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Advances in sanitation methods for fruit tree species through *in vitro* technologies: Possibilities and limits

Scientific Review

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Abstract – Viruses are considered of great economic importance, regarding their responsability for numerous epidemics in many crops over the world where they cause decrease in both yield and quality. In addition to the development of sensitive techniques for detection, identification and characterization of viruses, substantial progress has also been releazed in biotechnological methods for their efficiency in plant sanitation. This review of literature highlighted the advances on biotechnological methods devoted to the production of healthy plants in some virus-infected fruit species. These methods are essentially based on in vitro tissue culture like meristem culture, shoot-tip grafting and somatic embryogenesis. In order to improve the efficiency of those methods, additional tools such as thermotherapy, chemotherapy, electroptherapy and cryotherapy have been developed in combination with previously mentioned tissue culture techniques. Possibilities and limits of each sanitation method were discussed.

Keywords: Bibliographic research, meristem culture, somatic embryogenesis, viruseradication

1. Introduction

During the past several decades, the frequency of plant diseases caused by viruses increased worldwide and posed serious threat to the plant production and became a real barrier to productive development and sustainability of agriculture. Plant viruses are among the major factors that affect productivity and cause wide economic losses. Infected plants showing growth reduction, low yield and poor quality of fruits, may decline and die in few years (Wang et al. 2003a). Infection by viruses may cause physiological disorders resulting in specific symptoms on infected plants. At the molecular level, virus recognition by the host defense machinery results in the induction of plant defense responses. This includes mechanisms based on RNA silencing as well as others involving stress-response protein accumulation and their actions, to limit pathogen invasion and damages (Fang et al. 2015). Virus dissemination has been accentuated by the use of infected propagating material contributing to the spread of viruses in new areas. So, the acquisition of plant material with a high-healthy status is important for the germplasm and management. The control of viral diseases is also achieved by preventing methods such as quarantine measures (testing and isolation), or by use of resistant genotype and sanitation of virus-infected plant material (Roistacher and Moreno 1991). Resistant genotypes may be obtained via conventional breeding methods or biotechnological tools e.g. transgenic plants. Sanitation methods such as meristem culture and thermotherapy showed limited efficiency regardingsanitation rates and virus types. The development of new and more efficient technologies is required to produce healthy plants (Wang et al. 2009). Among these technologies, thermotherapy, chemotherapy, electrotherapy and cryotherapy coupled with mersitem culture and somatic embryogenesis gave better results in virus elimination from infected material. Nevertheless, the application of these techniques has been limited by the cost of requirements, special treatments during acclimatization, risk of somaclonal variation and other physiological abberations (Winkelmann et al. 2006). Mechanisms involved by these techniques in virus elimination and in virus/host plant interaction remain incompletely elucidated. The present review paper reports the main results obtained during the last twenty years in the field of sanitation against viral diseases to produce healthy fruit tree plants *(Vitis, Citrus, Malus, Pyrus, Prunus sp. and Ficus*). Various procedures based on tissue culture techniques (meristem culture, shoot-tip grafting, somatic embryogenesis) combined or not with thermotherapy, chemotherapy, electrotherapy or cryotherapy were presented and specific possibilities and limits of each method were highlighted.

