Plant Micropropagation

Introduction

Tissue culture is commonly used as a blanket term to describe all types of plant cultures, namely callus, cell, protoplast, anther, meristem, embryo and organ cultures. It relies on the phenomenon of cell totipotency, the latter being the ability of single cells to divide, to produce all the differentiated cells characteristic of organs, and to regenerate into a whole plant. The different techniques of culturing plant tissues may offer certain advantages over traditional methods of propagation. Growing plants *in vitro* in a controlled environment, with in-depth knowledge of the culture conditions and the nature of the plant material, ensures effective clonal propagation of genetically superior genotypes of economically important plants. Tissue cultures represent the major experimental systems used for plant genetic engineering, as well as for studying the regulation of growth and organized development through examination of structural, physiological, biochemical and molecular bases underlying developmental processes. Micropropagation has become an important part of the commercial propagation of many plants because of its advantages as a multiplication system. Several techniques for *in vitro* plant propagation have been devised, including the induction of axillary and adventitious shoots, the culture of isolated meristems and plant regeneration by organogenesis and/or somatic embryogenesis.

Fertile plants can be regenerated either by the growth and proliferation of existing axillary and apical meristems, or by the regeneration of adventitious shoots. Adventitious buds and shoots are formed *de novo*; meristems are initiated from explants, such as those of leaves, petioles, hypocotyls, floral organs and roots.

Application/Advantages of micropropagation in plant biotechnology

Micropropagation has become a suitable alternative to conventional methods of vegetative propagation of plants, and some of the application or advantages are as follows:

1. Micropropagation is the best technique for the production of millions of clones in one year. When it comes to conventional techniques, it takes years to produce an equal number of plants.
2. Micropropagation facilitates the growth, storage, and maintenance of a large number of plants in small spaces which makes it a cost-effective process.
3. Micropropagation is used for germplasm storage and the protection of endangered species. Seeds and vegetative organs of several species of plants have a limited storage life. Meristem cells are used for this purpose because they are stable, survive under cryopreservation procedures, and regenerate into pathogen-free plants whenever required.
4. Some plants are difficult or resistant to be grown on a large scale by conventional techniques. Micropropagation is a good alternative to cultivate these plants.

5. Micropropagation can be used to obtain disease or pathogen-free plants. Meristem tip culture is used to achieve this goal. For example, virus-free potatoes can only be obtained by the micropropagation of plants.
6. The micropropagation technique is the best alternative for clonal propagation at a large scale in a very short time. At times, the use of this technique has proven to reduce the time to culture by 50 %.
7. Micropropagation is used to increase the vigor and yield of floriculture species by multifold.
8. Micropropagation is popular for the production of synthetic seeds that analog to true seeds. These seeds match with the morphology, physiology, and biochemistry of the zygotic embryos.
9. Micropropagation also allows the production and regeneration of the stored stocks all over the year. It's best for nurseries that are willing to sell fruit, ornamental plants, and tree species any time of the year.
10. Micropropagation is the best technique to transfer different species of plants to different countries. This makes the international exchange processes easier and reduces the risk of contamination during the transfer.

Explants and their surface disinfection

Small pieces of plants (explants) are used as source material to establish cells and tissues *in vitro*. All operations involving the handling of explants and their culture are carried out in an axenic (aseptic; sterile) environment under defined conditions, including a basal culture medium of known composition with specific types and concentrations of plant growth regulators, controlled light, temperature and relative humidity, in culture room(s) or growth cabinet(s). The disinfection of explants before culture is essential to remove surface contaminants such as bacteria and fungal spores. Surface disinfection must be efficient to remove contaminants, with minimal damage to plant cells.

The composition of the culture medium depends upon the plant species, the explants, and the aim of the experiments. In general, certain standard media are used for most plants, but some modifications may be required to achieve genotype-specific and stage-dependent optimizations, by manipulating the concentrations of growth regulators, or by the addition of specific components to the culture medium.

Difference Between Direct and Indirect Organogenesis

1. Definition: Direct organogenesis refers to the process of plant regeneration and the formation of new organs (such as shoots or roots) directly from the explant (the piece of plant tissue used for propagation) without the intermediate formation of callus tissue. Indirect organogenesis, on the other hand, involves the formation of callus tissue as an intermediate step in the regeneration process.
2. Regeneration Pathway: In direct organogenesis, shoots or roots regenerate directly from the explant or tissue without forming an intermediate callus. In

contrast, indirect organogenesis involves forming a callus, an undifferentiated mass of cells, before the regeneration of shoots or roots occurs.

