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INVITED REVIEW

Plant cryopreservation: a continuing requirement for food and ecosystem security

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Abstract This issue of *In Vitro Cellular and Developmental Biology—Plant* is dedicated to current developments in liquid-nitrogen cryopreservation methods and their use in plant biology and germplasm preservation. The development of cryopreservation for storage of plant cells, tissues, and organs began in the 1960s and continues to this day. Long-term storage of *in vitro* cultures of secondary metabolite cell cultures, embryogenic cultures, clonal germplasm, endangered species, and transgenic products remains an important requirement for many scientists, organizations, and companies. The continued development of cryopreservation techniques and their application to new plants is the subject of this issue.

Keywords Cryopreservation · Cryotherapy · Food security · Genetic stability · Germplasm preservation

Introduction

Over the past 50 years, plant cryopreservation has developed from studies of the basic biology of freezing (Sakai 1965) and initial recovery of callus cells (Quatrano 1968) to standardized systems for storing plant organs, cells, and tissues in liquid nitrogen (Reed 2008b). Gradual improvements in the techniques and technology allowed the initial efforts involving controlled-rate cooling (Sakai *et al.* 1978; Withers and King 1980; Kartha *et al.* 1982; Towill 1983) to evolve and provide successful storage of the shoot tips of temperate plants (Reed and Hummer 1995; Sakai 1995; Reed and Chang 1997). Further improvements, including the development of various forms of vitrification techniques, resulted in the expansion of cryopreservation to a wider range of plant types and tissues that continue today (Fabre and Dereuddre 1990; Niino *et al.* 1992, 1997; Engelmann 2004; Staats *et al.* 2006, 2008; Yamamoto *et al.* 2011; Engelmann 2011; Engelmann and Dussert 2013).

Types of cryopreservation

Many techniques are used for the diverse plants and propagules that are stored. Often, more than one method is suitable (Table 1).

Dehydration Seed cryopreservation is well studied and the responses of seeds to dehydration and cold temperatures are used to categorize them as orthodox or recalcitrant. Orthodox seeds can be dried to a low moisture content and in that state, they are resistant to cold temperatures. Orthodox seeds are routinely stored at subfreezing temperatures and sometimes in liquid nitrogen or its vapor (Pritchard and Nadarajan 2008). Seeds that cannot withstand drying and/or cold temperatures are considered recalcitrant. These include large-seeded trees, seeds with high oil content, and the seeds of many tropical plants (Walters *et al.* 2008). These recalcitrant seeds can sometimes be stored as dehydrated isolated embryonic axes (Normah and Makeen 2008), or the seeds germinated and cryopreserved by the methods used for vegetative tissues (Normah *et al.* 2012). Dehydration is also a key aspect of dormant bud cryopreservation in addition to natural cold acclimation of the twigs (Harvengt *et al.* 2004; Towill and Ellis 2008; Jenderek *et al.* 2014).

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Table 1. Cryopreservation techniques that may be suitable for various plant or tissue types (modified from Reed 2011)

Plant type/technique	Controlled-rate cooling	Vitrification techniques (liquid)	Vitrification by encapsulation dehydration	Dormant buds	Desiccation only
Tropical plants desiccation tolerant		X	X		
Tropical plants desiccation sensitive		X			
Subtropical plants desiccation sensitive	X	X			
Subtropical plants desiccation tolerant	X	X	X		
Subtropical plants cold tolerant	X	X	X		
Temperate herbaceous plants desiccation tolerant	X	X	X		
Temperate herbaceous plants desiccation sensitive	X	X			
Temperate woody plants moderately cold hardy	X	X	X	X	
Suspension cultures, callus, somatic embryo	X	X	X		X
Embryonic axis, orthodox seed, pollen		X			X

Controlled-rate cooling The storage of cells, tissues, and organs in liquid nitrogen is based on the premise that all or nearly all metabolic activity is arrested at the temperature of liquid nitrogen, −196°C. The initial cryopreservation techniques involving controlled-rate cooling were based on the development of ice in the intercellular spaces which established an osmotic gradient, removing liquid water from the cells, and dehydrating them (Levitt 1980). Treatment with cryoprotectants and slow cooling were combined with nucleation of the solution at a high subfreezing temperature (~ − 5° to − 7°C) to form ice in the intercellular spaces (Sakai *et al.* 1978; Kartha *et al.* 1982) and allowed continued ice formation, precluding supercooling of the cells with resulting crystallization at low temperatures (Wisniewski and Fuller 1999). Due to osmotic dehydration, the cytoplasm became concentrated to the point where it vitrified (turned to a glass) when the samples were plunged into liquid nitrogen. The rate of cooling and the temperature at which the specimens were plunged regulated the movement of water and controlled the dehydration of the cells. Cell death was due to inadequate dehydration, resulting in ice crystal formation in the cells, or death by excessive dehydration.

