Problems Associated with Plant Micropropagation

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The production of micropropagated plants in plant-tissue-culture laboratories and nurseries is the most important method for propagation of many economic plants. Micropropagation based on tissue-culture technology involves large-scale propagation, as it allows multiplication of a huge number of true-to-type propagules in a very short time and in a very limited space, as well as all year round, regardless of the climate.

Keywords: acclimatization ; browning ; contamination ; delay of subculture ; hyperhydricity ; recalcitrance ; somaclonal variations ; totipotency

1. Introduction

Plant-tissue culture (PTC) refers to the *in vitro* culture of plant cells, tissues, organs, seeds, protoplasts, or embryos on a nutrient medium under aseptic conditions, where temperature, photoperiod, humidity, light, and the components of medium all supply the ideal, controlled growing environment [1]. PTC, as a crucial component of plant biotechnology, was and still is one of the optimum approaches, which can be applied to overcome many problems (i.e., global warming, climate change, desertification, salinization, the global water crisis) faced by agricultural and horticultural production, which are causing a global food crisis and famine [2][3]. According to the United Nations, the population of the world will increase to 9.9 billion by 2050, thus, food production must rise by 50% to feed billions more people. PTC can guarantee continuous production systems regardless of the environmental or geographical constraints to meet the food, feed, fiber, and energy supply needs of a growing population [2]. Several promising PTC techniques have been used, including *in vitro* micropropagation, organogenesis, and somatic embryogenesis [4]. PTC also has many benefits, such as production of pathogen-free plants [5][6]; somatic hybridization [7][8]; rapid propagation of difficult-to-propagate plants [9]; improving genetics of commercial plants as desired [10]; obtaining androgenic and gynogenic haploid plants for shortening breeding programs [11]; conserving rare and endangered plants [12]; producing different varieties tolerant to abiotic stresses such as drought, salinity, and heat [13][14]; and producing biological active compounds or secondary metabolites, especially through plant-cell-suspension culture [15][16].

PTC is considered the backbone of horticultural nurseries that can be used in propagating plants of forestry, vegetable, fruit, and ornamental species [5]. These nurseries have huge economic importance for many horticultural production systems, e.g., seedling production for tree-growing programs [17]; availability of high-quality tree planting materials [18]; and disease screening and discovery of new plant pathogens to maintain healthy nurseries [19], with the aim of producing high-quality, productivity as well as pathogen-free plants and/or trees. However, there are many obstacles, which may cause a loss for *in vitro* cultured plants in nurseries and *in vitro* culture laboratories, such as contamination of cultures [20], hyperhydricity phenomenon [21], browning of tissues or phenols exudation [22], shoot tip necrosis [23], delay of subculture, somaclonal variations [24][25], root hardening [20], and failure of acclimatization or limited planting [26].

2. Problems Associated with Plant Micropropagation

Any PTC laboratory needs some basic facilities or conditions, without them it is impossible to produce any micropropagated materials. These include the complete infrastructure and trained workers for proper controlled-environmental conditions [27]. The success of any PTC laboratory producing large-scale plant material depends mainly on these previous factors, besides the scientific team and their efficiencies (**Figure 1**). Making great progress in the different branches of biotechnology, there is a terrible race between different mega-companies and scientific centers in developing laboratories for PTC [27]. This reflects the significance of these laboratories and their role in the global bioeconomy.

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

2.1. Problems Originated Due to Technical Reasons

2.1.1. Contamination of Plant-Tissue Cultures

The contamination of *in vitro* plants is considered a crucial obstacle, which prohibits successful micropropagation protocol (**Figure 2**). Contamination may include many microorganisms, such as bacteria, fungi, molds, and yeasts. This contamination is the main factor in the losing of time and effort related to PTC, which increases the cost of production [28]. External contamination results from the laboratories and used materials (media; glassware; culture vessels, tools, explants), whereas internal contamination is related to the endophytic microbes in mother plants [29]. Several proper methods could be used to exclude and eliminate the contaminants through surface sterilization (**Table 1**), such as chemical agents (antiseptic agents, liquid detergent, mercuric chloride or sodium hypochlorite), ultraviolet (UV) sterilization, autoclaving of media and instruments, and improvement of cultural practices or handling [20]. Therefore, surface sterilization of the equipment and plant materials should be managed to improve the performance of the laboratories and, thus, acquire aseptic cultures [29]. On the other hand, antibiotics could be used as anti-microbial agents for eliminating endophytic bacteria in *in vitro* cultured plants [30].