2. Sanitation via meristem explants

2.1. Meristem culture and shoot-tip grafting

2.1.1. Possibilities

Meristem explant consists in the organized apex excised from donor plant shoot for subsequent in vitro culture. The explant includes the apical dome and a limited number of the youngest leaf primordia, it excludes differentiated provascular or vascular tissues (Grout 1990). Meristem culture is an efficient tool for regeneration, virus elimination and germplasm virus-free conservation of different plant species (Rout et al. 2006). This technique has been successfully used to obtain virus-free plants in some fruit trees such as grapevine, fig and apple (Table 1). For woody species especially fruit trees, meristem-tip culture is often difficult, so it is substituted by shoot-tip grafting (Faccioli and Marani 1998). That consists in the production of in vitro grafted-plants containing healthy meristem as a scion and healthy seedling as a rootstock. This techniqueoffers the advantages of rapid in vitro multiplication and the increased productivity that results from grafting superior rootstock and scion combinations (Gebhardt and Goldbach 1988). Micrografting also provides an alternative production technique when microshoots are difficult to root (Preece et al. 1989). Micrografting was developed by Murashige et al. (1972) in the early 1970's to rid citrus cultivars of viroids and viruses. It was known technique for the potential elimination of virus and viroid pathogens from citrus germplasm. It was generally used in quarantin and certification programs. Shoot-tip grafting has been totally efficient in eliminatingCitrus tristeza virus (CTV), Citrus exocortis viroid (CEVd) and cachexia-xiloporosis viroid from several varieties of citrus (Carvalho et al. 2002). This technique allows to overcome problems related to nucellar seedlings such as reversion to the juvenile state, excessive thorniness, vigorous and upright habit of growth, delay of fruiting, alternate bearing in early years and physical differences in fruit characteristics (Roistacher 2004). Viral sanitation by meristem is based on the fact that most viruses do not attack this organ, since the multiplication of meristem cells is faster than virus replication (Roistacher 2004) even viral particles detected in leaf primoridia and the basal part of the meristem. Only less differentiated cells in the apical dome of the meristem contain no detectable viral particles. Contrarely, neighbouring more differentiated cells showed positive signal indicating the presence of virus infection. In non-differentiated cells where virus were not detected, only few non-branched plasmodesmata crossing the cell walls could be observed, whereas branched plasmodesmata were frequently observed in the virus-infected tissues such as the base of first leaf primordia. These patterns suggest that virus distribution may be associated with the development of plasmodesmata and their ability to support virus movement (Wang et al. 2008). Indeed, Plasmodesmata are involved in the regulation of cell-to-cell movement of viruses (Oparka et al. 1999). Despite the ability of viruses for systemic infection, viral RNA and viral protein are often excluded from shoot and root apical meristem regions (Matthews 1992). These observations suggest an underlying molecular mechanism that protects shoot tips from viral invasion. In fact, plants possess RNA silencing mechanismthat protects shoot apex from viral infection (Roth et al. 2004). This is an important mean of defense against viruses and other nucleic acids invaders and it is triggered by double stranded RNA (dsRNA). Most known plant viruses have RNA genomes and replicate via dsRNA intermediates, thereby serving as potent inducers of RNA silencing, early in replication and as silencing targets later in infection (Roth et al. 2004). Pathogen-derived resistance in which RNA silencing directed against a viral transgene provide resistance to any virus carrying the targeted sequence (Baulcombe 1996). Thus, viruses could be targets of RNA silencing (Roth et al. 2004). Short-interfering RNAs (siRNA), the molecular markers of post-transcriptional gene silencing (PTGS), are powerful tools that interfer with gene expression and counter virus infection in plants (Chellappan et al. 2005). As a counter defense, viruses encode a number of factors that suppress PTGS to establish infection in plants (Voinnet et al. 2001). This data supports the thesis that PTGS is a defense mechanism against viruses (Chicas and Macino 2001). There are at least three different pathways in the gene-silencing mechanism: (i) the cytoplasmic siRNA silencing, (ii) the endogeneous mRNA silencing by microRNAs (miRNAs) and (iii) the transcriptional gene silencing by DNA methylation (Baulcombe, 2004). A unifying feature of these RNA silencing pathways is the cleavage of long double-stranded RNA (dsRNA) into short-interfering (21-24 nt) RNAs (siRNAs) (Hamilton and Baulcombe 1999) by a ribonuclease III-like enzyme termed DICER (Bernstein et al. 2001). In some case, the PTGS signal apparently may enter the shoot apex, but the virus itself does not gain entry and is not transmitted to the next (meiotic) generation. Thus, allowing entry of the PTGS signal to the shoot apex could serve as a double layer of protection against meiotic transmission of the virus. However, if it happens to gain entry, the PTGS signal is ready to activate the second line of defense. The presence of an RNA signal surveillance systemin the shoot apexnot only

excludes viral RNA from the apical meristem but also controls the entry and/or the distribution of endogenous RNAs that affect cell differentiation and organ development (Eckardt 2002).

