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Review paper

Virus Elimination from Ornamental Plants Using *in vitro* Culture Techniques

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SUMMARY

Viruses are responsible for numerous epidemics in different crops in all parts of the world. As a consequence of their presence great economic losses are being incurred. In addition to the development of sensitive techniques for detection, identification and characterization of viruses, substantial attention has also been paid to biotechnological methods for their elimination from plants. In this review article, the following biotechnological *in vitro* culture techniques for virus elimination from ornamental plants are presented: meristem culture, thermotherapy, chemotherapy, cryotherapy or a combination of these methods. The plant species, as well as the type of virus determine the choice of a most suitable method. The state of the art in investigation of virus elimination from *Impatiens* sp. in Serbia is summarized.

Keywords: Viruses; Ornamental plants; Epidemics; Biotechnology

INTRODUCTION

During the past several decades, the frequency of plant diseases caused by viruses has increased worldwide. The growing demands for high quality and disease-free planting material, increased volume of international seed and plant trade, high phytosanitary standards imposed by many countries for plant material imports, as well as the fact that a great number of viruses have a wide range of hosts, emphasize the significance of virus diseases of ornamentals.

The genus *Impatiens* includes over 900 species growing in tropical and subtropical regions of Africa, Madagascar, North India and Sri Lanka, East Himalayas and Southeast Asia. In South America and Australia, *Impatiens* spp. are introduced species. Some species have been grown as horticultural plants both in Serbia and worldwide and they have a significant role in improving public greeneries, gardens and balconies (Ferrante et al., 2006; Vujošević et al., 2008).

Viruses spread from mother plants to their progenies by infected cuttings, tubers and other plant vegetative propagation materials. Since the *Impatiens* species, like most ornamentals in commercial production, are reproduced by *vegetative propagation*, there is a great probability of virus transmission (Gera and Zeidan, 2006; Wang and Valkonen, 2008). Taking into account the fact that

the *Impatiens* genus is more and more popular in our country, while virus infection is a limiting factor in commercial production, the basic goal of this survey is to gain an insight into possibilities of practicing *in vitro* culture techniques to eliminate viruses from plant tissue.

Literature sources describe several viruses which are infective for *Impatiens*: *Tomato spotted wilt virus* (TSWV), *Impatiens necrotic spot virus* (INSV), *Tobacco streak virus* (TSV), *Tobacco ringspot virus* (TRSV), *Helenium virus S* (HSV) and *Cucumber mosaic virus* (CMV) (Gera and Dehan, 1992; Lembo Duarte et al., 2007). A great problem in greenhouse production of *Impatiens* species is caused by polyphagous pathogens, such as TSWV. Tehrani et al. (1990), Hausbeck et al. (1992), Ruter and Gitaitis (1993), Daughtrey (1996), as well as Krstić and Bulajić (2007), Đekić et al. (2008) and Milošević (2010) have described natural infections of *I. walleriana* with TSWV. Natural infections of *I. hawkerii* have been registered in the Netherlands (Verhoeven and Roenhorst, 1994), the USA (Daughtrey, 1996) and Serbia (Krstić and Bulajić, 2007; Đekić et al., 2008; Milošević, 2010; Milošević et al., 2011).

Techniques of *in vitro* culture include a group of significant biotechnological procedures which are successfully applied in many branches of agriculture, including horticulture, for the purpose of producing plants with improved features (Kumar Pati et al., 2006). Methods of plant regeneration in culture *in vitro* are being applied widely in commercial production of ornamentals worldwide, ensuring elimination of pathogens from infected material and production of disease-free plants (Akin-Idowu et al., 2009). Numerous commercial laboratories worldwide use the potential of *in vitro* propagation for massive plant production. On the other hand, the application of *in vitro* culture has been limited by the costs of required equipment, special treatments required during acclimatization and risk related to somaclonal variation and other physiological aberrations (Winkelmann et al., 2006).

Elimination of viruses from plant material includes the following stepdetection and identification of viruses in the initial plant material (by serological and molecular methods, electron microscopy, immunolocalization or biotest),

- therapy by applying biotechnological techniques (meristem culture, thermo- therapy, chemotherapy, cryotherapy, micrografting),
- testing of the treated plants for the presence of viruses using the same methods as described,
- micropropagation of virus-free plants with periodical testing for a possible presence of viruses.

