Impact of Plant Tissue Culture on Agricultural Sustainability

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Abstract Plant tissue culture is an important agricultural biotechnological tool that contributes in the production of crops with improved food, fiber, fuel, and feed. It is one way toward commercialization to face the food availability challenge in developing countries and allow them to cope with their fast-growing population in a restricted area of land. In addition, plant tissue culture enables some rare and nearly extinct plant species to be rescued and propagated. Conventional methods of propagation thus need to be supplemented with modern breeding techniques. In this way, higher levels of agriculture, afforestation, plant improvement as well as in vitro production of metabolites and plant secondary products can be reached and fulfilled on a year-round basis and under disease-free conditions. The main applications of plant tissue culture in the agricultural field, plant micropropagation, inducing new varieties and constrains of plant tissue culture and challenges this technique is facing as an industry helping the agricultural field, are discussed in this chapter.

Keywords Agriculture, Biotechnology, In vitro propagation, Industry, New varieties, Plant tissue culture

Contents

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1 Introduction

Plant tissue culture, as a modern biotechnology technique, is becoming nowadays very important for the development of mankind. It is considered one of the important breeding methodologies for many crops, vegetables and fruits, and it offers a substitute method for conventional vegetative propagation. It can also be considered as an efficient way of clonal propagation (also known as micropropagation); the prefix "micro" is used because this type of propagation is carried in a relatively small space in the lab. This technique produces an offspring totally like the mother plant.

Crops obtained through tissue culture are developed through time-saving and precise approaches compared to conventional plant breeding ones that take much longer. Plant tissue culture allows the rescue of embryos produced by incompatible crosses, prevents the phenomenon of "seed dormancy" observed in some plant species, and shortens the life cycle of some species known to have a relatively long life cycle.

Plant tissue culture is one way to face the food availability challenge in developing countries to cope with its fast-growing population in a restricted area of land. In addition, plant tissue culture enables some rare and nearly extinct plant species to be rescued and propagated. Conventional methods of propagation thus need to be supplemented with modern breeding techniques. In this way, higher levels of agriculture, afforestation, plant improvement, as well as in vitro production of metabolites and plant secondary products can be reached and fulfilled. Developing crops using the conventional ways face several problems such as low quality of the crop output and productivity fluctuations from year to year, which results in a deficit in the supply of the crop as well as its high price. The use of both tissue culture and genetic engineering techniques, combined, made possible the regeneration of plants with a novel character or two or more characters combined in a single plant species, thus saving time and effort of conventional plant breeding programs.

2 Definition and Intended Objectives of Plant Tissue Culture

Tissue culture means the cultivation or culture of specific plant tissues under aseptic conditions. Plants are grown in specific glassware or plastic containers with the addition of nutrients that suit each plant species. Plant tissue culture is based on a specific ability in plant cells, termed "totipotency" [1]. The concept of totipotency is based on the fact that all plant cells (except sperm and egg cells) contain the full complement of genes, which makes it possible to grow individual plant cells into full healthy plants that can be propagated inside the lab (in vitro). This process is also called "micropropagation."

Plant tissue culture is carried under specific conditions of complete sterilization of both the plant tissues as well as all the glassware and utensils used in the process. Plants are naturally contaminated mostly on their surfaces with microorganisms, so their surfaces are sterilized in chemical solutions (usually alcohol and sodium or calcium hypochlorite). This complete sterile medium helps to produce pest- and disease-free plant material [2]. During this process, plant hormones are added to the medium with specific ratios to each species and to each purpose, to control the plant tissue growth and proliferation. The medium can either be liquid, semisolid, or solid (by the addition of a gelling agent: agar).

3 Advantages of Plant Tissue Culture Technique

The following are the main advantages of the tissue culture technique:

- Small size of explant (the portion used in reproduction), used as starting material.
- Production of multiples of plants without the need for seeds or any pollinators.
- Production of mature plants with little space and time.
- Cultivation takes place under sterile conditions, under controlled environmental conditions (adequate temperature and light) which minimize the chance to transmit diseases, pests or pathogens.
- Production of plants from seeds that have a low germination rate.
- Regeneration of whole plants from genetically modified cells.
- Easy to maintain, move these plants and store them until needed, irrespective of the season and weather.
- This technique in plant breeding is characterized by increasing the plant yield and quality with lower production costs compared to traditional breeding methods.

