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Summary. The use of 'healthy' propagating material is of utmost importance in the control of CTV, which is the most serious virus affecting citrus trees worldwide. All the sanitation methods available are effective in CTV elimination; shoot-tip-grafting (STG) and somatic embryogenesis from stigma and style *in vitro* culture (SE) are the preferred ones for their numerous advantages. Both techniques are successful in regenerating CTV-free plants, identical to the mother tree, and they can also be applied in the safe international exchange of citrus healthy germplasm. SE is user friendly and can be extensively applied to all Citrus species whenever suitable explants are available; while STG can be applied in the sanitation of clementines and pomelos, which are not regenerated by SE.

Keywords. Citrus – Citrus tristeza virus – Sanitation – Somatic embryogenesis – Shoot-tip-grafting.

Méthodes d'assainissement des agrumes pour l'élimination du virus de la tristeza des agrumes (CTV)

Résumé. *L'utilisation du matériel de multiplication « sain » est d'une importance primordiale pour le contrôle du CTV, considéré comme le plus dangereux virus affectant les agrumes dans le monde. Toutes les méthodes d'assainissement disponibles sont efficaces pour l'élimination du CTV, cependant, considérant leurs nombreux avantages, le greffage d'apex et l'embryogenèse somatique, à partir de stigma et stylet en culture in vitro, sont les techniques préférées. Les deux techniques sont efficaces à régénérer des plantes indemnes de CTV, identique à la plante mère, et ils peuvent également être appliquées dans les échanges internationaux des ressources phytogénétiques d'agrumes sains. l'embryogenèse somatique est une technique simple, et peut être largement appliquée à toutes les espèces d'agrumes, lorsque les explants appropriés sont disponibles ; alors que le greffage d'apex peut être appliqué dans l'assainissement des clémentines et pomelos, qui ne sont pas régénérés par l'embryogenèse somatique.*

Mots-clés. *Agrumes – Virus de la tristeza des agrumes – Assainissement – Embryogénèse somatique, – Greffage d'apex.*

I – Introduction

One of the major constraints for Citrus cultivation is represented by the presence of *Citrus tristeza closterovirus* (CTV), a graft-transmissible agent, which may significantly cause the death of the trees or considerable losses in crop yield and quality, especially where sour orange (*Citrus aurantium* L.) is the predominant rootstock (Roistacher, 1991). CTV may often remain symptomless, thus representing a special risk in its spread over long distances through citrus infected propagating material. Once the infection arrives in a citrus growing area, its rapid spread is assured by different aphid species, which renders the virus control difficult. The worldwide movement of CTV and relative vectors, along with the infected citrus material, has increased in the last years due to the globalization and the lack of adequate quarantine measures (Moreno *et al.,* 2008). The production and use of 'healthy' citrus nursery plants is therefore the most efficient preventive control strategy and its use is also recommended in cross protection programmes to avoid possible synergy with other CTV strains and virus and virus-like agents. 'Healthy' citrus plants are produced in the framework of the clonal and sanitary selection programme, which

includes the recovery of all selected candidate trees (D'Onghia *et al.,* 1998). Several sanitation methods are available with different efficiency in virus elimination; the best procedure should be user friendly in terms of environmental conditions and skills and the following factors should also be considered: (i) time needed for plant regeneration; (ii) maintenance of true-to-type characters in produced plants; (iii) absence or rapid loss of juvenility characters.

II – Sanitation methods

Regenerated nucellar plants can be easily produced and they are considered free from CTV and most of infectious agents (Weathers and Calavan, 1959). Unfortunately, they show juvenility characters (e.g. excessive thorniness, vigorous and up-right habit, slow fruit growth etc.), which may persist for many years and over many budded generations (Roistacher, 1977). Moreover high variations among citrus nucellar budlines were also observed (Navarro *et al.,* 1985). These limitations were overcome using heat-treated buds by hot air at 35-42°C for 78-107 days with budwood preconditioning (Roistacher, 1977); thermotherapy was firstly applied for recovering citrus trees infected by tristeza and psorosis (Grant, 1967); it is effective for the elimination of most graft-transmissible agents but not of viroids and spiroplasmas (Calavan *et al.,* 1972). Disadvantages of nucellar plants and thermotherapy were finally solved by *in vitro* shoot-tip grafting (STG) (Navarro *et al.,* 1975; Navarro, 1981; 1988; 1992), which is currently the sanitation method of several extensive citrus improvement programmes for controlling CTV infections worldwide. Shoot tips of 0.14-0.18 mm in length, aseptically isolated from diseased citrus plants, are grafted (inverted T graft) onto young etiolated seedlings grown *in vitro* (Navarro *et al.,* 1975). About one month after grafting, plants are usually *in vivo* transferred and set fruits two, three years later. Produced plants are true-to-type and do not show juvenility characters. To increase explant survival, bigger shoot tips are collected from thermotreated buds at 32-35°C; this combination reduces virus replication and increase meristematic cell activity, thus obtaining clean shoot-tips to be used with STG. Indeed, the use of both methods is effective in the sanitation of some agents (e.g. *citrus psorosis virus*), which are difficult to eliminate using only STG (Carvalho *et al.,* 2002). Moreover, a safe introduction of citrus germplasm can be achieved by micrografting *in vitro* shoot-tips excised from imported budsticks *in vitro* cultured at 32°C (Navarro *et al.,* 1984; 1991). The number of successful grafts depends upon species, varieties and type of virus strain; however, the operator's skill is still a crucial point in the grafting survival and sanitation rate.

