CALLUS AND SUSPENSION CULTURES

Callus and Suspension Cultures

Callus is an unorganized, proliferative mass of differentiated plant cells, and usually occurs naturally as wound response. Tissues and cells cultured on an agar-gelled medium form an unorganised mass of cells is also called callus. It can be induced through culture of plant tissue on a medium usually containing relatively high levels of auxin, especially 2,4-D. However, because of the phase of disorganization that occurs, plants regenerating from callus, can be prone to genetic change.Callus cultures need to be sub-cultured every 3-5 weeks in view of cell growth, nutrient depletion and medium drying. Therefore, calluses are easy to maintain and are the most widely used. When explants are cultured on a suitable PGR(s) combination, many of its cells undergo division. Even mature and certain differentiated, e.g., parenchyma and often colenchyma, cells undergo changes to become meristematic; this is called dedifferentiation. Dedifferentiation involves, among other things, renewed and enhanced RNA and protein syntheses leading to the formation of new cellular components needed for meristematic activity. Initially, cell divisions are confined to the cut ends, but subsequently it covers the entire explant. The resulting cell mass is ordinarily unorganised, but it often consists of several cell types including fibers, and vascular elements.

Suspension Cultures:

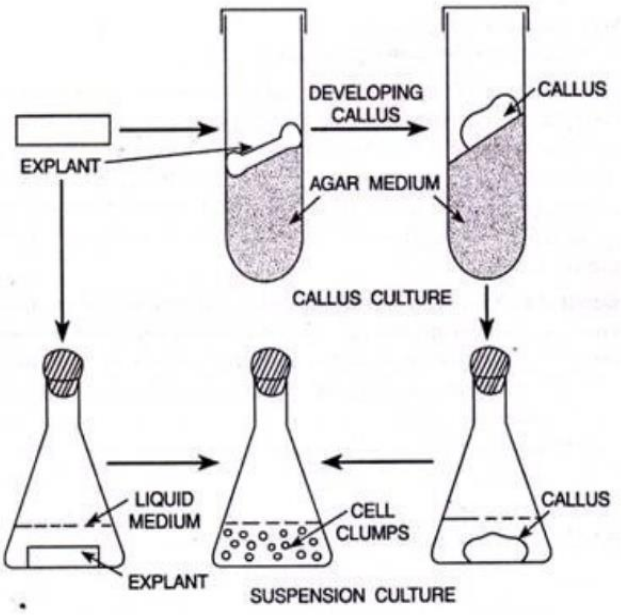
Tissues and cells cultured in a liquid medium produce a suspension of single cells and cells clumps of few to many cells; these are called suspension cultures. Liquid cultures must be constantly agitated, generally by a gyratory shaker at 100-250 rpm (revolution per minute), to facilitate aeration and dissociation of cell clumps into smaller pieces.

Suspension cultures grow much faster than callus cultures, need to be sub-cultured about every week, allow a more accurate determination of the nutritional requirements of cells and are the only system amenable to scaling up for a large scale production of cells and even somatic embryos (SEs). The suspension cultures are broadly grouped as follows: (1) batch cultures, (2) continuous cultures, and (3) immobilized cell cultures.

Batch Cultures:

In a batch culture, the same medium and all the cells produced are retained in the culture vessel, e.g., culture flasks (100-250 ml), fermenters (variable size), etc. The cell number or biomass of a batch culture exhibits a typical sigmoidal curve, having a lag phase during which the cell number or biomass remains unchanged, followed by a logarithmic (log) phase when there is a rapid increase in cell number and, finally, ending in a stationary phase during which cell number does not change.

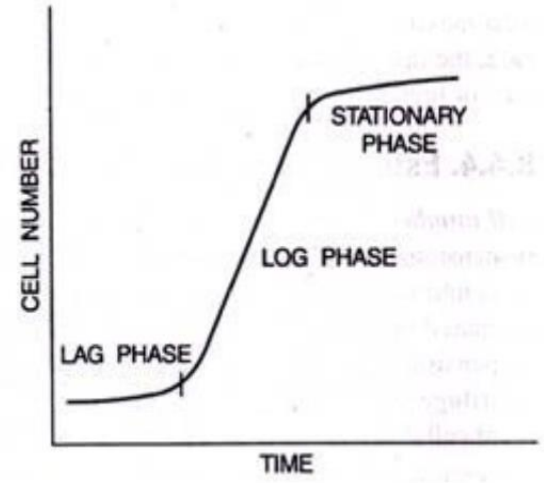
The lag phase duration depends mainly on inoculum size and growth phase of the culture from which the inoculum is taken. The log phase lasts about 3-4 cell generations (a cell generation is the time taken for doubling of cell number), and the duration of a cell generation may vary from 22-48 hr, depending mainly on the plant species. The stationary phase is forced on the culture by depletion of the nutrients and possibly due to an accumulation of cellular wastes. If the culture is kept in stationary phase for a prolonged period, the cells may die.



