ELISA CORRELATES WITH BIOLOGICAL INDEXING FOR THE DETECTION OF CITRUS PSOROSIS-ASSOCIATED VIRUS

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SUMMARY

An ELISA kit produced at the Istituto di Fitovirologia Applicata of CNR, Torino, Italy for the detection of citrus psorosis-associated virus (CPsAV) was successfully used to test 4,499 citrus plants of different varieties in nurseries, commercial groves and a germplasm collection of Apulia (Southern Italy). One hundred and seventy trees were indexed for psorosis using 'Madame Vinous' sweet orange and 'Dweet tangor'. From 60 plants that were positive by indexing, mechanical transmissions to herbaceous hosts were attempted. Psorosis was present in most of the citrus varieties, with a low incidence and distribution in commercial groves (7.4% of infected trees) and in the nurseries (4.6% of infected trees). In commercial groves, the local clementine cv. 'Fedele' (14.4% of infected trees) and 'Navelina' old clone sweet orange (9% of infected trees) were the most infected. The same rate of infection was shown by cv. 'Fedele' in the nurseries, whereas the highest infection rates (20.4% of infected trees) were observed in Satsuma 'Miyagawa'. All the plants that were psorosis-positive by indexing were also positive by ELISA with the sole exception of a cv. 'Fedele' source. By contrast, four citrus accessions that indexed negative, were found to be positive by ELISA. Serological tests revealed a high percentage of CPsAV infections in plants indexing positive for concave gum. Only from three plants out of the 60 that indexed positive was a virus with the filamentous particles typical of CPsAV recovered by mechanical inoculation to herbaceous hosts. The demonstration that ELISA can identify psorosis in a sensitive and reliable manner, represents a step forward in favour of serological assays, which can be used for routine testing and as a complement to biological indexing in sanitary selection programmes.

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RIASSUNTO

STRETTA CORRELAZIONE TRA ELISA E SAGGI BIOLOGICI NEL RILEVARE LE INFEZIONI DEL VIRUS ASSOCIATO ALLA PSOROSI DEGLI AGRUMI. Un corredo ELISA prodotto dall'Istituto di Fitovirologia Applicata del CNR di Torino per l'identificazione del virus associato alla psorosi degli agrumi (CPsAV) è stato usato per saggiare 4499 piante di differenti cultivar, in impianti commerciali e vivai pugliesi ed in una collezione agrumicola con accessioni di varia provenienza geografica. Centosettanta piante sono state saggiate sugli indicatori specifici "Madame Vinous" e "Dweet tangor", e da 60 piante che erano risultate positive all'indexaggio, sono state effettuate trasmissioni meccaniche ad ospiti erbacei. La psorosi è risultata presente sulla maggior parte delle cultivar saggiate con bassa incidenza sia negli impianti commerciali (7,4% di piante infette) che nei vivai (4,6% di piante infette). La cultivar locale di clementine "Fedele" e l' arancio "Navelina vecchio clone" sono risultati i più infetti in campo (rispettivamente 14,4% e 9% di alberi infetti) mentre, in vivaio, i più alti livelli d'infezione (20,4% di alberi infetti) sono stati riscontrati su mandarino Satsuma "Miyagawa". Tutte le piante positive all'indexaggio sono risultate positive a CPsAV anche in ELISA con la sola eccezione di un campione di "Fedele". Per converso, quattro accessioni negative all'indexaggio sono risultate positive in ELISA. I saggi sierologici hanno anche mostrato un'alta percentuale di infezioni di CPsAV in piante con concavità gommose. Solo da tre piante sulle 60 positive all'indexaggio è stato isolato su ospiti erbacei un virus con le tipiche particelle filamentose di CPsAV. La dimostrazione che l'ELISA può individuare la psorosi in maniera sensibile ed affidabile, rappresenta un importante passo in avanti a favore della diagnosi sierologica, che pertanto si propone come tecnica di routine, ancorché di supporto all'indexaggio, per l'identificazione di CPsAV nei programmi di selezione sanitaria.

