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Cryotherapy: An-innovative Tool for Eradication of Pathogens in Horticultural Crops

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***Abstract*:** Cryotherapy has a wide range of applications in agriculture, horticulture, biotechnology and breeding programs. Cryopreservation can be used as an alternative preservation method for various crops. Ultralow temperatures can be employed not only for germplasm storage but also for the eradication of plant infections. It may potentially have applications in the protection of endangered plant species germplasm. A wide variety of horticultural crops, including fruits, vegetables, and ornamental plants, have cryopreservation techniques that can be utilized as such or adapted for cryotherapy, which intensifies the eradication of pathogens in infected plant tissues. Several pathogens in some of the horticultural crops such as banana, *Citrus spp.,* grapevine*, Prunus spp.,* raspberry, potato and sweet potato have been eradicated using cryotherapy. Disease-free stock plants are necessary for propagation and healthy materials are required for the exchange of germplasm across nations or regions via quarantine programs. In addition, plant gene banks seek to keep germ-plasm collections free of pathogens. To tackle this challenge cryotherapy can act as an innovative method for the elimination of plant pathogens. This review analysed several methods and procedures for cryopreservation of plant germplasm and how it is helpful in eradicating pathogens.

***Keywords*:** Pathogen, Cryotherapy, Germplasm, Horticultural crops, Viruses

Cryopreservation is accomplished by immersing plant materials at extremely cold temperatures, typically liquid nitrogen at -196 °C or liquid nitrogen vapour at -165ºC to -190ºC (Zhao et al 2019) utilized to eradicate viruses, phytoplasmas, and bacteria from plant meristems. Cryopreservation has various advantages for storing live plants for long periods of time, including minimal storage space and maintenance requirements, as well as maximum stability of phenotypic and genotypic traits of saved germplasm. Cell dehydration is regarded as an important step before immersing tissues in liquid nitrogen. Cryopreservation has been used effectively on a wide variety of plant species, including herbaceous plants from both tropical and temperate regions, as well as woody species. Viruses are commonly recognized to spread unevenly throughout plants, and meristems may be virus-free or have a low viral concentration. Cryopreservation of shoot tips involves the use of liquid nitrogen, which causes the cells in the lower area of the apical dome to be destroyed. Only a few of the cells in the top layers of the dome are able to survive. Therefore, there is a possibility that viruses can be eradicated from cryopreserved shoot tips (Wang et al 2009).

Cryotherapy of shoot tips using cryopreservation: Cryotherapy of shoot tips is a potential strategy for eradicating plant pathogens that may be easily applied to a wide range of species and cultivars using existing cryopreservation protocols (Fig. 1). It can be performed in a tissue culture laboratory with minimal equipment, exhibits promising results in the creation of pathogen-free regenerants, and reduces the danger of genetic alterations during therapy compared to traditional approaches. More than 10,000 accessions of in vitro propagated crop plants are currently safely cryopreserved for the long term, with more than 80 percent of these belonging to five major crops (potato, cassava, bananas, mulberry, and garlic). Cryotherapy is a novel biotechnology method that is based on cryopreservation (Wang et al 2014, Wang et al 2009). According to Wang et al (2008) cryopreservation of shoot tips is a technique in which shoot tips are induced to ultra-low temperature, stored and regenerated for multiplication. Shoot tips subjected to cryopreservation or cryotherapy are typically the size consisting of apical or lateral shoot meristem (1–1.5 mm in size) with three to four leaf primordia (Benson, 2007). Meristem cells are subjected to highly packing to leave without intercellular cavities, are small and isodiametric in structure and consist of a large nucleocytoplasmic volume ratio. Sizes of cells and vacuoles increase and the nucleocytoplasmic ratio decreases with increasing distance from the apical dome (Wang et al 2008).

Need for plant pathogen eradication and preservation:

Since the dawn of time, pathogens such as viruses, viroids, and phytoplasmas have posed a threat to horticultural growth (Zhao et al 2019). All crops must have access to pathogen-free planting materials in order to produce excellent yields and quality. There are many economically important crops that are usually propagated through the vegetative method so it makes susceptible to pathogen attack (Skoric 2017). Many economically important horticultural crops, such as citrus, pome and stone fruit trees, berry crops, and many ornamental plants have been attacked by different pathogens namely viruses, mycoplasma, and rickettsia diseases of fruit trees. According to Hogenhout et al (2008), few infections attack only tissues that enable their independent mobility and are irregularly distributed. Infected plants domes or the youngest part of the meristematic tissue, are usually either pathogen-free or have a very low concentration of viruses and phytoplasmas.

