INVESTIGATING CRYOTHERAPY AS A NOVEL APPROACH TO VIRUS ELIMINATION IN ORCHIDS

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ABSTRACT

High quality orchids are important for Hawaii’s floriculture industry and a barrier to their production is viral infection, primarily from *Cymbidium mosaic virus* (CymMV) and *Odontoglossum ringspot virus* (ORSV). Prevention and elimination techniques, including virus screening, thermotherapy, and chemotherapy, exist as solutions. Each approach offers varying levels of success; it is easier to eliminate CymMV than ORSV due to differential movement in orchids. Cryotherapy is a new approach to virus elimination in crops and ornamentals, using liquid nitrogen submergence of explants to burst virus-infected cells while preserving undifferentiated cells with cryo solutions. Vitrification and Droplet-vitrification methods were assessed to determine explant regeneration success. From this a modified Droplet-vitrification protocol was created for cryotherapy studies involving *Dendrobium* and *Miltassia* (CymMV-infected) and *Oncidium* (ORSV-infected). ORSV was not eliminated in the two regenerated explants following the protocol presented here. CymMV-infected explants failed to regenerate and could not be assessed for virus elimination.
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CHAPTER 1

ORCHID VIRUS AND CRYOPRESERVATION LITERATURE REVIEW

Introduction

Orchids are widely associated with Hawaii, bringing to mind images of hula dancers draped in lei performing on the beach. These unique and diverse plants are not only an integral component of Hawaii’s tourism but have long been a prominent floriculture product for the state. Hawaii’s fascination with orchids as cultivated plants can be traced back to the late 1800s when a *Dendrobium* hybrid arrived from the Philippines (Kamemoto et al., 1999). From this point it was only a matter of time until an orchid craze spread throughout the islands resulting in breeding and cultivation of various new *Dendrobium* cultivars amongst professionals and hobbyists alike.

In an effort to better understand the breeding process and increase the number of developed cultivars, the University of Hawai‘i initiated a *Dendrobium* breeding program in 1950 led by Dr. Haruyuki Kamemoto (Kamemoto et al., 1999). Kamemoto explored the genetics and heritability characteristics in orchids, beginning with studies of cytogenetics and chromosomes to better understand how to develop a successful breeding program. After two decades of research on this subject, *Dendrobium* breeding began (Kamemoto et al., 1999). This breeding program has yielded numerous varieties now used by growers around the world for cut flower and potted plant production, including the popular UH amphidiploid varieties. This program continues today under the leadership of Dr. Teresita Amore.

Throughout the years Hawaii has continued to expand its floriculture market with regard to orchids. Today numerous genera and hybrids can be found in orchid nurseries throughout the islands and include *Dendrobium, Phalaenopsis, Oncidium, Vanda, Miltassia, Paphiopedilum* and many others. Despite the increased diversity of commercially available orchids, the Hawaiian floriculture industry has experienced a steady decline over the last 5 years.

Hawaii’s floriculture industry boasted record sales in 2007 at $108.8 million, $21.5 million of which was attributed to orchid sales, and accounted for 21% of the total state
floriculture products (USDA, NASS, 2012). Since then there has been a steady decrease in the state’s orchid revenues, with drops in both cut flowers and potted plants. Floriculture sales in 2012 totaled $69.1 million, down 36.4% from the 2007 statistic (USDA, NASS, 2013). Orchid sales in 2012 also dropped to $14.2 million and accounted for 21% of the state’s total floriculture products (USDA, NASS, 2013). In this same period, Dendrobiums, a long time staple of the Hawaiian orchid market, saw a slight increase in cut flower sales but decreases in potted plants and individual flowers, as well as an overall decline in the acreage dedicated for Dendrobium production (USDA, NASS, 2013).

The decline in the Hawaiian orchid industry may be attributed to competition from international and mainland orchid producers. International orchid growers have lower labor costs and Thai laboratories now dominate the orchid clonal propagation service, causing most Hawaii labs to close, being unable to compete (Kim and Sewake, 2013; R. Tokunaga, 2013, personal communication). Orchid growers on the mainland, particularly in California and Florida, have out competed Hawaii in orchid sales in recent years and will continue to be strong competitors in the future (Kim and Sewake, 2013). Among California and Florida’s competitive advantages are cheaper transportation costs for shipments to mainland retailers. To have a competitive edge, Hawaii orchid growers must produce a variety of high quality potted plant and cut flower orchids. While a diverse assemblage of orchids can be relatively easy to achieve, consistent production of high quality plants is another matter. Despite the effort and care growers put into their plants many are plagued by a silent threat, orchid viral infections.

_Cymbidium mosaic virus _and _Odontoglossum ringspot virus_

_Virus History and Description_  

Viral infection poses one of the largest threats to orchid health and marketability. There are over 30 different viruses known to infect orchids worldwide, and prevalent among them is _Cymbidium mosaic virus_ (CymMV), with _Odontoglossum ringspot virus_ (ORSV) a close second
Cymbidium mosaic virus was first described by Jensen and Gold in 1951. Prior to this time viral symptoms had been reported for a number of different orchid genera including Dendrobium, Cattleya, and Cymbidium but no virus particles had been documented. Using healthy and symptom-expressing leaves of Cymbidium seedlings, Gold and Jensen (1951) purified the samples for electron microscopy (EM) study. From their EM images it was determined that samples from the symptom-expressing Cymbidium leaves contained “thin, sinuous rods,” while the rod particles were absent in symptomless leaves (Gold and Jensen, 1951).

In 1951 Odontoglossum ringspot virus was also first described by Jensen and Gold. An Odontoglossum grande Lindl. orchid was investigated to understand what caused the symptoms of necrotic spots and reduced vigor in the plant (Jensen and Gold, 1951). Upon study with EM, Jensen and Gold (1951) described “rod-shaped particles, slightly sickle shaped” within the Odontoglossum leaf extracts. Further study revealed that the virus symptoms were not always present in infected plants and that when expressed, the most common symptoms were necrotic rings or spots and chlorotic leaves with the first few months of symptoms expression being the worst (Jensen and Gold, 1951).

The taxonomic classification of CymMV places it within the order Tymovirales, family Alphaflexiviridae, and the genus Potexvirus (ICTV, 2013). The Potexvirus genus is characterized based on the physical appearance, genomic structure, and life cycle of the viral species within it. All viruses of this genus are of the filamentous particle shape, as initially described by Gold and Jensen (1951) (Figure 1). There particles are 470-1000 nanometers (nm) long and 12-13 nm in diameter and consist of genomic RNA (ribonucleic acid) surrounded by coat proteins (ViralZone, 2008). ORSV is considered a member of the Tobacco mosaic virus group, being in the genus Tobamovirus and family Virgaviridae (ICTV, 2013; ViralZone, 2008). This genus, Tobamovirus, is characterized by rigid rods 18 nm in diameter and 300-310 nm long (ViralZone, 2008).
CymMV is a single-stranded positive sense RNA (ssRNA+) consisting of a monopartite genome (ViralZone, 2008) (Figure 1). The largest open reading frame (ORF) codes for the RNA-dependent RNA polymerase (RdRp) which is further used to transcribe the viral RNA during replication (Vaughan et al., 2008; ViralZone, 2008; Wong et al., 1997). The remaining four genes (TGB1-3 and Coat Protein) are thought to be expressed through subgenomic RNA and are utilized for replication and movement of virus particles within a host (Vaughan et al., 2008; ViralZone, 2008; Wong et al., 1997). Sequencing of different CymMV isolates has shown that there is minimal diversity amongst this species, indicating that CymMV isolates are quite similar to one another (Moles et al., 2007). The work by Moles et al. (2007) also indicated that there may be two centers of origin for CymMV, despite each original isolate having high sequence similarity to the other. *Cymbidium mosaic virus* replicates in the cytoplasm of the host cell. Once the viral RNA has entered the cell, the RdRp is coded and used to further translate the CymMV genome. The TGBs also assist with replication in organelles known as viral factories within the host cell. The coat protein (CP) and movement protein (MP), are subgenomic RNA transcribed by internal promoters of the CymMV genome. Once replication is complete and the proteins have been translated, these components are packaged into virions for further transmission both within the plant and to other plants (ViralZone, 2008). Like CymMV, ORSV is a monopartite ssRNA(+) genome (Figure 2) with a small replicase subunit that codes for the RdRp and three ORFs labeled 2-4 (ViralZone, 2008). Of these ORFs only the third and fourth are known to code for the movement protein and coat protein respectively (ViralZone, 2008). Replication of ORSV within a cell is similar to that of CymMV.

**Viral Infection**

**Symptoms**

The presence of CymMV in orchids can affect both the external characteristics of the orchid as well as its overall growth (Figure 3). External damages have been widely described to include brown streaks and spots on the petals and lips of flowers, black spotting, necrotic veins, mosaic pattern of leaves, sunken leaf patches, distorted flowers, and coloring breaking in flowers (Hu et al., 1993; Jensen, 1952; Kamemoto et al., 1999; Leonhardt and Sewake, 1999; Vaughan et
ORSV results in similar symptoms among which are color breaking, necrotic rings on leaves, and flower distortion (Hu et al., 1994). In cases of severe CymMV infection, symptoms can be readily observed in new orchid shoots with the early expression of yellowing and mosaic leaves (G. Yamada, 2013, personal communication). Despite the potential for noticeable virus symptoms, CymMV and ORSV do not always result in symptom expression in orchids and therefore should not be the sole factor in determining viral infection (Zettler et al., 1990).

Overall Plant Vigor

CymMV infection has been shown to affect an orchid’s overall health. Wannakrairoy (2007) showed that ‘Uniwi Pearl’ Dendrobium orchids infected with CymMV had a 9% reduction in flower sprays and an 18% reduction in flower yield. These reductions were deduced from averaging the flower yields and number of sprays produced by virus-free and virus-infected Dendrobium plants and represent the potential reductions for virused plants. CymMV has been shown to disrupt the crassulacean acid metabolism (CAM) photosynthetic pathway of both commercial and wild orchids (laboratory inoculation of wild orchids). Izaguirre-Mayoral et al. (1993) inoculated both wild and hybrid orchids with a strain of CymMV to analyze the viral impact on the CAM pathway. They found that CAM production of carbohydrates occurred two hours earlier in the infected wild orchids when compared to controls, and CAM was inhibited in the hybrid varieties, demonstrating that CymMV infection may reduce or halt an orchid’s ability to produce carbohydrates for survival. Similar effects on energy production of orchids was found with Oncidium orchids. Chia and He (1999) evaluated Oncidium growth and photosynthetic capacity when orchids were infected and after virus eradication from these orchids. They determined that virus-eradicated Oncidium had a 21% increase in photosynthetic capacity as well as an increase in plant height and flower size of 17% and 65% respectively (Chia and He, 1999).
Transmission

*Cymbidium mosaic* and *Odontoglossum ringspot viruses* have been shown to transmit primarily through mechanical processes. Most commonly, mechanical transmission occurs during propagation, harvesting of flower sprays, and pruning with tools (Hu et al., 1994; Kamemoto et al., 1999; Leonhardt and Sewake, 1999). Often orchid growers move from plant to plant harvesting sprays without disinfecting their tools, causing rapid transmission of the virus. Once an orchid has been inoculated or infected with CymMV, the virus spreads systemically through the phloem and results in a plant-wide infection within 30 days (Hu et al., 1994). For ORSV, the virus spreads from cell to cell and may take 7 months for a systemic infection to occur (Hu et al., 1994). In 1996 Porter et al. showed that CymMV could not be transmitted through seeds even when both parents were infected. There are no documented insect vectors for CymMV transmission, though the potential for these vectors exists. In 2010, Allen published findings that suggest the Australian Cockroach (*Periplaneta australasiae*) may spread CymMV through feeding damage. Cockroaches were placed in enclosures with one infected orchid and one healthy orchid and left for some time. As the cockroaches fed on both orchids, trace amounts of CymMV were found in the initially healthy orchid, indicating the potential for these insects to spread the infection (Allen, 2010).

Detection

Infection by CymMV can be detected through a variety of biological, serological, and molecular assays. When the virus is at a high concentration and/or well established within the orchid, symptom expression can indicate infection. In cases of very severe infection these symptoms are readily seen in new growth (Jensen, 1951; G. Yamada, 2013, personal communication). While the previously described symptoms are recognizable and indicate infection, they do not indicate a specific infection. Other orchid viruses and fungi cause similar symptoms such that visual detection is not always reliable. Symptoms of CymMV are not always expressed; for example white and pink flowers are more prone to symptom expression than are colored flowers (J.S. Hu, 2013, personal communication). Indicator plants are another option for biological detection of a viral infection. *Cassia occidentalis* used for CymMV
detection, is mechanically inoculated with tissue extracts from orchids suspected to be infected and the subsequent development of unique symptoms indicates infection (Vaughan et al., 2008). For the biological assay of ORSV infection it is recommended to use *Gomphrena* (Wisler, 1989).

A simple and easy method of CymMV and ORSV detection is through the use of lateral flow assays such as ImmunoStrips® (Agdia, Elkhart, IN). This assay is quick and can be completed in either the laboratory or field (Batchman, 2008). Using a lateral flow assay is straightforward and consists of a buffer filled pouch for grinding the leaf sample and a test strip which is inserted for 15-20 minutes to detect virus particles. These strips are capable of detecting both CymMV and ORSV. This assay allows for faster detection of virus without the use of complex laboratory equipment (Batchman, 2008). Due to its simplistic nature, this assay is best applied to orchids with a high concentration of the virus as trace amount of viral particles cannot be detected.

Another detection method is ELISA, or enzyme linked immunosorbent assay (Clark and Adams, 1977). This assay can readily be purchased as a kit (i.e. Agdia, Elkhart, IN). Testing for CymMV involves the indirect double-antibody sandwich (DAS-ELISA) approach as described by Grisoni et al. (2004). An array of CymMV antibodies which bind the CymMV virus particles present in a leaf sample were tested by Grisoni et al. (2004). Those antibodies with the highest binding were selected for use and can be detected by a color change which is quantitatively defined through absorbance readings. DAS-ELISA allows for processing multiple samples at once, though consecutive days are required to complete the assay.

