IN VITRO CONSERVATION OF HORTICULTURAL SPECIES

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Abstract

For medium term conservation of horticultural species, various routine techniques are now employed extensively. For long term conservation, cryopreservation (-196°C) is presently the only technique available. It has been applied to more than 40 horticultural species. However, its routine use still remains exceptional. The current development of new storage techniques is discussed.

Additional index words
medium term storage ; controlled atmosphere : long term storage ; cryopreservation ; encapsulation/dehydration ; vitrification ; domestic freezer

1. Introduction

During the last ten years, the importance of *in vitro* culture techniques increased dramatically, since they have been applied to more than 1 000 plant species. This way of propagation is indeed very interesting for large scale production as well as for genetic resources conservation, due to its numerous advantages : high multiplication rates, culture in aseptic conditions, production of virus-free material, reduction of surface and costs in labour and financial terms, etc...

In vitro culture is now used for industrial propagation in agriculture and horticulture. Indeed, 212 10^6 plants have been produced in Europe in 1988 using this method (Anon., 1990), including many ornementals. The storage of *in vitro* material becomes now an imperative necessity, in order to face the two major following problems : laboratory management, with the constant creation of new clones, the maintenance of those which are not multiplied for a given period of time and the seasonal production of many species ; risks of genetic variation, which increase with the culture duration in axenic conditions, and can lead to the production of plants which are not true to type.

In this paper, we will make a rapid survey of the various methods set up for the conservation of plant organs produced *in vitro*, focusing our attention on the horticultural species.

The techniques are different depending on the storage duration requested. For short to medium term conservation (several months to 2-3 years), growth reduction will be sought, in order to increase the intervals between subcultures. This storage can be carried out in a cold room or in a standard culture chamber. Various parameters, temperature being the most commonly used, can be modified, in order to limit organogenesis. They are employed together or separately. For long term conservation, cryopreservation, that is storage at ultra-low temperature, generally that of liquid nitrogen (LN, -196°C), is the only method currently available. At this temperature, all cell divisions and metabolic processes are stopped. The material can thus be conserved without alterations ~~or~~ modifications for theoretically illimited periods of time. Moreover, the cultures are stored in a small volume, sheltered from contaminations, with a very limited maintenance. This technique will be used for the conservation of material with important characteristics during extended periods.

2. Short and medium term storage

2.1 The main parameters

2.1.1 Temperature

Growth reduction is generally obtained by lowering the culture temperature to 0/5°C, for plants from temperate climates, which are cold resistant. Subcultures can thus be avoided for important durations, up to 51 months as in the case of *Malus domestica* plantlets (Druart, 1985). With sub-tropical or tropical plants, the storage temperature must be higher (8 to 20°C), depending on the cold sensitivity of the species. Light intensity reduction or suppression are oftenly used concomitantly with temperature reduction.

2.1.2 Culture medium

Various modifications can be made to the standard culture medium, such as : lowering the sugar and/or mineral elements concentration ; adding cryoprotective or osmotic agents (mannitol, sucrose) ; growth retardants (ABA, paclobutrazol). Finally, other substances such as activated charcoal can be used.

2.1.3 Modification of gaseous environment

Several methods exist, which allow to limit the quantity of oxygen available to the cultures. The easiest way consists in covering the explants with a layer of mineral oil (Caplin, 1959). However, its use with differentiated cultures poses some problems, the regrowth of the explants after the storage period being in one case suppressed (Chatti-Dridi, 1988) or delayed in the other (Jouve and Engelmann, 1991).

Another method consists in lowering the partial oxygen pressure, by reducing the atmospheric pressure in the culture chamber or by injecting a mixture of nitrogen and oxygen (controlled atmosphere).

The first experiments have been carried out by Bridgen and Staby (1981) with tobacco and chrysanthemum plants, which could be stored for 6 weeks under 1.3 % oxygen. More recently, this technique was successfully applied for a 4 month storage period under 1% oxygen of oil palm somatic embryos (Engelmann, 1990a). Regnard *et al.* (1990) could store peach shoots under hypoxia for up to 6 months. This method seems particularly interesting for tropical plants, for which temperature reduction cannot be applied, due to their cold sensitivity.

