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Plant Tissue Culture: A Comprehensive Overview

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Abstract

Plant tissue culture (PTC) is a technique of growing plant cells and organs *In vitro* which divide and regenerate into callus or specific plant parts. Micropropagation gained popularity after the discovery of Murashige and Skoog, also known as MS media in 1962. Using PTC a large number of identical, true-to-type plants can be produced in a short duration of time. Although, still there are a number of problems associated with PTC like Browning, Contamination, swollen base tube, albinism and delay of subculture. There is still a lot of work that need to be done in the domain of Plant tissue culture.

Keywords: Plant tissue culture, *In vitro*, Micropropagation

Introduction

Plant tissue culture plays a key function in numerous fields such as agriculture, horticulture, research, and conservation sectors (Bhatia *et al.*, 2015 and George *et al.*, 2008) ^[1,2]. For more than half century, *In vitro* tissue culture has proved essential in a wide range of applications, including plant reproduction, germplasm preservation, and bioenergy generation (Misra *et al.*, 2016 and Norougi *et al.*, 2022) ^[3]

Micropropagation is the most widely used PTC technology for producing large quantities of crop plants. The size of the tissue in culture is much smaller than in a traditional manner, the process has been called micropropagation. For the first time, in 1952, shoot meristems were cultured to produce virus-free plants. Later, the field of micropropagation was further expanded to include a wide variety of plant species, including fruit and plantation crops, with the discovery by Murashige and Skoog (1962) ^[4] of the hormone control of organogenesis and the most widely used tissue culture medium (MS media). The advantages of utilizing micropropagation are year-round availability of plantlets, quick multiplication of true-to-type planting material, multiplication of hybrid, and creation of female plants in dioecious fruit plants

Plant tissue culture (PTC) is a method of growing plant cells and organs *In vitro* that divide and regenerate into callus or specific plant parts. PTC is being widely utilized for generating huge number of plants at a very quick rate, with better genetic features, under controlled environmental circumstances. Also, the development of cell, tissue, and organ culture and regeneration of plantlets under *In vitro* conditions has opened up new paths in plant biotechnology (Dagla, 2012) ^[5]. As a result of *In vitro* propagation large numbers of identical plants can be produced within limited space and time, which can be used as planting materials, the technique is being used in the micropropagation for various plants. *In vitro* plant culture is an invaluable tool for mass multiplication of plants, eliminating plant diseases through meristematic tissue culture technique, plant conservation, and crop improvement through gene transfer (Sarasan *et al.*, 2011) ^[6].

PTC is capable of maintaining continuous production systems regardless of environmental or geographical limits in order to meet the food, feed, fibre, and energy supply needs of a growing population (Neumann *et al.*, 2020) ^[7]. One of the best methods for addressing the various issues facing agricultural and horticultural production—such as salinization, desertification, global warming, and climate change—is PTC. These issues are contributing to a global food crisis and famine (Viana *et al.*, 2022) ^[8]. PTC offers numerous other advantages, including the ability to produce pathogen-free plants (Rani *et al.*, 2019) ^[9]; quickly multiply hard-to-propagate plants (Park *et al.*, 2021) ^[10]; enhance the genetics of commercial plants to the desired extent (Naing *et al.*, 2021) ^[11]; obtain androgenic and gynogenic haploid plants to speed up breeding programs

(Sánchez *et al.*, 2020) ^[12]; preserve plants that are endangered or rare (Dhiman *et al.*, 2020) ^[13]; and produce various varieties tolerant to abiotic stresses like heat, salinity, and drought (Karakas *et al.*, 2020) ^[14].

This current review is intended to follow the typical sequence of plant tissue culture procedures, beginning with prerequisites and preparation, and commencing through initiation, proliferation, and regeneration processes. Thus, first, a quick overview of the various advances in micropropagation. Then, shifting onto PTC, the prerequisites of PTC and its methodologies.

Types of PTC

a) Meristem Tip Culture: This technique involves cultivating the meristem tip, which consists of one or two pairs of leaf primordia, in a medium. The plantlets regenerate in a few weeks, and once they have hardened off, they are placed in the soil in their natural habitat. Meristem tip-cultured plants give rise to polyploid plants instead of diploid plants. Additionally, meristem tip culture is a highly helpful method for getting rid of viruses from plant material that has been affected. Meristem culture also allows for the rapid multiplication of plants, which are otherwise difficult to propagate vegetatively. Produced plants are pathogen-free and may be kept for longer periods of time in fewer spaces.

b) Micrografting: Since it is challenging to regenerate entire plants from their meristem in woody species, such as the majority of fruit and forest plants, micrografting is used as a substitute to create virus-free plants. The process of micrografting involves several processes, such as preparing the scion, and rootstock, grafting *In vitro*, and acclimatizing/hardening the plants. The *In vitro*-raised nuclear seedlings are employed as rootstocks. The scion (meristem 0.1-0.4 mm) can be harvested from immature field-grown trees, defoliated glasshouse-grown plants, or nodal segments that have proliferated *In vitro* from older trees. The grafting is carried out in an aseptic environment with the use of a stereomicroscope. Many viruses, including *Citrus tristeza* virus, Peach Latent Mosaic Viroid, and Pear Vein Yellow Virus, have been eradicated in fruit plants using the process of micrografting

c) Ovule Culture: A crucial method in plant biotechnology, ovule culture entails cultivating ovules *In vitro* in order to trigger embryogenesis and subsequently plant regeneration. This technique has been successful in improving a number of crops, including cotton (*Gossypium* spp.), an economically significant crop. Ovule cultivation is especially useful for crops with intricate reproductive systems or those that are challenging to breed with conventional techniques. For instance, cotton greatly benefits from ovule cultivation procedures due to its complex floral structure and limited natural crossover. The effectiveness of ovule culture in cotton is demonstrated by earlier study by Zhang *et al.* (2016) ^[15], which demonstrates high rates of embryo production and plant regeneration. The study highlighted the possibility of ovule culture as a useful technique for crop development b reporting the cultivation of viable cotton plants with better fibre quality attributes. Breeders can expedite the breeding process and increase crop output by using this technology to quickly create homozygous lines and introduce desired features

d) Anther Culture: Anther culture, a crucial approach in plant biotechnology, involves the *In vitro* culture of anthers to induce embryogenesis before they regenerate into complete plants. When it comes to breeding and improving crops with recalcitrant or ineffective breeding systems, like some cereal

crops like barley (*Hordeum vulgare*), this approach has shown to be especially successful. Anther culture allows homozygous lines to be directly regenerated in a short amount of time, avoiding the slow and unpredictable procedure of conventional breeding. The effectiveness of barley anther culture has been supported by numerous studies, which show that it produces useful agronomic features and has high rates of regeneration. Svec *et al.* (2018) ^[16] demonstrated that barley plants may be successfully regenerated via anther culture, producing a large number of fertile plants with improved characteristics including grain quality and disease resistance. These results highlight the potential of anther culture as an important tool for crop development, giving breeders a quick and effective way to create new varieties with desired characteristics.