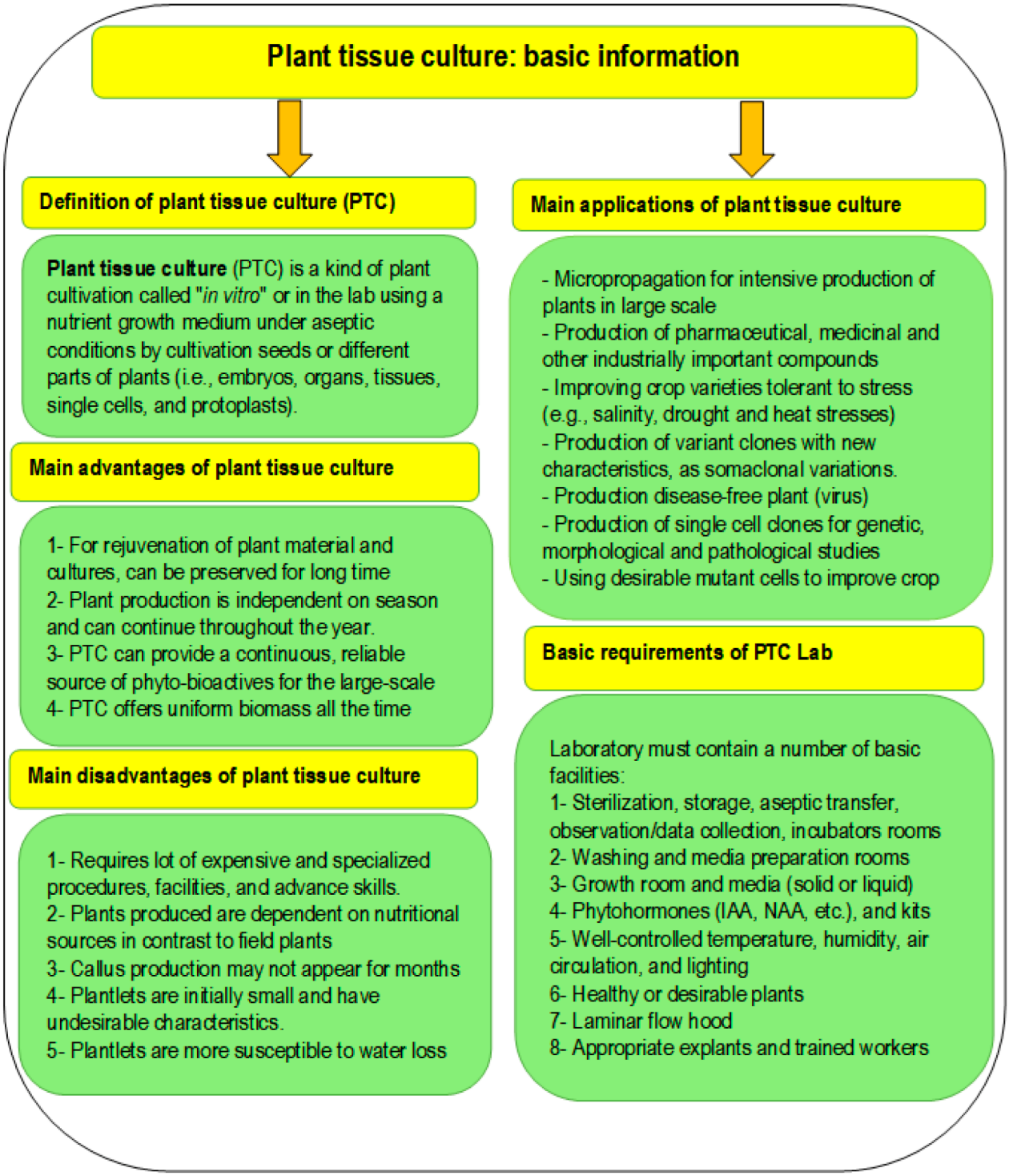
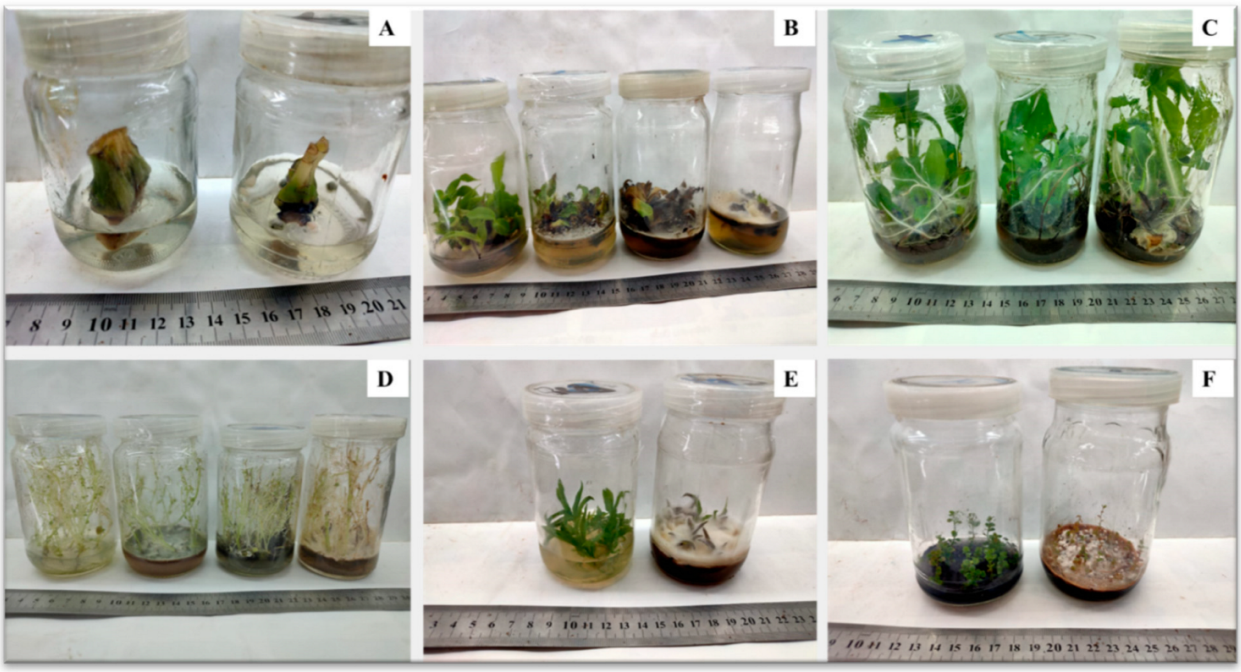
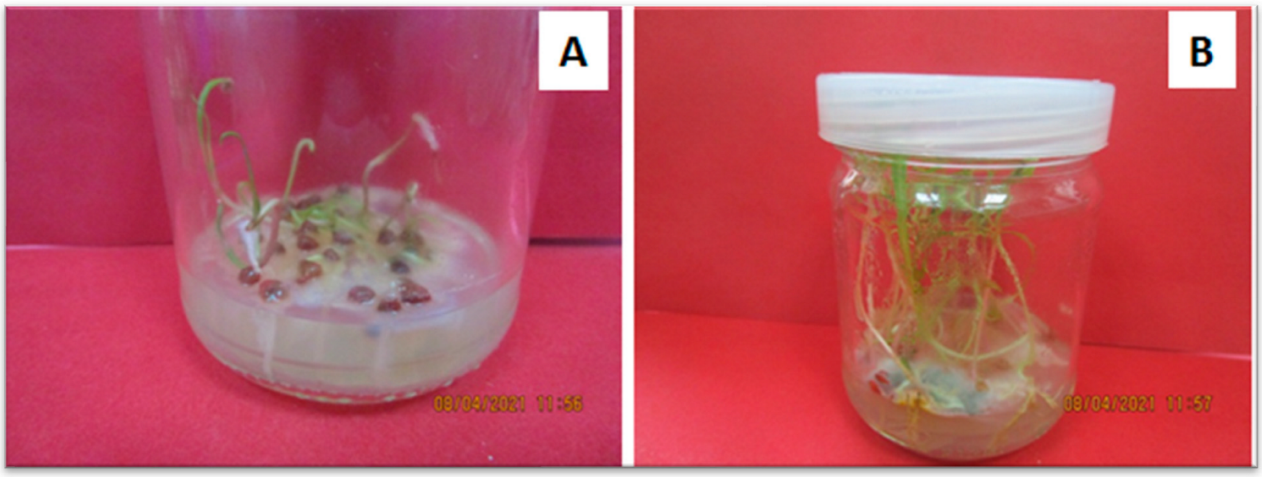


