development have been studying animal development for many years. This research yielded our first glimpse at a class of stem cells that can develop into any cell type in the body. This class of stem cells is called pluripotent, meaning the cells have the potential to develop almost all of the more than 200 different known cell types. Stem cells with this unique property come from embryos and fetal tissue.

In 1998, for the first time, investigators were able to isolate this class of pluripotent stem cell from early human embryos and grow them in culture. In the few years since this discovery, evidence has emerged that these stem cells are, indeed, capable of becoming almost all of the specialized cells of the body and, thus, may have the potential to generate replacement cells for a broad array of tissues and organs, such as the heart, the pancreas, and the nervous system. Thus, this class of human stem cell holds the promise of being able to repair or replace cells or tissues that are damaged or destroyed by many of our most devastating diseases and disabilities.

At about the same time as scientists were beginning to explore human pluripotent stem cells from embryos and fetal tissue, a flurry of new information was emerging about a class of stem cells that have been in clinical use for years—so-called adult stem cells. An adult stem cell is an undifferentiated cell that is found in a differentiated (specialized) tissue in the adult, such as blood. It can yield the specialized cell types of the tissue from

which it originated. In the body, it too, can renew itself. During the past decade, scientists discovered adult stem cells in tissues that were previously not thought to contain them, such as the brain. More recently, they reported that adult stem cells from one tissue appear to be capable of developing into cell types that are characteristic of other tissues. For example, although adult hematopoietic stem cells from bone marrow have long been recognized as capable of developing into blood and immune cells, recently scientists reported that, under certain conditions, the same stem cells could also develop into cells that have many of the characteristics of neurons. So, a new concept and a new term emerged-adult stem cell plasticity.

Are human adult and embryonic stem cells equivalent in their potential for generating replacement cells and tissues? Current science indicates that, although both of these cell types hold enormous promise, adult and embryonic stem cells differ in important ways. What is not known is the extent to which these different cell types will be useful for the development of cell-based therapies to treat disease.

Some considerations are noteworthy regarding this report. First, in recent months, there have been many discussions in the lay press about the anticipated abilities of stem cells from various sources and projected benefits to be realized from them in replacing cells and tissues in patients with various diseases. The terminology used to describe stem cells in the lay literature is often confusing or misapplied. Second, even among biomedical researchers, there is a lack of consistency in common terms to describe what stem cells are and how they behave in the research laboratory. Third, the field of stem cell biology is advancing at an incredible pace with new discoveries being reported in the scientific literature on a weekly basis.

This summary begins with common definitions and explanations of key concepts about stem cells. It ends with an assessment of how adult, embryonic and fetal stem cells are similar and how

they are different. In between lie important details that describe what researchers have discovered about stem cells and how they are being used in the laboratory.

3.4.4.2 Definitions and General Concepts About Stem Cells

In developing this report, some conventions were established to describe consistently what stem cells are, what characteristics they have, and how they are used in biomedical research. Here are some of the key definitions that are used throughout this report.

Stem cell. A stem cell is a cell from the embryo, fetus, or adult that has, under certain conditions, the ability to reproduce itself for long periods or, in the case of adult stem cells, throughout the life of the organism. It also can give rise to specialized cells that make up the tissues and organs of the body. Much basic understanding about embryonic stem cells has come from animal research. In the laboratory, this type of stem cell can proliferate indefinitely, a property that is not shared by adult stem cells.

Pluripotent stem cell. A single pluripotent stem cell has the ability to give rise to types of cells that develop from the three germ layers (mesoderm, endoderm, and ectoderm) from which all the cells of the body arise. The only known sources of human pluripotent stem cells are those isolated and cultured from early human embryos and from fetal tissue that was destined to be part of the gonads.

Embryonic stem cell. An embryonic stem cell is derived from a group of cells called the inner cell mass, which is part of the early (4- to 5-day) embryo called the blastocyst. Once removed from the blastocyst, the cells of the inner cell mass can be

cultured into embryonic stem cells. These embryonic stem cells are not themselves embryos. In fact, evidence is emerging that these cells do not behave in the laboratory as they would in the developing embryo—that is, the conditions in which these cells develop in culture are likely to differ from those in the developing embryo.

Embryonic Germ Cell. An embryonic germ cell is derived from fetal tissue. Specifically, they are isolated from the primordial germ cells of the gonadal ridge of the 5- to 10-week fetus. Later in development, the gonadal ridge develops into the testes or ovaries and the primordial germ cells give rise to eggs or sperm. Embryonic stem cells and embryonic germ cells are pluripotent, but they are not identical in their properties and characteristics.

Differentiation. Differentiation is the process by which an unspecialized cell (such as a stem cell) becomes specialized into one of the many cells that make up the body. During differentiation, certain genes become activated and other genes become inactivated in an intricately regulated fashion. As a result, a differentiated cell develops specific structures and performs certain functions. For example, a mature, differentiated nerve cell has thin, fiber-like projections that send and receive the electrochemical signals that permit the nerve cell to communicate with other nerve cells. In the laboratory, a stem cell can be manipulated to become specialized or partially specialized cell types (e.g., heart muscle, nerve, or pancreatic cells) and this is known as directed differentiation.

Adult stem cell. An adult stem cell is an undifferentiated (unspecialized) cell that occurs in a differentiated (specialized) tissue, renews itself, and becomes specialized to yield all of the specialized cell types of the tissue from which it originated. Adult stem cells are capable of making identical copies of themselves for the lifetime of the organism. This property is referred to as "self-renewal." Adult stem cells usually divide to generate progenitor or precursor cells, which then differentiate or develop

into "mature" cell types that have characteristic shapes and specialized functions, e.g., muscle cell contraction or nerve cell signaling. Sources of adult stem cells include bone marrow, blood, the cornea and the retina of the eye, brain, skeletal muscle, dental pulp, liver, skin, the lining of the gastrointestinal tract, and pancreas. The most abundant information about adult human stem cells comes from studies of hematopoietic (blood-forming) stem cells isolated from the bone marrow and blood. These adult stem cells have been extensively studied and applied therapeutically for various diseases. At this point, there is no isolated population of adult stem cells that is capable of forming all the kinds of cells of the body. Adult stem cells are rare. Often they are difficult to identify, isolate, and purify. There are insufficient numbers of cells available for transplantation and adult stem cells do not replicate indefinitely in culture.

Plasticity. Plasticity is the ability of an adult stem cell from one tissue to generate the specialized cell type(s) of another tissue. A recently reported example of plasticity is that, under specific experimental conditions, adult stem cells from bone marrow generated cells that resemble neurons and other cell types that are commonly found in the brain. The concept of adult stem cell plasticity is new, and the phenomenon is not thoroughly understood. Evidence suggests that, given the right environment, some adult stem cells are capable of being "genetically reprogrammed" to generate specialized cells that are characteristic of different tissues.

Clonality or clonally derived stem cell. A cell is said to be clonally derived or to exhibit clonality if it was generated by the division of a single cell and is genetically identical to that cell. In stem cell research, the concept of clonality is important for several reasons. For researchers to fully understand and harness the ability of stem cells to generate replacement cells and tissues, the exact identity of those cells' genetic capabilities and functional qualities must be known. Human pluripotent stem cells from embryos and fetal tissue are by their nature clonally

derived. However, very few studies have shown clonal properties of the cells that are developed from adult stem cells. It is crucial to know whether a single cell is capable of developing an array of cell types, or whether multiple stem cell types, that when grown together, are capable of forming multiple cell types. For instance, recent research has shown that a mixture of cells removed from fat tissue or umbilical cord blood are capable of developing into blood cells, bone cells, and perhaps others. Researchers have not shown that a single cell is responsible for giving rise to other cell types or, if so, what kind of cell it is. These results may well be attributable to multiple types of precursor cells in the starting tissue; such results from fat cells may, in fact, be due to the presence of hematopoietic stem cells in the fat tissue. The importance of showing that one cell type can reproducibly become another and self-replicate cannot be overemphasized.

Progenitor or precursor cell. A progenitor or precursor cell occurs in fetal or adult tissues and is partially specialized; it divides and gives rise to differentiated cells. Researchers often distinguish precursor/progenitor cells from adult stem cells in the following way: when a stem cell divides, one of the two new cells is often a stem cell capable of replicating itself again. In contrast, when a progenitor/precursor cell divides, it can form more progenitor/precursor cells or it can form two specialized cells, neither of which is capable of replicating itself. Progenitor/precursor cells can replace cells that are damaged or dead, thus maintaining the integrity and functions of a tissue such as liver or brain. Progenitor/precursor cells give rise to related types of cells-lymphocytes such as T cells, B cells, and natural killer cells, for example—but in their normal state do not generate a wide variety of cell types.

3.4.4.3 Challenges in Stem Cell Research

It is important to understand some of the difficulties that researchers have had in isolating various types of stem cells, working with the cells in the laboratory, and proving

experimentally that the cells are true stem cells. Most of the basic research discoveries on embryonic and adult stem cells come from research using animal models, particularly mice.

In 1981, researchers reported methods for growing mouse embryonic stem cells in the laboratory, and it took nearly 20 years before similar achievements could be made with human embryonic stem cells. Much of the knowledge about embryonic stem cells has emerged from two fields of research: applied reproductive biology, i.e., *in vitro* fertilization technologies, and basic research on mouse embryology.

There have been many technical challenges that have been overcome in adult stem cell research as well. Some of the barriers include: the rare occurrence of adult stem cells among other, differentiated cells, difficulties in isolating and identifying the cells (researchers often use molecular "markers" to identify adult stem cells), and in many cases, difficulties in growing adult stem cells in tissue culture. Much of the research demonstrating the plasticity of adult stem cells comes from studies of animal models in which a mixture of adult stem cells from a donor animal is injected into another animal, and the development of new, specialized cells is traced.

In 1998, James Thomson at the University of Wisconsin-Madison isolated cells from the inner cell mass of the early embryo, called the blastocyst, and developed the first human embryonic stem cell lines. At the same time, John Gearhart at Johns Hopkins University reported the first derivation of human embryonic germ cells from an isolated population of cells in fetal gonadal tissue, known as the primordial germ cells, which are destined to become the eggs and sperm. From both of these sources, the researchers developed pluripotent stem cell "lines," which are capable of renewing themselves for long periods and giving rise to many types of human cells or tissues. Human embryonic stem cells and embryonic germ cells differ in some characteristics, however, and do not appear to be equivalent.

Why are the long-term proliferation ability and pluripotency of embryonic stem cells and embryonic germ cells so important? First, for basic research purposes, it is important to understand the genetic and molecular basis by which these cells continue to make many copies of themselves overlong periods of time. Second, if the cells are to be manipulated and used for transplantation, it is important to have sufficient quantities of cells that can be directed to differentiate into the desired cell type(s) and used to treat the many patients that may be suffering from a particular disease.

In recent months, other investigators have been successful in using somewhat different approaches to deriving human pluripotent stem cells. At least 5 other laboratories have been successful in deriving pluripotent stem cells from human embryos and one additional laboratory has created cell lines from fetal tissue. In each case, the methods for deriving pluripotent stem cells from human embryos and embryonic germ cells from fetal tissue are similar, yet they differ in the isolation and culture conditions as initially described by Thomson and Gearhart, respectively. It is not known to what extent U.S.-based researchers are using these additional sources of embryonic stem and germ cells.

At present, there have been multiple human adult stem cell lines that have been created through a combination of public and private resources (e.g., hematopoietic stem cells). Substantial adult stem cell research has been underway for many years, and in recent years this has included basic studies on the "plasticity" of such cells.

3.4.4.4 What Kinds of Research Might Be Conducted with Stem Cells?

There has been much written about the new discoveries of various stem cell types and their properties. Importantly, these

cells are research tools and they open many doors of opportunity for biomedical research.

Transplantation Research—Restoring Vital Body Functions

Stem cells may hold the key to replacing cells lost in many devastating diseases. There is little doubt that this potential benefit underpins the vast interest about stem cell research. What are some of these diseases? Parkinson's disease, diabetes, chronic heart disease, end-stage kidney disease, liver failure, and cancer are just a few for which stem cells have therapeutic potential. For many diseases that shorten lives, there are no effective treatments but the goal is to find a way to replace what natural processes have taken away. For example, today, science has brought us to a point where the immune response can be subdued, so that organs from one person can be used to replace the diseased organs and tissues of another. But, despite recent advances in transplantation sciences, there is a shortage of donor organs that makes it unlikely that the growing demand for lifesaving organ replacements will be fully met through organ donation strategies.

The use of stem cells to generate replacement tissues for treating neurological diseases is a major focus of research. Spinal cord injury, multiple sclerosis, Parkinson's disease, and Alzheimer's disease are among those diseases for which the concept of replacing destroyed or dysfunctional cells in the brain or spinal cord is a practical goal. This report features several recent advances that demonstrate the regenerative properties of adult and embryonic stem cells.

Another major discovery frontier for research on adult and embryonic stem cells is the development of transplantable pancreatic tissues that can be used to treat diabetes. Scientists in academic and industrial research are vigorously pursuing all possible avenues of research, including ways to direct the specialization of adult and embryonic stem cells to become

pancreatic islet-like cells that produce insulin and can be used to control blood glucose levels. Researchers have recently shown that human embryonic stem cells to be directly differentiated into cells that produce insulin.

There are common misconceptions about both adult and human embryonic stem cells. First, the lines of unaltered human embryonic stem cells that exist will not be suitable for direct use in patients. These cells will need to be differentiated or otherwise modified before they can be used clinically. Current challenges are to direct the differentiation of embryonic stem cells into specialized cell populations, and also to devise ways to control their development or proliferation once placed in patients.

A second misconception is that adult stem cells are ready to use as therapies. With the exception of the clinical application of hematopoietic stem cells to restore the blood and immune system, this is not the case. The therapeutic use of this mixture of cells has proven safe because the mixture is place back into the environment from which it was taken, e.g., the bone marrow. In fact, many of the adult stem cell preparations currently being developed in the laboratory represent multiple cell types that are not fully characterized. In order to safely use stem cells or cells differentiated from them in tissues other than the tissue from which they were isolated, researchers will need purified populations (clonal lines) of adult stem cells.

In addition, the potential for the recipient of a stem cell transplant to reject these tissues as foreign is very high. Modifications to the cells, to the immune system, or both will be a major requirement for their use. In sum, with the exception of the current practice of hematopoietic stem cell transplantation, much basic research lies ahead before direct patient application of stem cell therapies is realized.

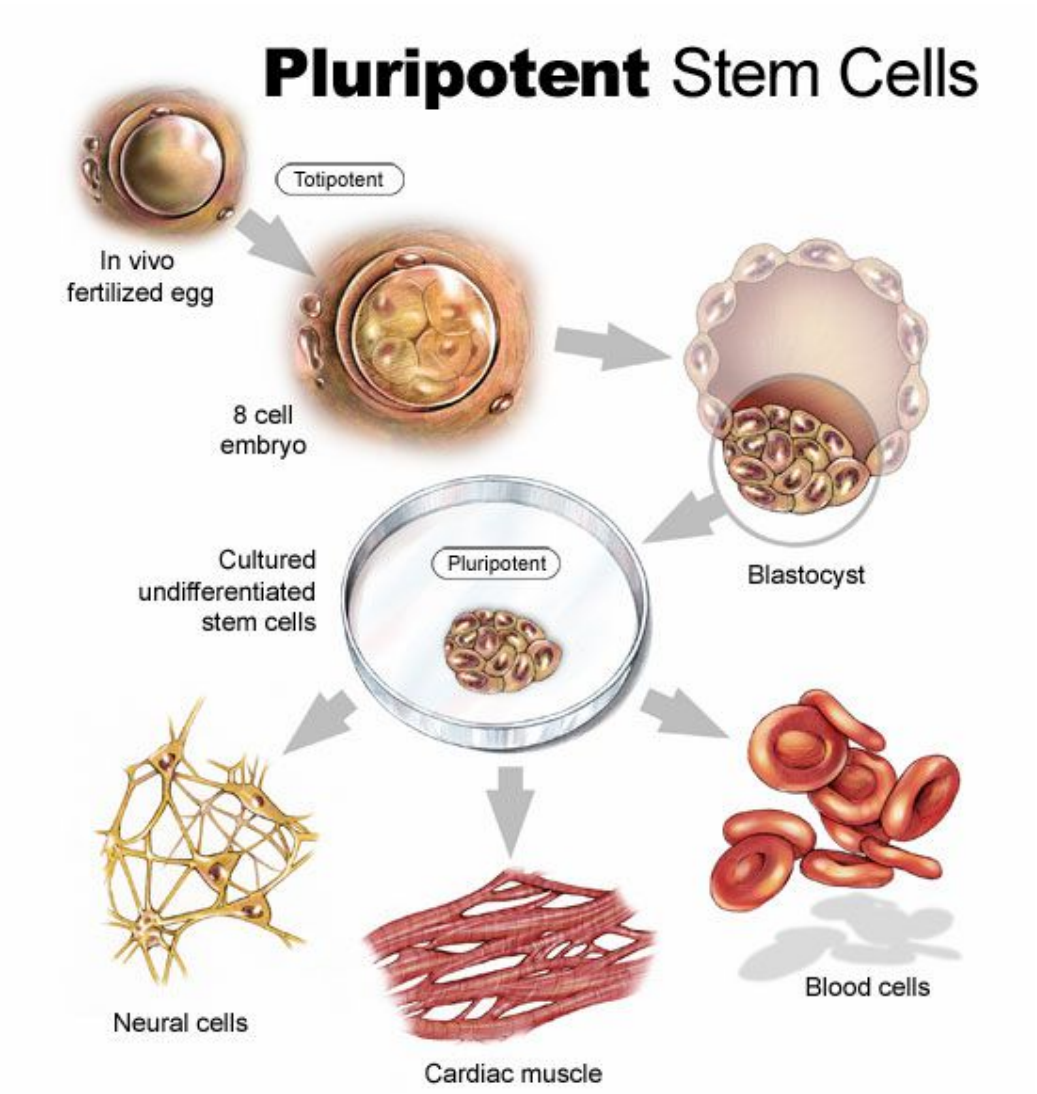
3.4.4.5 Stem cell cultivation

1) In vivo or in vitro fertilized egg – Invivo fertilized eggs are retrived from women's uterus or fertilized eggs can also be produced by invitro fertilization.In vitro fertilization (IVF) is a procedure in which eggs (ova) from a woman's ovary are retrived. They are fertilized with sperm in a laboratory procedure, and then the fertilized egg (embryo) is returned to the woman's uterus. In the Invitro fertilization the females are super ovulated by giving injections of gonodotrophin hormones.The eggs from the females are collected (egg retrival).At the same time a semen sample is collected from the male partner,processed and placed together with the healthy eggs and allowed to fertilize to produce embroyes

2) Blastocyst stage - The embryos are collected and allowed to grow in a specific medium in the co2 incubator at 370c and 5% co2.After 5 to 7 days the fertilized eggs begins to multiply .

3) Removal of stem cells.-From the multiplying embryos the inner mass of stem cells were collected and undifferentiated stem cells were separated .

4) Cultured undifferentiated stem cells – the undifferentiatedn stem cells were directed to a way to what they will become.or they may directed to become specialized cells like blood cells ,neural cells or muscle cells etc



3.4.4.6 Basic Research Applications

Embryonic stem cells will undoubtedly be key research tools for understanding fundamental events in embryonic development that one day may explain the causes of birth defects and approaches to correct or prevent them. Another important area of research that links developmental biology and stem cell biology is understanding the genes and molecules, such as growth factors and nutrients, that function during the development of the embryo so that they can be used to grow stem cells in the laboratory and direct their development into specialized cell types.

Therapeutic Delivery Systems

Stem cells are already being explored as a vehicle for delivering genes to specific tissues in the body. Stem cell-based therapies are a major area of investigation in cancer research. For many years, restoration of blood and immune system function has been used as a component in the care of cancer patients who have been treated with chemotherapeutic agents. Now, researchers are trying to devise more ways to use specialized cells derived from stem cells to target specific cancerous cells and directly deliver treatments that will destroy or modify them.

Other Applications of Stem Cells

Future uses of human pluripotent cell lines might include the exploration of the effects of chromosomal abnormalities in early development. This might include the ability to monitor the development of early childhood tumors, many of which are embryonic in origin. Another future use of human stem cells and their derivatives include the testing of candidate therapeutic drugs. Although animal model testing is a mainstay of pharmaceutical research, it cannot always predict the effects that a developmental drug may have on human cells. Stem cells

will likely be used to develop specialized liver cells to evaluate drug detoxifying capabilities and represents a new type of early warning system to prevent adverse reactions in patients. The coupling of stem cells with the information learned from the human genome project will also likely have many unanticipated benefits in the future.

3.4.4.7 Summary

Over the last few years research on stem cells has received much public attention both for its extraordinary potential and for the associated social, legal and public issues. The stem cells derived from various sources can be used to determine the pharmacokinetics of drugs and toxins.A stem cell is a cell from the embryo, fetus, or adult that has, under certain conditions, the ability to reproduce itself for long periods or, in the case of adult stem cells, throughout the life of the organism. It also can give rise to specialized cells that make up the tissues and organs of the body. Apart from the hematopoietic stem cells ,other adult stem cells that have been tested and put to clinical use include :myoblasts for cardiac and skeletal muscle loss or degradation:cultured beta cells for diabetes mellitus,and cultured limbal epithilial cells for recunstrution of ocular surface inpatients who have suferred extensive damage threatening vision. Stem cell cultivationincludes following steps1) In vitro fertilized egg (made in an artificial environment outside of a living organism.)2) Blastocyst stage (5 to 7 days after egg is fertilized) cells are beginning to multiply.3) Inner stem cell mass (removal of stem cells).4) Cultured undifferentiated stem cells (- stage when cells can be directed to what they will become.)5) Specialized cells - what the cells become.a) blood cellsb) neural cellsc) muscle cells

3.4.4.8 Model Questions

1) What are stem cells ?Discuss different applications of stem cell culture

3.4.4.9 References

1. Animal cell culture techniques by Ian Freshney.
2. Animal Cell culture by David son.

(Author

-P.Jaganmohan Rao

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Lesson 3.5.1

ENZYMES USED IN GENE CLONING

Objective

This lesson describe some important enzymes involved in Gene cloning and their importance.

3.5.1.1 Introduction

Before 1970 there was simply no method available for cutting a duplex DNA molecule in to discrete fragments. It became apparent that the

related phenomenon of host controlled restriction and modification. Present day DNA technology is totally dependant upon our ability to cut DNA molecules at specific sites with restriction endonucleases. The methods available for cutting DNA molecules we must consider the ways in which DNA fragments can be joined to create artificially recombinant molecule. The DNA ligase join covalently the annealed cohensive ends produced by certain restriction enzymes. The ability of DNA ligase from phage T4 – infected Ecoli to catalyze the formation of phosphodiester bond between blunt – ended fragments. The enzyme terminal deoxy nucleotide transferase to synthesize homopolymer 3' single stranded tails at the ends of fragments.

The fundamental unit of a genome that controls heridity is known as gene. Genetic engineering has been employed for the production of valuble polypeptides, insulin, interferon, growth hormones. Gene cloning alters the original sequence of the genes of an organism in a desired direction. Hence the process of gene cloning is also called genetic engineering. A new combination of genes is formed at the end of gene cloning. Thus the gene cloning results in the formation of a recombinant organism. The recombination has an altered sequence of genes in its genome. Hence this technology is often named Recombinant DNA technology. There are various biological tools which are used to carry out manipulation of genetic material and cells as well for example enzymes. Different types of enzymes are used in gene cloning.

3.5.1.2 Restriction endonucleases

Present day DNA technology is totally dependant upon our ability to cut DNA molecules at specific sites.

A batch of nucleic acid enzymes that cut DNA at highly specific sites were discovered in 1970 in Haemophill influenzae. They are called restriction endonucleases.

Roberts (1983) has given an extensive list of restriction enzymes and the sequence recognized by them.

(a) Host controlled restriction and modification

Restriction enzymes allow to monitor the origin of incoming DNA and to destroy if it is recognized as foreign.

Restriction endonucleases recognize specific sequences in the incoming DNA and cleave the DNA in to fragments either at specific sites or more randomly. Modification involves methylation of certain bases at a very limited number of sequences with in DNA which constitute the recognition sequences for the restriction endonuclease. This explains the phage that survive one cycle of growth upon the restrictive host can subsequently reinfect that host efficiently their DNA has been replicating in the presence of the modifying methylase and so it like the host DNA becomes methylated and protected from the restriction system restriction and modification of these processes can occur when DNA is transferred from one bacterial strain to another.

Restriction

A particular virus that plates effectively on one strain of host fails to plating effectively on another host strain its host range is said to be restricted.

Ex: If a λ phage is grown on E.coli strain C and this stock is titred upon E.coli C and E.coli K respectively then the efficiency of plating of λ is observed on these two strains will differ by several orders of magnitude. The titre on E.coli K is several fold lower. Thus the λ phage is said to be restricted by E.coli K.

Mechanism of restriction

Though the restricted phages adsorb to restrictive hosts and inject their DNA normally their DNA is degraded soon after injection.

The restriction endonuclease are a batch of enzymes that cut the foreign DNA and degrade it thus preventing the phage development.

Restriction is a host controlled phenomenon.

Modification

The process by which the restrictive host protects its own DNA from the potentially lethal effects of the restriction endonuclease is called modification.

Mechanism of Modification

Modification occurs in the in the host DNA by methylation of the base pairs which resemble the cutting sites or recognition sites of restriction endonucleases sequence specific methylation specific for same sequence as host methylated sequence cannot be cut by restriction enzymes of host.

Restriction endonucleases

(b) General characteristics and classification

The restriction endonucleases were isolated from E.coli K by an experiment in which they incubated the fractionated cell extract with a mixture of unmodified and modified λ DNA'S which were differentially labeled with ^{3}H and ^{32}P. After incubation and sucrose gradiant centrifugation showed degraded unmodified DNA and undegraded modified DNA in separate bands since then hundreds of restriction enconucleases were isolated and characterized.

General characters of restriction enzymes

They are encoded by the restrictive host (host controlled).

They perform the function of restriction and modification.

They recognize the foreign DNA and degrade it they cannot cut the hosts own DNA because it is modified.

They are always coupled process.

Classification of restriction enzymes

Depending upon the number of subunits, cofactor requirement, symmetry of cutting, cleavage sites and coupling of restriction and modification functions. The restriction enzymes are classified in to 3 types. Type I, II and III.

Type I

- ➔ It is a single multifunctional enzyme. Same enzyme acts as endonuclease and methylase.
- ➔ It has three different subunits.

- ➔ IT requires ATP Mg^{2+} for its activity.
- ➔ Recognazes a sequence of 8-10 base pairs and cuts randomly at least 1,000 base pairs away from recognition/host specificity site.
- ➔ It methylates host DNA at host specificity sites.
- ➔ It cannot be used in recombinant DNA technology because of non specificity cutting.

Type II

It has two separate enzymes for nuclease and methylase action.

- ➔ It has single subunit.
- ➔ It requires only Mg^{2+} for nuclease function.
- ➔ Recognizes GC rich sites and cuts at or near those recognized specificity sites.
- ➔ The methylase modifies host DNA at host specificity sites.
- ➔ Most widely used enzymes in recombinant DNA technology. Most abundant in nature.

Type III

A rare kind of restriction endonuclease which occurs as complex of two subunits. M and R.

- ➔ Subunit M is responsible for recognition and modification of specificity sites.
- ➔ Subunit R is responsible for nuclease action.
- ➔ Nuclease subunit requires Mg^{2+} and ATP as cofactors.
- ➔ Recognition sites are asymmetric and cleavage occurs by nicking one strand at a measured distance to one side of recognition 24-26 base pairs to 3' end of specificity site.
- ➔ Not used in recombinant DNA technology.

Mcr Systems

There are some restriction systems called Mcr modified cytosine restriction. The modified methyl cytosine of some phages may escape the normal endo nuclease action (Type I, II and III). But Mcr systems recognizes the modified or methyl cytosine sites also and restricts such phages.

- ➔ The genes for restriction enzymes are located in the main chromosome (or) in the plasmid. Some times they are also encoded by the Prophages.

(c) Nomenculature of restriction enzymes

The discovery of large number of restriction enzymes in the recent part called for a uniform nomenculature. The following rules are observed in nomenculature. The specific name of the host organism is represented by three letter abbreviation in Italics, the first letter being of generic name (in capital) and the second and 3rd letters of specific in small letters.

Ex: E.coli – Eco

Haemophilus influenza – Hin

Strai/type identification is represented by a subscript.

Ex: Eco_k Hin_d

- ➔ If a particular strain has several different restriction modification systems they are identified each by a Roman number not to be confused with type of restriction endonuclease.

Hsn_dI Hin_dII Hin_dIII

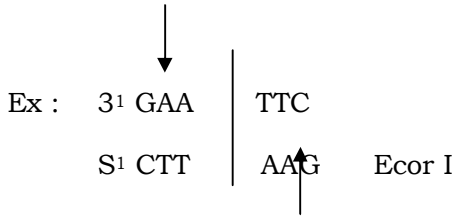
- ➔ In case where restriction and modification enzymes are separate the restriction enzyme is given a general name R followed by system name and the methylase is given a general name M follows by system name.

