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REVIEW

In vitro tissue culture of apple and other *Malus* species: recent advances and applications

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Abstract

Main conclusion **Studies on the tissue culture of apple have allowed for molecular, biotechnological and applied breeding research to advance. In the past 8 years, over 100 papers advancing basic biology, genetic transformation and cryobiology have emerged.**

Apple (*Malus* × *domestica* Borkh.; *Rosaceae*) is an important fruit crop grown mainly in temperate regions of the world. In vitro tissue culture is a biotechnological technique that has been used to genetically improve cultivars (scions) and rootstocks. This updated review presents a synthesis of findings related to the tissue culture of apple and other *Malus* spp. between 2010 and 2018. Increasingly complex molecular studies that are examining the apple genome, for example, in a bid to identify the cause of epigenetic mutations and the role of transposable elements in this process would benefit from genetically stable source material, which can be produced in vitro. Several notable or curious in vitro culture methods have been reported to improve shoot regeneration and induce the production of tetraploids in apple cultivars and rootstocks. Existing studies have revealed the molecular mechanism underlying the inhibition of adventitious roots by cytokinin. The use of the plant growth correction factor allows hypothetical shoot production from leaf-derived thin cell layers relative to conventional leaf explants to be determined. This updated review will allow novices and established researchers to advance apple and *Malus* biotechnology and breeding programs.

Keywords Epigenetics · Explants · Plant growth regulator · Phloroglucinol · Thin cell layer

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In vitro tissue culture for apple breeding and biotechnology

The tissue culture of domesticated apple (*Malus* × *domestica* Borkh.) has a rich and extensive history spanning approximately 60 years (Dobránszki and Teixeira da Silva 2010). Since the apple genome is highly heterozygous, a consistent genetic background in a given cultivar can be maintained only by vegetative propagation, i.e., cloning. This would be important in the production of genetically uniform scions and rootstocks for commercial apple production. In nurseries, apple plants are produced by grafting scions onto rootstocks. The rootstock determines some important traits of grafted trees, including growth vigour, yield and resistance or tolerance to biotic and abiotic stresses (Rom and Carlson 1987; St. Laurent et al. 2010). The Cornell-Geneva (Geneva® series) breeding program has bred several dwarf rootstocks that are resistant to diseases and pests and are also

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cold hardy.[1] Several of these rootstocks have been extensively researched in recent years (Tables 1, 2).

Epigenetic mutations can arise from the graft, resulting in altered fruit peel color (El-Sharkawy et al. 2015). In addition, bagging of fruits of a triploid cultivar 'Mutsu' was reported to induce epigenetic changes, as indicated by increases or decreases in DNA methylation and modifies levels of acetylation and trimethylation of histone proteins (Bai et al. 2016). One possible cause may be that a high proportion of the genome (57%) has transposable elements (Daccord et al. 2017). Epigenesis may lie at the heart of somaclonal variations that can arise spontaneously in vitro as a result of long-term culture or acclimatization (Gulyás et al. 2019 (unpublished)). This is an important issue as Webster and Jones (1989) discovered that increased cyclic shoot multiplication over a 21-month period induced rejuvenation of the in vitro cultured shoots of apple rootstock M9. In vitro culture may induce genetic variations, and thus an assessment of genetic stability is required for in vitro-based methods such as cryopreservation of plant germplasm, including apple (Wang et al. 2018a). Tissue culture thus continues to play an essential part of apple molecular breeding (Peil et al. 2011) and biotechnology.

In vitro propagation of apple

In vitro propagation of apple generally includes four stages, as in other plant species: (1) establishment of in vitro cultures from in vivo plants; (2) shoot regeneration and/or multiplication; (3) rooting of microshoots; (4) acclimatization of in vitro plantlets and the establishments of plant in an in vivo environment. The most frequently used explants in Stage 1 in apple in vitro propagation are shoot tips or axillary buds when propagated by axillary or terminal buds, or leaves when propagation is based on adventitious shoots (Fig. 1). Early studies were often hampered by tissue browning caused by oxidation of polyphenolics due to wounding during in vitro culture establishment (Dobránszki and Teixeira da Silva 2010). Reducing tissue browning is possible by timing the collection of explants, adding antioxidants to the culture medium such as ascorbic acid, activated charcoal (AC), or polyvinylpyrrolidine (PVP), singly or in combination, or the use of liquid culture or micrografting (Yepes and Aldwinckle 1994; Modgil et al. 1999; Dobránszki et al. 2000; Kaushal et al. 2005). Mert and Soylu (2010) found that timing of in vitro culture initiation and the use of axillary or terminal shoot tips affected medium browning during shoot regeneration, with a broad range of 10–84% browning across three rootstock cultivars, M9, MM106 and MM111. In Stage 2, the multiplication of shoots can be achieved by promoting the growth of axillary shoots or induction of adventitious shoots (Dobránszki and Teixeira da Silva 2010; Magyar-Tábori et al. 2010).

Availability of a highly efficient plant regeneration protocol is a prerequisite for studies of apple genetic transformation (Bhatti and Jha 2010). A couple of factors have been known to affect the establishments of such protocols, including genotype, explant type, type and concentration of plant growth regulators (PGRs), medium composition and culture conditions (Dobránszki and Teixeira da Silva 2010; Magyar-Tábori et al. 2010).

The objective of this review was to assess new advances in the literature related to the tissue culture of apple and *Malus* spp. that emerged between 2010 and 2018.

Explant infection and disinfection, and wounding-induced browning

There are a wide range of factors that need to be considered when disinfecting field- or ex vitro-derived plant material to be used as explants for in vitro culture, but some of the most important factors are genotype, explant type, disinfection procedure, age and physiological state of the mother plant and physical and chemical conditions applied in vitro (Teixeira da Silva et al. 2015, 2016a, b; Table 1). Paprštein and Sedlák (2015) reported 9% contaminated cultures for 'James Grieve Compact', 23% for 'Jarka', and 25% for 'Mivibe' and 2, 8 and 20% for 'Rubimeg', 'Bohemia' and 'Primadela' (Sedlák and Paprštein 2016), while Soni et al. (2011) noted that 8% of explants collected in summer were contaminated while explants collected in spring, autumn and winter showed 12, 32 and 54% contamination, respectively. These recent cases emphasize the importance of explant disinfection and contamination, even though many disinfection protocols are already available. Romadanova et al. (2016b) used 523 detection medium (Viss et al. 1991) to detect fungal and bacterial contamination in the in vitro culture of apple cultivars, clonal rootstocks and wild *M. sieversii* (Ledeb. M. Roem.), finding 22% contamination in laboratory-grown shoots across all cultivars versus 56% in field-grown shoots. In their study, as would be expected, levels of contamination were inversely related to in vitro shoot development or viability, while infection levels decreased as the exposure time or concentration of $HgCl_2$ increased.

Malus plants contain high levels of polyphenols (Volz and McGhie 2011). Wounding often causes apple explant browning, eventually leading to death of the in vitro tissue when established in vitro. Several methods are available to efficiently reduce browning (Dobránszki and Teixeira da

[1] https://www.ars.usda.gov/ARSUserFiles/80600500/Gennaro/GeneraldescriptionofTHEGENEVABreedingprogram.pdf; http://www.ctl.cornell.edu/plants/GENEVA-Apple-Rootstocks-Comparison-Chart.pdf.

Table 1 Disinfection procedures of tissues for in vitro culture of *Malus* species (2010-2018; alphabetical listing)*

Disinfection protocol	References
Nodal segments from 5-y-old tree → RTW → 70% EtOH 1 min → 1% $Ca(OCl)_2$ 10 min → SDW 4–5 × (5 min each)	Boudabous et al. (2010)
Shoot tips (1–2 cm) → 2.5% $CuSO_4$ 20 min → 70% EtOH 30 s → 20% bleach + Tween-20 1–2 drops 15 min → SDW 3×	Mert and Soylu (2010)
Apical buds from greenhouse → 70% EtOH 30 s → SDW 3 × → 0.1% $HgCl_2$ + 2–3 drops of Tween-20 6 min → SDW 5×	Amiri and Elahinia (2011a)
"Actively growing apical domes" → RTW 1 h → 10% NaOCl 30 min → rinses NR	Kepenek and Karoğlu (2011)
Terminal and axillary buds → RTW 1 h → 70% EtOH time NR → 0.1% $HgCl_2$ 3–4 min → 2% NaOCl 20–25 min → SDW number and time NR	Soni et al. (2011)
Use of Alayón-Luaces et al. (2008) protocol: receptacle tissue → 70% EtOH 1 min → 1.8% NaOCl + 2 drops Tween-20 30 min → SDW 4×	Alayón-Luaces et al. (2012)
Shoot tips from 10 cm scionwood cuttings → RTW time NR → 70% EtOH 1 min → 5% NaOCl + 0.1% Tween-20 + 150 mg/l AA 10 min → SDW + 150 mg/l AA 4×	Kereša et al. (2012)
Bud (0.8–1.5 cm) rinsed in water with detergent 10 min → 4% NaOCl 20 min → SDW 5×	Mitić et al. (2012); Stanišić et al. (2018)
Seeds → 70% EtOH 1 min → 0.1% $HgCl_2$ 10 min → SDW 3×	Dai et al. (2013, 2014)
Shoot tips (5–10 mm) → Tween-80 time NR → DW 3–4 × → meristems excised from shoot tips → 0.01% $HgCl_2$ 1 min → SDW 4–5×	Mir et al. (2013)
Single node cuttings → soapy RTW → fungicides (2 g/l each of Benomile + Ridomile) 20 min → 70% EtOH 1 min → SDW 3 × → 4% NaOCl 10–18 min → SDW 3 × → 0.1% $HgCl_2$ 2 min → SDW 3×	Ghanbari (2014)
Axillary buds → RTW 3 h → 0.1% $HgCl_2$ 6 min → SDW 3×	Jin et al. (2014)
Shoots (1–2 cm; age NR) → RTW time NR → 30% Tween-20 5 min → 50% bavistin + 0.5% streptomycin 10 min → 70% EtOH < 5 s → 0.2% $HgCl_2$ 4 min → DIW 3–4×	Mehta et al. (2014)
Shoot tips → 70% EtOH 1 min → 0.01% $HgCl_2$ 8 min → SDW 5×	Sun et al. (2014)
Axillary buds (0.2–0.6 cm) → tap water + 0.05% Tween-20 10 min → DW 3 × → 75% EtOH 30 s → SDW 3 × → 0.1% $HgCl_2$ 8 min → SDW 4×	Zhang et al. (2014)
Shoot tips → egg yolks + 20% NaOCl + 2 drops of Tween-20 20 min → SDW 3×	Castillo et al. (2015)
Partly defoliated shoot tips → 0.15% $HgCl_2$ + 0.05% Tween-20 1 min → SDW	Paprštein and Sedlák (2015)
Nodal segments → 70% EtOH 30 s → 0.6% NaOCl + few drops of Tween-20 20 min → SDW 3–5×	Geng et al. (2016)
Method 1: Cuttings (20–30 cm long) from 1 year-old laboratory-grown shoots → soapy water → bleach a few min → RW → forced (to stimulate dormant buds) in ½ MS + 1 mg/l GA_3 + 1 mg/l AA (pH 5.6) 2–4 w → shoots (1–2 cm) → 0.1% $HgCl_2$ 3, 5 or 7 min → bleach "Belizna" 2 min → DW; Method 2: shoot tips (2–3 cm) → soapy water → RW → 0.1% $HgCl_2$ 5, 7 or 10 min → rinses NR	Romadanova et al. (2016a)
Dormant cuttings → RTW 20 min → 8% (w/v) Capluq-50 fungicide solution time NR → drying and wrapping in plastic film and storage at 4 °C for 1 month → sprouting of cuttings in a phytotron (16-PP, 25/18 °C) → cutting new shoots → 10% NaOCl + 1 drop of Tween-20 15 min → SDW 3 ×	Lizárraga et al. (2017)
Axillary buds, terminal buds, shoot tips (spring and winter) → 1–2% NaOCl time NR → rinses NR	Modgil and Thakur (2017)
Cotyledons, stems, leaves → 2.6–5.3% NaOCl + Tween-20 5–10 min → DW 3 × (5 min each wash)	Rumiyati et al. (2017)
Mature fruits → surface wash with EtOH then flamed in laminar flow cabinet → cut open with sterile blade	Verardo et al. (2017)
Buds → 75% EtOH 30 s → SDW1 × → 10% bleach 5 min → SDW several ×	Vogiatzi et al. (2018)

AA ascorbic acid; *$Ca(OCl)_2$* calcium hypochlorite; *$CuSO_4$* copper sulphate; *DIW* deionized water; *DW* distilled water; *EtOH* ethyl alcohol (ethanol); *$HgCl_2$* mercuric chloride; *MS* Murashige and Skoog (1962) medium; *N/A* not applicable; *NaOCl* sodium hypochlorite; *NR* not reported; *RTW* running tap water; *RW* running water; *s* second(s); *SDW* sterilized (by autoclaving) distilled water. *No details are indicated for the other studies indicated in Table 2 as starting material was in vitro tissue/plants, for which surface disinfection was either not used, or not necessary. The following studies used in vitro cultures and thus did not require surface sterilization: Feng et al. (2013), Li et al. (2014, 2015, 2016), Wang et al. (2018c)

Table 2 Micropropagation and tissue culture of *Malus* species (2010–2018; alphabetical listing)

Species, cultivars or rootstocks	Explant(s) used, size and source	Culture medium, PGRs and additives*	Culture conditions	Remarks, experimental outcome and maximum productivity, acclimatization and variation	References
Cultivar (Douce de Djerba)	Nodes (1.0 cm) (shoot induction); shoot tips (1–3 cm) (shoot multiplication)	MS + 1 mg/l BA + 0.1 mg/l IBA (SIM). MS + 1–2 mg/l BA (SMM). ½ MS + 3 mg/l IBA + 2 g/l AC (RIM). pH 5.8. 3% sucrose. 0.8% agar	16-h PP. 83.6 μmol m^{-2} s^{-1}. 25–28 °C	85% of nodes formed shoots in SIM (89% in SMM). 67% of shoots rooted. Acclimatization in sand + peat (1:2) with 60% survival	Boudabous et al. (2010)
Rootstocks (M9, MM106, MM111)	Meristem tips (10–20 mm)	MS + 0.5 mg/l BA + 0.1 mg/l IBA + 0.1 mg/l GA_3 (shoot establishment). MS + 1 mg/l BA + 0.5 mg/l GA_3 (SMM). Shoot establishment – IBA (RIM). pH 5.8. 3% sucrose. 0.7% agar	Darkness 3 w → 16-h PP. 3500–4000 lx. 23 ± 2 °C	Generally, terminal shoot tips elongated more than axillary shoot tips. 95% shoot tips formed shoots in M9 (93% for MM106; 81% for MM111). For MM106, 69–74% of shoots from axillary shoot tips rooted (63–90% for shoots from terminal shoot tips), and lower values for M9 and MM106. Acclimatization NR	Mert and Soylu (2010)
Rootstock (EMLA111)	In vitro shoots (size, age NR)	½ MS + 0.3 mg/l IBA or 0.1 mg/l NAA + 2 g/l AC (RIM). 2% sucrose. pH, gelling agent NR	Conditions NR	The use of 100 mg/l PG induced callused roots. Max. 81–82% rooting in IBA (71% in NAA). 100% survival of acclimatized plants in coco-peat	Modgil et al. (2010)
Rootstock (MM106)	In vitro shoots (0.5–0.7 cm)	DKW + 2 mg/l BA + 0.2 mg/l GA_3 + 0.06 mg/l IBA + 150 mg/l PG + 4 g/l carrageenan (SMM). SMM but 0.2 mg/l BA (SEM). DKW + 0.8 mg/l IBA + 5 μg/l spermine and spermidine (RIM). pH 5.3. 3% sucrose. 0.6% agar	16-h PP. 40–50 μmol m^{-2} s^{-1}. 24 ± 2 °C	Purpose to assess effect of PGPR on acclimatized in vitro plants in organic substrate	Vettori et al. (2010)
Rootstocks (M9, M27, MM106)	Nodal explants (size NR)	MS/2 × MS + 2.0 μM BA + 1.5 μM IBA (initial single-node bud culture), subcultured every 30 d. MS + 4.4 μM BA + 2.27 μM TDZ (SIM, SMM). MS + 8.8 μM BA + 1.14 μM TDZ + 2.8 μM GA_3 (SEM). ½ MS + 5.4 μM IBA + 1.2 μM 2,4-D (RIM). 3% sucrose. pH 5.8. 0.7% agar (SIM, SEM, SMM) or 0.65% agar (CIM, RIM)	16-h PP. 50 μmol m^{-2} s^{-1}. 25 ± 2 °C	Max. shoot multiplication ratio (SN/month): in MS, 4.9 (M9), 5.7 (M27), 3.9 (MM106), and in 2 × MS, 5.1 (M9), 5.9 (M27), 3.8 (MM106). Max. rooting in 42, 51 and 64% of M9, M27, and MM106 shoots, respectively. Acclimatization NR	Amiri and Elahinia (2011a, b)
Cultivars (Royal Gala, Freedom)	tTCLs (0.1–0.3 mm thick) from "upper two, fully expanded young leaves of 3-week-old in vitro shoots"	MS + 0.2 mg/l NAA + 0.5/5.0 mg/l TDZ (Royal Gala/Freedom) + 0.25% gelrite (SIM). MS + 0.3 mg/l IBA + 0.2 mg/l GA_3 + 1.0/1.5 mg/l *m*T (Royal Gala/Freedom) + 0.7% agar–agar (SMM). 3% sucrose. pH NR	Darkness 3 w → 16-h PP. 35 (1 w) → 70 (1 w) → 105 μmol m^{-2} s^{-1}. 24.5 °C	AA and CA needed for preparation of tTCLs. Shoot regeneration from 100% of control first and second apical leaves (Royal Gala), but only 39% and 25% for Freedom (values for tTCLs: 92–97% and 71–78% (Royal Gala); 0–35% and 0–11% (Freedom), depending on light/dark condition) after 7 w. Rooting and acclimatization not performed	Dobránszki and Teixeira da Silva (2011)
Cultivar (Galaxy)	In vitro shoots	MS medium + 3% sucrose + 1 ml/l Humus® FW soil manure + 1.0 mg/l BA + 0.3 mg/l IBA + 0.2 mg/l GA_3. Gelling agents: 5.6 g/l agar–agar, 2.5 g/l phytagel, 2.8: 1.25 g/l agar–agar: phytagel, 2.8: 2.8 g/l agar–agar: guar gum, or 1.25: 2.8 g/l phytagel: guar gum. pH 5.8	16-h PP. 105 μmol m^{-2} s^{-1}. 22 °C	Guar gum, when added to the blend, speeded up diffusion. SN was highest (8.9 shoots/explant) and the level of shoot hyperhydricity decreased (from 59% to 12%) when the agar/guar gum blend was used. Using the agar/guar gum blend, production costs could be lowered by 42%	Dobránszki et al. (2011)

