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ELISA ASSAYS FOR THE DETECTION OF CITRUS INFECTIOUS VARIEGATION VIRUS (CVV)

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SUMMARY - ELISA testing was carried out in Greece using the ELISA kit against CVV from UCP-Morocco; 4 lemon sources were used as CVV positive controls at the Arboricultural Station in Poros, two of which showed CVV-like symptoms. An attempt was made to conduct a small-scale survey for CVV in Citrus groves in Paros Island and in 81 mother trees of a state nursery. Flower buds and young leaves proved to be the best explants, even if stored at 5°C for a couple of weeks. Background was high and results were often doubtful. The Greek CVV samples showed very low absorbance values if compared to the positive control.

Key words: Citrus, citrus infectious variegation virus, ELISA, Greece

RESUME *- Le test ELISA a été réalisé en Grèce en employant le kit pour le CVV fourni par l'UCP-Maroc. Quatre sources de citronnier ont été utilisées comme témoins positifs du CVV à la Station d'Arboriculture de Poros et deux de celles-ci ont montré des symptômes du type CVV. Parallèlement, on a essayé de conduire une enquête sur une petite échelle pour mettre en évidence le CVV dans les orangeraies de l'île de Paros et chez 81 pieds-mères d'une pépinière d'état. Les bourgeons à fleur et les jeunes feuilles se sont avérés être les meilleurs explants même si conservés à 5°C pendant environ deux semaines. Le bruit de fond était élevé et les résultats obtenus étaient souvent douteux. Les échantillons grecs de CVV ont affiché des valeurs très faibles d'absorbance par rapport au témoin positif.*

Mots-clés*: Agrumes, virus de la panachure infectieuse des agrumes, ELISA, Grèce*

INTRODUCTION

The causal agent of infectious variegation is an ilarvirus; it is the first citrus virus which has been mechanically transmitted from citrus to citrus and to herbaceous hosts (Trabut, 1913). Although the virus does not compromise the plant development and productivity, the major symptom induced, crinkly leaf in association with chlorotic variegation, can be very severe, especially in lemon.

The disease is present in some Mediterranean countries, both crinkly leaf and infectious variegation were detected in Poros (Greece); however, the extent of its distribution is not known yet (Zois, 1976; Keramidas, 1976; Kyriakopoulou, 1998; 2001).

MATERIALS AND METHODS

A first test was run on samples from negative and positive trees, to determine what material is best suited for the detection of CVV. The test was carried out in May and the samples had been freshly collected the day before to be processed at the Arboricultural Station at Poros, where positive and negative controls are kept for several citrus diseases. For CVV, the controls are lemons on *Citrus volkameriana*: - ten negative trees, three of which were sampled; - four positive trees, two of which showed only growth of the rootstock and were symptomless, while the other two displayed clear

CVV-like symptoms. All four trees were sampled. Then, an attempt was made to conduct a small-scale survey for CVV in Citrus groves. Unfortunately, last spring a wave of bad weather greatly damaged citrus groves in the western part of Greece, destroying almost completely all new growth. As a result, it was not possible to survey the Peloponnese and Paros Island was chosen, where in a state nursery 81 mother-trees are kept for the production of planting material serving the Cyclades Region. None of the mother-trees showed CVV symptoms, nor were symptoms found just in citrus groves in Paros, since these are small and scattered, and only a few could be visited.

Each sample was made up of at least four young shoots, and a young leaf or part of it from each shoot, was pooled to make one sample. Leaves, especially those from orange and mandarin, were not so tender as the lemon controls from Paros.

The precise testing procedure is described in Table 1, to facilitate the comparison with other laboratories. Essentially, the protocol from UCP Morocco was applied, but the following modifications were introduced :

1. The samples were homogenised in BIOREBA cotton-gauze lined plastic bags and therefore a larger amount of buffer had to be added. When less than 2 ml are used, a higher amount of liquid is absorbed by the cotton-gauze and pipetting the samples becomes very difficult. Samples were homogenised in 1/9 and 1/5 (w/v) instead of the recommended 1 gram in 2 ml buffer.
2. In the conjugate buffer, bovine albumin was used instead of ovalbumin. We use bovine albumin routinely in ELISA and have no high-grade ovalbumin.

