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Short communication

Occurrence of *Citrus psorosis virus* in Campania, southern Italy

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Abstract

Citrus psorosis virus (CPsV), genus *Ophiovirus*, is associated with a severe disease of citrus worldwide. Double antibody sandwich (DAS) ELISA using a polyclonal antiserum, and triple antibody sandwich (TAS) ELISAs, employing the IgG monoclonal antibody (mab) 13C5, and the IgM mab 2A3, were used to detect CPsV in orchards of different citrus varieties in Campania, southern Italy. TAS ELISA with 13C5 detected all the infections detected by DAS ELISA. Overall, 14% of trees younger than 15 years were positive, but only 1% of older trees, suggesting that infected propagating material has been increasingly used in recent years, in the absence of certification. Highest infection rates were in younger trees of sweet orange (22.8%) and clementine (18.6%). CPsV could easily be detected at all seasons of the year tested (June–January); these and earlier results indicate that TAS ELISA using 13C5 is a sensitive, broad-spectrum and reliable diagnostic method useful for routine tests and certification programmes. Of 44 field isolates responding strongly to DAS ELISA and 13C5-TAS ELISA, mab 2A3 gave similar results with 29 isolates, but gave low values with the others, thus providing a degree of differentiation among isolates. To confirm that the ELISA tests were indeed detecting CPsV, samples of 42 ELISA-positive plants were analysed by ISEM in a blind test, and in 38 of these, characteristic virus particles were clearly seen. Although CPsV was frequently and consistently detected in the area sampled, bark scaling symptoms were not seen: possible reasons for this are discussed.

Citrus psorosis is widespread, causing serious damage in many regions including South America, Mediterranean areas and probably Asia (Roistacher, 1993; Martelli and D'Onghia, 1998), though clear documentation for Asia is lacking. The disease is strongly correlated with presence of *Citrus psorosis virus* (CPsV), genus *Ophiovirus*, a virus with fine thread-like particles and a genome of 3 ssRNAs that appear to be entirely negative-stranded (Milne et al., 1996; 2000).

The classic symptoms of psorosis are bark scaling and internal wood staining of trunks and branches, flecking and ringspots in leaves, and decline; bark scaling often first appears in 10–12-year-old trees. However, in the absence of the severe B form of the disease, the bark-scaling symptom may be delayed or even absent. The most susceptible species are sweet orange, mandarin and grapefruit (Roistacher, 1991; 1993). In Italy the disease was first reported in Sicily (Fawcett, 1925) and later in all the citrus-growing areas (Servazzi et al., 1964), but was recognised only by symptomatology or occasionally by use of grafting to indicator plants.

Garcia et al. (1997) using an antiserum to CPsV in double antibody sandwich (DAS) ELISA, showed that results were positively correlated with data from biological indexing, for a selection of CPsV isolates worldwide, and the same antiserum was used to show that CPsV is widespread in orchards in Apulia in southern Italy (Djelouah and D'Onghia, 1998; D'Onghia et al., 1998). However, the method was not very sensitive and could only be used on newly flushed leaves, preferably from the greenhouse. Recently, improved protocols for

the DAS ELISA and the use of monoclonal antibodies (mabs) in triple antibody sandwich (TAS) ELISA have enabled reliable detection of the virus in field material at all seasons of the year; the mab 13C5 detected all sources of CPsV detected by the DAS ELISA, suggesting that it may recognise a 'universal' epitope, whereas mab 2A3 did not detect certain isolates (Alioto et al., 1999). Here the results of a survey of CPsV in citrus orchards in the Campania region of Italy, using the improved DAS and TAS ELISA, are presented. For convenience, the term 'isolate' is used for sources of virus from individual trees. However, coming directly from the field, some of these sources may well have been mixtures.

Orchards were surveyed from April to November 1999 in 6 major citrus-growing areas of Campania: the Sorrentina peninsula, Amalfi, Pontecagnano, Torre Annunziata, Nocera and Suio. At the time of survey, all trees sampled were examined for presence of bark-scaling and leaf symptoms. However, the different seasons at which the inspections were made were not always appropriate for seeing leaf symptoms.

