

# Citrus Psorosis Disease Agent Behaves as a Two Component ssRNA Virus

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**ABSTRACT.** Citrus seedlings were graft-inoculated with psorosis (isolate 90-1-1; Concordia, Argentina). When symptomatic young leaves were ground, homogenized and centrifuged at low speed, infectivity (local lesions on *Chenopodium quinoa*) remained in the supernatant. Polyethylene glycol precipitation and high-speed centrifugation yielded infective pellets. Infectivity was abolished by RNase treatment, even in 0.3 M NaCl, indicating that ssRNA(s) is essential for biological activity. Protein(s) is also required for infectivity, since it was sensitive to phenol extraction and proteinase K treatment. When high speed pellets were subjected to sucrose density gradient centrifugation, none of the single fractions accounted for loaded infectivity, but some of the two-fold combinations were much more highly infectious than expected from the contribution of single fractions. Therefore two components activate each other for infectivity. Protein analysis of slow- and fast-sedimenting components suggested that a protein of about 50 Kd MW is associated with infectivity. We interpret these and other results as indicating that a bipartite virus is associated with psorosis disease. The virus has ssRNA as its genetic material and a 50 Kd polypeptide as one of its structural protein(s).

Psorosis disease is an important limiting factor for citrus production in some countries, particularly for Argentina (4). Control of the disease is difficult at present because of the lack of a rapid and reliable test for propagation materials. The only way to index for psorosis is by inoculating appropriate biological indicators.

Development of any diagnostic method applicable to citrus, potential vectors or reservoirs requires the identification of the pathogenic agent, its characterization and the isolation of its constituents. Based on previous work (5) we report here on a further purification and partial characterization of Citrus Psorosis Disease Agent (CPDA). Results are in line with previous observations on the close relationship between CPDA from Argentina and the virus associated to Citrus Ringspot (2, 3). Results are also in agreement with preliminary work on the agent responsible for a similar disease in Spain (J. Navas, M. Cambra, and P. Moreno; personal communication).

## MATERIALS AND METHODS

**Plants and virus.** Psorosis isolate 90-1-1 from Concordia (Argentina) was used. It comes from a Pineapple sweet orange tree on trifoliate orange rootstock that had died because of

psorosis. This shoot proved to be free of citrus tristeza virus (CTV) by ELISA and by indexing on Mexican lime. Isolate 90-1-1 was propagated on Eureka lemon, Pineapple sweet orange, Temple mandarin and Rough lemon. Seedlings were inoculated by grafting using two bark chips each and cut back 20 cm above the graft to force new growth. Symptomatic new flush was collected and used as starting material for purification. *Chenopodium quinoa* plants 7 to 10 weeks old were used for biological assay. Fifteen  $\mu$ l of extracts were inoculated with a spatula on leaves pre-dusted with carborundum. Necrotic local lesions were counted 6 to 8 days after inoculation. Citrus plants were kept in the greenhouse at 28-30C with 16-hr light period; *C. quinoa* plants were kept at 23-26C with 18-hr light period. Lesions from *C. quinoa* were also used as starting material for purification.

**Partial purification.** The procedure is summarized in Fig. 3 (see Results for details). Temperature was kept at 4C throughout the procedure. All chemicals were of P.A. grade.

**Gel filtration through Sephacryl S-200.** P<sub>54</sub> was layered on top of a Sephacryl S 200 column (it fractionates particles with molecular weights ranging from 5,000 to 250,000 daltons)

and eluted with homogenization buffer. The eluted fractions—including the void volume—were concentrated by ultracentrifugation and inoculated on *C. quinoa*.

**SDS sensitivity assay.** P<sub>54</sub> was incubated for 1 hr at 4C with 0.1% sodium dodecyl sulfate (SDS).

**Proteinase K sensitivity assay.** P<sub>54</sub> was incubated for 1 hr at 4C with 10 µg/ml of proteinase K (Sigma).

**RNase sensitivity assay.** Two aliquots of P<sub>54</sub> were incubated at 4C for 30 min with 10 µg/ml of RNase (Type 1-A Bovine Pancreas, Sigma). NaCl was added up to 0.3M to one of the aliquots. Appropriate controls were run in parallel (6).