e) In vitro Mycorrhization: The utilization of arbuscular mycorrhizal fungus (AMF) in mycorrhization for micropropagation is becoming more popular because it has been shown to improve the post-transplant performance of *In vitro*-grown plants (Lovato *et al.* 1996; Rai 2001) ^[17, 18]. Recently, there has been a greater focus on AMF research due to its potential applications as bioregulators (Lovato *et al.*, 1996) ^[17], improved nutrient uptake, water relations, aeration, and soil pH balance (Sylvia, 1998) ^[19], as well as its potential for development of efficient AMF production methods and mycorrhization of *In vitro* plants. Mycorrhiza has been found to increase the survival rate of plants grown *In vitro*. Research has been done on bananas, sugarcane bamboo, etc.

f) Somatic Embryogenesis: Another essential component of clonal propagation is somatic embryogenesis. Somatic embryogenesis is a useful tool to accelerate the genetic improvement of commercial crop species when combined with traditional breeding programs and molecular and cell biology techniques (Stasolla and Yeung, 2003) ^[20]. Somatic embryogenesis offers a mechanism for totipotency expression in plant cells and an optimal experimental process for studying plant differentiation when compared to organogenesis (Litz and Gray, 1992) ^[21]. Therefore, superior germplasm of crops with respect to its vitamin content, disease, and other abiotic stress tolerant plants, low seed content, and extended shelf life can be multiplied at large scale by somatic embryogenesis. Elite germplasm storage and transit may be achieved by means of alginate encapsulation of somatic embryos or nonembryogenic vegetative propagules to generate synthetic seeds (Rai *et al.*, 2009) ^[22]

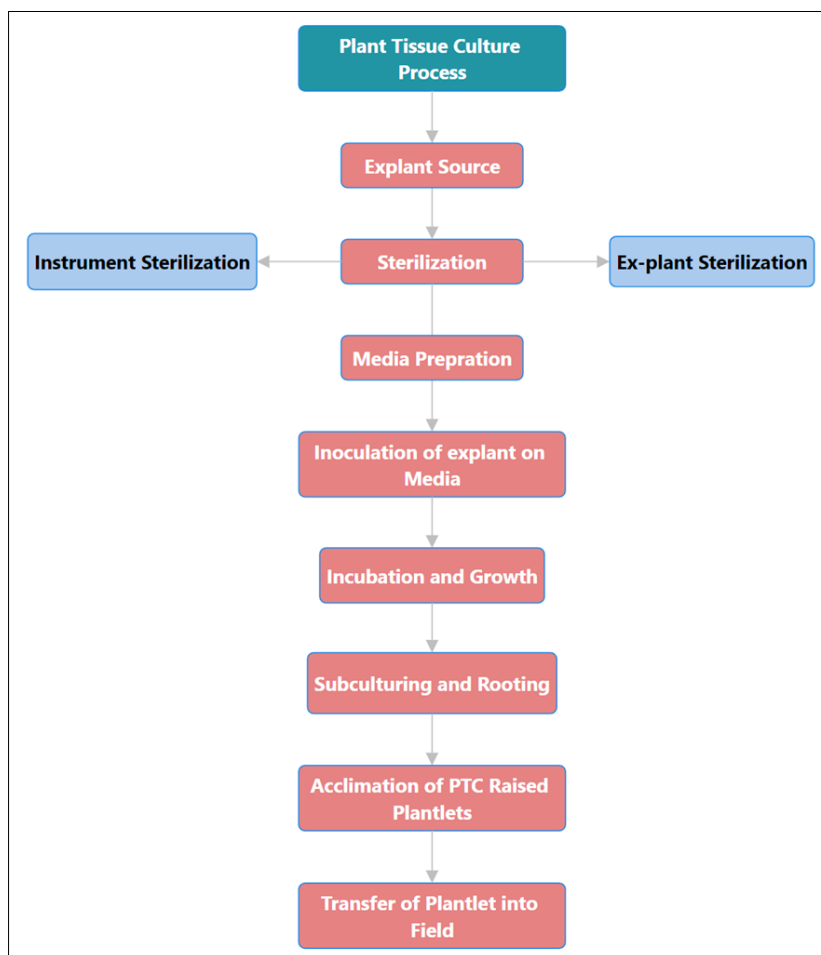
g) Embryo Rescue: According to Sage (2010) ^[23], one of the earliest and most effective methods of *In vitro* culture for assisting in the development of plant embryos that may not survive to become viable plants is embryo rescue. Modern plant breeding relies heavily on embryo rescue, which has made it possible to create several interspecific and intergeneric hybrids of crops used as ornamental plants. The technique nourishes the young or vulnerable embryo, therefore allowing it the chance to survive. Plant embryos are multicellular entities with the capacity to differentiate into new plants. The most extensively utilized embryo rescue process is referred to as embryo culture, which entails excising plant embryos and transferring them into media culture (Miyajuma, 2006) ^[24]. Utilizing embryo rescue allows for the creation of interspecific and intergeneric crossovers that would otherwise result in the loss of seeds. Interspecific incompatibility in plants can occur for several causes, but most typically embryo abortion occurs (Reed, 2005) ^[25]. Wide hybridization crossings in plant breeding can produce tiny, shrunken seeds that show fertilization has taken place but do not grow. Because remote hybridizations frequently fail to proceed with normal sexual reproduction, embryo rescue can

help avoid this issue. (Bridgen, 1994) ^[26] Embryo rescue transfers embryos with transplanted genes into tissue culture to complete their development into whole plant. In order to enable wide hybridization, embryo rescue is frequently utilized to create entire plants from embryos resulting from the crossing of two plants that would not typically produce progeny. (Singh and Shetty, 2011) ^[27].