Ex: R Hin_dIII, M Hin_dIII

(d) Target sites and cutting pattern

Rotational symmetry

Particular sequences of DNA containing tetra penta hexa or hepta nucleotides which have two fold axis of rotational symmetry are recognized by vast majority of Type II restriction endonuclease.



These sequences resemble palindromes that is words that read like backward and farword.

Sticky and cutting

Some restriction endonucleases makes single strands breaks four nucleotides apart in the opposite strands of its target sequence so generally fragments with protruding 5^1 termini which can associate by hydrogen bonds and by over lapping S^1 termini circularize by intermolecular ends. Hence the name sticky (or) cohesive ends. These cohesive ends in principle can offer possibly to ioin fragments from diverse source to be joined and by sealing the nicks. Some enzymes can produce S^1 fragments with cohesive ends.

Ex: Pst I

Providentia stuartii : S^1 T C G A 3^1

3^1 A G C T S^1

Blunt and cutting

Some restriction enzymes cut the DNA at the target sequences giving rise to blunt ends.

Ex: Hae III Haemophilus aegyptius

G G | C C
C C | G G

Some restriction enzymes and their cleavage sites

Microorganism	Enzyme abbreviation	Sequence	Note
Haemophilus aegyptius	Hae III	S^1 G G | C C 3^1 3^1 C C G G S^1	1
Staphylococcus aureus 3A	Sau 3AI	S^1 G A T C 3^1 3^1 C T A G S^1	2
Bacillus amyloliquefaciens H	Bam HI	S^1 G G A T C C 3^1 3^1 C C T A G G S^1	2

Thermus aquaticus	Taq I	S^1 T C G A 3^1 3^1 A G C T S^1	2
Escherichia coli RY 13	Ecor I	S^1 G A A T T C 3^1 3^1 C T T A A G S^1	2
Haemophalus influenia	Hind II	S^1 G T Py / Pu A C 3^1 3^1 C A Pu / Py T G S^1	1,5
	Hind III	S^1 A A G C T T 3^1 3^1 G A C G A S^1	2
Providentia stuarti	Pst I	S^1 C T G C A G 3^1 3^1 G A C G T C S^1	3

Haemophilus haemolyticus	Hha I	S^1 G C C ↓ G 3^1 3^1 C ↑ G G C S^1	3
Serratia marcescens	Sma I	C C C ↓ G G G G G G ↑ C C C	1
Xanthomane malvacearum	X maI	C ↓ C C G G G G G G C C ↑ C	2
Moranella bovis	Mbo II	G A A G A N8 ↓ C T T C T N7 ↑	4

Notes:

1. Produces blunt ends

2. Produces cohesive end with S^1 single stranded over hang.

3. Produces cohesive end with 3^1 single stranded over hang.

4. This is a type II S enzyme. It does not cut with in the recognition sequence but at whatever sequence lies to the right as shown N is any nucleotide.

5. Pu is any purine [A (or) G]

   Py is any pyramidine [C (or) T]

(e) Role of restrictive systems invivo

Analoqous to immune system in higher organisms to distinguish between self and non self DNA. However it is moderately effective in providing immunity to phage infections. This is because bacteriophage evolve some mechanisms to evade the action of restriction endonuclease system.

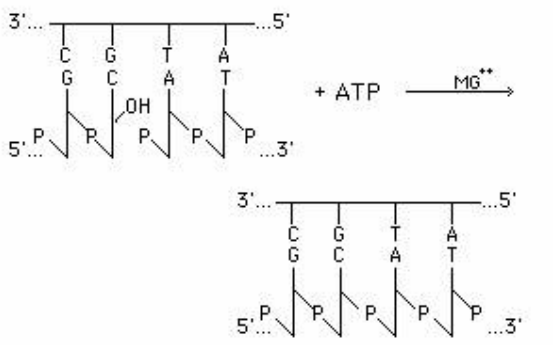
3.5.1.3 DNA ligase

Mertz and Davis (1972) for the first time demonstrated that cohensive termini of cleaved DNA molecules could be covalently sealed with E.coli DNA ligase and were able to produce recombinant DNA molecules. DNA ligase seals single strand ricks in DNA which hae $S^1 – 3^1$ – hydronyl termini. There are two enzymes which are entensively used ro covalently joining restriction fragments. The ligase from E.coli and that encoded by T_4 phage. The main source of DNA ligase is T_4 phage, hence the enzyme is known as T_4 DNA ligase.

For the joining reactions the E.coli DNA ligase uses nicotinanade adenine dinucleotide as a cofactor while T_4 DNA ligase requires ATP. In both cases cofactor breaks in to AMP which in turn adenylate the enzyme to form Enzyme AMP complex. This complex binds to nick containing 3^1 – OH and S^1 – phosphate ends on a double stranded DNA moledule. The S^1 phosphory terminus of the nick is adenylated by the Enzyme AMP complex with 3^1-04 terminus resulting in formation of phosphodiester and liberation of AMP. After the formation of phospho diester nick is sealed. T_4 enzyme has the ability to join the blunt ends of DNA fragments. Where as E.coli DNA ligase

join the cohesive ends produced by restriction enzymes. Additional advantage with T_4 enzymes is that it can quickly join and produce the full base pairs. Cohesive and ligation proceede about 100 times faster than the blunt end ligation. Action of DNA ligase shown in Fig.3.1.

Fig. 3.1 Action of DNA ligase.

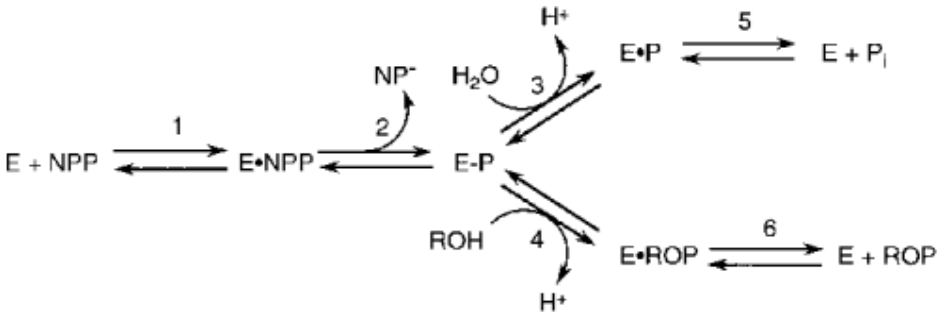


3.5.1.4 Alkaline phosphotase

When plasmid vector, for joining a foreign DNA fragment is treatment with restriction enzymes the major difficulty arises at the same time. Because the cohesive ends of broken plasmids instead of joining with foreign DNA join the cohesive end of the some DNA molecules and get recircubrazod. To overcome this problem the restricted plasmid is treated with an enzyme alkaline phosphotase, that digests the teomanal S^1 phosphoryl group. The restriction fragments of the foreign DNA to be cloned are not treated with alkaline phosphotase. Therefore the S^1 end of foreign DNA fragment can covalently join to 3^1 end of the plasmid. The hybrid (or) recombinant DNA obtained has a nick with 3^1 and 5^1 hydroxy ends. Ligase will only join 3^1 and S^1 ends of the recombinant DNA to gether if the S^1 end is phosphorylated, thus alkaline phosphotase and ligase prevent recircuarzation of the vector and increase the frequency of production of recombinant DNA molecules. The nicks between two 3^1 ends of DNA fragment and vector DNA are repaired inside the bacterial host

cells during the transformation. Mechanism of alkaline phosphotase as shown in Fig.3.2.

Fig.3.2 Mechanism of Alkaline phosphotase.



3.5.1.5 Reverse transcriphase

In addition to these enzymes reverse transcriptase is used to synthesize the copy DNA or complementary DNA (CDNA) by using mRNA as a template. Reverse transcriptase is very useful in the synthesis of CDNA and construction of CDNA alone bank. It was known that the genetic informations of DNA pas to protein through mRNA. During 1960's Temin and coworkers postulated that in certain cancer causing animal viruses which contain RNA as genetic material transcription of cancerous genes takes place most probably by DNA polymerase directed by virus RNA. Then DNA is used as template for synthesis of many copies of viral RNA in a cell. Reverse transcriptase, which is produced by certain RNA viruses uses an RNA strand as a template while directing deoxyribonucleotides in to the growing chain. Thus when an A,G,C or U residue of the temple RNA strand is encounters, the complementary deoxyribonucleotide is incorporated in to the growing DNA strand. The synthesis of DNA strand by reverse transcriptase in vitro is often incomplete, however before synthesis caeses the DNA stand usually turn back on it self for a few nucleotides.

The second DNA strand is synthesized by the addition of the klenow fragment of E.coli DNA polymerae which uses the first DNA strand as a template and adds deoxyribonucleotides to the growing strand starting from the end of the hair pin loop. After the reaction is complete the sample is treated with the enzyme Rnase which degrade the mRNA molecules and with S_1 nucleases which opens the hair pin loops and degrade single stranded DNA extensions. At the end of this

procedure the sample contains a minture of partial and complete double stranded complementary DNA copies of the more prevalent mRNAs in the original sample.

3.5.1.6 DNA Polymerase

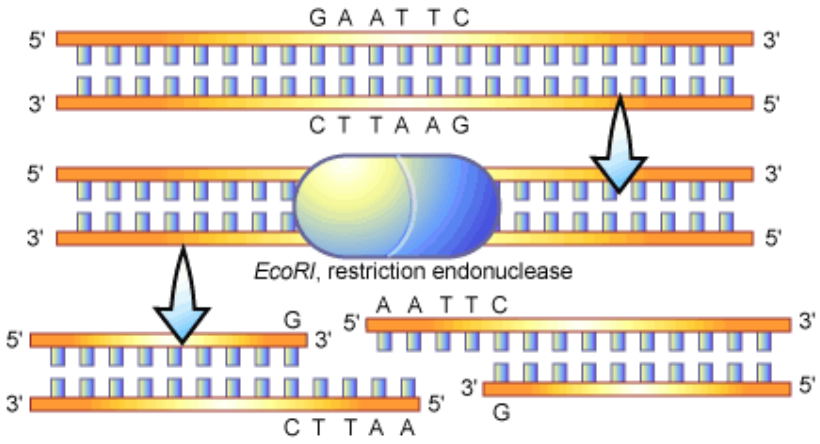
This enzyme polymerizes the DNA synthesis on DNA template and also catalyses a 5^1 - 3^1 and 3^1 – 5^1 enonucleolytis degradation of DNA. The DNA polymerase investigatedby A.Kornberg and coworkers in E.coli in 1956 is now known as DNA polymerase I. The other two enzymes are DNA polymerase II and DNA polymerase III. These have almost similar catalytic activity. DNA polymerase I mol. Wt 1,09,000 has a single polypeptide chain of about 1,000 aminoacid residues. The addition of mononucleotide to the free hydroxyl end of a DNA chain is catalyzed by this enzyme. It catalyses the reactions that is 3^1 – 5^1 exonuclease activity and 5^1 – 3^1 exonuclease activity. DNA polymerase useful for the growing of DNA strand. The incoming nucleotides is enzymatically joined by phosphodiester linkages to the 3^1 – 0 H group of the last nucleotide that was incorporated in the growing strand.

DNA polymerase have mol.wt 120,000. It catalyses 3^1 – 5^1 exonuclease activity. DNA pol III have mol.wt 140,000 is about several times more active than the other two polymerases. It is a dimer of DNA polymerase III it requires an auxillary protein co polymerase III and after combination yield a DNA polymerase III copolymerase III complex. It produces a parallel strand in the presence of ATP.

3.5.1.7 (a) Exonucleases

These enzymes act upon genome and digest the base pairs of on 5^1 (or) 3^1 ends of a single stranded DNA or at single strand nicks or gaps in double stranded DNA. The major difference between exonucleases and endonucleases. Enonucleases cut the DNA at the ends endonucleases cleaves the double stranded DNA at any point except the ends their action involves only one strand of the duplex.

Fig.3.6 Action of restriction endonuclease.



(b) S_2 nucleases

It degrades the single stranded DNA (or) single strand of double stranded DNA with cohesive ends. As a result of action of nuclease cohesive ends are converted in to blunk ends.

(c) Polynucleotide kinase

Polynucleotide kinase plays an very important role for the phosphorylotion. That means addition of phosphate group to bases. This phospharylation plays an very important role for the formation of recombinat DNA molecule.

(d) Terminal trnasferase

An enzyme purified from calf thymus, provides the means by which homopolymeric extensions can be synthesized. It will repeatedly add nucleotides to the 3^1 – OH termini of a polulation of DNA molecules.

3.5.1.8 Summary

Recombinant DNA technology comprises a battery of experimental procedures used to isolate clone pieces of DNA that contain specific genes. The success of gene cloning depends on being able to cut DNA molecules reproducibly in to fragments of discreted sizes. This precise cleaving of DNA is achieved by using type II restriction endonucleases. These enzymes bind to specific sequences

with in a DNA molecule and symmetrically cut phosphodiester bond of each strand at the recognition site.

DNA ligase plays an important role for the joining of DNA molecules. Alkaline phosphotase plays an important role for the removal of the phosphate groups of the terminal ends. This prevents the direct ligation of DNA molecules.

Reverse transcriptase plays an important role for the formation DNA from mDNA molecule. DNA polymerase plays animportant role for the synthesis of DNA.

3.5.1.9 Model Questions

1. What is the use of restriction endonucleases in gene cloning.
2. Write about nomenculature and types of restriction endonucleases.
3. Explain different types of enzymes used in genetic engineering and their importance.

3.5.1.10 Reference Books

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Lesson 3.5.2

VECTORS

Objective

3.5.2.1 Introduction

3.5.2.2 Plasmids
- (a) Sailent features and properties of plasmid DNA
- (b) Classification
- (c) Purification of plasmid DNA
- (d) Plasmid as a vector
- (e) PBR 322
- (f) PBR 327
- (g) PUC Vectors
- (h) PACYC 184 plasmid
- (i) PVN 121
- (j) Yeast Plasmid vectors
- (k) Ti and Ri plasmid

3.5.2.3 Bacteriophages
- (a) Sailent features of λ DNA
- (b) Life cycle of λ phage
- (c) Lambda phage vectors
  - (i) λgt 10, λgt11
  - (ii) EMBLS, EMPLY
  - (iii) Charon 34 and charon 35
  - (iv) M_{13} phage

3.5.2.4 Cosmids
- (a) Characteristic features of cosmids

3.5.2.5 Phasmids
3.5.2.6 PI cloning vectors for cloning large DNA segments
3.5.2.7 F-factor based vectors
3.5.2.8 Plant and Animal Viruses as vectors
3.5.2.9 Transposon as vectors
3.5.2.10 Binary (or) shuttle vectors
3.5.2.11 Summary
3.5.2.12 Model Questions
3.5.2.13 Reference Books

Objective

This lesson deals with the different types of vectors used in gene cloning.

3.5.2.1 Introduction

A variety of vectors have been developed which not only allow multiplication but also may be manipulated in such a way that the inserted gene may express in the host. Due to the importance of a variety of these cloning and expression vectors in genetic Engineering experiments. The cloning of DNA is possible only with the help of another DNA molecule, which is capable of replicating in a host. This other DNA molecule is often used in the form of a vector which could be plasmid, bacteriophage, a derived cosmid, phasmid, a transposon or even a virus. The desired feature of any cloning vector is that it should possess a site at which foreign DNA can be inserted without disrupting any essential function. Some times vectors are modified by inserting a DNA segment to create unique sites for one (or) more enzymes to facilitate its use in gene cloning.

Vectors

3.5.2.2 Plasmids

Plasmids are extrachromosomal circular DNA molecules found in bacterial cells. They are inherited sharply without the influence of chromosomal DNA. They replicate independently from the replication of chromosomal DNA. Bacterial plasmid DNA's are double stranded in nature. The bacterial plasmid DNA's exist in three forms namely covalently closed circular DNA, linear DNA and spherical DNA.

(a) Sailent features of plasmid DNA (or) Basic Properties of Plasmid DNA

The plasmid DNA is a circular double stranded DNA molecule found in the protoplasm of bacterial cells.

- ➔ It is inherited from organism to organism without the influence of chromosomal DNA.
- ➔ The repication fo plasmid DNA is independent from the replication of chromosomal DNA.
- ➔ In the bacterial cells plasmid DNA's exist in three forms. They are covalently closed circular DNA, linear DNA and supercoiled DNA. The twisting and supercoiling of DNA are caused by the enzyme topoisomerase. Types of plasmid are shown in Fig.3.2.

- ➔ The number of copies of plasmid DNA'S present in each bacterial cell is constant for many generations.

- ➔ Some plasmid DNA'S got integrated with the chromosomal DNA, these integrations results in the formation of episomes. The replication of these episomes depends on the replication of chromosomal DNA.

- ➔ The number of copies of plasmid present in a bacterial cell depends on the replication of plasmid DNA and the plasmid DNA replication go on side by side the plasmid number remains a few one (or) two but when the replication is free from chromosomal DNA the cells receive multiple copies of plasmide (10-20).

- ➔ The plasmid DNA'S replicate either through the rolling circle model (or) through the D-loop formation.

- ➔ Plasmids are widely distributed throughout the prokaryotos. Vary in size from less than 1 x 10^6 daltons to greater than 200 x 10^6 daltons.

- ➔ Plasmids can be categorized into one of two major types conjugative (or) non conjugative depending upon whether (or) not they carry a set of transfer genes called the "tra" genes that promotes bacterial conjugation.

- ➔ Plasmids can also be categorized on the basis of their being maintained as multiple copies per cell. (realaxed plasmide) (or) as a limited number of copies per cell (stringent plasmids).

(b) Classification

On the basis of the function of plasmids they are classified.

R-plasmids

These plasmids give resistance power to the organism against one (or) a few antibiotics. They transfer their resistance power from one organism to another organism during conjugation. They are important in field of medicine and Agriculture.

Virulance plasmid

These plasmids encode for the production of some specific to xins which increases the virulence of pathogens for example end plasmid is a virulence

plasmid which encode for the production of endotoxins. The endotoxins increases the pathogenicity of pathogens.

Sex plasmid

These plasmide transfer sexuatily from one organism to another organism. For example F-plasmid in bacteria encodes for maleness and transfers the maleness from one organism to another organism through conjugative transfer of this plasmid.

Beneficial plasmids

These plasmids carry some genes along with their original sequence and integrate them with the transfer of genes from one organism to another organism. Ex: Ti plasmid.

- ➔ Some plasmids give resistance to the organism against heavy metals example P 1258, and R6. Some other plasmids provide resistance to the organism against U.V. rays. They protect the cells against the destructive action of U.V. rays.

Some phenotypic traits enhibited by plasmid carrier genes :

Antibiotic resistance

Antibiotic Production

Degradation of aromatic compounds

Haemolysin production

Sugar fermentation

Heavy metal resistance

Induction of plant tumors

(C) Purification of plasmid DNA

A obvious prerequisite for cloning is the purification of the plasmid DNA. The isolation and separation of plasmids are mainly based on the shape and size of plasmids.

In general plasmids are isolated by breaking the cells with a detergent and the cellular contents is centrifuged with Cscl. But biotechnological works need cent percent pure plasmid DNA. So proper care must be taken during the

purification and seperation of plasmid DNA'S to avoid the unwanted chromosomal DNA fragments and proteins. Some times large plasmid DNAs also undergo breakdown and go along with the chromosomal DNA's during their isolation. So the breakage of plasmid must be avoided.

Pre treatment of the plasmid

The number of copies of plasmids in bacterial cells must be increased before the isolation of plasmid DNA. This process helps to isolate a large amount of plasmid DNA's from the bacterial culture. For this purpose the bacterial culture is treated with chloramphenicol which inhibit the protein synthetic machainory of bacterial cells. While the DNA'S replicate continuously. The blocking of protein synthesis results in a rapid increase in the number of plasmid DNA'S in the cells.

Isolation procedure

The cultured bacterial cells are treated with EDTA and lysozyme. The EDTA and iysozyme degrade the complex polysaccharide of bacterial cell wall some amount of sucrose solution is added to the culture to equilize the osmotic potential of the culture solution to that of the bacterial cells. Thus sucrose prevents the lysis of the wall less bacterial cells.

- ➔ The wall less cells are then lysed by treating them with sodium lauryl sulphate (SLS) solution. The cell lysate is centrifuged to remove the larger and smaller debris from the cell lysate. During the centrifugation of cell lysate the chromosomal proteins and the larger RNA molecules rapidly reach the bottom of the centrifuge tube and are removed carefully from the tube. The supernatent solution contains plasmid DNA'S along with some fragments of chromosomal DNA.

- ➔ This supernatent solution is again centrifuged with cscl. (Cesium chloride) to remove the chromosomal DNA fragments from the plasmid DNA.

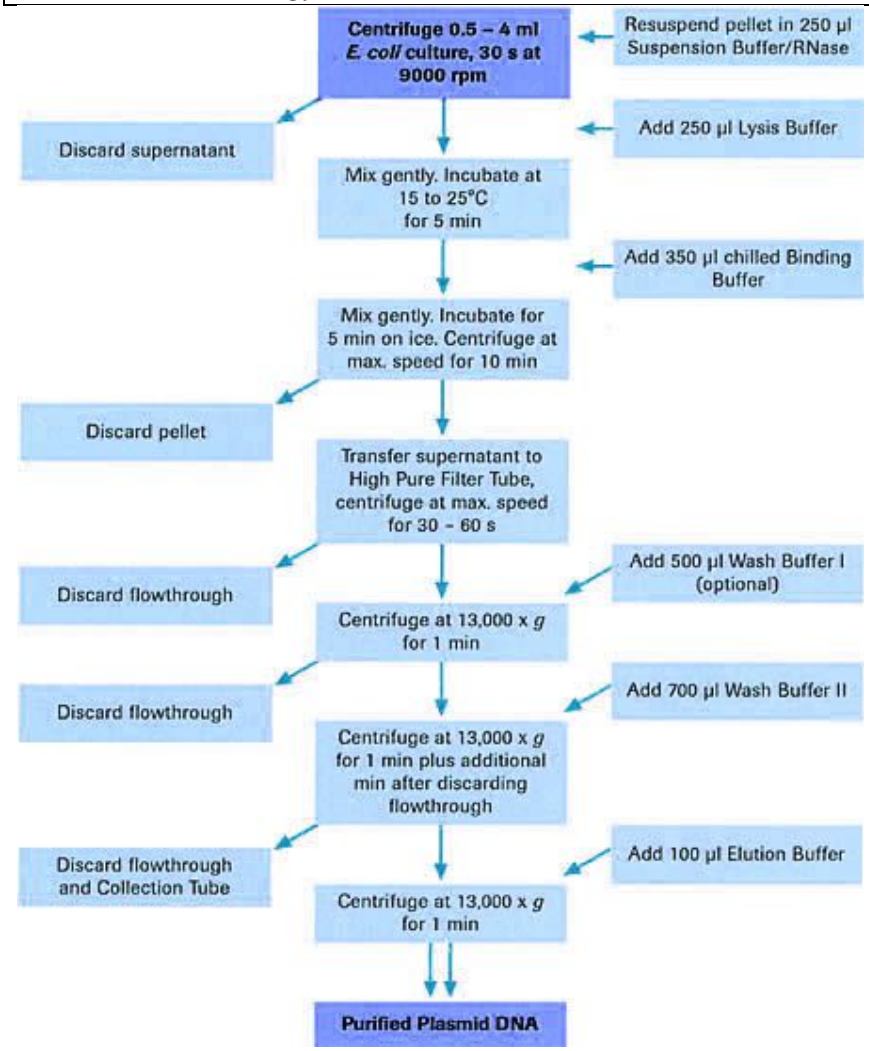
- ➔ The removal of chromosomal DNA fragments from the plasmid DNA'S is due to the difference in the number of CG base paire found in the plasmid DNAS and in the chromosomal DNAS. Hence two clear banks are formed each band is separately collected from the centrifuge tube. The plasmid DNA thus obtained are purified for further use.

- ➔ In some cases there is no difference in the number of C-G bases pairs in between the chromosomal DNA and plasmid DNA. Hence a red dye ethidium bromide is used for the seperation of plasmid from chromosomal DNA.

- ➔ The dye gets incorporated with in the gaps between the adjacent base pairs of large linear chromosomal DNA. But the circular plasmid DNA'S do not allow the dye to bind with them. The dye creates a difference in the molecular weight between the plasmid DNA and the chromosomal DNA fragment. Then they are separated by centrifuging the solution in the presence of cesium chloride density gradient.

- ➔ Separation of different forms of plasmid DNA'S from the mixture of plasmids. There are three forms of plasmid DNAS covalently closed circular plasmids linear plasmids and super coiled plasmids. In these three types of plasmids differ from each other in their sedimentation coefficient. This property is used for the seperation of one type of plasmids from other types of plasmids. The plasmid mixture is centrifuged with the help of sucrose density gradiant which separates all the three forms of plasmids according to their specific density.

- ➔ During the centrifugation the covalently closed circular DNAS more rapidly with great velocity and reach the bottom of the centrifuge tube the supercoiled plasmids move and form a layer just above the covalently closed circular DNAS and the linear plasmids form an upper layer in the centrifuge tube. These different forms of plasmids are carefully removed from the centrifuge tube and are used as vectors in gene cloning. The S value of a linear plasmid DNA is I5s, the S value of a supercoiled plasmid is 17S and that of covalently closed circular plasmid is 23S. The brief explanation of plasmid isolation as shown in Fig.3.8.



Gel electrophoresis

Gel electrophoresis is also used in the seperation of plasmid DNA's. The mixture of plasmid DNAS is electrophorsed through an agarose gel which separates the plasmids from one another due to the difference in their size and molecular weight. The smaller plasmids move rapidly along the gel while the larger plasmids move slowly. Thus clear bands are formed in the agarose gel. The agarose gel is treated with fluorescent dye is exposed to auto radiographic film. Dark bands appear on the autoradiogram. The corresponding regions in the agarose gel is removed and the plasmid DNA'S are separated from it for further use.

(d) Plasmid is a vector

Chang and cohen proved that the plasmid act as vectors which participates in the transfer of certain genes from one organism to another organism. Gene cloning alters the original sequence of the gene of an organism in a desired direction. Hence the process of gene cloning is also called genetic engineering.

All the plasmids present in the cells are not useful for the genetic engineering works because some plasmids lack the property of carrying the foreign gene along with their own sequences. Some plasmids successfully replicate only in the cells from which they were isolated but fail to replicate in the recipient cell.

Desirable properties of plasmid cloning vehicle

An ideal plasmid vector must have the following characteristic features for the successful gene cloning they are :

- ➔ The plasmid DNA must be smaller in size the smaller size of the plasmid help the easy uptake of chimeric plasmid by the recipient cell.
- ➔ The plasmid must have the capacity to transfer the foreign gene in to the recipient cell.
- ➔ The plasmid must be a shuttle vector which replicates both in the donar cell and in the recipient cell.
- ➔ It must integrate the foreign DNA segment in to the genome of the recipient cell.
- ➔ The plasmid must have one or a few genetic markers to detect the presence of cloned genes in the organism.
- ➔ The plasmid should not have any pathogenic property.
- ➔ The plasmid must have its own regulatory genes for the successful replication.

Usefulness of natural plasmids as cloning vehicles

The term nature is used to describe the plasmids which were not constructed invitro for the sole purpose of cloning.

Col E1 is a naturally occurring plasmid.

PSC ^{101}

RSF ^{2124}

Col E1 plasmid size 4.2 k.d restriction site ECORI immunity to colicin E1

Use – colicin E1 production

RSF ^{2124} – size 7.4 kd resriction sites – ECORI, BamHI

Use – colicin E1 production

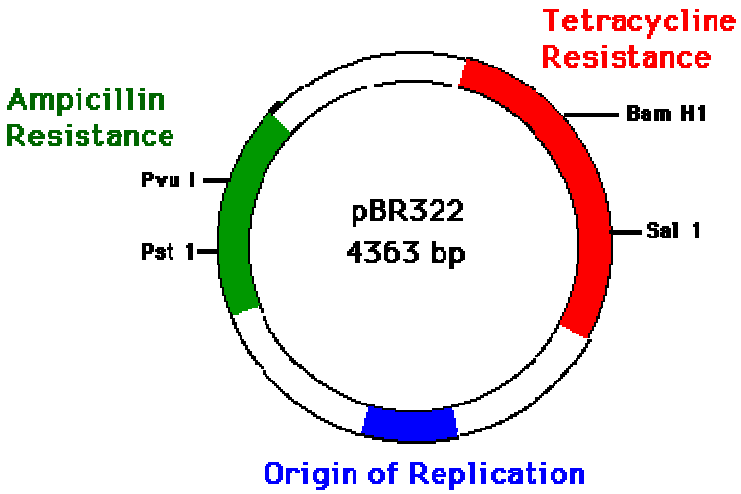
PSC – 101 plasmid size 5.8 kd restriction sites ECORI, Hind III Bam HI.