Figure 1. The basic information about the field of plant-tissue culture, including the definition, different applications, advantages, and disadvantages of this field. Sources: [9][31].



(a)



(b)

Figure 2. (**a**) Effect of contamination on *in vitro* plants (left jar = normal plant, right jar or jars = contaminated plant). (**A**) Banana plant (*Musa* sp.) at initiation stage of meristem culture, (**B**) banana plant at shoot multiplication stage, (C) banana plant at rooting stage, (**D**) potato plant (*Solanum tuberosum* L.), (**E**) *Cattleya* sp., and (**F**) blueberry (*Vaccinium corymbosum* L.) at rooting stage. (**b**) Effect of contamination on *in vitro* growth and shoot proliferation of spinach plant (*Spinacia oleracea* L.) (**A**) = Bacterial contamination and (**B**) = fungal contamination.

Table 1. Some surface-sterilization methods for external contamination used in tissue-culture laboratories.

Studied Plants	Surface-Sterilization Methods and Sterilized Items	Main Findings of the Study	Refs.
Potato (*Solanum tuberosum* L.)	Ultraviolet-C radiation to explants for 10 min	The external contamination of explants has been reduced.	[32]
Mulberry (*Morus alba* L.)	Mercuric chloride at 0.2% for 10 min to shoot tips and auxiliary buds	Minimum percentage of contamination and highly percentage of survival in correlation with shoot development were noticed for all mulberry cultivars under investigation.	[33]

