Chapter 1

Introduction

Plants and humans have a deep and interconnected relationship that has developed over thousands of years. From the earliest civilizations to modern times, plants have played an important role in human life, providing food, shelter, medicine, and countless other resources. Plants are the primary producers of the Earth's ecosystems, converting the energy of the sun into organic matter through photosynthesis. This process is fundamental to life on the Earth and provides the oxygen we breathe. Humans, in turn, rely on plants for their survival, as they provide us with the necessary nutrients, vitamins, and minerals that we need to maintain our health. Plants play a crucial role in sustaining human life on Earth, and humans have developed various techniques to make use of plant resources efficient. After 20^{th} century we are observed major revolution in life science. Research accumulated in specially agriculture filed with the help of biotechnology. One of the greatest significant applications of biotechnology in plant resource utilization is genetic engineering. Genetic engineering allows scientists to modify the genetic makeup of plants to produce desirable traits, such as increased yield or resistance to pests and diseases. This technology has led to the progress of genetically modified crops that can provide greater nutritional value and increased crop yield, helping to meet the growing demand for food worldwide. Another major application of biotechnology is plant tissue culture, which comprises the propagation of plants through the growth of individual plant cells or small tissue samples in a laboratory setting. This technology allows for the mass production of genetically identical plants with specific desirable traits, such as disease resistance or increased productivity.

1.1 Introduction of micropropagation

The concept of cell totipotency, which refers to the ability of a single cell to regenerate a whole organism, is the fundamental principle behind plant tissue culture with the help of this technique now it was possible to regenerant the plant from cells, tissues, or organs in a sterile, controlled environment outside the plant body (Fehér, 2019). Hence were this application micropropagation use a commercial, largescale production of the economic important plantlets (Twaij *et al.*, 2020). Micropropagation will help to multiple and largescale production of same genotypes in short time (Agarwal *et al.*, 2015). Use of micropropagation technique has numerous benefits, including the ability to rapidly reproduce plants that are hard to proliferate through conventional means. Such as long

maturation periods, low seed viability, self-incompatibility, and low rooting ability can be propagated efficiently using this technique (Chandana *et al.,* 2018). Micropropagation has become an essential tool in the commercial production of economically important plants and has created new opportunities for producers, farmers, and nursery owners worldwide (Hasnain *et al.,* 2022). Furthermore, the technology has also contributed to the conservation of rare and endangered plant species by enabling their rapid multiplication and reintroduction into the natural habitat. Overall, the significance of plant tissue culture and micropropagation in modern biotechnology is immense and cannot be overstated (Rohini, 2020).

Micropropagation derived from the Greek word 'micro' meaning small and the English word 'propagation' meaning to increase the number of propagules. Micropropagation involves the asexual multiplication of plants *in vitro* under carefully controlled sterile conditions, can be achieved through four distinct pathways. These pathways include (1) enhanced axillary branching, where multiple shoots develop from buds in the leaf axils; (2) adventitious shoot bud differentiation, which promotes shoot formation from non-meristematic tissues; (3) callus organogenesis, where undifferentiated cells develop into shoots or roots; and (4) somatic embryogenesis, which facilitates the direct formation of embryos from non-reproductive cells (Singh, 2015).

The objective of micropropagation is to produce viable plants in large numbers that can thrive in natural environments. Each pathway within micropropagation requires a series of carefully executed activities to achieve success. Unlike traditional propagation methods, micropropagation is a multi-stage process where every stage plays a critical role in achieving the ultimate goal of plant production in culture. Apart from somatic embryogenesis, every method of micropropagation consists of five distinct stages to accomplish the objective. every method has their advantages and disadvantages. These stages are in figure 1.

In the micropropagation techniques grown a cell and tissue on different artificial media under sterile condition with controlled environments. For the development of plant required necessary supplements like inorganic nutrients, organic supplements, vitamin, amino acid, gelling agent, and plant growth regulators with sugars (Gupta *et al.,* 2020). Mainly adding of plant growth regulator in medium because the role of auxins and cytokinin in plant differentiation by the investigating the interplay between these two plant

hormones and their impact on cellular processes, growth patterns, and developmental pathways (Su *et al.,* 2011). The addition of gelling agents to the culture medium serves to increase viscosity, facilitating the formation of a solid surface for supporting plant tissues and organs above the nutrient medium. Among the various gelling agents, agar is widely preferred due to its exceptional gelling properties, stability, and non-interference with plant metabolism (Madi Waheed Al-Mayahi and Hussian Ali, 2021). The culture vessels are usually small and tightly sealed during micropropagation resulting in significant decrease of CO_2 during the presence of light. This deficit is compensated in the tissue culture medium employing sugars as carbon and energy source (Zobayed, 2005). Moreover, it is widely recognized that the presence of sugar in the medium plays a pivotal role in the regulation of plant organ development and morphogenesis.

In this method, tissue culture plantlets are cultivated in culture vessels under controlled conditions. The plants are exposed to a 2000-3000 lux light intensity, equivalent to approximately 30-45 µmol m^{-2} s^{-1}. This is achieved by providing a 16/8-hour light and dark cycle. The temperature of the culture room is carefully maintained at 28±2°C, while the relative humidity is kept around 40-50%. To prevent contamination, tightly sealed culture vessels are utilized, which restrict the unwanted exchange of gases between the vessels and the surrounding atmosphere (Joshi *et al.,* 2006).

The hardening and acclimatization of *in vitro* plantlets generated through micropropagation is a crucial process to ensure their successful survival post-transplantation. This process involves gradually transitioning the plantlets from the sterile laboratory environment to polybags filled with soilless media. During this transition, the plantlets are exposed to lower humidity levels and high light intensity, replicating the conditions found in the natural environment. This hardening and acclimatization phase typically spans 2 to 4 weeks and plays a vital role in enabling the plantlets to adapt to the challenges of the external environment. By developing resilience to lower humidity and increased light levels, the plantlets become better equipped to establish and grow successfully outside the controlled laboratory setting. Thus, the hardening and acclimatization process is critical for ensuring the viability and long-term growth of micro propagated plantlets (Chandra *et al.,* 2010).

Exploring the economic factors of commercial banana production via tissue culture in India.

