Characteristics affecting the success of plant culture in vitro

1. Plant characteristics

a. Uniformity and size of the explant

Heterogeneity in the explant leads to the formation of heterogeneous callus, and the resulting shoots are heterogeneous in growth. The size and shape of the explant affect development, and it may fail to grow if its size is less than the appropriate limit. While if the size increases, the percentage of success and growth increases. This may be because it contains more nutrients and a more significant number of cells, which increases the production of many growths. Therefore, it is preferable to determine the explant's size, shape and composition. For example, the explant separated from the storage areas of tomatoes, potatoes, turnips, carrots, etc., it is preferable to separate a cylindrical portion with dimensions of 20×2.4 mm because the cylindrical shape provides a better opportunity for absorbing nutrients from the nutrient medium and exchanging gases. Growth regulators secreted from the wounded surfaces of the explant also have an influential role in stimulating division and callus formation. The minimum weight of a piece of carrot root is recommended to be 3.8 mg, as it contains approximately 25,000 cells. At the same time, the same mass of artichoke tubers has fewer cells due to their larger size. Therefore, it is recommended that the biomass of artichoke tubers be 8 mg, as they contain 20,000 cells. The meristematic tip should be separated from the growing tip of potato plants to ensure the production of virus-free plants.

b. Genotype

The ability of plants to grow and reproduce in vitro varies depending on their species and genetic makeup. It isn't easy to form adventitious stems on leafy parts of *Kalanchoe farinaceae* if grown in the field. At the same time, it is easy to do this by growing them in a suitable culture medium in vitro. Dicotyledons are generally easier to reproduce in vitro than monocotyledons. Plants that produce vegetatively efficiently in the field can quickly replicate in vitro. Also, some plant families and genera can reproduce, such as the Solanaceae family, including the Solanum, Nicotiana, Datura, and Lycopersicon. As well as other families such as Crueiferae, Gesneriaceae, Compositae, and Liliaceae.

c. Physiological age

Embryonic and neonatal tissues are more susceptible to morphogenesis when cultured in vitro compared to adult tissue. The ability of trees and shrubs to reproduce in vitro decreases as they age, so modern plants must be produced from them. For laboratory cultivation, it is preferable to use young explants such as meristematic tissue and recent seedlings. Some plant species differ in this regard, and explants separated from plants that are easy to reproduce vegetatively are better for laboratory cultivation than explants separated from plants that do not produce vegetatively. Dormant buds and dormant seeds are more challenging to grow in vitro than non-dormant ones. The growth and reproduction capacity of separated explants may increase during the flowering period of some plants, regardless of their age. In general, the growing tips separated from young stems remain the best in vitro cultivation because they are active and capable of dividing and multiplying with the increasing tips separated from the old branches of some plants.

2. Physical properties

a. Photoperiod

The first growth stage of the vegetative explant of many plants is not affected by light or darkness. In the following stages of development, it is necessary to emphasize the light and dark stages to form vegetative growth and roots. This was confirmed by the products resulting from planting fresh flower buds in Freesia. The presence of light may be necessary for the germination of some species of flowers. In contrast, the presence of darkness is essential for the germination of other species, such as orchid. The length of the photoperiod affects the growth of the explant and the formation of nitrate in vitro. This effect varies depending on the type of plant. 12 hours of light is considered the best for the emergence and formation of growths on separated explant of Helianthus tubers. While 16-15 hours of light is the best for embryo formation on the Geranium callus. While the number of embryos formed decreases as the photoperiod increases or shortens. The colour of the callus changes to green, and no embryos develop from it if it grows in the dark. The formation of new growths was not affected by cultivation of the apical meristem of Pharbitis nil under conditions of 16-24 h of light. It has also been proven that the level of oxygen and saptokinin within the tissue is affected by prolonging the photoperiod in meristematic tissues separated from plants that require a long photoperiod.

