# VIROIDS

## Citrus Exocortis and Cachexia Viroids Affect Growth, Yield and Fruit Quality of Lapithou Lemon on Sour Orange Rootstock in Cyprus

**A. Kyriakou[1], M. Ioannou[1], A. Hadjinicolis[1], R. Hoffman[2], E. Antoniou[1], L. Papayiannis[1], Th. Kapari-Isaia[1], and N. Ioannou[1]**

[1]*Agricultural Research Institute, P.O. Box 22016, 1516 Nicosia, Cyprus;*
[2]*Tolkowsky Laboratory, Volcani Center, Bet Dagan, Israel*

ABSTRACT. A selection (STG 267) of the local "Lapithou" lemon of Cyprus, which was freed from viroids by shoot-tip grafting *in vitro*, was grown in the field on sour orange rootstock, and compared to the maternal material (89-35), which contained citrus exocortis and cachexia viroids. Sour orange seedlings were grafted in the greenhouse in spring 1995 and planted at the Zygi Experimental Station, in the southern coastal plain of the island, in spring 1996. Twenty trees of each treatment were established in a replicated complete block design and tree performance was evaluated during 2000-2003. Growth of trees was seriously affected by viroids, with trunk circumference and tree canopy volume being reduced by 18% and 25%, respectively, when compared to shoot-tip grafted trees. Cumulative three-year yield of viroid-infected trees was about 50% the yield of healthy trees. With respect to fruit quality, sugar content and the ratio of total soluble solids to total acids were significantly higher in fruits from healthy trees. However, fruit size and juice content were not affected by viroids. The present study proved the significant superiority, in field performance, of the shoot-tip grafted Lapithou lemon, in comparison to the corresponding viroid-infected material.

In the early 1990s, within the context of a program for the production and distribution of healthy citrus material in Cyprus, six selections of the local Lapithou lemon were freed from virus problems by the technique of shoot-tip grafting *in vitro* (6). The main problems encountered in Lapithou lemon were the viroids which are widespread in all citrus grown on the island (8), as a result of the lack of a citrus certification scheme before 1994 (4), but also because of the widespread use of the sour orange rootstock, which is tolerant to viroids (13). Even at present, despite the threat faced by the citrus industry from *Citrus tristeza virus* (CTV) (7), sour orange, widely known for its susceptibility to CTV, is still used on the island as the main rootstock due to its excellent adaptation to the local environmental conditions.

The main objective of this study was to evaluate the field performance of the shoot-tip grafted Lapithou lemon, grown on sour orange rootstock, in comparison to the original maternal material, which contained two viroids: *Citrus exocortis viroid* (CEVd) and a cachexia variant (CCaVd) of *Hop stunt viroid* (HSVd).

### MATERIALS AND METHODS

A single Lapithou lemon tree (89-35) about 50 yr-old from Kouklia, Paphos, was used as the source of the material evaluated. This tree had originated from an 80-yr old tree which survived a serious infection by mal secco in the Ayios Neophytos area in Paphos. The source tree was found free of *Citrus psorosis virus* (CPsV), *Citrus variegation virus* (CIVV), concave gum and CTV, when tested by the methods described by Roistacher (12), but caused typical symptoms of CCaVd on Parson's special mandarin grafted on Volkamer lemon and severe epinasty symptoms on Etrog citron Arizona 861, indicating the possible presence of CEVd. Extraction of nucleic acids from the infected Etrog citron indicator and application of sequential electrophoresis (sPAGE), as described by Duran-Vila et al. (3) and Ben-Shaul et al. (1), revealed the presence of two viroids. Comparison of the relative mobilities of these viroids in denaturing PAGE to CEVd and *Avocado sunblotch viroid* (ASVd) confirmed the presence of CEVd and a CVIIb viroid or CCaVd.

The shoot-tip grafted material (STG 267), which was produced from lemon 89-35, was tested with the same methods used for the maternal material and was found free of locally known viruses and the two viroids infecting the source tree.