The banana plant (*Musa sp*.) is a type of flowering plant. It looks unique because its stem is made up of tightly packed leaves. This plant originally came from warm areas in South East Asia. People first started growing it in places like South East Asia and Papua New Guinea (Perrier *et al.,* 2011). Banana stands as a paramount food crop, holding the utmost significance. Over the course of centuries, numerous cultivable banana variants have been meticulously developed and perpetuated through vegetative propagation techniques (Hinge *et al.,* 2022). Banana exhibits a no abundance of potassium, with a solitary fruit capable of fulfilling approximately twenty three percent of our daily potassium requisites. Furthermore, it boasts significant quantities of vitamin A, B, B_6, C, and D. A consumption of 100 grams of mature fruit equates to approximately 116 kilocalories of energy, approx. 27 grams of carbohydrates, 1 gram of protein, 17 milligrams of calcium, 36 milligrams of phosphorous, and 358 milligrams of potassium (Ranjha *et al.,* 2022).

In the realm of global trade, the banana emerges as the fifth most prominent agricultural commodity, trailing only cereals, sugar, coffee, and cocoa. Its year-round availability underscores its significance. Principal banana-producing nations encompass China, India, Brazil, Ecuador, Mexico, Philippines, Indonesia and Angola. Notably, the worldwide banana production during the 2021 period stood at 113,918,763 tons, cultivated across an expanse of 5,637,508 hectares, yielding a productivity of 20.21 metric tons per hectare. Within this panorama, India's contribution reached 25.57%. Specifically in India, the banana production for the same period was 26,509.1 metric tons, cultivated across 776.0 hectares, reflecting a productivity rate of 34.2 metric tons per hectare (Mandal *et al.,* 2023). As per reports from the Food and Agriculture Organization (FAO), India harvests the foremost position globally in relations of banana cultivation area. Within the Indian context, notable states contributing significantly to banana production encompass Tamil Nadu, Gujarat, Maharashtra, Andhra Pradesh, Karnataka, Assam, Madhya Pradesh, and Bihar (Saxena *et al.,* 2017). A compilation of noteworthy banana cultivars in India is in Table 1.1

The significance of banana production extends to its substantial contribution to India's Gross Domestic Product (GDP), with both its domestic consumption and export capacity exerting a substantial influence on the overall national economy, figure 1.1 (Wardhan *et al.,* 2022)

Micropropagation industries assume a central role in furnishing pathogen-free propagules to the agricultural sector, thereby contributing to heightened agricultural productivity and the generation of rural employment, particularly within developing nations such as India (Patil *et al*., 2021). The inception of commercial plant tissue culture took root in Cochin, India, in 1987, marked by the establishment of a modest-scale production facility by A.V. Thomas and Company - Kerala (AVT). This endeavor was undertaken in partnership with the National Chemical Laboratory Pune, India, with the primary aim of growing greater genotypes of cardamom plants. Subsequently, this technology underwent iterative enhancements and expansion, facilitated by a partnership between AVT and a UK-based firm, aimed at augmenting production efficiency. In 1988, India-American Hybrid Seeds, based in Bangalore, imported a tissue culture unit and a greenhouse facility capable of producing up to 10 million plants annually (Salunkhe *et al.,* 2022).

The findings of a survey jointly conducted by the Department of Biotechnology (DBT) and the small farmer agri-business consortium (SFAC) in 2005 revealed that primary consumers of tissue culture plants (TCP) included State Agriculture Departments, private farmers, and Agri Export Zones (AEZ). In India, the prominent plant varieties propagated through tissue culture methodologies in the year 2003 encompassed banana, ginger, sugarcane, large cardamom turmeric, cardamom, vanilla, orchids, aloe, gerbera, anthurium, and stevia (Patil *et al.,* 2021). Up until July 2023, the Government of India, through the DBT, has officially recognized and sanctioned 78 commercial tissue culture units across the nation under the NCS-TCP program. As per DBT data compare to 2020 it is approx 20% reduced, List of State wise recognized companies given in Table No 1.2.

The utilization of planting material derived from tissue culture methodologies has precipitated a transformative shift, facilitating the implementation of high-density planting and substantial yield enhancements per unit of cultivated land. This revolutionary approach has found widespread adoption in Asian countries for banana cultivation (Suman, 2017). Despite the manifold advantages inherent to tissue culture-raised plants, their recognition as a viable agricultural practice in India was hindered by the considerable associated expenses. Numerous investigations have been undertaken to evaluate the cost of tissue culture banana propagules, delve into the determinants influencing production costs, and assess the risks inherent in the adoption of tissue culture techniques for banana cultivation (Muthee *et al.,* 2019; Thorne *et al.,* 2022). As indicated by the FAO survey, a substantial

majority of global banana growers, accounting for 92%, rely on suckers as their primary planting material, while a mere 8% opt for tissue culture-raised planting material. Within India, the ambition to transition one-third of banana cultivation to tissue culture-raised propagules necessitates an estimated minimum of 900 million propagules. However, the actual production only ranges from approximately 40 to 80 million propagules (Kumar, 2017). Furthermore, as per a survey orchestrated by the Department of Biotechnology (DBT), the market valuation of tissue culture banana plants stood at 15 Indian Rupees per plant in the year 2018. The demand for *in vitro* raised banana increase at the rate of 25%.

1.1.1 Limitations of traditional micropropagation methods

Large-scale micropropagation endeavors face specific problems that hinder the translation of laboratory-based protocols into viable technology for the production of desired clones (Cardoso *et al.*, 2018; Gulzar *et al.*, 2020; Kumari *et al.*, 2023).

Higher Cost of Production.

The expenses linked to micropropagation predominantly revolve around manual labor, as highlighted by (Lee *et al.*, 2019). Manual labor constitutes a significant portion of the costs associated with micropropagation, posing a significant challenge for the India to fully harness the potential of *in vitro* culture technology. In the India, the utilization of *in vitro* culture technology has been impeded by the substantial costs of labor in micropropagation, which accounted for 60-70% of the expenses related to the production of *in vitro* plants (Purohit *et al.*, 2011; Amare and Dugassa, 2022). The conventional micropropagation systems require labor-intensive tasks such as the excision and transplantation of plants and explants, contributing to the high labor costs involved (Cardoso *et al.*, 2018; Johnson *et al.*, 2023).

The formulation of culture media utilized for shoot propagation and rooting plays a crucial role in determining production costs. Among the various components of the medium, gelling agents like agar have a significant impact, accounting for 70% of the budget (Ebile *et al.*, 2022). Additionally, the inclusion of sucrose, which costs around INR 40/kg, significantly contributes to the overall expense of the culture media (Dhanalakshmi and Stephan, 2014). This highlights the economic significance of sucrose in the media composition. However, it is important to note that the incorporation of agar and sucrose in the medium also presents a major challenge in terms of contamination. These components

can serve as a breeding ground for contaminants, leading to further financial losses (Lu *et al.,* 2020).