Table 2 (continued)

Species, cultivars or rootstocks	Explant(s) used, size and source	Culture medium, PGRs and additives*	Culture conditions	Remarks, experimental outcome and maximum productivity, acclimatization and variation	References
Rootstock (M9), cultivars (Starking Delicious, Amasya)	Shoot tips (2–3 mm)	MS + 0.5 mg/l BA + 0.1 mg/l IBA (SMM). ½ MS + 0.5 mg/l IAA + 1% AC with or without growth retardants (0.5–5 mg/l PP333 or Alar-85) (RIM). pH 5.8. 2% sucrose. 0.4% agar	16-h PP. 3000–3500 lx. 21 ± 2 °C	SN/explant = 2.7 (M9), 3.5–3.7 (Amasya) and 2.5–2.7 (Starking Delicious) with no growth retardants, but surprising increase to 4.5 or 4.3, 5.4 or 6.5, and 3.6 and 4.7, respectively when Alar-8 and PP333 were applied. However, shoots were stunted. Rooting %/shoot, with or without PP333 or Alar-85: 29–36% (M9), 39–49% (Amasya) and 46–59% (Starking Delicious). Plantlets acclimatized in peat and agro perlite (1:2, v/v), but % survival NR	Kepenek and Karoğlu (2011)
Cultivar (Royal Gala)	Leaf segments (~ 5 mm) from in vitro shoots	MS + B_5 vitamins + 3% sucrose + 0.25% gelrite + 0.2 mg/l NAA + 0.5 mg/l TDZ, 5.0 mg/l BAR, or 5.0 mg/l BA, or 6.5 mg/l *m*TR (SIM). Subculture for 7 d before rooting: A: PGR-free medium; B: 0.5 mg/l BAR + 0.2 mg/l GA_3 + 0.3 mg/l IBA; C: 0.5 mg/l BAR + 0.5 mg/l GA_3 + 0.3 mg/l IBA; subculture for 4 w on medium D: 1.0 mg/l BAR + 0.2 mg/l GA_3 + 0.3 mg/l IBA. Rooting in two phases: ½ MS + 2.0 mg/l IBA for 5 d (RIM) and ½ MS + 2.0 ml/l Wuxal® for 2 w (REM). Gelling agent: 0.7% agar–agar	Shoot induction: dark, 24.5 °C for 3 w → 16-h PP. 22 °C for 4 w with increasing light intensity from 35 up to 105 µmol m^{-2} s^{-1}. Subculture in 16-h PP. 22 °C for 4 w at 105 µmol m^{-2} s^{-1}	Rooting of regenerated shoots was not successful directly after regeneration, i.e., a subculture was necessary. Rooting % was affected by both the hormone content of SIM and that of the subculture medium. Highest rooting percentage (76%) was achieved if shoots were regenerated on medium contained BAR and then subcultured for 4 w on medium with 1.0 mg/l BAR + 0.2 mg/l GA_3 + 0.3 mg/l IBA (D)	Magyar-Tábori et al. (2011)
Rootstock (Merton I. 793)	Terminal and axillary buds (0.5–2 cm); 2–3.5 cm shoots for SMM	Liquid MS 24 h → MS + 1 mg/l BA + 0.1 mg/l IBA + 10 g/l PVP for 4–6 w (SEM). MS + 0.5 mg/l BA + 0.01 mg/l IBA (SMM). ½ MS + 0.1 mg/l NAA (RIM). pH NR. 2.5% sucrose. 0.6% agar	16-h PP. 4000 lx. 25 ± 2 °C	87.5% bud break and elongation on SEM. Callus induced when BA (0.5–2 mg/l) was used in combination with 0.1 mg/l NAA or when 0.8 mg/l IAA was used at the rooting stage. 8-fold shoot multiplication on SMM. 67% of shoots rooted. 80–85% survival in coco-peat after drench in 5 g/l biocontrol agent	Soni et al. (2011)
M. baccata	In vitro shoots (1 cm; 3–4 w old)	MS + 2 µM BA + 0.6 µM NAA 4–5-w subcultures (SMM). MS + 12 µM BA + 0.6 µM NAA (CIM). pH 5.8. 3% sucrose. 0.7% agar	16-h PP (darkness for CIM). 40 µmol m^{-2} s^{-1}. 25 ± 2 °C	89% of explants induced callus (100% in wounded explants). 100% of shoots formed adventitious shoots from unwounded explants (78%, 61%, 21% from shoots tips cut into 2, 4 or > 6 fragments) with SN/explant = 1.0, 1.2, 2.3 and 1.6, respectively. Rooting and acclimatization not performed	Wu et al. (2011)
Cultivar (Orin)	In vitro shoots, suspension culture, callus	MS + 0.8 mg/l BA + 0.2 mg/l IAA (SMM). B5 (liquid/solid) + 0.4 mg/l BA + 1.5 mg/l 2,4-D (cell suspension/callus proliferation). pH NR. 3% sucrose. 0.7% agar (SMM, callus proliferation)	14-h PP (darkness for cell suspension). 600 µmol m^{-2} s^{-1}. 25 °C	Expression of the apple *cyMDH* gene (*MdcyMDH*), coding for a key enzyme in malic acid synthesis, was assessed. *MdcyMDH* was expressed in young or rapidly growing tissues such as callus or suspension cells, suggesting its link to plant growth, whereas it was downregulated in response to NaCl, low temperature stress (4 °C) and ABA	Yao et al. (2011)

Table 2 (continued)

Species, cultivars or rootstocks	Explant(s) used, size and source	Culture medium, PGRs and additives*	Culture conditions	Remarks, experimental outcome and maximum productivity, acclimatization and variation	References
Cultivar (Anna)	5 mm^3 of receptacle tissue from 75-d-old immature apple	MS + 1 mg/l 2,4-D + 0.1 mg/l BA for 1 or 10 30-d subcultures → PGR-free MS (CIM). pH 5.8. 3% sucrose. 0.65% agar	Darkness. 27 ± 2 °C	When 5 mg/l of PIC, ABA or GA_3 were added to CIM, and the cell wall fractions were analysed, PIC stimulated the production of neutral sugar and uronic acid relative to control callus grown on PGR-free medium	Alayón-Luaces et al. (2012) (based on Alayón-Luaces et al. (2008))
Rootstock (MM.106)	In vitro shoots (25 mm long)	Use of Bahmani et al. (2009) protocol: MS + 4.43 µM BA + 0.5 µM IBA (SMM). ½ MS + 5 µM IBA (RIM). pH 5.8. 90 mM sorbitol (SMM) or sucrose (RIM). 0.6% agar	16-h PP. 50 µmol m^{-2} s^{-1}. 25 ± 1 °C	The effect of NaCl-induced salt stress was assessed. As expected, shoot growth was significantly inhibited: SN/explant = 8.5 (control), 9.3 (20 mM NaCl), 5 (120 mM NaCl). Shoot length and fresh weight were significantly higher when 20 mM NaCl was used: 1.95 (control) vs 2.50 cm (NaCl), and 208 mg (control) vs 233 mg (NaCl). Rooting was also negatively impacted: 89.3% (control) vs 43.3% (100 mM NaCl). Longer roots formed at 20 mM NaCl (~ 6.9 cm) than in control (~ 5.2 cm)	Bahmani et al. (2012)
Cultivar (Topaz)	Shoot tips (1 cm)	MS + 1.5 mg/l BA + 0.2 mg/l IBA + 0.5 mg/l GA_3 + 150 mg/l AA + 1 g/l AC (shoot establishment). QL macroelements + MS microelements + 1 mg/l BA or [0.5 mg/l BA + 1.5 mg/l Kin] + 0.1 mg/l IBA + 0.5 mg/l GA_3 (SMM). PGR-free SMM + 2 mg/l IBA (RIM). pH 5.8. 3% sucrose. 0.8% agar	16-h PP. 40 µmol m^{-2} s^{-1}. 22 °C	SMM with BA alone or the BA + Kin combination formed 2.5 or 2.4 shoots/shoot. SN/shoot when SMM contained 1 mg/l TDZ was higher (3.6), but fasciation was observed. 69% of shoots rooted	Kereša et al. (2012)
Cultivars (Golden Delicious (GD), Melrose)	Shoot tips with pair of leaves (for culture establishment); leaves (5–8 mm long) from in vitro plants for SIM	MS + 5 µM BA + 0.5 µM NAA (establish shoot cultures for 4 w). MS + 2.2 µM BA + 0.26 µM NAA (SMM). 4-w subcultures → MS + 10 µM TDZ + 2.65 µM NAA (CIM). MS + 22 µM TDZ + 1.5 µM IBA (SIM, SMM; Seong and Song 2008). MS + 2.5 (GD) or 10 (Melrose) µM IBA (RIM). pH 5.8. 3% sucrose. 0.7% agar	16-h PP. 45 µmol m^{-2} s^{-1}. 25 ± 2 °C	In Melrose and GD, 97% of explants induced shoots when leaves were placed adaxial surface down on medium (SN = 11.8/explant for Melrose, 6.47 for GD), forming callus in 100% of explants. 74% of GD shoots rooted (62% for Melrose). Acclimatization in soil + sand (3:1, v/v) with 89% survival for GD (74% for Melrose)	Mitić et al. (2012); Stanišić et al. (2018)
Rootstock (M7)	In vitro cultures grown from axillary buds with expanded leaves; leaves used for CIM and SIM	MS + 1 mg/l BA + 5 mg/l NAA with 3-w subcultures (CIM). MS + 4 mg/l BA + 1 mg/l IAA (SIM). MS + 0.5 mg/l BA + 0.1 mg/l IBA + 0.5 mg/l GA_3 (SMM). ½ MS + 0.3 mg/l IBA (RIM). pH 5.6. 3% sucrose (2% for RIM). 0.7% agar (CIM), 0.7% Difco Bacto agar (SIM; 0.4% for RIM)	16-h PP. 40 µmol m^{-2} s^{-1}. 25 °C	59% of explants induced shoots (SN/explant = 3.66; 8.33 after 6 w). Higher SN/explant (9.33) after 4 w (or 24.33 after 6 w) when 1 mg/l TDZ + 0.5 mg/l NAA were used as SIM, but only 30% of explants formed shoots. When 70% fungal culture filtrate of *Dematophora necatrix* was added to the medium, 5.2% of callus and 40% of shoots survived. Five *D. necatrix*-resistant lines were developed	Modgil et al. (2012)

Table 2 (continued)

Species, cultivars or rootstocks	Explant(s) used, size and source	Culture medium, PGRs and additives*	Culture conditions	Remarks, experimental outcome and maximum productivity, acclimatization and variation	References
Rootstocks (M7, MM111)	Axillary shoots from 2-y-old in vitro cultures	MS + 2.2 μM BA (SMM). 4-w subcultures. pH NR. 3% sucrose. 0.7% agar (M7), 0.4% agargel (MM111)	16-h PP. 30 μmol m^{-2} s^{-1}. 25 °C (day)/20 °C (night)	Five carbon sources were tested, fructose, glucose, mannitol, sorbitol and sucrose, from 0% to 5% (w/v), in 1% increments. No growth was observed on medium without any carbon source. Quality of shoots was optimum at 4% sucrose, resulting in SN of 22.5/shoot for M7 and 4% sorbitol inducing SN of 13.3/shoot for MM111. Rooting and acclimatization not performed	Pathak and Dhawan (2012b)
Rootstock (*M. prunifolia* (Willd.) Borkh cv. Marubakaido)	Nodal segments (1–2 cm) from 30-d old in vitro plantlets	MS + 2.2 μM BA (SMM). 4-w subcultures. pH 5.7. 3% sucrose. 0.6% agar	16-h PP. 40 μmol m^{-2} s^{-1}. 26 ± 1 °C	In Pereira-Netto (2012), when 5 μl stigmasterol was applied to fully opened leaves, SN (main shoots) increased from 2.6 to 3.3/explant while SN of axillary shoots increased from 2.2 to 5.0/explant. Rooting and acclimatization not performed. In Pereira-Netto et al. (2012b), 12.5% of agar was replaced by galactomannan from the seeds of *Schizolobium paraybae*, resulting in a 2.7-fold in multiplication rate, "a 1.9-fold increase in the number of main branches and an 8.6-fold increase in the number of primary lateral branches."	Pereira-Netto (2012); Pereira-Netto et al. (2012b)
Cultivar (Royal Gala), *M. micromalus*	Zygotic embryos → seedling shoot tips → cloned plantlet leaves (2013) or cotyledons (2014) as explants	Sweet cherry medium (Dai et al. (2014) + 2.2 μM BA + 1.45 μM GA_3 (embryo germination). MS + 1.33 μM BA + 1.14 μM IAA + 0.29 μM GA_3 (SMM). MS + 9.10 μM TDZ + 2.69 μM NAA (SIM)	Darkness 20 d → light (14-h PP. 60 μmol m^{-2} s^{-1}. 23 ± 1 °C	In 2013 study, large differences in bud number/explant in seedling-derived clones (GL-1 to − 10), with highest in GL-3 (47.6/explant). Comparison of GL-3 and 'Royal Gala' and 'Hanfu' yielded 22.1, 8.4 and 7.8 buds/explant, respectively. In 2014 paper, shoot regeneration in 75–88% of nodal, proximal, and central parts of cotyledons (38% from distal parts) with SN/explant = 4.1, 7.3, 2.9, and 2.3, respectively	Dai et al. (2013, 2014)
Rootstock (M26)	Nodal explants (0.5–1.0 cm) with 2 axillary buds from in vitro plants	MS + 1.5 mg/l BA + 120 mg/l crab chitosan + 0.3 mg/l GA_3 (SIM). MS + 0.5 mg/l BA + 20 mg/l crab chitosan + 0.1 mg/l GA_3 (SEM). pH 5.7. 3% sucrose. 0.7% agar	16-h PP. 40 μmol m^{-2} s^{-1}. 24 °C	12.1 shoots/explant formed on SIM. Tweaking the concentration of BA, crab chitosan and GA_3 to 1.5, 60 and 0.1, respectively, increased number of leaves per plant (92 vs. 55 in additive- and PGR-free control) and biomass (plant fresh weight: 1.8 g/plant vs 0.7 g/plant in control). Rooting and acclimatization not tested	Dastjerd et al. (2013)
Cultivars (Gala, Fuji, Wangshanhong), rootstocks (M9, GM256), *M. micromalus*, and *M. robusta*	In vitro shoots	MS + 0.25 mg/l BA + 0.01 mg/l IBA (SMM). pH 5.8. 3% sucrose. 0.7% agar	16-h PP. 50 μmol m^{-2} s^{-1}. 24 ± 2 °C	Highest (75%) and lowest rates (36%) of shoot regrowth were obtained in cryopreserved shoot tips of 'Gala' and 'Wangshanhong', averaging 61% shoot regrowth across the 7 genotypes belonging to 4 *Malus* species	Feng et al. (2013)

