

CRYOPRESERVATION OF CITRUS GERMLASM

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Summary

Cryopreservation can give an important contribution to the long-term conservation of valuable citrus germplasm. In recent years, numerous reports have shown the possibility to store in liquid nitrogen (-196°C) various organs and tissues from *in vitro* culture of *Citrus* spp. The traditional cryogenic technique (“slow-cooling”) and innovative “vitrification/one-step freezing” procedures have all been successfully applied to citrus cryopreservation.

Keywords

Citrus, cryopreservation, embryogenic lines, shoot tips, seeds.

Traditionally, *Citrus* germplasm is conserved in clonal orchards belonging to botanical gardens or to scientific institutions. Genotypes of particular value are kept in greenhouses and screenhouses, where they can be more easily protected from losses due to pests, diseases and climatic hazards. However, the high costs of this traditional conservation system limit the number of accessions that can be preserved [4]. Moreover, although many citrus species are polyembryonic, meaning that several asexually-originated nucellar embryos are contained in the seed at maturation, the conservation of germplasm in clonal seed banks is difficult to apply, as many species have seeds recalcitrant to desiccation (i.e., non- or sub-orthodox seeds). Hence, conservation in liquid nitrogen (LN, at -196°C) should be considered as an alternative method for the long-term conservation of *Citrus* genetic resources.

The “slow-cooling” technique was the first approach to the cryopreservation of woody plant germplasm. Then, various technologically-innovative procedures were developed aiming at the vitrification and the direct plunging of specimens into LN (“one-step freezing”), such as the “use of vitrification solutions”, the “encapsulation-dehydration” and the “encapsulation-vitrification” techniques. Alternatively, a simple dehydration procedure can be used when seeds or isolated embryo axes have to be cryostored (see in this book “Status of cryopreservation technologies in plants: crops and forest trees” by B. Panis and M. Lambardi). Many reports are now available, showing that the cryogenic technology can be an important tool for the long-term preservation of *Citrus* explants, including embryogenic lines, somatic embryos, shoot tips, ovules, pollen, embryo axes and seeds.

Organs and tissues utilized in *Citrus* cryopreservation

Embryogenic callus lines and somatic embryos

Various procedures have been used for the cryopreservation of embryogenic callus lines from a wide range of citrus species and cultivars [4]. Many successful procedures (*C. aurantifolia*, *C. aurantium*, *C. deliciosa*, *C. limon*, *C. sinensis*, *C. paradisi*) are based upon the slow-cooling technique, consisting in the pre-treatment of embryogenic callus samples with a mixture of cryoprotectants (mainly, DMSO at 5-15% v/v and sucrose at 0.15-1.2 M concentrations), before being slowly cooled to -40°C (0.5-1°C/min) and then immersed in LN. The PVS2 (i.e., today the most widely used vitrification solution) was developed for the

first time working with embryogenic cell lines of navel sweet orange (*C. sinensis* [5]), and later on successfully used also with grapefruit (*C. paradisi*) and sudachi (*C. sudachi*).

Cryopreservation of somatic embryos, produced by *in vitro* cultured ovules of Washington navel sweet orange, was first achieved using a slow-cooling protocol (0.5°C/min up to -42°C), followed by immersion in LN [1]. However, also the encapsulation–dehydration technique has repeatedly been shown to be an effective approach to *Citrus* somatic embryo cryopreservation. High survival rates (up to 100%) were obtained after the beads (containing somatic embryos) had been pre-treated on media with high sucrose concentration, dehydrated below 25% moisture content and direct immersed in LN.

Shoot tips

Shoot tips are by far the most widely used explants for cryopreservation of vegetatively-propagated woody plants, as in this way the maintenance of genetic fidelity to the donor plant is assured. Over time, all the 3 main approaches to “vitrification/one-step freezing” have been successfully applied to the cryopreservation of *Citrus* species. Thirteen citrus cultivars have been cryopreserved by loading the shoot tips in PVS2, prior to their direct immersion in LN, obtaining 84% survival on average [6]. The encapsulation-dehydration technique has been reported for two *Citrus* rootstocks (*Poncirus trifoliata* and citrange), while an encapsulation-vitrification procedure has been developed with the sour orange (*C. aurantium*).

Ovules and pollen

In *Citrus*, the survival of ovules to cryopreservation has been shown to be very erratic. Differently, the pollen of 4 cultivars of *C. limon* has been successfully stored in LN for up to 3 and a half years.

Embryo axes and intact seeds

Embryo axes, isolated from seeds, have been shown to be an excellent explant for *Citrus* cryopreservation. Indeed, embryo axes of *C. aurantifolia*, *C. halimii*, *C. madurensis*, *C. sinensis*, *P. trifoliata* and *C. hystrix* survived storage in LN and germinated after thawing. One-hundred percent survival was obtained with *C. aurantifolia* and *C. halimii*, after the embryo axes were dehydrated to below 17% in air-flow and directly immersed in LN [3]. Successful cryopreservation of intact seeds of *Citrus* has been reported for *C. aurantifolia*, *C. halimii*, *C. sinensis*, *C. aurantium* and *C. limon*. The seeds are generally cryopreserved by direct immersion in LN, following a partial dehydration under air-flow or in silica gel. Highest survival rates (93%) are reported for seeds of *C. sinensis* and *C. aurantium*, provided that the intact seeds are dehydrated in the sterile air flow of a laminar flow hood down to a moisture content of 16% and 10%, respectively [2]. In the latter report, it was shown that, working with polyembryonic citrus species, the dehydration/cryopreservation procedure can promote the germination of also the zygotic embryo, together with the nucellar embryos.

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