b. Light intensity

The best lighting intensity suitable for the growth of different plant tissues has not yet been determined. This may be due to the diverse needs of plants depending on the stages of development and plant type. It has been found that 1000 Lux is the best lighting intensity in the first and second stages of secondary cultivation of plant tissues and cells, and it may increase to 10000-3000 Lux in the third stage. It requires increasing the lighting intensity after transferring the plants from the nutrient medium to the soil, and the light intensity in vitro must not reach the same level prevailing in the field, estimated between (30-70 watts/m^2) because increasing the light intensity in vitro is more than the optimum amount suitable for laboratory cultivation. It causes a lot of damage. The growth activity of explants grown in vitro was observed at low light intensity, estimated at 8-16 watts/m2. Growth in vitro may occur at lower light intensity. It is preferable to choose low light intensity because photosynthesis in cultivated explants and their new growth is weak due to the incomplete formation of chlorophyll, significantly if the concentration of carbon dioxide decreases. It is possible to compensate for the lack of sugar in the nutrient medium, but adding excess sugar may lead to weak growth of plants in vitro.

c. Wavelength

In the case of plants that are affected by the length of the photoperiod, it is controlled by adding the Phytochrome pigment because of its ability to absorb light with a wavelength of 660 nanometers. It turns into the Phytochrome-red (Pr) pigment. If the (Pr) pigment recognizes red rays, it turns into the Phytochrome far (Pfr) pigment, which can Absorption rays with a length of 730 nm. The transformation of the phytochrome pigment from Pr to Pfr occurs slowly in the dark and quickly when exposed to far-red rays. Pfr pigment controls plant formation by stimulating genes directly or indirectly. A study on tobacco plant tissues proved a difference in the ability of red and green light compared to blue light to destroy the IAA hormone added to the food medium, as blue light works to eliminate this hormone. At the same time, it does not affect the NAA hormone, which leads to weakened growth. These results are not similar to the effects of Chrysanthemum and Dahlia plants. It was observed that red light of 660 nm promoted the formation of adventitious stems on the separated explant of Helianthus tuberosis more than blue light. The growth of Pelargonium callus was better in the presence of white light (containing all colours of the polychromatic spectrum), and the presence of blue light was better than green and red light or complete darkness. It has also been shown that blue and violet light stimulate the formation of adventitious stems from tobacco callus, while green light encourages the formation of adventitious branches. In general, it has been shown that white light usually inhibits the formation of adventitious shoots and stimulates the formation of adventitious stems. In most cases, red light activates the formation of adventitious roots of Pseudotsuga menziesii and Brassica oleracea.

d. Light source

White fluorescent lamps are usually used to illuminate plant tissue culture laboratories. Some laboratories use 38-watt fluorescent lamps, although sodium lamps may give better results. The formation of roots on leafy parts of Kalanchoe was better by exposing them to light from these lamps because they contain a higher percentage of red-orange light. Lamps emitting a high proportion of ultraviolet (UV) radiation inhibited the formation of adventitious roots. It has been proven that the light emitted from fluorescent lamps weakens from the first moment they start operating. If the amount of light emitted at the beginning of operation is 100%, it decreases to 93% after 8 days. Then it reaches 86% after 4 months, then 70% after 12 mon.

e. Heat

The appropriate temperatures for the growth of explants vary depending on their types. Therefore, the growth room must be provided with a self-operating air conditioning device to stabilize the temperature, and different plants with their thermal needs must be isolated in other growth rooms. Low temperature is essential in breaking the dormancy of buds and seeds and improving their growth. Therefore, while they are in the dormant stage, they must be exposed to low heat until their dormancy is broken. 26-28 °C is considered the most appropriate temperature for the growth of most explants, 18 °C for species forming bulbs and tubers, and 29-28 °C for tropical species. Most of the separated explants of fruit trees are planted between 23-32 °C, as the callus of apple trees grows well at 20-32 °C, and their growth is weak at less than 20 °C. While the separated explant of the avocado retains its vitality at 55 °C. It was found that the most suitable temperature for the growth of isolated explant of potatoes grown in a liquid medium is 25-32°C. In comparison, the separated explant of Rhododendron and Narcissus plants are 20°C, and lilyum is 2°C.

f. Humidity

Although there are few studies on the effect of humidity inside the growth room, high humidity may lead to a high percentage of contamination. Increasing the cultivated nutrient medium's humidity increases water vapour condensation on its inner wall. Which leads to Vitrification. It is a phenomenon that leads to the death of tissue growing in vitro. This phenomenon can be controlled by increasing agar concentration in the nutrient medium.

g. Oxygen

Good ventilation is essential to help cells, tissues, etc. grow. This is achieved using shaking devices such as machines through conical flasks containing a liquid medium. To improve ventilation, explants are produced inside the jars on paper surfaces. Meanwhile, ventilation inside the tubes can be improved by covering them with metal caps only and not using cotton plugs. The explants are planted on the surface of the solid medium containing the seeds and not immersed in them.