Initiation of callus and suspension cultures

Batch cultures are maintained by sub-culturing. They are used for initiation of cell suspensions, which may be used for cloning, cell selection or as seed cultures for scaling up or for continuous cultures.

They are, however, unsuitable for studies on cell growth and metabolism because there is a constant change in cell density and nutritional status of the medium. But batch cultures are much more convenient than continuous cultures and, as a result, are routinely used.



A model curve for cell number in a batch culture

Continuous Cultures:

In a continuous culture, the cell population is maintained in a steady state by regularly replacing a portion of the used or spent medium by fresh medium. Such culture systems are of either (1) closed or (2) open type. In a closed continuous culture, cells are separated from the used medium taken out for replacement, and added back to the culture so that cell biomass keeps

on increasing. In contrast, both cells and the used medium are taken out from open continuous cultures and replaced by equal volume of fresh medium. The replacement volume is so adjusted that cultures remain at submaximal growth indefinitely.

The open cultures are of either turbidostat or chemostat types. In a turbidostat, cells are allowed to grow upto a preselected turbidity (usually, measured as OD) when a predetermined volume of the culture is replaced by fresh normal culture medium.

But in a chemostat, a chosen nutrient is kept in a concentration so that it is depleted very rapidly to become growth limiting, while 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.

Immobilized Cell Cultures:

Plant cells and cell groups may be encapsulated in a suitable material, e.g., agarose and calcium alginate gels, or entrapped in membranes or stainless steel screens. The gel beads containing cells may be packed in a suitable column or, alternatively, cells may be packed in a column of a membrane or wire cloth.

Liquid medium is continuously run through the column to provide nutrients and aeration to cells. Immobilization of cells changes their cellular physiology in comparison to suspension culture cells; this offers several advantages for their use in biochemical production, but they are usually not used for other studies.

Subculture:

After a period of time, it becomes necessary to transfer organs and tissues to fresh media chiefly due to nutrient depletion and medium drying. This is particularly true of tissue and cell cultures where a portion of tissue is used to inoculate new culture tubes or flasks; this is known as sub-culturing. In general, callus cultures are sub-cultured every 4-6 weeks, while suspension cultures need to be sub-cultured every 3-14 days. Plant cell and tissue cultures may be maintained indefinitely by serial sub-culturing.

In case of suspension cultures, sub-culturing should be done about or somewhat prior to the time of their maximum growth. The inoculums volume should be 20-25% of the fresh medium volume; in any case, the initial cell density of the fresh culture (just after inoculation) should be around 5×10^4 cells $m1^{-1}$ or higher otherwise the cells may fail to divide.

Estimation of Growth:

Cell number is the most informative measure of cell growth. This measurement is applicable to only suspension cultures, and even their cell aggregates must be treated, e.g., with pectinase, to dissociate them into single cells before counting the cell number in a haemocytometer.

Therefore, cell number is estimated only where information obtained justifies the efforts. In contrast, packed cell volume of suspension cultures is easily determined by pipetting a known volume into a 15 ml graduated centrifuge tube, spinning at 200 ×g for 5 min and reading the volume of cell pellet, which is expressed as ml cells/1 of culture.

Culture fresh and dry weights are the most commonly used measures of growth of both suspension and callus cultures. In case of callus cultures, the cell mass is placed on a preweighed dry filter paper or nylon filter and weighed to determine fresh weight.

Cells from suspension cultures are filtered onto a filter paper or nylon filter, washed with distilled water, excess water removed under vacuum and weighed along with the filter; the filter is preweighed in wet condition. For dry weight determination, the cells and the filter are dried in an oven at 60°C for 12 hr and weighed; the filter is pre-weighed in dry condition. Cell fresh and dry weights may either be expressed as per ml (suspension culture) or per culture.

