Viruslike Particles and Double-Stranded RNA in *Geotrichum candidum*, the Causal Agent of Citrus Sour Rot

Henia Mor, Rivka Steinlauf, and Isaac Barash

Department of Botany, The George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv, Israel.
The authors are grateful to E. E. Butler, Department of Plant Pathology, University of California, Davis, for supplying isolates of *Geotrichum candidum*.
This research was supported by Grant I-61683 from the United States-Israel Binational Agricultural Research and Development Fund.
Accepted for publication 3 March 1984.

ABSTRACT

Mor, H., Steinlauf, R., and Barash, I. 1984. Viruslike particles and double-stranded RNA in *Geotrichum candidum*, the causal agent of citrus sour rot. Phytopathology 74: 921-924.

A single kind of double-stranded RNA (dsRNA) with apparent molecular weight of 3.6×10^6 daltons was detected in isolates of *Geotrichum candidum* by agarose gel electrophoresis. The dsRNA molecule was present in 8 of 10 isolates of the citrus sour rot pathogen but absent in nonpathogenic isolates of *G. candidum*. Isodiametric (~40 nm) viruslike particles (VLPs) were purified from a pathogenic isolate. The dsRNA molecule of viral origin was identical in molecular size to the dsRNA obtained directly from cells. Two virus-associated proteins with estimated molecular weights of ~94,000 and 71,000 daltons were characterized by SDS polyacrylamide electrophoresis.

Many fungi contain segmented dsRNA (14). In almost all cases that have been carefully studied, the dsRNA has been found to be the genome of a mycovirus (14), although exceptions may exist (17). Viral infections have also been recorded in an increasing number of plant pathogenic fungi (3,9,11,14,15,17,18). A major question is whether mycoviruses or dsRNA segments may alter the phenotypic expression of fungi, including their pathogenicity. The killer systems in *Saccharomyces cerevisiae* and *Ustilago maydis* (5), as well as the "La France" disease of *Agaricus bisporus* (14), provide well documented examples of mycoviruses associated with phenotypic changes in fungi. However, the relationship between segmented dsRNA and changes in fungal virulence is still inconclusive.

Transmissible cytoplasmic factors that markedly diminish the vigor of growth in culture and/or pathogenicity to plants, have been reported for several phytopathogenic fungi (14). In *Endothia parasitica* (13,14) and *Rhizoctonia solani* (9), these traits were associated with the presence of dsRNA. DsRNAs were present in both more aggressive and less aggressive isolates of *Ceratocystis ulmi*, but the less aggressive isolates generally possessed more segments (17). The former and latter examples suggest that diminished pathogenicity might be caused by the presence of some dsRNA segments.

Geotrichum candidum Lk. ex Pers. comprises a complex (called "the complex" in this paper) of asexual fungi adapted to many different habitats (6). In contrast, isolates of the sour rot pathogen from widely separated geographical areas comprise a distinctly homogenous group among the populations of the complex and were, therefore, designated as the "citrus race" of *G. candidum* (7). Although the "citrus race" is morphologically indistinguishable from the noncitrus isolates, it is characterized by high virulence on lemon fruits, some specific nutritional requirements, and a high adaptation to the citrus environment (7). *G. candidum* had traditionally been classified with the Fungi Imperfecti until Butler and Petersen (8) discovered its sexual form, namely, *Endomyces geotrichum* Butler and Petersen. *E. geotrichum* is similar to *E. reessii* van der Walt (20), whose asexual stage may also form part of the complex.

The purpose of the present study was to determine whether mycoviruses can be detected in the sour rot pathogen and in the nonpathogenic isolates of *G. candidum*.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. § 1734 solely to indicate this fact.

MATERIALS AND METHODS

Isolates and cultures. Isolates of *G. candidum* were either obtained from E. E. Butler, Department of Plant Pathology, University of California, Davis, or isolated from citrus fruits infected with sour rot. Pathogenicity of these isolates was determined according to Schachnai and Barash (19). Cultures were maintained on PDA at 27 C or stored at 5 C. Mycelia and spores of *G. candidum* isolates were grown in liquid medium (5 g of yeast extract and 10 g of glucose per liter) for 72 hr at 27 C in shake culture. The cells were harvested by filtration and stored at −20 C.

Extraction and purification of dsRNA. Isolation and purification of dsRNA directly from cells of *G. candidum* was performed according to Morris and Dodds (16). Frozen cells (10 g) were disrupted in a French pressure cell (American Instrument Co., Silver Spring, MD 20910) (1) in the following extraction solution: 15 ml of GPS buffer (0.2 M glycine, 0.1 M Na_2HPO_4, and 0.6 M NaCl), 1% sodium dodecyl-sulfate (adjusted to pH 9.5 with 5 M NaOH), 0.1 ml of mercaptoethanol, 10 ml of redistilled water-saturated phenol, and 10 ml of chloroform. The homogenate was centrifuged for 20 min at 8,000 *g* and 4 C. The upper, aqueous phase was removed and reextracted twice with 20 ml of phenol-chloroform (1:1). The nucleic acids were precipitated from the aqueous phase by the addition of NaCl to a final concentration of 0.3 M and two volumes of cold absolute ethanol. The solution was placed at −20 C overnight to precipitate the dsRNA. The precipitate was collected by centrifugation, dried in a desiccator, and resuspended in STE buffer (0.1 M NaCl, 0.05 M tris-HCl, and 0.001 M Na_2EDTA, pH 7.0), adjusted to 15% ethanol (STE-15% ethanol). Then it was passed through a CF-11 cellulose column (Whatman Chemical Separation Ltd., London, England) (16), and the column was washed with 150 ml of STE-15% ethanol. The dsRNA was eluted with 50 ml of STE buffer and precipitated by the addition of two volumes of cold ethanol and NaCl as described earlier. The precipitate of dsRNA was resuspended in buffer solution suitable for electrophoresis.