2.1.2. Limits

Size of the excised shoot apices plays a dual role in virus elimination and plant regeneration. In fact, regeneration ability is positively correlated to the size of the shoot tip (Faggioli et al. 1997), but pathogen eradication is inversely correlatedto it (Wang et al. 2006a; Singh et al. 2008). Moreover, the efficiency of meristem culture in virus elimination is virus-dependent. As observed in apple tree, this technique is

Table 1. Main researchs and references on viral sanitation of some fruit tree species via meristem culture/shoot tip grafting

Fruit tree genus	Viral infection	References
Vitis	Grapevine fanleaf virus (GFLV), *Grapevine leafroll associated virus-1* (GLRaV-1)	Youssef et al. (2009)
Citrus	*Citrus tristeza closterovirus* (CTV) *Indian citrus ringspot virus* (ICRSV)	Abbas et al. (2008); Carimi et al. (2001); Fifaei et al. (2007);Singh et al. (2008)
Malus	*Apple stem pitting virus* (ASPV)	Wang et al. (2016)
Ficus	*Fig mosaic disease* (FMD)	Bayoudh (2016)

efficient in elimination ofApple stempitting virus (ASPV), but not for Apple stem grooving virus (ASGV) regardless of the size and developmental stage of excised shoot tips (Wang et al. 2016). It is well reported that the process of elimination of some viruses and viroids can be improved by using meristem culture or shoot-tip grafting coupled with thermotherapy, chemotherapy, electrotherapy and cryotherapy.

2.2. Thermotherapy

2.2.1. Possibilities

Thermotherapy is extensively used for elimination of different viruses from various fruit trees species such as grapevine, fig and Prunus (Table 2). Environmental factors have been shown to modify plant-virus interactions so much that virus-induced symptoms were attenuated (Challappan et al. 2005). A form of 'natural thermotherapy' may occur during summer when temperatures exceed 38°C. In vitro culture of shoot tips under these conditions was used with success in eliminating Grapevine leafroll-associated virus 3 (GLRaV-3) and Grapevine fanleaf virus (GFLV) (Valero et al. 2003). In general, thermotherapy consists of keeping plants, or more frequently a part of them, at temperatures between 35°C and 54°C, within the physiological tolerance limits of each plant, for an appropriate period (Spiegel et al. 1993). When meristem culture is ineffective per se, combination with thermotherapy has been revealed to be more effective in controlling viral diseases and establishing virus-free germplasm (Gribaudo et al. 2006; Milošević et al. 2012). In citrus, the combination of thermotherapy and shoot-tip grafting is recommended for the elimination of all known major citrus pathogens. Plants obtained by this method showed 100% success in eliminating tristeza, exocortis, psorosis and cachexia-xyloporosis. This combination is particularly effective in the sanitation of micrografted citrus against psorosis virus which are difficult to eliminate using shoot-tip graftingalone (Carvalho et al. 2002). Thermotherapy inhibits viral replication (Cooper and Walkey, 1978; Milošević et al. 2012) and movement (Milošević et al. 2012) by disrupting viral ssRNA and dsRNA synthesis (Carvalho et al. 2002). Virus degradation continues and results in subsequent elimination of the virus from shoot tips (Cooper and Walkey 1978). The RNA virus-induced symptom severity was found to be higher at low temperatures and decreased with rising temperature and elevated levels of virus-derived siRNA. So, the response of RNA silencing-mediated defense seems to be temperature dependent (Szittya et al. 2003). In this context, high temperature treatment may enhance the RNA silencing-mediated defense in the meristem tissue (Liu et al. 2016)by limiting the generation of small interfering RNA (siRNA) molecules, and promotes this innate immunity via increasing siRNA accumulation levels (Szittya et al. 2003; Velazquez et al. 2010).At high temperatures, the disappearance of viral symptoms in new leaves of infected plants, results from the higher temperature mediated acceleration of the host antiviral gene silencing system in the meristem (Wang et al. 2008; Smith et al. 2011).More recently, it has been reported that the elimination of Apple Stem Grooving Virus from Asian pear by thermotherapy is associated with the high temperature-induced mixed action of a number of miRNA-mediated target genes related to disease defense and hormone signal transduction pathways in the apical meristem of pear shoots (Juan et al., 2015). These data suggest that elevated temperatures may enhance vsiRNA-mediated antiviral gene

silencing activity, which in turn reduces the accumulation of viral RNA in the infected meristem tip cells of in vitro-cultured shoots (Liu et al. 2016).