Table 2 (continued)

Species, cultivars or rootstocks	Explant(s) used, size and source	Culture medium, PGRs and additives*	Culture conditions	Remarks, experimental outcome and maximum productivity, acclimatization and variation	References
Cultivars (Royal Gala, Freedom)	As for Dobránszki and Teixeira da Silva (2011)	As for Dobránszki and Teixeira da Silva (2011)	As for Dobránszki and Teixeira da Silva (2011)	In Royal Gala, shoots formed from 100% of both first and second apical leaves (conventional explants) when TDZ was 0.5 mg/l but 0% at 5 mg/l (the opposite trend was observed for Freedom, but values were 71% and 78% for first apical leaves at 7 and 9 w, and 42% and 55% for second apical leaves at the same time intervals). In Royal Gala, 88% of tTCLs formed shoots on SIM with 5 mg/l TDZ from the second apical leaf after 7 or 9 w (60% and 73% for the first apical leaf after 7 or 9 w). The four corresponding values for Freedom were: 19, 25, 52, and 54%. In terms of SN/explant, Royal Gala formed 6.3 or 10.2 shoots/regular first apical leaf explant after 7 or 9 w (4.4 and 6.3 from tTCLs) (equivalent values for the second apical leaf explant are 7.6 and 12.1). The eight corresponding values of SN/explant for Freedom are: 2.2, 3.2, 2.1, 2.4, 2.3, 2.6, 2.3 and 2.8. Percentage of explants forming shoots and SN/explant much lower at 4, 5 and 6 w for both leaf sources and both cultivars (Teixeira da Silva and Dobránszki, 2013). Alongside shoots, in both cultivars, callus also formed from both first and second apical leaves (standard explant and tTCLs) over a wide range of TDZ concentrations. When considering the actual volume of tissue (2 explants from conventional explants vs 50 tTCLs from the same conventional explant), i.e., a form of PGCF, SN/leaf was 12.6 and 15.2 for Royal Gala from the first and second apical leaves (conventional explant), respectively (equivalent values for Freedom were 3.1 and 1.9). However, when tTCLs were considered, the four equivalent values of SN/leaf were: 127.6, 193.6, 54.6, and 12.6. In other words, when the PGCF was considered, shoot productivity from tTCLs was many fold higher than from conventional leaf explants. Rooting and acclimatization not performed	Dobránszki and Teixeira da Silva (2013); Teixeira da Silva and Dobránszki (2013)
Rootstock (MM-106)	Apical meristems	MS + 2 mg/l BA + 0.5 mg/l IBA + 100 mg/l PG (SIM). MS + 1 mg/l BA + 0.5 mg/l GA_3 (SMM). MS + 3 mg/l IBA (RIM). pH, carbohydrate source and gelling agent NR	Conditions NR	SN = 13 shoots/explant. 80% of shoots rooted. 80% survival after acclimatization in vermiculite + cocopeat (ratio NR)	Mir et al. (2013)
Rootstock (Marubakaido)	In vitro shoots	Modified MS + *Rhizobia* or 1 mg/l IAA (RIM). pH 5.9. 3% sucrose. 0.6% agar	16-h PP. 25 µmol m^{-2} s^{-1}. 25 ± 2 °C	100% rooting achieved with IAA or two strains of *Adesmia latifolia*-derived *Rhizobia* (EEL16010B and EEL37810)	Muniz et al. (2013)
Rootstocks (M26, MM.106)	Single node stem cuttings (1 cm)	MS + 2 mg/l BA/2iP + 0.1 mg/l IBA (CIM, SIM). pH 5.8. 3% sucrose. 0.7% agar	16-h PP. 3000 lx. 25 ± 2 °C	More callus formed on MS than on DKW for both cultivars. MM.106 produced larger callus than M26. NS/explant = 7.1 (M26) and 5.8 (MM106). Rooting and acclimatization not performed	Noormohammadi et al. (2013)

Table 2 (continued)

Species, cultivars or rootstocks	Explant(s) used, size and source	Culture medium, PGRs and additives*	Culture conditions	Remarks, experimental outcome and maximum productivity, acclimatization and variation	References
Dwarf rootstocks (Azayesh-Esfahan (AE), Morabbaee-Mashhad (MM), M9)	Single node explants (size and plant age NR for SIM); 7-w-old in vitro shoots for SMM	MS + 0.5 mg/l BA + 0.5 mg/l IAA + 1 mg/l GA_3 (SIM). MS + 0.5 (MM) or 1.5 (AE, M9) mg/l BA (SMM). ½ MS + 1.5 mg/l IBA (RIM). pH 5.7. 3% sucrose. 0.8% agar	16-h PP. PPFD NR. 25 ± 2 °C	For all three cultivars, SN was higher on MS than on WPM. Max. SN/explant = 5.7, 8.0 and 5.0 for AE, M9 and MM, respectively (70, 55 and 18% of shoots rooted). Acclimatization not performed	Ghanbari (2014)
'Pingyitiancha' (*Malus hupehensis* var. *pinyiensis*)	Axillary buds from 1-y-old plants (size NR) for SMM; 30–35 d-old in vitro leaves for SIM	MS + 1 mg/l BA + 0.2 mg/l IBA (SIM for first *ex vitro*-derived shoots). MS + 0.3 mg/l BA + 0.1 mg/l NAA (SIM for first in vitro-derived shoots, 30-d old, and SMM). Subculture every 30–35 d. MS + 2 mg/l TDZ + 0.2 mg/l BA (SIM from leaf explants). MS + 0.3 mg/l BA + 0.1 mg/l NAA (SEM). ½ MS + 0.4 mg/l NAA (RIM). pH 5.7. 3% sucrose. 0.6% agar	21 d darkness → 16-h PP. 25 µmol m^{-2} s^{-1}. 25 ± 2 °C	97% of leaf explants formed shoots (max. 3.5/explant), but only after 21 d of initial darkness. 95% rooting of shoots. Plantlets acclimatized in soil, sand, and peat (1:1:1, v/v/v), but % survival NR. 100% monomorphism of SSR bands between mother plant and in vitro regenerants	Jin et al. (2014)
Cultivars (Gala, Fuji, Himekami, Wangshanhong, Greensleeves), rootstocks (M9, M26), *M. robusta*, *M. micromalus*	In vitro shoots	MS + 2–3 mg/l TDZ + 0.5 mg/l IBA (SIM from leaf segments). MS + 0.25 mg/l BA + 0.01 mg/l IBA (SMM). pH 5.8. 3% sucrose. 0.7% agar	21 d darkness → 16-h PP (50 µmol m^{-2} s^{-1} and 24 ± 2 °C) for shoot regeneration and cryopreservation	100% organogenesis and 4.5 shoots/leaf segment. Highest (79%) and lowest (28%) shoot regrowth obtained in cryopreserved shoot tips of 'Gala' and 'Himekami', respectively, with 57% shoot regrowth obtained across the 9 genotypes belonging to 4 *Malus* species	Li et al. (2014)
Rootstocks (Budagovsky 9 (B.9), MM106)	Nodal segments (2–3 cm)	MS + 2 mg/l BA + 0.02 mg/l NAA + 2 mg/l AC + 100 mg/l AA + 200 mg/l CH (SIM). PGR-free MS (SMM, RIM). pH 5.7–5.8. 2–3% sucrose. 0.8% agar	45 d darkness → 16-h PP. 70 µmol m^{-2} s^{-1}. 25 ± 2 °C	For MM106 and B9, 37% and 47% of explants formed shoots, with SN/explant = 5–6 and 6–7, and 98% and 96% rooting, respectively. When 20 ml of liquid medium was used, shoots were healthy, but 50 or 100 ml induced hyperhydricity in both cultivars, without impacting SN. Acclimatization in sand + soil (1:1, v/v) of agar-derived plantlets in a greenhouse resulted in 98% and 93% survival for MM106 and B9, respectively (82% and 81%, respectively when plantlets were derived from liquid-based medium)	Mehta et al. (2014)
Cultivars (Galaxy (2014); Golden Delicious (GD), double haploid (DH) derivative of GD, X9273 (2016))	In vitro shoots	MS + 0.5 mg/l BA + 0.1 mg/l IBA (SMM). MS + 5 mg/l TDZ + 0.5 mg/l IBA (SIM). pH, carbohydrate source and gelling agent NR. 0.3% gelrite (SIM)	Darkness: SIM. SMM: 16-h PP; 40–60 µmol m^{-2} s^{-1}. 23 ± 1 °C	2014 study: 100% of control leaves regenerated shoots (SN/explant = 14.9) while leaves of transgenic shoots carrying the *codA* and *nptII* genes showed 83% and 93% regeneration in transgenic lines 2 and 1 (SN/explant = 4.6 and 7.8, respectively). The application of 25 µM dexamethasone increased SN/explant to 18.3 without affecting regeneration efficiency 2016 study: the application of 1 mg/l mT reduced hyperhydricity by about 50% when BA was replaced by mT. GD shoot apex showed 97% survival (86% and 90% for GD apex and DH meristem, respectively). Shoot survival % decreased after cryostorage in liquid nitrogen of PVS2	Righetti et al. (2014); Poisson et al. (2016)

Table 2 (continued)

Species, cultivars or rootstocks	Explant(s) used, size and source	Culture medium, PGRs and additives*	Culture conditions	Remarks, experimental outcome and maximum productivity, acclimatization and variation	References
Dwarfing rootstocks (GM256, Budagovsky 71–3–150 (B1), Budagovsky 60–160 (B2))	Shoot tips (1–2 cm long)	MS + 1 mg/l BA + 0.2 mg/l IBA (shoot establishment). QL + 1 mg/l BA + 0.1 mg/l IBA (SMM). ½ QL + 0.5 mg/l IBA (RIM).pH 5.8. 3% (2% in RIM) sucrose. 0.6% agar	16-h PP. 40 µmol m^{-2} s^{-1}. 25 ± 2 °C	SN/shoot tip = 5.2 (GM256), 5.5 (B1), 3.7 (B2). Slightly lower productivity when QL was replaced by MS. 93% of GM256 shoots rooted when placed in the dark for 10 d (92% for B1 and 90% for B2 after shoot culture in the dark for 5 d). GM256 and B2 shoots showed shoot tip necrosis and hyperhydricity (B2). 90% survival of all three cultivars in soil + vermiculite + perlite (1:1:1, v/v/v), except for shoots with shoot tip necrosis (2% survival)	Sun et al. (2014)
Cultivars (Royal Gala, Freedom)	As for Dobránszki and Teixeira da Silva (2011)	As for Dobránszki and Teixeira da Silva (2011)	As for Dobránszki and Teixeira da Silva (2011)	The highest SN/explant was after 9 w of regeneration for Royal Gala: 12.1 and 6.5 from conventional explants and tTCLs, respectively (3.2 and 2.4 for Freedom). When the PGCF was applied, the SN/explant value for Royal Gala tTCLs was 11.8 and 13.0 for Freedom. Rooting and acclimatization not performed	Teixeira da Silva and Dobránszki, (2014)
Rootstock (G.41)	Axillary buds (0.2–0.6 cm) for shoot proliferation. In vitro leaves (wounded by crushing) for shoot induction.	PGR-free MS 8 w (shoot proliferation). MS + 4.4 µM BA + 2.46 µM IBA (SMM). MS + 1.8 µM TDZ + 0.9 µM NAA (SIM). ½ MS + 4.92 µM IBA (RIM). pH 5.8. 3% sucrose. 0.75% agar	16-h PP. 50 µmol m^{-2} s^{-1}. 25 °C	On SMM, highest multiplication index [(# survived shoots – # inoculated shoots)/# inoculated shoots] was 3.89, but 35% of shoots were hyperhydric, so suggested SMM = MS + 3.55 µM BA + 0.16 µM IBA. 96% of explants formed shoots (max. SN/explant = 5.55). 80% of shoots rooted. > 90% survival when acclimatized in sterile soil + vermiculite + perlite (1:1:1)	Zhang et al. (2014)
Rootstock (CG41)	Shoot tip? (size NR; plant age NR)	PGR-free MS + Staba (1969) vitamins (SIM). MS + 4.4 µM BA + 0.45 µM TDZ (SMM). MS + 0.049 µM IBA + 0.054 µM NAA + 0.46 µM Kin (SEM). ½ MS + 2.46 µM IBA (RIM). pH 5.8. 3% sucrose. 0.75% agar	16-h PP. 15 (first week) then 30 µmol m^{-2} s^{-1}. 23 ± 2 °C	4 shoots/shoot on SMM. SEMs that employed 2 g/l AC or MS + 4.4 µM BA gave statistically similar shoot elongation as established SEM. > 90% of shoots rooted. > 95% survival when plants were acclimatized in peat	Castillo et al. (2015)
Rootstock cultivars (B.9, Geneva 30 (G.30), Geneva 41 (G.41))	Nodal explants with 1–2 nodes (10–15 mm long) from softwood shoots (plant age NR)	MS + 1 mg/l BA + 1 mg/l IBA + 0.5 mg/l GA_3 (SIM, SEM). pH 5.8. 3% sucrose. 0.8% agar	16-h PP. 25 µmol m^{-2} s^{-1}. 27 ± 2 °C	The presence of GA_3 was essential for high SN in G.30. SN for B.9 was highest in red light (~ 6.5/explant) (SN = ~ 5.5 for G.30 in red light and ~ 2.1 for G.41 in blue light). Rooting and acclimatization not performed	Geng et al. (2015)
Cultivar (Orin)	Young embryo	MS + 1.5 mg/l BA + 0.5 mg/l IAA (CIM). MS + 0.5 mg/l BA + 1.5 mg/l 2,4-D (culture after genetic transformation; An et al. (2016). pH, carbon source, gelling agent NR	Darkness. 25 °C	The objective was to assess the response of callus to 100 and 200 mM NaCl in control and transgenic (harbouring the *MdVHA-B1* gene) lines. Fresh weight of transgenic callus was almost as high as control, untransformed callus in the absence of NaCl	Hu et al. (2015a); An et al. (2016)

Table 2 (continued)