**Phenol extraction.** P<sub>54</sub> was made 0.1M glycine-NaOH, pH 9, 0.1M NaCl, 1% sodium deoxycholate and 0.2% SDS and extracted with two volumes of phenol:chloroform (1:1) at 4C, until no denatured protein was visible in the interphase. After ethyl-ether extraction, nucleic acids were precipitated with 2.5 volumes of ethanol for 2 hr at -20C. The pellet was resuspended in 0.1M glycine-NaOH, pH 9, 0.1M NaCl, 0.1% SDS and ethanol precipitated. After resuspension in glycine buffer, three volumes of 4M sodium acetate pH 7.5 were added and nucleic acids were precipitated at 0C for 4 hr. The pellet was resuspended in glycine buffer, ethanol precipitated and the resulting pellet was dissolved in SSC (0.15M NaCl, 0.015M, sodium citrate) and inoculated. As a control for RNA infectivity, P<sub>54</sub>, equivalent to 1/6 of the amount subjected to phenol extraction, was inoculated in parallel.

**Polyacrylamide gel electrophoresis.** Mini-slab 14% polyacrylamide gels were run under denaturing conditions (0.1% SDS), for 1 hr at 200 volts (20-30 mA) (1). Gels were stained with Coomassie brilliant blue and subsequently silver stained.

## RESULTS AND DISCUSSION

**Assay and propagation hosts.** In previous work (5), we reported on the use of *C. quinoa* as local lesion host

for biological assay of CPDA. In an attempt to find a better assay host and/or an herbaceous systemic host, we inoculated several plant species with isolate 90-1-1 of CPDA. None of the tested plants showed systemic symptoms, but some of them developed necrotic local lesions. Fig. 1 and 2 show the symptoms on inoculated leaves of *C. quinoa*, *Gomphrena globosa*, and *Nicotiana megalosiphon*. The highest number of lesions were counted on *C. quinoa*, for example, when *C. quinoa*, *G. globosa*, *N. megalosiphon* and *Vigna sinensis* were inoculated with 90 µl aliquots of the same extract, 40, 23, 6, and 0 lesions were counted respectively. Although other species could be tried, *C. quinoa* remains at present as the species of choice for biological assay.

Because infectivity from citrus seedlings is usually low and variable for isolate 90-1-1, we tried lesions on



Fig. 1. Citrus psorosis disease inoculated (top) and mock inoculated (bottom) leaves of *Chenopodium quinoa*.