h) Callus culture: Callus culture is the induction of undifferentiated cell mass from explant under controlled laboratory conditions. It serves as an important tool for a number of applications like somatic embryogenesis, organogenesis, genetic transformation, and production of secondary metabolite. In 2020, callus culture of wheat (*Triticum aestivum*) was established by Gupta *et al.* The plants derived from callus culture exhibited enhanced tolerance to drought stress as compared to conventionally propagated plants, which indicated the importance of callus culture in breeding for stress tolerance. In 2009, Ahmed *et al.* conducted research on the *In vitro* synthesis of gymnemic acid with antidiabetic properties that was extracted from *Gymnema sylvestre* leaves using callus culture under abiotic stress conditions. Callus treated with 2, 4-dichlorophenoxyacetic acid (2, 4-D) ^[28, 29] and kinetin (KN) produced considerably more gymnemic acid. The blue light

increased gymnemic acid production up to 4.4- fold as compared with fluorescent light treatment.

i) Cell suspension culture: Cell suspension cultures, is growing of individual cells or groups of cells in a liquid media. For the investigation of biosynthetic pathways, cell suspensions are an excellent biological material. In fact, they make it possible to recover large quantity of cells from which enzymes can be extracted more readily than with callus cultures (Dougall 1981) ^[30]. Through these biosynthetic studies, the researcher can identify genes or enzyme activity that are limiting the synthesis of useful compounds. Liu and Saxena (2009) ^[31] reported flavonoid production in *Saussurea medusa* cell suspension culture. They studied a combination of treatment to induce callus from leaf explant. Results showed that MS + BA (0.5 mg/L) + NAA (2 mg/L) + sucrose (30 g/L) + agar (5 g/L) was most suitable for inducing callus. For the purpose of producing flavonoids, a fine cell suspension was created from the induced light-yellow calli in the MS liquid medium containing 30 g/L sucrose, 0.5 mg/L BA, and 2.0 mg/L NAA. According to Liu and Saxena (2009) ^[31], after 15 days, the greatest dry weight and flavonoid production were 17.2 g/L and 607.8 mg/L, respectively.



Methodology

Explant

Materials utilized for tissue culture propagation is known as explants. Due to varying totipotency in each plant, the age, kind, and location of explants are the primary determinants of tissue culture success (Gamborg *et al.*, 1976) ^[32]. The most often used sources of explants are shoot tips, nodal buds, and root tips. Also, the choice of explants primarily depends on their availability and the objective of the experiment. Explan

availability is mostly influenced by the stage at which plants develop, physiological factors, the existence of meristematic explants, explant handling techniques, and sterilizing protocols. It is more desirable to use immature explants over mature ones due to morphogenetic plasticity. For plant tissue culture, leaves are the best explant material since they are readily available year-round and the plant (mother stock) is neither harmed nor sacrificed during their harvest. According to Murashige *et al.* (1962) ^[4], small explants, such as meristems, occasionally

exhibit less growth, whereas large explants can raise the risk of contamination.

Plant Tissue Culture Media:

In PTC, preparing the culture media plays a crucial part in determining the effectiveness of the process. To support the growth and development of plant tissues, the culture media's nutritional, growth regulator, and pH compositions must be carefully balanced (Smith *et al.*, 2018) [33, 34, 35]. Generally, a base medium like Murashige and Skoog (MS) or Gamborgs B5 acts as the foundation and is supplemented with vitamins, macro- and micronutrients, and plant growth hormones like auxins and cytokinin. For optimal absorption and metabolic processes, the pH level should be kept between 5.5 and 6.5. For sterile tissue culture conditions, the media must be thoroughly sterilized by autoclaving or filtering to remove any impurities. Gelling agents such as agar or gelrite can be employed to solidify the medium and offer support for tissue growth. Prior to shifting explant in media, its sterilization is necessary. Since, fungal spores are lightweight, they are present throughout our environment. And when they come in contact with culture media used in PTC, they germinate and contaminate the media because the conditions are optimal for their germination. So, the two methods most commonly used for sterilization of the culture media are autoclaving and membrane filtration.

Composition of PTC media

The composition of the culture media is one of the most significant elements influencing the morphogenesis and development of plant tissues in culture. Plant tissue culture media is normally constituted of the following components; Micronutrient and Macronutrient. Micronutrients are needed at concentrations of less than 0.5 mM/l, whereas macronutrients are needed at concentrations of more than 0.5 mM/l. The macronutrients are Nitrogen (N), Phosphorus (P), Potassium (K), Calcium (Ca), Magnesium (Mg), and Sulphur (S). Whereas Micronutrients are Iron (Fe), Manganese (Mn), Boron (B), Copper (Cu), Zinc (Zn), Iodine (I), Molybdenum (Mo), Cobalt (Co) and Nickel (Ni). Apart from that, a Carbon or energy source is also needed. Since photosynthesis is impeded in cultured cells or tissues, carbon must be given to the media in the form of carbohydrates in order for the tissues to grow. The commonly used carbon and energy source is sucrose. The sucrose in the medium is rapidly transformed into glucose and sucrose. The glucose is subsequently consumed first followed by fructose. Sucrose is commonly utilized at a concentration of 2 - 3 %.

Organic Supplements

Vitamins and other organic supplements are necessary for a number of metabolic and catalytic processes. The most commonly used vitamin in PTC are thiamine (B1), nicotinic Acid (B3), pyridoxine (B6) and myo-inositol. The concentrations are in the range of 0.1 to 10 mg/L. Other key constituents are Amino acids, Gelling agents, and Growth regulators.