Samples were collected randomly from 894 trees, aged 4–200 years, of the main citrus species and cultivars. Between 10 and 40 g of mature leaves, plus young leaves if present, were sampled from different areas of each tree and stored at 4 °C for not longer than 5 days until processed for ELISA. Mixed subsamples of 1 g were prepared by taking small pieces of each leaf in the main sample. Following the method of Alioto et al. (1999), the subsample was sealed in a plastic bag with 9 volumes (w/v) of extraction buffer, homogenised, and used for DAS and TAS ELISAs. Samples were also taken from an isolated 30-year-old sweet orange tree, cv. Sanguinello, that had repeatedly proved ELISA-negative over a period of 2 years. Trees that were positive in initial tests were sampled again for a second test. If still positive, 10 of these trees were retested in different months in order to determine the best season for sampling.

Formally, values 3 times the optical density (OD) of the mean values for healthy sample were assumed to indicate infected samples. In practice, with the DAS ELISAs and 13C5-TAS ELISAs, experimental OD values were either high or closely similar to the values for healthy leaves, so that no borderline cases occurred. With mab 2A3, some intermediate values did emerge, as discussed below.

Biological indexing facilities were not available for confirming the ELISA results. We wished to send samples to IVIA, Valencia, Spain, for biological testing but quarantine considerations did not permit this. However, in a blind test using ISEM, fresh field samples of 42 plants already testing positive by 13C5-TAS ELISA and by DAS ELISA, randomised with 9 samples that were ELISA-negative, were sent to Turin in April 2000 for testing using mab 13C5. EM grids were coated for 15 min at 25 °C with the mab diluted 1/4000 in 0.1 M phosphate buffer, pH 7, then rinsed with the same buffer. Samples were extracted in 5 volumes (w/v) of 0.1 M phosphate buffer, pH 8, containing 2% polyvinylpyrrolidone, and incubated on the coated grids for 3 h at 25 °C. The grids were then rinsed with water, negatively stained with 1% uranyl acetate, and examined in a Philips CM 10 EM at 60 kV.

Of the 42 ELISA-positive samples, 38 were positive by ISEM (characteristic virus particles clearly seen), with 3 samples rated as dubious and 1 as negative. Of the 9 samples testing negative by ELISA and included at random, all were found negative by ISEM.

ELISA data for the 6 individual regions surveyed will be reported elsewhere; here we give the overall results. Numbers of healthy and infected trees found for each type of citrus are given in Table 1, referring to identical outcomes for the DAS and 13C5-TAS ELISAs. Overall, 4.9% of trees sampled were CPsV-positive. The proportion of infected plants was much higher (14.2%) in trees less than 15 years old, compared with older trees (0.9%). Further analysis showed that for trees aged 0–5, 6–10 and 11–15 years (total 268 trees), infections were 10%, 3% and 1.1% respectively.

Highest rates of infection for trees under 15 years old were found in sweet orange (22.8%) and clementine (18.6%). Among sweet orange cultivars, the most infected were Navelina, Washington navel and Tarocco, while in clementine the highest infection rate was in cv. Monreale. In trees 15 or more years old, Washington navel and Monreale appeared to be the most commonly infected, though absolute numbers were small. Only low rates of infection were found in lemon and mandarin, with no infection in sour orange, citron, kumquat or satsuma.

OD values for the CPsV-positive trees are shown in Table 2, recorded in parallel on the same samples for DAS ELISA and for TAS ELISA using each of the 2 mabs. The values showed that all samples that were positive in the DAS ELISA were also positive with 13C5 in TAS ELISA. They confirmed that 13C5-TAS ELISA was more sensitive than DAS ELISA, i.e. gave

Table 1. ELISA detection of CPsV in Campania, in citrus orchards of different varieties and ages

Citrus species	Cultivars	Trees under 15 years			Trees old 15 or more years		
		Total	Infected	%	Total	Infected	%
Lemon	Zagara bianca	22	0		13	0	
	Femminello	1	1		222	0	
	Sfusato amalfitano	37	0		17	0	
	Gloria d'Amalfi	8	0		2	0	
	Cetara apirene	2	0		0	0	
	S. Teresa	2	0		2	0	
	Lunario	4	1		1	0	
	Total	76	2	2.6	257	0	0
Sweet orange	Washington navel	8	3		6	1	
	Tarocco	36	12		22	0	
	Vaniglia	0	0		1	0	
	Biondo comune	25	4		156	0	
	Sanguinello	1	0		4	0	
	Valencia	22	0		11	0	
	Navelina	13	5		0	0	
	Total	105	24	22.8	200	1	0.5
Clementine	Monreale	56	11		51	4	
	Comune	3	0		30	1	
	ISA	0	0		18	0	
	Total	59	11	18.6	99	5	5
Mandarin	Avana comune	20	1		60	0	
Sour orange		8	0		4	0	
Kumquat		0	0		1	0	
Satsuma		0	0		2	0	
Citron		0	0		3	0	
Grand total		268	38	14.2	626	6	0.9

