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Improved biological indexing of *Citrus tristeza virus* (CTV)

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Abstract. Due to the low rooting ability of Mexican lime, the universal CTV indicator, buds were grafted onto Volkameriana lemon and Etrog citron. After the inoculation with 3 virus isolates from the MAIB collection and a short IBA treatment for rooting, the cuttings were kept in Jiffy pots under plastic bags at 22-24°C for virus detection. Traditional biological indexing was compared. Starting from 15-20 days after inoculation, clear-cut tristeza symptoms were observed on the new emerging leaves of the indicator, whereas the same results were delayed when inoculated seedlings were used. Results of biological indexing by using cuttings were confirmed by using serological and molecular assays.

Keywords. Biological indexing – Citrus – Citrus tristeza virus – Cuttings – ELISA.

Amélioration de l'indexage biologique de la tristeza des agrumes

Résumé. *Vu le faible pouvoir rhizogène de la lime mexicaine, utilisé comme indicateur universel du CTV, les bourgeons ont été greffés sur le citronnier Volkameriana et le cédratier Etrog. Après l'inoculation avec 3 isolats du virus originaires de la collection du MAIB, et un bref traitement à base d'AIB pour favoriser l'émission des racines, les greffons ont été placés dans des pots Jiffy, scellés dans des sachets en plastic et maintenus à 22-24°C pour la détection du virus. Cette technique à été comparée à l'indexage biologique traditionnel. Quinze à vingt jours après l'inoculation, des symptômes évidents de tristeza ont été observés sur les nouvelles feuilles développées par l'indicateur, alors que les mêmes résultats ont été obtenus plus tard quand on a utilisé des semis inoculés. Les résultats issus de l'indexage biologique utilisant des greffons ont été confirmés à travers des tests sérologiques et moléculaires.*

Mots-clés. *Indexage biologique – Agrumes – Virus de la tristeza des agrumes – Greffons – ELISA.*

I – Introduction

Citrus tristeza closterovirus (CTV) is the most destructive virus disease of citrus worldwide. It is spread by infected propagating materials, but its outbreaks are primary due to its transmission by aphids. Many laboratory techniques can be successfully applied for its detection by serological (Bar Joseph *et al.,* 1979; Garnsey *et al*., 1993) and molecular means (Mawassi *et al*., 1995; Pappu *et al*., 1993; Cevik *et al*., 1996; Nolasco *et al*., 2002) but none of these assays can totally replace the use of biological indexing on Mexican lime, the universal indicator of CTV. This index is still compulsory for a reliable detection of this pathogen when a primary source is produced in the framework of the certification program. Moreover, characterization of the virus strains are still mainly based on the use of biological indexing, even if laboratory assays may provide some indications.

Constraints of traditional indexing by graft transmission are space and time needed for the production of indicator seedlings and, after graft inoculation, for symptom expression. High skills are the major ingredient for the success of this technique which is based on the production of excellent indicator plants.

A new system of biological indexing based on the use of indicator cuttings instead of seedlings was developed for the detection of the main citrus virus and viroids (ElBacki *et al,* 2005; El Sayed, 2005).

In this study, the use of Mexican lime cuttings is applied in the detection of three CTV sources in comparison with the conventional method (Roistacher, 1991).

II – Materials and methods

Three virus sources, two from Italy and one from Egypt were provided from MAIB collection which represented typical CTV inoculum of Mediterranean origin.

Ten semi-hard wood and hard wood cuttings were used of Mexican lime for each indicator containing 4-6 nodes and were inoculated by chip budding using the bark tissue collected from the CTV sources. After labelling, all the cuttings were grafted onto Volkameriana lemon and Etrog citron as rootstock. then enclosed inside a plastic bag in order to keep high humidity inside the bag. The inoculated plants were placed in a Jiffy supporter and were maintained in an air conditioned greenhouse at cool temperatures (22-24°C). After ten days, the plastic bags were opened at the top in order to reduce the inside humidity and grafting success was evaluated. After 20 days, the Jjffy supporter was placed in a small flask inside plastic bags and were held for watering and fertilization (Fig. 1).

a **b** **c**

Figure 1. Grafting steps of indicator stem cuttings: (a) hard, semi-hard cuttings; (b) Volkameriana lemon cutting chip budded with M. lime bud and graft inoculated with CTV; (c) plants in the Jiffy pots inside the plastic bag.

Figure 2. Severe leaf vein clearing induced by CTV in M. lime grafted onto Volkameriana Lemon cutting.

Comparison with traditional biological indexing, as described by Roistacher (1991), was also performed using one year old Mexican lime seedlings. These indicator plants were chip budded

with two blind buds from each selected CTV source. After sealing the graft with parafilm and labelling, the inoculated plants and the negative controls were grown with the inoculated cuttings in our air conditioned greenhouse.