In the subsequent tests, the following modifications were made to reduce the background:

a) After coating, the plates were blocked with 1% bovine albumin in PBS-tween (incubation at room temp. for 1 hour) to prevent binding of the second Ab directly to the plates.
b) The second Ab was applied at a higher dilution.
c) The second Ab was added together with the samples and incubated overnight in the refrigerator, to reduce the effect of non-specific binding of Ab and the second Ab.

RESULTS

The results of the preliminary test on known positive and negative samples are presented in Table 2. The average A_{405} value of each sample tested is given, along with the standard deviation (θ) between repetitions. Standard deviations exceeding 15% of the average A_{405}, are printed in bold italics. The positive/negative threshold (T) is calculated for 9 negative repetitions as A_{405} +3.36 θ and for 6 negative repetitions as A_{405} +4.O3 θ. The positive/negative discrimination factor of each sample is presented and expressed as A_{405}/T.

Similarly, in Table 3 the test results are presented for several of the unknown samples tested. These were 90 in all, samples 1 - 81 coming from mother trees of Paros, while the remaining 9 had been taken from citrus groves in Poros and near Athens.

When the unknown samples were tested, none was clearly positive. However several samples gave doubtful results (one positive and one negative reading or both readings below the threshold value). Many of these samples were then re-tested. These results are reported in Table 3.

CONCLUSIONS

1. The best material is represented by flower buds and young and very young leaves. The leaves should be well stretched, but still very tender. Older, firm leaves from the last shoots seem unreliable, either because of a lower virus titer or probably, because of insufficient homogenisation with the HOMEX method. Bark, prepared as for CTV testing, proves also to be unreliable and consequently, samples for CTV and CVV testing must be prepared separately.
2. CVV is very stable in plant extracts kept at 5°C (easily detectable after two weeks' storage), but less

stable in cool-stored leaves. Samples, kept in unsealed plastic bags, wilted after a few days, especially the most tender parts.

3. Background was rather high and it was possible to discriminate between negative and positive samples at best after substrate incubation of 1/2 and 1 hour. Background could be reduced by:
   a) Diluting the samples. 1/5(w/v) extracts gave much higher background and higher deviations between repetitions than extracts half its strength.
   b) Blocking plates before adding the samples. Blocked plates had hardly any rise in background even after almost 3 hours' incubation at room temperature and can be kept for days in the refrigerator to detect very low virus titres.
   c) Diluting the second Ab. Using second Ab at half-strength, the reaction was somewhat slower, but discrimination was as good or better than with full-strength second Ab.
4. The second Ab, when incubated overnight at 5°C together with the samples, has a low specific binding capacity for the test which is run in this way.
5. The positive sample (lemon mother-tree) from Paros had a much lower reading than the positive control and could not be detected with the protocol applied, due to high background. All samples with one positive and one negative reading at first proved negative. It is therefore possible that one more lemon mother-tree (not re-tested) is actually positive. The leaves of the samples from Paros were somewhat more mature compared to controls from Paros. This might explain the relatively low virus titer in the Paros sample that tested positive.

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Table 1. ELISA procedure for CVV detection

Plates	Costar 9018 high binding capacity
Outer wells	Buffer
Volume	200 μl
Repetitions	2 (triples only in the first test)
Coat	As protocol file
Wash (after every step)	Asys Hitech Flexiwash I plus, 3 cycles saline-tween (not buffered)
Block	1% bovine albumin Sigma A-7030 in PBS-tween, 1 hour room temperature (only last test)
Controls	Negative (-) Lemon trees in Paros on *Citrus volkameriana*, four branches from each tree pooled and tested as one sample Positive (+) Lemon trees 1 and 2 in Paros on *Citrus volkameriana*, four branches from each tree tested separately, trees 3 and 4 only rootstock surviving (symptomless), three branches from each tree tested separately
Samples	Approximately 0.5 gr in 4.5 ml sample buffer as protocol sheet
Preparation	***Material***: Bark (ba), full grown flower buds (fb) and leaves from last flush; Young leaves, fully stretched but still reddish(yl), green leaves still tender (te), or firm (lf) sliced bark and leaves homogenized with Bioreba Homex 6 in cotton-gauze lined plastic bags and incubated for 2 hours in common fridge (5°C) before transfer to plates
Incubation	Overnight in fridge
Second ab	As protocol file (bovine albumin Sigma A.7030 instead of ovalbumin)
Conjugate	As protocol file (bovine albumin Sigma A.7030 instead of ovalbumin)
Substrate	Sigma 104-105 1 tablet (5 mg) in 10 ml substrate buffer as protocol file

