A New Perspective on Cryotherapy: Pathogen Elimination Using Plant Shoot Apical Meristem via Cryogenic Techniques

Ergun Kaya, Selin Galatali, Sevinc Guldag, and Onur Celik

Abstract

Plant pathogens cause different diseases on crops and industrial plant species that result in economic losses. Pathogen-free plant material has usually been obtained by traditional procedures such as meristem culture, thermotherapy, and chemotherapy. However, there are many limitations of these procedures such as mechanical challenges of meristem excision and low regeneration rate, low resistance to high temperatures, phytotoxicity, and mutagenic effects of the chemicals used in the procedures. Cryotherapy is a newly developed biotechnological tool that has been very effective in virus elimination from economically important plant species. This tool has overcome the abovementioned limitations. This chapter aims to highlight the importance of the cryogenic procedures (vitrification, encapsulation-vitrification, droplet vitrification, two-step freezing, dehydration, encapsulation-dehydration) in order to generate virus-free germplasm.

Key words Plant biotechnology, Dehydration, Liquid nitrogen, Plant viruses, Vitrification

1 Introduction

Plant pathogens such as bacteria, fungus, and viruses cause harmful diseases on plants, and some of them can cause direct and/or indirect losses of billions of dollars every year. Plant viruses causing diseases on plants can destroy crops and industrial plant species; therefore, they have negative effect on food security and crop industry [1, 2]. Chemical therapies or physical treatments are not sufficient to be directly controlled of them. There are many different traditional ways to prevent for virus contaminations such as biological and chemical control of the vector being often an insect transmitting viruses, growing virus resistant crop varieties being made via genetic transformations, using virus-free planting material and the protection of disease placement in fields where viruses do not yet occur [3, 4].

Muhammad Naseem and Thomas Dandekar (eds.), *Plant Stem Cells: Methods and Protocols*, Methods in Molecular Biology, vol. 2094, https://doi.org/10.1007/978-1-0716-0183-9_15,

Pathogen-free (especially virus-free) plant material has generally been obtained using meristem culture and/or thermotherapy methods [5, 6]. For successful viral elimination via meristem culture, it is usually necessary to excise shoot tips that are 0.1–0.5 mm (depend on plant species) in size [7, 8]. There are some limitations for virus elimination using these methods such as mechanical challenges of meristem excision and low regeneration rate [9]. Thermotherapy method connected with meristem culture is also a difficult process requiring specific conditions such as virus-specific treatments and specific equipment. However, all of viruses cannot be eliminated via thermotherapy, and sometimes the infected plants are not resistant to high temperatures [10].

An alternative method is chemotherapy for plant virus elimination. This method based on the usage of antiviral chemicals associated with thermotherapy or meristem culture was successfully used for virus elimination of some infected plants such as apple [10]. The antiviral chemicals such as quercetin and ribavirin prevent virus nucleic acid synthesis (replication), and thus the virus concentration cannot increase in infected plants [11]. However, phytotoxicity and mutagenic effects of these antiviral chemicals are reported for some plant species and/or cultivar [12].

Because of some limitations of the traditional methods, it is beneficial to develop different kind of efficient biotechnological procedures for obtaining virus-free plants. Cryotherapy—newly developed biotechnological tool—has been very effective for virus elimination for economical important plant species such as sweet potato [13], strawberry [14], raspberry [15], potato [16], grape [17], and apple [18].

2 Cryotherapy

Pathogen-free (virus-free) plant materials are the most important for agricultural and horticultural crop productivity and for ornamental plant quality [19]. The plants especially vegetatively propagated are inclined to pathogen infections, which are transported to new plants in infected steels, tubers, roots, and other vegetative parts of plants. Conservation of plant genetic resources is one of the most important tools for breeding new species and plant cultivars for future requirements. However, germplasm collections need to be established from pathogen-free species and cultivars. Therefore, development of efficient methods for pathogen elimination is a critical point of gene banks for maintenance of their collections [20].

Cryotherapy is a new method used for pathogen elimination from infected plant shoot tips [13]. There are many reports for successfully pathogen elimination from plants infected by different kind of virus and bacteria like pathogens via cryotherapy of shoot

Table 1
Different cryotherapy treatments for pathogen elimination for vegetatively propagated and economically important plant species [21]