An accurate method of detecting CymMV and ORSV at any concentration is through the use of one of many forms of the Polymerase Chain Reaction (PCR), such as Reverse Transcription PCR, Immunocapture PCR, and others. PCR involves the amplification of a piece of, or entire, DNA (deoxyribonucleic acid) fragment. As these are ssRNA(+) viruses it is necessary to first convert the RNA genome into a complementary DNA (cDNA) form for further amplification through PCR. Amplified cDNA of CymMV or ORSV after PCR allows for a more accurate detection of infection and provides opportunities to closely examine the genetic makeup of the virus (Barry et al., 1996; Vaughan et al., 2008; Wong, 2010).
Methods of Control and Elimination

In order to achieve the production and maintenance of healthy orchids, growers must overcome one of the largest threats to orchid health and marketability, viral infection. There are a variety of preventative measures and virus elimination protocols that can be utilized for this purpose. These methods of virus prevention and elimination have been successfully applied to numerous orchid species for the control of both CymMV and ORSV. It is important to note that these methods are not restricted to orchids and their viruses, these methods have been applied to other ornamental and crop plants with success (Milosevic et al., 2012; Wang et al., 2009).

Seed Propagation

One method of obtaining virus free plant material is through seed propagation, a readily accepted option as CymMV is not transmitted through seeds. Porter et al. (1996) crossed two parent orchids infected with CymMV and grew the resulting seeds. Out of 7,000 seedlings produced and sampled, none tested positive for viral infection. In 2006, Khentry et al. conducted a survey of CymMV and ORSV in Thailand and found that no seedlings were positive for either virus. To date no study has found CymMV to be seed transmitted. While this is useful information, seedling populations are variable and have limited market potential except for the UH amphidiploid crosses. Growers often prefer clonal material to produce large numbers of a selected plant with desirable qualities.

Tool Cleaning

Current efforts to control the spread of CymMV and ORSV rely on preventative sanitation methods. Due to the mechanical transmission of the viruses, following proper sanitation practices when handling orchids is important. It is highly recommended to clean tools when moving from plant to plant during pruning, flower spray harvesting, etc. Tool cleaning to remove virus particles can be accomplished through dipping the tools in solutions of skim milk, 70-90% ethanol, or a 1-5% bleach solution (Hu et al., 1994). Chang et al. (2010) have proposed a new method of disinfecting tools using a liquid culture filtrate of a Streptomyces isolate. This
Streptomyces isolate has the capability to degrade proteins and inactivate a virus (Chang et al., 2010). Derived from muddy soils in Taiwan, Streptomyces was used to degrade CymMV coat proteins present on tools, labeling tape, human hands, and Phalaenopsis orchid leaves (Chang et al., 2010). ORSV could be successfully eliminated from tools in this manner as well. This isolate provides a less toxic approach for tool decontamination as opposed to using bleach solutions. In tissue culture laboratories flaming of tools is a means of decontamination of bacteria, fungi, and virus.

Screening of Plant Material

Since Gold and Jensen’s (1951) report of virus particles in infected plants, prevention has been widely acknowledged in helping to stop the spread of virus. Another preventative measure is to test orchids prior to clonal propagation and only use plants that test negative for CymMV/ORSV infection (Jensen, 1952; Khentry et al., 2006; Loi et al., 1990). In this way only virus-free plants are utilized which allows growers to start with clean stock, thus being better able to implement strong sanitation practices. This practice is commonly employed by orchid growers on Oahu like Mr. Stams Wu of Lake View Orchids, who routinely tests his orchids for both CymMV and ORSV infections before sending plant material out for propagation (S. Wu, 2013, personal communication). Another Oahu grower, Mr. Gavin Yamada, burns any plant materials that are infected or that he suspects to be infected in order to cut down on the spread of virus in his nursery (G. Yamada, 2013, personal communication).

Meristem Culture

Many treatments for eliminating CymMV from orchid tissues are also available and are well reviewed by Milosevic et al. (2012). One approach for virus elimination is through meristem or shoot tip culture, where only the top most cells of the apical meristem are taken from orchids to serve as propagation material (Zettler et al., 1990). These small, undifferentiated cells have a lower chance of being infected and are thus more likely to serve as virus-free starting material (J.S. Hu, 2013, personal communication; Milosevic et al., 2012). As tissue
culture propagation has become more widely used, this precision is less often practiced, meaning larger shoot tips are being used and these larger pieces are more likely to be infected as there are also differentiated cells present (Zettler et al., 1990). Ishii (1974) looked at meristem culture as a means of virus elimination of *Laeliocattelya* infected with both CymMV and ORSV. This work determined that culture of meristems less than 0.5 millimeters long could result in the elimination of CymMV but not ORSV. In the end, Ishii (1974) concluded that meristem culture alone was not a feasible means of virus elimination and should be investigated for use in conjunction with other virus elimination techniques.

**Thermotherapy**

Some have recommended heat or thermotherapy to treat infections of CymMV in orchids. With this method, plants are grown at higher than normal temperatures; temperatures that disrupt the synthesis of viral RNA (Milosevic et al., 2012). However a 1990 study by Loi et al., where infected *Oncidium* were subjected to heat treatments, determined that while heat could eliminate some of the CymMV particles, traces of the virus could still be found in the plants. Often thermotherapy is used in conjunction with other methods of virus elimination, such as chemotherapy, to increase successful production of virus free materials (MiSeon et al., 1997).

**Chemotherapy**

Chemotherapy has shown considerable success and involves the addition of antivirals to the growing medium of plants to target the virus as the plant develops (Milosevic et al., 2012). Ribavirin and Dithiouracil are most commonly used to treat viral infections, not only in plants but also in humans (Crotty et al., 2002; Dawson and Lozoya-Saldana, 1984). In addition to treating CymMV, Ribavirin has also been used effectively against *Tobacco mosaic virus*, *Tomato-white necrosis virus*, and *Cucumber mosaic virus* (Dawson and Lozoya-Saldana, 1984). During the late 1980s and 1990s many studies were conducted utilizing Ribavirin to effectively eliminate CymMV and ORSV in *Dendrobium*, *Mokara*, and *Cymbidium* orchids (Albouy et al., 1988; Lim et al., 1993; Loi et al., 1991; Toussaint et al., 1993). Albouy et al. (1988) concluded...
that numerous consecutive subcultures onto Ribavirin medium are necessary to increase the number of virus-free protocorms generated, but that increasing the exposure to the virucide increases phytotoxic effects. The work of Lim et al. (1993) compared the use of Ribavirin in thin section culture to that of meristem culture for the production of virus-free Mokara orchids. Through the different treatments it was found that the addition of Ribavirin into the growing medium was successful in eliminating both CymMV and ORSV but that it came at the cost of low tissue survival rates. In 1997 Porter and Kuehnle compared the virucides Ribavirin and Dithiouracil in eliminating CymMV from Dendrobium. They found that both virucides were effective at similar dosages for virus elimination, and that the best results were obtained when virucides were added to both the liquid and solid growing media.

Transgenics

Attempts have been made to create transgenic orchids that are resistant to CymMV. Borth et al. (2006) used three CymMV sequences: coat protein, movement protein, and mutated movement protein to transform Nicotiana benthamiana and Dendrobiums. They found varying levels of resistance in N. benthamiana plants but no resistance was documented in the Dendrobiums. In 2004 Liao et al. were able to successfully transform Phalaenopsis orchids with the coat protein gene of CymMV through particle bombardment. Through this work it was discovered that there existed enhanced RNA-mediated resistance of Phalaenopsis (Liao et al., 2004). Chang et al. (2005) had similar success using the CymMV coat protein gene in the transformation of Dendrobium protocorms via particle bombardment. These Dendrobium protocorms experienced lower levels of virus accumulation four months after inoculations and had milder CymMV symptom expression (Chang et al., 2005).

Cryotherapy

A more recent approach to virus elimination in plants has been through the use of cryotherapy, or the submersion of plant materials in liquid nitrogen. The principle of this
method is simple, use freezing temperatures to kill plant cells infected with virus. However, the mechanisms behind this principle are still under investigation. It is hypothesized that the cells most often killed through liquid nitrogen submersion are those that are differentiated with large vacuoles (Wang and Valkonen, 2009; Wang et al., 2009). These cells are also where the virus is most likely to reside, having needed distinct organelles to move into a new cell and replicate (Feng et al., 2013; Hu et al., 1994; Wang and Valkonen, 2009). The large vacuole size of these differentiated cells leads to these cells having a higher water content which in turn makes them more likely to freeze during submersion in liquid nitrogen (Wang and Valkonen, 2009). An important attribute of cryotherapy for virus elimination is that the frequency of virus elimination and size of plant material (with regard to shoot tips), are independent of one another (Feng et al., 2013). This differs considerably from meristem culture for virus elimination were the highest frequency of elimination is achieved by using the smallest shoot tips (Feng et al., 2013). Further support for this mode of action comes from work with Argyranthemum. Zhang et al. (2014) subjected Argyranthemum shoot tips infected with Chrysanthemum stunt viroid (CSVd) to cryotherapy by droplet-vitrification. Of the 50% of regenerated shoot tips, all were still infected with CSVd; further analysis showed that CSVd can invade meristematic cells, which must survive cryo treatments for plant regeneration (Zhang et al., 2014).

Cryotherapy in Crops and Ornamentals

A few important crops and ornamentals have undergone cryotherapy with successful tissue regeneration and virus elimination as seen in Table 1. One of the first uses of cryotherapy comes from the work of Brison et al. (1997). Their work utilized Prunus shoot tips excised from plants infected with Plum pox virus. These shoot tips were cryopreserved using controlled rate cooling protocols. Upon thawing and regeneration Brison et al. (1997) found 50% survival and successful eradication of the virus in Prunus shoot tips. While it was expected that some shoot tips would not survive the treatment, these losses were thought to be exacerbated by the use of very small shoot tips. While small shoot tip sizes (around 0.5 millimeters) are necessary for meristem culture of virus-free plants, cryopreservation allows for the use of larger explants (Brison et al., 1997). Larger explants increase the chance of survival during cryotherapy.
Despite this low survival rate, virus free explants of *Prunus* were obtained, demonstrating the potential of cryotherapy for virus elimination.

In 2002, Helliot et al. used cryopreservation as a means of eliminating virus in banana plants. Vitrification methods of cryotherapy led to 30% elimination of *Cucumber mosaic virus* and 90% elimination of *Banana streak virus*. These are promising results especially when compared to the spontaneous elimination through meristem culture alone that yielded 0% *Cucumber mosaic virus* free cultures and 52% *Banana streak virus* free cultures (Helliot et al., 2002). Throughout this work shoot tips 1.5-3 mm in length were used.

Wang et al. (2003) developed methods for the elimination of *Grapevine virus A* (GVA) in *Vitis vinifera* L. Their methods explored both encapsulation-dehydration and vitrification techniques, the results being 97% elimination of virus in regenerated tissues. This experiment used shoot tips of approximately 1 mm in size and did not observe the same regeneration problems as did Brison et al. (1997). Higher rates of regeneration could be attributed to using a different set of cryopreservation methods. Further exploration of regenerated tissues under the microscope revealed that large cells (with large vacuoles) burst after submersion in liquid nitrogen while the younger, denser cells survived (Wang et al., 2003). This provides support that the mode of action for virus elimination during cryotherapy is through the destruction of larger, differentiated cells where the virus is present.

In 2008 Ding et al. attempted to eliminate *Candidatus Liberobacter asiaticus*, one of the citrus huanglongbing (HLB)-associated pathogens, from citrus shoot tips. Using the vitrification method, 80.1% of adult citrus shoot tips were successfully regenerated and of these 96.3% were free of the bacteria. This work further supported the cryotherapy mode of action hypothesis as the differentiated cells, with large vacuoles where the pathogen is likely to reside, were destroyed during freezing, prompting the regeneration of undifferentiated meristematic cells free of HLB-associated bacteria (Ding et al., 2008).

Elimination of virus in sweet potato was undertaken by Wang and Valkonen in 2008b. Shoot tips of sweet potato infected with *Sweet potato chlorotic stunt virus* and *Sweet potato feathery mottle virus* were subjected to cryotherapy by encapsulation-vitrification. For both viruses, 100% virus elimination was achieved (Wang and Valkonen, 2008b). Large shoot tips
(1-1.5 mm) had the best regeneration of 87% following submersion in liquid nitrogen. Wang and Valkonen (2008b) discovered that longer exposure to the plant vitrification solution assisted in the recovery of tissues with the best regenerate in treatments of 120 minutes exposure to the solution. This same year Wang and Valkonen (2008a) were also able to eliminate a bacterial disease in sweet potato shoot tips. Sweet potato infected with little leaf phytoplasma were subjected to encapsulation-vitrification with 85% of shoot tips regenerating and 100% of those shoot tips with eliminated phytoplasma (Wang and Valkonen, 2008a).

*Raspberry bushy dwarf virus* was successfully eliminated from *Prunus idaeus* (Wang et al., 2008). Here shoot tips were first subjected to thermotherapy for a few weeks before being cryopreserved following the encapsulation-vitrification method (Wang et al., 2008). Following the combined treatments, Wang et al. (2008) found 30-40% of shoot tips were able to successfully regenerate and of these, 35% were virus-free.

In 2014, Yi et al. published their findings on the elimination of *Potato virus Y* (PVY) and *Potato leaf roll virus* (PLRV) from potato shoot tips. These *in vitro*-grown shoot tips were subjected to the droplet-vitrification and encapsulation-vitrification methods of cryotherapy. Regeneration of shoot tips ranged from 65% in the droplet-vitrification method to 70% in encapsulation-vitrification, with both methods yielding over 90% elimination of PVY and PLRV (Yi et al., 2014). This same year saw the elimination of a virus complex from garlic (Vieira et al., 2014). Using garlic shoot tips, Vieira et al. (2014) was able to successfully eliminate *Onion yellow dwarf virus*, *Leek yellow strip virus*, and *Garlic common latent virus* with 87.5%, 93.8% and 62.5% elimination respectively. This work utilized the vitrification method and overall 64% regeneration of shoot tips was achieved (Vieira et al., 2014).

While most of the cryotherapy work has been geared toward crop plants, an ornamental has also been investigated. Gallard et al. (2011) investigated the use of cryotherapy for virus elimination in *Pelargonium*. Using shoot tips infected with *Pelargonium flower break virus* (PFBV) and *Pelargonium line pattern virus* (PLPV), droplet-vitrification procedures were applied. Of the three varieties to regenerate following cryotherapy, 18-44% had elimination of PFBV and 50% were free of PLPV (Gallard et al., 2011).
Shin et al. (2013) were able to eliminate *Yam mosaic virus* from infected shoot tips of *Dioscorea opposita*. Utilizing encapsulation-dehydration 40% of shoot tips regenerated and of those 90% were virus-free 3 months post-cryotherapy. From these virus-free shoot tips Shin et al. (2013) were able to propagate additional virus-free yams.

