

CRYOPRESERVATION AS A TOOL FOR THE LONG-TERM CONSERVATION OF WOODY PLANT GERMPLASM: DEVELOPMENT OF THE TECHNOLOGY AT THE CNR/IVALSA INSTITUTE OF FLORENCE

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Summary

Recent advances in cryopreservation have paved the way to the safeguarding of woody plant biodiversity with a biotechnological approach, which can be regarded as complementary to the traditional clonal orchards and seed banks. Here, the main results obtained at the CNR/IVALSA Institute of Florence are reported, with reference to the cryopreservation of shoot tips of poplar, plum, olive, chestnut and fruit rootstocks, dormant buds of persimmon and olive, seeds of *Citrus* spp., and embryogenic lines of olive, horsechestnut and ash.

Keywords

Cryopreservation, encapsulation-dehydration, germplasm, vitrification, woody species

Introduction

The preservation of plant biodiversity combats the risk of “genetic erosion”, i.e., the risk that species, forms and plant varieties may become extinct, producing a definitive loss of the genetic variability which they contain. As concerns fruit and timber trees, the cultivation of which is characterized by the use of a more and more restricted number of selected cultivars or provenances, the establishment of in-field clonal collections (for vegetatively propagated species) and seed banks (for seed propagated species) are the traditional approaches to *ex situ* germplasm preservation. However, clonal orchards require vast areas of lands, their management is very expensive, and the system runs the risks arising from biotic and abiotic stress. Moreover, many woody species have non-orthodox seeds which cannot be preserved in seed banks. Hence, the cryopreservation of germplasm can be an important complementary approach to the safeguarding of woody plant biodiversity, assuming that the development of a low-input and widely-applicable technology is pursued. At a cryogenic temperature, the rate of chemical and biophysical reactions is so slow that the biological growth and development of the ultra-frozen organ/tissue are hampered, while, if a proper technique of ultra-rapid freezing is applied, cell survival is not affected. In theory, germplasm cryopreservation can be considered unlimited in terms of time.

Cryopreservation by vitrification/one-step freezing

Cryopreservation is the storage at ultra-low temperature (mainly -196°C , i.e., the temperature of liquid nitrogen) of organs and tissues from *in vitro* culture, such as buds, shoot tips, zygotic and somatic embryos, pollen and cell cultures. Although the technique was first introduced in plants in the '70s, it has never been applied on a wide scale due to the high cost of cryo-freezers; indeed, in order to escape the formation of lethal intracellular ice crystals, time-consuming and laborious slow-cooling procedures have had to be used. New cryogenic techniques are now available, aiming at the direct immersion in liquid nitrogen (“one-step freezing”) of plant specimens from tissue culture, without resorting to expensive apparatus for slow cooling and with a considerable simplification of procedures. This technology is based on the induction of cell vitrification during a very fast decrease of temperature. “Vitrification” of cells and tissues is the physical process

which avoids intracellular ice crystallization during ultra-freezing by the transition of the aqueous solution of the cytosol into an amorphous glassy state. As a consequence of this process, plant tissues are protected from damage and remain viable during their long-term storage at -196°C . In the most recent approaches to cryopreservation, vitrification can be induced in two different ways, i.e., (i) by treating the explants with a highly concentrated vitrification solution (mainly, a mixture of glycerol, ethylene glycol and DMSO, named “PVS2”), or (ii) by partial dehydration over silica gel desiccant of explants, naked or encapsulated in calcium-alginate beads. It is noteworthy that, following these procedures, the plant specimens can be directly plunged into liquid nitrogen, where they can be stored for an indefinite period of time without undergoing the risks of contamination or genetic alteration.

Procedures developed at the CNR/IVALSA Institute of Florence

Taking advantage from a long expertise in tissue culture, in the year 1996 a research line on cryopreservation was initiated at the CNR/IVALSA Institute of Florence, “Istituto per la Valorizzazione del Legno e delle Specie Arboree” (*Trees and Timber Institute*), laboratory of “*In vitro* conservation and cryopreservation”. The study aims at the development and exploitation of the cryogenic technology for the long-term preservation of vegetatively- and seed-propagated woody plants. In time, various vitrification/one-step freezing procedures have been investigated (i.e., loading of explants with the PVS2 solutions, dehydration, encapsulation-dehydration, encapsulation-vitrification) and applied to the cryopreservation of different explants, such as shoot tips, nodal segments, dormant buds, seeds, embryogenic callus. Considerable results have already been achieved with the cryopreservation of shoot tips of white poplar (*Populus alba*; [3]), grey poplar (*P. canescens*; [2]), and plum (*Prunus domestica*; [1]), while similar investigations are in progress with apple and pear rootstocks, olive (*Olea europaea*), chestnut (*Castanea sativa*) and redwood (*Sequoia sempervirens*). Moreover, a recently undertaken research line concerns the cryopreservation of dormant axillary buds, directly collected in the field, from persimmon (*Diospyros kaki*) and olive.

As for seed cryopreservation, an effective procedure by dehydration and direct immersion in liquid nitrogen has been developed for seeds of polyembryonic *Citrus* species; the method promoted the recover of both the zygotic and the nucellar embryos [5].

The storage in liquid nitrogen of cells from embryogenic callus lines is another application of relevant scientific interest. For instance, the establishment of “cryobanks” of embryogenic cultures would allow for the safe and long-term storage of transgenic lines, during the time required for the verification of transgenic plant characteristics; as well as, for the preservation of stock cell cultures used for secondary metabolite production, in order to avoid their decline due to repeated subculturing. At the IVALSA’s cryopreservation laboratory, very promising results have been already obtained with the storage at -196°C of embryogenic lines of olive [4], horsechestnut (*Aesculus hippocastanum*) and ash (*Fraxinus angustifolia*).

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