Species, cultivars or rootstocks	Explant(s) used, size and source	Culture medium, PGRs and additives*	Culture conditions	Remarks, experimental outcome and maximum productivity, acclimatization and variation	References
Cultivars (Gala, Fuji, Wangshanhong), rootstocks (M9, GM256), *M. micromalus*, *M. robusta*	In vitro shoots	MS + 0.25 mg/l BA + 0.01 mg/l IBA (SMM). pH 5.8. 3% sucrose. 0.7% agar	16-h PP. 50 µmol m^{-2} s^{-1}. 24 ± 2 °C	Comparison of shoot regrowth of shoot tips cryopreserved by encapsulation-dehydration and droplet-vitrification showed the former produced higher shoot regrowth (61%) than the latter (48%) across 7 genotypes belonging to 4 *Malus* species. Analysis by ISSR and RAPD did not detect any polymorphic bands, and FCM did not detect changes in ploidy levels in plants recovered after both cryopreservation methods	Li et al. (2015)
Rootstock cultivars (M7, M9)	Single nodes	MS + 2 mg/l BA + 0.1 mg/l IBA (SMM1), MS + 2 mg/l 2iP + 0.1 mg/l IBA (SMM2), DKW + 2 mg/l BA + 0.1 mg/l IBA (SMM3), DKW + 2 mg/l 2iP + 0.1 mg/l IBA (SMM4). pH 5.8. 3% sucrose. 0.7% agar	16-h PP. 3000 lx. 25 ± 2 °C	ISSR used to assess genetic variation between mother plants plantlets derived from in vitro culture. The % polymorphism in M7 for subculture 1 (42%, 28%, 17%, 14% = SMM1, SMM2, SMM3, SMM4) and subculture 2 (33%, 53%, 33%, 7% = SMM1, SMM2, SMM3, SMM4). The 8 values for M9 (subculture 1, subculture 2) = 13%, 18%, 15%, 17%, 46%, 41%, 30%, 18%)	Noormohammadi et al. (2015)
Cultivars (James Grieve Compact (JGC), Jarka, Mivibe)	Shoot tips (5–10 mm long)	PGR-free MS 4 w → MS + 1.5 mg/l BA (shoot establishment). MS + 4 mg/l BA (SMM). ½ MS + 1 mg/l NAA (Jarka) or 1 mg/l IAA (Mivibe) (RIM). pH 5.8. 3% sucrose. 0.7% agar	16-h PP. PPFD NR. 22 ± 1 °C	9%, 14%, 26% of uncontaminated JGC, Jarka and Mivibe explants formed shoots (shoot proliferation rate = 1.6, 3.6, and 2.3, respectively). TDZ induced callus. 44% of Jarka shoots rooted (22% for Mivibe). JGC cultures died, so rooting not tested. Jarka and Mivibe plantlets acclimatized (survival not quantified)	Paprštein and Sedlák (2015)
Rootstock cultivars (G.30, G.41)	As in Geng et al. (2015)	MS + 1 mg/l BA + 0.1 mg/l IBA + 0.5 mg/l GA_3 (SMM, SEM). pH 5.8. 3% sucrose. 0.8% agar	As in Geng et al. (2015)	Shoots collected in spring and chilled for 6 w formed greater SN/explant than shoots collected in autumn or winter: 3.4, 2.7, and 2.9. BA stimulated shoot growth and elongation more than TDZ and zeatin. Shoots were stunted when TDZ was used at 0.5–2 mg/l. SN/explant, shoot multiplication and shoot length were comparable when SMM had 2 mg/l BA with (at 0.5 mg/l) or without GA_3: SN/explant = 6.6 or 6.3, respectively. *Epi*-brassinolide decreased SN/explant at 0.1 mg/l (2.7) or at 1 mg/l (1.8) compared to the control (4.1). After a first sub-culture, SN/explant was 1.5, but increased to 4.2 after a second sub-culture	Geng et al. (2016)
Cultivar (Gala)	In vitro shoots	MS + 0.8 mg/l BA + 0.2 mg/l IAA (shoot maintenance). MS + 0.1 mg/l IAA (RIM). pH, carbon source, gelling agent NR	16-h PP. 30 µmol m^{-2} s^{-1}. 25 °C	Transgenic plants harbouring the *MdSOS2L1* gene had higher tolerance to 300 mM NaCl and a higher amount of procyanidins and malate and lower levels of ROS	Hu et al. (2016)

Table 2 (continued)

Species, cultivars or rootstocks	Explant(s) used, size and source	Culture medium, PGRs and additives*	Culture conditions	Remarks, experimental outcome and maximum productivity, acclimatization and variation	References
Rootstocks (M9, M26)	In vitro shoots	MS + 0.25 mg/l BA + 0.01 mg/l IBA (SMM). pH 5.8. 3% sucrose. 0.7% agar	16-h PP. 50 µmol m^{-2} s^{-1}. 24 ± 2 °C	Shoot tip culture and cryotherapy were applied to eradicate ASPV and ASGV from infected in vitro shoots of apple rootstocks M9 and M26. Although larger shoot tips (1.0 mm) produced higher shoot regrowth rate (90–92%) than those (31–35%) by smaller shoot tips (0.5 mm), 100% of plants regenerated from the latter were ASPV-free, while none of the plants were free of ASPV from the former. Following cryopreservation, shoot tips smaller than 1.0 mm were not able to regrow. Although larger shoot tips (1.5 mm) produced higher shoot regrowth (73–75%) than smaller (1.0 mm) shoot tips (42–45%), similar ASPV-free frequencies (80–85%) were obtained, regardless of the size. Both shoot tip culture and cryotherapy could not eradicate ASGV, regardless of the shoot tip size used. Many cells in the AD and some cells in LPs 1–3 survived following cryopreservation. Immunohistological localization did not find ASPV in the AD and LPs 1–3, but found ASGV across AD and all LPs. These results explain why shoot tip culture and cryopreservation can eradicate ASPV, but failed to eradicate ASGV	Li et al. (2016)
14 cultivars, 5 rootstocks, 10 *Malus sieversii*	Shoot tips: laboratory (1–2 cm); field (2–3 cm)	Liquid MS + 0.5 mg/l BA + 0.01 mg/l IBA + 1 mg/l GA_3 + 1 mg/l AA (subculture daily for 2–4 w) → MS (solid) + 0.5 mg/l BA + 0.01 mg/l IBA (shoot establishment). MS (solid) + 0.5 mg/l BA + 0.01 mg/l IBA (SMM). pH 5.7. 3% sucrose. 0.4% agar + 1.75% gelrite	Conditions NR.	Values indicate averages across germplasm. 55% shoot regeneration with 7 min $HgCl_2$ (31% with 5 min). 18% shoot viability with 10 min $HgCl_2$ (11% with 7 min). 55% survival of laboratory grown and forced winter shoots (19% from field-derived shoot tips). Daily subculture and addition of AA reduced negative impact from polyphenols. Low percentage of callusing and hyperhydric shoots observed. Cryotherapy (Wang et al. 2018b) is being used to eliminate viruses from shoot clones	Romadanova et al. (2016a)
Cultivars (Rubimeg, Bohemia, Primadela)	Shoot tips (5-15 mm long)	MS + 1.5 mg/l BA 2 mo → MS + 2 mg/l BA 4–6 × (shoot establishment). MS + 4 mg/l BA (SMM). pH 5.8. 3% sucrose. 0.7% agar	16-h PP. 40 µmol m^{-2} s^{-1}. 22 ± 1 °C	66%, 92%, 94% of uncontaminated Primadela, Bohemia and Rubimeg explants formed shoots (shoot proliferation rate = 1.3, 4.1, and 2.1, respectively). Rooting not tested. Acclimatization not performed	Sedlák and Paprštein (2016)

Table 2 (continued)

Species, cultivars or rootstocks	Explant(s) used, size and source	Culture medium, PGRs and additives*	Culture conditions	Remarks, experimental outcome and maximum productivity, acclimatization and variation	References
Cultivar (Gala)	In vitro shoots	MS + 2 mg/l TDZ + 0.5 mg/l IBA (adventitious bud regeneration medium). MS + 0.25 mg/l BA + 0.01 mg/l IBA (shoot tip culture medium). pH 5.8. 3% sucrose. 0.7% agar	16-h PP. 50 µmol m^{-2} s^{-1}. 24 ± 2 °C	Shoot tips of adventitious buds derived from leaf segments at different developmental stages were cultured for eradication of ASPV and ASGV. Shoot regrowth rates increased from 10% to 15% in 0.3 mm shoot tips containing two LPs excised after 2–3 w of adventitious bud induction, to 53–55% in those containing three LPs excised after 3–4 w. Highest shoot regrowth (82%) was obtained in 0.4 mm-shoot tips containing four LPs excised after 4 w. ASPV-free frequency (95–100%) was high in 0.2–0.4 mm shoot tips containing 2–3 LPs excised after 2–4 w, but low (20%) in 0.4 mm shoot tips containing four LPs excised after 4 w. No shoots regenerated were free of ASGV, regardless of size and developmental stage. Immunonohistological virus localization did not find ASPV in 0.2–0.4 mm shoot tips containing 2–3 LPs after 2–4 w of culture, but found ASGV inside buds of the same size and developmental stage	Wang et al. (2016)
8 cultivars (Cacharela, Camoesa, Repinaldo, Tres en Cunca, Gravillán, Ollo Mouro, José Antonio, Príncipe Grande)	In vitro culture establishment from dormant cuttings. In vitro shoots (10–15 mm, 3–5 leaves/bud) for in vitro propagation.	In vitro culture establishment: MS + 1 mg/l BA + 0.2 mg/l GA_3 + 0.3 mg/l IBA. MS + BA, ZEA, 2iP, or TDZ (0, 0.25, 0.5 or 1.0 mg/l) with or without IBA (0.1 mg/l) (SMM). After 6 subcultures: MS + 0.1 mg/l IBA, or without IBA (M0 medium) for 28 d (RIM). pH 5.8. 3% sucrose. 0.7% agar	16-h PP. 40 µmol m^{-2} s^{-1}. 25/18 ± 1 °C day/night	Optimal cytokinin concentration for shoot multiplication depended on the cultivar. ZEA and 2iP induce new shoots with very low efficiency. Highest multiplication rate with 1.0 mg/l TDZ in José Antonio and Tres en Cunca, but this also resulted in highest hyperhydricity. For other cultivars 0.25 and 0.5 mg/l TDZ were best. For high rooting percentage (91%; 5.67 and 4.12 roots/shoot, respectively) RIM + IBA was necessary in two cultivars (Camoesa and Repinaldo). Cacharela and Príncipe Grande needed IBA in RIM for rooting while Ollo Mouro rooted only on auxin-free medium. Tres en Cunca did not develop roots in any RIM. Acclimatization in peat: crust: coconut fibre: black peat (6: 2: 1: 1, v/v); 24 ± 2 °C. Survival (50–100%) was cultivar-dependent	Lizárraga et al. (2017)
Rootstock cultivar (G. 814)	Nodal segments (1.5–2.5 cm long in first phase, 2.5–3.5 cm long in second SMM phase) from in vitro plants	Modified QL (Leblay et al., 1991) + 1 mg/l BA (SMM). SMM + 1.5 mg/l IBA (RIM). pH 5.8. 3% sucrose. 0.6% agar	16-h PP. 27 µmol m^{-2} s^{-1}. 25 ± 2 °C	SN = 9.7 shoots/explant. 94% of shoots rooted. Concentrations of IBA exceeding 1.5 mg/l induced callus. Acclimatization not performed	Meneguzzi et al. (2017)

Table 2 (continued)

Species, cultivars or rootstocks	Explant(s) used, size and source	Culture medium, PGRs and additives*	Culture conditions	Remarks, experimental outcome and maximum productivity, acclimatization and variation	References
Rootstocks (MM106, M7, MM111, M793, M26)	Axillary buds, terminal buds, shoot tips	MS + 100 mg/l AA (± 50 mg/l cysteine, and/or 500 mg/l AC) (initial shoot culture, 4–5 w). MS + 2 mg/l BA + 1 mg/l GA_3 + 0.2 mg/l IBA/NAA (shoot establishment). MS + 0.5–1.5 mg/l BA + 0.05–0.1 mg/l IBA ± 0.1–0.5 mg/l GA_3 + 100 mg/l PG (for MM111, MM106) (SMM). ½ MS + 0.3-1 mg/l IBA + 0.2-0.5 mg/l NAA (RIM). pH NR. 3% sucrose (2%, RIM). 0.8% agar	Conditions NR	After 6 subcultures, % shoot development from explants: 40-50% (MM106), 65-75% (M7), 70-80% (MM111), 70-90% (M793), 14-20% (M26); SN/explant: 3-7 (MM106), 4-7 (M7), 4-10 (MM111), 3-5 (M793), 3-9 (M26); rooting: 70-80% (MM106), 80-90% (M7), 80-90% (MM111), 60-70% (M793), 90-100% (M26). 90-95% survival of hardened plants. In Modgil et al. (2017), RAPD analysis between mother plants and clonal propagules resulted in same profiles, i.e., no genetic variation	Modgil and Thakur (2017); Modgil et al. (2017)
Cultivars (Free Redstar, Gala Must, Pinova, Co-op 32, Redchief, Sander)	Shoot clumps and leaves from in vitro plants	MS + 4.5 µM BA + 0.3 µM GA_3 + 0.5 µM IBA (SMM1). Pre-culture for 4 w on MS + 4.5 µM BA (Free Redstar) or 4.4 µM BA (other 5 cultivars) → 8 w (2 × 4-w subcultures) on MS + 18 µM BA or 4.5 µM TDZ + 2.5 µM NAA (SIM) → MS + 2.25 µM BA + 1.5 µM GA_3 + 0.25 µM IBA (SMM2) → SMM1. Leaves from 4-w pre-cultures onto MS + 4.5 µM TDZ + 2.5 µM NAA + antimitotic agent. pH 5.6. 3% sucrose. 0.6% agar	16-h PP. 30 µmol m^{-2} s^{-1}. 21 °C	Histological analysis was only performed for Co-op 32. Max. SN/explant and % explants forming shoots for Free Redstar, Gala Must, Pinova, Co-op 32, Redchief, Sander: (52%; 3.4), (89%; 14.2), (56%; 2.7), (60%; 5.5), (88%; 14.8), (27%; 2.6), respectively. After treatment with four antimitotic agents (colchicine, trifluralin, oryzalin or APM), max. SN/explant for same cultivars = 2.2 in control, 7.2 at 10 mg/l APM, 8.7 at 5 mg/l oryzalin, 6.1 in control, 25 at 5 mg/l oryzalin, and 2.5 at 10 mg/l oryzalin, respectively	Podwyszyńska et al. (2017)
M. sylvestris	Cotyledons	MS + 2 µM 2,4-D (CIM). pH NR. 3% sucrose. 0.2% gellan gum.	2 w darkness → 16-h PP. 2000 lx. 25/16 ± 1 °C (day/night).	327 mg of callus formed per explant. Callus was not induced from stems and leaves. Shoot induction, rooting and acclimatization not performed	Rumiyati et al. (2017)
Cultivars (Golden Delicious (GD), Mela Rosa Marchigiana (MRM))	Fruit pulp discs (5 mm diameter, 3 mm thick)	MS + 2 mg/l BA + 2 mg/l NAA (CIM, GD). B5 + 2 mg/l BA + 0.2 mg/l 2,4-D (CIM, MRM). Subcultures every 2 w. pH 5.8. 3% sucrose. 0.8% agar	Darkness. 25 ± 2 °C.	Highest callus biomass (12.45 g) from GD on MS after 2 months (11.66 g for MRM on B5). Methanolic followed by ethanolic extraction yielded qualitatively similar compounds (β-sitosterol; phloridzin; oleanolic, ursolic, maslinic, corosolic, pomolic, tormentic, and annucoic acids), but yield was different for each cultivar. Levels were comparable to in vivo pulp compounds	Verardo et al. (2017)
Cultivars (Pink Lady, Huafu)	In vitro shoots	MS + 1.0 mg/l BA + 0.2 mg/l IBA (SMM), subcultured every 30-40 d. pH 5.8. 3% sucrose. 0.56% agar	16-h PP. 2000 lx. 24 ± 1 °C.	44% (Pink Lady) and 65% (Huafu) shoot survival after pre-culture for 13 d	Hu et al. (2018)
Rootstock (M26)	In vitro stem cuttings (size NR)	½ MS + 1 mg/l IBA (RIM). pH NR. 2% sucrose. 0.8% agar	Conditions NR	To assess the effect of changing explants from RIM to medium containing a cytokinin, one-third of explants were transferred to RIM with 2 mg/l BA after 3 or 7 d. The initiation of adventitious root formation was between 3 and 7 d	Mao et al. (2018)

Table 2 (continued)

Species, cultivars or rootstocks	Explant(s) used, size and source	Culture medium, PGRs and additives*	Culture conditions	Remarks, experimental outcome and maximum productivity, acclimatization and variation	References
Tetraploid cultivars (Free Redstar, Gala Must, Pinova, Redchief)	Microcuttings obtained from Podwyszyńska et al. (2017)	SIM + SMM (Podwyszyńska et al. 2017). MS (minus adenine sulphate and NH_4NO_3) + 2.5 µM IBA or 1.3 µM NAA + 5 µM IAA + 50 µM putrescine (RIM). REM = RIM minus auxins	16-h PP. 30 µmol m^{-2} s^{-1}. 21 °C. In some experiments, 7 d of darkness → REM (18 d)	The objective of the study was to improve rooting ability of neo-tetraploid cuttings. Except for 'Redchief', rooting was about 20-40% when only IBA at 2.5-7.5 µM was used, but close to 0% for the other three cultivars. Use of a two-step rooting system (RIM → REM) increased rooting % from 17% to 57% in 'Gala Must'. Use of darkness and 26 °C increased rooting of 'Free Redstar' to 48% and of 'Pinova' to 78%. Except for 'Pinova', the use of BA or *m*T improved rooting of shoots and resulted in high acclimatization % (as high as 100% in some treatments)	Podwyszyńska and Cieślińska (2018)
Cultivars (Holsteiner Cox, Maglemer, Prima)	Axillary buds	MS + 0.5 mg/l BA + 0.05 mg/l NAA (SMM), subcultured after 2 d, then every 4 w. pH 5.7. 2% sucrose. 0.5% agar	16-h PP. 52 µmol m^{-2} s^{-1}. 25 ± 2 °C.	This study assessed bud viability via CO_2 gas exchange (i.e., respiratory output) and tissue culture. 76% of control buds formed shoots (0% in cryopreserved buds), i.e., were viable (vs. 85% viability in gas exchange assay and 91% of grafted shoots). The difference between the in vitro assay index % and other % values reflects the unresponsive buds to in vitro culture	Vogiatzi et al. (2018)
Cultivar (Gala)	In vitro shoots	MS + 0.25 mg/l BA + 0.01 mg/l IBA (shoot tip culture medium; SMM). pH 5.8. 3% sucrose. 0.7% agar	16-h PP. 50 µmol m^{-2} s^{-1}. 24 ± 2 °C	Although ASGV-infected stock shoots multiplied more shoots/explant (7.2) than healthy stock shoots (5.1), the number of shoots ≥ 1.0 cm was less in the former (1.8) than in the latter (2.9). There were no significant differences in total shoot regrowth percentages in cryopreserved shoot tips between the healthy and virus-infected shoots. All regenerated shoots were normal in cryopreserved shoot tips of healthy stock shoots, while only about 45% of regenerated shoots were normal and 55% were abnormal shoots from cryopreserved shoot tips of virus-infected shoots. Alternation in levels of endogenous IAA in the virus-infected stock shoots was most likely responsible for reduced length of shoots, while cell damage and alternations in mitochondria shape in the virus-infected shoots resulted in formation of abnormal shoots in cryopreserved shoot tips	Wang et al. (2018c)