Studied Plants	Surface-Sterilization Methods and Sterilized Items	Main Findings of the Study	Refs.
Guayusa (*Ilex guayusa* Loes.)	70% ethanol for 2 min + 2.5% sodium hypochlorite + five drops of Tween-20 for 25 min to apical stem segment	Surface sterilization with ethanol and sodium hypochlorite resulted in 100% surface-sterilized stakes.	[34]
Eucalyptus (*Eucalyptus obliqua*)	Active chlorine added to culture media at 0.005% for establishment and 0.003% for shoot multiplication and elongation	Active chlorine at 0.005% led to the lowest fungal contamination rate in the establishment stage, where at 0.003% resulted in maximum number of shoots per explant and the greatest shoot length in the multiplication stage.	[35]
Guava (*Psidium guajava* L.)	Silver nanoparticles (AgNPs) at 50 mg L^{-1} directly to shoot tips for 5 min or at 5 mg L^{-1} added to culture medium	AgNPs at 50 mg L^{-1} yielded a contamination rate of 40%, where at 5 mg L^{-1} reduced shoot contamination rate to 50% compared to controls (80%) and enhanced multiplication rate of the shoots by 180%, as an alternative method for surface sterilization of explants which are easily damaged by commonly used surface sterilizing.	[36]
Rosmarinus officinalis L.	Bio-synthesized of silver nanoparticles via *Rubia tinctorum* L. using cell culture were applied for surface sterilization of stem explants	Sterile explant percentages varied between 40 and 97% and no browning was observed. This method could be used in surface sterilization of explants which have a browning problem caused by their phenolic contents.	[37]
Sargassum fusiforme	A crude extract of a medicinal herbal plant Tarragon *(Artemisia dracunculus*) was used for surface sterilization of explants (leaf, stipe, and stolon) cultured *in vitro*	The crude extract of A. *dracunculus* showed a high microbial sterilization effect with (90, 80, and 20%) for leaves, stipes and stolons, respectively. It has very low toxicity to plant tissues compared to chemical sterilants.	[38]
Chinaberry (*Melia azedarach* L.)	Dipping leaf explants in 2 g L^{-1} benomyl for 2 h + 7% hydrogen peroxide (H_2O_2) for 10 min + 2% NaOCl for 12 min for surface sterilization	The lowest contamination percentage of explants and browning as well as the highest percentage of callus induction and growth were observed.	[39]
Carnation (*Dianthus caryophyllus*)	Sodium dichloroisocyanurate (NaDCC) was applied as a medium sterilizer to culture medium at 0.02 g L^{-1} instead of autoclave sterilization	Contamination rate recorded below 5%, sodium isocyanurate has the potential to substitute media autoclaving in plant-tissue culture.	[40]
Butterfly pea *Clitoria ternatea* L.	0.1% Bavistin solution + 70% ethanol, and 0.1% $HgCl_2$ was used for surface sterilization of nodal explants	Microbial contamination was eliminated and then surface sterilized nodal explants were used for shoot multiplication induction.	[41]

Studied Plants	Surface-Sterilization Methods and Sterilized Items	Main Findings of the Study	Refs.
Orchid (*Angraecum rutenbergianm* Kraenzl)	0.5% (*w*/*v*) NaDCC solution + 2 mL L^{-1} Plant Preservative Mixture (PPM™) was used for surface sterilization of Seed capsules	87.5% of the total number of capsules was disinfected and the seeds inside them were clean after 3 months of culture. Both NaDCC and PPM were essential to suppress microbial growth.	[42]

In this regard, banana axillary shoots contaminated by internal bacteria were cultured on MS medium supplemented with different filter-sterilized antibiotics (ampicillin, penicillin, ticarcillin) added separately at various concentrations (25, 50, 100, 200 mg L^{-1}). The results showed that the studied antibiotics recorded zero contamination at 100 or 200 mg L^{-1}, however, a reduction in shoot-multiplication parameters was noticed. The most effective concentration of a single added antibiotic for eliminating the bacterial contamination was 100 mg L^{-1} [43].

The external contamination by epiphytic microbes could be inhibited by surface sterilization with running water with/without detergent, chemical substances (ethanol, mercuric chloride, sodium hypochlorite), and plant-preservative mixtures [32]. However, there are some materials could be added to the culture media to inhibit the external contamination, such as plant-preservative mixtures [32] and benomyl fungicide [39]. The investigation showed that inclusion benomyl at 100 and 500 mg L^{-1} in culture medium significantly decreased the fungal-contamination percentage in *Melia azedarach* L. *in vitro* cultures from leaf explants, minimized browning, and recorded the highest percentage of callus induction and growth [39]. Endophytic microbes that are present within the explants are considered a major constrain to the establishment and growth of tissue-cultured plants, as they are more difficult to remove by normal surface sterilization. However, the internal bacterial contamination could be eliminated by supplying the culture media with different substances (**Table 2**), for example, antibiotics [44][45], copper sulfate [44][46], or fungicides [47]. After identification of the contaminants, a low-phytotoxicity antibiotic should be selected [47].

Table 2. Some antiseptic substances used for eliminating endophytic contamination in plant-tissue cultures.

Applied Substance (Name and Concentration)	Plant Species/Cultivar	Success of Decontamination	Refs.
Kanamycin and streptomycin sulphate at 10 µg ml^{-1} each were added to shoot multiplication medium +2 mg L^{-1} BAP +10 mg L^{-1} adenine sulfate	*Guadua angustifolia* Kunth	Bacterial growth was inhibited and intensive formation of high-quality shoots was observed.	[48]
Antibiotics (timentin at 150 mg L^{-1} + gentamycin at 30 mg L^{-1} were added to culture medium	*Camellia sinensis* var. *sinensis*	They were effective to eliminate bacterial endophytic up to 24 days with 0% contamination.	[49]
Antibiotic, cefotaxime at 62.5 mg L^{-1} was supplemented to ½ Murashige and Skoog (MS) medium for establishment	Jerusalem artichoke (*Helianthus tuberosus* L.)	It recorded 0% contamination 100% survival of stem nodes cultures.	[45]
Copper sulfate ($CuSO_4$ $5H_2O$) at 60 mg L^{-1} was added to MS medium +3 mg L^{-1} BA + 1 mg L^{-1} KIN for shoot multiplication	Banana (*Musa* sp.)	The growth of the endophytic bacteria was inhibited by recording 0% contamination.	[46]
Copper sulfate ($CuSO_4$ $5H_2O$) at 70 mg L^{-1} was supplemented to MS medium + 5 mg L^{-1} BA for shoot multiplication	*Philodendron selloum*	It eliminated the endogenous bacteria contamination to 0%, without decline in growth of *in vitro* shoots.	[44]