h. Carbon dioxide

The accumulation of carbon dioxide leads to toxicity for plants growing in vitro culture containers, especially if they are tightly sealed. Therefore, excess carbon dioxide must be eliminated from the pots to avoid harm to the plants while adding sucrose to the medium as an essential carbon source.

i. Ethylene

Using an alcohol or gas flame to sterilize the mouth of tubes or flasks during cultivation in vitro. This leads to an increase in ethylene accumulation inside it, especially if it is tightly sealed. Excessive ethylene causes the following damages:

- The formation of unspecialized plant cells (callus) leads to obstruction of plant formation or production of soft, waxy plants.
- Decrease in the concentration of green colour or its absence in explant and increase in the IAA content of the food medium.

3. Characteristics of the growing medium

a. Components of the growing medium

All explants separated from dicotyledonous or monocotyledonous plants can produce callus when grown in a suitable medium. It is preferable to maintain the components of the medium suitable for each growth stage, and any change in the composition of the medium, even if it is simple, leads to a significant difference, like the growth of callus culture.

b. pH-Value

pH value of 5-6.5 is considered suitable for growing in vitro. It is preferable to set it at 5.7. The consistency of the medium to which Agar is added changes when the pH drops below 5.4 or rises above 7.0. Autoclaving causes a pH drop of 0.3-0.5. Also, the pH of the liquid medium is subject to change after cultivation. This may be due to the secretions of explants during their growth. The medium's acidity is also affected by the speed of N absorption. Therefore, we must stabilize the pH to maintain a balance in the concentration of NO3 and NH4 in the culture medium. A severe decrease in acidity leads to the following:

- It decreased iron absorption in the acidic medium (4.5-5.4). Therefore, raising the acidity number towards the neutralization point is necessary by adding EDTA, vitamin B1, and pantothenic acid, which is less stable and makes non-cohesive agar.
- Precipitation of some salts such as phosphate and iron. Therefore, the absorption of ammonia ions decreases, and growth stops.
- It is easy for sugars to decompose during sterilization of the medium with an autoclave into glucose and fructose, and the glucose is partially converted to fructose after sterilization with an autoclave if the medium's acidity is pH 6.

c. Osmotic potential

Many factors affect the osmotic potential of the culture medium, such as the concentration of agar, mineral elements, the molecular weight of mineral salts, and the ease of their ionization. Sucrose has a relative effect in increasing the osmotic potential. Sucrose is a disaccharide that decomposes during autoclave sterilization into two monosaccharide units, which causes an increase in the osmotic potential of the culture medium. Major mineral salts also have an effect, as the osmotic potential varies greatly depending on the medium's content of sugars and salts. The osmotic potential is estimated in Bar or Pascal. Studies have shown that increasing the osmotic potential more than 105×3 Pa, which is equal to 3 bar, leads to the cessation of growth due to stopping water absorption. Mannitol is considered one of the compounds that causes an increase in the osmotic potential of the medium and is a physiological inhibitor of growth. Adding one molar of mannitol leads to an osmotic potential equivalent to (22.4 bar). Recently, polyethylene glycol (PEG) has been added to change the osmotic potential of the medium instead of mannitol. Table 4 shows the osmotic potential of some culture media.

Table 4. Osmotic potential of some food media

Culture media	Sugar (bar)	Nutrient elements (bar)
White	1.46	0.43
Hilderbrandt	1.46	0.61
Heller	4.05	0.96
MS	2.20	2.27

d. Explant exudates

The separated explant of some woody trees and shrubs secrete brown or black pigments from their wounds. They are Polyphenols and Tannins. They are toxic compounds that lead to stunted growth in tissue cultures. Researchers have suggested some treatments to overcome these secretions, which are as follows:

i. They reduce wounds on the explant or soak them in water before planting them in the growing medium. Or add activated charcoal to the medium at a 0.2 - 3.0% concentration.
ii. Add the PVP compound to the medium at a 250 - 1000 mg/L concentration. It is a polymer that can adsorb phenolic compounds.
iii. Add antioxidant compounds such as citric acid, ascorbic acid, and cysteine, which prevent the oxidation of phenols.
iv. We are adding three amino acids: glutamine, arginine, and asparagine.
v. Reducing the concentration of mineral salts in the medium reduces osmotic potential and reduces secretions.
vi. Replanting on a liquid medium leads to the spread of toxic secretions in a diluted manner around the roots.