Cryopreservation of plant germplasm: There are many ways to preserve plant germplasm including seeds, tubers, roots, bulbs, corms, rhizomes, buds and cuttings. Since all biochemical and most physiological processes are halted at -196°C, cryopreservation or freeze-preservation, which entails storing the germplasm at ultra-low temperatures, is the approach of choice for long-term conservation. Recently, several plant species have been tested for this technology with a specific protocol like banana, citrus spp Grapevine, raspberry, sweet potato, etc.

Cryopreservation methods: The existing cryogenic strategies rely on air-drying, freeze dehydration, osmotic dehydration, the addition of penetrating cryoprotective substances and adaptive metabolism (hardening) or combinations of these processes.

Air drying: This is the most frequent approach for reducing cell water content in hydrated tissues, and it is sometimes referred to as the desiccation technique. This technique was mostly used on pollen, seeds, and embryogenic axes. The second common method of dehydration is air-drying, in which water is eliminated by airflow. In any case, dehydration is necessary for efficient cryopreservation to prevent intracellular freezing and permanent cell damage caused by ice crystal formation (Kaviani et al 2011).

Classical freezing protocol and preculture: Slow freezing and one-step freezing are the two most prevalent methods of cryopreservation (Kalaiselvi et al 2017). Samples must be prepared with cryoprotectants such as DMSO, sucrose, etc. before freezing slowly (also known as the two-step freezing procedure). They are then instantly submerged in liquid nitrogen and chilled to 40°C at the rate of 0.03–0.5°C/min. One-step freezing, on the other hand, does not necessitate any additional equipment. Cryogenic techniques like vitrification, encapsulation and cryoplates have recently been created despite the fact that older methods have been employed successfully on a variety of plant materials.

Vitrification: This approach is one of the most commonly used for plant cryopreservation since it is relatively simple to implement, does not require specialized equipment, and typically yields a high percentage of recovery. The formation of an amorphous glassy structure from intracellular solutes is the cornerstone of vitrification (Kaviani et al 2011). The dried material is then immersed in liquid nitrogen and rapidly frozen. PVS1, PVS2, and PVS3 are the three most commonly reported glycerol-based vitrification solutions. Two basic vitrification-based techniques, encapsulation vitrification and droplet vitrification have been developed

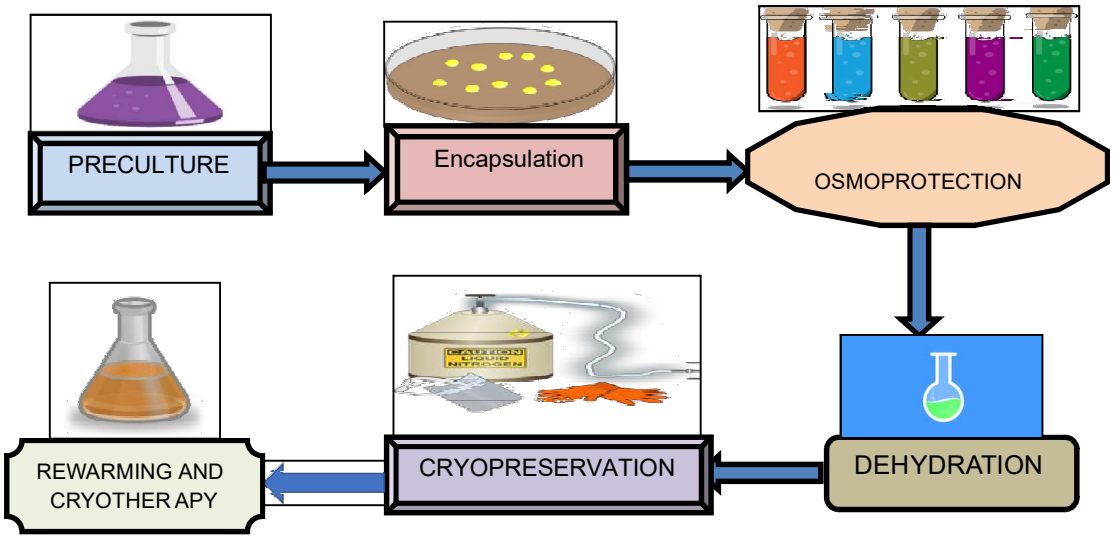

Fig. 1. Cryopreservation protocols for the cryotherapy of shoot tips

through the modification and optimization of vitrification procedures.