Not all attempts at cryotherapy are successful. Zhang et al. (2014) were unable to eliminate viroids from *Argyranthemum* following droplet-vitrification despite 50% regeneration of shoot tips. In addition to this, Nukari et al. (2014) could not eliminate *Apple mosaic virus* (ApMV) from hops. Encapsulation-dehydration of hop shoot tips yielded low regeneration (2%) and no elimination of ApMV (Nukari et al., 2014).

**Cryopreservation of Orchids**

No published research has been found to indicate that cryotherapy has been successfully applied to the elimination of virus in orchids. Although no cryotherapy has been documented, cryopreservation techniques have been published for numerous orchids. Cryopreservation is a highly investigated means of germplasm long term storage due to its minimal space and maintenance requirements (Teixeira da Silva et al., 2014; Wang et al., 2009). Protocols use orchid materials ranging from shoot tips to protocorms (germinated orchid seeds) to protocorm-like bodies or plbs (clonal propagation output in liquid culture). Small explant material is important as shoot tips and plbs have a higher percentage of undifferentiated cells, meaning smaller vacuoles and lower overall water content (Normah et al., 2013).

**Cryopreservation**

At its most basic premise, cryopreservation serves as an option for the long term storage of plant material whereby the material is subjected to extreme freezing temperatures (-196°C) to arrest cellular functions. Bukhov et al. (2006) evaluated the functions of photosystems I and II in *Bratonia* orchids prior to and following cryopreservation in liquid nitrogen. Their work found
that immediately after thawing of plant materials, the photosystems are inhibited through an interruption of the electron transport pathways. This provides evidence of the arrest of cellular functions as well as the need to optimize protocols to promote the highest levels of survival following cryopreservation. Upon thawing, and given adequate regeneration time, these plant systems will function normally and normal plant growth will resume.

In order to successfully survive cryopreservation, plant materials are prepared for liquid nitrogen submersion with pretreatment, osmoprotective, and vitrification solutions (Benson, 2008). Vitrification is a crucial component of cryopreservation and refers to the phase transition from a liquid to an amorphous glass without crystallization; allowing the plant cells to freeze instantly without damage to their cellular components (Benson, 2008; Volk and Walters, 2006). Recent work with Acer embryos found that after exposure to liquid nitrogen, fully hydrated cells were full of ice crystals while those cells subjected to dehydration with vitrification solutions had minimal, if any, ice crystal formation (Wesley-Smith et al., 2013). Based on their Acer studies, Wesley-Smith et al. (2013) suggest that dehydrated cells not only have less water available to form crystals, but that dehydration also promotes faster cooling rates which allows for a faster transition of liquids to a glassy state. With vitrification approaches plant materials are sufficiently dehydrated, often with highly viscous solutions, prior to rapid freezing so that the increased viscosity within the cells inhibits water molecules from crystallizing (Gonzalez-Arnao et al., 2008; Volk and Walters, 2006). In this way the plant material can be thawed and resume normal growth after a period of recovery.

As mentioned above, different media solutions are crucial to the success of cryopreservation. These solutions, or cryoprotectants, serve to simulate the natural freezing tolerance of many cold climate plants (Fujikawa and Jitsuyama, 2000). Natural freezing tolerance is obtained through the storage of soluble carbohydrates to offset cellular water; cryoprotectants function similarly by displacing water and adding a level of antifreeze protection to cells (Fujikawa and Jitsuyama, 2000). The first of these, a pretreatment solution, consists of a basic growth media with an enhanced sucrose content. The particular growth media is dependent on the plant material and/or researcher’s preference and varies from protocol to protocol and may be in either liquid or semi-solid form. By culturing the selected plant material on media with increased sucrose content, the plant material begins to displace its cellular water content as the
water is exchanged for sucrose in the media. Initial sucrose increases within cells also aids in the regrowth of plant materials after treatments, especially when used with tropical species (Sakai and Engelmann, 2007; Sakai et al., 2008). The solution next applied to the plant material is an osmoprotectant, glycerol combined with a liquid form of the growth media. Glycerol functions as an ‘antifreeze agent,’ due to its stabilizing properties of allowing more water to be displaced from cells without significantly decreasing the overall cell volume (Benson, 2008; Meryman and Williams, 1985; Normah et al., 2013; Sakai and Engelmann, 2007; Sakai et al., 2008).

Osmoprotective solutions use a combination of 2M glycerol and 0.4M sucrose (Sakai and Engelmann, 2007).

After soaking in the osmoprotective solution, the plant material is subjected to a Plant Vitrification Solution (PVS). This vitrification solution is considered the ‘dehydration’ step for preparing plant material for liquid nitrogen submersion (Normah et al., 2013; Sakai et al., 2008; Volk and Walters, 2006). Volk and Walters (2006) studied shoot tips of garlic and mint subjected to PVS2 and found that not only did cellular water decrease, but the internal liquid of cells did not undergo ice crystal formation; instead the internal liquid vitrified immediately to a glassy state. Different variations of PVS have been created but the most widely used is PVS2, a combination of 30% glycerol, 15% ethylene glycol, 15% dimethylsulfoxide (DMSO), and 0.4M sucrose (Benson, 2008; Normah et al., 2013; Sakai and Engelmann, 2007; Sakai et al., 2008). PVS2 is first loaded to dehydrate the plant material, and is then replaced with a fresh solution of PVS2 in which the plant material remains during exposure to liquid nitrogen. The glycerol and DMSO ingredients of PVS2 both serve to dehydrate the plant material though they permeate the cells differently; glycerol moves slowly into plant cells while the permeation of DMSO is far faster (Kartha and Engelmann, 1994). Although PVS plays a key role in preparing and protecting the plant material, it is potentially toxic and thus the duration and temperature of exposure must be monitored to decrease toxicity effects (Benson, 2008; Gonzalez-Arnao et al., 2008; V. Pence, 2014, personal communications; Sakai and Engelmann, 2007).

After PVS and subsequent submersion in liquid nitrogen, plant materials are treated with a rinsing solution. The rinsing solution is often 0.8-1.2M sucrose (in a liquid growth media) and aids in the rehydration of cells (Normah et al., 2013; Sakai et al., 2008). With optimized
exposure times and concentrations for these cryoprotectant solutions, plant materials have an increased chance of survival and regeneration following liquid nitrogen submersion.

Orchid Cryopreservation

Different approaches to the cryopreservation of orchids have been successful as seen in Table 2, and include vitrification, encapsulation-dehydration, droplet-vitrification, and encapsulation-vitrification (Gonzalez-Armao et al., 2009; Mohanty et al., 2013; Poobathy et al., 2013a; Popova et al., 2010; Teixeira da Silva et al., 2014; Yin and Hong, 2009; Yin et al., 2011). These approaches are based upon the vitrification technique with modifications to improve cellular survival and recovery. Orchid materials used for cryopreservation include seeds, protocorms, protocorm-like bodies, seedlings, and protoplasts (Teixeira da Silva et al., 2014).

Vitrification

Vitrification is a method of cryopreservation whereby plant materials are treated with cryoprotectant solutions prior to rapid freezing in liquid nitrogen (Bhojwani and Dantu, 2013; Normah et al., 2013; Reed, 2008). This method was successfully applied to the orchid *Bratonia* by Popova et al. (2010) where protocorm-like bodies (plbs) were soaked in an osmoprotective loading solution, transferred to PVS2, sucked up into a straw and then immediately submerged in liquid nitrogen. Regeneration of plbs after cryopreservation was possible, though not all tissues regenerated. Popova et al. (2010) reported that incomplete regeneration of all tissues may be due in part to the toxicity of the vitrification solution, especially when plbs were submersed for too long a duration. Their research also concluded that the addition of growth regulators resulted in stable regrowth for plbs, but were not necessary for regeneration.

Poobathy et al. (2013a) obtained similar results using this technique to cryopreserve *Dendrobium* Sonia-28 and reported that there is a lag phase during the regeneration of plbs subjected to submersion in liquid nitrogen compared with plbs that undergo all steps of cryopreservation except for submersion. Johari et al. (2009) employed vitrification to the cryopreservation of *Brassia* shoot tips ranging from 0.5-1.5 centimeters in length. They
determined that increased sucrose concentrations (compared to sucrose concentrations of normal growth media) enhanced viability of shoot tips so long as the concentration was not in excess of 0.5M sucrose and that longer shoot tips provided the best survival (Johari et al., 2009).

Encapsulation-dehydration

Cryopreservation by encapsulation-dehydration refers to the method of enclosing small plant material in ‘beads’ and then drying these ‘beads’ to dehydrate the cells (Reed, 2008). This allows for water to be removed from the cells to lower the chances of freezing while protecting the plant material from direct contact with liquid nitrogen. Yin et al. (2011) first encapsulated *Brassidium* plbs by coating them with sodium alginate and then placing them into calcium chloride where the coating hardens into a ‘bead’ surrounding the plb. These ‘beads’ are then placed into an osmoprotective solution, surface dried, and then placed in a laminar flow cabinet until a water content of 19.9% is achieved. From here the ‘beads’ can be placed in cryovials and immersed in liquid nitrogen (Yin et al., 2011). From this technique Yin et al. (2011) determined that larger plbs (3-4 mm diameter) had the highest regeneration rates but that regeneration was still possible for smaller plbs.

Similar work applied this technique to protocorms of *Cleisostoma*, a rare Thai orchid (Maneerattanarungroj et al., 2007). This work saw a 49% survival rate of protocorms and has established this protocol as a reliable means of cryopreserving *Cleisostoma*. Lurswijidjarus and Thammasiri (2004) used encapsulation-dehydration to preserve shoot tips of *Dendrobium* Walter Oumae. They evaluated the survival of shoot tips 1-3 mm in length and found that the 0.3M sucrose pretreatment provided the highest recovery of plant materials after exposure to liquid nitrogen (Lurswijidjarus and Thammasiri, 2004). These works with *Cleisostoma* and *Dendrobium* Walter Oumae used protocols similar to those described for *Brassidium* plb cryopreservation by encapsulation-dehydration.
Encapsulation-vitrification

Encapsulation-vitrification cryopreservation is quite similar to encapsulation-dehydration except that cryoprotectants are used in place of a dehydration step. Rather than lowering the moisture content of cells through laminar air flow chambers or silica beads, the cryoprotectants displace water in the cells to keep cell volumes relatively intact while removing water that may freeze (Reed, 2008). Once again, keeping the plant material enclosed in ‘beads’ minimizes the toxic effects due to direct contact with the vitrification solution (Normah et al., 2013).

*Dendrobiums* have been successfully cryopreserved and regenerated through the encapsulation-vitrification method. Here plbs undergo the same encapsulation procedures detailed above, are transferred to an osmoprotective loading solution (some first have a culture phase while in ‘bead’ form), and then are placed directly into a PVS (Mohanty et al., 2013; Yin and Hong, 2009). Soaking in PVS occurs for different time durations before immersion in liquid nitrogen. After immersion, ‘beads’ are placed on solid media for regrowth and after a time the plbs are extracted to continue growth and proliferation.

Droplet-vitrification

One of the newest forms of cryopreservation is that of droplet-vitrification. In this method plant materials undergo the normal procedures outlined in the above methods including pretreatment, osmoprotective solution and PVS2. Once the PVS2 soaking time is completed, the plant material is placed within droplets of PVS2 on thin pieces of aluminum foil which are then plunged directly into liquid nitrogen (Normah et al., 2013). The aluminum foil, once submerged in liquid nitrogen, serves as a conductor to pull heat out of the plant material which results in the vitrification of solutions as there is not enough energy (produced through heat) to allow ice crystals to form (Benson et al., 2006). Gonzalez-Arnao et al. (2009) used droplet-vitrification to cryopreserve shoot tips of *Vanilla planifolia* and found 30% survival and 10% regeneration rates for these explants.
Cryopreservation Similarities

All researchers involved in orchid cryopreservation stress the importance of pre-culturing plant materials in the presence of higher sucrose concentrations and utilizing dark incubation following liquid nitrogen thawing. Pre-culturing with higher sucrose causes the cells to take up more sugar which reduces the water content of cells and reduces the chances of crystallization during freezing (Yin et al., 2011). Dark incubation enhances regrowth after liquid nitrogen as it is believed to give the tissues time to repair and decrease toxic effects from photo oxidation (Mohanty et al., 2013). Despite possible toxic effects during cryopreservation, no differences in plant material form or growth were reported between control samples and those that underwent cryopreservation.
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Wu, S. 2013. Personal communications.

Yamada, G. 2013. Personal communications.


Table 1. Crops and ornamental plants subjected to cryotherapy as a means of eliminating virus since 1997. In some cases viroids, phytoplasma, and bacteria were investigated in place of a virus.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Virus, viroid, phytoplasma or bacterium</th>
<th>Cryotherapy Method</th>
<th>Regeneration (%)</th>
<th>Virus Elimination (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Allium sativum</em></td>
<td>Onion yellow dwarf virus</td>
<td>V</td>
<td>64</td>
<td>87.5</td>
<td>Vieira et al. 2014</td>
</tr>
<tr>
<td></td>
<td>Leek yellow strip virus</td>
<td>V</td>
<td>64</td>
<td>93.8</td>
<td></td>
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<tr>
<td></td>
<td>Garlic common latent virus</td>
<td>V</td>
<td>64</td>
<td>62.5</td>
<td></td>
</tr>
<tr>
<td><em>Argyranthemum maderense</em></td>
<td>Chrysanthemum stunt viroid</td>
<td>DV</td>
<td>50</td>
<td>0</td>
<td>Zhang et al. 2014</td>
</tr>
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<td><em>Citrus</em></td>
<td>Candidatus Liberobacter asiaticus</td>
<td>V</td>
<td>80.1</td>
<td>96.3</td>
<td>Ding et al. 2008</td>
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<td><em>Dioscorea opposita</em></td>
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<td>ED</td>
<td>45</td>
<td>90</td>
<td>Shin et al. 2013</td>
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<td><em>Humulus lupulus</em></td>
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<td>ED</td>
<td>2</td>
<td>0</td>
<td>Nukari et al. 2014</td>
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<td><em>Ipomea batatas</em></td>
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<td>EV</td>
<td>87</td>
<td>100</td>
<td>Wang and Valkonen 2008b</td>
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<td></td>
<td>Sweet potato feathery mottle virus</td>
<td>EV</td>
<td>87</td>
<td>100</td>
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<td></td>
<td>Little leaf phytoplasma</td>
<td>EV</td>
<td>85</td>
<td>100</td>
<td>Wang and Valkonen 2008a</td>
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<td><em>Musa spp.</em></td>
<td>Banana streak virus</td>
<td>V</td>
<td>50</td>
<td>90</td>
<td>Helliot et al. 2002</td>
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<td></td>
<td>Cucumber mosaic virus</td>
<td>V</td>
<td>50</td>
<td>30</td>
<td></td>
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<td><em>Pelargonium cultivars</em></td>
<td>Pelargonium flower break virus</td>
<td>DV</td>
<td>-</td>
<td>18-44</td>
<td>Gallard et al. 2011</td>
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<td></td>
<td>Pelargonium line pattern virus</td>
<td>DV</td>
<td>-</td>
<td>50</td>
<td></td>
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<td><em>Prunus</em> rootstock</td>
<td>Plum pox virus</td>
<td>V</td>
<td>50</td>
<td>50</td>
<td>Brison et al. 1997</td>
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<td><em>Rubus idaeus</em></td>
<td>Raspberry bushy dwarf virus</td>
<td>EV (+thermotherapy)</td>
<td>30-40</td>
<td>35</td>
<td>Wang et al. 2008</td>
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<td>65.3</td>
<td>91.3</td>
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<td></td>
<td></td>
<td>EV</td>
<td>70.5</td>
<td>97.9</td>
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<td>Potato leaf roll virus</td>
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<td>98.4</td>
<td>Yi et al. 2014</td>
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<td>EV</td>
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<td>99.7</td>
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<td>ED</td>
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<td>97</td>
<td>Wang et al. 2003</td>
</tr>
<tr>
<td></td>
<td></td>
<td>V</td>
<td>50</td>
<td>97</td>
<td></td>
</tr>
</tbody>
</table>

DV, droplet-vitrification; EV, encapsulation-vitrification; ED, encapsulation-dehydration; V, vitrification
Table 2. Selection of cryopreservation methods applied to various orchid genera since 2002.