Table 2. Results of the ELISA test (1) for CVV average A_{405} values with standard deviation (), pos./neg threshold value (T = A_{405} negs. + 3.36) and discrimination factor A_{405}/T

TEST 1											
Incubation 23° C		7 min		15 min		29 min		67 min		135 min	
		A_{405} σ	**A_{405}/T**	**A_{405} σ**	**A_{405}/T**	**A_{405} σ**	**A_{405}/T**	**A_{405} σ**	A405/T	A405 σ	A405/T
Blank	(4)	0.038 ± 0.001		0.045 ± 0.004		0.067 ± 0.009		0.116 ± 0.026		0.208 ± 0.059	
Neg. 1	(3)	0.038 ± 0.004		0.042 ± 0.005		0.074 ± 0.019		0.161 ± 0.063		0.347 ± 133	
Neg. 2	(3)	0.038 ± 0.002		0.046 ± 0.002		0.073 ± 0.003		0.164 ± 0.014		0.355 ± 0.027	
Neg. 3	(3)	0.045 ± 0.003		0.057 ± 0.005		0.095 ± 0.007		0.220 ± 0.029		0.500 ± 0.087	
Neg. average	(9)	0.040 ± 0.005		0.049 ± 0.007		0.081 ± 0.015		0.182 ± 0.046		0.401 ± 0.110	
Threshold		0.057		0.073		0.131		0.337		0.771	
Pos. 1 1 fb	(3)	0.154 ± 0.006	2.7	0.348 ± 0.004	4.8	0.947 ± 0.086	7.2	2.184 ± 0.054	6.5	> 2.500	3.2
1 lf	(2)	0.039 ± 0.004	0.7	0.053 ± 0.007	0.7	0.102 ± 0.022	0.8	0.220 ± 0.052	0.7	0.489 ± 0.144	0.6
2 yl	(2)	0.163 ± 0.006	2.9	0.360 ± 0.042	4.9	0.980 ± 0.003	7.5	2.164 ± 0.023	6.4	2.495 ± 0.078	3.2
2 lf	(2)	0.096 ± 0.035	1.7	0.169 ± 0.081	2.3	0.441 ± 0.207	3.4	1.143 ± 0.576	3.4	1.879 ± 0.506	2.4
3 yl	(3)	0.151 ± 0.024	2.6	0.325 ± 0.042	4.5	0.980 ± 0.083	7.5	2.303 ± 0.032	6.8	2.430 ± 0.039	3.2
3 lf	(3)	0.052 ± 0.004	0.9	0.067 ± 0.008	0.9	0.123 ± 0.029	0.9	0.315 ± 0.039	0.9	0.756 ± 0.041	3.1