MERISTEM CULTURE

The most important technique in micropropagation is meristem proliferation wherein apical buds or nodal segments harbouring an axillary bud are cultured to regenerate multiple shoots without any intervening callus phase (Kumar Pati et al., 2006). Meristem culture is an efficient tool for regeneration, elimination of viruses from infected plants and production of virus-free seed material of different plant species (Grout and Brian, 1999; Faccioli, 2001; Rout et al., 2006). *Viruses* do not have their own *metabolism, and* virus replication, which occurs in a host cell, is dependent on the host cell's protein and nucleic acid synthesizing machineries and metabolic pathways. Virus control is difficult because virus replication inhibitors have shown to be toxic to the host cell as well. So, effective control agents (usually chemical agents) exist for most plant diseases, except those caused by viruses. The main reason for use of the meristem culture is the fact that most viruses do not attack the meristem of shoots, since the multiplication of meristem cells is faster than replication of viruses, although more recent studies have shown that the mechanism of gene silencing is in fact the main reason (Foster et al., 2002).

Meristem culture is a procedure which does not differ in general from procedures for shoot reproduction *in vitro.* However, an important requirement is that initial explants be as small as possible in order to be able to isolate only the apical meristem, rather than permanent cells containing viruses. Morel and Martin (1952) first demonstrated the elimination of viruses from *Dahlia* using *meristem culture*. Since then, the use of *meristem culture* to obtain *virus*-free ornamental plants has been widely used by numerous groups of researchers (Table 1).

Methods of meristem culture are more appropriate than callus culture because continuous proliferation of undifferentiated tissue during subculturing can increase the risk of genetic and/or epigenetic variations (Nesi et al., 2009). Dahlia, freesia, geranium, lily, etc., have been grown virus-free using meristem culture (Ram et al., 2005). However, production of virus-free plants by using meristem culture has numerous obstacles, such as: meristem size, meristem position on a plant, protocol applied during detection (different sensitiveness of the applied methods), endogen infections and the period of recovery and growth of produced plants (Cha-um et al., 2006). Furthermore, the distribution of viruses in shoot tips may vary depending on a virus-host combination. Therefore, various sizes of shoot tips must be excised and tested to determine the optimal size range supporting efficient virus elimination and also a high rate plant regeneration.

Table 1. Elimination of viruses from ornamental plants by shoot meristem culture or its combination with other biotechnological procedures

Species	Procedure	Virus	Reference
Alstromeria sp.	meristem culture	*Alstroemeria mosaic virus* (AlMV)	Chiari and Bridgen, 2002
Chrysanthemum sp.	meristem culture	*Cucumber mosaic virus* (CMV)	Verma et al., 2004
Chrysanthemum morifolium cv. Regol Time	meristem culture, chemotherapy and thermotherapy	*Chrysanthemum B Carla virus* (CVB)	Ram et al., 2005
Chrysanthemum morifolium	meristem culture	mixed infection by CMV and *Tomato aspermy virus* (TAV)	Kumar et al., 2009
Chrysanthemum sp.	meristem culture	*Tomato spotted wilt virus* (TSWV), *Impatiens necrotic spot virus* (INSV), *Iris yellow spot virus* (IYSV)	Balukiewicz and Kryczynski, 2005
Dianthus gratianopolotanus	meristem culture	*Carnation mottle virus* (CarMV), *Carnation latent virus* (CLV), potyviruses	Fraga et al, 2004a
Lilium sp.	meristem culture	*Lily symptomless virus* (LSV)	Allen, 1975
L. x elegans	meristem culture and thermotherapy	LSV	Nesi et al., 2009
New Guinea Impatiens (*I. hawkerii*)	meristem culture	mixed infection by TSWV and CMV	Gera and Dehan, 1992
I. hawkerii	meristem culture	TSWV	Milošević et al., 2011
Phlox paniculata	meristem culture and thermotherapy	CLV, CarMV, CMV, *Tobacco mosaic virus* (TMV), *Tospoviruses* (subgroups I, II and III), *Potyviruses*	Fraga et al., 2004b
Viola odorata	meristem culture	*Viola mottle virus* (VMV), CMV, *Bean yellow mosaic virus* (BYMV)	Van Caneghem et al., 1997

Regeneration ability is positively proportional to the size of the shoot tip, but pathogen eradication is more efficient using small shoot tips (Wang et al., 2006). Meristems of greater dimensions regenerate a greater number of plants, while the obtained number of virus-free plants is inversely proportional to the size of meristem (Verma et al., 2004). The risk of infection by a virus also increases with explant size. Apart from the indicated factors, elimination of viruses also depends on their concentration in plant tissue and the physiological condition of the mother plant from which a meristem has been isolated (Verma et al., 2004). Meristem culture is a widely used system of plant virus elimination and conservation of virus-free germplasm.