In this chapter, we will discuss four applications of plant tissue culture technique that are being used in the agricultural field; these are (1) micropropagation, (2) organogenesis, (3) somatic embryogenesis, and (4) protoplast culture.

4 Micropropagation

Basically, micropropagation is like rooting of plant cuttings and might also be considered as another method of vegetative propagation of plants. However, it differs from the conventional method in that it is carried out in complete aseptic conditions and requires unique conditions, i.e., an artificial nutrient medium supplemented with specific growth factors.

It is used to sustain agriculture and is an example of direct laboratory to soil transfer of biotechnological benefits. A small plant cutting or explant (usually an axillary bud) is surface sterilized and inoculated into a culture vessel containing a solid or semisolid nutrient medium and supplemented with the proper ratio of auxin/cytokinin. The inoculated culture vessel is incubated at room temperature, (Fig. 1) [3].

In a day or 2, many shoots develop from the axillary bud in a process known as axillary bud proliferation. After that, each growing point is subcultured (i.e., cultured in a new fresh medium) to give rise to a new shoot. This phenomenon is known as adventitious shoot formation (adventitious refers to an organ grown in a place different from its normal position). Auxin stimulates each shoot to develop roots. After the root emerges, the new plantlet is transferred to the field.

5 Organogenesis

Organogenesis refers to the differentiation of organs, such as shoot and root, from an undifferentiated mass of cells. The process depends on the fact that the cells of an explant (the part of the plant to be used in culture) are highly differentiated because

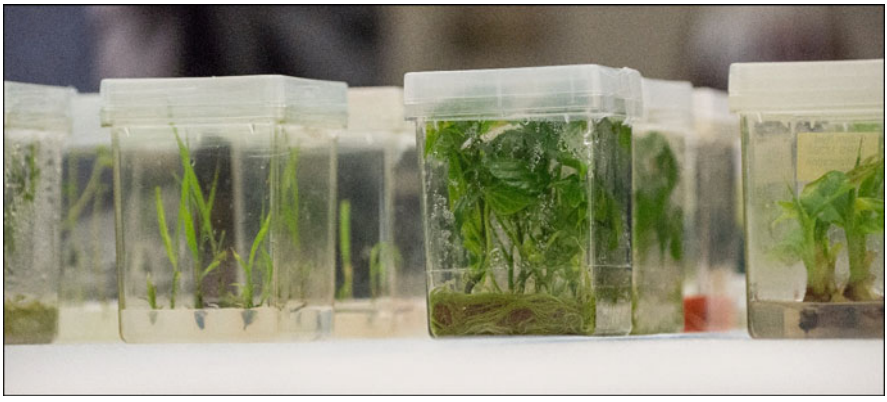

Fig. 1 Plant tissue cultures being grown at a USDA seed bank, the National Center for Genetic Resources Preservation. By USDA, Lance Cheung – Flickr, Public Domain, (https://commons.wikimedia.org/w/index.php?curid=44757726)

they are taken from a differentiated plant part such as a root, a stem, or a leaf. When an explant is placed in an artificially enriched nutrient medium, its differentiated cells start to dedifferentiate (which means to return to an undifferentiated state) to form a mass of unorganized cells known as "callus."

The cells of the callus then redifferentiate and produce the desired tissue in response to specific growth regulators added into the medium (plant hormones). This tissue develops then into an organ.

Single cells can also be cultured and induced to develop shoot and root one followed by the other by the addition of proper plant hormones combinations. Plant growth regulators (hormones) relative amounts to each other play an important role in the differentiation process.

There are two important groups of plant hormones that play the most important roles in plant tissue culture: auxins and cytokinins. Auxins, such as indole acetic acid (IAA) and naphthalene acetic acid (NAA), promote root differentiation and cytokinins such as kinetin and adenine promote shoot differentiation. A balance of both auxin and cytokinin usually produces a callus [4].

It has been established that root and shoot differentiation depends on the ratio or quantitative interaction between cytokinins and auxins [5]. This principle is applied to plant cells or tissues cultured in vitro. Kinetin and IA are added to the in vitro culture in required specific amounts and ratios, one following the others to promote shoot and root differentiation.