Nine-month-old sour orange seedlings, grown in the greenhouse, were budded in April 1995 with either lemon 89-35 or the shoot-tip grafted material STG 267. Top grafting was by inserting a 40 mm budstick with 2-3 buds on the decapitated sour orange seedling at the height of about 35 cm. In March 1996, 21 plants successfully grafted with 89-35 and an equal number of plants grafted with STG 267 were planted in the field at Zygi Experimental Station, in a replicated complete block design. The experiment comprised 7 rows, each composed of 6 plants, 3 infected and 3 healthy, planted alternately. Distance between trees was 5 m within a row and 6 m between rows. In February 1997 the young lemon trees suffered serious damage from frost. Twelve trees (eight 89-35 and four STG 267) died and were replaced with plants of the same age in March of the same year. One 89-35 tree failed to grow after replacement and this tree and the adjacent STG 267 tree were excluded from the experiment. Thus, only 20 pairs of experimental trees were used for the study.

The soil was of medium to fine texture with 40% clay and 45-50% $CaCO_3$, with pH 8.0-8.3 (11). Mean daily minimum/maximum air temperatures of the area were 6.4°C/17.5°C in January and 19.4°C/33.6°C in July. Relative humidity ranges from 56% to 83% in the winter months and from 55% to 66% during summer. Average annual precipitation is 374 mm (Cyprus Metereological Services, 1991-2000). Irrigation of the trees was by drippers and the average amount of water applied annually was about 350 mm the first 3 yr and 700 mm thereafter. Weeds were controlled by hand the first 2 yr and by the use of herbicides after the second year. Trees were pruned (mainly sucker removal, always by the same technician to ensure uniformity) and sprayed with insecticides as needed. Strict measures were taken to prevent accidental mechanical infection of the shoot-tip grafted trees, including fencing of the plot in order to avoid unauthorized use of tools on the trees.

Tree growth was measured in September 2000, 2002 and 2003, as the increase in height of each tree and the increase in trunk circumference at a point fixed at 10 cm above the bud union. Canopy volume was estimated (at the same time intervals) from the tree radius and the height of the tree minus the height of the trunk, assuming that the tree canopy is conical. Yields were recorded as total fruit weight and number of fruits per tree, harvested in late November during 2001-2003. During the same time period fruit samples were taken annually for quality studies. They consisted of 10 fruits collected at random from each tree and quality characteristics measured included juice content, total soluble solids and acid contents. Each fruit was cut in half and juice was extracted with an electric juice extractor. Total soluble solids were determined by refractometer, while acidity, expressed as anhydrous citric acid, was determined by titrating with 0.1 N NaOH, using phenolphthalein as indicator. Data were statistically analyzed using Student's *t* test.

To confirm freedom from viroids and/or viroid infection of the field-grown trees, in April, 2003, seven trees of STG-267 and seven trees which originated from lemon 89-35, were tested on indicators of Etrog citron selection Arizona 861, grafted on sour orange, in the virology glasshouse with temperatures 15-35°C. Three to twelve months later citron plants were observed for symptoms.