Hosokawa et al. (2004a, 2004b) presented a new method for production of virus-free plants, for which a tip of chrysanthemum, petunia, cabbage or carnation root is used apart from the apical shoot meristem. Root apex is considered to be a suitable organ for meristem culture, since root meristem has a high potential of cell division.

Standardization of medium composition is most important for regeneration of virus-free plants (Kumar et al., 2009). The history of morphogenesis *in vitro* mostly refers to an optimization of composition of mediums and defining different factors affecting the efficiency of *in vitro* plant regeneration. In an experiment for *I. hawkerii,* shoot formation rate was the highest in MS medium (Murashige and Skoog, 1962) with 0.1 μM N-(2-chloro-4-pyridil)-N'-phenylurea–forchlorfenuron (CPPU) (Milošević et al., 2011). A similar result was found by Subotić et al. (2008). They investigated the regeneration of axillary buds of *I. walleriana* in culture *in vitro*. The media with 0.1 μM CPPU induced the highest number of axillary buds and roots per explant. According to that result, the responses of *Impatiens* plants to high concentration of 1-phenyl-3-(1,2,3-tidiazole-5-) urea (TDZ) included fascinating malformations of the stem, manifested as enlargements and flattenings as if several stems were fused (Subotić et al., 2008; Milošević et al., 2011). On the other hand, the effect of CPPU and TDZ on shoot hyperhydricity was significant for *I. walleriana* and *I. hawkerii*. Higher concentrations of both cytokinins resulted in the formation of shoots of changed habitus which does not meet the criteria of commercial production. Differences regarding the effect of different cytokinins on the induction and number of adventitious buds may result from their different activities, i.e. different tissue sensitivities to

the adenine and urea type of cytokinins (Mok and Mok, 2001). Also, differences in the activity of cytokinins may be due to their various translocation rates to meristematic regions and metabolic processes, in which the cytokinins may be degraded and conjugated with physiologically inert compounds, such as sugars or amino acids. Vitrification of carnation shoots in the presence of a high concentration of 6-benzyleaminopurin (BAP) was described by Radojević et al. (2010) and other research groups. According to their results, BAP concentration higher than 1 mgL^{-1} increased the number of vitrified shoots. Growth and morphogenesis *in vitro* are regulated by an interaction and balance between the hormone provide in the medium and those produced endogenously by an explanted tissue (Subotić et al., 2008). These results clearly indicated that the choice of medium was a key step in regeneration of a normal phenotype (Fraga et al., 2004b).

Evidence is growing that the main *endogenous* plant growth regulators, the cytokinins, are involved in the formation of visible disease symptoms. Cytokinins affect cell division, ageing, development of chloroplast and transportation of nutritive matters (Horgan, 1984), and they also affect the processes of virus infection and symptom development (Jerman et al., 1995). Also, Walkey (1991) and Clarke et al. (1998) suggested that the addition of *plant growth regulators* to media could be related to a development of plant resistance to virus infection since high concentrations of cytokinins stimulate the host to interfere with the synthesis of virus proteins. Cybularz-Urban and Hanus-Fajerska (2006) monitored the effect of elimination of *Cymbidium mosaic virus* (CyMV) and *Odontoglossum ringspot virus* (ORSV) from an orchid hybrid using meristem culture. Meristems with two leaf primordia grown in MS media supplemented with 0.5 mg/l zeatin, 3.2 mg/l kinetin or 0.4 mg/l kinetin were used to induce regeneration, while 0.2 mg/l zeatin or 5 mg/l BAP or 0.4 mg/l kinetin was added to the medium during multiplication. The elimination of CyMV was achieved in the medium for induction with 0.5 mg/l zeatin and 3.2 mg/l kinetin, while ORSV was not eliminated in any treatment. The authors indicated that other researchers (Dhingra et al., 1991; Hu et al., 1994; Mauro et al., 1994) had reported *the same conclusion* that a concentration of viruses in plant tissue would be reduced by adding growth regulators to the culture media.

Virus elimination from ornamental plants in Serbia

Among ornamental plants, *I. walleriana*, *I. hawkerii* and *I. balsamina* are particularly popular and widely cultivated in Serbia. Their production is substantial, and a significant part of *Impatiens* plants produced in Serbia originate from imported seeds and planting material. Despite the fact that active quarantine measures are regularly employed, a small part of the imported planting material and potted plants of *Impatiens* have been proved to be infected with TSWV (Krstić et al., 2005a, 2005b). During a survey for TSWV on ornamentals in greenhouse production in Serbia (Krstić et al., 2006) growers have been observed to produce their own *Impatiens* planting material sometimes by using diseased mother plants. For that reason an investigation was conducted in Serbia to establish a successful protocol for virus elimination from TSWV infected *Impatiens* plants.

