

Review. Elimination of viruses in plants: twenty years of progress

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Abstract

To shed light on trends about elimination of viruses from plants, a bibliographic research was conducted to identify thermotherapy, chemotherapy and tissue culture trials published from 1991 through 2010. Among woody plants, grapevine, apple and peach are the most frequent targets of sanitation protocols because their health status is strictly regulated. Even if thermotherapy represents the preferred method for the host, grapevine viruses can also be eliminated with chemotherapy and tissue culture; apple viruses respond to chemotherapy as well. Although a similar trend was reported among herbaceous plants, chemotherapy was the most frequently used technique in potato. With regard to virus, thermotherapy was successfully applied against viruses belonging to 13 families and an unassigned genus. Instead, chemotherapy and tissue culture techniques eradicated viruses belonging to fewer families (nine). An interpretation of thermotherapy effects considers the new metabolic “pathways” triggered by the natural antiviral response emitted by the infected plant, with particular reference to virus-induced gene silencing. With regard to chemotherapy, several groups of antiviral drugs belong to inosine monophosphate dehydrogenase inhibitors, S-adenosylhomocysteine hydrolase inhibitors, neuraminidase inhibitors. Tissue culture, usually adopted to regenerate plantlets in biotechnological breeding programs, represents the less used tool for eliminate viruses from plants.

Additional key words: bibliographic research; chemotherapy; plant virus, thermotherapy; tissue culture.

Introduction

Looking at plant health from a holistic perspective, it is obvious that planting material must meet high sanitary standards, particularly with regard to viral infections. While it is widely believed that in many seed-propagated crops this is not the case or that, at any rate, it is of limited importance, the health status of plants subjected to vegetative propagation is critical. Historically good health of the produced plants, in terms of viruses, could only be assured by carefully selecting mother plants that were virus-free plants according to accepted diagnostic procedures and then propagating material that was guaranteed. Indeed, beginning in the 1960s, states or unions of states, such as the former European Economic Community, were involved in defining legal regulations regarding the health status of grapes (68/93/EEC), ornamental plants (77/93/EEC), and fruit

trees (92/34/EEC); a similar approach was also undertaken by the North American Plant Protection Organization (NAPPO). The most dangerous viruses are addressed by these regulations which then lead to the production of plants characterized by good health status, and their use can be considered safe. Mother plants are critical as they are used by nurseries to produce propagation material and distribute it, perhaps worldwide: strict regulatory measures are important to protect agricultural systems and environments from the spread of viruses. Generally the selection of germplasm, thanks to diagnostic tools and procedures recently developed in plant virology, leads to the identification of healthy wild types that can be included in vegetative production systems. However in some cases, identification of healthy plants among selected local varieties with limited diffusion can be difficult or impossible, making sanitation procedures necessary to recover useful healthy plants.

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Abbreviations used: CMV (*Cucumber mosaic cucumovirus*); IMP (inosine monophosphate); IMPDH (inosine monophosphate dehydrogenase); SAH (S-adenosylhomocysteine hydrolase); SAM (S-adenosylmethionine); TMV (*Tobacco mosaic virus*); VIGS (virus-induced gene silencing).

Attempts to sanitize virus-infected plants or portions of diseased plants to obtain new plants has not led to miraculous results, even if some recent technologies seem to offer new opportunities for facing difficulties that occur during sanitation procedures. The culture of meristems, combined with thermotherapy or chemotherapy offers encouraging results. However application of these approaches, as well as other techniques, has not led to a definitive clarification of the mechanisms of action involved, probably due to incomplete knowledge about the target virus and the mechanism of resistance activated by plants. Furthermore, the impact of sanitation techniques on the virus/host interaction still needs elucidation.

The aim of this paper is to provide a comprehensive and systematic survey of the literature pertaining to plant sanitation-related research issues in order to ascertain the current “state of the art” of thermotherapy (including cryotherapy or associated techniques such as meristem culture), chemotherapy and tissue culture (including techniques such as meristem tip and embryogenesis) techniques. All documents used in this study were accessed from the database of the Science Citation Index (SCI) (ISI, Web of Science, Philadelphia, USA), Science Direct (SD) (Elsevier, Amsterdam, Holland) and Google Scholar (Google Inc., Mountain View, USA). In this paper, we discuss trials (techniques used to get virus elimination) published from 1991, when the use of diagnostic assays such as immunoenzymatic or molecular tests were widely applied, until December 2010. The timeline and trials have been divided into four periods of five years: 1991-1995 (28 trials), 1996-2000 (34 trials), 2001-2005 (55 trials), and 2006-2010 (59 trials).