Growth regulators help blacken the medium and oxidize phenolic compounds. Failure to add it leads to the cessation of oxidation processes. It has been proven that adding the compound Diethyl-dithiol carbonate (DIECA) to the water-washing explants after sterilizing them with sterilizing compounds at a concentration of 2 mg/L or adding drops of this compound while cutting explants contributes to stopping the oxidation of phenolic compounds. It has been observed that a brown colour appears at the base of the branches in tissue cultures due to Photoactivation. This phenomenon can be reduced by keeping the bases of the stems in the dark during their growth or preventing the penetration of light by painting the outer sides of the pots black up to the level of the nutrient medium, or wrapping their bases with foil, or adding a thin layer from charcoal and particles on the surface of the nutrient medium.

Callus culture

A callus is a mass of unspecialized and irregular cells. Tumor tissue forms on wounds of differentiated tissues and organs. Callus cells are parenchymal and are not entirely homogeneous. Upon microscopic examination, a small amount of differentiated tissue is observed next to a large volume of undifferentiated tissue. It can be observed that callus tissue has formed on the tissues of non-cultivated plants, significantly when plant cuttings are cut and planted in the soil, where callus first arises from the cut areas of the cutting or root.

Explant used in creating calli cultures

The explant in creating callus cultures are differentiated tissues separated from any part of the plant (root, stem, leaf, scale leaf, anther, shoot tip, etc.). The explant primarily contains tissues in different stages of cell division and preparation for organ specialization with various functions. Cell division and multiplication rates are rapid if the explant contains meristematic cells. Therefore, it is possible to choose the growing tip as a source for the emergence of callus and benefit from it for various purposes or to propagate the plant vegetatively indirectly, that is, to divide the callus into branches and plants.

Stimulating callus formation

In most experiments, the first step is to induce callus to emerge from the previously selected explant or from seedlings that have been sterilized and planted in the appropriate nutrient medium. It must be taken into account that not all plant cells are competitive, but rather, meristematic cells can often form regular structures and then a complete plant. While other cells are not qualified and do not express the presence of totipotency, they fail to differentiate and create a whole plant. Visual selection under a dissecting microscope can be checked once a week, and observations recorded. The callus arises from different areas on the explant, such as the root cambium tissue, and several somatic embryos may appear directly surrounding the seed when grown as an explant. Many activities not visible to the naked eye occur during callus development and do not appear until about six weeks later as a mass of callus tissue. Types that stimulate callus cultures to emerge from cell suspension cells can be identified, especially in studies related to somatic embryos, callus growth curves, selection experiments such as stress tolerance, Isolation of pure cell lines with dyes, etc. Callus cultures contain a heterogeneous mixture of heterogeneous cells in size, shape, colour, metabolic processes, chromosome number, etc. A section of the cells appears carrying dyes of different colours, which makes it easier to isolate them and select cell lines of the same colour.

Sometimes, these lines contain helpful secondary metabolites. The levels of Endogenous hormones and the polar transport characteristic of the hormone within the explant influence the stimulation of callus formation on that part. Perhaps one of the most important factors affecting callus development, which has been studied extensively, is the concentrations of growth regulators supplied to the medium, which vary according to the plant species and the explant type. Environmental and nutritional conditions also play an essential role in developing callus in tissue culture cells under an optical microscope.

Figure 7. Indication and Subculturing of embryonic calli derived from rice seeds.

Callus growth curve:

Callus tissues are similar in their growth to single-celled microorganisms. The growth curve of a callus takes the shape of a letter or a signoid (Fig. 8), and callus growth goes through five phases. The composition and biochemical behaviour of callus cells differs as they pass through the five phases of development (lag phase, log phase, linear phase, deceleration phase, and stationary phase), as there are cells that differ in their phenotypic and physiological features in each phase. The components of the medium affect the duration of callus survival at a certain phase, so it is necessary to deal with callus cultures at a specific developmental phase.