Growth Regulators:

Plant growth regulators (PGRs) are synthetic or natural substances that are added to basal media with the purpose of either promoting or inhibiting the morphogenesis for processes like organogenesis, callogenesis, and somatic embryogenesis *In vitro*. PGRs control and direct cell division and expansion as well as the initiation and development of shoots and roots on

explants and embryos. There are several categories of plant growth regulators, such as ethylene, abscisic acid, gibberellins, auxins, and cytokinin. The balance between auxin and cytokinin is often required for the development of adventitious shoots and roots. High ratios of auxin to cytokinin promoted the growth of roots, while high ratios of cytokinin to auxin promoted the creation of shoots.

The basal media is supplemented with various PGRs, either individually or in combination, including auxins, gibberellins, and cytokines. According to Shahnawaz *et al.* (2011), the fortification of PGRs affects shoot organogenesis and multiplication as well as rhizogenesis, with higher auxin to low cytokinin ratios promoting rhizogenesis. Therefore, adding different PGR types and combinations that correspond to the endogenous hormone levels needed for a particular explant would cause an *In vitro* response or morphogenesis. PGR that are primarily used are cytokinin and auxin. Cytokinin stimulates protein synthesis, and cell division, induces shoot formation, induces axillary shoot proliferation, inhibits root formation, and controls morphogenesis, while auxin is linked to cell division, cell elongation, meristem formation, and maintenance of apical dominance. Widely utilized Auxin synthetics are IBA, NAA, 2, 4-D, etc and Cytokinin synthetics are - 6-BAP/ BA. Other than these gibberellins, ethylene and abscisic acid are not of great importance.

Sterilization of instruments and explant

This process is employed to eliminate microorganisms and avoid contamination. The most common contaminant observed in OTC are Bacteria and fungi. Sustaining aseptic (free from any microorganisms) is crucial for the achievement of good tissue culture processes. All culture vessels, media, and tools used to handle tissues, as well as the explant itself, must be disinfected in order to meet asepsis requirements.

Different methods for Sterilization of instruments:

a) Dry sterilization: It is carried out in Hot Air oven. This technique involves sanitizing metallic devices and glassware at 160-180 degrees Celsius for three hours on dry heat. To keep dry items sterile, they can be wrapped in aluminium foil, brown paper, or metal containers that are sealed. The main drawback of dry sterilization are slow heat penetration and poor air circulation.

b) Flame sterilization: Flame sterilization involves passing the instruments through a flame until they turn red hot to remove any contaminants. This process is commonly used for instruments, like scalpels, forceps, spatulas and needles used in handling plant materials (Jones and Smith, 2019) [36]. By producing a high temperature during the flame sterilization process, bacteria, fungi, and other microorganisms are successfully eliminated, assisting in the prevention of contamination of plant tissues and culture media. Flame Sterilization ensures the integrity of PTC and contributes to the reliability of experimental results in PTC.

c) Steam sterilization: It can be done in a pressure cooker or an autoclave. Autoclaving is typically done at 121 degrees Celsius for 20 minutes at 15 psi of pressure. It is used to sterilize a variety of items, including pipettes, water, cotton plugs, plastic caps, and media. It is usually preferred over dry sterilization.

d) Ultra violet sterilization: An essential method for getting rid of microbiological contamination from culture surfaces and equipment in plant tissue culture labs is UV sterilization. This technique uses short-wavelength ultraviolet (UV-C) radiation to

damage the DNA of bacteria, fungi, and viruses. Studies (Smith *et al.*, 2018; Jone and Patel, 2020) ^[33, 37] have demonstrated the effectiveness and consistency of UV sterilization in protecting plant cultures from contamination. Additionally, UV sterilization reduces the need for strong chemical disinfectants, which encourages tissue culture facilities to adopt environmentally friendly methods (Gomez and Sanchez, 2019) ^[38] In order to maintain aseptic conditions in the laboratory by preventing airborne contamination, laminar airflow is also utilized together with UV sterilization to pass filtered air (Brown and White, 2017) ^[39]. Laminar flow hood can be easily sterilized by turning on the hood and wiping down all surface with 95% ethyl alcohol 15 minutes before initiating any operation under the hood.

Sterilization of Explant

An important prerequisite for effective *In vitro* micropropagation, is sterilization of explants. Plant tissue collected from field or which have a probability of microbial contamination needs surface sterilization. The concentration and exposure duration depends on the type of explant used for PTC. The removal of impurities during the disinfection process is influenced by the concentration and length of exposure to various sterilizing agents during the surface sterilization of explants. Agents like calcium hypochlorite, sodium hypochlorite, ethanol, mercuric chloride, hydrogen peroxide, or silver nitrate are used for sterilization (Mihaljevic *et al.*, 2013) ^[40]. The choice of sterilizing agent is based on the kind of explants and their morphological attributes, such as tissue hardness and softness (Yadav *et al.*, 2011) ^[41]. Additional explant kinds, whether juvenile or mature, have an impact on sterilization processes since the former are more delicate, react *In vitro* faster and need less time to be exposed to sterilizing agents.

Inoculation of Explant on Media

Explants are then utilized as source for culture after being chopped into little pieces (leaves). The organic and inorganic salts, vitamins, minerals, gelling agents, carbohydrates, and numerous additives needed to effectively induce an *In vitro* plant tissue culture response is all included in the plant tissue culture-based media. The basal medium favours the fundamental nutritional basis necessary for the growth of *In vitro* plantlets. Different forms of carbohydrates (fructose, glucose, sorbitol, and sucrose) are utilized as carbon sources to support the production of energy, preserve osmotic balance, and promote the process of growth (Yeo *et al.*, 2023) ^[42]. Because sucrose is easily translocated and resistant to enzymatic breakdown, it is commonly employed as a source of carbohydrates (Kaur *et al.*, 2022) ^[43].

Incubation and Growth

Incubation refers to the time period, when explants are placed in a controlled environment to facilitate their growth and development on a nutrient rich media. The ideal plant tissue parameters for a good explant-based culture response under controlled circumstances are temperature, pH, relative humidity, and light intensity. These parameters are therefore standardized to establish controlled conditions required to manage photosynthesis, photomorphogenesis, and photoperiodism (Gantait *et al.*, 2015) ^[44]. The explant is incubated in a regulated environment with respect to humidity, light, and temperature. It begins to split over time and develop into a mass of undifferentiated cells known as a callus. To

produce larger quantities of desired products or plant biomass, it is essential to select fast-proliferating *In vitro* environments.