In addition, material from asymptomatic and symptomatic plants was subjected to RNA extraction and RT-PCR. Total RNA was extracted from fresh, young leaves of Etrog citron using TRIzol® reagent from Invitrogen life technologies (Cat. No. 15596-026). The primer pair CEV-AM3(-) (5'-CCGGGGATC-CCTGAAGGACTT-3') and CEV-AP3(+) (5'-GGAAACCTGGAGGAA-GTCGAG-3') (5) was used to amplify a 371bp product from CEVd samples. For the detection of CCaVd, primers CV2-AM(-) (5'-CCGGGGCTCCTTT-CTCAGGTAAGT-3') and CV2-AP(-/) (5'-GGCAACTCTTCTCAGAATCCA-GC-3') (15) were used to amplify a 302bp fragment. For cDNA synthesis and polymerase chain reaction (PCR) amplification, 5 µl of each antisense primer (4 µM) was added to 5 µl total RNA extracted from each sample. The mixture was incubated for 5 min at 90°C using a dry heating block and immediately put on ice until further use. Ten microliters of reverse transcription reaction buffer containing 10 mM Tris-HCl [pH 8.8], 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.25 mM of each deoxyribonucleoside triphosphate (dNTP), 40 units Superscript II Virus Reverse Transcriptase (Invitrogen life technologies), 15 units RNAguard RNase Inhibitor and 0.1 M DTT were added to each tube and incubated at 42°C for 60 min and 94°C for 5 min in an MJ RESEARCH PTC-200 thermal cycler. An aliquot of 2 µl of this preparation was PCR amplified in a 20 µl reaction buffer containing 1× PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl2), 0.2 mM each of the four dNTPs, 1 U Taq DNA polymerase (Invitrogen life technologies) and 0.5 µM each of sense and antisense primers specific to CEVd and CCaVd, respectively. The cycling profile was 94°C for 40 s, 60°C for 40 s and 72°C for 45 s, with a final extension step of 72°C for 5 min. The reaction products were analyzed by electrophoresis in 1.5% agarose gels in TAE buffer (1×: 0.04 M Tris-acetate, 0.001 M EDTA), stained with ethidium bromide, and visualized under UV light.

## RESULTS

**Tree growth.** The growth of Lapithou lemon, expressed as trunk circumference, tree height and canopy volume, was significantly affected by CEVd and CCaVd (Table 1). The effect of the viroids was greater at the young age of the trees. Thus, at the age of 3-4 yr, tree girth, height and canopy volume were reduced by 24%, 14% and 29%, respectively, in comparison to shoot-tip grafted trees, whereas three years later these differences in the three above parameters were 18%, 8% and 25%, respectively, but still statistically significant and obvious to the visitor's eye at first glance.

**Fruit yield.** Numbers of fruit and annual fruit weight of "Lapithou" lemon were both reduced between 40% and 65% by viroids during the first 3 yr of harvest (Table 2). As with tree growth, the effect of viroid infection was higher on the first year of harvest, with cumulative fruit number and yield from infected trees were lower by 50% and 54%, respectively, than in shoot-tip grafted trees. Mean fruit weight was not seriously affected by viroid infection, although there was a trend of a slight increase of fruit weight from the infected trees, and during 2002 this difference was significant.

**Fruit quality.** Results of the effect of the two viroids on fruit quality of "Lapithou" lemon are pre-

TABLE 1
EFFECT OF VIROIDS ON GROWTH OF LAPITHOU LEMON AT ZYGI

| Year | | Trunk circumference (cm) | Tree height (cm) | Tree canopy volume ($m^3$) |
|---|---|---|---|---|
| 2000 | Healthy | 26.12 | 268.05 | 4.04 |
| | Infected | 19.90 | 229.15 | 2.88 |
| | LSD ($P$ = 0.05) | 3.93* | 25.12* | 0.98* |
| 2002 | Healthy | 35.13 | 327.50 | 12.59 |
| | Infected | 29.13 | 289.50 | 8.54 |
| | LSD ($P$ = 0.05) | 3.96* | 20.16* | 2.56* |
| 2003 | Healthy | 42.54 | 353.00 | 12.67 |
| | Infected | 34.91 | 324.00 | 9.48 |
| | LSD ($P$ = 0.05) | 3.94* | 24.53* | 2.28* |

*Difference significant at $P$= 0.05.

sented in Table 3. Juice content was not affected by viroids, whereas total soluble solids and total acid contents were both consistently higher in fruits from shoot-tip grafted trees. In addition, except for 2003, the ratio of total soluble solids to total acid was significantly higher in fruit from healthy trees.