<table>
<thead>
<tr>
<th>Orchid</th>
<th>Material type</th>
<th>Cryopreservation Method</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aerides odorata</em></td>
<td>plb</td>
<td>ED</td>
<td>Hongthongkham and Bunnag 2014</td>
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<td><em>Ascocenda</em> Wangsa Gold</td>
<td>plb</td>
<td>V</td>
<td>Rajasegar et al. 2015</td>
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<td><em>Brassia rex</em></td>
<td>st</td>
<td>V</td>
<td>Johari et al. 2009</td>
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<td><em>Brassidium</em> Shooting Star</td>
<td>plb</td>
<td>ED</td>
<td>Yin et al. 2011</td>
</tr>
<tr>
<td></td>
<td></td>
<td>V</td>
<td>Mubbarakh et al. 2014</td>
</tr>
<tr>
<td><em>Bratonia</em> hybrid</td>
<td>plb</td>
<td>V</td>
<td>Popova, et al. 2010</td>
</tr>
<tr>
<td><em>Calanthe davidii</em></td>
<td>st</td>
<td>DV</td>
<td>Lin et al. 2014</td>
</tr>
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<td><em>Cymbidium</em> hybrid</td>
<td>plb</td>
<td>EV, ED</td>
<td>Teixeira da Silva 2013</td>
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<td><em>Dendrobium candidum</em></td>
<td>plb</td>
<td>V</td>
<td>Junhui et al. 1999</td>
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<td></td>
<td></td>
<td>De</td>
<td>Bian et al. 2002; Lin et al. 2004</td>
</tr>
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<td></td>
<td></td>
<td>EV</td>
<td>Yin and Hong 2009</td>
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<tr>
<td><em>Dendrobium chrysanthemum</em></td>
<td>plb</td>
<td>EV</td>
<td>Mohanty et al. 2013</td>
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<td><em>Dendrobium</em> Bobby Messina</td>
<td>plb</td>
<td>V, ED</td>
<td>Anthony et al. 2010; Zainuddin et al. 2011</td>
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<td><em>Dendrobium nobile</em></td>
<td>plb</td>
<td>ED, EV</td>
<td>Mohanty et al. 2012</td>
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<td>plb</td>
<td>ED</td>
<td>Subramaniam et al. 2011</td>
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<td>plb</td>
<td>V</td>
<td>Hwa et al. 2009; Poobathy et al. 2013c; Sin et al. 2010</td>
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<td></td>
<td></td>
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<td><em>Phalaenopsis bellina</em></td>
<td>plb</td>
<td>ED</td>
<td>Khoddamzaden et al. 2011</td>
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<td><em>Vanda coerule</em></td>
<td>plb</td>
<td>DV</td>
<td>Jitsupakul et al. 2011</td>
</tr>
<tr>
<td><em>Vanda Kaseem's Delight</em></td>
<td>plb</td>
<td>V</td>
<td>Poobathy et al. 2012</td>
</tr>
</tbody>
</table>

De, dehydration; DV, droplet-vitrification; ED, encapsulation-dehydration; EV, encapsulation-vitrification; plb, protocorm-like body; st, shoot tip; V, vitrification
Figure 1. Illustrative depiction of *Cymbidium mosaic virus* (ViralZone 2008). The upper image illustrates the particle shape while the lower image details the genome. RdRp, RNA-dependent RNA polymerase (transcribes viral RNA); TGB1-3, replication and movement of virus particles; CP, coat protein.

Figure 2. Illustrative depiction of *Odontoglossum ringspot virus* (ViralZone 2008). The upper image shows the particle shape while the lower image details the viral genome. RdRp, RNA-dependent RNA polymerase (transcribes viral RNA); ORF2-4, open reading frame; MP, movement protein (aids in movement of virus); CP, coat protein.
Figure 3. Comparison of healthy and symptom-expressing virused orchids. A) Healthy dendrobium flower  B) Black spotting on a symptom-expressing dendrobium flower  C) Healthy orchid leaves  D) Black and necrotic spots on a symptom-expressing orchid leaf.
CHAPTER 2
EXAMINATION OF THE VITRIFICATION AND DROPLET-VITRIFICATION CRYOPRESERVATION METHODS FOR ORCHID HYBRIDS

Abstract

Cryopreservation is an important means of germplasm storage allowing for plant tissues and seeds to be stored for an indefinite period of time with minimal space and maintenance requirements. The vitrification and droplet-vitrification methods of cryopreservation were assessed to determine the best approach for the preservation of Dendrobium and Miltassia hybrids. Five Dendrobium and Miltassia orchid hybrids were evaluated for regeneration of shoot tips and protocorm-like bodies (plbs) following cryopreservation by vitrification and/or droplet-vitrification. Dendrobium UH1427 shoot tips and Miltassia plbs were subjected to vitrification and were assessed for regeneration under 3, 5, and 7 day pretreatment durations at sucrose concentrations of 0.3, 0.4, and 0.5M for each pretreatment period. These same pretreatment parameters were also tested using droplet-vitrification for the same orchid hybrids. In addition, Dendrobium UH232 shoot tips, Dendrobium Spanish Eyes plbs, and Miltassia Phoenix Rising ‘White Light’ shoot tips were cryopreserved using droplet-vitrification with a 3 day, 0.3M sucrose pretreatment. Neither the Miltassia plbs nor the UH1427 shoot tips survived the vitrification treatment. The droplet-vitrification method resulted in the regeneration of UH1427 (6.7% and 3.3%), UH232 (7.5%), and ‘White Light’ (2.1%) shoot tips. A 3 day pretreatment with 0.3M sucrose yielded the highest regeneration with 2 shoot tips of UH1427 and 3 shoot tips each of UH232 and ‘White Light.’ Histological observations of Dendrobium UH1427 shoot tips and Miltassia plbs showed treated cells with burst interior cells, enlarged nuclei, and intact cells; damage was experienced when explants were exposed to cryoprotectant solutions and/or liquid nitrogen. The successful regeneration of three orchid hybrids demonstrates the potential of the droplet-vitrification method to be a useful tool for the long-term storage of orchid germplasm.
Introduction

Cryopreservation offers a means of long-term germplasm storage with minimal space and maintenance requirements (Teixeira da Silva et al., 2014; Wang et al., 2009). Protocols for long term storage in liquid nitrogen use a variety of orchid explants including seeds, protocorms (germinated orchid seeds), protocorm-like bodies or plbs (clonal material resembling germinated seeds), and shoot tips. Such orchid explants have been cryopreserved and regenerated with varying levels of success (Gonzalez-Arnao et al., 2009; Teixeira da Silva et al., 2014). Plbs of *Dendrobium candidum*, *D. chrysanthemum*, *D. nobile*, *D. Bobby Messina*, and *D. Sonia 17* were successfully regenerated following cryopreservation via vitrification, encapsulation-dehydration, and encapsulation-vitrification (Anthony et al., 2010; Ching et al., 2012; Hwa et al., 2009; Junhui et al., 1999; Mohanty et al. 2012,2013; Poobathy et al., 2013c; Pouzi et al., 2011; Sin et al., 2010; Subramaniam et al., 2011; Yin and Hong, 2009; Zainuddin et al., 2011). Poobathy et al. (2013b) utilized the droplet-vitrification method to regenerate *D. Sonia 28* plbs. Shoot tips of *D. Walter Oumae* were regenerated following encapsulation-dehydration (Lurswijidjarus and Thammasiri, 2004). A *Bratonia* hybrid (synonymous with *Miltassia*) was successfully cryopreserved utilizing plbs and the vitrification approach (Popova et al., 2010). Additional orchids of the Oncidiinae Alliance, from which *Miltassia* arises, have been cryopreserved and include *Brassia*, *Brassidium*, and *Oncidium* (Johari et al., 2009; Miao et al., 2005; Mubbarakh et al., 2014; Yin et al., 2011). Cryopreservation of Oncidiinae Alliance orchids has utilized the vitrification and encapsulation-dehydration approaches with both plbs and shoot tips. The use of small explant material is critical as these explants have large numbers of undifferentiated cells which translates to smaller vacuoles and lower overall water content (Normah et al., 2013).

As encapsulation-dehydration and encapsulation-vitrification have both been widely applied to *Dendrobium* and Oncidiinae Alliance orchids, and this study assessed the use of vitrification and droplet-vitrification as cryopreservation methods. This work utilized the plant vitrification solution 2 (PVS2) as described by Sakai et al. (1990). In the case of vitrification, plant material is suspended in PVS2 inside a cryovial which is then submerged in liquid nitrogen. Droplet-vitrification involves plant material being suspended in droplets of PVS2 on thin strips of aluminum foil which are then placed directly into liquid nitrogen. In addition to these
approaches, different pretreatment solutions and durations were evaluated to determine the approach with the highest regeneration rate. This pretreatment step, where explants are exposed to high sucrose concentrations, is important for the displacement of cellular water and regrowth following exposure to liquid nitrogen (Sakai and Engelmann, 2007; Sakai et al., 2008).

The objective of this study is to assess the regeneration of Dendrobium and Miltassia explants following cryopreservation by the vitrification and/or droplet-vitrification protocols. Finding a protocol that allows for high rates of successful regeneration will be necessary for applying cryopreservation as a long-term storage option for these plant materials.

Materials & Methods

Five Dendrobium and Miltassia orchids were selected for cryopreservation based on the availability of plant material. The Dendrobium orchids include two cultivars developed by the University of Hawaii, UH1427 and UH232, and the hybrid Spanish Eyes developed and registered by Dr. Bernie Cagauan of Kiilani Gifts and Gardens. Two Miltassia hybrids were acquired from growers on the Big Island of Hawaii. One Miltassia, an undisclosed hybrid, was received in plb form and retained as such. The second hybrid, received as in vitro plantlets, was a Miltassia Phoenix Rising ‘White Light.’

All cryopreservation work was conducted under sterile tissue culture room conditions. Tools, plant media, and cryo solutions were autoclaved prior to use and aseptic practices were implemented throughout. Flasked orchids were grown on either shelves or a rotary shaker. When shelving was utilized, petri dishes (100 millimeters x 15 mm) were placed 19-21 centimeters (cm) below grow lights and 250 milliliter (mL) flasks were 9 cm from the flask top to the bottom of the lights. Plants were grown under 24 hour light conditions produced by 40 watt Gro-Lights (Sylvania, Westfield, Indiana), with an average air temperature of 26.5°C, average illumination of 79 µmol m⁻² s⁻¹, and average humidity of 37%. Four shelves of 0.44 square meters each were utilized on two metal racks. When orchids were grown on the rotary shaker, 125 mL flasks were 18 cm from the overhead lights. Again the previously described
light, temperature, illumination, and humidity values were used for the shaker. Handling of orchid materials was done inside a modified laminar flow hood of wood and plexiglass construction with a Merv 13 Green Pleat filter (Filtration Group, Downers Grove, Illinois).

Steps of Vitrification Method for *Dendrobium* UH1427 and *Miltassia*

*Dendrobium* UH1427 shoot tips 2-4 millimeters in length were excised from *in vitro* plantlets and 1-4 mm plbs of a *Miltassia* hybrid were taken from *in vitro* cultures. All plant materials were cultured on VW media (Vacin and Went, 1949) modified with 15% coconut water and 2% sucrose for *Dendrobium* and 1% sucrose for *Miltassia* (pH 4.9 for both) (Sagawa and Kunisaki, 1984). *Dendrobium* orchids were grown on semi-solid media and the *Miltassia* in liquid media on a rotary shaker at 126 rpm. Excised shoot tips and plbs were placed on semi-solid VW media modified with 15% coconut water and sucrose concentrations of 0.3, 0.4, and 0.5M (pH 5.8 for all). From each step of this process (excision only, pretreatment, osmoprotective, PVS2) 5-15 shoot tips/plbs were kept as controls. For *Dendrobium* shoot tips 3 replicates of 10 shoot tips each were evaluated and for *Miltassia* 5 replicates of 10 plbs each were evaluated for the full treatment and exposure to liquid nitrogen.

