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VIRUS ERADICATION FROM PLANTS VIA NOVEL BIOTECHNOLOGICAL PROCESSES: ONE STEP FREEZING METHODS BASED ON VITRIFICATION OF CRYOTHERAPY TECHNIQUES

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Abstract

Plant diseases are caused by microscopic pathogens including viruses, viroids, phytoplasmas, bacteria, and fungi. Pathogenic microorganisms are difficult to diagnose and treat as a consequence of their small size and thus rely on technologically advanced methodologies for recognition of chemicals, proteins or molecular fragments specific to different organisms. Cryotherapy is an coming out method stemming from the use of cryopreservation, a cold treatment used for the long-term storage and preservation of all plant materials. It has recently been accepted that cryotherapy is an effective method for elimination viruses from plant shoot tips. This review covers different one step-freezing methods based on vitrification of cryotheraphy for virus eliminations from plants. In addition, the advantages and disadvantages as well as future prospects of each methods have also been discussed.

Keywords: Cryotherapy, ELISA, Plant viruses, RT-PCR, Virus Eradication, Vitrification.

YENİ BİYOTEKNOLOJİK YÖNTEMLERLE BİTKİLERDEN VİRÜS ELİMİNASYONU: KRİYOTERAPİ TEKNİKLERİNİN VİTRİFİKASYON TEMELLİ TEK AŞAMALI DONDURMA YÖNTEMLERİ

Özet

Bitki hastalıkları, virüs, viroid, fitoplazma, bakteri ve mantarları kapsayan mikroskobik patojenler tarafından oluşturulur. Oldukça küçük organizmalar oldukları için, patojenik mikroorganizmaların tanısı ve tedavisi zordur ve bu nedenle, farklı organizmalara özgü kimyasalların, proteinlerin veya moleküler fragmentlerin tanısı için ileri teknolojili yöntemler gerekir. Kriyoterapi, tüm bitki materyallarinin korunması ve uzun süreli saklanması için kullanılan, bir soğuk uygulamalı yöntem olan kriyoprezervasyonun kullanımını temel alan bir metot olarak geliştirilmiştir. Yakın zamanda kabul gören kriyoterapi, bitki sürgün uçlarından virüslerin eliminasyonu için etkin bir yöntemdir. Bu derleme çalışması, bitkilerden virüs eliminasyonu için, kriyoterapinin vitrifikasyon temelli farklı tek aşamalı dondurma tekniklerini kapsamaktadır. Ek olarak, her bir tekniğin gelecekteki uygulamalarda, avantaj ve dez avantajları da tartışılmıştır.

Anahtar Kelimeler: Bitki virüsleri, ELISA, Kriyoterapi, RT-PCR, Virüs Eliminasyonu, Vitrifikasyon

1. Introduction

Viruses are contagious pathogens that are too small to be detected with a light microscope, but despite their small size they can cause confusion. The simplest viruses are formed a small piece of nucleic acid covered by a protein coat. As is the case with other organisms, viruses carry genetic information in their nucleic acid which typically specifies three or more proteins. All viruses are obligate parasites that depend on the cellular machinery of their hosts to reproduce. Viruses are not active outside of their hosts, and this has led some people to suggest that they are not alive. All types of living organisms including animals, plants, fungi, and bacteria are hosts for viruses, but most viruses infect only one type of host. Viruses cause many important plant diseases and are responsible for losses in crop yield and quality in all parts of the World [1].

A plant virus is a microparasite, a nucleoprotein that is entirely dependent upon plant cells for its survival and multiplication. It must also have the ability to move between plant cells and ultimately between-plants if it is to persist [2]. When a virus enters a living cell of a susceptible plant host, a series of biochemical events occur that result in plant infection. The nucleic acid is freed from its protein coat and a process of replication occurs, which depends on whether the virus is single- or double-stranded RNA or DNA [3]. In very general terms, the nucleic acid codes for the production of new viral nucleic acid, and then new viral protein, utilising the enzyme constituents of the host cell. The first new virions appear in plant cells about 10 hours after inoculation. Although there may be multiplication of a virus in a single cell, plant infection is usually considered to occur when there is movement of the virus to cells throughout the plant, and multiplication of the virus in most of these cells [4].