Key words: citrus, psorosis, ophiovirus, ELISA, diagnosis.

INTRODUCTION

Psorosis is a widespread disease of citrus, characterized by different forms, *i.e.* psorosis A, psorosis B, and ringspot, related by cross-protection (Roistacher and Calavan, 1965; Derrick *et al.*, 1991; Roistacher, 1993). There are large variations among psorosis isolates with respect to their mode of transmission, apart from grafting, since some are mechanically transmissible whereas others, like the severe Argentine and Uruguayan strains, may be possibly vector-transmitted (Roistacher, 1991, 1993), although the putative vector is unknown.

Until recently, the only effective method for diagnosis was biological indexing on differential indicators (Roistacher, 1993) which is slow, labour-intensive, and requires special skills and controlled glasshouse conditions. Thus, the need was felt for developing simpler and more rapid detection methods.

Derrick *et al.* (1988) were able to partially purify citrus ringspot virus (CtRSV), the best characterized isolate of CPsAV, laying the basis for further understanding of virus particle morphology, and development of serological (ELISA) and molecular (RT-PCR) methods of diagnosis (Garcia *et al.*, 1997).

Because psorosis and ringspot appear to be caused by the same virus (Navas-Castillo *et al.*, 1993; Roistacher, 1993) the putative causal agent of these diseases is now referred to as citrus psorosis-associated virus (CPsAV) (Milne *et al.*, 1996). This virus has particle morphology and properties of a kind (Garcia *et al.*, 1994; Milne *et al.*, 1996) that prompted the proposal of its assignment to a novel genus denoted *Ophiovirus* (Milne *et al.*, 1996).

In this paper we report on the use in DAS-ELISA of an antiserum to CtRSV produced at IFA, Torino (Garcia *et al.*, 1997). First we tested the correlation between ELISA results and those of biological indexing on a collection containing psorosis accessions of different Italian and Mediterranean origin. Obtaining a good correlation, we went on to assess by ELISA the incidence of psorosis in citrus nurseries and groves in Apulia.

MATERIALS AND METHODS

Sampling. Investigations were carried out in the spring of 1996 in 15- to 20-year-old commercial groves, and in the nurseries along the Ionian coast of Apulia. In the groves, samples were taken from the main citrus species and cultivars ('Navelina' sweet orange, 'Common' and local clementine ecotypes) while in the nurseries, a wider range of citrus species and their cultivars was sampled. In the groves, sampling was carried out at random regardless of whether the plants showed symptoms or not, along the periphery of square areas about 2 ha in size (D'Onghia *et al.*, 1998). Three 10-15 cm long young shoots were collected from each tree. Five trees were grouped in each sample. A total of 1,533 trees were sampled from commercial plantings in 12 selected areas and 2,966 trees were sampled at random from 17 different nurseries representing both main commercial and minor varieties. In addition, we tested by ELISA about 20 psorosis sources of different origins (Italy, Lebanon, Spain and USA) and three concave gum isolates (CG301, CG302 and CG306 courtesy of C.N. Roistacher), in collection at IAM Bari. We included the concave gum isolates because a high proportion of trees with concave gum symptoms also tested ELISA-positive for CPsAV, and we wished to be sure that this was simply due to mixed infection, and not because the ELISA was detecting the concave gum agent itself.

Trees that gave positive reactions were inspected for symptoms and sampled for a second trial in September. Whenever a sample gave a positive ELISA reaction, the five trees from which the composite sample originated were tested individually. In trials preliminary to large-scale testing it was repeatedly found that by using extracts from a sample made up of a leaf each from a psorosis-infected reference source and four healthy seedlings, ELISA responses were consistently positive.