Thermotherapy

Thermotherapy treatment 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. In practice, the selected temperature represents the best compromise between virus degradation and plant survival, taking into account that the threshold of thermal sensitivity of some viruses is 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). The thermal cycles most frequently reported in 1991-2010 trials were set between

35°C and 38°C. Kassanis (1949) provided an interpretation of the results obtained with the treatment, basing it on identification of the infected cell as the environment where virus particles are in a dynamic equilibrium between newly formed particles and degraded ones. Thermal treatment, therefore, produces a shift in this balance towards greater viral degradation which, when repeated over time, can lead to elimination (Kassanis, 1957; Cooper & Walker, 1978). The principal alterations in viral particles as a result of thermal treatment above 35°C are related to the rupture of hydrogen and disulfide bonds of capsid protein, followed by nucleic acid phosphodiester covalent bonds, and consequently, even deterioration of viral infectivity which can include selective inhibition of viral replicase, changes in pH and cellular ionic strength, increase of lytic enzymes, competition between viral RNA and messenger for ribosome bonds.

In the field of thermotherapy, cryotherapy represents a whole new approach (Nukari *et al.*, 2009; Wang & Valkonen, 2009; Wang *et al.*, 2009). The freezing of shoot tips (*i.e.* in liquid nitrogen, and subsequent thawing and regeneration to shoots) was found to result in virus-free plants with high efficiency. Moreover cryotherapy takes only a few days: a minor addition to the whole procedure of virus elimination which requires several months. Meristem culture of shoot tips was also used to enhance thermotherapy virus elimination as the elimination ratio of viruses is higher when the size of isolated tissue (*i.e.* shoot tip) is smaller (Mori & Hosokawa, 1977).

From our literature survey, thermotherapy is the technique most frequently applied in sanitation protocols: in the time frame 1991-2010, trials relative to thermotherapy were the most frequently published considering each period (Fig. 1).

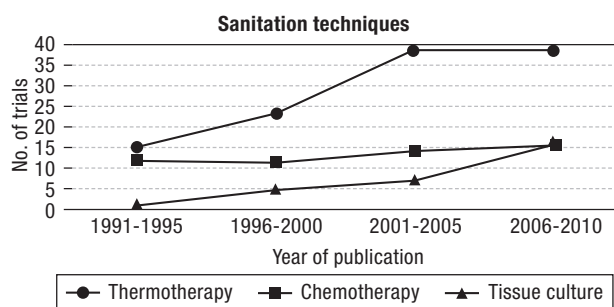


Figure 1. Distribution by antiviral techniques (thermotherapy, chemotherapy or embryogenesis) in trials published during 1991-2010.

Thermotherapy applications

Plant thermotherapy was applied in woody and herbaceous plants in, respectively, 60.3% and 39.7% of published trials from 1991 to 2010 (Table 1). Among woody plants, grapevine sanitation was largely investigated (17.4%), as well as apple (9.1%) and peach sanitation (7.4%). Among herbaceous plants, garlic was sanitized from various viruses (9.1%), as well as potato (6.6%). Thermotherapy was successfully applied against viruses belonging to 13 families and an unassigned genus (Table 1).

Thermotherapy applications can cause phenotypical modification such as double nodes and modified leaf shape (Koruza & Jelaska, 1993). In addition, specific effects were reported in grapevine, such as an increment in grape quality (Mannini *et al.*, 1996) or in phenolic concentration in leaves and berries (Guidoni *et al.*, 1997).

Mechanism of action

Plant thermotherapy is described as achieving a cellular environment which is progressively less adequate for virus vitality (Pennazio, 1995). Similar interpretations are also reported by Mink *et al.* (1998) who discussed the effects of heat treatment on the functionality of viral movement proteins able to produce a restriction of the infected tissues. In fact, the different ability for movement of viral particles in plant tissues influenced the choice of elimination treatment, with thermotherapy as the most effective against viruses characterized by parenchymatic localization, compared to meristem culture technique which is more suitable for phloematic viruses that are limited to vascular tissues and rarely found in parts of the plant where differentiated tissues are absent (Grout, 1990). However, up to now, differences in localization of phloem and parenchymatic viruses in the host tissue have not fully explained their different susceptibilities to thermotherapy elimination. A study carried out by subjecting a homogeneous collection of germplasm differently infected by phloematic viruses to thermotherapy, found different levels of susceptibility to heat stress by different viral agents, thus suggesting other influential parameters on the mechanism of elimination besides that of the tissue localization (Panattoni & Triolo, 2010).

