LUCAS BATISTA DE SOUZA

PROPAGAÇÃO *in vitro* E APLICAÇÃO DE TERMOTERAPIA E CRIOTERAPIA VISANDO LIMPEZA VIRAL EM *Hippeastrum hybridum* Hort. cv. 'APPLE BLOSSOM'

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Agronomia/Fisiologia Vegetal, área de concentração em Fisiologia Vegetal Aplicada, para a obtenção do título de Doutor.

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In vitro PROPAGATION AND APPLICATION OF THERMOTHERAPY AND CRYOTHERAPY AIMING VIRAL CLEANING IN *Hippeastrum hybridum* Hort. cv. 'APPLE BLOSSOM'

Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Fisiologia Vegetal, para a obtenção do título de Doutor.

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Ao meu círculo familiar mais próximo dedico esta produção.
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Truly**,** "*little knowledge makes people feel pride,*
much knowledge who feel humble."

Leonardo da Vinci

RESUMO GERAL

Dentre as espécies ornamentais em destaque no setor de floricultura brasileiro, está o hippeastrum (*Hippeastrum hybridum* Hort.) uma herbácea originária da América do Sul. Atualmente, os preços praticados no mercado brasileiro para a comercialização de algumas variedades de hippeastrum, destacando-se a cv. 'Apple Blossom' são considerados elevados. Os preços elevados são principalmente devido à baixa taxa de propagação vegetativa e as perdas relacionadas à contaminação dos bulbos por vírus. Como os sintomas se manifestam comumente após a brotação, levam à proliferação viral em toda a produção através de insetos vetores e instrumentos de corte. Considerando a ineficiência dos métodos tradicionais para eliminação viral, a crioterapia surge como uma alternativa para intensificar a eliminação de patógenos em tecido vegetal infectado. Durante o processo da crioterapia, o material biológico é imerso em nitrogênio líquido (NL) por um curto período, normalmente 90 minutos e nessas condições as células infectadas são eliminadas, conservando-se apenas células altamente citoplasmáticas na região meristemática, as quais permanecem vivas devido ao uso de procedimentos que evitam a cristalização de água intracelular. Assim, visando a multiplicação em larga escala de genótipos elite, em espaço físico e tempo reduzidos, torna-se essencial a aplicação de técnicas de cultura de tecidos para propagação *in vitro* da espécie, além do estabelecimento de um protocolo para criopreservação de gemas de hippeastrum cv. 'Apple Blossom', visando a eliminação de complexos virais.

Palavras-chave: Amarílis. Multiplicação *in vitro*. Encapsulamento-vitrificação. Crioterapia. Estabilidade genética.

GENERAL ABSTRACT

Hippeastrum (*Hippeastrum hybridum* Hort.), an herbaceous plant native to South America, is among the outstanding ornamental species in the Brazilian florist sector. Currently, the prices practiced in the Brazilian market for some amaryllis varieties commercialization, standing out the cv. 'Apple Blossom' are considered high. The high prices are mainly due to the low rate of vegetative propagation and losses related to bulbs virus contamination, where the symptoms usually manifest themselves after budding, leading to viral proliferation throughout the production by means of insect vectors. Considering the inefficiency of the traditional method for viral elimination, cryotherapy appears as an alternative to intensify the elimination of pathogens in infected plant tissue. In cryotherapy, the biological material is immersed in liquid nitrogen (NL) for a short period, usually 90 minutes and in these conditions, the infected cells are eliminated, conserving only highly cytoplasmic cells in the meristematic region, which remain alive due to the use of procedures that prevent intracellular water crystallization. Thus, in order to multiply large-scale elite genotypes in reduced physical space and time, it is essential to apply tissue culture techniques for *in vitro* species propagation, in addition to establishing a protocol for cryopreservation of amaryllis buds cv. 'Apple Blossom', aiming at the elimination of viral complexes.

Keywords: *Hippeastrum hybridum*. *In vitro* multiplication. Encapsulation-vitrification, Cryotherapy. Genetic stability.

SUMÁRIO

FIRST PART: General introduction

1 INTRODUCTION

Brazilian flower and ornamental plants production acquired a remarkable development over the years and stood out as a fertile field for the national agribusiness (SHIROTO *et al.*, 2016). Brazil has reached an annual turnover of more than US$ 1.4 billion since 2013, an average annual growth of 9% since then, with revenues expected to be over US$ 2 billion in 2019 (SCHOENMAKER, 2018). However, to remain in the sector, the producer needs to specialize and pursue strategies to reduce costs and offer high-quality seedlings to meet the growing demand for an increasingly competitive market (SILVA *et al.*, 2015).

Among the outstanding ornamental species in the floriculture sector is hippeastrum (*Hippeastrum hybridum* Hort.), popularly mistaken for the name amaryllis, an herbaceous plant native to Central and South America belonging to the Amaryllidaceae family (TOMBOLATO *et al.*, 2007). *Hippeastrum* means “knight of the stars” derived from the junction of the Greek words *Hippos* (horse) and *Astron* (star) (DUNCAN, 2016). Herbert, who named this genus, called it “star knight lily” (MEEROW e SNIJMAN, 1998).

Its vegetative propagation is traditionally performed through bulb division, where each bulb normally produces two stalks, forming sets of eight to twelve flowers (KHARRAZI *et al.*, 2017). The flowers produced are marketed with interest in landscaping, gardening, besides cut and pot flower industries (AMARAL, 2006). The Brazilian production of hippeastrum bulbs is concentrated in the region of Holambra, in the State of São Paulo, and in Paraipaba, Ceará (ESPERANÇA *et al.*, 2011). However, bottlenecks have compromised crop productivity on Brazilian soil, resulting in high expenditures on the import of germplasm, main bulbs from the Netherlands, representing a considerable share of the total production cost (NEVES e PINTO, 2015).

Brazilian imports of bulbs jumped from US$ 4.7 million in 2008 to US$ 7.3 million in 2010 (ESPERANÇA *et al.*, 2011). The growing Brazilian dependence on imports of genetic material is linked to the difficulty in propagation and the presence of pathogens in the national materials, mainly viruses in propagated bulbs, which spread rapidly and can be transmitted through insect vectors and contaminated tools (AMARAL, 2006). Currently, the control and viral cleaning in hippeastrum occur through the application of thermotherapy. This technique consists in the control of pathogens by treatments in certain combinations between time and

temperature (TORRES *et al.*, 2000) associated with tissue culture (PARMESSUR *et al.*, 2002). The hippeastrum cultivar known as 'Apple Blossom' already has a protocol for thermotherapy, in which bulb incubation occurs at 37 °C for 40 days. However, this protocol does not guarantee a high reduction in the presence of viruses, since the values did not exceed 55% (AMARAL *et al.*, 2007).

Research has shown that the probability of obtaining plants without virus is inversely proportional to the size of the excised meristem (ANIS e AHMAD, 2016), and a size ranging from 0.1 to 1 mm is recommended for most species (SILVA *et al.*, 2013). The difficulty in tissue excision and, mainly, the non-guarantee of recovery and removal of its infection have challenged the pathogen elimination using meristems. In addition, the extended duration of thermotherapy under high temperature has a severe effect on plant growth and survival, frequently having a low success rate with recurrence of the infection in the field (CONCI e NOME, 1991; HU *et al.*, 2012). An alternative to attenuate these problems would be the development of more efficient methods, focused on the quality and phytosanitary resistance, that guarantee the complete elimination of viral complexes.

Cryopreservation is based on the immersion of live plant material into liquid nitrogen at -196 °C (PETTINELLI *et al.*, 2017). When applied 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), in addition to the use of relatively larger explants without compromising the successful elimination of the virus, which makes the preparation of the material more feasible (WANG e VALKONEN, 2009; PRUDENTE *et al.*, 2018).

Cryotherapy has been successfully applied to many species of commercial and ornamental use. In *Chrysanthemum morifolium*, cryopreservation has been shown to be the efficacy determinant of the viral complex *Chrysanthemum stunt viroid* (CSVd) elimination (JEON *et al.*, 2016). Other positive results were observed using cryotherapy for *Vitis vinifera* (WANG *et al.*, 2003; BAYATI *et al.*, 2011; PATHIRANA *et al.*, 2016), *Fragaria ananassa* (CAI *et al.*, 2008), *Solanum tuberosum* (BAI *et al.*, 2012; KUSHNARENKO *et al.*, 2017), *Rubus idaeus* (WANG *et al.*, 2008; WANG e VALKONEN, 2009), *Ipomea batatas* (WANG e 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, no information regarding the potential use of this technique for the removal of viral complexes in hippeastrum

has been reported so far. Its use is further justified by the growing demand of this species in the world flower market accompanied by the low efficiency of traditional techniques for viral elimination (AMARAL *et al.*, 2007).

Therefore, this study was based on research on the optimization of *in vitro* propagation, on the physiological study of thermotherapy in bulbs, and on the development of a cryopreservation protocol for the subsequent application of cryotherapy in the treatment of 'Apple Blossom' *H. hybridum*, aiming at the elimination of some viral complexes. Thus, contributing to increasing the competitiveness of the Brazilian producers of this species, obtaining micropropagated seedlings with high quality and virus-free, generating technological independence of Brazil in the flower market, and in a short period will contribute to the development and expansion of the flower sector in the national and international scenario.

2 THEORETICAL FRAMEWORK

2.1 *Hippeastrum hybridum* historical and economic importance

Hippeastrum Herb. is a genus of the Amaryllidaceae family with approximately 70-90 species from Central and South America and more than 600 hybrids and cultivars (POGGIO *et al.*, 2007; POGGIO *et al.*, 2014). Two centers of origin are recognized, one in eastern Brazil, and the other in the center of the Andes along the Peruvian and Bolivian borders (MEEROW e SNIJMAN, 1998). Its species have economic value as ornamental plants and are used in the pharmaceutical industry due to its alkaloid content (POGGIO *et al.*, 2014).

As a major center of origin, Brazil has about 34 species described with widespread occurrence throughout its territory, 25 of which are endemic (REFLORA, 2019). Recently, *H. idimae, H. lunaris*, and *H. mauroi* were discovered in the Brazilian Cerrado (OLIVEIRA et al., 2017; CAMPOS-ROCHA et al., 2018).

In turn, *H. hybridum* is a bulbous species with exuberant flowering, which fascinates those who observe it. Since it was part of the genus *Amaryllis* in the past, *Hippeastrum* sp. plants are still mistakenly called amaryllis (SEALY, 1939; BRANTS e VAN DEN HEUVEL, 1965), possibly due to the fact that the hybrids market has not struggled to call them by the correct name. Amaryllis is the common name of *H. belladonna,* native to South Africa, which differs from *H. hybridum,* which originates in the Americas, and should be called hippeastrum (SULTANA *et al.*, 2010).

Much of its attractiveness and economic interest derives from its breeding history of more than 200 years (READ, 2004). Breeding made the flowers as diverse in color as facilitated their cultivation, attracting many worshipers and breeders (MEEROW, 2009). Historically, breeding began in England in 1799, where the first hybrid was produced from *H. vittatum* and *H. reginae* (TRAUB, 1934), both native to Brazil (TOMBOLATO *et al.*, 2007). However, the establishment of the modern hippeastrum primary production center took place in the Netherlands in the early 20th century, with additional Dutch stock grown in Brazil (MEEROW, 2009). The dominant genotypes at this time were the cultivars ‘Apple Blossom’, ‘Dazzler’, ‘Dutch Belle’, and ‘White Christmas’ (MEEROW, 2009).

The hippeastrum bulb is an important product for the international flower market, while the flower stalk has a less economic expression (TOMBOLATO, 2004). It was noted as promising economically in the early 2000s (EPHRATH *et al.*, 2001), confirming its prominent position in the market a few years later. It is among the 20 most commercialized crops in the

market for ornamental plants, highlighting the high value and price appreciation of 14% in recent years (HANKS, 2018). Ornamental horticulture moved more €5.8 billion in 2016, with only its two largest representatives, Royal FloraHolland (Netherlands) and Landgard (Germany) (HANKS, 2018). This is a growing market, especially Brazil (Veiling Holambra, São Paulo), which increased by 153% between 2009 and 2016, moving €170 million only in 2014 (HANKS, 2018).

2.2 Propagation methods

Prior to its prominence among the flower and ornamental plant market, hippeastrum producers had to solve technical problems regarding the propagation method, which required a great deal of manual labor (EPHRATH *et al.*, 2001). There are four common methods for its propagation: seed, bulbil development, double scales, and *in vitro* (EPHRATH *et al.*, 2001; ZHANG *et al.*, 2013; ANDRADE-RODRÍGUEZ *et al.*, 2015; KHARRAZI *et al.*, 2017).

Propagation through seeds usually results in the flowers characteristic variation, being more used by breeders for new cultivars production (EPHRATH *et al.*, 2001). In turn, the development and subsequent separation of bulbils are considered slow, since it takes four to five years from bulb formation to flowering, only feasible for cultivars that yield more than three bulbils at a time (EPHRATH *et al.*, 2001; ANDRADE-RODRÍGUEZ *et al.*, 2015).

Most of the time, propagation is carried out by scaling and may be single or tween scales, where tween scales have been shown to be the most productive (HUANG *et al.*, 1990b; KHARRAZI *et al.*, 2017). Still, bulbils obtained by tween scales take three years to mature (OKUBO *et al.*, 1999). In the effort to improve the yield of plant propagation, other bulb fragmentation methods have been tested and it was pointed out that cutting into eight fragments without scale separation is the best method (KHARRAZI *et al.*, 2017). However, depending on the cultivar, other forms of fragmentation are indicated, as is the case of ‘Pasadena’, where the four-part split and tween-scale separation was more productive (ANDRADE-RODRÍGUEZ *et al.*, 2015).

Although *in vivo* propagation by bulb fragmentation is preferred, in order to continue to meet the growing demand of the ornamental plant market, *in vitro* propagation has been stimulated by the development of several studies (MII *et al.*, 1974; SEABROOK e CUMMING, 1977; HUANG *et al.*, 1990a; TOMBOLATO *et al.*, 1994; DE BRUYN, 1997; MUJIB *et al.*, 2007; SULTANA *et al.*, 2010; AMANI *et al.*, 2015). Thus, tissue culture has become increasingly an option, once it provides high multiplication rates, is a process conducted under

aseptic conditions, requires little space, and allows the production of plants free of pathogens (DE BRUYN, 1997; PRUDENTE *et al.*, 2018). However, micropropagation in hippeastrum is not commercially used (TOMBOLATO *et al.*, 2010), although developments that are more recent have applied *in vitro* techniques to a lesser extent (DÖRING, 2019).

Undoubtedly, the use of tween scales is preferred even for *in vitro* propagation, given the number of studies that used this explant. In general, a combination of cytokinins (6-benzylaminopurine (BAP) or benzyl adenine (BA)) and auxins (indoleacetic acid (IAA) or 1-naphthaleneacetic acid (NAA)) is used (DE BRUYN, 1997; TOMBOLATO *et al.*, 2001). The concentration for any of the phytoregulators added to the medium does not exceed 10 mg L^{-1}, where the highest propagation rates normally occur with a higher proportion of cytokinin (TOMBOLATO *et al.*, 2001). Induction of *in vitro* shoots may also occur spontaneously, without the addition of growth regulators, but at a much lower rate (AMANI *et al.*, 2015).

In addition to tween scales, other explants have already been tried for *in vitro* multiplication of hippeastrum, such as single scales, basal plate, ovaries, pedicels, and leaves (DE BRUYN, 1997; HUANG *et al.*, 2005b; a; AMANI *et al.*, 2015). The results for multiplication rates in tissue culture propagation studies varied according to species and cultivar, and the best result was obtained with *H. vittatum*, of eight bulbils per double scale (15 mm) (ZAYED *et al.*, 2011). However, for most protocols, the number of bulbils or recovering shoots per explant is between two and five (HUANG *et al.*, 1990a; TOMBOLATO *et al.*, 2001; SULTANA *et al.*, 2010; AMANI *et al.*, 2015).

As the tween scales size used in the experiments of the different authors is not similar, the comparison between the obtained yield becomes complex. Thus, a good estimate is reached considering the maximum number of bulbils and recovering structures (shoots, protocorm, somatic embryo) obtained from a single bulb. Thus, the average yield of the *in vitro* multiplication of hippeastrum can reach 200 clones per bulb, in the best of scenarios (ZAYED *et al.*, 2011). This number is up to 3 times higher than that achieved by traditional *ex-vitro* bulb fragmentation methods, which is 60-70 clones (TOMBOLATO *et al.*, 2001).

Evidence from the literature shows that tissue culture accelerated the propagation of most hippeastrum cultivars. However, one of the most commercially important cultivars, ‘Apple Blossom’, does not yet have a desirable *in vitro* propagation rate (TOMBOLATO *et al.*, 2001). In this context, the development of new *in vitro* propagation methodologies has been using Light emitting diodes (LEDs) (DUTTA GUPTA e JATOTHU, 2013; MILER *et al.*, 2018).

LEDs have the fundamental advantage of emitting a specific wavelength that can yield faster or more favorable results for researchers and growers (MILER *et al.*, 2018). Other advantageous characteristics of LEDs include low power consumption, long life cycle, and low heat radiation (MILER *et al.*, 2018).

Ornamental plants, like the others, respond to light quality, as demonstrated for tulips, where flowering, bulb quality, and yield were affected by the light spectrum maintained during cultivation (AMIRI *et al.*, 2018). However, the effects are species- and plant-dependent, and adjustments should be fitted for best results (OUZOUNIS *et al.*, 2018).

In this context, the *in vitro* multiplication and plant growth of gerbera (*Gerbera jamesonii*) was more efficient under red/blue LEDs (70/30%), supporting the replacement of traditional lighting with fluorescent lamps by LEDs (PAWŁOWSKA *et al.*, 2018). Above all, as LED illumination has not been studied for the propagation of any *Hippeastrum* sp. species, it may contribute to the viability of its reproduction and growth *in vitro* or *ex vitro*.

2.3 Crop major phytosanitary problems

The greatest threat to hippeastrum production in Brazil is pathogens, mainly viruses (AMARAL, 2006). What favors the spread of viruses is the fact that the species only manifests the symptoms of viral contamination after sprouting; during that period, it can infect vector insects and host plants (AMARAL, 2006). Due to the persistence and wide dissemination of these vectors, Brazil imports first-line genetic material originating in the Netherlands, and with the heating of the Brazilian domestic market, bulb imports jumped from US$ 6.74 million in 2004 to US$ 46.81 million in 2014 (NEVES e PINTO, 2015).

Viruses rarely kill plants, but they reduce their market value (ALEXANDRE *et al.*, 2011). In this context, depreciation occurs especially due to the obvious symptoms in leaves, bulbs, and flowers, which reduce the product attractiveness (AMARAL, 2006). The major viruses to infect hippeastrum are *Hippeastrum mosaic virus* (HiMV), *Sunflower mosaic virus* (SuMV), *Cucumber mosaic virus* (CMV), and *Nerine latent virus* (NeLV) (BRANTS e VAN DEN HEUVEL, 1965; DE LEEUW, 1972; ALEXANDRE *et al.*, 2011).

The first report of the mosaic by HiMV occurred in 1922. However, no virus was found at that time (BRANTS *et al.*, 1970). The symptoms of HiMV include, in addition to the mosaic aspect (yellow and dark green bands) on the leaves, the presence of inclusion bodies, which cannot be visualized before the symptoms clearly manifest (BRANTS e VAN DEN HEUVEL,

1965). This virus is not transmitted by seeds, but by aphids and mechanically (BAKARDJIEVA e DENKOVA, 1996), and its symptoms are suppressed by low temperatures (KAHN, 1960; BRANTS e VAN DEN HEUVEL, 1965). In turn, SuMV is a member of the genus *Potyvirus* as well as HiMV (BERGER e PARRISH, 2011). This fact makes these viruses have characteristics in common, such as non-persistent aphid transmission and intracellular formation of inclusions (BRUNT, 1992)

CMV is a virus widely spread around the world due to its capacity to infect more than 1200 species between monocotyledons and dicotyledons of different climatic zones (JACQUEMOND, 2012). Its symptoms can be either leaf mosaic or necrosis and tissue death (PALUKAITIS e CARR, 2008). An aggravating factor for CMV is the synergism with viruses of other genera, which frequently causes their numbers to increase in the host (JACQUEMOND, 2012). This *Cucumovirus* is transmitted by aphids, non-persistently, and by seeds (JACQUEMOND, 2012). In addition, several wild and weedy plants serve as CMV hosts, where it remains asymptomatic, making weed removal one of the main components of integrated management of viral diseases (JONES, 2004).

Regarding the genus *Carlavirus*, NeLV is an asymptomatic representative in hippeastrum (MAAT *et al.*, 1978). Due to similarities in host responses and serological tests, NeLV and *Hippeastrum latent virus* (HLV) were thought to be the same virus, which was proven by their respective sequencing to be untrue (WYLIE e JONES, 2012). The whitefly is considered one of the main *Carlavirus* vectors (ROSARIO *et al.*, 2014)

Other viruses already described for infecting hippeastrum include *Hippeastrum chlorotic ringspot virus* (HCRV), responsible for causing concentric chlorotic rings, leaf misalignment, chlorosis, and local necrosis (XU *et al.*, 2017); *Bean yellow mosaic virus* (BYMV), causing dark and yellowish green areas that intensify with the plant age (SCHULZE *et al.*, 2017); *Tomato spotted wilt virus* (TSWV), of the genus *Tospovirus*, which is capable of infecting more than 900 plant species (XU *et al.*, 2017), causing lesions in necrotic and pigmented patterns, chlorosis and non-necrotic mosaic (BEST, 1968); *Tobacco mosaic virus* (TMV), a *Tobamovirus* that causes the traditional yellowing of plant tissues and survives even after the death of the host (DE LEEUW, 1972; MPHUTHI, 2017); in addition to at least 26 other viruses of the genera *Carlavirus*, *Maculavirus*, *Nepovirus*, *Potexvirus* and *Potyvirus* (WYLIE e JONES, 2012).

2.4 Viral elimination techniques

Viral infections frequently cause systemic diseases, so that viral particles, or viroids, cannot be controlled in the field (BARBA *et al.*, 2017). In addition, making a plant species resistant to viroids is considered difficult (MATSUSHITA *et al.*, 2012). Thus, the elimination of viral infections in important crops has been a challenge in the last four decades, at the same time that several approaches have been developed (BARBA *et al.*, 2017; WANG *et al.*, 2018a).

In any case, virus-free plants are grown around the world to control viral diseases from economically important crops, due to phytosanitary requirements for the import of new cultivars and exchanges between breeding programs (KRCZAL, 1998; PREVIATI *et al.*, 2008; BARBA *et al.*, 2015). Germplasm conservation is another reason for the maintenance of virus-free plants (SENULA *et al.*, 1999; POSTMAN e SUGAR, 2000).

In this context, thermotherapy was the first approach used for viral elimination in plants (BARBA *et al.*, 2017). In this technique, the infected material, *in vivo* or *in vitro*, is exposed to high temperatures, normally 37-40 °C in the case of hippeastrum, for different times depending on the virus (AMARAL *et al.*, 2007; BARBA *et al.*, 2017).

Temperatures above 35 °C cause disruption of the protein capsule hydrogen and disulfide bonds, followed by nucleic acids phosphodiester bonds and, consequently, deterioration of viral infectivity, which may include selective inhibition of viral replicase, pH changes, and cellular ionic strength, increase in lytic enzymes, competition between viral and messenger RNA by the ribosome binding site (PANATTONI *et al.*, 2013).

Thermotherapy has already resulted in the production of virus-free plants in bulbous species such as garlic (*Allium sativum*) (TORRES *et al.*, 2000) and lily (*Lily* x *elegans*) (NESI *et al.*, 2009). In both cases, thermotherapy was combined with shoot culture (meristematic tissue) to obtain 100% viral elimination. On the other hand, in cucumber (*Solanum muricatum*), thermotherapy was inefficient to eliminate *Tomato mosaic virus* (ToMV) (PROHENS *et al.*, 1998), as well as in chrysanthemum (*Chrysanthemum* sp.) for *Chrysanthemum stunt viroid* (CSVd) and *Chrysanthemum chlorotic mottle viroid* (CChMVd) (HOLLINGS e STONE, 1970; PALUDAN, 1980).

Shoot culture is another approach, which can be effective in viral elimination when applied alone or in combination with thermotherapy, chemotherapy or cryotherapy (BARBA *et al.*, 2017; WANG *et al.*, 2018a). This technique involves the extraction of meristematic tissue present in the apical dome, or the bud of the infected plant itself. Since many viroids are

generally not seen in the apical meristem, recovery of this material may mean viral elimination (PANATTONI *et al.*, 2013; BARBA *et al.*, 2017).

The size of the shoot (5-10 mm) or the meristematic tissue (0.2-0.7 mm) is especially important for viral elimination efficiency through shoot culture, since the lower the tissue, the lower the viral concentration (PANATTONI *et al.*, 2013). In contrast, the smaller the tissue, the more difficult the plant recovery (PANATTONI *et al.*, 2013; WANG *et al.*, 2018a). Recovery requires a combination of specific nutrients and phytohormones in the culture medium to ensure optimum growth of each species at this stage. In addition, exposure to different components of the medium may pose a risk for genetic stability and progeny performance (PANATTONI *et al.*, 2013).

Isolated, shoot culture has already been shown to be efficient in garlic (*A. sativum*) eliminating, at the same time, *Leek yellow stripe virus* (LYSV), *Onion yellow dwarf virus* (OYDV), *Garlic latent virus* (GLV) (AYABE e SUMI, 2001), besides *Potyvirus*, when combined with thermotherapy (RAMÍREZ-MALAGÓN *et al.*, 2006). In another bulbous species, chincherinchee (*Ornithogalum thyrsoides*), bud culture eliminated *Ornithogalum mosaic virus* (OrMV) (WANGAI e BOCK, 1996).

The latest viral elimination technique developed was cryotherapy. Cryotherapy is considered innovative and is based on cryopreservation, where the infected material is treated for a short time in liquid nitrogen (LN), using a pre-established protocol for the species (BARBA *et al.*, 2017; WANG *et al.*, 2018a).

The literature describes several types of cryopreservation protocols, based on techniques such as vitrification, encapsulation-dehydration, encapsulation-vitrification, and droplet-vitrification (SAKAI e ENGELMANN, 2007; BI *et al.*, 2017). Among these, encapsulation-dehydration and encapsulation-vitrification have the advantage of facilitating the manipulation of small shoots due to the large size of the capsules. However, the second one is more rapid in execution due to the vitrification process being faster than dehydration (SAKAI e ENGELMANN, 2007). Thus, encapsulation-vitrification has been more suitable for the cryopreservation of large quantities of different genotypes shoots (CIRINGER *et al.*, 2018)

When the buds are frozen in LN, only the cells at the top of the apical dome are apt to survive, while those in the lower parts are dead (WANG *et al.*, 2008). In addition, the use of procedures that prevent crystallization of intracellular water works best in healthy cells (PRUDENTE *et al.*, 2018). This is due to the fact that infected cells have larger vacuoles, higher water content and a lower nucleus/cytoplasm ratio than healthy ones. Therefore, they are more

sensitive to ice damage and die during cryotherapy which, in turn, operates a kind of "selection" for healthy cells (BARBA *et al.*, 2017).

Shoot cryotherapy has proven to be much more efficient than traditional methods, such as thermotherapy and shoot culture, for the elimination of viruses that do not infect the apical dome meristematic cells (WANG *et al.*, 2009; WANG e VALKONEN, 2009). However, cryotherapy cannot eliminate viruses that can infect the meristematic cells of the tips of the apical domes, since in this technique the viroids are not deactivated, but eliminated along with the dead host cells (WANG *et al.*, 2018a). For this reason, shoot cryopreservation has also been proposed as new biotechnology for the conservation of viral germplasm (WANG *et al.*, 2018b).

Among the bulbous species, OYDV, LYSV, and *Garlic common latent virus* (GCLV) were eliminated from garlic (*A. sativum*) by cryotherapy in combination with thermotherapy (VIEIRA *et al.*, 2015). On the other hand, in *Argyranthemum madeirense*, this technique alone failed to eliminate *Chrysanthemum stunt viroid* (CSVd), once this virus is able to invade meristematic cells, which survive to freeze in LN (ZHANG *et al.*, 2014).

Despite the relatively large number of species in which some type of viral elimination approach was undertaken, a limited number of studies subsequently assessed the genetic stability of the recovered plant (WANG *et al.*, 2018a). Since they are common in viral elimination techniques, high temperatures, cuts and high osmolarity cause stress in the plant (KEVERS *et al.*, 2004; HATFIELD e PRUEGER, 2015). These conditions create a risk of genetic variation, while the purpose of growing virus-free plants is to improve quality and yield while maintaining the genomic stability of the original cultivar (WANG *et al.*, 2018a). Therefore, it is necessary to access the genetic stability and observe the field behavior of virus-free plants obtained by the described techniques (WANG *et al.*, 2018a).

Cynara scolymus plants heat-treated and recovered by shoot culture did not demonstrate genetic alterations determined by simple sequence repeat (SSR) (ACQUADRO *et al.*, 2010). However, in the same study, polymorphic bands were detected by amplified fragment length polymorphism (AFLP) markers, indicating that thermotherapy may induce genetic variation (WANG *et al.*, 2018a). Likewise, *V. vinifera* somaclones recovered *in vitro* were genetically different from the original cultivars (POPESCU *et al.*, 2002). These data reinforce the need for attention regarding the somaclonal variation derived from viral elimination methods.