Plant material remained on pretreatment media for 3, 5, or 7 days. After the pretreatment, excised material was transferred to 2 mL cryovials filled with 1.8 mL osmoprotective solution [VW + 15% coconut water + 0.4M sucrose + 0.4M glycerol, pH 5.8] and held for 20 minutes at room temperature. The osmoprotective solution was then removed and 1.8 mL PVS2 [VW + 15% coconut water + 0.4M sucrose + 30% glycerol + 15% ethylene glycol + 15% dimethyl sulfoxide (DMSO), pH 5.8] placed in the vial for 20 minutes at room temperature. The PVS2 solution was then replaced with 1.8 mL of fresh PVS2, cryovials were sealed and incubated in liquid nitrogen for 1 hour. Cryovials were removed and plunged into a 40°C water bath for 3 minutes. Once thawed, the PVS2 was removed and replaced with an unloading solution [VW +15% coconut water + 1.2M sucrose, pH 5.8] for 20 minutes. Orchid explants were then removed from the cryovials, blotted on sterile filter paper, plated on semi-solid recovery media [VW + 15% coconut water + 0.3M sucrose] and placed in the dark. After 24 hours the plant material was transferred to VW + 15% coconut water + 2% sucrose (pH 4.9)
and returned to the dark for 6 days. Plated material was then moved to filtered light conditions (two racks away from grow lights and under four layers of cheese cloth) for one week before returning to normal lighting conditions. Orchid explants were monitored for 5 months after being returned to normal lighting conditions to assess regeneration.

Steps of Droplet-vitrification Method for Dendrobium UH1427 and Miltassia

Dendrobium UH1427 shoot tips 2-4 mm in length were cut from in vitro plantlets and 1-4 mm plbs of a Miltassia hybrid were taken from in vitro cultures. All plant materials were cultured on VW media modified with 15% coconut water and 2% sucrose for Dendrobium and 1% sucrose for Miltassia (pH 4.9 for both). Dendrobium orchids were grown on semi-solid media and the Miltassia in liquid media on a rotary shaker at 126 rpm. Excised shoot tips of UH1427 and plbs of Miltassia were placed on semi-solid VW media modified with 15% coconut water and sucrose concentrations of 0.3, 0.4, and 0.5M (pH 5.8 for all). All media and solutions used for cryopreservation were adjusted to pH 5.8. From each step of this process (excision only, pretreatment, osmoprotective, PVS2) 5-15 shoot tips/plbs were kept as controls. For Dendrobium shoot tips 3 replicates of 10 shoot tips each were evaluated and for Miltassia 5 replicates of 10 plbs each were evaluated for regrowth following full treatment and submersion in liquid nitrogen.

Plant material remained on pretreatment media for 3, 5, or 7 days. After the pretreatment, explants were transferred to 2 mL cryovials filled with 1.8 mL osmoprotective solution [VW + 15% coconut water + 0.4M sucrose + 0.4M glycerol, pH 5.8] and held for 20 minutes at room temperature. The osmoprotective solution was then removed and 1.8 mL PVS2 [VW + 15% coconut water + 0.4M sucrose + 30% glycerol + 15% ethylene glycol + 15% dimethyl sulfoxide, pH 5.8] placed in the vial for 20 minutes at room temperature. Orchid explants were then transferred to aluminum foil strips (20 x 7 millimeters) with two 10-20 microliter drops of PVS2 per strip and one explant per drop. These foil strips were plunged into liquid nitrogen and held for 1 hour. At the end of the hour foil strips were removed and placed directly into an unloading solution [VW +15% coconut water + 1.2M sucrose, pH 5.8] for 40 minutes at room temperature. Plant materials were then removed from the cryovials, blotted on
sterile filter paper, plated on semi-solid recovery media [VW + 15% coconut water + 0.3M sucrose] and placed in the dark. After 24 hours the explants were transferred to VW + 15% coconut water + 2% sucrose (pH 4.9) and replaced in the dark for 6 days. Plated material was then moved to filtered light conditions (two racks away from grow lights and under four layers of cheese cloth) for one week before returning to normal lighting conditions. Orchid explants were monitored for 5 months after being returned to normal lighting conditions to assess regeneration.

**Microtome preparation for Dendrobium UH1427 shoot tips and Miltassia plbs**

To understand the cellular level impacts of the cryopreservation process on UH1427 shoot tips and *Miltassia* plbs, microtome slides were prepared and analyzed based on the protocol outlined by Ruzin (1999). Samples were taken from each treatment group (pretreatment (0.3, 0.4, and 0.5M; durations of 3, 5, and 7 days), osmoprotective, PVS2, full treatment + freezing) for each method (vitrification and droplet-vitrification) and for both *Miltassia* and UH1427 samples. All orchid explant samples were placed in vials containing a killing solution [95% EtOH + glacial acetic acid + 40% formaldehyde] for 24 hours at a volume of 10x the volume of samples. Explants were then subjected to a dehydration series of alcohol for one hour at each of the following concentrations: 50% EtOH, 80% EtOH, 100% EtOH, 75% EtOH + 25% tert-Butyl alcohol (TBA), 50% EtOH + 50% TBA, 25% EtOH + 75% TBA, 25% EtOH + 75% TBA + erythrosine dye, and ending with 3 changes of 100% TBA. Paraffin chips were added at the third 100% TBA change to allow the paraffin to begin infiltrating the orchid tissues. After two days the remaining TBA was poured off and replaced with melted paraffin. Vials were then placed in a dry oven at 63°C for two days with two paraffin changes. Orchid explants infiltrated with paraffin were then placed into wax moulds, filled with melted paraffin, and allowed 24 hours to harden before affixing to balsam blocks for cutting.

Wax blocks containing orchid plbs and shoot tips were cut with a Spencer Rotary Microtome (Model 820, Buffalo, New York) at a thickness of 10 micrometers. Three to five sections of ribbon were mounted per microscope slide with 5 replicates per orchid sample. Microscope slides were dried for a minimum of 24 hours in a fume hood prior to staining following Johansen’s protocol with a modification of CitriSolv® being substituted for xylene.
(Ruzin, 1999). To begin the staining process, microscope slides were placed in Coplin jars and underwent two changes of CitriSolv® (15 minutes per change) to deparaffinize the slides. Microscope slides were then rehydrated with 95, 90, 80, and 75% EtOH for 1 hour at each concentration. Following the final rehydration step, slides were stained in Safranin O for two hours and then thoroughly rinsed with distilled water. Slides were then dehydrated in 95% EtOH for 10 seconds and 100% EtOH for 10 seconds. Counterstaining in Fast Green FCF was then done (5-30 seconds per slide, duration of exposure varied based on quickly the stain was absorbed), slides were dipped into two clearing solutions and then placed into CitriSolv® for two 15 minute changes. Coverslips were mounted on slides and allowed to dry for 24 hours in a fume hood.

Microscope slides were analyzed using a Celestron PentaView microscope (Model 44348, Torrance, California). Cells were examined to determine where and to what extent damage occurred during each step of the cryopreservation process.

**Cryopreservation of orchid material**

In the subsequent evaluations of orchid material during cryopreservation the following cryo solutions were utilized (all at pH 5.8) and were prepared in half-strength MS media (Murashige & Skoog, 1962):

- Pretreatment: 0.3M sucrose + 15% coconut water
- Osmoprotective solution: 0.4M sucrose + 2M glycerol + 15% coconut water
- Plant Vitrification Solution 2 (PVS2): 0.4M sucrose + 15% coconut water + 30% glycerol + 15% ethylene glycol + 15% DMSO
- Rinsing solution: 1.2M sucrose + 15% coconut water

For each orchid variety 10-20 shoot tips/plbs were removed at each step of the cryopreservation process (excision only, pretreatment, osmoprotective solution, PVS2) to assess the impacts of these steps on regeneration.
Steps of a modified Droplet-vitrification protocol

*Dendrobium* UH232 and *Miltassia* Phoenix Rising ‘White Light’ plantlets were cultured *in vitro* on a semi-solid half-strength MS (15% coconut water + 2% sucrose for *Dendrobium* or 1% sucrose of *Miltassia*, pH 5.0) for 2 weeks. Concurrently, *Dendrobium* Spanish Eyes plbs were cultured in a liquid half-strength MS (15% coconut water + 2% sucrose, pH 5.0) for 2 weeks on a rotary shaker at 126 rpm. Excised shoot tips 2-3 mm in length of UH232 (70 explants) and *Miltassia* ‘White Light’ (136 explants) and *Dendrobium* Spanish Eyes plbs (240 explants) were placed in a pretreatment media on a rotary shaker (126 rpm) for 3 days. An additional 136 shoot tips of *Miltassia* ‘White Light’ were placed in pretreatment media on a rotary shaker (126 rpm) for 1 day. Orchid materials were then transferred to an osmoprotective solution for 20 minutes which was then replaced with PVS2 for 20 minutes. After exposure to these solutions, explants were placed individually into PVS2 droplets (10 µL) on aluminum foil strips (20 mm x 7 mm) and plunged into liquid nitrogen for 1 hour. Foil strips containing frozen orchid materials were subsequently removed and placed in a rinsing solution for 40 minutes at room temperature. Plbs and shoot tips were then blotted on sterile filter paper, plated on a semi-solid half-strength MS (15% coconut water + 2% sucrose for *Dendrobiums* or 1% sucrose for *Miltassia*, pH 5.0) and placed in dark recovery for one week. Petri dishes were then moved to filtered light (two racks away from grow lights and under four layers of cheese cloth) for one week and then returned to normal lighting conditions to assess regeneration.

Results

Assessment of Vitrification Method for *Dendrobium* UH1427 and *Miltassia*

Vitrification of *Dendrobium* UH1427 shoot tips

After 5 months of observation no regeneration was seen in the UH1427 shoot tips regardless of pretreatment parameters (Table 3). Under the parameters of a 3 day 0.3M sucrose pretreatment, 7 of the 30 treated shoot tips retained a yellow-green coloring for the first three months following submersion in liquid nitrogen. However none of these 7 shoot tips showed any
new growth 5 months post-cryopreservation, and had lost the yellow-green coloring. Regeneration of controls from each step of the process was variable as seen in Table 3.

Vitrification of *Miltassia* hybrid plbs

None of the *Miltassia* plbs subjected to liquid nitrogen via vitrification regenerated 5 month post-cryopreservation (Table 4). None of the PVS2 controls from the 3 day pretreatment (0.3M, 0.4M, or 0.5M sucrose concentrations) regenerated which indicates that the pretreatment may not have been long enough to provide support against the PVS2 toxicity. These parameters also had the lowest plb regeneration of osmoprotective solution controls (Table 4).

Assessment of Droplet-vitrification Method for *Dendrobium* UH1427 and *Miltassia*

Droplet-vitrification of *Dendrobium* UH1427 shoot tips

Following 5 months of observation post-cryopreservation, 3 shoot tips of UH1427 successfully regenerated and formed plantlets (Table 5). Of these regenerated shoot tips, one came from the pretreatment parameter of 3 days at 0.4M sucrose and the other two came from the 3 day pretreatment on 0.3M sucrose. The regenerated plantlets demonstrated the normal growth and physical characteristics seen in excision-only controls. Latent mortality of shoot tip controls from each step of cryopreservation can be seen in Figures 4 and 5. Latent mortality is defined here as the initial regeneration of explants with subsequent tissue death.

None of the shoot tips from the 5 and 7 day pretreatment durations (0.3, 0.4, and 0.5M sucrose) regenerated following submersion in liquid nitrogen. As seen in Table 5, the 0.5M sucrose pretreatment for 5 and 7 days resulted in the lowest regeneration amongst shoot tip controls with none of the PVS2 controls from either treatment regenerating.
Droplet-vitrification of *Miltassia* hybrid plbs

*Miltassia* plbs subjected to droplet-vitrification for liquid nitrogen submersion showed no regeneration following 5 months of recovery (Table 6). Regeneration of control plbs taken throughout the protocol was also low with the highest regeneration seen in the pretreatment controls from each sucrose concentration (0.3, 0.4, and 0.5M) during the 7 day pretreatment and the 0.4M and 0.5M sucrose pretreatment for 5 days.

**Microtome Analysis of *Dendrobium* UH1427 shoot tips and *Miltassia* plbs**

Analysis of the UH1427 and *Miltassia* explant cells following the steps of cryopreservation found three similarities. One similarity, shown in Figure 6A, is the absence of material within cells. These cells kept their polyhedral form but the nuclei and other cellular components have been lost from the cell interior. A second commonality found in many cells is the presence of enlarged nuclei. As shown in Figure 6B these nuclei, can be 2-3 times larger than the normal nuclei of nearby cells. The third commonality was observed regardless of treatment where large numbers of dense cells are seen around the edges of the orchid shoot tips and plbs. These dense cells are defined here as being small with tightly packed intracellular components. Such cells rarely show any destruction unless that damage was sustained during the preparation of slides.

Analysis of *Miltassia* plbs subjected to freezing for both vitrification and droplet-vitrification showed burst cells in the interior of plbs. Burst cells presented both as missing areas of the plbs and as destroyed cell walls. The burst cells occupied interior areas where the cells were generally larger than the exterior cells as shown in Figure 7D. This internal bursting of plb cells may explain why none of these plant materials regenerated following submersion in liquid nitrogen. In contrast to the larger cells, the small, dense cells on the perimeter remained intact (Figure 7C). Plbs also exhibited enlarged nuclei and distorted cell walls shown Figures 7A and 7B.

Interpretation of *Dendrobium* UH1427 shoot tips was not as clear as that of the *Miltassia* plbs. Shoot tip samples sustained damage during microtome preparation that obscured cellular
integrity. Despite the damage, it appears that the majority of cells remained intact throughout the cryopreservation process and simply failed to regenerate. The smaller the cell, the better the polyhedral cell wall that survived liquid nitrogen submersion. Figure 8A shows intact, normal cells following exposure to the pretreatment media and enlarged nuclei following exposure to the osmoprotective solution (Figure 8B).

Assessment of a modified Droplet-vitrification protocol

Modified Droplet-vitrification for Dendrobium UH232

After 4 months of recovery, 3 shoot tips of UH232 regenerated following cryopreservation by droplet-vitrification (Table 7). The resulting plants exhibited the normal growth and physical characteristics seen in excision-only controls. Controls exhibited latent mortality during the 4 month recovery period. Figure 9 shows the decrease in surviving shoot tip controls 11, 31, and 131 days following treatment. It is likely that the latent mortality was a result of toxic effects of the osmoprotective and PVS2 solutions remaining in the shoot tip tissues.

