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Biochemical Characterization and Diagnosis of Different Citrus Tristeza Virus Strains

D.G. Gumpf, M. Bar-Joseph, S. Garnsey, R. Lee

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Principal Investigator: D. J. Gumpf

Cooperating Investigators: M. Bar-Joseph, S. M. Garnsey and R. F. Lee

Grantee Institution: The Regents of the University of California
University of California
Riverside, CA 92521

Cooperating Institutions: Volcani Center
Agricultural Research Organization
Bet Dagan, Israel

U. S. Horticultural Research Laboratory
USDA/SEA
2120 Camden Road
Orlando, FL 32803

Agricultural Research and Education Center
University of Florida
P. O. Box 1088
Lake Alfred, FL 33850

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Principal Investigator

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ABSTRACT

A standardized protocol was developed to compare the biological activities of different CTV strains. Isolates were compared separately in the U. S. and Israel and directly at a single location in the United States. A series of experiments were also conducted to determine which of 12 different hosts resulted in the highest virus titer. Results from these experiments indicated that Citrus hystrix, citron and lime should make the best virus propagating hosts. The CTV strains which were biologically characterized were then used for the additional studies proposed by this project. Purification procedures which yielded intact infections 2000 nm particles were developed. The coat proteins of several purified CTV isolates were characterized further as to amino acid composition, polypeptide maps, and molecular weight estimates. Only very minor differences were observed between the various strains.

Both polyclonal antisera and monoclonal antibodies were produced against a variety of CTV isolates. Tests using the polyclonal antisera indicated that several different determinants must be involved in the CTV-antibody reactions. The monoclonal antibodies produced are highly reactive, specific for CTV, and will react in the presence of crude sap with no healthy reaction. Analysis of clones for CTV strain specificity is still under study.

Single stranded RNA of $5.4 - 6.5 \times 10^6$ daltons with messenger RNA activity was separated from dissociated purified CTV by electrophoresis on 1.2% agarose gels. Using this extracted RNA, cDNA could be synthesized, converted to double stranded DNA, and cloned in Escherichia coli. The resulting clones all with virus specific sequences could be used as probes for molecular hybridization. Three of the nine cloned probes hybridized differentially with CTV RNA isolates with differing biological properties.

Introduction

Citrus tristeza virus (CTV) is the most serious virus problem affecting citrus (Bar-Joseph et al., 1979a). A CTV-induced decline in orange, mandarin and grapefruit trees grafted on sour orange rootstocks has destroyed approximately 50 million trees worldwide. The threat of CTV-induced decline remains for an additional 50 million trees on intolerant rootstocks in the U.S. and Israel alone.

In addition to this decline of scions on sour orange rootstocks, severe isolates of CTV cause slow declines and/or serious stem pitting injury to some oranges, grapefruit and limes regardless of rootstock (Bar-Joseph et al., 1979a). Recently, natural spread of severe isolates has occurred in southern California, and it threatens large citrus acreages if it is not contained (Roistacher et al., 1979).

The conventional approach for controlling the CTV decline involving sour orange rootstock is to use CTV-tolerant or CTV-resistant rootstocks such as rough lemon and Troyer or Carrizo citrange. However, some of these commercially acceptable rootstocks are affected by the severe isolates of CTV, seedling yellows and stem-pitting, as well as by other diseases such as citrus blight in Florida. Stem pitting problems caused by CTV in Pera sweet orange, grapefruit, and limes in Brazil have been controlled successfully by mild strain cross protection (Müller and Costa, 1977). This control approach was developed largely by empirical means, and there are problems which interfere in the further practical use of mild strain cross protection. Isolates that are mild in one host are often severe in others, and not all mild isolates are equally protective. Cross protection may also break down after a period of time as it is influenced by challenge pressure and environment. Mild strain cross protection has not been tested or used extensively to prevent CTV decline problems, a factor of considerable interest to areas where CTV may spread if not contained by eradication or other means. The ability to solve these problems before wide scale employment of mild strain cross protection for the control of CTV, emphasizes the need for a thorough understanding of this complex virus system. This can result only from a detailed biochemical and biophysical study of the virus particle, virus particle components and the various isolate and strain relationships.

Citrus tristeza virus is a member of the closterovirus group (Bar-Joseph et al., 1979b). It is phloem-limited, vectored semi-persistently by several aphid species with varying efficiency, and has been mechanically transmitted experimentally with some difficulty. The infectious virion is assumed to be a 2000 X 10-12 nm rod, but infectivity has not been directly associated with specific nucleoproteins and RNA species. Low yields of purified virus, susceptibility of the CTV particle to shearing and lack of efficient infectivity assays have hindered extensive investigation on CTV properties.

The objectives of this proposal were the following:

1. Determine the infectious nucleoprotein and nucleic acid species of different strains of CTV.
2. Develop efficient purification procedures for whole virus, virus protein and viral RNA.

3. Chemically characterize the virus proteins and differences in proteins that are strain related.
4. Develop specific antisera for testing strain relationships.
5. Characterize the CTV RNA, and to test for RNA homology between different strains.
6. Based on analysis of information obtained from the above objectives, develop practical procedures to identify specific biological isolates of CTV singly and in mixed infections.