a higher ratio of OD (infected sample) to OD (healthy sample). When reacting, 2A3 usually gave higher OD ratios than 13C5; however, some samples (Na 6, 7, 8, 10, 18, 19, 20, 24, 25 and 26) did not react with 2A3 even when the DAS and 13C5-TAS values were high, and isolates Na 22, 23, 35, 38 and 39 gave low responses (values from 5 to 23).

Periodical retesting of the 10 selected ELISA-positive field trees in June, July, September, November and January showed that the virus was readily detected by DAS ELISA and 13C5-TAS ELISA over the whole period, and suggested that there is no time of year especially favourable or unfavourable to detection by this method.

Inspection of ELISA-positive trees did not reveal the presence of classical symptoms of the disease either on trunks and branches or leaves, with the exception of sample Na 4, 12 and 13 which showed interveinal flecking, vein clearing and oak-leaf pattern on young leaves. The oak-leaf symptom is, however, an indication of the presence of concave gum disease, not psorosis (Roistacher, 1991).

Leaf symptoms of CPsV are usually evanescent, appearing only on young leaves in cool conditions. As ELISA-positive trees were not all inspected at such times, our failure to see leaf symptoms is probably not significant. However, all trees 15 or more years old were re-examined for symptoms on young leaves in April 2000, but showed none.

The results show that CPsV is widespread in Campania, with an especially high incidence in some varieties and in younger plantings. Propagating material used in this area is acquired from other Italian regions, especially from Sicily. Our results suggest that the virus has been introduced on a large scale in nursery material in the recent past; the current situation is probably unchanged. A certification scheme for this virus is not in operation.

Table 2. Results of DAS ELISA, using polyclonal antiserum, and TAS ELISA with 2 different mabs (13C5 and 2A3) on 44 isolates of CPsV from Campania, tested in November 1999. OD-S, optical density of the sample; S/H, ratio of sample OD to healthy OD. S values are means of 2 wells and H values means of 4 wells. H values were set at +0.001 if they fell below this value