Modified Droplet-vitrification for Dendrobium Spanish Eyes

Two months post-cryopreservation none of the 200 D. Spanish Eyes plbs subjected to freezing via droplet-vitrification regenerated. As seen in Table 7 the regeneration of plbs from the control groups has been quite high, with 100% of the osmoprotective controls regenerating. The pretreatment and PVS2 controls also showed high numbers of regenerating shoot tips. This could indicate that toxicity of the cryo solutions was not an issue or that the chemicals did not penetrate deep enough into the plbs for toxicity to occur. If the latter is true, then plbs subjected to the full treatment + freezing would have retained higher cellular water leading to more bursting upon submersion in liquid nitrogen and may explain why no plbs regenerated.
Modified Droplet-Vitrification protocol for *Miltassia* Phoenix Rising ‘White Light’

Within 2 months of freezing in liquid nitrogen, 2 shoot tips of *Miltassia* ‘White Light’ regenerated (Table 7) from the 3 day pretreatment. The regenerating shoot tips were first observed 40 days post-cryopreservation. Pretreatment, osmoprotective, and PVS2 controls have shown low regeneration (1-2 shoot tips per control group). No shoot tip regeneration occurred in the 1 day pretreatment of ‘White Light’ shoot tips that underwent liquid nitrogen submersion. Controls in this 1 day pretreatment group have also shown low regeneration as shown in Table 7. Excision-only controls of *Miltassia* shoot tips had a 50% recovery indicating that excision problems may be affecting regeneration following subsequent steps of cryopreservation for this orchid.

Discussion

The results of orchid cryopreservation show that the droplet-vitrification method can be successfully applied to both *Dendrobium* and *Miltassia* hybrids. In the cases of *Dendrobium* UH232 and *Miltassia* Phoenix Rising ‘White Light,’ three shoot tips of each regenerated following exposure to cryoprotectants and submersion in liquid nitrogen. Droplet-vitrification of *Dendrobium* UH1427 resulted in successful regeneration of two shoot tips from the 0.3M sucrose pretreatment and one shoot tip from the 0.4M sucrose pretreatment. This low regeneration rate, 7.5% UH232, 2.1% ‘White Light,’ 6.7% 0.3M and 3.3% 0.4M UH1427, demonstrates the need for further research into the cryopreservation parameters of this method and how best to optimize them. These three orchid varieties also exhibited latent mortality of the shoot tip controls removed from each step of cryopreservation. Latent mortality was most pronounced for the UH232 variety, and may be a result of toxicity of chemicals and/or sucrose remaining in the orchid tissues. In particular DMSO, a primary ingredient in the vitrification solution, can be toxic (Sarasan et al., 2006). Similar patterns of latent mortality were observed for the explant controls of the other genera.
Early work with *Miltassia* and *Dendrobium* using the vitrification approach yielded no regeneration of frozen explants. However the same *Dendrobium* variety was successfully regenerated when the droplet-vitrification method was used. The success of the droplet-vitrification approach may be due to the ability of the aluminum foil strips to quickly transfer heat out of the orchid explants, allowing faster freezing and lowering the chance of cellular destruction (Gonzalez-Arnao et al., 2009; Sakai and Engelmann, 2007). It is important to recognize that while droplet-vitrification yielded the only regeneration, it does not mean that it is a better method, but rather, circumstances of these experiments led to it being a more successful approach to cryopreservation (Towill and Bajaj, 2002).

The histological observations of *Miltassia* hybrid plbs and *Dendrobium* UH1427 shoot tips subjected to the vitrification and droplet-vitrification approaches are consistent with findings by Poobathy et al. (2013a). Their investigation of cryopreserved plbs noted most cellular damage occurring within the interior of the plbs while the exterior cells remained intact. In addition enlarged nuclei were noted in treated cells and their appearance interpreted as potential chromatin structure damage (Poobathy et al., 2013a). These enlarged nuclei were observed in both the treated and control shoot tips and plbs of *Dendrobium* UH1427 and the *Miltassia* hybrid, indicating that the exposure to high levels of sucrose, cryoprotectant chemicals, and liquid nitrogen may have damaged the nuclei in a similar manner. This cellular observation was also noted by Mubbarakh et al. (2014). As with these studies, the microtome analysis of plb and shoot tip cells showed rupturing of cell walls of frozen plant materials which may have inhibited regeneration of the orchid explants.

Regeneration of shoot tips in these experiments and not plbs suggests that adjustments may be necessary before and/or after subjecting the plbs to liquid nitrogen. Poobathy et al. (2013a) also reported no regeneration of vitrification cryopreserved *Dendrobium* plbs. In the work by Mubbarakh et al. (2014) *Brassidium* plbs had 17.5% regeneration when exposed to 0.25M sucrose pretreatment but when lower and higher sucrose concentration were used, no regeneration followed freezing in the liquid nitrogen. One alteration to the droplet-vitrification was changing the media by switching from VW to MS. This change was the result of discussions regarding nutrient availability in the media post-cryopreservation. In general there are more nutrient sources in the MS media that may allow for better explant regeneration (K.
An additional improvement to the protocol was the use of a liquid pretreatment media in place of the semi-solid media, as a liquid pretreatment may allow for better cellular penetration (K. Cheah, 2015, personal communication). This alteration was implemented for the *Miltassia* ‘White Light’ shoot tips but did not result in a significant increase in regeneration.

Cryopreservation by droplet-vitrification is a promising means of germplasm storage as indicated by the success with *Dendrobium* UH1427 and UH232 and *Miltassia* ‘White Light.’ From each hybrid three shoot tips were successfully regenerated within one month of freezing. Further study and refinement of this protocol will be necessary to achieve high and consistent rates of regeneration. Cryopreservation also holds potential for virus elimination, referred to as cryotherapy. Efficient regeneration protocols will allow for the best chances of recovering virus-free explants following liquid nitrogen submersion during cryopreservation.

Acknowledgement

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Table 3. Results of vitrification method applied to *Dendrobium* UH503 shoot tips. Controls were removed at each step of the process (excision, pretreatment (1), osmoprotective(1+2), PVS2(1, 2 +3)) to determine the effects on shoot tip regeneration. Final observations and data collection was completed 5 months after the start of UH503 vitrification experiments. Full treatment included all steps (1, 2 + 3) as well as submersion in liquid nitrogen.

<table>
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<tr>
<th>Pretreatment length (days)</th>
<th>Pretreatment Sucrose Concentration (M)</th>
<th>Excised Shoot Tips</th>
<th>Pretreatment Controls</th>
<th>Osmoprotective Controls</th>
<th>PVS2 Controls</th>
<th>Full Treatment (+ Freezing)</th>
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</table>

#/#, number of regenerated shoot tips out of the total number of shoot tips for that group

n/a, contamination of samples resulted in no regeneration and necessity of sample destruction
**Table 4.** Results of vitrification method applied to *Miltassia* hybrid plbs. Controls were removed at each step of the process (excision, pretreatment (1), osmoprotective(1+2), PVS2(1, 2 +3)) to determine the effects on plb regeneration. Final observations and data collection was completed 5 months after the start of *Miltassia* vitrification experiments. Full treatment included all steps (1, 2 + 3) as well as submersion in liquid nitrogen.

<table>
<thead>
<tr>
<th>Pretreatment length (days)</th>
<th>Pretreatment Sucrose Concentration (M)</th>
<th>Excised Shoot Tips</th>
<th>Pretreatment Controls</th>
<th>Osmoprotective Controls</th>
<th>PVS2 Controls</th>
<th>Full Treatment (+ Freezing)</th>
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#/#, number of regenerated plbs out of the total number of plbs for that group
Table 5. Results of droplet-vitrification method applied to *Dendrobium* UH51427 shoot tips. Controls were removed at each step of the process (excision, pretreatment (1), osmoprotective (1+2), PVS2 (1, 2 +3)) to determine the effects on shoot tip regeneration. Final observations and data collection was completed 5 months after the start of UH1427 droplet-vitrification experiments. Full treatment included all steps (1, 2 + 3) as well as submersion in liquid nitrogen.

<table>
<thead>
<tr>
<th>Pretreatment length (days)</th>
<th>Pretreatment Sucrose Concentration (M)</th>
<th>Excised Shoot Tips</th>
<th>Pretreatment Controls</th>
<th>Osmoprotective Controls</th>
<th>PVS2 Controls</th>
<th>Full Treatment (+ Freezing)</th>
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#/#, number of regenerated shoot tips out of the total number of shoot tips for that group

n/a, shoot tips contaminated resulting in no regeneration and the necessity of sample destruction
Table 6. Results of droplet-vitrification method applied to *Miltassia* hybrid plbs. Controls were removed at each step of the process (excision, pretreatment (1), osmoprotective(1+2), PVS2(1, 2 +3)) to determine the effects on plb regeneration. Final observations and data collection was completed 5 months after the start of *Miltassia* droplet-vitrification experiments. Full treatment included all steps (1, 2 + 3) as well as submersion in liquid nitrogen.

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<thead>
<tr>
<th>Pretreatment length (days)</th>
<th>Pretreatment Sucrose Concentration (M)</th>
<th>Excised Shoot Tips</th>
<th>Pretreatment Controls</th>
<th>Osmoprotective Controls</th>
<th>PVS2 Controls</th>
<th>Full Treatment (+ Freezing)</th>
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#/#, number of regenerated plbs out of the total number of plbs for that group

n/a, contamination of plbs led to no regenerate and subsequent destruction of samples
Table 7. Regeneration results from the modified droplet-vitrification protocol applied to *Dendrobium* Spanish Eyes, *Dendrobium* UH232, and *Miltassia* Phoenix Rising 'White Light.' Controls were removed at each step of the process (excision, pretreatment (1), osmoprotective(1+2), PVS2(1, 2 +3)) to determine the effects on regeneration of plbs and shoot tips. Full treatment included all steps (1, 2 + 3) as well as submersion in liquid nitrogen.

<table>
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<tr>
<th>Orchid Material</th>
<th>Pretreatment length (days)</th>
<th>Excised Shoot Tips</th>
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<th>Osmoprotective Controls</th>
<th>PVS2 Controls</th>
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<td>1/10</td>
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<td>1/10</td>
<td>1/10</td>
<td>0/96</td>
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</table>

#/#, number of regenerated shoot tips or plbs out of the total number of shoot tips/plbs for that group.
Figure 4. Dendrobium UH1427 shoot tips following droplet-vitrification with a 3 day pretreatment on 0.4M sucrose VW media. Controls from the cryopreservation steps demonstrated latent mortality. Only one shoot tip successfully regenerated following submersion in liquid nitrogen.
Figure 5. *Dendrobium* UH1427 shoot tips following droplet-vitrification with a 0.3M sucrose VW media for a 3 day pretreatment. Shoot tips controls removed after each step of the cryopreservation process show latent mortality. Of the shoot tips subjected to the full treatment including liquid nitrogen, two regenerated.
Figure 6. Histological observation of cellular similarities of *Miltassia* and *Dendrobium* orchid samples used in cryopreservation. A) Cells show normal polyhedral shapes with some cells appearing to have very large vacuoles. B) Red stained nuclei are enlarged. C) Cells along the exterior of the tissue are denser than those closer to its center.
Figure 7. *Miltassia* protocorm-like body cells after cryopreservation by vitrification and droplet-vitrification. A) Enlarged nuclei stained red. B) Misshapen cell walls. C) Small, dense cells along the exterior remain intact after freezing. D) Burst cell walls.
**Figure 8.** Histological observation of *Dendrobium* UH1427 shoot tip cells. A) After exposure to pretreatment media of 0.3M sucrose for 7 day all cells have retained normal polyhedral shapes. B) Exposure to the osmoprotective results in the nuclei of larger cells becoming enlarged (stained red) while the denser cells retain normal nuclei (stained blue).
Figure 9. Assessment of *Dendrobium* UH232 shoot tip regeneration and latent mortality following droplet-vitrification. Shoot tips from the steps prior to freezing demonstrated latent mortality while frozen shoot tips show regeneration. In this assessment of droplet-vitrification a 3 day pretreatment on 0.3M sucrose MS media was used.
CHAPTER 3
CRYOTHERAPY OF ORCHID HYBRIDS FOR THE ELIMINATION OF CYMBIDIUM MOSAIC VIRUS AND ODONTOGLOSSUM RINGSPOT VIRUS

Abstract

Cryotherapy was applied to orchid genera for the elimination of Cymbidium mosaic virus (CymMV) and Odontoglossum ringspot virus (ORSV). Infected Dendrobium, Miltassia, and Oncidium orchids were subjected to droplet-vitrification starting with a one and/or three day 0.3M sucrose pretreatment. Explants were subsequently exposed to an osmoprotective solution [2M glycerol + 0.4M sucrose] and a plant vitrification solution 2 [0.4M sucrose + 30% glycerol + 15% dimethylsulfoxide + 15% ethylene glycol] before submersion in liquid nitrogen for one hour. Explants were placed in a rinsing solution [1.2M sucrose] and then plated on recovery media [1-2% sucrose + 15% coconut water; Murashige & Skoog media; pH5.0]. Three months after cryotherapy the Dendrobium and Miltassia explants failed to regenerate. One shoot tip of Oncidium from each of the one and three day pretreatments regenerated and was tested for ORSV elimination via reverse-transcription polymerase chain reaction (RT-PCR). This assay found the treated shoot tips to still be infected with ORSV following cryotherapy treatments. Elimination of CymMV could not be determined due to a lack of regeneration in treated shoot tips.
Introduction

Cultivated orchids are commonly found to be infected with *Cymbidium mosaic virus* (CymMV) and *Odontoglossum ringspot virus* (ORSV) (Hu et al., 1993). These viruses may manifest as brown spots on flowers and leaves, color breaking of flowers, and reduced plant growth (Hu et al., 1993; Jensen, 1952; Kamemoto et al., 1999; Leonhardt and Sewake, 1999). In many instances ORSV has been difficult to eliminate and thus much attention has focused on CymMV (J.S. Hu, personal communication, 2013). Approaches to control and eliminate CymMV include cleaning propagation tools, screening material for infection prior to propagation, meristem culture, thermotherapy, chemotherapy, and transgenics (Chang et al., 2005; Hu et al., 1994; Jensen, 1952; Milosevic et al., 2012; Porter and Kuehnle, 1997; Zettler et al., 1990). These prevention and elimination techniques have been applied to orchids with varying levels of success.

