Virus Elimination in Plants

Subjects: Biotechnology & Applied Microbiology

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Virus elimination from plants is mostly based on the in vitro culture of isolated meristem, and in addition thermotherapy, chemotherapy, electrotherapy, and cryotherapy can also be applied. Treatments can result in low rate of survival, inhibited growth, incomplete development, or abnormal morphology. The rate of destruction depends on the genotypes and physiological condition of plants. There are several ways to decrease the harmful effect of treatments.

virus eradication meristem isolation thermotherapy chemotherapy electrotherapy cryotherapy phytotoxicity

1. Introduction

The phytopathogen viruses can cause very significant economic losses in crop yield and quality [1][2]. In vegetatively propagated plants (tuberous plants, bulbs, fruits, etc.), they can be of particular importance, as they are more likely to be passed on to the offspring with the propagating material [3][4][5].

Viruses are very simple “organisms”, and to this day there is a debate about whether we can consider them to be living things at all [6]. They are very small in size (mostly 5–300 nm), and are constructed of a hereditary material (nucleic acid template molecule(s)) that is in general protected by a protein/lipoprotein envelope. Viruses are obligate parasites [7].

Due to the failure of own specific metabolic processes (protein synthesis, etc.), there is no pesticide to control viruses [8][9]. Cultivation of resistant cultivars may be one solution to the problem [2], but in the case of vegetatively propagated plant species, the planting of virus-free propagating material is the most effective method of control [10].

Virus elimination in general occurs under in vitro conditions based on meristem isolation. Production of planting material of vegetatively propagated crops is worldwide based on the micropropagation of virus- and pathogen-free in vitro shoot cultures [5][11], which can originate from non-adventitious (axillary or apical) or adventitious buds [12][13]. Beside organogenesis the micropropagation can also occur by somatic embryogenesis [11][14].

During the virus elimination process, it is possible to separate the virus-free part (meristematic region of shoot/root tips) from the virus-infected plant parts, since the distribution of viruses in the plant is not uniform [15][16]. Many viruses are not found in the apical dome of meristem [17], and their presence in the meristematic region can be

affected by exogenous (environmental conditions) and endogenous (developmental stage of plants) factors [18]. At least four reasons are known why meristem can be free of some viruses, as follows:

(1) Intensive metabolic processes and the raised auxin concentration accompanying active cell division in meristems inhibit viral replication as well [9].
(2) The RNA silencing involved in the plant defense mechanism against viruses [19], could also play an important role in prevention of accumulation of viruses and viroids in the apex [20][21][22].
(3) The spread of viruses in plants from cell to cell is relatively slow, and their long-distance movement in plant mostly occurs via the vascular system [7] that does not exist in the meristem yet [23][24][25].
(4) Wang et al. [26] supposed a relationship between the presence of viruses and the plasmodesmata development, since they observed a few (non-branched) plasmodesmata in the cell walls of tissues where the virus was not detected, while they occurred frequently in the tissues infected by virus such as the base of first leaf primordium.

Meristem isolation and culture are often combined with other treatments (thermo- and chemotherapy) to increase the effectiveness of virus eradication [27][28]. There are other methods to obtain virus-free plantlets (electrical treatment, cryotherapy, embryogenesis) [29][30][31][32][33][34].

Even though the environmental condition for in vitro culture is highly controlled, the explants and developing plantlets suffer from several stress factors. These factors are injury occurring during meristem isolation, incomplete plant development (i.e., shoot without root), high humidity and high level of gases in vessel, supra-optimal levels of nutrients, sucrose and growth regulators in medium, etc. [35][36]. Virus elimination treatments applied in tissue culture can result in further stress effects including high or very low temperature and toxicity of chemicals leading to changes in physiology and/or morphology of plantlets [36]. Apart from inhibited growth of in vitro shoots, hyperhydricity is one of the most common morpho-physiological anomalies in in vitro cultures as a response to inappropriate environmental factors, i.e., high humidity, inadequate plant growth regulator (PGR) levels, etc. [35].

2. Meristem Excision and Culture

2.1. The Background

Usually the apical meristem of the shoot is very small (about 100 µm in diameter), dome shaped, and consists of about 800–1200 cells. However, the size and the shape are various depending on the species and developmental stage of plant. The shoot apex includes also the other surrounding tissues of the meristems and organs found at the distal end of the shoot [37].

Localization of viruses in the plant varies depending on the virus and plant species infected. For example, the Apple stem grooving virus (ASGV) was detected in the whole shoot tip of apple (*Malus domestica* Borkh.), even in the youngest region [38]. Wang et al. [26] found that the Raspberry bushy dwarf virus (RBDV) in raspberry (*Rubus ideaus* L.) existed in all tissues including leaf primordia (LP) and the base of meristem; virus did not present only in the least differentiated cells of the apical dome (AD). However, the smallest part of meristem, which could be

mechanically isolated, was the 0.1 mm in length with the youngest leaf primordium in the case of raspberry (*Rubus idaeus* L., 'Z13') [26].

