Conservation strategies of citrus germplasm: *in vitro* and *in vivo*

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SUMMARY Conservation of citrus genetic resources needs an integrated *in situ* and e*x situ* approach. In view of preventing dissemination of diseases, it is necessary to resort to *ex situ* conservation for maintenance of clean material, whereas reinfection may be avoided by using *in vivo* and *in vitro* conservation. Following specific guidelines, the collection field fulfilling specific requirements or the insect-proof screenhouse can ensure *in vivo* conservation of a healthy gene pool as well as pomological traits. For several species, *in vitro* culture may play a major role as a conservation strategy or even be the only option available. Despite their several advantages, *in vitro* techniques still display considerable constraints such as genetic instability and length of tissue storage.

Key words: citrus, germplasm, conservation, *in vitro* culture, regeneration, diseases.

RESUME *La conservation des ressources génétiques des agrumes nécessite une approche intégrée in situ et ex situ. En vue de prévenir la dissémination des maladies, il est nécessaire d'avoir recours à la conservation ex situ pour la maintenance du matériel sain, alors que la recontamination peut être évitée par une conservation in vivo et in vitro. Si on adopte des indications spécifiques, la collecte au champ en prenant des précautions particulières, ou bien la cage grillagée pouvant assurer la conservation d'un pool de gènes sains in vivo, de même que les caractéristiques pomologiques.*
Pour diverses espèces, la culture in vitro peut jouer un rôle prépondérant comme stratégie de conservation ou elle pourrait même être la seule option disponible. Malgré leurs avantages, les techniques in vitro continuent à montrer des contraintes considérables comme l'instabilité génétique et la durée de conservation des tissus

Mots-clés *Agrumes, ressources génétiques, conservation, culture in-vitro, régénération, maladies.*

Introduction

Citrus genetic resources including modern and obsolete varieties, genetic stocks, breeding lines and wild genotypes - form the basis for citrus industry development. The creation of germplasm collections ensures the long-term preservation of this important biodiversity. The value of conserved germplasm can be assessed in terms of useful traits for citrus breeding and of the economic impact that the germplasm utilisation has on citrus productivity (cultivars and rootstocks). There are two approaches to conservation: *in situ* and *ex situ*. Conservation of plant diversity using protected areas, on-farm and home gardens is considered to be *in situ*. *Ex situ* conservation comprises the following methods: seed storage, field gene banks, *in vitro* storage, pollen storage, DNA storage and botanical gardens (Ramanatha and Arora, 1997). If conservation is carried out in the area where diversity has evolved, the evolutionary potential of species and populations will be maintained. However, this must be complemented by *ex situ* conservation, due to the habitat destruction and loss of biodiversity by human activities.

Therefore, the movement of citrus germplasm for *ex situ* conservation is of utmost importance but, unfortunately, the exchange of citrus germplasm between different citrus areas entails the risk of spreading diseases. Clean rootstocks and scions are essential and the establishment of gene banks might depend on the production of disease-free materials. Maintaining these gene banks for conservation and use of healthy citrus genetic resources requires the development and transfer of new technologies which are mainly designed for *in vivo* and *in vitro* conservation.

In vivo conservation

In order to avoid the risk of reinfection of a clean gene pool and to maintain pomological traits for trueness-to-type, *in vivo* conservation proves to be essential and it may be carried out in appropriate facilities, such as the insect-proof screenhouse and the collection field, fulfilling the same technical requirements as certification facilities (D'Onghia *et al*., 1998) .

The *insect-proof screenhouse* must guarantee the complete isolation from air- and soil-born pathogens. The main characteristics are the double insect-proof net, the double entrance, the isolation from the flow of surface water and a separation of the plants from the floor. Under the screenhouse, plants (minimum 2/genotype) can easily bear and be kept in container for 10 years before being renewed.

An insect control should be routinely applied as prevention method.

The *field* must be: (i) located in areas free from vector-transmitted pathogens (i.e. citrus tristeza virus) and from the inoculum of destructive fungi (*Phoma tracheiphila*), preferably far (at least 100 mt) from citrus commercial orchards; (ii) established on a good arable land, free from citrus stumps, nematodes and fungi of agronomic importance, where no citrus species has been grown for at least 5 years; (ii) isolated from the flow of surface water, with a borderline of at least 3 mt, constantly tilled and kept free from vegetation.

At least 4-8 plants for genotype, grafted on suitable rootstocks (a minimum of 2), well mapped and labelled, should be grown establishing homogeneous plots within a single specie.

The application of a sound integrated pest management (IPM) to the conservation field should also guarantee protection against pathogens and pests during the plot life, which should not last more than 40 years, before being renewed.