Nuclear Cytology:

Callus and suspension cultures show both numerical (polyploidy and aneuploidy) and structural (deletions, translocations, etc.) chromosome changes. The frequency of these changes tends to increase with the duration of in vitro culture so that some cultures may become predominantly or even completely polyploid or aneuploid.

Explants contain endopolyploid cells, which may give rise to a portion of the polyploid cells in cultures. But most polyploid cells appear to originate through endoreduplication (additional rounds of DNA replication without intervening cell division) although selection for such cells cannot be ruled out.

Aneuploid cells originate mainly due to anaphase irregularities like unequal chromatid separation, lagging chromatids or chromosomes, anaphase bridges giving rise to breakage-fusion-bridge cycle, chromosome fragmentation, etc.

The cytogenetic status of cultured cells is influenced by several factors of the culture system, e.g., GR concentrations and combination, culture age, liquid or agar medium, subculture interval, sucrose concentration, etc. Suspension cultures of many diploid species show a selection for diploid cells so that they remain predominantly diploid for long periods, e.g., in case of Vicia hajastana and Haplopappus gracilis cultures remained predominantly diploid for over 300 days.

Secondary metabolite production through Cell suspension cultures

Plant cell and tissue cultures can be established routinely under sterile conditions from explants, such as plant leaves, stems, roots, and meristems for multiplication and extraction of secondary metabolites. Strain improvement, methods for the selection of high-producing cell lines, and medium optimizations can lead to an enhancement in secondary metabolite production. The capacity for plant cell, tissue, and organ cultures to produce and accumulate many of the same valuable chemical compounds as the parent plant in nature has been recognized almost since the inception of *in vitro* technology. The strong and growing demand in today's marketplace for natural, renewable products has refocused attention on *in vitro* plant materials as potential factories for secondary phytochemical products and has paved the way for new research exploring secondary product expression *in vitro*. There is a series of distinct advantages to producing a valuable secondary product in plant cell culture, rather than *in vivo* in the whole crop plant.

These include the following:

- Production can be more reliable, simpler, and more predictable.
- Isolation of the phytochemical can be rapid and efficient, when compared with extraction from complex whole plants.

- Compounds produced *in vitro* can directly parallel compounds in the whole plant.
- Interfering compounds that occur in the field-grown plant can be avoided in cell cultures.
- Tissue and cell cultures can yield a source of defined standard phytochemicals in large volumes.
- Tissue and cell cultures are a potential model to test elicitation.
- Cell cultures can be radiolabeled, such that the accumulated secondary products, when provided as feed to laboratory animals, can be traced metabolically.

While research to date has succeeded in producing a wide range of valuable secondary phytochemicals in unorganized callus or suspension cultures, in some cases production requires more differentiated micro plant or organ cultures. This situation often occurs when the metabolite of interest is only produced in specialized plant tissues or glands in the parent plant. A prime example is ginseng (*Panax ginseng*). Because saponin and other valuable metabolites are specifically produced in ginseng roots, root culture is required in vitro. Similarly, herbal plants such as *Hypericum perforatum* (St. John's wort), which accumulates the hypericins and hyperforins in foliar glands, have not demonstrated the ability to accumulate phytochemicals in undifferentiated cells. As another example, biosynthesis of lysine to anabasine occurs in tobacco (*Nicotiana tabacum*) roots, followed by the conversion of anabasine to nicotine in leaves. Callus and shoot cultures of tobacco can produce only trace amounts of nicotine because they lack the organ-specific compound anabasine. In other cases, at least some degree of differentiation in a cell culture must occur before a product can be synthesized (e.g., vincristine or vinblastine from *Catharanthus roseus*). Reliance of a plant on a specialized structure for production of a secondary metabolite, in some cases, is a mechanism for keeping a potentially toxic compound sequestered. Intensive activities have been centered on production of natural drugs or chemoprotective compounds from plant cell culture by one or more of the following strategies:

Accumulation of secondary metabolites in plant cell cultures for plant cell culture techniques to become economically viable, it is important to develop methods that would allow for consistent generation of high yields of products from cultured cells. Careful selection of productive cells and cultural conditions resulted in accumulation of several products in higher levels in cultured cells.