DAS-ELISA. An antiserum produced at IFA-CNR Turin, against CtRSV-4 (Garnsey and Timmer, 1980) was used in DAS-ELISA (Clark and Adams, 1977). Plate wells (Falcon 3911) were coated with IgG at 1.4 μg ml^{-1} in 0.1 M sodium carbonate buffer, pH 9.6 plus 0.01% sodium azide, then incubated for 2 h at 37°C. Extracts were prepared from freshly collected young citrus leaf samples, tenfold diluted in phosphate-buffered saline with 0.05% Tween 20 (PBS-T) containing 2% polyvinylpyrrolidone, and incubated overnight at 4°C. Tenfold dilution of the extracts was used because preliminary trials in which several extract dilutions were tested, had shown its suitability for ELISA, without appreciable loss of sensitivity. Conjugated alkaline phosphatase, diluted 1/1000 in PBS-T, was added and incubated for 2 h at 37°C. The substrate (1 mg ml^{-1} of paranitrophenyl phosphate) was then added, and the colour permitted to develop for 2 h at room temperature. Optical densities were measured at 405 nm in a Titertek Multiskan photometer. The results were based on the mean absorbance values of the 2 wells loaded with each sample. A sample was considered positive if its value was 3 times or more higher than the healthy citrus plants extracts used as controls (0.020 to 0.025).

Ten American psorosis isolates (P200, P203, P203M, P205, P208, P209, P213, P215M, P216, P216M, courtesy of C.N. Roistacher), from the IAM-B collection, were used as positive controls. These controls gave mean absorbance values ranging from 0.090 to 0.210.

Biological indexing. Budwood from 170 trees, chosen at random and not necessarily showing symptoms, was used for indexing by the method of Roistacher (1991). A total of eight indicator seedlings (four of 'Madame Vinous' sweet orange and four of 'Dweet tangor') were graft-inoculated from each tree, both positive and negative controls. Plants were grown in a greenhouse at about 27°C and inspected at least once each week for symptoms, beginning one month after grafting. Samples showing any type of leaf symptom were mechanically inoculated to *Chenopodium quinoa* and *C. amaranticolor* using the following extraction media : (*i*) 0.05 M Tris-TME pH 7.5, containing 0.01 M $MgCl_2$ and 0.02 M EDTA (Vaira *et al.,* 1997); (*ii*) 0.05 M Tris-TACM pH 8.0, containing 0.1% ascorbic acid, 0.1% cysteine and 0.5% 2-mercaptoethanol (Derrick *et al.,* 1988); (*iii*) 0.05 M K_2HPO_4 pH 7.7, containing 0.2% DIECA. The herbaceous plants were kept in the glasshouse at a temperature averaging 25°C.

Electron microscopy. Crude extracts for negative staining and immunosorbent electron microscopy (ISEM) were prepared from symptomatic citrus leaves by grinding one weight of tissue in 2 vol. of 0.05 M Tris buffer, pH 8.0 and centrifuging at 10,000 *g*. ISEM was carried out as described by Garcia *et al.* (1994), using IgGs diluted 1:500 and incubated for 30 min at 37°C, and for 3h with the sample preparation. The grids were negatively stained with 1% aqueous uranyl acetate for 3 min.

RESULTS

Indexing. Observations made on the leaves of the emerging young flushes of growth in the indexed seedling plants showed symptoms of shock, interveinal flecking, mottling and oak leaf patterns These symptoms were consistently shown by 'Dweet tangor' but not always by 'Madame Vinous'. Shock symptoms were not always given by sources that were ELISA-positive for CPsAV. Oak leaf patterns, with the only exception of two Satsuma trees, were always associated with 'Navelina' old clone which, as determined in a previous study, was affected by concave gum. However, according to results of indexing and ELISA the same sources proved to be infected also by CPsAV. Of the 170 citrus accessions indexed, 60 (35.2%) induced symptoms on the indicators. All the trees that indexed positive were also ELISA positive. However three accessions of 'Navelina' old clone and one of Satsuma 'Miyagawa', that indexed negative, gave a positive ELISA reaction. Conversely, only one accession of 'Fedele' clementine, that showed bark scaling and wood staining in the field and mottling in 'Dweet tangor', was ELISA negative (Table 1).