Materials and Methods

Selection of CTV strains and propagation hosts. Isolates of CTV which ranged in severity from only very mild reactions in Mexican lime to one showing a severe seedling yellows reaction were chosen for the biological characterization studies. The CTV isolates of differing severity were compared separately in California and in Florida using a standardized five plant host range, Mexican lime, grapefruit, sweet orange, sour orange, and sweet orange on a sour orange rootstock. A single source of seed, collected in California, was used for all test plants.

In addition, arrangements were made to use the quarantine facilities of the USDA Beltsville Agricultural Research Center in Maryland, to compare the CTV strains from different areas under common conditions. Permits were obtained to import key isolates from different areas of the world for comparative purposes. Direct comparisons on the five plant host range described above were initiated on eighteen (18) isolates.

Purification of infectious virus. Two methods for the purification of citrus tristeza virus (CTV) were developed for and used for additional studies. California and Israel isolates were purified by the procedures of Gumpf et al., (1981) and Bar-Joseph et al. (1985). Purification of Florida isolates was carried out by the procedure of Garnsey et al. (198_), Lee et al. (1981), and Lee et al. (in press).

Characterization of coat protein. Coat protein from sodium dodecyl sulfate (SDS)-degraded CTV isolates was purified by gel electrophoresis and isoelectric focusing and then used for peptide mapping and amino acid composition. The protein subunits were enzymatically digested with Staphylococcus aureus V8 protease and trypsin according to the procedures of Cleveland et al. (1977) and Casey et al. (1979). Cyanogin bromide cleavage of 2.0 M LiCl dissociated coat protein was performed according to the methods of Gross and Witkop (1962). Two-dimensional peptide maps of the ¹²⁵I-labeled tryptic digestion products and high pressure liquid chromatography (HPLC)-separated peptides were used to obtain characteristic catalogs of the various CTV isolates.

Amino acid compositions were determined on 12, 24 and 72 hour acid hydrolyzed samples using an automated Beckman 120-C amino acid analyser. Computer analysis of the data was done in order to detect differing relationships among the virus isolates.

Preparation of polyclonal antisera. Antisera to undegraded unfixed CTV, undegraded formaldehyde fixed CTV and SDS-degraded CTV coat protein were prepared by the method of Gonsalves et al. (1978) and Garnsey et al. (1979). Gamma globulins (IgG) separated from the whole serum by the sodium sulfate procedure of Kekwick (1940) or by the protein A-Sepharose affinity chromatography procedure of Miller and Stone (1978) were used for in situ immunofluorescence (ISIF) and enzyme linked immunosorbent assay (ELISA). The concentration of IgG was estimated spectrophotometrically using $E_{0.1\%/280\text{ nm}} = 1.4$. In some tests IgG fractionated by ammonium sulfate $(\text{NH}_4)_2\text{SO}_4$ precipitation and cellulose chromatography (Bar-Joseph et al., 1979a) was used.

Preparation of monoclonal antibodies. Procedures for the production of CTV monoclonal antibodies are described by Zheng et al., 1985.

Hybridomas were prepared from the mouse myeloma cell line P3-X63-Ag8.653 and spleen cells from BALB/C mice immunized with partially purified CTV. The mice were given two 0.5 ml subcutaneous injections of antigen mixed with an equal volume of Freund's complete adjuvant one week apart followed two weeks later with an intravenous injection of the antigen diluted in saline. Within 72 hours of the final intravenous injection, cell fusions were performed by standard procedures using polyethylene glycol as the fusing agent. Hybrids were cloned by limiting dilution and screened for reactivity to CTV infected and healthy Etrog citron (Citrus medica) sap by solid-phase ELISA with alkaline phosphatase conjugated affinity purified goat anti-mouse IgG (Boehringer Mannheim, Indianapolis, Indiana).

Conjugation of antisera. Antisera for ISIF were conjugated with either fluorescein isothiocyanate (FITC) (Miles Laboratories, Elkhart, IN 46515) or with tetramethylrhodamine isothiocyanate (TRITC) (Research Organics, Inc., Cleveland, OH 44125). The IgG was conjugated with FITC by the method of Blakeslee and Baines (1976) and with TRITC as described by Brlansky et al. (1982).

The IgG for double sandwich ELISA was conjugated with alkaline phosphatase Type VII (Sigma Chemical Co., St. Louis, MO 63178) as previously described (Bar-Joseph et al., 1979a).

Conjugated antibodies used for monoclonal studies were affinity purified goat anti-mouse or goat anti-rabbit IgG conjugated with alkaline phosphatase available from Boehringer Mannheim, Indianapolis, Indiana.

In situ immunofluorescence. Both FITC- and TRITC-labeled IgG were used in this study with a modification of the technique of Brlansky et al. (1982). Sections of bark and petiole material of virus-infected and healthy citrus were cut using a Harris Model WRC cryostat (Harris Model WRC cryostat, Harris manufacturing Inc., North Billerica, MA 01862). Longitudinal and transverse sections 30 to 40 μm thick were immersed in labeled IgG to CTV. Labeled normal serum IgG and labeled IgG specific to Pierce's disease bacteria were used as controls. Sections were incubated at room temperature for 1 hour or at 37 C for 30 minutes, washed in phosphate-buffered saline for 10 to 20 minutes, and mounted on microscope slides in Aqua Mount.[®] The slides were observed with a Leitz Dialux[®] fluorescence microscope (E. Leitz, Inc., Rockleigh, NH 07647) equipped with a 50-V mercury lamp and viewed in the 560- to 590-nm range for TRITC (Leitz filter block N2) and in the 380- to 420-nm range (Leitz

filter block H2) for FITC. Photographs were taken with Kodak Ektachrome EC 400® color film and/or Kodak Technical Pan 2415® black and white film.