Studies examining the chromosomal structure of callus cells have shown that most cells enter the metaphase during the exponential phase of cell growth, where division speeds up and the amount of callus increases. The callus cells are transferred to a new medium at the end of the linear phase and before entering the decelerating phase, noting the transfer of callus that appears healthy and the exclusion of brown masses. Slow-growing callus cultures with an opaque white colour are preferred for differentiation and to exclude green and fast-growing callus masses because they do not differentiate into organs.

The stage of callus growth must be determined that produces the most significant amount of secondary metabolites or the required compound, which is often the phase of growth cessation as a result of exhaustion of media nutrients, hardening of the matrix, accumulation of toxic intermediates, and depletion of CO from within the callus masses. Determining the callus growth curve requires sacrificing replicates of cultures at regular time intervals and recording the weights of wet and dry calli after removing the agar residue. Cells can be counted after being treated with 4% Chromium tioxide at a temperature of 70°C for 2-15 minutes after mixing the mixture and measuring the number of cells using a hemocytometer blood cell count slide. The growth curve of the cell suspension is determined by withdrawing 5-10 ml and placing it in graduated test tubes to be centrifuged, and the compressed volume of the cells is recorded.

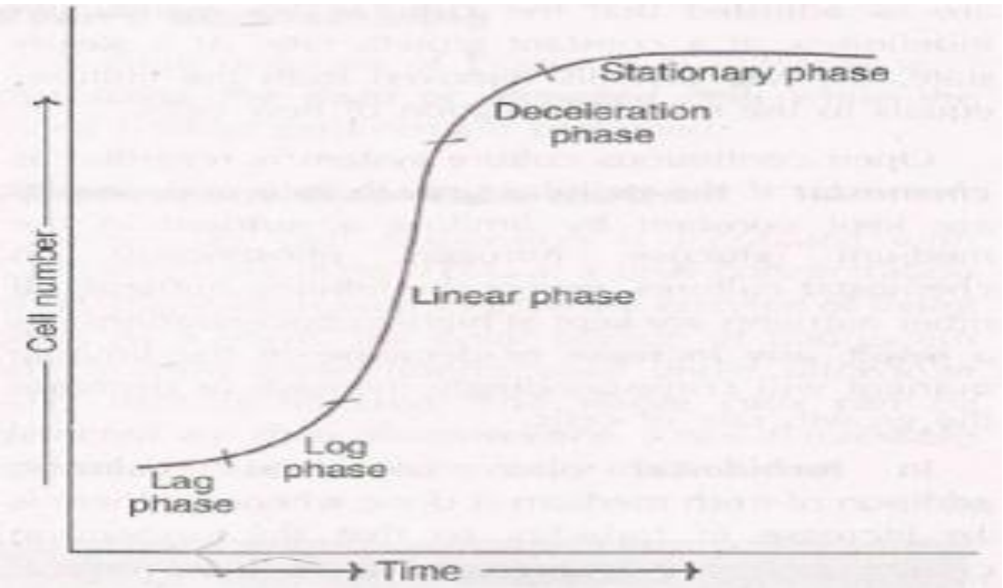

Figure 8. The five phases of growth of calli cultures.

Principles of callus culture

To successfully stimulate callus to develop, it is necessary to consider the following: Preparing a surface-sterilized explant ready for planting, selecting the appropriate medium and the appropriate combination of growth regulators, and incubating the crops under controlled conditions of light, temperature, humidity, etc. Prefer explant in the early stage and those separated from seedlings, recent vegetative growths and buds. Various other explants respond to callus formation, such as root tips, phloem tissue, developing embryos, flower and leaf parts, fruits, tubers, bulbs, and others, considering excluding lignified cells. Callus cells enter three developmental stages: (A) Induction, (B) Cell division, and (C) Differentiation. Intracellular metabolic processes are stimulated and increased in the first developmental stage, the length of which depends on the physiological state of the explant, nutritional requirements, endogenous hormonal content, the polar transport of growth regulators, and environmental factors. As a result of increased cell metabolic rates, the cell accumulates factors that encourage division to form a cell mass of large numbers of cells. The lower cells are in contact with the middle, and the upper ones are nourished by the diffusion of nutrients from the cells below them. Cell differentiation begins in the third stage when specific vital pathways start to express, and secondary metabolic substances accumulate. Sometimes, the callus appears in different colours (yellow, green, white) at this stage with a genetic disorder resulting in phenotypic variations that may be attributed to epigenetic or genetic developmental factors. The callus is divided (Subculture) after it reaches a suitable size for a period that may reach 21-28 days. If the mass is small, it is transferred back to a new medium (Reculture) to allow it to get a suitable size. The callus is generally replanted on a new medium for 3 to 4 weeks by dividing the callus pieces into weights of 250-500 mg. Habituation occurs as a result of the continued growth of the callus in the perpetuation medium in the presence of growth regulators, as the callus pieces are able, over time, to grow in a medium devoid of regulators, and this is what It's called hormone habituation. It is difficult to distinguish between the two types of callus, standard and those that grow without hormones, except for the inability of the latter to grow without them. This helps reduce or eliminate the costs of growth regulators, reduce work steps, eliminate the possibility of error in preparing concentrations of growth regulators, and other benefits.