Plant	Pathogen	Cryotherapy method	Reference
Banana (Musa)	*Cucumber mosaic virus* (CMV)/ *Banana streak virus* (BSV)	Vitrification	[22]
Beijing lemon, mandarin, pummelo, sweet orange (*Citrus*)	*Huanglongbing bacterium* (HLB)	Vitrification	[23]
Grapevine (*Vitis vinifera*)	*Grapevine virus* (GVA)	Encapsulation-vitrification	[24]
Grapevine (*Vitis vinifera*)	*Grapevine virus* (GVA)	Encapsulation-dehydration	[25]
Potato (*Solanum tuberosum*)	*Potato leaf roll virus* (PLRV)/*Potato virus* Y (PVY)	Encapsulation-vitrification Droplet vitrification	[26]
Prunus hybrid	*Plum pox potyvirus* (PPV)	Vitrification	[27]
Raspberry (*Rubus idaeus*)	*Raspberry bushy dwarf virus* (RBDV)	Thermotherapy followed by cryotherapy (Encapsulation-vitrification)	[8]
Sweet potato (*Ipomoea batatas*)	*Sweet potato chlorotic stunt virus* (SPCSV)/*Sweet potato feathery mottle virus* (SPFMV)	Encapsulation-vitrification	[13]
Yam (*Dioscorea opposita*)	*Yam mosaic virus* (YMV)	Encapsulation-dehydration	[28]

tips, such as potato [16] and sweet potato [13]. Cryotherapy can be used for a wide range of plant species and cultivars because it is based on plant cryopreservation methods being available for many additional vegetatively propagated and economically important plant species (Table 1).

In cryotherapy technique, infected plant cells are eliminated by the fatal efficacy of liquid nitrogen (−196 °C, the ultralow temperature) and/or following warming; mechanical removal is not required. After cryotherapy treatments, shoot tip regeneration rates might be lower than traditional meristem culture treatments; however, larger shoot tips can be used for easier excision, and obtained pathogen-free materials are much more via cryotherapy [13].

2.1 Cryogenic Procedures

Cryotherapy involving physical dehydration and chemical vitrification treatments of shoot tips are not needed special equipment in addition to that used in a basic plant tissue culture laboratory.

Liquid nitrogen being the main material for cryotherapy is usually easy available in laboratories found in almost all countries. Cryogenic treatments are divided into two major groups including traditional techniques (classical slow cooling) and one-step freezing techniques [29].

2.1.1 Traditional Techniques: Classical Slow Cooling

Classical slow cooling methods cover two-step cooling down to a specified prefreezing temperature (−40 °C), followed by direct immersion in liquid nitrogen. During reduction of temperature by slow cooling, cells and the other medium firstly supercool, followed by ice formation in the medium [30]. The plant cell membrane behaves as a physical barrier and prevents the fatal ice nucleation from the cell inner, and the cells stay unfrozen but supercooled. As the temperature is further reduced, an increasing volume of the extracellular solution is transformed into ice form, in this way resulting in the concentration of intracellular solutes. whereby the plant cells continue supercooled and hydrated vapor pressure of them passes over that of the frosted other compartment, plant cells balance via loss of water to other ice formation. Before the intracellular components solidify, different amounts of cell water content will be removed depending upon the prefreezing temperature and the cooling rate. In ideal conditions, big volume or almost all intracellular water causing ice nucleation is removed, in this way decreasing or preventing fatal intracellular ice nucleation during liquid nitrogen immersion. But sometimes dehydration causing more intense ice nucleation can induce a series of damaging cases due to intracellular salt concentration and modifies in the plant cell membrane [31]. Rewarming process should be as fast as possible to prevent the fatal re-ice nucleation cases in which ice reforms at a thermodynamically suitable, bigger, and more harm ice nucleation form [30].

Classical slow cooling processes contain different consecutive steps: cold hardening (pre-cold culture at +4 °C) and sucrose preculture (on preculture medium supplemented with different concentrations of sucrose) of plant materials, cryoprotection (chemical vitrification or physical dehydration), slow cooling (0.5–1 °C/min) to transferring a prefreezing temperature (approximately −80 °C), immersion of samples rapidly in liquid nitrogen, storage process, rapid rewarming, and recovery. Classical slow cooling methods are usually operationally complicated since they require the use of specific and costly programmable freezers. Sometimes, it can be used cheap and a specific tool named Mr. Frosty® freezing container based on usage of propanol (allows 1 °C/min temperature reduction) with a -80 laboratory freezer [32, 33].

Classical slow cooling techniques have been successfully applied to many plant culture types especially in cell suspension cultures and callus cultures [33, 34].