4 yl	(2)	0.037 ± 0.003	0.6	0.045 ± 0.002	0.6	0.075 ± 0.001	0.6	0.172 ± 0.006	0.5	0.340 ± 0.007	0.4
4 te	(2)	0.042 ± 0.004	0.7	0.052 ± 0.006	0.7	0.080 ± 0.013	0.6	0.198 ± 0.071	0.6	0.449 ± 0.088	0.6
4 lf	(2)	0.046 ± 0.004	0.8	0.058 ± 0.007	0.8	0.098 ± 0.008	0.7	0.206 ± 0.023	0.6	0.495 ± 0.122	0.6
Pos. 2 1 ba	(3)	0.038 ± 0.002	0.7	0.043 ± 0.003	0.6	0.076 ± 0.007	0.6	0.148 ± 0.006	0.4	0.292 ± 0.027	0.4
1 yl	(3)	0.142 ± 0.008	2.5	0.302 ± 0.015	4.1	0.868 ± 0.146	6.6	2.094 ± 0.151	6.2	2.398 ± 0.041	3.1
1 lf	(2)	0.040 ± 0.004	0.7	0.049 ± 0.006	0.7	0.081 ± 0.015	0.6	0.186 ± 0.040	0.6	0.383 ± 0.102	0.5
2 ba	(2)	0.054 ± 0.002	0.9	0.088 ± 0.004	1.2	0.202 ± 0.011	1.5	0.626 ± 0.042	1.9	1.541 ± 0.068	2
2 yl	(2)	0.136 ± 0.004	2.4	0.320 ± 0.012	4.4	0.926 ± 0.155	7.1	2.227 ± 0.014	6.6	2.394 ± 0.050	3.1
2 lf	(3)	0.058 ± 0.002	1	0.098 ± 0.004	1.3	0.261 ± 0.072	2	0.739 ± 0.145	2.2	1.709 ± 0.045	2.2
3 lf	(3)	0.073 ± 0.005	1.3	0.140 ± 0.019	1.9	0.357 ± 0.124	2.7	1.035 ± 0.212	3.1	2.109 ± 0.064	2.7
4 lf	(2)	0.037 ± 0.002	0.6	0.043 ± 0.002	0.6	0.068 ± 0.006	0.5	0.123 ± 0.006	0.4	0.247 ± 0.026	0.3
Pos. 3 1 lf	(2)	0.049 ± 0.006	0.9	0.058 ± 0.009	0.8	0.149 ± 0.055	1.1	0.345 ± 0.122	1	0.718 ± 0.213	0.9
2 yl	(2)	0.088 ± 0.004	1.5	0.198 ± 0.021	2.7	0.618 ± 0.037	4.7	1.586 ± 0.048	4.7	2.357 ± 0.075	3.1
2 te	(2)	0.103 ± 0.005	1.8	0.241 ± 0.030	3.3	0.810 ± 0.041	6.2	1.745 ± 0.075	5.2	2.351 ± 0.060	3
3 te	(2)	0.135 ± 0.022	2.4	0.311 ± 0.019	4.3	0.734 ± 0.064	5.6	1.849 ± 0.105	5.5	2.397 ± 0.020	3.1