**Viroid content of trees.** All Etrog citron indicators inoculated with material from the micrografted (STG 267) experimental trees remained symptomless 12 mo after inoculation, whereas all citron indicators, which were inoculated with material from seven trees produced from the original 89-35 Lapithou lemon exhibited severe leaf malformation symptoms 2-6 mo post-inoculation. RNA extraction and RT-PCR proved the presence of CEVd (Fig. 1) and CCaVd in all symptomatic citron plants. All symptomless citron plants were free of the two above viroids.

## DISCUSSION

The micrografted, virus-tested Lapithou lemon was shown to be superior to the maternal viroid-infected lemon in a field performance trial on sour orange rootstock at the southern coastal part of Cyprus, with respect to tree growth, productivity and fruit quality. The viroids present in the original infected lemon were CEVd and CCaVd.

Viroid-infected lemon trees, at the age of 7 yr showed a significant reduction in girth, height and canopy

TABLE 2
EFFECT OF VIROIDS ON YIELD AND FRUIT SIZE OF LAPITHOU LEMON

| Year | | Number of fruits per tree | Yield per tree (Kg) | Single fruit weight (g) |
|---|---|---|---|---|
| 2001 | Healthy | 66 | 11.54 | 175 |
| | Infected | 23 | 4.36 | 190 |
| | LSD ($P$ = 0.05) | 29.70* | 4.23* | 39.66 |
| 2002 | Healthy | 206 | 33.87 | 165 |
| | Infected | 116 | 20.62 | 178 |
| | LSD ($P$ = 0.05) | 70.15* | 12.63* | 13.77* |
| 2003 | Healthy | 390 | 60.06 | 154 |
| | Infected | 190 | 27.76 | 146 |
| | LSD ($P$ = 0.05) | 86.87* | 12.92* | 11.22 |
| Total | Healthy | 662 | 105.47 | 159 |
| | Infected | 329 | 52.74 | 160 |
| | LSD ($P$ = 0.05) | 91.66* | 15.35* | 7.47 |

*Difference significant at $P$ = 0.05.

TABLE 3
EFFECT OF VIROIDS ON FRUIT QUALITY OF LAPITHOU LEMON

| Year | | Juice content by weight (%) | Total soluble solids (%) | Total acid (%) | Total soluble solids/ Total acid |
|---|---|---|---|---|---|
| 2001 | Healthy | 42.23 | 9.91 | 6.00 | 1.65 |
| | Infected | 38.83 | 8.94 | 5.63 | 1.59 |
| | LSD ($P$ = 0.05) | 11.26 | 0.36* | 0.21* | 0.05* |
| 2002 | Healthy | 30.93 | 12.92 | 7.85 | 1.64 |
| | Infected | 32.68 | 10.81 | 6.87 | 1.57 |
| | LSD ($P$ = 0.05) | 2.27 | 0.48* | 0.24* | 0.04* |
| 2003 | Healthy | 39.75 | 10.57 | 6.93 | 1.52 |
| | Infected | 41.58 | 9.58 | 6.28 | 1.52 |
| | LSD ($P$ = 0.05) | 2.26 | 0.36* | 0.27* | 0.04 |

*Difference significant at $P$ = 0.05.

volume compared to the micrografted trees. Similar results were obtained by Nauer et al. (9) and Roistacher et al. (13) in a field trial in the Central Valley of California in which they compared Washington navel orange infected by CEVd with micrografted healthy trees on sour orange and Troyer citrange rootstocks. Vernière et al. (16, 17) received also analogous results in Corsica in a field trial in which they compared healthy to CEVd-infected Clementine trees on trifoliate orange rootstock. In the same trial it was found that Citrus viroid III also retarded tree growth. In addition, in a field trial in New South Wales, CEVd and another dwarfing agent, most probably another viroid, also reduced growth of 7-yr old Lisbon and Eureka lemon on rough lemon, smooth Seville, Bowman citrange and trifoliate orange rootstocks (2). In the present study it was shown that the effect of the viroids on the growth of lemon was reduced with increasing tree age (Table 1) and as the trial is being continued, it will be interesting to see if with time the viroid effect will persist and/or diminish further. The senior author noticed previously that the effect of other diseases, such as stubborn on sweet orange and impietratura on Marsh seedless grapefruit, became less apparent with increasing tree age in coastal areas of Cyprus. Broadbent et al. (2) also reported that differences in yield of Lisbon and Eureka lemon due to inoculation with viroids were less in 1980 than in 1979.