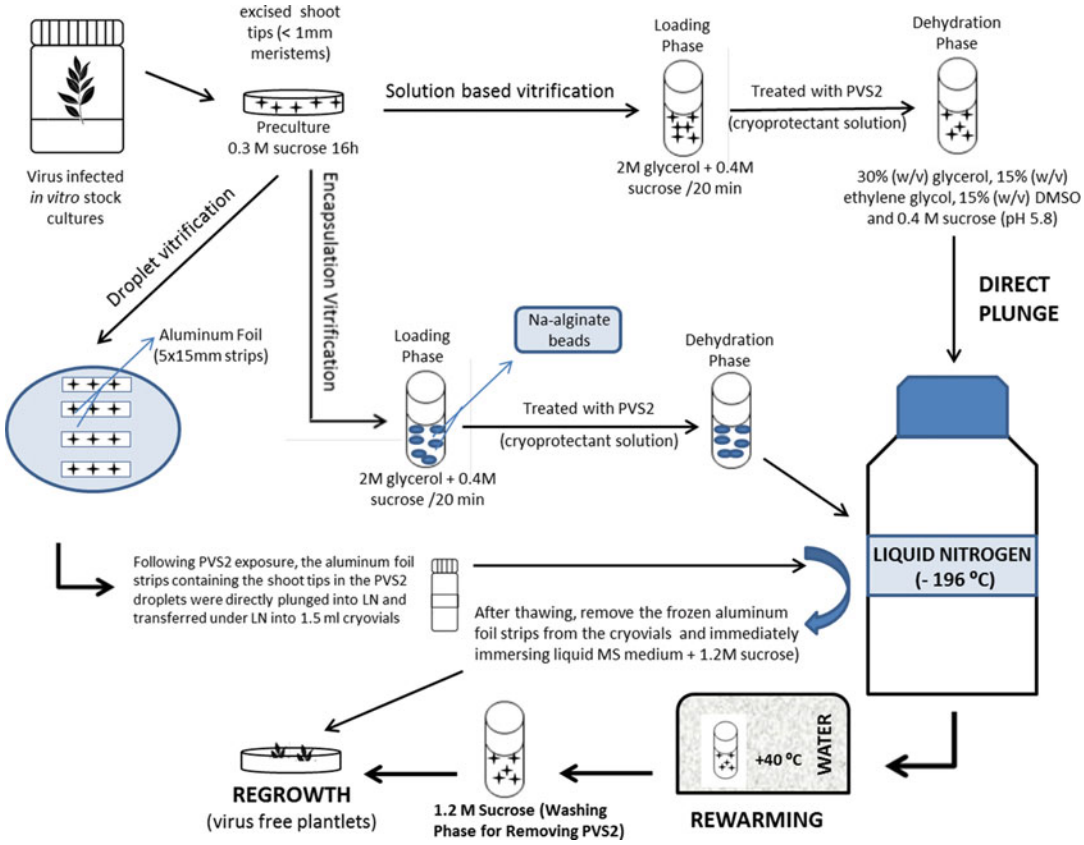

Fig. 1 Schematic presentation of three different vitrification methods based on one-step freezing techniques: cryotherapy, vitrification, encapsulation-vitrification, and droplet vitrification [21]

2.1.2 One-Step Freezing Techniques

One-step freezing techniques can be achieved via direct immersion in liquid nitrogen without slow cooling process (Fig. 1), which is changed by exposure of plant materials to physical dehydration or chemical vitrification using a cryoprotectant solution. This technique is divided into five main procedures: vitrification, dehydration, encapsulation vitrification, encapsulation-dehydration, and droplet vitrification [29].

Vitrification

Vitrification processes are based on the physical treatments, of which a high concentration of cryoprotectant solution prevents fatal ice nucleation in cells during direct immersion in liquid nitrogen [35]. Because of amorphous glass formation of cell water content, all metabolic reactions requiring molecular diffusion stop, and this amorphous formation leads to metabolic inactivity and stability during immersion of liquid nitrogen [36].

Vitrification method based on cryoprotectant solutions combines a classical cooling procedure (combining the cryoprotectant treatment and dehydration steps). After cold hardening and sucrose

preculture, in cryoprotectant treatment, a chemical solution of a concentrated diffusing cryoprotectant solution is applied, followed by a vitrification solution. Treatment time, application temperature, and solution concentration may differ for plant species and cultivars [37–41].

Different cryoprotectant solutions for vitrification have been successfully used for cryotherapy and cryopreservation studies [42, 43]. However, two of them frequently used are the glycerol-based cryoprotectant solutions named plant vitrification solution 2 (PVS2) [42, 44] and PVS3 [45]. The PVS2 solution contains 30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) dimethyl sulfoxide (DMSO), and 0.4 M sucrose (pH 5.8). PVS3 consists of 40% (w/v) glycerol and 40% (w/v) sucrose in basal culture medium.

In the vitrification process, the plant material such as shoot tips and cells must be sufficiently dehydrated by the cryoprotectant solution (which hardly diffuses into the tissue during the dehydration process) without causing damage, in order to be able to vitrify during fast cooling in liquid nitrogen. Consequently, to achieve successful regrowth after cryopreservation using vitrification methods, it needs to optimize dehydration tolerance of the plant material to be cryopreserved to the vitrification solution. There are many reports representing that cells and shoot tips have dehydration tolerance to cryoprotectant solution such as PVS2 resistance following fast cooling in liquid nitrogen with small or no additional loss in survival [46, 47].