Milošević et al. (2011) examined a possibility for obtaining virus-free plants from *I. hawkerii* shoots infected with TSWV using meristem culture. For the investigation, DAS-ELISA and RT-PCR positive plants were selected. Meristem explants (0.3-1.5 mm) from virus-infected shoots were cultured on MS media supplemented with different concentrations of the cytokinins, CPPU or TDZ (0.01-1.0 μM), respectively. Using this protocol, a large number of *in vitro* shoots were successfully produced from a single explant. Cytokinins showed a stimulatory effect on the length, fresh and dry weights, as well as on the content of plant pigments. By DAS-ELISA and RT-PCR, 80% of the *in vitro* plantlets were shown to be virus-free, recommending the presented protocol for practical implementation in production of virus-free *Impatiens* planting material.

Figure 1. Symptoms on *I. walleriana* plants infected with TSWV – (a) necrotic spots, (b) necrotic flecks, line pattern and deformation of leaf, (c) necrosis of petals and sepals, (d) necrosis of steam

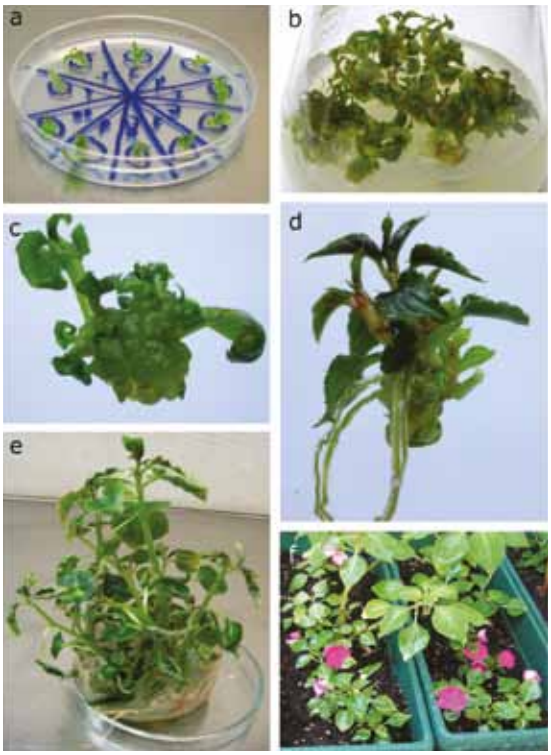

Figure 2. *In vitro* propagation of virus-free *I. Walleriana* – (a) shoot formation on MS media after 7 days, (b) multiplication of shoots on MS supplemented with 0.01 μM CPPU, (c) development of axillary shoot on MS with 1 μM TDZ, (d) axillary shoots on MS with 0.1 μM CPPU, (e) shoot elongation on MS cytokinins-free medium, (f) plants in acclimatization stage

A similar protocol was tested with ELISA and RT-PCR positive *I. walleriana* plants bearing characteristic TSWV symptoms (Figure 1) (Milošević et al., unpublished). After employing a slightly modified protocol for virus elimination, 75% of produced regenerated plants proved to be virus-free (Figure 2). It was shown that *in vitro* culture is an efficient method for elimination of TSWV from *Impatiens* species. The effectiveness of this virus removal treatment depends on the genotype. The established protocol with both *Impatiens* sp. is one of the first attempts to obtain virus-free *Impatiens* plants from infected mother plants in Serbia, and its implementation could be of great importance for the production of these species and ornamentals in general. Domestic production of safe and virus-free planting material of different ornamentals will help reduce the imports of planting material, thus raising biosecurity in Serbia and reducing the number of introduced quarantine and economically harmful organisms.