DAS-ELISA From Table 2, where the results of ELISA testing of individual trees are reported, it appears that 5.6 % of a total of 4,499 plants sampled in the groves and nurseries were CPSaV-positive. This represented 114 out of 1,533 trees from groves (7.4%) and 137 out of 2,966 (4.6%) trees from nurseries. In the groves the most infected cultivar was the local clementine 'Fedele' (14.4%), many of whose trees showed severe psorosis-A bark scaling in the field. However, strangely enough, these samples gave the lowest ELISA readings (0.058-0.060). 'Navelina' old clone, known to be seriously infected by concave gum (D'Onghia *et al.,* 1992), exhibited a 9% infection by ELISA and gave readings as high as some of the positive controls (0.245-0.250), whereas the three concave gum sources from USA were clearly ELISA-negative. Only two sources out of 200 of 'Navelina' ISA, a virus-tested clone, were infected.

In the nurseries, most of the varieties surveyed were ELISA-positive. Satsuma 'Miyagawa' (ELISA readings from 0.170 to 0.185) was the most infected (20.4%) followed, with a 16% infection, by 'Diamante' citron (ELISA reading from 0.085 to 0.090). As in the groves, a similar rate of infection was shown by 'Fedele' clementine (13.7%) and 'Navelina' old clone orange (7.2%).

The 20 psorosis sources held in the virus collection were all ELISA-positive with the exception of one isolate of Italian origin. In a repeated ELISA index test done in September, the same results were obtained.

Field symptoms. There was a fairly good correlation between ELISA response and field symptoms, for 88% of the ELISA-positive trees observed in the field in September showed symptoms. The remaining 12% of the trees were completely symptomless. Various types of leaf mottling occurred in young flushes of the local clementine cvs of 'Fedele' and 'Precoce di Massafra' and in 'Navelina' ISA. Ringspot symptoms, interveinal flecking and yellow etching patterns along the minor veins, similar to those described by Wallace and Drake (1968), were observed on mature leaves, but rarely on

Table 1. Comparative results of CPsAV detection using different diagnostic tests.

No. of samples	Species	Diagnostics tests	
		ELISA	Graft-transmission
	Sweet oranges		
36	Navelina old clone*	20	17
10	Navelina ISA	2	2
6	Washington. Navel	3	3
4	Valencia	0	0
4	Tarocco	2	2
	Mandarin		
11	Avana seedless	1	1
	Clementine		
21	Common	3	3
8	Fedele	6	7
8	Gentile	4	4
5	Precoce di Massafra	3	3
	Tangelo		
3	Orlando	0	0
	Satsuma		
19	Miyagawa	13	12
	Lemon		
4	Sfusato	1	1
3	Monachello	0	0
3	Femminello	1	1
2	Interdonato	0	0
2	*Kumquat*	1	1
16	*Citron*	3	3
2	*Lime*	0	0
1	*Chinotto*	0	0
2	*Grapefruit*	0	0

*CPsAV was isolated by mechanical inoculation only from three accessions of this variety.

the fruit of 'Navelina' old clone. Severe psorosis-A bark scaling and wood staining were present in many of the 'Fedele' and 'Common' clementine trees. Oak leaf patterns were observed in the leaves of the young flush of 'Navelina' old clone and on 'Gentile' clementine. In some 'Navelina' and 'Fedele' groves, the infections detected by visual inspections ranged from 50 to 70%. A range of symptoms expression from mild to severe was found in all groves surveyed.

Mechanical inoculation. A virus was recovered by mechanical inoculation to herbaceous hosts only from three 'Navelina' old clone plants out of the 60 sources that had given psorosis reactions on the indicators (Table 2). The inoculum had been prepared in 0.05 M K_2HPO_4, pH 7.7. *C. quinoa* reacted with chlorotic/necrotic local lesions (Fig. 1A) whereas systemic mottling and ringspots developed in *C. amaranticolor* (Figs 1B, C).