Subculturing

Subculturing is the step, in which small fraction of cultured tissue or cells are periodically moved into a new nutritional medium (Murashige and Skoog, 1962) ^[4]. The elimination of accumulated metabolites, nutritional replenishment, and inhibition of tissue senescence or overgrowth are some of the functions of this process. Moreover, subculturing facilitates the expression of desired features and eliminates genetic or somaclonal variants that might emerge from prolonged cultivation. The success of tissue culture propagation is greatly influenced by the frequency and timing of subculturing since these factors directly affect the growth and physiological state of the cultured material (Murashige *et al.*, 1962) ^[4].

Rooting

For any type of plant to regenerate *In vitro*, root growth is essential (Mutanda *et al.*, 2019) ^[45]. For any study that aims to cultivate plants in greenhouses, the field, or their natural habitats, rooting individual micro shoots obtained through micropropagation is an essential step. When rooting does not occur spontaneously or after subculture on a PGR-free media, the key parameters influencing the root induction are auxin type and concentration. The most often used PGR for Rooting is IBA, which is followed in use by NAA and IAA. These compounds are widely employed alone, in combination with BAP, Kin, or GA3. Additives that are added as nutrients, elicitors, antioxidants, or in studies of salt requirement or tolerance include CNH, Gln, myo-inositol, glycine, AC, ascorbic acid, carbon dioxide (CO₂), NaCl, and magnesium chloride (MgCl₂).

Acclimation of PTC Raised Plantlets

PTC raised plantlets are acclimatized in the greenhouse. The hardening of the fully formed plantlets is the penultimate and most challenging step in micropropagation research (Puchooa *et al.*, 2005) ^[46]. When transplanted straight into such field conditions, sensitivity of the *In vitro*-regenerated plants to environmental factors causes wilting and scorching of the leaves. There are several reasons why most immature plantlets may die during the hardening or acclimatization stage. As they are exposed to abiotic stresses like temperature, light intensity, humidity, and biotic stress like soil microflora (microbes living in soil). Plantlets are also vulnerable to microbial invasion and dehydration during acclimatization, it is necessary to move them gradually so they can adjust to changing conditions. So, to increase the survival rate of the micro propagated plantlets, several environmental parameters, including as temperature, lighting intensity, and humidity, required to be adjusted (Jahan *et al.*, 2009) ^[47].

Shifting of PTC Raised Plantlets in Field

Field transfer is ultimate steps in PTC after acclimation of raised plantlets. While field transferring of acclimatized plantlets, various factors (Environmental conditions, Timing, Handling, Transportation, Watering, and protection from disease and pest) must be taken care of.

4) Factors affecting PTC

PTC depends on several factors, such as (i) totipotency—the innate ability of individual cells to develop into a complete organism—(ii) explants, which are small tissues removed from

any part of the plant, (iii) an aseptic environment to prevent microbial contamination, and (iv) nutrient media, which have a significant influence on the growth and morphogenesis of plant tissues (Anonymous, 2020).

Light

Light is important for the growth and development of plant. Three aspects of light—wavelength, flux density, and photoperiod—affect *In vitro* growth traits such as stem elongation, leaf size, and plant structure.

Anigozanthos bicolor and *Zieria fraseria* produced *In vitro* was studied by Tapingkae to determine the impact of light amount and quality on their growth and development. Three white light intensities of 40, 80, and 200 $\mu\text{mol m}^{-2}\text{s}^{-1}$, five light-dark cycles (4L2D, 6L6D, 12L12D, 16L8D, and 24L), and light of five spectral values (white (390-760 nm), blue (450-492 nm), green (492-550 nm), yellow (550-588 nm), and red (647-770 nm)) were applied. After six weeks of growth, strong light intensities of 80 to 200 $\mu\text{mol m}^{-2}\text{s}^{-1}$ and a short light-dark cycle of 4L2D favorably affected the total number of rhizomes and the dry weight of *A. bicolor* plantlets. However, the light spectrum was not crucial for rhizome formation in *A. bicolor*. The plantlets of *Z. fraseria* growing under 200 $\mu\text{mol m}^{-2}\text{s}^{-1}$ irradiance had the maximum shoot dry weight. Shoot length was positively affected by blue light. Shorter photoperiods resulted in shorter shoot lengths, but the light cycle did not affect productivity. When growing *Rosa* sp. *In vitro*, Red (R) and Blue (B) LED lights were utilized, and the results were compared to explants grown under 16/8 hours of light/dark fluorescent light. The highest growth and elongation were seen in shoots under RB LED (16:4) (Azadi *et al.*, 2017).

Temperature

Numerous physiological processes, including as respiration and photosynthesis, are influenced by temperature. Although optimal temperatures vary widely depending on genotype, the most common temperature range for cultivation has been found to be between 20 °C and 27 °C. Air temperature is mentioned as a crucial factor in affecting the growth and development of *Phalaenopsis* plantlets *In vitro* before they are transplanted *in vivo*. *Phalaenopsis* plantlets that had been acclimated to air temperatures of 152, 252, and 352 °C were promptly moved to *in vivo* environments and left there for 14 days. Compared to plantlets acclimated to high temperatures, those adapted to low temperatures maintained greater levels of total carotenoid content, chlorophyll a, and chlorophyll b (Kaushal *et al.*, 2023)^[48].

Relative Humidity and Gas Exchange

The culture vessel is usually a closed system, but some gas exchange may happen based on the type of vessel, the closure, and how tightly they are sealed together. Sealing vessels must allow ample ventilation to prevent significant ethylene accumulation and CO₂ depletion. Using tightly closed containers that hinder gas exchange may deleteriously impact appropriate plant growth and development during *In vitro* cultivation. By allowing gas exchange, these methods improve photosynthetic capacity, rate of multiplication, and plant survival after being transferred to *ex vitro* environments (Miri *et al.*, 2018)^[49].