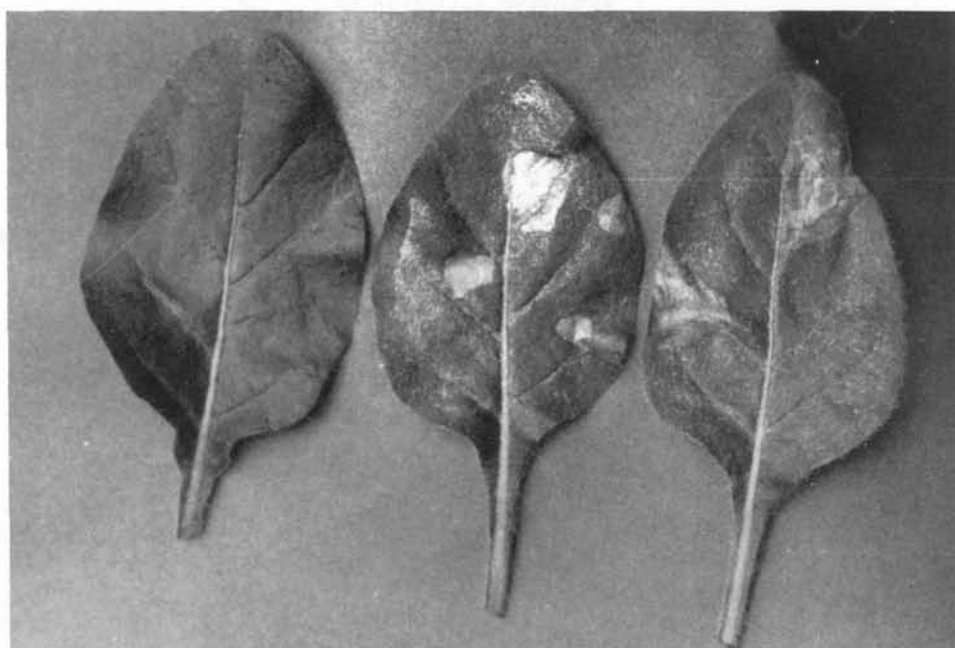
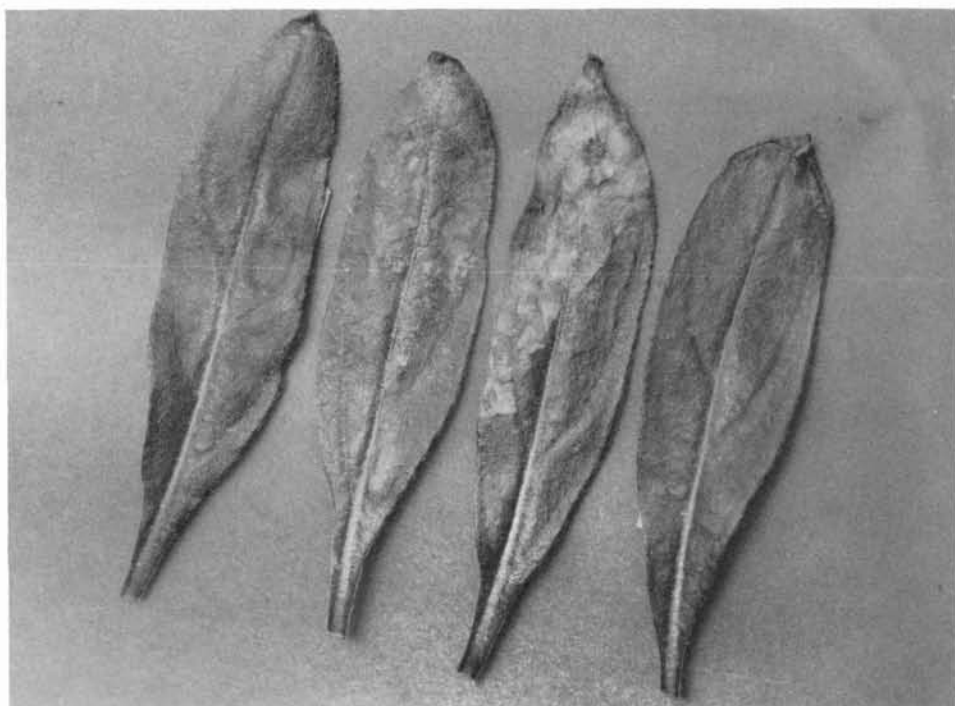


Fig. 2. Citrus psorosis disease inoculated (right leaves) and mock inoculated (left leaf) of *Gomphrena globosa* (top) and *Nicotiana megalosiphon* (bottom).

*C. quinoa* as an alternative starting material for CPDA purification. Necrotic lesions were cut out and pro-

cessed as indicated in Fig. 3. Infectivity of the resulting  $P_{54}$  represented a  $2.6 \pm 0.9$ -fold ( $n=8$ ) increase over