(e) Construction and characterization of a new cloning vehicle : PBR ^{322}

PSC ^{101}, Col EI and RSF ^{2124} can be used to clone DNA. They suffer from a number of disadvantages. For this reason considerable effort has been expended on constructing invitro superior cloning vehicles.

The most versatile and widely used of these artificial plasmid vector is PBR ^{322} plasmid PBR ^{322} contains the AMP^{R} and TC^{R} genes genes of RSF ^{2124} and PSC ^{101} respectively.

PBR ^{322} palsmid vector as shown in Fig.3.9.



Plasmid PBR 322 is small in size it is made up of 4362 base paire long. The sequence was revised by the inclusion of an additional CG base pair at

position 526, thus increasing the size of the plasmid to 4363 base pairs. The most useful aspect of the DNA sequence is that it totally characterizes PBR 322 in terms of its restriction sites such that the cract length of every fragment can be calculated.

- ➔ The plasmid has a number of restriction sites for the action of different types of restriction enzymes. The restriction enzymes break the DNA at their restriction sites in the plasmid. As a result the linear plasmid DNA with cohesive tail is formed. The foreign gene can be inserted at the restriction site. There are over 20 enzymes with unique cleavage sites on the PBR 322 genome.

The target sites of seven of these enzymes (ECOR I, Bam HI, Sal I, Xma III, Nhe I, Nru I Sph I) linc with the Tc^R gene and there are sites for a further two (Cla I and Hind III) with in the promotor of that gene.

There are unique sites for three enzymes (Pst I, Pvu I and Sca I) with in the AP^R gene. Thus cloning in PBR 322 with the aid of any one of those 12 enzymes will result in insertional inactivation of either the AP^R or the TC^R markers. However cloning in the other unique sites does not permit the easy selection of recombinants because neither of the antibiotic resistance determinants is inactivated.

Plasmid PBR 322 is the most widely used cloning vehicle. In addition it has been widely used as a model system for the study of prokaryotic transcription and translation as well as investigation of the effects of topological changes on DNA confirmation.

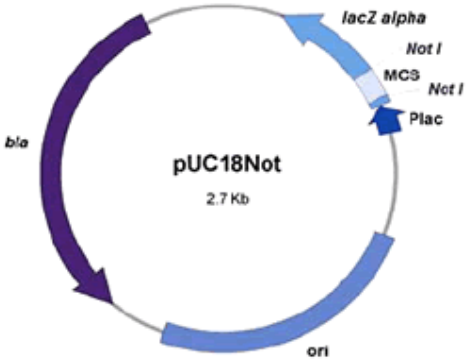
(f) PBR 327

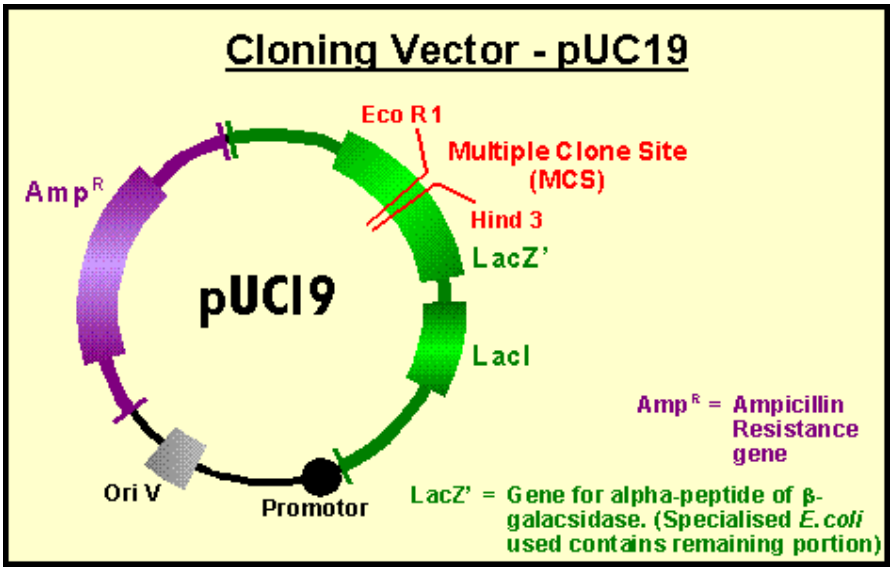
Another vector derived from PBR 322 by deletion of nucleotides between 1,427 to 2,516. These nucleotides are deleted to reduce the size of the vector and to eliminate sequences that were known to interfere with the expression of the cloned DNA in eukaryotic cells. PBR 327 still contains genes for resistance against two antibiotics (tetracycline and amphicilin). Both PBR 322 and PBR 327 are very common plasmid vectors.

(g) PUC vectors

Another series of plasmids that are used as cloning vectors belong to puc series. These plasmids are 2,700 base paire long and possess amphicillin resistance gene and the origin of replication derived from PBR 322. The Lac Z gene derived from E.coli with in the lac region is also found a polyinker sequence

having unique restriction sites when DNA fragments are cloned in this region of PUC the lac gene is inactivated. These plasmids when transformed in to an appropriate E.coli strain which is lac and grown in the presence of IPTG (Isopropyl thiogalactoside) which behaves like lactose and induces the synthesis of β-galactosidase enzyme and x-gal substrate for the enzyme will give rise to white (or) clear colonies on the other hand PUC having no inserts and transformed in to bacteria will have an active lac-z gene and therefore will produce blue colonies thus permitting identification of colonies having PUC vector with cloned DNA segments. The cloning vectors belonging to PUC family are available in pairs with reversed orders of restriction sites relative to lac z promotor. PUC 8 and PUC 9 other similar pairs include PUC 12 and PUC 13 (or) PUC 18 & PUC 19 PUC vector structure as shown in Figure 3.10.





(h) PACYC 184 plasmid

Plasmid contains two sequences for giving existence to the organism against drugs, one sequence gives it resistance against tetracycline while the other sequence gives it existence against chloramphenicol. If the gene is cloned at ECORI restriction site, the plasmid fails to give the organism resistance against chloramphenicol.

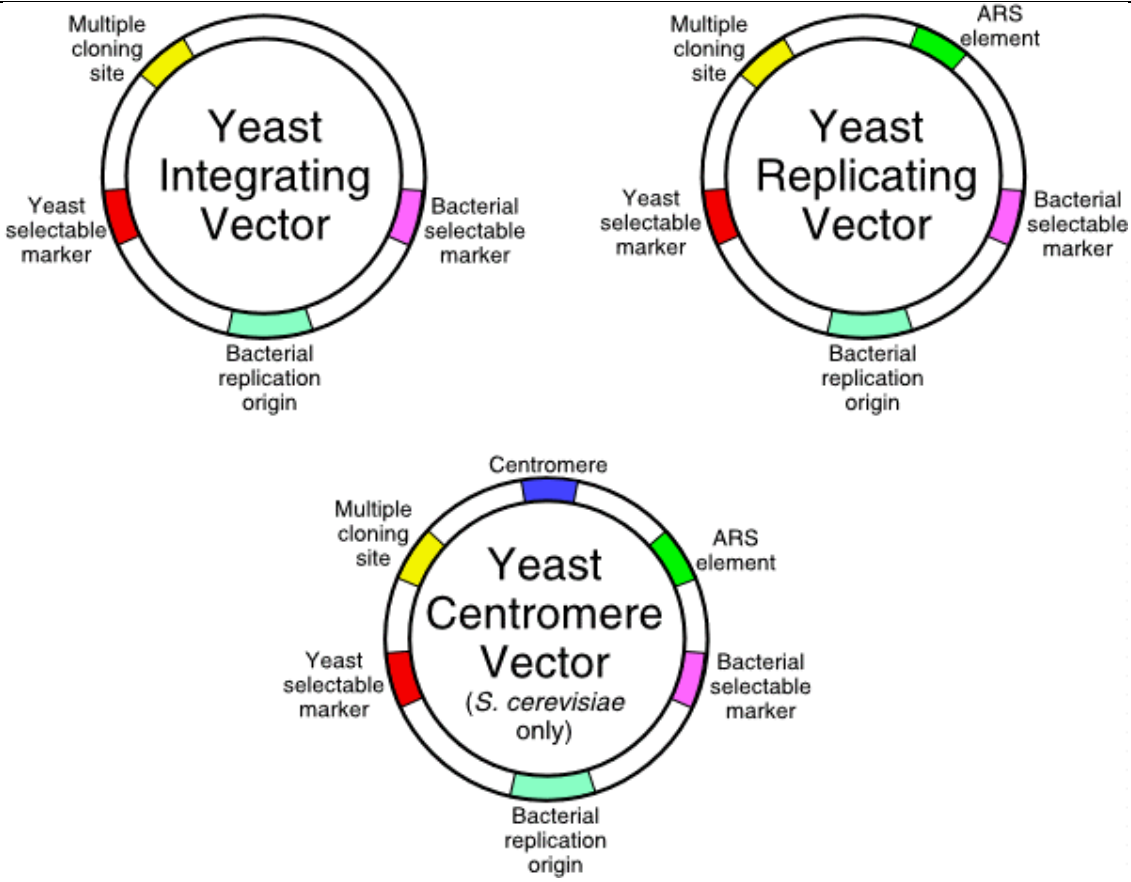
Sal I, Bam HI cut the plasmid at a sequence which gives the organism resistance against tetracycline. So if the gene is cloned at the restriction sites, the plasmid fails to give resistance to the organism against tetracycline. Hind III cuts the plasmid at a sequence which is free from those sequences giving resistance to the organism against tetracycline and chloramphenicol so if the gene is cloned at Hind III restriction site, the transformed organisms grow successfully in the medium containing both of these drugs.

(i) PVN – 121 Plasmid

Contains two sequences for giving resistance to the organism against the drugs. One sequence gives it resistance against amphicillin and the other sequence gives it resistance against tetracycline.

(j) Yeast plasmid vectors

Special vectors are also known for introducing DNA segments in yeast cells, a Eukaryotic system that has been used extensively for developing genetically engineered yeast cells. Although E.coli plasmids or phages can be used for transfer of genes to yeast cells. The possible examples of the yeast plasmid vectors are YIP (or) Yest Integrative plasmids, YEP (or) Yeast Episomal Plasmids.



(k) Ti and Ri plasmids as vector for higher plants

In higher plants Ti plasmid of Agrobacterium tumefaciens (or) Ri plasmid of Agrobacterium rhizogenes is the best known vector. T-DNA from Ti (or) Ri plasmid of Agrobacterium is considered to be a very potential vector for cloning Experiments with higher plants. The foreign DNA has to be first cloned in to T-DNA of Ti (or) Ri plasmid modified hybrid T-DNA can be transferred to the genome of plant cells by Agrobacterium infection. These recombinant Ti plasmids can then be used for transformation of higher plants. This system can be widely used. Agrobacterium infects nearly all dicotyledonous plants. Such cloning in plants has proved to be of immense use to modify agricultural plants to increase their productivity.

3.5.2.2 Bacteriophage λ

Bacteriophage provide another source of cloning vectors since usually a phage has a linear DNA molecule, a single break will generate two fragments which are later joined to with foreign DNA to generate a chimeric phage particle.

Coli phage λ is one of the temperate phages, it infects the E.coli cells. It was first isolated by lederberg in 1951. The life cycle of λ phage is a lysogenic cycle in which the host bacterium is not broken. This is because λ DNA does not interfere with the synthetic machinery of the host cells. The viral genes get integrated with the chromosomal DNA of bacteria and replicate along with the chromosomal DNA of the host cell. In this condition the phage DNA is called prophage and the bacterium containing the prophage is called lysogenic bacterium. The λ DNA is expressed in the host bacterium. The λ phage has the molecular wt of 57 x 10^6 daltons but its DNA is 32 x 10^6 daltons in its molecular weight.

(a) Sailent features of λ DNA

It is a double stranded DNA which may exist in linear form or circular form the specific form of λ DNA depends upon the specific stroan of phage from which it is isolated. The linear λ DNA has cohesive ends, each of the ends is complementary to another end. The cohesive end or the single stranded tail ends with free 5^1 phosphate group.

- ➔ The circular form of DNA is formed due to the base pairing between the complementary bases found in its cohensive ends. Some times a number of λ DNA'S join together end to end and form a large sized nucleic acid multimer.
- ➔ The base composition of λ DNA differs from species to species.
- ➔ The λ DNA contains about 40 genes of which only 18 genes are essential for lytic (or) lysogenic cycle of phages. The remaining genes may participate in some other cellular functions. The λ DNA contains two sequences for viral assembly of which one sequence participates in the synthesis of head protein while the other participates in the synthesis of tail proteins.
- ➔ The λ DNA has three regullter genes, three promotor genes and a few termination sites for the regulation of its structural and functional genes.

(b) Life cycle of λ phage

In general both lytic life cycle and lysogenic life cycle occur in λ phages. But the frequency of lytic life cycle is very low when compared with that of lysogenic life cycle. The frequency of lytic life cycle is increased by exposing the phage culture towards U.V. rays or by treating the culture with mitomycin. The different stages of λ phage life cycle are explained below.

The phage attaches it self to the cell wall of E.coli. cells with the help of tail fibres. The phage injects its DNA in to the bacterial cells. The λ DNA is a double stranded DNA containing the complementary single stranded tails.

- ➔ Inside the bacterial cell, the two ends of λ DNA get joined together and form a circular DNA. It is carried by complementary base pairing between the sticky ends of λ DNA.
- ➔ The circular λ DNA gets integrated with the chromosomal DNA of the bacterial cell. Thus a new combination of genes is formed. The host DNA containing the λ DNA is named prophage DNA. The bacterium containing the prophage DNA is called lysogenic cell.
- ➔ Thus the λ DNA gets integrated with the chromosomal DNA of the bacterial cell. Thus a new combination of genes is formed. The host DNA containing the λ DNA is named prophage DNA. The bacterium containing the prophage DNA is called lysogenic cell.
- ➔ Thus the λ DNA multiplies rapidly and survives with in the bacterial cells for a number of generations along with the bacterial DNA. This process is called lysogenic life cycle.
- ➔ Rarely the prophage DNA gets disintegrated from the chromosomal DNA. As a result, the λ phage enters the lytic life cycle. This phage DNA synthesizes its head and tail proteins more rapidly and the phage multiplies rapidly in the host cells finally the virus particles break the host cell and they are then released in to the culture. Here the λ DNA interferes with the host's synthetic machinery. This method of multiplication of bacteriophage is called lytic life cycle.

(c) Lambda phage vectore

λ gt 10, λ gt 11, EMBL 3, EMBL 4, charon.

(i) λ gt 10 & it 11

λ gt 10 and λ gt 11 are modified lambda phages designated to clone CDNA fragments. The major difference between these two vectors is that λ gt 11 is an expression vector where inserted DNA is expressed as β-galactosidase fusion protein.

λgt 10 is a 43 kb double stranded DNA for cloning fragments that are only 7 kb in lenth. λ gt 11 is a 43.7 kb double stranded λ phase for cloning DNA fragments which are less than 6 kb in length.

(ii) EMBL3, EMBL4

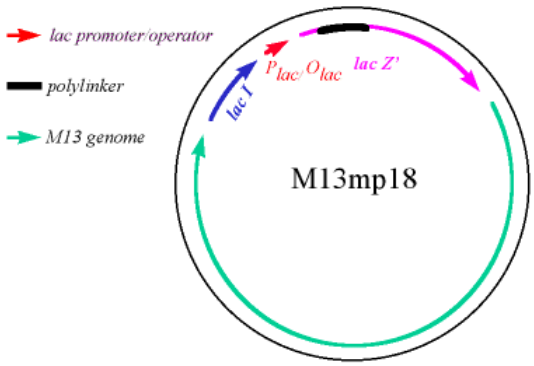
EMBL3, EMBL4 are two vectors that are designed so that a central non essential part of 44 kb long phage can be replaced by a foreign DNA. Cleavage of the phage with an appropriate enzyme generates three fragments (left arm, right arm and a central fragment called stuffer). The central fragment representing 40% of the phage genome is non essential for propagation of the phage and can be replaced by foreign DNA that may be as long as 20 – 23 kb. EMBL3, EMBL4 are two such replacement vectors used for preparing genomic libraries in eukaryotes with cloned fragments 15-25 kb in size. The two vectors have poly linkers with reverse orders of restriction sites with respect to each other.

(iii) Charon 34 and charon 35

Charon 34 and charon 35 differ from each other only in their central fragment and will accept fragments 9-20 kb long.

(iv) M 13 phage as cloning vector for DNA sequencing

M 13 is a filamentous bacteriophage of E.coli and contains a 7.2 kb long single stranded circular DNA. M 13 phage has been variously modified to give rise to a M 13 modified phage series of cloning vectors which can be used for the purpose of sequencing through sanger's metod of dideoxy chain termination.



3.5.2.4 Cosmids

Cosmids are plasmid particles into which certain specific DNA sequences namely those for cos sites are inserted. Cos mids are the constructed vector

DNA's. The cos sites is excited from the DNA of λ phage and cloned in the plasmid DNA. Thus the resulting DNA shares the properties of plasmid DNA and λ phage DNA such a modified plasmid DNA is called cosmid DNA.

This type of DNA is not naturally found in living cells. They are constructed vectors useful in genetic engineering.

- ➔ Col E1 plasmid is generally used in genetic engineering works. It is constructed from col E1 plasmid of E.coli cells. This plasmid DNA is cut with a restriction enzyme which removes a portion of the DNA from the plasmid.

- ➔ The same restriction enzyme is used to cut the λ DNA to get a DNA fragment containing cos – site. These two DNA fragments are then mixed to gether in the presence of enzyme DNA ligase which links to gether the two DNA fragments and to end. Thus a recombinant plasmid is formed. The newly constructed DNA is called col E1 cosmid.

(a) Characteristics feature of cosmid DNA

Cosmids are the constructed vectors of DNAs the characteristic features of an idela cosmid DNA are.

The cosmid is a circular double stranded DNA. The cos site consists of two complementary single strands held to gether by complementary base pairing between these two strands.

- ➔ At the cos site 3^1 end of each of the DNA strand does not establish covalent bond with the 5^1 end of the same chain. That is a definite nick is present in each of the two strands.

The nicks are retained in the cosmid for a number 9 of generations.

The cosmid does not participate in the multiplication of phage particles.

The cosmid DNA packed with in the protein coat of bacteriophage thus the transformed virus particle is formed.

The phage particles thus formed is transferred to another organism through the induction of the phage particle to infect the organism. This method of gene transfer from one organism to another organism is often named transduction.

3.5.2.5 Phasmids

Plasmids are prepared artificially by combining features of phages with plasmids as the name suggests one such phasmids which is commonly used in molecular biology laboratories is P Blue Script II KS which is derived from PVC 19 and is 2961 base pairs long. The KS designation indicates the orientation of polylinker such that the transcription of lac z gene proceeds from the restriction site for Kpn I.

The following features of P Blue script II KS. It has a multiple cloning sites flanked by T_3 and T_7 promoters to be read in opposite direction on the two strands.

- ➔ An origin of replication (col E1 Dri) derived from plasmid.
- ➔ A gene for amphicillin resistance for antibiotic selection of chimeric phagemid vector.
- ➔ Phagemids has been developed that consists of hybrid between plasmid and filamentous phage cloning vehicle. There are generally smaller than the M 13 vectors and were formed by combining sequences from PBR 322 with the M 13 origin of replication. These vectors can be propagated normally as double stranded plasmids with in the cell.

3.5.2.6 PI cloning vectors for cloning large DNA segments

The bacteriophage PI cloning vectors can allow cloning of 100 kbp long DNA segments with an efficiency of 10^5 clone per ug of insert DNA. Therefore in their capability, the fall between YACs and cosmids. The vector with insert DNA is amplified in E.coli and several microorganisms of cloned DNA can be recovered from 5-10ml of exponential phase of E.coli cells.

3.5.2.7 F-factor based vectors

F-factor based vectors have recently been developed for cloning large DNA segments in E.coli. The cloning of large DNA segments is achieved by a method called chromosomal building in which through repeated recombination size of cloned segment can be increased. These bacterial vectors will complement the YAC vectors for cloning segments larger than 100 kbp in length and offers some advantages over YAC system. These vectors have already been used for cloning large DNA segments from the bithron gene of drosophila.

3.5.2.8 Plant and animal viruses as vectors

A number of plant and animal viruses have also been used as vectors both for introducing foreign genes into cells and for gene amplification and expression in host cells.

plant viruses

Cauliflower mosaic virus (CaMV) tobacco mosaic virus (TMV) and Gemini viruses are three groups of viruses that have been used as vectors for cloning of DNA segments.

CaMV infects particularly the members of crucifierace and has a double stranded DNA molecule. Following infection the virus spreads simultaneously through out the plant in a very high copy number reaching up to 105 virus particles/cell. These features make cauliflower mosaic virus a suitable vector for transformation of higher plants and produce transgenic plants.

Gemini viruses comprise a group of single stranded DNA. Plant viruses causing important diseases in maize and other cereals.

Animal viruses

A number of animal viruses are also used as vectors either for the delivery of nucleic acids in to cultured cells followed by its integration with host genome or for the amplication and high level expression of foreign gene using the promoters from virus genes. These cloned genes can have a variety of uses including gene therapy in mammalian cells and synthesis of important proteins by cloned genes in cell cultures.

3.5.2.9 Transposons are vectors

Transposons of higher plants. AC and DS are popular transposons in corn and were earlier known to represent activator – dissociation system. Each represents a transposon with short terminal repeats enclosing a long DNA segment which measures more than 4,500 bp in AC and 400 base pairs in DS. Each possesses genes including the gene for transposase enzyme responsible for transposition. Part of this region can be deleted and the transposon can be used for cloning of foreign DNA segment in the same way as in other cases.

3.5.2.10 Binary and shuttle vectors

For the purpose of gene transfer in higher plants a number of binary vectors were developed. These were based on the PCV (plant cloning vectors) series of plasmids these vectors contain a conditional mini – RK 2 replicon which

is maintained and mobilized by trans – acting functions derived from the plasmid RK 2 replicon. The plasmid RK 2 was introduced in to both E.coli as well as agrobacterium to facilitate replication of binary vectors in both these hosts so that the vector can be maintained and shuttle between both – hence the name shuttle vector also. Similarly binary (or) shuttle vectors for maintanance and transfer between E.coli and yeast cells have also been designed.

Properties of shuttle vectors

- ➔ The vector must replicate in many organisms to facilitate the isolation and characterization of genes.
- ➔ The vector must be easily recognized by selectable markers.
- ➔ The vector should be small in size to accomdate DNA inserts.
- ➔ Cloned genes should be easily detected.
- ➔ The vector must be stable, non pathogenic, and non stress inducing.
- ➔ The vector must effectively deliver genetic information for stable maintainance in alternate derived recipients.
- ➔ The introduced genetic information should be stable maintained as a new heritable determinant

3.5.2.11 Summary

Vectors plays an very important role in genecloning. Plasmid is nothing but extrachromosomal circular DNA. It contains resistance markers and origin of replication. Small DNA fragement is inserted into plasmids. Bacteriophage is another important vector. It contains cos sites in between the two cos sites packaging of large sized DNA. Cosmid having the both characteristics of plasmid and bacteriophage. Ti plasmids and plasmids are useful for transfer of genes to plants. Shuttle vectors having the characteristics of generate the genes in different organisms. Animal viruses and plant viruses are also act as vectors.

3.5.2.12 Model Questions

1. What is plasmid & explain its importance in gene cloning?
2. Why cosmid is more advantage than plasmids.
3. What is bacteriophage and its importance.
4. Explain the different types of vectors used in gene cloning.

3.5.2.13 Reference Books

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3. Old and Primrose (1994), Principles of gene manipulation 5th edition, Blackwell Scientific Publications Oxford, United Kingdom.
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P. Sudhakar

Lesson 3.5.3

IDENTIFICATION AND EXPRESSION OF CLONED GENES

Objective

Objective

This lesson deals with the study of identification and expression of cloned vectors.

3.5.3.1 Introduction

The isolation of genes that encode proteins is often the goal of a biotechnology experiment. In prokaryotic organisms these structural genes each having a continuous coding domain in the genomic DNA, in eukaryotes however the coding regions of structural genes are seperated by noncoding regions consequently different cloning strategies have to be used for prokaryotic and eukaryotic genes.

The gene is introduced into the vector to construct a recombinant DNA. This recombinant DNA enter in to host cell. After entering identification of the cloned gene is very important. Isolating a particular single – copy gene sequence from a complete mammalian genomic library requires techniques in which hundreds of thousands of recombinant can be screened. The general principles employed in recombinant selection and screening procedures. Some popular methods of identification are used. DNA hybridization with a labeled DNA probe followed by radiographic screening for the problable mmunological screening for the protein product immunochemical, south – western methods nucleic acid hybridization with labeled probes is the most generally applicable method.

Synthesis of a functional protein depends upon transcription of the appropriate gene, efficient translation of the mRNA and in many cases post translational processing.

Many proteins when made in their natural host under go post translational modification.

Ex: Glycosylation, phosphorylation. Such modifications to proteins do not occur in E.coli.

Another important aspect of gene expression is the stability of the protein. Expression systems are generally useful for producing recombinant proteins.

3.5.3.2 Identification

(a) Screening by DNA hybridization

The presence of a target nucleotide sequence in a DNA sample can be determined with a DNA probe. This procedure is called DNA hybridization and depends on the formation of stable base pairs between the probe and target sequence. DNA hybridization is feasible because double stranded DNA by heat

(or) alkali treatment. Heating DNA, breaks the hydrogen bonds that hold the bases together but does not affect the phosphodiester bonds of the DNA backbone. If the heated solution is rapidly cooled, the strands remain single stranded. However if the temperature of a heated DNA solution is lowered slowly the double stranded helical confirmation of DNA can be reestablished due to the base pairing of complementary nucleotides (renaturation). The process of heating and slowly cooling double stranded DNA is called annealing. Some of the products of this process contain molecules of hybrid DNA that is double stranded DNA in which the two strands come from different DNA molecule.

In general for a DNA hybridization assay the target DNA is denatured and the single strands are irreversibly bound to a matrix. This binding process is often carried out at a high temperature. Then the single strands of a DNA probe which are labeled with either radioisotope (or) another tagging system are incubated with the bound DNA sample. If the sequence of nucleotides in the DNA probe is complementary to a nucleotide sequence in the sample then base pairing occurs. The hybridization can be detected by autoradiography (or) other visuatization procedures depending on the nature of the probe lable. If the nuceotide sequence of the probe does not base pair with a DNA sequence in the sample then no hybridization occurs and the assay gives a negative result. Generally probes range in length from 100 to more than 1000 base pairs although both larger and smaller probes can be used.

DNA probes can be labeled in various ways one strategy which is called the random primer method utilizes a mixture of synthetic random nucleotides containing all possible combinations of sequences of six nucleotides that act as primers for DNA synthesis on the basis of the chance occurance of complementary sequences on the unlabeled probe DNA template. After the digomer sample is mixed with the denatured probe template DNA, the four deoxy ribo nucleotides and a portion of E.coli DNA polymerase I called the klenow fragment retains both DNA polymerase and 3^1 exonuclease activates but lacks the S^1 exonuclease activity that is normally associated with E.coli, DNA polymerase I. This S^1 exonuclease activity would degrade some of the newly synthesized DNA with the available 3^1 – hydroxyl groups of the bound random primers and the strands of the probe as templates, new DNA synthesis occurs. If a radioactive lable is used then one of the dNTP'S contain the isotope 32P in the α-position phosphate. Autoradiography can be used later to determine whether the labeled proble sequences hybridize to sequences of a target DNA sample Labelled DNA probe production

For non isotopic detection of hybridization biotin can be attached to one of the four dNTP'S that is incorporated during the DNA synthesis step when probe with this kind of tag (label) hybridizes to the sample DNA. Detection is based on the binding an intermediary compound that carries an appropriate enzyme.

Depending on the assay system the enzyme can be used for the formation of either a chromogenic (colored) molecule that can be visualized directly or a chemiluminiscent response that can be detected by autoradiography.

Transformed host cells are often screened by plating out the transformed cells on the growth medium of a master plate and then transferring samples of each colony to a solid matrix such as nitrocellulose (or) nylon membrane lysing the cells deproteinizing and denaturing the DNA and binding the DNA to matrix. At this stage a labeled probe is added. If hybridization occurs signals are observed on anautoradiograph. The colonies from master plate that correspond to samples containing hybridized DNA are then isolated and cultured.

(b) Screening by immunological assay

If a DNA probe is not available alternative methods can be used to screen a transformed host cells for example if a cloned DNA sequence is transcribed and translated, the presence of the protein, or even part of it, can be determined by an immunological assay. Technically this procedure has much in common with a DNA hybridization assay. All cell lines are grown on master plates. A sample of each colony is transferred to a matrix. Where the cells are lysed and the released protein attach to the matrix. The matrix with the bound proteins is treated with an antibody that specifically binds to the proteins encoded by the target gene. Following the interaction of the primary antibody with the target protein (Ag) any unbound antibody is present washed away and the matrix is treated with a second antibody that is specific for a primary antibody. In many assay systems the second antibody has an enzyme such as alkaline phosphotase attached to it. After the matrix is washed a colorless substrate is added. If the secondary antibody has bound to the primary antibody the colourless substrate is hydrolyzed by the attached enzyme and produces a coloured compound that accumulates at the site of the reaction. Immunological assay as shown in Figure 3.19.