Dehydration

Dehydration method based on physical process using activated silica or laminar airflow for removing water content of tissues and cells is a very simple procedure, and it only consists of explant dehydration, then freezing them in fast direct immersion in liquid nitrogen. Zygotic embryos or embryonic parts extracted from seeds are usually used for explant sources in this technique. And this technique has also been applied to a large number recalcitrant and intermediate species [48–50]. Dehydration process is usually performed in a sterile laminar airflow cabinet; however, more specific and effective dehydration conditions are achieved by using a flow of sterile compressed air or silica gel. Ultrafast drying by using a compressed dry airstream allows freezing of samples with a relatively high water content, thus decreasing desiccation damages [51]. The water content of tissue and cells reducing between 10 and 20% (basis on fresh weight) supports optimal survival, and regrowth rate is generally obtained when samples are frozen during liquid nitrogen treatment [50].

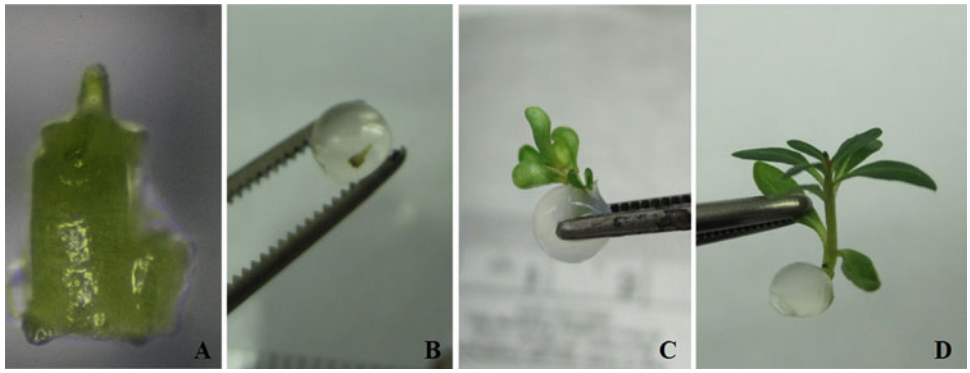

Fig. 2 Naked 0.1 mm shoot tip (**a**) and encapsulated meristem (**b**) of *Eucalyptus* spp. for cryotherapy via encapsulation-vitrification method; regrowth stages of after 4 weeks (**c**) and 6 weeks (**d**) of encapsulated meristems of frozen *Eucalyptus* spp. [38]

Encapsulation-Vitrification

Vitrification process allows the explants freezing in a short period of time. However, this process is difficult to apply for a large number of samples at the same time, as the duration of the consecutive stages of a vitrification protocol is usually too short; on the one hand, these stages require a very definite period, and small-sized plant materials are difficult to manipulate. On the other hand, the encapsulation-dehydration methods take a much longer time to perform; however, encapsulated plant materials are very easy to manipulate, by using optimum size of the calcium alginate beads (Fig. 2a–d). Thus, encapsulation-vitrification method combines the advantages of vitrification having fast application and encapsulation-dehydration having easy manipulation of encapsulated plant materials [52].

Encapsulation-Dehydration

The encapsulation-dehydration method is based on physical dehydration process of encapsulated plant materials. This method includes similar application with simple dehydration process; however, the main difference from simple dehydration is usage of encapsulated shoot tips. Explants encapsulated in calcium alginate beads desiccates in a laminar airflow cabinet or with activated silica gel for reducing water content, and then they are fast immersed directly in liquid nitrogen [53]. This technique has been used for shoot tips of many species from tropical and subtropical origin as well as to cell suspensions and somatic embryos of several species [54].

Droplet Vitrification

The droplet vitrification method based on chemical vitrification and one-step freezing treatments was first reported by Schäfer-Menuhr et al. [55] using potato shoot tips. In this technique, 01–03 mm explants (Fig. 3a) are treated with the cryoprotectant solution (usually PVS2) put individually in 3–10 μL droplets of cryoprotectant solutions (depending on explant size) placed on a piece of