In order to obtain yields in high concentrations for commercial exploitation, efforts have focused on the stimulation of biosynthetic activities of cultured cells using various methods - Culture productivity is critical to the practical application of plant cell culture technology to production of plant-specific bioactive metabolites. Until now, various strategies have been developed to improve the production of secondary metabolites using plant cell cultures. The tissue culture cells typically accumulate large amounts of secondary compounds only under specific conditions. That means maximization of the production and accumulation of secondary metabolites by plant tissue cultured cells requires

(i) manipulating the parameters of the environment and medium,

(ii) selecting high yielding cell clones,

(iii) precursor feeding, and

(iv) elicitation.

Optimization of cultural conditions:

Number of chemical and physical factors like media components, phytohormones, pH, temperature, aeration, agitation, light affecting production of secondary metabolites has been extensively studied. Several products were found to be accumulating in cultured cells at a higher level than those in native plants through optimization of cultural conditions. Manipulation of physical aspects and nutritional elements in a culture is perhaps the most fundamental approach for optimization of culture productivity. For example, ginsenosides by Panax ginseng, rosmarinic acid by Coleus bluemei, shikonin by Lithospermum erythrorhizon, ubiquinone-10 by Nicotiana tabacum, berberin by Coptis japonica, were accumulated in much higher levels in cultured cells than in the intact plants.

Selection of high-producing strains:

Plant cell cultures represent a heterogeneous population in which physiological characteristics of individual plant cells are different. Synthesis of several products in high amounts using selection and screening of plant cell cultures have been already described by many workers. Cell cloning methods provide a promising way of selecting cell lines yielding increased levels of product.

Precursor feeding:

Exogenous supply of a biosynthetic precursor to culture medium may also increase the yield of the desired product. This approach is useful when the precursors are inexpensive. The concept is based on the idea that any compound, which is an intermediate, in or at the beginning of a secondary metabolite biosynthetic route, stands a good chance of increasing the yield of the final product. Attempts to induce or increase the production of plant secondary metabolites, by supplying precursor or intermediate compounds, have been effective in many cases. For example, amino acids have been added to cell suspension culture media for production of tropane alkaloids, indole alkaloids etc. Addition of phenylalanine to Salvia officinalis cell suspension cultures stimulated the production of rosmarinic acid. Addition of the same precursor resulted stimulation of taxol production in Taxus cultures. Feeding ferulic acid to cultures of Vanilla planifolia resulted in increase in vanillin accumulation. Furthermore, addition of leucine, led to enhancement of volatile monoterpenes in cultures of Perilla frutiscens, where as addition of geraniol to rose cell cultures led to accumulation of nerol and citronellol.

Elicitation:

Plants produce secondary metabolites in nature as a defense mechanism against attack by pathogens. Elicitors are signals triggering the formation of secondary metabolites. Use of elicitors of plant defense mechanisms, i.e. elicitation, has been one of the most effective strategies for improving the productivity of bioactive secondary metabolites. Biotic and abiotic elicitors which are classified on their origin are used to stimulate secondary metabolite formation in plant cell cultures, thereby reducing the process time to attain high product concentrations. Production of many valuable secondary metabolites using various elicitors were also reported

Conclusions

The use of plant cell culture for the production of chemicals and pharmaceuticals has made great strides building on advances in plant science. The increased use of genetic tools and an emerging picture of the structure and regulation of pathways for secondary metabolism will provide the basis for the production of commercially acceptable levels of product. The increased appeal of natural products for medicinal purposes coupled with the low product yields and supply concerns of plant harvesation has renewed interest in large-scale plant cell culture technology. Knowledge of biosynthetic pathways of desired compounds in plants as well as in cultures is often still in its infancy, and consequently, strategies are needed to develop an information based on a cellular and molecular level. Because of the complex and incompletely understood nature of plant cells in in vitro cultures, case-by-case studies have been used to explain the problems occurring in the production of secondary metabolites from cultured plant cells. These results shows that plant cell culture systems have potential for commercial exploitation of secondary metabolites. The introduction of the techniques of molecular biology, so as to produce transgenic cultures and to effect the expression and regulation of biosynthetic pathways, is also likely to be a significant step towards making cell cultures more generally applicable to the commercial production of secondary metabolites.

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