Excision and culture of meristem in this size (0.1–0.3 mm) are feasible only under sterile conditions, because the wounded area is too large in relation to the volume of explant, and any kind of contamination would be fatal for it [39]. Even under sterile conditions, the loss due to contamination can be very high in overly susceptible species, as there are many small sites in phylotaxis or they may be contaminated with soil [40]. It seems that normal plant development requires an apical shoot tip containing at least 1–2 leaf primordia that can ensure the production of auxins and cytokinins [9].

Even though the ability of meristems to survive was proven to be better under drought [35], freezing [41], or heat [26] stress than other tissues (leaves, cotyledons), the excised meristem suffers from several stresses during isolation and cultivation. The wounding accompanied by excision of meristem resulted in a stress similar to that caused by herbivorous insects, and reactive oxygen species (ROS) are produced in a very short time, which damages the meristems [35][39]. Browning of explants may also be a serious problem in species producing polyphenols [9]. Polyphenol oxidase (PPO) is one of the enzymes responsible for wounding induced browning of explant, which can even lead to death of those [42]. In wounded cells, PPO and its substrate (polyphenols) are released from the vacuoles and from plastids/chloroplast, where they are localized. Polyphenols can react with molecular oxygen; this reaction is catalyzed by PPO, and finally dark pigment (melanin) will be produced. Antioxidant enzymes (peroxidases (POX), superoxide dismutase (SOD)) also play role in explant browning [43].

In addition to wounds, chemical stress also affects the plant during the disinfection process using standard chemicals such as sodium hypochlorite, ethanol, and mercuric chloride [3][35]. However, both the extent of wound damage and the contamination can be reduced by use of hypodermic needle for meristem isolation [44][45][46][47]. Mother plant can be pre-treated by fungicide and bactericide chemicals in order to decrease the rate of contamination of shoot tips; thus, a less harmful disinfection procedure can be used [9].

Although shoot initiation could be observed after about 10 days in sweet potato (*Ipomoea batatas* (L.) Lam) [44][47] or 7–21 days in summer squash (*Cucurbita pepo* L., 'Bulum', 'Rumbo') [48], even several months can be taken for obtaining fully-developed healthy plantlets, during which time the in vitro cultures may become contaminated or the medium may dry out [3]. In vitro plant regeneration from isolated meristem/shoot tip takes place through organogenesis, as a response to wounding; in general, new organs or whole plantlets can develop. Even though plant cells have a very strong regeneration capability due to their developmental flexibility [49], the survival and regeneration rate of explants are significantly different, which depends on several factors.

2.2. Effect of the Meristem Size on the Regeneration Ability of Explant

The size of the excised part is crucial: as the size of the excised meristems increased from less than 0.1 to 0.5 mm gradually, the regeneration rates increased from 10–20% up to 44–50% in cardamom (*Amomum subulatum* Roxb.) [50] (Table 1). Similarly, survival rates of carnation (*Dianthus caryophyllus* L.) shoot meristems increased from 20%

to 80% with an increase of explant size from 0.1 to 0.4 mm [51]. Wang et al. [26] also detected positive correlation between the shoot tip length of raspberry (*Rubus idaeus* L., 'Z13') and its survival rate and regeneration capacity. The explant excised in 0.1 mm survived in 25%, and 40.0% of them regenerated to shoots, while 40% of shoot tips in 0.2 mm length survived with 65% regeneration rate. Significantly more shoots (95%) survived in the case of the 0.3 mm shoot tip, and each of them was able to regenerate the shoot. Similarly, sweet potato (*Ipomoea batatas* (L.) Lam.) meristems in the length of longer than 0.3 mm were only able to survive [52].

Meristems between 1 and 2 mm in size showed at least 79.2% regeneration rate with 100% virus elimination rate in the case of both Sugarcane mosaic virus (SCMV) and Sugarcane yellow leaf virus (ScYLV) in sugarcane (*Saccharum* spp. L., 'NCo376') virus elimination experiments. Plantlets developed from the larger shoot apex remained all infected by viruses, while smaller explants responded with shoot development only at a 46.4–53.9% rate [53]. Meristems 0.3–0.6 mm in size were proven to be the most sui0le for the establishment of in vitro culture in apple (*Malus domestica* Borkh.), although some shoots initiated from explants sized 0.5–0.6 mm remained infected by Apple chlorotic leaf spot virus (ACLSV). A high rate of loss (44.4%) could be observed in the case of meristems smaller than 0.3 mm, due to their dehydration, while the culture of meristem explants excised in larger than 0.6 mm size failed in virus removal. Moreover, the presence of phenolic browning was also more frequent in meristem explants > 0.5 mm [40]. The size of the meristem can also affect the time required for regeneration; in the meristem culture of cardamom (*Amomum subulatum* Roxb., 'Golsahi' and 'Ramsahi'), the shoot initiation occurred within 14–18 weeks on explants larger than 0.3 mm, while regeneration on smaller meristems could be observed after 18–24 weeks [50].