(c) Screening by protein acivity

DNA hybridization and immunological assays work well for many kinds of genes and gene products. If the target gene produces an enzyme that is not normally made by the host cell a plate assay can be devised to identify members that carry the functional gene encoding the enzyme/ For example the genes for α - amylase, endonuclease and β - glucosidase from various organisms have been isolated by plating techniques. The medium is supplemented with a specific substrate and then using a selective stain to identify those colonies that are capable of utilizing the substrate.

If the gene that is being sought encodes a product that is essential for the growth of a mutated host cell, the cells that are able to grow on minimal medium

in the absence of the required substrate must carry a functional form of the target gene on the cloned vector variations of this form of genetic complementation have been used to isolate a variety of important genes, including those for the biosynthesis of antibiotics and the formation of nitrogen fixing nodules on the roots of certain plants.

3.5.3.3 **Genetic methods**

(a) Selection for presence of vector

Genetic selection is a very powerful tool since it can be applied to large populations. All useful vector molecules carry a selectable genetic marker (or) property. Plasmid and cosmid vector carry drug resistance or nutritional markers and in the case of phage vectors plaque formation is itself the selected property. Genetic selection for presence of the vector is a pre requisite stage in obtaining recombinant population. This can be defined to distinguish recombinant molecules and non recombinant parent vector. Insertional inactivation of a drug resistance marker.

(b) Selection of inserted sequences

If an inserted foreign gene in the recombinant is expressed. Then genetic selection may provide the simplest method for isolating the clones containing the gene.

It has been found that certain eukaryotic gene are expressed in E.coli and can complement autotrophic mutations in the host bacterium. The frgments of yeast DNA obtained by mechanical shearing in to the plasmid col E1 using a homopolymer tailing procedure. They transformed E.coli this B mutants with recombinant plasmid and by selecting for complementation isolated clones carrying an expressed yeast this gene.

(c) Immuno chemical methods

Immunochemical detection of clones synthesizing a foreign protein has also been successful in cases where the inserted gene sequence is expressed. A particular advantage of the method is that genes that do not confer any selectable property on the host can be detected, but it does of course require that specific antibody is available.

During the early development of recombinant DNA technology, a number of laboratories published similar immunochemical detection methods. These

properties are exploited in the following way first transformed cells are plated on agar in a convenient petridish.

- ➔ The bacterial colonies are then lysed in one of a number of ways.
- ➔ By exposure to chloroform vapour by spraying with aerosol of virulent phage (or) by using a host bacterium that carries a thermoinducible prophage.

This releases the antigen from positive colonies a sheet of polyvinyl that has been coated with the appropriate antibody (unlabelled) is applied to the surface of the plate where upon the antigen complexes with the bound IgG. The sheet is removed and exposed to 125 I-labelled Igr. The 125 I – IgG can react with the bound antigen via antigenic determinants at site other than those involved in the initial binding of antigen to the IgG coated sheet. Positively reacting colonies are detected by washing the sheet and making the autoradiographic image. The required clones can then be recovered from the replica plate.

Immunochemical screening can be carried out upon colonies of induced lysogenic bacteria. The screening is carried out on plagues of the recombinant phage. The original approach with induced lysogen. In this approach the library of recombinant igtll is first used to lysogenize E.coli. This is efficiently carried out with a hfla (high frequency of lysogeny) mutant of E.coli. Lysogeny produce detectable amounts of hybrid proteins upon induction. It has been claimed that up to 10^6 colonies can be screened on a single 8.2 cm diameter filter. The immunochemical method is much more convenient method that is carried out on phage plaques. .

(e) South – Western screening for DNA binding protein

A closely related approach has been used very successfully for screening and isolation of clones expressing fusion protein where the foreign sequence encodes a DNA binding protein that binds specifically to a particular DNA sequence. The method involves a nitrocellulose membrane on which expressed fusion proteins are adsorbed. The screening is carried out by incubating the membrane with a radiolabelled duplex DNA oxigonucleotide containing the sequence for which the DNA binding protein is specific. Positively reacting plaques are identified by autoradiography of the filter. This technique therefore uses a radiolabelled DNA to detect polypeptide on the nitrocellulose and has been called a south – western procedure. It has been spectacularly successful in the isolation of clones expressing CDNA sequence corresponding to certain mammalian transcription factors. The procedure has been found most efficient when the oligonucleotide containing the binding sequence has been ligated in to

multimeric form. This may mean that a single DNA multimer may be bound by more than one fusion polypeptide molecule on the filter.

(e) Southern blotting

The original method of blotting was developed by southern for detecting fragments in an agarose gel that are complementary to a given RNA (or) DNA sequence. In this procedure referred to as southern blotting, the agarose gel is mounted on a filter paper wick which dips in to a reservoir containing transfer buffer.

The hybridization membrane is sand witched between the gel and a stack of papers towels (or) other absorbant material which serve to draw the transfer buffer through the gel by capillary action. The DNA molecules are carried out of the gel by the buffer flow and immobilized on the membrane. Initially the membrane material used was nitrocellulose. The main draw back with this membrane is its fragile nature supported nylon membranes have since been developed with an alternative fixation method utilizes ultraviolet cross linking. It is based on the formation of cross links between a small fraction of the thyamin residues in the DNA and positively charged amino sugars on the surface of nylon membrane. A calibration experiment must be performed to determine the optimal fixation period.

Following the fixation step the membrane is placed in a solution of labeled RNA, single stranded DNA (or) oligodeoxy nucleotide which is complementary in sequence to the blot transferred DNA band (or) bands to be detected. Conditions are chosen so that the labeled nucleic acid hybridizes with the DNA on the membrane since this labeled nucleic acid is used to detect and locate the complementary sequence is called probe. After the hybridization reaction has been carried out the membrane is washed to remove the unbound radioactivity and regions of hybridization are detected autoradiographically by placing the membrane in contact with x-ray film. Autoradiography following each washing stage will reveal any DNA bands that are related to but not perfectly complementary with the probe and will also permit and estimate of the degree of mismatching to be made. Which have greater binding capacity for nucleic acids in addition to high tensile strength.

For efficient southern blotting, gel pre treatment is important large DNA fragments require a longer transfer time than short fragments. To allow uniform transfer of a wide range of DNA fragment sizes the electrophoresed DNA is exposed to a short depurination treatment followed by alkali. This shortens the DNA fragments by alkaline hydrolysis at depurinated sites. It also denatures the fragments prior to transfer ensuring that they are in the single stranded state and accessible for probing. Finally the gel is equilibrated in neutralizing solution prior to blotting.

An alternative method uses +vely charged nylon membranes which remove the need for extended gel pre treatment with them the DNA is transferred in native (non denatured) form and then alkali denatured insite on the membrane and a number of methods for nitrocellulose membranes and this also can be used with nylon membranes. Due to the flammable nature of nitrocellulose. It is important that it is backed in a vaccum oven.

The southern blotting methodology can be extremely sensitive. It can be applied to mapping restriction sites around single copy gene sequence in a complex genome such as that of man.

(f) Western blotting

The term western blotting refer to a procedure which does not directly involve nuclei acids but which is importance in gene manipulation. It involves the transfer of electrophoresed protein bands from a polyacrylamide gel on to a membrane of nitrocellulose (or) nylon to which they bind strongly. The bound proteins are then available for analysis by a variety of specific protein – ligand interactions. Most commonly antibodies are used to detect specific antigens. Lectine have been used to identify the glycoproteins. In these cases the probe may itself be labeled with radioactivity (or) some other `tag' may be employed often however the probe is unlabelled and is itself detected in a sand witch reaction using a second molecule which is labeled, for instance a species specific second antibody or protein A of staphylococcus aureus (or) streptavidin. These second molecules may be labeled in a variety of ways. With readioactive enzyme (or) fluorescent tags. An advantage of the sandwitch approach is that a single. Preparation of labeled second molecule can be employed as a general detector for different probes. For example an antiserum may be raised in rabbits which reacts with a range of mouse immunoglobulin. Such a rabbit antimouse (RAM) antiserum may be radiolabelled and used in a number of different applications to identify polypeptide bonds probeed with different specific monoclonal antibodies each monoclonal antibody being of mouse origin. The sand witch method may also give a substantial increase in sensitivity owing to the multivalent binding of antibody molecules.

(g) Differential screening

This is a variant of nucleic acid hybridization method that is particularly suitable for isolating tissue specific or developmentally regulated cDNA sequences (or) clones derived from mRNA'S that are induced by particular treatments.

Isolation of cDNAS derived from mRNAS which are abundant in the gastrula embryo of the frog xenopus but which are absent or present at low abundance in the egg. A cDNA clone library is prepared from gastrula. MRNA replica filters carrying initial sets of recombinant clones are then prepared. One of these filters is then probed with 32 P labeled mRNA (or) cDNA from gastrula embryos and one with 32 P labeled mRNA from the egg. Some colonics will give a positive signal with both probes. These represent cDNAS derived from mRNA types that are abundant at both stages of development. Some colonies will not give a positive signal with both probes. These represent cDNAS derived from mRNA types that are abundant at both stages of develoment. Some colonies will not give a positive signal with either probe these correspond to mRNA types present at undetectably low abundance in both tissues. This is a feature of using probes derived from mRNA populations. Importantly some colonies give a positive signal with the gastrula probe but not with the egg probe. Such differential screening has been applied to the analysis of the development of xenopus and to the slime mould dictyostelium. In both instances it was estimated that a five fold difference in mRNA abundance could be detected in this procedure.

(h) Recombinational probe

This ingenious and powerful method is based upon homologous recombination in the E.coli host. The probe sequence here is inserted in to a sepecially constructed plasmid vector ∏ Vn. This is a very small plasmid of 902 base pairs derived from the Col E1 replicon which contains a convenient polylinker sequence and a suppressor tRNA gene, SUPF. Genomic phage λ libraries are propagated on recombinant – proficient E.coli containing the probe. ∏ Vn recombinant plasmid. Phase carrying sequences homologous to the probe acquire an integrated copy of the plasmid by homologous recombination. Phage bearing integrated probe ∏ Vn can then be recovered and isolated by growth under appropriate selective conditions. This is most easily achieved by using a phage λ vector carrying an amber mutation suppressible by SUPF. By finally plating on a non suppressing E.coli only those phage that have integrated a sup F gene, by virtue of homology with the probe can form plagues.

This method can be applied readily to a very large numbers of phage in a genome library and has the advantage of being very quick, providing that the probe - ∏ Vn recombinant plasmid has been constructed at a prior stage. The shortest probe segment giving high recombination has been found to be about 60 base pairs long.

3.5.3.4 Expression of cloned genes

The minimal requirement for an effective gene expression system is the presence of a strong and regulatable promoter suquence up tream from a cloned gene. A strong promoter is one that has a high affinity for RNA polymerase, with the consequence that the adjacent downstream region is highly transcribed. The ability to regulate a promoter enables the cell to control the extent of transcription in a precise manner. The promoter from the well studied lac operon of E-coli has been used extensively for expressing cloned genes.

Prokaryotic Expression Systems are generally useful for producing heterologous proteins from cloned eukaryotic complementary DNAS. In some cases, however eukaryoic proteins that have been synthesized in bacteria either are unsteable (or) lack biological activity. In eukaryotes there are a number of modifications that may occur at the post translational stage, after protein synthesis is complete. Correct disulplide bond formation, proteolytic cleavage of a precurrsor form. Selected segments of aminoacid sequences are removed to yield a functional protein.

- ➔ Glycosylation, this reaction is major modification that endows a protein with stability and in some instances its distinctive properties. The most common protein glycosylations occur by the addition of specific sugar residues to serine (or) threonine (or) to asparagines. More over no single eukaryotic host cell system is capable of performing all of these post translational modifications for every potential heterologous protein. Therefore, if a particular protein requires a specific set of modifications, then it may be necessary to examine different eukaryotic expression systems to find the one that can produce a biologically authentic product.

In general eukaryotic expression have the same kind of features as their prokaryotic counterpart.

A selectable eukaryotic marker gene.

A eukaryotic promoter sequence.

The appropriate eukaryotic transcriptional and translational stop signals.

A sequence that signals polyadenylation of the transcript messenger RNA

(a) Saccharomyces cerviceae expression systems

The common yeast saccharomyces cerviceae has been used extensively as a host cell for the expression of cloned eukaryotic genes. First, it is single celled,

it is extensively well known genetically and physiologically, and it can be grown readily in both small culture vessel and large scale bioreactors. Second, several strong promoters have been isolated from this yeast and characterized, and a naturally occurring plasmid called zum plasmid, can be used in endogenous yeast expression vector systems. Third, saccharomyces cerviceae is capable of carrying out many post translational modifications. Fourth, it normally secretes so few proteins that when it is engineered for extracellular release of a heterologous protein, the product can be readily purified fifth, because of its years of use in backing and brewing industries saccharomyces cerviceae has been listed by the U.S. Food and Drug administration as a "generally recognized as safe" organism. A number of proteins that have been produced in saccharomyces cerviceae are currently being used commercially as vaccines, pharmaceuticals and diagnostic agents.

(b) Saccharomyces cervisiae vectors

There are three classes of saccharomyces cerviceae vectors (2) episomal or plasmid vectors (2) Integrating vectors (3) Yeast artificial chromosome vectors (YACS) of these episomal vectors have been used extensively for the production of either intra on extra heterologous proteins. However such plasmid based expression systems are often unstable under conditions of large scale growth. Although a integration of an expression vector or transcription unit in to the chromosomal DNA is anexperimental option that yields a stable genetically engineered organism.

A YAC is designed to clone a large segment of DNA which is then maintained as a separate chromosome in the host yeast cell. The YAC system is highly stable and has been used for the physical mapping of human genomic DNA the analysis of large transcription units, and the formation of genomic libraries containing DNA from individual human chromosomes. A YAC vector mimics a chromosome because it has a sequence that acts as an origin of DNA replication. In some cases the inserted DNA is cloned in to a site that disrupts a cloned yeast gene. In the absence of product of this gene, a colorimetric response is observed when recipient cells are grown on a specialized medium. Alternatively some YAC vectors contain a selectable marker gene that is independent of the cloning site. However gene that is independent of the cloning site. However YACS have not yet been used as expression systems for the commercial production of heterologous protein.

(c) Secretion of heterologous protein by saccharomyces cerviceae

In yeast only secreted proteins are glycosylated, so secretion system must be used for recombinant proteins that require either O-linked or N-linked sugars for biological activity. To facilitate protein secretion in yeast, the pre pro α gene

is cloned infront of the CDNA that encodes the desired protein. This gene fusion creates a protein that can be efficiently secreted by yeast.

A properly processed and active form of the protein hirudin was synthesized and secreted by an sacchoromyces cervisiae strain containing an episomal expression vector that induced pre pro α factor. The gene for hirudin came from an invertebrate the leech hirudo medicinalis. This protein is a powerful blood anticoagulant that does not have immunological side effects in humans. The availability of large quantities of active hirudin has facilitated the testing of its efficacy in removing venous blood clot and allevating other thrombic conditions. In one clinical trail with 12,142 patients, of whom 4,131 had heart disease, recombinant hirudin showed a small advantage as a treatment over heparin. However because of the higher cost of recombinant hirudin, the slight therapeutic benefit may not warrant its adoption as a standard treatment for acute coronary syndromes.

(d) Other yeast expression systems

Recombinant prot expression in saccharomyces cerviceae has been successful with many proteins. But despite a few high yields expression generally has been low, in addition there are other limitations. During scale up plasmid loss is a frequent occurrence, even when inducible promoters are used.

The heterologous protein is often hyperglycosylated containing more than 100 mannose residues in each N-linked oligosaccharide side chain. Therefore, researches have been examining other yeast species and eukaryotic systems that can act as host cells for the production of heterologous protein.

(e) Expression of hepatitis B surface antigens

The methylotrophic yeast pidia pastoris can be grown easily and economically in large bioreactors. The development of pichia pastoris as a host organism for the production of heterologous protein would provide a means for achieving high yields of fully active products. Fire, the HBS Ag sequence was cloned between the promoter region of alcohol oxidase gene I (AOXIP) and the termination and polyadenylation signal region (AOXIT) of the same gene. The AOXI gene is regulated by methanol in pichia pastoris. In the presence of methanol, alcohol oxidase can represent as much as 30% of the total cellular protein because of the strength of the AOXI promoter, in the absence of methanol, no alcohol oxidase is synthesized.

The complete plasmid developed for the study has the AOXIP – HPS Ag – AOXIT unit, an origin of replication that functions in pichia pastoris, a DNA fragment containing the E.coli PBR 322 origin of replication and an E.coli selectable marker a segment of DNA that lies downstream from the AOXIT

sequence and facilitates integration of input DNA in to a specific chromosomal site and a functional histidine dehydrogenase gene which encodes an enzyme that is required for the synthesis of aminoacid histidine. The PBR 322 sequence enables the vector to be maintained in E.coli so that cloning steps can be carried out easily and when required, large amounts of vector DNA can be prepared

To avoid problems due to plasmid instability the strategy in this experiment was to integrate the AOXIP – HBS Ag – AOXIT unit in to the genome of pichia pastoris. To do this a histodinol dehydrogenase deficient (HISY) strain of pichia pastoris was transformed with the segment of the vector that contained the AOXIP – HBS Ag – AOXIT, His 4 and 3^1 – AOXI sequence. A double cross over event between the AOXIP and 3^1 – AOXI sequences of the input DNA and complementary sequences in the chromosomal DNA led to the integration of the AOXIP – HBS Ag – AOXIT and His 4 sequence in to the genome with the concomitant loss of the chromosomal AOXI gene cells that have an integrated form of the HIS 4 gene can grow in medium lacking histidine. A second mode of selection is slow growth in the presence of methanol, because a second but less efficient gene, AOX 2 is the only one that is present and functional after the loss of the AOXI gene as a result of the double crossover events. Thus, cells that have integrated the input DNA fragment and lost the AOXI gene will be histidine independent and grow slowly on methanol.

In the presence of methanol, which activates the AOXI promoter, a clone carrying an integrated DNA unit synthesized large quantities of authentic HBS Ag which was retained in the cytoplasm. This protein product was assembled in to the same multisubunit complex that occurs in hepatitis B virus infected human cells and was capable of eliciting neutralizing antibodies against hepatitis B virus. When this clone was grown in 240 liter batch cultures, the yield was equivalent to approximately 9×10^6 doses of vaccine. Further more, the genetic construct was stable and remained unaltered after 200 hours of culture in the presence of methanol

(f) Expression of Bovine Lysozyme C2

The ability of pichia pastoris to secrete a heterologous protein was examined in a system using bovine lysozyme C2 CDNA which encodes the complete protein and its own leader peptide. Bovine lysozyme is a stomach enzyme that attacks bacterial cell walls, is resistant to proteases, and has a narrow pH range for activity. These properties may enable bovine lysozyme to be used as an additive to animal feed stocks to enhance digestion by cows and other ruminants to be used with organisms that are given bacteria as a food source.

The vector constructed for this secretion study was identical to the AOXIP – AOXIT vector described except that the lysozyme CDNA sequence replaced the

HBS Ag coding sequence. In this experiment the entire plasmid was integrated in to a defective genomic copy of the HIS 4 gene of pichia pastoris. The product of this integration had a bovine lysozyme CDNA expression unit flanked by one functional and one defective histidinol dehydrogenase gene. The bovine lysozyme precureor was accurately processed by pichia pastoris and secreted in to the medium. The secreted protein had a specific activity equal to that of the authentic enzyme with a 200 hour continuous, 10 liter, high cell density fermentation system about 20 g of lysozyme was produced.

Yeast expression system have begun to play an important role in the production of heterogenous proteins for research, industrial and medical applications.

3.5.3.5 Cultured insect cell expression systems

Baculovirus infect insects. They have large double strand circular DNA genomes with in a rod shapted capsid. The baculovirus which have been exploited as vectors are the autograph California nuclear polyherosis virus (ACMVPV) and the silk worm virus bombyx mori nuclear polyhedrosis virus (BMNPV).

During normal infection the viruses produce nuclear inclusion bodies which consist of virus particles embedded in a protein matrix component of which is a virus encoded protein called polyhedrin. Large amounts of virus and polyhedrin are produced. Transcription of polyhedrin gene is driven by an extremely active promotor which is therefore ideally suited as a promoter for driving expression of foreign genes.

The promotor for the polyhedrin gene is exceptionally strong but the viral reproduction cycle does not depend on the presence of the polyhedorin gene consequently it was reasoned that replacement of the polyhedrin gene with a gene for a heterologous protein, followed by infection of cultured infect cells with the genetically engineered bacculovirus would result in the production of large amounts of a heterologous protein that because of the similarity of post translational modification systems between insects and mammals, would mimic closely, if not precisely, the authenbic form of the target protein on the basis of these promises investigatore developed bacculovirus as an expression vector for both mammalian proteins and animal virus proteins.

The most commonly used cell line for genetically engineered ACMVPV is derived from the fall army worm, spodoptera frugiperda. IN these cells, the polyhedrih promotor is exceptionally active and during infections with wild type baculovirus, high levels of polyhedrin are synthesized.

(a) Baculovirus expression vector system

The first step in the production of a recombinant baculovirus ACMNPV is to create a transfer vector. The transfer vector is an E.coli based palsmid that carries a segment of DNA from ACMNPV. The polyhedrin promotor region and an adjacent portion of upstream ACMNPV DNA which provides a region for homologous recombination with AIMNPV. A cloning site for the input DNA and the polyhedrin termination and polyadenylation signal regions and an adjacent portion of down stream ACMNPV DNA, which provides a second region for homologous recombination with ACMNPV. The coding region for the polyhedrin gene has been detected from this block of DNA. A gene of interest is cloned between the polyhedrin promoter and termination sequences and the construct is propagated in E.coli.

Insect cell in culture that have been transfected with ACMNPV DNA are transfected with a transfer vector carrying a cloned gene. Within some of the doubly transfected cells, a double cross over event occurs and the cloned gene with polyhedrin promotor and termination region then become integrated into the ACKNPV DNA with the consomitant loss of the polyhedrin gene. Viruons lacking the polyhedrin gene produce distinctive zenes of cell lysis from which recombinant baculovirus can be isolated.

The visual identification of occulusion – negative plagues is tedious and subjective – consequently DNA hybridization or a polymerase chain reaction assay can be used to detect recombinant beculovirus. Moreover if the E.coli lac 2 gene, which encode β-galactosidase is put under the control of a baculovirus promoter that is turned on during the early to late stages of the lytic cycle and this construct is made part of the DNA unit that is incorporated in to the ACMNPV genome, recombinant plaques turn blue when chromogenic substrate for β-galactosidase is added to the medium.

When host insect cells in culture are infected with recombinant baculovirus heterologous protein can be harvested after 4 to 5 days. The baculovirus expression vector system has been used to produce more than 500 different heterologous proteins, of which 95% had the correct post translational modifications.

3.5.3.6 Mammalian cell expression vectors

Extrachromosomal mammalian expression vectors are helpful for studying the function and regulation of mammalian genes. In addition authentic recombinant proteins with the potential to be therapeutic agents can be produced by mammalian cell lines in culture for use in human clinical trails. Although a variety of different mammalian expression vectors have been

developed they all tend to have common features and are very similar in design to other eukaryotic expression vectors.

A representative mammalian expression vector contain a eukaryotic origin of replication from an animal virus (Ex: Simian virus 40 SV 40). The promotor sequences that drive both the cloned gene and the selectable marker genes and the transcription termination sequences for these genes most be eukaryotic and are usually taken from either animal viruses (or) mammalian genes (Ex: β-action, thymidin kinase (or) borine growth hormone). Strong promoters and efficient poly adynilation signals are usually preferred. The sequences that are required for selection and propagation of a mammalian expression vector in E.coli are derived from a standard.

(a) Selectable marker systems for mammalian expression vectors

The bacterial gene (Neo^r) that encode neomycin phosphotransferase is often used to select transfected mammalian cells. The tonic agent for this system is the compount G-418 which kills non transfected mammalian cells by blocking translation. However with in a transfected cell, G-418 is phosphorylated by neomycin phosphotransferase. The addition of a phosphate group inactivates G-418 consequently only these cells that synthesize Neo^r gene product survive and proliferate.

Another system for selecting transfected mammalian cells uses the gene that encodes the enzyme dihydrofolate reductase (DHFR) with this protocol this recipient cells most have a defective DHFR. After transfection of DHFR deficient cells with a mammalian expression vector carrying an active DHFR gene. The chemical compound methotrenate (MTX) is added to the medium. The DHFR deficient cells that were not transfected cannot grow in the presence of MTX but the cells not that synthesize dihydrofolate reductase survive. After the initial selection for cells with the DHFR gene, the concentration of MTX in the medium is increased, which select for cells with large numbers of copies of the vectors. These cells are perpetuated and produce high levels of recombinant protein. Other dominent marker selection schemes have been developed for ex the enzyme glutamine synthetase confers resistance to the cytotoxic effects of the chemical compound methionine sulfonimine (MSX). In this GS – MSX system, a vector carrying a GS gene is prepared and introduced in to cultured mammalign cells. Selection of these cells that have a high vector copy number is achieved by increasing the concentration of MSX in the medium. In this case the host cell line need not lack GS because only multiple copies of GS gene can confer resistant to MSX. This selection scheme gives the GS – MSX system, a potential advantage over the DHFR – MTX system.

(b) Expression of two cloned genes in one mammalian cell

The active form of some commercially important proteins consists of two different protein chains for ex human thyroid stimulating hormone is a two chain protonand hemoglobin is a tetramer with two copies of each subunit. It is possible to clone the gene (or) CDNA for each subunit of a multimeric protein synthesize and purify each subunit separately and then mix the chains to gether in a test tube. Unfortunately relatively few multichain proteins are properly assembled invitro. By contrast invivo assembly of dimeric and tetrameric proteine is quite efficient consequently various strategies have been devised for the production of two different recombinant proteins with in the same cell.

Two mammalian expression vectors each with the gene (or) CDNA for one of the subunits and a different selectable marker gene are cotransfected in to host cells. The transfected cells are treated with both selecting agents and the cell can survive carry both vectors.

(c) Double cassette vectors

Two vector systems have been used successfully for the production of authentic dimeric and tetrameric recombinant proteins. However loss of one of the two vectors in doubly transfected cells is common. More over the two vectors are not always maintained with in the same copy number so one subunit may be over preduced relative to the other and yields of the final product may be reduced. To overcome these problems single vectors that carry two cloned genes have been developed. In some instances the two genes are placed under the control of independent promoter and polyadenylation signals (double cassette vectors).

(d) Dicistronic expression vector

Alternatively to ensure that equal amounts of the recombinant proteins are synthesized. Vectors have been constructed with the two cloned genes separated from each other by a DNA sequence that contains an internal ribosomal entry site (IRES) IRES found in mammalian virus genome and after transcription allow stimulatous translation of different proteins from a polycistronic mRNA molecule. Transcription of the gene IRES – gene construct is controlled by one promotor and polyadynalation signal. A single two gene transcript is synthesized and translation proceeds from the S^1 end of the mRNA to produce one of the chains and internally from the IRES element to produce the second chain.

3.5.3.7 Summary

Identification of cloned genes is very important. In this DNA hyboridization method DNA probes are used. Immunological methods, southern, western screening plays an important role for the identification of cloned genes. Prokaryotic expression systems work well for the production of a number of proteins however some proteins require specific post ranslational modifications such as glycosylation phosphorylation and acetylation. The yeast saccharomyces cerviceae which is well characterized genetically and can be grown in large fermenters has been used for the production of a number of different proteins from cloned genes. Saccharomyces cerviccae vectors that support secretion of heterologous proteins have been devised to simplify the purification process.

The baculovirus AcMNPV which infects insect cells, has been developed as a eukaryotic expression vector, a transfer vector, that carried the cloned gene flanked by AcMNPV specific sequence was transfected into an insect cell that had been infected with AcMNPV. A double cross over event between the transfer vector and the AcMNPV genome inserted the cloned gene into the AcMNPV genome and put it under the control of a strong promoter that is active during the late stage of lytic cycle. A heterologous protein is produced by infecting insect cells with recombinant baculovirus.

Mammalian expression systems have been devised for the invivo assembly of proteins that consist of two different subunits. The production and assembly of two chain and four chain proteins were achieved by cotransfection of host cells with two vectors that each carry a gene for one of the subunits. Alternative approaches include using one vector that carries the two genes as separate transcription units (or) forming a single transcription unit with the two genes. With one of the proteins being translated from the S^1 end of the mRNA and the other one from an IRES.

3.5.3.8 Model Questions

1. Write about the identification procedures of cloned gene?
2. Write about prokaryotic expression systems.
3. Write about mammalian expression systems.