Abbreviations: Benzylaminopurine (BAP), Murashige and Skoog (MS), benzyladenine (BA).

2.1.2. Delay of Subculture and Burned Plantlets

The *in vitro* micropropagation technique has a lot of benefits; the most important one is represented in producing true-to-type plantlets, which are genetically and physiologically uniform. These plant materials can be used in the intensive production of several plants such as cucumber (*Cucumis sativus* L.) [24], olive trees (*Olea europaea* L.) [49], strawberry (*Fragaria* × *ananassa* Duchesne) [50], cardamom (*Elettaria cardamomum* Maton) [51], and *Flemingia macrophylla* (Willd.) Merr [52]. *In vitro* propagation protocol includes certain stages that should be followed (1) pre-establishment stage, (2) establishment stage (3) multiplication stage, (4) rooting stage, and (5) acclimatization stage, as reported in several articles [47][48][53][54][55][56]. However, under heavy work and a lot of tasks in PTC laboratories, the lack of facilities, and a limited number of expert workers and technical specialists, a delay of subculture definitely happens (**Figure 3**). This delay will for sure cause a great loss or damage in the production of *in vitro* plantlets and even in the maintenance of stock cultures. This situation may differ in the laboratories of developing and developed countries, as it can be clearly observed and spread in the first case for the reasons mentioned earlier. Generally, the effect of time of subculture on the shoot-proliferation rate of *in vitro* cultures varyies from one species to another [57]. In this regard, the long-term incubation period on a culture medium of constant hormonal composition had a negative effect on the multiplication rate of six ornamental species and cultivars of the Rosaceae family [58] and two cultivars of *Dasiphora fruticose* (L.) *Rydb.* [59], but it was found that the longer incubation period (75 days) of pineapple (*Ananas comosus* L. Merr.) *in vitro* shoots on a culture medium resulted in a higher multiplication rate and total number of shoots than a shorter one (30 days) [60]. Somaclonal variation, which resulted due to prolong incubation period in the culture medium (delay of subculture), hinders supplying clonally identical plantlets, which is considered the main target of plant micropropagation [61][62]. The effect of subculture times on genetic fidelity of *Tetrastigma hemsleyanum* Diels and Gilg callus cultures under a long-term tissue culture was studied [61]. The obtained results clearly indicated that the frequency of somaclonal variation has been increased by increasing the subculture time. Moreover, long-term cultures during clonal multiplication of *Moringa oleifera* shoots resulted in high somaclonal variation [62]. It was proven that supplementation of a culture medium with 50 µM salicylic acid (SA), an anti-ethylene compound, decreased hyperhydricity as well as somaclonal variation under a long-term culture. So, SA was recommended for moringa clonal micropropagation [62]. Burned plantlets are a very common phenomenon during *in vitro* handling, which results from using hot planting tools (mainly forceps and scalpels) during transfer the plantlets (**Figure 4**). This problem can be avoided when the workers in the PTC laboratory are trained and have enough experience in this field.

Figure 3. Effect of subculture delay on *in vitro* growth and shoot multiplication (in each photo, left jar = normal plant, middle jar = plant in the first stage of degradation, right jar = dead plant). Blackberry (*Rubus ulmifolius* Schott) (**A**), banana (Musa sp.) (**B**), *Philodendron selloum* (**C**), blueberry (*Vaccinium corymbosum* L.) (**D**), potato (*Solanum tuberosum* L.) (**E**), and *Gypsophila paniculata* (**F**).

Figure 4. Effect of too-high temperature forceps and scalpels during sterilization on *in vitro* shoot multiplication and growth of burned plants. Photo (**A**) represents banana plant (*Musa* sp.) and (**B**) blueberry plant (*Vaccinium corymbosum* L.).