Pathogen-free stocks of planting materials are pivotal for productivity of agricultural and horticultural crops and for the value of ornamental plants [5]. Vegetatively propagated plants

are particularly prone to accumulate pathogens, which are transmitted to new crops in infected cuttings, tubers and other vegetative propagules. Preservation of plant genetic resources is one of the cornerstones for breeding new cultivars for future needs but germplasm collections should not inadvertently constitute a source of pathogens disseminated to new areas in the germplasm supplied to breeders. Hence, gene banks also need efficient methods for enhancing the phytosanitary status of their collections [6-7].

Cryotherapy of shoot tips is a new method for pathogen eradication based on cryopreservation techniques. Cryopreservation aims to the storage of biological samples at ultra-low temperature, usually that of liquid nitrogen (-196 °C), and is considered as an ideal means for long-term storage of plant germplasm. In cryotherapy, plant pathogens such as viruses, phytoplasmas and bacteria are eradicated from shoot tips by exposing them briefly to liquid nitrogen [8].

2. Determination of Plant Viruses

Plant viruses cause major losses to several agricultural and horticultural crops around the world. Unlike other plant pathogens, there are no direct methods available yet to control viruses and, consequently, the current measures rely on indirect tactics to manage the viral diseases. Hence, methods for detection and identification of viruses, both in plants and vectors, play a critical role in virus disease management. Diagnostic techniques for viruses fall into two broad categories: biological properties related to the interaction of the virus with its host and/or vector (e.g., symptomatology and transmission tests) and intrinsic properties of the virus itself (coat protein and nucleic acid). Detection methods based on coat protein include precipitation/agglutination tests, enzyme-linked immunosorbent assays, and immunoblotting. Viral nucleic acid-based techniques like dot-blot hybridization assays and polymerase chain reaction are more sensitive than other methods. Availability of these diagnostic methods provides greater flexibility, increased sensitivity, and specificity for rapid diagnosis of virus diseases in disease surveys, epidemiological studies, plant quarantine and seed certification, and breeding programs [9].

Recent advances in biotechnology and molecular biology have played a significant role in development of rapid, specific and sensitive assays for detection of plant viruses. Production of monospecific polyclonal antibodies, monoclonal antibodies have enabled to group isolates of viruses and distinction of closely related strains. In cDNA hybridization applications, there is an increasing interest to employ non-radioactive probes for detection of nucleic acids. Detection limit of nucleic acid is remarkably comparable to those of radioactive labelled probes. Application of polymerase chain reaction (PCR) has made it possible to amplify the low numbers of viral RNA/DNA molecules and their subsequent detection [10].

Elisa Tests

For long time after discovery, diagnostis and detection of plant viruses was mainly based on their biological properties (host range, typical symptoms). Detection and diagnosis of plant viruses has included serological laboratory tests since the 1960s. Serological assays were originally developed to detect viruses by utilizing antibodies to detect epitopes of protein antigens. The different formats for immunological diagnostic techniques include enzyme-linked immunosorbent assay (ELISA), immunofluorescence (IF) and immuno-strip tests [11]. Enzyme-linked immunosorbent assays are by far the most commonly used immunodiagnostic technique for virus detection since the 1970s [12-14]. 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 (Figure 1).

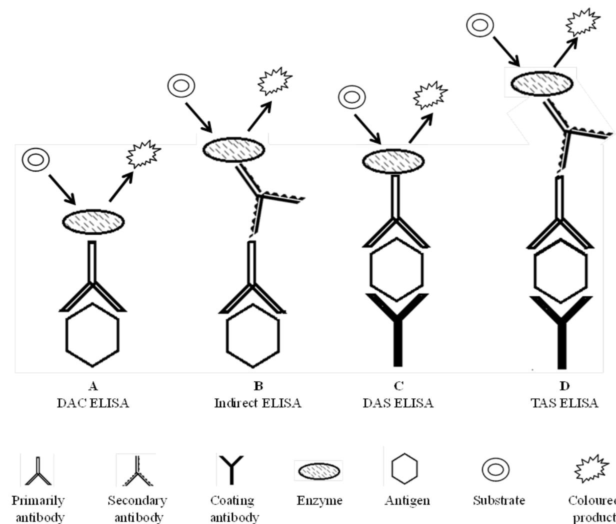

Figure 1. Four types of enzyme-linked immunosorbent assay (ELISA) commonly used for virus detection. A. Direct antigen-coating (DAC) ELISA. The microtiter plate wells are directly coated with the test sample and then incubated with a primary antibody, which binds to the target antigen if the virus is present in the test sample. The primary antibody is conjugated to an enzyme (e.g. alkaline phosphatase) that converts the added substrate (e.g. p-nitrophenyl phosphate) leading to the development of a color change. B. Indirect ELISA. Similar to DAC, but the primary antibody is detected via a secondary antibody that is conjugated to the enzyme. C. Double antibody sandwich ELISA (DAS-ELISA). Similar to DAC, except coating antibodies are used to coat the microtiter plate, which then trap the target antigen within the test sample. D. Triple antibody sandwich (TAS) ELISA. Similar to DAS-ELISA, except that before adding the detecting antibody-enzyme conjugate, a monoclonal antibody is added [15-19].