6 Somatic Embryogenesis

In flowering plants, the fusion of two gametes leads to the production of an embryo. The resulting plant embryo follows a preprogrammed development through a series of differentiation events to develop into a mature embryo that results in the formation of a plantlet [6]. Under normal conditions, embryos result from sexual reproduction through the formation of a zygote and are thus known as "zygotic embryos."

A different type of embryos can be developed by plant tissue culture techniques; the embryo is like a zygotic embryo in shape, but it is formed from a somatic cell, not from reproductive ones thus bypassing sexual reproduction. Such embryos are known as "somatic embryos." They are bipolar [7]. The formation of somatic embryos is known as somatic embryogenesis.

Somatic embryo formation starts with a mass of single cells or a tissue grown on a semisolid nutrient medium. A cell keeps dividing and forms a cell aggregate. The cell aggregate passes through different stages including globular, heart-shaped, and torpedo stages.

The torpedo stage is the mature stage. The culture is initially started on a semisolid medium, and the obtained callus is transferred to a liquid well-agitated and aerated medium. The callus is broken down into cells that will each develop into a somatic embryo. Mature stages of somatic embryos are sorted out, transferred to a semisolid medium, and grown to maturity to obtain a regenerated plant (Fig. 2).

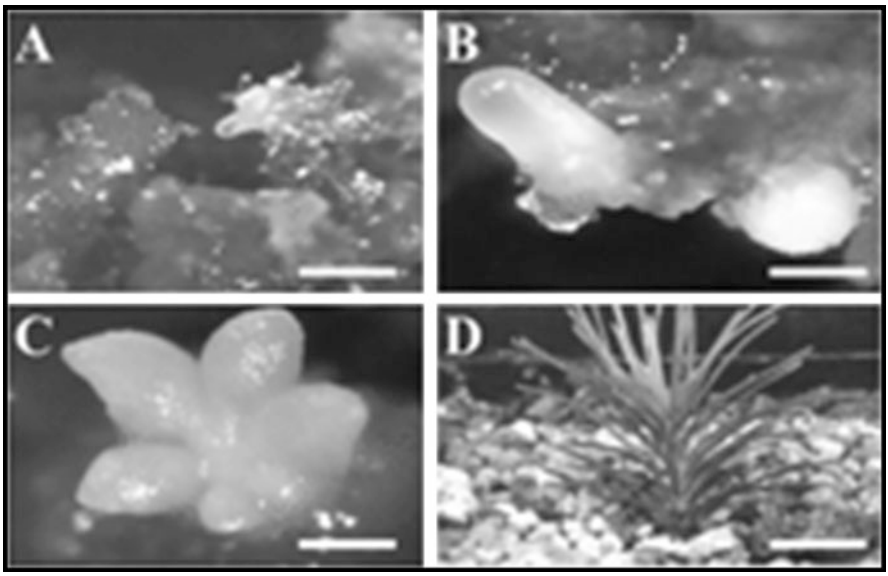

Fig. 2 Regeneration through somatic embryogenesis in slash pine. Different stages of embryo development are noticed, (**a**) embryogenic callus, obtained from zygotic embryo (bar = 0.1 cm), (**b**) globular somatic embryo (bar = 0.5 cm), (**c**) cotyledonary somatic embryo (bar = 0.5 cm), (**d**) regenerated plant, established in soil (bar = 0.8 cm) (adapted from [8])

There are four ways by which somatic embryos could be transferred to the field. These methods are briefly explained below:

1. Germination of somatic embryos takes place in the laboratory; they are then transplanted into pots and then transferred to the field.
2. Encapsulation of dormant embryos takes place in a gel, containing a proper nutrient for the embryo. These encapsulated embryos are known as "artificial/synthetic seeds." These seeds have the advantage to be easily planted in the field.
3. Germination of embryos under controlled conditions: Emerged seedlings are mixed with a gel like medium. The seedling-gel mix is then sown in the field.
4. The embryos are germinated and then used in a viscous carrier gel, supplemented with growth regulators, sucrose and nutrients, a process known as "fluid drilling." The major advantages of this process are (1) the rescue of zygotic embryos initiated by distant incompatible crosses and (2) the ability to overcome seed sterility and dormancy.