Fruit production of Lapithou lemon was significantly reduced by viroids and the cumulative 3-yr yield of infected trees was only 50% that of 'healthy' trees. A similar effect was found for two isolates of CEVd on the 8-yr cumulative yield of clementine trees grafted on trifoliate rootstock (14), while the 6-yr total yield of Washington navel was reduced by CEVd by 32% on Troyer citrange and 23% on sour orange rootstock (9). The cumulative 5-yr yield of Eureka and Lisbon lemon on five different rootstocks in New

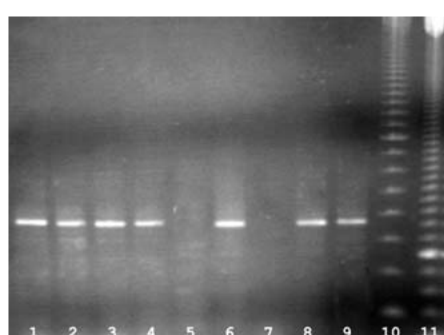


**Fig. 1. Gel electrophoresis of amplification products generated by RT-PCR from CEVd-infected Etrog citron. Lanes 1, 2, 3, 4, 6, 8, and 9 are CEVd positive samples from lemon trees 89-35. Lanes 5 and 7 are negative control samples. Lane 10: 100bp Molecular marker. Lane 11: 50 bp Molecular marker.**

South Wales was adversely affected by viroids and the decrease was up to 80% (2). In the same trial inoculation with one dwarfing agent did not affect yield of Lisbon and Eureka on Benton and Bowman citranges, respectively. In the present study viroids had no consistent effect on average fruit weight of Lapithou lemon, although a trend of bigger fruits was apparent on infected trees (Table 2), perhaps as a result of the lower productivity. On the contrary, in New South Wales, the weight of fruit of Lisbon and Eureka lemon on five rootstocks decreased from 9% to 16% as a result of infection by viroids (2).

With regard to fruit quality, juice content of Lapithou lemons was not affected by viroids, which was also found by Nauer et al. (9) for Washington navel. However, total soluble solids and total acid contents, contrary to what was previously found for Washington navel (9), were significantly affected in lemons, and the ratio of total soluble acids to total acid, which is an index of fruit quality, was significantly higher in micrografted lemons in two out of three years in which the analysis was conducted. It will be interesting to find out if the quality values will continue to be higher in the micrografted lemon trees as they grow older. It is not known which of the two viroids affected the trees more, but most probably it is the CEVd which induced the greatest effect on the infected lemon, as was shown by Nauer et al. (9) for Washington navel. Nevertheless, the trial proved once more the superiority of the micrografted healthy to the original infected citrus material (10).