The most common serological technique for routine diagnosis is double antibody sandwich-enzyme-linked immunosorbent assay (DAS-ELISA). ELISA is one of the most widely used tests for plant viruses. A rapid, sensitive and reliable serological laboratory test that requires only 2 days to complete. Since reliable ELISA protocols for diagnostic purposes were described [16], grapevine has been one of the first species to take benefits from this technique. The presence of the antigen in infected sap is indirectly detected through a colorimetric reaction that develop because of the reaction of an enzyme (e.g., alkaline phosphatase, horseradish peroxidase) conjugated to antibodies in the presence of an appropriate substrate (pnitrophenylphosphate, tetrametylbenzidine) [20]. ELISA is currently employed for routine detection of GFkV but cannot be used for any of the other members of the complex due the unavailability of antisera [21].

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Nucleic acid-based methods have increasingly been used in recent years to develop diagnostic assays for plant pathogens. These methods have the potential to be very sensitive and highly specific and are based on the unique nucleic acid sequence of the pathogen [18-19]. Inexpensive and effective nucleic acid extraction methods have already been described, including total RNA, double-stranded RNA (dsRNA) and DNA extractions from plant material (Figure 2) [22-23].

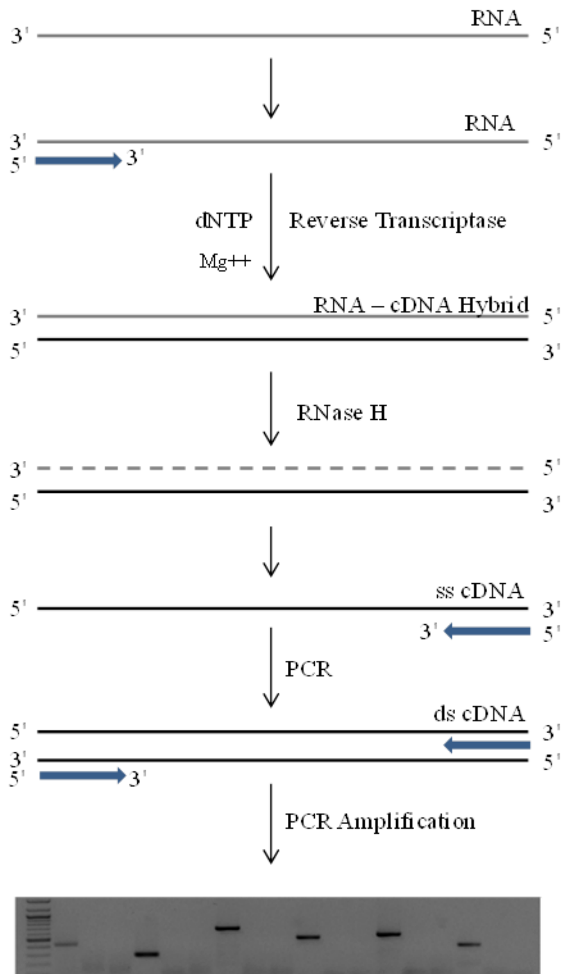

Figure 2. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR).

3. Cryotherapy

Cryopreservation, the storage of biological material at ultralow temperature, usually that of liquid nitrogen (-196 °C), is the only technique currently available to provide the safe and inexpensive long-term conservation of these different types of germplasm. At this temperature, all cellular divisions and metabolic processes are stopped. The plant material can thus be stored without alteration or modification for a theoretically unlimited period of time. Furthermore, cultures are stored in a small volume, protected from contamination, and need a very limited maintenance. Classical cryopreservation techniques, which are based on freeze-stimulated dehydration, are mainly operated for freezing undifferentiated cultures and apices of cold-tolerant species. New cryopreservation techniques, which are based on vitrification of internal solutes, are successfully employed with all explant types, including cell suspensions and calluses, apices, and somatic and zygotic embryos of temperate and tropical species [24-26].