Developments over the last 20 years in research aimed at investigating the metabolic processes involved in defense mechanisms of plants have suggested an interpretation of the heat treatment effects according to new

metabolic “pathways” triggered by the natural antiviral response produced by the infected plant, with particular reference to Virus-Induced Gene Silencing (VIGS) induced by the presence of viral RNA in infected plants (Ruitz *et al.*, 1998). The process was first observed and described during studies of healthy transgenic plants where silencing is activated by plant cells as a means of genic control against all sequences that have no homology with those of their genome, including those of viral origin which in this context would trigger the same reaction (Mourrain *et al.*, 2000; Carrington *et al.*, 2001; Dalmay *et al.*, 2001; Vance & Vaucheret, 2001; Voinnet, 2001). RNA silencing was described as such an effective defense as to constitute an immunity mechanism at the genomic level. It is characterized by adaptability, specificity and mobility also in consideration of the system’s own RNA signal to the most distant parts in terms of infection (Carrington *et al.*, 2001; Voinnet, 2001). Over the course of investigations on infected plants, a correlation between VIGS and the thermal regimes to which a plant is submitted has emerged. Studies included treatments with temperatures lower (30°C) than those set out in standard thermotherapy protocols (36°C), allowing more precise investigation of possible relationships between gene silencing and thermal increase (Szittyá *et al.*, 2003; Qu *et al.*, 2005). Moreover, in research conducted by Szittyá *et al.* (2003), *Nicotiana benthamiana* plants infected with *Cymbidium ringspot virus* were exposed to different thermal regimes between 15°C and 27°C. For each heat treatment concentrations of short interfering RNA were determined (elevated at 27°C and undetectable at 15°C), while an increasing gradient, starting from 21°C, was observed in reference to the treatments. In relation to the different spread of viral particles observed in the temperature range tested, the authors identified a hyper activity of the system of temperature-dependent gene silencing, as a mechanism of antiviral protection of the plant. VIGS was defined by these authors as a defense system that operates ineffectively at low temperature, therefore increasing the plant’s susceptibility to virus infections that do not encounter blocking gene systems. In contrast, increased heat stress induces an increase in the host defense system’s capacity by creating a barrier to infection. Chellapan *et al.* (2005) continued investigations to better define the mechanisms that determine the influence of temperature on the antiviral silencing, also for *Geminivirus* (ssDNA), by applying heat treatment (25-30°C) to cassava (*Manihot esculenta*) and tobacco