Cryo-Plate methods: Cryoplates are utilized in the most advanced cryogenic methods. On the basis of the dehydration operation, two basic cryoplate approaches may be identified: the V and D cryoplate methods (Matsumoto 2017). Explants are dehydrated using the V technique on cryoplates utilizing PVS2 vitrification (Yamamoto et al 2011), whereas explants are dehydrated using air using the D technique on cryoplates (Ninno et al 2013). Shot tips are put into tiny wells of an aluminum cryoplate loaded with alginate beads and dehydrated using either a PVS2 solution or air flow in a laminar flow cabinet, depending on the technique. The cryo plate is then submerged in liquid nitrogen instantly. Cryoplate methods are mostly user-friendly since manipulating samples on aluminum plates is straightforward (Yamamoto et al 2011, Ninno et al 2013). Several plant species, including the strawberry (Yamamoto et al 2012) and date palm (Salma et al 2014), have been successfully cryopreserved utilizing cryoplate techniques.

Dehydration: The first stage in cryopreservation is dehydrating tissues to remove water that can freeze. The water content of less than 0.25 g H_2O g/dm (dm; dry mass) is typically referred to as "unfreezable" water, and plant cells holding 0.25-0.4 g H_2O g/dm (dry mass) typically survive exposure to liquid nitrogen (Volk et al 2006). Proper dehydration can be achieved osmotically by administering extremely concentrated solutions; in this scenario, the concentration gradient between the solution and intracellular fluid provides the driving force for dehydration. The second common method of dehydration is air-drying, in which water is extracted by airflow. In any case, dehydration is necessary for efficient cryopreservation to prevent intracellular freezing and permanent cell damage caused by ice crystal formation (Kaviani et al 2011).

Pathogen eradication from economically important horticultural crops: There has been a lot of research done on cryotherapy employing shoot tips for pathogen eradication (Table 1). All *Prunus* spp. are susceptible to Sharka pox virus (Damsteegt et al 2007). The success rate was higher in cryo treated shoot tips, with 96 percent of the regenerated plants virus-free, compared to only 12 percent in meristem culture (Wang et al 2008). They also created a new technique for virus elimination in which RBDV-infected shoot tips (1 mm) were first treated to thermotherapy under *in-vitro* conditions, followed by cryotherapy. After 28 days of heat treatment, the survival (36%) and regeneration (30%) of cryo treated shoot tips were obtained using this method. Wang et al (2008) discovered that thermotherapy followed by cryotherapy resulted in virus-free plantlets in 33-35 percent of cases. According to studies, Ribavirin is the most important antiviral agent for controlling potato plant viruses when used alone or in combination with other treatments such as meristem culture, cryotherapy, and electrotherapy (Kushnarenko et al 2017). According to Waswa et al (2017), thermotherapy is one of the efficient strategies for eradicating the potato virus, which relies on the kind of virus, potato cultivar, and the length of heat treatment. Chemotherapy is ineffective when administered at low doses (Yang et al 2013), but when used at high doses, it generally inhibits potato plant development. As a result, by combining the two therapies, the regeneration capacity of plantlets can be improved.