3 GENERAL CONSIDERATIONS

Undoubtedly, the market for flowers and ornamental plants has great economic importance at national and world level, besides being in expansion. One way of sustaining this growth and ensuring competitive prices is by improving propagation methods, as well as ensuring the phytosanitary nature of the species of greatest interest. *Hippeastrum hybridum* cv. 'Apple Blossom' is one of these species. However, its propagation is still below expected in techniques of great potential such as *in vitro* multiplication, and it is frequently threatened by pathogens, especially viruses.

Therefore, studies that seek to aggregate new technologies to optimize the productivity of hippeastrum clonal propagation would greatly aid producers. These, in turn, would be guaranteed to deliver products of assured quality at increasingly affordable prices, generating income and expanding the industry. Likewise, studies that develop methodologies that can prevent viral threats, while bringing greater understanding of the interaction between pathogens, the host plant, and viral elimination techniques, would contribute maintaining the quality of the plant material and would allow the development of preventive and phytosanitary monitoring measures for crops.

In this context, the subsequent chapters aim to meet the above-mentioned demands, so that: a new and highly productive *in vitro* multiplication protocol of *H. hybridum* cv. 'Apple Blossom' through LED lighting was developed and described until the acclimatization stage; studies on the physiological effects of thermotherapy on recovered bulbs, a protocol for gemstone cryopreservation, as well as its application to cryotherapy along with thermotherapy and its effects on genetic stability, were conducted.

REFERENCES

ACQUADRO, A. et al. Production and fingerprinting of virus-free clones in a reflowering globe artichoke. **Plant Cell, Tissue and Organ Culture (PCTOC),** v. 100, n. 3, p. 329-337, 2010.

ALEXANDRE, M. A. V. et al. *Hippeastrum mosaic virus* diagnosed in *Hippeastrum* and *Eucharis* in Brazil. **Journal of Plant Pathology,** v. 93, n. 3, p. 643-649, 2011.

AMANI, S. et al. Micropropagation of *Hippeastrum hybridum*. **Cumhuriyet Science Journal,** v. 36, n. 4, p. 594-605, 2015.

AMARAL, L. The production of amaryllis in the brazilian flower market. **Bioikos,** v. 20, n. 2, p. 71-74, 2006.

AMARAL, L. et al. Conservação *in vitro* de germoplasma indexado de três cultivares de amarílis (*Hippeastrum* Herb.). **Ornamental Horticulture,** v. 13, n. 2, 2007.

AMIRI, A. et al. Tulip response to different light sources. **J Anim Plant Sci,** v. 28, n. 2, p. 539-545, 2018.

ANDRADE-RODRÍGUEZ, M. et al. Bulb cutting methods to propagate Hippeastrum hybridum Hort. **Revista Chapingo, Serie Horticultura,** v. 21, n. 1, p. 57-61, 2015.

ANIS, M.; AHMAD, N. **Plant Tissue Culture: Propagation, Conservation and Crop Improvement**. Springer, 2016.

AYABE, M.; SUMI, S. A novel and efficient tissue culture method–" stem-disc dome culture"–for producing virus-free garlic (*Allium sativum* L.). **Plant Cell Reports,** v. 20, n. 6, p. 503-507, 2001.

BAI, J. et al. Can cryopreservation eliminate the *potato virus X* (PVX) and *potato spindle tuber viroid* (PSTVd)? **Bioscience Methods,** v. 3, 2012.

BAKARDJIEVA, N.; DENKOVA, S. *Hippeastrum mosaic virus* isolated in Bulgaria. **Biotechnology & Biotechnological Equipment,** v. 10, n. 1, p. 41-43, 1996.

BARBA, M. et al. Viroid elimination by thermotherapy, cold therapy, tissue culture, *in vitro* micrografting, or cryotherapy. In: HADIDI, A.; FLORES, R., *et al* (Ed.). **Viroids and Satellites**. Boston: Academic Press, p.425-435, 2017.

BARBA, M.; ILARDI, V.; PASQUINI, G. Control of pome and stone fruit virus diseases. In: (Ed.). **Advances in virus research**: Elsevier, v.91, p.47-83, 2015.

BAYATI, S.; SHAMS-BAKHSH, M.; MOINI, A. Elimination of *Grapevine virus A* (GVA) by cryotherapy and electrotherapy. **Journal of Agricultural Science and Technology,** v. 13, p. 442-450, 2011.

BERGER, P. H.; PARRISH, J. C. *Potyvirus*‡: Potyviridae. **The Springer Index of Viruses**, p. 1425-1437, 2011.

BEST, R. J. *Tomato spotted wilt virus*. In: (Ed.). **Advances in virus research**: Elsevier, v.13, p.65-146, 1968.

BI, W. L. et al. Cryopreservation of grapevine (*Vitis* spp.)-a review. **In Vitro Cellular & Developmental Biology-Plant,** v. 53, n. 5, p. 449-460, 2017.

BRANTS, D. H.; FOKKEMA, N.; DE BODE, R. Further identification of *Hippeastrum mosaic virus*. **Netherlands journal of plant pathology,** v. 76, n. 3, p. 171-173, 1970.

BRANTS, D. H.; VAN DEN HEUVEL, J. Investigation of *Hippeastrum mosaic virus* in *Hippeastrum hybridum*. **Netherlands Journal of Plant Pathology,** v. 71, n. 5, p. 145-151, 1965.

BRISON, M. et al. Effect of cryopreservation on the sanitary state of a cv *Prunus* rootstock experimentally contaminated with Plum Pox Potyvirus. **Plant Science,** v. 123, n. 1, p. 189-196, 1997.

BRUNT, A. The general properties of potyviruses. In: (Ed.). ***Potyvirus*** **Taxonomy**: Springer, p.3-16, 1992.

CAI, B.-H. et al. Preliminary study on the elimination of *mild yellow-edge virus* from *in vitro* shoot tips of Meihou strawberry cultivar by vitrification-cryopreservation treatment. **Journal of Fruit Science,** v. 25, n. 6, p. 872-876, 2008.

CAMPOS-ROCHA, A.; MEEROW, A. W.; DUTILH, J. H. A. Two new critically endangered species of *Hippeastrum* (Amaryllidaceae) from the Brazilian Cerrado. **Phytotaxa,** v. 360, n. 2, p. 91-102, 2018.

CIRINGER, T. et al. Cryopreservation of an endangered *Hladnikia pastinacifolia* Rchb. by shoot tip encapsulation-dehydration and encapsulation-vitrification. **In Vitro Cellular & Developmental Biology-Plant,** v. 54, n. 6, p. 565-575, 2018.

CONCI, V.; NOME, S. F. Virus free garlic (*Allium sativum* L.) plants obtained by thermotherapy and meristem tip culture. **Journal of Phytopathology,** v. 132, n. 3, p. 186-192, 1991.

DE BRUYN, M. Micropropagation of amaryllis (*Hippeastrum hybridum*). In: (Ed.). **High-Tech and Micropropagation VI**: Springer, p.3-13, 1997.

DE LEEUW, G. *Tobacco mosaic virus* in *Hippeastrum hybridum*. **European Journal of Plant Pathology,** v. 78, n. 2, p. 69-71, 1972.

DÖRING, M. *Hippeastrum* Herb. in English Wikipedia - Species Pages. Wikimedia Foundation. 2019. Disponível em: < https://www.gbif.org/species/144107351 >. Acesso em: 26 mar 2019.

DUNCAN, G. **The Amaryllidaceae of Southern Africa**. Umdaus Press, 2016.

DUTTA GUPTA, S.; JATOTHU, B. Fundamentals and applications of light-emitting diodes (LEDs) in *in vitro* plant growth and morphogenesis. **Plant Biotechnology Reports,** v. 7, n. 3, p. 211-220, 2013.

EPHRATH, J. E. et al. Various cutting methods for the propagation of hippeastrum bulbs. **Biotronics,** v. 30, p. 75-83, 2001.

ESPERANÇA, A. A.; LÍRIO, V. S.; DE MENDONÇA, T. G. Análise comparativa do desempenho exportador de flores e plantas ornamentais nos estados de São Paulo e Ceará. **Revista econômica do Nordeste,** v. 42, n. 2, p. 259-286, 2011.

HANKS, G. A review of production statistics for the cut flower and foliage sector, updated 2018 (part of AHDB Horticulture funded project PO BOF 002a). **The National Cut Flower Centre, AHDB Horticulture**, p. 132, 2018.

HATFIELD, J. L.; PRUEGER, J. H. Temperature extremes: Effect on plant growth and development. **Weather and climate extremes,** v. 10, p. 4-10, 2015.

HELLIOT, B. et al. Cryopreservation for the elimination of cucumber mosaic and banana streak viruses from banana (*Musa* spp.). **Plant Cell Reports,** v. 20, n. 12, p. 1117-1122, 2002.

HOLLINGS, M.; STONE, O. M. Attempts to eliminate chrysanthemum stunt from chrysanthemum by meristem-tip culture after heat-treatment. **Annals of applied biology,** v. 65, n. 2, p. 311-315, 1970.

HU, G. et al. Efficacy of virus elimination from *in vitro*-cultured sand pear (*Pyrus pyrifolia*) by chemotherapy combined with thermotherapy. **Crop protection,** v. 37, p. 20-25, 2012.

HUANG, C. L.; CHANG, K. C.; OKUBO, H. *In vitro* morphogenesis from ovaries of *Hippeastrum* x *hybridum*. **Journal of the Faculty of Agriculture Kyushu University,** v. 50, n. 1, p. 19-25, 2005a.

_____. *In vitro* morphogenesis from pedicels of *Hippeastrum* x *hybridum*. **Journal of the Faculty of Agriculture Kyushu University,** v. 50, n. 1, p. 27-33, 2005b.

HUANG, C. W.; OKUBO, H.; UEMOTO, S. Comparison of bulblet formation from twin scales and single scales in *Hippeastrum hybridum* cultured *in vitro*. **Scientia Horticulturae,** v. 42, n. 1, p. 151-160, 1990a.

_____. Importance of two scales in propagating *Hippeastrum hybridum* by twin scaling. **Scientia Horticulturae,** v. 42, n. 1, p. 141-149, 1990b.

JACQUEMOND, M. *Cucumber mosaic virus*. In: (Ed.). **Advances in virus research**: Elsevier, v.84, p.439-504, 2012.

JEON, S. M. et al. Elimination of *chrysanthemum stunt viroid* and *chrysanthemum chlorotic mottle viroid* from infected chrysanthemum by cryopreservation. **Protoplasma,** v. 253, n. 4, p. 1135-1144, 2016.

JONES, R. A. Using epidemiological information to develop effective integrated virus disease management strategies. **Virus Research,** v. 100, n. 1, p. 5-30, 2004.

KAHN, R. The present status of the Amaryllis mosaic disease in the United States. **Louisiana Soc. Hort. Res,** v. 5, p. 24-30, 1960.

KEVERS, C. et al. Hyperhydricity of micropropagated shoots: a typically stress-induced change of physiological state. **Plant Cell, Tissue and Organ Culture,** v. 77, n. 2, p. 181-191, 2004.

KHARRAZI, M. et al. Vegetative propagation of amaryllis (*Hippeastrum* × *johnsonii*) by different cutting methods. **Korean Journal of Horticultural Science and Technology,** v. 35, n. 3, p. 373-380, 2017.

KRCZAL, G. **Virus certification of ornamental plants—the European strategy**: St. Paul**:** p. 277-287, 1998.

KUSHNARENKO, S. et al. Combined ribavirin treatment and cryotherapy for efficient *Potato virus M* and *Potato virus S* eradication in potato (*Solanum tuberosum* L.) *in vitro* shoots. **In Vitro Cellular & Developmental Biology-Plant,** v. 53, n. 4, p. 425-432, 2017.

MAAT, D.; HUTTINGA, H.; HAKKAART, F. *Nerine latent virus*: some properties and serological detectability in *Nerine bowdenii*. **European Journal of Plant Pathology,** v. 84, n. 2, p. 47-59, 1978.

MATSUSHITA, Y.; AOKI, K.; SUMITOMO, K. Selection and inheritance of resistance to Chrysanthemum stunt viroid. **Crop Protection,** v. 35, p. 1-4, 2012.

MEEROW, A.; SNIJMAN, D. Amaryllidaceae. In: (Ed.). **Flowering Plants· Monocotyledons**: Springer, p.83-110, 1998.

MEEROW, A. W. Tilting at windmills: 20 years of *Hippeastrum* breeding. **Israel Journal of Plant Sciences,** v. 57, n. 4, p. 303-313, 2009.

MII, M.; MORI, T.; IWASE, N. Organ formation from the excised bulb scales of *Hippeastrum hybridum in vitro*. **Journal of Horticultural Science,** v. 49, n. 3, p. 241-244, 1974.

MILER, N. et al. Application of wide-spectrum light-emitting diodes in micropropagation of popular ornamental plant species: a study on plant quality and cost reduction. **In Vitro Cellular & Developmental Biology-Plant**, p. 1-10, 2018.

MPHUTHI, P. *Tobacco mosaic virus* symptoms, transmission and management. **Farmer's Weekly,** v. 2017, n. 17014, p. 60-61, 2017.

MUJIB, A.; BANERJEE, S.; GHOSH, P. D. Callus induction, somatic embryogenesis and chromosomal instability in tissue culture-raised hippeastrum (*Hippeastrum hybridum* cv. United Nations). **Propagation of Ornamental Plants,** v. 7, n. 4, p. 169-174, 2007.

NESI, B. et al. Production of lily symptomless virus-free plants by shoot meristem tip culture and *in vitro* thermotherapy. **HortScience,** v. 44, n. 1, p. 217-219, 2009.

NEVES, M.; PINTO, M. Mapeamento e quantificação da cadeia de flores e plantas ornamentais do Brasil. **São Paulo: OCESP**, 2015.

OKUBO, H.; HUANG, C. W.; KISHIMOTO, F. Effects of anti-auxins and basal plate on bulblet formation in scale propagation of amaryllis (*Hippeastrum* x *hybridum* hort.). **Journal of the Japanese Society for Horticultural Science,** v. 68, n. 3, p. 513-518, 1999.

OLIVEIRA, R. S.; URDAMPILLETA, J. D.; DUTILH, J. H. A. A new *Hippeastrum* (Amaryllidaceae) species from Brazil. **Phytotaxa,** v. 307, n. 2, p. 147-152, 2017.

OUZOUNIS, T. et al. LED or HPS in ornamentals? A case study in roses and campanulas. **European Journal of Horticultural Science,** v. 83, n. 3, p. 166-172, 2018.

PALUDAN, N. *Chrysanthemum stunt* and *chlorotic mottle*. Establishment of healthy chrysanthemum plants and storage at low temperature of chrysanthemum, carnation, campanula and pelargonium in tubes. **V International Symposium on Virus diseases of Ornamental Plants**, p.303-314, 1980. .

PALUKAITIS, P.; CARR, J. Plant resistance responses to viruses. **Journal of Plant Pathology**, p. 153-171, 2008.

PANATTONI, A.; LUVISI, A.; TRIOLO, E. Elimination of viruses in plants: twenty years of progress. **Spanish Journal of Agricultural Research**, n. 1, p. 173-188, 2013.

PARMESSUR, Y. et al. Sugarcane yellow leaf virus and sugarcane yellows phytoplasma: elimination by tissue culture. **Plant Pathology,** v. 51, n. 5, p. 561-566, 2002.

PATHIRANA, R. et al. Pre-treatment with salicylic acid improves plant regeneration after cryopreservation of grapevine (*Vitis* spp.) by droplet vitrification. **Acta Physiologiae Plantarum,** v. 38, n. 1, p. 11, 2016.

PAWŁOWSKA, B. et al. Impact of LED light sources on morphogenesis and levels of photosynthetic pigments in *Gerbera jamesonii* grown *in vitro*. **Horticulture, Environment, and Biotechnology,** v. 59, n. 1, p. 115-123, 2018.

PETTINELLI, J. D. et al. Cryopreservation of somatic embryos from *Petiveria alliacea* L. by different techniques based on vitrification. **In Vitro Cellular & Developmental Biology-Plant,** v. 53, n. 4, p. 339-345, 2017.

POGGIO, L.; GONZALEZ, G.; NARANJO, C. A. Chromosome studies in *Hippeastrum* (Amaryllidaceae): variation in genome size. **Botanical journal of the Linnean Society,** v. 155, n. 2, p. 171-178, 2007.

POGGIO, L. et al. Genome downsizing and karyotype constancy in diploid and polyploid congeners: a model of genome size variation. **Aob Plants,** v. 6, p. 11, 2014.

POPESCU, C.; FALK, A.; GLIMELIUS, K. Application of AFLPs to characterize somaclonal variation in anther-derived grapevines. **VITIS-GEILWEILERHOF-,** v. 41, n. 4, p. 177-182, 2002.

POSTMAN, J.; SUGAR, D. Elimination of viruses from the USDA *Pyrus* germplasm collection. **VIII International Symposium on Pear**, p.529-530, 2000.

PREVIATI, A. et al. *In vitro* production of virus-free chrysanthemum stock plants for cut flowers. **Propag Ornam Plant,** v. 8, p. 167-9, 2008.

PROHENS, J. et al. *Tomato mosaic tobamovirus*, causal agent of a severe disease of pepino (*Solanum muricatum*). **Plant Disease,** v. 82, n. 11, p. 1281-1281, 1998.

PRUDENTE, D. O. et al. Cryotherapy as a technique for virus elimination in ornamental species. **Plant Cell Culture & Micropropagation,** v. 13, n. 2, p. 29-33, 2018.

RAMÍREZ-MALAGÓN, R. et al. Differential organ infection studies, potyvirus elimination, and field performance of virus-free garlic plants produced by tissue culture. **Plant cell, tissue and organ culture,** v. 86, n. 1, p. 103-110, 2006.

READ, V. M. **Hippeastrum, the gardener's amaryllis. Royal Horticultural Society plant collector guide**. Timber Press. Cambridge. UK.: 2004.

REFLORA. Amaryllidaceae in Flora do Brasil 2020 em construção. **Jardim Botânico do Rio de Janeiro**, 2019. Disponível em: < http://reflora.jbrj.gov.br/reflora/floradobrasil/FB4354 >. Acesso em: 26 mar. 2019.

ROSARIO, K. et al. RNA viral metagenome of whiteflies leads to the discovery and characterization of a whitefly-transmitted *carlavirus* in North America. **PLOS ONE,** v. 9, n. 1, 2014.

SAKAI, A.; ENGELMANN, F. Vitrification, encapsulation-vitrification and droplet-vitrification: a review. **Cryoletters,** v. 28, n. 3, p. 151-172, 2007.

SCHOENMAKER, K. O mercado de flores no Brasil. São Paulo, 2018. Disponível em: < https://docs.wixstatic.com/ugd/875639_f02d8909d93a4f249b8465f7fc0929b4.pdf >. Acesso em: 21 mar. 2019.

SCHULZE, A.; ROBERTS, R.; PIETERSEN, G. First report of the detection of Bean yellow mosaic virus (BYMV) on *Tropaeolum majus*; *Hippeastrum* spp., and *Liatris* spp. in South Africa. **Plant Disease,** v. 101, n. 5, p. 846-847, 2017.

SEABROOK, J. E. A.; CUMMING, B. G. The *in vitro* propagation of amaryllis (*Hippeastrum* spp. Hybrids). **In Vitro,** v. 13, n. 12, p. 831-836, 1977.

SEALY, J. Amaryllis and Hippeastrum. **Bulletin of Miscellaneous Information (Royal Botanic Gardens, Kew),** v. 1939, n. 2, p. 49-68, 1939.

SENULA, A.; KELLER, E.; LESEMAN, D. Elimination of viruses through meristem culture and thermotherapy for the establishment of an *in vitro* collection of garlic (*Allium sativum*). **International Symposium on Methods and Markers for Quality Assurance in Micropropagation** 530, p.121-128, 1999.

SHIROTO, C. S.; PERES, N. V.; SABBAG, O. J. Economic viability of potted chrysanthemums production in Atibaia, São Paulo state. **Ornamental Horticulture,** v. 22, n. 2, p. 130-137, 2016.

SILVA, L. C.; PAIVA, P. D. O.; SANTOS, A. C. Flower and ornamental plants wholesale markets in Brazil. **Ornamental Horticulture,** v. 21, n. 1, p. 53-62, 2015.

SILVA, L. C. et al. Shoot-tip cryopreservation by droplet vitrification of *Byrsonima intermedia* A. Juss.: a woody tropical and medicinal plant species from brazilian Cerrado. **Cryoletters,** v. 34, n. 4, p. 338-348, 2013.

SULTANA, J. et al. *In vitro* bulb production in *Hippeastrum* (*Hippeastrum hybridum*). **Journal of Central European Agriculture,** v. 11, n. 4, p. 469-474, 2010.

TOMBOLATO, A. et al. Brazilian descriptor for *Hippeastrum* hybrids. Building a sustainable future: the role of botanic gardens. Proceedings of the 3rd Global Botanic Gardens Congress, Wuhan, China, **Botanic Gardens Conservation International**. p.1-11, 2007.

TOMBOLATO, A. F. C. **Cultivo comercial de plantas ornamentais**. Instituto Agronômico, 2004.

TOMBOLATO, A. F. C.; AZEVEDO, C.; NAGAI, V. Effects of auxin treatments on *in vivo* propagation of *Hippeastrum hybridum* Hort. by twin scaling. **HortScience,** v. 29, n. 8, p. 922-922, 1994.

TOMBOLATO, A. F. C.; COSTA, A. M. M.; EGLIT, A. C. Micropropagação de *Hippeastrum hybridum* 'Apple blossom', mediante escamas duplas. **Ornamental Horticulture,** v. 7, n. 1, p. 35-40, 2001.

TOMBOLATO, A. F. C. et al. Bulbosas ornamentais no Brasil. **Ornamental Horticulture,** v. 16, n. 2, 2010.

TORRES, A. C. et al. Shoot tip culture and thermotherapy for recovering virus-free plants of garlic. **Horticultura Brasileira,** v. 18, n. 3, p. 192-195, 2000.

TRAUB, H. A preliminary amaryllis (*Hippeastrum*) checklist. **Yearbook Am. Amaryllis Soc,** v. 1, p. 45-51, 1934.

VIEIRA, R. L. et al. Efficient elimination of virus complex from garlic (*Allium sativum* L.) by cryotherapy of shoot tips. **Acta Physiologiae Plantarum,** v. 37, n. 1, p. 11, 2015.

WANG, M. R. et al. *In vitro* thermotherapy-based methods for plant virus eradication. **Plant methods,** v. 14, n. 1, p. 87, 2018a.

WANG, M. R. et al. Cryopreservation of virus: a novel biotechnology for long-term preservation of virus in shoot tips. **Plant Methods,** v. 14, p. 10, Jun 2018b.

WANG, Q. et al. Elimination of *grapevine virus A* (GVA) by cryopreservation of *in vitro*-grown shoot tips of *Vitis vinifera* L. **Plant Science,** v. 165, n. 2, p. 321-327, 2003.

WANG, Q. et al. Cryotherapy of shoot tips: a technique for pathogen eradication to produce healthy planting materials and prepare healthy plant genetic resources for cryopreservation. **Annals of Applied Biology,** v. 154, n. 3, p. 351-363, 2009.

WANG, Q. C. et al. Combined thermotherapy and cryotherapy for efficient virus eradication: relation of virus distribution, subcellular changes, cell survival and viral RNA degradation in shoot tips. **Molecular Plant Pathology,** v. 9, n. 2, p. 237-250, 2008.

WANG, Q. C.; VALKONEN, J. P. T. Elimination of two viruses which interact synergistically from sweetpotato by shoot tip culture and cryotherapy. **Journal of Virological Methods,** v. 154, n. 1, p. 135-145, 2008.

_____. Cryotherapy of shoot tips: novel pathogen eradication method. **Trends in Plant Science,** v. 14, n. 3, p. 119-122, 2009.

WANGAI, A.; BOCK, K. Elimination of *Ornithogalum mosaic virus* from chincherinchee (*Ornithogalum thyrsoides*) by meristem tip culture, and field trials of reinfection. **Plant pathology,** v. 45, n. 4, p. 767-768, 1996.

WYLIE, S. J.; JONES, M. G. K. Complete genome sequences of seven *carlavirus* and *potyvirus* isolates from *Narcissus* and *Hippeastrum* plants in Australia, and proposals to clarify their naming. **Archives of Virology,** v. 157, n. 8, p. 1471-1480, 2012.

XU, Y. et al. Identification of *Taeniothrips eucharii* (Thysanoptera: Thripidae) as a Vector of *Hippeastrum chlorotic ringspot virus* in Southern China. **Plant Disease,** v. 101, n. 9, p. 1597-1600, 2017.

ZAYED, R. et al. *In vitro* micropropagation and alkaloids of *Hippeastrum vittatum*. **In Vitro Cellular & Developmental Biology-Plant,** v. 47, n. 6, p. 695-701, 2011.

ZHANG, W. et al. Effects of temperature, plant growth regulators and substrates and changes in carbohydrate content during bulblet formation by twin scale propagation in *Hippeastrum vittatum* 'Red lion'. **Scientia Horticulturae,** v. 160, p. 230-237, 2013.

ZHANG, Z. B. et al. Cryotherapy could not eradicate *chrysanthemum stunt viroid* from infected *Argyranthemum maderense* 'Yellow empire'. In: REED, B. M. (Ed.). **II International Symposium on Plant Cryopreservation** (Acta Horticulturae).. Leuven 1: Int Soc Horticultural Science, v.1039, p.201-208, 2014.

SECOND PART: Paper 1

Norms from the journal PLANT CELL, TISSUE AND ORGAN CULTURE – PCTOC (Preliminary version)

Alta frequência de regeneração de gemas de bulbos de amarílis expostos a diodos emissores de luz (LEDs)

High–frequency shoot regeneration of amaryllis bulbs exposed to light-emitting diodes (LEDs)

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Authors Contribution Statement

MSc Souza conducted all the experiments and participated in writing the manuscript. Dr. Paiva is Mr. Souza's (Ph.D. candidate) supervisor and provided the facilities for the study conduction. Dr. Prudente is co-supervisor, provided all technical support for micropropagation experiments and wrote the final version of the manuscript. Mr. Ferraz participated in the execution of the experiments. Dr. Paiva and Domiciano provided all support and facilities for the execution of the histological analyses and wrote part of the discussion.

High–frequency shoot regeneration of amaryllis bulbs exposed to light-emitting diodes (LEDs)

Key Message: The amaryllis cv. 'Apple Blossom' micropropagation was highly improved after the exposure of explant under LED light sources.

Abstract The *in vitro* propagation protocols for amaryllis cv. 'Apple Blossom' have low yield due to excessive explant oxidation. Therefore, the aim was to optimize the *in vitro* propagation of amaryllis cv. 'Apple Blossom' using antioxidants and light-emitting diodes (LEDs). Thus, the bulbs were sectioned in explants consisting of twin scales joined by the basal plate. The twin scales were immersed in ascorbic acid solutions and inoculated in MS medium supplemented with 10 µM 6-benzylaminopurine (BAP) and 2.5 µM indole-3-acetic acid (AIA), 1 g L^{-1} PVP, 30 g L^{-1} sucrose, 8 g L^{-1} agar and different activated charcoal concentrations. The best combination of activated charcoal and ascorbic acid was then used to evaluate multiplication under the white fluorescent lamp or mixed treatment of RED/BLUE (RB) LED lamps. For each experiment, the evaluations were performed after 30 days of *in vitro* cultivation, analyzing shoot number and recovery percentage. Subsequently, the shoots were individualized and acclimatized with commercial substrate or vermiculite in a greenhouse, and leaf number, length, and width were evaluated 30 days after acclimatization. Ascorbic acid at a concentration of 500 mg L^{-1} and activated charcoal at 2 g L^{-1} resulted in a higher shoot number, besides hindering tissue browning. Shoot development was 37.85% higher under a blue and red LED lamp. Acclimatization was successfully performed on a commercial substrate with 100% survival of *ex vitro* plants. With the control of explant browning and the use of RB LED lamps, it is estimated that each bulb produced on average 310 clones, which is fourfold more than the previous methods for propagation of amaryllis cv. 'Apple Blossom'.

Keywords: *Hippeastrum hybridum*, *in vitro* multiplication, antioxidants, light spectrum, acclimatization.

Introduction

Amaryllis is an ornamental bulbous species of the genus *Hippeastrum*, which is commercially appreciated due to the intense color of its flowers (De Bruyn 1997). After hybridization in breeding programs, more than 300 *Hippeastrum hybridum* cultivars were developed, establishing it among the 20 cut flowers sold by Royal FloraHolland over the years (AbdelKader 2012; Hanks 2015). The main commercial cultivars are cv. 'Apple Blossom', 'Ster van Holland', 'Orange Souvereign', 'Overingen', 'Red Lyon', 'Telstar' and 'Rilona' (Tombolato et al. 2007). However, the prices for its commercialization in the flower market for some amaryllis cultivars, especially cv. 'Apple Blossom', are considered high (Hanks 2015), mainly due to the low propagation rate of commercial hybrids by scaling of *ex vitro* matrix bulbs in the specific substrate, which generates on average between 60-70 bulbs per matrix bulb (Tombolato et al. 2001).

