Biological Assay and Preliminary Isolation of Citrus Psorosis Disease Agent from Argentina

A. N. Sarachu, E. L. Arrese, C. Casafús, N. B. Costa, M. L. García,
O. Grau, G. M. Marcó, and A. Robles

ABSTRACT. Psorosis has expanded steadily in Argentina and at present it causes the loss of 5-8% of the orange and grapefruit trees per year. As a first step towards a better control of the disease, we have recently started a project aimed at the purification and characterization of the putative psorosis virus. It was mechanically transmitted from citrus to *Chenopodium quinoa*, inducing local necrotic rings which were roughly proportional in number to the relative concentration of the inoculated extract. Graft transmission to several citrus hosts followed by assay on *C. quinoa* showed that the most infective extracts were those obtained from Pineapple sweet orange and Eureka lemon. Symptomatic young leaves from these two citrus hosts were ground and homogenized in buffer. Polyethylene glycol precipitation and high-speed centrifugation yielded infective pellets, although much of the infectivity was lost during the process.
Index words. host range, purification, citrus ringspot virus.

Psorosis has been known as a citrus disease for a long time in several countries (5). In Argentina, psorosis is the most important viral disease in the Concordia (Entre Ríos Province), San Pedro (Buenos Aires Province) and Bella Vista (Corrientes Province) citrus-producing regions, where it has steadily expanded causing at present the loss of up to 8% of the orange and grapefruit trees per year.

Previous work has indicated that the type of psorosis occurring in the Concordia region shows similarities with the citrus ringspot virus (CRSV) in symptomatology, natural diffusion, host range and mechanical transmission to herbaceous plants (2). Although the viral nature of the psorosis agent has been proposed by several authors (1, 4, 5, 6), it has never been purified and information on its physical, biological and biochemical properties is lacking.

Partial purification of CRSV has been recently achieved (3); the virus apparently consists of two components which depend on each other for infectivity. As a first step towards a better control of the disease, we have recently started a project aimed at the purification and characterization of the putative psorosis virus.

MATERIALS AND METHODS

The citrus seedlings used in the experiments reported here were grown in plastic pots in a greenhouse. The potting soil received an initial fertilization and additional fertilization was added periodically by irrigation. Insecticides and fungicides were applied weekly as needed. *Chenopodium quinoa* plants were grown in sterilized potting mix in a greenhouse; supplemental light was supplied in winter.

Citrus indicator seedlings were inoculated by grafting using two bark chips each. Seedlings were cut back 20 cm above the graft in order to force new growth. Symptoms on the new flush were recorded periodically.

C. quinoa plants were kept in the dark for 16 hr before inoculation. Inoculum was applied with cotton swabs (about 30 μl/leaf) or glass spatulas (15 μl/leaf) to leaves predusted with carborundum.

The inoculum used in this work was from a trifoliate orange shoot from a Pineapple sweet orange tree 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.

Except as noted, extracts were prepared by grinding tissues in a mortar and pestle at 4C. The standard homogenization buffer (HB) was 0.05 M Tris-HCl, pH 8.0 with 0.1% ascorbic acid, 0.1% cysteine and 0.5% 2-mercaptoethanol. Extracts were treated with other chemicals as indicated in table 2 and figure 2. All chemicals were of P.A. grade.

RESULTS

For the purification and subsequent characterization of the citrus psorosis disease agent (CPDA), it is desirable to have a reproducible starting material not contaminated with other viruses, particularly CTV which is widespread in the region. In this respect, we first tested seedlings of several citrus species and varieties which were inoculated with our CPDA isolate under greenhouse conditions, to select those which produced abundant symptomatic tissue.

Thirty different species and varieties were inoculated and symptoms were recorded periodically. Table 1 shows the results obtained with seven species which gave good symptoms. Pineapple sweet orange and Eureka lemon showed the largest diversity in symptom type and the highest incidence of infection. Extracts of symptomatic leaves from these two species were highly infectious on *C. quinoa* confirming the presence of CPDA. When asymptomatic leaves were employed, extracts from Pineapple sweet orange showed no in-

fectivity. In the case of Eureka lemon results were not consistent, i.e., in some experiments they were as infectious as those from symptomatic leaves, whereas in others infectivity from asymptomatic leaves was much lower than that obtained with symptomatic material. Therefore, symptomatic young leaves from Pineapple sweet orange and Eureka lemon seedlings were used routinely as starting material for purification. When possible, Eureka lemon was preferred because more leaf material per seedling could be obtained and infectivity per gram of tissue was somewhat higher.

The development of a purification procedure for CPDA requires some method to monitor its presence in different samples. Because CPDA has not been characterized and sap inoculation of citrus host has been unsuccessful, we tried to assay its biological activity on herbaceous hosts. Only *C. quinoa* appeared to be a useful assay host. Local lesions were first visible as incomplete rings 4 days postinoculation (p.i.) and increased in number until 8-10 days p.i. (fig. 1); at that time rings became necrotic.