In vitrification-based procedures, cell dehydration is applied prior to freezing by exposure of samples to concentrated cryoprotective media and/or air desiccation. This is followed by rapid cooling. Consequently, all factors that influence intracellular ice formation are prevented. Glass transitions (changes in the structural conformation of the glass) during cooling and rewarming have been enrolled using thermal analysis [27]. Vitrification based procedures allow practical benefits in comparison to classical freezing techniques [28].

Cryotherapy is a novel application of plant cryopreservation techniques that allows pathogen eradication at a high frequency. It eliminates plant pathogens such as viruses, phytoplasmas and bacteria by briefly treating shoot tips in liquid nitrogen using cryopreservation protocols (Table 1). This technique is a coming out science originated from the use of cryopreservation, a cold treatment used for the long-term storage and preservation of valuable plant materials. It has recently been confirmed that cryotherapy is an effective method for removing viruses from plant shoot tips [29] [30]. Especially, cryotherapy includes the excision of shoot tips which are frozen in liquid nitrogen then cultured in suitable conditions until cells regenerate into plants after thawing and a period of regrowth. With cryotherapy, select conditions are used to provide the survival of a limited number of undifferentiated cells while eliminating the remaining differentiated and larger cells which have a higher probability of containing virus. Because of this precision, cryotherapy can result in a higher level of virus free regenerations than traditional methods [31] [32]. Healthy plants are regenerated from the surviving pathogen-free meristematic tissue. The method facilitates treatment of large numbers of samples and is independent of shoot tip size. It has the potential to replace more traditional methods like meristem culture (Figure 3) [7].

3.1. One-Step Freezing Based on Vitrification

Vitrification refers to the physical process which a highly concentrated cryoprotective solution supercools to very low temperatures and eventually solidifies into a metastable glass, without undergoing crystallization at a practical cooling rate [33]. Thus, vitrification is an effective freeze-avoidance mechanism. As a glass is exceptionally viscous and stops all chemical reactions that need molecular diffusion, its formation leads to metabolic inactivity and stability over time [34].

In the traditional freezing method, slow freezing to about -40°C results in the concentration of the unfrozen fraction of the suspending solution and cytosol, which is sufficient to allow vitrification upon relatively rapid cooling in liquid nitrogene (LN; partial vitrification) [35]. Vitrification can also be achieved by direct immersion in LN without the freze concentration step, which is changed by exposure of cells and shoot tips to an highly concentrated (7 to 8 M) cryoprotectant solution. Such a procedure is referred to as vitrification (complete), which is dissimilar from the traditional slow freezing method [36].

Table 1. List of plant species cryopreserved using the vitrification techniques for pathogen elimination.

Plant	Family	Pathogen	Cryopreservation Method	Efficacy (%)	Reference
Banana (Musa)	*Musaceae*	*Cucumber mosaic virus* (CMV) / *Banana streak virus* (BSV)	Vitrification	30 / 90	Helliot et al. (2002)
Bejing lemon, mandarin, pummelo, sweet orange (Citrus)	*Rutaceae*	*Huanglongping bacterium* (HLB)	Vitrification	91 - 98	Ding et al. (2008)
Grapevine (*Vitis vinifera*)	*Vitaceae*	*Grapevine virus* (GVA)	Encapsulation - vitrification	97	Wang et al. (2003)
Grapevine (*Vitis vinifera*)	*Vitaceae*	*Grapevine virus* (GVA)	Encapsulation - dehydration	40 / 62	Bayati et al. (2011)
Potato (*Solanum tuberosum*)	*Solanaceae*	*Potato leaf roll virus* (PLRV) / *Potato virus Y* (PVY)	Encapsulation - vitrification	83 / 93	Wang et al. (2006)
Potato (*Solanum tuberosum*)	*Solanaceae*	*Potato leaf roll virus* (PLRV) / *Potato virus Y* (PVY)	Encapsulation - vitrification	85 / 91	Wang et al. (2006)
Potato (*Solanum tuberosum*)	*Solanaceae*	*Potato leaf roll virus* (PLRV) / *Potato virus Y* (PVY)	Droplet - vitrification	86 / 95	Wang et al. (2006)
Prunus hybrid	*Rosaceae*	*Plum pox potyvirus* (PPV)	Vitrification	75	Brison et al. (1997)
Raspberry (*Rubus idaeus*)	*Rosaceae*	*Raspberry bushy dwarf virus* (RBDV)	Thermotherapy followed by cryotherapy (Encapsulation – vitrification)	33 - 35	Wang et al. (2008)
Sweet potato (*Ipomoea batatas*)	*Convolvulaceae*	*Sweet potato chlorotic stunt virus* (SPCSV) / *Sweet potato feathery mottle virus* (SPFMV)	Encapsulation - vitrification	100 / 100	Wang and Valkonen (2008a)
Sweet potato (*Ipomoea batatas*)	*Convolvulaceae*	*Sweet potato chlorotic stunt virus* (SPCSV) / *Sweet potato feathery mottle virus* (SPFMV)	Encapsulation - vitrification	100	Wang and Valkonen (2008b)
Yam (*Dioscorea opposita*)	*Dioscoreaceae*	Yam mosaic virüs (YMV)	Encapsulation - dehydration	40	Shin et al. (2013)