Protocol Potency

One of the most crucial factors to consider during large-scale production in micropropagation is the multiplication rate, with a minimum three-fold increase being deemed acceptable (Purohit *et al.,* 2011). A high multiplication rate is pivotal for the success of large-scale production, as it reduces the need for frequent subculturing, resulting in cost savings in terms of labor. A high rate of shoot multiplication can partially compensate for losses incurred due to contamination and challenges encountered during the rooting, hardening, and acclimatization processes. It serves as a valuable strategy to mitigate setbacks and streamline the production workflow. However, the micropropagation industry has not experienced the anticipated rapid growth primarily due to decreased all over multiplication rates. This phenomenon is evident in the production of ornamental pot plants, where the current annual output is 40 million plants globally, despite having an installed capacity of 110 million plants (Patil *et al.,* 2021).

Loss due to contamination

Apart from the decline in multiplication rates over time, contamination poses a significant problem during the multiplication phase of micropropagation (Cardoso *et al.,* 2018). Contamination can be highly detrimental, as it has the potential to destroy months of work and turn into a nightmare for micropropagation practitioners. Bacteria and fungi are commonly encountered contaminants in cultures of many plant species, especially in large-scale commercial operations (Cassells, 2012; Okoroafor, 2022). These microorganisms can either originate from the explant itself or enter the laboratory environment as natural or man-made contaminants (Dubey and Babel, 2022), it is worth noting that the incorporation of agar and sucrose in the culture medium also contributes significantly to contamination issues, leading to further economic losses (Kumari *et al.,* 2023).

Hyperhydricity

Shoots cultivated *in vitro* are exposed to a unique microenvironment characterized by abundant sugar and nutrient levels, low light intensity, aseptic conditions, and high humidity. While these conditions promote rapid growth and multiplication, they can also induce various morphological, anatomical, and physiological abnormalities in plants (Hazarika, 2006). One significant issue that arises during *in vitro* plant culture is

hyperhydricity, which has a direct impact on commercial production. Hyperhydric shoots, despite continuing to grow and multiply at acceptable rates, often encounter difficulties in rooting. Even when they can be rooted, they struggle to establish successfully in soil (Makunga *et al.,* 2006). Consequently, hyperhydricity poses a significant challenge in micropropagation as it hampers the successful establishment of plants in subsequent stages of growth. This issue needs to be addressed to ensure the efficient production of healthy plants for commercial purposes (Modi *et al.,* 2009).

Liquid culture system for batter growth of culture

In vitro micropropagation of plant is highly depended on a composition of media (Gonçalves and Romano, 2013). The practice of plant tissue culture primarily occurs on semi-solid media containing agar. However, this method's reliance on expensive materials amplifies the overall costs of plant cultivation. The additional expense of labor-intensive subculturing further adds to the financial burden. An alternate strategy involves the complete exclusion of gelling agents, opting instead for a liquid medium for cultivating plants. Liquid media are particularly advantageous in micropropagation, effectively reducing the costs of producing plantlets, expediting multiplication rates, and enabling automation (Dutta Gupta, 2006; Mehrotra *et al.,* 2007). Certainly, utilization of liquid culture systems presents a notably enhanced degree of uniformity in culturing conditions. The renewal of culture medium can be achieved conveniently without necessitating alterations to the container structure. Feasible sterilization can be attained through microfiltration, simplifying the subsequent cleaning of culture containers. In contrast to the practice of cultivating on semi-solid media, the potential employment of substantially larger containers becomes viable, thereby offering the advantage of decreased transfer durations (Jyoti Sahu and Ram Kumar Sahu, 2013).

Micropropagation from many species have performed better in liquid medium rather than on semi–solid medium. For instance, a high number of shoots was produced *Solanum tuberosum* L. (Karyanti *et al.,* 2022), and more somatic embryos were produced in *Arabica coffee* (Aguilar *et al.,* 2022). The better growth effect of liquid medium on shoot elongation in *Turnera ulmifolia* L. (Shekhawat *et al.,* 2014), and biomass production in *Lycium barbarum* L, (Ruta *et al.,* 2020) has been reported. Nowadays *in vitro* propagation with the help of liquid medium has been carried out as an innovative and cost-effective method in a large number of plants (Melaku *et al.,* 2016; Mohapatra and Batra, 2017; Pijut,

2018; Daneshvar Royandazagh, 2019; Premkumar *et al.,* 2020; Stevens and Varutharaju *et al.,* 2021; Vyas *et al.,* 2021; Muhammet Dogan, 2022; Shekhawat *et al.,* 2022) See table 1.3 for more references.

To ensure cultures remain upright in the liquid medium, it's essential to use a support matrix consistently. This matrix greatly aids robust shoot growth, especially with increased aeration, ensuring effective nutrient uptake. Furthermore, it disperses harmful phenolic exudates throughout the medium. The inherent stability of these supports effectively mitigates the negative impacts of shear stress and mechanical damage, which are often linked to aeration and agitation in shake flask cultures (Dutta Gupta and Prasad, 2010). A solid matrix is crucial for many plant systems, serving to enhance multiplication, facilitate proper rooting, and ensure improved anchorage in various types of culture vessels (Shahzad *et al.,* 2017). Employing support matrices also offers economic benefits by eliminating the need for expensive gelling agents, thereby lowering costs. Furthermore, it minimizes expenses associated with washing and cleaning (Cardoso *et al.,* 2018). Maintenance of cultures in this manner can lead to decreased contamination risks since subculturing is limited to adding sterile liquid media only (Nirmal *et al.,* 2023). Several mechanical supports are currently accessible and their successful application has been demonstrated across various plant systems by different researchers.

Moreover, the imperative for achieving high-quality plants at competitive prices underscores the necessity for *in vitro* culture automation. Advance has been made in automating micropropagation through techniques such as organogenesis or somatic embryogenesis within a bioreactor, offering a promising avenue for cost reduction (Neumann *et al.,* 2020). Bioreactors are commonly recognized as self-contained, aseptic surroundings that utilizing liquid nutrient solutions. Effective micropropagation of plants within these systems, aiming for heightened plant yield, necessitates an enhanced grasp of plant physiological and biochemical reactions to cues in the culture microenvironment. Furthermore, precise manipulation of distinct physical and chemical factors is pivotal in leading plant morphogenesis in liquid culture setups. Bioreactors prove especially adept for substantial-scale tissue culture, enabling the generation of multiple plantlets within a solitary batch (Lee *et al.,* 2019).