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## LITERATURE CITED

1. Ben-Shaul, A., Y. Guang, N. Mogilner, R. Hadas, M. Mawassi, R. Gafny, and M. Bar-Joseph
1995. Genomic diversity among populations of two citrus viroids from different graft-transmissible dwarfing complexes in Israel. Phytopathology 85: 359-364.
2. Broadbent, P., P. Nicholls, and B. Freeman
1988. Effect of graft-transmissible dwarfing sgents on lemons. In: *Proc. 10th Conf. IOCV*, 211-218. IOCV, Riverside, CA.
3. Duran-Vila, N., J. A. Pina, J. F. Ballester, J. Juarez, C. N. Roistacher, R. Rivera-Bustamante, and J. S. Semancik
1988. The citrus exocortis disease: A complex of viroid-RNAs. In: *Proc. 10th Conf. IOCV*, 152-164. IOCV, Riverside, CA.
4. Gavriel, I.
2002. Citrus certification program of Cyprus. In: *Proc. 15th Conf. IOCV*, IOCV, Riverside, CA.
5. Gross, H. J., G. Krupp, H. Domdey, M. Raba, P. Jank, C. Lossow, H. Alberty, K. Ramm, and H. L. Sänger
1982. Nucleotide sequence and secondary structure of citrus exocortis and chrysanthemum stunt viroid. Eur. J. Biochem. 121: 249-257.
6. Ioannou, M., A. Kyriakou, and N. Ioannou
1991. Production of healthy Lapithou lemons by shoot-tip grafting in vitro. Tech. Bull. No. 139, Agric. Res. Inst., Min. Agric. & Nat. Res., Nicosia, Cyprus, 7 pp.
7. Kapari, Th., A. Kyriakou, N. Ioannou, J. Gavriel, M. Bar-Joseph, G. Savva, D. Polycarpou, N. Loizias, E. Iosephidou, A. Hadjinicolis, and Chr. Papayiannis
2000. A six-year fight against citrus tristeza virus in Cyprus. In: *Proc. 14th Conf. IOCV*, 335-337. IOCV, Riverside, CA.
8. Kyriakou, A. P.
1992. Incidence in Cyprus of citrus exocortis viroid and its mechanical transmission. Plant Pathol. 41: 20-24.
9. Nauer, E. M., C. N. Roistacher, E. C. Calavan, and T. L. Carson
1988. The effect of citrus exocortis viroid (CEV) and related mild citrus viroids (CV) on

field performance on Washington navel orange on two rootstocks. In: *Proc. 10th Conf. IOCV*, 204-210. IOCV, Riverside, CA.

10. Navarro, L., J. Juarez, and J. A. Pina
2001. Strategies and problems for the production of high quality nursery citrus trees worldwide. *Proc. 6th World Congr. Int. Soc. Citrus Nurserymen*, 1-10.
11. Orphanos, P. I. and Ph. Kokkinos
1983. The soil of Zyghi station. Miscellaneous Report No. 11, Agric.Res.Inst., Min. Agric. & Nat. Res., Nicosia, Cyprus, 7 pp.
12. Roistacher, C. N.
1991. *Graft Transmissible Diseases of Citrus. Handbook for Detection and Diagnosis*. FAO, Rome.
13. Roistacher, C. N., H. Canton, and P. S. Reddy
1995. Severe decline of citrus on exocortis-susceptible rootstocks in Belize and the costs incurred by the use of infected budwood. In: *Proc. 3rd Int. Workshop on Citrus tristeza virus and the brown citrus aphid in the Caribbean Basin: Management Strategies*, 130-138. Lake Alfred, Florida.
14. Roistacher, C. N., J. E. Pehrson, and J. S. Semancik
1991. Effect of citrus viroids and the influence of rootstocks on field performance of navel oranges. In: *Proc. 11th Conf. IOCV*, 234-239. IOCV, Riverside, CA.
15. Sano, T., T. Hataya, and E. Shikata
1988. Complete nucleotide sequence of a viroid isolated from Etrog citron, a new member of hop stunt viroid group. Nucleic Acids Res. 16: 347.
16. Vernière, C., L. Botella, A. Dubois, C. Chabrier, and N. Duran-Vila
2002. Properties of citrus viroids: Symptom expression and dwarfing. In: *Proc. 15th Conf. IOCV*, 240-248. IOCV, Riverside, CA.
17. Vernière, C., X. Perrier, C. Dubois, A. Dubois, L. Botella, C. Chabrier, J. M. Bové, and N. Duran-Vila
2004. Citrus viroids: symptom expression and effect on vegetative growth and yield of Clementine trees grafted on trifoliate orange. Plant Dis. 88: 1189-1197.