2,4-D 2,4-dichlorophenoxyacetic acid, *2iP* 6-(γ,γ-dimethylallylamino)purine, *AA* ascorbic acid; *ABA* abscisic acid, *AC* activated charcoal, *AD* apical dome, *Alar-85* daminozide, *APM* amiprophos methyl, *ASGV* apple stem grooving virus, *ASPV* apple stem pitting virus, *B5* medium (Gamborg et al. 1968), *BA* N^6-benzyladenine (BA is used throughout even though BAP (6-benzylamino purine) may have been used in the original (Teixeira da Silva 2012a), *BAR* N^6-benzyladenine riboside, *CA* citric acid, *CH* casein hydrolysate, *CIM* callus induction medium; *d* day(s), *DKW* Driver and Kuniyuki (1984) medium, GA_3 gibberellic acid, *IAA* indole-3-acetic acid, *IBA* indole-3-butyric acid; *ISSR* inter simple sequence repeat, *Kin* kinetin (6-furfuryl aminopurine); *LP* leaf primordium; *mo* month(s); *MS* Murashige and Skoog (1962) medium, *mT meta*-topolin (6-(3-hydroxybenzylamino)purine) or N^6-metahydroxy-benzyladenine, *mTR meta*-topolin riboside or N^6-metahydroxy-benzyladenine ribozide, *NAA* α-naphthaleneacetic acid, *NR* not reported in the study, *PG* phloroglucinol, *PGCF* plant growth correction factor (Teixeira da Silva and Dobránszki 2011, 2014), *PGR* plant growth regulator, *PGPR* plant growth-promoting rhizobacteria, *PIC* picloram (4-amino-3,5,6-trichloro-pyridine-2-carboxylic acid), *PP* photoperiod, *PP333* paclobutrazol, *PPFD* photosynthetic flux density, *PVP* polyvinyl pyrrolidone, *QL* (Quoirin and Lepoivre) macroelements, Quoirin and Lepoivre (1977), *RAPD* random amplified polymorphic DNA; *REM* root elongation medium, *RIM* root induction medium, *ROS* reactive oxygen species, *SEM* shoot elongation medium, *SIM* shoot induction medium, *SMM* shoot multiplication medium, *SN* shoot number (or number of shoots), *SSR* simple sequence repeat, *TDZ* thidiazuron (*N*-phenyl-*N*'- 1,2,3-thiadiazol-5-ylurea), *tTCL* transverse thin cell layer, *w* week(s), *WPM* woody plant medium (Lloyd and McCown, 1980), *y* year(s), *ZR* zeatin riboside

*Even though the term "calli" was used in the original, the term callus has been used here based on recommendation of Teixeira da Silva (2012b)

Silva 2010). Inclusion of 0.25% ascorbic acid, 0.5% citric acid and 3 g/l activated charcoal (AC) in the medium was found to reduce browning of apple rootstocks MM106 and MM111 (Jafarkhani Kermani et al. 2009) and 'Douce de Djerba' (Boudabous et al. 2010). Treatment of axillary and terminal buds in Murashige and Skoog (1962) medium (MS) containing 100 mg/l ascorbic acid reduced browning of apple rootstocks MM106, M7, MM111, M793 and M26 (Modgil and Thakur 2017). Adding 0.15 g/l citric acid and 0.1 g/l ascorbic acid into the medium was necessary to avoid explant oxidation when preparing leaf transverse thin cell layers (tTCLs) (Dobránszki and Teixeira da Silva 2011, 2013). Working on apple 'Remo', 'Rewena' and 'Reanda', Dobránszki et al. (2005a) reported the successful development of micrografts when shoot tips excised from field-grown plants were used, while use of in vitro shoot tips caused death of rootstocks, due to phenolic browning.

Explant type: conventional explants or thin cell layers

Leaf segments are the most widely used explants to induce adventitious shoots in *Malus* spp. Adventitious shoots can be induced directly without callus formation (Pawlicki and Welander 1994) or indirectly through callus (Dufour 1990). Thidiazuron (TDZ) is more effective than N^6-benzyladenine (BA) for shoot regeneration (Gamage and Nakanishi 2000; Dobránszki et al. 2004, 2006; Mitić et al. 2012; Li et al. 2014). The optimal TDZ concentration varies with apple genotypes (Fasolo et al. 1989; Sarwar and Skirvin 1997; Dobránszki et al. 2004, 2006; Magyar-Tábori et al. 2010; Mitić et al. 2012). Fasolo et al. (1989) noted that in three cultivars ('McIntosh', 'Paladino Spur McIntosh' and 'Triple Red Delicious') 10 μM TDZ induced shoots efficiently but other cultivars ('M7', 'M9', 'M26', 'Orine', 'Golden Delicious', 'Melrose', 'Macspur', 'Strakrimson' and 'Fuji') induced shoots when TDZ concentration exceeded 10.0 μM (Gamage and Nakanishi 2000; Magyar-Tábori et al. 2010; Mitić et al. 2012; Li et al. 2014), although the vast majority of remaining cultivars responded best when TDZ was applied at less than 5.0 μM (Sarwar and Skirvin 1997; Magyar-Tábori et al. 2010; Li et al. 2014). A number of cultivars such as 'Royal Gala' and 'Dayton' responded similarly to a wide range of TDZ concentrations (5–20 μM) (Magyar-Tábori et al. 2010). Protocols using TDZ to induce adventitious buds have recently been developed for several *Malus* species and genotypes (Li et al. 2014; Podwyszyńska et al. 2017). It is worth noting that TDZ has been reported to induce abnormal shoots in a wide range of plants (Dewir et al. 2018). Given the genomic instability of apple and apparent ease with which epigenetic changes take place, the use of TDZ is cautioned as there are some documented cases of TDZ-induced abnormalities in apple in vitro culture such as hyperhydric shoots (Fig. 2) and dwarfing, or shoot fasciation (Kereša et al. 2012), although the development of these phenomena depend strongly on the level of TDZ applied as well as the genotype used (Dobránszki et al. 2001, 2006; Magyar-Tábori et al. 2010). Mert and Soylu (2010) observed hyperhydric shoots in 38–45% of axillary shoot tips and in 31–38% of terminal shoot tips for rootstock M9, but almost no hyperhydric shoots in rootstocks MM106 and MM111 at the shoot establishment stage, but 75–100%, 1–3% and 9–100% hyperhydric shoots for M9, MM106 and MM111 axillary shoot tips, respectively, across three sampling dates (53–83%, 1–11% and 1–65% for M9, MM106 and MM111 terminal shoot tips, respectively). Shoot elongation is effectively achieved with the inclusion of gibberellic acid (GA_3), but here, too, the needed concentration is genotype-dependent (Dobránszki and Teixeira da Silva 2010).

In tTCL-based studies of apple, *meta*-topolin (N^6-metahydroxy-benzyladenine; *m*T), an aromatic cytokinin was essential in pre-treatment for adventitious shoot induction, as was assessed for 'Royal Gala' (Dobránszki et al. 2005b) and 'Freedom' (Magyar-Tábori et al. 2010). About 5.6 and 8.0 adventitious shoots formed from the first and second leaves of in vitro shoots of apple 'Royal Gala', while the value was 2.1 from both leaf sources of apple 'Freedom' (Dobránszki and Teixeira da Silva 2011). In the study of Dobránszki and Teixeira da Silva (2011), tTCLs formed significantly fewer shoots per explant than regular leaf explants: 3.2 and 2.7-5.1 for first and second leaves, respectively for 'Royal Gala', and 0–1.5 and 0–1.4 for first and second leaves, respectively for 'Freedom'. This result is not unexpected when one considers the difference in explant size and surface area, but when the plant growth correction factor (PGCF) is considered, tTCLs can in fact form more shoots than regular leaf explants (Teixeira da Silva and Dobránszki 2014) (see Table 2 for details).

Explant size, in particular the surface area, affected the quantitative outcome of adventitious shoot regeneration in apple, and the use of tTCLs resulted in several dozen-fold more shoots from a designated surface area once the PGCF was considered, i.e., relative to a regular explant, tTCLs have the ability to produce vastly more shoots (Dobránszki and Teixeira da Silva 2013; Teixeira da Silva and Dobránszki 2014) (see Table 2 for details). This makes tTCLs a preferred explant for adventitious shoot regeneration in apple although they can be laborious to produce due to their size. Scientists working on any aspect of apple tissue culture are alerted to the fact, as for many other plant species, that the timing of sampling (reflecting different developmental stages) impacts the quantitative outcome, primarily the number of adventitious shoots (Dobránszki and Teixeira da Silva 2013; Teixeira da Silva and Dobránszki 2013) (see Table 1 for details). Mehta et al. (2014) observed the same phenomenon, i.e.,

dependence of quantification of shoot and root induction on timing of sampling, for two rootstocks, MM106 and B9.

Shoot induction, elongation and multiplication

As had already been reviewed (Dobránszki and Teixeira da Silva 2010; Magyar-Tábori et al. 2010), medium composition affects the success of both axillary and adventitious shoot induction. Ghanbari (2014) found that three dwarf apple rootstocks performed equally well in terms of shoot production, on MS medium rather than on woody plant medium (WPM; Lloyd and McCown 1980). However, Ciccoti et al. (2008), when studying the multiplication rate and shoot height of 11 apomictic genotypes of *Malus sieboldii*, found that the optimal basal medium was cultivar-dependent. MS medium was the best for six genotypes while QL (Quoirin and Lepoivre 1977) basal medium resulted in the highest multiplication rate for four genotypes and only one genotype performed optimally on DKW (Driver and Kuniyuki 1984) medium. In apple, the shape and orientation of the explant affects shoot productivity (Dobránszki and Teixeira da Silva 2010). Zhang et al. (2014) showed that cut-based wounding of leaf explants and their orientation with the abaxial surface down on the medium was important for adventitious shoot production, and 96% of explants formed shoots versus 39% when explants were adaxial surface down on the medium.

Plant growth regulators

The most important factor, which governs the in vitro development and growth of apple shoots, is the choice of PGRs applied in the medium (Dobránszki and Teixeira da Silva 2010). Amiri and Elahinia (2011a) showed how the choice of PGR, in particular the use of TDZ and 6-benzyladenine (BA), affected the outcome of shoot induction in three rootstocks, while GA_3 was essential for shoot elongation (Table 2). In fact, the vast majority of apple shoot multiplication studies had employed either BA or TDZ (Yepes and Aldwinckle 1994). As in some earlier studies in apple, Castillo et al. (2015) found that TDZ caused explant and leaf deformation when used continuously for the tissue culture of apple rootstock CG41. The advantages and disadvantages of the application of TDZ have also been discussed above (see adventitious shoot induction in ‘In vitro propagation of apple’ section). Dastjerd et al. (2013) noted that effective induction and elongation of M26 rootstock shoots was only possible when the medium contained a combination of BA, crab chitosan and GA_3. Curiously, the relative concentration of these three compounds could be used to manipulate the growth form in vitro, for example, to increase bushiness as a result of the production of more or less leaves or more or less elongated shoots, which has potential applications to ornamental in vitro cultures. That study reported for the first time the use of chitosan in apple tissue culture, indicating chitosan as a promising additive in tissue culture of woody species. Chitosan has been widely used in orchid tissue cultures and has a wide range of biological properties, including antimicrobial and promotion of plant growth (Uthairatanakij et al. 2007). TDZ multiplied shoots more effectively than BA in eight Galician traditional apple cultivars, but its optimal concentration depended on the cultivar (Table 2; Lizárraga et al. 2017). However, the percentage of hyperhydric shoots was highest in five out of eight cultivars while in two cultivars (‘Ollo Mouro’, ‘José Antonio’), it exceeded 50% when TDZ was added to the shoot multiplication medium (Lizárraga et al. 2017). Geng et al. (2015) noted the importance of GA_3 for shoot development in three rootstocks released from the Cornell-Geneva (Geneva® series) breeding program, depending on the light source, while red light induced significantly more shoots, and longer shoots, than when white or blue light was used, but this depended on the cultivar.

Studying the effects of cytokinin supply (BA, BAR, *m*T and a dual application of BA + *m*T) on the quality of axillary shoots of apple ‘Royal Gala’ and ‘Freedom’, Dobránszki and Mendler-Drienyovszki (2014, 2015) noted that the choice of cytokinin affected the chlorophyll (chl) content as well as the functionality of the photosynthetic apparatus (maximum quantum yield F_v/F_m and maximum efficiency of the photochemical process, F_v/F_0, light chl fluorescence, Y(II)) in the leaves of axillary shoots after three weeks of culture. The F_v/F_m values varied between 0.683 and 0.861 in ‘Royal Gala’ and between 0.709 and 0.790 in ‘Freedom’ corresponded to the chl fluorescence values measured in field-grown plants. The effects of cytokinins were genotype-dependent. Total chl content of leaves was highest when BA was supplied to ‘Royal Gala’ and when *m*T was supplied to ‘Freedom’. In ‘Royal Gala’, 0.5 µM BA or 2.0–25.0 µM BAR (Dobránszki and Mendler-Drienyovszki 2014), while in ‘Freedom’ the supply of two cytokinins (2.2 µM BA + 2.0 µM *m*T) favoured dark and light chl fluorescence measured in the third, developed apical leaves (Dobránszki and Mendler-Drienyovszki 2015).

Kepenek and Karoğlu (2011) increased shoot number/explant in rootstock M9 and apple ‘Starking Delicious’ and ‘Amasya’ when two growth retardants (paclobutrazol and daminozide) were applied, but shoots were stunted while there was no effect on rooting.

The effects of antibiotics on shoot growth and development can be important when the objective is to produce transgenic plants. Stanišić et al. (2018), using the protocol of Mitić et al. (2012) for ‘Golden Delicious’, noted that when 300 mg/l cefotaxime was used, shoot growth improved, increasing shoot number per explant from 7.67 to 9.69 in the control, whereas other β-lactam antibiotics (meropenem and timentin) stunted shoot growth, a similar response that

chrysanthemum and tobacco had displayed when several aminoglycoside antibiotics were tested (Teixeira da Silva and Fukai 2003).

Carbohydrate source

Pathak and Dhawan (2012b) found that the growth of rootstock M7 and MM111 shoots could be supported on medium containing between 1 and 5% fructose, glucose, mannitol, sorbitol and sucrose, with the most effective concentration resulting in longest shoots and greatest productivity arising from 4% sucrose and sorbitol for M7 and MM111, respectively. However, they did not assess the in vitro rooting in response to these five carbon sources. Their shoot-related results correspond to the well-known, special carbon metabolism of apple in which the main products of photosynthesis are sucrose and sorbitol (Cheng et al. 2005), as was described by Karhu (1997) for in vitro apple culture. The choice of carbohydrate affects shoot regeneration in herbaceous plants such as chrysanthemum (Teixeira da Silva 2004) and in apple (Bahmani et al. 2009), but can also affect rooting as in peony (Wang et al. 2012), an important issue for woody plants, which tend to be difficult to root in vitro.

Gelling agents

Pereira-Netto et al. (2012b) partially replaced agar with galactomannan and managed to increase shoot productivity several fold. The use of alternative gelling agents may be a way to reduce the cost of micropropagation (Purohit et al. 2011). When 50% of agar–agar was replaced by guar gum in the medium, the diffusion speed of the blend improved, the solidity and elastic character of the agar-guar gum blend decreased, its dynamic elasticity decreased to one-third of the agar gel and shoot multiplication rate increased to 8.9, while the rate of hyperhydric shoots decreased to 12% from 59% on agar gel (Table 2; Dobránszki et al. 2011; Table 2; Fig. 2).