The size of isolated meristem significantly affected the shoot length in potato (*Solanum tuberosum* L.): meristems were excised in 100, 200, and 300 µm, and the length of shoots in the average of two cultivars were 5.2, 7.2, and 9.7 cm, respectively, after 60 days of culture initiation [54]. The optimal size of the meristem depended on the genotype in the case of fig (*Ficus carica* L.) varieties. The highest regeneration rates (96%) were achieved by isolation of meristem in 0.5 mm length in the case of Capri fig 'Assafri'. Two of the Tunisian local figs showed the best results when a meristem of 1.0 mm length was used for culture in the case of 'Zidi' and 'Bither Abiadh', resulting in 79% and 73.3% regeneration rates, respectively. The third Tunisian local fig ('Soltani') showed the highest regeneration rate (95.2%) when meristem of 1.5 mm length was isolated [55]. However, it was observed in each variety that culture initiation by larger shoot apex (1.5 mm) was accompanied by death of the explant base in high rate (up to 76.45%, depending on genotype).

2.3. Effect of the Genotypes, Explant Source, and Age on the Regeneration Ability of Isolated Meristems

The survival of the meristem explants and the success of shoot regeneration was still also affected by many factors, including the genotypes and physiological stage of the mother plant, or other environmental factors such as plant growth regulators (PGR), nutrient supply, light condition, etc., reviewed by Bidabadi and Jain [56]. Interaction between the factors can often be detected; however, one of the most important factors proved to be the PGR content of initiation medium, e.g., in sweet potato (*I. batatas* (L.) Lam) [57] and in grapevine (*Vitis vinifera* L.) [58].

The regenerative capacity of genotypes can be very different; for example, in potato (*S. tuberosum* L.), it varied between 40% and 80% [59]; in summer squash (*Cucurbita pepo* L.) between 69% and 75% [48]; and in fig (*Ficus carica* L.) between 51.7–78.3% [60]. Other factors, however, can also play role in the rate of responsiveness, as was observed when 100 genotypes of garlic (*Allium sativum* L.) were involved in virus elimination experiments based on meristem isolation. Although very high regeneration rates (90–100%) were observed in garlic meristems in length of 1.0 mm (2–3 leaf primordia), when 0.3–0.8 mm sized meristems were isolated, their survival and regeneration rate varied between 1% and 80%, and six genotypes did not regenerate the shoot at all [61].

In another experiment, 10 genotypes of tested 51 garlic accessions showed better results on PGR-free medium, while the others performed better on media containing PGRs (0.1 mg/L β-indolyl-acetic acid (IAA) + 0.1 mg/L kinetin (KIN) or 0.01 mg/L α-naphthalene acetic acid (NAA) + 0.01 mg/L 6-benzyladenine (BA)), and the majority of them preferred the latter medium [61]. Although the same PGRs were used for meristem cultures of fig (*F. carica* L.), the best levels of them (BA and gibberellic acid, GA_3) were different for fig cultivars [60].

Table 1. Results and details of meristem cultures experiments.

Plant Species, Cultivar, Virus	Methods	Survival and/or Regeneration [Reference]
Fig, *Ficus carica* L., 'Bursa Siyahi', 'Alkuden', FMV	Meristems (0.5–0.8 mm) were in D for 1 wk, transfer weekly on MS with various PGR combinations (mg/L): A: 0.1 GA_3 + 0.2 BA + 0.1 IBA; B: 0.1 GA_3 + 0.5 BA + 0.1 IBA, C: 0.2 GA_3 + 0.2 BA + 0.1 IBA, D: 0.2 GA_3 + 0.2 BA + 0.1 IBA, for 8 wks, transfer to MS with various PGR for shoot development: A: 0.1 GA_3 + 1.0 BA + 0.1 IBA; B: 0.1 GA_3 + 2.0 BA + 0.1 IBA, C: 0.2 GA_3 + 1.0 BA + 0.1 IBA, D: 0.2 GA_3 + 2.0 BA + 0.1 IBA. Rooting on MS: 1: 0.1 GA_3 + 0.0 IBA; 2: 0.1 GA_3 + 1.0 IBA, 3: 0.1 GA_3 + 2.0 IBA, 4: 0.0 GA_3 + 0.0 IBA.	Survival rates on A/B/C/D: 'Bursa Siyahi': 73.3%/73.3%/80%/86.7%, 'Alkuden': 73.3%/40%/46.7%/46.7%. Shoot development on A/B/C/D: 'Bursa Siyahi': 44.4%/63.9%/58.9%/70%, 'Alkuden': 63.9%/70%/44.4%/50%. Rooting rate/root number on A/B/C/D: 'Bursa Siyahi': 66.6%/6.3; 44.4%/5.3; 44.4%/4.3; 22.2%/1.6. 'Alkuden': 44%/30; 83.3%/40; 33.4%/0.7; 16.7%/1.3. [60]
Raspberries, *Rubus idaeus* L., 'Z13', RBDV	Meristems of 0.1 mm (1LP), 0.2 mm (2LP), 0.3 mm (2LP) cultured for 3 days on solid MS with 100 mg/L myo-inositol, 30 g/L sucrose, 0.5 mg/L BA, 0.05 mg/L IBA, 3.5 g/L Bacto agar, 1.2 g/L Gelrite, and 2.5 g/L AC for 3 ds, then transfer to the same medium without AC. Culture at 22 ± 2 °C, 16 h L., 45 µE s^{-1} m^{-2}	Survival/regeneration rates: 0.1 mm: 25%/40%; 0.2 mm: 40%/65%; 0.3 mm: 95%/100%. [26]
Grapevine, *Vitis vinifera* L., 'Flame Seedless', GLRaV-1, GFLV	Meristems (0.5 mm, 1.0 mm with 2 LP), on WP without PGR or with 0.5, 1.0 or 1.5 mg/L BA, 0.04 mg/L IBA.	Shoot number per explant: 0.5/1.0 mm explant on different BA (mg/L): BA 0: 0.8/1.0; BA 0.5: 3.7/6.8; BA 1.0: 5.8/12.2; BA 1.5: 5.3/13.1 in GFLV infected plants. BA 0: 0.9/1.0; BA 0.5:

Plant Species, Cultivar, Virus	Methods	Survival and/or Regeneration [Reference]
	Culture for 2 wks at 25 ± 2 °C, 16 h L. Then sub-culture: 4 wks.	3.9/6.2; BA 1.0: 5.8/10.1; BA 1.5: 7.3/12.8 in GLRaV-1 infected plants. Shoot length (cm): 0.5/1.0 mm explant on different BA (mg/L): BA 0: 6.4/8.5; BA 0.5: 8.9/11.6; BA 1.0: 9.3/10.4; BA 1.5: 9.8/10.9 in GFLV infected plants. BA 0: 5.3/8.9; BA 0.5: 7.7/11.5; BA 1.0: 8.2/9.6; BA 1.5: 7.1/8.5 in GLRaV-1 infected plants. [58]
Sugarcane, *Saccharum* spp. L., 'NCo376', SCMV, ScYLV	AP meristems in sizes from 0.5 to 10 mm on the liquid MS with 20 g/L sucrose, 10 g/L agar, 3.5 g/L AC, 1 mg/L methylene blue. PGR treatment: A: 2 mg/L BA, 1 mg/L KIN, 0.5 mg/L NAA; B: 0.5 mg/L BA; C: 2 mg/L BA; D: 0.1 mg/L BA, 0.015 mg/L KIN. Culture in D for 1 wk, then16 h L., 28 °C, after 1 wk sub-culture on medium without AC. Shoot proliferation on liquid MS medium with 0.1 mg/L BA. Sub-cultures: fortnight. Shoots (4 cm) rooted in ½ MS with 5 g/L sucrose, 8 g/L agar, 0.25 g/L casein-hydrolysate, for 2–3 wks.	Regeneration rate of different sized meristems, explants from field/node shoot. ≤1 mm: 46.4%/53.9%; >1 ≤2 mm: 79.2%/100%; >2 ≤10 mm: 69.2%/100%. Regeneration rates/shoot number on different PGR: A: 50%/5.9; B: 55%/4.1; C: 100%/3.8; D: 100%/11.1. [53]
Summer squash, *Cucurbita pepo* L., 'Bulum', 'Rumbo', ZYMV, CMV, AMV, BYMV	Meristem 0.3 mm, from 25–30 ds old shoot onto filter paper bridge on liquid MS with various PGR content: KIN or BA (0.5/1.0/1.5/2.5 mg/L), or 0.5 mg/L NAA with KIN (1.0/1.5/2.5 mg/L), or 0.5 mg/L GA_3 with KIN (1.5/2.0/2.5 mg/L), or GA_3 (0.5–2.0 mg/L). Culture at 25 ± 2 °C, 16 h, 2000–3000 lux, for 28 ds. Then onto MS with 8.0 g/L agar, and combinations of BA, KIN, IBA, IAA.	Regeneration rates: 'Bulum'/'Rumbo': Control: 14.4%/11.3%, best results: 2.0 mg/L KIN + 0.5 mg/L GA_3: 75.6%/69.3%. Shoot length (cm): 'Bulum'/'Rumbo': Control: 3.1/2.97, best results: 2.0 mg/L KIN: 4.7/4.24. Number of roots: 'Bulum'/'Rumbo': Control: 2.9/2.8, best results: 1.0 mg/L BA: 3.4/3.3. Number of shoots (42 ds): 'Bulum'/'Rumbo': Control: 2.6/2.5, best results: BA 2.0 mg/L: 4.8/4.1. [48]
Okra, *Abelmoschus esculentus* L. (Moench.), 'Parbhani Kranti', 'SL-444', OMV, YVMV	Meristems 0.3–0.5 mm on filter paper bridge on liquid MS with combinations of BA: (0.1; 0.5; 1.0; 1.5; 2.0 mg/L) and GA_3 (0.1; 0.5 mg/L) or NAA (0.1; 0.5 mg/L). Culture for 3–4 wks. Then sub-cultured on MS with various PGR (+8 g/L agar). Micropropagation from nodal segments. Rooting on MS with NAA or IBA (in 0.5, 1.0, 2.0 or 3.0 mg/L). Culture conditions: 24 ± 1 °C, 16 h L., 28–34 µmol m^{-2} s^{-1}.	Survival results on PGRs (mg/L): 'Parbhani Kranti'/'SL-444': BA 0.1: 32.7/28.8%; BA 0.5: 7.9/45.8%; BA 1.0: 72.3/67.4%; BA 1.5: 58.2/52.5%; BA 2.0: 40.74/35.9%. BA 0.5 + GA_3 0.1: 49.4/42.6%; BA 1.0 + GA_3 0.1: 53.5/47.3%; BA 1.0 + GA_3 0.5: 60.3/58.8%; BA 1.5 + GA_3 0.5: 55.6/50.5%; BA 0.5 + NAA 0.1: 40.2/38.1%; BA 1.0 + NAA 0.5: 56.5/51.6%; BA 1.5 + NAA 0.5: 50.7/45.9%. Best multiplication rates: on 1.0 mg/L BA + 0.5 mg/L GA_3: 'Parbhani Kranti' 8.9 shoots/explant, 'SL-444': 6.8 shoot/explant. [46]