Table 2. Main researchs and references on viral sanitation of some fruit tree species via meristem culture/shoot tip grafting coupled with thermotherapy

Fruit tree genus	Viral infection	References
Vitis	*Grapevine fleck maculavirus* (GFkV) *Grapevine fanleaf virus* (GFLV) *Grapevine leafroll associated virus* (GLRaV-Pr), *Grapevine rupestris stem pitting associated virus* (GRSPaV), *Grapevine vitivirus A* (GVA)	Bota et al. (2014) ; Křižan et al. (2009) Maliogka et al (2009) ; Valero et al. (2003) ; Panattoni et al. (2007)
Citrus	*Citrus tristeza closterovirus* (CTV) *Citrus psorosis virus* (CPsV), *Indian citrus ringspot virus* (ICRSV)	Arif et al. (2005) ; Carvalho et al. (2002) ; Velazquez et al. (2010) ; Sharma et al. (2008)
Malus	*Apple stem grooving virus* (ASGV), *Apple chlorotic leaf spot virus* (ACLSV), *Apple stem pitting virus* (ASPV)	Paprštein et al. (2008)
Pyrus	*Apple chlorotic leaf spot virus* (ACLSV), *Apple stem grooving virus* (ASGV), *Apple stem pitting virus* (ASPV)	Cielinksa et al. (2000) ; Tan et al. (2010) ; Wang et al. (2006a)
***Prunus* sp.**	*Apple chlorotic leaf spot virus* (ACLSV), *Prunus Dwarf Virus* (PDV), *Prunus necrotic ringspot virus* (PNRSV), *Plum pox virus* (PPV)	Cielinksa et al. (2007) ; Dziedzic et al. (2008) ; Laimer et al. (2006); Manganaris et al. (2003); Jacab-Ilyefavi et al. (2012), Koubouris et al. (2007) ; Křižan and Ondrušiková (2009); Polák and Hauptmanová (2009)
Ficus	*Fig leaf-mottled associated virus* (FLMaV), *Fig mosaic virus* (FMV), *Fig latent virus* (FLV), *Fig latent badnavirus* (FBV), *Fig mosaic disease* (FMDs)	Chalak et al. (2015); Chiumenti et al. (2013); Sahraroo et al. (2009)

2.2.2. Limits

The major limitation of thermotherapy technique is the selection of an appropriate temperature/duration of thermotherapy application that must ensure the best compromise between virus degradation and plant survival. Indeed, the threshold of heat sensitivity of some viruses must be lower than that of plant cells and that damage caused to plant tissues by the thermal stress can more easily be reversed than viral damage (Spiegel et al. 1993). Fruit trees, especially stone fruits species, are particularly sensitive to high temperatures and their survival rate after the heat treatment is low (Stein et al. 1991). Heat treatment must be applied in plants within their physiological tolerance for period of time ranging from several weeks to months. For exemple, a maximum of survival shoots (100%) of apricot was obtained in shoot tips excised from shoots incubated at 37°C for 3 weeks. Decline in survival was obtained with prolonged incubation and totally inhibited after 4 weeksof incubation (Gül Şeker et al. 2015). Efficiency of thermotherapy combined with shoot-tip culture is also limited in the case of mixed infections (Wang et al. 2006a; Paprštein et al. 2008). It depends on genotype, virus species and specific virus-genotype interactions (Maliogka et al. 2009). This technology is an expensive process that requires specific and proper equipement (Bhojwani and Dantu 2013).