Positive and negative samples, 2 or 3 repetitions per sample (indicated in brackets) no blocking step with bovine albumin material:
ba = bark; fb = flower buds; lf = firm leaves; yl = very young leaves (reddish); te = green, still tender leaves

Table 3. Results of the ELISA test (2-3) for CVV by optical readings after 35' average A405 values with standard deviation (), pos./neg threshold value (T = A405 negs. + 4.03 θ) and discrimination factor A405/T

TEST 2 - 3										
Incubation 25° C	Samples 1.5 gr in 7.5 ml buffer		Plates blocked with 1% bovine albumin Samples 1.5 gr in 15 ml buffer							
	Ab 1/1000 37°C, 4 hours		**Ab 1/1000** 37°C, 4 hours		**Ab 1/2000** 37°C, 4 hours		**Ab 1/1000** 5°C, overnight together with samples		**Ab 1/2000** 5°C, overnight together with samples	
	31 min		**35 min**		**35 min**		**35 min**		**35 min**	
	A_{405} σ	**A_{405}/T**	**A_{405} σ**	**A_{405}/T**	**A_{405} σ**	**A_{405}/T**	**A_{405} σ**	**A_{405}/T**	**A_{405} σ**	**A_{405}/T**
Blank (4)	0.032 ± 0.006		0.043 ± 0.002		0.038 ± 0.001		0.049 ± 0.003		0.039 ± 0.002	
Neg. 1	0.060 ± 0.022		0.051 ± 0.004		0.045 ± 0.001		0.040 ±0.004		0.042 ± 0.003	
Neg. 2	0.048 ± 0.002		0.045 ± 0.001		0.046 ± 0.002		0.046 ±0.001		0.044 ± 0.002	
Neg. 3	0.047 ± 0.003		0.049 ± 0.003		0.046 ± 0.002		0.041 ±0.001		0.045 ± 0.004	
Neg. average	0.051 ±0.012		0.048 ± 0.004		0.045 ±0.001		0.042 ±0.003		0.043 ± 0.003	
Threshold	0.099		0.064		0.049		0.054		0.055	
+ 1 (yl-sap fridge)	0.131 ± 0.025	1.3	0.403 ± 0.078	6.3	0.233 ± 0.031	4.8	0.071 ± 0.005	1.3	0.055 ± 0	1
+ 1 yl leaves fridge	0.054 ± 0.020	0.5								
+ 2 (yl-sap fridge)	0.149 ± 0.006	1.5								
+ 2 te leaves fridge	0.114 ± 0.045	1.2								
+ 4-1 te leaves fridge	0.109 ± 0.053	1.1								
+ 4-2 yl leaves fridge	0.080 ± 0.004	0.8								
+ 4-3 yl leaves fridge	0.057 ± 0.004	0.6								
Sample 4	0.072 ± 0.018	0.7	0.046 ± 0	0.7	0.046 ± 0.004	0.9	0.045 ± 0.003	0.8	0.043 ± 0.002	0.8
Sample 8	0.047 ± 0.001	0.5	0.046 ± 0.002	0.7	0.044 ± 0.001	0.9	0.042 ± .002	0.8	0.045 ± 0	0.8
Sample 12	0.051 ± 0.001	0.5	0.045 ± 0.001	0.6	0.043 ± 0.002	0.9	0.044 ± 0.003	0.8	0.044 ± 0.004	0.8
Sample 13	0.054 ± 0.001	0.5	0.050 ± 0.001	0.8	0.048 ± 0.002	1	0.048 ± 0.004	0.9	0.046 ± 0.002	0.8
Sample 14	0.053 ± 0.002	0.5	0.045 ± 0.004	0.7	0.042 ± 0.002	0.9	0.041 ± 0.001	0.8	0.045 ± 0.001	0.8
Sample 15	0.046 ± 0.002	0.5	0.045 ± 0.001	0.7	0.046 ± 0.002	0.9	0.044 ± 0.001	0.8	0.043 ± 0.002	0.8
Sample 16	0.053 ± 0.001	0.5	0.051 ± 0.001	0.8	0.047 ± 0.001	1	0.046 ± 0.001	0.9	0.043 ± 0.002	0.8
Sample 17	0.050 ± 0.004	0.5	0.042 ± 0.002	0.7	0.043 ± 0.001	0.9	0.041 ± 0.001	0.8	0.043 ± 0.004	0.8
Sample 20	0.053 ± 0.007	0.5	0.048 ± 0.004	0.8	0.047 ± .001	1	0.042 ± 0.002	0.8	0.047 ± 0.004	0.9
Sample 24	0.049 ± 0.001	0.5	0.048 ± 0.001	0.8	0.045 ± 0.002	0.9	0.047 ± 0.001	0.9	0.046 ± 0.001	0.8
Sample 27	0.061 ± 0.006	0.6	0.117 ± 0.001	1.8	0.103 ± 0.009	2.1	0.040 ± 0.003	0.7	0.043 ± 0.003	0.8

Controls as test 1, samples of unknown status, fresh leaves, young/tender

Table 4. Results of the ELISA test (2-3) for CVV by optical readings after 75' average A405 values with standard deviation (), pos./neg threshold value (T = A405 negs. + 4.03 θ) and discrimination factor A405/T