3. Morphological Characteristics: Direct organogenesis often results in the formation of shoots or roots that closely resemble the original plant tissue in terms of morphology, structure, and genetic makeup. However, in indirect organogenesis, the regenerated shoots or roots may exhibit variations in morphology compared to the original tissue. The callus phase can lead to somaclonal variations, which are genetic and phenotypic variations resulting from tissue culture processes.
4. Efficiency and Speed: As it skips the callus phase, direct organogenesis typically shows faster and more efficient regeneration than indirect organogenesis. Indirect organogenesis involves forming and manipulating a callus, which can be time-consuming and less efficient than direct organogenesis.
5. Genetic Stability: Generally, direct organogenesis maintains a higher level of genetic stability than indirect organogenesis. This is because it bypasses the callus phase, reducing the opportunity for genetic and epigenetic changes to occur. Due to prolonged exposure to the callus phase, indirect organogenesis can result in somaclonal variations caused by cellular reprogramming and genetic rearrangements during this stage.
6. Preferred Applications: Direct organogenesis is often preferred for applications aiming to regenerate true-to-type plants with minimal genetic variations. It is commonly used for clonal propagation, micropropagation of elite plant varieties, and multiplication of valuable horticultural crops. Indirect organogenesis is more suitable for applications such as somatic embryogenesis, where the formation of embryos is desired, or for genetic transformation experiments where the callus can serve as a target tissue for gene transfer.
7. Conclusion: The main difference between direct and indirect organogenesis is that direct organogenesis involves the direct development of shoots or roots from the explant without an intermediate callus phase. In contrast, indirect organogenesis involves the formation of a callus followed by the regeneration of shoots or roots.

Stages of micropropagation

The following distinct stages are recognized for the micropropagation of most plants (Fig. 10):

Stage I: Establishment of axenic cultures – introduction of the surface disinfected explants into culture, followed by initiation of shoot growth. The objective of this stage is to place selected explants into culture, avoiding contamination and providing an environment that promotes shoot production. Depending on the type of explant, shoot formation may be initiated from apical and axillary buds (pre-

existing meristems), from adventitious meristems that originate on excised shoots, leaves, bulb scales, flower stems or cotyledons (direct organogenesis), or from callus that develops at the cut surfaces of explants (indirect organogenesis). Usually 4–6 weeks are required to complete this stage and to generate explants that are ready to be moved to Stage II. Some woody plants may take up to 12 months to complete Stage I, termed 'stabilization'. A culture is stabilized when explants produce a constant number of normal shoots after subculture.

Stage II: Multiplication – shoot proliferation and multiple shoot production. At this stage, each explant has expanded into a cluster of small shoots. Multiple shoots are separated and transplanted to new culture medium. Shoots are subcultured every 2–8 weeks. Material may be subcultured several times to new medium to maximise the quantity of shoots produced.

Stage III: Root formation– shoot elongation and rooting. The rooting stage prepares the regenerated plants for transplanting from *in vitro* to *ex vitro* conditions in controlled environment rooms, in the glasshouse and, later, to their ultimate location. This stage may involve not only rooting of shoots, but also conditioning of the plants to increase their potential for acclimatization and survival during transplanting. The induction of adventitious roots may be achieved either *in vitro* or *ex vitro* in the presence of auxins. The main advantage of *ex vitro* compared to *in vitro* rooting is that root damage during transfer to soil is less likely to occur. The rates of root production are often greater and root quality is optimized when rooting occurs *ex vitro.*

Stage IV: Acclimatization – transfer of regenerated plants to soil under natural environmental conditions. Transplantation of *in vitro*-derived plants to soil is often characterized by lower survival rates. Before transfer of soil-rooted plants to their final environment, they must be acclimatized in a controlled environment room or in the glasshouse. Plants transferred from *in vitro* to *ex vitro* conditions, undergo gradual modification of leaf anatomy and morphology, and their stomata begin to function (the stomata are usually open when the plants are in culture). Plants also form a protective epicuticular wax layer over the surface of their leaves. Regenerated plants gradually become adapted to survival in their new environment.