2.3. Chemotherapy

2.3.1. Possibilities

Chemotherapy is another approach to in vitro virus elimination in plants. It consists in the addition of chemicals of a known ability to prevent virus replication in the culture medium.Some contributions have been provided and showed the efficacy of this method in the elimination of viruses fromsome fruit tree species (Table3).Chemotherapy combined with thermotherapy could greatly improve the efficiency of Apple stem grooving virus and Apple chlorotic leaf spot virus eradicationfrom sand pear as it was compared with the separated application of both methods (Hu et al. 2012). Several groups of antiviral agents such as ribavirin (Leyssen et al. 2005) and quercetin (Mlahotra et al. 1996) that have shown significant therapeutic potential against plant viruses, belong to inosine monophosphate dehydrogenase

(IMPDH) inhibitors, S-adenosylhomocysteine hydrolase (SAH) inhibitors and neuraminidase (NA) inhibitors (Panattoni et al. 2013). Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) is one of the most used chemotherapeutic agent. It is a guanosine analogue that produces broad-spectrum activity against several RNA and DNA viruses (Leyssen et al. 2005). Various mechanisms of action have been suggested to be responsible for the antiviral activity of ribavirin. These mechanisms include depletion of intracellular guanosine 5'-triphosphate GTP pools (by inhibition of the cellular IMP dehydrogenase [IMPDH] by the 5'-monophosphate metabollite of ribavirin), inhibition of viral polymerase activity by the 5'-triphosphate metabolite of ribavirin, inhibition of viral capping (by inhibition of [viral or cellular] guanylyltransferase activity by ribavirin 5'-triphosphate), and induction of error catastrophe as a result of mutations accumulation (some of them lethal) in the viral genome (Crotty et al. 2001). However, none of these mechanisms may be predominantly responsible for the antiviral activity of ribavirin (Leyssen et al. 2005). The presence of inhibitors acts by reducing the intracellular pool of guanosine and also preventing the synthesis of viral RNA (Franchetti et al. 1996). The effectiveness of antiviral molecules belonging to SAH hydrolase inibitors has been known for some time and centers on the mechanism of action of SAH hydrolase, another key enzyme for viral replication (Panattoni et al. 2013). S-adenosylmethionine (SAM) is used in transmethylation reactions, in which this molecule donates methyl groups to a wide range of acceptors including nucleic acids, viral proteins and phospholipds, and is then converted to S-adenosylhomocysteine. Methylation is regulated negatively by both an increase in SAH and in reduction of SAM or SAM/SAH ratio. The removal of SAH plays an essential role and it is mediated by SAH hydrolase that is able to convert this molecule into homocysteine and adenosine (De Clercq 2005).The mechanism of action of NA inhibitors is based on the inhibition of neuraminidases, glycoprotein found in the membrane lining of flu virus, and these inhibitors have provided very interesting results with regard to some Orthomyxoviridae with ssRNA-genome and innovative ones for phytoviruses. NA inhibitors are molecules that act by binding to the active site of viral neuraminidas, preventing the release and spread of newly-generated virion progeny fom infected cellsto healthy ones (Gubareva 2004). Quercetin is a natural product flavonoid that has been shown to be effective antivirals against several plant viruses (Mlahotra et al. 1996). It enhances CAMP levels by inhibiting cAMP phosphodiesterase (Mucsi and Pragai 1985) and this may reduce virus replication.

Table 3. Main researchs and references on viral sanitation of some fruit tree species via meristem culture/shoot tip grafting coupled with chemotherapy

Fruit tree genus	Viral infection	References
Vitis	*Grapevine fleck maculavirus* (GFkV), *Grapevine vitivirus A* (GVA) *Grapevine rupestris stem pitting associated virus* (GRSPaV)	Gutá et al. (2014); Panattoni et al. (2007); Skiada et al. (2013)
Malus	*Apple stem grooving virus* (ASGV), *Apple chlorotic leaf spot virus* (ACLSV), *Apple stem pitting virus* (ASPV)	Paprštein et al. (2008)
Pyrus	Apple Stem Grooving Virus (ASGV), *Apple chlorotic leaf spot virus* (ACLSV), *Hop Stunt Viroid* (HSVd)	Cho et al. (2016) ; Cielinksa et al. (2000) ; El Dougdoug et al. (2010)
Prunus	*Apple chlorotic leaf spot virus* (ACLSV), *Prunus necrotic ringspot virus* (PNRSV), *Prunus Dwarf Virus* (PDV), *Hop Stunt Viroid* (HSVd) *Plum pox virus* (PPV)	Cielinksa et al. (2007) ; El Dougdoug et al. (2010) ; Gül Şeker et al. (2015), Hauptmanová and Polák (2011) ; Jacab-Ilyefavi et al. (2012), Paunovic et al. (2007)

