Plant Cell Culture & Micropropagation ISSN:1808-9909

Review

Cryotherapy as a technique for virus elimination in ornamental species

Crioterapia como técnica para a eliminação de vírus em espécies ornamentais

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ABSTRACT

Pathogens disseminated among crops pose a great threat to the maintenance of the production of ornamental species in Brazil. The traditional methods used for virus elimination of infected plant material have limitations and, therefore, the application of new techniques such as cryotherapy, which is based on the use of cryopreservation methods to intensify the elimination of pathogens in infected plant tissues, may be the key to production optimization and consequent technological and productive independence of Brazil in the international flower market.

Index terms: Cryopreservation; *in vitro* conservation; *in vitro* culture.

RESUMO

Os patógenos disseminados entre as culturas representam uma grande ameaça para a manutenção da produção de espécies ornamentais no Brasil. Os métodos tradicionais utilizados para a limpeza viral de materiais vegetais infectados possuem limitações e, portanto, a aplicação de novas técnicas como a crioterapia, que se baseia na utilização de métodos de criopreservação para intensificar a eliminação de patógenos em tecido vegetal infectado, pode ser a chave para a otimização da produção e consequente independência tecnológica e produtiva do Brasil no mercado internacional de flores.

Termos para indexação: Criopreservação; conservação *in vitro*; cultura *in vitro.*

INTRODUCTION

The Brazilian production of flowers and ornamental plants has developed remarkably over the years and has emerged as a fertile field for national agribusiness (Shiroto; Peres; Sabbag, 2016). Brazil reached an annual turnover of R$ 5.22 billion in 2013, an increase of 8.3% over the results obtained in 2012, providing financial growth for several regions of the country (Junqueira; Peetz, 2014). However, to stay in the industry, the producer needs to specialize and seek strategies to reduce costs and offer high quality seedlings to meet the growing demand of an increasingly competitive market (Silva; Paiva; Santos, 2015; Birk et al., 2017).

Most ornamental species featured in the Brazilian flower sector are propagated vegetatively. However, some bottlenecks have compromised the productivity of some crops in Brazilian soil, which results in high expenditures on germplasm imports, mainly from the Netherlands, representing a considerable portion of the total production cost (Junqueira; Peetz, 2008). Brazilian imports of genetic material are linked to the presence of pathogens in national plant materials, mainly viruses, which spread rapidly and can be transmitted through vector insects and contaminated tools (Amaral, 2006). When compared to large crops, ornamental plants are less studied and their viruses are little known. Some families are known to affect the main ornamental species traded in Brazil, such as the Bromoviridae, Potyviridae and Bunyaviridae (Van Regenmortel, 2016).

Virus elimination and control is currently performed through the application of thermotherapy. This technique consists in the control of pathogens by treatments in certain relationships between time and temperature (Torres et al., 2000), associated with tissue culture (Parmessur et al., 2002). Research has shown that the probability of obtaining plants without virus is inversely proportional to the size of the excised meristem (Anis; Ahmad, 2016), and a size ranging from 0.1 to 1 mm is recommended for most species

Received in January 21, 2018 and approved in March 12, 2018
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(Silva et al., 2013). Pathogen elimination using meristems has been challenged by the difficulty in tissue excision and, mainly, in the non-guarantee of regeneration and removal of infection. In addition, the extended duration of thermotherapy at a high temperature has a severe effect on plant growth and survival, frequently with a low success rate, with recurrence of the infection in the field (Conci; Nome, 1991; Hu et al., 2012). An alternative to attenuate these problems would be the development of more efficient methods, focused on quality and phytosanitary resistance, that guarantee the complete elimination of virus complexes.

Cryopreservation is based on the immersion of the plant material in liquid nitrogen at -196 °C (Engelmann, 2011). When used in order to eliminate pathogens from plant meristems, it is called cryotherapy (Brison et al., 1997). In this technique, conditions are selected to allow the survival of less differentiated cells and to eliminate a large proportion of tissue infected with viruses (Brison et al., 1997), besides the use of relatively large explants, without compromising the successful elimination of the virus, which makes the preparation of the material more viable (Wang; Valkonen, 2009). Cryotherapy is being successfully applied to many species of commercial and ornamental use. Em *Chrysanthemum morifolium*, Jeon et al. (2016) demonstrated that cryopreservation was determinant in the efficacy of elimination of the virus complex *chrysanthemum stunt viroid* (CSVd). In addition, a number of positive results were observed for several species using cryotherapy: *Vitis Vinifera* (Wang et al., 2003; Bayati; Shams; Moieni, 2011; Pathirana et al., 2016), *Fragaria ananassa* (Cai et al., 2008), *Solanum tuberosum* (Wang et al., 2006; Bai et al., 2012), *Rubus idaeus* (Wang et al., 2008; Wang; Valkonen, 2009), *Ipomea batatas* (Wang; Valkonen, 2008), *Allium sativum* (Vieira et al., 2015) and Banana (*Musa spp*.) (Helliot et al., 2002).