Applications of callus tissue culture in biotechnology

Callus cultures grow slowly in a static medium, which allows many studies related to growth, differentiation, metabolism, etc., as shown below:

1. Study the nutritional needs of plants by studying them at the cellular level first.
2. We are studying the differentiation of cells and organs and the physiological and molecular processes accompanying them.
3. Establishment of cell suspension cultures and protoplasts and obtaining single cells
4. They are investigating and taking advantage of the physical variations resulting from indirect callus replacement.

5. The genetic transformation made it easier for many callus cells to isolate single cells and genetically engineer them.
6. Callus farms were used to study and regulate the production of primary and secondary metabolic compounds.

Cell suspension cultures

Most cell suspension cultures originate from callus cultures due mainly to mechanical impact in agitated liquid media. In stationary cultures on agar, a suspension can commonly be produced using a sterile glass rod or squeezing with a scalpel. In particular, with 2.4D in an agar medium, a loosely connected cell population develops on the opposite side of the agar, which can be easily scraped off with a scalpel. Enhanced production of recombinant proteins from plant cells is an important application.

An improvement can often be obtained by using ammonia as a nitrogen source, probably due to the excretion of protons in exchange for its uptake by the cells.

Callus cultures in a liquid nutrient medium are usually agitated, and after 10–14 days, this mechanical impact results in the development of cell suspensions consisting of cells from the periphery of the explants. Besides healthy cells that continue to grow, such a suspension also contains dead or decaying cell material. If the methods described above fail to succeed, then an enzymatic maceration of callus material should be attempted (0.05% crude macerozyme, 0.05% crude cellulose Onozuka P-1 500, and 8% sorbitol. Another possibility to produce a cell suspension is first obtaining protoplasts, as described later.

The definition of a cell suspension still provokes controversial discussions. The original aim in the 1950s was to establish culture systems in which, similarly to algae cultures, a suspension of cells of higher plants would consist solely of single cells. In practice, this aim was reached only for a few systems using a hanging drop method. All other attempts failed. Even in experiments starting with a population of single cells in a liquid medium, cell aggregations of various sizes will develop soon after initiation of growth, coexisting with some free cells.

Methods to Establish a Cell Suspension

As done for callus cultures, the description of obtaining a cell suspension shall be illustrated in a practical example that can be easily adapted to many other systems. In this example, shoot explants of *Datura innoxia* were originally used to produce callus cultures to study the synthesis of secondary metabolites. The establishment of callus cultures, now from shoot tissue, will be briefly described for a better understanding.

Explants of the uppermost (youngest) internode are cut using an extremely sharp scalpel. For sterilization, cut ends are briefly dipped into liquid paraffin to prevent the entrance of the agent used for surface sterilization for 5–6 min in the hypochlorite solution already described.

Using a laminar flow (aseptic working bench) for all further handling, internode segments 1–2 cm long are rinsed four to five times with sterilized distilled water. After this, the paraffin cover and the epidermis are removed with the help of a sterilized scalpel, and with a second sterilized scalpel, the tissue is cut into segments about 1 mm thick. These discs are cut into halves, which then serve to establish callus cultures. These segments are more significant than those used to develop primary carrot cultures, weighing about 7–8 mg and 30,000 cells each. If the diameter of the disc is even more significant, then even more explants can be obtained. The nutrient medium is given in (NL medium) and is suitable for stationary and liquid cultures. Within 3 weeks of culture, a highly increased callus develops from which peripheral cells can easily be scraped off with the help of a scalpel. This cell material is transferred to the MS medium with agar supplemented with kinetin for growth at 27 ℃ at a 12 h light/dark rhythm. After two subcultures at an interval of 3–4 weeks, the subsequent subcultures are initiated every 2 weeks. Also, only peripheral cell material from newly developed callus pieces is used for subculturing.

