COSMOS: Journal of Engineering & Technology
A Refereed Research Journal
Vol 4 / No 1 / Jan - Jun 2014 ISSN: 2231-4210

THE DISCUSSION ON CRYOPROTECTION AND CRYOPRESERVATION USING VACUUM INFILTRATION VITRIFICATION

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Abstract

The present paper explain exposure to PVS must be controlled to enable sufficient cellular dehydration whilst limiting injury from chemical toxicity and establishment of a simple and high-throughput cryopreservation method using cryoprotectant is highly desirable.

Keywords: Cryoprotection, Cryopreservation, Vacuum Infiltration.

Introduction

The vitrification process plays a key role in cryopreservation for the long term conservation of plant genetic resources. Vitrification is defined as a physical process by which a concentrated aqueous solution solidifies into a stable amorphous glass without the formation of ice crystals when the temperature is decreased. Vitrification of plant specimens can be achieved in many ways, including air drying of embryos, and more recently through the use of highly concentrated plant vitrification solutions (PVS) that readily form glasses on cooling and inhibit crystallization. However, exposure to PVS must be controlled to enable sufficient cellular dehydration whilst limiting injury from chemical toxicity and establishment of a simple and high-throughput cryopreservation method using cryoprotectant is highly desirable. Plant vitrification solutions combine cryoprotectants that vary in permeability [e.g. dimethyl sulphoxide (DMSO) and glycerol], such that cellular water is replaced, cell viscosity is increased and the freezing behaviour of the remaining water is altered. PVS2 is probably the most commonly used cryoprotectant (CP) for plant cells, tissues and embryos; for example, the cryopreservation of embryonic axes of citrus and in vitro shoot-tips of Parkia speciosa, a tropical species with recalcitrant (desiccation sensitive) seeds.

The conventional approach to verification generally involves a tissue pre-culture step on sucrose-enriched medium, followed by treatment with a loading solution and dehydration, before cooling, with highly concentrated verification solution for a period that varies with species, tissue and temperature. For example, 60 min PVS2 treatment was optimal for cryopreserving embryonic axes of Parkia speciosa. Moreover, protocorm-like bodies of Dendrobium orchid and Citrusmadurensis embryonic axes required shorter PVS exposure times at 25°C than 0°C, i.e. 20 versus 60 min respectively. Whilst the time window for optimum PVS treatment is wider at lower temperatures, tropical species tend to respond better with warmer temperature treatment, e.g. Colocasia esculenta.

The physical dimensions (geometry) and permeability characteristics of the tissue under investigation profoundly affect the outcome of the

cryoprotection and cryopreservation procedures. Smaller apices (1.5 or 3 mm in diameter) of garlic displayed higher regeneration after cryopreservation than large ones (4.5 mm in diameter). Similarly, Nephelium ramboutan-ake shoot-tips of c. 2 mm tolerated cryopreservation well, as did 0.8 mm diameter axillary buds of Colocasia esculenta . The cryopreservation of mature zygotic embryos of recalcitrant seeds generally requires a reduction in tissue mass to facilitate cryoprotectant uptake. Usually, this involves the excision of the embryonic axis. In axes of recalcitrant seeds of sweet chestnut (Castanea sativa) such surgical intervention results in a burst of superoxide (O_2), with further oxidative stress during subsequent desiccation. In this context, the free radical scavenging capability of CPs is additionally crucial for survival of cryopreservation procedures, assuming that the protectants permeate sufficiently. Permeation of chemicals into the intercellular spaces and cells of plant tissues is compounded by many features (e.g. mass, morphology, cellular anatomy and chemical composition). To enable the rapid permeation of the viability stain, triphenyl tetrazolium chloride (TTC), into oily tissues of pine seed, we previously used vacuum infiltration. Similarly, this system has been used to improve efficient gene transformation, the delivery of pathogenic bacteria into the intercellular spaces of plants to study pathogen-plant cell

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interactions and the diffusion of an inhibitor of ethylene action so that pear fruits have prolonged storage. In addition, preliminary studies have shown that vacuum-assisted glycerol cryoprotectant infiltration can preserve the normal histology of rat leg muscle with no ice crystal formation after 3 weeks storage at −80°C . In this study, we developed and compared the efficacy of vacuum infiltration vitrification (VIV) using PVS_2 for the cryopreservation of seed embryos of three species with varying morphology, stress physiology and chemistry: Carica papaya (Caricaceae); Passiflora edulis (Passifloraceae); and Laurus nobilis (Lauraceae).