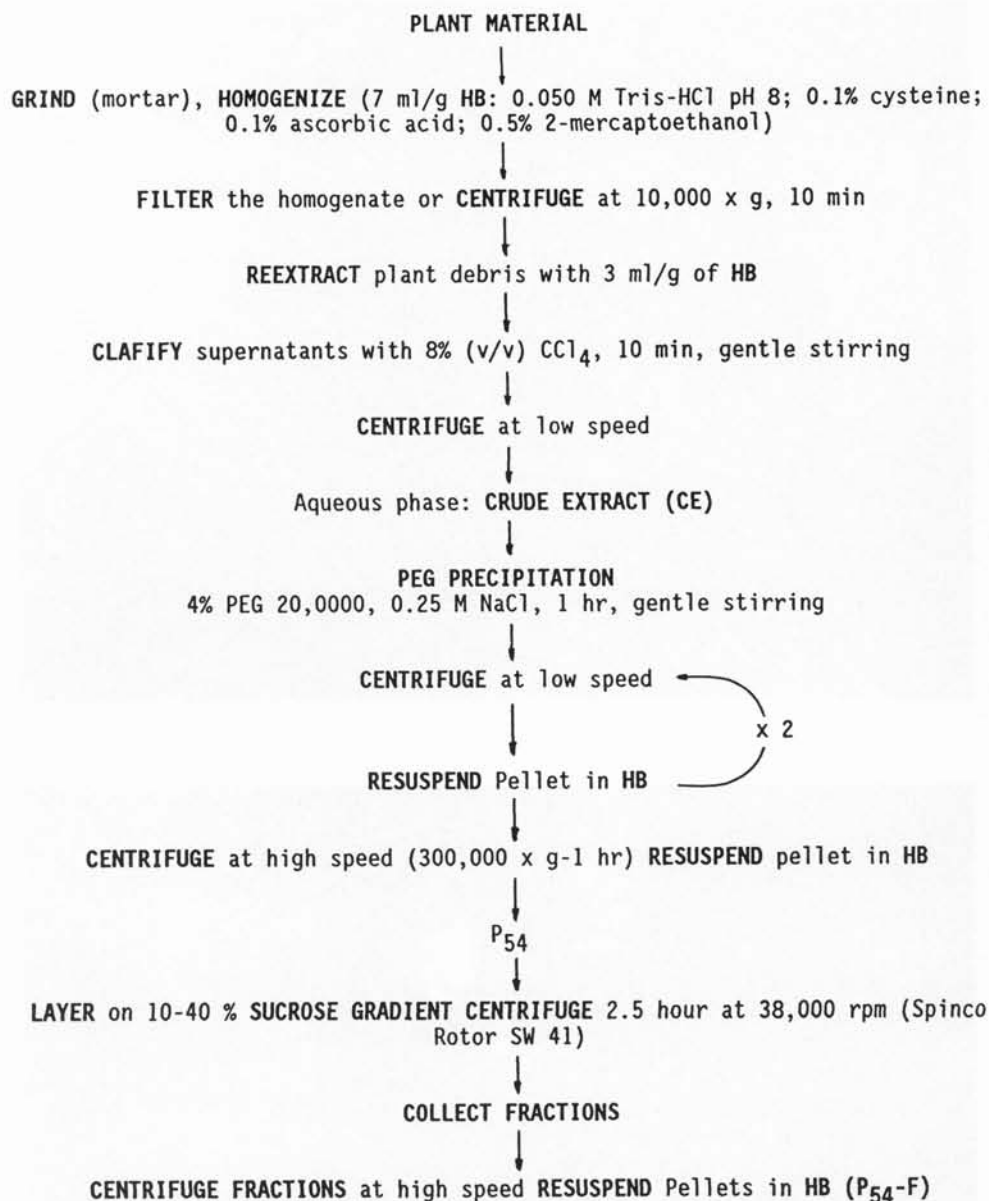


Fig. 3. Purification procedure used for isolation of the citrus psorosis disease agent.

the initial infectivity. Therefore, we included necrotic lesions of *C. quinoa* as another starting material for CPDA purification, because it allowed close prediction of the infectivity expected from the material being processed. This is of particular interest in enzyme sensitivity and RNA infectivity tests, which require initial high infectivity values to have biological significance.

**Purification.** A procedure for the partial purification of CPDA was previously reported by this laboratory (5). That procedure has been modified and extended and is shown in Fig. 3. Major modifications include: i) High speed centrifugation at 73,500 x g for 3 hr was replaced by centrifugation at 300,000 x g for 1 hr, shortening the procedure. This is important for saving the infectivity which is unstable

especially when several rounds of high speed centrifugation are needed. In addition, the infectivity recovered was significantly higher when the crude extract (CE) was centrifuged at 300,000 x g for 1 hr. ii) For further purification of infectious high speed pellets ( $P_{54}$ ) a sucrose density gradient centrifugation step was added. Analysis of protein composition of  $P_{54}$  and gradient fractions indicated that plant proteins were indeed removed by the gradient. However, gradient fractions still contained many polypeptides of suspected plant origin, since they are present in healthy and CPDA-infected samples (Fig. 4). iii) The PEG precipitation step is optional; it is included for large volumes of CE to avoid additional high speed centrifugations. In addition,  $P_{54}$ s from the resuspended PEG pellets are smaller and easier to resuspend than those from CE. Alternatively, it may be omitted for small volumes of CE, in order to shorten the whole procedure.

The purification scheme outlined in Fig. 3 yields infective gradient fractions and allows separation of fast and slow sedimenting components after 9 hr. Nevertheless, purification is still partial, as judged by protein analysis (Fig. 4).