In this context, the application of plant tissue culture through micropropagation techniques has become an important tool for the large-scale multiplication of elite genotypes, in reduced physical space and time, seeking high yield and/or plants resistant to diseases and pests (Shahzad et al. 2017). Among the *in vitro* culture techniques, the technique called "double scale" or "scaling" (Luyten 1926) offers a number of advantages over other *in vitro* propagation methods for amaryllis, including: high multiplication rate; elimination of the dependence on specific availability periods of propagating material, allowing to establish the desired period for obtaining the explants; production of synthetic seeds and conservation of genetic resources in the long term through cryopreservation techniques (Benson 2014).

However, the protocols for the *in vitro* propagation of amaryllis have a number of obstacles, such as severe explant oxidation, causing significant losses in the shoot multiplication from double scales (Mii et al. 1974). *Hippeastrum* sp. bulbs, when sectioned, exude mucilaginous compounds capable of inducing the oxidation of lipids and other molecules, initiating the production of free radicals potentially harmful to *in vitro* explants (Mii et al. 1974).

Another important factor to consider is the luminous quality in the organogenic development, influencing as much as phytohormones in the expression of regulatory genes (Li et al. 2017). The literature has shown that the properties of the light emitting diode (LED) allow an expressive efficiency in the *in vitro* photomorphogenic process since LEDs can increase shoot development by increasing the concentration of photosynthetic pigments such as chlorophyll and carotenoids in plant tissues (Shin et al. 2008). For this reason, the study of spectral combinations from the introduction of LEDs has been increasingly applied, once they produce shoots with longer length in a short period of time, since they do not develop signs of stress when taken to the *ex vitro* environment (Shin et al. 2008). However, there are no reports in the literature relating the light spectrum and the *in vitro* propagation of amaryllis.

Given the above and given the need for improvement in the current protocol for large scale *in vitro* production of amaryllis cv. 'Apple Blossom', the refinement of tissue culture techniques and applied growing conditions is essential since it will optimize the micropropagation with high quality, resulting in best competitive

values for their commercialization. Thus, the objective of this study is to standardize the extraction of double scales and subsequent shoot induction in amaryllis cv. 'Apple Blossom' by minimizing the negative effects of explant oxidation, in addition to maximizing the frequency of shoot regeneration *in vitro* for acclimatization through the use of blue and red LEDs.

Material and methods

Origin of botanical material and disinfestation of explants. Bulbs of amaryllis cv. 'Apple Blossom' were supplied by Terra Viva (Holambra/SP, Brazil; https://www.terraviva.agr.br) and stored in a cold chamber at 4 °C until explant removal. The bulbs were removed from their commercial packaging and their outermost roots and scales were then excised with a knife before washing in running water for approximately 15 minutes (Fig. 1a-c). Subsequently, the bulbs were sectioned in 4 equal parts transverse to their base (Fig. 1d) and in a laminar flow chamber; the sections were immersed in 70% alcohol for 1 minute, with subsequent immersion in 3% calcium hypochlorite (Fig. 1d) containing 5 drops of Tween® 20 for each 100 mL of solution for 10 minutes under stirring. The sections were drained and immersed in distilled water so as not to dry during explant removal in a laminar flow chamber.

Explant excision. The sections were cut again with a scalpel (slides nº 22) longitudinally to the basal plate to reduce the height of the scales, and transversely until scales of 4 to 12 mm in length (Fig. 1e) were obtained. Each explant consisted of a pair of scales joined by the basal plate (Fig. 1f). During the excision process prior to inoculation, the explants were immersed in 100 or 500 mg L^{-1} ascorbic acid solution for 15 minutes to prevent tissue dehydration and subsequent explant oxidation in the culture medium.

***In vitro* multiplication.** The growth medium consisted of the MS culture medium (Murashige and Skoog 1962), supplemented with 30.0 g L^{-1} sucrose, 1.0 g L^{-1} PVP, 2.5 µM indoleacetic acid (AIA), 10.0 µM 6-benzylaminopurine (BAP), 8 g L^{-1} agar, as well as different concentrations of activated charcoal (0.0, 2.0 and 4.0 g L^{-1}). The pH of the medium was adjusted to 5.8 before autoclaving at 121 ºC and 1.5 atm for 20 minutes. 50-mL aliquots of medium were dispensed into 75 x 150-mm flasks and 3 explants were inoculated per vial. The material was conditioned in a growth room under an irradiance of 36 µmol $m^{-2}s^{-1}$ with a 16-hour photoperiod and temperature of 25 °C ± 2 °C. *In vitro* multiplication was set in a double factorial scheme (ascorbic acid x activated charcoal concentrations); after 30 days of *in vitro* culture, recovery percentage (% of explants showing at least one shoot) and shoot number per explant were evaluated. The study of the correlation between explant size and shoot number was conducted after determination of ascorbic acid and activated charcoal concentrations that maximized *in vitro* multiplication. Thus, explants of 4, 6, 8, 10 or 12 mm measured at baseline were excised and left in 500 mg L^{-1} ascorbic acid for 15 minutes and inoculated in MS culture medium. The material was conditioned in a growth room under an irradiance of 36 µmol $m^{-2}s^{-1}$ with a 16-hour photoperiod and temperature of 25 °C ± 2 °C. After 30 days of *in vitro* culture, recovery percentage and shoot number per explant were evaluated.

Histological analyses. Histological analyses were performed to observe the internal morphology of the explants (double scales with 6 mm) after cell differentiation and shoot growth at 15 days after inoculation in the culture medium. For light microscopy, the chemical fixation of the explants in 2.5% paraformaldehyde in 0.1M sodium phosphate buffer solution (pH 7.2) was carried out for 24 hours at room temperature. After fixation, the samples were washed in phosphate buffer 3 times, for 20 minutes each, followed by dehydration in ethyl series (30%, 50%, 70% and 90% for 25 minutes each and 100%, 25 minutes, 2 times each). The samples were then infiltrated as follows: in absolute alcohol and historesin (Leica Historesin®) at the ratio of 1:1 for 24 hours, and in pure historesin for 24 hours, both steps at room temperature. Soon after infiltration, the samples were placed in the molds in different orientations and immediately placed in pure historesin. The molds were left on a bench for 24 hours and then packed in an oven at 38 °C to complete the polymerization period. After polymerization, the blocks were removed from the molds and 5-µm thick sections were done on a microtome (Leica, RM 25) and stained with toluidine blue safranin (1%) for visualization of cell nuclei (Gurr 1965). The sections were fixed on slides with Canada balm and observed under a Zeiss Scope AX10® light microscope coupled to an AxioVision R.L. 4.8® digital camera.

Use of LEDs on *in vitro* culture. The effect of the blue and red LED lamp on *in vitro* multiplication was evaluated in the same medium described above and left in 500 mg L^{-1} ascorbic acid for 15 minutes prior to inoculation; 10-mL aliquots of medium were dispensed into 20 x 150 mm test tubes. A 6-mm explant was inoculated per tube. The treatments consisted of two types of light source: (i) 20 W white fluorescent lamp with a white light (400–700 nm) 36 µmol m^{-2} s^{-1} irradiance (Osram, Brazil) and (ii) mixed treatment of RED LED (peak at 630 nm) and BLUE LED (peak at 460 nm) (RB) with 86 µmol m^{-2} s^{-1} irradiance (Osram, Brazil). The spectral distributions in relative energy of the white fluorescent and RB LED lamps were determined with a light quantum meter (Model 1400, LI-COR, USA) (Fig. 6). After inoculation, the explants were kept at a temperature of 25 °C ± 2 °C, with a 16-hour photoperiod. The variables evaluated after 30 days of *in vitro* cultivation were recovery (%) and shoot number per explant.

***Ex vitro* acclimatization.** Shoots obtained *in vitro* at 60 days of cultivation were used for acclimatization. Vermiculite and Tropstrato® (Genfértil, Brazil) were compared as substrates. Approximately 30 mL of each type of substrate were distributed in 500-mL plastic cups, which were previously moistened to their field capacity. A shoot was placed in each cup, covered with a plastic bag, to decrease the water loss of the system, totaling 30 replications per treatment. Each week, one edge of the plastic bag was removed together with the replenishment of the field capacity of the substrate. By the end of the third week, the plastic bag was completely removed. The acclimatization process was considered complete at the end of four weeks. The evaluated variables were leaf number, sum of leaf length and sum of leaf width (cm).

Statistical analysis. The experimental design was completely randomized for all experiments. The data were submitted to analysis of variance (ANOVA) using the statistical software R 3.2.5, a package of experimental analysis ExpDes.pt (Ferreira et al. 2013). According to the ANOVA results, the data were compared using the Tukey test ($p<0.05$) or modeled in polynomial regressions ($p<0.05$).

Results

***In vitro* multiplication.** When initiating the micropropagation experiments through bud cultivation, described in the literature by Tombolato et al. (2001), a deficiency in the detailing of the procedures was observed, besides the current need for optimization of the protocol for this species, including new compounds in the culture media that could induce to the maximum bud formation and adequacy of the acclimatization protocol, leading to more satisfactory results.

Thus, the thorough preparation sequence of the plant material was illustrated (Fig. 1). Shoots purchased in commercial packages are devoid of most of the substrate residues where they were originally cultivated and undergo a vernalization period to induce flowering, which leaves the outer scales and the remaining roots dry (Fig. 1a). Therefore, the dry parts were removed and the thinning of the surplus parts, such as the superficial layer of the basal plate and the upper part of the scales (Fig. 1b-c), began. Once cleaned, the shoot was divided into four sections, facilitating the disinfestation step in a laminar flow chamber (Fig. 1d). The disinfested sections were sectioned again, reducing the size of the scales to facilitate shoot induction (Fig. 1e). The newly obtained double scale has a whitish appearance and is covered by a mucilage that is involved with the pink oxidation of the *in vitro* explant, and its immersion in 500 mg L^{-1} ascorbic acid solution prior to inoculation is indicated to prevent oxidation increase (Fig. 1f-g). According to the establishment of *in vitro* double scales, they become gradually greenish, and the addition of activated carbon in the culture medium is also important to avoid the damage caused by scale oxidation (Fig. 1h). After 10 days, the appearance of the first shoots between the double scales appears in a magnifying glass; however, the visualization is more apparent after 15 days of *in vitro* culture (Fig. 1i).

There was no interaction between the factor evaluated (ascorbic acid x activated charcoal concentrations). The use of activated charcoal at 2 g L^{-1} resulted in a higher shoot number than the other concentrations tested; on the other hand, recovery was only distinguished between the control and the other concentrations of activated carbon added to the medium, with an increase of 16% (Fig. 2).

The two tested concentrations of ascorbic acid also resulted in a different shoot number, which was 23% higher at a concentration of 500 mg L^{-1} (Fig. 3).

The basal length of the double scales was determinant for shoot number. According to the prepared model, 2-mm explants are required to obtain at least one shoot and, for every 2 mm in which the basal length of the double scales is increased, an additional shoot is expected (Fig. 4).

Use of LEDs on *in vitro* culture. Shoot development was 37.85% higher under a blue and red LED lamp, compared to the white fluorescent lamp, although the recovery of these shoots was not significantly affected by the type of light source (Fig. 5).

In general, the multiplication process begins at 14 days of *in vitro* culture (Fig. 6a). Shoots emerge between the two scales joined by the basal plate (Fig. 6b). However, it is possible that they originate in less than 10% of the cases in the region outside a scale (Fig. 6c). Although shoot emergence occurs simultaneously in the same explant, its subsequent growth is uneven (Fig. 6d). Rooting occurs naturally during multiplication, from the third week of *in vitro* culture, without the requirement of an additional step (Fig. 6e). During development, shooting changes from a whitish color to green, as leaf growth begins (Fig. 6f). Explants inoculated in the medium without the addition of antioxidants acquire an intense pink coloration at the edges where they were excised and release substances that render the medium yellowish (Fig. 6g). The addition of activated carbon to the medium and immersion of the explants in ascorbic acid solution prior to inoculation minimize these effects (Fig. 6g).

Histological analyses. The longitudinal sections of the basal plates and the double scales showed cell differentiation patterns using the dye toluidine blue after 15 days in the culture medium (Fig. 7).

Acclimatization. Acclimatization on two substrate types (Tropstrato® and Vermiculite) was planned so that regression models could be established to describe the performance of the responses evaluated in each of them (Fig. 8). In the fourth week, it was evident that Tropstrato® yielded the highest plant growth under acclimatization: a new leaf releases every 25 days on Tropstrato® against 34 days on Vermiculite (Fig. 8a); a leaf growth of approximately 5.36 cm per week on Tropstrato® versus 4.36 cm on Vermiculite (Fig. 8b); and a leaf width of 2.1 cm on Tropstrato® versus 1.82 cm on Vermiculite (Fig. 8c).

Discussion

Previous studies on amaryllis propagation describe in a simplified way the obtention process of double scales, in addition to reporting different size patterns for the explants used (Huang et al. 1990a; Huang et al. 1990b; Mii et al. 1974; Tombolato et al. 2001). Therefore, the description made in this study on the procedures for the obtention of a standardized form of the double scales used in the *in vitro* multiplication of amaryllis was of significant importance (Fig. 1). The first standardization adopted in this study was the reduction in the height of leaves of the double scale since it was verified that these leaves offer a physical barrier to shoot development. In this context, it was evidenced that double scales that exceed 2 cm in height tend to oxidize more, bend to their base and/or remain closed, instead of opening in a shape that resembles the letter V, which causes less resistance to the emergence of multiple shoots.

The use of antioxidant compounds directly in the explants in the form of an ascorbic acid solution or dissolved in the culture medium in the form of activated carbon and PVP considerably reduced the browning of the explant caused by exudates as phenolic compounds (Aga and Khillare 2017), which reduces or delays morphophysiological responses due to the reduced organogenic competence of oxidized cells (Oliveira et al. 2017; Liu et al. 2015). In agreement with this response, both shoot number and recovery percentage were higher when the culture medium was supplemented with 2 g L^{-1} activated carbon. On the other hand, the immersion of explants in ascorbic acid solution at 500 mg L^{-1} showed a significant effect only on shoot number.

In this study, it was observed that there is a linear relationship between the basal length of double scales and the number of shoots obtained, with the average value of a 2-mm double scale shoot. On the other hand, in other studies with *Hippeastrum hybridum*, where basal length was not considered, each double scale produced at most four 10 mm double-scale shoots (Huang et al. 1990a; Huang et al. 1990b; Okubo et al. 1999; Sultana et al. 2010; Tombolato et al. 1994; Tombolato et al. 2001). Other factors may have contributed to this result, not just the lack of standardization in the length of double scales. Among them, it is possible to consider the age and physiological stage of donor bulbs, the reduced height of the double scales used in this study, which physically facilitated the development of multiple shoots, as well as the use of antioxidant agents.

During the experiments conducted, and after confirmation through anatomical analyses, it was shown that the shoots became apparent after 14 days of *in vitro* culture, approximately the same time reported for the cultivar ‘Akamaruben’ (Huang et al. 1990b), but less time than previously reported for cv. ‘Apple Blossom’ (Tombolato et al. 2001).

When the double-scale multiplication method is used, bud growth is expected inside (Fig. 6b). However, shoots originated in the outer region of the double scale (Fig. 6c) were observed. The importance of the two scales for the propagation of amaryllis by double scales has been well discussed previously (Huang et al. 1990b). The basal plate acts both physiologically and mechanically, joining the tissues in shoot formation, but is not essential for the shooting process (Okubo et al. 1999). Thus, in spite of the relevant joint effect of the scales and the basal plate, the absence of a basal plate on the external side of the double scale does not prevent shoot emergence on its external side.

The multiple shoots grew unevenly after appearing at the same time, indicating a reallocation of the energetic compounds to the shoot most apt to develop (Fig. 6d). During shoot growth, it is known that there is a reduction in soluble and insoluble sugars, mainly starch, in the scales and their increase in shoots (Zhang et al. 2013). However, it is not yet known how these carbohydrates are partitioned between the various shoots and how much this phenomenon impairs the development of other shoots. Therefore, shoot individualization is suggested, since they naturally root in the culture medium after 21 days (Fig. 6e), before the growth contrasts are perceived, around 24 days, to promote greater uniformity during acclimatization.

The results show that shoot number is higher under blue and red LED lamps than under fluorescent lamps, both of which are similar in terms of percent recovery. As the wavelengths emitted by LEDs are specific, they can be used to better regulate the photomorphogenic radiation required by development, fostering optimal development (Dutta Gupta and Jatothu 2013; Kim et al. 2004). Many other studies highlight the efficiency of LED lamps, compared to fluorescent lamps on *in vitro* culture (Dutta Gupta and Jatothu 2013). For example, the red LED promoted a higher rate of induction, propagation, and fresh matter, while the blue LED led to greater organogenic differentiation, compared to the fluorescent lamp at 25% (Mengxi et al. 2011). Thus, a well-defined balance between the luminous spectra in the red and blue regions justifies the higher shoot number obtained. The greening

of scales and shoots after *in vitro* establishment had already been reported before, indicating that they are capable of photosynthesis (Mii et al. 1974; Stancato and Mazzafera 1995).

The acclimatization of amaryllis, or even its *ex vitro* propagation, is generally performed in Vermiculite (De Bruyn 1997; Kharrazi et al. 2017; Stancato and Mazzafera 1995; Zhang et al. 2013). Acclimatization was 100% in both substrates (vermiculite and Tropstrato®). However, it was observed that Tropstrato® yielded higher shoot growth, leaf number and leaf growth at the end of the 4-week evaluation. Vermiculite is an inert material which has relatively large particles capable of retaining large portions of water and air necessary for plant growth. In turn, Tropstrato® is also able to retain large amounts of water and air, keeping the available moisture for a longer time period. In addition, the commercial substrate is a composite material and may provide minerals, even if a few, sufficient to sustain the higher growth in acclimatization.

Ultimately, considering the established standardization, a 25-cm shoot makes it possible to obtain up to 50 double scales of 10 mm of basal length, on average. Of these, 90% (45 double scales) recover 5 shoots, which means that 225 clones are obtainable from a single shoot multiplied under a fluorescent lamp. Under the blue and red LED lamps, the expected number is 37.85% higher, that is, 310 clones per shoot, which represents a significant advance, considering the economic importance of the species.

Conflict of interest

The authors declare that they have no conflict of interest.

Authors Contribution Statement

MSc Souza conducted all the experiments and participated in writing the manuscript. Dr. Paiva is Mr. Souza's (graduate candidate) supervisor and provided the facilities for the study conduction. Dr. Prudente is co-supervisor, provided all technical support for micropropagation experiments and participated in writing the final version of the manuscript. Mr. Ferraz participated in the execution of the experiments. Dr. Paiva and Domiciano provided all support and facilities for the execution of the histological analyses and wrote part of the discussion.

References

AbdelKader HH (2012) Postharvest physiology of cut hippeastrum (*Hippeastrum hybridum* Herb.) inflorescences. World Applied Sciences Journal 19:943-950

Aga E, Khillare Y (2017) *In vitro* multiplication of *Coffea arabica* L. from leaf explants through indirect somatic embryogenesis. International Journal of Botany Studies 2:17-22

Benson EE (2014) Cryopreservation. In: Plant conservation biotechnology. CRC Press, pp 109-122

De Bruyn M (1997) Micropropagation of amaryllis (*Hippeastrum hybridum*). In: High-Tech and Micropropagation VI. Springer, pp 3-13

Dutta Gupta S, Jatothu B (2013) Fundamentals and applications of light-emitting diodes (LEDs) in *in vitro* plant growth and morphogenesis. Plant Biotechnology Reports 7:211-220

Ferreira E, Cavalcanti P, Nogueira D (2013) ExpDes.pt: Experimental Designs package. R package (Português) version 1

Gurr E (1965) The rational use of dyes in biology and general staining methods. Leonard Hill, London

Hanks G (2015) A review of production statistics for the cut flower and foliage sector 2015 (part of AHDB Horticulture funded project PO BOF 002a). The National Cut Flower Centre, AHDB Horticulture:102

Huang CW, Okubo H, Uemoto S (1990a) Comparison of bulblet formation from twin scales and single scales in *Hippeastrum hybridum* cultured *in vitro*. Scientia Horticulturae 42:151-160

Huang CW, Okubo H, Uemoto S (1990b) Importance of two scales in propagating *Hippeastrum hybridum* by twin scaling. Scientia Horticulturae 42:141-149

Kharrazi M, Tehranifar A, Nemati H, Bagheri AR (2017) Vegetative propagation of amaryllis (*Hippeastrum* × *johnsonii*) by different cutting methods. Korean Journal of Horticultural Science and Technology 35:373-380

Kim S-J, Hahn E-J, Heo J-W, Paek K-Y (2004) Effects of LEDs on net photosynthetic rate, growth and leaf stomata of chrysanthemum plantlets *in vitro*. Scientia Horticulturae 101:143-151

Li CX, Xu ZG, Dong RQ, Chang SX, Wang LZ, Khalil-Ur-Rehman M, Tao JM (2017) An RNA-Seq analysis of grape plantlets grown *in vitro* reveals different responses to blue, green, red LED light, and white fluorescent light. Frontiers in Plant Science 8:16

Liu C-P, Yang L, Shen H-L (2015) Proteomic analysis of immature *Fraxinus mandshurica* cotyledon tissues during somatic embryogenesis: effects of explant browning on somatic embryogenesis. Int J Mol Sci 16:13692

Luyten I (1926) Het voortkweeken van Hippeastrum langs vegetatieven weg I. Meded. no 20 vh Laboratorium voor Plantenphysiologisch Onderzoek te Wageningen en Versl. Kon. Akad. v. Wet. A'dam. Afd Nat Dl 35:115

Mengxi L, Zhigang X, Yang Y, Yijie F (2011) Effects of different spectral lights on *Oncidium* PLBs induction, proliferation, and plant regeneration. Plant Cell Tiss Organ Cult 106:1-10

Mii M, Mori T, Iwase N (1974) Organ formation from the excised bulb scales of *Hippeastrum hybridum in vitro*. Journal of Horticultural Science 49:241-244

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia Plantarum 15:473-497

Okubo H, Huang CW, Kishimoto F (1999) Effects of anti-auxins and basal plate on bulblet formation in scale propagation of amaryllis (*Hippeastrum* x *hybridum* hort.). Journal of the Japanese Society for Horticultural Science 68:513-518

Oliveira C, Degenhardt-Goldbach J, de França Bettencourt GM, Amano E, Franciscon L, Quoirin M (2017) Micropropagation of *Eucalyptus grandis* × *E. urophylla* AEC 224 clone. J For Res 28:29-39

Shahzad A et al. (2017) Plant tissue culture: applications in plant improvement and conservation. In: Plant Biotechnology: Principles and Applications. Springer, Singapore, pp 37-72

Shin KS, Murthy HN, Heo JW, Hahn EJ, Paek KY (2008) The effect of light quality on the growth and development of *in vitro* cultured *Doritaenopsis* plants. Acta Physiol Plant 30:339-343

Stancato GC, Mazzafera P (1995) Effects of light on the propagation and growth of bulbs of *Hippeastrum hybridum* cv. Apple Blossom (Amaryllidaceae). Scientia Agricola 52:331-334

Sultana J, Sultana N, Siddique MNA, Islam AKMA, Hossain MM, Hossain T (2010) *In vitro* bulb production in Hippeastrum (*Hippeastrum hybridum*). Journal of Central European Agriculture 11:469-474

Tombolato A, Dutilh J, Barbosa W, Veiga R, Lucon T Brazilian descriptor for *Hippeastrum* hybrids. In: Building a sustainable future: the role of botanic gardens. Proceedings of the 3rd Global Botanic Gardens Congress, Wuhan, China, 2007. Botanic Gardens Conservation International, pp 1-11

Tombolato AFC, Azevedo C, Nagai V (1994) Effects of auxin treatments on *in vivo* propagation of *Hippeastrum hybridum* Hort. by twin scaling. HortScience 29:922-922

Tombolato AFC, Costa AMM, Eglit AC (2001) Micropropagação de *Hippeastrum hybridum* 'Apple blossom', mediante escamas duplas. Ornamental Horticulture 7:35-40

Zhang W, Song L, Teixeira da Silva JA, Sun H (2013) Effects of temperature, plant growth regulators and substrates and changes in carbohydrate content during bulblet formation by twin scale propagation in *Hippeastrum vittatum* 'Red lion'. Scientia Horticulturae 160:230-237

Figures

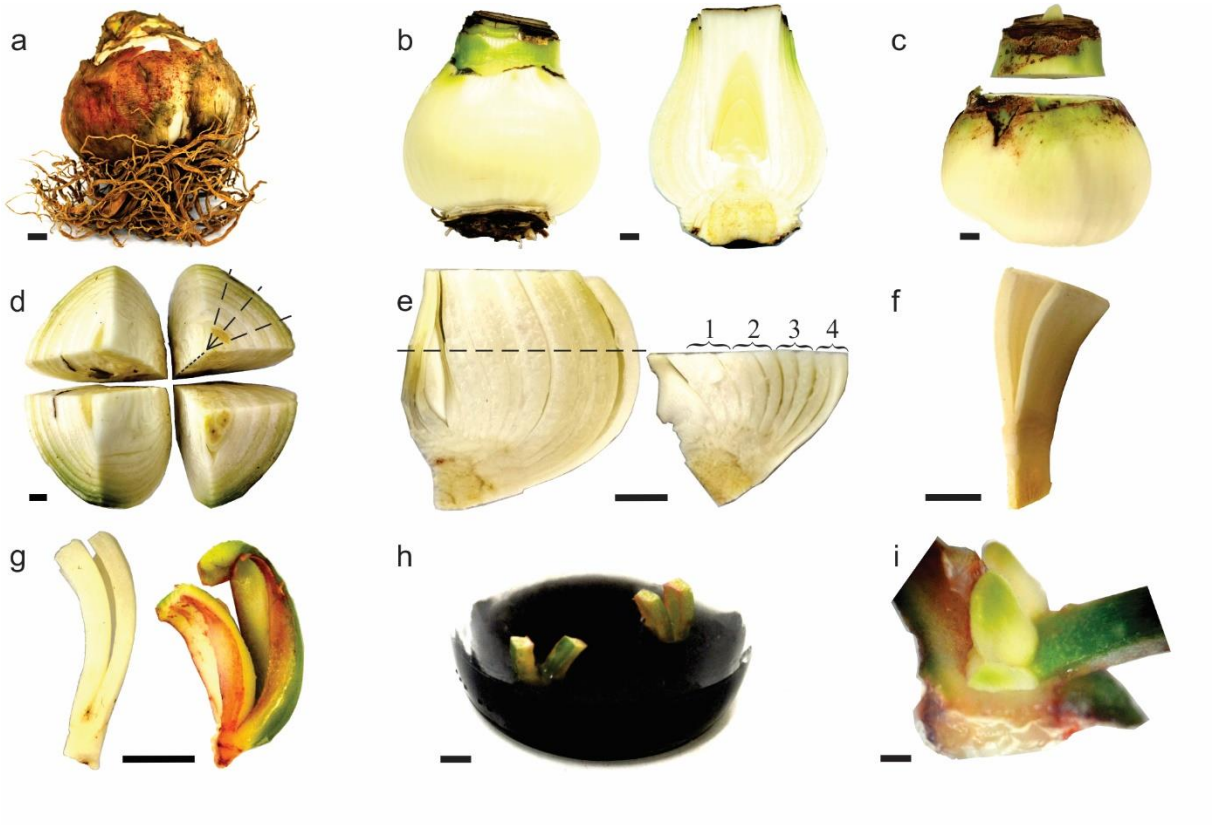

Fig. 1 Steps for the obtention of double scales in amaryllis. a: fresh commercial bulb; b: bulb sectioned in half; c: bulb with the upper and lower parts excised; d: bulb sectioned in four, indicating the next cuts; e: lateral view of the section used to obtain double scales, indicating the region to be discarded (left) and number of double scales obtained in section (right); f: isolated double scale; g: double scale before (left) and after one week of inoculation (right); h: pair of double scales inoculated in a flask; i: two shoots sprouting between double scales after 15 days of *in vitro* culture. Bars 1.0 cm (a-h), 0.2 cm (i).

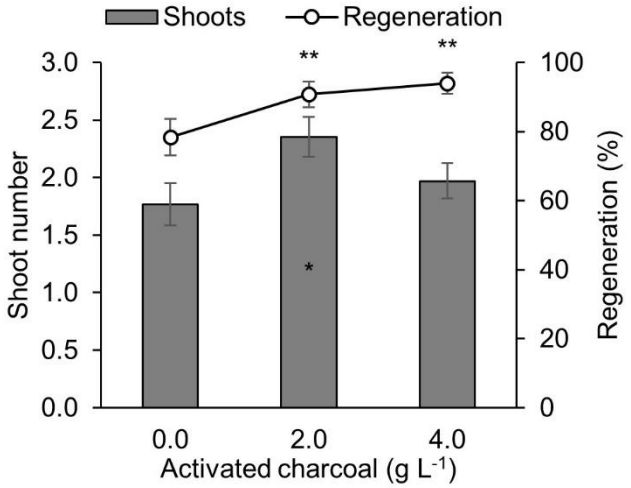

Fig. 2 Influence of activated carbon on the number of shoots recovered in amaryllis. Bars indicate the standard error.