Different is the case of citrus somatic embryogenesis from stigma and style *in vitro* culture (Carimi *et al.,* 1995), which is the most recent sanitation technique for the total recovery of the most infected Citrus spp., except for clementines and pomelos. It is user friendly and can regenerate a number of healthy plants from different embryo lines (D'Onghia *et al.,* 1997). Styles and stigmas are dissected from freshly collected closed flowers under laminar flow. Explants are vertically placed in Petri dishes with the cut surface in contact with medium supplemented with BAP. A creamy-white callus appears on the cut surface of the style base about two weeks after culture initiation, while green somatic embryos develop 2-7 months after culture initiation on the callus surface after several subcultures. After germination, embryos develop into plantlets which are *in vivo* transferred. Regenerated plants begin fruiting on some branches after three years with different grade according to the species. Flowering usually occurs 3-4 years later in plants growing in the field.

Unlike other techniques, stigmas and styles are considered better antigen sources than other tissues normally used for viral detection as for CTV and CPsV; even if the callus obtained is still highly infected (D'Onghia *et al.,* 2000; Djelouah *et al.,* 2002), all the embryos formed are totally free from CTV as for most graft-transmissible agents. Juvenility in regenerated plants is lost after the first year of *in vivo* growth and plants are virtually identical to the original source (D'Onghia *et al.,* 2000). It is successfully used in the safe exchange of citrus germplasm with very little

manipulation. The major limit in the success of regeneration by SE is the flower explant, which must be freshly collected before opening or stored at 4°C for 5-6 days. The storage period can reach 20 days, depending on species and varieties.

A crucial phase of *in vitro* sanitation techniques is the acclimatization of regenerated plants. Plants can be *in vivo* transplanted directly into plastic pots containing sterilized soil or the apical portion can be grafted onto a 4-6 month-old rootstock seedling. Pots are closed into polyethylene bags and maintained in a greenhouse at 25°C; after 1 week bags are opened and left 10 days more before being removed (De Pasquale *et al.,* 1999).

III – Sanitary and genetic analyses

Whatever the sanitation method used is, regenerated plants must undergo sanitary and genetic controls, to assess virus elimination and their trueness-to-type.

Several sanitary assays are nowadays available for CTV detection. A preliminary virus detection should be performed by serological (DAS-ELISA or DTBIA) or molecular assays (probe hybridization or PCR-based assays) (Bar Joseph *et al.,* 1979; Garnsey *et al.,* 1993; Cevik *et al.,* 1996; Cambra *et al.,* 2000; Bertolini *et al.,* 2008); if results are negative, biological indexing by graft transmission onto the universal indicator, 'Mexican lime' [*C. aurantifolia* (Christm.) Swing.] should also be applied (Roistacher, 1991) in thermo-conditioned greenhouse at 18-24°C.

Morpho-pomological traits and genetic assays by flow cytometric analysis and DNA-based techniques (ISSR and RAPD) are used to detect the genetic fidelity in regenerated plants (Coletta *et. al.* 1998; Fang and Roose, 1997).

IV – Conclusion

CTV is easily eliminated by most of the available sanitation methods. Nevertheless, somatic embryos from style and stigma *in vitro* culture seem to meet most of the criteria of selection which are considered important in the choice of the method. Unlike STG, it is user friendly, it doesn't need the combination with thermotherapy to increase the success and all regenerated plants are totally free from CTV. Even if juvenility characters usually appear in some species or varieties, they are easily lost in the first years of growth and the plants produced are identical to the mother trees. As for STG, the time needed for regenerating plants ready to set fruits can vary from 3 to 4 years. This technique is also applicable in the safe exchange of citrus germplasm. However, limitations are still the need for fresh or flowers stored for a short period and the unsuccessful regeneration in clemetines and pomelos. Hence, the improvement of protocols is always desirable and STG can remain the alternative whenever these limitations are present.

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