Serological detection was carried out on the symptomatic and symptomless plants using DAS ELISA and DTBIA (Bar Joseph *et al.,* 1979; Garnsey *et al*., 1993).

ELISA Plates were coated with polyclonal antibodies using the Agritest-Italy commercial kit; at the concentration indicated by the Company; samples were grinded in extraction buffer at 1/10 concentration, using bark or petiole tissue

Diluted linked antibodies (as reported by the company) were added to each well. P-nitrophenyl phosphate in substrate buffer was used and readings of the absorbance values were made by using automatic plate reader at 405 nm with a Titertek Multiskan plus MKII reader; CTV sources were considered positive if the OD405 values were more than 2,5 times above the values of healthy extracts.

Samples were also analyzed by DTBIA for the detection of CTV using the commercial kit of Plantprint-Spain (Garnsey *et al*., 1993). Five tender shoots from each indicator were cut transversely with a sterile razor and the sections were pressed carefully on the nitrocellulose membrane. After blocking with 1% bovine serum albumin (BSA), the membrane was incubated with the Mabs 3DF1+3CA5 mixture conjugated with alkaline phosphatase (PlantPrint). Membranes were developed by using the BCIP-NBT (Sigma fast tablets), then read under a light microscope at 10x and 20x magnification. The positive reaction was revealed by the presence of purple–violet blots in the region of phloem tissue cells.

Molecular detection was carried out on the symptomatic and symptomless plants using RT-PCR Test (Nolasco *et al*., 2002)

Total nucleic acid from about 100mg of young citrus infected barks were extracted by using the RNeasy Mini Kit, Qiagen, according to the manufacturer's instructions. cDNA was synthesized using TNA extracted as template and the PCR mix reaction contained 10mM Tris (pH:8.8), 50 mM KCl, 3mM MgCl2, 4U RNA Guard ribonuclease inhibitor, 7.5U MuLV reverse transcriptase (Applied Biosystems, Roche), 1U Taq polymerase (Promega), 0.2 mM of each dATP, dTTP, dGTP and dCTP, 200nM CTV1 forward primer, 200nM CTV10 reverse primer (Nolasco *et al*., 2002) and 3μl of extracted TNA.

cDNA synthesis was performed at 38°C for 45 min followed by a denaturation step and inactivation of reverse transcriptase at 94°C for 2 min. The amplification process consisted of 30 cycles at 92°C for 30 sec, 52°C for 30 sec and 72°C for 30 sec, followed by a 10 min elongation cycle at 72°C. The amplification products were analysed in 1% agarose gel electrophoresis.

III – Results and discussion

Graft success was higher in both rootstocks (Volkameriana lemon and Etrog citron) and shoot flushing usually occurred in 20 – 30 days after inoculation. Symptoms development differed in terms of time of symptom appearance and the number of symptomatic plants for each CTV source. Most of the symptoms with rooted cuttings developed after 20-23 days, while the the inoculated one year old seedlings of the tested indicators showed the first symptoms in some plants one month after inoculation.

Results of biological indexing by cuttings were confirmed by ELISA using plant tissue from symptomatic and symptomless indicator plants after 5 weeks of growth. All inoculated indicator stem cuttings were CTV ELISA-positive.

Twenty days from inoculation clear-cut infectious variegation symptoms were observed on the new emerging leaves of both indicators. Results of biological indexing were confirmed by ELISA, DTBIA and PCR on the indicator leaves.

About 15-20 days after inoculation, clear-cut CTV symptoms were observed on the new emerging leaves of Mexican lime using cuttings, whereas the same results were delayed where seedlings were used. These results of biological indexing by cuttings were also confirmed by using serological and molecular assays.

IV – Conclusion

Based on these preliminary results, CTV can be successfully detected by the use of inoculated indicator cuttings instead of seedlings. Due to the low rooting ability, Mexican lime must be chip-budded onto Volkameriana lemon cuttings.

This technique could be used during the entire year, because the production of stem cuttings under warm conditions overcomes the seasonal rooting variability of most citrus species. The biological assay using indicator cuttings can be readily concluded one month after grafting without any transplanting.

This method overcomes all constraints of traditional biological indexing in citrus without a decrease in reliability of symptom expression. However the use of this technique in combination with laboratory assays, mainly in asymptomatic cuttings, is always recommended for a reliable sanitary assessment of a citrus genotype.

Further research should also be carried out using mild and moderate pathogen strains, enlarging the use of stem cuttings to other citrus indicator species needed for virus biological characterization.

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