Vitrification

Solution-based vitrification method incorporates a two-step protocol (combining the loading and dehydration phase). After preculture, in loading phase, a dilute solution of a permeating cryoprotectant is applied, followed by a vitrification solution. Vitrification temperature and solution concentration may vary for different species [33, 37-40].

Several vitrification solutions have been improved by various resarchers worldwide [27] [41]. On the contrary, the most frequently used solutions are the glycerol-based vitrification solutions described plant vitrification solution PVS2 [27] [42] and PVS3 [43]. 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.

The PVS2 solution easily supercools below -100°C upon rapid cooling and solidifies into a metastable glass at about -115°C. Following upon slow warming, it has been recorded, using differential scanning calorimetry (DSC), that the vitrified PVS2 solution displays a glass transition (Tg) at about -115°C, with an exothermic devitrification (crystallization) at about -75°C and an endothermic melting at about -36°C [44]. The successive steps of a complete vitrification procedure employed for freezing shoot tips are presented in Figure 3.

In the vitrification method, cells and shoot tips must be sufficiently dehydrated by the vitrification solution (which hardly penetrates into the cells during the dehydration process) without causing injury, in order to be able to vitrify upon rapid cooling in LN. As a result, the key for successful cryopreservation by vitrification is to quantify dehydration tolerance of the samples to be cryopreserved to the PVS2 solution. A lot of research papers have represented that cells and shoot tips which acquire dehydration tolerance to PVS2 solution withstand subsequent rapid cooling in LN with little or no additional loss in survival [45-49]

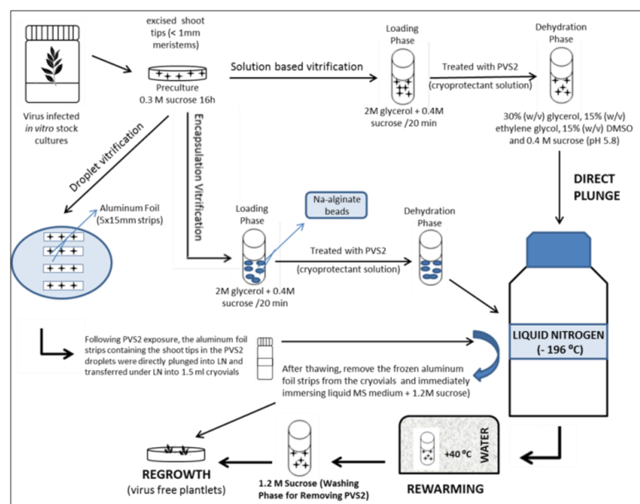

Figure 3. Schematic repsentation of three different vitrification based methods of chryotheraphy; vitrification, encapsulation-vitrification and droplet vitrification.

Encapsulation Vitrification

Vitrification allows the freezing of explants within a short time period. On the contrary, it is hard to treat simultaneously a large number of samples with this technique, as the duration of the successive steps of a vitrification protocol is often very short; these steps need a very precise duration and small sized explants are difficult to manipulate. By contrast, the encapsulation-dehydration technique takes a much longer time to implement; however, encapsulated explants are very easy to manipulate, thanks to the relatively large size of the alginate beads. Thus, a new technique termed encapsulation-vitrification, which combines the advantages of vitrification (rapidity of implementation) and of encapsulation-dehydration (ease of manipulation of encapsulated explants) has been established [50].