Serologically specific electron microscopy (SSEM). The procedure for SSEM was that previously described for plant viruses by Derrick and Brlansky (1976) and specifically for CTV by Garnsey *et al.* (1979). Unfractionated antisera were used throughout at dilutions of 1:500 in 0.05 M Tris buffer, pH 8.0. Parlodion-coated copper grids were commonly used; although freshly prepared formvar-coated grids were also used. In the latter case, grids were coated with 0.5% formvar in ethylene dichloride. After the IgG-sensitized grids were exposed to test extracts and rinsed, the grids were positively stained with a 5% solution of uranyl acetate in 50% ethanol for 1 minute and washed in 95% ethanol for 30 seconds. The grids were viewed with a Philips 200 transmission electron microscope.

Enzyme-linked immunosorbant assays (ELISA). The double sandwich ELISA procedure developed for CTV by Bar-Joseph *et al.* (1979a) was used. Immulon II Microtiter® plates (Dynatech Laboratories, Inc., Alexandria, VA 22314) were coated with 1 µg IgG per ml for 4 hours at 37 C. Antigen preparations were prepared as previously described or by grinding either healthy or CTV-infected tissue at a ratio of 1 g fresh weight per 24 ml of 0.05 M Tris pH 8.0. Antigen preparations were then additionally diluted 1 to 50 (w/v) with either Tris buffer or Tris buffer containing SDS so that the final SDS concentration was 0.1%. Antigen preparations were incubated overnight at 4 C in the microtiter plates. Conjugates were diluted 1 to 1,000 (about 1 µg IgG per ml) in a phosphate-buffered saline solution (PBS) containing 0.5% (v/v) Tween 20; 2% (w/v) polyvinylpyrrolidone (40,000 molecular weight, Sigma) and 0.02% (w/v) bovine serum albumin (fraction V powder, Sigma) and incubated in the plates for 24 hours at 4 C. After rinsing, substrate (p-nitrophenylphosphate, 1.0 mg/ml in 10% triethanolamine, pH 9.8) was added. After 30 minutes at room temperature, the A_{405 nm} was read on each well using a Bio-Tek EIA Reader®, Model EL 307 (Bio-Tek Instruments, Inc., Burlington, VT 05401). Some samples were diluted with water and read in a spectrophotometer.

SDS immunodiffusion (SDS-ID). The SDS-immunodiffusion method used was that described by Garnsey *et al.* (1979).

Dot immunobinding assay. A detailed protocol for this assay was developed for this work and has been described by Zheng *et al.* (1985). Crude sap from infected plants or purified virus in sample buffer was spotted onto nitrocellulose membranes for the dot-immunobinding assay. When the spots were dry unoccupied sites on the nitrocellulose were blocked with polyoxyethylene sorbitan monolaurate (Tween 20). The membrane was then incubated with alkaline phosphatase conjugated antibody and the immuno complexes subsequently detected by the reduction of the tetrazolium salt to diformazan when hydrogen ions are released from the formation of indigo by the reaction of the phosphatase on the indoxyl phosphate. A positive reaction is observed as a purple dot against the white background of the nitrocellulose.

RNA extractions. Extraction of RNA from CTV suspension and their subsequent electrophoresis on agarose gels was performed by the methods of Bar-Joseph *et al.* (1985) and Rosner *et al.* (1983).

Isolation of dsRNA. Frozen bark tissue from graft-inoculated Egyptian lime (*C. aurantifolia* S.) seedlings was ground to powder in liquid N₂ with a

mortar and pestle. The nucleic acids were extracted from the powdered tissue with phenol and the dsRNA was isolated by chromatography on CF-11 (Whatman) columns (Dodds and Bar-Joseph, 1983; Bar-Joseph et al., 1983).

Electrophoresis of RNA and blotting onto nitrocellulose. Fractionation of RNA in a denaturing agarose gel by electrophoresis and the transfer of RNA bands onto nitrocellulose were carried out as detailed by Fellows et al. (1982).

Dot-spots. Aliquots of RNA (3 μ l) were spotted on nitrocellulose paper presoaked in 20 X SSC (Rosner et al., 1983).

Preparation of recombinant plasmids containing CTV DNA sequences and selection of clones containing inserts complementary to CTV RNA. All the procedures employed for the production of CTV cDNA were carried out as described by Rosner et al. (1983).

Nick-translated labelling. DNA of plasmids containing cloned cDNA sequences complementary to the genomic RNA of CTV was labelled with 32 P by the nick-translation procedure (Ginzburg et al. 1980).

Polynucleotide-kinase labelling. Total RNA extracted from CTV infected and uninfected plant tissue was labelled with 32 P using the polynucleotide-kinase end-labelling procedure (Maizels, 1976).

Hybridization conditions. The labelled DNA or RNA probes were hybridized with the RNA blots and dot-blots as described in Thomas (1980).