* Significant difference in shoot number according to the Tukey test ($p<0.05$)

** Significant difference in recovery according to the Tukey test ($p<0.05$)

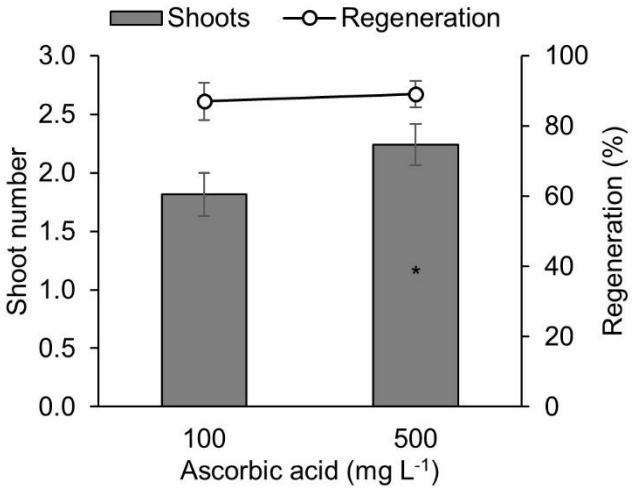

Fig. 3 Influence of ascorbic acid on the number of shoots recovered in amaryllis. Bars indicate the standard error.
* Significant difference in shoot number according to the Tukey test ($p<0.05$)

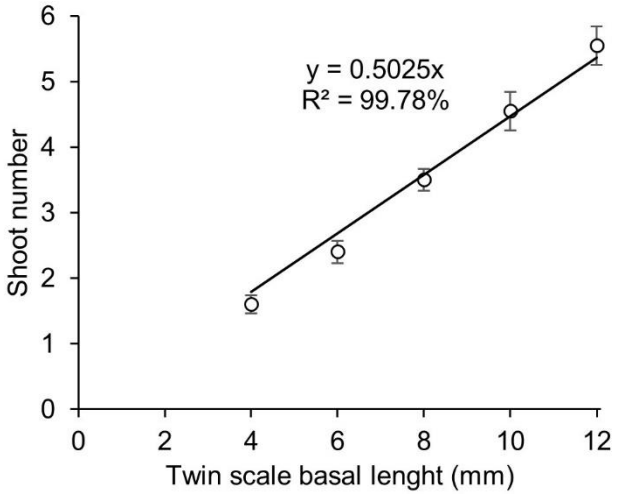

Fig. 4 Polynomial regression model that describes the shoot number as a function of the basal length of inoculated double scales. Bars indicate the standard error. Parameters estimated with $p<0.05$

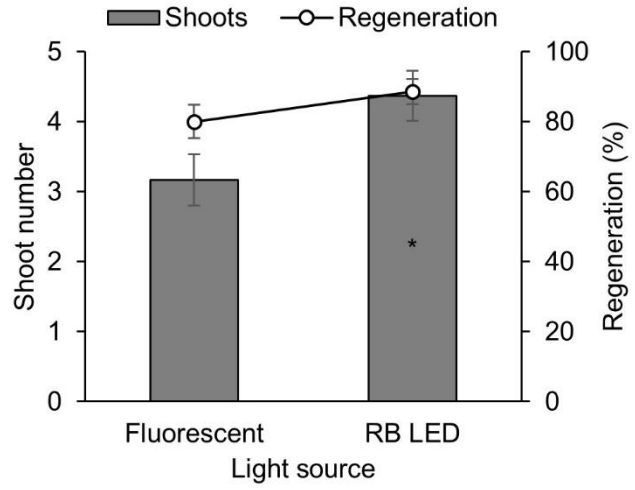

Fig. 5 Influence of the type of lamp used as a light source on the number of shoots recovered in amaryllis. Bars indicate the standard error.
* Significant difference in shoot number according to the Tukey test ($p<0.05$)

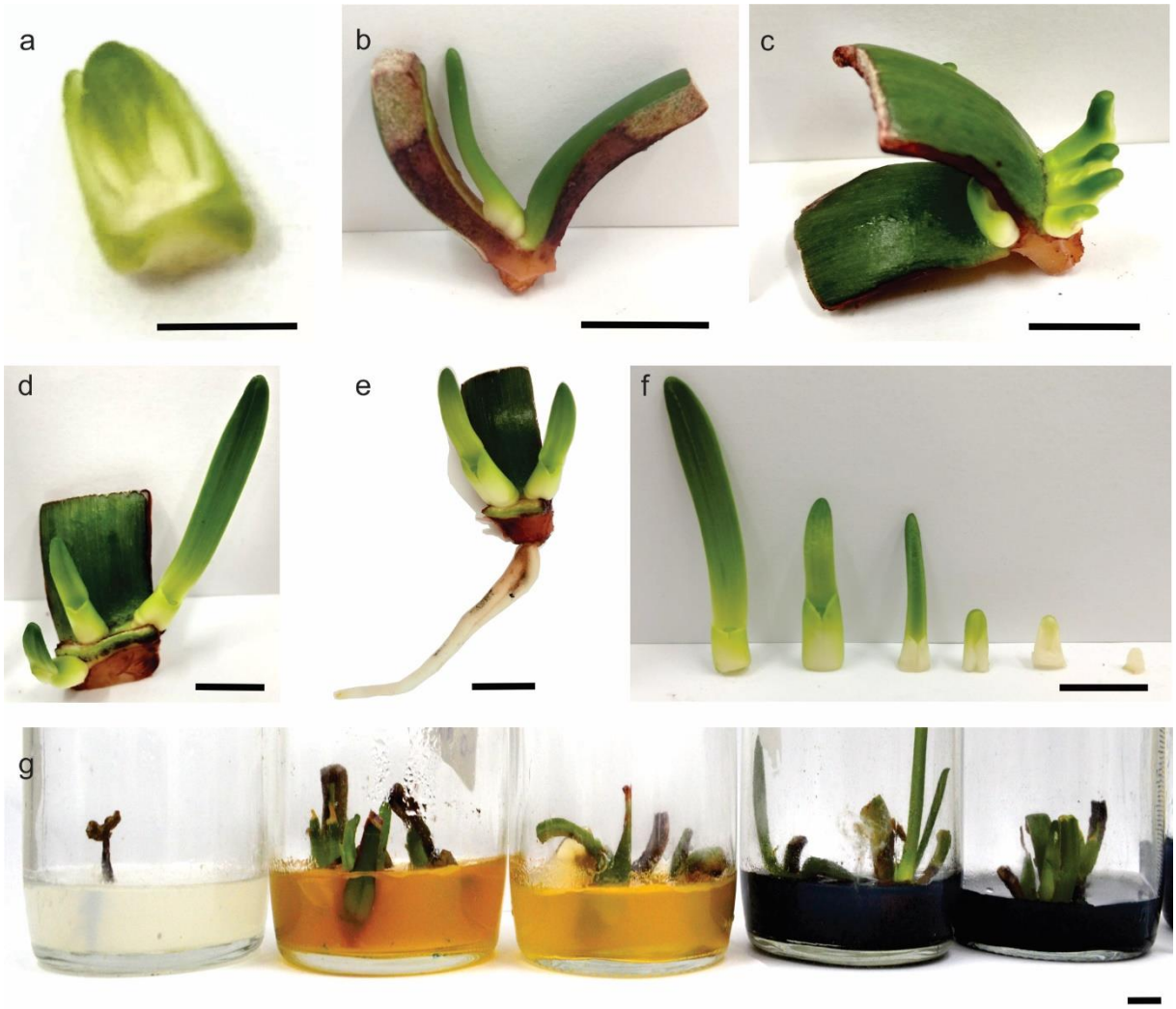

Fig. 6 *In vitro* shoot development in amaryllis. a: sprouting at 14 days of *in vitro* culture; b: shoot growth between double scales; c: multiple shoots emerging from the interior and exterior of double scales; d: ununiform shoot growth; e: natural rooting during shoot induction; f: growth stages of shoots at 10, 14, 16, 21, 24, 28 days of *in vitro* culture (from right to left); g: details of the colors acquired by the culture media under the conditions (from left to right), initial, 100 mg L^{-1} ascorbic acid, 500 mg L^{-1} ascorbic acid, 2 and 4 g L^{-1} activated carbon, respectively. Bars 0.5 cm (a), 1.0 cm (b-g).

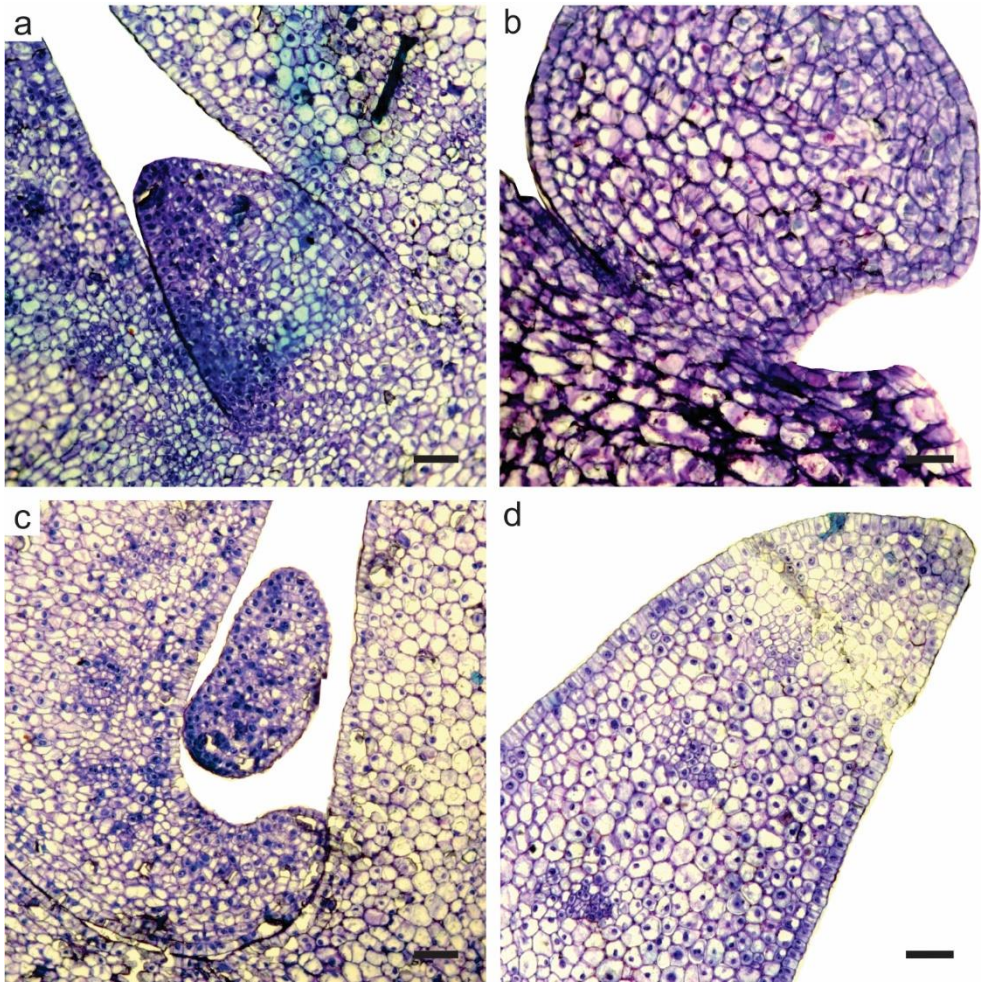

Fig. 7 After 15 days of *in vitro* culture, it is observed that the cells start the differentiation process and already have a central and evident nucleus with high cytoplasmic density, forming a spherical region between the proximal and distal portions of the initial cells.

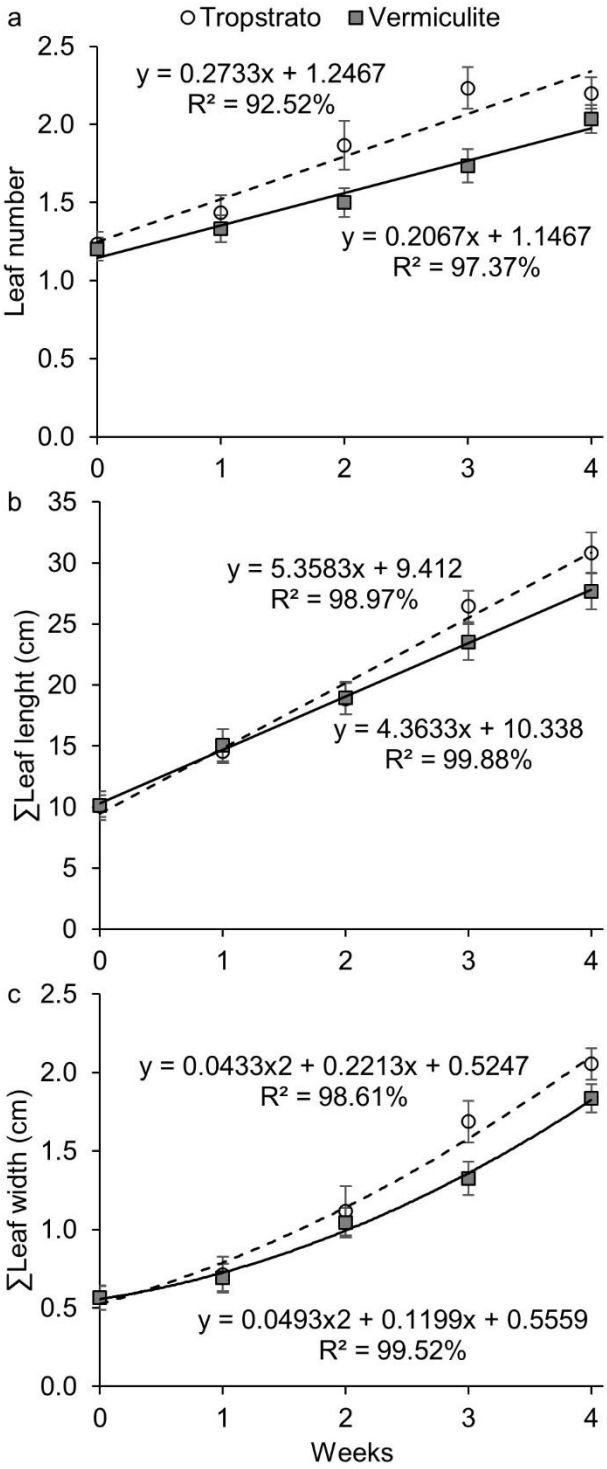

Fig. 8 Performance of responses associated with the acclimatization of amaryllis over four weeks. Polynomial regression models for leaf number (a); for the sum of leaf length (b); and for the sum of leaf width (c). Bars indicate the standard error. Parameters estimated with $p<0.05$.

Annexes

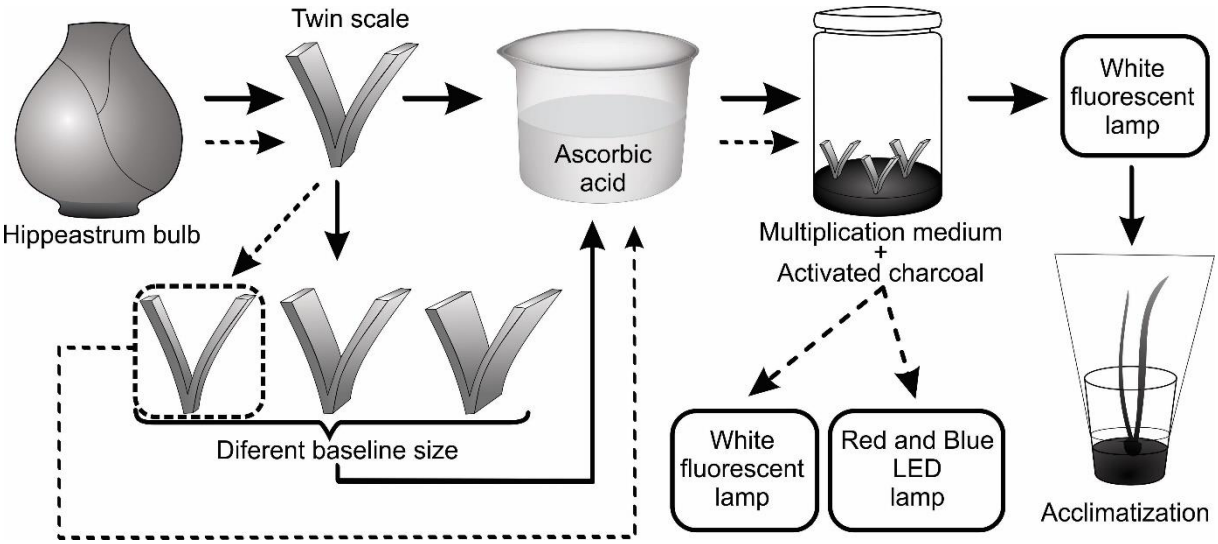

Fig. S1 Schematic experimentation overview. At first, ascorbic acid and activated charcoal concentrations were fitted; subsequently, this fitting was applied to fit twin scale baseline size; then the light quality influence provided by a fluorescent or a LED lamp was evaluated; ultimately, acclimatization was achieved.

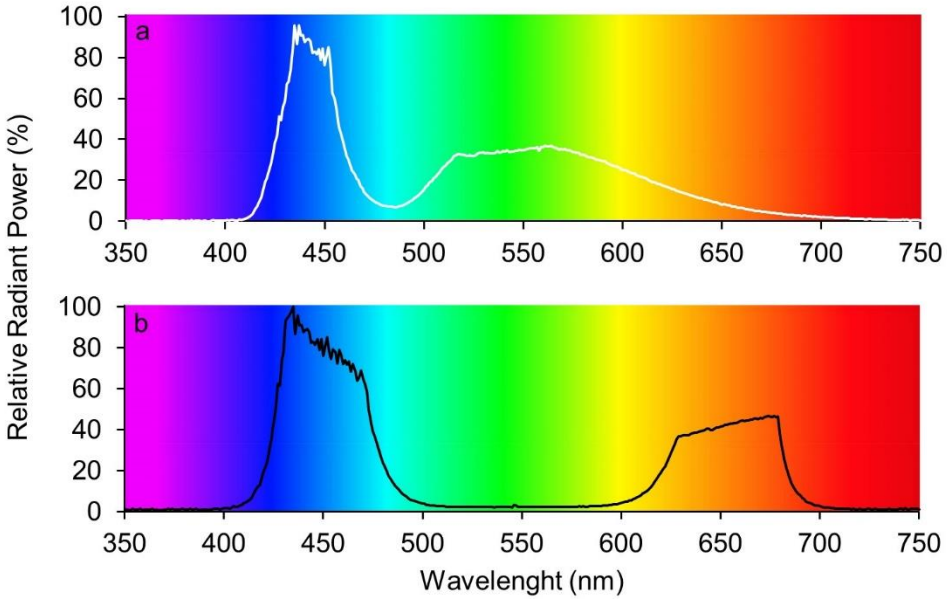

Fig. S2 Spectral distribution of the relative energy of the white fluorescent lamp (a) and the red and blue LED lamp (b).

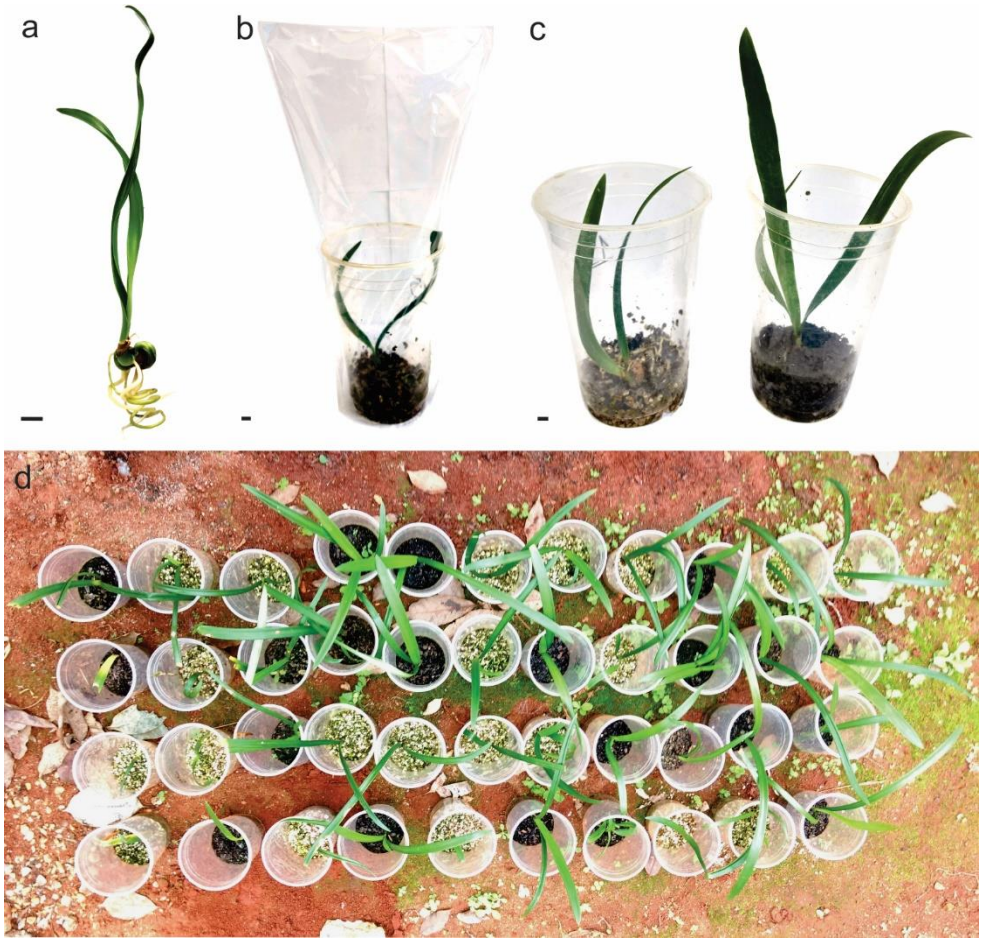

Fig. S3 General aspect of amaryllis seedlings at 60 days of *in vitro* culture (a); during the acclimatization process, at 15 days of *ex vitro* cultivation on commercial substrate Tropstrato® (b); at 30 days of *ex vitro* culture on vermiculite commercial substrate Tropstrato® (c); and top view of the plants after the acclimatization process in a greenhouse (d). Bars 1.0 cm.

SECOND PART: Paper 2

Norms from the journal Plant Cell Reports

(Preliminary version)

A eliminação viral após termoterapia e crioterapia depende do órgão da planta e estágio de desenvolvimento em *Hippeastrum hybridum*

Viral elimination after thermotherapy and cryotherapy depends on the plant organ and developmental stage in *Hippeastrum hybridum*

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Authors Contribution Statement

MSc Souza conducted all the experiments and participated in writing the manuscript. Dr. Paiva is Mr. Souza's (Ph.D. candidate) supervisor and provided the facilities for the study conduction. Dr. Prudente is co-supervisor, provided all technical support for cryopreservation experiments and wrote the final version of the manuscript. Dr. Rivas participated in the execution of viral diagnostics and participated in writing the manuscript. Dr. Paiva, Dr. Rivas, and Dr. Domiciano provided all support and facilities for the execution of the histological, gas exchanges and biochemical analyses and wrote part of the discussion.

Key Message

The plant organ and developmental stage determine the viral elimination and detectability by the means of RT-PCR in *Hippeastrum hybridum*.

A eliminação viral após termoterapia e crioterapia depende do órgão da planta e estágio de desenvolvimento em *Hippeastrum hybridum*

Resumo

Hippeastrum hybridum é uma espécie ornamental bulbosa de grande importância econômica. Contudo, a contaminação por vírus tem comprometido a sua produção. A principal técnica utilizada pelos produtores como medida para combater a contaminação viral é a termoterapia. Está técnica não garante a eliminação de todos os vírus hospedados em *H. hybridum*, além de afetar negativamente a fisiologia dos bulbos. Recentemente, a crioterapia vem ganhando expressivo destaque como uma alternativa para a erradicação de viroses. Assim, objetivou-se estudar os efeitos fisiológicos da termoterapia no crescimento e desenvolvimento dos bulbos, bem como desenvolver um protocolo de criopreservação baseado na encapsulamento-vitrificação para aplicação na crioterapia, sozinha ou em combinação com a termoterapia, e verificar a dependência entre a detecção viral com o órgão vegetal e o estágio de crescimento avaliado. Foram feitas análises de crescimento, trocas gasosas, quantificação de pigmentos fotossintetizantes, carboidratos e atividade de invertases ácida e neutra, além da comparação entre os tempos de exposição aos crioprotetores PVS2 e PVS3. A diagnose viral foi feita histologicamente e por RT-PCR, sendo a estabilidade genética avaliada por citometria de fluxo. Os resultados evidenciaram que a termoterapia atrasou o crescimento e o desenvolvimento subsequente dos bulbos por reduzir o conteúdo de clorofilas *a*, *b* e carotenoides, levando a uma menor taxa fotossintética. Além disso, desenvolveu-se o primeiro protocolo de criopreservação para a espécie, baseado no encapsulamento-vitrificação com 60% de regeneração ao se utilizar o PVS3. A termoterapia e a crioterapia não induziram alterações genéticas. Contudo, os tratamentos aplicados foram ineficazes na eliminação viral, possivelmente devido ao elevado tamanho das gemas utilizadas. Sobretudo, a detecção foi dependente do órgão e do estágio de desenvolvimento.

Palavras-chave: ‘Apple Blossom’, citometria de fluxo, amarílis, ornamental, encapsulamento-vitrificação.

Viral elimination after thermotherapy and cryotherapy depends on the plant organ and developmental stage in *Hippeastrum hybridum*

Key Message
The plant organ and developmental stage determine the viral elimination and detectability by the means of RT-PCR in *Hippeastrum hybridum*.

Abstract *Hippeastrum hybridum* is a bulbous ornamental species of great economic importance. However, virus contamination has compromised its production. The main technique used by producers as a measure to combat viral contamination is thermotherapy. This technique does not guarantee all viruses elimination hosted in *H. hybridum*, in addition to negatively affecting the bulbs physiology. Recently, cryotherapy has gained significant prominence as an alternative to the virus elimination. Then, the aims were to study the physiological effects of thermotherapy on the bulb's growth and development, as well as to develop a cryopreservation protocol based on encapsulation-vitrification for application in cryotherapy, alone or in combination with thermotherapy, and verify the dependence between viral detection with the plant organ and the growth stage evaluated. Growth analyzes, gas exchanges, photosynthetic pigments quantification, carbohydrates and acid, and neutral invertase activity, as well as the comparison between the exposure times to the PVS2 and PVS3 cryoprotectants, were performed. The viral diagnosis was made histologically and by RT-PCR, and genetic stability was assessed by flow cytometry. The results evidenced that the thermotherapy delayed bulbs growth and subsequent development by reducing the content of chlorophyll *a*, *b* and carotenoids, leading to a lower photosynthetic rate. In addition, the first cryopreservation protocol for the species was developed, based on encapsulation-vitrification with 60% regeneration when using PVS3. Thermotherapy and cryotherapy did not induce genetic alterations. However, the treatments applied were ineffective in viral elimination, possibly due to the high sized shoots used. Above all, the detection was organ and stage dependent.

Keywords: 'Apple Blossom', flow cytometry, amaryllis, ornamental, encapsulation-vitrification.

Introduction

Among the 20 most traded crops in the flower and ornamental plants market, *Hippeastrum* sp. can be highlighted, due to its high value and price appreciation of 14% in recent years (Hanks 2018). Ornamental horticulture moved more than €5.8 billion in 2016 with only two of the most prominent representatives, Royal FloraHolland (Netherlands) and Landgard (Germany) (Hanks 2018). Besides, this is a rising market, emphasizing Brazilian participation (Veiling Holambra, São Paulo), which increased its profits by 153% between 2009 and 2016, moving alone €170 million in 2014 (Hanks 2018).

Hippeastrum hybridum, popularly known as amaryllis, is a bulbous species noted as economically promising in the early 2000s (Ephrath et al. 2001), confirming its prominent position on the market a few years later (Zhang et al. 2013; Hanks 2018). Before *H. hybridum* gain the current economic importance, producers had to solve technical problems regarding the propagation method, which required a lot of manual labor; long growth period in relation to other crops and difficulties involving the regulation and flowering control to coincide with the best marketing season (Ephrath et al. 2001).

Once the initial technical problems were solved, the amaryllis crop established itself in the world market. However, the emergence of mosaic viruses began to threaten their maintenance in nurseries (Tombolato et al. 2001). Viruses frequently do not kill ornamental plants but reduce their value by delaying the flowering cycle and impairing the plant's visual appearance (Alexandre et al. 2011).