2.2. Problems Originated Due to Physiological Reasons

2.2.1. Browning of Plant-Tissue Cultures

The browning of explants or phenolic browning is a phenomenon that results naturally from enzymatic oxidation of the polyphenolic compounds, which are well-known inhibitors in PTC (**Figure 5**). Phenols released from injured or cut explants are then oxidized to quinones by polyphenol oxidases (PPOs) and peroxidase (POD), causing browning of the tissues and medium as well [32]. These quinones bind with cell proteins or polymerizes by dehydration, causing the disruption of cell metabolism, inhibition of growth, and, ultimately, death of explants [63]. Browning of tissues could be reduced by timing the explants' collection, supplementing the culture medium with antioxidants, such as citric acid, ascorbic acid, activated charcoal (AC), and polyvinyl pyrrolidine (PVP), alone or in combination, or using liquid culture or micrografting [64][65][66]. In addition, this phenomenon can be overcome by decreasing the biosynthesis of phenolic compounds and by inhibiting the activity of the phenylalanine ammonia lyase enzyme during the *in vitro* propagation. Moreover, 2-aminoindane 2-phosphonic acid (AIP), an inhibitor polyphenol production, could be added to the culture medium to inhibit oxidative browning [48]. Nitric oxide (NO) was applied to the growth medium to reduce callus browning, which allowed the tissues to recover and regenerate [22]. A comparative study was done among three browning inhibitors that were supplemented to the calli induction medium of bamboo (*Dendrocalamus sinicus* L.C. Chia and J.L. Sun) to prevent browning [67]. These browning inhibitors were sterilized and added to the medium at different concentrations: citric acid (200–600 mg L^{-1}), vitamin C (100–300 mg L^{-1}), and AC (400–1200 mg L^{-1}). Citric acid ($C_6H_8O_7$) at 400 mg L^{-1} was the best inhibitor, significantly inhibiting and reducing callus browning to 17.59%.

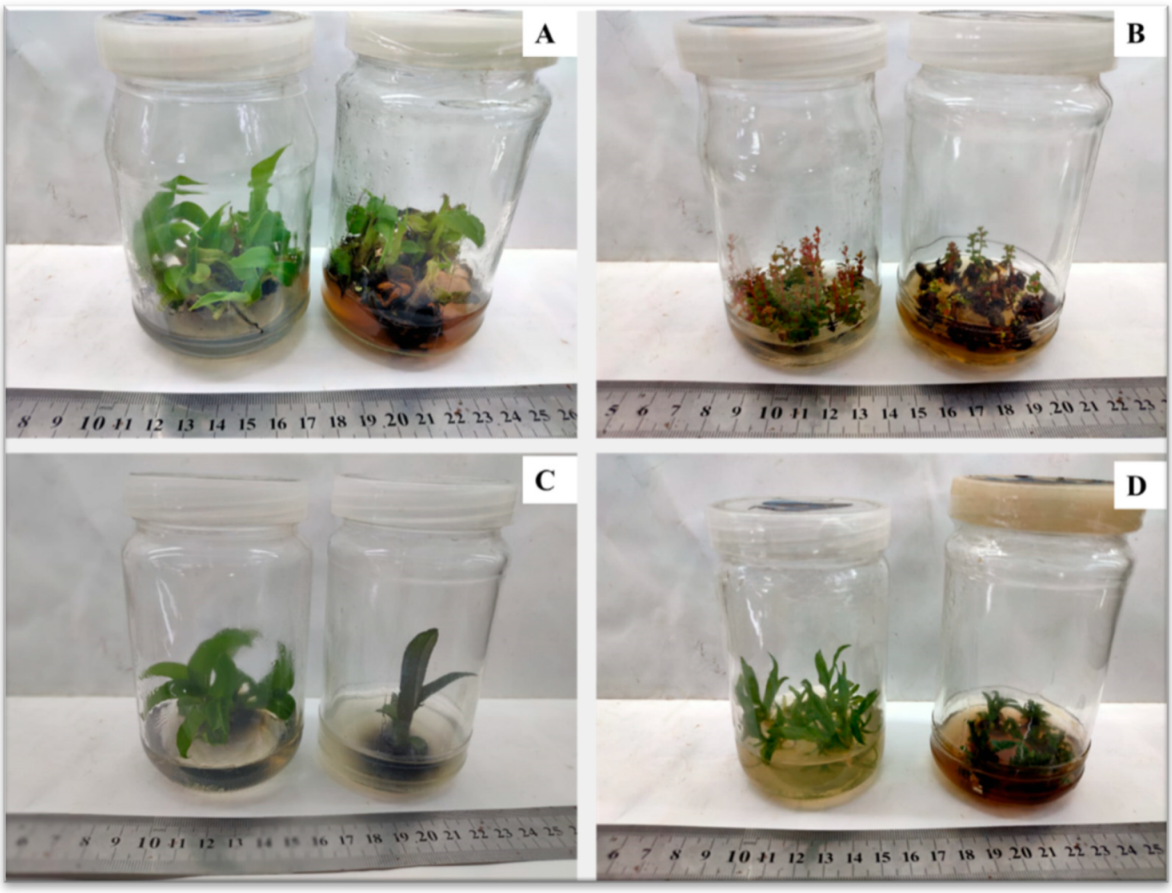


Figure 5. The symptoms of browning at the stage of shoot multiplication of *in vitro* plantlets. Left jar = normal plant, right jar = browned plant. Banana plant (*Musa* sp.) (**A**), blueberry plant (*Vaccinium corymbosum* L.) (**B**), *Phalaenopsis* sp. (**C**), and *Cattleya* sp. (**D**).