Furthermore, when plant tissues are submerged in a liquid medium, they often experience oxidative stress, leading to observable alterations in plant anatomy, physiology,

and survival (Pasternak *et al.,* 2005). Other strategy is use of temporary immersion of shoot in liquid media then after transfer plant in to semi solid medium (Lee *et al.,* 2019). Temporary immersion system has been successfully tested and beneficial in *Capparis spinosa* L. (Gianguzzi *et al.,* 2019), Apple (Kim *et al.,* 2020), Sugarcane (J. D. da Silva *et al.,* 2020), *Lycium barbarum* L. (Ruta *et al.,* 2020), *Larix* × *eurolepis Henry* (Le *et al.,* 2021). *Brassavola nodosa* L. (Vendrame *et al.,* 2023), *Carludovica palmata* (Minchala-Buestán *et al.,* 2023). For the commercial method of micropropagation Malang (*Colocasia esculenta* L.) with the high efficiency and cost effective were develop (Arano-Avalos *et al.,* 2020), see table 1.5 for more references.

1.1.2 Factors affecting micropropagation

Photoautotrophic Micropropagation

However, conventional micropropagation techniques often rely on exogenous sources of energy, primarily sugars, to support plant growth. Such methods may inadvertently lead to suboptimal growth and physiological outcomes, limiting the full potential of micropropagation in producing robust and acclimatization-ready plants (Afreen, 2005). The growth and development of plantlets in the context of micropropagation are significantly influenced by pivotal factors, such as carbon dioxide concentration and light conditions (Kozai, 1991). These factors play a crucial role in shaping the success of micropropagation techniques. Notably, the presence of an optimal concentration of carbon dioxide and appropriate light conditions are vital for fostering robust plant growth during the micropropagation process (Batista *et al.,* 2018).

When carbon dioxide concentration becomes depleted during periods of light exposure, and when the irradiance levels are low, the photosynthetic activity of plants cultivated in a controlled culture environment is detrimentally impacted. This phenomenon, as elucidated by (Nguyen *et al.,* 1999), underscores the significance of maintaining adequate carbon dioxide levels and providing optimal light conditions throughout the micropropagation process. The adverse effects of suboptimal carbon dioxide concentration and insufficient light exposure on the photosynthetic processes of plants grown under controlled conditions emphasize the necessity of carefully managing these parameters to ensure successful growth and development during micropropagation. The addition of sucrose often leads to photomixotropic growth in plantlets, which complicates their hardening and acclimatization after tissue culture. To address this, adopting a

photoautotrophic approach by minimizing or removing saccharides from the growth medium, as suggested by (Kozai, 1991), proves effective. This strategy reduces reliance on external energy sources like sucrose, aligning growth with natural photosynthesis. Consequently, it mitigates photomixotrophy-related challenges, improving the success and efficiency of plantlet micropropagation and establishment in *ex vitro* conditions (Soni *et al.,* 2021). Carbon dioxide levels in plantlet cultivation advantages. Elevated carbon dioxide boosts resistance to contamination (Mitra *et al.,* 1998), enhances vigor and root systems (M. R. Mohamed *et al.,* 2021) and improves photosynthesis, eliminating post *in vitro* acclimatization (Saldanha *et al.,* 2013; Pinheiro *et al.,* 2021). This simplifies transitioning micro propagated plantlets from controlled to external conditions, benefiting cultivation practices. The investigation into the realm of photoautotrophic micropropagation has unveiled a compelling connection between the presence of a carbon dioxide and light-enriched environment during *in vitro* culture and the facilitation and support of shoot growth in plantlets. Remarkable strides have been made in the augmentation of plantlet growth and multiplication through the cultivation of controlled carbon dioxide-enriched environments. These advancements have been demonstrated across a diverse array of plant types, including herbaceous species as observed in studies by (Joshi *et al.,* 2009) and (Norikane *et al.,* 2010), as well as in woody plants, was reported by (Voelker *et al.,* 2016; de Jesus Santana *et al.,* 2022; Luis and Jabín, 2023), See Table 1.6 for more references.

The impact of a carbon dioxide-enriched environment on the *in vitro* shoot growth and multiplication of *Feronia limonia* has been examined, revealing a noteworthy finding Joshi *et al.,* (2010) discovered that the. This finding underscores the autonomy of carbon dioxide's positive influence on shoot development, shedding light on a potentially novel avenue for optimizing micropropagation techniques. In a parallel (Aragón *et al.,* 2010) arrived at similar conclusions in their report. Carbon dioxide (CO_2) enrichment plays a pivotal role in enhancing plant growth across micropropagation systems.

Notably, it benefits semi-solid and bioreactor-based liquid cultures. Recent research highlights its significance in various plant species like, Plum (Gago *et al.,* 2022) vanilla (Luis and Jabín, 2023), *Salix vamilanis* (Gago *et al.,* 2021) investigated elevated CO_2 levels in liquid medium bioreactors during shoot growth for Plum, vanilla, *Salix vamilanis*. This yielded notable growth enhancement and healthy after (Afreen *et al.,* 2002) plantlet

production. Higher CO_2 levels in the liquid medium likely optimize conditions for plant growth, boosting photosynthesis and carbohydrate synthesis. This benefits overall physiological processes for robust development.

Extending photoautotrophic conditions to rooting, rooted (Hung *et al.,* 2016) blueberry in liquid medium. Similarly, Vives *et al.,* (2017) achieved stevia' rooting via temporary immersions in liquid medium under photoautotrophic conditions. These instances illustrate that photoautotrophic micropropagation extends beyond shoot growth, benefiting root establishment.

Culture vessel environment

Lately, researchers have found out different things about the containers used for growing plants in labs. These things matter a lot for how well the plants grow. Some of these container features include the type of container, how much space they have inside, the size of the opening at the top, whether the top is covered or not, and how clear the container (Huang and Chen, 2005). These features influence how much moisture, light, and heat stay inside the container where plants are grown, as well as how well air can move in and out. All of these factors are really important for helping the shoots of the plants grow and multiply as much as possible (Huang and Chen, 2005; Islam *et al.,* 2005).