**Infectivity of density gradient fractions.** When  $P_{54}$  was subjected to sucrose density gradient centrifugation, infectivity from single fractions accounted for only 19% of the loaded infectivity (Table 2). Either infectivity survives centrifugation across the sucrose gradient poorly or it requires two or more components which are not present in any single fraction. The second alternative turned out to be true: some two-fold combinations were much more highly infectious than expected from the contribution of the two individual fractions (Table 2). A clear activation of the infectivity of fraction 4 was observed upon addition of fraction 2 (11-fold increase; Table 2) which showed no infectivity when assayed alone. When infectivity from all of the two-fold combinations

was considered, it accounted for the total loaded infectivity.

We interpreted this result as an indication that infectivity on *C. quinoa* is the result of the contribution of two distinguishable components. The fast sedimenting one is present in fractions 4 and 3 and the slow sedimenting component is distributed over fractions 2 and 3. As a consequence, only fraction 3 is infective alone (Table 2). A more detailed analysis of this part of the gradient (from fraction 2 to 4) would allow a better separation of components, as an intermediate region, free of infectivity-contributing components, is ex-

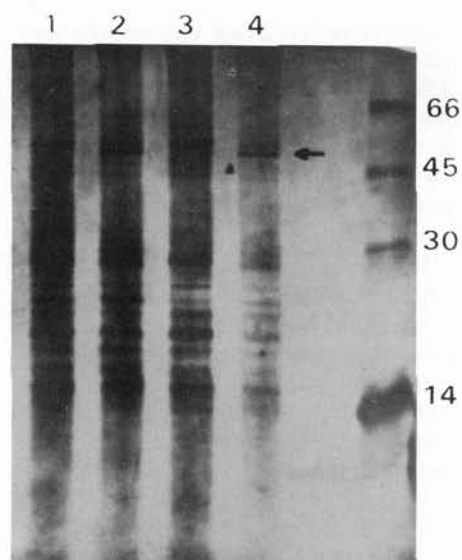


Fig. 4. Protein analysis of sucrose gradient fractions. Healthy and Citrus Psorosis Disease Agent-infected *C. quinoa* leaves were processed as indicated in Fig. 3. High speed pellets of gradient fractions ( $P_{54}$ -F) were electrophoresed. Lane 1: healthy material; fractions from the central part of the gradient; Lane 2: CPDA-infected; fractions from of the central part of the gradient (mixture of top and bottom components); Lane 3: healthy material; fraction from the lower (fast sedimenting) part of the gradient; Lane 4: CPDA-infected; fraction from the lower part of the gradient (mainly bottom component); Lane 5: MW markers (bovine serum albumin, 66 Kd; egg albumin, 45 Kd; carbonic anhydrase, 29 Kd; RNase, 14 Kd). The position of the 50 Kd polypeptide associated to CPDA is indicated.

pected. Biochemical characterization of components will require such separation. The possibility that more than two components are involved can not be excluded from the results shown here; it could be also explored by means of a further fractionation of the central part of the gradient.

#### Nucleoprotein nature of CPDA.

The viral nature of CPDA was suspected from the initial purification experiments because infectivity on *C. quinoa* did not sediment at low speed but sedimented at high speed, making it unlikely that CPDA could be a bacterium, a fungus or a viroid. The distribution of CPDA infectivity over sucrose gradient (Table 2) strongly supports those initial observations. While bipartite plant viruses are common, all known viroids are single RNA molecules which would not be expected to sediment at the position at which infectivity is recovered from the gradient. The following experimental results provide additional support to the nucleoprotein (viral) nature of CPDA and indicate the structural requirements for biological activity: i) When an infectious extract was applied on a Sephacryl S-200 column (see Materials and Methods), infectivity was recovered only from the void volume (results not shown), indicating that infectivity is associated to a particle larger than 250 Kd; viroids would be retained on such a column; ii) Treatment of infectious P<sub>54</sub> with 0.1% SDS completely abolished infectivity (Table 1) suggesting that it depends on interactions or structures involving proteins (protein-protein, protein-nucleic acid, membrane); iii) Infectivity of P<sub>54</sub> disappeared upon incubation with proteinase K (Table 1), indicating that protein(s) is essential for biological activity; iv) The genome of most plant viruses consists of RNA. When P<sub>54</sub> was incubated with RNase infectivity was totally abolished, either in the absence or in the presence of 0.3 M NaCl (Table 1). Therefore, ssRNA is essential for infectivity. In addition, this result indicates that the ssRNA is accesible for