2.1.4. Type and physiological stage of the explants

The explants must have a minimal size, generally more than 1 cm. The presence of roots increases oftenly the survival potentialities, as observed with various materials.

2.2. Results

The techniques described above are now routinely employed in the laboratories, but only a few papers are published on this subject. Table 1 presents a list of horticultural crops for which research on slow growth storage has been published. For more complete references, see notably Aitken-Christie and Singh (1987), Wilkins *et al.* (1989), Engelmann (1991).

3. Long term conservation

3.1. Classical protocols

A standard cryopreservation protocol is a sophisticated process which comprizes several successive steps : choice and obtainement of starting material, pretreatment, freezing, storage, thawing, and post-treatment, for which optimal conditions have to be defined with each new species. All these procedures have already been described in details in many review articles (see Kartha, 1985 ; Dereuddre and Engelmann, 1987).

In the case of horticultural species, cryopreservation has been applied to more than 50 different species, in the form of cell suspensions, calluses, meristems, somatic, pollinic and zygotic embryos (table 2). However, in many cases, resistance to freezing in LN has been proved at the laboratory level, but it does not necessarily imply that this technique is effectively used for the germplasm storage of many species. The first two examples of experimenting cryopreservation as a routine technique concern oil palm (Engelmann, 1990b) and cassava (Roca *et al.*, 1991).

3.2 New cryopreservation techniques

Standard cryopreservation techniques are very oftenly complicated. The aim of these new freezing techniques is to look for eventual simplifications of the standard protocols.

3.2.1 Encapsulation/dehydration

This technique is adapted from the "synthetic seed" technology. It has been developed with pear and potato meristems (Dereuddre *et al.*, 1990 ; Fabre and Dereuddre, 1990). The explants which have to be frozen are encapsulated in alginate beads. The beads are pretreated with high sucrose concentrations, dehydrated under the laminar flow, frozen either slowly or rapidly. After slow thawing, direct regrowth of the embedded meristems is obtained on standard medium.

This technique may prove interesting in two situations : for material which are recalcitrant to standard freezing techniques, it seems that the protection conferred by the beads allows to submit the embedded material to pretreatment conditions which would otherwise be detrimental. It may be beneficial also if the encapsulation allows to carry out rapid freezing, thus avoiding the use of a programmable freezing apparatus and simplifying the process.

3.2.2 Vitrification

Vitrification is a physical mechanism which can be defined as the transition of water from the liquid phase directly into an amorphous phase or glass, at low temperature, whilst avoiding the formation of crystalline ice (Grout, 1990). Vitrification requires very high concentrations of cryoprotective substances and ultra-rapid freezing and thawing rates. Only a limited number of papers have been published concerning the vitrification of plant species . The main advantage of this technique is the simplification of the freezing step. However, the use of very high cryoprotectant concentrations requires a very precise timing during their addition and their dilution, presently making it far from being extensively and easily usable in a near future.

3.2.3 Use of a domestic freezer

The last technique consists in the utilization of a domestic freezer as replacement for the programmable freezing apparatus. It has been developed by Tessereau *et al.* (1990) using carrot and coffee cell suspensions and somatic embryos. After the pretreatment, the samples are placed for 24 hours in a commercial freezer, before immersion in LN. Rapid regrowth of the material has been obtained.

4. Conclusion

Medium term conservation techniques are now routinely used in commercial laboratories, due to their immediate advantages and the low necessary investments. Cryopreservation has been developed for numerous species at the experimental level, but its utilization still remains exceptional. Indeed, the setting up of a cryopreservation protocol is oftenly time consuming and sophisticated equipment is oftenly necessary. However, its use becomes obligatory in the case of large collections. Ongoing researches aim at simplifying the standard freezing procedures, by developing alternative techniques.

The integration of conservation techniques in the management of a tissue culture laboratory requires preliminary researches. These works are developed currently, notably in international centers, which are in the process of setting up *in vitro* collections for various species.