When several leaves of different *C. quinoa* plants were inoculated with the same extract, the number of lesions was obviously variable, but the variation was judged reasonable for plant viruses (i.e., higher and lower figures differed by a maximal factor of 3). In addition, the number of lesions induced by dilutions of the ex-

TABLE 1
SYMPTOMS ON CITRUS SPECIES AND VARIETIES INDUCED BY THE
CITRUS PSOROSIS DISEASE AGENT

Host	Symptom				
	Shock	Flecking	Spots	Variegation	Rings
Duncan grapefruit	$1/6^z$	0/6	3/6	0/6	0/6
Rough lemon	0/5	1/5	1/5	1/5	0/5
Pineapple sweet orange	1/5	1/5	3/5	1/5	1/5
Eureka lemon	3/5	2/5	1/5	2/5	0/5
Etrog citron	3/5	0/5	2/5	0/5	0/5
Mexican lime	0/5	2/5	1/5	0/5	0/5
Swingle citrumelo	0/5	0/5	2/5	0/5	2/5

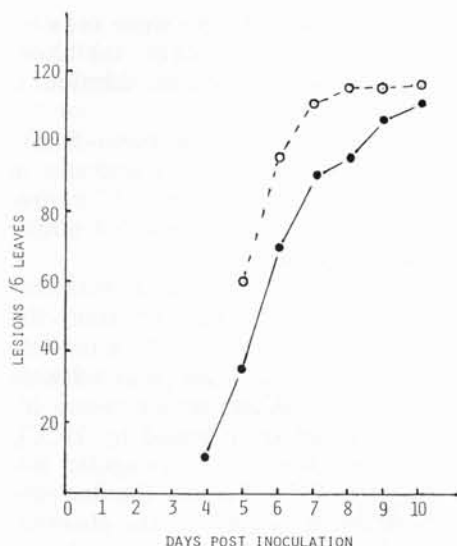^{z}No. of plants positive/no. of plants inoculated.

Fig. 1. Time course of appearance of local lesions induced by the citrus psorosis disease agent on *Chenopodium quinoa*. Leaves were inoculated with crude extracts (o) or with a 1:5 dilution of P25 (●) (see fig. 2). Inoculation was performed with cotton swabs.

Fig. 2. Isolation and partial purification procedure of citrus psorosis disease agent. All manipulations were performed at 4C. See text for further details.

tracts closely followed the dilution factor: in two experiments the undiluted extract and the 1:5 dilution produced 93 vs. 19 and 285 vs. 57 lesions per 6 leaves, respectively.

Isolation and partial purification of CPDA was performed following the procedure outlined in fig. 2.

The addition of ascorbic acid and thiols to the homogenization buffer (HB) was essential for biological activity (table 2). Clarification of the initial extract was performed by a mild treatment with CCl_4 followed by low-speed centrifugation (10,000 x g, 10 min at 4C). Infectivity was drastically reduced when CCl_4 was replaced by $HCCl_3$ but only slightly by Freon (table 2). Biological activity was also affected by the addition of Triton X-100 to the homogenization buffer. After low-speed centrifugation infectivity remained in the supernatant.

However, some infectivity was retained in the pellet when grinding was not exhaustive. To avoid losses, reextraction of this pellet with some HB was incorporated into the routine procedure.
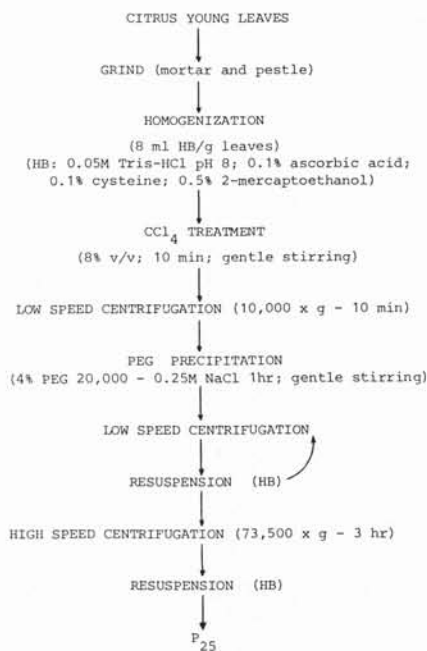
Attempts to concentrate the extract by polyethylene glycol (PEG) precipitation were unsuccessful in initial trials. By making the extract 0.25 M in NaCl and 4% (w/v) in PEG 20,000 infectivity could be completely recovered from the pellet (table 2). This pellet was resuspended in HB and centrifuged at low speed; most of the infectivity went to the supernatant but some could be recovered after a second resuspension of the pellet.