The culture vessels usually have very high relative humidity, which leads to a poorly developed epicuticular wax layer and malfunctioning plantlet stomata. Therefore, different techniques to lower the relative air humidity inside the vessel

have been tried. These techniques include opening culture containers a few days before acclimatization, utilizing special closures that make it easier for water to escape, or cooling the bottom of the containers, which causes more water vapor to condense on the gel surface. Additionally, controlling relative humidity during *In vitro* acclimation plays a critical role in enhancing the morphological traits of plantlets that are transplanted into *in vivo* (Bayanati *et al.*, 2015)^[50].

Applications of PTC

Plant tissue culture, or PTC, has become an important tool for modern plant biology, providing hitherto unseen possibilities for the manipulation, multiplication, and conservation of plant species. Applications for plant tissue culture now extend far beyond micro- and clonal propagation. Routine procedures now encompass virus removal, somatic hybridization, somatic embryogenesis, and the use of bioreactors for mass replication (Aitken-Christie *et al.* 1995; Paek *et al.* 2001)^[51, 52]. This method can be used for a wide range of projects; producing secondary metabolites, genetic modification, and mass propagation of elite plant types. Furthermore, PTC is essential to conservation efforts since it makes it possible to preserve rare and endangered plant species using methods like cryopreservation. PTC is a vital tool in modern plant research and biotechnology, with applications spanning a wide range of industries, including horticulture, forestry, agriculture, and pharmaceuticals.

Haploid and Double Haploid Production

In plant tissue culture, haploid and double haploid production relate to the creation of plant cells or tissues with one haploid set of chromosomes or two identical sets of chromosomes, respectively. Haploids offer a comparatively simpler way of inducing mutations, and disease-resistant mutants are crucial for enhancing agricultural productivity. Many techniques can be used to create haploid and double haploid plants. But the most frequent approach to produce haploid and double haploid plants is *In vitro* culture of male (*i.e.* microspores and anthers) or female (*i.e.* ovules or ovaries) gametophyte, known as androgenesis or gynogenesis, respectively. When embryos or calli are produced from the gametophyte, these can be regenerated into haploid and double haploid plants. In wheat and maize, haploid and diploid somatic cell lines of pollen plants have been effectively established by the application of the anther culture. Guha and Maheshwari (1964)^[53] were the first to describe androgenesis in *Datura* using anther culture. Also, Gynogenic methods for haploid generation have been shown for at least 24 species (Bohanec 2006)^[54]. These procedures are usually less efficient than androgenesis, and, thus, fewer investigations have been carried out in gynogenesis. While gynogenic techniques are useful in cases where plants do not react to androgenic techniques, there is a problem with regenerating albino plants from anther culture, or the donor plants are male sterile.

Biotransformation

Biotransformation can be described as a process in which the functional groups of organic compounds are transformed by living cultures, entrapped enzymes or permeabilized cells to a chemically different product (Ramachandra and Ravishankar 2002)^[55]. Cell suspension cultures, immobilized cells, enzyme preparations, and hairy root cultures can be employed for the creation of food additives or pharmaceuticals by the biotransformation process. György and Hohtola (2009)^[56] explored the synthesis of cinnamyl glycosides from the compact

callus of *Rhodiola rosea* by the biotransformation of cinnamyl alcohol.

Secondary Metabolite Production

Plants produce different substances that are helpful as medications, food, flavoring compounds, and cosmetics, for example, phytochemicals like *L*-DOPA, morphine, codeine, reserpine, vincristine, vinblastine, and taxol are used as

medicines. The poor yields of secondary metabolites in suspension cultures definitely were an obstacle for commercialization. But advancement in Plant tissue culture technology has been shown as a profitable technique for the generation of plant compounds. Below are several advantages of secondary metabolite production through plant cell/tissue culture, rather than *in vivo* in the complete crop plant.

Table 1: Transgenic cultures and plant systems used to produce secondary metabolites

Species	Uses	Transformation method	Marker gene used	Resulting transgenic tissue	References
<i>Catharanthus roseus</i>	Anticancer	<i>A. rhizogenes</i>	gfp	Hairy root	Rizvi <i>et al.</i> (2013) ^[57]
<i>Dracocephalum kotschy</i>	Against stomach and liver disorder	<i>A. rhizogenes</i>	rolB	Hairy root	Sharafi <i>et al.</i> (2014) ^[58]
<i>Gentiana scara</i>	Used in gastric infection	<i>A. rhizogenes</i>	No marker gene selected	Hairy root	Huang <i>et al.</i> (2014)
<i>Helicteres isors</i>	Source of diosgenin	<i>A. rhizogenes</i>	rolB	Hairy root	Kumar <i>et al.</i> (2014) ^[59]
<i>Pterocarpus marsupium</i>	Cytotoxic, antiproliferative	<i>A. tumefaciens</i>	gus gene	Shoot	Radhika <i>et al.</i> (2013) ^[60]
<i>Maackia amurensis</i>	Hepatoprotective effect	<i>A. tumefaciens</i>	rolC	Callus	Grishchenko <i>et al.</i> (2013) ^[61]
<i>Rauwolfia serpentina</i>	CNS disorder treatment, hypertension, insomnia	<i>A. rhizogenes</i>	crtDC	Hairy root line	Mehrotra <i>et al.</i> (2015) ^[62]
<i>Tribulus terrestris</i>	In sexual dysfunction	<i>A. rhizogenes</i>	rolB	Hairy root	Sharifi <i>et al.</i> (2012) ^[63]
<i>Leptadenia pyrotechnica</i>	Medicinal use	<i>A. tumefaciens</i>	gus/gfp	Hypocotyls	Dutta <i>et al.</i> (2013) ^[64]
<i>Picrorhiza kurroa</i>	Liver ailments, dyspepsia	<i>A. tumefaciens</i>	gfp/hpt	Leaf	Bhat <i>et al.</i> (2012) ^[65]
<i>Terminalia bellerica</i>	Antimalarial and antibacterial	<i>A. tumefaciens</i>	nptII/gus	Cotyledon	Dangi <i>et al.</i> (2012) ^[66]
<i>Centaurea montana</i>	Anticancer	<i>A. tumefaciens</i>	ipt	Leaf	Wissam <i>et al.</i> (2011) ^[67]
<i>Cuminum cyminum</i>	Medical used	<i>Microprojectile bombardment</i>	hpt II	Embryo callus	Singh <i>et al.</i> (2010) ^[68]
<i>Ricinus communis</i>	Castor oil	Particle gun-mediated	gus/hptII	Embryo callus	Sailaja <i>et al.</i> (2008) ^[69]
<i>Rauwolfia micrantha</i>	Antiarrhythmic	<i>A. rhizogenes</i>	No marker gene selected	Plant	Sudha <i>et al.</i> (2003) ^[70]