Plant Species, Cultivar, Virus	Methods	Survival and/or Regeneration [Reference]
Sweet potato, *Ipomoea batatas* (L.) Lam), 'Awassa local', 'Awassa-833', 'Guntute', SPFMV, SPCSV	Meristems on MS with 30 g/L sucrose, 7 g/L agar, and 13 PGR combinations (GA_3, NAA, and BA). Culture at 24 ± 2 °C, 12 h L., 40 µmol m^{-2} s^{-1}. Sub-culture: 4 wks.	The best regeneration rates were: (1): 66.7% on medium with 1 mg/L BA, 0.01 mg/L NAA, and 1 mg/L GA_3 in 'Awassa-833' and in 'Guntute'. (2): 63.33% on medium with 1 mg/L BA, 0.01 mg/L NAA, and 2 mg/L GA_3 in 'Awassa local'. The highest number of shoots per explant: 'Awassa-833': 5.26, 'Awassa local': 5.12 both on medium with 2 mg/L BA. 'Guntute': 2.5 on medium with 3 mg/L BA. [57]
Carnation, *Dianthus caryophyllus* L., CLV, CarVMV	Meristems in sizes of 0.1; 0.2; 0.3; and 0.4 mm with 1–2 LP, cultured on MS with 0.1 mg/L NAA, 2.0 mg/L KIN, grown at 25℃, 16 h L. Shoot clump proliferation on MS with 30 g/L sucrose, 8 g/L agar, 0.2 mg/L BA. Multiplication on MS with 1.0 mg/L BA, 0.5 mg/L KIN, sub-culture: for 3wks. Rooting: MS with 1.5 mg/L NAA.	Survival rates of meristem in size of 0.1/0.2/0.3/0.4 mm with 1–2 LP: 20%/35%/65%/80%. [51]
Potato, *Solanum tuberosum* L., 'Burren', 'Binella', PVY	AP meristems (100, 200, 300 µm) cultured on MS, with 2 mg/L glycine, 5 mg/L nicotinic acid, 5 mg/L pyridoxine, 5 mg/L thiamine, 5 mg/L ascorbic acid, 200 mg/L myo-inositole, 2.0 mg/L GA_3, 0.2 mg/L KIN, 3% sucrose, 0.6% agar. Culture: 25 ± 2 °C, 16 h, 2.5 µmol m^{-2} s^{-1}.	Survival rates of 100/200/300 µm meristems: 'Burren': 88%/100%/100; 'Binella': 86%/94%/100%. Shoot length (cm) after 60 ds: 'Burren': 5.4/7.7/9.9; 'Binella': 4.9/6.6/9.6. [54]
Sweet potato, *Ipomoea batatas* (L.) Lam., 'Bellela', 'Temesgen', 'LO-3233', 'Zapallo', SPCSV, SPFMV, SPMMV, SPCFV, SPCaLV, SPMSV, SwPLV, SPVG, CMV	Meristems 0.5–0.7 mm on MS with 30 g/L sucrose, BA (0.1; 0.5; 1.0; 2.0; 5.0 mg/L) combined with 0 or 0.01 mg/L NAA, and 0 or 1.0 mg/L GA_3. Culture at 25 ± 2 °C, 16 h L., 51 µmol m^{-2} s^{-1}. Sub-culture on same medium 4 wks. Multiplication: MS with PGR combinations: KIN, BA, IAA. Rooting: ½ MS with 0, 1, 2, 3, 4, or 5 mg/L IBA.	Regeneration rates of 'Bellela'/'Temesgen'/'LO-3233'/'Zapallo' on medium without PGR: 5.4/17.1/13.0/21.6%, on medium with 0.01 mg/L NAA + 1.0 mg/L GA_3 + 0.1 mg/L BA: 6.7/30/20/30%; or +0.5 mg/L BA: 63.3/53.3/40/16.7%, or +1.0 mg/L BA: 63.3/70/60/70%; or +2.0 mg/L BA: 73.3/93.3/90/80%; or +5.0 mg/L BA: 100/100/76.6/70%. The best shoot proliferation on MS + 0.5 mg/L BA + 0.5 mg/L KIN. The best rooting was on PGR-free medium. [44]
Sweetpotato, *Ipomoea batatas* (L.) Lam., 'BARI-11', 'BARI-22', 'BARI-33', 'BARI-44', 'BARI-55',	AP meristems (0.3–0.5 mm, 1–2 LP) on filter paper bridge, on liquid MS with combinations of KIN and GA_3. Culture at 25 °C, 16 h L., 50–60 µmol m^{-2} s^{-1} for 4 wks. Sub-culture on semisolid medium for 4–6 wks.	Regeneration rates in a range of 7 genotypes: KIN 1.0 mg/L: 37.5–50%; KIN 2.0 mg/L: 45.8–66.7%, KIN 2.5 mg/L: 54.7–70.8%; KIN 3.0 mg/L: 41.7–58.3%; GA_3 1.0 mg/L: 33.3–45.8%; GA_3 1.5 mg/L: 41.7–54.2%; GA_3 2.0 mg/L: 45.8–62.5%; GA_3 3.0 mg/L: 37.5–50%;