Electron microscopy. A few particles with the typical open circular form [O configuration as described by Milne *et al.,* (1996)] for CPsAV were detected by negative staining in leaf extracts from herbaceous hosts (Fig. 2A), but not in citrus leaf extracts. Particles with a L form (*sensu* Milne *et al*., 1996), composed of two intertwined threads with a loop at each end, were revealed by ISEM in both citrus and herbaceous host preparations (Fig. 2B). In symptomatic *C. amaranticolor* plants, no particles other than those resembling CPsAV were found.

DISCUSSION

The present survey gives some perspective on the occurrence and distribution of CtRSV-psorosis in citrus groves and nurseries in Apulia, particularly in local ecotypes of clementine and 'Navelina' old clone.

The very high correlation of positive ELISA results with indexing demonstrates the feasibility of using serological assays for large scale diagnosis of psorosis disease. Moreover, the four ELISA-positive samples which indexed negative on indicators suggest that ELISA can be more sensitive than indexing for detecting CPsAV. The low absorbance values found for all 'Fedele' clementines, which were severely affected by the bark scaling form of psorosis, is difficult to explain unless the virus titre was low, or these sources were infected by a different virus strain serologically related but not identical to the CtRSV-psorosis strain used to raise the antiserum employed. The high proportion of local concave gum samples testing psorosis-positive, indicates a high level of mixed infection with the two agents. This is in line with the observation that in the 'Fedele' and 'Navelina' old clone plantings, the growers had top worked plants showing severe shell bark lesions or concave gum symptoms in the attempt to overcome the disease, not realizing that in this way new emerging grafts would be re-infected with viruses present in the sour orange rootstock.

The results of sap transmission tests were not satisfactory because of the exceedingly low level of successful virus recovery. However, this is in line with the notion that CPsAV detection by mechanical transmission is still not uniform or readily accomplished. Roistacher

Table 2. Results of ELISA test for the detection of CPsAV in Apulian citrus nurseries and groves.

Citrus species	Cultivars	Trees		
		Tested	Infected	%
NURSERIES				
Sweet orange	Navelina old clone	249	18	7.20
	Navelina ISA	218	0	0.00
	Valencia Late	18	0	0.00
	Washington navel	79	9	11.40
	Vaniglia	10	0	0.00
	Tarocco	38	3	7.90
		612	**30**	**5.00**
Mandarin	Avana	188	3	1.60
	Avana Seedless	35	1	2.80
	Avana Tardivo di Ciaculli	5	0	0.00
	Fortuna	19	1	5.20
		247	**5**	**2.00**
Clementine	Common	139	15	10.80
	ISA	104	0	0.00
	Oroval	26	0	0.00
	Nules	5	0	0.00
	Local			
	- Fedele	124	17	13.70
	- Gentile	106	8	7.50
	- Precoce di Massafra	530	6	1.20
		1034	**59**	**5.70**
Tangelo	Mapo	11	0	0.00
	Nova	11	0	0.00
		22	**0**	**0.00**
Satsuma	Miyagawa	103	21	20.40
		103	**21**	**20.40**
Lemon	Femminello	124	8	6.50
	Lunario	694	1	0.15
	Zagara Bianca	5	0	0.00
	Sfusato Amalfitano	9	1	11.10
	Local Seedless	5	0	0.00
	Monachello	3	0	0.00
	Interdonato	2	0	0.00
		842	**10**	**1.20**
Grapefruit		8	0	0.00
Citron	Diamante	44	7	16.00
Bergamot		5	0	0.00
Chinotto		5	0	0.00
Kumquat		42	5	12.00
Lime		2	0	0.00
		106	12	11.30
	Total	**2966**	**137**	**4.60**
GROVES				
Sweet orange	Navelina old clone	640	58	9.00
	Navelina ISA	200	2	1.00
		840	**60**	**7.00**
Clementine	Common	161	5	3.10
	Local			
	- Gentile	179	10	5.60
	- Fedele	236	34	14.40
	- Spinoso	32	1	3.10
	- Precoce di Massafra	85	4	4.70
		693	**54**	**7.80**
	Total	**1533**	**114**	**7.40**
	GRAND TOTAL	**4499**	**251**	**5.60**