The plot should be routinely sanitary assayed for the presence of vector and mechanical transmissible pathogens.

In vitro conservation

Conservation of citrus germplasm in the field requires greater space, labour and costs and, aside from all the above-mentioned prevention measures, the risk of damage by natural calamities and pathogen infection may be always incurred. Therefore, *in vitro* conservation can easily overcome these difficulties, ensuring the maintenance of healthy citrus germplasm in all countries. Moreover, for several citrus species, *in vitro* culture may play a major role as a conservation strategy or even be the only option available.

For an efficient *in vitro* conservation of healthy germplasm, we need suitable protocols of plant regeneration. Fortunately, there exist many reports on organogenesis from different types of explants of *Citrus* and citrus rootstocks. The morphogenic responses of *Citrus* cultured *in vitro* are influenced by the genotype, the explant type and the culture medium. Explants include shoot tips (Barlass and Skene, 1986), stem sections (Grinblat, 1972; Chaturvedi and Mitra, 1974; Raj Bhansali and Arya, 1979; Barlass and Skene, 1982), root sections (Sauton *et al*., 1982; Burger and Hackett, 1986; Sim *et al*., 1989; Bhat *et al*., 1992), leaf sections (Chaturvedi and Mitra, 1974, Hu and Kong, 1987), stem internodes (Duran-Vila and Navarro, 1989), epicotyl segments (Edriss and Burger, 1984) and transverse thin cell layer (tTCL) explants excised from stem

internodes (Van Le *et al*., 1999). The regeneration of adventitious shoots has been obtained either directly from the explant or from an intermediate callus phase. The regeneration of *Citrus* and related species by organogenesis has been described in many works.

Marin and Duran-Vila (1991) proposed a micropropagation protocol for conservation of citrus germplasm *in vitro*. This method was based on establishment of primary cultures to recover *in vitro* plants from nodal stem segments (dissected from 1-2 year-old plants) and the maintenance of successive cycles of secondary cultures with the recovery of rooted plantlets in each subculture cycle. The subculture cycle would last 8-12 months and would include several operations (culture of nodal segments, rooting of shoots and elongation of the rooted shoots). This protocol allowed the long-term maintenance of lemon, lime, sweet orange and trifoliate orange; the plants regenerated *in vitro* could be maintained for up to 12 months on the same medium before subculture to fresh medium.

Recently, Van Le *et al*., (1999) have reported a new effective regeneration method leading to a high frequency of plant regeneration by using transverse thin cell layer (tTCL) explants excised from the stem internodes of 1-year-old trifoliate orange (*P. trifoliata*). The Authors found that the optimal combination of growth regulators for shoot regeneration was obtained with MS medium supplemented with 10 µM BA and 1 µM N-phenyl-N'-1,2,3-thidiazol-5ylurea (thidiazuron, TDZ), leading to 90% of responsive tTCL, forming on average 37 buds per tTCL. The highest percentage (100%) of shoot elongation was obtained on MS medium supplemented with 1 µM gibberellic acid (GA_3), 60% of shoots formed roots on MS medium containing 5 µM -naphthaleneacetic acid (NAA) and 100% of these plants survived. The plants regenerated from tTCL had no morphological alterations; this could be due to the direct regeneration of plants from the explant without an intermediate callus phase and therefore the reduction of somaclonal variation (Van Le *et al.,* 1999). According to the Authors**,** as regards the high efficiency of bud formation, in tTCL the transport of nutrients and growth regulators from the medium to the cells of the explant is faster. Moreover, a higher quota of responsive cells can be directly exposed to the medium (Van Le *et al*., 1999).

The main steps of *in vitro* conservation of healthy germplasm can be summarized as follows: (i) selection of virus-free plants tested for pomological traits; (ii) collection and sterilisation of shoots; (iii) production of rooted shoots; (iv) selection of non-contaminated material (fungal and bacterial contamination); (v) growth of non-contaminated plantlets in test tubes; (vi) periodical sub-culturing (6 months intervals) of germplasm maintained *in vitro*.

For *in vitro* conservation of healthy germplasm, a plant tissue culture laboratory with basic equipment (refrigerator, freezer, technical and analytical balances, pH meter, magnetic stirrer, washing machine, oven, autoclave, laminar flow hood, growth chamber) is needed.

Conclusion

Although *in vivo* conservation has its limitations, this method represents a compulsory step in the use of healthy citrus genetic resources for trueness-to-type assessment. Indeed, despite their several advantages, *in vitro* techniques still display considerable constraints such as genetic instability and length of tissue storage.

Given the advantages and disadvantages of the two methods, an integrated approach should be applied for appropriate conservation.

In both cases, skilled personnel surely represent the basic ingredient for the success of this activity.

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