A recent approach in the elimination of plant viruses has been through the use of cryotherapy. Through this method, infected plant materials are subjected to cryoprotectant solutions, frozen in liquid nitrogen (-195.8°C), and regenerated as virus-free plants. It is hypothesized that the mechanism for this elimination relies on the plant cells destroyed during liquid nitrogen submersion being differentiated with large vacuoles, as these cells are most likely to harbor virus particles since they possess the distinct organelles needed by the virus to move and replicate (Feng et al., 2013; Hu et al., 1994; Wang and Valkonen, 2009). The large vacuole size of these differentiated cells leads to a higher intracellular water content which increases the chance of the water freezing during submersion in liquid nitrogen (Wang and Valkonen, 2009). When these large vacuole cells, with virus and a high water content, freeze and burst, the virus is destroyed as it is no longer contained within the cell; additionally the virus particle may be damaged during the process. An important attribute of cryotherapy is that the frequency of virus elimination and the size of the plant material (with regard to plant shoot tips), are independent of one another whereas in meristem culture virus elimination, higher elimination frequency results from using the smallest of shoot tips (Feng et al., 2013). Cryotherapy has been successfully applied to eliminating virus in crops and ornamentals (Brison et al., 1997; Ding et al., 2008;
Gallard et al., 2011; Helliot et al., 2002; Shin et al., 2013; Vieira et al., 2014; Wang and Valkonen, 2008a, 2008b; Wang et al., 2008; Wang et al., 2003; Yi et al., 2014). A few viruses with high rates of elimination following cryotherapy include: Plum pox potyvirus, Grapevine virus A, Cucumber mosaic virus, and Banana streak virus (Brison et al., 1997; Helliot et al., 2002; Wang et al., 2003). To date there are no reports of cryotherapy being used to eliminate virus in orchids.

The objective of this study was to determine if cryotherapy can be applied for the elimination of CymMV and ORSV in Dendrobium, Miltassia, and Oncidium orchids using the droplet-vitrification method; this method refers to the specific way in which explants are placed in liquid nitrogen, in this case the explants are individually suspended in 10-20 microliter (µL) droplets of Plant Vitrification Solution 2 (PVS2) on small aluminum foil strips which are then placed directly into liquid nitrogen. Droplet-vitrification has been successfully applied to orchid genera for cryopreservation and these genera include Dendrobium, Vanilla, and Vanda (Gonzalez-Arnao et al., 2009; Jitsupakul et al., 2011; Poobathy et al., 2013). Eliminating orchid viruses in this manner is not only a novel approach but also means that a wider variety of orchid germplasm could be stored through cryopreservation regardless of viral infections.

Materials & Methods

All cryotherapy work was conducted in a tissue culture room. Tools, plant media, and cryo solutions were autoclaved prior to use and aseptic practices were implemented throughout. Orchids were grown on either shelves or a shaker. When shelving was utilized, petri dishes (100 millimeters x 15 mm) were 19-21 centimeters (cm) below grow lights and 250 milliliter (mL) flasks were 9 cm from the flask top to the bottom of the lights. Plants were grown under 24 hour light conditions produced by 40 watt Gro-Lights (Sylvania, Westfield, Indiana), with an average air temperature of 26.5°C, average illumination of 79 µmol m⁻² s⁻¹, and average humidity of 37%. Four shelves of 0.44 square meters each were utilized on a metal rack. When grown on the shaker, 125 mL flasks were 18 cm from the overhead lights. Again the previously described light, temperature, and humidity values were used for the shaker. Handling of orchid materials
was done inside a modified laminar flow hood of wood and plexiglass construction with a Merv 13 Green Pleat filter (Filtration Group, Downers Grove, Illinois).

Testing for virus infection in orchid materials prior to cryotherapy

In vitro plantlets of a Thai Dendrobium hybrid and Oncidium Pacific Sunrise ‘Hakalau’ underwent ImmunoStrip® testing for both CymMV and ORSV. Forty leaf samples of the Thai Dendrobium hybrid and 2 leaf samples of Oncidium were tested using ImmunoStrip® test kits (Agdia, Inc Elkhart, Indiana). Leaf samples approximately 1 square inch where placed into testing bags filled with a buffer solution and ground. Testing strips were inserted for 15-20 minutes before results were read.

Seven orchid hybrids were selected for enzyme linked immunosorbent assay or ELISA (Agdia, Inc Elkhart, Indiana) to test for both CymMV and ORSV. These hybrids include Dendrobium UH306 (two selections A & B), Miltassia hybrid, Dendrobium Spanish Eyes, and three Dendrobium hybrids (457, 662, 541). CymMV testing was completed as a compound ELISA and ORSV as a direct ELISA. Two 96 well testing plates were coated with a carbonate coating buffer [5 milliliter distilled water + 1 mL carbonate concentrate per sample], 25 microliters (µL) of CymMV and ORSV capture antibodies were placed in the wells of the respective test plates, incubated for 4 hours in a humid box, and then washed 3 times with distilled water (DW). For each orchid sample 0.5 grams of tissue were ground in 5 mL of extraction buffer. Negative and positive controls for each virus were diluted from concentrated stocks using the extraction buffer. Samples were dispensed into testing plates and incubated overnight in a humid box in the dark.

The next day plates were washed 8 times with DW water. Enzymes for ORSV [5 mL + 25 µL enzyme conjugate] and CymMV [5 mL ECI buffer + 25 µL enzyme A + 25 µL enzyme B] were distributed into the respective well plates. Plates were then incubated for 2 hours at room temperature in a humid box and then washed 8 times with DW water. PNP buffer was added to each well and plates were incubated in the dark for 1 hour. Well plates were then read with an ELISA plate reader at 405 nanometers to obtain virus results. Results were considered positive for infection when ELISA values were greater than three.
Orchid materials that tested positive for CymMV and/or ORSV were used in subsequent cryotherapy studies.

Cryotherapy of orchid materials

In the subsequent evaluations of virus elimination during cryopreservation the following cryosolutions were utilized and prepared in half-strength MS media at pH 5.8 (Murashige and Skoog, 1962):

- **Pretreatment:** 0.3M sucrose + 15% coconut water
- **Osmoprotective solution:** 0.4M sucrose + 2M glycerol + 15% coconut water
- **Plant Vitrification Solution 2 (PVS2):** 0.4M sucrose + 15% coconut water + 30% glycerol + 15% ethylene glycol + 15% DMSO
- **Rinsing solution:** 1.2M sucrose + 15% coconut water

For each orchid hybrid 10 shoot tips or 20 plbs were removed at each step of the cryopreservation process (pretreatment, osmoprotective solution, PVS2) to assess the impacts of these steps on regeneration and virus elimination.

Steps of CymMV-infected explant Droplet-vitrification

Virus infected *Dendrobium* UH306A and B plbs, *Miltassia* hybrid plbs, and Thai *Dendrobium* plantlets were cultured on a half-strength MS [15% coconut water + 2% sucrose for *Dendrobiums* or 1% sucrose for *Miltassia* and *Oncidium*] for 2-4 weeks. Shoot tips of the Thai *Dendrobium* 2-3 mm in length (1640 shoot tips) were excised from plantlets and plb samples of UH306 (165 plbs of each A and B), and *Miltassia* (560 plbs) were selected for treatments. Plbs and shoot tips were placed in a liquid pretreatment on a rotary shaker at 126 rpm for 3 days. Plant materials were then moved to an osmoprotective solution for 20 minutes after which time the osmoprotective solution was replaced with PVS2 for an additional 20 minutes. Orchid materials were then transferred to 10 µL droplets of PVS2 (one shoot tip or plb per droplet) on aluminum foil strips (20 mm x 7 mm) with two PVS2 droplets per foil strip. Foil strips were then plunged into liquid nitrogen and held for 1 hour. After, foil strips containing frozen orchid material were removed and placed in a rinsing solution at room temperature for 40 minutes.
Orchid materials were then blotted on sterile filter paper, plated on a semi-solid half-strength MS [15% coconut water + 2% sucrose for Dendrobium or 1% sucrose for Miltonia, pH 5.0] and placed in the dark for one week. Following dark recovery, petri dishes were moved to filtered lighting (two racks away from grow light and under four layers of cheese cloth) for one week and then returned to normal lighting conditions for assessment of regeneration.

**Steps of ORSV-infected explant Droplet-vitrification**

*In vitro* plantlets of Oncidium Pacific Sunrise ‘Hakalau’ were cultured on a semi-solid half-strength MS [1% sucrose + 15% coconut water, pH 5.0] for two weeks. Shoot tips 2-3 mm in length were excised and placed in a pretreatment media for the durations of one (136 shoot tips) and three (136 shoot tips) days. Following pretreatment, shoot tips were placed in the osmoprotective solution for 20 minutes, PVS2 for 20 minutes and then placed onto foil strips (20 mm x 7 mm) with PVS2 (10 µL droplets) and subsequently plunged into liquid nitrogen for 1 hour. After freezing, foil strips containing shoot tips were placed into a rinsing solution at room temperature for 40 min. Shoot tips were then blotted on sterile filter paper, plated on a semi-solid half-strength MS [1% sucrose + 15% coconut water, pH 5.0], placed in the dark for one week, filtered lighting (two racks away from grow lights and under four layers of cheese cloth) for one week, and then moved to normal lighting where regeneration was monitored.

**Testing for ORSV elimination following cryotherapy**

Creation of sample complimentary DNA

Preliminary assessment of virus elimination of ORSV was conducted via the reverse transcription polymerase chain reaction (RT-PCR) for regenerated Oncidium ‘Hakalau’ orchid materials. Samples tested included the two regenerated ‘Hakalau,’ three untreated ‘Hakalau,’ and one water control. Additionally, three virus-free Dendrobium UH503 samples were used during the determination of an appropriate RT-PCR annealing temperature.
Total RNA extraction was completed with an RNeasy Mini Kit (Qiagen, Valencia, CA) and followed the supplied protocol. Approximately 0.15 g of tissue were ground to a fine powder in liquid nitrogen. Each sample received 450 µL of Buffer RLT and was vigorously vortexed. In the case of the two ‘Hakalau’ samples (<10 mg of material each) that regenerated post-cryotherapy tissue grinding was done in 2 mL centrifuge tubes with 50 µL Buffer RLT and a Kontes pestle used to grind the tissues. Additional RLT Buffer was then added for a final volume of 450 µL as used for the other samples. Each solution was transferred to a QIAshredder spin column and centrifuged at full speed for two minutes. Supernatant was transferred to a fresh tube and combined with 200 µL 95% ethanol (per sample). The resulting lysate and precipitate was moved to a pink spin column and centrifuged at 8,000 x g for 30 seconds. Flow through was discarded and 700 µL Buffer RW1 added to the spin column (per sample). Columns were then centrifuged for 30 seconds at 8,000 x g. Again flow through was discarded, 500 µL Buffer RPE (per sample) added to spin column, and columns centrifuged at 8,000 x g for 30 seconds; this step was then repeated as described with a one minute centrifuge. Spin columns were then moved to new tubes and centrifuged for two minutes at full speed to dry the spin column membrane. Spin columns were placed in new collection tubes, 50 µL RNase-free water added to each tube, and tubes centrifuged at 10,000 rpm for one minute.

Reverse transcription was then completed to create complimentary DNA (cDNA) strands of the extracted orchid RNA following a modified protocol of Barry et al. (1996). The heat-denature template consisted of 2 µL total orchid RNA + 2 µL random primers + 5.5 µL water for each sample. Samples were incubated for eight minutes at 72°C and then placed immediately on ice. For the reverse transcription reaction the following template was used for each sample: 9.5 µL denature template + 4 µL RT 5X buffer + 5 µL dNTPs (2.5mM each) + 0.5 µL RNasin + 1.0 µL MMLV (Moloney murine leukemia virus). Samples were incubated at 42°C for 45 minutes and then 95°C for 10 minutes in a GeneAmp PCR System (Applied Biosystems, Carlsbad, CA).
Quantification of Total RNA purity and concentration

For each orchid sample of extracted total RNA the purity and concentration were examined. Two microliters of each sample were run through a Nanodrop 2000 Spectrophotometer (ThermoScientific, Waltham, MA) for purity (A260/A280 ratio) and nucleic acid concentration (nanogram per microliter).

Determination of appropriate RT-PCR annealing temperature

Three untreated *Oncidium* ‘Hakalau,’ three *Dendrobium* UH503, and one water control were used to determine the appropriate annealing temperature to use during the polymerase chain reaction (PCR) a modified protocol based on Barry et al. (1996) was used. For the polymerase chain reaction each orchid cDNA was added to a PCR master mix. The master mix solution consisted of 10 µL GoTaq + 1.0 µL 811 primer (10pmol/µL) + 1.0 µL 812 primer (10pmol/µL) + 7.0 µL RNase-free water + 1.0 µL cDNA. Primer 811 utilized the sequence 5’ - TTT GTACCAATTCTCTGGGT – 3’ and primer 812 utilized the sequence 5’ – CTGCGGATTGTCTACCTCGA – 3’ both of which represent the ORSV coat protein. Once all samples were prepped, the tubes were loaded into a Veriti 96 Well Thermal Cycler PCR machine (Applied Biosystems, Carlsbad, CA). The PCR reaction profile was 95°C for 7 minutes; 35 cycles of 95°C for 30 seconds, a gradient of 46, 48, 50, 52, 54, and 56°C for 30 seconds, and 72°C for 1 minute. Final extension at 72°C was for 10 minutes.

PCR of treated *Oncidium* ‘Hakalau’ shoot tips

Samples of ‘Hakalau’ that regenerated following cryotherapy along with one untreated, one pretreatment control, one osmoprotective control, and one PVS2 control of ‘Hakalau’ and one water control were assayed for the presence of ORSV by RT-PCR. The protocol follows that outlined in the ‘Determination of appropriate annealing temperature’ section with the only difference being the use of 54°C as the annealing temperature.
Gel Electrophoresis of PCR products

All products resulting from PCR were run through gel electrophoresis to determine the presence or absence of ORSV in samples. For each sample, 5 µL was loaded into a well in a 1% agarose gel. Molecular weight markers, M111-1 (Lamda Biotech, St. Louis, MO) and Easy Ladder II (Bioline, Taunton, MA), were used and represent a weight range of 1000-100 base pairs (bp) for M111-1 and 5000-500 bp for Easy Ladder II. Samples were run at 4 volts per centimeter for 45 minutes or until dye had moved sufficiently down the gel before assessing the presence/absence of ORSV.

Results

Testing for virus infection in orchid materials before cryotherapy

ImmunoStrip® testing revealed all tested Thai *Dendrobium* to be positive for CymMV and all *Oncidium* ‘Hakalau’ to be positive ORSV. Of the six orchid hybrids tested by ELISA, three were CymMV positive, *Dendrobium* UH306A and B and *Miltassia* hybrid plbs, and none were positive for ORSV. Table 8 illustrates the presence or absence of viral infection in each tested orchid hybrid.