Plant Species, Cultivar, Virus	Methods	Survival and/or Regeneration [Reference]
'BARI-66', 'BARI-77', SPFMV, SPMMV		KIN 2.0 + GA_3 0.1 mg/L: 54.2–66.7%; KIN 2.0 + GA_3 0.5 mg/L: 62.5–79.2%; KIN 2.5 + GA_3 0.1 mg/L: 50–62.5%; KIN 2.5 + GA_3 0.5 mg/L: 54.2–75%. [47]
Fig, *Ficus carica* L., 'Zidi', 'Soltani', 'Bither Abiadh', 'Assafri', FMD	ST (0.5, 1.0, and 1.5 mm) on MS with 30 g/L sucrose, 7 g/L agar, 90 mg/L PG. PGRs: (M1): 0.2 mg/L BA, 0.1 mg/L NAA, 0.1 mg/L KIN; (M2): 0.2 mg/L BA, 0.1 mg/L NAA, 0.1 mg/L IPA; (M3): 0.2 mg/L BA, 0.1 mg/L NAA, 0.1 mg/L GA_3, (M4): 0.2 mg/L BA 0.1 mg/L 2,4-D. Culture at 25 ± 1 °C, 16 h L., 40 µmol m^{-2} s^{-1}	Regeneration rates of different sized meristems: 0.5/1.0/1.5 mm: 'Zidi': 61.1%/79%/70.5%; 'Bither Abiadh': 67.8%/73.3%/56.7%; 'Soltani': 90%/55.7%/95.2%; 'Assafri': 96%/92.6%/87.96%. [55]
Large Cardamom, *Amomum subulatum* Roxb., 'Golsahi', 'Ramsahi', CBDV, LCCV	Meristems 0.2–0.7 mm on MS with 30 g/L sucrose and various PGRs: BA, 0.5–1.0 mg/L, GA_3, 0.1 mg/L, IBA or NAA 0.01–0.1 mg/L, or IAA, 0.12–0.15 mg/L, PVP, 0.5 g/L or AA 100 mg/L, 7 g/L agar, for 6 wks. Then transfer to same MS. Sub-culture: MS with PGRs: BA (0.5–1.0 mg/L), IBA (0.01–0.1 mg/L), and GA_3 (0.1–0.5). [62][57]	Survival rates of meristems: 0.2–0.3 mm: 20.7%; 0.3–0.4 mm: 25.7%; 0.4–0.5 mm: 32.1%; 0.5–0.6 mm: 32.9%; 0.6–0.7 mm: 36.4%. Survival rates on medium with different PGR content: (1): 1.0 mg/L BA + 0.05 mg/L IBA + 0.1 mg/L GA_3: 56.6%; (2):0.5 mg/L BA + 0.08 mg/L IBA + 0.1 mg/L GA_3: 37.5%; (3): 0.5 mg/L BA + 0.58 mg/L NAA + 0.1 mg/L GA_3: 9.5%. [63][45][50]
Potato, *Solanum tuberosum* L., 8 cultivarsPVY, PVM, PVS, PVX	Meristems on liquid MS with 20 g/L sucrose, 1 g/L casein, 0.1 mg/L IBA, 1 mg/L GA_3, and 40 mg/L adenine hemisulphate. Culture at 20 ± 2 °C, 16 h L., 50 µmol $s^{-1}m^{-2}$. Sub-culture: 3 wks (2×). Then transfer to MS with 30 g/L sucrose, 1 g/L casein, 0.5 mg/L IBA, 9 g/L Bacto agar. [64]	Regeneration rates: 'Truls': 70%, 'Kerrs Pink blatt skall': 60%, 'Gammelraude': 60%, 'Abundance': 50%, 'Gjernespotet': 40%, 'Hroar Dege': 75%, 'Iverpotet/Smaragd': 80%; 'Sverre': 75%. [59]