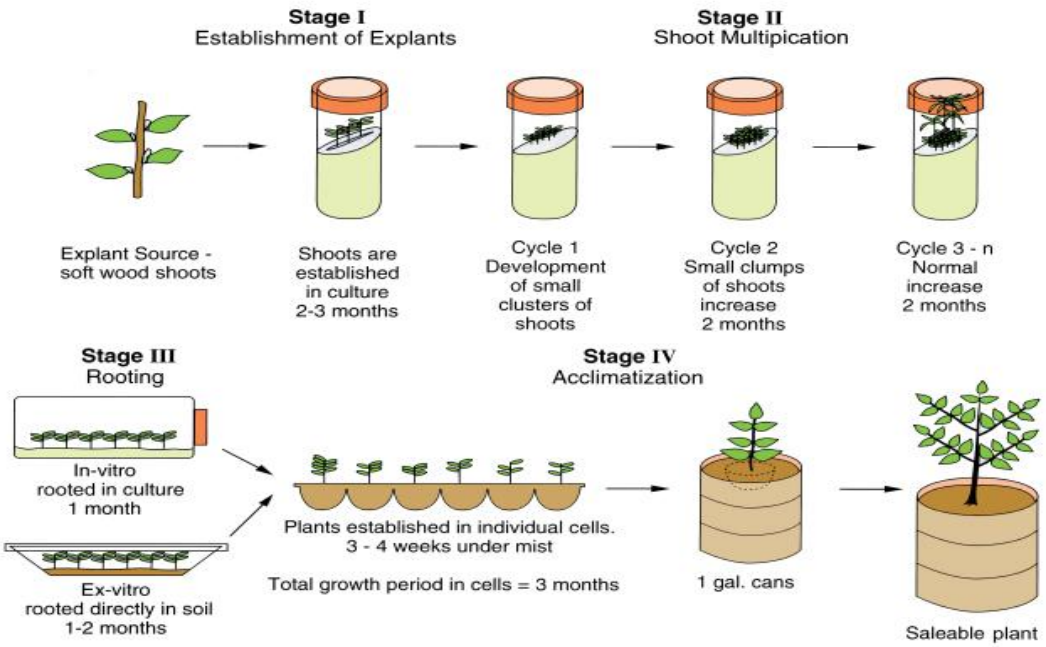

Figure 10. Stages of micropropagation in the plants.

1. Cultures of apical and axillary buds

Currently, the most frequently used micropropagation method for commercial mass production of plants utilizes axillary shoot proliferation from isolated apical or axillary buds under the influence of a relatively high concentration of cytokinin. In this procedure, the shoot apical or axillary buds (Fig. 11) contain several developing leaf primordia. Typically, the explants are 3–4 mm in diameter and 2 cm in length. Development *in vitro* is regulated to support the growth of shoots, without adventitious regeneration.

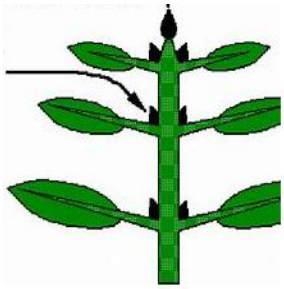

Figure 11. apical and axillary buds in the plant

2. Meristem and single- or multiple-node cultures (shoot cultures)

Meristems are groups of undifferentiated cells that are established during plant embryogenesis. Meristems continuously produce new cells which undergo differentiation into tissues and the initiation of new organs, providing the basic structure of the plant body. Shoot meristem culture is a technique in which a dome-shaped portion of the meristematic region of the stem tip is dissected from a selected donor plant and incubated on culture medium. Each dissected meristem comprises the apical dome with a limited number of the youngest leaf primordia*a*, and excludes any differentiated provascular or vascular tissues. A major advantage of working with meristems is the high probability of excluding pathogenic

organisms, present in the donor plant, from cultures*b*. The culture conditions are controlled to allow only organized outgrowth of the apex directly into a shoot, without the formation of any adventitious organs, ensuring the genetic stability of the regenerated plants.