It is interesting to observe that research works concerning conservation are more oftenly carried out in connection with technical institutes, which are directly involved in conservation. This evolution should allow in a near future for the possible utilization of proven storage techniques in commercial laboratories.

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CONSERVATION IN VITRO DES ESPECE HORTICOLES

Résumé

La conservation de matériel végétal produit in vitro peut être envisagée pour le moyen ou le long terme. Pour une conservation à moyen terme, les techniques classiques utilisent principalement, séparément ou conjointement, l'abaissement de la température, de l'intensité lumineuse, et d'eventuelles modifications du milieu de culture. Ces techniques sont couramment employées dans les laboratoires de recherche et de production. Pour la conservation à long terme, seule la cryoconservation (stockage à la température de l'azote liquide, -196°C) est actuellement utilisable. La résistance à la congélation dans l'azote liquide a été obtenue pour plus de 50 espèce horticoles différentes, sous forme de suspensions cellulaires, cals, méristèmes et embryons. Cependant, son utilisation en routine reste encore exceptionelle.

Table 1 - List of horticultural species for which medium term storage experiments have been reported in the litterature.

Actinidia chinensis
Actinidia chinensis cv. Hayward
Citrus spp.
Chrysanthenum morifolium
Colocasia
Cryptocorine
Cymbidium
Dendrobium
Dianthus caryophyllus
Eucaplytus spp
Eucalyptus dalrympleana
Eucalyptus gunii
Fuschia hybrida cv. Swingtime
Lolium
Lotus corniculatus
Malus domestica
Malus domestica cv golden
Malus domestica spp (M27/26/111)
Malus domestica baccata
Malus domestica prunifolia
Pinus radiata
Prunus avium
Prunus avium cv Schneider
Prunus avium x pseudocerasus
Prunus avium cv. Hedelfinger
Prunus avium cv. Starkrimson
Prunus canescens
Prunus cerasifera
Prunus domestica
Prunus incisa x serrula
Prunus insititia
Prunus Kursar
Prunus nipponica
Prunus padus
Prunus persica x amygdalus
Prunus persica cv
Populus alba x P. grandidentata
Punica granatum
Pyrus communis var. Caucasia
Pyrus pashia
Rhododendron var. Purple Splendour
Xanthosoma

Table 2 - List of horticultural species which have been cryopreserved in the form of cell suspensions (a), calluses (b), meristems (c), somatic (d), pollen (e) and zygotic embryos (f).

(a) cell suspensions

Acer saccharum
Acer pseudoplatanus
Atropa belladonna
Berberis dictyophilla
Catharantus roseus
Coleus blumei
Corydallis sempervirens
Datura innoxia
Datura stramonium
Digitalis lanata
Dioscorea deltoidea
Glaucium flavium
Glycine max
Hyosciamus muticus
Myrtillocactus geometrizans
Nicotiana plombaginifolia
Nicotiana sylvestris
Nicotiana tabacum
Onobrychis viciifolia
Populus euramericana
Pseudotsuga menziesii
Rhazia orientalis
Rhazia stricta
Rosa Paul's scarlet

(b) calluses

Coleus blumei
Gossypium arboreum
Lavandula vera
Phoenix dactylifera
Populus americana
Ulmus americana

(c) meristems

Dianthus caryophyllus
Digitalis lanata
Haplopapus gracilis
Lilium multiflorum
Malus domestica
Malus spp.
Mentha spp.
Morus bombycis
Pyrus serotina
Pyrus communis
Rubus spp.
Vaccinium
Vanda hookeriana
Xanthosoma

(d) somatic embryos

Citrus sinensis
Picea abies
Picea glauca
Pinus taeda
Xanthosoma

(e) pollen embryos

Atropa belladonna
Citrus spp.
Nicotiana tabacum
Petunia hybrida
Primula obconica

(f) zygotic embryos

Aesculus hypocastanea
Araraucaria excelsa
Castanea
Cocos nucifera
Fagus
Howea fosteriana
Juglans
Quercus
Veitchia merrillii

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