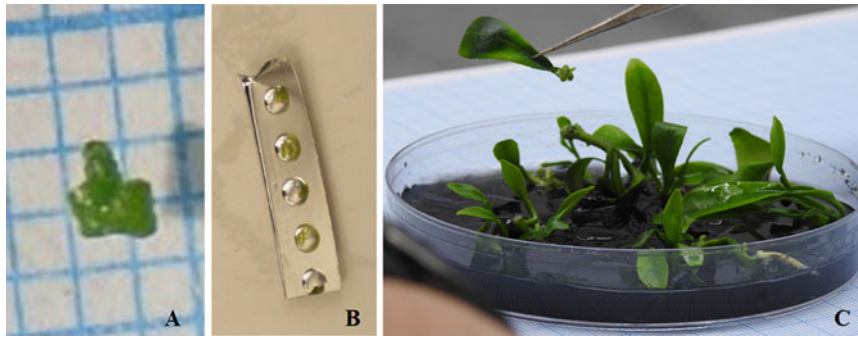

Fig. 3 Treatment of samples with the cryoprotectant solution and their placement on aluminum foil strip with subsequent transfer on regrowth medium

aluminum foil strip (Fig. 3b), which is then directly immersed in liquid nitrogen. For rewarming, the aluminum foils are directly plunged in liquid medium supplemented with 1–1.2 M sucrose, and after 20 min of unloading, shoot tips are transferred on regrowth medium (Fig. 3c). The main advantage of this method is the possibility of achieving very high cooling/warming rates due to the very small volume of cryoprotectant solution which the explants are placed. Although this is a newly developed technique, there are many reports obtained with a high regrowth percentage after immersion of liquid nitrogen [38, 39, 56–58].

3 Confirmation of Pathogen-Free Plants

3.1 Immunodiagnostic Techniques: ELISA

Determination of plant viruses is usually based on their biological characteristics (host range, typical symptoms), and this process has been achieved via serological tests since the 1960s. Serological laboratory tests were originally developed for determination of viruses by using antibodies to detect epitopes of protein antigens. The immunological diagnostic methods include enzyme-linked immunosorbent assay (ELISA), immunofluorescence (IF), and immuno-strip tests [59]. ELISA is by far the most traditional used immunodiagnostic method for virus determination since the 1970s [60]. Variations on this technique exist that differ from each other in the way the antigen-antibody complex is detected, but the underlying mechanism is the same.

3.2 DNA-Based Techniques: Reverse Transcriptase-PCR (RT-PCR)

Plant virus diagnostics and detection of polymerase chain reaction-based techniques have progressively been used in recent years to improve diagnostic assays for plant pathogens. These techniques have the potential to be very sensitive and highly specific and are based on the unique nucleic acid sequence of the pathogens [61]. Cheap and effective nucleic acid extraction methods have already been described, including total RNA (Fig. 4), double-

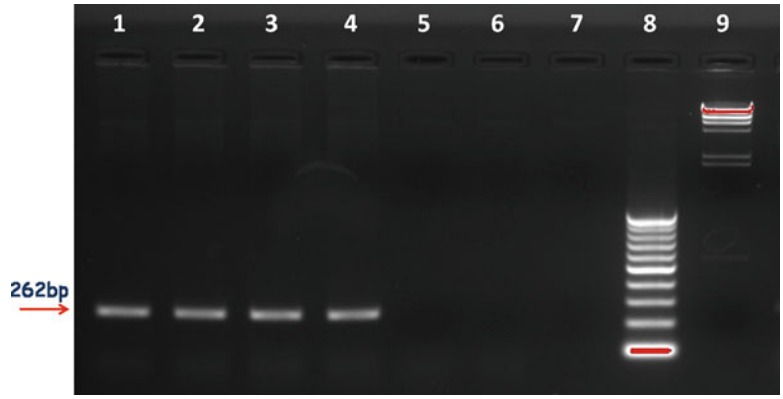

Fig. 4 Agarose gel electrophoretic analysis of one-tube RT-PCR of ApMV-infected and non-infected Turkish hazelnut cultivars. (1–4) Symptomatically ApMV-infected *Corylus* cultivars, (5, 6) non-infected *Corylus* cultivars, (7) negative control, (8) 100 bp ladder molecular size marker, (9) Lambda/HindIII Marker

stranded RNA (dsRNA), and DNA extractions from plant material [62]. Additionally, these techniques provide an efficient and rapid tool for large-scale early screening of plant material, especially in virus elimination programs [63].

4 Conclusions

Plant pathogen elimination using cryotherapy techniques is a newly developing method that can be readily tested with different plant species and cultivars for which cryogenic processes are available. Cryotherapy-based procedures could also be easily applied in basic tissue culture laboratories related in pathogen-free plant production, where they could simplify the application of wide numbers of plant materials, result in notable density of pathogen-free plants, and prevent the difficulties associated with the excision of small shoot tips. Such protocols do not require any specific tools and only marginally add to the time and cost of the traditional procedures of shoot tip culture for pathogen elimination. Furthermore, the use of cryogenic procedures based on vitrification and optimized regrowth of shoot tips should reduce the risk of genetic stability of treated plants.