Somaclonal variation and Crop improvement

Somaclonal variation is described as variation originating in cell and tissue cultures, and the people are called as “somaclone” (Larkin and Scowcroft 1981)^[71]. The process of growth, development, and morphogenesis occurs *In vitro* and is influenced by the external supply of growth regulators. Increased PGR dosages over time affects mitotic activity, which frequently resulted in spontaneous changes in daughter cells. As a result, genotypic and phenotypic differences may be seen in the regenerated plants made from such tissues that were obtained from organ, protoplast, and callus cultures. There have been reports of both genetic and epigenetic modifications, although most of them only indicate epigenetic changes. In most cases, the conditions in the culture room and the different kinds of external stress induce the variability. Therefore, the nature of the permanent changes are heritable, while the transient changes are reversible.

Synthetic Seed Production

Murashige in 1977 defined Synthetic sees as “an encapsulated single somatic embryo, *i.e.*, a clonal product that could be handled and used as a real seed for transport, storage and sowing, and that, therefore, would eventually grow, either *in vivo* or *ex vitro*, into a plantlet” (Murashige 1977)^[72]. PTC produced somatic embryos, shoots, or other tissues which can be used for sowing are encapsulated in encapsulating agent.

Following a thorough screening of coating materials for the manufacturing of synthetic seeds, Redenbaugh *et al.* (1988)^[73] advised using Naalginate solution coating, which included propagules that were to be dipped in calcium chloride solution to produce hardened Ca-alginate gel through an ion-exchange reaction. The capsule also contains artificial matrix (artificial endosperm) composed of minerals, vitamins, and PGRs to sustain the artificial seed. The development of artificial seeds has opened up novel possibilities for *In vitro* plant biotechnology, including large-scale clonal propagation, delivery of clonal plantlets, the preservation of germplasm, breeding of plants for situations in which normal seed propagation is not feasible, genetic homogeneity, and simple storage and transportation. The use of artificial seed technology has the following possible benefits: ^[74] better handling, ^[75] cheap production cost, ^[29] easier exchange of germplasm between various countries, ^[51] genetic uniformity of recovered plantlets, ^[76] direct delivery to the field, ^[50] to shorten the life cycle and ^[65] reduction of the storage space (Sharma *et al.* 2013)^[77].

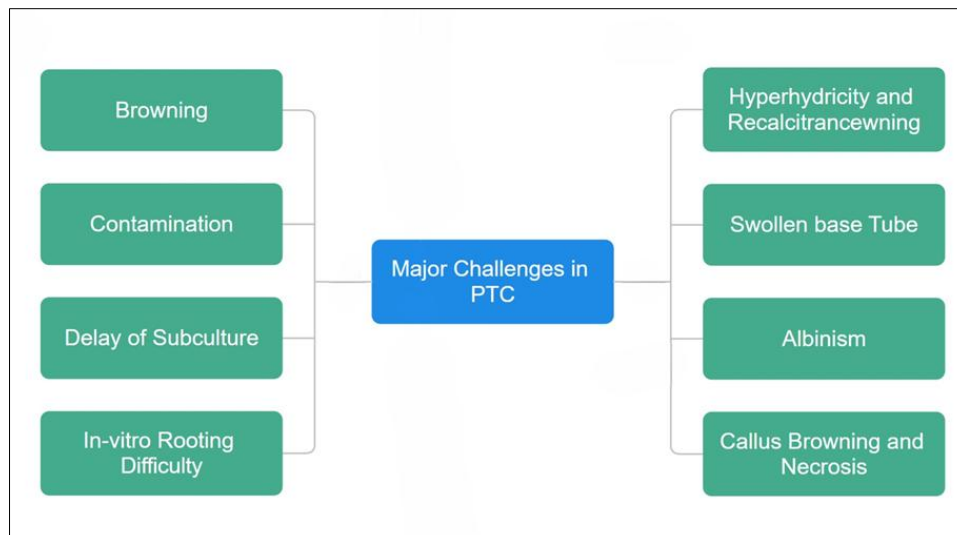
Problems in PTC

Plant tissue culture, is beset with numerous issues. These include technical (contamination, subculture delay, burned plantlets), physiological (browning of plant tissue cultures, difficulty establishing roots *In vitro*, failure of subsequent

acclimatization), and genetic (primarily somaclonal variations). Hyperhydricity, shoot tip necrosis, albino plantlets, recalcitrance in clonal micropropagation, abnormalities in the shoots, and *In vitro* habituation are examples of disorders and abnormalities in plant micropropagation that are substantial issues in this subject.

Apart from the previously mentioned problems, socioeconomic factors are sometimes cited as the main obstacles. These factors

include, according Moyo *et al.*, 2011^[78], the loss or deficiency of skilled human resources, inadequate infrastructure, and the high cost of technology development and its transfer. In plant-tissue-culture facilities, contamination is thought to be the single biggest and most significant factor influencing the success of micropropagation (Singh, 2018)^[79, 78]. Depending on the type of plant, choosing the right micropropagation protocol is also essential.