Table 1. Plants subjected to thermotherapy experiences during 1991-2010

| Plant thermotherapy | % of trials ¹ | Family/genus ² | References |
|--------------------------|--------------------------|--|---|
| Woody plants | 60.3 | | |
| Grapevine | 17.4 | <i>Bromoviridae; Closteroviridae; Comoviridae; Flexiviridae; Secoviridae; Tymoviridae</i> | Hatzinikolakis & Roubelakis-Agelakis, 1993; Kuniyuki <i>et al.</i> , 1994; Spiegel <i>et al.</i> , 1995; Guidoni <i>et al.</i> , 1997; Leonhardt <i>et al.</i> , 1998; Gribaudo <i>et al.</i> , 1999; Buciumeanu & Visoiu, 2000; Milkus <i>et al.</i> , 2000; Valero <i>et al.</i> , 2003; Bertamini <i>et al.</i> , 2004; Gribaudo <i>et al.</i> , 2006; Komar <i>et al.</i> , 2007; Panattoni <i>et al.</i> , 2007a; Salami <i>et al.</i> , 2009; Skiada <i>et al.</i> , 2009a; Panattoni & Triolo, 2010 |
| Apple | 9.1 | <i>Betaflexiviridae; Bromoviridae; Flexiviridae; Potyviridae</i> | Yamaga & Munakata, 1991; Knapp <i>et al.</i> , 1995; Bhardwaj <i>et al.</i> , 1998; Chen & Li, 2001; Wang <i>et al.</i> , 2006; Cieslinska, 2002; Manganaris <i>et al.</i> , 2003b; Freitas <i>et al.</i> , 2004; Paunovic & Jevremovic, 2006; Sedlak <i>et al.</i> , 2007; Talacko <i>et al.</i> , 2007; Paprstein <i>et al.</i> , 2008; Wang LP <i>et al.</i> , 2010 |
| Peach | 7.4 | <i>Bromoviridae</i> | Stein <i>et al.</i> , 1991; Gella & Errea, 1998; Zilkah <i>et al.</i> , 2001 |
| Pear | 6.7 | <i>Flexiviridae; Tombusviridae</i> | Gella & Errea, 1998; Refatti <i>et al.</i> , 1999; Saponari <i>et al.</i> , 1999; Postman & Sugar, 2002; Zilka <i>et al.</i> , 2002; Tan <i>et al.</i> , 2010 |
| Apricot | 3.3 | <i>Potyviridae</i> | Manganaris <i>et al.</i> , 2003a; Laimer <i>et al.</i> , 2006; Koubouris <i>et al.</i> , 2007; Polak & Hauptmanova, 2009 |
| Plum | 3.3 | <i>Bromoviridae</i> | Dziedzic, 2008 |
| Raspberry | 3.3 | <i>Idaeoviruses</i> | Karesova <i>et al.</i> , 2002 |
| Others* | 9.8 | <i>Alphaflexiviridae; Bromoviridae; Caulimoviridae; Closteroviridae; Comoviridae; Flexiviridae</i> | Cheema <i>et al.</i> , 1999; Helliot <i>et al.</i> , 2002, 2004; Arif <i>et al.</i> , 2005; Saponari <i>et al.</i> , 2007; Kenganal <i>et al.</i> , 2008; Previati <i>et al.</i> , 2008; Sharma <i>et al.</i> , 2008 |
| Herbaceous plants | 39.7 | | |
| Garlic | 9.1 | <i>Flexiviridae; Potyviridae</i> | Le <i>et al.</i> , 1991; Bruna, 1997; Ghosh <i>et al.</i> , 1997; Robert <i>et al.</i> , 1998; Senula <i>et al.</i> , 2000; Fajardo <i>et al.</i> , 2002; Bertaccini <i>et al.</i> , 2004; Conci <i>et al.</i> , 2005; Patena <i>et al.</i> , 2005; Ramírez-Malagón <i>et al.</i> , 2006 |
| Potato | 6.6 | <i>Alphaflexiviridae; Flexiviridae; Potyviridae</i> | Faccioli & Colombarini, 1991, 1996; El-Amin <i>et al.</i> , 1994; Pozzer <i>et al.</i> , 1994; Horackova <i>et al.</i> , 1999; Kryszczuk, 1999; Nascimento <i>et al.</i> , 2003; López-Delgado <i>et al.</i> , 2004 |
| Artichoke | 2.5 | <i>Comoviridae; Potyviridae</i> | Barba, 2001; Navacchi <i>et al.</i> , 2005; Pace <i>et al.</i> , 2008 |
| Chrysanthemum | 2.5 | <i>Bromoviridae; Flexiviridae</i> | Ram <i>et al.</i> , 2005, 2009 |
| Sugarcane | 2.5 | <i>Potyviridae</i> | Victoria <i>et al.</i> , 1999; Balamuralikrishnan <i>et al.</i> , 2003; Ramgareeb <i>et al.</i> , 2010 |
| Sweet potato | 2.5 | <i>Geminiviridae; Potyviridae</i> | Green <i>et al.</i> , 1992; Jeeva <i>et al.</i> , 2004 |
| Others** | 14.0 | <i>Alphaflexiviridae; Bromoviridae; Bunyaviridae; Flexiviridae; Geminiviridae; Idaeoviruse; Potyviridae; Secoviridae</i> | Chen & Sherwood, 1991; Dunbar <i>et al.</i> , 1993; Ahiabu <i>et al.</i> , 1997; Petrzik & Svoboda, 1997; Postman, 1997; Malaurie <i>et al.</i> , 1998; Shibolet <i>et al.</i> , 2001; Li <i>et al.</i> , 2002; Mangal <i>et al.</i> , 2002; Uchanski <i>et al.</i> , 2002; Cieslinska, 2003; Fraga <i>et al.</i> , 2004; Verma <i>et al.</i> , 2005; Zhang <i>et al.</i> , 2006; Tomassoli <i>et al.</i> , 2008; Nesi <i>et al.</i> , 2009; Nukari <i>et al.</i> , 2009; Ling, 2010; Wasswa <i>et al.</i> , 2010 |

¹ Percentage of trials out of total. ² Family/genus of virus eliminated by thermotherapy. * Banana, cherry, citrus, fragaria, mandarin, olive, rose. ** Begonia, blueberry, caper, carnation, cassava, hop, horseradish, lilly, peanut, phlox, strawberry, taro, tomato, ulluco, yam.

(*Nicotiana benthamiana*) plants infected by Cassava mosaic disease. They achieved similar results and confirmed the close relationship between temperature and VIGS. For their part, Qu *et al.* (2005) considered plants of *N. benthamiana* infected by *Potato virus X* exposed to different thermal regimes (up to 33°C) with particular attention to the involvement of RNA-dependent RNA polymerase (RdRp), which is sensitive to temperature changes and thus induces the silencing complex, thereby highlighting its role. Wang *et al.* (2008) conducted combined thermotherapy (38°C) and cryotherapy treatments associated with the removal of meristem tips on raspberry plants infected by *Raspberry dwarf virus*. From their results, the authors noted the close relationship between temperature and RNA silencing which seems to act as a means to increase the degradation of virus RNA.

Chemotherapy

The development of research in the field of chemotherapy has not been as lively as the work conducted on thermotherapy, but valuable contributions have been provided by the most extensive investigations of antiviral chemotherapy performed in clinical medicine. In this regard, the discovery of ribavirin (Sidwell *et al.*, 1972; Huffman *et al.*, 1973) represented a defining moment in the research, marking a different route of investigation in the study of new chemical synthesis analogues of nucleoside or precursors of RNA bases. In fact, knowledge of the complex interactions that develop between virus and host cell provides a guide for selecting the potentially most suitable treatment. To date, more than 40 antiviral molecules, synthesized in this way, are available on the market for clinical application. Keeping in mind the relevant differences between animal and plant hosts, the potential similarities between metabolic pathways present in both have been the starting point for experimentation on phytoviruses, making possible to highlight the effectiveness of these antiviral drugs in the botanical field. However, in plant virology, the fact that less resources are available and that there has been a delay in knowledge of the molecular characteristics of many phytoviruses, means that fewer results than in medicine are available.