Assessment of CymMV-infected explant Droplet-vitrification

Following one to two months of post-cryopreservation recovery neither the *Dendrobium* shoot tips or plbs nor the *Miltassia* plbs regenerated. The Thai *Dendrobium* shoot tip controls displayed poor regeneration with excision-only controls faring the best at 80% regeneration. Of the pretreatment, osmoprotective, and PVS2 controls only one shoot tip from the PVS2 control group regenerated. Though none of the frozen *Dendrobium* UH306 A or B plbs regenerated, the controls fared better as shown in Table 9. All excision-only and pretreatment UH306 A and B controls regenerated, 80-90% of osmoprotective controls, and 50-70% of PVS2 controls. *Miltassia* plbs saw no explants survive freezing. Excision-only plb controls had 100%
regeneration but that dropped off upon exposure to pretreatment, osmoprotective solution, and PVS2 (Table 9).

**Assessment of ORSV-infected explant Droplet-vitrification**

Two months post-cryopreservation two of the *Oncidium* ‘Hakalau’ shoot tips have regenerated as shown in Figure 10. These regenerated materials represent one shoot tip from each of the one day and three day pretreatments. Shoot tip controls have resulted in 90-100% regeneration except for the one day PVS2 controls with 60% regeneration.

**Testing for CymMV and ORSV elimination following cryotherapy**

As none of the CymMV-infected explants regenerated, testing for virus elimination could not be done.

In the case of ORSV, two infected *Oncidium* shoot tips were tested three months post-cryopreservation by RT-PCR. The initial RT-PCR with untreated ‘Hakalau,’ UH503, and water control revealed that all annealing temperatures used (46, 48, 50, 52, 54, and 56°C) successfully amplified the ORSV coat protein as shown in Figures 11 and 12, demonstrating that any of these temperatures are suitable for this protocol. Based on the results of the annealing temperature gradient, 54°C was chosen as the annealing temperature for subsequent RT-PCR. Quantification of total RNA extracted revealed high nucleic acid concentrations and purity relative to amount of tissue used as shown in Table 10. RT-PCR of shoot tips that regenerated following cryotherapy found them to still be infected with ORSV as shown in Figure 13. Controls of ‘Hakalau’ from the pretreatment, osmoprotective, and PVS2 groups all showed the presence of ORSV.

**Discussion**

The orchid hybrids investigated here showed different responses to the Droplet-vitrification treatments. Thai *Dendrobium* plantlets fared the worst with most controls and all
frozen explants failing to regenerate as shown in Table 9. The three day liquid pretreatment may have resulted in too much exposure to sucrose and thus a failure to regenerate based on the fact that none of the pretreatment controls survived. Other cryopreservation works have noted the differences in survival that result from a change in sucrose concentration and the same may be true for liquid versus semi-solid media (Johari et al., 2009; Mubbarakh et al., 2014). The size of shoot tips used (2-3 mm) may have caused regeneration issues. Brison et al. (1997) noted that their use of 0.3-2 mm Prunus shoot tips resulted in low recovery and exacerbated losses following freezing. Additionally the three day pretreatment may have been too long where the subsequent overexposure to sucrose plasmolyzed the cells (R. Paull, 2015, personal communication).

Though neither of the Dendrobium UH306 selections regenerated following cryopreservation, the controls exhibited high levels of recovery shown in Table 9. Freezing damage during submersion in liquid nitrogen may have been too great for explants to overcome. Many reports of successful plb cryopreservation utilize the encapsulation-dehydration or encapsulation-vitrification method where the encasing of plbs of calcium alginate beads may offer extra protection against freezing damage (Ching et al., 2012; Mohanty et al., 2012, 2013; Yin and Hong 2009). Such an approach to cryopreservation should be considered in future experiments.

Despite assessing both one and three day pretreatment durations with Miltassia plbs, none regenerated following exposure to liquid nitrogen. As shown in Table 9, the ability of control plbs to regenerate diminished following each step of cryotherapy. As noted earlier, such inability to survive exposure to cryoprotectants may contribute to the lack of regeneration following submersion in liquid nitrogen.

Initial RT-PCR work determined that wide ranges of annealing temperatures are suitable for the amplification of the ORSV coat protein. An annealing temperature of 54°C was selected for subsequent PCR work as a higher annealing temperature will provide better specificity for primer binding (W. Borth, 2015 personal communications). All orchid samples prepared for RT-PCR showed high total RNA concentrations and purities relative to the amount of tissues used. This indicated enough RNA to proceed with the assay and to expect accurate results.
Oncidium ‘Hakalau’ shoot tips, one from each of the one day and three day pretreatments, were the only explants to survive exposure to liquid nitrogen. After three months of successful regeneration and regrowth, these explants were tested for ORSV infection and found to still be infected. The presence of virus after cryotherapy in this research is supported by work by Nukari et al. (2014) who found hops infected with Apple mosaic virus (ApMV) to remain infected after cryotherapy. Nukari et al. (2014) determined that cryotherapy could not eliminate ApMV because the virus moved from cell-to-cell within the plant and that the likelihood of a cell surviving freezing and not being infected was very low. ORSV also exhibits cell-to-cell movement and it is therefore likely that the same explanation for the lack of virus elimination applies here. ORSV is notoriously difficult to eliminate in orchids and this work demonstrates preliminary evidence that cryotherapy may not be an effective means of elimination.

In conclusion, ORSV was not eliminated from infected shoot tips of Oncidium that underwent this cryotherapy protocol and CymMV elimination could not be assessed due to the failure of shoot tips to regenerate following cryotherapy. Further refinement of protocols and additional experiments with CymMV-infected orchid explants will be necessary to determine if cryotherapy is a viable means of elimination for this virus. Preliminary investigations indicate that ORSV was not eliminated using this protocol, this may be a result of the small number of regenerated plantlets resulting in a small sample size.

Acknowledgments
This research was funded by a Monsanto Research Fellowship. Special thanks to Dr. Wayne Borth and Dr. Kishore Dey for their assistance with virus testing.
Literature Cited


Table 8. Presence or absence of virus in *Dendrobium*, *Miltassia*, and *Oncidium* orchids tested by ELISA or lateral flow assay.

<table>
<thead>
<tr>
<th>Orchid material</th>
<th><em>Cymbidium</em> mosaic virus</th>
<th><em>Odontoglossum</em> ringspot virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thai <em>Dendrobium</em> hybrid</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>Dendrobium</em> UH306</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>Miltassia</em> hybrid</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>Oncidium</em> Pacific Sunrise 'Hakalau'</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

-, negative for virus; +, positive for virus
Table 9. Results of explant regeneration following cryotherapy for *Dendrobium*, *Miltassia*, and *Oncidium* varieties. Controls were removed at each step of the Droplet-vitrification process (excision, pretreatment (1), osmoprotective (1 +2), and PVS2 (1, 2, +3)) to determine the effects of that step on explant regeneration. Full treatment refers to explants that went through all steps of cryotherapy (1 - 3) and were subsequently submerged in liquid nitrogen. Assessment of regeneration was completed two months after experiments.

<table>
<thead>
<tr>
<th>Orchid</th>
<th>Pretreatment Duration (days)</th>
<th>Excised Explants</th>
<th>Pretreatment Controls</th>
<th>Osmoprotective Controls</th>
<th>PVS2 Controls</th>
<th>Full Treatment + Freezing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thai <em>Dendrobium</em> hybrid</td>
<td>3</td>
<td>10/10</td>
<td>0/10</td>
<td>0/10</td>
<td>1/10</td>
<td>0/1600</td>
</tr>
<tr>
<td><em>Dendrobium</em> UH306 A</td>
<td>1</td>
<td>10/10</td>
<td>9/10</td>
<td>8/10</td>
<td>8/10</td>
<td>0/125</td>
</tr>
<tr>
<td><em>Dendrobium</em> UH306 B</td>
<td>1</td>
<td>n/a</td>
<td>10/10</td>
<td>9/10</td>
<td>5/10</td>
<td>0/125</td>
</tr>
<tr>
<td><em>Miltassia</em> Hybrid</td>
<td>1</td>
<td>20/20</td>
<td>18/20</td>
<td>11/20</td>
<td>0/20</td>
<td>0/200</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>20/20</td>
<td>9/20</td>
<td>6/20</td>
<td>3/20</td>
<td>0/200</td>
</tr>
<tr>
<td><em>Oncidium</em> Pacific Sunrise 'Hakalau'</td>
<td>1</td>
<td>10/10</td>
<td>9/10</td>
<td>10/10</td>
<td>5/10</td>
<td>1/96</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>10/10</td>
<td>9/10</td>
<td>9/10</td>
<td>9/10</td>
<td>1/96</td>
</tr>
</tbody>
</table>

#/#, number of regenerated explants out of the total number of explants removed at that step; n/a, samples destroyed due to contamination
Table 10. Quantification of nucleic acid concentration and purity of total RNA extracted from *Dendrobium* and *Oncidium* orchids. RNA purity is the ratio of A260/A280 wavelength absorption. Despite the use of minimal tissues for the full treatment *Oncidiums*, high purities were obtained during extraction.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Starting tissue weight (mg)</th>
<th>Nucleic acid concentration (ng/µL)</th>
<th>A260</th>
<th>A280</th>
<th>A260/A280</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Dendrobium</em> UH503 A</td>
<td>100</td>
<td>15.100</td>
<td>0.377</td>
<td>0.171</td>
<td>2.200</td>
</tr>
<tr>
<td><em>Dendrobium</em> UH503 B</td>
<td>100</td>
<td>12.400</td>
<td>0.309</td>
<td>0.147</td>
<td>2.110</td>
</tr>
<tr>
<td><em>Dendrobium</em> UH503 C</td>
<td>100</td>
<td>16.300</td>
<td>0.407</td>
<td>0.192</td>
<td>2.120</td>
</tr>
<tr>
<td><em>Oncidium</em> 'Hakalau' untreated A</td>
<td>100</td>
<td>43.300</td>
<td>1.082</td>
<td>0.514</td>
<td>2.110</td>
</tr>
<tr>
<td><em>Oncidium</em> 'Hakalau' untreated B</td>
<td>100</td>
<td>70.100</td>
<td>1.753</td>
<td>0.804</td>
<td>2.180</td>
</tr>
<tr>
<td><em>Oncidium</em> 'Hakalau' untreated C</td>
<td>100</td>
<td>63.000</td>
<td>1.580</td>
<td>0.741</td>
<td>2.130</td>
</tr>
<tr>
<td><em>Oncidium</em> 'Hakalau' pretreatment control</td>
<td>60</td>
<td>75.100</td>
<td>1.880</td>
<td>0.879</td>
<td>2.140</td>
</tr>
<tr>
<td><em>Oncidium</em> 'Hakalau' osmoprotective control</td>
<td>60</td>
<td>92.000</td>
<td>2.300</td>
<td>1.080</td>
<td>2.130</td>
</tr>
<tr>
<td><em>Oncidium</em> 'Hakalau' PVS2 control</td>
<td>60</td>
<td>77.000</td>
<td>1.930</td>
<td>0.898</td>
<td>2.140</td>
</tr>
<tr>
<td><em>Oncidium</em> 'Hakalau' full treatment 1</td>
<td>&lt;10</td>
<td>11.700</td>
<td>0.290</td>
<td>0.164</td>
<td>1.770</td>
</tr>
<tr>
<td><em>Oncidium</em> 'Hakalau' full treatment 2</td>
<td>&lt;10</td>
<td>12.400</td>
<td>0.310</td>
<td>0.161</td>
<td>1.920</td>
</tr>
</tbody>
</table>

mg: milligrams; ng: nanograms; µL: microliters
Figure 10. Regenerated Oncidium 'Hakalau' shoot tips following each step of cryotherapy. Controls represent shoot tip excision only$^1$, pretreatment$^2$ (1+2), osmoprotective solution$^3$ (1+2+3), PVS2$^4$ (1+2+3+4), and the full treatment$^5$ (1+2+3+4+5) and liquid nitrogen freezing. For each control group initially 10 shoot tips were used, while each of the two full treatment + freezing groups initially had 96 shoot tips. Despite two different pretreatment durations the same number of shoot tips in each category regenerated after exposure to liquid nitrogen.
Figure 11. Agarose gel electrophoresis of reverse-transcription polymerase chain reaction (RT-PCR) amplification products derived from virus-free *Dendrobium* (lanes 2-4; 11-13) and *Odontoglossum ringspot virus* (ORSV)-positive *Oncidium* (lanes 5-7; 14-16) orchid plants. Lane 1: Easy Ladder molecular weight markers; lanes 2-8: RT-PCR products obtained using ORSV primers at 46°C; lanes 9-10: Easy Ladder molecular weight markers (5000-500 base pairs); lanes 11-17: RT-PCR products obtained using ORSV primers at 56°C; lane 18: Easy Ladder molecular weight markers (5000-500 bp). Water controls placed in lanes 8 and 17. ORSV is expected to amplify at 290-300 base pairs.
Figure 12. Examination of annealing temperatures for *Odontoglossum ringspot virus* (ORSV) primers. Agarose gel electrophoresis of reverse-transcription polymerase chain reaction (RT-PCR) amplification products from virus-free *Dendrobium* (lanes 2-4; 10-12; 19-21; 27-29) and ORSV-infected *Oncidium* (lanes 5-7; 13-15; 22-24; 30-32) orchids. Lane 1: M111-1 molecular weight markers; lanes 2-8: RT-PCR products obtained using ORSV primers at 48°C; lane 9: M111-1 molecular weight markers; lanes 10-16: RT-PCR products obtained using ORSV primers at 50°C; lanes 17-18: M111-1 molecular weight markers; lanes 19-25: RT-PCR products obtained using ORSV primers at 52°C; lane 26: M111-1 molecular weight markers; lanes 27-33: RT-PCR products obtained using ORSV primers at 54°C; lane 34: M111-1 molecular weight markers. ORSV-positive *Oncidium* leaf extracts showed bands across all annealing temperatures. Lanes 32 and 33 show the misplacement of positive and water controls. Water controls placed in lanes 8, 16, 25, and 32. ORSV is expected to amplify at 290-300 base pairs.
Figure 13. Reverse-transcription polymerase chain reaction (RT-PCR) results for *Odontoglossum ringspot virus* (ORSV) infection in Oncidium ‘Hakalau’ following cryotherapy. Agarose gel electrophoresis amplification products show the continued presence of ORSV in ‘Hakalau’ shoot tips following cryotherapy (lanes 3 and 4). Lane 1: M111-1 molecular weight markers; lane 2: ORSV positive ‘Hakalau’ control; lane 3: 1 day pretreatment regenerated ‘Hakalau’; lane 4: 3 day pretreatment regenerated ‘Hakalau’; lane 5: pretreatment only ‘Hakalau’ control; lane 6: osmoprotective control ‘Hakalau’; lane 7: PVS2 control ‘Hakalau’; lane 8: negative water control. ORSV is expected to amplify at 290-300 base pairs.