2.3.2. Limits

Chemotherapy as other techniques for virus eradicationpresents some limits and needs to be optimized. Phytotoxicity of antiviral agents when used at high concentrations is the major disadvantage (Cielinksa 2002, 2007; Skiada et al. 2013; Gül Şeker et al. 2015). Degree of this phytotoxicity depends on the genotype sensivity to these virucides (Paprstein et al. 2013). Another limit consists in the possibility of the mutagenic alteration caused by antiviral compounds that is not still verified (Skiada et al. 2013).

2.4. Electrotherapy

2.4.1. Possibilities

Traditional techniques applied to eradicate viral diseases in plants, i.e. meristem culture alone or combined with thermotherapy or chemotherapy fail sometimes to produce clean material in some species. Alternative procedure using electric current treatments is proposed as more efficient tool to overcome this problem (González et al. 2006). Electrotherapy is a simple method of sanitation without the need to use any special or expensive equipment. It seems to be more effective, faster and easier than the previous methods in regenerating virus-free plants. It can be more effective if combined with chemotherapy in some cases (Sabry et al. 2009). This procedure is based on the disruption or the degradation of viral nucleoprotein and elimination of its virulence activity by electric shock (Sabry et al. 2009). This denaturation of viral particles preferentially occurs during transport through the plasmodesmata of the apoplastic space than during their stay inside the cell (Gonzalez et al. 2006). Inactivation of specific nucleoprotein involved in cell-to-cell movement of viral particles leads to their blockage, and prevents their further penetration into healthy cells (Hormozi-Nejad et al. 2010). The basis of this observation is still poorly understood (Bădărău et al. 2014). Application of this technique for virus elimination in fruit trees seems to be limited to the eradication of *Grapevine leafroll-associated virus* from infected grapevine plants (Bayati et al. 2011).

2.4.2. Limits

Exposing plant tissues to electricity increases the temperature inside the cells. Consequently, the success of this increment strongly depends on age, volume, hydric potential, thickness of tissues and cellular constitution (Gonzales et al. 2006). Temperature elevation in the targeted cells may affect protein synthesis and may cause morphological abnormalities in regenerated plants from electrotherapy-treated shoot tips. Therefore, it is important that the temperature-induced denaturation of virus coat protein is irreversible; this should not affect the infectivity, because RNA is unlikely to be affected (Bayati et al. 2011).