The major viruses hosted by *H. hybridum* are *Hippeastrum mosaic virus* (HiMV) and *Sunflower mosaic virus* (SuMV), genus *Potyvirus* (Duarte et al. 2009; Raj et al. 2009; Alexandre et al. 2011; Wylie and Jones 2012), *Cucumber mosaic virus* (CMV), genus *Cucumovirus* (Gutierrez-Villegas et al. 2004), *Nerine latent virus* (NeLV), genus *Carlavirus* (Wylie and Jones 2012), besides those less common, *Tomato spotted wilt tospovirus* (TSWV) and *Impatiens necrotic spot tospovirus* (INSV), genus *Tospovirus (*Dong *et al. 2013)*, in addition to *Tobacco mosaic virus* (TMV), genus *Tobamovirus* (De Leeuw 1972).

In an effort to eliminate viral contamination, methods of bud culture and thermotherapy, or their combination, were applied and suggested in *Hippeastrum* sp. (Yanagawa and Osaki 1996; Amaral et al. 2007). Recently, the cryopreservation techniques diversification has allowed its exploration for the development of cryotherapy, which allowed obtaining vírus-free plants with success (Wang et al. 2014; Prudente et al. 2018).

Several cryopreservation techniques have been successfully applied for cryotherapy, including the encapsulation-vitrification one, which was efficient in raspberry for the elimination of *Raspberry bushy dwarf virus* (RBDV) (Wang et al. 2008), and in sweet potato for the elimination of *Sweet potato chlorotic stunt virus*

(SPCSV) and *Sweet potato feathery mottle virus* (SPFMV) (Wang and Valkonen 2008). However, cryotherapy by encapsulation-vitrification has not been studied in *Hippeastrum* sp. to date.

Despite efforts, thermotherapy remains the most widespread alternative to viral elimination in bulbous species (Walkey 2018). However, its application may mean the loss of bulb vigor, and the success of viral cleaning largely depends on the type of virus, the species and the plant part treated, as well as the growth stage in which it is found (Hu et al. 2015; Lizárraga et al. 2017).

There are approximately 300 *H. hybridum* cultivars in commercial use, being 'Apple Blossom' one of the main ones (Tombolato et al. 2007), the chosen as the object of this study. Therefore, the objective was to evaluate the physiological mechanisms by which thermotherapy can affect bulb growth and development; to develop a bulb cryopreservation protocol by encapsulation-vitrification to be used for cryotherapy, besides verifying the dependence between viral detection with the plant organ and the growth stage evaluated.

Material and methods

Botanical material origin. *H. hybridum* cv.'Apple Blossom' bulbs were supplied by the company Terra Viva (Holambra/SP, Brasil; https://www.terraviva.agr.br) and stored in a cold room at 4 °C until explant removal or planting. The bulbs were removed from their commercial packaging and their outermost roots and scales were then excised with a knife before washing in running water for approximately 15 minutes.

***Ex vitro* experiment:**

Thermotherapy. In order to test the viral cleaning protocol and to perform the material phytosanitary cleaning, 48 bulbs were selected from a batch previously analyzed positively for the presence of virus by RT-PCR. In the thermotherapy treatment, the 48 bulbs were exposed to a constant temperature of 37 ºC in a Biochemical Oxygen Demand (BOD) chamber for 40 days (Conci and Nome 1991). After this period, 40 thermotherapy-treated bulbs and 40 fresh bulbs were potted in vermiculite (5L), one bulb/pot. The bulbs were kept under greenhouse conditions, at an average temperature of 25 ± 2 ºC, for physiological evaluations for 30 days.

The remaining eight thermotherapy-treated bulbs were used for the extraction of double scales, with subsequent inoculation in *in vitro* culture medium for the virus cleaning experiments.

Growth analysis. During 10 weeks, fresh bulbs and bulbs from the thermotherapy treatment were evaluated for leaf number, length, and width, as well as floral stalk length. The percentage of survival and bulbs flowering was determined at 30 days of cultivation. The fresh bulbs were weighed at the beginning and, at the end of 10 weeks of cultivation, the fresh matter (FM) of roots, bulbs, and leaves was determined. Subsequently, the same samples were oven-dried with forced air at 65 °C for 72 hours and then again weighed for dry matter (DM) calculation.

Gas exchange measurements. For gas exchange evaluations, leaves were selected from control bulbs and after the thermotherapy treatment at 30 days in a greenhouse. Measurements were performed on the middle third of the fully expanded leaf between 9 and 11 o'clock in the morning. The CO_2 net assimilation rate (*A*), stomatal conductance (*gs*), transpiratory rate (*E*), instantaneous water use efficiency (WUE = *A*/*E*), the carbon content in the substomatal chamber and external carbon (*Ci*/*Ca*) and instantaneous carboxylation efficiency (*A*/*Ci*) were considered. Measurements were obtained with the aid of a portable gas exchange analyzer, LI6400XT IRGA (LI-COR, Lincoln, USA).

Quantification of photosynthetic pigments: chlorophyll *a* and *b* and carotenoids. For the determination of pigment contents, nine leaves were selected from control bulbs and after the thermotherapy treatment at 30 days in a greenhouse; 0.1 mg of leaf tissue in 80% acetone was macerated. After filtration, the final volume was completed to 5 mL. Subsequently, spectrophotometric readings were carried out at 45 and 663 nm for chlorophylls *a* and *b*, respectively while, for carotenoid contents, readings were carried out at 445 nm (Lichtenthaler and Buschmann 2001).

Quantification of total soluble sugars, reducing sugars and starch. For the quantification of total soluble sugars (TSS), reducing sugars (RS) and starch, leaves, roots and bulbs of five control and after the thermotherapy treatment plants were harvested, at 30 days in the greenhouse. The plant material was oven-dried with forced air at 65 °C for 72 hours and then stored on craft paper until analysis. The homogenate from phosphate buffer (pH 7) was centrifuged at 5,000 *g* for 10 minutes and the supernatant was collected. The procedure was repeated and the supernatants were combined to make a final volume of 10 mL. The crude extract was used for the quantification of TSS and starch by the Anthrone method (Dische 1962), while RS was quantified according to the protocol described for 3,5-dinitrosalicylic acid (DNS) (Miller 1959).

Invertase isoforms activity. The activities of two invertase (EC 3.2.1.26) isoforms was evaluated: vacuole acid (IAV) and cytosol neutral (INC) were evaluated in control and treated samples of bulb and root tissues, collected at 30 days after the transfer of the plants to the greenhouse. The invertase isoforms were extracted by homogenizing 0.4 g of fresh tissue in 1.5 mL of potassium phosphate buffer (100 mM pH 7.5) added with phenylmethylsulfonyl fluoride (PMSF) (1 mM), $MgCl_2$ (5 mM) and dithiothreitol (DTT) (1 mM). Subsequently, the homogenate was centrifuged at 18,000 *g* at 4 °C for 20 minutes (Cairo et al. 2009). The supernatant was used to evaluate the activity of soluble invertases. The enzymatic activities were evaluated by the quantification of RS produced using DNS (Miller 1959).

***In vitro* experiments:**

Cryopreservation protocols applied in bulbs. In order to establish an efficient cryopreservation protocol that could be used for cryotherapy, protocols were developed through buds encapsulation-vitrification. Before buds encapsulation, the explants were disinfected.

Explant disinfestation and excision. The bulbs were sectioned in 4 equal parts transverse to their base, in a laminar flow chamber, and the sections obtained were immersed in 70% alcohol for 1 minute. Subsequently, the fragments were immersed in 3% calcium hypochlorite solution, containing 5 drops of Tween® (Sigma Aldrich®, St. Louis, USA) 20 for each 100 mL of solution, for 20 minutes under stirring. The bulb sections were transferred to distilled water to avoid drying during explant removal in a laminar flow chamber.

The bulb sections were again sectioned with a scalpel longitudinally to the basal plate to reduce the height of the scales, and transversely until ~8 mm scales were obtained. Each explant consisted of a pair of scales joined by the basal plate (double scales). During the excision process prior to inoculation, the explants were immersed in 500 mg L^{-1} ascorbic acid solution for 15 minutes to prevent tissue dehydration and subsequent explant oxidation in the culture medium.

***in vitro* multiplication.** For explant multiplication, MS (Murashige and Skoog 1962), culture medium was used, supplemented with 30.0 g L^{-1} sucrose, 1.0 g L^{-1} polyvinylpyrrolidone (PVP; Sigma Aldrich®), 2.5 µM indoleacetic acid (IAA; Sigma Aldrich®), 10.0 µM of 6-benzyl aminopurine (BAP; Sigma Aldrich®), 8 g L^{-1} of agar and 2.0 g L^{-1} activated charcoal (Sigma Aldrich®). The pH of the medium was adjusted to 5.8 before autoclaving at 121 ºC and 1.5 atm for 20 minutes. Aliquots of 50 mL medium were dispensed into 75 x 150 mm flasks and 3 explants were inoculated per flask. The material was conditioned in a growth room under mixed red (630 nm) and blue (460 nm) (RB) LED light (Osram, Barueri-SP, Brazil), under irradiance of 86 µmol m^{-2} s^{-1}, with a 16-hour photoperiod and temperature of 25 °C ± 2 °C for up to 14 days. From 14 days, the buds become very large for encapsulation and cryopreservation.

Bud encapsulation. For the application of cryopreservation techniques, buds from *in vitro* multiplication with approximately 0.2-0.5 cm were transferred to MS (Murashige and Skoog 1962) preculture medium, added with 0.3 M sucrose for 24 hours. Subsequently, the buds were immersed in MS (Murashige and Skoog 1962) medium supplemented with 0.09 M sucrose and 2.5% alginate (Sigma Aldrich®), individually rescued and dripped in calcium chloride solution (100 mM), in which they remained for 20 minutes, characterizing the complexation phase. The beads were immersed in distilled water autoclaved three times for the removal of excess calcium chloride.

Encapsulation-vitrification. The beads were treated with loading solution (2M glycerol + 0.5M sucrose) for 20 minutes before immersion in Plant vitrification solution 2 (PVS2) or Plant vitrification solution 3 (PVS3) at 0 °C for different periods (0, 30, 60, 120, 180 and 240 minutes). After treatment in PVS2 or PVS3, the beads were inserted into 2 mL cryotubes and immersed in liquid nitrogen, where they remained for 90 minutes. Buds thawing was carried out in a water bath at 40 ºC for three minutes and the beads were then immersed in discharging solution (1.2 M sucrose) at 25 °C for 15 minutes.

After cryopreservation, the beads were partially de-complexed in potassium nitrate solution (100 mM) for 15 minutes and inoculated into the multiplication medium. The beads were maintained in a growth room with 16-hour photoperiod under irradiance of 36 µmol $m^{-2}s^{-1}$ and temperature of 25 ± 2 °C. The variables evaluated after 30 days of cultivation were: percentage of survival and percentage of regrowth.

Cryotherapy for viral cleaning. In order to evaluate the efficiency of viral disinfection methods, *Hippeastrum* buds, previously disinfested as previously described, were divided into 4 treatment groups: (i) *in vitro* bud culture (control); (ii) thermotherapy combined with *in vitro* bud culture; (iii) bud cryotherapy and (iv) thermotherapy combined with bud cryotherapy. All groups were maintained in the multiplication medium previously described after receiving their treatments, and the percentage of survival and regrowth were evaluated after 30 days of cultivation.

Genetic stability. For the determination of the DNA content by flow cytometry, the plant material in the acclimatization phase of the seedlings was ground in Petri dishes along with the reference standard - 9.09 pg of pea (*Pisum sativum* L.) DNA (Baranyi and Greilhuber 1995), and 1 mL of nuclei extraction buffer LB01 (Dolezel et al. 1989). The suspension obtained was filtered on a 50 µm gauze screen and then stained with 25 µL propidium iodide. The whole process was carried out on an ice container.

The DNA analysis was performed using a Facscalibur™ flow cytometer (Becton Dickinson, Nova Jersey, EUA) cytometer and the histograms obtained with the Cell Quest software were analyzed statistically with WinMDI 2.8 software. Nuclear DNA content (pg) of the plants was estimated using the ratio between the fluorescence intensities of the G1 nuclei (nuclei in the G1 phase of Interphase) of the reference standard (*Pisum sativum*) and the G1 nuclei of the sample, multiplying this ratio by the amount of DNA of the reference standard at 9.09 pg (Baranyi and Greilhuber 1995).

Detection of cytological alterations and viral inclusions. The observation of cytological alterations in semi-fine paradermal sections of leaf tissues, previously fixed in 70% alcohol and stained with 2% acetic carmine (Shunmugam et al. 2018), was performed under light microscopy.
For the observation of viral inclusions, under light microscopy, semi-fine paradermal sections of fresh leaf tissues were stained with 1% Azure A on Cellosolve, with the Luxol brilliant green 1% blend in Cellosolve/Calcomine Orange 1% in Cellosolve (Christie and Edwardson 1986) or with Floxin 1% in water reverse osmosis (Rubio-Huertos 1972).

The obtained sections, with the inner face that was in contact with the downward-facing mesophyll, were immersed in Azure A with 0.2 M Na_2HPO_4 at the ratio 9:1 (v/v), in the Luxol brilliant green/Calcomine Orange/water reverse osmosis water (LCA) at the ratio 8:1:1 (v/v), or in Floxin. After 10-15 minutes, the excess dyes were removed by 5 passes in 95% ethanol and the sections were mounted on 60% glycerin.

The coverslips were sealed with enamel to prevent glycerin evaporation. The sections were observed on a Zeiss Scope AX10® light microscope (Carl Zeiss, Oberkochen, Germany) coupled to a digital camera and the images were obtained by AxioVision R.L. 4.8® software.

Viral diagnosis
Total RNA extraction. Three procedures were used to obtain total RNA from control and treated *Hippeastrum* samples: Trizol LS Reagent, according to the instructions of the manufacturer Life Technologies Salzman et al. (1999) protocol; and with RNeasy Plant Mini Kit, as described by the manufacturer Qiagen (Qiagen, Hilden, Germany). In all procedures, 100 mg of each material were previously ground in the presence of liquid nitrogen.

RT-PCR (Reverse Transcriptase – Polymerase Chain Reaction). Reverse transcription reactions of RNA (cDNAs) were performed with 2.5 µL of RNA and GoScript Reverse Transcription System, according to the manufacturer's instructions (Promega; Madison, Wisconsin, USA). Enzyme amplification (PCR) was performed from 3 µL of cDNA and GoTaq G2 Hot Start Green Master Mix (Promega) and amplification conditions were those described by the authors of the primers used (Table 1) for the detection of viruses already reported in *Hippeastrum* sp., according to the literature: *Cucumber mosaic virus*, genus *Cucumovirrus* (Gutierrez-Villegas et al. 2004); *Tomato spotted wilt virus*, *Impatiens necrotic spot virus* and *Hippeastrum chlorotic ringspot virus*, genus *Tospovirus* (Dong et al. 2013; Berniak 2016); *Sunflower mosaic virus* (Smith 2012) and *Hippeastrum mosaic virus* (Alexandre et al. 2011), genus *Potyvirus*; *Nerine latent vírus* (Maat et al. 1978), genus *Carlavirus*; and *Tobacco mosaic virus* (De Leeuw 1972), genus *Tobamovirus*. For internal control of the reactions and validation of the obtained results, primers directed to the mitochondrial NADH dehydrogenase gene were used (Lee and Chang 2006).

Subsequently, enzymatic amplification (PCR) was performed from 3 µL cDNA, 1 µL sense primer, 1 µL antisense primer, 12.2 µL GoTaq Master Mix (Promega) and 7.8 µL 'nuclease free'. After initial denaturation at 95 °C/2 minutes, the cDNA was amplified in 30 cycles of 95 °C/30s, 58 °C/50s, 73 °C/50 °C, and a final extension was made at 73 ° C/5min, in an MJ Research PTC-100 (Bio-Rad Laboratories, Hercules, USA) thermal cycler.

The amplified products were analyzed in 1.5% agarose gel in horizontal electrophoresis prepared in TAE buffer (Tris + glacial acetic acid + 0.5 M EDTA, pH 8.0) containing ethidium bromide (0.05 µL/mL) (Seal & Coates, 1998). The electrophoretic run was performed at 80 V for 30-40 minutes and the gel was visualized and documented in a Major Science UVDI-365 transilluminator (UV) (Major Science, Saratoga, EUA).

Table 1. Primers used in RT-PCR assays with RNA extracted from samples of *Hippeastrum* sp. and their respective labor concentrations

Virus	*Primers*	Reference
Cucumber mosaic virus (CMV)	Sense (CM1 - 25μM): 5' TATGATAAGAAGCTTGTTTTCGCG 3' Antissense (CM2 - 25μM): 5' GCCGTAAGCTGGATGGACAA 3' Target: Protein coat	(Wylie et al. 1993)
Tospovirus	Sense (S1 Univ - 10μM): 5' TGTARTGRTCCATWGCA 3' Antissense (S2 Univ - 10μM): 5' AGAGCAATYGTGTCA 3' Target: Nucleocapsid	(Mumford et al. 1996)
Potyvirus	Sense (M4T - 50μM) for RT: 5' GTTTTCCCAGTCACGAC$(T)_{15}$ 3' PCR: Sense (S - 20μM): 5' GGXAAYAAYAGYGGXCAZCC 3' PCR: Antissense (M4 - 20μM): 5' GTTTTCCCAGTCACGAC 3' Target: NIb/protein coat	(Chen and Adams 2001)
Carlavirus	Sense (M4T - 50μM) for RT: 5' GTTTTCCCAGTCACGAC$(T)_{15}$ 3' PCR: Sense (pCar-1- 50μM): 5' ATGCCXCTXAXXCCXCC 3' PCR: Antissense (M4 - 50μM): 5' GTTTTCCCAGTCACGAC 3' Target: ORFs 3, 4, 5, 6 e 3' UTR	(Chen et al. 2002)
Tobamovirus	Sense (Tobamo1 - 25μM):5'TGATHAARMGDAAYWTBAAYDCDCC 3' Antissense (Tobamo2 - 25μM): 5' TTBGCYTCRAARTTCCA 3' Target: Viral polymerase	(Gibbs et al. 1998)
Hippeastrum mosaic virus (HiMV)	Sense (HiMV-R - 10μM) Antisense (HiMV-F - 10μM) Target: Protein coat	Rivas *et al.*, 2012 (not published)

Statistical analysis. The experimental design was completely randomized for all experiments. The data were submitted to analysis of variance (ANOVA) using the statistical software R 3.2.5, a package of experimental analysis ExpDes.pt (Ferreira et al. 2013). According to the ANOVA results, the data were compared using the Tukey test ($p<0.05$) or modeled in polynomial regressions ($p<0.05$). All experiments were repeated completely at least three times.

Results

Thermotherapy: growth analysis. The plants from thermotherapy-treated bulbs showed a greater number of leaves until the third week of cultivation when the control plants reached similar values; the control plants presented a higher number of leaves than those treated from the sixth week (Fig. 1a). After the sixth week of cultivation, the flower stalk had higher growth in the control plants and a stagnation of this response in the plants coming from thermotherapy-treated bulbs (Fig. 1b). Leaf length and width were lower in the control plants; however, lower growth rates were observed in those derived from thermotherapy-treated bulbs, resulting in both treatments presenting similar values for these responses after ten weeks of cultivation (Fig. 1c-d).

Both bulb and blooming survival percentages were lower (47% and 78%, respectively) in plants from thermotherapy-treated bulbs (Fig. 2).

Bulbs lose 13% fresh matter and 28% dry matter when passing through the thermotherapy process (Fig. 3a-b). In addition, subsequent growth was affected, so that the fresh root, bulb and leaf matter decreased by 35, 17 and 38%, respectively, while the dry matter of the same parts decreased by 48, 31 and 33%, respectively (Fig. 3a-c).

Gas exchange measurements/Quantification of photosynthetic pigments, TSS, RS, and starch. In relation to gas exchanges, only photosynthesis differed significantly between the control and thermotherapy-treated plants: photosynthesis was 19.22% lower in those thermotherapy-treated (Fig. 4). Thermotherapy-treated plants had a reduction in the levels of chlorophyll *a* and *b* and carotenoids of 49.88; 52.97 and 70.19% in relation to the control, respectively (Fig. 5). Thermotherapy-treated plants had a lower TSS content in root and bulb, 65.23 and 61.55%, respectively (Fig. 6a). While the content of RS was lower in thermotherapy-treated roots and in the control bulb, 54.25 and 79.37%, respectively (Fig. 6b). The starch content was lower only in the root (47.65%) of thermotherapy-treated plants (Fig. 6c). However, the content of TSS, RS, and starch was at the same level in the leaves of both treatments (Fig. 6a-c).

Activity of invertase isoforms. The activity of the cytosolic neutral invertase was higher in the root and in the bulb of thermotherapy-treated plants (Fig. 7a), whereas the activity of the vacuole acid invertase was higher only in the roots of these plants (Figure 7b).

Cryopreservation protocols. During the toxicity test of the vitrification solutions, a high survival rate was observed with the use of the PVS3 cryoprotectant in comparison to PVS2, since it caused an increase of 40% in the regrowth percentage after 240 minutes of exposure (Fig. 8a). Similarly, PVS3 proved to be more efficient as a cryoprotectant, yielding more than 60% bud regrowth, whereas PVS2 yielded a maximum of 30% regrowth (Fig. 8b).

The buds used as the explant source in cryopreservation protocols were extracted between 10 and 14 days of cultivation of the explants in the multiplication medium, with a size between 0.2 and 0.5 cm when transferred to the preculture medium (Fig. 9a and b). The buds within the capsules maintained a green coloration at their apexes after cryopreservation or acquired this coloration during the first days of cultivation, when viable (Fig. 9c). The surviving cryopreserved buds were fully recovered and suitable for acclimatization after 60 days in the multiplication medium (Fig. 8d).

Feasibility of viral cleaning techniques. The cryotherapy technique by means of encapsulation-vitrification yielded survival percentage 33% lower than the control, but a similar regrowth. Nevertheless, survival and regrowth were much higher than those of the thermotherapy and those of thermotherapy followed by cryotherapy (Table 2).

Table 2. Comparison of the effects of buds culture, cryotherapy, thermotherapy, and thermotherapy + cryotherapy on survival and regrowth

Methods	Survival (%)	Regrowth (%)
Buds culture (control)	100a	95a
Encapsulation-vitrification (cryotherapy)	67b	90a
Thermotherapy	32c	78b
Thermotherapy + cryotherapy	27d	70c

Values followed by the same letter in the column did not differ according to the Tukey test ($p < 0.05$)

Genetic stability. The explants recovered after undergoing thermotherapy or cryotherapy treatments or their combination showed stability in the DNA content, relative to the control (Fig. 10), as determined by flow cytometry. Nuclear DNA contents for control and other treatments ranged from 55.27-55.87 pg (Table S1).

Detection of cytological alterations and viral inclusions. Cytological changes were clearly detected between an asymptomatic infected leaf (Fig. 11a, b) and a leaf showing symptoms of viral infection (Fig. 11c, d). Directly on the leaf, it is possible to observe chlorotic depigmentation throughout the leaf blade characteristic of HiMV mosaic, indicating its interference with chlorophyll synthesis (Fig. 11c). Internally, densely stained structures are observed on the symptomatic leaf, indicating small necrotic areas of intense red color absent in the asymptomatic leaf (Fig. 11b, d).

In the young leaf of the infected and untreated bulb (Fig. 12a), no viral inclusions were observed (Fig. 12a-d). On the other hand, in the bud leaf after *in vitro* regrowth (Fig. 12e), it was possible to detect the presence of fibrillary viral inclusions around the nucleus, mainly observable in stomatal cells (Fig. 12f-i). Similarly, the recovered bud leaf that underwent the cryotherapy process at growth stages 2 and 3 (Fig. 12i, m; Fig. S1d, e) had opaque inclusion bodies within the nucleus (Fig. 12n, o), in addition to fibrillary inclusions also in stomatal cells (Fig. 12l, p), indicating that the virus was not eliminated during cryotherapy.

At growth stage 1 (Fig. S1), it was not possible to make paradermal sections. Likewise, due to the use of the thermotherapy material and the combination of thermotherapy with cryotherapy for the physiological and virus detection analyses, it was not possible to have material in conditions for performing the histological preparations.

Viral diagnosis. More specifically, considering all controls, the viruses HiMV, CMV and *Carlavirus* (probably *Nerine latent virus*) were detected in the bulb scale, as well as in the fully expanded leaf; the latter was not detected in bulbs recovered *in vitro* (Table 3). It was also found that the viruses *Tospovirus* and *Tobamovirus* were absent in the samples. It is important to note that in the young bulb leaf and cryo-treated buds at growth stage 1 (Fig. S1), it was not possible to detect any of the viruses by RT-PCR, thus suggesting a very low viral load level in these materials.

However, in cryo-treated bulbs from growth stage 2, in heat-treated bulbs, and in those that underwent thermotherapy followed by cryotherapy, HiMV and *Potyvirus* were detected. CMV was detected even after cryotherapy at growth stages 2 and 3, while *Carlavirus* was detected after cryotherapy, and the combination of

thermotherapy and cryotherapy, indicating that the thermotherapy reduces viral load but does not eliminate the virus (Table 3).

Table 3. Results of RT-PCRs from RNA extracted from *Hippeastrum* submitted to the different treatments

Treatment	HiMV	CMV	*Carlavirus*	*Potyvirus*	*Tospovirus*	*Tobamovirus*
Control (completelly expanded leaf)	+	+	+	+	−	−
Scale	+	−	−	+	−	−
Young leaf	−	−	−	n/e	−	−
In vitro multiplication	+	+	−	n/e	−	−
Thermotherapy	+	−	−	+	−	−
Cryotherapy (growth stage 1)	−	−	−	n/e	−	−
Cryotherapy (growth stage 2)	+	+	−	n/e	−	−
Cryotherapy (growth stage 3)	+	+	−	n/e	−	−
Cryotherapy (all stages)	+	−	+	+	−	−
Thermotherapy + Cryotherapy	+	−	+	+	−	−

−: negative result for virus analyzed; +: positive result for virus analyzed; n/e: not evaluated; CMV: *Cucumber mosaic virus*; HiMV: *Hippeastrum mosaic virus*; *Carlavirus* (*Nerine latent virus*); *Potyvirus* (*Sunflower mosaic virus* and *Hippeastrum mosaic virus*); *Tospovirus* (*Tomato spotted wilt tospovirus*, *Impatiens necrotic spot tospovirus*, and *Hippeastrum chlorotic ringspot virus*); *Tobamovirus* (*Tobacco mosaic virus*)

Discussion

The international trade in plants and seeds for planting and consumption and the regional and international exchange of plants for *in vitro* multiplication or genetic breeding presuppose that these plant materials are free from diseases, especially those of viral origin since there is no chemical control that can currently be applied in the crop for control. Thus, the strategy for virus control or avoiding the introduction of viruses into a crop is the use of virus-free plant material (Panattoni et al. 2013).

In vitro techniques associated with thermotherapy, chemotherapy and cryotherapy have yielded worthy results in obtaining plants of economic interest and free of viruses (Wang et al. 2018b). However, thermotherapy, which is the most applied technique for the treatment of virus-infected amaryllis bulbs (Amaral et al. 2007) can dramatically affect bulb vigor during growth recovery, as evidenced in this study.

Thermotherapy causes disruption of hydrogen and disulfide bonds of the protein coat, followed by phosphodiester bonds of nucleic acids and, consequently, deterioration of viral infectivity, which may include selective inhibition of viral replicase, changes in pH and cellular ionic strength, increase in lytic enzymes, competition between viral and messenger RNA by the ribosome binding site (Panattoni et al. 2013). However, it was observed in this study that thermotherapy has negative effects on the growth and development of hippeastrum cv. 'Apple Blossom'. The lower number of leaves, flowering, and length of the floral stalk were observed, as well as reduced survival and biomass of treated bulbs.

The negative physiological performance of the bulbs undergoing the heat treatment can be explained by their lower photosynthetic rate due to the reduction in the concentrations of chlorophyll *a* and *b* and carotenoids. As a consequence, there is less availability of carbohydrates to support plant growth and development.

In terms of growth, it was noted that plants derived from thermotherapy-treated bulbs had a higher initial response than control plants. However, when the growth trend was observed over ten weeks, growth stabilization was clear, especially in relation to the number of leaves and the length of the flower stalk. The apparent better initial development of thermotherapy-treated plants may be an indication of a higher respiration and carbohydrate consumption triggered by the high temperature, which does not necessarily imply biomass accumulation (Brestic et al. 2016).

With respect to these data, it is possible to infer that each species has an optimal temperature for its development, which reflects the most stable form of its proteins and when grown at suboptimal temperature, it shows a rapid development after being transferred to higher temperatures (Hatfield and Prueger 2015). Even so, the higher growth sustained at high temperatures does not, by itself, exceed the maximum growth characteristic of a species (Hatfield and Prueger 2015).

Most significantly, the percentage of survival and flowering were lower in the plants derived from thermotherapy-treated bulbs, demonstrating that thermotherapy affected the capacity of the bulbs to complete their life cycle. In this aspect, literature data showed that the temperature of 32 ºC, considered high, was sufficient to reduce flowering, as well as other growth responses of the ornamental plants *Antirrhinum majus*, *Calendula officinalis*, *Impatiens wallerana*, *Mimulus hybridus* and *Torenia fournieri* (Warner and Erwin 2005). On the other

hand, it is also known that a hot-cold-hot temperature alternation is required for bulbous plants to complete their life cycle (Khodorova and Boitel-Conti 2013). Therefore, thermotherapy interrupts this alternation, contributing to lower plant growth and the appearance of physiological disorders during flowering.