These species have purported differences in seed storage characteristics. C. papaya has a high level of desiccation tolerance to about 5% moisture content, limited (months) storability at −20°C, but tolerance of cryopreservation. P. edulis seeds may show reduced viability after drying to 5–6% moisture content, but the majority of dry seeds tolerate cryopreservation. Both species have spatulate embryos in copious endosperm. Finally, L. Nobilis has seeds with a lowest safe moisture content of 24%, below which they are desiccation sensitive and successful moist storage at 0°C is limited to about 4 months. L. Nobilisembryos are linear and bigger (c. 5 mg dry mass) than both C. papaya and P. Edulis. An unifying feature of these three species is that their seeds contain c. 25% oil. However, fatty acid composition varies greatly. Whilst L. Nobilis seed has c. 50% saturated fat, mainly laurate (12:0), C. Papaya and P. Edulis seeds are about 80% unsaturated fat with either oleic (18:1) or linoleic (18:2) as the main (c. 80%) fatty acid, respectively . Consequently, the thermal behavior of the lipids, and visco-elastic properties of the specimens, should vary between species and this has been correlated with poor dry seed storage performance at c. −20°C in some species, e.g. in an orchid and a few Cuphea sp. Our primary objective in this study is to investigate whether vacuum infiltration vitrification (VIV)-cryopreservation is effective at preserving embryos of species from different provenances (i.e. Mediterranean to tropical), and with disparate morphology, chemistry and physiology. Given that seed desiccation sensitivity in plants correlates strongly with tropical moist forest habitats, in which the majority of the world's plant species grow and which are under continuing threat of deforestation, resolving the ex situ conservation options for such species is an urgent imperative.

Materials and Methods

Plant Material

The varieties of Solanum tuberosum 'Desiree', 'Ackersegen', 'King Edward', as well as the material of S. Acaule, and S. Demissum were received from the Cultivated Potato Collection, Germany, situated in the Northern Branch Station of IPK in Grob Lusewitz. 'Desiree', 'Ackersegen', and 'King Edward' were supplied as in vitro plants, S. Acaule as tuber and S. Demissum as seeds.

Explant Sterilization

Tubers of S. Acaule were put in the dark for sprouting for 8 weeks. Dark-grown sprouts were cut in nodal segments and rinsed in 70% ethanol. Afterwards segments were placed into an Erlenmeyer flask and shaken in sodium hypochlorite solution with 3% active chlorine for 10 min. Then segments were washed three times with sterile water under sterile conditions. Nodal segments were dried on filter paper and bleached ends were cut off. At last segments were put onto MS medium (Murashige and Skoog, 1962) with 3% sucrose for the establishment of in vitro culture.

S. Demissum seeds were also washed in sodium hypochlorite solution with 3% active chlorine for 10 min and rinsed with sterile water afterwards. Seeds were placed on MS medium with 3% sucrose for germination.

Conclusion

The aim of this study was to get deeper insight into the function of the DMSO droplet cryopreservation method and the changes in biochemical characteristics which occur during pre-culture with constant and alternating temperatures. Cold tolerances were determined by electrolyte leakage tests and revealed, that no change in cold tolerances and, therefore, no cold acclimation takes place in wild and cultivated potato cultures after AT. The better regeneration results after AT of donor plants and cryopreservation can, therefore, not be directly related to cold acclimation. After AT biochemical changes in shoot tips consisted of slight increase in soluble sugars, slight decrease in starch and amino acid concentration. The increase in soluble sugars could explain the better cryopreservation results. Slight decrease in starch and amino acid concentration confirm the fact that no cold acclimation occurs. The proteome analysis does not give clearly interpretable results, because of the low number of proteins identified. But the change in protein expression between shoot tips of plants treated with CT and AT, respectively, was small. This is in accord with the other biochemical compounds, which do not show strong changes after AT in comparison to CT. Also in the ultra structure

there were no significant changes between shoot tips isolated directly after CT and AT, which is in concordance with the biochemical results. However, cry preserved and rewarmed shoot tips were slightly less damaged when precultured with AT in contrast to CT. TEM revealed that the DMSO cryoprotectant incubation is the step, after which ultra structure is changed strongly. DSC analysis showed that incubation in DMSO solution is necessary to depress the freezing point and to lower the amount of freezable water. These analyses made clear, that the incubation of shoot tips in DMSO is the critical step of the method.

Immediately after rewarming, shoot tips showed similar structure as before cooling. The influence of LN on ultra structure can, therefore, not be seen directly after this stage. Microscopic studies revealed that survival was, however, detectable 2 days later. Only small areas of the original shoot tips survived, mainly in leaf primordia and sometimes in the meristematic dome. Here cells showed mitotic activity and regrowth of shoot tips. Donor plants subjected to AT had mainly significant higher shoot regeneration than plants precultured in CT.

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