The effect of vessel has been studied and advantages of vented closures have been reported in a number of species like *Hemidesmus indicus* (Manokari *et al.,* 2022), *cork ork* (Jiménez *et al.,* 2011), *Carnations* (Casanova *et al.,* 2008; Majada *et al.,* 2000) *Chlorophytum borivilianum* (Joshi and Purohit, 2012), *Mentha* spp. (Islam *et al.,* 2005), *Capsicum annuum* (M. A. H. Mohamed and Alsadon, 2011), *Wrightia tomentosa* (Joshi *et al.,* 2009) *Stevia rebaudiana* (A. R. Modi *et al.,* 2012) have shown enhanced rate of shoot multiplication and higher fresh weight contents.

Morpho–physiological and biochemical status

Plants cultivated using liquid mediums frequently exhibit a propensity for atypical apoplastic water accumulation, leading to anatomical, physiological and gross morphological irregularities referred to as hyperhydricity. This anomaly manifests in characteristics such as leaves exhibiting diminished photosynthetic capacity and deficient or absent cuticular wax development (Gaspar, 1991; Isah, 2015). For the hyperhydrate shoot and root solved this problem various supporting material was used in liquid culture system (Nirmal *et al.,* 2023). Use temporary immersion for growing *in vitro* plant (Ramírez-

Mosqueda *et al.,* 2019; García-Ramírez, 2023) and agitated liquid (Karalija *et al.,* 2017; Malik *et al.,* 2017) in order to make micropropagation methods better, it's really important to understand these unusual things that happen during different stages of plant growth. We also need to compare them to how things happen in semi-solid conditions. This is a very basic thing we have to do before we can make micropropagation methods work even better.

Morphological and anatomical studies

To understand the changes that happen inside cells while plants regeneration, several parameters are studies to have looked at how the cells' shapes and structures change. by using electron microscope (Zafar *et al.,* 2019; Jayappa *et al.,* 2020). The research has involved looking at different parts of the plants, like the outer skin cells, tiny openings called stomata, the cells that control these openings, and the waxy layer on the surface. The findings have shown interesting connections between how close the stomata are and their numbers, and the conditions where the plants are growing. These conditions include things like how much light they get, the amount of carbon dioxide, a gas they use, how much ethylene, another gas, is present, the humidity around them, and the environment outside their usual growth setting (Vahdati *et al.,* 2017; Neto *et al.,* 2020; García-Ramírez, 2023).

In recent years, a lot of focus has been put on examining the inner structure of plant leaves that are grown in controlled environments. The leaves formed in these conditions often have less developed middle layers and fragile transport systems for nutrients. Because of these features, when these plants are moved to different places, they tend to react strongly to the change, which we call transplantation shock (Isah, 2015; Mani and Shekhawat, 2017). The leaves of plants that are grown in controlled environments have a thinner upper layer with a poorly developed section known as the palisade layer. These leaves also have a significantly amount of mesophyll air (Isah, 2015; Ma *et al.,* 2015). Differences in the way leaves are structured between plants grown naturally (*in vivo*) and those grown in controlled environments (*in vitro*) have also been recorded in (Yanyou *et al.,* 2006; Jogam *et al.,* 2020;). Stems of plantlets grown in controlled environments were thin and had much less of the supportive tissues called collenchyma and sclerenchyma compared to plants that were grown in natural outdoor conditions (Pinheiro *et al.,* 2021: Darwesh, 2022).

Physiological studies

The use of culture media with high amounts of sucrose and salt, commonly used to cultivate cultures, along with inadequate light conditions, can limit the ability of leafy

shoots to carry out photosynthesis effectively. As a result, plants cultured *in vitro* either have low levels of chlorophyll, the pigment essential for photosynthesis, or the enzymes responsible for the photosynthesis process might not function properly or could be completely absent (Malik *et al.,* 2017).

The technique of measurement of chlorophyll fluorescence which exhibit the health of photosynthetic system of any leaf has become an important parameter to study the photosynthetic performance of plants. The fluorescence measurements can exhibit the extent of stress and related damage to photosystems. Chlorophyll fluorescence during *in vitro* growth has been measured in several species (Jiménez *et al.,* 2011; Habibi and Purohit, 2019; Li *et al.,* 2020).

To Determination the levels of carbonic anhydrase (CA) enzymes in plantlets grown in controlled environments can provide insights into their rates of growth. Research into the activity of this enzyme in various plants cultivated *in vitro* has shown that the speed of growth in plantlets is directly linked to their CA enzyme activity (Yanyou *et al.,* 2006). Moreover, the findings imply that within controlled environments, plantlets with higher carbonic anhydrase activity tend to exhibit elevated net photosynthetic rates and accelerated growth rates. These outcomes provide a basis for making informed decisions when choosing a suitable growth medium for plant tissue culture. The assessment of carbonic anhydrase enzyme activity as a physiological indicator to gauge relative growth rates has been performed on Paulownia tomentosa plants (Lazova *et al.,* 2004).

Frequently, plantlets that have been grown in controlled environments encounter difficulties in regulating water loss when they are taken out of their culture containers. This challenge leads to low survival rates when these plantlets are moved to conditions outside of the controlled environment, known as ex vitro conditions (Hazarika, 2006). To develop improved protocols for successful transplantation, investigations have focused on the water condition of plantlets. This includes studying factors like the total fresh weight, dry weight, percentage of water content, and percentage of dry weight under various *in vitro* conditions. Additionally, researchers have examined the percentage of water loss at different stages of micropropagation to address the issue of wilting. These studies aim to establish measures that can effectively prevent wilting during the transplant process (Joshi *et al.,* 2006; Habibi and Purohit, 2019).

Biochemical Studies

Plants face challenges due to being exposed to factors like oxidative stress, excessive moisture, low temperatures, varying light intensity, and different chemicals present in the growth media (Hasanuzzaman *et al.,* 2012). When plants experience oxidative stress, they generate active oxygen species, which can be damaging to plant growth because they negatively affect the internal components and metabolism of the plant. Specifically, if environmental stresses disrupt the dark phase of photosynthesis, it leads to the production of a particular active oxygen species known as superoxide (O^2) (van Rossum *et al.,* 1997). The conversion of superoxide (O^{2-}) to hydrogen peroxide (H_2O_2) is commonly understood to occur through the action of an enzyme called superoxide dismutase (SOD) within the chloroplasts of plants. This underscores the importance of plants maintaining the proper functioning of these enzymes to cope with oxidative stresses effectively. Alterations in the levels of SOD activity occurring during the growth process in controlled environments has been reported (Faisal and Anis, 2010).