The single-or multiple-node technique involves production of shoots from cultured stem segments, bearing one or more lateral buds, positioned horizontally or vertically on the culture medium*c*. Axillary shoot proliferation from the buds in the leaf axils is initiated by a relatively high cytokinin concentrationd. Meristem and node cultures are the most reliable for micropropagation to produce true-to-type plants*e.*

3. Adventitious shoot formation

Adventitious shoot formation is one of the plant regeneration pathways *in vitro*, and is employed extensively in plant biotechnology for micropropagation and genetic transformation, as well as for studying plant development. Adventitious meristems develop *de novo* and *in vitro* they may arise directly on stems, roots or leaf explants, often after wounding or under the influence of exogenous growth regulators (direct organogenesis). Cytokinins are often applied to stem, shoot or leaf cuttings to promote adventitious bud and shoot formation. Adventitious buds and shoots usually develop near existing vascular tissues enabling the connection with vascular tissue to be observed. Adventitious organs sometimes also originate in callus that forms at the cut surface of explants (indirect organogenesis). Somaclonal variation, which may be useful or detrimental, may occur during adventitious shoot regeneration.

4. Somatic embryogenesis

Somatic embryogenesis was defined by Emons as the development from somatic cells of structures that follow a histodifferentiation pattern which leads to a body pattern resembling that of zygotic embryos (Fig. 12). This process occurs naturally in some plant species and can be also induced *in vitro* in others species. There is considerable information available on *in vitro* plant regeneration from somatic cells by somatic embryogenesis. Somatic embryogenesis may occur directly from cells or organized tissues in explants or indirectly through an intermediate callus stage.

It has been confirmed in many species that the auxins 2,4-D and NAA, in the correct concentrations, play a key role in the induction of somatic embryogenesis. Application of the cytokinins, BAP or kinetin, may enhance plant regeneration from somatic embryos after the callus or somatic embryos have been induced by auxin treatment. However, in some species (such as *Abies alba*) cytokinins on their own induce somatic embryogenesis.

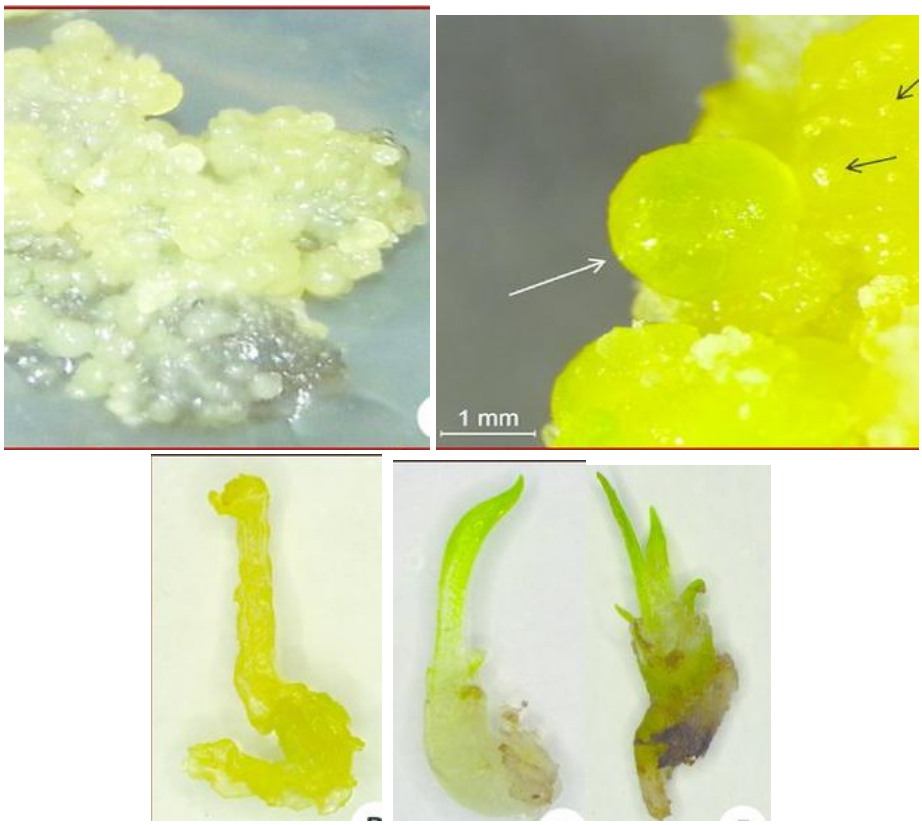

Figure 12. Stages of somatic embryogenesis formation

Problems Associated with Plant Micropropagation

Due to the great potential of plant tissue culture in both scientific research laboratories and commercial companies, several applications for this vital field could be achieved, but this sector still face a lot of problems, especially regarding plant micropropagation, which causes a lot of economic crises for these laboratories and companies. These problems are highlighted in the following:

1. Contamination of Plant-Tissue Cultures

The contamination of *in vitro* plants is considered a crucial obstacle, which prohibits successful micropropagation protocol. Contamination may include many microorganisms, such as bacteria, fungi, molds, and yeasts. External contamination results from the laboratories and used materials (media; glassware; culture vessels, tools, explants), whereas internal contamination is related to the endophytic microbes in mother plants. Several proper methods could be used to exclude and eliminate the contaminants through surface sterilization, such as chemical agents (antiseptic agents, liquid detergent, mercuric chloride or sodium hypochlorite), ultraviolet (UV) sterilization, autoclaving of media and instruments, and improvement of cultural practices or handling. On the other hand, antibiotics (ampicillin, penicillin, ticarcillin) could be used as anti-microbial agents for eliminating endophytic bacteria in *in vitro* cultured plants. The external contamination by epiphytic microbes could be inhibited by surface sterilization with running water with/without detergent, chemical substances (ethanol, mercuric chloride, sodium hypochlorite), and plant-preservative mixtures. However, there are some materials could be added to the culture media to inhibit

the external contamination, such as plant-preservative mixtures and benomyl fungicide. Endophytic microbes that are present within the explants are considered a major constrain to the establishment and growth of tissue-cultured plants, as they are more difficult to remove by normal surface sterilization. However, the internal bacterial contamination could be eliminated by supplying the culture media with different substances, for example, antibiotics, copper sulfate, or fungicides. After identification of the contaminants, a low-phytotoxicity antibiotic should be selected.

2. Delay of Subculture and Burned Plantlets

The *in vitro* micropropagation technique has a lot of benefits; the most important one is represented in producing true-to-type plantlets, which are genetically and physiologically uniform. These plant materials can be used in the intensive production of several plants. *In vitro* propagation protocol includes certain stages. under heavy work and a lot of tasks in plant tissue culture laboratories, the lack of facilities, and a limited number of expert workers and technical specialists, a delay of subculture definitely happens. This delay will for sure cause a great loss or damage in the production of *in vitro* plantlets and even in the maintenance of stock cultures. Generally, the effect of time of subculture on the shoot-proliferation rate of *in vitro* cultures varyies from one species to another. Somaclonal variation, which resulted due to prolong incubation period in the culture medium (delay of subculture), hinders supplying clonally identical plantlets, which is considered the main target of plant micropropagation. It was proven that supplementation of a culture medium with 50 µM salicylic acid (SA), an anti-ethylene compound, decreased hyperhydricity as well as somaclonal variation under a long-term culture. So, SA was recommended for moringa clonal micropropagation. Burned plantlets are a very common phenomenon during *in vitro* handling, which results from using hot planting tools (mainly forceps and scalpels) during transfer the plantlets. This problem can be avoided when the workers in the plant tissue culture laboratory are trained and have enough experience in this field.

3. Browning of Plant-Tissue Cultures

The browning of explants or phenolic browning is a phenomenon that results naturally from enzymatic oxidation of the polyphenolic compounds, which are well-known inhibitors in PTC (Fig. 13). Phenols released from injured or cut explants are then oxidized to quinones by polyphenol oxidases (PPOs) and peroxidase (POD), causing browning of the tissues and medium as well. These quinones bind with cell proteins or polymerizes by dehydration, causing the disruption of cell metabolism, inhibition of growth, and, ultimately, death of explants.

Figure 13. Browning of Plants

4. *In Vitro* Rooting Difficulty and Failure of Subsequent Acclimatization

A successful micropropagation protocol requires the appropriate conditions for *in vitro* root initiation and the development of regenerated shoots. Healthy, rooted *in vitro* micro shoots could be acclimatized successfully *in vitro* and/or *ex vitro*; when they are transferred to *ex vitro* conditions, then they can be established in the soil. It was observed that the type and concentration of cytokinin added to the shoot-multiplication medium highly affects the subsequent rooting stage, where rooting frequency has been reduced in many species by supplementation of thidiazuron (TDZ) or benzyladenine (BA) to the shoot-multiplication medium, while it strongly inhibited rooting in other species. Moreover, by increasing TDZ concentration in the last shoot-multiplication medium, the number of roots per shoot decreased until it reached 0% at 0.45 μM TDZ.