Effects of combined treatments: Thermal, cold and chemotherapeutic treatments have been utilized in the past to remove infections from various plant species, and cryotherapy has also been used to enhance pathogen eradication (Jeon et al 2016, Barba et al 2017, Kushnarenko et al 2017, Zhao et al 2018). Combining thermotherapy and cryotherapy improves pathogen eradication because the pathogen-free regions are larger and fewer shoot tip cells

Table 1. Cryotherapy techniques' effectiveness in eliminating viruses from horticultural plant species

Plant species	Pathogen	Pathogen-free (%)		References
		Cryopreserved shoot tips	Shoot tip culture	
Malus x domestica	Apple stem grooving virus (ASGV)	30-100[a]	22	Zhao et al (2018)
Malus x domestica	Apple stem pitting virus (ASPV)	100	0	Bettoni et al (2018)
Vitis vinifera	Grapevine fanleaf virus (GFLV)	78	82	Marković et al (2015)
Prunus armeniaca	Plum pox virus (PPV)	100	-	Sekar et al (2015)
Solanum tuberosum	Potato virus M (PVM)	100[b]	-	Kushnarenko et al (2017)
Solanum tuberosum	Potato virus S (PVM)	100[b]	-	Kushnarenko et al (2017)
Allium sativum	Garlic common latent virus (GCLV)	80	13	Vieira et al (2015)
Allium sativum	Leek yellow strip virus (LYSV)	100[b]	35	Vieira et al (2015)

[a]Thermo+cryo; [b]Chemo+cryo

survive the cryotreatments (Wang 2008, Zhao et al 2018). Thermotherapy has some additional advantages as it reduces virus tire and also enhances virus-induced gene silencing (Liu et al 2016, Zhao et al 2018). As discussed earlier the combination of cryotherapy and thermotherapy resulted in 33 to 36% of the recovered plants being free of RBDV (Raspberry bushy dwarf virus (Wang et al 2008). Around 30 to 100% ASGV-free plants for four apple cultivars and rootstocks were obtained in a combination of thermotherapy and cryotherapy but this was not possible when shoot tips were treated only with cryotherapy treatment (Zhao et al 2018). Cold therapies have advantages when combined with thermotherapy because they limit viroid replication and reduce viroid mobility in the hosts (Barba et al 2017; Jeon et al 2016, Zhang et al 2016). Jeon et al (2016) discovered that cold therapy followed by cryotherapy improves CSVd (Chrysanthemum stunt viroid) removal from in vitro shoot tips. This combination (cold and heat treatment) resulted in 20% CSVd-free plants. However, only 13% of plants were viroid-free after being treated with cryotherapy alone (Zhao et al 2019). Aside from thermotherapy and cold therapy, chemotherapy has the potential to be useful since it suppresses viroid RNA production, reduces viroid titer, and expands viroid-free zones in the shoot tips (Barba et al 2017). 'Asana' and 'Nikitka' potato cultivars were free of PVM in 20% and 57 % of cases, respectively, however both cultivars were not free of PVS. It was estimated that 100 percent of the plants that had been regenerated were free of PVM and PVS after 135 days of in vitro ribavirin treatment (Kushnarenko et al 2017).

Advantages and limitations of cryotherapy: Using cryotherapy to remove diseases from numerous plant species can improve or perhaps replace some of the more traditional approaches. Studies have shown that cryotherapy methods are simple to implement and do not require specific tools or equipment beyond those found in a plant tissue culture facility. Furthermore, it makes it easier to process vast quantities of samples (Bhojwani and Dantu, 2013). It produces pathogen-free plants with a high frequency, minimizing the challenges associated with the excision of extremely small meristems. The vegetative and root growth of virus-free plants was substantially greater than that of virus-infected plants (Li et al 2018a).

CONCLUSION

Cryotherapy is a viable, well-organized approach for the successful removal of plant diseases in a wide range of horticulture species. Cryogenic techniques limit the amount of viable contaminated tissue in several fruits, vegetables, and ornamental plants, cryopreserved shoot tips may be regarded as safer for exporting germplasm between regions and countries. For instance, post-thaw regeneration of major agricultural species remains quite low. In this study, we demonstrate that much of the work in the field of plant cryobiotechnology has already been completed. However, significant obstacles continue to impede the expansion of cryopreservation's use. Variations in morphological and yield variance in micro propagated bananas have been found under *in vitro* conditions. As a result, the phytosanitary status of regenerated plants must still be determined. Plants regenerated after cryotherapy should be tested for true-to-type since tissue culture can cause genetic instability in plants. More plant species and cultivars will necessitate the optimization and modification of existing techniques in the foreseeable future.

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