In response to stresses like extreme temperatures, drought, and salinity, the accumulation of proline is observed in various plant species. While its function in enhancing plant tolerance to changes in osmotic conditions is debated, proline is believed to play a role in adjusting osmotic balance, neutralizing harmful reactive oxygen species, and safeguarding the integrity of cell membranes (Caverzan *et al.,* 2016). The ability of proline to improve plant resistance against abiotic stresses like drought and salinity has been reported in several studies (Ashraf and Foolad, 2007), *Ailanthus altissima (Filippou et al., 2014), Ipomoea batatas (Fan et al., 2012)* and *Macrotyloma uniflorum* (Tejavathi *et al.,* 2010) have been reported increase in stress condition.

Investigations into the levels of soluble sugars provide insights into sugar metabolism and its absorption by plants (Rabot *et al.,* 2012). When the culture medium contains elevated amounts of sucrose, it leads to significant buildup of soluble sugars in the plantlets. This phenomenon occurs because *in vitro* plantlets rely more on nutrients obtained from the culture medium than those derived from photosynthesis (Wilson *et al.,* 2001). The accumulation of sugars additionally results in reduced osmotic potentials within the plants (Kaur *et al.,* 2021). Research focused on the levels of soluble sugars at various stages of micropropagation has been conducted in Lily (Wu *et al.,* 2021). An upsurge in the overall carbohydrate content was record from *in vitro* shoots to tissue culture plantlets

in natural surroundings. Similar outcomes have been recorded in *Physocarpus opulifolius* L. maxim. (Jagiełło-Kubiec *et al.,* 2021) and *Lavandula viridis* (Mansinhos *et al.,* 2022) concerning carbohydrate levels during the process of *ex vitro* rooting.

Furthermore, plants grown *in vitro* exhibit limited photosynthetic capacity a phenomenon attributed to chlorophyll abnormalities and diminished pigment concentrations (Eckstein *et al.,* 2012).

1.2 Problem statement based on literature review

In India, the widespread challenges associated with tissue culture-based propagation of banana plants, particularly in the context of large-scale micropropagation efforts, have posed numerous obstacles. While tissue culture offers several advantages, the limitations imposed by traditional micropropagation methods hinder its widespread adoption as a conventional agricultural practice in India. Current constraints, such as labor-intensive procedures, high production costs due to the extensive use of culture media components like sucrose, elevated cost of inputs, contamination issues, and the occurrence of hyperhydricity, collectively impede its universal application and precision. Tissue culture-based propagation in the banana industry is hampered by these constraints, which affect both cost-efficiency and reproducibility.

Because of these limitations we need cost effective and more productive micropropagation protocol for the banana plant production. It will help us to sustain tissue culture industry Basically we are depended on the traditional method of the micropropagation and need to further improvement on it to develop good quality plantlets with higher multiplication rate of healthy plantlets with low cost.

The primary experimental results of the liquid culture medium showed improvements in plant growth. These findings support the enhancement of the current protocol for better growth in plants. Additionally, we aim to explore alternative gelling agents, culture vessels, and CO_2 enrichment methods, which can further increase plant production and foster the health of plantlets in *in vitro* conditions. Studying morphological and physiological parameters under these treatments will help us understand how plants respond to different growth conditions in *in vitro* as well as improve their survival rate in ex vitro conditions. These efforts are directed towards achieving our desired objectives, which are outlined as follows:

1.3 Objectives

1. To establish *in vitro* shoot cultures of banana on Semi Solid **(SS)** medium.
2. To multiply shoot cultures in **liquid culture system (LM)** using variety of support matrix, **under CO_2 enriched** environment, **altered gelling agent** and **different culture vessels** and their stoppers to study morphological and biochemical parameters.
3. To induce *in vitro* rooting in elongated shoots grown on **SS medium** as well as **LM** and study of all the morphological, physiological and biochemical parameters in rooted shoots.
4. *In vitro* and *ex vitro* hardening of rooted shoots
5. To test the genetic fidelity in tissue culture-derived plantlets using molecular marker (*viz.* RAPD.)

Hypotheses

- The utilization of cost-effective modifications, including the use of alternative gelling agents, culture vessels, and CO_2 enrichment in both semi-solid and liquid culture mediums, will result in a significant increase in banana plant growth and the enhancement of morpho-physiological characteristics during the micropropagation process.
- The implementation of these modifications will positively impact the production of a higher quantity of healthy banana plants, demonstrating improved growth and quality during the micropropagation process compared to traditional methods.

Table 1.1 Statistics of banana production in India 2021-2022: Source National Horticulture Board (NHB)

Sr. No	Name of state	Production in MT	% Contribution in total production
1	Andhra Pradesh	5,838.88	17.99
2	Maharashtra	4,628.04	14.26
3	Gujarat	3,907.21	12.04
4	Tamil Nadu	3,895.64	12.00
5	Karnataka	3,713.79	11.44
6	Uttar Pradesh	3,391.01	10.45
7	Bihar	1,968.21	6.06
8	West Bengal	1,147.79	3.54
9	Assam	1,108.00	3.41
10	Chhattisgarh	585.52	1.80
	Remaining other state	2270.02	6.99
	Total Production in India	32,454.11	

Table 1.2 List of state wise DBT recognized tissue culture industries in India

Sr. No	State	Total	Recognized
1.	Andhra Pradesh	3	2
2.	Bihar	2	1
3.	Chhattisgarh	6	6
4.	Gujarat	22	15
5.	Haryana	3	2
6.	Himachal Pradesh	3	2
7.	Jharkhand	1	1
8.	Karnataka	12	8
9.	Maharashtra	24	20
10.	Madhya Pradesh	6	5
11.	Orissa	3	2
12.	Punjab	4	3
13.	Rajasthan	1	1
14.	Tamil Nadu	9	4
15.	Telangana	6	3
16.	Uttar Pradesh	4	1
17.	West Bengal	2	2

Source: www.dbtncstco.nic.in

Table 1.3 Use of liquid culture system for the micropropagation of different plant species.