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

A successful micropropagation protocol requires the appropriate conditions for *in vitro* root initiation and the development of regenerated shoots. Healthy, rooted *in vitro* micro shoots could be acclimatized successfully *in vitro* and/or *ex vitro*; when they are transferred to *ex vitro* conditions, then they can be established in the soil. Previously, the literature investigated the post effect of cytokinins in shoot-proliferation medium on subsequent rooting ability of micro shoots. It was observed that the type and concentration of cytokinin added to the shoot-multiplication medium highly affects the subsequent rooting stage, where rooting frequency has been reduced in many species by supplementation of thidiazuron (TDZ) or benzyladenine (BA) to the shoot-multiplication medium [68][69], while it strongly inhibited rooting in other species. Moreover, by increasing TDZ concentration in the last shoot-multiplication medium, the number of roots per shoot decreased until it reached 0% at 0.45 µM TDZ [70]. Similarly, the subsequent rooting of 'Royal Gala' apple regenerated shoots was inhibited at a high concentration of meta-topolin-riboside (TOPR, 6.5 mg L^{-1}) in the regeneration medium. Although BA, the most frequently applied cytokinin to the proliferation medium for shoot multiplication, has an inhibitory effect on the subsequent rooting. It was reported that adding BA to the proliferation medium resulted in the decreased rooting ability of 'Red Fuji' apple micro shoots compared to meta-topolin (TOP) [68].

Reducing the salt strength to a half or a third in the rooting medium has been proven to be useful for enhancing root induction, development, and number as well in many leguminous species, fruits, medicinal plants, and trees [65][69][71], while a full-strength MS medium has been widely used for *in vitro* rooting in most herbaceous non-shrub plants that do not have problem in rooting. In this regard, *in vitro* growth of *Typhonium flagelliforme* L., a valuable medicinal herb, is improved by increasing MS medium strength, while the maximum number of roots was recorded with full-strength MS rooting medium [72]. Moreover, *in vitro* regenerated shoots of *Cordyline fruticosa* and *Philodendron selloum* were successfully rooted by 100% after four weeks on full MS medium free-growth regulators [44]. Unlike plants known to be hard-rooting species, it is better to reduce the strength of medium to half, to decrease the osmotic pressure [68]. From that, micro shoots of sweet potato (*Ipomoea batatas* (L.) Lam. were *in vitro* rooted with 100% rooting after three weeks culturing on half-strength MS medium [73]. It was proven that the optimal medium for *in vitro* rooting of *Arnica montana* L. and Cattleya orchids hybrid was half-strength MS supplemented by 0.5 and 2.5 mg L^{-1} indole 3 butyric acid (IBA), respectively [71][74]. It is widely accepted that auxin is needed for root induction, but its absence or continued presence in

the medium inhibits the adventitious roots of apple micro shoots [65]. Moreover, root quality may be improved by addition of auxins [69].

In addition, the type of auxin added to the rooting medium is mainly dependent on several factors; the most important one is the protocol used for *in vitro* rooting, if it is one-phase or two-phase, while the latter one is widely applied for most horticultural plants such as apple (*Malus domestica* Borkh.). It was reported that the rooting of micropropagated apple shoots includes a short phase of one week for root induction, in which IBA was frequently applied, followed by a longer phase of several weeks where naphthaleneacetic acid (NAA) was the most-common auxin supplied to the medium for root elongation [65][66]. Moreover, the optimal auxin for rooting was plant-species-dependent. Therefore, the choice of auxin is a very important issue for woody plants, which show difficulty in *in vitro* rooting. It was found that IBA has been commonly used, rather than indole-3-acetic acid (IAA) and NAA, as the most effective auxin to induce root formation in woody species such as apple and *Cassia angustifolia* Vahl.; whereas, 1, 2-benzisoxazole-3-acetic acid (BOA) showed very poor effect on root induction in woody plants [66]. In this regard, IBA was the most efficient among the other auxins tested for root induction from the *in vitro* shoots of *Cymbidium aloifolium*, *Dendrobuim aphyllum*, *Dendrobuim moschatum*, and Cattleya hybrid [74][75]. Moreover, the IBA applied method may be effective on *in vitro* root initiation. It was recorded that shoots could be cultured directly on rooting medium supplemented with IBA or dipped in IBA-filter sterilized solutions, then cultured on MS medium for rooting induction. It was stated that chickpea *(Cicer arietinum* L.) 'Giza4' *in vitro* shoots had the highest rooting potential, rooting percentage, number of roots, and root length, when shoots were dipped in 50 mM L^{-1} IBA and cultured in liquid medium [76].