In vitro translation of virus RNA. Protein synthesis in a rabbit reticulocyte lysate cell free system directed by virus RNA was carried out as in Pelham and Jackson (1976). The 35 S labelled translation products were fractionated in SDS-10% polyacrylamide gel (Laemmli, 1970) and visualized by autoradiography following sodium salicylate treatment of the gel.

Results and Discussion

Biological characterization of CTV strains. We have developed a standardized protocol to compare severity of different CTV strains which will allow a more objective comparison of strain severity and provide a common reference between researchers in different areas (Fig. 1). The use of a numerical index score will facilitate communication of a variety of strain-host interactions.

Isolates of differing severity were compared separately in California, Florida and Israel using the standardized five plant host range all propagated from seed sources in California. Following examination of the results from these individual tests, it was decided that a single location with no variables in conditions, host materials or interpretation of symptoms would be of great advantage. With this in mind, arrangements were made to use the quarantine facilities at USDA Beltsville Agricultural Research Center, Beltsville, Maryland, and the direct comparison on the five plant host range was initiated on 18 isolates from several citrus growing areas of the world including California, Florida, and Israel. The following preprint which will be presented at a CTV workshop in South Africa is appended:

Garnsey, S. M., D. J. Gumpf, E. L. Civeralo, R. F. Lee, C. N. Roistacher, and M. Bar-Joseph. 1985. Recent progress toward a standardized evaluation of the biological properties of CTV. South African CTV Workshop (In press).

At the present time, however, we have a bank of 25 isolates from four countries and we are negotiating to obtain additional isolates from four other countries. This bank of CTV isolates will provide a valuable resource for future studies on strain characterization such as aphid transmissibility, nucleotide sequence homology, and serological homology.

Purification of infectious virus. We have developed purification procedures which have yielded intact 2000 nm particles with which we can also associate infectivity. The details of these procedures have been published as part of this project and will therefore not be repeated. The reader may examine the following appended reprints and preprints for this information:

Bar-Joseph, M., D. J. Gumpf, J. A. Dodds, A. Rosner, and I. Ginzburg. 1985. A simple purification method for citrus tristeza virus and estimation of its genome size. *Phytopathology* 75: 195-198.

Garnsey, S. M., R. F. Lee and R. H. Bransky. 1981. Preparation and stability of infectious citrus tristeza virus (CTV). *Phytopathology* 71: 218.

Garnsey, S. M., R. F. Lee and R. H. Bransky. 1981. Rate zonal gradient centrifugation of citrus tristeza virus. *Phytopathology* 71: 875.

Gumpf, D. J., M. Bar-Joseph, and J. Allan Dodds. 1981. Purification of citrus tristeza virus (CTV) on sucrose-cesium sulfate cushion gradients and estimation of its RNA size. *Phytopathology* 71: 878.

Lee, R. F., S. M. Garnsey, and R. H. Bransky. 1981. An improved purification procedure for citrus tristeza virus. *Phytopathology* 71: 235.

Lee, R. F., S. M. Garnsey, and R. H. Bransky. 1985. A purification procedure for enhancement of citrus tristeza virus yields and its application to other phloem-limited viruses. *Phytopathology* 75: (submitted).

Citrus tristeza virus particles shorter than 2000 nm were not infectious and addition of short particles to moderately infectious whole virus preparations did not enhance infectivity.

Serological titer was not necessarily closely correlated with infectivity titer in gradient assays. Fractions containing subgenomic fragments often had high ELISA values even though the OD₂₆₀ was less than that for whole virus peak fractions and there was no infectivity. Seedling yellows (SY) symptoms were obtained in plants inoculated with rate zonal gradient whole virus bands and from partially purified preparations passed through an agarose bead column with molecular cutoffs of 15 and 50 X 10⁶ daltons. Both results suggest that

SY is encoded on the whole virus RNA and subgenomic species are not required in the inoculum to induce SY effects. See the following appended reprint for these results:

Garnsey, S. M. 1984. Separation of citrus tristeza virus strains and strain variants by stem-slash inoculation of citrus receptors. *Phytopathology* 74: 848.

Use of macerating enzymes, especially Driselase, improved extraction of CTV from tissue extracts, when yield was measured serologically or by other quantitative means. Particle integrity looked good in Driselase-treated preparations, but we failed to achieve the desired enhancement of specific infectivity. In fact, in many tests infectivity was lower in Driselase-treated preparations than in nontreated preparations even though the total amount of virus was lower in nontreated preparations. We tried several variations in extraction conditions, but results were generally inconclusive. In at least one case, passage of step gradient fractions of Driselase-treated extracts through an Agarose A-15 column did yield greater than expected infectivity.

The use of Driselase was also evaluated as a procedure for liberating CTV inclusion bodies from infected tissue. The inclusion bodies were extracted by the use of Driselase followed by a series of low speed centrifugations and treatment with Triton X 100 and finishing with centrifugation on a step Cs_2SO_4 gradient containing sucrose. The inclusion bodies can be detected by the use of SDS-immunodiffusion and ELISA. Results of this work are given in the following appended reprint:

Lee, R. F., S. M. Garnsey, R. H. Bransky, and L. A. Calvert. 1981. Purification of inclusion bodies of citrus tristeza virus. *Phytopathology* 71: 953.