7 Protoplast Culture and Fusion

Protoplasts can be defined as the spherical plasmolyzed content of plant cells whose cell wall has been removed. The cell remains bound by a plasma membrane [9]. Figure 3 illustrates the culture of protoplast isolated from embryogenic callus of date palm [10]. Protoplast fusion enables combining useful characters from two plant species in one species. The concept is that isolated protoplasts from two different species of plants are induced to fuse to produce a single protoplast containing both the genetic material and the cytoplasm of both fused protoplasts.

Fusing two protoplasts is not direct or straightforward; it should be induced by some agents, called "fusogens." There are two types of fusogens: chemical and electrical. Polyethylene glycol (PEG) is a chemical fusogen. However, it cannot be considered a universal fusogen, since it is toxic to some types of plant protoplasts. Another way is the use of a direct electric current applied to the fusing protoplasts. This method is known as electro-fusion. The product of the fusion of two protoplasts is called "a synkaryon."

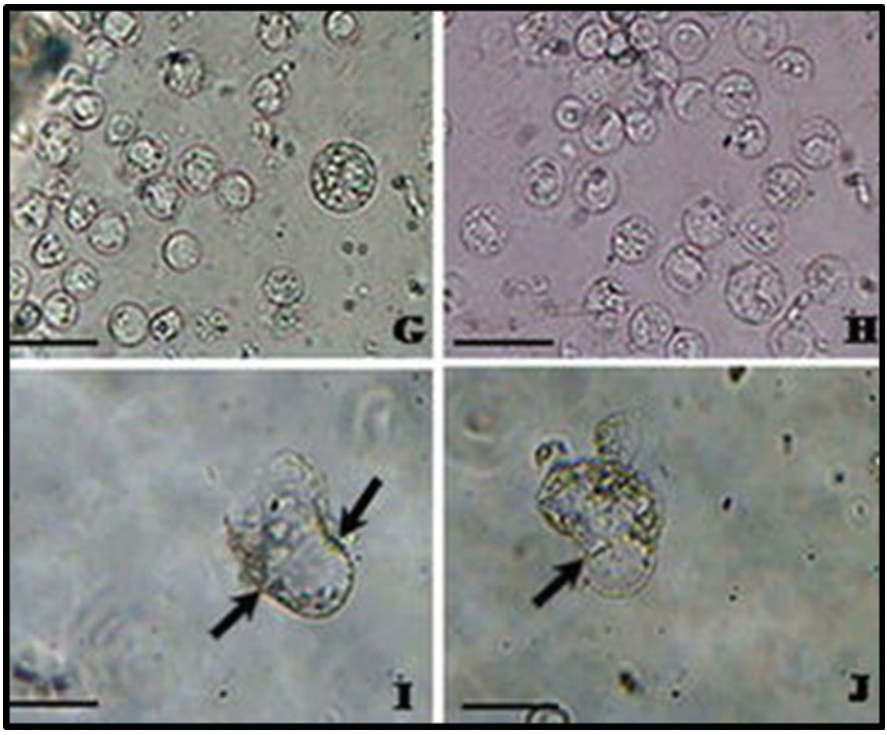

Fig. 3 Date palm protoplast culture as a method of palm micropropagation: (**g–h**) isolated protoplasts (bar = 30.5 μm), (**i–j**) first cell divisions of protoplasts after 3 days of culture (bar = 9.5 μm; *arrows* indicate separation of the cells after division and thickening of the protoplast periphery due to regeneration of a new cell wall) (adapted from [10])

Protoplast fusion takes place in three steps. First, two protoplasts are laid close to each other. Second, the plasma membranes of both cells fuse, and then the two nuclei lie in the mixed cytoplasm. This stage is known as "a heterokaryon." In the third and last stage, the two nuclei fuse leading to the formation of a synkaryon.

After synkaryon formation, a cell wall is regenerated around the fused protoplasts, and the cell is cultured in a defined artificially enriched nutrient medium. This sequence of events is very like what happens in the case of callus culture. This process is also known as somatic hybridization, and the products are known as "somatic hybrids."