On the other hand, auxin concentrations in rooting medium influence the rooting ability in many species. Low auxin concentrations were optimal for improving the rooting of legumes for root development forming adventitious roots, and it increased the number of roots per shoot. Furthermore, the carbohydrate source and its concentration can affect the *in vitro* rooting in many species where sugar is essential for root formation, by supplying the micro shoots with energy. No roots could be developed in apple 'Merton Malling' rootstocks on a sugar-free medium [65]. It was demonstrated that a low sucrose concentration (20 mg L^{-1}) is preferable to enhance the rooting of *Astragalus chrysochlorus* to reach 93% [77], whereas 1% (10 mg L^{-1}) sucrose was the optimal concentration for rooting of the 'Granny Smith' apple in liquid culture medium containing 10 µM IBA. There are other factors that can impact the *in vitro* rooting of micro shoots [65], such as cultivars or genotypes, because they are different in their rooting ability and, thus, their optimal rooting medium. The orientation of micro shoots was reported to be a factor altering the efficiency of *in vitro* rooting. It was reported that the rooting percentage of apple micro shoots significantly depended on the interaction between the micro shoots' orientation, if it is upright or inverted in rooting medium, genotype, and auxin level [78]. Moreover, the number of subcultures and shoot orientation for the last shoot-multiplication medium prior to the rooting stage could influence the efficiency of the subsequent rooting process. The best result for rooting percentage (≈89%) was recorded for apple micro shoots placed horizontally on the last medium for shoot multiplication one week before transferring on rooting medium [79]. It was observed that rooting could be improved in several cultivars by increasing the number of subcultures [65]. Activated charcoal as organic compound could be employed to enhance root length, if it is added to root elongation medium. These previous factors should be taken in consideration during *in vitro* rooting, especially for difficult-to-root plant species as woody plants.

Due to many reasons, such as low humidity, high level of irradiance, low root uptake of water under *in vitro* conditions [80], rapid desiccation of *in vitro* plantlets, and their easy infection by fungal and bacterial diseases; *in vitro* rooted plants might fail when they are transferred to *ex vitro* conditions during acclimatization. To overcome those obstacles, *in vitro* plants should be supported gradually by following the right acclimatization strategy and right selection of the acclimatization medium [81]. Thus, the ultimate success of the *in vitro* micropropagation of plants depends on the successful establishment of plantlets in the soil through the acclimatization process [82]. Application of biostimulators was proven to be used for successful acclimatization. The fungus of *Piriformospora indica* has been used as a biological stimulator to enhance plant growth and root development during the acclimatization process, allowing *in vitro* hard rooting plants such as orchid to be transferred to the field [83]. Another plant-growth-promoting microorganism (*Pseudomonas oryzihabitans*) has been applied to the *in vitro* rooted plantlets of pear to boost the growth efficiency in soil [84]. The physiological aspects of *in vitro* rooted banana plants (*Musa* sp.) have been improved through inoculation of *Buttiauxella agrestis* and *Bacillus thuringiensis* (plant growth-promoting bacteria that produce auxins) into the root system of banana at the acclimatization stage. Hence, high quality seedlings have been acquired [85]. In addition, many supporting materials (e.g., agar, rockwool, perlite, vermiculite) have been used to improve the growth and survival rates of *in vitro* plants in *ex vitro* conditions [86].

2.3. Problems Originated Due to Genetical Reasons

2.3.1. Somaclonal Variation

The somaclonal variation point to culture-induced, unexpected, and undesired variations or anomalies, which eventually is inherited by the clonal progenies, becomes genetic and happens during tissue cultures; this is, thus, noticed in the *in vitro* regenerated plants, and is an often an unwanted phenomenon, especially in mass micropropagation programs that are highly desirable for the production of plant material of the same type as the mother (genetically uniform) by maintaining the genetic stability of the *in vitro* regenerated plantlets [24][25]. Somaclonal variation is genotype-dependent, where some genotypes or species are more amenable to these changes than others when they are cultured *in vitro* [87]. There are other reasons for genetic variability, including the period of the *in vitro* culture of the explant [88], composition of the culture medium in particular auxins and cytokinins as plant-growth regulators, type of sugar [87], proliferation rate of tissues, interval between subcultures, natural selection, mutations in *in vitro* cultures [32], ploidy level, and mode of regeneration used. *In vitro* culture of the explant causes stress on the plant cells and makes them undergo a genomic shock or change [25]. Furthermore, mechanical factors, such as damage of explant or its exposure to sterilizing substances, as well as instability in temperature, humidity, and lighting, can promote somaclonal variation [89].

Many molecular studies on somaclonal variation have been published on different crops, such as orchid (*Dendrobium* sp.) [90], olive (*Olea europaea* L.) [91], date palm (*Phoenix dactylifera* L.) [92], cucumber (*Cucumis sativus* L.) [24], pineapple (*Ananas comosus* L.) [93], and flax (*Linum usitatissimum*) [94]. The great potential of uses of somaclonal variations in agriculture may include producing new generations of new agronomic variants with favorable traits and generations of disease resistance varieties. Somaclonal variation in different plant species can be assessed by biochemical, morphological, and molecular-based markers methods [25]. The main problems of somaclonal variations may include (1) generation of unpredictable and uncontrollable variations particularly non-agricultural use. (2), generation of somaclonal variants that is limited only to those plants that have ability to multiply in culture medium and regenerate into whole plants, (3) "*somaclones may be having low growth rate as well as reduced fertility*" [25], and (4) somaclonal variations prevent obtaining true to type plants, which is considered a main issue in micropropagation protocols. To overcome somaclonal variation, we have to avoid long-term *in vitro* cultures [61][62] and overexposure to phytohormones in culture medium as well as regularly reinitiate clones from new explants, plus the number of subcultures should be kept at a minimum and the use of 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin (KIN) in tissue cultures should be rationalized, because they are known to induce variations [95].

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