After a subsequent high speed centrifugation, infectivity was recovered from the pellet and was absent in the supernatant. However, only part of the initial infectivity was recovered.

DISCUSSION

In previous experiments with our CPDA isolate, Pineapple sweet orange behaved as a sensitive indicator (2). In this work, Eureka lemon proved to be as efficient as Pineapple sweet orange for the multiplication of CPDA to supply infected starting ma-

TABLE 2
CITRUS PSOROSIS DISEASE AGENT
PURIFICATION PROCEDURE: EFFECT
OF BUFFER COMPOSITION, SOLVENT
TREATMENT AND POLYETHYLENE
GLYCOL (PEG) PRECIPITATION

Expt. no.	Treatment[z]	Lesions/ 6 leaves[y]
1	HB	55
	TA	14
	T	0
2	HB	90
	HB/X-100	36
	HB/HCCl$_3$	0
3	HB	38
	HB/CCl$_4$	44
4	HB/CCl$_4$	330
	HB/Freon	166
5	Control	72
	P$_{PEG}$	75
	S$_{PEG}$	0

[z]Tissue homogenized with a mortar and pestle in standard homogenization buffer 0.05M Tris-HCl, pH 8.0 + 0.1% ascorbic acid + 0.1% cysteine + 0.5% 2-mercaptoethanol (HB); in 0.05M Tris, pH 8 (T); in 0.05M Tris, pH 8.0 + 0.1% Ascorbic acid (TA); in HB plus 0.1% Triton X-100 (HB/X-100); in HB and treated with HCCl$_3$, CCl$_4$ or Freon; in HB, treated with CCl$_4$ and PEG (P$_{PEG}$: pellet; S$_{PEG}$: supernatant).
[y]Results correspond to inoculation of *Chenopodium quinoa* with P25 (see fig. 2) from each preparation.

terial for purification. In addition, Eureka lemon is more vigorous and produces higher amounts of symptomatic leaves than Pineapple sweet orange. Moreover, extracts from asymptomatic leaves of Eureka lemon were infectious on *C. quinoa* in some experiments and this was never observed with asymptomatic leaves of Pineapple orange.

The local lesion assay on *C. quinoa* proved reliable and allowed us to compare the biological activity of different samples, i.e., those obtained from different citrus hosts or at different steps of the purification procedure. Since the number of lesions was proportional to the concentration of the extract, one might conclude that infectivity is due to a single component agent. However, only crude or par-

tially purified extracts were assayed and they may contain inhibitors whose behavior towards dilution is unknown.

Grinding of liquid nitrogen-frozen leaves did not show any increase in infectivity over grinding at 4C of precooled leaves using precooled buffer (data not shown).

Treatment of extracts with organic solvents suggested that the biological activity is due to a particle which cannot withstand polar solvents (i.e. HCCl$_3$). Alternatively, some inhibitors could be released by HCCl$_3$ treatment, but not by nonpolar solvents (i.e. CCl$_4$, Freon). Similar considerations may apply to the observation that addition of nonionic detergent led to the loss of infectivity (table 2).

The presence of ascorbic acid and mercaptoethanol in the homogenization buffer was essential for infectivity. Other reducing agents (i.e. Na$_2$SO$_3$) and thiol reagents (e.g. diethyldithiocarbamic acid) did not give any increase in infectivity.

By applying the procedure outlined in figure 2, infectivity was recovered from the high-speed pellet (P25). However, only part of the initial biological activity remained at that point. Data in figure 1 show that infectivity of crude extracts was similar to that found in a 1:5 dilution of P25; considering the volumes of the crude extract and P25 the recovery of infectivity was below 20%. This low recovery may be ascribed to damage produced by the procedure itself and/or to the lability of the infective agent. In this respect, it should be noted that crude extracts kept at 4C also showed a rapid decrease in infectivity.

Electron microscope observation of high-speed pellets derived from infected and healthy leaves did not show consistent differences, which may be taken as an indication that an elongated virus is not involved; any (roughly) isometric virion would look similar to normal cell components at the present stage of purification.

The similarities in symptomatology, natural spread, host range and mechanical transmission to herbaceous plants between our CPDA isolate and CRSV have been previously reported (2, 5). Two additional analogies are in line with these reports: (a) both CRSV and CPDA rapidly lose infectivity *in vitro;* (b) our partial purification scheme closely resembles that described by Derrick *et al.* (3) for CRSV.

The procedure described here must be extended to achieve further purification. High-speed pellets still contain many protein bands which are probably mostly host polypeptides. Several strategies to achieve further purification are envisaged and include density gradient and isopycnic centrifugation, gel filtration and isoelectric precipitation.

ACKNOWLEDGMENTS

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