Sr. No	Plant	Result	Reference
1.	*Pogostemon erectus*	Multiplication rate high compare to solid medium.	Muhammet Dogan, 2022
2.	*Spathoglottis plicata*	Better shooting and rooting response.	Shekhawat *et al.,* 2022
3.	*Orcid*	Cost effective	Deb and Pongener, 2022
4.	*Gentiana kurroo Royle*	1.41fold Higher biomass	Alphonse and Thiagarajan, 2021
5.	*Aerva lanata (L.)*	Higher multiplication rate.	Varutharaju *et al.*, 2021
6.	*Juncus rigidus*	Best shoot bud induction and high rooting.	Vyas *et al.*, 2021
7.	*Steinernema jeffreyense*	Higer mass production and high recovery rate.	Dunn *et al.,* 2020
8.	*Vaccinium vitis-idaea ssp.*	2-3 time more multiplication rate compares to solid medium.	Arigundam *et al.,* 2020
9.	*Scoparia dulcis L*	Better plant growth compares to semi solid medium.	Premkumar *et al.*, 2020
10.	Bamboo plants	2-3-fold shoot generation increase.	Ara *et al.,* 2020
11.	*Mentha × piperita L*	2.5-fold shoot production increase.	Vaidya *et al.,* 2019
12.	*Anthurium andraeanum Lind*	Rate of Somatic Embryo increase.	Wang *et al.,* 2019
13.	*Citrus × latifolia*	High number of shoot recorded.	Bulbarela-Marini *et al.,* 2019

Sr. No	Plant	Result	Reference
14.	*lilium candidum*	Increase rate of survive in acclimatization.	Daneshvar Royandazagh, 2019
15.	*Jatropha Curcas*	Highest number of multiple shoot buds was recorded.	Singh, 2018
16.	*Rosa canina*	Shoot multiplication was 1.5-2 times better than in other systems.	Malik *et al.*, 2018
17.	*Plectranthus bourneae Gamble*	Successfully hardened and transferred to greenhouse condition with 83% survival.	Thaniarasu *et al.*, 2018
18.	*citrus epicotyl*	High-quality shoots were produced from explants.	Niedz and Marutani-Hert, 2018
19.	*Juglans nigra L.*	Long-term survival and proliferation of micro shoots was achieved.	Stevens and Pijut, 2018
20.	Pineapple	Increased salinity or drought tolerance.	Gómez *et al.*, 2017
21.	*Typhonium flagelliforme*	Liquid culture media were more effective for all the growth parameters.	Rezali *et al.*, 2017
22.	*Rosa tomentosa*	Beter growth compare to solid medium.	Malik *et al.*, 2017
23.	*Quercus robur*	Increase axillary bud proliferation.	Gatti *et al.*, 2017
24.	sugarcane	*in vitro* rooting rate increase.	Nápoles Borrero *et al.*, 2017
25.	*Bacopa monnieri L*	Increase antioxidant property.	Wangdi and Sarethy, 2016

Table 1.4 List of different type of Temporary Immersion system used during of micropropagation of different plant species.

Sr. No	Plant	System	Result	References
1.	*Eucalyptus globulus*	Immersion system	Increase multiplication rate	González *et al.*, 2011
2.	*Tectona grandis L*	TIS	Increase the rooting rate and survive rate in field.	Quiala *et al.*, 2012
3.	*Pistacia vera* L.	TIB (RITA)	Significantly increase shoot number and root numbers.	Akdemir *et al.*, 2013
4.	*D. lutea* × *purpurea*	TIS	Weight increase	Welander *et al.*, 2014
5.	*Echinacea purpurea*	BIT	Shoot number increase and health plant growth	Welander *et al.*, 2014
6.	*Rubus idaeus*	TIS, BIT	Increase shoot length and shoot number.	Welander *et al.*, 2014
7.	*Eleocharis dulcis*	TIBS	Increase the number of rooting	Gao *et al.*, 2015
8.	*Castanea sativa* × *C. crenata*	CIS	Increase photosynthetic rate and increase shoot number	Cuenca *et al.*, 2017
9.	*Prunus avium* L	TIS	Significantly increase number of shoot and root growth	Godoy *et al.*, 2017
10.	*Olea europaea* L.	Bioreactor	Shoot length and Shoot no. 2 time Increase	Benelli and Carlo, 2018
11.	*Bletilla striata*	TIBS	Increase seed germination rate and shoot length increase	Zhang *et al.*, 2018
12.	*Salix viminalis*	RITA	Hight shoot multiplication rate	Regueira *et al.*, 2018

Sr. No	Plant	System	Result	References
13.	*Capparis spinosa* L.	TI	Increase of shoot num 3 time	Gianguzzi *et al.*, 2019
14.	*Anthurium andreanum* Lind	PI TI	Shoot number increase 2 time. Shoot number increase 8 time	Martínez-Estrada *et al.*, 2019
15.	*Anthurium andreanum*	Bioreactor	Significantly increase shoot no.	Ramírez-Mosqueda *et al.*, 2019
16.	*Tectona grandis Linn. F.*	TIBS (RITA)	Decrease Hyperhydricity increase shoot length	Aguilar *et al.*, 2019
17.	*Musa* AAA	TIS, TIB, MATIS, SETIS	Plant health increase and leaf number and shoot number	Bello-Bello *et al.*, 2019
18.	*Bambusa vulgaris*	TIS	Increase stomatal index and shoot length	García-Ramírez *et al.*, 2019
19.	*Alnus glutinosa*	TIS, Platform, RTA	increased the proliferation rates	José *et al.*, 2020
20.	*Malus domestica*	(CIB TIB	Significantly increase shoot number and root numbers.	Kim *et al.*, 2020
21.	*Saccharum officinarum* L.	Bioreactor	Increase Root and shoot num.	da Silva *et al.*, 2020
22.	*Colocasia esculenta*	TI, TIB	Shoot number and shoot length 2-time increase	Arano-Avalos *et al.*, 2020
23.	*Agave tequilana*	TIS (BioMINT)	Increase leaves number	Monja-Mio *et al.*, 2020)
24.	*Musa sp. (*silk AAB)	TIB	Shoot num 3 time increase	Uma *et al.*, 2021

Sr. No	Plant	System	Result	References
25.	*Larix* × *eurolepis* Henry	TIB	Increase somatic embryo proliferation rate	Le *et al.,* 2021
26.	*Agava angustifolia* Haw	TIS	bigger and better quality of plant	Monja-Mio *et al.,* 2021
27.	*Colocasia esculenta*	TI, TIB	Shoot number and shoot length 3-time increase	Mancilla-Álvarez *et al.,* 2021
28.	*Salix viminalis* L	TIBS (RITA)	Shoot and leaf growth significantly increase	Gago *et al.,* 2021
29.	*Castanea sativa* (Mill)	TIB, CIB	Increase shoot length	Gago, Bernal, *et al.,* 2022
30.	*Prunus domestica* L	TIBS (RITA)	Significantly increase number of shoot and decrease plant nacrosis	Gago, Sánchez, *et al.,* 2022
31.	*Cannabis sativa* L	RITA, Plant form bioreactors	Increase Shoot length in less concentration of sucrose	Rico *et al.,* 2022

PI- Partial immersion, TIB – temporary immersion bioreactor, RITA- Recipient for Automated Temporary Immersion, BIT - temporary immersion bioreactor, TIBS - temporary immersion bioreactor system, CIS- continuous immersion system, TIS – Temporary immersion system, TI- temporary immersion, MATIS^{R} – Company name Bioreactor, SETIS^{TM} - Company name Bioreactor, BioMINT- Modular Temporary Immersion Bioreactor, CIB- Continues immersion bioreactor.