Chemotherapy applications

Herbaceous plants were more frequently investigated than woody plants, with 66.0% and 34.0% respectively

of published trials in 1991-2010 (Table 2). Potato (18.9%), orchid and tobacco (9.4%) were subjected to many treatments, while grapevine sanitation represented the main treated host among woody plants (11.3%) as well as apple and plum (7.5%).

Chemotherapy, mainly with well-known pro-drugs such as ribavirin, was successfully performed against viruses belonging to 9 families and an unassigned genus (Table 2).

Synthetic nucleoside such as tiazofurin, selenazofurin (2-pD-ribofuranosylselenazole-4-carboxamide) and benzamide riboside (3-(1-deoxy-pD-ribofuranosyl) benzamide, and non-nucleosides such as micophenolic acid [6-(4-hydroxy-6-methoxy-7-methyl-3-oxo-1,3-dihydroisobenzofuran-5-yl)-4-methyl-hex-4-enoic acid] were tested against *Cucumber mosaic cucumovirus* (CMV) and *Grapevine leafroll-associated virus 3* (GLRaV-3) (Panattoni *et al.*, 2005, 2007a). Interesting results were obtained using dihydroxypropyladenine [(RS)-9-(2,3-dihydroxypropyl) adenine] in combination with ribavirin and resulted in the elimination of *Grapevine vitivirus A* (Panattoni *et al.*, 2007b). Surprising positive results were achieved by supplying oseltamivir to *in vitro Nicotiana tabacum* explants infected by CMV and *V. vinifera* explants infected by GLRaV-3; high rates of sanitation in both combinations were noted (D'Anna *et al.*, 2006; Panattoni *et al.*, 2006; Guta *et al.*, 2010). Moreover, replication of *Tobacco mosaic virus* (TMV) was inhibited by bitriazolyl compounds (Xia *et al.*, 2006), tylophorine B (Xi *et al.*, 2006), phenanthrene-based tylophorine derivatives (Wang *et al.*, 2010a), derivatives of thiadiazoleacetamide (Zhao *et al.*, 2006), cyanoacrylate derivatives (Chen *et al.*, 2008), and racemic phenanthroindolizidine alkaloids or pure alkaloids (Wang *et al.*, 2010b): no sanitized plants were reported for these novel compounds.

Mechanism of action

In contrast to thermotherapy, chemotherapy in plants was poorly investigated considering the mechanism of action involved. The inhibition of replication of TMV was reported using nucleobase or nucleoside analogues (Schulze & Kluge, 1994), bitriazolyl compounds (Xia *et al.*, 2006), tylophorine B (Xi *et al.*, 2006), and derivatives of thiadiazoleacetamide (Zhao *et al.*, 2006).

Several groups of antiviral drugs, that have shown significant therapeutic potential against plant viru-