3.5.3.9 Reference Books

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P. Sudhakar

Lesson - 3.5.4

GENE THERAPY

3.5.4.1 Objective:

The main objective of the lesson is to explain in detail the different approaches of gene therapy and it importance

3.5.4.2 Introduction:

Genes contain instructions that direct the normal growth, development and function of our bodies. These instructions are encoded into the structure of DNA (Deoxyribonucleic

Acid).Our bodies are made of many billions of cells, each of which contains a full set of the estimated 30,000 different genes that make up the human genetic blueprint. Genes issue instructions to cells in the form of chemically coded "messages".

Cells translate these messages into proteins that perform all the critical functions of the cell. Some of these proteins form building blocks for structures within the cells, others are enzymes which help carry out chemical reactions and still others form communication networks within and between cells. There are many different proteins and individual genes may be involved in the production of more than one protein. The body has many different cell types (eg. skin, muscle, liver, brain). Not all genes are switched on in every cell. Different genes are active in different cell types, tissues and organs.

A change in a gene that makes the gene faulty is called a mutation. In some cases mutations in genes are inherited when the mutation is present in the germ cells (*eggs and sperm*) of a parent; in other cases, the mutations occur in the genes in the body cells (*somatic cells*) and are not inherited. Mutations can result in a genetic condition. Changes in genes are also involved in other types of illness as well. Cancer, for example, is caused by genetic errors in cells that result in uncontrolled cell growth. Infections also involve genes, as infectious agents such as bacteria and viruses have their own unique sets of genes and our susceptibility to infections may differ according to our genetic make-up.

In 1977 scientists began the task of mapping, isolating and decoding every gene in the human genetic make-up. This task was accelerated by the establishment of the Human Genome Project in 1995 and was declared complete in 2003. Work now turns towards discovering how each gene functions and the protein or proteins for which it codes. As a result of the Human Genome Project, it is expected that there will be a dramatic increase in both our knowledge of and ability to treat (and in some cases, cure) conditions with a genetic basis.

3.5.4.3 What is Gene Therapy?

Gene therapy is "the use of genes as medicine". It involves the transfer of a therapeutic or correct gene into specific cells of an individual in order to repair a faulty gene. Thus it may be used to replace a faulty gene, or to introduce a new gene whose function is to cure or to favorably modify the clinical course of a disease.

Genes, which are carried on chromosomes, are the basic physical and functional units of heredity. Genes are specific sequences of bases that encode instructions on how to make proteins. Although genes get a lot of attention, it’s the proteins that perform most life functions and even make up the majority of cellular structures. When genes are altered so that the encoded proteins are unable to carry out their normal functions, genetic disorders can result.

Gene therapy is a technique for correcting defective genes responsible for disease development. Researchers may use one of several approaches for correcting faulty genes:

- A normal gene may be inserted into a nonspecific location within the genome to replace a nonfunctional gene. This approach is most common.
- An abnormal gene could be swapped for a normal gene through homologous recombination.
- The abnormal gene could be repaired through selective reverse mutation, which returns the gene to its normal function.
- The regulation (the degree to which a gene is turned on or off) of a particular gene could be altered.

How does gene therapy work?

In most gene therapy studies, a "normal" gene is inserted into the genome to replace an "abnormal," disease-causing gene. A carrier molecule called a vector must be used to deliver the therapeutic gene to the patient's target cells. Currently, the most common vector is a virus that has been genetically altered to carry normal human DNA. Viruses have evolved a way of encapsulating and delivering their genes to human cells in a pathogenic manner. Scientists have tried to take advantage of this capability and manipulate the virus genome to remove disease-causing genes and insert therapeutic genes.

Target cells such as the patient's liver or lung cells are infected with the viral vector. The vector then unloads its genetic material containing the therapeutic human gene into the target cell. The generation of a functional protein product from the therapeutic gene restores the target cell to a normal state. See a diagram depicting this process.

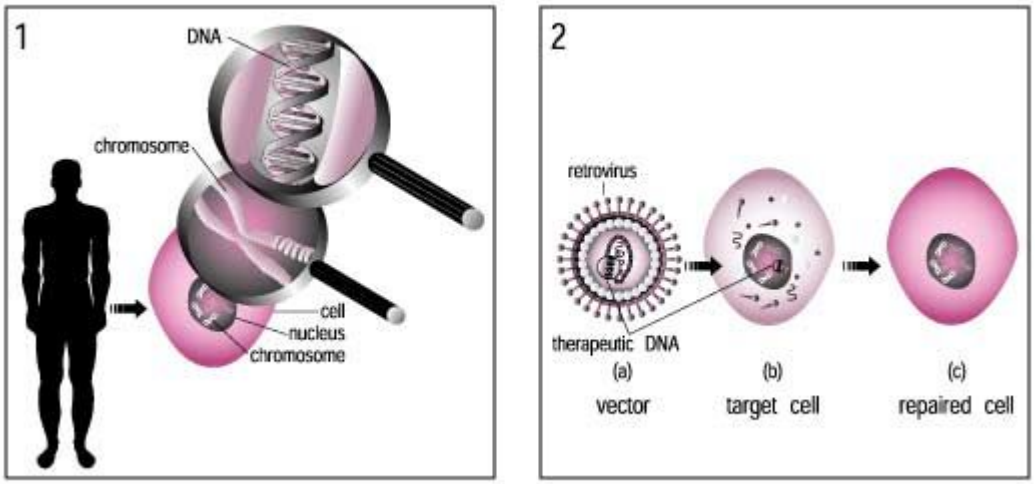

Figure 3.5.4.1 To reverse disease caused by genetic damage, researchers isolate normal DNA and package it into a vector, a molecular delivery truck usually made from a disabled virus. Doctors then infect a target cell —usually from a tissue affected by the illness, such as liver or lung cells—with the vector. The vector unloads its DNA cargo, which then begins producing the missing protein and restores the cell to normal

3.5.4.4 Types of Gene Therapy

There are two forms of gene therapy:

Somatic gene therapy :

It involves introducing a "good" gene into targeted cells with the end result of treating the patient - but not the patient's future children because these genes do not get passed along to offspring. In other words, even though some of the patient's genes may be altered to treat a disease, it won't change the chance that the disease will be passed on to the patient's children. This is the more common form of gene therapy that's being done throughout the world.

Germ line gene therapy:

It involves modifying the genes in egg or sperm cells, which will then pass any genetic changes to future generations as well. In experimenting with this type of therapy, scientists injected fragments of DNA into fertilized mouse eggs. The mice grew into adults and their offspring had the new gene. Scientists found that certain growth and fertility problems could be corrected with this form of therapy, which led them to think that the same could be true for humans. However, although it has potential for preventing inherited disease, this form of gene therapy is controversial and currently very little research is being done in this area, both for technical and ethical reasons.

Successful development of gene therapy may result in cures for diseases as varied as hemophilia, diabetes, hypercholesterolemia, and lysosomal storage disorders. In fact, it is difficult to imagine an area of medicine that would not be affected by gene therapy. Two basic distinctions can be used to characterize this field.

The first distinction is viral versus non-viral. Viral gene therapy approaches use genetically modified viruses to introduce genes into human cells by infection. Non-viral approaches use noninfectious (e.g. chemical or physical) means to introduce the genes.

The second distinction is in vivo versus ex vivo. In vivo gene therapies are based on the administration of DNA-based drugs directly to the patient. Ex vivo gene therapies are based on removing a small number of cells from a patient, introducing a gene into the cells and implanting the engineered cells into the patients.

Strategies used for the delivery of the genes

Among the strategies being pursued, perhaps the most prominent and straightforward is gene therapy. The fundamental idea is to administer a functional gene, so as to give targeted cells one that would have been present but is missing or defective, or sometimes one never meant to be there. In the first instance, the treatment addresses single-gene, or mendelian, disease; in the second, it becomes applicable especially to acquired illnesses, including heart disease and cancer (Figure 1), where ultimately gene therapy may have its greatest public-health impact. In all cases, success is measured by how well an added gene functions. The major problems lie in delivering the gene to the correct destination and in having it act with a useful duration, producing sufficient amounts of the desired protein.

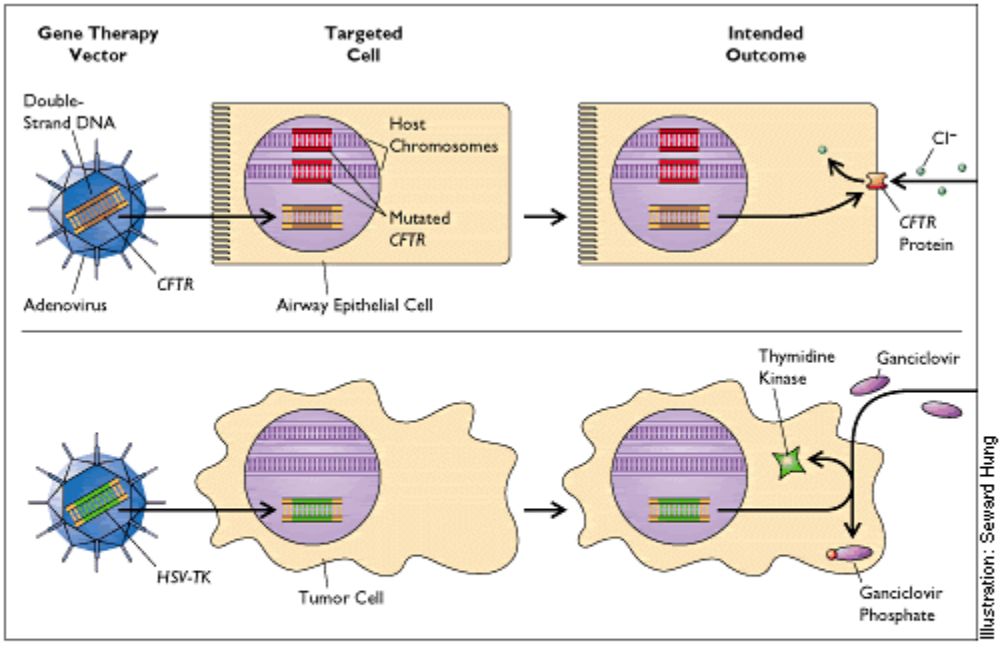

FIGURE 1. Varying purposes of gene therapy are illustrated by two forms of therapy now in clinical trials. In both instances, the therapeutic vector (an adenovirus) delivers a single gene. Against disorders such as cystic fibrosis (top), the intent is gene replacement. In the targeted epithelial cells, the delivered gene substitutes for the patient's defective gene—in this case, two mutated copies of *CFTR*—in restoring natural expression of a chloride channel. Against disorders such as cancer (bottom), the intent may be to induce a novel cellular function. Hence no gene is replaced. Instead, a novel gene is introduced, in this case a herpes simplex gene coding for a viral thymidine kinase. When the gene is expressed, the tumor cell can convert the prodrug ganciclovir into its active form, facilitating the cell's "suicide." For simplicity, each cell's genome has been symbolized by dual chromosomes, each a double-stranded DNA whose helical twining has likewise been omitted.

Vectors: Non viral and Viral

Gene-therapy vectors can be placed in two broad categories. In one class, the nonviral vectors, a gene is given a more or less artificial carrier: in some cases, a lipid encapsulation, so as to facilitate passage across cell membrane; in others, a tiny gold bead on which the gene is plated so it can be shot into the skin; in still others, a protein to which the gene is attached. (The protein is selected for its capacity to bind to a receptor expressed on the surface of specific cell types.)

In the other broad class, viral vectors, the gene is engineered into a modified virus in hopes of capitalizing on the infectivity of such a vector. Over long spans of viral and host evolution, viral vectors indeed have refined a wide range of gene-packaging and cell-entry mechanisms. Through highly specific means, typically involving receptors, they gain entrance to cells. There they are able to evade intracellular degradation and to induce a cell to express the viral genes. In some instances they splice their own DNA into a host-cell chromosome. Short of causing illness, the program is precisely what one wants a vector to achieve for a therapeutic gene.

Perhaps not surprisingly, viral vectors are currently more effective than nonviral ones. Of several types in clinical trials, the major ones are retroviruses, adenoviruses, adeno-associated viruses, and herpesviruses.

Some of the different types of viruses used as gene therapy vectors:

Retroviruses - A class of viruses that can create double-stranded DNA copies of their RNA genomes. These copies of its genome can be integrated into the chromosomes of host cells. Human immunodeficiency virus (HIV) is a retrovirus.

Adenoviruses - A class of viruses with double-stranded DNA genomes that cause respiratory, intestinal, and eye infections in humans. The virus that causes the common cold is an adenovirus.

Adeno-associated viruses - A class of small, single-stranded DNA viruses that can insert their genetic material at a specific site on chromosome 19.

Herpes simplex viruses - A class of double-stranded DNA viruses that infect a particular cell type, neurons. Herpes simplex virus type 1 is a common human pathogen that causes cold sores

For therapeutic use, they share several forms of genomic alteration. Essential viral genes are removed, leaving the virus incapable of replicating or, in some cases, restricting viral replication to the cells targeted by the therapy. The viral genes' place is taken by a therapeutic gene or genes. Viral vectors also share the same general technique of manufacture (Figure 2). Viruses, unlike bacteria, cannot replicate autonomously but require the services of host cells. In particular, viral vectors with deletions of genes controlling viral replication must be grown in a cell line engineered to contain the missing viral genes (transcomplementation). Having been purified from such cells, the virus can be administered therapeutically. Within the recipient, it may infect and deliver therapeutic genes but cannot reproduce unless it is engineered to have limited reproductive potential before being cleared by the patient's immune system

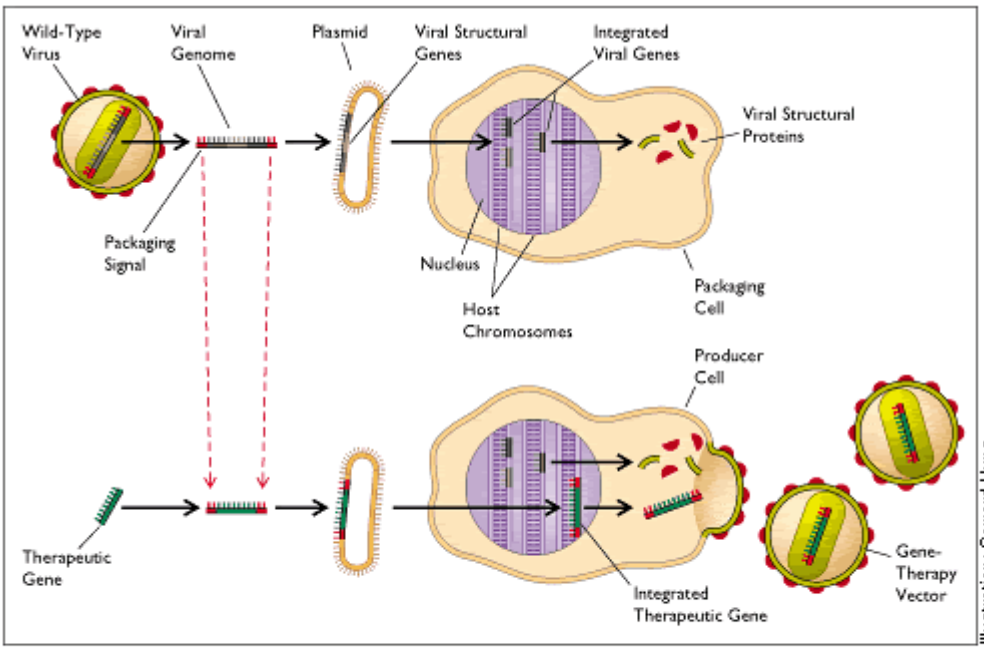

FIGURE 2. General strategy for manufacturing a viral vector suitable for delivering gene therapy is to delete some genes from the virus and give them to a cell. The virus loses some or all of its structural genes (top sequence), so that a so-called packaging cell can be made capable of synthesizing the viral proteins. In the viral genome, a therapeutic gene is placed where the structural genes had been (bottom sequence). Equipped with this construct, the cell becomes a producer cell, able both to make virions and to fill them with the therapeutic gene.

For use as a therapeutic vector, each type of virus has its own idiosyncrasies (Figure 3). Classic retroviruses, for instance, can integrate their genome, and any therapeutic genes, into that of a host cell only if the cell is dividing (and the nuclear membrane has dissolved). Because viral infection is needed for gene expression, retroviruses cannot productively infect postmitotic cells. Likewise, cells that divide infrequently, such as airway epithelial cells or totipotent hematopoietic stem cells, are poor targets for in vivo administration of a retrovirus. Lentiviral retroviruses, including HIV, do not share this limitation. Nor do adenoviruses, which in fact have a mechanism by which the viral nucleocapsid advances the viral genome through nuclear pores. On the other hand, adenoviruses tend to be recognized as foreign and therefore to trigger a host immune response. Whereas retroviral vectors have been stripped of all viral genes, leaving them incapable of inducing host-cell expression of any viral proteins, most adenoviral vectors in current use have lost only some of their genes

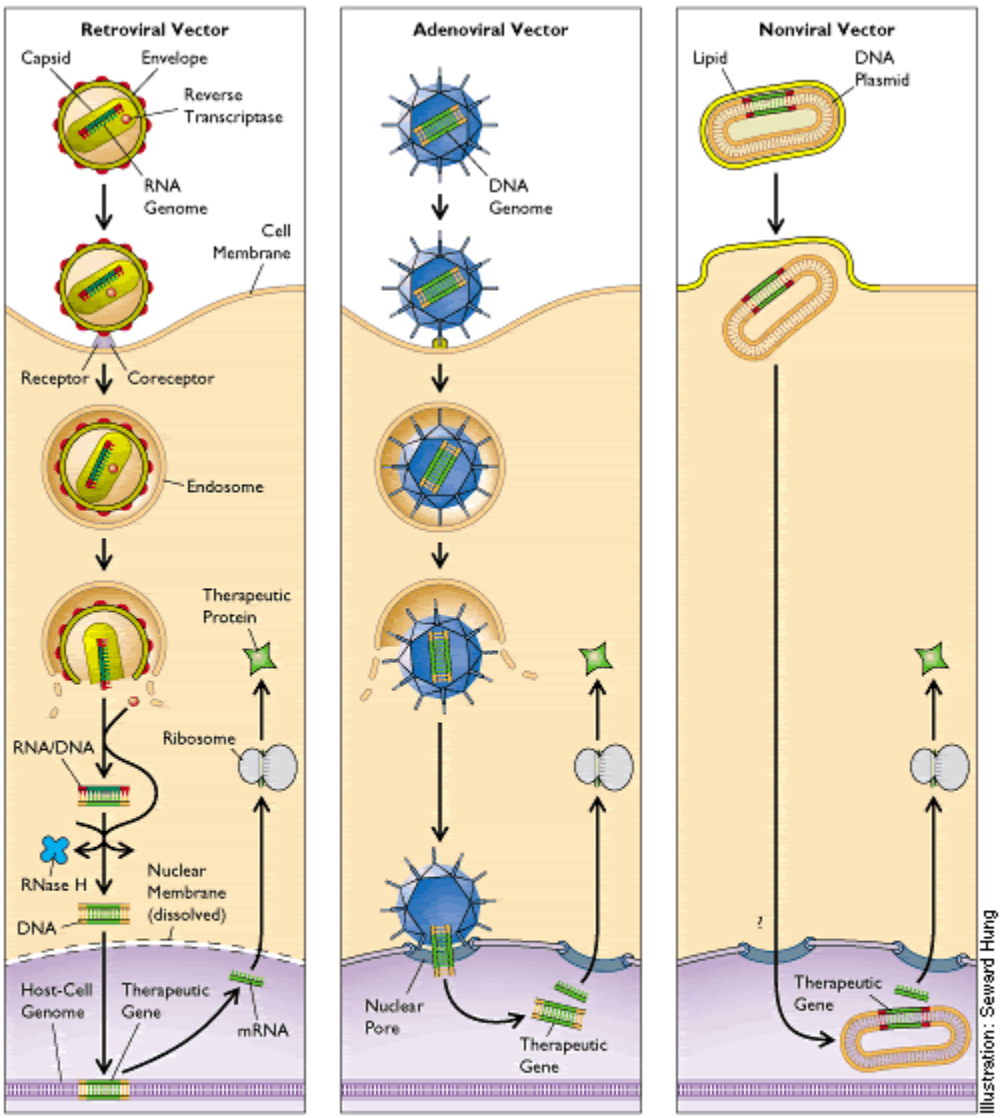

Figure 3. Idiosyncrasies of a given viral vector arise from differences in viral life cycles. Retroviruses (left) and adenoviruses (center) are alike in entering cells by binding to specific receptors. The retroviral genome is, however, a single-stranded RNA, requiring conversion into double-stranded DNA for insertion into the host-cell genome. Unable to pass through nuclear pores, the genetic material gains access only when the nuclear membrane dissolves during mitosis. The adenoviral genome is itself double-stranded DNA , which enters the nucleus at any time via formation of a capsid-pore complex. To be expressed, it does not require integration into host-cell DNA. Artificial vectors such as lipid-coated DNA (right) cannot rely on natural means of entering cells. The vector's intracellular trafficking and even the details of its own structure are not yet well understood.

Across all viral types, there are further limitations. As noted, the vectors tend not to disperse well in a targeted tissue. Even when injected directly into a tumor, they are prone to miss some of the targeted cells. In addition, their use does not guarantee long-term gene expression. Such problems are being addressed. In some vectors, therapeutic genes have been augmented by genetic control elements, or promoters, that make the gene governable by a systemically administered drug. In one example, expression of the erythropoietin gene is controlled by orally administered rapamicin. In other vectors, the virus retains an ability to replicate. Several are now in clinical trials, including a

herpesvirus engineered to replicate selectively in tumors but poorly or not at all in normal neural tissue (its natural environment). A replication-conditional adenovirus has likewise been developed. It, too, is designed to replicate in tumors but poorly, if at all, elsewhere. Replicating vectors may lyse tumor cells, even apart from any therapeutic genes they might carry, an idea harking back to therapies first tested in the middle part of this century, when a variety of wild-type viruses, including adenoviruses, were administered to cancer patients. The viruses indeed killed tumor cells but none of the patients was cured. Therapies based on replicating viruses will have to address limitations imposed by immune-mediated clearance of the vector.

Meanwhile, novel classes of viruses are being added to those under study for possible use as gene-therapy vectors. Among the most recent is a chicken virus that occasionally infects human chicken handlers (Newcastle disease virus).

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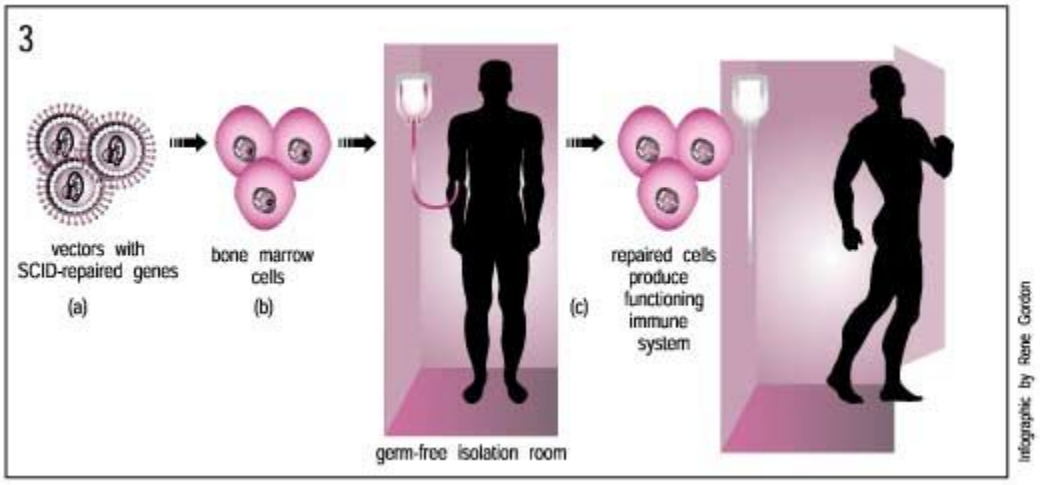
Besides virus-mediated gene-delivery systems, there are several nonviral options for gene delivery. The simplest method is the direct introduction of therapeutic DNA into target cells. This approach is limited in its application because it can be used only with certain tissues and requires large amounts of DNA.

Another nonviral approach involves the creation of an artificial lipid sphere with an aqueous core. This liposome, which carries the therapeutic DNA, is capable of passing the DNA through the target cell's membrane.

Therapeutic DNA also can get inside target cells by chemically linking the DNA to a molecule that will bind to special cell receptors. Once bound to these receptors, the therapeutic DNA constructs are engulfed by the cell membrane and passed into the interior of the target cell. This delivery system tends to be less effective than other options.

Researchers also are experimenting with introducing a 47th (artificial human) chromosome into target cells. This chromosome would exist autonomously alongside the standard 46 --not affecting their workings or

causing any mutations. It would be a large vector capable of carrying substantial amounts of genetic code, and scientists anticipate that, because of its construction and autonomy, the body's immune systems would not attack it. A problem with this potential method is the difficulty in delivering such a large molecule to the nucleus of a target cell.

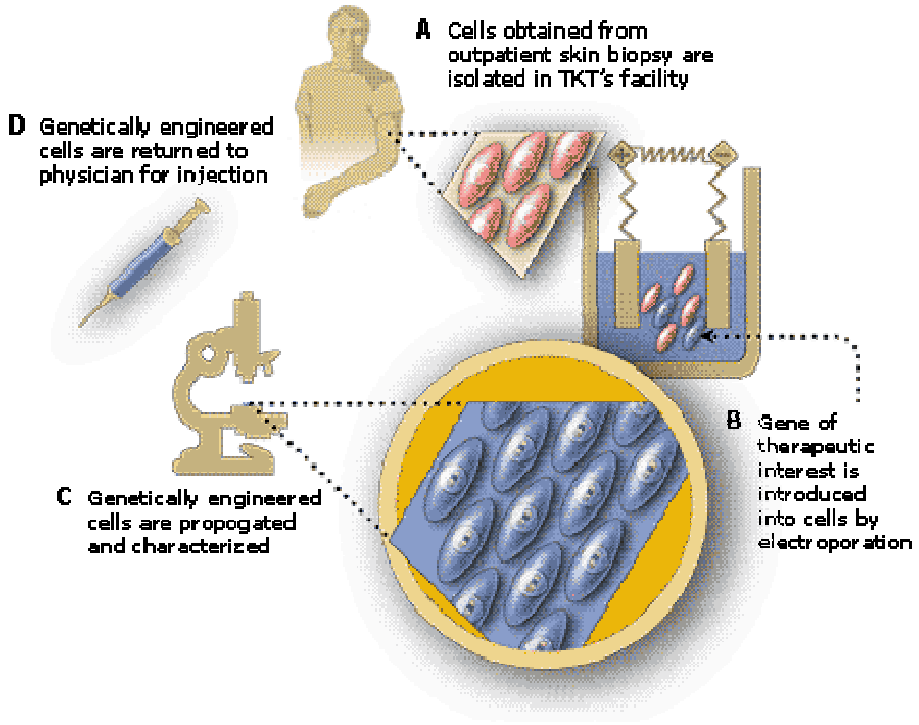


Recently, French researchers reported dramatic results in treating a disease called severe combined immune deficiency (SCID), the disorder suffered by David, The Boy in the Bubble. A broken gene eliminates the production of an enzyme essential for the development of a normal immune system. Scientists isolated the normal copy of the gene and packaged it into a vector. In the laboratory, they then used the vector to transport the gene into the patient's own bone marrow cells. Bone marrow cells create the immune system. The treated bone marrow cells are then given back to the patient in a germ-free isolation room, where they reconstitute a normal, functioning immune system, freeing the patient from the need to remain in isolation.

Ex vivo Gene Therapy:

TKT's enabling gene therapy technology, known as Transkaryotic Therapy, is a non-viral ex vivo, cell-based system which is designed to genetically modify a patient's own cells to produce and deliver therapeutic proteins within the body. TKT believes Transkaryotic Therapy is well-suited to allow safe and long-term delivery of therapeutic proteins for the treatment of chronic protein deficiency states.
TKT scientists have successfully delivered therapeutic proteins for the lifetime of experimental animals. Much of TKT's work has focused on gene therapy using fibroblasts, a cell type present in the skin (and throughout the body) that is readily obtained from patients and propagated in culture. The company has developed a variety of methodologies for the stable transfection of normal human cells. "Stable transfection" means that the introduced DNA fragment becomes part of a chromosome in the treated cell. One such methodology is electroporation, a technique based on subjecting cells to a

brief electrical pulse. The pulse transiently opens small pores in the cell membrane that allow the DNA fragments of interest to enter the cell. The technique is simple and reproducible (it works for a variety of cell types and for cells derived from newborns to the elderly).
Over the past decade, TKT believes it has developed the basic technologies required for a safe and effective gene therapy approach which can be refined and optimized for patient use. In patients, TKT envisions that the system would function as follows



ETHICAL CONSIDERATIONS

While the body has many billions of cells, only a very small proportion of these cells are involved in reproduction, the process by which our genes are handed on to future generations. In males these cells are located in the testes and in females, in the ovaries. These special reproductive cells are called **"germ cells"**.