Problems and Disorders

a) Browning: In plant tissue culture, browning is a complex biochemical process that has significant effects on the viability and quality of the culture. The main cause of it is believed to be the enzyme-mediated oxidation of phenolic compounds, resulting in the development of brown pigments and the subsequent degradation of tissue. Research on this phenomenon has been carried out on a wide range of plant species. The results have shown the role of reactive oxygen species (ROS) in oxidative browning pathways and complex regulatory networks involving enzymes like polyphenol oxidase (PPO) and peroxidase (POD) (Dixon and Paiva, 1995; Mayer, 2006)^[80, 81]. Furthermore, it has been found that key parameters affecting browning sensitivity in plant tissue cultures are genotype, explant type, growth hormone concentrations, light intensity, and culture medium composition (Yao *et al.*, 2018; Lata *et al.*, 2020)^[82, 83]. To mitigate the effects and increase the efficacy and reliability of plant tissue culture systems, focused efforts to understand the fundamental mechanisms of browning and its regulatory variables must be developed.

b) Contamination: Plant tissue culture contamination is a recurring problem that frequently challenges the success and purity of the culture. Plant growth and development can be seriously hampered in the presence of microorganisms in the culture medium, such as bacteria, fungus, or viruses. Contamination concerns are caused by things like insufficient sterilizing methods and poor aseptic procedures (George *et al.*, 2008)^[84, 2]. Reyad and Abdelhameed (2019)^[85] list stringent aseptic conditions, periodic monitoring of cultures for indicators of contamination, and stringent sterilization processes as examples of mitigating techniques. For plant tissue cultures to be established and maintained in good condition, effective management is necessary.

c) Delay of subculture: In plant tissue culture, the growth and production of the cultures can be greatly impacted by the delay of the subculture. Suboptimal subculture intervals may lead to

overcrowding of explants, nutritional depletion, and buildup of growth inhibitors, resulting in lower proliferation rates and poor-quality cultures. Subculture delays may be caused by things like scarce resources, or bad scheduling (George *et al.*, 2008)^[84, 2]. Timely subculturing is vital to maintaining healthy and vigorous cultures, providing ideal growth conditions, and preventing contamination. To reduce downtime and increase the performance of plant tissue culture systems careful planning and adherence to preset subculture schedules are crucial.

d) *In vitro* rooting difficulty: When plants transferred from *In vitro* to ex vitro conditions, rooting is an important step that must be completed, and its failure can compromise the overall efficacy of plant tissue culture. Hormonal imbalances, poor culture conditions, genotype-specific responses, and the physiological age of the explants are some of the factors that lead to rooting difficulties (Ahmad *et al.*, 2016; Jain *et al.*, 2017)^[75, 86]. Furthermore, root initiation and elongation may be impeded by insufficient auxin concentrations or inappropriate auxin-to-cytokinin ratios in the rooting medium. Optimizing culture conditions is necessary to overcome *In vitro* rooting challenges. This includes choosing the right rooting hormones, adjusting nutrition levels, and adjusting environmental elements like temperature and light intensity. Furthermore, the success of plant tissue culture procedures can be increased by developing genotype-specific protocols and utilizing cutting-edge methods such as temporary immersion systems to promote rooting efficiency.

e) Hyperhydricity: Hyperhydricity, commonly known as vitrification, is a physiological condition often observed in plant tissue culture (PTC) systems. The cultured tissues have a transparent, gelatinous appearance as a result of the explants' abnormally high water uptake. The basic mechanisms responsible for hyperhydricity are still being investigated despite their prevalence. The typical signs and symptoms of hyperhydricity have been linked to abnormal tissue differentiation and water homeostasis being disturbed by

excessive cytokinin concentrations in comparison to auxins. Plant growth regulators (PGRs), especially cytokinin and auxins imbalance, leads to hyperhydricity in tissue culture (Jones *et al.*, 2016) ^[87]. Additionally, ethylene plays a part in aggravating hyperhydricity; higher ethylene levels encourage tissue senescence and water retention (Smith and Deb 2018) ^[33, 34, 35].

f) Callus Browning and Necrosis: Common physiological abnormalities in plant tissue culture (PTC) systems include callus browning and necrosis. The buildup of phenolic chemicals and oxidative stress in the cultured tissues are the main causes of callus browning and necrosis (Li *et al.*, 2018) ^[88]. Brown pigments and tissue necrosis are caused by the release of phenolic compounds from injured explants and the subsequent enzymatic oxidation mediated by polyphenol oxidase (PPO) and peroxidase (POD) enzymes. Furthermore, oxidative damage can be made worse by unfavourable culture conditions, such as severe osmotic stress or nutritional deficits, which can hasten callus browning and necrosis (Wang *et al.*, 2019) ^[89].

g) Recalcitrance: Recalcitrance to some plant species or tissues that display difficulties or incapacity to undergo successful *In vitro* culture. Genetic, physiological, and environmental factors are major contributors to the multifactorial nature of recalcitrance in PTC (Santos *et al.*, 2019) ^[90]. Genetic variables, such as the existence of endogenous inhibitors or the absence of essential regeneration-associated genes, might hamper the onset and advancement of tissue differentiation in recalcitrant species. Furthermore, the overall recalcitrance phenotype is influenced by physiological factors, such as the incapacity of regenerated tissues to undergo correct morphogenesis or the inability of explant tissues to undergo dedifferentiation (Guo *et al.*, 2020) ^[91].

h) Albinism: The absence of chlorophyll and subsequently the lack of colour in plant tissues is known as Albinism. Lacking functioning chloroplasts, albino plants have a characteristic white or pale yellow colour and are frequently linked to decreased viability and poor photosynthesis. Albino regeneration is aesthetically pleasing, but it poses serious challenges to genetic transformation and tissue culture-based plant propagation. According to Feng *et al.* (2017) ^[92], the albino phenotype may result from mutations in important genes related to plastid formation or chlorophyll biosynthesis. Moreover, suboptimal culture conditions may increase the occurrence of albinism by interfering with the processes involved in pigment synthesis and chloroplast differentiation. These conditions include excessive concentrations of growth regulators, osmotic stress, and insufficient light intensity (Wang *et al.*, 2021) ^[93].

i) Swollen base tube: The abnormal expansion and deformation of the basal portion of plant shoots is swollen shoot base syndrome. It is thought to result from a complicated interaction between environmental, physiological, and hereditary factors. Patel *et al.* (2019) ^[94] reveals that hormonal abnormalities, notably alterations in auxin and cytokinin signalling pathways, can contribute to aberrant shoot growth and differentiation, resulting to the creation of inflated shoot bases. Additionally, unfavourable culture conditions can worsen tissue stress and cause physiological abnormalities such cell hypertrophy and hyperplasia. These factors include high humidity, inadequate aeration, and pathogen contamination (Gupta *et al.*, 2020) ^[95, 28].

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