If on the one hand, thermotherapy may favor viral elimination; on the other hand, high temperatures can mean the creation and maintenance of an environment conducive to the development of phytopathogenic fungi for 40 days (Sexton and Howlett 2006). *Botrytis cinerea* is an example of this type of fungus, which infects *Hippeastrum* and is responsible for major economic losses by causing host death (Elad et al. 2016). This data may explain, in part, the lower survival percentage of thermotherapy-treated plants, since the lethality of this treatment was associated to the development of necrosis associated with a fungal infestation (data not shown), although the fungus identity was not the target of this study.

When analyzing the gas exchange responses, it was observed that only photosynthesis was significantly affected by thermotherapy. At the same time, the content of chlorophyll *a* and *b* and carotenoid pigments were lower. Thus, it is suggested that the negative effects of thermotherapy are partially overcome at the expense of carbon skeletons that would later be used in the biosynthesis of pigments, or even affecting the enzymatic capacity responsible for the biosynthesis of these pigments. In fact, studies point to the negative influence of the high temperature on the chlorophyll biosynthesis capacity, and the damage is in both biosynthesis and acceleration of degradation (MATHUR et al., 2014).

Because of the lower pigment content and reduction in photosynthesis, thermotherapy-treated *Hippeastrum* plants presented lower TSS content in the root and bulb, as well as lower contents of RS and starch in the root. Bulbous species such as *Hippeastrum* accumulate the carbohydrates produced during photosynthesis in underground organs, resulting in an increase in bulb bulk and determining the ability to bloom (Khodorova and Boitel-Conti 2013). Therefore, the lower percentage of flowering in heat-treated plants was more a reflection of sugar depletion.

However, more important than the carbohydrate reserve itself is its mobilization and transport to other organs, as it indicates the instant plant demand due to its physiological state (Khodorova and Boitel-Conti 2013). Thus, it was noticed that TSS, RS, and starch remained at the same levels in the control leaves and thermotherapy-treated plants, while there was a clear contrast in the distribution of sugars in the bulb and root.

One of these contrasts was the lower content of RS in the control bulb which, together with the higher content of TSS in the same organ, allows to infer that the sucrose content is also high in the bulb. Thus, it is possible to say that the control plants were in the storage phase, since the amount of sucrose was always higher than that of RS during this phase, as observed in more than 30 other geophytes (Ranwala and Miller 2008a). In addition, the activity levels of sucrose cleavage enzymes also remained low (Fig. 7), as also observed in tulip (*Tulipa gesneriana*) plants (Balk and de Boer 1999).

Differently from the control, thermotherapy-treated plants did not enter the storage stage at 30 days of cultivation, since they have a higher content of RS and possibly less sucrose, besides sustaining greater activities of invertases. In this context, it is known that correctly stored bulbs at low temperatures result in plants with high expression of invertase genes and high activity of these enzymes in growing stems, which optimizes the hexoses metabolism (Lambrechts and Kollöffel 1993; Balk and de Boer 1999; Ranwala and Miller 2008b).

Sucrose cleavage enzymes, such as invertase and sucrose synthase (SuSy), are activated in response to the demand for carbohydrates and energy (Zhang et al. 2016; Wan et al. 2018). It is believed that, in the deprivation of treatment with low temperature, the demand for carbohydrates in the shoots does not occur and, consequently, there is no activation of invertases and SuSy (Lambrechts et al. 1994; Ranwala and Miller 2008b). Nevertheless, sucrose cleavage enzymes play an important role in development control (Koch 2004; Gao et al. 2018), being the low activity of these enzymes in the stalks of bulbs stored at high temperatures (Lambrechts and Kollöffel 1993) a possible factor of delay of leaf development and subsequent carbohydrate storage (Khodorova and Boitel-Conti 2013).

The knowledge about the physiological effects of thermotherapy on *Hippeastrum* bulbs was important, as it revealed the potential of this treatment to affect subsequent growth and development of recovered plants. Moreover, it allows differentiating the physiological consequences of a combined treatment of thermotherapy and cryotherapy.

However, prior to initiating the cryotherapy experiments, it was necessary to establish a cryopreservation protocol with cryoprotection and vitrification appropriate to *Hippeastrum* to reduce harmful tissue damage, since intracellular water content is a critical factor for the efficiency of the protocol related to this technique (WANG et al., 2018).

Cryotherapy provided the survival and recovery of amaryllis bulbs by means of the use of the encapsulation-vitrification technique. The use of the cryoprotectant PVS3 led to more satisfactory results when compared to the PVS2 cryoprotectant. The main difference may be the composition of the PVS3 solution (50% glycerol and 50% sucrose), considered highly concentrated, allowing slow and uniform dehydration of bulbs that present high percentage of water in their vacuoles (Volk and Caspersen 2017).

In turn, the PVS2 cryoprotectant (30% sucrose, 15% ethylene glycol, 30% glycerol and 15% DMSO) also provides excellent protection against the damaging effects caused by freezing; however, DMSO is moderately toxic to plant cells, depending on the treatment temperature, duration of use and concentration (Fahy and Wowk 2015). This happens because after thawing, excess DMSO may result in inhibition of mitochondrial respiration and increase in cytosolic calcium (Galvao et al. 2014), which would explain the lower bulb survival.

Another important factor is the explant size. The explants used in this study can be considered relatively large, varying from 0.2 to 0.5 cm (Fig. 9a). Based on the high sized explants, sensitivity to biochemical toxicity and high osmolality, the literature postulates that PVS3 is the most indicated cryoprotectant (Kim et al. 2009).

When comparing the four groups of treatments, it was observed that the application of thermotherapy or cryotherapy, aiming at viral cleaning, reduced the percentage of survival and recovery, and the two techniques used had an additive effect when combined (Table 2). The much lower survival rate after thermotherapy was largely due to the high level of *in vitro* contamination. As previously mentioned, not only did the increase in the storage temperature of the bulbs affect their growth and development (Fig. 3) but also favored the appearance of pathogenic fungi, reducing the viability and health of the plant material during *in vitro* establishment.

From a practical point of view, it is essential that any viral elimination method is both capable of eliminating the samples viral load and of allowing the normal development of the material undergoing the treatment (Milosevic et al. 2012; Panattoni et al. 2013). Thus, cryotherapy showed a more productive character, allowing more than double survival, compared to thermotherapy.

However, viral cleaning techniques and the tissue culture itself present the risk of inducing genetic variation, which should be taken into account (Bota et al. 2014; Wang et al. 2018a). To date, there is no record in the literature on the genetic stability of *Hippeastrum* sp. submitted to thermotherapy, cryotherapy, or *in vitro* culture. Thus, this is the first study regarding the somaclonal variation of both cryopreservation and the application of thermotherapy and cryotherapy.

In this context, the use of large explants (> 1 cm) and the use of PVS3 instead of PVS2 in the experiments minimized the induction of phenotypic and genetic variations by the treatments (Akdemir et al. 2013; Bota et al. 2014). Thus, genetic stability in *H. hybridum* was observed after the treatments (Fig. 10).

Cynara scolymus plants thermotherapy-treated and recovered by buds culture also did not demonstrate genetic alterations determined by simple sequence repeat (SSR) (Acquadro et al. 2010). However, in the same study, polymorphic bands were detected by amplified fragment length polymorphism (AFLP) markers, indicating that thermotherapy may induce genetic variations (Wang et al. 2018a). Likewise, *Vitis vinifera* somaclones recovered *in vitro* were genetically different from the original cultivars (Popescu et al. 2002).

In turn, bud cryotherapy did not involve genetic instability of *Platycladus orientalis* (Ahn and Choi 2017), *Asparagus officinalis* (Carmona-Martin et al. 2018), *Arachis glabrata* (Dolce et al. 2018), *Allium sativum* (Liu et al. 2017), and *Phoenix dactylifera* (Alansi et al. 2017). But it induced modifications influenced by the cultivar genotype of *Mentha* x *piperita* and *Chrysanthemum* x *morifolium* (Martin et al. 2014), in addition to small modifications in the genome of *Hedeoma todsenii* (Pence et al. 2017), and *Pistacia vera* due to the toxic effect of PVS2 (Akdemir et al. 2013).

Because a virus-infected plant does not always manifest symptoms clearly, it is important to use histological techniques that may aid in viral diagnosis. Thus, it was shown in the study that the internal structure of the fully expanded leaf infected and without the manifestation of symptoms differed from the leaf that clearly manifests the symptoms (Fig. 11).

In this aspect, the coloration with acetic carmine was not sufficient to indicate the presence of the infection in an asymptomatic leaf. Originally, this dye is applied for nucleic acid staining, due to its general specificity for all weakly acidic cellular components (Chu 1946; Rattenbury 1952).

Even with the aid of specific dyes for viral inclusions, it was not possible to detect them in young leaves (Fig. 12a-e); on the other hand, it was possible to detect the viral inclusion bodies in recovered bulb leaf *in vitro* (Fig. 12e-h). Since bulb culture is a technique that even alone has the potential to eliminate viral infection, as demonstrated in another bulbous plant, *A. sativum* (Murkute and Gawande 2018), this result may indicate that the bud size of *Hippeastrum* sp. (Fig. 10a) was too large and carried with it some infected cells.

Likewise, the leaf of a bud recovered after cryopreservation at growth stage 2 and 3 also showed viral inclusions (Fig. 12i-p). These findings demonstrate that cryotherapy was ineffective at eliminating all remaining infected cells in the treated buds. The cryotherapy technique was also not successful in eliminating the viroids of *C. morifolium*, believing the authors to be due to the initial high concentration of the pathogen and to the plant genotype (Jeon et al. 2016). On the other hand, in *Vitis* sp. the failure was attributed to bud size and plant development stage (Wang et al. 2016). As the initial concentration of viroids is directly proportional to the size of the explant, in *Hippeastrum* sp. it is likely that the size was so large that cryotherapy was also unable to eliminate all remaining cells with the virus.

The viral diagnosis by RT-PCR confirmed the infection of the bulbs used by HiMV, CMV, *Carlavirus*, and *Potyvirus* (Table 3), which are the most common in *Hippeastrum* plants with mosaic symptoms (De Leeuw

1972). On the other hand, the results showed the absence of *Tospovirus* and *Tobamovirus*, whose genera have viral species that infect *H. hybridum* (De Leeuw 1972; Xu et al. 2014).

Although the viruses were confirmed, their detections were dependent on the plant organ and development stage of plant material. For example, only in fully expanded leaf were detected HiMV, CMV, *Carlavirus*, and *Potyvirus* at the same time, and in the young leaf, none of them were detectable. This observation suggests that viral concentration may vary as tissue grows and develops, with viral concentration being lower in younger tissues. Therefore, it is recommended to carry out detection tests after a certain culture time to minimize false-negative tests.

The viral concentration in the sap determines the plant growth limitations in the midst of a viral population translocating within the host and therefore the effective size of the infection and the rate at which the viroids multiply in each plant tissue (Gutiérrez et al. 2012). As the new tissues are vascularized (Mazur and Friml 2017), the viral load in them tends to be much lower.

Similarly, in bulbs that underwent cryotherapy and at growth stage 1 the viral infection was also not detectable, possibly due to its reduced development. Even so, at higher growth stages, the viruses were detected, reiterating the inefficacy of cryotherapy for viral cleaning in *H. hybridum*. The same was true for thermotherapy and the combination of thermotherapy followed by cryotherapy, with exception to CMV, which may have been eliminated by double treatment. However, the low bud survival rate after thermotherapy followed by cryotherapy makes the procedure not recommended.

CMV is a cosmopolitan virus that affects many horticultural, fruit and ornamental crops, and the development of resistance to CMV is a valuable contribution to the containment of this and other pathogens (Zitter and Murphy 2009). This virus, transmitted by aphids, can infect more than 1200 plant species and the losses vary according to the intensity of the infection (Hooks and Fereres 2006). Despite the recurrence, CMV is not so highlighted by the productivity losses since it can remain asymptomatic in *Hippeastrum* sp. (Zitter and Murphy 2009).

Irrespective of the treatments applied for viral elimination were successful, the presented results point to possible strategies that could be used in order to eliminate the main viruses in *H. hybridum*. However, the percentage of viral elimination, the time required for the execution of the treatments and the costs should be taken into account when evaluating the viability of the procedure, in relation to the renovation of the nursery with absent virus matrices.

This study demonstrated that thermotherapy, despite being a technique applied for viral elimination in *Hippeastrum hybridum* cv. 'Apple Blossom', affected the physiology of the species, delaying the growth and subsequent bulb development. These physiological consequences were in part due to the reduction in the concentration of photosynthetic pigments and, consequently, of photosynthesis. In addition, an efficient and reproducible cryopreservation protocol was developed by the means of encapsulation-vitrification and use of PVS3. This protocol, when applied to cryotherapy, ensured genetic stability, as well as thermotherapy and the combination of both. However, both thermotherapy and cryotherapy, or their combination, have not been effective in eliminating the viruses that infect hippeastrum.

Particularly noteworthy is the evidence that samples analyzed in young tissues did not allow virus detection by a technique as sensitive as RT-PCR. This reinforces the need for care regarding the completion of viral elimination results reported for other species by thermotherapy or cryotherapy, where non-attention to this detail may have produced false-negative tests. In any case, subsequent studies should also be encouraged to access genetic stability and observe the field performance of virus-free plants obtained by heat-based methods. These studies would accelerate the transposition of virus-free plants into agricultural production on a large scale, improving crop productivity and quality.

Conflict of interest

The authors declare that they have no conflict of interest.

Authors Contribution Statement

MSc Souza conducted all the experiments and participated in writing the manuscript. Dr. Paiva is Mr. Souza's (Ph.D. candidate) supervisor and provided the facilities for the study conduction. Dr. Prudente is co-supervisor, provided all technical support for cryopreservation experiments and wrote the final version of the manuscript. Dr. Rivas participated in the execution of viral diagnostics and participated in writing the manuscript. Dr. Paiva, Dr. Rivas, and Dr. Domiciano provided all support and facilities for the execution of the histological, gas exchanges and biochemical analyses and wrote part of the discussion.

References

Acquadro A et al. (2010) Production and fingerprinting of virus-free clones in a reflowering globe artichoke. Plant Cell Tiss Organ Cult 100:329-337

Ahn C-H, Choi Y-E (2017) *In vitro* clonal propagation and stable cryopreservation system for *Platycladus orientalis* via somatic embryogenesis. Plant Cell Tiss Organ Cult 131:513-523

Akdemir H, Süzerer V, Tilkat E, Yildirim H, Onay A, Çiftçi YO (2013) *In vitro* conservation and cryopreservation of mature pistachio (*Pistacia vera* L.) germplasm. Journal of Plant Biochemistry and Biotechnology 22:43-51

Alansi S, Al-Qurainy F, Khan S, Nadeem M, Tarroum M, Alshameri A, Gaafar ARZ (2017) Genetic fidelity testing in regenerated plantlets of cryopreserved and non-cryopreserved cultivars of *Phoenix dactylifera*. Pak J Bot 49:2313-2320

Alexandre MAV, Duarte LML, Rivas EB, Cilli A, Harakava R, Galleti SR, Kitajima EW (2011) *Hippeastrum mosaic virus* diagnosed in *Hippeastrum* and *Eucharis* in Brazil. Journal of Plant Pathology 93:643-649

Amaral L, de Arruda Veiga RF, Tombolato AFC, Barbosa W (2007) Conservação *in vitro* de germoplasma indexado de três cultivares de amarílis (*Hippeastrum* Herb.). Ornamental Horticulture 13

Balk PA, de Boer AD (1999) Rapid stalk elongation in tulip (Tulipa gesneriana L. cv. Apeldoorn) and the combined action of cold-induced invertase and the water-channel protein γTIP. Planta 209:346-354

Baranyi M, Greilhuber J (1995) Flow cytometric analysis of genome size variation in cultivated and wild *Pisum sativum* (Fabaceae). Plant Systematics and Evolution 194:231-239

Barba M, Hosakawa M, Wang Q-C, Taglienti A, Zhang Z (2017) Viroid Elimination by Thermotherapy, Cold Therapy, Tissue Culture, *In Vitro* Micrografting, or Cryotherapy. In: Hadidi A, Flores R, Randles JW, Palukaitis P (eds) Viroids and Satellites. Academic Press, Boston, pp 425-435

Berniak H (2016) Characterization of a new *Tomato spotted wilt virus* isolates found in *Hippeastrum hybridum* (Hort.) plants in Poland. Journal of Horticultural Research 24:5-12

Bota J, Cretazzo E, Montero R, Rosselló J, Cifre J (2014) *Grapevine fleck virus* (GFkV) elimination in a selected clone of *Vitis vinifera* L. cv. Manto Negro and its effects on photosynthesis. OENO One 48:11-19

Brestic M, Zivcak M, Kunderlikova K, Allakhverdiev SI (2016) High temperature specifically affects the photoprotective responses of chlorophyll b-deficient wheat mutant lines. Photosynth Res 130:251-266

Cairo PAR, Oliveira LEMd, Mesquita AC (2009) Activity of rubisco and enzymes of sucrose synthesis and hydrolysis associated to latex productivity, in rubber tree clones [*Hevea brasiliensis* (Willd ex. Adr. de Juss.) Muell.-Arg] cultivated in Lavras, MG. Ciência e Agrotecnologia 33:369-376

Carmona-Martin E, Regalado JJ, Peran-Quesada R, Encina CL (2018) Cryopreservation of rhizome buds of *Asparagus officinalis* L. (cv. Morado de Huetor) and evaluation of their genetic stability. Plant Cell Tissue Organ Cult 133:395-403

Chen J, Adams M (2001) A universal PCR primer to detect members of the Potyviridae and its use to examine the taxonomic status of several members of the family. Arch Virol 146:757-766

Chen J, Chen JP, Adams M (2002) Characterisation of some carla-and potyviruses from bulb crops in China. Arch Virol 147:419-428

Christie RG, Edwardson JR (1986) Light microscopic techniques. Plant disease 70:273-279

Chu J (1946) Reaction of Nucleic Acid to Aceto-carmine. Nature 157:513

Conci V, Nome SF (1991) Virus free garlic (*Allium sativum* L.) plants obtained by thermotherapy and meristem tip culture. Journal of Phytopathology 132:186-192

De Leeuw G (1972) *Tobacco mosaic virus* in *Hippeastrum hybridum*. Eur J Plant Pathol 78:69-71

Dische Z (1962) General color reactions. Methods in carbohydrate chemistry 1:478-512

Dolce NR, Faloci MM, Gonzalez AM (2018) *In vitro* plant regeneration and cryopreservation of *Arachis glabrata* (Fabaceae) using leaflet explants. In Vitro CellDevBiol-Plant 54:133-144

Dolezel J, Binarova P, Lucretti S (1989) Analysis of nuclear DNA content in plant cells by flow cytometry. Biologia plantarum 31:113-120

Dong JH, Yin YY, Fang Q, McBeath JH, Zhang ZK (2013) A new tospovirus causing chlorotic ringspot on *Hippeastrum* sp in China. Virus Genes 46:567-570

Duarte L, Alexandre M, Harakava R (2009) Identificação molecular do *Hippeastrum mosaic virus* em Amarílis (*Hippeastrum* sp.). Tropical Plant Pathology 34:S274

Elad Y, Pertot I, Prado AMC, Stewart A (2016) Plant hosts of Botrytis spp. In: *Botrytis*–the fungus, the pathogen and its management in agricultural systems. Springer, pp 413-486

Ephrath JE, Ben-Asher J, Baruchin F, Alekperov C, Dayan E, Silberbush M (2001) Various cutting methods for the propagation of hippeastrum bulbs. Biotronics 30:75-83

Fahy GM, Wowk B (2015) Principles of cryopreservation by vitrification. In: Cryopreservation and freeze-drying protocols. Springer, pp 21-82

Ferreira E, Cavalcanti P, Nogueira D (2013) ExpDes.pt: Experimental Designs package. R package (Português) version 1

Galvao J, Davis B, Tilley M, Normando E, Duchen MR, Cordeiro MF (2014) Unexpected low-dose toxicity of the universal solvent DMSO. The FASEB Journal 28:1317-1330

Gao M et al. (2018) Transcriptome analysis of starch and sucrose metabolism across bulb development in *Sagittaria sagittifolia*. Gene 649:99-112

Gibbs A, Armstrong J, Mackenzie AM, Weiller GF (1998) The GPRIME package: computer programs for identifying the best regions of aligned genes to target in nucleic acid hybridisation-based diagnostic tests, and their use with plant viruses. Journal of Virological Methods 74:67-76

Gutierrez-Villegas C, Ruiz-Medrano R, Piedra-Ibarra E, De La Torre-Almaraz R (2004) Characterization of a *cucumber mosaic virus* (CMV) strain associated with yellow mottle symptoms of amaryllis (*Hippeastrum* x *hybridum* Leopoldii) in Mexico. Agrociencia 38:343-354

Gutiérrez S, Yvon M, Pirolles E, Garzo E, Fereres A, Michalakis Y, Blanc S (2012) Circulating virus load determines the size of bottlenecks in viral populations progressing within a host. PLOS Pathogens 8:e1003009

Hanks G (2018) A review of production statistics for the cut flower and foliage sector, updated 2018 (part of AHDB Horticulture funded project PO BOF 002a). The National Cut Flower Centre, AHDB Horticulture:132

Hatfield JL, Prueger JH (2015) Temperature extremes: Effect on plant growth and development. Weather and climate extremes 10:4-10

Hooks CR, Fereres A (2006) Protecting crops from non-persistently aphid-transmitted viruses: a review on the use of barrier plants as a management tool. Virus research 120:1-16

Hu G, Dong Y, Zhang Z, Fan X, Ren F, Zhou J (2015) Virus elimination from *in vitro* apple by thermotherapy combined with chemotherapy. Plant Cell Tiss Organ Cult 121:435-443

Jeon SM, Naing AH, Kim HH, Chung MY, Lim KB, Kim CK (2016) Elimination of *chrysanthemum stunt viroid* and *chrysanthemum chlorotic mottle viroid* from infected chrysanthemum by cryopreservation. Protoplasma 253:1135-1144

Khodorova NV, Boitel-Conti M (2013) The role of temperature in the growth and flowering of geophytes. Plants (Basel, Switzerland) 2:699-711

Kim H-H, Lee Y-G, Shin D-J, Ko H-C, Gwag J-G, Cho E-G, Engelmann F (2009) Development of alternative plant vitrification solutions in droplet-vitrification procedures. CryoLetters 30:320-334

Koch K (2004) Sucrose metabolism: regulatory mechanisms and pivotal roles in sugar sensing and plant development. Current opinion in plant biology 7:235-246

Lambrechts H, Kollöffel C (1993) Soluble and insoluble invertase activity in elongating *Tulipa gesneriana* flower stalks. Physiologia Plantarum 89:830-834

Lambrechts H, Rook F, Kolloffel C (1994) Carbohydrate status of tulip bulbs during cold-induced flower stalk elongation and flowering. Plant Physiology 104:515-520

Lee S-C, Chang Y-C (2006) Multiplex RT-PCR detection of two orchid viruses with an internal control of plant nad5 mRNA. Plant Pathology Bulletin 15:187-196

Lichtenthaler HK, Buschmann C (2001) Chlorophylls and carotenoids: Measurement and characterization by UV-VIS spectroscopy. Current protocols in food analytical chemistry 1:F4. 3.1-F4. 3.8

Liu X-X, Wen Y-B, Cheng Z-H, Mou S-W (2017) Establishment of a garlic cryopreservation protocol for shoot apices from adventitious buds *in vitro*. Scientia Horticulturae 226:10-18

Lizárraga A, Ascasíbar J, González ML (2017) Fast and effective thermotherapy treatment for *in vitro* virus eradication in apple and pear trees. American Journal of Plant Sciences 8:2474

Maat D, Huttinga H, Hakkaart F (1978) *Nerine latent virus*: some properties and serological detectability in *Nerine bowdenii*. Eur J Plant Pathol 84:47-59

Martin C, Gonzalez I, Kremer C, Gonzalez-Benito ME (2014) Cryopreservation and genetic stability of shoot cultures in vegetatively propagated species: mint and chrysanthemum. In: Reed BM (ed) Ii International Symposium on Plant Cryopreservation, vol 1039. Acta Horticulturae. Int Soc Horticultural Science, Leuven 1, pp 127-132

Mazur E, Friml J (2017) Vascular tissue development and regeneration in the model plant Arabidopsis. In: Plant Engineering. IntechOpen,

Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Chemistry 31:426-428

Milosevic S, Cingel A, Jevremovic S, Stankovic I, Bulajić A, Branka K, Subotic A (2012) Virus elimination from ornamental plants using *in vitro* culture techniques vol 27

Mumford R, Barker I, Wood K (1996) An improved method for the detection of Tospoviruses using the polymerase chain reaction. Journal of Virological Methods 57:109-115

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia Plantarum 15:473-497

Murkute A, Gawande S (2018) Production of virus free planting material through meristem culture in short day garlic cultivars Bhima Omkar and Bhima Purple. Journal of Environmental Biology 39:286-290

Nishizawa S, Sakai A, Amano Y, Matsuzawa T (1993) Cryopreservation of asparagus (*Asparagus officinalis* L.) embryogenic suspension cells and subsequent plant regeneration by vitrification. Plant Science 91:67-73

Panattoni A, Luvisi A, Triolo E (2013) Elimination of viruses in plants: twenty years of progress. Spanish Journal of Agricultural Research:173-188

Pence VC et al. (2017) Survival and genetic stability of shoot tips of *Hedeoma todsenii* RSIrving after long-term cryostorage. In Vitro Cell Dev Biol-Plant 53:328-338

Popescu C, Falk A, Glimelius K (2002) Application of AFLPs to characterize somaclonal variation in anther-derived grapevines. Vitis-Geilweilerhof- 41:177-182

Prudente DO, Paiva R, Souza LB, Paiva PDO (2018) Cryotherapy as a technique for virus elimination in ornamental species. Plant Cell Culture & Micropropagation 13:29-33

Raj S, Snehi S, Kumar S, Khan M (2009) First molecular detection and identification of a potyvirus associated with severe mosaic disease of amaryllis (*Hippeastrum hybridum* Hort.) in India. Australasian Plant Disease Notes 4:50-53

Ranwala AP, Miller WB (2008a) Analysis of nonstructural carbohydrates in storage organs of 30 ornamental geophytes by high-performance anion-exchange chromatography with pulsed amperometric detection. New Phytologist 180:421-433

Ranwala AP, Miller WB (2008b) Gibberellin-mediated changes in carbohydrate metabolism during flower stalk elongation in tulips. Plant Growth Regul 55:241

Rattenbury J (1952) Specific staining of nucleolar substance with aceto-carmine. Stain technology 27:113-120

Rubio-Huertos M (1972) Inclusion bodies. In: Principles and techniques in plant virology. Kodo Agrawal, pp 66-75

Sakai A, Hirai D, Niino T (2008) Development of PVS-Based vitrification and encapsulation-vitrification protocols. In: Plant cryopreservation: a pratical guide. Corvalis, Springer, pp 33-58

Salzman R, Fujita T, Zhu-Salzman K, Hasegawa P, Bressan R (1999) An improved RNA isolation method for plant tissues containing high levels of phenolic compounds or carbohydrates. Plant Mol Biol Rep 17:11-17

Sexton AC, Howlett BJ (2006) Parallels in fungal pathogenesis on plant and animal hosts. Eukaryotic cell 5:1941-1949

Shunmugam AS et al. (2018) MeioCapture: an efficient method for staging and isolation of meiocytes in the prophase I sub-stages of meiosis in wheat. BMC plant biology 18:293

Smith KM (2012) A textbook of plant virus diseases. Elsevier,

Tombolato A, Dutilh J, Barbosa W, Veiga R, Lucon T Brazilian descriptor for *Hippeastrum* hybrids. In: Building a sustainable future: the role of botanic gardens. Proceedings of the 3rd Global Botanic Gardens Congress, Wuhan, China, 2007. Botanic Gardens Conservation International, pp 1-11

Tombolato AFC, Costa AMM, Eglit AC (2001) Micropropagação de *Hippeastrum hybridum* 'Apple blossom', mediante escamas duplas. Ornamental Horticulture 7:35-40

Volk GM, Caspersen AM (2017) Cryoprotectants and components induce plasmolytic responses in sweet potato (*Ipomoea batatas* (L.) Lam.) suspension cells. In Vitro Cell Dev Biol-Plant 53:363-371

Walkey DG (2018) Virus diseases. In: Onions and allied crops. CRC Press, pp 203-224

Wan H, Wu L, Yang Y, Zhou G, Ruan Y-L (2018) Evolution of sucrose metabolism: the dichotomy of invertases and beyond. Trends in plant science 23:163-177

Wang B et al. (2014) Potential applications of cryogenic technologies to plant genetic improvement and pathogen eradication. Biotechnology Advances 32:583-595

Wang MR, Cui ZH, Li JW, Hao XY, Zhao L, Wang Q-C (2018a) *In vitro* thermotherapy-based methods for plant virus eradication. Plant methods 14:87

Wang MR, Li BQ, Feng CH, Wang QC (2016) Culture of shoot tips from adventitious shoots can eradicate *Apple stem pitting virus* but fails in *Apple stem grooving virus*. Plant Cell Tissue Organ Cult 125:283-291