Vitrification Cryopreservation obtained in a manner that eliminated all ice formation was a major goal of basic research during the 1980s and 1990s. The addition of concentrated cryoprotective solutions is a critical step that provides increased viscosity, suitable cellular dehydration, and suppression of ice formation (Volk and Walters 2010). Treatment with these solutions in combination with osmotic or cold temperature *in vitro* pretreatments allows successful cryopreservation of many plant types. The type of container used, its size, and the amount of vitrification solution can each affect the outcome of the procedure as they affect the cooling rate (Wesley-Smith *et al.* 2001). Vitrification techniques are now available in many forms including liquid solutions such as the plant vitrification solution (PVS) series developed by A. Sakai (Sakai *et al.* 1991; Matsumoto *et al.* 2001) (PVS2 and 3 are most used), Steponkus (1985), or Kim *et al.* (2009). These same solutions are used as a single droplet on aluminum foil for very rapid cooling in droplet vitrification protocols. In addition, encapsulation in alginate beads followed by sucrose loading and desiccation (encapsulation dehydration), and combinations of encapsulation and vitrification solutions are commonly used. The most recent modifications involve the use of aluminum cryoplates with encapsulation dehydration or encapsulation vitrification (Yamamoto *et al.* 2011, 2012).

Protocols

Cryopreservation success is heavily dependent on well-developed protocols and how they are implemented (Reed 2001). Protocols may specify pre-growth conditions, the type of plant material used, size of propagules, pretreatments, cryoprotectants, cooling and rewarming procedures, type of vial used, and regrowth conditions and procedures (Reed 2008a). Most depend on specific tissues and detailed requirements for *in vitro* culture or pre-cryopreservation treatments. Explant size, physiological condition, and acclimation to osmotic stress or cold conditions are all important to the outcome of a protocol. In addition, careful attention to all steps of a protocol is required as each optimized step can have a large impact on the recovery of living cells or tissues. Each step of a protocol has an important function, from the pre-growth steps to cryoprotection to rewarming to regrowth. Deviations from the original protocol should be avoided unless recovery is poor (Reed 2008a).

Recovery

Regrowth of cells or tissues is the true test of a successful protocol. Only the full recovery of the required cell cultures or a multiplying shoot culture is adequate for judging the success of a protocol. The use of early "survival" data distracts from the fact that these data rarely reflect the final outcome of useable cultures or plants. Recovery involves all factors of *in vitro* growth. For seeds, optimized germination conditions are important. Growth-medium optimization (Chang and Reed 1999; Pennycooke and Towill 2000), room temperature, light exposure (Benson 1990), plant growth regulators (Chang and Reed 2001; Volk and Walters 2010), and antioxidants (Uchendu *et al.* 2009, 2010, 2013; Uchendu and Keller 2016) all have an effect on *in vitro* recovery.

Importance of cryopreservation

The twentieth century of cryobiological research was devoted to basic studies of ice formation, vitrification of solutions, and the beginnings of cryopreservation as a long-term storage technique. The twenty-first century is when the bulk of the storage will take place based on those scientific principles. The myriad of plant species and cultivars that have been or are being stored highlight the success of these techniques and the clear need for continued support for long-term storage. Orthodox seeds, tolerant of drying and cold temperatures, have long been stored in liquid nitrogen or at subfreezing temperatures, but new cryopreservation techniques now allow storage of many recalcitrant seed types and vegetatively propagated plants in one form or another. Engelmann (2011; Engelmann and Dussert 2013) cited numerous examples of cryostorage of orthodox and recalcitrant seeds, callus cultures for pharmaceutical production, forestry and crop embryogenic cultures, lower plants such as bryophytes and ferns, tropical plants, a wide range of endangered species from orchids to wild citrus, as well as large collections of shoot cultures of vegetables, fruit, and ornamental plants.

These cryopreserved collections are invaluable to the future of plant breeding and ecosystem restoration. They provide important backup collections for vegetatively propagated plants and those with small natural populations or those threatened by human development, environmental change, or development of new diseases. Cryopreservation allows the storage of unique genotypes at a lower long-term cost than field collections or constant regeneration of seed collections (Engelmann 2011). Additional value comes from the use of cryotherapy to remove viruses from vegetatively propagated crops (Brison *et al.* 1997; Waterworth and Hadidi 1998; Wang *et al.* 2003). While several therapies are available for eliminating viruses from plants, cryotherapy provides an additional technique that may be more effective for some crops. In general, cryopreserved collections provide long-term security for plant genetic resources of all types. They provide a secure backup for field collections, insure that little-used but unique genotypes are preserved, store research materials that would otherwise be discarded, save important disease resistance genes, and save genes that may be important in combating future challenges.

The contributions in the present issue illustrate the wide diversity of projects in progress throughout the world and the value of cryostored collections to a multitude of countries. They include the work of national and international genebanks, universities, research institutes, and botanical gardens. The plants studied are as diverse as the countries and institutions represented, covering a wide range of unique wild flora, ornamentals, fruit and vegetable crops, medicinal herbs, and endangered species. The propagules stored include pollen, embryos, cell suspensions, embryogenic callus, and shoot cultures.

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