Table 2. Plants subjected to chemotherapy experiences during 1991-2010

| Plant chemotherapy | % of trials ¹ | Family/genus ² | References |
|--------------------------|--------------------------|--|--|
| Woody plants | 34.0 | | |
| Grapevine | 11.3 | <i>Closteroviridae; Comoviridae; Flexiviridae</i> | Weiland <i>et al.</i> , 2004; Panattoni <i>et al.</i> , 2006, 2007a,b; Skiada <i>et al.</i> , 2009b |
| Apple | 7.5 | <i>Flexiviridae</i> | Yamaga & Munakata, 1991; James <i>et al.</i> , 1997; Cieslinska, & Zawadzka, 1999; Cieslinska, 2002; O'Herlihy <i>et al.</i> , 2003 |
| Plum | 7.5 | <i>Betaflexiviridae; Bromoviridae; Potyviridae</i> | Janeckova, 1993; Gabova, 1995; Chen & Li, 2001; Paunovic <i>et al.</i> , 2007 |
| Bamboo | 1.9 | <i>Flexiviridae</i> | Chen & Lu, 2000 |
| Black currant | 1.9 | <i>Secoviridae</i> | Kolbanova <i>et al.</i> , 2004 |
| Citrus | 1.9 | | Iwanami & Ieki, 1994 |
| Fragaria | 1.9 | <i>Secoviridae</i> | Cieslinska, 2003 |
| Herbaceous plants | 66.0 | | |
| Potato | 18.9 | <i>Flexiviridae; Geminiviridae; Potyviridae</i> | Conrad, 1991; Faccioli & Colombarini, 1991; Green <i>et al.</i> , 1992; Park <i>et al.</i> , 1994; Kim <i>et al.</i> , 1996; Truskinov & Rogozina, 1997; Faccioli & Zoffoli, 1998; Horackova, 1998; Nascimento <i>et al.</i> , 2003; Fang <i>et al.</i> , 2005 |
| Orchid | 9.4 | <i>Flexiviridae</i> | Loi <i>et al.</i> , 1991 |
| Tobacco | 9.4 | <i>Tobamovirus</i> | Schulze & Kluge, 1994 |
| Lilly | 5.7 | <i>Bromoviridae; Potyviridae</i> | Kim <i>et al.</i> , 1994; Xu & Niimi, 1999; Xu <i>et al.</i> , 2000 |
| Chrysanthemum | 3.8 | <i>Bromoviridae; Flexiviridae</i> | Ram <i>et al.</i> , 2005, 2009 |
| Garlic | 3.8 | <i>Potyviridae</i> | Bertaccini <i>et al.</i> , 2004; Ramírez-Malagón <i>et al.</i> , 2006 |
| Tomato | 3.8 | <i>Tobamovirus</i> | Xu <i>et al.</i> , 2004 |
| Others* | 11.2 | <i>Alphaflexiviridae; Bromoviridae; Comoviridae; Flexiviridae; Tobamovirus</i> | Chen & Sherwood, 1991; Lim <i>et al.</i> , 1993; Toussaint <i>et al.</i> , 1993; Fletcher <i>et al.</i> , 1998; Freitas & Rezende, 1998; Yap <i>et al.</i> , 1999; Fletcher & Fletcher, 2001; Ling <i>et al.</i> , 2003; Jiang <i>et al.</i> , 2005; Navacchi <i>et al.</i> , 2005; Verma <i>et al.</i> , 2005; Singh <i>et al.</i> , 2007; Ling, 2010 |

¹ Percentage of trials out of total. ² Family/genus of virus eliminated by chemotherapy. * Artichoke, begonia, gladiolus, onion, peanut, sweet potato, ulluco, cymbidium.

ses, belong to inosine monophosphate dehydrogenase (IMPDH) inhibitors, S-adenosylhomocysteine hydrolyase (SAH) inhibitors, neuraminidase (NA) inhibitors. IMPDH inhibitors represent a class of molecules derived from the structure of ribavirin and they are characterized by a pronounced antiviral activity, as shown by the large number of molecules tested. Ribavirin is a synthetic nucleoside analogue of guanosine, synthesized by Sidwell *et al.* (1972) during testing in clinical medicine against the *Respiratory syncytial virus*. It subsequently proved to be effective also against other viruses such as *Influenza viruses type A and B* and *Lassa virus*, the agent of the fever of the same name (Stein *et al.*, 1987). The initial hypothesized mechanism of

action lay in its potential to inhibit inosine monophosphate dehydrogenase but, in light of recent acquisitions, its mechanism appears to be more complex and articulated in several ways, and it has not yet been fully described (Jayaram *et al.*, 1999). IMPDH inhibitors are directly involved in the process of transcription of triphosphate ribonucleotides and thus lead to inhibition of viral nucleic acid replication. In fact, the principal target of antiviral activity of these molecules is the inosine monophosphate dehydrogenase, an enzyme that catalyzes the conversion of inosine 5'-monophosphate (IMP) in xantosine 5'-monophosphate and is able to alter the pathway for the production of guanosine mono- di- and triphosphate. The presence of its inhibitor acts on this

path by reducing the intracellular pool of guanosine and thus also preventing the synthesis of viral RNA (Franchetti *et al.*, 1996). In addition, guanosine triphosphate is responsible for converting IMP to succinyladenine monophosphate catalyzed by adenosylsuccinate that leads to the production of adenosine triphosphate and then its reduction leads to reduction of the ATP potentially needed for viral synthesis (Streeter *et al.*, 1973). Moreover, ribavirin's antiviral effect is by forcing RNA viruses into error catastrophe (Crotty *et al.*, 2001).

The effectiveness of antiviral molecules belonging to SAH hydrolase inhibitors has been known for some time and centers on the mechanism of action of SAH hydrolase, another key enzyme for viral replication. 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 phospholipids, and is then converted to S-adenosylhomocysteine. Methylation is regulated negatively by both an increase in SAH and a reduction of SAM or SAM/SAH ratio. The removal of SAH plays an essential role and it is mediated by SAH hydrolase able to convert this molecule into homocysteine and adenosine. The accumulation of SAH thus makes their conversion and consequent blocking of the maturation of viral RNA impossible, in particular without terminating formation of the "cap" (De Clercq, 2005).