Rooting and acclimatization

The in vitro rooting of apple microshoots can be induced using different auxins, such as indole-3-butyric acid (IBA, 0.3-3.0 mg/l), α-naphthaleneacetic acid (NAA; 0.1–1.0 mg/l) or indole-3-acetic acid (IAA; 1 mg/l) (Table 2). A series of methods were developed and the majority of them used a two-phase protocol for in vitro rooting (De Klerk et al. 1999; reviewed in Dobránszki and Teixeira da Silva 2010). It is widely accepted that auxin is necessary for root induction, but its continued presence in the medium inhibits the development of adventitious roots (Dobránszki and Teixeira da Silva 2010). Rooting of microshoots involves a short root induction phase (up to one-week long) followed by a longer root elongation phase (several weeks). IBA was frequently applied in the root induction phase, while NAA was mainly applied or an auxin-free medium was used in the root elongation phase (reviewed in Dobránszki and Teixeira da Silva 2010). IBA has been the most commonly applied auxin for rooting in different apple genotypes, with the rooting percentage varying between 18 and 100%, depending on the concentration applied and genotype (Table 2). The success of rooting also depends on the type of explant used for rooting: 63–90% of MM106 terminal shoot tips rooted compared to 69–74% from axillary shoot tips (Mert and Soylu 2010). In some cases (Modgil and Thakur 2017; Modgil et al. 2017), the combination of IBA and NAA was effective, such as 0.3 mg/l IBA and 1 mg/l NAA for EMLA111 rootstock. Modgil et al. (2010) achieved higher rooting percentage by using IBA (81–82%) than by using NAA (71%). Amiri and Elahinia (2011a) noted that the presence of IBA was essential for rooting three rootstocks (M9, M27, MM106), but only when the concentration of cytokinins was decreased, i.e., rooting performance increased when cytokinin level was decreased or when auxin level was increased. In contrast to this finding, Mehta et al. (2014) reported 98% and 92% rooting for MM106 and B9 rootstocks on PGR-free medium. In 'Jarka' and 'Mivibe', IBA was not an effective auxin to induce roots (8% and 1% rooting of in vitro shoots, respectively), rather 1 mg/l NAA and 1 mg/l IAA successfully induced rooting in 'Jarka' and 'Mivibe' (44% and 22%, respectively) (Paprštein and Sedlák 2015). A study by Lizárraga et al. (2017) on the in vitro propagation of eight traditional apple cultivars from CIAM (Centro de Investigaciones Agrarias de Mabegondo) Germplasm Bank in Spain effectively demonstrates the strong genotype dependence of cultivars in terms of their auxin demand for in vitro adventitious rooting. They noted that two out of eight Galician apple cultivars ('Camoesa', 'Repinaldo') showed the highest rooting percentage and root number when rooting medium contained 0.1 mg/l IBA, that two cultivars ('Cacharela', 'Príncipe Grande') rooted only in the presence of exogenously added IBA while one cultivar ('Ollo Mouro') developed roots only on auxin-free medium. One cultivar ('Tres en Cinca') did not develop roots either on IBA-containing or auxin-free medium (Table 2). When two strains (EEL16010B and EEL37810) of *Rhizobia* were used, 100% rooting of Marubakaido rootstock was achieved (Muniz et al. 2013). Podwyszyńska and Cieślińska (2018) found that the use of putrescine, a polyamine, when used in combination with NAA and IBA, was able to improve the rooting and subsequent acclimatization of neo-tetraploids of four apple cultivars, 'Free Redstar', 'Gala Must', 'Pinova' and 'Redchief' (more details in Table 2).

Phloroglucinol (PG), a phenol derivative, has been successfully employed for the in vitro rooting of many plants including apple, a species that is generally considered to be difficult to root (Webster and Jones 1989; Sharma et al. 2000; Teixeira da Silva et al. 2013) and is as or even more effective than commonly used auxins (Dobránszki and Teixeira da Silva 2010). The effect of PG, however, depends on the genotype as described in earlier studies (reviewed in Dobránszki and Teixeira da Silva 2010; Teixeira da Silva et al. 2013). The combination of PG (162 mg/l) with IBA (2 mg/l) resulted in a significant increase in the rooting (from 5.5% up to 69%) of M.9 apple rootstock (James and Thurbon 1979). Webster and Jones (1989) achieved 93% rooting on in vitro shoots of M.9 rootstock when only 162 mg/l PG was added to the rooting medium, after 21 subcultures. The rooting percentage was 77% on PG-free medium, but only 69% when PG was added only to shoot-induction medium. Similarly, if PG was applied to rooting medium in the case of the Tydeman Early Worcester scion, rooting percentage increased from 50% in PG-free medium to 68% (Modgil et al. 1999). The opposite effect was detected in MM.106 rootstock when PG was applied to rooting medium because rooting percentage decreased to 53% on PG-free medium from 66%. However, PG applied to shoot proliferation medium had a favourable after-effect on rooting, increasing it to 81% (Sharma et al. 2000). Acclimatization of in vitro rooted microshoots can be achieved in a wide range of substrates such as vermicompost, coco-peat, sand and perlite, usually in a mixed ratio, while the use of arbuscular mycorrhizal fungi (AMF) has been shown to improve the acclimatization of in vitro-derived apple, mainly with endomycorrhizae from the *Glomus* genus, although, until 2010, only about half a dozen studies on apple had been published using AMF (Dobránszki and Teixeira da Silva 2010).

In 'Royal Gala' leaf segments, the ability of adventitious shoots to root depends on the choice of cytokinin used in the shoot regeneration medium previously. No rooting was achieved if TDZ or a *m*T riboside (*m*TR) was used in shoot regeneration and rooting was only 10% and 25% after regeneration of shoots using regeneration media contained BA or a BA riboside (BAR), respectively (Magyar-Tábori et al. 2011). Therefore, to achieve a high percentage of rooting (76%), a four-week-long subculture on medium supplemented with 1 mg/l BAR as the source of cytokinin was necessary prior to rooting of newly developed shoots (Magyar-Tábori et al. 2011; Table 2; Figs. 3, 4).

Shoots that had been rooted in vitro were successfully (80–100%) hardened in different substrates such as cocopeat, peat, soil: sand (3:1 v/v), a mixture of vermiculite and cocopeat, or in soil: vermiculite: perlite (1:1:1 v/v/v) (Table 2). Vettori et al. (2010) found that when a rhizobacterium (*Azospirillum brasilense* Sp245) was applied during the acclimatization phase, the biomass and stem weight of rootstock MM106 improved.

In the study of Mao et al. (2018), cuttings were placed in IBA-containing medium then transferred to medium with BA after either 3 or 7 days. The analysis of roots indicated that endogenous auxin levels decreased while the genes related to auxin signalling were suppressed as were genes related to cell cycle in adventitious root formation in the 3-day group, but not in the 7-day group. This suggests that exposure to an auxin for at least 7 days cannot reverse the fate of adventitious root formation, even if a cytokinin is added to the auxin-containing rooting medium. The apparent key to the determination of adventitious root formation lies in the timing of the application of a cytokinin, either before, during or after the initiation stage of adventitious roots, which is between days 3 and 7 in apple (De Klerk et al. 1999). *MdDRB1*, a gene coding for a double-strand RNA-binding protein in apple, controls adventitious root formation in 'Gala' (You et al. 2014).

Other biotechnological advances and applications of apple

Genetic stability of tissue cultured apple

Like other plant species, there may be genetic variation in plants derived from apple in vitro culture (Dobránszki and Teixeira da Silva 2010). Molecular markers are an effective method to verify the genetic stability of micropropagated plants (Kacar et al. 2006). The DNA amplification profile of molecular markers such as inter simple sequence repeats (ISSR) was very similar in in vitro-derived plants and mother shoots of rootstocks 'MM111' and 'Merton 793' (Pathak and Dhawan 2010, 2012a, c), and 'Gala' (Li et al. 2014, 2015). No genetic variation was found in 'Gala' plantlets derived from leaf segments (Montecelli et al. 2000) or from axillary buds of rootstock 'EMLA 111' (Gupta et al. 2009) when material was analyzed by random amplified polymorphic DNA (RAPD). Using RAPD, Viršcek-Marn et al. (1998) found no variation in leaf-derived plantlets of 'Golden Delicious Bovey' and 'Goldspur', Modgil et al. (2005) detected genetic variation in rootstock 'MM106' plants derived from axillary buds, while Caboni et al. (2000) detected polymorphism in leaf-derived 'Golden Delicious Bovey' and 'Goldspur' plants but not in plants originating from apical meristems of rootstock 'Jork 9'. Modgil et al. (2017) used RAPD to assess whether in vitro propagated M7 rootstock plantlets differed genetically from mother plants, claiming 0% polymorphism. In contrast, Noormohammadi et al. (2015), using ISSR, found significant differences between mother plants and in vitro cultured plantlets that had been grown on four different media, finding as much

as 53% or 46% polymorphism in rootstocks M7 and M9. In the same study, select lines of M9 tissue cultured plants displayed a significant increase in genome size, as assessed by flow cytometry, with variation ranging between 1.35 and 4.12 pg in a comparison of 16 samples encompassing mother plants and in vitro clones of M7 and M9. Pathak and Dhawan (2012a) found 6% polymorphism in rootstock MM106 in vitro plantlets that had been subcultured over 4 years. Unlike Noormohammadi et al. (2015), Pathak and Dhawan (2010) found no genetic variation among two-year-old tissue cultured clones of rootstock MM111 when assessed by ISSR markers, nor in rootstock Merton 793 (Pathak and Dhawan 2012c). These data indicate that genetic stability of offspring can depend on genotype, explant type and shoot multiplication method, or the number of subcultures (Dobránszki and Teixeira da Silva 2010). However, the correct choice of molecular method is also an important factor in detecting genetic polymorphism, if there is any.

Cryopreservation

Malus is an extensively studied plant genus in terms of cryopreservation of in vitro shoot tips (Wang et al. 2018a). Earlier studies mainly used two-step freezing (Katano et al. 1983; Kuo and Lineberger 1985; Stushnoff 1987) and vitrification (Niino et al. 1992; Wu et al. 1999). Over the past two decades, various cryopreservation protocols have been developed, including droplet-vitrification (Halmagyi et al. 2010a, b; Li et al. 2015; Poisson et al. 2016), DMSO-droplet (Zhao et al. 1999; Halmagyi et al. 2010b) and encapsulation-dehydration (Wu et al. 1999; Zhao et al. 1999; Paul et al. 2000; Hao et al. 2001; Kushnarenko et al. 2009; Feng et al. 2013; Li et al. 2015). Comprehensive information on cryopreservation of in vitro *Malus* plants can be found in recent comprehensive reviews (Benelli et al. 2013; Wang et al. 2018a). Therefore, this topic will not be addressed in the present review.

Virus eradication

In vitro culture is a successful method to eradicate apple viruses and detailed information on this subject before the twenty-first century can be found in several comprehensive reviews (Laimer and Barba 2011; Panattoni et al. 2013; Barba et al. 2015). Advances on this subject since 2000 reported improvements of in vitro-based methods for apple virus eradication, including thermotherapy (Paprštein et al. 2008; Hu et al. 2015b, 2017; Vivek and Modgil 2018; Wang et al. 2018b), chemotherapy (James et al. 1997; Paprštein et al. 2013; Hu et al. 2015b), combining chemotherapy with thermotherapy (Hu et al. 2015b), shoot tip culture from adventitious buds (Wang et al. 2016), cryotherapy (Li et al. 2016; Romadanova et al. 2016a; Bettoni et al. 2018) and combining thermotherapy with cryotherapy (Zhao et al. 2018) (Fig. 5).

Thermotherapy

In thermotherapy applied to virus eradication (Paprštein et al. 2008), in vitro diseased shoots of two apple cultivars ('Idared' and 'Sampion') were thermo-treated at 39 °C for 6 days. Shoot tips (1–2 mm long) with 1–2 leaf primordia (LPs) were excised from heat-treated shoots and cultured, to regenerate shoots. About 63% of 'Idared' and 44% of 'Sampion' shoot tips excised from heat-treated shoots regenerated into shoots and no shoot tips survived when thermotherapy was applied for 10 days. Four of five clones of 'Idared' were free of apple chlorotic leaf spot virus (ACLV), apple stem pitting virus (ASPV) and apple stem grooving virus (ASGV), but no virus-free plants were found in 'Sampion', as analyzed by reverse transcription-polymerase chain reaction (RT-PCR). Hu et al. (2017) reported a thermotherapy procedure for apple virus eradication. Pot-grown diseased plants were heat-treated at 38 °C for 30 days. Shoot segments (1 cm long) were excised from heat-treated plants and surface-disinfected, followed by excision of the shoot tip (1 mm long) and cultured for shoot regeneration. With this procedure, the total level of survival and virus-free frequency (ACLSV, ApMV, ASPV and ASGV, analyzed by RT-PCR) were about 30% and 25%, respectively, across the five apple cultivars including 'Yanfu 9', 'Xinyanfu 3', 'Huafu', 'Apple 123' and 'Zhengzhou no. 5'. In the study of Vivek and Modgil (2018), infected shoots were exposed to 37–40 °C for 4 weeks using hot air. Shoot tips (0.3–0.5 mm long) containing 1–4 LPs were excised from heat-treated shoots that had been surface-disinfected and cultured for shoot regeneration. This procedure resulted in about 26–32% shoot regeneration and efficiently eradicated ACLSV, ASPV, ApMV and ASGV from apple 'Oregon Spur-II'.

Chemotherapy

Paprštein et al. (2013) reported a two-cycle chemotherapy for apple virus eradication. Infected in vitro shoots (5–10 mm long) of apple 'Fragrance' were cultured for the first cycle (4 weeks) on medium containing 20 mg/l ribavirin then to medium containing 100 mg/l ribavirin for the second cycle (4 weeks). Shoot tips (3 mm long) containing 2–3 LPs were excised from chemo-treated shoots, and 100% of shoot tips developed shoots without any visible variation. About 35% and 65% of plantlets were free of ASPV and ASGV, but all plantlets were still infected with ACLSV, following the first cycle of chemotherapy. About 76% of plantlets were free of ACLSV, following the second cycle of chemotherapy. Ribavirin concentration higher than 40 mg/l was found to be phytotoxic to in vitro apple cultures (James et al. 1997; Hu

et al. 2015). Genotype-specific responses to ribavirin concentration may be responsible for such differences (Paprštein et al. 2013).

Combining chemotherapy with thermotherapy

Combining chemotherapy with thermotherapy was recently reported to be more efficient than a single use of either method for apple virus eradication (Hu et al. 2015b). After virus-infected in vitro shoots of apple 'Xinhongjiangjun' were cultured for 40 days on medium containing 25 mg/l ribavirin and then subjected to heat treatment at 36 °C for 20 days, 1.0 mm-long shoot tips were excised and cultured, with ~ 90% shoot survival. Shoot regeneration levels and total virus elimination frequencies (ACLSV, ASPV and ASGV) were about 60% and 100%, and 94% and 100% in terminal and axillary shoot tips, respectively. These data indicate that virus eradication was more efficient when axillary shoot tips were used than terminal ones.

Culture of shoot tips from adventitious buds

Wang et al. (2016) attempted to eradicate ASPV and ASGV by culturing shoot tips derived from adventitious buds that had been induced from leaf segments. In that study, 0.24 cm^2 leaf segments were excised from young terminal leaves of one-month-old virus-infected in vitro apple 'Gala' shoots and used to induce adventitious buds. Shoot tips (0.4 mm long) with four LPs were excised from 4-week-old adventitious buds and cultured for shoot regeneration. This procedure produced 82% shoot regeneration and 95–100% ASPV-free frequencies. However, none of the plants regenerated were free of ASGV. In many previous studies on shoot tip culture for apple virus eradication, only a single shoot tip could be harvested from each shoot from a stock plant (O'Herlihy et al. 2003; Paprštein et al. 2008; Sedlak et al. 2011; Li et al. 2016). In the study of Wang et al. (2016), three leaves from one stock shoot were used for the induction of adventitious buds and produced at least 24 buds, resulting in a much higher efficiency of bud production than previous studies (O'Herlihy et al. 2003; Paprštein et al. 2008; Sedlak et al. 2011; Li et al. 2016).