However, even with the success of cryotherapy in several species, the use of the technique for commercial ornamental species in Brazil is still scarce. This review, therefore, will be based on studies on the application of cryotherapy for virus elimination in ornamental species, contributing to intensify the application of this technique, generating technological independence of Brazil in the flower market.

VIRUS ELIMINATION THROUGH TISSUE CULTURE ASSOCIATED WITH THERMOTHERAPY

Since there is a high virus incidence rate in the field for ornamental plants, phytosanitary quality is obtained mainly through *in vitro* meristem culture techniques, associated with accessory techniques such as thermotherapy, in order to maximize the efficiency of virus elimination (Kovalskaya; Hammond, 2014). Tissue culture, applied through *in vitro* meristem culture, is one of the oldest techniques for mass clonal propagation and disease-free plants (Murashige, 1977; Bhojwani; Dantu, 2013). This technique consists in the collection, under aseptic conditions, of apical meristems, since they present great organogenetic competence, once they consist of undifferentiated embryonic cells (Smith; Murashige, 1970). The apical meristem must have a size ranging from 0.1 to 1 mm, due to the fact that the lower the explant, the greater the chance of obtaining regenerators free from contamination (Torres et al., 2000). The efficacy in virus elimination through stem apex culture is due to the lower pathogen distribution in young and avascularized portions of plants and their difficulty of multiplication in these regions (Debener; Byrne, 2014).

On the other hand, thermotherapy consists of exposing part of the plant, or the entire plant, to high temperatures for a defined period of time (Conci; Nome, 1991). According to Walkey (1991), thermotherapy causes virus inactivation by denaturing their nucleic acids. However, the small size of the explants required for the *in vitro* meristem culture, associated with the long period required for thermotherapy, have been pointed out as limitations of the techniques (Parmessur et al., 2002).

APPLICATION OF CRYOTHERAPY FOR ELIMINATION OF VIRUS COMPLEXES

Cryotherapy is based on the use of cryopreservation methods to enhance pathogen elimination in infected plant tissues (Brison et al., 1997). Cryopreservation consists in the maintenance of living biological material under aseptic conditions in the long term, using ultra-low temperature (-196 ºC) (Dulloo et al., 2009; Coelho et al., 2017). This ultra-low temperature does not allow the occurrence of thermally directed metabolic

reactions, guaranteeing viability in the storage of the biological material without undergoing genetic alterations (Engelmann, 2011). In cryotherapy, the biological material is immersed in liquid nitrogen (NL) for a short period, frequently 90 minutes (Cejas et al., 2012) and, under these conditions, infected cells are eliminated, conserving only highly cytoplasmic cells in the meristematic region (Wang; Valkonen, 2009), which remain alive due to the use of procedures that prevent crystallization of intracellular water (Silva et al., 2013).

Considering the results of more than a decade of research, cryotherapy may be considered a rapid method that can aid or even replace some traditional methods to eradicate microorganisms (Wang; Valkonen, 2009). Its main advantages are the ability to simultaneously treat a large number of samples (Bhojwani; Dantu, 2013), leading to a higher speed in the method, decrease in cost and increase in frequency of virus-free plants after regeneration. (Wang; Valkonen, 2009; Feng et al., 2013). Before starting the experiments with cryotherapy, it is necessary to establish adequate dehydration of the plant material to reduce tissue damage, since the intracellular water content is a critical factor for the efficiency of the protocols related to this technique (Engelmann, 2011). Thus, before plant tissue exposure to NL, some of its intracellular water is removed by the addition of cryoprotectants, such as Plant Vitrification Solution (PVS2) (Kobayashi; Sakai; Oiyama, 1990), or physically withdrawn using silica gel or air flow in laminar flow chamber (Wang et al., 2000). Several cryopreservation techniques have been successfully used for cryotherapy, including vitrification techniques (Matsumoto; Sakai, 2003; Ganino et al., 2012; Marković et al., 2013), encapsulation-dehydration (Wang et al., 2000; Wang et al., 2003; Bayati; Shams; Moieni, 2011), Encapsulation-vitrification (Jeon et al., 2015) and droplet vitrification (Pathirana et al., 2016).

FUTURE PERSPECTIVES

Considering the results of more than a decade of research, cryotherapy may be considered a rapid method that can aid or even replace some traditional methods to eradicate microorganisms. Its main advantages are the ability to simultaneously treat a large number of samples, leading to a higher speed in the method, decrease in cost and increase in frequency of virus-free plants after regeneration. However, the challenge remains, since the few studies in the literature on its application to ornamental species reveal a bottleneck that limits studies and comparisons in order to extend its application to evaluate the productive potential of regenerated plants through this technique, so that they can be used for commercial production in the same way as those produced from conventional methods.

ACKNOWLEDGEMENTS

This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq; Brasília, DF–Brazil), Fundação de Amparo à Pesquisa de Minas Gerais (FAPEMIG; Belo Horizonte, MG–Brazil) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES; Brasília, DF–Brazil).

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