Reducing the salt strength to a half or a third in the rooting medium has been proven to be useful for enhancing root induction, development, and number as well in many leguminous species, fruits, medicinal plants, and trees, while a full-strength MS medium has been widely used for *in vitro* rooting in most herbaceous non-shrub plants that do not have problem in rooting.

In addition, the type of auxin added to the rooting medium is mainly dependent on several factors; the most important one is the protocol used for *in vitro* rooting, if it is one-phase or two-phase, while the latter one is widely applied for most horticultural plants. It was reported that the rooting of micropropagated apple shoots includes a short phase of one week for root induction, in which IBA was frequently applied, followed by a longer phase of several weeks where naphthaleneacetic acid (NAA) was the most-common auxin supplied to the medium for root elongation. Moreover, the optimal auxin for rooting was plant-species-dependent. Therefore, the choice of auxin is a very important issue for woody plants, which show difficulty in *in vitro* rooting. It was found that IBA has been commonly used, rather than indole-3-acetic acid (IAA) and NAA, as the most effective auxin to induce root formation in woody species such as apple and *Cassia*

angustifolia Vahl.; whereas, 1, 2-benzisoxazole-3-acetic acid (BOA) showed very poor effect on root induction in woody plants. In this regard, IBA was the most efficient among the other auxins tested for root induction from the *in vitro* shoots.

Due to many reasons, such as low humidity, high level of irradiance, low root uptake of water under *in vitro* conditions, rapid desiccation of *in vitro* plantlets, and their easy infection by fungal and bacterial diseases; *in vitro* rooted plants might fail when they are transferred to *ex vitro* conditions during acclimatization. To overcome those obstacles, *in vitro* plants should be supported gradually by following the right acclimatization strategy and right selection of the acclimatization medium. Thus, the ultimate success of the *in vitro* micropropagation of plants depends on the successful establishment of plantlets in the soil through the acclimatization process. Application of biostimulators was proven to be used for successful acclimatization. The fungus has been used as a biological stimulator to enhance plant growth and root development during the acclimatization process, allowing *in vitro* hard rooting plants such as orchid to be transferred to the field. The physiological aspects of *in vitro* rooted banana plants (*Musa* sp.) have been improved through inoculation of *Buttiauxella agrestis* and *Bacillus thuringiensis* (plant growth-promoting bacteria that produce auxins) into the root system of banana at the acclimatization stage. Hence, high quality seedlings have been acquired. In addition, many supporting materials (e.g., agar, rockwool, perlite, vermiculite) have been used to improve the growth and survival rates of *in vitro* plants in *ex vitro* conditions.

5. Problems Originated Due to Genetical Reasons Somaclonal Variation

The somaclonal variation point to culture-induced, unexpected, and undesired variations or anomalies, which eventually is inherited by the clonal progenies, becomes genetic and happens during tissue cultures; this is, thus, noticed in the *in vitro* regenerated plants, and is an often an unwanted phenomenon, especially in mass micropropagation programs that are highly desirable for the production of plant material of the same type as the mother (genetically uniform) by maintaining the genetic stability of the *in vitro* regenerated plantlets. Somaclonal variation is genotype-dependent, where some genotypes or species are more amenable to these changes than others when they are cultured *in vitro*. There are other reasons for genetic variability, including the period of the *in vitro* culture of the explant, composition of the culture medium in particular auxins and cytokinins as plant-growth regulators, type of sugar, proliferation rate of tissues, interval between subcultures, natural selection, mutations in *in vitro* cultures, ploidy level, and mode of regeneration used. *In vitro* culture of the explant causes stress on the plant cells and makes them undergo a genomic shock or change. Furthermore, mechanical factors, such as damage of explant or its exposure to sterilizing substances, as well as instability in temperature, humidity, and lighting, can promote somaclonal variation.

To overcome somaclonal variation, we have to avoid long-term *in vitro* cultures and overexposure to phytohormones in culture medium as well as regularly

reinitiate clones from new explants, plus the number of subcultures should be kept at a minimum and the use of 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin (KIN) in tissue cultures should be rationalized, because they are known to induce variations.