Characterization of coat protein. The coat proteins of several aphid-transmitted isolates of CTV which varied widely in their biological properties were purified and examined. A major coat protein (CP1) and a minor coat protein (CP2) with estimated molecular weights of 23,000 and 21,000 daltons, respectively, were found in all Florida isolates. Both CP1 and CP2 react with CTV specific antisera. The amino acid composition of the coat proteins were low in methionine and high in lysine, aspartic acid, and glycine. When CP1 and CP2 were digested with trypsin, V8 protease, or thermolysin and the resultant polypeptides examined by SDS polyacrylamide gel electrophoresis, the polypeptide maps were similar. The coat proteins from five different Florida CTV isolates were compared by comparison of the polypeptide maps after proteolytic enzyme digestions. All isolates produced similar maps with only some minor differences. Results of this work are given in the following appended reprint:

Lee, R. F., L. A. Calvert, and J. D. Hubbard. 1984. Characterization of the coat proteins of citrus tristeza virus. *Phytopathology* 74: 801.

When the proteins which are translated in vitro from RNA extracted from purified CTV virions are examined with CTV antisera, there is a polypeptide of

about 28,000 daltons which is immunoprecipitated but no proteins corresponding to CP1 and CP2 as found in purified virions. Based on the similarity of amino acid composition and polypeptide maps after digestion by proteolytic enzymes, it seems that CP1 and CP2 are derived by post-translational processing from the 28,000 MW protein found in the in vitro translates.

Serological determinants. Studies with various polyclonal antisera were conducted using different techniques. Tests with antisera to fixed, undegraded virus; unfixed, undegraded virus and SDS-degraded viral coat protein were conducted using various procedures including ELISA, SSEM, SDS-ID and immunofluorescence. These tests indicated that at least several different determinants must be involved in CTV antigen-antibody reactions. See the appended reprint listed below for complete results of this work:

Brlansky, R. H., S. M. Garnsey, R. F. Lee and D. E. Purcifull. 1984.
Application of citrus tristeza virus antisera in labeled antibody, immuno-electron microscopical, and sodium dodecyl sulfate-immunodiffusion tests. Pages 337-342 in: Proc. 9th Conf. Int. Organ. Citrus Virol. S. M. Garnsey, L. W. Timmer and J. A. Dodds, eds. IOCV, Gainesville.

Host and isolate effects on serological titer of CTV in plant extracts. Various observations have suggested that CTV replicates in higher concentration in some hosts than others and that the titer of some isolates is higher than others in the same host.

A series of experiments were conducted to overcome the technical problems associated with controlling various extraneous variables which can affect CTV titer measurements. Samples were collected at a constant point in the growth cycle (just when a new leaf became fully expanded) to avoid age effects on titer. Collection time on a calendar basis were not possible because of the different growth habits of the various hosts and because severe CTV isolates also drastically reduce growth rates in reactive hosts. A single source of gamma globulin and conjugate was used for all ELISA tests and common controls were used for all plates to provide for plate-to-plate correction.

Twelve different hosts were inoculated with each of 5 isolates of varying severity. Additional hosts were inoculated with one or more isolates. After systemic infection was verified by ELISA, plants were put in randomized groups, and pruned. Shoots were analyzed separately and as composite collections in two separate tests. Samples were collected, extracted to a standard dilution, freeze dried and then tested by ELISA under standard conditions with standards for each test. Samples were replicated in each ELISA plate, the paired values averaged and corrected to the plate value for the standard. Results are summarized in Table 1. These values are for a three-sample composite per plant and in most cases five replications. The following appended preprint presents these results:

Garnsey, S. M., R. F. Lee, R. H. Brlansky, and R. K. Yakomi. 1985.
Serological titer of citrus tristeza virus (CTV) isolates of varying severity in different citrus hosts. Phytopathology 75: (submitted).

Lee, R. F. and J. P. Agostini. 1985. Relative titers of Florida citrus tristeza virus isolates in sweet orange and etrog citron. Proc. Int. Soc. Citriculture (in press).

The general conclusions that can be made from this work are summarized by the following statements:

1. Variation within treatments was often as great as variation between treatments.
2. Citrus hystrix was the best host overall, but other reactive hosts such as citron and lime are also good.
3. Eureka lemon and sour orange were poor hosts in general for SY isolates.
4. Satsuma and Cleopatra mandarin had very low CTV titers.

Monoclonal antibody production. Monoclonal antibody producing hybridoma clones have been produced against a California CTV isolate T519, a California SY isolate SY568 and against the Florida isolate T30. The hybrids were cloned by limiting dilution and screened for reactivity to sap from CTV infected and healthy citron. The resulting monoclonal antibodies are highly reactive and specific for CTV with no healthy reaction.

Further characterization of the reacting components is underway by protein slab gel electrophoresis and western blot analysis. Analysis of clones for CTV strain specificity is still under study using a solid phase ELISA using alkaline phosphatase conjugated affinity purified goat anti-mouse IgG. The solid phase ELISA technique we are using was developed for this project. See the following appended reprint and preprint for details of the technique.

Gumpf, D. J., Wichai Kositratana and Guang-Yu Zheng. 1984. Dot-immunobinding assay for virus detection. Phytopathology 74: 847.