Cryotherapy

Shoot tip cryotherapy can be used to eradicate viruses from apple. In a study by Romadanova et al. (2016b), shoot tips were excised from cold-hardened (22 °C for 8 h/day, then − 1 °C for 16 h/night, for 3 weeks) in vitro shoots and subjected to vitrification cryotherapy, as described by Reed and Yu (1995). Virus-free frequencies were 60–100% for ACLSV in six of the nine genotypes, 25–67% for ASPV in five of the six genotypes, 50% for ASGV in one genotype and 89% for ApMV in one genotype. Bettoni et al. (2018) successfully eradicated ACLSV, ASPV and ASGV from infected in vitro shoots of apple 'Marubakaido'. Using the Feng et al. (2013) encapsulation-dehydration cryotherapy method, they achieved 72% shoot regrowth of cryo-treated shoot tips, 100% virus-free frequencies for ACLSV and ASPV and 90% for ASGV. Li et al. (2016) reported that encapsulation-dehydration cryotherapy could eradicate viruses from apple rootstocks 'M9' and 'M26'. Shoot tips (1.5 mm long) with 3–4 LPs were excised from 1-month-old in vitro shoots and were cryo-treated using the encapsulation-dehydration method indicated by Feng et al. (2013). After cryotherapy, during post-culture, about 74% of 'M9' and 75% of 'M26' shoot tips regenerated into shoots after 8 weeks. About 80–85% of the regenerants survived, but none of them were free of ASGV in the two apple rootstocks. Histology indicated that many cells in the apical dome (AD) survived as did some cells in LPs 1–3, but cells in LP 4 and older tissue were killed or damaged following cryotherapy (Li et al. 2016). Immunohistological virus localization did not detect ASPV in the AD or in LPs 1–3, rather in LP4 and older tissue, bud found ASGV in the AD and in all LPs (Li et al. 2016). These data explained why shoot tip cryotherapy could efficiently eradicate ASPV but failed to eradicate ASGV. Although shoot tip culture produced higher rates of shoot regeneration (90% for 'M9' and 92% for 'M26') than shoot tip cryotherapy, none of the regenerants were free of ASPV in 1.5 mm shoot tips (Li et al. 2016). It is well-known that the size of shoot tips is critical for virus eradication in shoot tip culture (Laimer and Barba 2011; Panattoni et al. 2013; Barba et al. 2015). However, similarly high virus-free frequencies were produced in shoot tip cryotherapy, regardless of the size of shoot tips, thus enabling much easier handling of shoot tip cryotherapy than shoot tip culture (Wang and Valkonen 2009; Wang et al. 2009, 2014).

Combining thermotherapy with cryotherapy

Cryotherapy failed to eradicate viruses that are able to infect meristematic cells of shoot tips (Wang et al. 2008; Li et al. 2016). More recently, Zhao et al. (2018) reported thermotherapy followed by shoot tip cryotherapy for efficient eradication of ASGV. In that study, an alternating temperature (36 °C/32 °C, day/night) was applied to treat ASGV-infected in vitro shoots for 4 weeks. Shoot tips (1.5 mm long) containing 4 to 5 LPs were taken from heat-treated stock shoots and used for droplet-vitrification cryotherapy, according to Li et al. (2015). All shoots following thermotherapy survived. About 47% of shoot tips following thermotherapy and cryotherapy regenerated into shoots, and all regenerated shoots were free of ASGV. When applied to three other apple cultivars ('Fuji', 'Ruixue' and 'Nongguo 25')

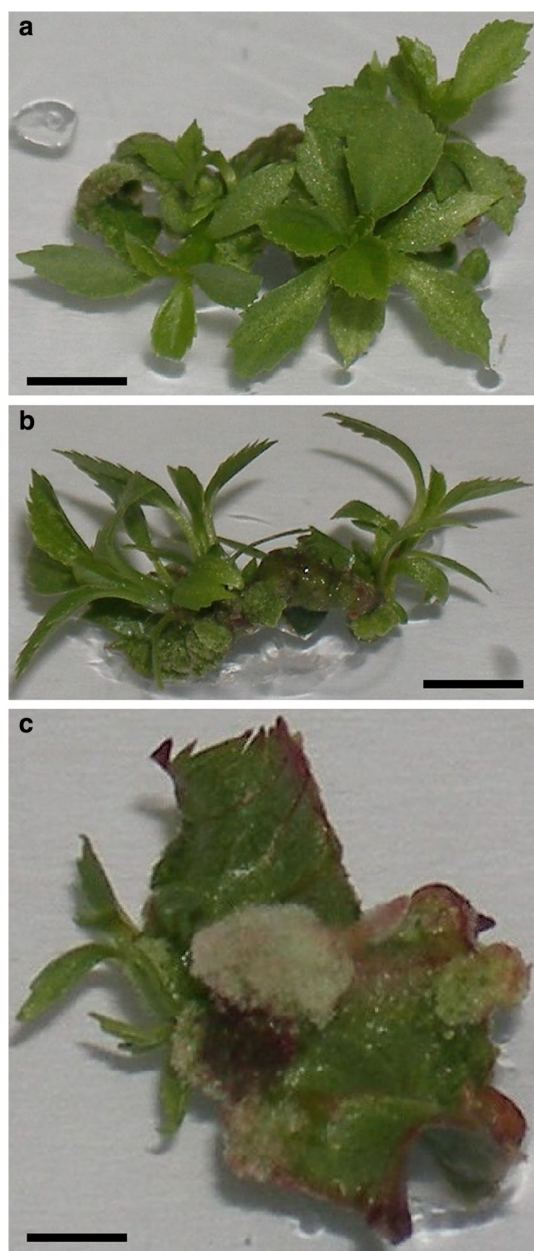

Fig. 1 Adventitious shoot regeneration of 'Royal Gala' on MS medium supplemented with (**a**) 5.0 mg/l BA, 5.0 mg/l BAR (**b**) or 0.5 mg/l TDZ (**c**) after 7 weeks (each). Scale bars: **a** 4.0 mm; **b** 5.0 mm; **c** 2.5 mm. BA, N^6-benzyladenine; BAR, N^6-benzyladenine riboside; MS, Murashige and Skoog (1962) medium; TDZ, thidiazuron. Photos A and B were presented as a poster (Magyar-Tábori et al. (2010) Post-effects of cytokinins on the rooting capacity of adventitious apple shoots. In: XXVIII International Horticultural Congress on Science and Horticulture for People: International Symposium on Environmental, Edaphic, and Genetic Factors Affecting Plants, Seeds and Turfgrass. Lisbon, Portugal, 2010. 08. 22–2010. 08. 27. Leuven: ISHS International Society for Horticultural Science

and apple rootstock 'M9', this procedure produced shoot regeneration levels ranging from 33 to 76% and ASGV-free frequencies ranging from 30 to 100% (Zhao et al. 2018). These results suggest that combining thermotherapy with cryotherapy may be the most efficient method reported thus far for plant virus eradication (Wang et al. 2008; Zhao et al. 2018).

Induction of polyploidization in vitro

Polyploids can be induced by two mechanisms, mitotic polyploidization, which is based on the doubling of somatic tissues and meiotic polyploidization, which generates 2n gametes (Ramsey and Schemske 1998), which can be used immediately in crossing experiments, thereby reducing the breeding process by one generation. Murashige and Nakano (1966) reported the first in vitro polyploidization experiment in tobacco. In vitro plant propagation offers a great opportunity to improve the efficiency of chromosome doubling since in vitro cultures offer a more contained and standardized environment than greenhouse treatments. Some studies reported spontaneous chromosome doubling as a side effect of in vitro multiplication (Barow and Jovtchev 2007; Chen et al. 2009; Meyer et al. 2009).

Podwyszyńska et al. (2016) examined the ploidy of 70 apple genotypes and found that polyploids had larger vegetative (stomata, leaves) and reproductive (flowers) characters, and fruits, suggesting that there is practical value to inducing polyploids in vitro. The polyploidization of apple in vitro has been achieved with colchicine and oryzalin (Bartish et al. 1999; Ou et al. 2008), but given the toxicity associated with the former (Spiller 2014), several polyploidization agents were compared by Podwyszyńska et al. (2017), namely colchicine, trifluralin, oryzalin or amiprophos methyl (APM). After 8 weeks of culture, tetraploids could be obtained in 'Gala Must' in response to 125 mg/l colchicine, in 'Pinova' in response to 125 or 250 mg/l colchicine, 100 mg/l trifluralin and 5 or 10 mg/l APM, in 'Redchief' in response to 125 or 250 mg/l colchicine, 50 mg/l trifluralin, 5 mg/l oryzalin and 5 mg/l APM, and in 'Sander' in response to 125 mg/l colchicine and 50 mg/l trifluralin. In that study, numerous mixoploids were also obtained when shoot explants were used for polyploidisation, but only sporadic occurrence of mixoploids was observed when leaf explants were used. The kind of antimitotic agent and its concentration as well as cultivar influenced explant viability and shoot production (Table 2).

In vitro assays for testing abiotic or biotic stress resistance

Apple in vitro culture has been used to test for biotic stress resistance/tolerance, including to diseases, since the 1980's. Similarly, resistance/tolerance to drought and salt stress were studied in in vitro apple shoot cultures (reviewed in Dobránszki and Teixeira da Silva 2010). Bahmani et al. (2012) noted

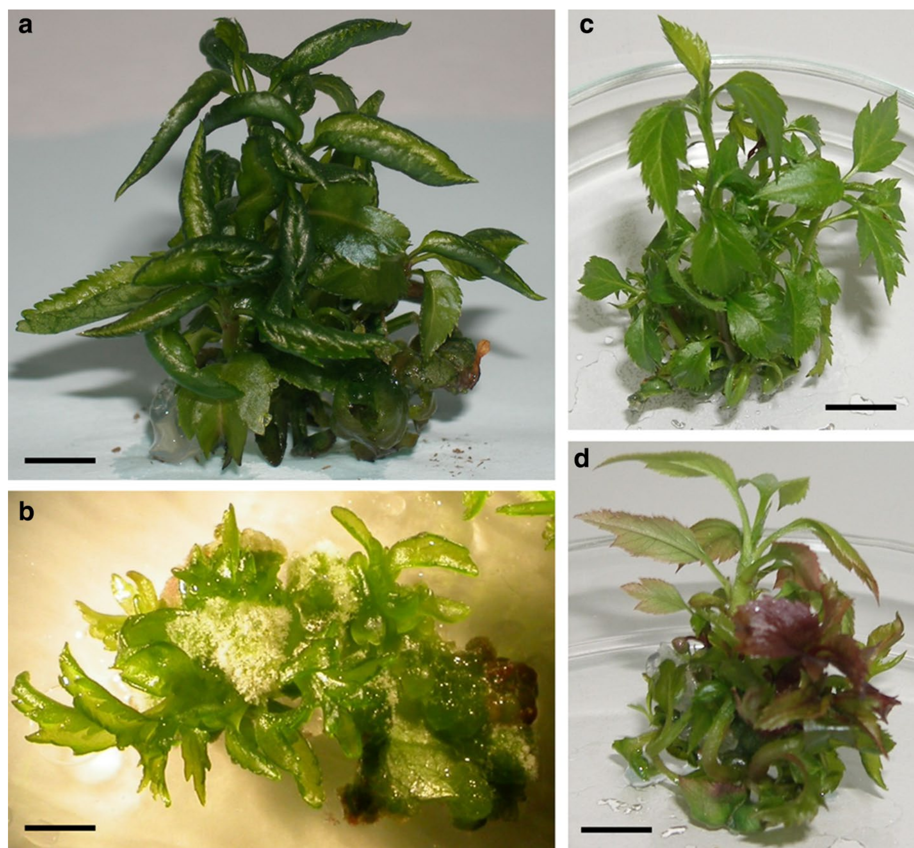

Fig. 2 Incidence of hyperhydricity in vitro and effect of gelling agent on shoot regeneration. **a** Hyperhydric shoots of apple rootstock M26 cultured on MS medium with 0.5 mg/l BA and 0.1 mg/l IBA. **b** Hyperhydric adventitious shoots of 'Royal Gala' regenerated on MS medium with 0.5 mg/l TDZ, also inducing some callus formation. 'Galaxy' in vitro shoots cultured on MS medium with 5.6 g/l agar (**c**) or 2.5 g/l phytagel (**d**). Notice the accumulation of anthocyanins in in vitro leaves when shoots are cultured in phytagel. Scale bars: **a** 4 mm; **b** 1 mm; **c** 10 mm; **d** 8 mm

that a high salt concentration (> 100 mM NaCl) inhibited the growth of MM106 in vitro shoots and decreased the number of regenerated shoots, rooting percentage and root number while a low NaCl concentration (20 mM) significantly increased shoot length and fresh weight. In vitro apple callus cultures serve as excellent systems for testing abiotic stress and for assessing the molecular mechanisms underlying this process, through an understanding of gene expression and function. Yao et al. (2011) used apple 'Orin' in vitro plantlets, callus and suspension cultures to understand the role of the *MdcyMDH* (*Md* = *Malus* × *domestica*) gene, which codes for cytosolic NAD-dependent malate dehydrogenase, a key enzyme in malic acid synthesis, in growth and responses to two abiotic stresses, salinity and cold. The latter is important to understand since apple and other *Rosaceae* plants accumulate dehydrin proteins in response to cold stress (Haimi et al. 2017). An et al. (2016) scratched a part of the surface area of leaf margins of 'Gala' in vitro plantlets with a blade to induce wounding. In a separate stress treatment, tissues (presumably leaves) were transferred to medium containing 100 mM methyl jasmonate. Both stress treatments activated a homolog of the *Arabidopsis thaliana MYC2* gene, *MdMYC2*, which encodes a transcription factor, and increased its expression levels, suggesting the importance of this gene in jasmonic acid (JA) signalling. MYC2 is mainly a positive regulator of abscisic acid signalling (Lorenzo et al. 2004). An et al. (2016) also used 10 day-old 'Orin' callus, which was transformed with *Agrobacterium tumefaciens*. Both over-expression and antisense constructs were introduced and callus in the former were red, having genes related to anthocyanin biosynthesis over-expressed while the latter had under-expressed genes, indicating that *MdMYC2* plays a role in JA signalling. Meng et al. (2016) found that 'Orin' callus grown on solid medium or liquid medium (i.e., water-logging stress) showed a differential expression of 34 *WRKY* genes, which code for transcription factors, and that the expression level of 127 *MdWRKY* genes had a much higher level of expression in callus (as much as 2- to 10-fold) than in shoot tips, leaves and fruit of 'G.41' rootstock trees that had been micropropagated and grown in a greenhouse. WRKY proteins regulate gene expression and are involved in cell signalling and JA-mediated plant immunity in response to pathogens (biotic stress signalling) and abiotic stress (salinity, drought, heat, freezing) signalling (Eulgem and Somssich, 2007; Phukan et al. 2016). Hu et al. (2016) found that the *MdSOS2L1* (coding for a calcineurin B-like protein (CBL)-interacting protein kinase, MdCIPK24-LIKE1) gene positively improved salt tolerance in apple 'Gala' by increasing the level of antioxidant metabolites such as procyanidin and malate after interaction with three calcineurin B-like proteins, MdCBL1, MdCBL4 and MdCBL10. Meng et al. (2018) developed two antisense