The mechanism of action of NA inhibitors is based on the inhibition of neuraminidases 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 neuraminidase, preventing the release and spread of newly-generated virion progeny from infected cells to healthy ones (Gubareva, 2004). Neuraminidase is a glycoprotein found in the membrane lining of flu virus. Cutting the terminal residues of sialic acid found on the surface of infected cells, it can activate multiple effects that promote the release of new virus particles and also prevent the formation of viral aggregates after release from the host cell, preventing virus inactivation, virus spread in the respiratory tract and inducing apoptosis (McClellan & Perry, 2001). In light of the importance of this enzyme in viral replication and pathogenesis, medical research has focused on the development of selective inhibitors, in particular sialic acid analogues, for the prophylaxis and treatment of flu. Currently, two NA inhibitors are commercially

available: zanamivir (Relenza[®], GlaxoSmithKline) and oseltamivir (Tamiflu[®], Gilead/Roche) (McClellan & Perry, 2001). Unfortunately, plant viruses sensitive to Oseltamivir are not involved with replication steps related to neuraminidase protein, and the mechanism of action of these inhibitors in plants are yet unknown.

Tissue culture

Plant tissue culture is a technique based in the isolation of small parts of plants (tips, meristems and somatic embryos) and growing them on artificial media in adequate conditions so the parts of plants can grow and develop into complete plants (Hollings, 1965). Moreover, this technique can be used to produce virus-free plants. The size of tissue like shoot tip (5.0-10.0 mm) or meristem portion (0.2-0.7 mm) is the critical point for the achievement of virus eradication, considering than smaller portion of tissue can be characterized by a lower virus concentration. The size of the meristematic dome determines the ability of explant to survive on a specific nutrient medium and the time required to establish a new plant. Starting from a small meristem several months are needed to obtain the new plantlets, while 1-2 months can be enough if the starting material is a shoot-tip culture (Faccioli, 2001). Moreover, considering that this technique is time-consuming, it is common to use different media supplemented with several hormones. Anyway, these media could represent a critical step for the stability of genetic and performance profile of the progeny (Jayasinghe & Salazar, 1997).

Somatic embryogenesis, usually adopted to regenerate plantlets in biotechnological breeding programs, has been used to eliminate viruses from plants. Explants such as anthers, ovaries or leaves, were cultivated on a callus induction medium, and the kind of infected tissue used interfered with elimination rates (Popescu *et al.*, 2010). The calli were transferred to an embryo differentiation medium, producing embryo-derived plantlets able to be micropropagated by cultural apical cuttings.

The presence of virus particles in callus and the regeneration of healthy embryos or plantlets is 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, Popescu *et al.* (2003) showed how sanitation rates, and genetic variations, could be

also related to the length of time required for callus induction. For example, very short time of subcultures could not allow virus particles spreading from infected to healthy tissue.

Considering the complex of 1991-2010 research, 55.2% of trials dealing with tissue culture were published between 2006 and 2010 (Fig. 1).

Tissue culture applications

Tissue culture research was almost equally focused on woody or herbaceous plants (48.3 and 51.7%, respectively) (Table 3). Among woody plants, grapevine sanitation was largely investigated (34.5%). Sanitation of herbaceous plant was mainly referred to sugarcane (13.8%), garlic (10.3%) and potato (6.9%). Tissue culture was performed against viruses belonging to 9 families (Table 3).

Mechanism of action

The recovery of pathogen-free clones from source infected plants through the use of tissue culture techniques is based on the premise that pathogen concentration is not uniform throughout the infected plant such as shoot tips. In particular, the meristematic tissue

from roots and terminal sprouts could be pathogen-free. Due to the fact that the most differentiated vascular tissue is far away from the meristems, the vascular elements of the primordium leaves are incipient and are not yet in contact with the principal part of the stem's vascular system. For this reason, virus particles present in the mature vascular system can only reach the top of the meristem zone by moving slowly from cell to cell. Instead viruses that infect non-vascular tissues are disseminated from cell to cell through plasmodesmata that represent a slow process which makes it relatively difficult for viruses to infect that apical zones (Faccioli, 2001).

The mechanism whereby regenerated somatic embryos are freed of some viruses is not clear. It was reported that phloem-limited viruses are able to invade initially the callus derived from anther and ovary cultures (Gambino *et al.*, 2006), but translocation of these viruses from infected tissue to somatic embryos was not observed (Goussard *et al.*, 1991; Gambino *et al.*, 2006) or, in some cases, translocation depends on the genotype and the length of time necessary for regeneration of tissues (Popescu *et al.*, 2003).