Gumpf, D. J., Guang-Yu Zheng and J. M. Diaz. 1985. Production and evaluation of specific monoclonal antibodies to CTV strains. South African CTV Workshop (submitted).

Zheng, Guang-Yu, Wichai Kositratana and D. J. Gumpf. 1985. Detection of viruses in sap using alkaline phosphatase conjugated antibody with dot-immunobinding assays. Phytopathology 75: (submitted).

Serological detection of CTV in crude plant sap. A simple and rapid technique was developed to detect and identify viruses applied to nitrocellulose membranes as spots of crude sap. The immobilized antigens are then reacted with monoclonal antibodies followed by reaction with anti-immunoglobulin conjugated with alkaline phosphatase. Results of this procedure can be seen by examining the following preprints which are appended.

Gumpf, D. J., Wichai Kositratana and Guang-Yu Zheng. 1984. Dot-immunobinding assay for virus detection. Phytopathology 74: 847.

Kositratana, Wichai, Guang-Yu Zheng, D. J. Gumpf, and L. G. Weathers.
1985. Comparative sensitivity of three serological methods for plant virus detection. *Phytopathology* 75: (submitted).

Zheng, Guang-Yu, Wichai Kositratana and D. J. Gumpf. 1985. Detection of viruses in sap using alkaline phosphatase conjugated antibody with dot-immunobinding assays. *Phytopathology* 75: (submitted).

A radio-immunosorbant assay (RISA) has also been developed for the detection of CTV. Using the double-antibody sandwich procedure, purified immunoglobulins were adsorbed to polystyrene beads. The beads were washed, incubated with the test sample, washed again and incubated in ^{125}I -labeled immunoglobulin. The amount of ^{125}I -immunoglobulin bound to the beads was proportional to the amount of CTV in the sample. When compared to ELISA, RISA did not give increased sensitivity in detection of CTV, but it was easier to quantitate. Results of this work can be found in the following appended reprint:

Lee, R. F., L. W. Trimmer, D. E. Purcifull, and S. M. Garnsey. 1981.
Comparison of radioimmunosorbent assay and enzyme linked immunosorbent assay for detection of Rickettsia-like bacteria and citrus tristeza virus. *Phytopathology* 71: 889.

Extraction and characterization of CTV-RNA. Single stranded RNA from dissociated purified CTV could be resolved by electrophoresis on 1.2% agarose gels. Molecular weight of non-denatured CTV-RNA was estimated to be $5.4 - 6.5 \times 10^6$ daltons. More complete information can be found in the following reprint which is appended:

Bar-Joseph, M., D. J. Gumpf, J. A. Dodds, D. Rosner, and I. Ginzburg.
1985. A simple purification method for citrus tristeza virus and estimation of its genome size. *Phytopathology* 75: 195-198.

The RNA from several CTV isolates has been translated in an in vitro rabbit reticulocyte lysate system. Major products were polypeptides of about 28,000, 33,000, 50,000, and 65,000 daltons. The 28,000 polypeptide was the only major product immunoprecipitated with antiserum specific for CTV coat protein. These results suggest that the full size genomic RNA of the virus has a messenger RNA activity. See the following reprint and preprint which are appended for further details:

Nagel, J., E. Hiebert and R. F. Lee. 1982. Citrus tristeza virus RNA translated with a rabbit reticulocyte lysate: capsid protein identified as one of the products. *Phytopathology* 72: 953.

Rosner, Arie, Moshe Bar-Joseph, and Irith Ginzburg. 1985. Analysis of cloned DNA sequences complementary to the citrus tristeza virus (CTV) genomic RNA. (submitted).

We were unable to demonstrate infectivity of RNA preparations extracted from infectious nucleoprotein preparations. There are several possible reasons. Only limited tests have been run with good preparations and these may not have been in sufficient concentration. The extraction procedure may also have rendered the RNA noninfectious or the assay procedure (stem slash inoculation) is not well suited for assay of RNA preparations.

Association of infectivity with CTV RNA preparations will remain a difficult problem until easier means are developed to obtain intact RNA's in quantity.

Diagnosis of specific viral RNA sequences in plant extracts with a ³²P-labelled double-stranded RNA probe. Methods for screening of virus related nucleic acids had to be developed prior to the production of cloned complementary DNA (cDNA) which was to be used to study sequence homology of serologically indistinguishable CTV strains. The first of these methods was a procedure for the extraction of double-stranded RNA (dsRNA) from virus infected plants. The method is based on grinding plant tissue in 4-6% p-aminosalicylic acid and recovery of dsRNA by phenol extraction and precipitation with 30-33% ethanol. The complete procedure is outlined in the following reprint which is appended:

Bar-Joseph, M., A. Rosner, M. Moscovitz, and R. Hull. 1983. A simple procedure for the extraction of double-stranded RNA from virus-infected plants. J. Virol. Methods 6: 1-8.

Lee, R. F. 1985. Use of double stranded RNAs to diagnose citrus tristeza virus strains. Proc. Fla. State Hort. Soc. (in press).