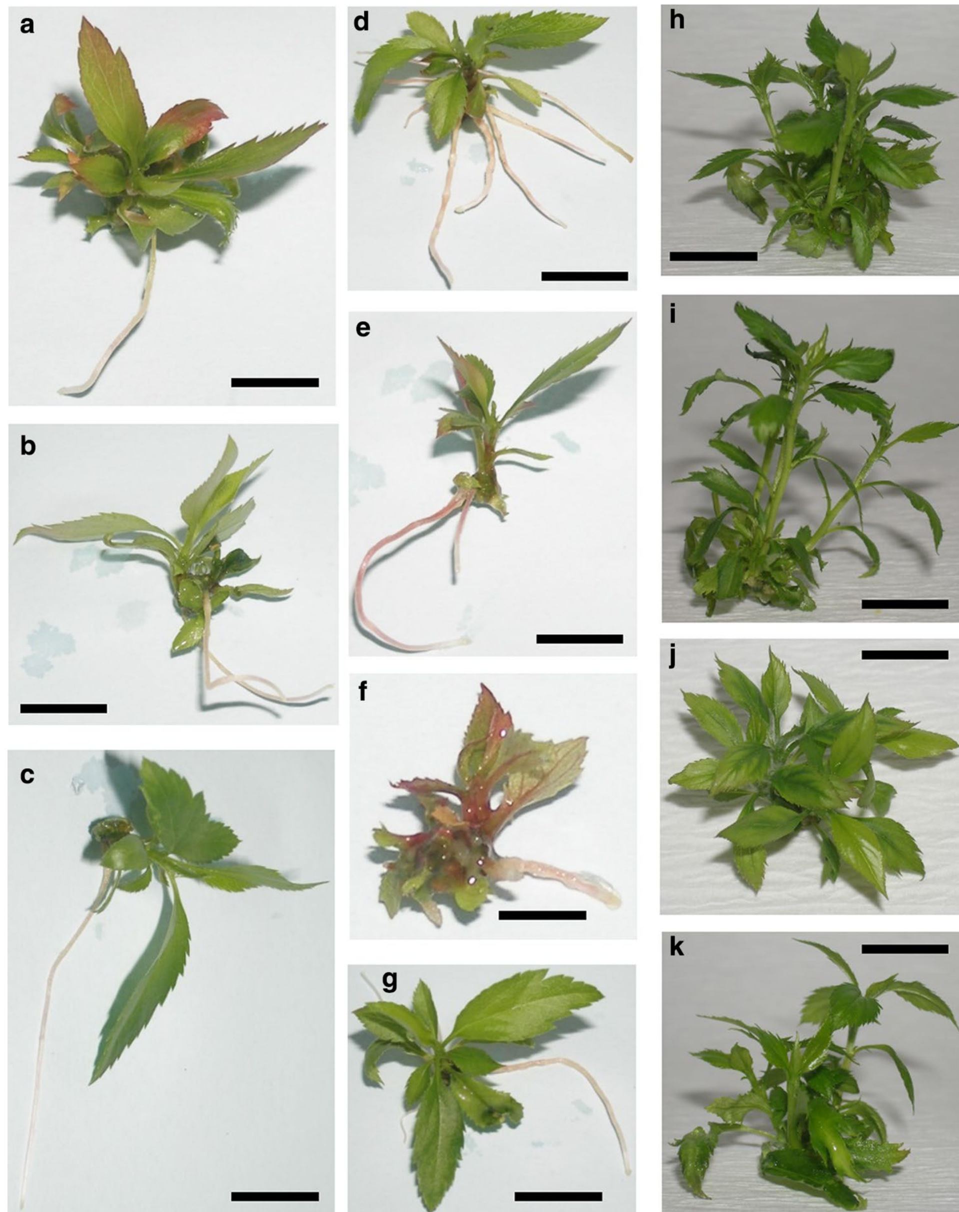

Fig. 3 Rooting of adventitious 'Royal Gala' shoots. Shoots were regenerated on different media (*A* = MS + 5.0 mg/l BA; *B* = MS + 5.0 mg/l BAR; *C* = MS + 6.5 mg/l *m*TR; *D* = MS + 5.0 mg/l BA; *E* = MS + 5.0 mg/l BAR; *F* = MS + 0.5 mg/l TDZ; *G* = MS + 6.5 mg/l *m*TR; *H* = MS + 5.0 mg/l BA; *I* = MS + 5.0 mg/l BAR; *J* = MS + 0.5 mg/l TDZ; *K* = MS + 6.5 mg/l *m*TR) and photographed 1 week after subculture on MS medium in three rooting trials: I (*A* − C) = 0.5 mg/l BAR, 0.3 mg/l IBA, and 0.2 mg/l GA_3; II (*D* − *G*) = 0.5 mg/l BAR, 0.3 mg/l IBA, and 0.5 mg/l GA_3; III (*H* − *K*) = newly developed shoots after 4-week sub-culture on medium 1.0 mg/l BAR, 0.3 mg/l IBA, and 0.2 mg/l GA_3, before rooting process. Scale bars: **a** 5.0 mm; **b** 7.0 mm; **c** 5.0 mm; **d** 14 mm; **e** 10 mm; **f** 4 mm; **g** 10 mm; **h** 7 mm; **i** 7 mm; **j** 7 mm; **k** 7 mm. *BA* N^6-benzyladenine; *BAR* N^6-benzyladenine riboside; *IBA* indole-3-butyric acid; GA_3, gibberellic acid, *MS* Murashige and Skoog (1962) medium, *TDZ* thidiazuron; mTR *meta*-topolin riboside. Root induction was performed for 1 week on medium containing MS salts (half-strength; ½MS), 100 mg/l *myo*-inositol, 0.5 mg/l vitamin B_1, 2% sucrose, 0.7% agar–agar and 2.0 mg/l IBA. Root elongation took 2 weeks on ½MS medium with 50 mg/l *myo*-inositol, 3% sucrose, 2.0 ml/l Wuxal®, and 0.7% agar–agar (Magyar-Tábori et al., 2011). Photos **d**, **e**, **f** and **g** were presented as a poster (Magyar-Tábori et al. (2010) Post-effects of cytokinins on the rooting capacity of adventitious apple shoots. In: XXVIII International Horticultural Congress on Science and Horticulture for People: International Symposium on Environmental, Edaphic, and Genetic Factors Affecting Plants, Seeds and Turfgrass. Lisbon, Portugal, 2010. 08. 22–2010. 08. 27. Leuven: ISHS International Society for Horticultural Science

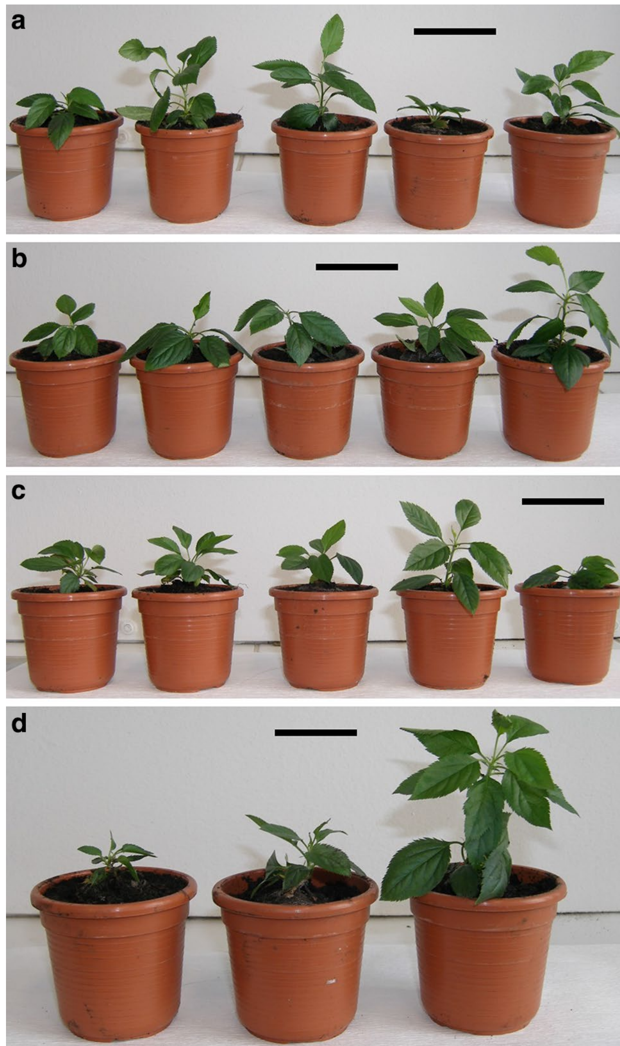

Fig. 4 Acclimatization of 'Royal Gala' adventitious shoots that were regenerated on different media (MS + 5.0 mg/l BA (**a**), 5.0 mg/l BAR (**b**), 0.5 mg/l TDZ (**c**) or 6.5 mg/l *m*TR (**d**)) then multiplied on shoot multiplication medium (1.0 mg/l BAR, 0.3 mg/l IBA, and 0.2 mg/l GA_3). Newly developed shoots were rooted in root induction medium (½MS + 2.0 mg/l IBA) and roots were elongated on PGR-free ½MS medium. The acclimatization process was based on the method reported by Bolar et al. (1998). Plantlets were planted into Jiffy-7® pellets which were previously soaked in 1/10 MS solution containing 1.5 ml/l Previcur 607 SL, then placed into a VEG-box and sealed completely to ensure high relative humidity. After 3 weeks, plants were potted in a mixture of perlite and peat (1:3, v/v) and covered with plastic bags. When new leaves appeared, a small hole was made in each bag then gradually increased. Plants were grown under a 16-h photoperiod (105 µmol m^{-2} s^{-1}) and at 22 + 2 °C throughout the entire acclimatization period. Plants shown are 3 months old. Scale bars: **a** 6 cm; **b** 5.7 cm; **c** 6 cm; **d** 3.9 cm. BA, N^6-benzyladenine; BAR, N^6-benzyladenine riboside; *IBA* indole-3-butyric acid; *GA_3* gibberellic acid; *MS* Murashige and Skoog (1962) medium; *PGR* plant growth regulator; *TDZ* thidiazuron; mTR meta-topolin riboside

lines (A4, A10) of apple 'Greensleeves' and 'Orin' in which the *ALDOSE-6-PHOSPHATE REDUCTASE* (*A6PR*) gene was suppressed, making plants more susceptible to the fungus *Alternaria alternata*. A6PR oversees the conversion of glucose 6-phosphate to sorbitol 6-phosphate and sorbitol results from the dephosphorylation of sorbitol 6-phosphate by sorbitol-6-phosphate phosphatase. Using tissue-cultured plants, when one *nucleotide-binding/leucine-rich-repeat* (*NLR*) gene, *MdNLR16*, was overexpressed in the antisense lines, resistance to *A. alternata* increased.

Bioassays and biofertilizers

Apple 'Royal Delicious' was used as a novel bioassay to assess the amount of browning and polyphenol oxidase activity in response to bacterially synthesized volatile organic compounds (Gopinath et al. 2015). A γ-proteobacterium (*Luteibacter rhizovicinus* MIMR1) that had originally been isolated from contaminated in vitro cultures of 'Golden Delicious' (Piagnani et al. 2007) produced high levels of IAA (127 mg/l), inducing roots in barley (*Hordeum vulgare* L.) tissue cultures, suggesting that this apple-derived plant growth-promoting bacterium could serve as a biofertilizer (Guglielmetti et al. 2013). Muniz et al. (2013) isolated 20 strains of *Rhizobium* from the root nodules of a legume, *Adesmia latifolia*, and found that they produced 13–51 mg/l of IAA, the highest by strain EEL16110, although another two dead strains, EEL16010B and EEL37810, induced rooting in 100% of in vitro shoots of Marubakaido apple rootstock, equivalent to when 1 mg/l IAA, was used. Using the protocol developed by Ciccoti et al. (2008), Giorno et al. (2013) and Guerriero et al. (2013) developed an in vitro assay for 'Golden Delicious' to better understand the pathogenesis-related genes involved in infection of plantlets by '*Candidatus* Phytoplasma mali'. Giorno et al. (2013) found that the content of glucose, fructose and sorbitol was lower while the content of sucrose and starch was higher. Guerriero et al. (2013) found that cellulose and callose synthase genes involved in cell wall biosynthesis were up-regulated. Verardo et al. (2017) used callus cultures derived from pulp tissue for producing important secondary metabolites such as phloridzin and triterpenic acids.

Conclusions

A key objective of apple tissue cultures is to multiply pathogen-free clones, as evidenced in the past decade by extensive studies about virus eradication using several methods. In vitro techniques are very costly since skilled manual labour is needed, as is specific equipment and the high price of plant material, so the effectiveness of the in vitro protocol should always be optimized, and improved, where possible. Several technological developments that have been reported in the past decade include the application of additives such as crab chitosan, phloroglucinol and methods such as tTCls

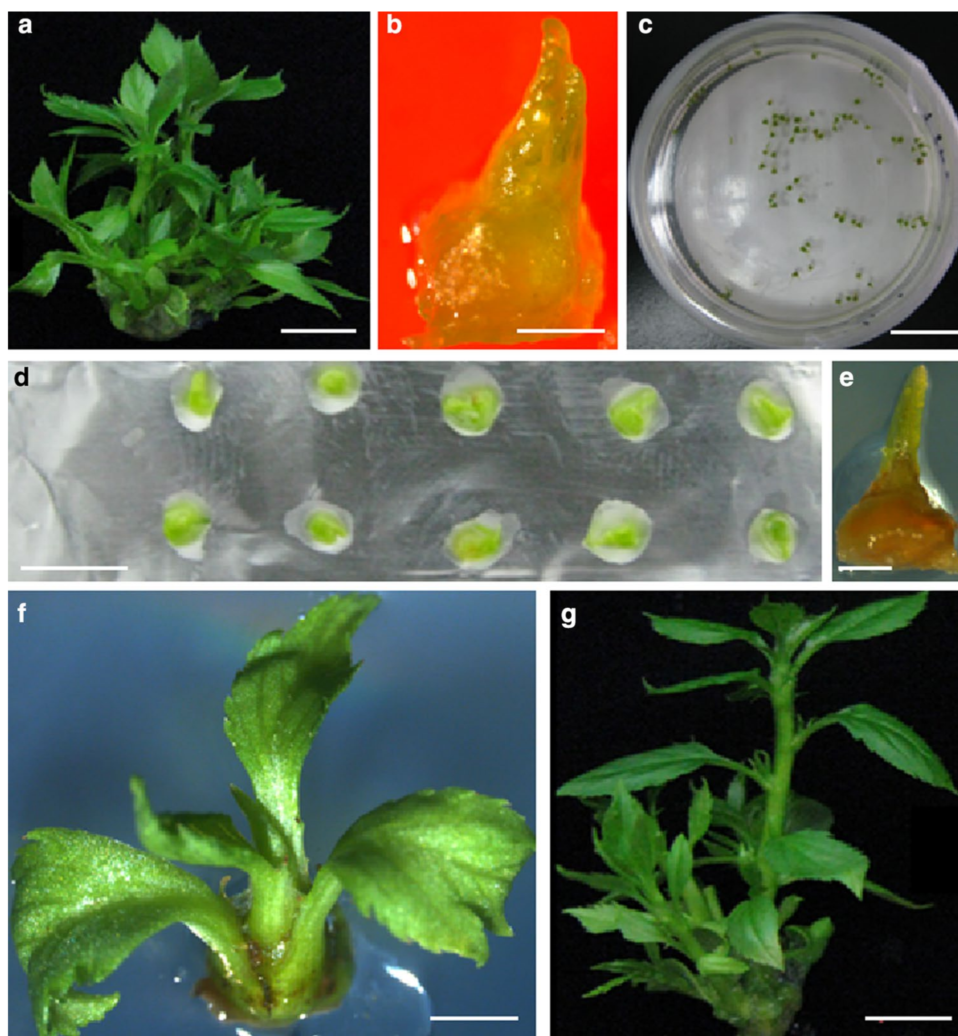

Fig. 5 Droplet-vitrification cryotherapy for eradication of apple stem pitting virus (ASPV) from in vitro shoots of apple 'Gala'. Infected in vitro stock shoots (**a**). Shoot tip excised from infected in vitro shoots and used for cryotherapy (**b**). Exposure of shoot tips to plant vitrification solution 2 (PVS2) (**c**). PVS2 droplets on aluminum foil strips (**d**). Surviving shoot tips after 3 weeks of post-culture following cryotherapy (**e**). Shoot regrowth after 6 weeks of post-culture following cryotherapy (**f**). Elongated shoot after 12 weeks of post-culture following cryotherapy (**g**). These shoots showed a negative response to RT-PCR for ASPV detection, and will be tested again for the virus after established in a net-proof greenhouse for at least 10 months. Bars: 1 cm (**a**, **c**, **d**, **f**, **g**), 1 mm (**b**, **e**)

or alternative illumination (red light) in a bid to improve the growth response and productivity of in vitro plants. Growth retardants and antibiotics were also tested with varying success during shoot multiplication, while microorganisms such as bacteria and fungi were shown to enhance the acclimatization of apple.

Micropropagation of pathogen-free in vitro apple shoot cultures is a vegetative process and clones have to be true-to-type, although epigenetic modifications can appear easily, possibly due to large-scale transposable elements in the apple genome. Tissue cultures provide a very good method and basis for physiological and genetic studies in apple, and the genes controlling adventitious root formation, genes involved in responses to abiotic/biotic stress and the genetic stability of in vitro cultured apples have all been summarized in this review.

The primary role of cytokinins in adventitious shoot regeneration has still room for improvement because the most effective type and level of cytokinin will depend on the apple genotype and ineffective concentrations might lie very close to effective concentrations, so a small deviation from the optimum may cause an adverse effect in vitro. Cytokinins can also affect the functionality of the photosynthetic apparatus. Similarly, the effect of auxins in the rooting process has also been extensively studied in apple. Important interactions were detected in in vitro experiments but the responses to a wide range of treatments or conditions were often genotype-dependent.

Biotechnological methods also allow the storage of germplasm and help breeding work by methods such us polyploidization. There is very little available literature about somatic embryogenesis in apple in the past decade. In fact, somatic embryogenesis in *Malus* spp. is lacking.

Author contribution statement All authors are co-corresponding authors. All authors contributed to the intellectual discussion underlying this paper, literature exploration, writing, reviews and editing, and accept equal responsibility for the content.

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Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest of relevance to this topic.

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