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Sanitation of citrus germplasm by somatic embryogenesis and shoot-tip grafting

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SUMMARY *Citrus* can be infected with several graft-transmissible pathogens, which can be eliminated by the use of *in vitro* shoot-tip-grafting and by the recently developed technique of somatic embryogenesis from stigma and style culture. An account of the use, advantages and limitations of these sanitation methods is given in order to ensure the movement of healthy citrus genetic resources in appropriate conditions.

Key words: citrus, *in vitro* culture, plant regeneration, sanitation, shoot-tip grafting, somatic embryogenesis , viruses.

RESUME *les agrumes peuvent être infectés par divers pathogènes transmissibles par greffage, ces derniers peuvent être éliminés par l'utilisation du micro greffage in vitro et récemment par une nouvelle technique d'embryogenèse somatique à partir de la culture de stigma et stylet, un compte rendu de l'utilisation, l' avantage et les limites de cette méthode d'assainissement est rapporté, ceci afin de faciliter le mouvement des agrumes sains comme ressource phytogénétique dans des conditions appropriées.*

Mots clés - *Agrumes, culture in vitro, régénération de plants, assainissement, microgreffage, embryogenèse somatique, virus.*

Introduction

There is no doubt that the uncontrolled movement of citrus germplasm for any purpose involves the risk of accidentally introducing plant quarantine pests, increasing the extent of the infected areas, deteriorating the

development and productivity of *Citrus* and compromising the successful maintenance and management of citrus field gene banks. In most countries, this is due to the absence or non-application of plant quarantine and certification regulations. In particular, those pathogens that are often symptomless, such as viruses, pose a special risk. The major citrus virus and virus-like diseases occurring in the Mediterranean are: tristeza, psorosis, infectious variegation, concave gum, cristacortis, impietratura, exocortis, cachexia, gummy bark, stubborn. Satsuma dwarf and citrus chlorotic dwarf in Turkey, tatterleaf in Morocco have also been reported.

The production of clean material is therefore essential for the conservation and use of citrus genetic resources. Besides *in vitro* shoot tip grafting as described by Navarro *et al.* (1975), which is routinely used for the production of healthy material, often in combination with heat therapy, somatic embryogenesis from stigma and style has recently been developed (D'Onghia *et al*. 1997; 2001 a, b, c).

Sanitation by *in vitro* shoot-tip-grafting

Sanitation by *in vitro* shoot tip grafting has proved to be very effective in eliminating all citrus graft-transmissible diseases but the success rate can vary from 60 (i.e. tatterleaf, psorosis) up to 100% (i.e. citrus viroids, *S. citri*). This method involves different steps: rootstock and scion preparation, *in vitro* grafting and growing, *in vivo* acclimatation .

Rootstock and scion preparation

After removing the integuments, seeds of trifoliate oranges are surface-sterilized in aseptic conditions and incubated in test tubes containing Murashige and Skoog (MS) medium. Seeds are then kept in the dark at 30°C for germination.

Source plants are transferred to a greenhouse (or in a heat chamber when heat treatment is needed) and new shoots are induced by defoliating the entire plants. Shoots can also be produced from lateral buds of *in vitro* cultured budsticks (Navarro *et al.*, 1991).

Grafting procedure

Disinfected shoots, collected from defoliated plants, or shoots from *in vitro* grown budsticks are used for micrografting. Under a steromicroscope, lateral leaves are removed from the shoot and the meristem tip, with two primordia

leaves (about 0.2 mm in size), is excised. The shoot tip is inserted into an inverted-T incision made on a sixteen-day-old seedling. Grafted plants are placed into a test tube containing a MS liquid medium and incubated at 27 °C under 16- hour light exposure.

Acclimatation

About one month after grafting, plants are *in vivo* re-grafted onto six-month-old seedlings, grown in greenhouse conditions for acclimatation.

Sanitation by somatic embryogenesis from stigma and style culture

The regeneration of somatic embryos from citrus floral tissues was firstly described by De Pasquale *et al*. (1994). This new technique allows the regeneration of plantlets which are genetically identical to the original clone. It was improved by using different explant types (thin cell layers of stigma and style or entire organs), excised from flowers of several citrus species (De Pasquale *et al*., 1994; Carimi *et al*., 1994, 1995, 1998, 1999). As reported in Table 1, most genotypes are regenerated by somatic embryogenesis from the floral tissue culture, except clementines (D'Onghia *et al*., 2001a).

Table 1. List of genotypes regenerated by somatic embryogenesis from floral tissue culture

Species	Genotypes
Citrus limon	Femminello Zagara Bianca, Femminello Santa Teresa, Lunario, Monachello, Berna, Limone di Massa Pilone, Limone Pera
C. sinensis	Bonanza, Atwood, Navelina, Washington Navel, Navelate, Valencia Late, Ugdulena 6, Brasiliano NL 92, Sanguinello, Tarocco Gianduia, Tarocco Puglia
C. medica	Cedro di Diamante, Cedro di Trabia
C. aurantifolia	Limetta messicana
C. limetta	Limetta romana
C. deliciosa	Avana, Tardivo di Ciaculli
C. myrtifolia	Chinotto Comune
C. madurensis	CNR P9, Scillipoti, Continella
C. paradisi	Marsh Seedless
C. aurantium	Consolei, AA CNR 12, AA CNR 30, AA CNR 31, AA CNR 32
C. tardiva	CNR P6
C. othaitensis	Arancio pinocchio
C. sinensis x C. reticulata	Dweet tangor
C. meyerii	Meyer
Fortunella margarita	Kumquat ovale

This method proved to be very effective in eliminating the main graft-transmissible diseases (tristeza, psorosis, infectious variegation, concave gum, cristacortis, impietratura, citrus viroids), with a 100% sanitation rate of the regenerated plants.

Somatic embryogenesis involves the following steps: flowers are collected in the field before opening and surface-sterilized; stigmas and styles are dissected under sterile conditions and cultured in Petri dishes containing MS medium, supplemented with 0-13 μM BAP, to induce callus formation; 2-5 months after culture initiation, somatic embryos are formed on the surface of the callus; germinated embryos are transferred into test tubes containing MS medium at 27 °C and exposed to 16 hours light to induce plant development; after 2 months' incubation, regenerated plants are *in vivo* grafted onto six-month-old seedlings (De Pasquale *et al.*, 1999).

In order to assess whether other diseases not included in previous reports (satsuma dwarf, tatterleaf, citrus mosaic, vein enation, stubborn, witches' broom, citrus canker, citrus variegated chlorosis and greening) may be eliminated by this technique, further investigations need to be conducted. Moreover, this technique should be improved by developing specific protocols for citrus genotypes which are unable to regenerate through *in vitro* somatic embryogenesis (i.e. clementine).

Conclusion

With the only exception of clementines, *in vitro* somatic embryogenesis from floral tissues seems very promising for the production of citrus plants, true-to-type, free from the main graft-transmissible pathogens and able to bear fruits in few years (D'Onghia *et al.*, 1997; 2001a,b,c).

Given the advantages and the limits of sanitation techniques (*in vitro* shoot-tip grafting and somatic embryogenesis) and taking into account that sanitary assays and assessment of pomological traits after the use of a sanitation method are compulsory, *in vitro* somatic embryogenesis from floral tissues seems very desiderable. The apparently no risk of spreading diseases by the use of this technique is of utmost importance because, in some countries, indexing procedures are not properly applied. The genetic variability, probably greater with somatic embryogenesis, but also observed in *Citrus* spp. under natural conditions, can be evaluated during the tests for the assessment of the trueness-to-type.

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