2.5. Cryotherapy

2.5.1. Possibilities

Cryotherapy is a relatively new application of plant cryopreservation techniques that consists in a promising tool, coupled with mersitem culture, for achieving in a short time, high frequency of regenerating plants free of viruses (Engelmann 2004; Wang et al. 2014). Cryotherapy has been used successfully in eradicating virus infections by brief treatment of shoot tip in liquid nitrogen (−196°C) (Englemann 2004) in some species with economic importance such as *Vitis and Prunus* (Table 4). According to the results from numereous researchs, cryotherapy can be considered as a fast developing method that helps and/or replaces traditional methods in eradicating microorganisms from infected plant tissues(Wang and Walkonen 2009).Compared to traditional procedures, cryotherapy has numerous advantages in pathogen eradication (Wang et al. 2003a), (i) treatment of large number of samples quikly and simultaneously (Bhojwani and Dantu 2013),(ii) low cost and high frequency of plants free from viruses after recovery (Wang et al. 2003a),(iii) efficient virus elimination independently to shoot-tip size (Wang et al. 2009). To understand the action mode of cryopreservation for the eradication of viral particles, ahistological study carried out on cryo-treated meristematic clumps of banana showed that only highly cytoplasmic cells located in the meristematic dome and at the base of leaf primordia survived cryotherapy by vitrification pretreatment (Helliot et al. 2002). However, infected cells in the vascular region and vacuolated cells of the apical dome are eliminated by the ultra-low temperature due to ice crystallisation (Wang and Valkonen 2008). So, before the exposure of plant tissue to liquid nitrogen, part of the intracellular water must be removed by the addition of plant vitrification solution 2 (PVS2) (Sakai et al. 1990; Gribaudo et al. 2012), or they are physically dehydrated using silica gel or by exposing to the air flow of the laminar hood (Wang et al. 2000). Several different techniques have been applied for cryotherapy. These include vitrification (Pennycooke and Towill 2001; Wang et al. 2003a; Ganino et al. 2012; Markovic et al. 2013), encapsulation-dehydratation (Wang et al. 2000; Pennycooke and Towill 2001; Wang et al. 2003a; Bayati et al. 2011; Markovic et al. 2013), encapsulation-vitrification (Wang et al. 2006b)and droplet vitrification (Pathirana et al. 2015). Regardless of the cryotherapy method, it is important that after cryotherapy in vitro culture condition must be favorable to survival and regeneration of the cryopreserved material. Optimising the culture conditions is essential for good regeneration of cryo-preserved buds (Wang et al. 2000; Pennycooke and Towill 2001). For exemple, optimum concentration of growth regulators in regeneration medium for cryo-preserved material differs

among cryogenic methods (Wang et al. 2003b) and genotypes (Wang et al. 2000). Therefore, investigations need to be performed on selected genotypes in each species, in combination with vitrification, encapsulation-dehydration, encapsulation-vitrification and droplet vitrifictaion methods, in order to establish efficient protocols that can result in high survival rates with high frequency of virus eradication (Bettoni et al. 2016). Cryotherapy preceeded by thermotherapy of shoot tips can be used to enhance virus eradication (Wang et al. 2009).

2.5.2. Limits

The greatest challenge to the broader application of cryotherapy technique is the differential response that genotypes of the same specie can show under cryotreatment (Wang et al. 2014; Wang and Walkonen 2009). Results concerning survival and regeneration vary among the cryotherapy methods and even between laboratories, limiting its generalization (Benelli et al. 2013; Benson 2008; Wang et al. 2003a; Matsumoto and Sakai 2003). Intracellular water content is a critical factor for the efficiency of cryotherapy protocols. The prerequisite to this efficiency is achieving proper dehydration of plant material, reducing harmful damage to tissues (Englemann 2004).Excessive dehydration causes negative effects on survival rate and, likewise, water in excess in the tissue causes irreversible damage because water crystallization inside the cell causes rupture of cell membrane system, cell collapse and death (Wang et al. 2009).Regarding the above-mentioned limits, published researchs propose further improvements to this technique (Wang et al. 2003b; Gribaudo et al. 2012; Bettoni et al. 2016).

Table 4. Main researchs and references on viral sanitation of some fruit tree species via meristem culture/shoot tip grafting coupled with cryotherapy

Fruit tree genus	Viral infection	References
Vitis	*Grapevine vitivirus A (*GVA) *Grapevine leafroll-associated virus* (GLRaV)	Bayati et al. (2011) ; Wang et al. (2003a) ; Pathirana et al. (2015)
Prunus	*Plum pox virus* (PPV) *Hop Stunt Viroid* (HSVd)	Brison et al. (1997); Gül Şeker et al. (2015); El Dougdoug et al. (2010)
Pyrus	*Hop Stunt Viroid*	El Dougdoug et al. (2010)