TEST 2 - 3										
Incubation 25° C	Samples 1.5 gr in 7.5 ml buffer		Plates blocked with 1% bovine alb min Samples 1.5 gr in 15 ml buffer							
	Ab 1/1000 37°C, 4 hours		**Ab 1/1000** 37°C, 4 hours		**Ab 1/2000** 37°C, 4 hours		**Ab 1/1000** 5°C, overnight together with samples		**Ab 1/2000** 5°C, overnight together with samples	
	75 min		**2¾ hours**		**2¾ hours**		**2¾ hours**		**2¾ hours**	
	A_{405} σ	**A_{405}/T**	**A_{405} σ**	**A_{405}/T**	**A_{405} σ**	**A_{405}/T**	**A_{405} σ**	**A_{405}/T**	**A_{405} σ**	**A_{405}/T**
Blank (4)	0.056 ± 0.005		0.097 ± 0.003		0.091 ± 0.004		0.145 ± 0.037		0.166 ± 0.045	
Neg. 1	0.228 ± 0.171		0.146 ± 0.055		0.088 ± 0.012		0.173 ± 0.130		0.089 ± 0.033	
Neg. 2	0.220 ± 0.115		0.102 ± 0.001		0.089 ± 0.005		0.071 ± 0.011		0.112 ± 0.021	
Neg. 3	0.123 ± 0.003		0.092 ± 0.005		0.085 ± 0.006		0.090 ± 0.001		0.123 ± 0.013	
Neg. average (6)	0.190 ± 0.106		0.113 ± 0.036		0.087 ± 0.006		0.111 ± 0.076		0.108 ± 0.024	
Threshold	0.617		0.258		0.113		0.417		0.205	
+ 1 (yl-sap fridge)	0.183 ± 0.386	1.9	2.347 ± 0.037	9.1	2.202 ± 0.033	19.5	0.389 ± 0.053	0.9	0.341 ± 0.021	1.7
+ 1 yl leaves fridge	0.289 ± 0.049	0.5								
+ 2 (yl-sap fridge)	0.133 ± 0.239	1.8								
+ 2 te leaves fridge	0.720 ± 0.148	1.2								
+ 4-1 te leaves fridge	0.647 ± 0.152	1								
+ 4-2 yl leaves fridge	0.473 ±0.363	0.8								
+ 4-3 yl leaves fridge	0.260 ±0.055	0.4								
Sample 4	0.294 ± 0.129	0.5	0.083 ± 0.002	0.3	0.081 ± 0.004	0.7	0.153 ± 0.016	0.4	0.110 ± 0.008	0.5
Sample 8	0.587 ± 0.451	1	0.120 ± 0.004	0.5	0.095 ± 0.001	0.8	0.138 ± 0.014	0.3	0.118 ± 0.016	0.6
Sample 12	0.894 ± 0.948	1.4	0.111 ± 0.023	0.4	0.080 ± 0.003	0.7	0.105 ± 0.035	0.3	0.094 ± 0.035	0.5
Sample 13	1.604 ± 0.716	2.6	0.099 ± 0.004	0.4	0.084 ± 0.005	0.7	0.245 ± 0.064	0.6	0.144 ± 0.021	0.7
Sample 14	0.376 ± 0.302	0.6	0.093 ± 0.014	0.4	0.076 ± 0.001	0.7	0.089 ± 0.014	0.2	0.082 ± 0.005	0.4
Sample 15	0.338 ± 0.261	0.5	0.102 ± 0.013	0.4	0.089 ± 0.002	0.8	0.133 ± 0.053	0.3	0.097 ± 0.018	0.5
Sample 16	0.347 ± 0.122	0.6	0.100 ± 0.008	0.4	0.084 ± 0.001	0.7	0.104 ± 0.008	0.2	0.081 ± 0.006	0.4
Sample 17	0.575 ± 0.614	0.9	0.090 ± 0.006	0.3	0.090 ± 0.006	0.8	0.077 ± 0.016	0.2	0.092 ± 0.004	0.4
Sample 20	0.827 ± 0.440	1.3	0.097 ± 0.017	0.4	0.084 ± 0.002	0.7	0.074 ± 0.009	0.2	0.069 ± 0.008	0.3
Sample 24	0.638 ± 0.495	1	0.089 ± 0.001	0.3	0.084 ± 0.003	0.7	0.148 ± 0.022	0.4	0.101 ± 0.016	0.5
Sample 27	0.395 ± 0.088	0.6	0.964 ± 0.112	3.7	0.765 ± 0.023	6.8	0.075 ± 0.005	0.2	0.100 ± 0.008	0.5

Controls as test 1, samples of unknown status, fresh leaves, young/tender