Table 1.5 Reports on use of CO_2 Enrichment during of micropropagation of different plant species.

Sr. No	Plant	Effect	Reference
1.	*Cannabis sativa L. (cannabis)*	Increase in vitro rooting, Increase photosynthesis rate	Pepe *et al.,* 2022
2.	*Lippia dulcis*	High growth rate, increase photosynthesis pigment and increase antioxidant defense response	Rocha *et al.,* 2022
3.	*Aristotelia chilensis [Mol.] Stuntz*	Increase biomass production	Trentini *et al.,* 2021
4.	*Pfaffia glomerata*	accumulation of dry mass, in addition to increasing the photosynthetic rate	Louback *et al.,* 2021
5.	*Wasabia japonica (Wasabi)*	Plant all over growth increase and photosynthesis rate increase	Hoang *et al.,* 2019
6.	*Alnus glutinosa (Black alder)*	acclimatization percentages, with survival percentages greater than 85% being achieved.	Arencibia *et al.,* 2018
7.	*Salix viminalis (basket willow)*	Increase leaf number, dry weight increase and rate of acclimatization	Kaur and Minhas, 2016
8.	*Hevea brasiliensis (rubber tree)*	Increase acclimatization rate	Tisarum *et al.,* 2018
9.	*Castanea sativa×Castanea crenata (hybrid chestnut)*	High proliferation rates, multiplication, rooting and acclimatization increase	Chaari-Rkhis *et al.,* 2015
10.	*Protea cynaroides*	Rooting and leaf number increase 2.7% as compare to control plant	Wu and Lin, 2013
11.	*gerbera*	Increase rooting number leaf num and fresh weight and dry weight	Cardoso *et al.,* 2013
12.	*Cynara scolymus*	Water contain increase, Water use efficiency increase	Pérez-Jiménez *et al.,* 2015
13.	*'Golden' papaya*	Improve potential photosynthetic rates (Apot) and improved biomass accumulation	Schmildt *et al.,* 2015
14.	*Pfaffia glomerata*	Higher dry weight	Corrêa *et al.,* 2015

Sr. No	Plant	Effect	Reference
15.	*Solanum tuberosum)*	Significant different observed Shoot length, root length, leaf area and fresh weight	Kaur and Minhas, 2016
16.	*Grammatophyllum scriptum*	Increase average root length and shoot length	Sasongko *et al.*, 2017
17.	*Prunus avium*	Increase photosynthetic rates, height, and leaf number	Pérez-Jiménez *et al.*, 2017
18.	*Lippia alba*	Increase physical parameters with the increased stomatal density and lignin content for all chemotypes	Batista *et al.*, 2017
19.	*Sorbus commixta Hedl*	promoted axillary shoot length, number of nodes, axillary shoot fresh weight, axillary shoot dry weight, leaf length, leaf width, and total chlorophyl.	Park *et al.*, 2018
20.	*1) Cymbidium* *2) Phalaenopsis*	Increase photosynthesis rate and plant total weight increase	Nhut *et al.*, 2018
21.	*Cannabis sativa L*	A 97.5% rooting rate	Kodym and Leeb, 2019
22.	*Camellia sinensis (L.) O. Kuntze*	Increase dry weight and shoot length	Bag *et al.*, 2019
23.	*Strawberry (Fragaria x Ananassa cv. Festival)*	shoots rooted best on the same medium of elongation	Kepenek, 2019
24.	*Physalis angulata*	Increase chlorophyll contain and plant growth	Santos *et al.*, 2020

Figure 1.1 Economics of banana plant is represented through its various products used in food and other industries.

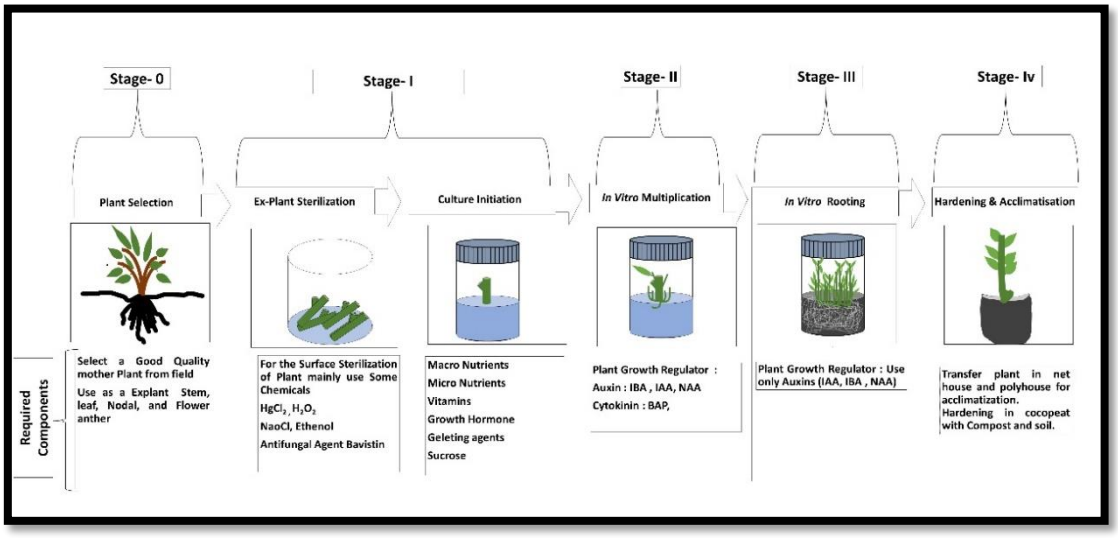

Figure 1.2 Schematic diagram of different stages of micropropagation