323' and 'Zapallo') or to 5.0 mg/L ('Belella' and 'Temesgen'). Although 100% regeneration rate were observed in 'Belella' and 'Temesgen' cultured on medium with 5.0 mg/L BA, the shoots were dwarf and highly multiplied. In the case of 'Lo-323' and 'Zapallo' genotypes, application of 2.0 mg/L BA resulted in the best shoot initiation rates (90% and 80%, respectively) and shoots were of good quality [44]. Lower level of BA (1.0 mg/L) was found to be optimum for 'Awassa-83', 'Guntute', and 'Awassa local' sweet potato (*I. batatas* (L.) Lam.) cultivars, which resulted in shoot induction at more than 60.0% of the rate of isolated meristems [57]. High regeneration rate (83%) could be achieved by using 1.0 mg/L BA and 0.5 mg/L IAA for culture of gentian (*G. kurroo* Royle) meristems [45]. The same level of BA applied alone as PGR resulted in the best survival rates (72% and 67%) in meristem cultures of both okra (*A. esculentus* L. (Moench.)) genotypes 'Parbhani Kranti' and 'SL-44' [46]. In meristem cultures of grapevine (*Vitis vinifera* L.) with increase of both cytokinins (BA and KIN) level (from 0.0 to 1.0 mg/L), the amount of formed callus increased, especially in the case of BA [65]. Combination of 0.1 mg/L BA with 0.015 mg/L KIN was the best in the shoot induction medium also for sugarcane (*Saccharum* spp. L., 'NCo376') meristems, resulting in a 100% regeneration rate and high shoot number per meristem (13.7), while those meristems cultured on medium without

KIN (0.5 and 2.0 mg/L BA) yielded less than five shoots per meristem, although their regeneration rates were different: 55% and 100%, respectively. However, the lowest regeneration response (50%) and quite a few shoots (six per explant) were detected on meristems grown on medium with a combination of 2.0 mg/L BA and 1.0 mg/L KIN, supplemented with 0.5 mg/L NAA [52]. Sweet potatoes (*I. batatas* (L.) Lam) tended to respond to unfavorable cytokinin content of the medium with undesirable callus formation: use of thidiazuron (TDZ) or BA led to callus formation and failed in shoot induction [52]. When cultivars responded to BA with abundant callus development, the KIN also could be used efficiently for meristem cultures in several sweet potato (*I. batatas* (L.) Lam) genotypes; when 2.0 mg/L KIN was added to medium with 0.5 mg/L GA_3, the survival rates of explants varied between 62.5% and 79.2% depending on genotypes [47]. The best shoot initiation responses (75% survival rate of isolated meristems with high vigor) were obtained by application of liquid Murashige–Skoog medium (MS) [62] with 2.0 mg/L KIN and 0.5 mg/L GA_3 without any kind of callus development [52]. The same PGR combination (2.0 mg/L KIN and 0.5 mg/L GA_3) was the most effective for summer squash (*Cucurbita pepo* L.) regeneration (75.5% and 69.27% regeneration rates) from meristem in the case of 'Bulum' and 'Rumbo', respectively [48]. Addition of 0.5 mg/L GA_3 to meristem culture medium containing 2.0 mg/L KIN increased the survival rate by about 14% in average of seven sweet potato (*I. batatas* (L.) Lam.) genotypes, compared those cultured on the medium without GA_3 [47]. GA_3 level had to be increased from 1.0 to 2.0 mg/L to achieve the best regeneration rate (63.3%) in 'Awassa local' sweet potato (*I. batatas* (L.) Lam.) cultivar [57]. Even a much higher level GA_3 (up to 20 mg/L) also enhanced the shoot regeneration in the case of 'Brondal' sweet potato (*I. batatas* (L.) Lam.) cultivar; regardless, there were no significant differences between shoot regeneration capability of meristems cultured on media with 5.0, 10.0, and 20.0 mg/L GA_3 content. However, the most shoots were obtained on the medium containing 10.0 mg/L GA_3 due to multiplied shoots developed on meristems [64].