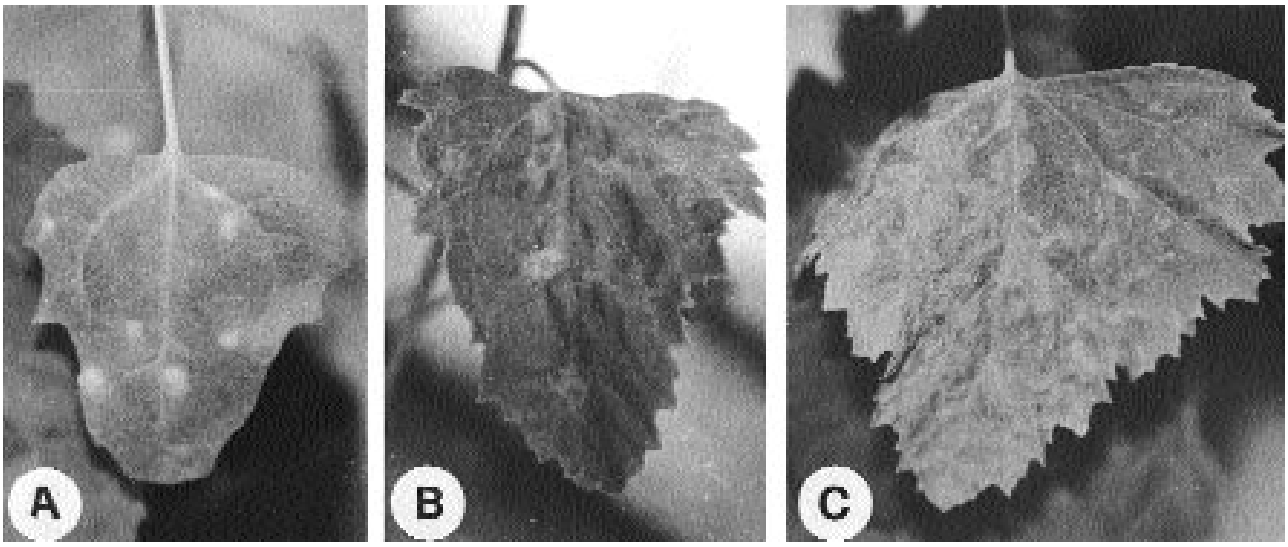

Fig. 1. (**A**) Chlorotic local lesion on the inoculated leaf of *Chenopodium quinoa*. Systemic rings (**B**) and mottling (**C**) and deformation of the leaves of *Chenopodium amaranticolor.*