THERMOTHERAPY AND CHEMOTHERAPY

H*eat* treatment was originally applied by Kassanis in 1949 (Parmessur and Saumtally, 2001) to *eliminate viruses* from *plant* tissue. Since then, thermotherapy has been extensively used for elimination of different viruses from various plants. Growing host plants at higher temperatures significantly reduces replication of many plant viruses by disrupting viral ssRNA and dsRNA synthesis. Thermal inactivation point differs for different viruses, and it ranges between 40 and 46^{0}C in TSWV (depending on isolates), while it is 90^{0}C in TMV. Since high temperature can inhibit virus replication and movement, thermoterapy combined with meristem culture can greatly improve virus elimination efficiency by augmenting the virus-free region of treated shoot tips. Thermotherapy along with meristem culture has been used to raise virus-free carnation, narcissus, chrysanthemum and others (Hakkaart and Quak, 1964). Ram et al. (2005) presented *in vitro* production of CVB-free chrysanthemum cv. Regol Time by meristem culture, chemotherapy and thermotherapy. No plant was found virus-free using meristem culture alone. Meristem culture and thermotherapy did not give a satisfactory number of virus-free plants. The maximum percentage of CVB-free chrysanthemum was obtained with 2-thiouracil and thermotherapy for 30 days. These results are in contrast with earlier findings of other research groups (Hakkaart and Quak, 1964; Asatani, 1972; Goethals et al., 1973; Paludan, 1973), who had produced 90-100% CVB-free plants using these methods. One of the reasons may be that earlier testing of virus-free plants had mainly been based on biological inoculation. Biological indexing was not found to be a reliable method as most plants that were found negative for the virus were tested positive with RT-PCR (Ram et al., 2005). Another approach to *in vitro* virus elimination in plants is to supplement the nutrient medium with a chemical of a known ability to prevent virus replication. Incorporation of antiviral compounds into explant and meristem culture media has resulted in a higher percentage of virus-free progeny plants originating from virus-infected explant or meristem donor plants than are produced in tissue cultures that have no chemicals such as ribavirin (virazole), acycloguanosine, azidothymidine, and 2-thiouracil.

CRYOTHERAPY

More recently, prolonged exposure to a low temperature (-196^{0}C) followed by shoot tip culture has proved quite successful in virus elimination. Cryotherapy of

shoot tips can result in virus-free plants at a high frequency. Thermotherapy had other effects which together with subsequent cryotherapy resulted in virus elimination. When used for cryotherapy, conditions are selected to allow survival of only a limited number of the least differentiated cells and to eliminate a large proportion of virus-infected tissues. Therefore, cryotherapy can result in virus-free regenerants with a much greater frequency than what is typically obtained with a conventional meristem culture (Wang and Valkonen, 2008). Cryotherapy has successfully eliminated pathogens (viruses and bacteria) from numerous plant species: *Solanum tuberosum*, *Ipomea batatas*, *Vitis vinifera*, *Citrus* spp., *Rubus idaeus*, *Musa* spp. (Wang and Valkonen, 2009). The elimination of TSWV by cryotherapy was performed in tobacco (Yordanova et al., 1998), and the procedure is currently developing for the *Impatiens* species in the Laboratory for Plant Physiology of the Institute for Biological Researches "Siniša Stanković", University of Belgrade. A successful procedure has been developed for immunolocalization of TSWV in *I. walleriana* plants (Trifunović et al., 2011). Immunolocalization carried out on *Pelargonium* apices originating from cryopreserved shoot tips, sampled from DAS-ELISA negative plants, showed that they were still virus-infected (Gallard et al., 2011). Using immunolocalization, *Pelargonium flower break virus* (PFBV) and *Pelargonium line pattern virus* (PLPV) could be detected in *Pelargonium* apices, even in the meristematic dome (Gallard et al., 2011). However, viral particles were more numerous in the basal zone cells than in meristematic cells. These results demonstrate that PFBV and PLPV are present within meristematic cells and that cryopreservation can partly reduce the quantity of these viruses in *Pelargonium* plants but not eliminate them totally.

CONCLUSION

Diseases caused by viruses are a significant problem constantly present in commercial production of plants, horticultural species in particular. The need for virus-free stocks has therefore become essential. In order to obtain virus-free material, the following methods can be applied: meristem culture, thermotherapy, chemotherapy, cryotherpy or a combination of these several techniques as virus elimination is more effective when different techniques are combined. Investigations in Serbia have begun and the first successful virus elimination protocol using meristem culture has been tested for *I. hawkerii* and *I. walleriana*. Efforts should now be made to develop an optimized cryotherapy system that could be useful for elimination of TSWV from *Impatiens* and other ornamentals species.

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Primena tehnika *in vitro* kulture za eliminaciju virusa iz ukrasnih biljaka

REZIME

Virusi su odgovorni za brojne epidemije na različitim usevima u svim delovima sveta. Posledica njihovog prisustva su velike ekonomske štete, pa osim razvoju osetljivih tehnika za detekciju, identifikaciju i karakterizaciju virusa, velika pažnja se poklanja i biotehnološkim metodama za njihovu eliminaciju. U ovom preglednom radu predstavljene su tehnike *in vitro* kulture za eliminaciju virusa iz biljnog materijala: kultura meristema, termoterapija, hemoterapija, krioterapija ili kombinacija ovih metoda. Koja će metoda biti primenjena zavisi od biljne vrste, kao i od vrste virusa. U radu je dat pregled istraživanja na eliminaciji virusa iz *Impatiens* sp. u Srbiji.

Ključne reči: Virusi; ukrasne biljke; epidemija; biotehnologija