6. Hyperhydricity

The term of hyperhydricity (HH) mainly describes a physiological disorder or malformation resulting from the immoderate hydration, little lignification, and, thereby, diminished mechanical strength of *in vitro* cultured plants. It occurs in herbaceous, woody plants and succulent plants. This term is also called glassiness, glauciness, translucency, and vitrescence, and it used to describe this physiological disorder (Fig. 14). The hyperhydrated or vitrified *in vitro* plants appear to be turgid or hyperhydric and hypo-lignified, and their surfaces are watery. Somehow, their organs are translucent, less green in some cases, and they break easily.

The main reasons for the HH in PTCs may include high relative humidity, high salt concentration of the medium, low light intensity, gas accumulation in the jar, length of time intervals between subcultures where a lengthy subculture enhanced ethylene accumulation in culture vessels that led to HH, number of subcultures where upon increasing the number of subcultures of *in vitro* cultured plants the occurrence of HH increases, low calcium content, and high ammonium content, which may trigger oxidative stress. There are different factors that impact HH, which can be classified as follows: (1) explant, including physiological age, genotype, organ type, and size; (2) media components, such as basal medium, gelling agents, and plant-growth regulators; (3) culture conditions, such as ventilation and light intensity; and (4) exogenous additive substances, such as salicylic acid (SA), polyethylene glycol (PEG 6000), hydrogene peroxide (H_2O_2), and Ag^+. This phenomenon could be controlled, as reported in some studies, by supplementing the culture medium with $AgNO_3$ or ascorbic acid, salicylic acid, spermidine, and H_2O_2. Gelling agents are one of the components in the culture medium, and the type and concentration of them significantly affects the quality of the *in vitro* plant, as they cause HH. So, HH could be prevented in shoots by replacing gelrite with one of the other gelling agents such as Agar-Agar, at 7 g/ L. It was noticed that using gelrite as a gelling agent caused a lower multiplication rate and almost four-fold HH (65%) in *Aloe polyphylla* and two times higher HH in teak (*Tectona grandis* L.), higher than that in an agar-solidified medium, due to its physical properties that lead to a higher water percentage in *in vitro* cultured tissues, as well as because of the small amount of gelrite put in the medium; thus, the quantity of water is greatly causing HH. Moreover, this phenomenon could be minimized by ensuring adequate gas exchange with ventilation and light-emitting diodes facilitation or supplementation of the growth media with polyamines.

Figure 14. in vitro hyperhydricity

7. Shoot Tip Necrosis

Shoot-tip necrosis (STN) is defined as a physiological status that leads to death of the shoot tip of *in vitro* shoots and is caused by the culture conditions. Moreover, STN results in shoot tip burn, injury, or dieback. STN may occur when the shoot tip of a plantlet has some abnormal features, such as in the elongation and/or rooting stages or browning and death during multiplication, although the growing of plantlets may be appeared as ideal conditions for both *in vitro* and *ex vitro* cases. The precise mechanism of STN still needs more clarification, but some reasons have been proposed such as the deficiency of some minerals (e.g., Ca, Mg, K, B, or NO^{-}_{3}), an imbalance of nutrients, accumulations of phenolic compounds in shoot tip, a lack of antioxidants, a low level of Ca^{+}_{2}, the presence of high concentrations of PGRs in the culture medium, or the kind and concentration of cytokinins in the medium. Several methods have been employed to relieve STN, i.e., the supplementation of 50–100 mg/L calcium chloride to MS medium, which allows recovery of 90% of banana and plantains (*Musa* spp.) shoots, or the addition of 0.50 mM fructose + 1.0 mM calcium chloride to culture medium, which gave 100% success to control STN in plum (*Prunus salicina* L.). Adding adenine sulphate at 100 mg/L with 10 µM BA was the most effective to inhibit STN in 90% of *Syzygium cumini* L. cultures. Applying ethylene-inhibitor compounds to the shoot-regeneration medium, it has been proven to enhance apical shoot initiation and multiplication rates as well as reduce the leaf senescence (yellowing) and shoot necrosis of *in vitro* roses through ethylene biosynthesis inhibition.