All other cells in the body, irrespective of whether they are brain, lung, skin or bone cells, are known as **"somatic cells"**.

In gene therapy, *only* somatic cells are targeted for treatment. So any changes to the genes of a person by gene therapy will only impact on the cells of their body and cannot be passed on to their children. Changes to the somatic cells cannot be passed on to future generations (inherited).

Gene therapy treats the individual and has no impact on future generations.

An example of gene therapy
Imagine, for example, a little boy with hemophilia, a condition that is caused by a faulty gene that makes his liver unable to make blood clotting factor VIII (see Genetics Fact Sheet 38).

Gene therapy would involve putting a correct copy of the gene which codes for factor VIII into his liver cells so that his liver could then produce adequate levels of factor VIII. While the little boy himself would be cured, the altered genes in his germ cells would remain unchanged and he could still pass the faulty gene on to future generations.

Concerns with gene therapy of the eggs or sperm

The possible genetic manipulation of the egg or sperm cells (germ cells) remains the subject of intense ethical and philosophical discussion.

The strong consensus view at present is that the risks of germ-line manipulation far

exceed any potential benefit and should not be attempted.

3.5.4.5 Summary Of Gene Therapy

Gene therapy is a new and largely experimental branch of medicine that uses genetic material (DNA) to treat patients. Researchers hope one day to use this therapy to treat several different kinds of diseases. While rapid progress has been made in this field in recent years, very few patients have been successfully treated by gene therapy, and a great deal of additional research remains to be done to bring these techniques into common use.

Many diseases seen today are the result of a defective gene in the DNA of the patient and can not be cured using the traditional methods such as antibiotics and antiviral medication. The victims are now looking to gene therapy as a potential cure for their problems Along with the appearance of the recombinant DNA technology, it becomes possible for human beings to isolate, study, and change gene in the laboratory. Gene Therapy is the process of replacing a defective gene inside a patient's DNA with a working gene that will produce the correct gene products The genetic diseases "in which a single known gene does not function properly", such as sickle cell anaemia, thalassaemia and Lesch-Nyhan syndrome, are most suitable to be treated with the gene therapy

Whether given as pills or injections, most conventional drugs simply need to reach a minimal level in the bloodstream in order to be effective. In gene therapy, the drug (DNA) must be delivered to the nucleus of a cell in order to function, and a huge number of individual cells must each receive the DNA in order for the treatment to be effective. The situation is further complicated by the fact that a given gene may normally function in only a small portion of the cells in the body, and **ectopic expression** may be toxic. Thus, successful gene therapy often requires highly efficient delivery of DNA to a very restricted population of cells within the body.

To achieve these goals, many researchers have turned to viruses. Viruses are parasites that normally reproduce by infecting individual cells in the human body, delivering their DNA to the nucleus of those cells. Once there, the viral DNA takes over the cell, converting it to a factory to make more viruses. The cell eventually dies, releasing more viruses to continue the cycle. Scientists can remove or disable some of the genetic material of the virus, making it unable to reproduce outside of the laboratory. This genetic material can then be replaced by the gene needed to treat a patient. The modified (or recombinant) virus can then be administered to the patient, where it will carry the therapeutic gene into the target cells. In this way, scientists can take advantage of the virus's ability, gained over millions of years of evolution, to deliver DNA to cells with tremendous efficiency. One of the most commonly used is a cold virus called adenovirus. Recombinant adenoviruses have been used in experimental gene therapy for muscle diseases, and can deliver genes to almost all of the cells in a small region surrounding the site of injection. Unfortunately, while adenoviruses excel at gene delivery, evolution is a double-edged sword, and the many mechanisms our own bodies have evolved to combat harmful viral infections are also used against therapeutic viruses

3.5.4.6

Model Questions.

1. What is Gene Therapy add a note on different vectors used for delivering of genes in Gene Therapy?

2. What is Anti sense Therapy?

3. What is somatic Gene Therapy and Germ Therapy?

4. Describe Exvivo and Invivo Gene Therapy?

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Lesson- 3.1.1

MEDIA PREPARATION

Contents

Objective

Objective

The excised plant tissues and organs can grow only on artificially prepared nutrient medium which is known as culture medium. In this chapter discussions relating to the composition of nutrient medium, preparation of a nutrient medium, and selection of nutrient medium for a particular type of explant are made.

3.1.1.1 Introduction

Excised plant tissues and organs require a proper nutrient medium for their growth and development, which is known as culture medium. Many workers have proposed the composition of a nutrient medium for the growth of plant tissue. But no single medium is capable of maintaining optimum growth of all plant tissues. Therefore the most suitable medium for a particular tissue must be determined by trial and error. The proposed composition of a culture medium will be often modified to stimulate the growth of a particular plant material.

Mostly all tissue culture media are synthetic. A synthetic medium consists of only chemically defined compounds. The medium must have a carbon source, a nitrogen source, inorganic salts, organic supplements, vitamins and growth regulators.

Different types of media have been used by different workers for culturing different types of explants. The different types of tissue – culture media are, M.S medium, B5 medium, Whites medium, S.H. medium, L.S. medium etc. The M.S. medium was formulated by Murashige and Skoog in 1962. B5 medium was formulated by Gamborg et al in 1968. S.H. medium was formulated by Schenk and Hildebrandt in 1972. The L.S. medium was formulated by Linsmaier and Skoog in 1965.

The M.S. medium and B5 medium are generally used in plant cell and tissue culture. The composition of M.S. and B5 media are listed in the table.

3.1.1.2 Composition of plant tissue – culture media

Inorganic Nutrients

All nutrient media provide the 15 essential elements necessary for plant growth. They are categorized into macronutrients and micronutrients.

Macro Nutrients

These are needed in concentration >0.5 m mol.

They include C, H, O, N, P, K, Ca, S, Mg

Micro Nutrients

There are required in concentrations <0.5 m mol.

Different tissue culture media provide different concentrations of the inorganic nutrients. Iron is provided as iron. EDTA complex to keep it available at higher pH i.e. above 5.8. MS and B5 are the most commonly used media.

Carbon Source

Sucrose 20 – 50 gms/lit is the most commonly used carbon source for all cultured plant materials. Autoclaving hydrolyses sucrose and enhances its availability to plant cells. Plant tissue can utilize other sugars like maltose, galactose, lactose, mannose and starch. But these are rarely used.

Vitamins

For optimum callus growth vitamins are essential. Inositol, thiamine, pyridoxine and nicotinic acid are the vitamins used it plant tissue culture of these thiamine is essential and the rest are promotory.

Growth Regulators

Auxins, cytokinins and Gibberellins are the growth regulators commonly used in tissue culture.

Auxins

They are commonly used to support cell division and callus growth, somatic embryo induction and rooting.

Eg:- IAA - Indole – 3 – acetic acid

IBA - Indole – 3 – butyric acid

NAA - Naphthalene acetic acid

NOA - Naphthoxy acetic acid

2,4-D - 2,4 – dichlorophenoxy acetic acid

Cytokinins

They are used to promote cell division, regeneration of shoots, somatic embryo induction and to enhance proliferation and growth of axillary buds.

Eg:- Kinetin (furfuryl aminopurine)

BAP (benzyl amino purine)

Zeatin

2-ip (Isopentenyl adenine)

TDZ – Thidiazuron (a compound having cytokinin activity)

- Abscisic acid promotes somatic embryo and shoot bud regeneration in many species and also improves somatic embryo maturation.

- Among the 20 gibberellins known, GA_3 is used in plant tissue culture. It promotes shoot elongation and somatic embryo gemrination.

- The common range of concentrations used are,

Auxins - 0.1 – 3 mg/l

Cytokinins - 0.1 – 3 mg/l

ABA	-	0.2 mg/l
GA_3	-	0.1 – 1 mg/l

Amino acids

Cultured tissues are capable of synthesizing the aminoacids necessary for their metabolism. Inspite of this the addition of aminoacids to media is important for stimulating cell growth in protoplast culture and for establishing cell lines.

Eg:- L – Glycine – 2 m mol/l

Casein hydrolysate – 0.05 – 0.1%

L – Glutamine – 8 m mol/l

L – Cysteine – 10 m mol/l

L – Asparagines – 100 m mol/l

L – Arginine – 10 m mol/l

Tyrosine – 100 m mol/l

Aminoacids added singly are found to be inhibitory to cell growth and their mixtures are found to be beneficial.

Activated Charcoal

Addition of activated charcoal to the medium stimulates growth and differentiation in orchids, carrot, ivy and tomato. Its effect is found to be inhibitory in tobacco, soyabean and camellia. Inhibition of growth is due to the absorption of phytohormes by activated charcoal. The stimulation may be due to the adsorption of inhibitory compounds to activated charcoal and darkening of the medium. It also absorbs toxic compounds produced during the culture and promotes cell growth. Activated charcoal is acid washed and neutralized before adding to the medium at a concentration of 0.5 – 3%.

Antibiotics

Addition of antibiotics to tissue culture media is generally avoided because their presence retards cell growth. But, some plant cells have a systemic infection of microorganisms. To prevent the growth of these microbes the media are to be enriched with antibiotics.

Streptomycin (or) Kanamycin at low concentration effectively controls systemic infection and the media supplemented with these antibiotics do not adversely inhibit the growth of cell cultures.

Complex Organic Additives

The organic extracts like yeast extract, coconut milk, casein hydrolysate, corn milk, malt extract and tomato juice were used to support plant tissue growth.

In tissue cultures the success achieved with the use of coconut milk and casein hydrolysate are significant. For anther culture, potato extract has been found to be suitable.

In many cases their effects could be reproduced by a single amino acid. For example L-asparagine could replace yeast extract and tomato juice in the medium for callus culture of maize endosperm. L-glutamine alone has demonstrated favourable tissue responses in several species and enrichment by fruit extracts was found unnecessary. Such additives should be used only when synthetic media fails.

Solidifying Agents

Solidifying agents are commonly used for preparing semi solid or solid tissue culture media. The most commonly used solidifying agents are Agar, Gelatin, Alginate, Phytagel, Gelrite and Seaplaque (K) (highly purified agarose).

- Agar, a polysaccharide obtained from sea weeds, has several advantages over the other gelling agents. These gels donot react with media constituents, they are not digested by plant enzymes and remain stable at all incubation temperatures.

- Generally 0.5 – 1% agar is used in plant cell culture media preparation.

- In nutritional studies the use of commercially available agar is to be avoided because it contains impurities in the form of Ca, Mg, K, Na and trace elements. These impurities can be removed by washing agar in double distilled water for 24 hours, then rinsing in ethanol and drying at 60^{O} C for 24 hours. This can be used for such critical experiments.

pH

Plant cells and tissue require optimum pH for growth and development in cultures. pH effects the uptake of ions. For most of the culture media pH 5 – 6 before sterilization is considered optimal. Higher pH gives a hard medium and a lower pH results in unsatisfactory solidification of the agar.

3.1.1.3 Media Preparation

Now a days plant tissue culture media most commonly used are available in the market as dry powders. These powders contain inorganic and organic nutrients. The medium can be prepared by dissolving these powders in some quantity of distilled water. After mixing the contents thoroughly in water, sugar, agar (melted) and other organic supplements are added.

Finally, the volume is made up to one litre the pH is adjusted and the medium is autoclaved.

For the preparation of the culture medium by using conventional methods, we have to prepare the stock solutions, of (1) macro nutrients, (2) micronutrients, (3) iron and (4) organic nutrients except sucrose. For each growth regulator a separate stock solution is prepared by dissolving it in a small quantity of suitable solvent and then adjusted with distilled water to the desired volume to get the final concentration of 1 m mol/l (or) 10 m mol/l. All the stock solutions are stored in proper plastic (or) glass containers and kept at low temperatures. Iron stocks should be stored in coloured bottles. The containers should be shaken well before use and if there is any detection of contaminant (or) precipitate the stocks must be discarded. In storing coconut milk we have to take extra care. The liquid extract

from the fruit is boiled to deproteinise it, filtered and stored in plastic bottles in a deep – freeze at – 20^{O}C.

In experiments in which changes in the quantity and quality of media constituents is needed, it is better to weigh and dissolve each ingredient separately before mixing them together.

1. Preparation of Stock Solutions

It is not possible to weigh and mix all the constituents just before the preparation of medium, which is a time consuming and tedious job. If we want to prepare very little volume of the medium, then also it is very difficult to weigh some constituents that are used in very small quantity for one litre medium. So, it is convenient to prepare the concentrated stock solutions of macro – salts, micro-salts, vitamins, amino acids, hormones etc. All stock solutions should be stored in a refrigerator and should be checked visually for contamination with microorganisms (or) precipitation of ingredients. Stock solutions of vitamins, amino acids and hormones should not be stored for indefinite period and should be kept in a deep freezer chamber.

The widely used culture medium was formulated by Murashige and Skoog (MS – medium). The procedure for the preparation of stock solutions of MS – medium is given as follows. Macro salts should be made at 10 or 20 times of their final concentration in the medium and the micronutrients can be prepared at 1000 times of their final concentration. While making the stock solutions, it is advisable to dissolve each constituent completely before adding another. Otherwise precipitation of salts may occur.

Stock solution of macro salts

To make 1000 ml of this stock solution, dissolve the salts one after another in 800 ml of double distilled water and then make up the volume to 1000 ml. The solution is filtered and can be stored in the refrigerator (10 – 16^{O}C) for a long period.

Stock Solution of KI (X 1000)

Dissolve 83 mg of KI in 100 ml of double distilled water and store in the refrigerator. (0.83 mg/litre present in the original medium).

Table of Macro Salts

Constituents	Amount (mg/l) present in the original medium	Amount (gm) to be taken for stock solution (X20)	Final volume of stock (ml)
$NH_4 NO_3$	1650	33.0	
KNO_3	1900	38.0	
$Cacl_2,2H_2O$	440	8.8	1000
KH_2PO_4	170	3.4	
$MgSo_4$, $7H_2O$	370	7.4	

Stock Solution of Micro Salts (X 1000)

To make 1000 ml of this stock solution dissolve the salts one after another in 800 ml of double distilled water and then make up the final volume.

Table of Micro Salts

Constituents	Amount (mg/l) present in the original medium	Amount (X100) to be taken for stock solution (value expressed in mg)	Final volume of stock (ml)
H_3BO_3	6.2	620	
$Na_2 MOO_4$, $2H_2O$	0.25	25	
$COCL_2$, $6H_2O$	0.025	2.5	
$CuSO_4$, $5H_2O$	0.025	2.5	100
$NzSO_4$, $7H_2O$	8.6	860	

$MnSo_4, 4H_2O$	22.3	2230	

Stock Solution of Iron (X200)

Dissolve 45 mg of Na_2EDTA (37.25 mg/lit in original medium) in 75 ml of boiling double distilled water. Then add gradually 557 mg of $FeSo_4$, $7H_2O$ (27.85 mg/lit in original medium). Keep the magnetic stirrer on for at least 1 hour in hot condition until the colour of the solution changes to golden yellow. Finally make the volume to 100ml and store in the refrigerator (5^OC). This solution must be kept in an amber coloured bottle.

To make 100 ml of this solution, dissolve the salts sequentially as mentioned above, one by one in 80 ml of double distilled water. Make up the final volume. Filter and store the solution at 5^OC in a refrigerator for long periods.

Stock Solution of Meso – Inositol (X500)

Dissolve 1 gm of Meso – Inositol in 20 ml of double distilled water. Store at O^OC for 15 days.

Stock Solution of Glycine (X1000)

Dissolve 40mg glycine in 20 ml of double distilled water and store at O^OC for 15 days.

Stock Solution of MS Vitamins, (X1000)

Table

Constituent	Amount (mg/l) present in the original medium	Amount (x50) to be taken for stock solution (value in mg)	Final volume (ml)	Storage tempera-ture (^OC)	Duration of storage (in days)
Thiamine HCL	0.1	5			
Nicotinic acid	0.5	25	50	0	15

Pyridoxine HCL	0.5	25			

Stock Solution of Hormones

Stock solutions of hormones are general and can be used for any medium at any combinations and concentrations.

Auxins and cytokinins are not directly dissolved in water. They are first made soluble in water miscible solvents and then water is added to get the final volume.

Table for stock solutions of hormones

Hormone	Required amount for stock solution	Amount of solvent required to dissolve	Amount of water to be added	Final concen-tration	Storage tempe-rature (^{O}C)	Duration of storage (days)
Auxins						
2,4-D	10	1ml abs. Ethyl alcohol	9 ml	0.5 mg/ml	0	7
IAA	10	- do -	- do -	- do -	- do -	- do -
NAA	10	- do -	- do -	- do -	- do -	- do -
IBA	10	- do -	- do -	- do -	- do -	- do -
Cytokinins						

Kinetin (6-furfuryl amino purine)	10	1ml (1N) HCL	- do -	- do -	- do -	- do -
BAP (6-Benzyl aminopurine)	10	- do -	- do -	- do -	- do -	- do -
Zeatin	10	- do -	- do -	- do -	- do -	- do -
2iPA (2,iso-pentenyl-adenine)	10	- do -	- do -	- do -	- do -	- do -

2. Procedure for the preparation of culture medium

Media should be prepared with care. To make 1 litre of MS medium.

- 30 gms of cane sugar is dissolved in 200 ml of double distilled water.
- 1-2 gms of activated charcoal is added to this and filtered through a filter paper.
- Take double distilled water in another flask and add the appropriate amount of stock solutions in sequence as shown in the table.

Stock solution of macro salts	→ 50 ml
Stock solution of micro salts	→ 1 ml
Stock solution of KI	→ 1 ml

Stock solution of Fe-EDTA	→ 5 ml
Stock solution of MS-vitamins	→ 1 ml
Stock solution of Glycine	→ 1 ml
Stock solution of Meso-inositol	→ 2 ml

- Desired concentration of auxin and cytokinin are added from stock solution according to the formula given below,

$$\text{Amount of stock solution to be taken in ml. For one litre medium} = \frac{\text{Desired concentration}}{\text{Stock concentration}}$$

- If the quantity of the medium is less than one litre, then the hormones are added using another formula,

$$\text{Amount of stock solution to the medium} = \frac{\text{Required concentration x volume of medium in ml}}{\text{Stock concentration x 1000}}$$

- Pour the filtered sucrose solution and the solution mixture of salts, vitamins, aminoacids and hormones into a one litre measuring cylinder. Make the final volume to

one litre with double distilled water. Shake well to mix up uniformly.

- Adjust the pH of the liquid medium in between 5.6 to 5.8 by using 0.1N HCl or 0.1N NaOH. This operation is done by using pH meter.

- 5 – 8% agar is added to the liquid medium to make it solid medium. Heat it to $60^{O}C$ to dissolve the agar completely.

- Distribute the culture medium into culture tubes or conical flasks. Close their mouths with non-adsorbent cotton plug wrapped in a gauge cloth.

- Cover the plug with brown paper with the help of a rubberband.

- The medium is sterilized finally by autoclaving.

3.1.1.4 Selection of a suitable medium

A suitable medium may be deviced for a new system in several ways. All components of the medium are divided in to four categories, viz. minerals, auxins, cytokinins and organic nutrients. Now each group of substances are prepared in 3 concentrations: low (L), medium (M) and High (H).

In 1974 De Fossard et al. have provided a broad spectrum experiment for this purpose. According to them the various combinations of these four categories of components of three different concentrations give us an experiment with 81 treatments. The best of these treatments may be selected as a new medium suitable for an untested system.

3.1.1.5 Summary

Excised plant tissues and organs can only grow *invitro* on a suitable artificially prepared solid (or) liquid nutrient medium which is known as culture medium. The Murashige and Skoog (MS) based culture media are commonly used or plant tissue culture and have proven effective for growth promotion of both monocotyledons and dicotyledons.

A culture medium is composed of inorganic salts, an iron source, vitamins, aminoacids, plant hormones and a carbon source.

The inorganic salts are supplied in two groups viz. macrosalts and microsalts. The most commonly used phytohormes are synthetic auxins and cytokinins. The auxin are 2,4-D, NAA, IBA etc. and the cytokinins are kinetin, 6-BAP, Zeatin, etc. The concentration and ratio of hormones may vary from plant to plant and should be standardized for a particular plant tissue. Some plant tissues grow in the presence of complex natural additive such as coconut milk, casein hydrolysate, yeast extract, water melon extract, malt extract, potato extract, ripe tomato extract, orange juice extract etc. Diphenyl urea a growth factor found in coconut milk, exhibits cytokinin – like responses.

Based on the constituents, there are two types of culture media Viz. chemically defined and chemically undefined media. In the former one the composition and concentration of all constituents are known, whereas in the latter the exact composition and the concentration of all constituents are not known due to the addition of natural products like coconut milk.

It is not possible to weight and mix all the constituents just before the preparation of the medium. It is convenient to prepare the concentrated stock solutions of macrosalts, microsalts, vitamins, aminacids, hormones etc. All stock solutions should be stored in a refrigerator for a limited period.

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3.1.1.6 Model questions

1. What is culture medium? Give the basic composition of a general plant tissue culture medium? How can you prepare and sterilize the culture medium?

3.1.1.7 Reference books

1. An introduction to plant tissue culture Dr.M.K. Razdan, Oxford & IBH publishing Co. Pvt., Ltd.

2. Plant cell and tissue culture, Narayana Swamy, Tata Mcgrow-hill.

3. An introduction to plant tissue culture, Dr.Kalyan Kumar De, New Central Book Agency.

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Lesson 3.1.2

STERILIZATION

Contents

Objective

Objective

All the materials that are used in tissue culture must be free from microbes. The culture medium contains sugar, which supports the growth of microorganisms like bacteria, fungi etc. when they come in contact with the medium either in cellular form (or) in spore form, they grow faster than the higher plant tissue due to their brief life cycle and will kill the tissue. So, to avoid this situation maintenance of complete aseptic conditions inside the culture vial is essential. In this chapter, the different sterilization techniques used in plant tissue culture are discussed.

3.1.2.1 Introduction

The most important aspect of *invitro* techniques is the necessity to carry out various operations under aseptic conditions. Bacteria and fungi are the most common contaminants observed in cultures. When the microbes come in contact with the culture media from different sources viz. glass , instruments, nutrient medium and also from the plant material itself, they grow faster than the cultured tissue. So, the tissue is killed due to contamination. The contaminants may also release metabolic wastes which are toxic to the plant tissues. So, to avoid this, the maintenance of complete aseptic environment inside the culture vial where the tissue grows is very much essential. Therefore, the surface of plant tissue and all non-living articles including the nutrient medium should be sterilized.

3.1.2.2. Different sterilization techniques used in plant tissue culture

All the materials viz. vessels, instruments, medium, plant materials etc. that are used in culture work must be freed from microbes. This can be achieved by the following techniques of sterilization.

1. Dry heat
2. Flame sterilization

3. Autoclaving
4. Filter sterilization
5. Wiping with 70% ethanol
6. Surface sterilization

1. Dry heat

Glass wares and Teflon plastic wares, instruments and aluminium foil can be sterilized by exposure to hot dry air in an oven at 160^{O} – 180^{O}C or 2 – 4 hr. All the items should be properly sealed before sterilization. Sealing with paper is not advisable as it decomposes at high temperatures. The disadvantage of dry heat sterilization is poor circulation of air and slow penetration of heat, so sterilization of the glassware by autoclaving and flame sterilization of the instruments like forceps is recommended. More recently, glass bead sterilizers are being employed for the sterilization of forceps, scalpels etc. These devices use dry heat and the temperature is maintained around 300^{O}C.

2. Flame Sterilization

Instruments like forceps, scalpels, needles etc. are flame sterilized by dipping them in 95% alcohol followed by flaming. These instruments are repeatedly sterilized during the operation to avoid contamination. It is customary to flame the mouths of the culture vessels prior to inoculation/subculture. During flame sterilization the main problem is with alcohol, because it is inflammable and if spilled near a flame will cause an instant fire. Nowadays, in place of flame sterilization, dry sterilization of instruments using sterile pots is practiced in order to avoid instant fires.

3. Autoclaving

The culture vessels which may be empty (or) containing the medium are sterilized by heating in an autoclave (or) a pressure cooker to 121^{O+}C at a pressure of 15 pounds per square inch for 15 minutes for 20-50 ml medium. So the larger the volume of

the medium the more will be the time of sterilization. The pressure should not exceed 20 PSI as higher pressure may lead to the decomposition of carbohydrates and other components of the medium. Care should be taken such that all the culture vessels are sealed properly with cotton plugs. The autoclave should be opened only when its pressure gauge indicates zero pressure.

4. Filter Sterilization

Some growth regulators, like GA_3, Zeatin, ABA, Urea, some vitamins and enzymes thermo labile and may decompose during autoclaving. The solutions of these compounds are filter sterilized by passing through a membrane filter under positive pressure. A Millipore filter with a pore size of not more than 0.2 μm is generally used in filter sterilization. The membrane filter is held in a suitable assembly. The assembly together with the fitter is sterilized by autoclaving before use. The filter sterilized solutions are then combined with other nutrient substances which are sterilized in the autoclave and cooled, to give a complete medium. In the case of agar medium they are added when the medium has cooled to about $40^{O}C$.

Laminar air flow cabinets are used to create an aseptic working space by blowing filter sterilized air through an enclosed space which is opened on one side. The air is first filtered through a coarse pre filter to remove large particles, it is then passed through a HEPA (high efficiency particulate air) filter which filters out all particles larger than 0.3 μm. This sterilized air blows through the cabinet at 1.8 mg/hr which is sufficient to keep the enclosed working area aseptic.

5. Wiping with 70% Ethanol

The surfaces that cannot be sterilized by other techniques, viz. culture room platform of the laminar air flow cabinet and the hands of the operator are sterilized by wiping them thoroughly with 70% alcohol and the alcohol is allowed to dry. The culture rooms are initially cleaned by gently washing the floor and walls with a detergent. This is followed by wiping with 95% ethanol.

6. Surface Sterilization

All plant materials that are to be used for culture are treated with an appropriate sterilizing agent to inactivate the

microbes present on their surface. This is called surface sterilization. The excised piece of plant material that is used for culture is called explant. The surface sterilization protocol mainly depends on the source and the type of tissue of the explant which will determine the extent of contamination and tolerance to the sterilizing agent.

In the process of sterilization living materials should not loose their biological activity and only the bacterial (or) fungal contaminants should be eliminated. So, the plant organs or tissues are surface – sterilized by treatment with a disinfectant solution at suitable concentrations for a specific period. The most widely used disinfectants and their concentrations in the solution are given in the table.

- Hard explants are treated directly with disinfectants for example in the culture of mature seeds (or) mature endosperm, the whole seeds (or) re coated seeds are surface sterilized.

- An explant that carries a heavy load of microorganisms is to be washed in running tap water for 1-2 hr prior to its treatment with disinfectant solution.

- In case of the delicate tissues such as shoot apices, pollen grains and shoot or flower buds, ethyl alcohol (or) isopropyl alcohol are used for surface sterilization. These explants are given a rinse in 70% ethanol for few seconds and then left exposed in the sterile hood until the alcohol evaporates.

- Usually shoot apices (or) pollen grains are fee from micro contaminants and may be used for inoculation without surface sterilization.

Addition of a few drops of surfactants like Triton-X (or) tween – 80 to the solution (or) treating the plant material in a solution of cetavlon for 2 minutes before exposing to sterilant may enhance sterilization efficiency.

A general protocol for the sterilization of seeds will be as follows: Explants obtained from the field should be first thoroughly washed under running tap water to remove the dirt and epiphytotic microbes then they are treated with 1% cetarlon for 10 – 15 minutes to reduce bacterial contamination. Then they are rinsed with 70% ethyl alcohol for 30 sec. Then they are treated with 0.2% $HgCl_2$ for 10 – 15 minutes. This step is followed by a rinse with 70% ethanol and finally 4 – 6 rinses with sterilized distilled water. The entire protocol is carried out in an aseptic area provided by a laminar airflow cabinet.

Most of the explants are large and can be sterilized directly. But in the case of small explants like ovules, embryos, pollen grains etc., which are small, the organs containing them i.e. ovaries for ovules and ovarales (or) developing seeds for embryos are surface sterilized followed by which the desired explants are dissected out aseptically.

3.1.2.3 Summary

The various operations of tissue culture technique are to be carried out under aseptic conditions. Bacteria and fungi are the common contaminants that we will come across in *in vitro* cultures. The contaminants may also release metabolic wastes which are toxic to plant tissues. So, to avoid this the maintenance of complete aseptic environment in the culture vessel is very much essential.

To achieve this objective all the materials that are used in culture work must be freed from microbes. This can be achieved by following different methods of sterilization viz. dry heat, flame – sterilization, filter – sterilization, autoclaving, wiping with 70% ethanol and surface sterilization. Glass wares, Teflon plastic wares and instruments are sterilized by dry heat in an oven at 160 – 180^{O}C for 3 hours. Instruments like forceps, scalpels, needles etc. are flame sterilized by dipping them in 95% alcohol followed by flaming.