References

1. Soosaar JL, Burch-Smith TM, Dinesh-Kumar SP (2005) Mechanisms of plant resistance to viruses. Nat Rev Microbiol 3:789–798
2. Lazarowitz SD (2006) Plant viruses. In: Fields BN, Knipe DM (eds) Fields virology, 5th edn. Lippincott, Williams & Wilkins Publishers, Philadelphia, PA, pp 641–706
3. Wren JD, Roossinck MJ, Nelson RS, Scheets K, Palmer MW, Melcher U (2006) Plant virus biodiversity and ecology. PLoS Biol 4:80

4. Hogenhout SA, Ammar E, Whitfield AE, Redinbaugh MG (2008) Insect vector interactions with persistently transmitted viruses. Annu Rev Phytopathol 46:327–359
5. Faccioli VC, Marani F (1998) Virus elimination by meristem tip culture and tip micrografting. In: Hadidi A et al (eds) Plant virus disease control. American Phytopathological Society, Saint Paul, MN, pp 346–380
6. Maliogka VI, Skiada FG, Eleftheriou EP, Katis NI (2009) Elimination of a new ampelovirus (GLRaV-Pr) and Grapevine rupestris stem pitting associated virus (GRSPaV) from two Vitis vinifera cultivars combining in vitro thermotherapy with shoot tip culture. Sci Hortic 123:280–282
7. Benson EE (2007) Cryopreservation of shoot-tips and meristems. In: Day JG, Pennington MW (eds) Cryopreservation and freezing-drying protocols, methods in molecular biology. Humana Press, Totowa, NJ, pp 121–132
8. Wang QC, Cuellar WJ, Rajama ML, Hiraka Y, Valkonen JPT (2008) Combined thermotherapy and cryotherapy for virus eradication: relation of virus distribution, subcellular changes, cell survival and viral RNA degradation in shoot tips to efficient production of virus-free plants. Mol Plant Pathol 9:237–250
9. Wang MR, Li BQ, Feng CH, Wang QC (2016) Culture of shoot tips from adventitious shoots can eradicate apple stem pitting virus but fails in apple stem grooving virus. Plant Cell Tissue Organ Cult 125:283–291
10. Hu GJ, Dong Y, Zhang Z, Fan X, Ren F, Zhou J (2015) Virus elimination from in vitro apple by thermotherapy combined with chemotherapy. Plant Cell Tissue Organ Cult 121:435–443
11. Parker WB (2005) Metabolism and antiviral activity of ribavirin. Virus Res 107:165–171
12. Skiada FG, Maliogka VI, Katis VI, Eleftheriou EP (2013) Elimination of Grapevine rupestris stem pitting-associated virus (GRSPaV) from two vitis vinifera cultivars by in vitro chemotherapy. Eur J Plant Pathol 135:407–414
13. Wang QC, Valkonen JPT (2008) Elimination of two synergistically interacting viruses from sweet potato using shoot tip culture and cryotherapy. J Virol Methods 154:135–145
14. Cai BH, Zhang JY, Qu SC, Gao ZH, Quiao YS, Zhang Z, Zhu F (2008) Preliminary study on the elimination of strawberry mild yellow edge virus from in vitro shoot tips of strawberry cv. Meihou by vitrification cryopreservation. J Fruit Sci 25:872–876
15. Wang QC, Valkonen JPT (2009) Improved recovery of cryotherapy-treated shoot tips following thermotherapy of in vitro-grown stock shoots of raspberry (Rubus idaeus L.). CryoLetters 30:171–182
16. Yi JY, Lee GA, Jeong JW, Lee SY, Lee YG (2014) Elimination potato virus Y (PVY) and potato leaf roll virus (PLRV) using cryotherapy of in vitro grown potato shoot tips. Korean J Crop Sci 59:498–504
17. Pathirana R, McLachlan A, Hedderley D, Carra A, Carimi F, Panis B (2015) Removal of leafroll viruses from infected grapevine plants by droplet vitrification. Acta Hortic 1083:491–498
18. Li BQ, Feng CH, Hu LY, Wang MR, Wang QC (2016) Shoot tip culture and cryopreservation for eradication of apple stem pitting virus (ASPV) and apple stem grooving virus (ASGV) from apple rootstocks 'M9' and 'M26'. Ann Appl Biol 168:142–150
19. Waterworth HE, Hadidi A (1998) Economical losses due to plant viruses. In: Hadidi A et al (eds) Plant virus disease control. American Phytopathological Society, Saint Paul, MN, pp 1–13
20. Esquinas-Alca´ zar J (2005) Protecting crop genetic diversity for food security: political, ethical and technical challenges. Nat Rev Genet 6:946–953
21. Kaya E, Yilmaz Gokdogan E (2015) Virus eradication from plants via novel biotechnological processes: one step freezing methods based on vitrification of cryotherapy techniques. Mugla J Sci Technol 1(2):34–40
22. Helliot B, Panis B, Poumay Y, Swenen R, Lepoivre P, Frison E (2002) Cryopreservation for the elimination of cucumber mosaic and banana streak viruses from banana (Musa spp.). Plant Cell Rep 20:1117–1122
23. Ding F, Jin S, Hong NI, Zhong Y, Cao Q, Yi G, Wang G (2008) Vitrification-cryopreservation, an efficient method for eliminating Candidatus Liberobacter asiaticus, the citrus Huanglongbing pathogen, from in vitro adult shoot tips. Plant Cell Rep 27:241–250
24. Wang QC, Mawassi M, Li P, Gafny R, Sela I, Tanne E (2003) Elimination of Grapevine virus A (GVA) by cryopreservation of in vitro-grown shoot tips of Vitis vinifera L. Plant Sci 165:321–327
25. Bayati S, Shams-Bakhsh M, Moieni A (2011) Elimination of Grapevine Virus A (GVA) by cryotherapy and electrotherapy. J Agr Sci Technol 13:443–450
26. Wang Q, Liu Y, Xie Y, You M (2006) Cryotherapy of potato shoot tips for efficient elimination of Potato leaf roll virus (PLRV) and Potato virus Y (PVY). Potato Res 49:119–129