TABLE 1  
SENSITIVITY OF THE CITRUS PSOROSIS DISEASE AGENT TO RNase, PROTEINASE K, AND SDS AND TO PHENOL EXTRACTION

Treatment	No. of lesions <sup>2</sup> /90 µl
Control	185
RNase	0
Control	64
RNase-NaCl	0
Control	379
Proteinase K	0
Control RNA (P <sub>54</sub> )	163
RNA (equivalent to 6 times P <sub>54</sub> )	0

<sup>2</sup>On *Chenopodium quinoa*

exogenous RNase, implying that the ssRNA-containing particle is lax enough to allow the entry of protein molecules (RNase MW = 14 Kd) or to permit the exposure of the nucleic acid to interact with external macromolecules; v) RNA extracted from infectious P<sub>54</sub> was not infective on *C. quinoa*. Table 1 shows that infectivity of RNA was less than 0.1% of expected from infectivity of the control not subjected to phenol extraction. This result could be interpreted in two ways: infectivity depends on the

TABLE 2  
INFECTIVITY OF INDIVIDUAL AND TWO-FOLD COMBINATIONS OF SUCROSE DENSITY GRADIENT FRACTIONS<sup>2</sup>

	F1	F2	F3	F4	F5	F6
F1	0	0	40	10	0	0
F2		0	81	82	3	0
F3			70	135	45	18
F4				14	3	16
F5					0	0
F6						0

<sup>2</sup>After centrifugation (see Fig. 2), gradient was separated into five fractions of 2.5 ml (plus a pellet, F6). Fractions were concentrated by high speed centrifugation and resuspended in 0.35 ml of HB. Figures are the number of lesions on five *C. quinoa* leaves inoculated with 15 µl/leaf of individual fractions or 1:1 combinations. From the infectivity of P<sub>54</sub> (117 lesions/5 leaves), we estimated that a total of 2100 lesions were loaded on the gradient. From the infectivity of the three more infectious combinations (F2 + F3; F2 + F4; F3 + F4) we estimated a minimal recovery of 1650 lesions (78%).

structural integrity of a nucleoprotein or on some minimal protein-nucleic acid interaction (as is the case for Ilarviruses) or genome RNA is negative sense and not infective by itself. Whatever the real cause, this result confirms that protein(s) is essential for infectivity; vi) In view of the structural requirements for biological activity and its rapid drop under "in vitro" (HB) conditions, we tested the ability of divalent cations to stabilize it. Addition of 5 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub> or 1 mM MnCl<sub>2</sub> to HB during homogenization failed to increase infectivity; on the contrary, infectivity was reduced to about 50% of the control. The same results were obtained when P<sub>54</sub> was resuspended in HB containing any of these cations. The possibility that divalent cations (either endogenous or added) might be deleterious to infectivity appeared unlikely as addition of EDTA to HB drastically reduced infectivity.

**A 50 Kd protein associated to CPDA.** Denaturing polyacrylamide gel electrophoresis analysis of healthy and psorosis infected crude extracts (CE) and high speed pellets (P<sub>54</sub>) showed no differences. In addition, uninfected P<sub>54</sub> samples contain many proteins, making it difficult to detect differences and reflecting the low level of purification reached at that step (results not shown).

After sucrose density gradient centrifugation, fractions from uninfected tissue still contain many proteins. However, in their infected counterparts a protein of approximately 50 Kd is clearly seen; a protein of apparently the same mobility is found in much lower amount in healthy samples (Fig. 4, compare lanes 1 and 3 with lanes 2 and 4). This result indicates that a 50 Kd polypeptide is associated with psorosis disease; it could be a CPDA structural protein or a plant protein induced in some way by the pathogen. The following observations favour the first alternative: i) The 50 Kd protein is detected in those gradient fractions that combined are infectious (Fig. 4); in other

words, it is associated with infectivity; ii) This protein has been found not only in gradient fractions from the indicator herbaceous host *C. quinoa* (Fig. 4) but also in gradient fractions from the systemic citrus hosts (not shown); iii) The 50 Kd protein was detected in fractions containing top and bottom components, suggesting that both particles contain the same polypeptide and favouring the idea that it represents a viral rather than a host protein, because it appears unlikely that a non viral protein would be found in different zones—but not in the upper part—of the gradient.

Taken together, results presented in this paper indicate that the virus associated with Citrus Psorosis Disease is of the bipartite (two component) type. The virus contains ssRNA as genetic material and a 50 Kd structural protein.

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