The second procedure which was specifically developed for this project was a method for screening virus-related RNAs in plant extracts based on the hybridization of a polynucleotide kinase-mediated ³²P-labeled dsRNA probe. Additional information about this procedure may be obtained from the following reprint which is appended:

Rosner, A., M. Bar-Joseph, M. Moscovitz, and M. Mevarech. 1983. Diagnosis of specific viral RNA sequences in plant extracts by hybridization with a polynucleotide kinase-mediated, ³²P-labeled, double-stranded RNA probe. Phytopathology 73: 699-702.

Cloning of complementary DNA of CTV-RNA. Complementary DNA (cDNA) of CTV RNA, synthesized using calf thymus DNA random primers, was converted to a double-stranded form and inserted into the PstI site of the *Escherichia coli* pBR322 plasmid by the G-C tailing method. Bacterial clones harbouring viral-specific sequences were detected by colony hybridization with a ³²P-labelled viral RNA probe. Hybridization patterns of denatured virus RNA revealed the presence of three types of specific clones: those hybridizing with a distinct narrow band corresponding to the full-length virus RNA, those hybridizing with a broader band of virus RNA sequences, and those hybridizing with several distinct virus-related RNA bands. Similar patterns were obtained when these clones were hybridized to purified double-stranded RNA from CTV-infected plants. None of these cDNA clones hybridized with similarly treated preparations extracted from healthy plants. Complete results of this work are discussed in the following reprint which is appended:

Rosner, A., Irith Ginzburg, and M. Bar-Joseph. 1983. Molecular cloning of complementary DNA sequences of citrus tristeza virus RNA. J. gen. Virol. 64: 1757-1763.

Further analyses of the recombinant molecules of pBR322 plasmid carrying cDNA sequences specific for CTV were performed. Four out of the seven clones

tested contained virus specific sequences only, while three clones hybridized to virus sequences as well as to sequences of RNA isolated from healthy plants. A virus specific sequence was used as a probe for further analysis of the virus genome by molecular hybridization. Additional results may be obtained from the following preprint which is appended:

Rosner, A., M. Bar-Joseph, and I. Ginzburg. 1985. Analysis of cloned DNA sequences complementary to the citrus tristeza virus (CTV) genomic RNA. (submitted).

Cloned cDNA for typing of CTV strains. Cloned cDNA sequences 500-2000 base pairs long, derived from a severe citrus tristeza isolate, were used to study sequence homology with RNA of nine other serologically indistinguishable CTV isolates which differed in their biological properties. Six of the nine CTV isolates hybridized positively with the tested cDNA clones, while three others hybridized differentially with these cDNA clones. Complete results of this work are reported in the following reprint which is appended:

Rosner, A. and M. Bar-Joseph. 1984. Diversity of citrus tristeza virus strains indicated by hybridization with cloned cDNA sequences. Virology 139: 189-193.

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Fig. 1.

CTV - BIO CHARACTERIZATION

ISOLATE: _____

I. PRIMARY TEST PLANTS

	REACTION	TEST CONDITIONS	WEIGHT FACTOR	REACTION SCALE				TOTAL INDEX
				0 (NONE)	1 (MILD)	2 (MOD.)	3 (STRONG)	
MEX. LIME	VEIN CLEARING STEM PITTING	GH (70-80°F)	1 X					
SWT/SOUR	STUNT. PHLOEM NEC. AT B.U.	FIELD	2 X					
SOUR SDLG	SDLG YELLOWS	GH (70-80°F)	3 X					
DUNCAN GRFT	STUNTING, STEM PITTING	GH (70-80°F)	4 X					
MADAM VINOUS SWT.	STUNTING, STEM PITTING	GH (70-80°F)	5 X					

PRIMARY INDEX SCORE: _____

II. SECONDARY TEST PLANTS

HOST	REACTION SCALE			
	0 (NONE)	1 (MILD)	2 (MOD.)	3 (SEVERE)
RANGPUR LIME				
ROUGH LEMON				
VALENCIA				
EUREKA LEMON				
TRIFOLIATE ORANGE				
SATSUMA				

Table 1. Serological titer of 5 CTV isolates in different hosts as measured by ELISA (OD₄₀₅)

Host	Isolate					Average all isolates
	T-3	T-4	T-26	T-30	T-36	
<u>First test</u>						
<u>C. mac.</u>	0.911	0.881	0.754	0.783	1.137	0.893
<u>C. excelsa</u>	1.070	0.902	0.761	0.891	1.216	0.968
Calamondin	0.880	1.227	0.714	0.769	0.784	0.875
<u>C. shaddock</u>	0.395 (1)	0.856	0.856	0.761	0.930	0.760
Sweet lime	0.775	1.011	0.816	0.684	0.809	0.819
H 7	1.042	0.939	0.842	0.814	1.140	0.955
Sour #2	0.649	0.957	0.770	0.828	0.835	0.808
Citron 861	1.122 (1)	1.104	0.870	0.869	1.122	1.071
<u>C. hystrix</u>	1.084	1.077	0.883	0.986	1.127	1.031
Duncan grapefruit	0.680	1.006	0.899	0.898	0.975	0.892
Lab sweet	0.940	0.870	0.844	0.792	0.940	0.877
Eureka lemon	0.608	1.045	0.757	0.666	0.730	0.761
Average	0.864	0.990	0.814	0.812	0.979	
<u>Second test</u>						
<u>C. hystrix</u>	0.881	0.927	0.645	0.713	0.846	0.824
Duncan grapefruit	0.536	0.788	0.684	0.704	0.680	0.678
Lab sweet	0.706	0.563	0.558	0.659	0.546	0.633
Eureka lemon	0.472	0.671	0.556	0.494	0.502	0.539
Sour #2	0.340	0.684	0.473	0.608	0.560	0.533
Valencia/Rangpur	0.648	0.504	0.676	0.698	0.543	0.533
Average	0.597	0.690	0.599	0.646	0.613	