Culture vessels, culture medium, micro pipettes etc. are sterilized by autoclaving in an autoclave (or) Pressure Cooker at

121^{O}C temperature and 15 pounds per square inches pressure for 15 – 40 minutes basing on the volume of the medium.

Heat labile compounds such as growth regulators, urea, certain vitamins and enzymes are filter sterilized by passing their solution through a membrane filter of 0.45 μ (or) lower pore size.

Laminar flow cabinets are used to create an aseptic working space by blowing fitter sterilized air through an enclosed space.

The surfaces that cannot be sterilized by other techniques, viz. platform of laminar flow cabinet and the hands of the operator are sterilized by wiping them thoroughly with 70% alcohol.

All the plant materials that are to be used for culture are treated with an appropriate sterilizing agent to inactivate the microbes present on their surface. This is surface sterilization.

3.1.2.4 Model questions

1. What is sterilization? Discuss the different sterilization techniques used in plant tissue culture.
2. Write short notes on the following?
   (a) What is surface sterilization?
   (b) What is autoclaving?
   (c) What is filter sterilization?

3.1.2.5 Reference books

1. An introduction to plant tissue culture Dr.M.K. Razdan, Oxford & IBH publishing Co. Pvt., Ltd.
2. Plant cell and tissue culture, Narayana Swamy, Tata Mcgrow-hill.

3. An introduction to plant tissue culture, Dr.Kalyan Kumar De, New Central Book Agency.

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Lesson 3.1.3

CALLUS CULTURE

Objective

Objective

Callus is an undifferentiated mass of cells produced from isolated plant cells, tissues (or) organs when grown aseptically on the nutrient medium under controlled experimental conditions. In this chapter different aspects related to the callus viz. initiation, culture, maintenance, morphology, anatomy and other characteristics and its importance.

3.1.3.1 Introduction

Higher plant body is multicellular and different tissue systems present in different organs function in a highly coordinated manner. In nature, sometimes callus or callus like tissue is found to be formed in various parts of the plant either due to deep wound (or) due to some diseases. Deep wound in branches and trunks of the intact plants results in the formation of soft mass of unorganized parenchymatous tissue below the injured surface. Such callus tissue is known as wound callus. Wound callus is formed by the division of cambium tissue. The main function of such callus is to heal up the injured part of the plant. In some higher plants, some times callus like tissue masses are formed on the stem, leaf, root by the stimulation of cell division due to some diseases caused by fungi, bacteria, viruses, insects or genetic factors. Such – callus like out growths are known as galls (or) tumours. But the callus in tissue culture is produced from the excised plant tissues. The explants are cultured aseptically *invitro* under controlled condition on the nutrient medium. In culture, the excised plant tissue looses its structural integrity and changes completely to a rapidly proliferative unorganized mass of cells which is called the callus tissue. The development of callus tissue is first reported by a French scientist R.J.Gatheret (1934-1937) from the excised cambial tissue of Salix capraea and other woody species.

3.1.3.2 Principles of Calls Culture

For inducing the callus production, the explant is first inoculated in a flask containing proper nutrient medium. Then the culture flask is maintained properly by keeping it in a room where the temperature is maintained constant. This process is

called incubation. During incubation, the explant starts to produce callus tissue.

The important stages of callus induction are, inoculation of the explant, incubation of culture flasks, and formation of callus.

1. Inoculation of the explant

The culture room is sterilized properly before the worker enters the room. All the glassware are sterilized by autoclaving. The inoculation bench is sterilized by wiping it with ethyl alcohol. Thus the maintenance of aseptic condition inside the workroom is the first step in callus induction.

Then the sterilized medium is distributed in to the conical flasks and their mouths are plugged with sterilized cotton to prevent contamination. Then the surface sterilized explants are transferred to the semisolid medium in the flask and they are gently pressed in the medium. The pressing of the explants in the medium brings about good contact between the explant and the nutritional medium. The inoculation of the explants is done by keeping the mouths of the culture flasks near the flame of a spirit lamp. The mouths of the flasks are plugged with sterilized cotton.

2. Maintenance of Culture Flasks

The flasks inoculated with the explants are transferred in to a culture room where the temperature is maintained constant (or) in an incubator. The temperature of the incubator should be adjusted to 25 $\pm$ 2^{o}C which is favorable for callus production. The culture room should be provided with proper light and air for the normal growth and development of explants. So, the culture rooms must be fitted with florescent light to supply sufficient light to the flasks. But some people are carrying out this callus culture even in darkness.

3. Callus Formation

During the incubation period, the cells of the explant undergo growth and division, resulting in callus with in 3-8 days after incubation. The callus is an undifferentiated mass of actively growing parenchymatous cells. The cells are soft and they are tightly held and it is a brittle mass of cells which are liable to breakdown due to mechanical disturbance. During culture the radicle tip emerges first if the explant is kept horizontally on the nutrient medium and the shoot tip emerges first if the explant is placed vertically on the medium.

4. Proliferation of callus tissue

The well developed callus is cut in to small pieces with the help of a sterile knife. Then the callus pieces are transferred to another medium containing an altered composition of hormones. This medium induces the growth of some more callus tissues. This process of production of more callus tissues is called callus proliferation. The medium used for callus proliferation is known as proliferation medium. The callus tissues multiply more rapidly in this medium due to their fast growth and cell division. 2,4-D is a synthetic plant growth hormone which induces callus production in most of the tissues. 2,4-D is avoided in the proliferation medium.

The callus pieces are cultured in the medium containing growth hormones like IAA, NAA, Kinetin, 6-Benzyl aminopurine etc. IAA induces callus proliferation in dicots, but high concentration of IAA reduces callus formation in the monocots.

5. Sub-culture of Callus

After the proper growth of callus tissues, they are to be transferred to a fresh medium at regular intervals. This transfer helps to maintain the cells in a viable condition. During sub culturing the previously cultured tissues act as explant for establishing the secondary culture. This process of culture is known as subculture. Generally subcultures are practiced at a regular interval of every 4 weeks.

Fig-1

3.1.3.3 How the callus tissue is formed

Formation of callus tissue is the out come of cell expansion and division of the cells of the explant. During the formation of callus tissue, the explant looses its original characterstic. Under the influence of exogenously supplied growth hormone, cell enlargement and cell division will take place and forms an unorganized mass of cells. As a result, the explant undergoes an irreversible change in its shape, size, symmetry, structural organization and cellular integrity.

For the initiation of callus culture, tissues from young seedling (or) from juvenile part of the mature plant are generally taken. In such tissue a growth momentum is already present among the cells due to the presence of maximum number of physiologically active meristematic cells. There will be a nutrient gradient among the different cells of the explant basing on their position in the explant with reference to the nutrient medium. So, the cells divide asynchronously depending upon the availability of the nutrient and hormones and the callus tissue is formed. With in few days after inoculation either enlargement in size (or) the swelling followed by rupture of the tissue takes place, basing on the type of explant viz. Leaf, stem segment, root segment, anther etc. This change indicates the response of explant for callus formation, which will be followed by the appearance of little irregular cellular masses around the cut edges (or) from the ruptured surface. It is explained that the formation of cellular mass particularly at the cut end first may be due to injury during excision. Some endogenous growth substances oozes out through the injured tissue at the cut end and stimulates the cell division which is simultaneously induced by the exogenously supplied hormones.

There is another explanation that both endogenous product and exogenous hormones make a threshold level and their interaction results in the formation of an unorganized cell mass growth at the extend. It is generally accepted that exogenous hormones play the major role for the formation of callus in tissue culture. Both auxin and cytokinin are required for indefinite growth and cell division in callus culture.

When the explant are cultured on a suitable medium, many of its cells undergo division. Mature and differentiated cells like parenchyma and collenchyma cells undergo changes to become meristematic. This is called dedifferentiation. During the process of dedifferentiation there will be renewed and enhanced RNA and protein synthesis leading to the formation of new cellular components needed for meristematic activity.

Regarding the growth regulators, some times only 2,4-D (Auxin) alone is sufficient for callus formation. Auxins are required for growth and cytokinins are required for cell division. This concept is applicable for callus formation also.

After the initiation of visible unorganized mass of cells at the cut end, gradually the whole tissue is involved in callus formation therefore, initially the cell divisions are confined to the cut ends, but gradually it covers the entire surface of the explant. The callus tissue gradually increases in mass as the new cells are added by mitosis. Estimation of callus growth can be made on the basis of changes in fresh weight (or) dry weight.

The type of tissue (or) cell present in the explant is an important factor for the rapid formation of callus tissue. If any meristematic tissue is present in the explant such as vascular cambium, the dividing cells of the vascular cambium continue the rapid growth under *invitro* condition, and proliferate into callus tissue. The vacuolated and highly differentiated cells of pith and cortex are also stimulated to divide and proliferate in the irregular mass of cells which spread over most of the surface and inner part of the explant.

3.1.3.4 Morphology, Anatomy and other characterstics of Callus Tissue

Morphology

Callus tissue proliferates as an irregular mass of cells. It is very difficult to describe its external morphology. The calli derived from different plants look alike. But they can be distinguished on the basis of other characteristics texture, colouration, hormone requirements etc. So the calli initiated

from explants of the some plant species may show considerable variation.

Anatomy

The anatomical structure of callus tissue is revealed by light microscopy and electron microscopy.

Microtome section (or) squash preparation of the callus tissue shows that the cellular composition of callus tissue is extremely heterogenous ranging from small cells with dense cytoplasm to large cells with vacuolated cytoplasm. The shape of the cells with in the callers tissue varies from spherical to markedly elongated. Large elongated cells are generally non-dividing cells having a large central vacuole where as the small actively dividing cells are with dense cytoplasm and small vacuoles. Elongated cells with in the callus tissue may be differentiated into lignified xylem trachieds (or) phloem – like cells. Formation of xylem and phloem with in the callus tissue is known as cytodifferentiation. It is also observed that some localized groups of meristematic cells, constituting the active loci will arise in the callus tissue. These are called meristemoids. The meristemoids may differentiate into either shoot (or) root primordia (or) embryoids.

Electron microscope reveals that the cells at their non-dividing state have a large central vacuole and thin peripheral cytoplasm. The no. of organellae is also minimum in the cytoplasm. The synthesis of new cytoplasm and reduction in the size of the central vacuole takes place when the cells enter into the dividing state. There is an increase in the no. of endoplasmic reticulum, mitochondria, golgibodies and ribosomes. The endo plasmic reticulum occurs as sheets running parallel with the cytoplasm. Microtubules are associated with the walls of actively growing cells. Cells from the green coloured zone of the callus contains chloroplasts, but the grana are poorly developed. Mostly the plastids act as amyloplasts. Cultured cells contain membranous myelin like bodies and membrane bound groups of vesicles which are known as multivesicular bodies.

Other Characteristics

Texture

Callus tissue shows variability in its appearance and texture. Basing on its texture callus tissue can be divided into two categories viz. soft callus and hard callus. Soft callus is friagile in nature and is made up of heterogenous mass of cells having minimal contact. Hard callus consists of closely packed cells. It is compact in nature.

Colouration

Generally callus tissue is creamish yellow (or) white in colour. Sometimes it may be pigmented. Pigmentation may be uniform or patchy. It may be green in colour, sometimes white callus tissue grown under dark conditions turns green after transferring it to light. Green colour develops due to the development of chloroplast in the cells of callus tissue Eg:- Callus tissue from the cotyledons of Soyabean.

Callus may be yellow due to the synthesis of carotenoid pigments. Eg:- Callus tissue of *Nigella sativa* grown under dark condition.

Purple coloured callus is reported from cauliflower cultures due to the accumulation of anthocyanin in the vacuoles (or) due to the production of DOPA (3,4 – dihydroxy phenyl alanine).

Brown colour frequently develops in the explant and subsequently in the callus tissue. This is due to the excretion of phenolic substances. Plant tissues contain large no. of phenolic compounds and also contains polyphenol oxidase which is specially separated from the phenols. When the plant tissue is cut during explant preparation, the enzyme comes in contact with phenols which are then oxidized to quinines. These quinones will subsequently polymerize to form brown products. The formation of quinines is responsible for the browning of the callus tissue. Excretion of phenols inhibit the growth of callus tissue.

3.1.3.5 Habituation of Callus

Generally callus tissue needs growth hormones in the nutrient medium in order to grow as long as they are maintained

through serial sub-cultures. It has been observed that callus tissue in some plant species may become habituated after prolonged culture. So, the callus tissue is able to grow on a standard maintenance medium or basal medium which is devoid of growth hormones. This property of the callus tissue is known as habituation and that callus tissue is known as habituated callus. The actual cause of this habituation is not clearly known. But, it is suggested that the cells in habituated callus tissue appear to have developed the capacity to synthesise adequate amounts of auxins and cytokinins which may account for their independence of exogenously supplied hormones. Habituated callus tissue cannot be distinguished from the normal callus tissue except in their hormone independence.

The plant tumour tissue can be isolated from the plant and cultured aseptically. In culture, the tumour tissue is capable of growing on simple basal medium i.e. hormone free medium like the habituated callus. These tissues differ from the habituated callus in their mode of origin. In case of wound tumour, the virus remains with in the cells and multiplies and it may disappear after prolonged periods of culture.

In crowngall tumour tissue probably, the presence of bacterial DNA in the genome of the crown gall tumour cells may make them hormone – independent.

3.1.3.6 Chromosomal variation in callus tissue

Chromosomal variation in callus may occur genetically (or) epigenetically in the cells of the callus tissue.

1. Genetical basis of chromosomal variation

Callus tissue is raised from root, shoot, leaves and other organs. These explants are made up of numerous cells which remain in different states of differentiation. Normally, the meristematic diploid cells undergo selective division for the growth of an organ. Some times endoreduplication occurs frequently in the differentiated tissues of higher plants and the endoreduplicated cells remain in mitotically blocked condition.

The degree of endoreduplication depends upon the degree of cellular differentiation. Therefore, the genomic constitution becomes heterogenous in the original explant. The callus tissue may get such genomic heterogeneity due to non-selective induction of asynchronous division of both diploid and endoreduplicated cells. So, the pe-existing genomic heterogeneity of the explant may be a source chromosomal variation in the callus tissue. This variation of chromosomal number ranges from aneuploidy to different levels of polyploidy viz. tetraploid, hexaploid and so on. Occurrence of both haploid and different level of polyploid cells in the same callus tissue is known as mixoploid cell population.

2. Epigenetic basis of Chromosomal Variation

At the early stage of callus growth the percentage of diploid cells is higher than that of the polyploid cells. The no. of polyploid cells may increase (or) decrease through serial subcultures. All the meristematic cells of the callus are expected to be diploid. But, the callus tissue derived from meristem may also show variation in chromosome number. The variation generated during tissue culture is called epigenetic variation. Eg:- In the plant Crepis, cellular differentiation occurs without endoduplication. So, all the cells of the explants taken form that plant are diploid. But the cells of the callus tissue derived from such plant are not diploid. Thus it appears that the variation in chromosome number is not always derived from the original explant. But it may arise due to the interaction of both genome and cytoplasm. Their interaction may bring about mitotic disturbance. In pea plants it is found that kinetin encouraged the development of polyploid cells in culture. It is also observed that strong auxins like 2,4-D induces the polyploidy in callus culture. It is also found that prolonged sub-culture may lead to the establishment of one karyotype and others are gradually eliminated. In most of the cases polyploid cells are found.

Sometimes structural changes of chromosomes like deletion, translocation etc. may occur in culture. Cells of the callus tissue may be haploid if it is derived from microscope culture. An ideal callus culture is characterized by the possession of numerical (or) structural stability in long term culture but it is very rare.

3.1.3.7 Significance of Callus Culture

1. The whole plant can be regenerated from callus tissue through the manipulation of nutrient and hormonal constituents. This phenomenon is known as plant regeneration (or) organogenesis (or) morphogenesis.

2. A large no. of embryos can be produced, from the somatic cells of the callus tissue by manipulating the nutrient and hormonal constituents.

3. Callus tissue is a good source of genetic variability. So, it may be possible to regenerate a plant from genetically variable cells of the callus tissue.

4. From the callus tissue we can initiate cell suspension culture.

5. Callus culture is very much useful to obtain commercially important secondary metabolites. Eg:- If an explant from a medicinally important plant is grown invitro, and the callus is produced, then the secondary metabolites (or) drug can be directly extracted from the callus tissue without sacrificing the whole plant. So, this technique is an alternative method for the conservation of medicinal plants in nature.

6. Many biochemical assays can be performed from callus culture.

3.1.3.8 Summary

Callus is an unorganized proliferative mass of cells without any differentiation. In nature, sometimes callus (or) callus like structure is found to be formed in various parts of intact plant either due to deep wound (or) due to some diseases. Callus tissue developed from the injured part of the plant is known as wound callus. It is formed by the division of either cortical cells (or) cambial cells. Callus – like out growth caused by some diseases is commonly called gall (or) tumour. The callus tissue which is important to plant tissue culture, is produced through tissue culture technique from a small explant of the plant. The

explant is cultured aseptically on a solid (or) liquid nutrient medium under controlled conditions.

For successful initiation of callus tissue from the explants, three important criteria should be accomplished. 1. Aseptic prepartion of the plant material. 2. Selection of suitable nutrient medium with growth regulators. 3. Incubation of culture under controlled physical conditions. The callus grows in presence of artificial light (2,000 – 3,000 lux for 16 hrs) or in complete dark conditions. The suitable temperature for the growth of callus tissue is usually $25 \pm 2^{O}C$. Generally 55% to 60% relative humidity is maintained in the culture room. Callus culture can be maintained continuously by serial sub-cultures.

The callus formation is an outcome of cell expansion and cell division of the explant. Under the influence of exogenourly supplied hormones, the explant is triggered off a growth sequence in which cell enlargement and cell division predominate to form an unorganized mass of cells. Both the endogenously produced and exogenously added hormones make a threshold level and their interaction results in the formation of an unorganized mass of cells from the cut end of the explant. There is a general concept that auxin is required for cell growth and cytokinin for cell division. This is applicable for invitro callus formation callus tissue generally increases in mass as the new cells are added by mitosis.

Callus shows variation appearance and texture. The shape of individual cells of the callus mass ranges from nearly spherical to elongated. Sometimes callus mass undergoes differentiation and forms phloem cell and lignified xylem cells. Generally the callus tissue is creamish yellow or white in colour. Sometimes callus tissue may be pigmented. Brown colour develops frequently in the explant and subsequently in the callus tissue due to the excretion of phenolic substances.

In some plants species the callus tissue may become habituated to grow on the culture medium which is devoid of hormones, after prolonged culture. Callus tissue derived from the plant tumour tissue (or) crown gall tissue is also capable of growing on growing on simple basal medium like the habituated callus.

Chromosomal variation may occur genetically (or) epigenetically in the cells of the callus tissue. Sometimes numerical (or) structural changes of chromosomes may occur in culture.

The whole plants can be regenerated from the callus tissue through the manipulation of nutrient and hormonal constituents in the culture medium, embryoids can also be produced by the callus by the process of `somatic embryo genesis'.

Callus tissue is a good source of genetic variability, so it may be possible to regenerate the plant lets from the cells showing genetic variability. Callus culture is very important to obtain commertially important secondary metabolites.

3.1.3.9 Model Questions

1. What is callus tissue? Describe the principle and discuss the significance of callus culture?

2. How the callus tissue is formed *invitro*? Discuss the morphology, internal structure and other characterstics of callus tissue.

3. Write short notes on the following:

   (a) What is habituated callus tissue? How is it formed?

   (b) What are the main reasons of chromosomal variation in callus tissue? State its significance in callus culture?

   (c) Why do the explant and the callus tissue of some plants develop brown colour in culture?

4. Write short notes on the following :

   (a) Internal structure of callus tissue.

   (b) Significance of callus culture.

   (c) Difference between soft and compact callus.

3.1.3.10 Reference books

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Lesson 3.1.4

CELL SUSPENSION AND SINGLE CELL CULTURES

Objective

3.1.4.1 Introduction

3.1.4.2 Suspension Cultures

1. Isolation of Single Cells
2. Types of Suspension Cultures
3. Culture Medium for Suspension Cultures
4. Conditioning of Medium
5. Agitation of Medium
6. Synchronization of Suspension Cultures
7. Growth Patterns in Suspension Cultures
8. Measurement of Growth in Suspension Cultures
9. How to test the viability of Cultured Cells
10. Importance of Suspension Cultures

3.1.4.3 Single Cell Culture

1. Principle of Single Cell Culture
2. Methods of Single Cell Culture
3. Factors Effecting Single Cell Culture
4. Importance of Single Cell Culture

3.1.3.4 Summary

3.1.3.5 Self Assessment Test

3.1.3.6 References

Objective

Cell suspension and single cell culture provides an opportunity to investigate the properties and potentialities of

plant cells. In this chapter different aspects related to those cultures Viz. basic principles of culture, different types of cell suspension cultures, methods of single cell culture, growth pattern and estimation of growth in suspension cultures, test for cell viability, different factors effecting the cultures and the applications are discussed.

3.1.4.1 Introduction

Callus proliferates as an unorganized mass of cell. So it is very different to follow many cellular events during its growth and developmental phases. To overcome such limitations of callus culture, the cultivation of free cells and small cellular aggregates in a chemically defined liquid medium as a suspension was initiated to study the morphological and biochemical changes during their growth and developmental phases.

Cell suspension cultures were first reported by W.H. Muir (1953) from the callus of Tagetes erecta and Nicotiana tabacum. Later in 1956 F.C. Steward and E.M.Shantz reported the suspension cultures from carrot root explant and obtained very large no. of plant lets from the culture.

Establishment of cell suspension and single cell cultures provide an opportunity to investigate the properties and potentialities of plant cells. Single cell systems have a great potential for crop improvement. Through the cell culture of an intact organ (or) whole plant we can synthesize natural products. Free cells in culture permit quick administration and withdrawal of diverse chemicals. So, the free cell cultures are the easy targets for mutant selection.

During cell culture variability arises. Such variability is called `special heterogeneity'. This is very important because the differences between the cells in their karyotype and the ability to accumulate secondary metabolites, would manifest during morphogenesis in the clones regenerated from single cells. Thus the cell line selection technique can be applied to produce high yielding cultures and also the plants with superior agronomic traits.

3.1.4.2 Isolation of Single Cells for the Initiation of cell suspension and single cell culture

For the initiation of suspension and single cell culture we should be provided with single cells, which can be obtained from the pre established callus (or) from the explant directly. Single cells can be isolated either from the cultured tissues (or) from the plant organs.

1. Isolation of single cells from plant organs

The most suitable material for the isolation of single cells is the leaf tissue. From the leaf the single cells can be isolated using mechanical (or) enzymatic method.

(a) Mechanical Method

The procedure for mechanical method involves mild maceration of 10 gms of leaves in 40 ml of the grinding medium (20 μ mol sucrose, 10 μ mol $mgcl_2$) and 20 μ mol tris –Hcl buffer pH 7.8) with a mortor and pestle. The homogenate is filtered through two layers of muslin cloth and the cells released thus are washed by centrifugation at low speed using the same medium. Pipette out the supernatant and suspend the cells in a volume of the medium sufficient to achieve the required cell density.

(b) Enzymatic Method

Takebe in 1968, treated the tobacco leaf with pectinase and retained a large no. of metabolically active cells. Potassium dextran sulphate added to the enzyme mixture improved the yield of free cells. Isolation of single cells by the enzymatic method gives high yields from the preparations of spongy parenchyma with minimum injury to the cells. This can be accomplished by providing osmotic protection to the cells while the enzyme macerozyme degrades the middle lamella and cell wall of parenchymatous tissue.

2. From Cultured Tissue

This is the most widely followed approach to obtain a single cell system from cultured tissues. Surface sterilized explants are

placed on a nutrient medium consisting of a suitable proportion of auxins and cytokinins to initiate cultures. Then the explant starts callusing from its cut ends, which gradually extends to the entire surface of the tissue. The callus is separated from the explant and transferred to a fresh medium to get more amount of callus tissue. Repeated subculture on agar medium improves the friability of the callus. Friability is prerequisite for raising a fine cell suspension in a liquid medium. Pieces of undifferentiated and friable calli are transferred in to liquid medium dispensed in autoclaved flasks, which is continuously agitated in autoclaved flasks, which is continuously agitated to obtain a **Suspension Culture**. Agitation of pieces breaks them into free cells and small aggregates and also maintains uniform distribution of cells and cell clumps in the medium. Agitation also allows gaseous exchange between the culture medium and culture air.

Fig-1

3.1.4.3 Suspension Culture

It is a type of culture in which single cells (or) small aggregates of cells are suspended in agitated liquid medium and allowed to multiply.

Principle: It is very different to follow many cellular events during its growth and development through callus culture. So, the cultivation of free cells as well as small aggregates of cells in a chemically defined liquid medium as a suspension allows to study the morphological and biochemical changes during their growth and development.

Note: The suspension culture can be initiated from pre established callus (or) from the explant directly which is continuously agitated in a liquid medium on a Rotating Shaker. Agitation facilitates proper aeration and uniform distribution of nutrients in the medium and also allows the callus to break up.

An ideal cell suspension can be obtained by transferring a more friable callus to agitated liquid medium. It breaks up and

disperses into single cells and small cell aggregates. Eliminate the large callus pieces and transfer only single cells and small cell aggregates to fresh medium. After two (or) three weeks a suspension of actively growing cells is produced. The suspension cultures are clonally maintained by the routine transfer of cells in the early stationary phase to a fresh medium.

An ideal culture should consist of only single cells which are physiologically and bio chemically uniform. It can be achieve through synchronization of suspension culture. A synchronous culture is one in which the majority of cells proceed through each phase of the cell cycle simultaeneously (G_1, S, G_2, α M), More friable callus is an ideal material for dispersion of cells. The dispersion of less friable callus can be improved by increasing the concentration of auxins (or) by adding low concentrations of cellulase and pectinase enzymes to the liquid medium.

The time interval taken for the development of suspension culture from the callus tissue is called initiation passage. During this passage the callus tissue breaks up and the cells grow and divide until the depletion of some nutrient in the medium.

The suspension culture that is obtained at the end of the initiation passage is filtered through nylon mesh to remove larger pieces of callus tissue. Now the filtrate containing single cells and small aggregates is transferred in to a fresh liquid medium to initiate passage I suspension. In the subsequent passages the cell suspension is sub-cultured by pipetting aliquots of the suspension in to fresh culture medium.

3.1.4.2.1 Types of suspension culture

The suspension cultures are broadly classified as, 1. Batch cultures 2. Continuous cultures and 3. Immobilized cultures.

3.1.4.2.2 Culture medium for suspension cultures

Generally, the media suitable for growing callus culture for a particular species is also suitable for growing suspension culture of that particular species provided such that the agar is

omitted. In some cases in concentration of auxin and cytokinins is often critical for the growth of cell suspension culture and the concentration of auxins and cytokinins used for callus culture is generally reduced for suspension cultures. But in some cases it differs. The culture medium for tobacco cell suspension requires an increase in the concentration of 2,4-D from 0.3 mg/lit to 2 mg/lit, followed by supplementing the callus medium with additional vitamins and case in hydrolysate. The inorganic phosphate is rapidly utilized in the actively growing suspension cultures and consequently it becomes a limiting factor.

Many media have very little buffering capacity and the pH can change with an increase in the cell biomass. This necessitates monitoring and adjustment of pH in suspension culture. B_5 and ER (Erikson – 1965) media specially recommended for the suspension culture of higher plants. These media are used for initial population density 5 x 10^4 cell/ml (or) more. With lower cell density, the medium requires to be conditioned (or) enriched with various other compounds.

3.1.4.3.2.2. Conditioning of Medium

A conditioned medium is a medium in which plant cells have been grown for about 48 hrs and the cells are then filtered out. For the initiation of cell suspension cultures at a low inoculum density a conditioned medium is used. A conditioned medium can be retained by filtering out the cells growing at high density from 4-6 week old liquid culture. This medium can be used in drops (or) as thin layers to culture cells at low population density/single cells. In 1989 – Torres gave the principle of conditioning. This principle involves the separation of a high density cell culture from a low density culture medium by a barrier that permits the diffusion of solutes and air. A high density cell suspension kept inside a dialysis tube is suspended by means of a thread in the flask containing the low cell density culture. The metabolites produced by the nurse culture (high density culture) diffuse in to the low density medium and increases the growth of low cell density. This meets the conditions of growth for low cell density populations since the necessary substances that may not be found in the low density medium are released in to it by the biosynthetic activity of the nurse cells.

3.1.4.3.2.1 Agitation of the Medium

Suspension cultures require constant agitation of the medium for adequate aeration. This also facilitates dispersion of cells. This is done by using a shaker and suitable flaks. In 1953 Muir introduced the orbital platform shaker for growing suspension cultures of tobacco and Tagetes eracta. The platform of the shaker is fitted with interchangeable clips for holding the flaks. Generally a shaking speed of 30-150 rpm is optimum for most of the tissue. Rotary shakers are also used which can rotate at slow speed (1-2 rpm) and they will have a disc which can be rotated by a shaft. Both orbital and rotary shakers have a control for regulating the speed.