27. Brison M, de Boucaud MT, Pierronnet A, Dosba F (1997) Effect of cryopreservation on the sanitary state of a cv. Prunus rootstock experimentally contaminated with Plum Pox Potyvirus. Plant Sci 123:189–196
28. Shin JH, Kang DK, Sohn JK (2013) Production of yam mosaic virus (ymv)-free Dioscorea opposita plants by cryotherapy of shoot-tips. Cryo Letters 34(2):149–157
29. Engelmann F (2004) Plant cryopreservation: progress and prospects. In Vitro Cell Dev Biol Plant 40:427–433
30. Mazur P (1984) Freezing of living cells: mechanisms and applications. Am J Physiol Cell Physiol 247:125–142
31. Meryman HT, William RT, Douglas MSTJ (1977) Freezing injury 2solution effects and its prevention by natural or artificial cryopreservation. Cryobiology 14:287–302
32. Engelmann F (1997) In vitro conservation methods. In: Ford-Lloyd BV, Newburry JH, Callow JA (eds) Biotechnology and plant genetic resources: conservation and use. CABI, Wallingford, pp 119–162
33. Ozudogru EA, Capuana M, Kaya E, Panis B, Lambardi M (2010) Cryopreservation of Fraxinus excelsior L. embryogenic callus by one-step freezing and slow cooling techniques. CryoLetters 31(1):63–75
34. Withers LA, Engelmann F (1998) In vitro conservation of plant genetic resources. In: Altman A (ed) Biotechnology in agriculture. Marcel Dekker Inc., New York, pp 57–88
35. Fahy GM, MacFarlane DR, Angell CA, Meryman HT (1984) Vitrification as an approach to cryopreservation. Cryobiology 21:407–426
36. Burke MJ (1986) The glass state and survival of anhydrous biological systems. In: Leopold AC (ed) Membrane, metabolism and dry organisms. Cornell University Press, Ithaca, NY, pp 358–364
37. Fahy GM, Levy DI, Ali SE (1987) Some emerging principles underlying the physical properties, biological actions and utility of vitrification solutions. Cryobiology 24:196–213
38. Kaya E, Alves A, Rodrigues L, Jenderek M, Hernandez-Ellis M, Ozudogru A, Ellis D (2013) Cryopreservation of eucalyptus genetic resources. CryoLetters 34(6):608–618
39. Ozudogru EA, Kaya E (2012) Cryopreservation of Thymus cariensis and T. vulgaris shoot tips: comparison of three vitrification-based methods. CryoLetters 33(5):363–375
40. Ozudogru EA, Kaya E, Kirdok LM (2011a) Comparison of different PVS2-based procedures for cryopreservation of Thymus spp. European Cooperation in Science and Technology, Food and Agriculture, Cryoplanet, COST Action 871:86–92
41. Ozudogru EA, Kirdok E, Kaya E, Capuana M, Benelli C, Engelmann F (2011b) Cryopreservation of redwood (Sequoia sempervirens (D. Don.) Endl.) in vitro buds using vitrification-based techniques. CryoLetters 32 (2):99–110
42. Sakai A, Kobayashi S, Oiyama I (1990) Cryopreservation of nucellar cells of navel orange (Citrus sinensis Osb. var. brasiliensis Tanaka) by vitrification. Plant Cell Rep 9:30–33
43. Steponkus PL, Langis R, Fujikawa S (1992) In: Steponkus PL (ed) Advances in low temperature biology, vol 1. JAI Press, Hamptomill, UK, pp 1–16
44. Sakai A, Kobayashi S, Oiyama I (1991) Cryopreservation of nucellar cells of navel orange (Citrus sinensis Osb.) by a simple freezing method. Plant Sci 74:243–248
45. Nishizawa S, Sakai A, Amano Y, Matuzawa T (1993) Cryopreservation of Asparagus (Asparagus officinalis L.) embryogenic suspension cells and subsequent plant regeneration by vitrification method. Plant Sci 91:67–73
46. Matsumoto T, Sakai A, Yamada K (1994) Cryopreservation of in vitro-grown apical meristems of wasabi (Wasabia japonica) by vitrification and subsequent high plant regeneration. Plant Cell Rep 13:442–446
47. Reinhoud PJ (1996) Cryopreservation of tobacco suspension cells by vitrification. Doctoral Paper, Rijks University, Leiden, The Netherlands
48. Engelmann F (2000) Importance of cryopreservation for the conservation of plant genetic resources. In: Engelmann F, Takagi H (eds) Cryopreservation of tropical plant germplasm—current research progress and applications. JIRCAS/IPGRI, Tsukuba/Rome, pp 8–20
49. Kaya E (2016) Long-term germplasm conservation of two economical important Musa species via cryopreservation-dehydration technıque. Biological Diversity and Conservation 9(3):178–182
50. Kaya E, Souza FVD, Yılmaz Gökdoğan E, Ceylan M, Jenderek M (2016) Cryopreservation of citrus seed via dehydration followed by immersion in liquid nitrogen. Turk J Biol 41:242–248
51. Berjak P, Farrant JM, Mycock DJ, Pammenter NW (1989) Homoiohydrous (recalcitrant) seeds: the enigma of their desiccation sensitivity and the state of water in axes of Landolphia kirkii Dyer. Planta 186:249–261

