ELIMINATION OF *CITRUS VARIEGATION VIRUS* (CVV) BY SEED CRYOTHERAPY

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Introduction

Citrus variegation ilarvirus (CVV) is one of the oldest viruses reported in Citrus, which produces crinkling, puckering and variegation of leaves in field trees and, occasionally, variegated and deformed fruits. This virus is primarily transmitted by grafting, but leaf sap inoculation is also efficient. However, no consistent data are available concerning CVV transmission by seed, which is apparently low by literature. The elimination of CVV in citrus genotypes is usually carried out by shoot-tip-grafting. In the case of seed sources, virus elimination from seeds could be more promising for seedlings production to be used as rootstocks and self rooted varieties.

In cryotherapy, plant pathogens such as viruses, phytoplasmas and bacteria are eradicated by exposing explants to liquid nitrogen. It allows treatment of a high number of samples and results in a high frequency of pathogen-free regenerants.

In this work, three polyembryonic Citrus genotypes (sour orange, mandarin and lemon), which were found infected by CVV, were used for trials of virus seed transmission and its elimination by dehydration/cryopreservation of the seeds.

Material & Methods

Fresh ripe fruits, obtained from open-pollination, were harvested from three polyembryonic Cirus genotypes from the collection of the Istituto di Genetica Vegetale - CNR of Palermo, Italy: sour orange 'Canaliculata', mandarin 'Bombajensis' and lemon 'Zagara bianca' clone LCNR8B.

Seed extraction. Seeds were extracted from fruits, washed, dried at room temperature. Seeds were surface-sterilized in ethanol (70% v/v in water) and sodium hypochlorite (2% w/v in water) and then stored at 4°C for 20 days in darkness before to be used for the trials.

CVV transmission by seeds. One hundred fifty seeds per genotype were individually tested (seed coats and peeled seeds) before cryoperservation for assessing the presence of CVV by TAS-ELISA (Davino et al., 1984), whereas 20 seeds/genotype were also tested by RT-PCR (Bennani et al., 2002) using CVVa/CVV4 primers.

Seed cryopreservation and germination. The dehydrated seeds were placed in cryovials which were then plunged into liquid nitrogen at -196°C. After thawing, seeds were germinated *in vitro* into Petri dish on MS semi-solid medium (7g l^{-1} phyto agar, supplemented with 50 gl-1 sucrose and 500 mg l-1 malt extract) and kept at 26±1°C in the dark for one week (Fig. 1a). Plantlets were transferred into Jiffy® pots (Fig. 1b) within plastic boxes, under a 16 h photoperiod at 60 µmol m-2 s-1 provided by cool-white fluorescent tubes.

After 45 days, the seedlings were transplanted into pots with soil mixture and incubated in the growing chamber.

Molecular characterisation of zygotic and nucellar embryos. Six ISSR primers, (ACC)6CC, CC(ATG)6, (GA)8GG, CCA(TG)7T, GCA(AC)7, GGG(CA)7 were used to discriminate between zygotic and nucellar plants after treatment (Doyle and Doyle, 1987).

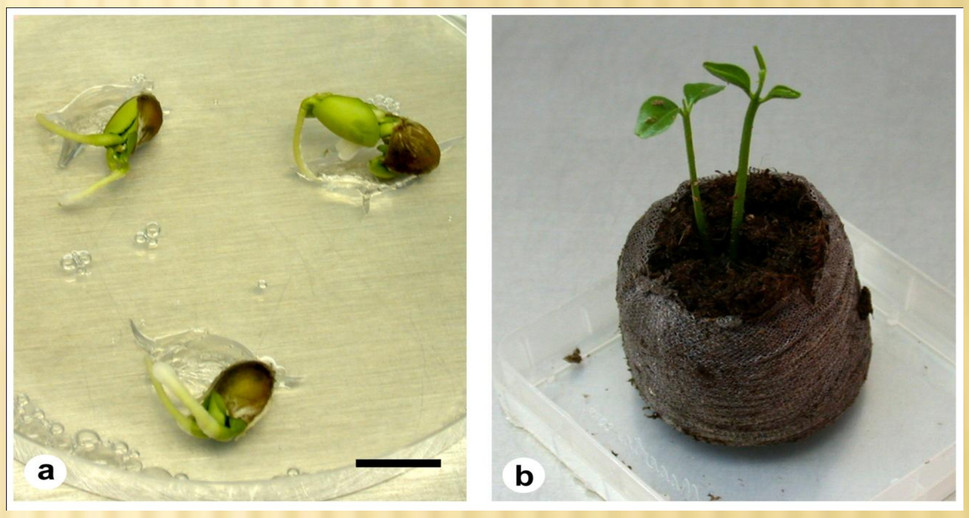

Figure 1. Germination *in vitro* (a) and related plantlets transferred into Jiffy® pots (b) of sour orange 'Canaliculata' developed from cryopreserved seeds. Bar = 1 cm.