EVALUATION OF RESEARCH ACHIEVEMENTS WITH RESPECT TO PROPOSED RESEARCH

The first step toward satisfying the objectives of this project was to select a number of strains from the varying locations and compare them biologically as well as to determine the most appropriate hosts for virus increase. The results discussed previously indicate that not only did we accomplish the biological characterization but we have decided, because of a continuing interest, to pursue the idea of comparing these strains further. Toward this end we have made arrangements to continue these studies at a single location, Beltsville, Maryland, under quarantine. As a result we are putting together a bank of CTV isolates which will provide a valuable resource for future studies on strain characterization.

As a result of the studies to determine the most appropriate host-virus combinations for virus increase, we were able to make considerable improvements on the purification of CTV, which was one of the major objectives of the proposal. As evidence of this progress, we were subsequently able to perform the proposed characterizations of both the protein coat and nucleic acid of CTV.

Using the improved methods for CTV purification, rabbit polyclonal antibodies were raised against whole virus and degraded coat protein. Subsequent serological tests again verified that the many differing biological strains of CTV can not be differentiated by conventional serology. Differences were apparent, however, when antiserum prepared against whole virus is reacted with coat protein and vice versa in SSEM studies. Therefore, the use of monoclonal antibodies may well reveal small serological differences or the presence of additional antigen species not currently recognized. After some initial problems of technique which unfortunately delayed our progress considerably, we have produced monoclonal antibodies to several CTV strains. These antibodies are highly reactive and for the first time, we have antibodies specific for CTV with no healthy reaction. In addition, we have developed a dot immunobinding procedure for the evaluation of our clones as far as their ability to differentiate between strains.

Also, as a result of the improved purification procedures, high quality coat protein preparations from several CTV strains which varied in their biological properties were prepared and examined as proposed. A complete characterization of the coat protein was completed, including amino acid composition, molecular weight estimation, polypeptide mapping, and serological studies.

Using the newly developed procedures, virus was purified and viral RNA extracted and characterized. Unfortunately, we were not able to show that these RNA preparations were infectious. Association of infectivity with CTV RNA will undoubtedly remain a difficult problem until easier means are developed to obtain intact RNA in quantity. The extracted RNA was of sufficient quality, however, that it could function as messenger RNA in a cell free translation system by producing a protein of about 28,000 daltons which was immunoprecipitable. The isolated CTV RNA genome also was of sufficient size and quality to serve as template for the production of randomly primed cDNA. As proposed, the cDNA was converted to double-stranded form cut with S_1 nuclease to molecules of about 600 nucleotides which were tailed with poly(dc) and inserted into the PstI site of pBR322 plasmid. The recombinant plasmids

were introduced into E. coli. The cloned viral cDNA sequences were used for hybridization experiments with genomic CTV RNA as proposed. The differential hybridization of several cDNA clones indicates the possible advantages of using small cloned cDNA segments as probes for viral typing. The further construction of a cDNA library derived from a large variety of viral strains should improve the resolving power of this method for typing purposes, one of the major objectives of this project.

DESCRIPTION AND RESULTS OF COOPERATION

The degree of cooperation is without question the reason for the progress and success of this project. Research on some objectives was performed at all locations, for example developing more efficient purification procedures. Each laboratory worked on their own CTV strains but as a result of the cooperation, the strains were all compared biologically using the same hosts all planted from a single lot of seed. This comparative work has even expanded further into a large cooperative work which will be continued after the project has expired (see main body of this report for details of this continuing work). The extraction and characterization of CTV-RNA were also performed at all locations.

Certain aspects of the work were done at specific locations because that is where the equipment and expertise were located, thus to avoid duplication of expensive equipment this cooperative approach was taken. The coat protein analyses were performed in Florida, the production of monoclonal antibodies in California, and the cDNA probe work primarily in Israel although some was likewise performed in Florida.

As a result of this project, there has been a great deal of communication between the labs involved. A feeling of cooperation and commitment to solving the problem has resulted. As evidence for this spirit of cooperation, a number of review papers or papers dealing with epidemiology and CTV management have been co-authored by the cooperators on this project. I realize these papers are not directly related to the objectives of this proposal, but they certainly offer proof of the spirit of interest and cooperation displayed by the research personnel on this project. The co-authored papers which are appended are as follows:

Bar-Joseph, M., C. N. Roistacher, S. M. Garnsey, and D. J. Gumpf. 1981.
A review on tristeza, an ongoing threat to citriculture. Proc. Int. Soc. Citriculture 1: 419-423.

Bar-Joseph, M., C. N. Roistacher, and S. M. Garnsey. 1983. The
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Fishman, Sveltano, Ruth Marcus, H. Talpay, M. Bar-Joseph, Y. Oren, R.
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