52. Matsumoto T, Sakai A, Takahashi C, Yamada K (1995) Cryopreservation of in vitro-grown apical meristems of wasabi (Wasabia japonica) by encapsulation vitrification method. CryoLetters 16:189–196
53. Dereuddre J, Hassen M, Blandin S, Kaminski M (1991) Resistance of alginate-coated somatic embryos of carrot (Daucus carota L.) to desiccation and freezing in liquid nitrogen: 2. Thermal analysis. CryoLetters 12:135–148
54. Engelmann F, Takagi H (2000) Cryopreservation of tropical plant germplasm-current research progress and applications. JIRCAS/IPGRI, Tsukuba/Rome
55. Schäfer-Menuhr A, Schumacher HM, Mix-Wagner G (1997) Long-term storage of old potato varieties by cryopreservation of shoot-tips in liquid nitrogen. Plant Genet Resour Newsletter 111(1):19–24
56. Panis B, Piette B, Swennen R (2005) Droplet vitrification of apical meristems: a cryopreservation protocol applicable to all Musaceae. Plant Sci 168(1):45–55
57. Souza FVD, Kaya E, Vieira J, Hilo de Souza E, Amorim O, Skogerboe D, Matsumoto T, Alves C, Ledo S, Jenderek M (2015) Droplet-vitrification and morphohistological studies of cryopreserved shoot tips of cultivated and wild pineapple genotypes. Plant Cell Tissue Org Cult 124(2):351–360
58. Kaya E, Souza FVD (2017) Comparison of two PVS2-based procedures for cryopreservation of commercial sugarcane (Saccharum spp.) germplasm and confirmation of genetic stability after cryopreservation using ISSR markers. In Vitro Cell Develop Biol Plant 53(4):410–417
59. Schaad NW, Frederick RD, Shaw J, Schneider WL, Hickson R, Petrillo MD, Luster DG (2003) Advances in molecular-based diagnostics in meeting crop biosecurity and phytosanitary issues. Annu Rev Phytopathol 41:305–324
60. Van Weemen BK, Schuurs AHWM (1971) Immunoassay using antigen-enzyme conjugates. FEBS Lett 15:232–236
61. Ward E, Foster SJ, Fraaije BA, McCartney HA (2004) Plant pathogen diagnostics: immunological and nucleic acid-based approaches. Ann Appl Biol 145:1–16
62. White EJ, Venter M, Hiten NF, Burger JT (2008) Modified Cetyltrimethylammonium bromide method improves robustness and versatility: the benchmark for plant RNA extraction. Biotechnol J 3(11):1424–1428
63. Kaya E (2015) Using reverse transcription-polymerase chain reaction (RT PCR) for determination of apple mosaic ilarvirus (ApMV) in Hazelnut (Corylus avellana L.) Cultivars. JSM Biochem Mol Biol 3(1):1011