This method overcomes the difficulty of the fusion of gametes of two unrelated plant species. Carlson et al. [12] obtained the first somatic hybrid by fusing the isolated protoplasts of *Nicotiana glauca* with *N. langsdorffii*. However, in some cases, two nuclei cannot coexist due to cellular incompatibility. As a result, one nucleus is eliminated, and the result is a protoplast containing the cytoplasm of both species but the nucleus of only one species. In this case, the resulting hybrid is known as a cytoplasmic hybrid or cybrid.

Somatic hybridization is carried in sexually incompatible plant species. A well-known example of a somatic hybrid is "pomato." Pomato was obtained by fusing the protoplasts of potato and tomato. However, this hybrid is of little commercial value [12].

8 Success in Developing In Vitro Protocols for Crop Plants

Biotechnological application of crop improvement programs requires a reproducible and efficient system for in vitro regeneration. An in vitro plant regeneration technique refers to culturing, cell division, cell multiplication, and dedifferentiation and differentiation of cells, protoplasts, tissues, and organs on specific defined solid or liquid medium under sterile conditions and physically controlled environment [13]. A reliable successful in vitro regeneration technique is of great importance to obtain complete whole plants starting from simple cells either through clonal propagation methods or genetic engineering of plants (Genetic Engineering of Plants: Agricultural Research Opportunities and Policy Concerns (1984).

In its beginning, tissue culture was most commonly used for high-value horticultural crops. However, today, tissue culture propagation has also been very successful in producing improved self-sufficient crops widely used in developing countries [14]. One notable advance and improvement made possible by tissue culture was the development of disease-free bananas in East Africa. Bananas are considered a major source of nutrition and income in many countries of Africa. Rice is another crop whose demand increases rapidly in West Africa and cannot be fulfilled by local production only. The region imports around 6 million tons of rice annually (half of the region's requirements) at a cost of about US$1 billion. To address this decrease in product, Monty Jones, a Sierra Leone scientist working at

the Africa Rice Center (previously WARDA), started a breeding program based on tissue culture techniques to develop crosses between an African species of rice (*Oryza glaberrima*) and an Asian species (*Oryza sativa*). The former is better adapted to local environments with a drawback of producing lower yields (around 1 ton per hectare), whereas the latter has the advantage to yield around 5 tons per hectare.

Numerous embryos were obtained by crossing the two species, but these were grown to maturity only with the use of tissue culture. The obtained "new rice for Africa" were called "NERICAs." They have the advantage of being tolerant to many different severe conditions and yield a much higher product [15].

Cassava is a major staple crop for millions of people in tropical countries in East and Central Africa, supplying a great portion of the energy need mostly in the agricultural areas, and it is the second most important staple crop in Africa after maize [16]. Cassava is highly susceptible to many diseases and pests especially African cassava mosaic disease which can result in a huge yield loss reaching 100% [17]. Frog-skin disease, which affects cassava, has been eliminated from five different cassava cultivars using a combination of heat treatment and tissue culture. Tissue culture may enable the development of disease-free cassava varieties in Africa as innovations seek to lower the cost of application of the technology [18].

Date palms, potatoes, citrus, and stone fruits are also grown in many areas of the world including Egypt using tissue culture techniques. A study concluded by Khaled et al. [19] showed that banana grown by tissue culture outperformed banana grown under traditional farming at the level of all the studied economic variables, where average profit from tissue-cultured banana reached around 591% of the profit realized from traditionally produced banana. In addition, it was found that tissue-cultured banana is better in terms of shape, taste, and nutritional value, in addition to obtaining a virus-free crop. Therefore, the study recommended supporting and improving banana production by tissue culture, as a replacement for traditional production to raise the crop productivity and exports of Egyptian banana [19].

Date palm is also a major agricultural crop that has great nutritional value and health benefits. It is propagated either sexually by seeds or vegetatively by off-shoots. Seed propagation has some limitations such as high percentage of male plants and slow growth [20].

The use of tissue culture in case of date palm has proven to be a very convenient method for large-scale multiplication. It enables the production of a high-quality and uniform planting material under sterile disease-free conditions on a year-round basis, irrespective of the weather or season. Two main tissue culture methods are used for date palm micropropagation, somatic embryogenesis, and organogenesis. Inflorescences were also used as good promising explants of elite cultivars of date palm [20].