Wang MR et al. (2018b) Cryopreservation of virus: a novel biotechnology for long-term preservation of virus in shoot tips. Plant Methods 14:10

Wang QC, Cuellar WJ, Rajamaki ML, Hirata Y, Valkonen JPT (2008) Combined thermotherapy and cryotherapy for efficient virus eradication: relation of virus distribution, subcellular changes, cell survival and viral RNA degradation in shoot tips. Mol Plant Pathol 9:237-250

Wang QC, Valkonen JPT (2008) Elimination of two viruses which interact synergistically from sweetpotato by shoot tip culture and cryotherapy. Journal of Virological Methods 154:135-145

Warner RM, Erwin JE (2005) Prolonged high temperature exposure and daily light integral impact growth and flowering of five herbaceous ornamental species. J Am Soc Hortic Sci 130:319-325

Wylie S, Wilson C, Jones R, Jones M (1993) A polymerase chain reaction assay for *cucumber mosaic virus* in lupin seeds. Australian Journal of Agricultural Research 44:41-51

Wylie SJ, Jones MGK (2012) Complete genome sequences of seven carlavirus and potyvirus isolates from *Narcissus* and *Hippeastrum* plants in Australia, and proposals to clarify their naming. Arch Virol 157:1471-1480

Xu Y et al. (2014) Molecular characterization of the *hippeastrum chlorotic ringspot virus* L segment and its protein. Arch Virol 159:2805-2807

Yanagawa T, Osaki T (1996) *In vitro* propagation of bulblets and elimination of viruses by bulb-scale cultures of *Hippectstrum hybridum* Bulbs. Plant tissue culture letters 13:147-152

Zhang C, Zhang H, Zhan Z, Liu B, Chen Z, Liang Y (2016) Transcriptome analysis of sucrose metabolism during bulb swelling and development in onion (*Allium cepa* L.). Frontiers in Plant Science 7:1425

Zhang W, Song L, Teixeira da Silva JA, Sun H (2013) Effects of temperature, plant growth regulators and substrates and changes in carbohydrate content during bulblet formation by twin scale propagation in *Hippeastrum vittatum* 'Red lion'. Scientia Horticulturae 160:230-237

Zitter T, Murphy J (2009) *Cucumber mosaic virus*. The Plant Health Instructor

Figures

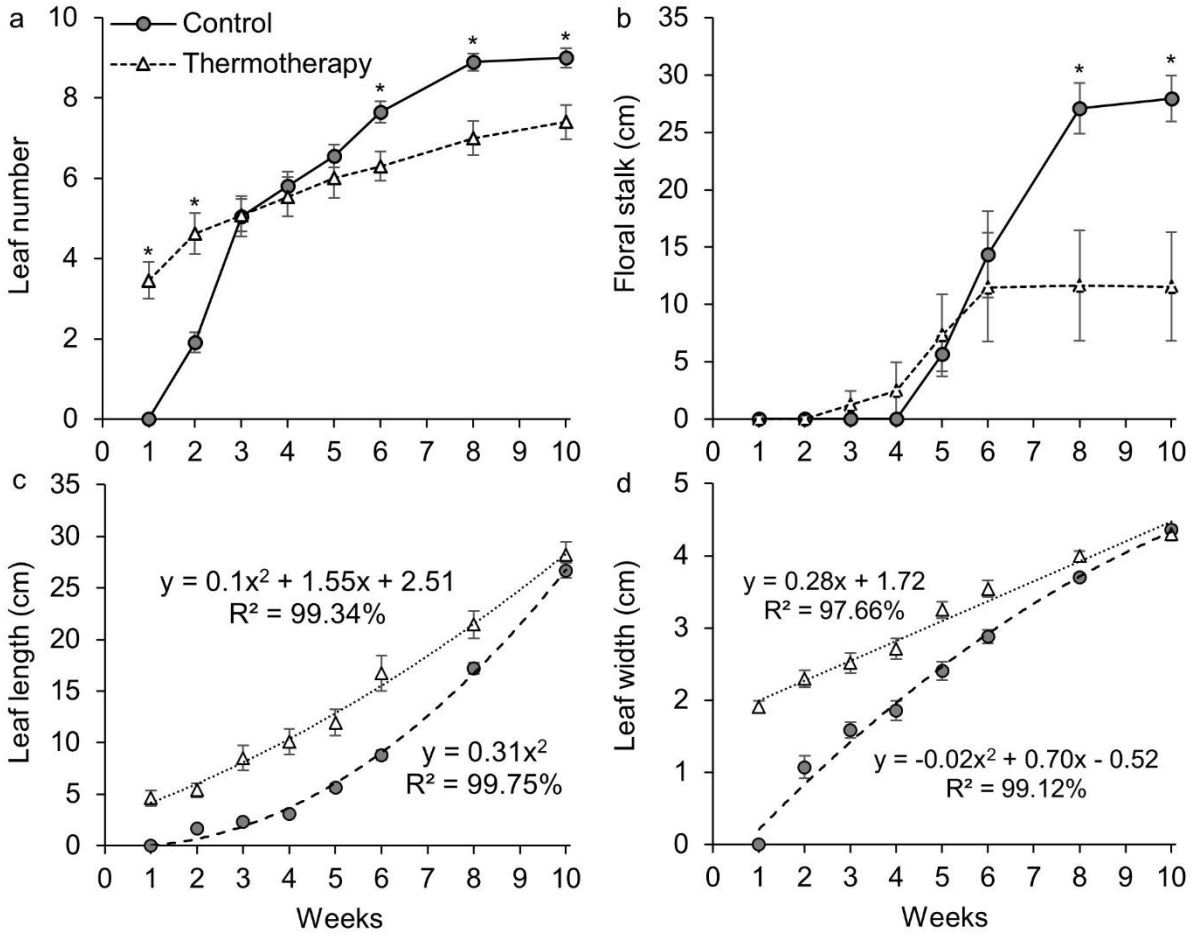

Fig. 1 Bulb growth responses subjected to thermotherapy for 10 weeks in a greenhouse; leaf number (a); floral stalk length (b); leaf length (c) and leaf width (d); bars indicate the standard error; *significant difference according to the Tukey test (p<0.05)

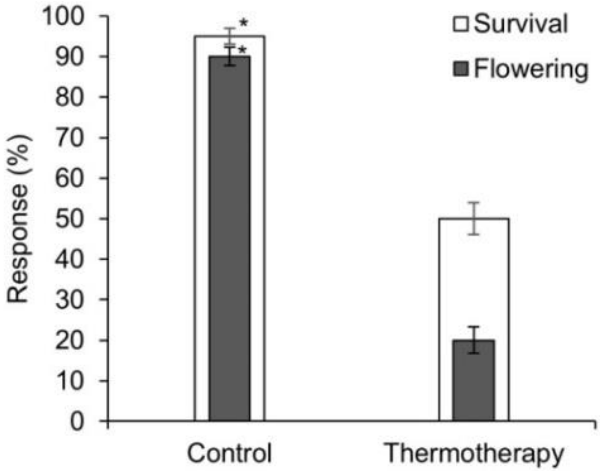

Fig. 2 Survival and flowering of control and thermotherapy treated plants after 30 days in a greenhouse; bars indicate the standard error; *significant difference according to the Tukey test (p<0.05)

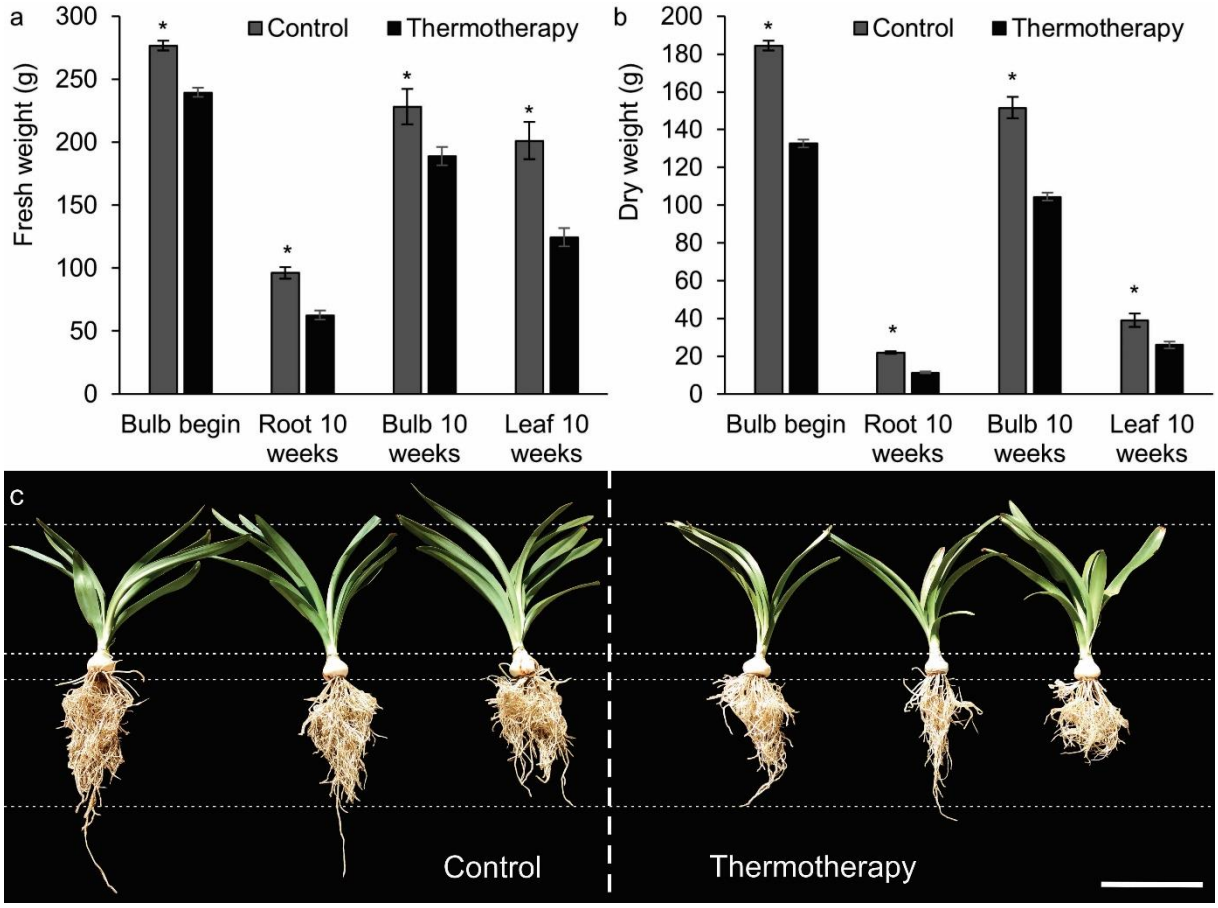

Fig. 3. Biomass allocation among the control and thermotherapy treated plants parts after 10 weeks in a greenhouse; fresh weight (a); dry weight (b); samples of the control and thermotherapy treated plants (c); bars indicate the standard error; scale bar = 30 cm; *significant difference according to the Tukey test (p<0.05)

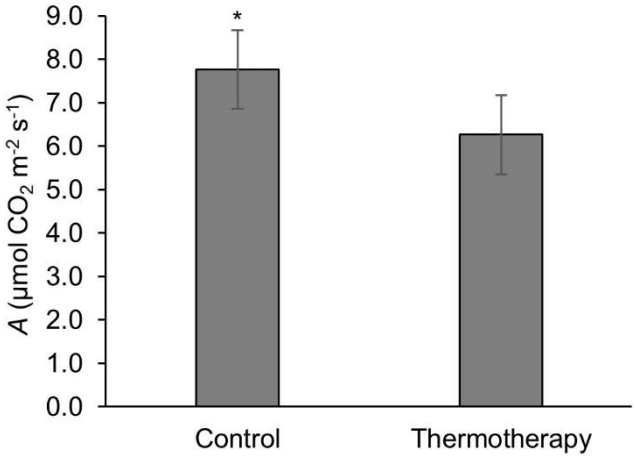

Fig. 4. Photosynthesis (A) of control and thermotherapy treated plants measured after 30 days in a greenhouse; bars indicate the standard error; *significant difference according to the Tukey test (p<0.05)

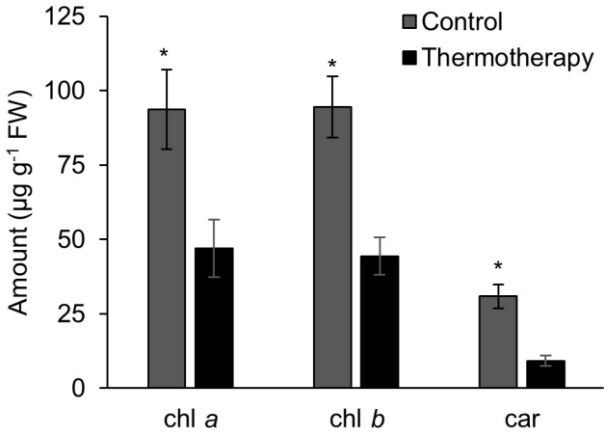

Fig. 5. Concentration of chlorophyll a, b and carotenoids in control and thermotherapy treated plants after 10 weeks; bars indicate the standard error; *significant difference according to the Tukey test (p<0.05)

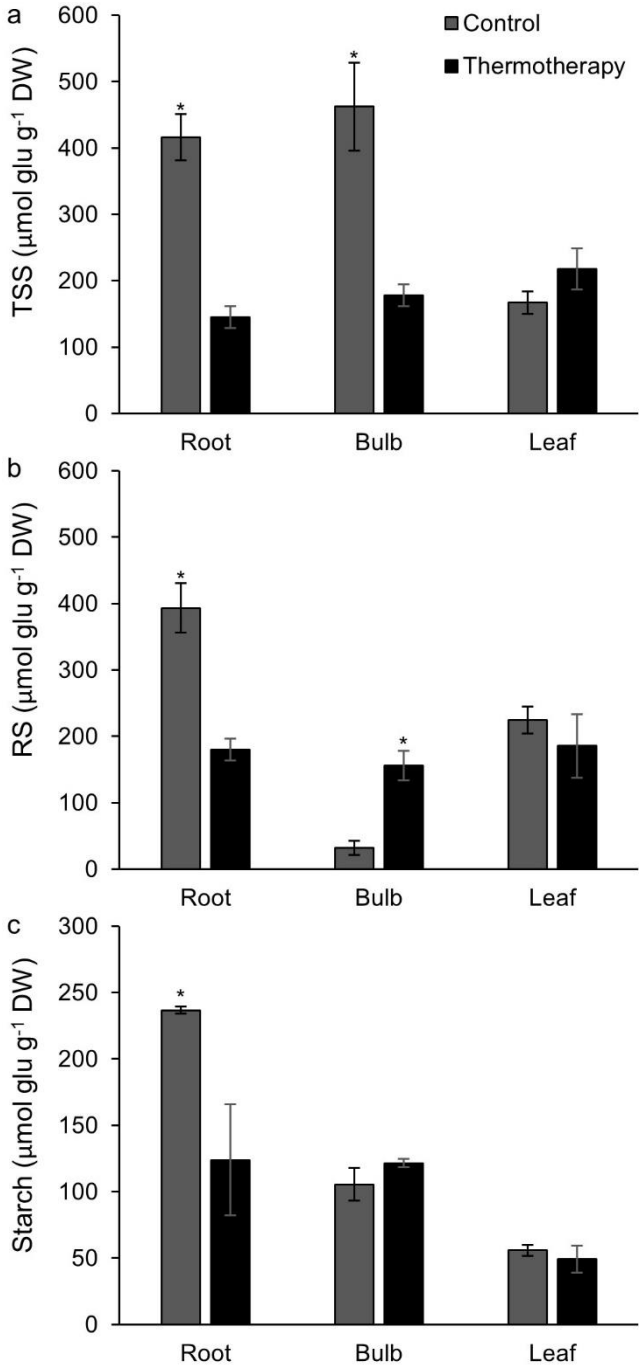

Fig. 6. Carbohydrates concentrations in control and thermotherapy treated plants after 30 days in a greenhouse; TSS: total soluble sugars (a); RS: reducing sugars (b); starch (c); bars indicate the standard error; *significant difference according to the Tukey test ($p<0.05$)

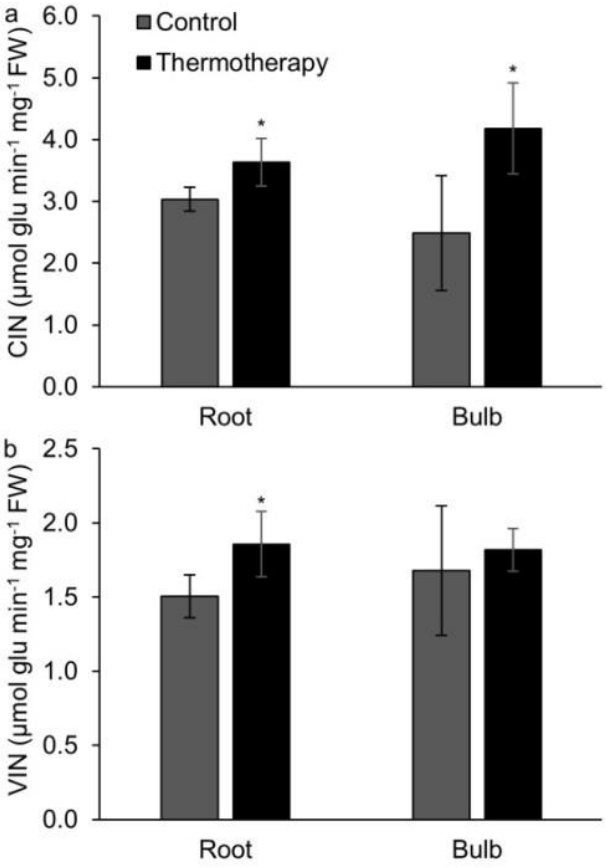

Fig. 7. Invertase isoforms activity after 10 weeks of cultivation; neutral cytosolic (a) and acid vacuolar (b); bars indicate the standard error; *significant difference according to the Tukey test (p<0.05)

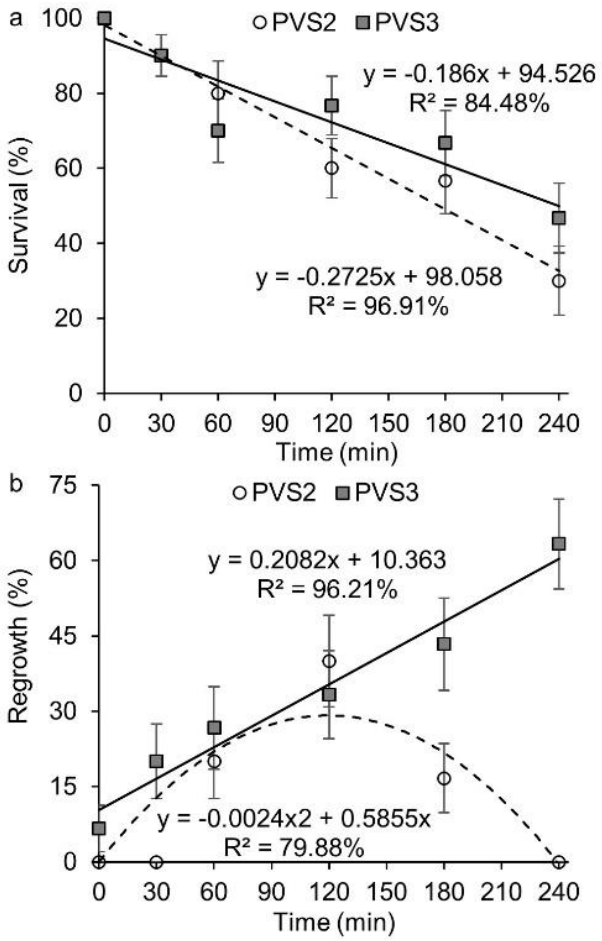

Fig. 8. Survival percentage prior to liquid nitrogen immersion (a) and encapsulated shoot regrowth percentage exposed to different time in PVS2 and PVS3 solutions (0, 30, 60, 120, 180 e 240 minutes) after immersion in liquid nitrogen (b) at 30 days of *in vitro* culture; parameters estimated with $p<0.05$

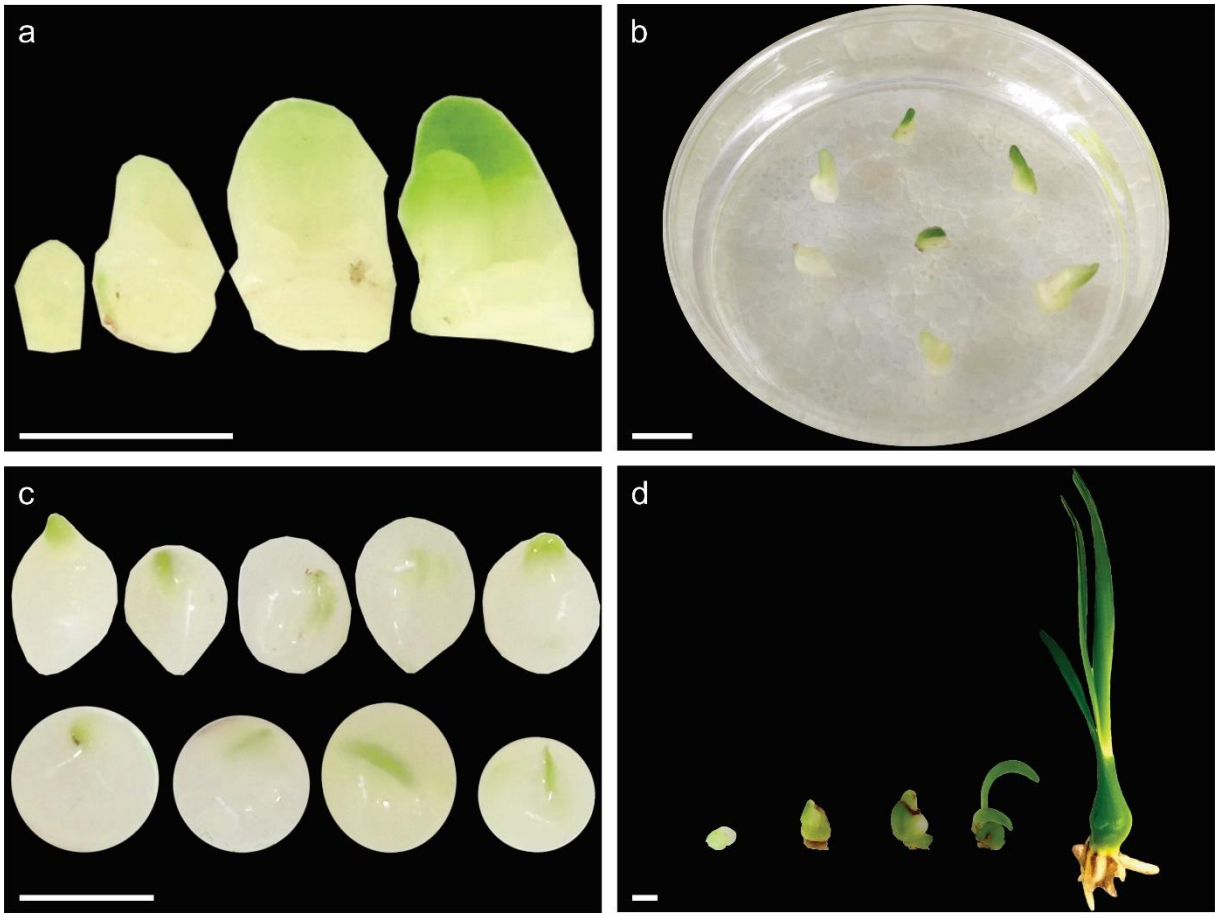

Fig. 9. Hippeastrum shoots isolated from multiplication explants (a); shoots in pre-culture medium (b); hippeastrum beads after cryopreservation (c); shoots regeneration stages of hippeastrum beads after 1, 2, 3, 4, e 8 weeks (d); scale bar = 0.5 cm

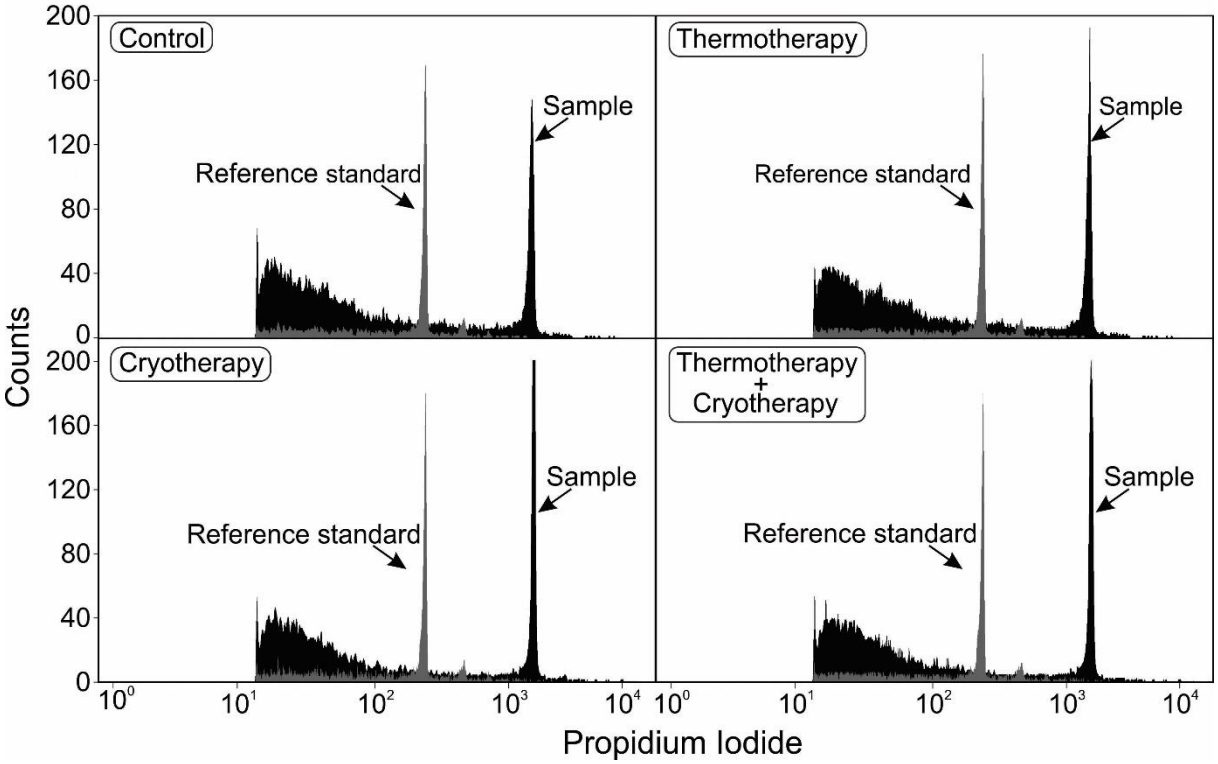

Fig. 10. DNA content profiles as determined by flow cytometry in the control and after treatments

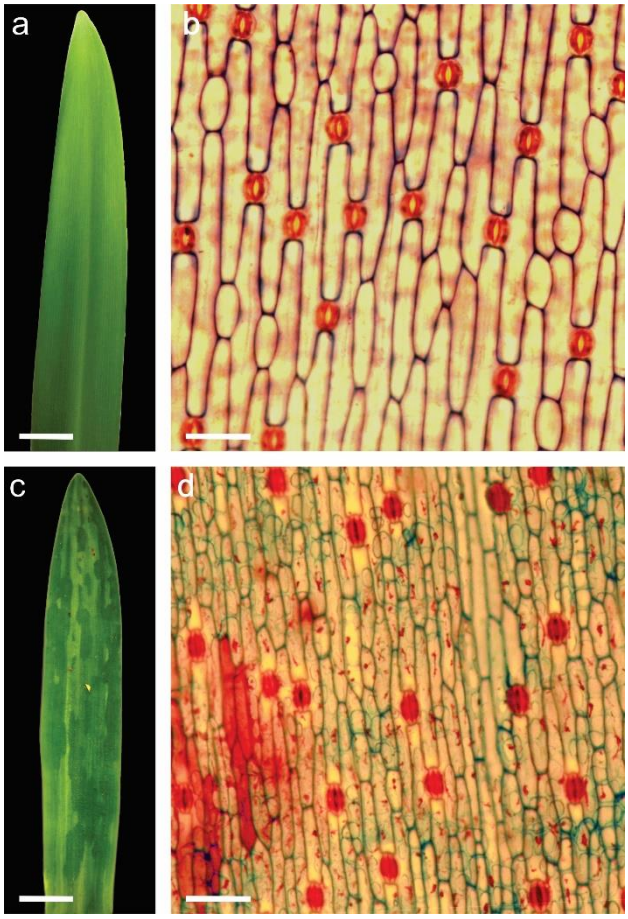

Fig. 11. Asymptomatic infected leaf (a) and respective paradermal section without apparent cytological alterations (b); symptomatic chlorotic mosaic leaf (c), and respective paradermal section showing apparent cytological alterations (d); acetic carmine staining; scale bars = 1 cm (a, c), 100 µm (b, d)