After the production of sufficient cell material, the loosely connected clusters are transferred into a liquid medium of the same composition in Erlenmeyer flasks on a shaker. Within 1 week, a dense cell suspension develops. An injection of 5 g fresh weight corresponds to a cell density of 40,000 cells per ml of nutrient medium, sufficient for optimal proliferation of the cell population.

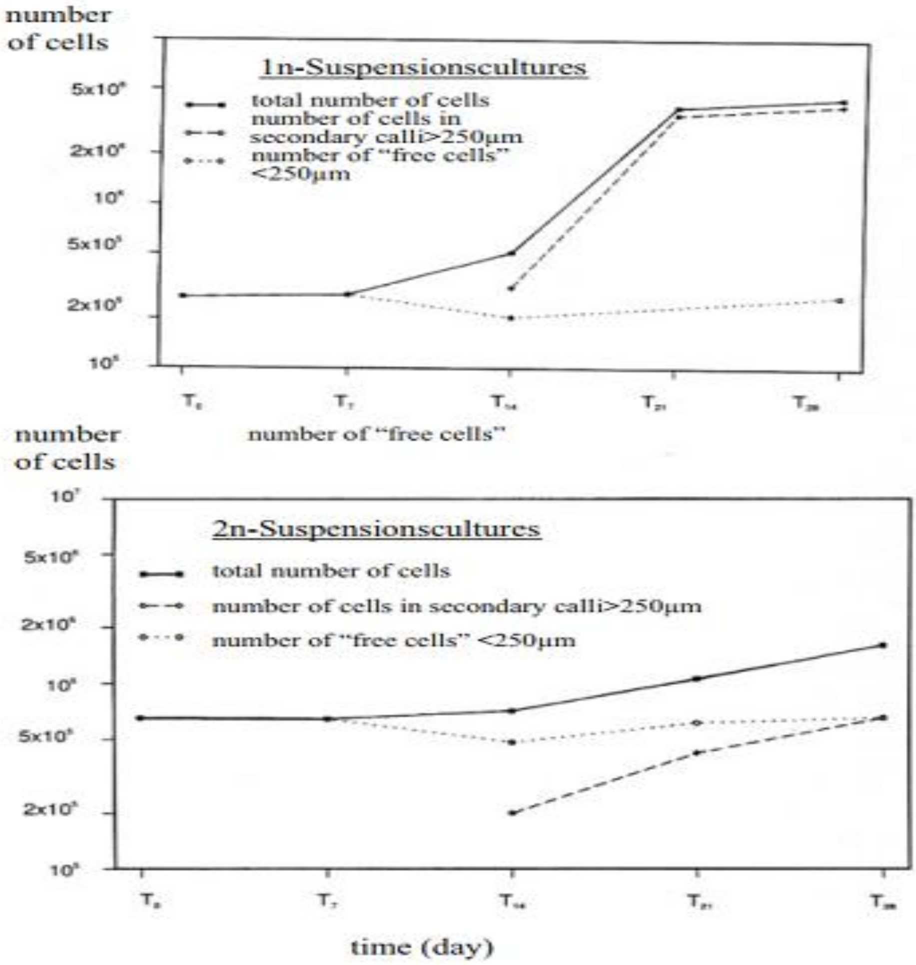

Figure 9. Growth of haploid (top) and diploid cell suspensions of *Datura innoxia*

Cell Population Dynamics

A cell suspension usually consists roughly of three fractions, i.e., free single cells of various shapes, cell aggregates of up to ten cells or more, and cell groups with a threadlike morphology. Suitable sieving techniques can isolate these fractions. Investigations to characterize these three fractions indicated that cell proliferation by division occurs predominantly in cell aggregates comparable to the meristematic nests of callus cultures. In both, very small cells can be seen in the center, and cell size increases toward the periphery. Highest cell division activity occurs in the center of these structures.