Application of more than 0.05 mg/L of NAA in the medium of sweet potato (*I. batatas* (L.) Lam) meristem culture led to formation of abundant calli without the ability of shoot regeneration [57]. Addition of 0.1 mg/L isopentyl adenosine (IPA) to the culture initiation medium containing 0.2 mg/L BA and 0.1 mg/L NAA also resulted in significantly higher rate of fig (*Ficus carica* L.) explants (58.3–81.3%), showing callus development on three of the four varieties tested, compared to the medium without IPA (20.4–62.2%). However, no callus development was observed in 'Soltani' cultivar on medium with IPA, this variety was also characterized by poor callus development (0–8.75%) on other media [55]. Excised sweet potato (*I. batatas* (L.) Lam.) meristems preferred the culture on the liquid medium instead of the semi-solid medium, where the majority of explants were not responsive enough, maybe due to the higher accessibility of the nutrients and water in the liquid medium compared to the semi-solid medium [52]. Application of the liquid medium was also preferred for species that suffered from polyphenolic browning, because the toxic molecules (quinone compounds) are less able to accumulate around the explants [40]. Inhibition of production of phenolic molecules by addition of 2-aminoindane-2-phosphonic acid (AIP) into the medium can reduce the rate of browning as was reported in *Artemisia annua* L., *Ulmus americana* L., and *Acer saccharinum* L. in vitro cultures [66]. Addition of antioxidants to the medium or as pre-treatment for mother plants, such as phloroglucinol (PG), ascorbic acid (AA), citric acid (CA), etc., can prevent the browning of meristem cultures [55][67][68]. Adsorption of toxic materials by application of activated charcoal (AC) in the medium also frequently used solution in tissue

cultures [69]. Application of 2.0 g/L AC in culture initiation medium can also enhance the regeneration ability of meristems isolated from several grapevine (*V. vinifera* L.) cultivars [70].

Shoot length of sweet potato (*I. batatas* L. Lam) cultivars varied depending on the genotypes and BA level added to the shoot initiation medium. After three months of culture period, the longest shoots (8.8 cm) developed on 'LO-323' explants on medium supplemented with 0.5 mg/L BA, while similar growth was observed in 'Belella' (8.2 cm) on the medium with 1.0 mg/L BA. The shortest shoots developed on 'Zapallo' explants, where the best results (2.9 cm) were obtained by application of 1.0 mg/L BA [44]. Although significantly more shoots developed on the meristems of grapevine (*V. vinifera* L.) cultured on media supplemented with BA (0.2–1.0 mg/L) compared to those induced by media with the same levels of KIN, the shoot elongation was greatly inhibited [65]. In the case of different sized grapevine (*V. vinifera* L., 'Flame Seedless') meristems explants (0.5 and 1.0 mm), the number of shoots were almost the same (0.9–1.0 shoots per explant) when meristems were cultured on cytokinin-free medium. However, when they were grown on media containing BA (0.5–1.5 mg/L), significantly more shoots developed on the larger explant. Increasing the BA concentration from 0.5 to 1.0 mg/L in media also significantly increased the number of shoots, while a further increase to 1.5 mg/L no longer resulted in a further significant change. In any case, the higher number of shoots was accompanied by a decrease in the length of the shoots [58]. In the case of 'Brondal' sweet potato (*I. batatas* L. Lam) cultivar, the significantly longest shoots (up to 20 mm) developed on medium supplemented with 10.0 mg/L GA_3, compared to those shoots (<14 mm) grown on media with 0.0 and 5.0 mg/L GA_3, each medium contained also 1.0 mg/L BA, while the length of shoots regenerated on the medium with 20.0 mg/L GA_3 did not differ from either [64]. The PGR content in the medium can be supposed to be the most determining factor affecting the rate of shoot growth from the isolated meristem.

2.5. Effect of the Season and In Vitro Culture Condition on the Regeneration Ability of Explants

April and May were the best seasons for the establishment of grapevine (*Vitis vinifera* L., 'Flame seedless') meristem culture, because the phenolic content of plant was low [58]; the beginning of summer is still appropriate for the establishment of grapevine tissue culture, during the period of rapid shoot growth [71]. Similarly, in the case of temperate trees, the spring months are best suited for starting in vitro culture when they are in the active growth phase [9][43]. After initiation cultures of plants producing polyphenols, they should be stored under dark and cool conditions for a short period, in order to reduce the activity of enzymes [9][43][60][65]. Frequent transfer to fresh medium is also required for these species [60][72]. Photoperiod also can affect the success of shoot regeneration. Murashige [73] reported that *Calanchoe* sp. regenerated better under short day illumination, which is required for its flowering, while walnuts (*Juglans* sp.) preferred a long day photoperiod for shoot proliferation.

2.6. Solutions for Improvement of Survival and Regeneration Ability of Explants after Meristem Isolation

Pre-treatment of the mother (donor) plant by chemicals to reduce contamination, and timing of the shoot tip/meristem collection and excision can improve the survival of explants. Technically, the use of sharp and thin

tools for meristem isolation (hypodermic needle) can reduce the rate of injury. Decisions about the size of the meristem to be excised should be made considering the virus type (its localization) and plant species and cultivar; moreover, the positive correlation between the survival rate and the size of meristem should be taken into account. Application of an adequate medium for in vitro culture initiation from meristems is also necessary to enhance the responsiveness of explants and their regeneration capacity; liquid medium may be preferred, proper balance of PGRs is necessary, and the type and level of cytokinins and auxins are especially crucial. Additives applied in the initiation media such as antioxidants (AA, CA, polyvinylpyrrolidone (PVP)), or absorbents (AC) play an important role in prevention losses due to phenolic browning. Growing conditions are also to be fitted to the requirements of genotypes, and if needed, a dark and cool environment should be ensured.

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