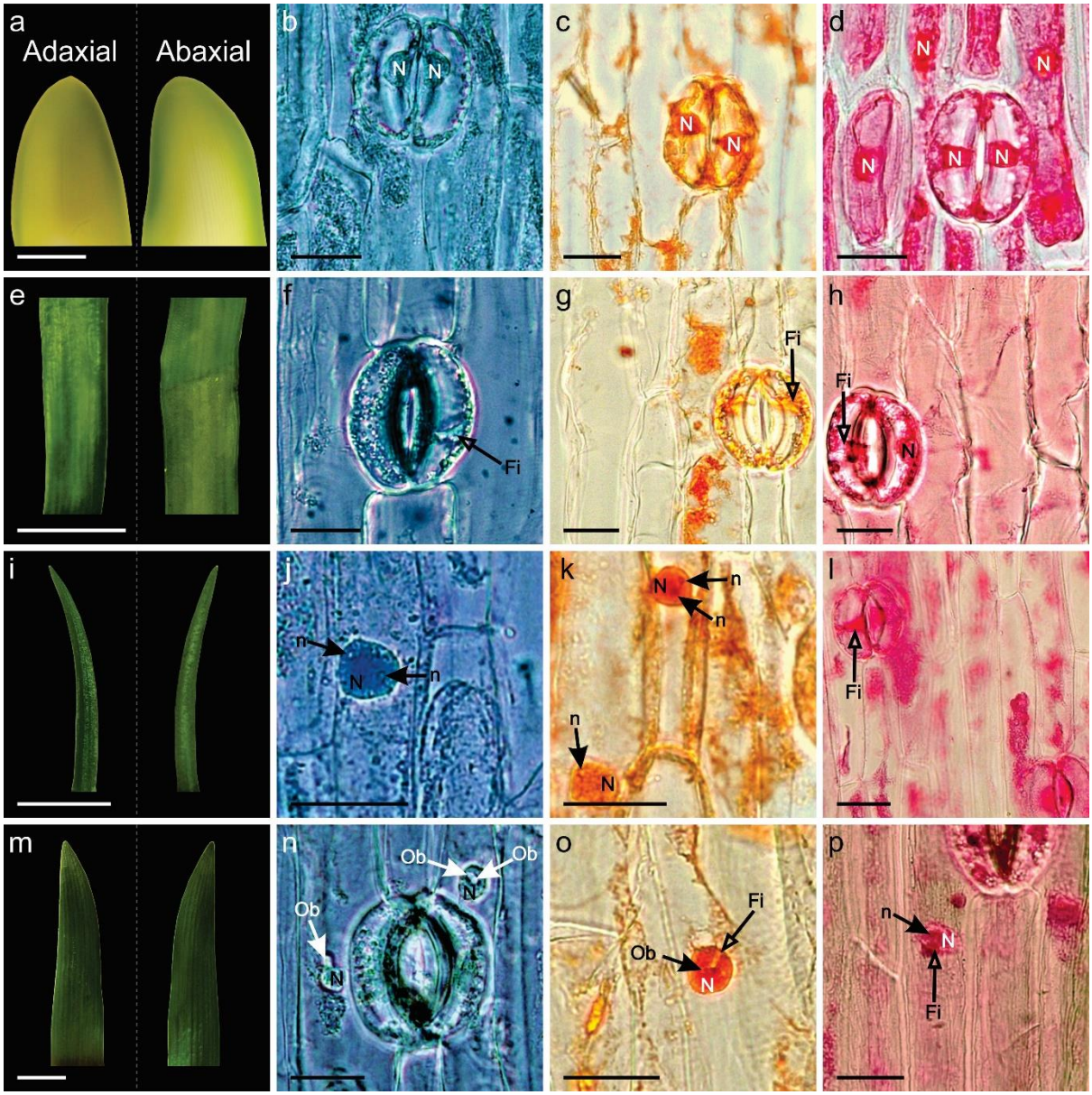

Fig. 12 Adaxial and abaxial surface of bulb young leaf in the beginning of regrowth (a); paradermal sections showing integral nucleus and absence of viral inclusions (b, c, d); adaxial and abaxial surface of *in vitro* regenerated shoot leaf (e); paradermal sections showing fibrillary inclusion in stomatal cell (f, g, h); adaxial and abaxial surface of *in vitro* regenerated shoot leaf after cryotherapy in growth stage 2 (i); paradermal sections showing nucleolus inside nucleus (j, k); paradermal sections showing fibrillary inclusion in stomatal cell cytoplasm (l); adaxial and abaxial surface of *in vitro* regenerated shoot leaf after cryotherapy in growth stage 3 (m); paradermal sections showing opaque bodies inside nucleus (n, o) and fibrillary inclusion (o, p); N: nucleus; n: nucleolus; Ob: opaque bodies; Fi: fibrillary inclusion. Paradermal inclusions were stained with Azure A (b, f, j, n), LCA (c, g, k, o), and Floxin (d, h, l, p); scale bars: white = 1 cm e black = 25µm

Annexes

Table S1. DNA content estimated by flow cytometry for hippeastrum (*Hippeastrum hybridum*) cv. 'Apple Blossom' under different treatments

Treatment	DNA (pg)	SD
Control	55.27 a	1.99
Thermotherapy	55.44 a	1.94
Cryotherapy	55.87 a	1.58
Thermotherapy + Cryotherapy	55.83 a	1.81

*Mean followed by the same letter did not differ among each other, accordingly Tukey test at 5%

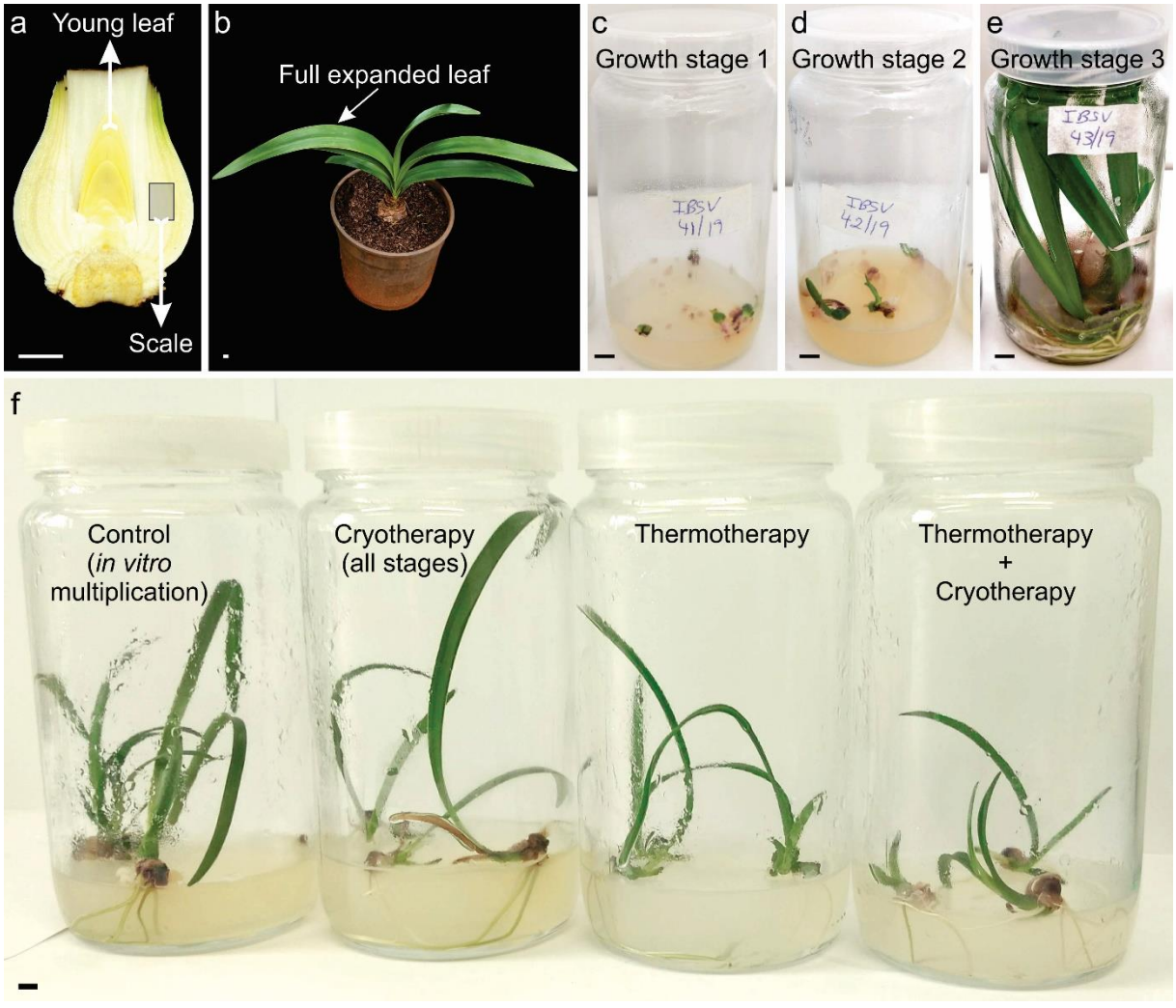

Fig. S1. Control bulb indicating young leaf and scale (arrow) led to analysis (a); control plant indicating completely expanded leaf (b); cryopreserved shoot in growth stage 1 (c); cryopreserved shoot in growth stage 2 (d); cryopreserved shoot in growth stage 3 (e); samples of further treatments analyzed as indicated (f); scale bar = 1 cm

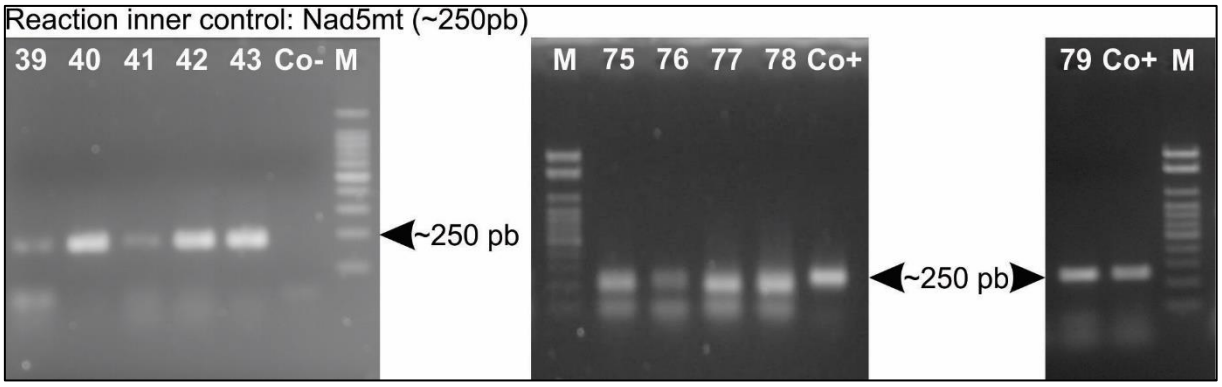

Fig. S2. Electrophoretic analysis, in agarose gel 1.5%, of PCR products for Nad5mt detection (reaction inner control). Bulb young leaf (39); *in vitro* cultivated shoot (40); cryotherapy stage 1 (41); cryotherapy stage 2 (42); cryotherapy stage 3 (43); scale (75); thermotherapy (76); cryotherapy all stages (77); thermotherapy + cryotherapy (78); completely expanded leaf (79); negative control (Co-); positive control (Co+). Marker (M) - DNA Ladder 100pb e 1 Kb (Norgen)

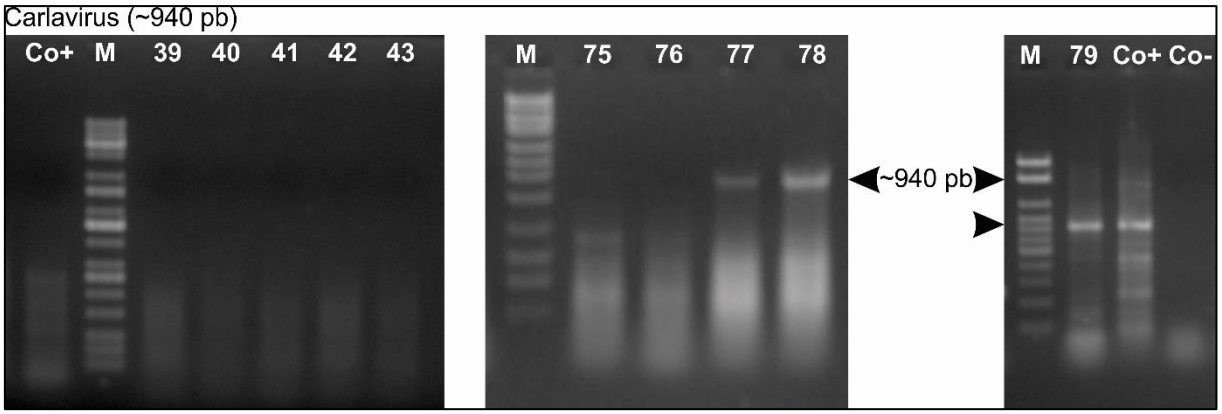

Fig. S3. Electrophoretic analysis, in agarose gel 1.5%, of PCR products for *Carlavirus* (*Nerine latent virus*) detection. Bulb young leaf (39); *in vitro* cultivated shoot (40); cryotherapy stage 1 (41); cryotherapy stage 2 (42); cryotherapy stage 3 (43); scale (75); thermotherapy (76); cryotherapy all stages (77); thermotherapy + cryotherapy (78); completely expanded leaf (79); negative control (Co-); positive control (Co+). Marker (M) - DNA Ladder 100pb e 1 Kb (Norgen)

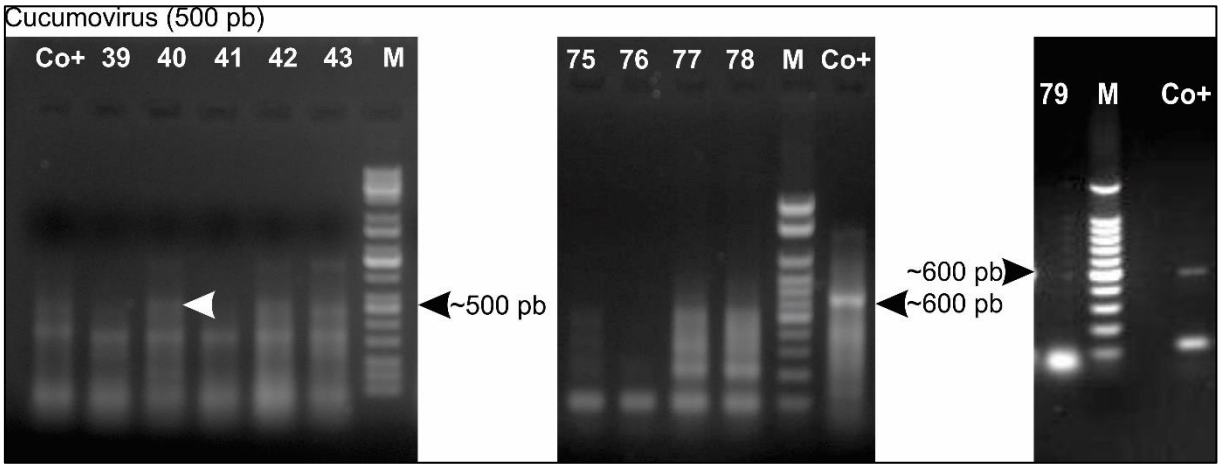

Fig. S4. Electrophoretic analysis, in agarose gel 1.5%, of PCR products for CMV (*Cucumber mosaic virus*) detection. Bulb young leaf (39); *in vitro* cultivated shoot (40); cryotherapy stage 1 (41); cryotherapy stage 2 (42); cryotherapy stage 3 (43); scale (75); thermotherapy (76); cryotherapy all stages (77); thermotherapy + cryotherapy (78); completely expanded leaf (79); positive control (Co+). Marker (M) - DNA Ladder 100pb e 1 Kb (Norgen)

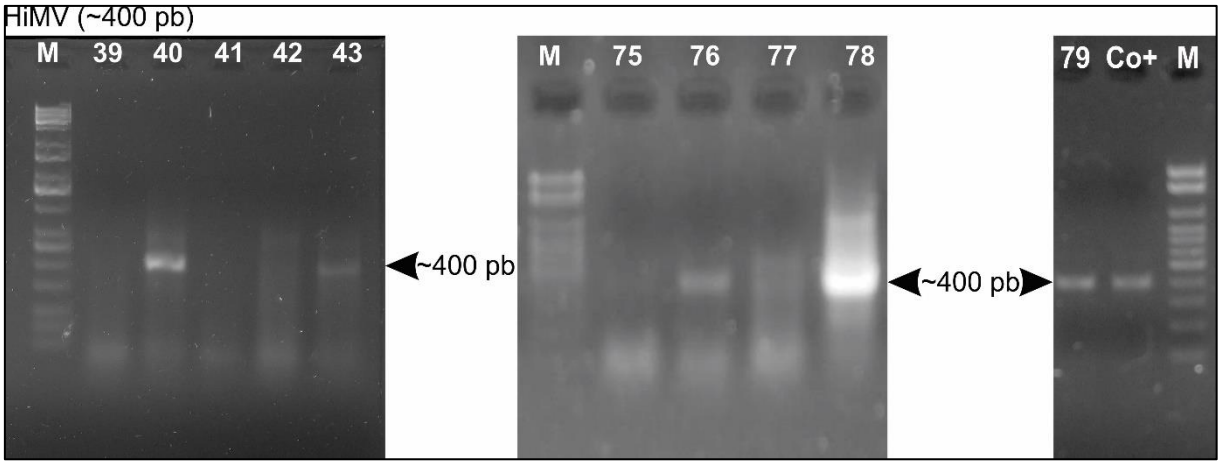

Fig. S5. Electrophoretic analysis, in agarose gel 1.5%, of PCR products for HiMV (*Hippeastrum mosaic virus*) detection. Bulb young leaf (39); *in vitro* cultivated shoot (40); cryotherapy stage 1 (41); cryotherapy stage 2 (42); cryotherapy stage 3 (43); scale (75); thermotherapy (76); cryotherapy all stages (77); thermotherapy + cryotherapy (78); completely expanded leaf (79); positive control (Co+). Marker (M) - DNA Ladder 100pb e 1 Kb (Norgen)

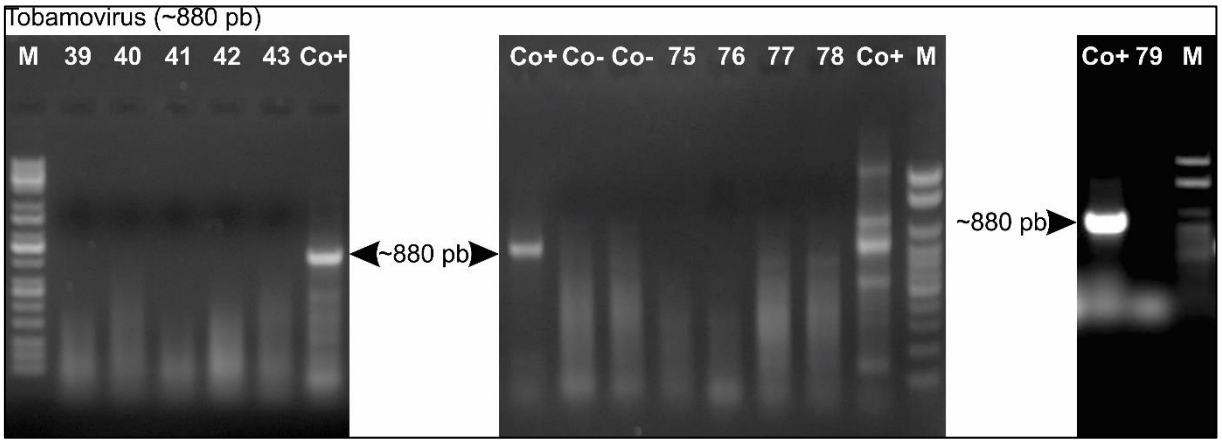

Fig. S6. Electrophoretic analysis, in agarose gel 1.5%, of PCR products for *Tobamovirus* (*Tobacco mosaic virus*) detection. Bulb young leaf (39); *in vitro* cultivated shoot (40); cryotherapy stage 1 (41); cryotherapy stage 2 (42); cryotherapy stage 3 (43); scale (75); thermotherapy (76); cryotherapy all stages (77); thermotherapy + cryotherapy (78); completely expanded leaf (79); negative control (Co-); positive control (Co+). Marker (M) - DNA Ladder 100pb e 1 Kb (Norgen)

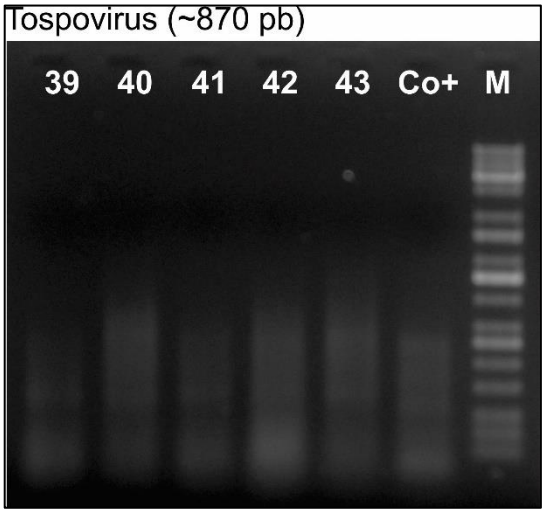

Fig. S7. Electrophoretic analysis, in agarose gel 1.5%, of PCR products for *Tospovirus* (*Tomato spotted wilt virus*, *Impatiens necrotic spot virus* and *Hippeastrum chlorotic ringspot virus*) detection. Bulb young leaf (39); *in vitro* cultivated shoot (40); cryotherapy stage 1 (41); cryotherapy stage 2 (42); cryotherapy stage 3 (43); positive control (Co+). Marker (M) - DNA Ladder 100pb e 1 Kb (Norgen)

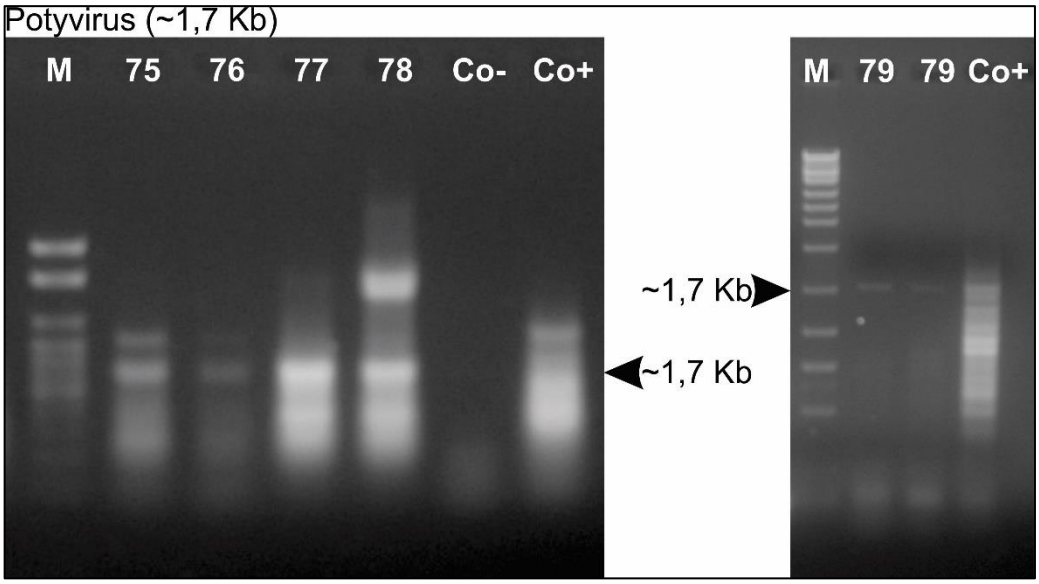

Fig. S8. Electrophoretic analysis, in agarose gel 1.5%, of PCR products for *Potyvirus* (*Hippeastrum mosaic virus* and *Sunflower mosaic virus*) detection. scale (75); thermotherapy (76); cryotherapy all stages (77); thermotherapy + cryotherapy (78); completely expanded leaf (79); negative control (Co-); positive control (Co+). Marker (M) - DNA Ladder 100pb e 1 Kb (Norgen)

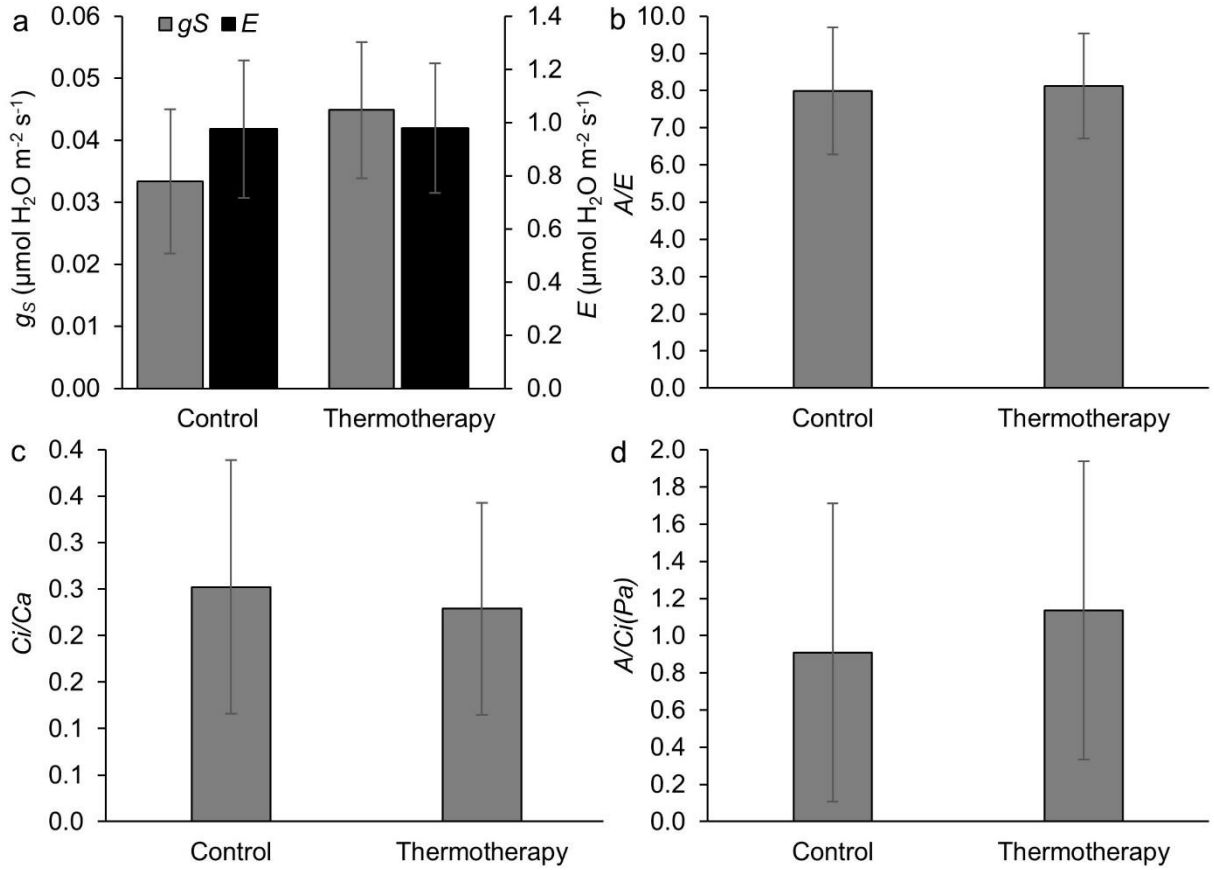

Fig. S9. Gas exchange responses of control and thermotherapy treated plants after 30 days in a greenhouse; g_s: stomatal conductance and *E*: transpiration (a); *A/E*: instant water use efficiency (b); *Ci/Ca*: inner carbon by atmospheric carbon ratio (c); *A/Ci(Pa)*: carboxylation instant efficiency (d); bars indicate the standard error

CONCLUSIONS

In conclusion, this thesis highlighted the economic importance of the ornamental plants market. The ornamental plants business is responsible for employing thousands of people and generates, annually, a billionaire turnover all over the world. As a worth bulbous ornamental species, *Hippeastrum hybridum* needs a continuous improvement of its commercial production to achieve the growing demand. In particular, the 'Apple blossom' cultivar had poor *in vitro* multiplication rate due to the lack of standard and explant oxidation. Hence, a highly productive protocol for quick propagation was developed by the means of explant size standardization, ascorbic acid, activated charcoal, and red and blue LED lamp use.

Besides, this work demonstrated that thermotherapy affected the hippeastrum bulbs physiology, causing severe negative effects on growth and development due to the photosynthetic pigments and photosynthesis reduction. Moreover, the encapsulation-vitrification with PVS3 showed to be the more efficient procedure for this species cryopreservation. This protocol, when applied to cryotherapy, ensured genetic stability, as well as thermotherapy and the combination of both. However, both thermotherapy and cryotherapy, or their combination, decreased viral contamination, but did not eliminated the viruses in hippeastrum. Nevertheless, the finding reported are crucial to support new studies that surely will result in hippeastrum virus-free plants.