3.1.4.3.3. Synchronization of Suspension Cultures

Cells in suspension cultures vary greatly in size, shape, DNA and nuclear content. The cell cycle time also varies with in individual cells. So, the cell cultures are mostly a synchronous. This variation complicates the study of biochemical, genetic physiological aspects of cell metabolism. So, it is essential to manipulate the growth conditions in an asynchronous culture to achieve high degree of synchronization. In a synchronous culture all the cells will be in the same phase of cell cycle (G_1, S, G_2 x M). Synchronisation is expressed as percentage synchrony of cells in suspension cultures. Synchronization can be achieved by physical and chemical methods.

3.1.4.3.3.1. Physical Methods

Physical properties of cells like the size of individual cells (or) growth environmental conditions like light and temperature are monitored to achieve synchronization. Some of them are,

A. Selection by Volume

Synchronization may be achieved by selecting on the basis of size of cell aggregates. This method was proved successful for carrot suspension cultures, where 90% of cell aggregates that are isolated were in early embryonic stages.

B. Temperature Shock

Low temperature shocks combined with nutrient starvation are found to induce synchronization of suspension cultures.

3.1.4.3.3.2. Chemical Methods

Cell cultures are starved of a nutrient (or) supplied with a biochemical inhibitor to prevent the cells from completing a cell cycle. Through this approach the cells are first arrested at a particular stage of cell cycle and then they are allowed to undergo division simultaeneously either by supplementing the starved chemical (or) by withdrawing the inhibition.

A. Starvation

This is based on depriving the suspension cultures from an essential growth compound leading to a stationary growth phase. Resupply of the missing compound induces the resumption of synchronous cell growth. This procedure has been found to be effective in Acer pseudoplatanus suspension cultures.

In the cultures which are starved of nitrogen, phosphorus (or) carbonate, the arrest of cell growth during the G_1 (or) G_2 phase of cell cycle is observed. After a period of starvation, when these growth limiting compounds are supplied to the medium, the stationary cells undergo synchronous division. Growth hormone starvation is also reported to induce synchronization of cell cultures.

B. Inhibition

Synchronization can be temporarily achieved by blocking the progression of events of cell cycle so that all the cells will be arrested in a specific phase, using a biochemical inhibitor. After releasing this biochemical block synchronous divisions can be achieved. This procedure is used in Tobacco, Tomato and Soya bean. Inhibitors of DNA synthesis like 5-aminouracil, Fud R-Fluoro deoxy uracil (or) 5-fluro deoxy purine, hydroxy urea, TDR (or) excess thymidine will bring the cells to G_1/S boundary. After the removal of the inhibitor synchronous divisions will take place.

C. Mitotic Arrest

Colchicine arrests the cell division at metaphase. Suspension cultures in exponential growth are supplied with 0.02% (W/V) colchicine for 4-8 hours. It inhibits spindle formation. Longer colchicine treatment may lead to an increased frequency of abnormal mitosis and chromosome stickiness. So, only shorter duration of treatment is recommended. This technique is used in case of zea maize. As colchicines can induce genomic changes, there is also possibility of obtaining asynchronous cultures.

3.1.4.3.5. Measurement of growth in suspension cultures

Growth assessment in cell suspension cultures can be accomplished by following selected parameters at regular intervals. These include; (a) cell counting, (b) packed cell volume and (c) Fresh/dry weight increase of cells and cell colonies.

1. Cell Counting

It is a more accurate measure to determine the growth of cultures. Increase in cell number depends on the rate of mitotic index (MI) of cells in suspension cultures. Determination of cell number is a tedious procedure since suspension cultures carry cell aggregates. There are two methods generally employed for macerating the cell masses found in culture, They are 1. Chromic trioxide treatment, 2. Pectinase enzyme treatment.

1. Chromic Trioxide Treatment

1. A small volume of the sample suspension is treated with anaqueous chromic trioxide solution. (2 parts of the chromic trioxidet 1 part of sample).

2. The test solution is heated to about 70^{O}C for 2-15 min.

3. Then the suspension is allowed to cool by keeping it in a water bath.

4. The cooled test suspension is shaken well (or) agitated on a shaker to separate the individual cells from the mass of cells.

5. The suspension is now centrifuged, the chromic acid is poured off and the pellet is resuspended in 8% Nacl solution.

6. After 10-15 min the free cells are counted on a naemocytometer.

2. Pectinase Enzyme Treatment

In this method the sample suspension is treated with 0.25% pectinase enzyme which dissolves the middle lamella present in between the two adjacent cells. As a result, the individual cells are released from the cell mass. Then the suspension is used to count the cell number.

Packed Cell Volume

It is a more effective and the easiest method for the determination of cell growth in the suspension culture. For the determination of packed cell volume, 10 ml of culture suspension is transferred to a graduated measuring tube. This sample is centrifuged for favouring the deposition of cells at the bottom of the tube. After the settlement of all cells in the tube, the total volume of packed cells can be measured against the readings of the graduated measuring tube. The packed cell volume is expressed as ml of cell pellet per ml of culture.

Cell Fresh Weight

The total weight of cells in the suspension depends on the weight of all individual cells and the number of cells found in the suspension. The weight of cells in the sample can be measured by using the following procedure :

1. A desired volume of the sample is filtered through whattman no.1 filter paper with the help of Buchnar funnel under slight vacuum conditions. After the completion of filtration, only the cells will remain on the filter paper.

2. The cells that remain on the filter paper are washed properly with distilled water and again filtered through the filter paper and the excess water found along with the cells is allowed to drain-off.

3. The filter paper containing the cells is weighed properly with the help of a sensitive balance.

4. The weight of a wet filter paper alone is deducted from the weight of the filter paper with cells. The resulting value gives us the total weight of living cells found in the sample.

5. The weight of cells in the sample can be expressed in grams/liter. This weight is calculated with reference to the total volume of culture suspension.

Cell Dry Weight

A procedure similar to that for fresh weights is followed for determining cell dry weight except that the filter paper is dried in an oven for 12 hours at $60^{O}C$. After cooling in a desiccator containing silica gel, the dried filter is reweighed the cell weight is expressed as gms/litre of culture.

3.1.4.3.6. How to test the viability of cultured cells

The growth of cultures is largely dependent on the viability of the cells. There are different methods which help to decide whether the cultured plant cells in the medium are alive (or) not. This testing of viability is necessary to know the living cells for proceeding to further steps in plant tissue culture. The different methods used for testing cell viability are as follows :

1. Phase Contrast Microscopy

The cell viability can be determined by direct observation of cells under a phase – contrast microscope. Cytoplasmic streaming and the present of healthy nucleus indicate that the cells are viable. Phase contrast microscopy is recommended as it is different to observe these aspects in unstained cells under a bright field.

2. Reduction of Tetrazolium Salts

This method is used to measure the respiratory efficiency of living cells. The cell masses are stained with 2,3,5-tri-phenyl tetrazolium chloride (TTC). Living cells reduces TTC to formazon

which gives red colour. Formazon can be extracted and measured spectrophotometrically.

3. Fluorescein Diacetate (FDA) Method

This method offers quick visual assessment of the viability of cells. Stock solution of FDA (0.5% w/v) is prepared in acetone and stored at $O^{O}C$. The cells are treated with 0.01% solution of FDA. Live cells cleave FDA by esterase activity and produce fluorescein, which cannot cross the plasma membrane and it accumulates mainly in the cytoplasm of the living cells. What UV exposure, fluorescein gives green fluorescence so that the cells appear green. In deed cells the fluorescein is lost and they do not fluoresce.

4. Evan's Blue Staining

A dilute solution of (0.025%) Evan's blue dye stains the dead (or) damaged cells. It freely enters the damaged cells and it is not taken by live cells. So, the dead cells stain blue and the living cells remain unstained. Evan's blue is usually taken in conjunction with FDA.

3.1.4.3.3. Growth Patterns in Suspension Culture

Under appropriate light, temperature, aeration and nutrient medium the growth of suspension culture follows a growth curve. The growth of suspension culture can be monitored by counting the cell number per unit volume of culture in relation to days of culture. From growth curve for a typical higher plant suspension culture consists of lag phase, logarithmic (or) exponential phase, linear phase and stationary phase.

A lag phase is the period where the cells adjust themselves to the nutrient medium and prepares for cell division. This is followed by very rapid cell division, causing a logarithmic increase in cell number. This phase is called logarithmic phase. It will be followed by a further period of rapid cell division, which results in a linear increase in number. This phase is called linear phase. As the nutrients are depleted and some of the cells of the culture show senescent characteristics, the rate of cell

division with in the culture declines and it passes through the stationary phase. At this stage the growth curve forms a plateau. If the cells are removed just before (or) just after the entry in to the stationary phase in each growth cycle and are sub cultured to fresh medium, then identical patterns of growth of the cell line can be maintained in each culture passage. Dry weight, total protein, DNA synthesis etc. can also be considered as other parameters for the preparation of identical growth curves. The chemical composition of the cell changes throughout the growth cycle and such changes are closely coupled to the cell division in most of the plant material. However in some materials there is no correlation between the rate of cell division and the rate of dry weight accumulation. From these studies, it has been concluded that there are independent mechanisms and many biosynthetic pathways for controlling cell division. In synchronized cell populations, continuous changes in physiological property may also cause the divergence between the rate of cell division and the biochemical changes of the cell. It is also important to note that the degree of cellular aggregation is not constant but changes significantly during the growth cycle of the cell suspension culture. As the culture enters the period of most active growth, the cell aggregation is maximum and during the stationary phase cell aggregation is minimum.

For experimental studies on the growth of cell suspension the inoculum cell density is an important factor. Very low density (or) high density of cells in liquid medium are unable to grow. So, to induce the growth an initial cell density of 2×10^6 to 2×10^8 cells/ml are inoculated in liquid medium. This initial cell density increases during the growth and attains a higher density at the stationary phase. The particular initial cell density that is able to grow in liquid medium is called critical initial density (CID). The CID may vary from plant to plant.

3.1.4.3.1. Types of Suspension Culture

The suspension cultures are broadly classified as, 1. Batch Cultures, 2. Continuous Cultures and 3. Immobilized Cultures.

1. Batch Culture

In the batch culture, the cells are nourished in a fixed volume of the medium and the amount of cell material increases until the nutrient elements are depleted and inhibitory substances get accumulated; as a result of which the growth ceases. In a batch culture the same medium and all the cells produced are retained in the culture vessel. The culture vessels may be culture flasks (or) fermenters. The cell number (or) biomass of a batch culture exhibits a typical sigmoidal curve, having a log phase during which the cell number remains unchanged, followed by a logarithmic phase (log phase) during these is a rapid increase in cell number and finally ending in a stationary phase during which cell number does not change. The log phase duration mainly depends on inoculum size and growth phase of the culture from which inoculum is taken. The log phase lasts for 3-4 cell generations. The duration of a cell generation may vary from 22-48 hours which depends upon the plant species. The stationary phase is forced on the culture by a depletion of the nutrients and also due to accumulation of cellular wastes. If the culture is kept in stationary phase for a prolonged period the cells may die.

Batch cultures are maintained by sub-culturing. They are used for the initiation of cell suspensions which may be used for cloning, cell selection (or) as seed cultures for scaling up (or) for continuous cultures. Batch cultures are chracterised by a constant change in the pattern of cell growth and metabolism and there will be a constant change in cell density and nutritional status of the medium. So, they are not ideal systems for studies on cell growth and metabolism. Batch suspension cultures are most commonly maintained in conical flask incubated on orbital platform shakers at the speed of 80-120 rpm.

1. Slowly Rotating Cultures

Single cells and cell aggregates are grown in a specially designed flask, the nipple flask. Each nipple flask possesses eight nipple – like projections. The capacity of each flask is 250 ml. There are 10 nipple flasks which are loaded in a circular manner on the large flat-disc of a vertical shaker. When the flat disc rotates at a speed of 1-2 rpm, the cells with in each nipple

of the flask are bathed in culture medium and exposed to air alternately.

Fig-2

2. Shake Cultures

It is a simple and effective system of suspension culture. In this method, single cells and cell aggregates present in a fixed volume of liquid medium are taken in conical flasks. The conical flasks are mounted on a horizontal large square plate of an orbital platform shaker. The square plate moves by a circular motion at a speed of 60-180 rpm.

Fig-3

3. Spinning Cultures

Large volumes of cell suspension may be cultures in 10 litre bottles which are rotated in a culture spinner at 120 rpm at an angle of 45^{O}. Here the culture vessel is rotated.

Fig-4

4. Stirred Culture

This system is used for large scale batch culture of 1.5 to 10 litres. Here the large culture vessel is not rotated but the cell suspension inside the vessel is kept dispersed continuously by bubbling sterile air through the culture medium. An internal magnetic stirrer is the most convenient way to agitate the culture medium safety. The magnetic stirrer revolves at 200 – 600 rpm. The culture vessel is a 5-10 litres round – bottom flask.

Fig-5

3.1.4.3.1.2. Continuous Cultures

In continuous culture, the cell population is maintained in a steady state by regularly replacing a portion of the spent medium by fresh medium. There are two types of continuous culture systems. Viz. closed type and open type. In a closed continuous culture, the cells are separated from the used medium that is taken out for replacement and they are added

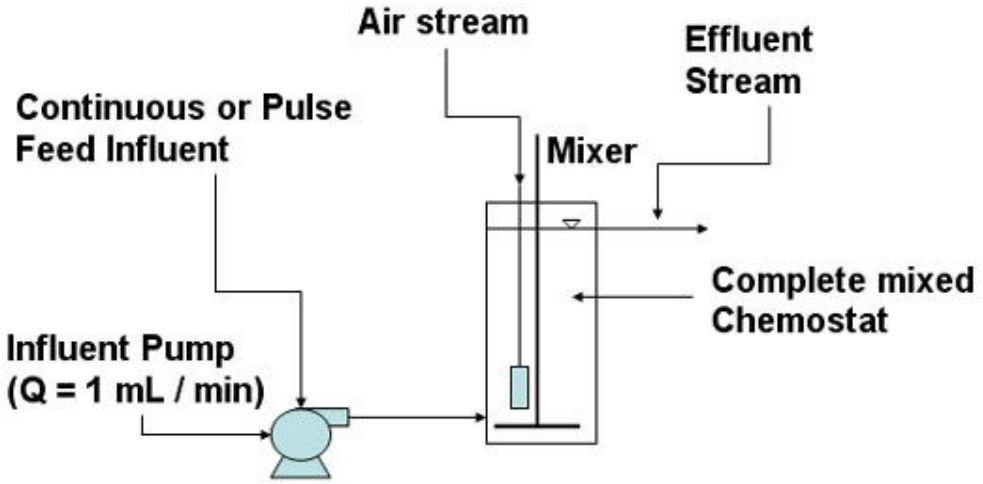
back to the cultures so that the cell biomass keeps on increasing. In open continuous cultures, both cells and the used medium are taken out and replaced by an equal volume of fresh medium. The replacement volume is so adjusted that cultures remain at sub maximal growth indefinitely.

Note:- In continuous systems the nutrient depletion does not occur due to continuous replacement of nutrient medium and the cells always remain in the steady state of active growth phase.

There are two major types of open continuous cultures viz. chemostat and turbidostat.

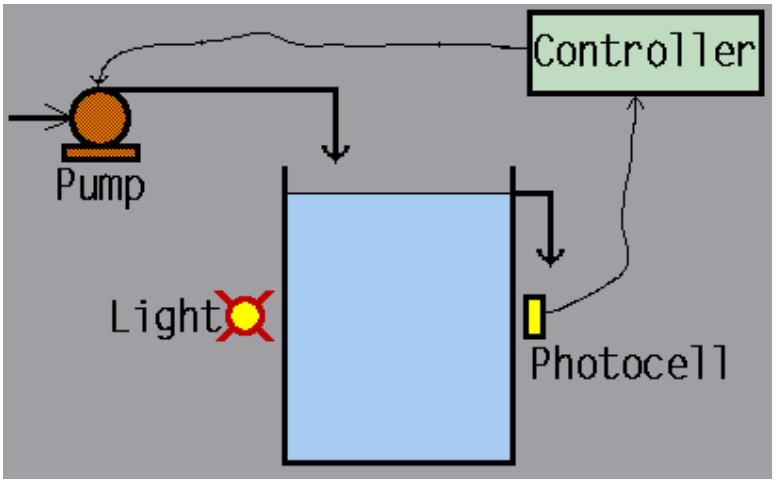
Chemostat

In chemostats the cell growth is maintained steady by a constant inflow of fresh medium consisting of nutrients. A chosen nutrient is kept in a concentration so that it is depleted very rapidly to become growth limiting, while the other nutrients are still in concentrations higher than required. In such a situation, any addition of the growth limiting nutrient is reflected in cell growth. Chemostats are ideal for the determination of effects of individual nutrients on cell growth and metabolism.



Turbidostat

The turbidity of a suspension culture medium changes rapidly when the cells increase in number due to their steady state growth. The changes of turbidity of the culture medium can be measured by the changes in the optical density of medium. In a turbidostat, the cells are allowed to grow up to a pre selected turbidity. PH of the medium also changes due to the increase in cell density. In the turbidostats, an automatic monitoring unit is connected with the culture vessel and that unit adjusts the medium flow in such a way as to maintain the optical density (or) pH at a chosen, present level.



Advantages of Continuous Cultures

1. Ease of maintaining sterility over a long period of time.
2. Less detrimental effects during mechanical failures.
3. A degree of automation.
4. Versatality with regard to growth conditions such as temperature, aeration, stirring speed, illumination, nutrient and growth regulator levels.

3.1.4.3.7. Importance of Suspension Culture

1. Important information about cell physiology, biochemistry, and metabolic events at the level of individual cells and

small cellular aggregates can be obtained from suspension cultures.

2. It is also important to have an understanding of an organ formation or embryoid formation starting from single cell (or) small cell aggregates.

3. The technique of plating out cell suspension on agar plate is useful to obtain single cell clones.

4. Suspension culture derived from medicinally important plants can be studied for the production of secondary metabolites such as alkaloids.

5. Mutagenesis studies may be facilitated by the use of cell suspension cultures to produce mutant cell clones from which mutant plants can be raised. The cell population in a suspension culture can be subjected to a range of chemical mutagens, viz. Ethylmethane sulphonate (EMS), N-nitroso-N-methyl urea etc. The mutagens can be added directly in the liquid medium. After the mutagen treatment cells are plated on agar medium for the selection of mutant cell clones from which the plants can be raised.

3.1.4.4. Single Cell Culture

Single cell culture is a method aseptically on a nutrient medium under controlled conditions. Free cells isolated either from plant organs (or) cell suspensions are grown as single cells under in vitro conditions using a suitable medium. This process is called plating. The advantage of single cell culture over callus culture (or) cell suspension culture is that single cell culture is an ideal system fro studying cell metabolism, the effects of various substances on cellular responses and to obtain single cell clones.

In 1902, Gibberlandt made an attempt to isolate and culture single cells from the leaves of flowering plants.

In 1934, W.H. Muir described paper raft nurse culture technique for the culture of single cells.

In 1968, L.Bergmann – Grew the single cells first using petridish plating technique.

3.1.4.4.1. Principle of Single Cell Culture

The basic principle of single cell culture is the isolation of large no. of intact living cells and culture them on a suitable nutrient medium for their requisite growth and development. Single cells can be isolated from a variety of tissue and organs of green plant as well as from callus tissue and cell suspension. Single cells from the intact plant tissue are isolated either mechanically (or) enzymatically. The single cells are traditionally isolated from the established friable callus tissue and cell suspension culture. The isolated single cells can be cultured either in the liquid medium or on solid medium. There are five different methods that are used for culturing single cells such as paper raft nurse technique, Bergmann petridish plating technique, The microchamber techqniue, The microdroplet technique, The plating with nurse tissue technique.

In culture, the single cells divide repeatedly to form a callus tissue. Such callus tissue also retains the capacity to regenerate the plant lets through organogenesis and embryogenesis.

3.1.4.4.2. Methods of Single Cell Culture

There are five different methods which are widely employed for culturing single cells. Isolated single cells are the essential prerequisite item for all these methods.

1. The Paper Raft Nurse Technique

Single cells are placed on small (8 x 8 mm) piecies of stabilized filter paper which are inturn placed on the top of an established callus cultures several days in advance. This allows the filter papers to be wetted by the exudates from callus tissues. The single cells placed on the filter papers derive their nutrition from the callus exudates diffusing through the filters. The cells divide and form macroscopic colonies on the filters. When the cell colony reaches a suitable size, it is transferred to fresh

medium, where it gives rise to the callus tissue. A callus tissue originating from a single cell is known as a `single cell clone'.

2. Microchamber Technique

A microchamber can be created either by using a microscope slide and cover slips. The coverslips are held in place by sterile mineral oil. A cavity slide can also be used for creating a microchamber. Single cells are suspended in conditioned medium and a drop of medium having a single cell is placed in the microchamber which is coverd by a coverslip. In case of cavity slide, the drop is placed on to a coverslip which is then inverted in to the slide cavity. Microchambers allow microscopic observation and they can be kept in a petridish for incubation; under 16 hrs illumination (3000 lux) at 25^{O}C. When the cell colony becomes sufficiently large, the cover glass is removed and the tissue is transferred to fresh solid (or) semi solid medium.

3. Microdrop Method

A specially designed dish, called cuprak dish is used in this method. This dish is having a smaller outer chamber and a larger innerchamber. The outer chamber is filled with sterile distilled water to avoid desiccation of cells and the larger chamber is having many microwells.

Microdrops of 0.25 – 0.5 ml are distributed in the microwells and the dish is sealed with parafilm. The dish is incubated under 16hrs white cool light (3000 lux) at 25^{O}C. The cell colony derived from the single cell is transferred on to fresh solid (or) semisolid medium in a culture tube for further growth.

4. Nurse Callus Technique

This method is actually a combination of petridish plating method and paper raft nurse culture method. In this method the growth of single cell is induced by nurse callus. Here the single cells are plated on to agar medium in a petridish. Two or three callus masses (Nurse tissue) derived from the same plant tissue are also embedded along with the single cells in the same medium. The cells near the nurse callus will begin to divide first

indicating that the single cells closer to the nurse callus in the solid medium gets essential growth factors that are liberated from the callus mass. The developing colonies growing near to the nurse callus also stimulate the division and colony formation of other cells.

Free cells isolated from mesophyll tissue (or) cell suspensions are grown as single cells under invitro conditions using a suitable medium.

5. Bergmann Plating Technique

This process is called plating. This is important to obtain single cell clones. This technique is developed by Bergmann in 1960. It is the most popular technique for plating single cells. In this technique the free cells are suspended in liquid medium at a density twice the finally desired plating cell density. Sterilized agar medium (0.6-1% w/v) is kept melted in a water bath at 35^{O}C. Equal volumes of the liquid and agar media are mixed thoroughly and rapidly spreadout in petridishes, in such a manner that the cells become fixed in an evenly distributed thinlayer (Ca 1 mm thick) after the agar has cooled and solidified.

The dishes are sealed with parafilm and incubated in the dark (or) diffused light at 25^{O}C. The cells remain embedded in the soft agar medium are observable under a microscope. When microscopic colonies develop they are isolated and cultured separately on a fresh medium. Free cells can also be plated in the liquid medium but follow up of individual cells (or) their derivatives is difficult in this procedure because the cells do not remain in a fixed position. The plating efficiency (PE) can be calculated from the counting of cell colonies by the following formula.

$$PE = \frac{\text{Number of colonies per plate}}{\text{Number of total cells per plate}} \times 100$$

Figure -6

Factors affecting single cell culture

1. The composition of the medium for the growth of single cell culture is generally more complex than callus and cell suspension culture.

2. Paper raft nurse culture technique indicates that isolated cells get the exact essential nutrients from the cells mass for the induction of division of single cells.

3. In case of petridish plating technique the initial plating cell density is very critical.

3.1.4.4.3. Importance of single cell culture

1. Single cell culture could be used successfully to obtain single cell clones.

2. The occurrence of high degree of spontaneous variability in the cultured tissue and their exploitation through single cell culture is very important in relation to crop improvement programmes.

3. Single cells can be grown on a medium containing mutagenic compounds and the proliferating cell lines are isolated. The mutant nature of the selected cell line can be confirmed by regenerating the plants and comparing their phenotypes with a normal plant. Many cell lines resistant to amino acid analogues, antibiotics, herbicides, fungal toxins etc. have been selected by this method.

4. Many plants synthesize the various natural compounds in the form of alkaloids, steroids etc. Some of these of medicinally important. It is reported that single cell cultures can synthesise several times higher amounts of alkaloid than the alkaloid content in the intact plant. So single cell culture could become a valuable technique for the industrial production of such important natural compounds.

5. Single cell culture is an ideal system for the study of biotransformation.

6. Induction of polyploidy has been found to be very much useful for plant breeding to overcome the problem of sterility associated with hybrids of unrelated plants. Polyploidy can be easily achieved by single cell culture.

3.1.4.4.4. Summary

Cell suspension culture is a type of culture in which cells (or) small aggregates of cells multiply while suspended in agitated liquid medium. To achieve an ideal cell suspension, a friable callus is transferred to agitated liquid medium where it breaks up and readily disperses. An ideal suspension culture should consist of only single cells which are physiologically and biochemically uniform. Movement of cells in relation to nutrient medium facilitates gaseous exchange, removes any polarity of cells due to gravity and eliminates the nutrient gradient within the medium and at the surface of cells suspension culture can be initiated either from pre established friable callus (or) from the explant directly in to the liquid medium, which is continuously agitated on a moving rotary shaker. The concentration of auxins and cytokinins used for callus culture is generally reduced for suspension culture. To facilitate easy separation of cells the concentration of auxin is increased (or) enzymes such as cululase (or) pectinase are added to the medium. Critical initial density (or) the minimum effective density is the smallest inoculum from which a new suspension culture can be successfully grown.

There are two types of suspension cultures viz. 1. Batch cultures and 2. Continuous cultures. Batch culture is a type of

suspension culture where the cell material (or) inoculum grows in a limited volume of agitated liquid medium. It is again divided into, 1. Slowly rotating cultures 2. Shake culture 3. Spinning culture and 4. Stirred culture.

In continuous culture system the old liquid medium is continuously replaced by the fresh liquid medium to stabilize the physiological states of the growing cells. In this method nutrient depletion does not occur due to continuous flow of nutrient medium and the cells always remain in the steady state of active growth phase. Continuous culture systems can be divided in to two categories Viz. open continuous culture and closed continuous culture. Chemostat and turbido stats represent open continuous systems. In a chemostat the growth rate and cell density are held constant by a fixed rate of input of growth – limiting nutrient. In a turbidostat the fresh medium flows in to it in response to an increase in the turbidity of the culture.

Under suitable conditions, the growth of suspension culture follows a predictable pattern (or) a growth curve. The growth curve for a typical suspension culture consists of lag phase, logarithmic phase (or) exponential phase, linear phase and stationary phase. In cell suspension culture the cells grow by cell division and the no. of cells increases. The cell growth can be measured by various ways viz. (1) by counting the cell number in haemocytometer under a microscope (2) detecting the changes of optical density of the cell suspension by a colorimeter (3) by measuring the packed cell volume (PCV) (4) by measuring the dryweight of cell mass per unit volume of culture at regular intervals (5) by measuring the fresh weight. For the studies on growth test for viability of cells is very important. It can be determine by using some stains like fluorescein diacetate, Evan's blue etc.

Suspension culture can be sued for obtaining single cell clones by planning cell suspension on agar plates. Plants can be regenerated from such clones by the process of embryo genesis. Cell suspention culture may be used for the synthesis of secondary plant products like alkaloids, Glycosides etc. Mutagenic studies may be facilitated by cell suspension cultures to produce mutant cell clones from which mutant plants can be raised.

Single cell culture is a method of growing isolated single cells aseptically on a nutrient medium under controlled conditions. Single cells can be obtained from the tissues of intact plants (or) from friable callus tissue. The isolated single cell can be cultured either in liquid medium (or) on solid medium. There are five important methods of single cell culture viz. 1. The paper raft nurse culture techique 2. Petridish plating technique 3. Nurse callus technique 4. Micro chamber technique 5. Micro droplet technique.

The composition of culture medium for the growth of single cell is generally more complex than callus and cell suspension culture. In case of Bergmann plating technique the initial plating cell density is very critical. Single cell culture technique is very important for the fundamental and mutation studies and it has wide industrial applications.

3.1.4.5. Self Assessment Test

1. What is cell suspension culture? Give a general account of cell suspension culture.

2. Give an outline of the principle of cell suspension culture and its applications in biological studies.

3. Write short notes on the following :

   (a) CID

   (b) Turbidostat

   (c) Chemostat

   (d) Batch Culture

   (e) PCV

4. Give a general account of the principle and importance of single cell culture.

5. Write shorts notes on the following :

   (a) What is the advantage of a single cell culture over callus culture and cell suspension culture.

   (b) Importance of single cell culture in mutation breeding.

   (c) How are the single cells isolated from the infact plant tissue and cultured tissue.

   (d) Paper raft nurse culture technique.

   (e) Bergmann plating technique.

3.1.4.6. Reference books

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