Encapsulation-vitrification is a combination of encapsulation and vitrification procedures, and has been successful with shoot tips of a lot of species. The procedure for encapsulation-vitrification is shown in Figure 3. Encapsulated shoot tips are added to the culture bottle containing loading solution (LS) for osmoprotection. Beads in the bottles are osmoprotected for 16 hours at room temperature (25ºC). LS is the liquid culture medium in which sucrose and the glycerol were contained. After loading, LS is removed from a bottle, and PVS is added newly for the dehydration of plant tissues. The same as with vitrification, the dehydration using PVS2 is performed at 0ºC in light of the toxicity to plant cells [36, 38, 51].

After dehydration of PVS2, encapsulated samples are moved to a cryotube containing fresh PVS2, and immersed in LN. Cryopreserved tubes are warmed using hot water (40ºC) for 1-

2 min, and the vitrification solution is removed from the tube. After removal of the solution, unloading solution is added to a tube, and cryoprotectants are removed from plant tissues for 30 min at 25ºC. After unloading, samples are moved from the cryotube, and recultured [38, 51].

Droplet Vitrification

The droplet method was first reported by Schäfer-Menuhr et al. [52] [53] using potato apices. In the droplet method, in order to make a plant sample cool quickly, Wesley-Smith et al. [54] used not liquid nitrogen but a slush nitrogen (-210ºC) and an isopentane (-160ºC). In addition, the droplet method can reportedly obtain a high regrowth percentage after cryopreservation in tropical plants difficult to cryopreserve [38, 55-58].

In the droplet vitrification protocol, shoot tips are loaded, treated with the PVS2 vitrification solution, put individually in 5-10 µl droplets of PVS2 placed on a piece of aluminium foil, which is then immersed in LN. For warming, the aluminium foils are plunged in liquid medium containing 1.2 M sucrose and after 20 min unloading, shoot tips are retrieved and placed on recovery medium (Figure 3). The main interest of this technique is the possibility of achieving very high cooling/warming rates due to the very small volume of cryoprotective medium in which the explants are placed. This is a very recent technique which has been applied until now to a limited number of plant species only. However, the very promising results achieved call for a broader utilization of droplet-vitrification [36].

4. Concluding Remarks

Virus elimination by cryotherapy of shoot tips from infected plants is an coming out method that can be easily tested with species and genotypes for which cryopreservation protocols are available. Regulations of the method might be need for expanding cryotherapy to additional genotypes and for increasing the percentage of pathogen-free regenerants. In gene banks practising cryopreservation the expertise is easily available, and cryotherapy could be adopted in pathogen-eradication schemes for species and genotypes that are going to be cryopreserved.

5. Future Prospects

Traditional procedures like meristem culture and thermotherapy followed by meristem culture have been set up and are now widely performed to produce pathogen- free plants. On the other hand, to meet the requirements for increased food production and developed productivity of crops, more efficient methods for production of pathogen-free plants are needed. To date, cryotherapy of shoot tips has been successfully applied to eradicate viruses, phytoplasmas and some bacterial pathogens from plants. The current logical understanding of mechanisms behind pathogen elimination by cryotherapy of shoot tips is useful for designing pathogen eradication schemes for new pathogen–host combinations but cellular and molecular details of the mechanism deserve to be addressed also in future studies.

Cryotherapy-based protocols are simply to apply and do not need special tools in addition to those typically available in a plant tissue culture laboratory. Cryotherapy enables processes of large numbers of samples. It products pathogen-free plants at a high frequency avoiding also the complexities associated with excision of very small meristems. Functional cryopreservation protocols are available for many economically important monocot, herbaceous and woody plant species in addition to those mentioned above [59]. There is hence a lot of scope to use cryotherapy more widely than reported to date. The main limitation lies in genotype-specificity of many cryopreservation protocols, which may preclude wider application of the available method to all genotypes or cultivars of the species. Similar limitations are also experienced with tissue culture techniques used for traditional meristem culture for pathogen eradication. These limitations will be alleviated and overcome by further development and adjustment of cryoprotocols for plants.

Cryotherapy provides alternative, efficient strategy for eradication of plant pathogens in many species. Cryopreserved shoot tips may also be considered to be safer for exchange of germplasm between countries and regions because the cryogenic procedure reduces the amount of viable infected tissue [60]. However, the phytosanitary status of regenerated plants still needs to be tested. Tissue culture may induce genetic instability in plants and therefore plants regenerated following cryotherapy should be checked for the true-to-typeness, which is performed also in the conventional pathogen eradication schemes based on meristem culture. However, as no callus formation occurs using the optimised meristem regeneration protocols [24], the risk for somaclonal variation is considered minimal.

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