9 Problems Associated with Plant Micropropagation

Plant tissue culture is known to generate a range of variability between cells of the same tissue. This variability is known as "somaclonal variation." This variation between cells might be due to spontaneous gene mutation or changes in epigenetic marks. This variation might be a disadvantage for in vitro cloning when one's objective is to obtain a true-to-type plant progeny.

On the other hand, somaclonal variation is very beneficial when the breeder's goal is to obtain genetic variation, particularly in the case of plants that are propagated asexually, those that are hard to breed or those with a narrow genetic base, having only few cultivars available [21]. Genetic variations occur in various types of cells such as calli, undifferentiated cells, and isolated protoplasts [22].

10 Production of New Plant Varieties with Tissue Culture

In tissue cultures, variations are detected between cells of the same tissue. These variations are attributed to the phenomenon of somaclonal variation [23]. There are many factors that might cause these variations such as wounding, oxidative stress, imbalance of media components, sterilization processes, and improper physical conditions of the culture such as light and humidity. Plants are exposed to oxidative stress that results in accumulation of reactive oxygen species (ROS), such as hydrogen peroxide, superoxide, peroxyl, hydroxyl, and alkoxyl which may result in genetic mutations such as changes in chromosome number, rearrangements, or epigenetic modifications, not due to any change in DNA sequence. Epigenetic modifications include DNA hyper or hypomethylation. This is the reason explaining why sometimes somatic embryogenesis is a preferred method to obtain uniform plants because DNA in the initial stages of embryogenesis normally contain lower levels of methylation [24]. Also, the number of subcultures and the duration of each one influence the rate of somaclonal variations [25].

Also, in oil palm, in vitro proliferation was found to induce DNA hypermethylation, and changes in DNA methylation may change the expression of embryogenic capacity during tissue culture [26]. Several plant growth regulators (PGRs) used during tissue culture such as 2,4-dichlorophenoxy acetic acid (2,4-D), naphthalene acetic acid (NAA) and BAP (6-benzylaminopurine), and synthetic phenylurea derivatives (4-CPPU, PBU, and 2,3-MDPU) have also been found to be involved in somaclonal variations. Kinetin was found to cause severe hypomethylation of DNA in proliferating cultures of carrot root explants within 2 weeks [27], and auxins, including NAA, on the other hand, were found to cause hypermethylation [28].

Some in vitro multiplication techniques were found to be associated with "somaclonal variation" [29]. Regenerated plants with some aberrations can develop from genetic- and/or epigenetic-mediated gene expression alterations and can sometimes result in significant economic losses. For example, around 5% of commercial oil palm (*Elaeis oleifera*) plants regenerated via somatic embryogenesis bore somaclonal abnormalities

that included the mantled inflorescence syndrome [30]. This disease was later found to be associated with changes in DNA methylation status [31] and was also associated with the use of specific plant growth regulators, hormones, and nutrients that were added to the culture media [32, 33].

The nature of the in vitro propagation system used to produce regenerated plants can influence the chance of producing significant quantities of somaclonal variant plants. A higher chance to produce genetic and/or epigenetic changes in regenerated plants is usually linked to in vitro propagation systems that depend on a two-stage process to generate new plants by passing through an intermediate callus stage (such as in somatic embryogenesis) [34].

First, cells from the explant material must dedifferentiate to form unspecialized callus cells. Second, some of these callus cells must redifferentiate to allow for the development of the specialized cells needed to form tissues and organs. Rodríguez López et al. [35] showed that the *C*-methylation profiles of leaves from plants developed by somatic embryogenesis in cocoa (*Theobroma cacao*) had many of the features of the explant tissue as well as some of those in the leaves of the mother plant. This finding suggests that the epigenetic DNA methylation landscape (and therefore the global gene regulation patterns) is not entirely dedifferentiated in the callus cells before new adventitious plant tissues develop.