Comparison of techniques

Thermotherapy, even in association with other techniques such as tissue culture, represents the most fre-

Table 3. Plants subjected to tissue culture experiences during 1991-2010

| Plant tissue culture | % of trials ¹ | Family/genus ² | References |
|--------------------------|--------------------------|--|--|
| Woody plants | 48.3 | | |
| Grapevine | 34.5 | <i>Closteroviridae</i> ; <i>Comoviridae</i> ; <i>Flexiviridae</i> ; <i>Tymoviridae</i> | Goussard & Wiid, 1992; Popescu <i>et al.</i> , 2003, 2010; Gambino <i>et al.</i> , 2006, 2010; Gribaudo <i>et al.</i> , 2006; Borroto-Fernández <i>et al.</i> , 2009; Youssef <i>et al.</i> , 2009 |
| Others* | 13.8 | <i>Bromoviridae</i> ; <i>Caulimoviridae</i> ; <i>Ophioviridae</i> ; <i>Potyviridae</i> | Ramos & Zamora, 1999; D'Onghia <i>et al.</i> , 2001; Golino <i>et al.</i> , 2007; Quainoo <i>et al.</i> , 2008 |
| Herbaceous plants | 51.7 | | |
| Sugarcane | 13.8 | <i>Luteoviridae</i> ; <i>Potyviridae</i> | Fitch <i>et al.</i> , 2001; Parmessur <i>et al.</i> , 2002; Ramgareeb <i>et al.</i> , 2010 |
| Garlic | 10.3 | <i>Potyviridae</i> | Ebi <i>et al.</i> , 2000; Ayabe & Sumi, 2001; Ramírez-Malagón <i>et al.</i> , 2006 |
| Potato | 6.9 | <i>Potyviridae</i> | Truskinov & Rogozina, 1997; Mahmoud <i>et al.</i> , 2009 |
| Others** | 20.7 | <i>Bromoviridae</i> ; <i>Caulimoviridae</i> ; <i>Flexiviridae</i> ; <i>Potyviridae</i> | Wangai & Bock, 1996; Morris <i>et al.</i> , 1997; Mangal <i>et al.</i> , 2002; Šedivá <i>et al.</i> , 2006; Kumar <i>et al.</i> , 2009; Kabir <i>et al.</i> , 2010 |

¹ Percentage of trials out of total. ² Family/genus of virus eliminated by thermotherapy. * Banana, citrus, cocoa, rose. ** Carnation, chinchinchee, chrysanthemum, dahlia, peanut, pumpkin.

Table 4. Distribution by antiviral techniques (thermotherapy, chemotherapy or tissue culture) in most frequently sanitized woody and herbaceous plants in trials published during 1991-2010

| | Thermotherapy | Chemotherapy | Tissue culture |
|--------------------------|---------------|--------------|----------------|
| Woody plants | | | |
| Grapevine | 56.8 | 16.2 | 27.0 |
| Apple | 73.3 | 26.7 | 0.0 |
| Peach | 100.0 | 0.0 | 0.0 |
| Herbaceous plants | | | |
| Garlic | 68.7 | 12.5 | 18.8 |
| Potato | 40.0 | 50.0 | 10.0 |
| Sugarcane | 42.9 | 0.0 | 57.1 |

quently used technique for sanitation over the last 20 years, while applications of methods such as chemotherapy and tissue culture represent the main topic in plant sanitation research.

Among woody plants, grapevine, apple and peach are the most frequent targets of sanitation protocols because their sanitary status is strictly regulated by legal provisions (Mink *et al.*, 1998). Even if thermotherapy represents the preferred method for each host, grapevine viruses can be eliminated even with chemotherapy and tissue culture, whereas apple viruses can be removed by chemotherapy as well (Table 4). Among herbaceous plants, chemotherapy was the technique most frequently used in potato, while tissue culture was the preferred one for sugarcane (Table 4).

Discussion

The high level of specialization attained by many viruses due to their replication and pathogenetic mechanisms towards the host make them an extremely variable and complex target. This complexity is the background upon which a strategy can be optimized, as reported in clinical research (De Clercq, 2002, 2005). Therefore, the outcome of therapeutic action is strongly influenced by the ontological properties of the virus to be eliminated as well as the characteristics expressed by the plant as well as the trans-membrane transport of drugs (Luvisi *et al.*, 2012a; Rinaldelli *et al.*, 2012). The application of chemotherapy or thermotherapy should aim at stopping the synthesis of new virions, but elimination can be achieved only if viral particles formed prior to treatment are completely eliminated, as suggested in research dated before 1991 (Kassanis,

1957; Cooper & Walker, 1978). For example, in many cases the chemotherapy target is an enzyme that can be efficiently blocked for the synthesis of new virus particles, but it is generally ineffective against already formed virus particles, which can only naturally degrade according to specific virus properties and host characteristics. Conversely, thermotherapy treatment is potentially effective in degrading viral particles present in cells, but its performance is poor with regard to the synthesis of new ones. In any case, this topic was rarely reported in virus elimination research on plants.

In conclusion, it is not sufficient just to choose drug or thermal exposure. Other parameters have to be taken into account as well, for example 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, even if the applied treatment is actually capable of blocking the activity of viral replication (Luvisi *et al.*, 2012b). Furthermore, the complex interaction between host and virus has been underlined by recent evidence, such as gene silencing and silencing-suppressor proteins, leading to new tools and improved antiviral therapies.

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