Results and discussion

TAS-ELISA and RT-PCR results before cryoperservation proved the presence of CVV in the coat seeds, peeled seeds and seedlings at different rate of infection (Fig. 2). No CVV infection was detected in the peeled seeds of the sour orange, while a 4% of infection rate was found in the peeled seeds of mandarin and lemon (Table 1). RT- PCR assays conducted in 3 months seedlings evidenced an infection rate varying from 3.2% and 3.5% in sour orange and lemon. Interestingly, no CVV infection was detected in the mandarin seedlings by both sanitary assays.

Table 1. Results of TAS-ELISA assays for CVV detection in seed components and seedlings before cryoperservation

Seed sources	Seed coats		Peeled seeds		Seedlings	
	Infected/tested N.	%	Infected/tested N.	%	Infected/tested N.	%
Sour orange	54/150	36	0/150	0	5/171	2.9
Mandarin	45/150	30	6/150	4	0/170	0
Lemon	55/150	36	6/150	4	3/130	2.3

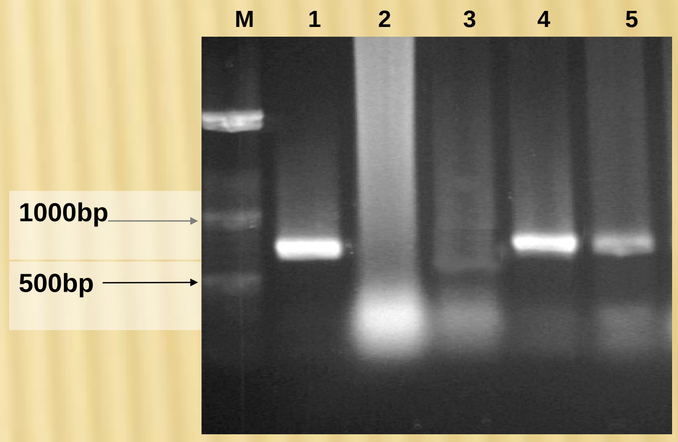

Figure 2. Electrophoretical profile of RT-PCR product amplified from Citrus leaf extract of the three seed sources. Lane M: Marker; lane 1, 4, 5: CVV-infected samples; lane 2: Negative sample; lane 3: Water control .

Zygotic plantlets developed from cryopreserved seeds occurred in sour orange (Fig. 3a) with 27% and in lemon (Fig. 3b) with 68%, whilst in mandarin only nucellar plantlets were obtained; the mandarin seedlings generated from cryopreserved seeds did not show any differences with the mother plant.

After cryopreservation, CVV was detected by TAS ELISA and RT-PCR in the seed coats with an infection rate of 22%, 12% and 4% in sour orange, lemon and mandarin, respectively. In the peeled seeds the infection rate was lower in sour orange, mandarin, while no infection was detected in the peeled seeds of lemon.

However, all plantlets developed from cryopreserved seeds were CVV-free 2 years after transplanting (Tab. 2).

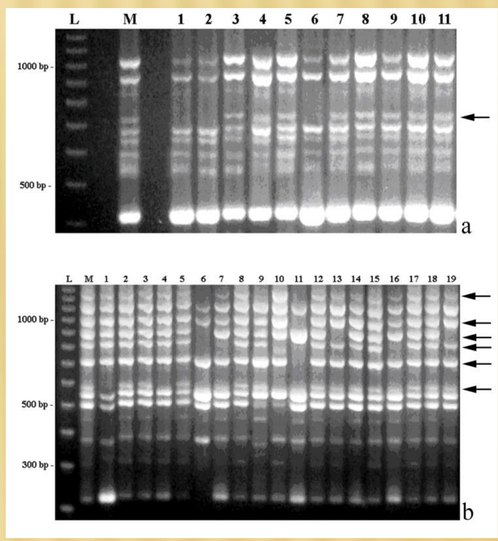

Figure 3 (a, b). ISSR profiles amplified from DNA of sour orange 'Canaliculata' (a) and of lemon 'Zagara bianca' clone LCNR8B (b). The arrow show the polymorphic bands.
Lane L, 100 bp DNA ladder; lane M, mother plant; lanes 1-11 (a) and lanes 1-19 (b) seedlings developed from dehydrated/cryopreserved seeds.

Table 2. TAS-ELISA results of CVV detection in seed components and seedlings after cryopreservation.

Seed sources	Seed coats		Peeled seeds		Seedlings	
	Infected/tested N.	%	Infected/tested N.	%	Infected/tested N.	%
Sour orange	11/50	22	2/50	4	0/30	0
Mandarin	2/50	4	1/50	2	0/30	0
Lemon	6/50	12	0/50	0	0/30	0

CVV transmission by seed was detected for the first time by serological and molecular means in the produced seedlings of sour orange and lemon. No CVV transmission was assessed in mandarin seedlings.

Liquid nitrogen did not eliminate CVV infection in seed components of all tested genotypes; the infection rate was higher in seed coats than in peeled seeds. However, plantlets obtained from cryopreserved seeds were all CVV-free after several serological and molecular assays during two years of growth.

The germination of zygotic plantlets was very frequently in lemon, while it was very little in sour orange and it never occurred in mandarin.

This study showed that he dehydration/cryopreservation of *Citrus* seeds is promising in the production of CVV-free *Citrus* plants in the framework of *Citrus* improvement programme.