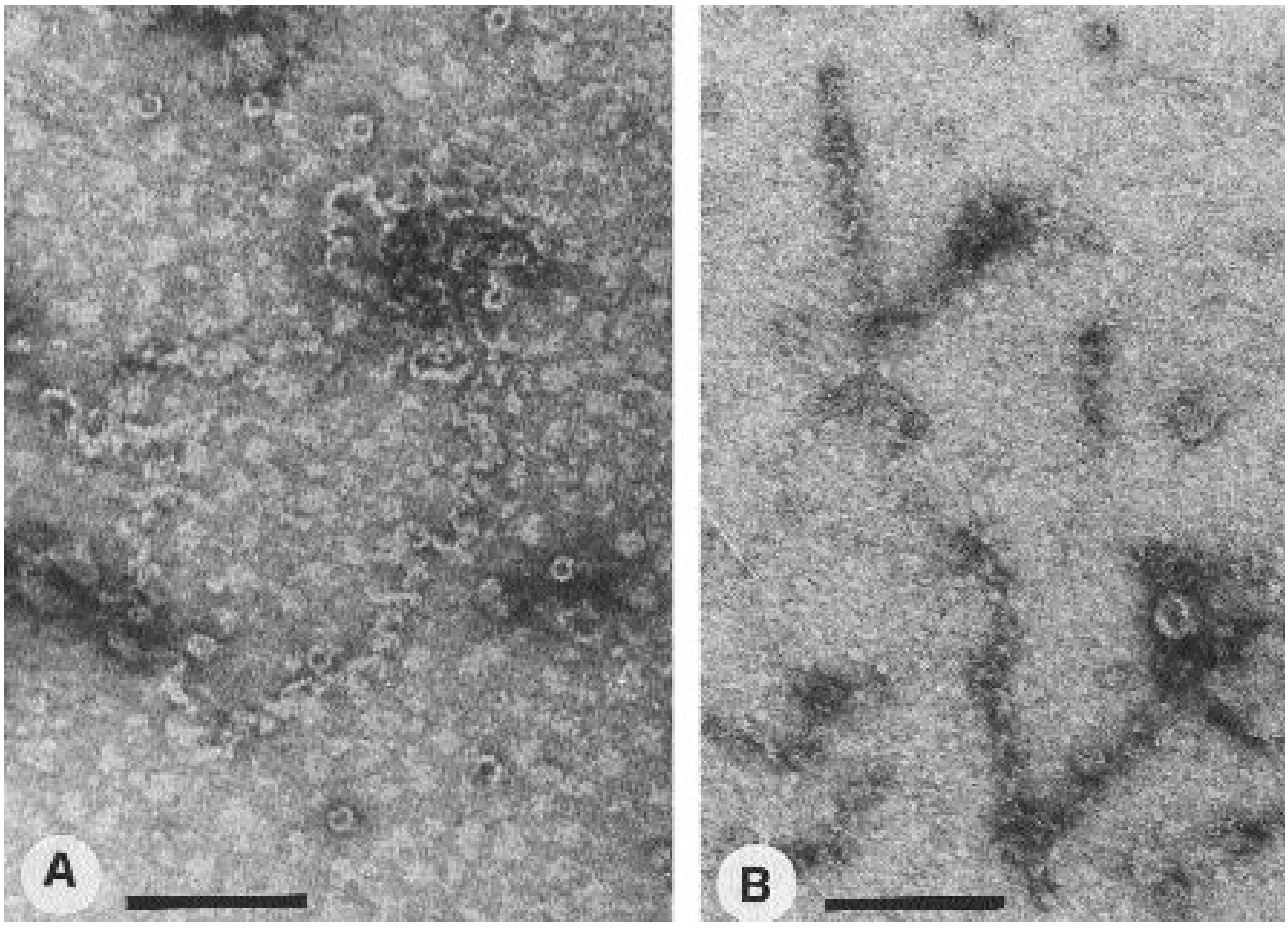

Fig. 2. Negatively stained CPsAV-like particles in the single-stranded open configuration (**A**) and in the duplex linear form (**B**) (*sensu* Milne *et al.*, 1996). Bars = 100 nm.

(1993) reported that 'it is important to recognize that many psorosis-A isolates are difficult to be mechanically transmitted or perhaps do not transmit mechanically from infected sweet orange or citron to other citrus or herbaceous hosts'.

Finally, our findings confirm the presence of filamentous particles resembling those of CPsAV (Milne *et al.*, 1996) in both mechanically inoculated herbaceous hosts and symptomatic citrus sources from Southern Italy. The low number of particles observed under the electron microscope may be due to the fact that no polyvinylpyrrolidone was used for the preparation of samples for electron microscopy (Milne *et al.*, 1996).

The possibility of ready detection of CPsAV by ELISA, even in symptomless and young plants, suggests that this technique can be used not only for massive field diagnosis but also as complementary to biological indexing in the selection of virus-free candidate clones for inclusion in certification programmes.

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