11 Advantages of Somaclonal Variation in Plants Produced by Tissue Culture

Somaclonal variation is the variation resulting from chromosomal rearrangements in plants grown by tissue culture and specially those regenerated through a callus phase. The resulting variation can be genotypic or phenotypic. Phenotypic variation might be genetic (pre-existing in the somatic cells of the explant) or epigenetic in origin (caused by temporary phenotypic changes and not cause by any change in DNA sequence). Somaclonal variation may have physiological, genetic, or biochemical cause/s. Physiological causes include exposure of the culture to plant growth regulators with different ratios. Genetic causes include changes in chromosome number and/or structure as well as gene mutation and transposable element activation. Biochemical causes include lack of photosynthetic ability due to alteration in carbon metabolism, starch biosynthesis via carotenoid pathway, nitrogen metabolism, and antibiotic resistance [21]. Somaclonal variation in plants may have its advantages in crop improvement, creation of additional beneficial genetic variations, increased and improved production of secondary metabolites, and selection of plants resistant to various toxins, herbicides, high salt concentration, and mineral toxicity.

12 Hardening and Field Establishment of In Vitro Grown Plants

Hardening is the acclimatization of tissue culture grown plants and their gradual exposure to soil which is the ultimate success of in vitro propagation [36]. Hardening or ex vitro acclimatization of plants grown by tissue culture is considered the bottleneck for commercializing these plants. Researchers use different approaches to establish these plants in soil with the maximum possible efficiency. Rooting is very important for hardening. In addition, when in vitro grown plants are transferred to soil and exposed to ex vitro conditions, they suffer from losses due to environmental changes which sometimes might lead to plant mortality. The level of plant acclimatization and photosynthetic apparatus was found to be affected by the type of media, hormone amount and concentration, concentration of sucrose and the gelling agent, temperature, and pH [37, 38]. Different types and combination of substratum have been used as potential possible ways for a better hardening process. For example, a combination of charcoal pieces and mosses was found optimum for epiphytic orchids, and a mixture of moss and decayed food was preferred for terrestrial ones. In addition, manipulation in salt solution added to a matrix or substrate was used for hardening of in vitro raised *Carica papaya* [39], and soaked cotton was used for successful hardening of *Saccharum officinarum* [40]. Bacterial inoculations are sometimes effective in improving the survival rate of tissue culture grown plants. The term "biotization" refers to a technique analogous to vaccination where physiological changes take place in the plants by plant growth-promoting bacteria when they are transferred into soil. This process enhances the tolerance of these plants to both abiotic and biotic stress which helps the plants to better survive during hardening. Cost-effective approaches for hardening of plants constitute a challenge that the scientists need to approach and try continuously to find new alternatives for plants grown in vitro to adjust to the new growth conditions ex vitro [41].

13 Constraints of Plant Tissue Culture

Plant tissue culture in vitro is a powerful technology that has a promising role in sustaining agriculture and a great potential to produce elite plants with superior quality, with the use of little chemicals. However, this industry is technology driven and requires labor, energy, trained personnel, and specific equipment which are not always available. Acclimatization of in vitro grown plants is often an expensive part of the technology and requires greenhouses to obtain suitable end products. In summary, this technique must be handled very carefully. Otherwise, non-desired unproductive products will be obtained.

14 Conclusion

Plant tissue culture has many applications in the field of agriculture and has proven to be a successful industry allowing the increased production of important crop plants and has thus contributed to the Second Green Revolution. Increased rate of crop production as well as improved crop varieties will be facilitated with plant tissue culture techniques, providing all necessary treatments, equipment, and personnel that are available. Improving and investing in plant tissue culture will thus likely have a great effect in agriculture sustainability and in creating several employment opportunities in the field of agriculture industry.

15 Recommendations

Future research in the field of plant tissue culture should focus on the production and propagation of genetically homogenous disease-free plants and specially the important economic crops to meet the continuously increasing world demand. Somaclonal variation is considered an important source of genetic variability that should be exploited to obtain new stable genotypes that can be grown in different types of soil. In vitro culture of zygotic embryos should be used to recover plants obtained from intergeneric crosses that do not yield fertile seeds. Plant tissue culture is an indispensable tool for genetic engineering in plants to grow plants that are tolerant to both biotic and abiotic stress factors. Decision and policy makers are highly encouraged to invest in new plant tissue culture techniques that suit the agriculture of different crop varieties and in different parts of the land.

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