Isolates	Citrus species	Cultivars	DAS		TAS			
					13C5 (IgG)		2A3 (IgM)	
			OD-S	S/H	OD-S	S/H	OD-S	S/H
NA3	Lemon	Femminello	1.500	167	1.720	1720	2.000	2000
NA4	Sweet orange	Biondo comune	0.914	48	0.388	194	1.157	1157
NA5	Sweet orange	Tarocco	1.002	111	0.645	645	1.877	1877
NA6	Clementine	Monreale	0.769	85	0.475	475	−0.004	−4
NA7	Clementine	Monreale	0.929	103	0.485	485	−0.010	−1
NA8	Sweet orange	Biondo comune	1.017	113	0.512	512	0.000	0
NA10	Clementine	Comune	0.425	47	0.200	200	−0.012	−1.2
NA11	Lemon	Lunario	0.917	101	1.154	1154	2.290	2290
NA12	Mandarin	Avana	1.831	203	1.131	1131	2.428	2428
NA13	Sweet orange	Washington navel	0.970	108	0.504	504	1.581	1581
NA14	Sweet orange	Washington navel	0.580	64	0.299	299	1.156	1156
NA15	Sweet orange	Washington navel	1.883	209	0.878	878	2.349	2349
NA16	Sweet orange	Biondo comune	0.804	89	0.458	458	1.508	1058
NA17	Sweet orange	Biondo comune	0.998	111	0.729	729	2.106	2106
NA18	Clementine	Monreale	0.807	90	0.530	530	−0.006	−6
NA19	Clementine	Monreale	1.111	123	0.694	694	−0.002	−2
NA20	Clementine	Monreale	1.106	123	0.624	624	0.002	2
NA21	Clementine	Monreale	0.709	79	0.394	394	1.247	1247
NA22	Clementine	Monreale	0.821	91	0.447	447	0.007	7
NA23	Clementine	Monreale	0.850	94	0.543	543	0.005	5
NA24	Clementine	Monreale	1.398	155	1.008	1008	−0.014	−1.4
NA25	Clementine	Monreale	0.761	85	0.430	430	−0.005	−5
NA26	Clementine	Monreale	0.795	88	0.539	539	−0.014	−1.4
NA27	Sweet orange	Washington navel	0.274	30	0.164	164	0.652	652
NA28	Sweet orange	Tarocco	0.831	92	0.542	542	1.728	1728
NA29	Sweet orange	Tarocco	0.962	107	0.466	466	1.425	1425
NA30	Sweet orange	Tarocco	0.838	93	0.615	615	1.868	1868
NA31	Sweet orange	Tarocco	0.996	111	0.888	888	2.128	2128
NA32	Sweet orange	Tarocco	1.095	122	0.751	751	2.048	2048
NA33	Sweet orange	Tarocco	0.801	89	0.541	541	1.598	1598
NA34	Sweet orange	Tarocco	0.980	109	1.530	1530	2.110	2110
NA35	Sweet orange	Tarocco	0.784	87	0.357	357	0.009	9
NA36	Sweet orange	Tarocco	0.703	78	0.489	489	1.749	1749
NA37	Sweet orange	Tarocco	0.984	109	0.745	745	2.176	2176
NA38	Sweet orange	Tarocco	0.846	94	0.664	664	0.011	11
NA39	Sweet orange	Navelina	0.716	80	0.623	623	0.023	23
NA40	Sweet orange	Navelina	0.943	105	0.686	686	2.082	2082
NA41	Sweet orange	Navelina	0.936	104	0.663	663	2.033	2033
NA42	Sweet orange	Navelina	0.631	70	0.407	407	1.538	1538
NA43	Sweet orange	Navelina	1.448	128	0.945	945	2.306	2306
NA44	Clementine	Monreale	0.490	54	0.228	228	0.543	543
NA45	Clementine	Monreale	0.618	69	0.292	292	0.763	763
NA46	Clementine	Monreale	0.286	32	0.112	112	0.363	363
NA47	Clementine	Monreale	0.145	16	0.080	80	0.272	272

In contrast to reports from Apulia, where the 88% of ELISA-positive trees showed symptoms, both on bark and leaves (D'Onghia et al., 1998), the ELISA-positive trees in Campania showed no bark-scaling. There is no clear explanation of this difference. However it may be in part because D'Onghia et al. (1998) sampled 15–20 year-old trees, whereas in our study most ELISA-positive trees were very young. It is also possible that the CPsV strains involved are different, and do not include or express the more severe B form of psorosis (Roistacher, 1991). A further possibility is that the citrus varieties or rootstocks may differ somewhat in the 2 regions. Nevertheless, the finding of relatively mature but symptomless trees carrying an agent indexing as psorosis is not unusual (personal communications, Dr. P. Moreno, IVIA, Valencia, Spain; Dr. C.N. Roistacher, University of California, Riverside).

The ELISA-positive but symptomless trees found in our survey may develop the disease as they mature, but meanwhile they are assumed by the grower to be healthy, and could be used as a source of propagating material. The virus may also spread naturally in orchards, a possibility for which there is some evidence in the USA (Timmer and Garnsey, 1980) and Argentina (Beñatena and Portillo, 1984), but which has not been examined carefully in Italy.

The present study has shown that DAS and TAS ELISA can now readily detect CPsV in field trees, even at supposedly 'unfavourable' times of the year such as mid-summer or mid-winter. Thus indexing by this method can be carried out year-round. The data obtained show that mab 13C5 reacted with all isolates detected by DAS ELISA. This is consistent with a previous report (Alioto et al., 1999) and confirm that 13C5 could be a 'universal' mab for CPsV. Mab 2A3 did not detect 10 isolates and reacted weakly with 5 others, a first indication that different strains are present in the Campania region.

Our results and those of Djelouah and D'Onghia (1998) and D'Onghia et al. (1998) underline the importance of establishing a certification and eradication programme for CPsV.

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