Due to the agitation of the shaker, the outermost cells of the cell aggregates are mechanically removed and then represent the fraction of free single cells. These cells should be older and mostly quiescent in terms of cell division activity. However, some of these cells preserve the ability to divide, or this is re-induced. Such cells are possibly the origin of the third fraction, the cellular threads. A similar organization can be observed in carrot cell suspensions. As an example, the threadlike structure in observed in a carrot suspension seems to be the result of three cell divisions. One terminal cell differentiates into a tracheidlike structure, the other accumulates anthocyanin, and the four central cells showing chlorophyll accumulation would be the youngest cells derived from the last rounds of cell division. The great differences in the structure of the two terminal cells point to an unequal first cell division, with differences in the distribution of cytoplasm. The nutrient medium can be regarded as identical for both cells.

A determination of DNA concentration indicated a near-cytogenetic homogeneity only for cells in the aggregates (secondary calli). In the population of free single cells, a strong inhomogeneity exists, sometimes with very high DNA content per cell. This observation is consistent with results obtained from callus material. Here, also the lowest C-values of a ploidy level can be found in the center of the meristematic nests with high cell division activity.

In both cases, these small cells in haploid cultures were found to have a DNA content essentially identical to that of microspores of the same species (G1-phase cells) or twice that of G2-phase cells. In diploid cultures, the DNA content was either twice that of G1-phase cells of haploids or four times the value of microspores in G2-phase cells. In older cells located between meristematic nests in callus material, which would be comparable to the fraction of free cells in the suspension, a broad variation in C-values was determined. Apparently, cytogenetic stability is linked to the age of the cells, i.e., the length of time elapsed since the last division. In young material with high cell division activity, a high percentage of cells contains DNA characteristic of the ploidy level. A supplement of kinetin, which increases cell division activity, results in a higher cytogenetic stability and homogeneity of the cell population.

In cell suspensions, many cell structures occur that are morphologically difficult to classify. However, some well-defined cell types can also be observed, e.g., tracheids, as described above. In a cell suspension, all free single cells are bathed in the same nutrient solution, and therefore the morphological diversification of its components should be based on the origin of the individual cell. The significance of unequal cell divisions has already been mentioned above—whatever the cause of this phenomenon may be. A direct relation between cell shape and vitality has not been observed.

In cell suspensions of some species like Daucus in an IAA-supplemented medium, after some weeks of culture, the formation of early stages of embryo development can be observed, and these can eventually be raised to intact plants.

Using the methods described above, only limited amounts of cell material can be produced, usually not sufficient to study physiological or biochemical problems of primary or secondary metabolism or, e.g., somatic embryogenesis. If greater amounts of material are required, fermenter cultures are performed. As an example, fermenter cultures of Datura innoxia shall be described. Here, within 2 weeks it was possible to produce 1 g of dry weight per day in a liquid nutrient medium of 3.5 l originally inoculated with a cell suspension of 30 g fresh weight. The cell suspension was obtained by a method used to raise cytogenetically stable material, as described later. Pre-culture is carried out in 200 ml nutrient medium MS+kinetin, in a 750 ml Erlenmeyer flask on a shaker. For initiation of the pre-culture, the vessel is inoculated with 1–2 g fresh weight (90–250 μm fraction). The main aim of the pre-culture is to propagate the cells. After 10–14 days of pre-culture, the content of the vessel (cells and nutrient medium) is transferred to the fermenter, as described above. In the fermenter, cell aggregates as well as free single cells occur.

The principle to distinguish between a propagation phase and a production phase is also applied to fermenter cultures used for biotechnological purposes. Here, fermenters of much larger volume are used; to produce cell suspensions for inoculation, however, smaller laboratory fermenters are used initially, as described later. Usually, the cell suspension is transferred with some nutrient medium from the smaller to the next bigger fermenter. For a semi-continuous culture, it is common practice to remove part of the cell material in certain intervals of time for processing and to apply fresh nutrient medium. As described later, plant cell suspensions are already today cultured in fermenters with a volume of thousands of liters (Mitsu Petrochem. Ind. Ltd.), e.g., to produce shikonin derivatives using cultures of Lithospermum officinale. Also propagation via somatic embryogenesis has been carried out in a fermenter (e.g., Daucus).

To maintain cell strains in a healthy condition for prolonged periods, subcultures have to be made frequently, usually at 1- or 2-week intervals, and with a dilution of 1:5 after 1 week and 1:10 after 2 weeks with the fresh nutrient medium. The optimal dilution and the subculture frequency have to be determined for each individual strain. As described above, also cryopreservation is often used to maintain cell suspensions.