8. In Vitro Habituation

It is the natural phenomenon, whereby tissue cultures continue to develop in the absence of auxins or cytokinins, because the endogenous biosynthesis by the cultured tissue leads to an autotrophic or independent state (auxins or cytokinins-sufficiency), which is called habituation (Fig. 15). These cultures do not need the supplementation of external auxins or cytokinins, when they lose their requirements for these specific hormones. This phenomenon resulted from a

prolonged culture period. Early studies explain that cytokinin independence may be due to overproduction by the cultured tissues. Habituation can be transferred to the new cultures, when the callus derived from the habituated ones keeps the cytokinin-independent state. A specific gene (the cytokinin receptor *CRE1*), whose overexpression may responsible for the habituation as a naturally occurring phenomenon. The definition of habituation as "the ability of plant tissues to grow or regenerate on plant growth regulators-free medium". It was noticed that the habituated rice calli (H-calli) showed decreasing levels of lipids and starch, the two major storage metabolites in higher plants, compared to non-habituated ones (NH-calli). This is attributed to their elevated consumption of lipids and starch in the absence of an exogenous addition of hormones, to ensure their requirements of energy to maintain an optimum growth and development, so they can survive and grow under an exogenous growth regulator free medium. The two types of rice calli (H-calli and NH-calli) were reported to have completely different endogenous hormonal status. The indole-3-acetic acid (IAA) content in habituated calli was seven-fold higher than that in non-habituated ones. On the other hand, there was a decrease in abscisic acid (ABA), ethylene, and gibberellins (GA_{12}, GA_{19}, GA_{29}) in the habituated calli. This explains why the habituated rice calli can grow faster than the non-habituated ones.

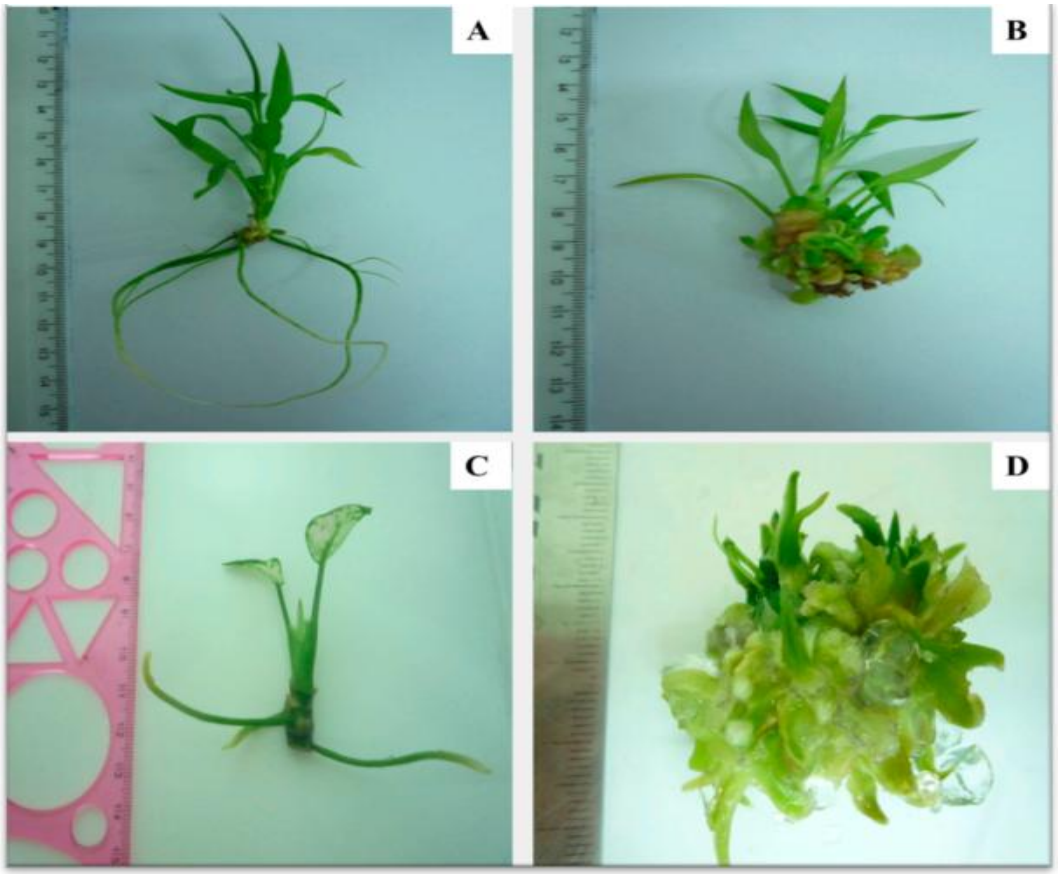

Figure 15. In Vitro Habituation