3. Sanitation via somatic embryogenesis

3.1. Application

Somatic embryogenesis mainly considered as a support for plant breeding programs, was also adopted as an alternative method for sanitation of virus-infected plants. In tree species, somatic embryogenesis has been exclusively used to eliminate viruses from grapevine and citrus (Table 5). In grapevine, floral tissues like anthers (Popescu et al. 2002; Gambino et al. 2006) or ovaries (Gambino et al. 2006) of infected plants were used as explants for induction of somatic embryogenesis. RT-PCR realized on embryogenic calli and regenerated embryos revealed the presence of virus particles in callus, but not in somatic embryos and plantlets. This difference may be related to the virus distribution and mechanisms of virus movement in the tissues, and most probably to the characteristics of the callus and its evolution after several months of culture. Moreover, sanitation rates could be also related to the duration of subculture required for callus induction. A very short time of subcultures could not allow virus particles spreading from infected to healthy tissue (Popescu et al. 2003). For Citrus, somatic embryogenesis constitutes the most recent sanitation technique for the total recovery of the infected Citrus ssp., except for clementines and pomelos (Carimi et al. 1995). It is considered to be a very promising technique for the eradication of citrus viroids and viruses like psorosis (D'Onghia et al. 2000, 2001; Djelouah et al. 2002) and tristeza (D'Onghia et al. 2000; Djelouah et al. 2002). Style and stigma used as explants for embryogenic process, gave better results than other floral parts and became more and more useful for their specific advantages concerning sanitation and juvenility traits (D'Onghia et al. 2000; Meziane et al. 2012). The mechanism whereby regenerated somatic embryos are healthy is not well understood. Gambino et al. (2006) suggested that it could be related to the rapid proliferation of embryogenic cells within the callus or to the origin of the embryogenic callus from virus-free cells within the original explant. The lack of vascular connections between callus tissue and embryos can be the reason for the sanitation occured during cultures, especially for phloem-limited virus that are able to invade initially

the callus derived from anther and ovary explants (Gambino et al. 2006), but their translocation from infected tissues to somatic embryos was not possible (Gambino et al. 2006). In general, translocation of viruses depends on the genotype and the length of time necessary for tissues regeneration (Popescu et al., 2003).

Table 5. Main researchs and references on viral sanitation of some fruit tree species via somatic embryogenesis

Fruit tree genus	Viral infection	References
Vitis	*Arabis mosaic virus* (ArMV), *Grapevine rupestris stem pitting associated virus* (GRSPaV), *Grapevine leafroll-associated virus 1* (GLRaV-1), *Grapevine vitivirus A (*GVA) *Grapevine fanleaf virus* (GFLV), Citrus vitroids	Borroto-Fernández et al. (2009), Gambino et al. (2006), Gribaudo et al. (2006) Gambino et al. (2009) Gambino et al. (2011)
Citrus	*Citrus psorosis virus* (CPsV), *Citrus dawrfing viroid* (CDVd), *Citrus bark cracking vitoid* (CBCVd)	Carimi et al. (2001), D'Onghia et al. (2000, 2001), El-Sawy et al. (2013) Ben Mahmoud et al. (2017)

3.2. Limits

The use success of somatic embryogenesis as a tool of sanitation depends on the success of the regeneration protocol that is generally laborious and time-consuming (Popescu et al. 2003; Gribaudo et al. 2006). Moreover, the possibility of somaclonal variation should also be considered. It has been reported that direct somatic embryogenesis from anther cultures of grapevine was accompanied by rearrangement of genetic material, whereas indirect somatic embryogenesis and/or long-term culture were accompanied by changes in the methylation status of DNA (Popescu et al. 2002).

4. Conclusion

Diseases caused by viruses are a significant problem constantly present in commercial production of plants, and particularly in fruit tree species. The need for virus-free production has therefore become essential. In order to obtain virus-free material, tissue culture techniques (meristem culture and somatic embryogenesis), usually adopted to regenerate plantelts in biotechnological breeding programs, have been used to eliminate some viruses. For more efficiency, these techniques could be combined with more advanced technologies such as thermotherapy, chemotherapy, electrotherapy and cryotherapy. The choice of suitable sanitation method depends generally on plant species as well as the type of virus.

Apart from the choice of sanitation method, other parameters have to be taken into account as well, such as the structural and biological characteristics of a virus can strongly interfere with the results of treatment and are important for the final outcome of elimination. Limited or partial knowledge of some of these parameters can lead to incomplete elimination of the pathogen (Luvisi et al. 2012).

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