Extraction and purification of VLPs. Frozen cells (50 g) were used for purification of VLPs according to Bozarth (2) with an additional precipitation step using 6% polyethylene glycol (PEG 8000) and 0.15 M NaCl as described by Bozarth et al (4). Negative staining and electron microscopy of virions were made with Formvar-coated 49-μm (300-mesh) grids which were floated on drops of purified viral components for 1−5 min (4). The grids were then drained, washed with H_2O, touched briefly to a drop of 2% uranyl acetate, and examined in a Jeol 100B electron microscope. DsRNA from the purified virions was extracted with phenol:chloroform (1:1) as described earlier and used for

electrophoresis.

Electrophoresis. Samples for agarose gel electrophoresis were dissolved in buffer solution containing 0.04 M tris-HCl, 0.02 M acetic acid, and 0.002 M Na_2EDTA, pH 8.1. Electrophoresis was performed according to Davis et al (10) in 1% agarose. DsRNA was stained with ethidium bromide (1 μg/ml) and photographed under short-wave UV light (10). Molecular weight estimates of the dsRNA were carried out by extrapolation of the values reported for dsRNA of *Ustilago maydis* strain P-1 on agarose (12).

The presence of dsRNA in electrophoresis gels was confirmed by measuring the sensitivity of RNA to ribonuclease A (RNase) and deoxyribonuclease I (DNase) obtained from Sigma Chemical Co., St. Louis, MO 63178. For reaction with RNase, samples of dsRNA (5–10 μg) were dissolved in 300 μl of buffer, consisting of either 15 mM NaCl, 1.5 mM Na-citrate, pH 7.6 (low-salt buffer) or 300 mM NaCl, 30 mM Na-citrate, pH 7.6 (high-salt buffer). Following addition of RNase (1 μg/ml), the reaction mixture was incubated for 30 min at 37 C. The reaction was terminated by adding 300 μl of phenol:chloroform (1:1) and dsRNA was extracted and purified as described previously. The reaction with DNase was according to Pusey and Wilson (17).

Characterization of viral proteins. The virus particles were centrifuged in a CsCl density gradient (1.39 g/ml) for 42 hr at 45,000 rpm in an SW 56 rotor. Fractions of 0.3 ml were collected and the absorption at 254 nm in each fraction was determined. The peak from the gradient was pooled and pelleted by centrifugation for 18 hr at 35,000 rpm in a type Ti 65 rotor. The particles were treated with 3% SDS and 2% mercaptoethanol, boiled for 2 min, and loaded on 10% polyacrylamide gel. Electrophoresis was performed with tris-glycine buffer, pH 8.9. The voltage was regulated to 150V for 4 hr. Molecular weight estimates of the protein were carried out by extrapolation with standard proteins.

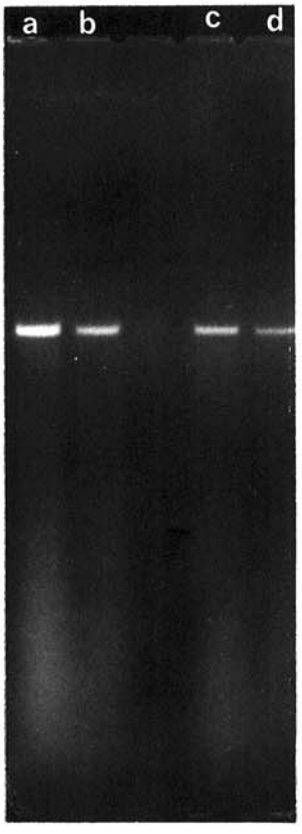

Fig. 1. Agarose gel electrophoresis of dsRNA preparations from isolates of *Geotrichum candidum* pathogenic to citrus fruits. The dsRNA sample applied to the gel for each isolate was about 2 μg/ml. Electrophoresis was carried out for 3 hr at 100 V; (lane a) strain 210; (lane b) GI-3; (lane c) GI-9; and (lane d) 223.

TABLE 1. Detection of dsRNA by agarose gel electrophoresis in preparations from isolates of *Geotrichum candidum*

Isolate	Origin	Pathogenicity[a]	dsRNA
223[b]	Israel	+	+
220[b]	Rhodesia	+	+
219[b]	Rhodesia	+	−
210[b]	Egypt	+	+
178[b]	California	+	−
GI-20	Israel	+	+
GI-7	Israel	+	+
GI-3	Israel	+	+
GI-12	Israel	+	+
GI-9	Israel	+	+
PR-47[b]	Puerto Rico	−	−
PR-27[b]	Puerto Rico	−	−
$31A_1$[b]	...	−	−
$31A_2$[b]	...	−	−
30 A_1nc[b]	...	−	−
60-21[b]	...	−	−

[a] Pathogenicity is based on inoculation of lemon fruits (7).
[b] Isolate supplied by E. E. Butler. The perfect stage of isolate PR 47 is *Endomyces geotrichum* and of isolates PR 27, $31A_1$, $31A_2$ nc and 60-21 is *E. reessii*. All other isolates are citrus races of *G. candidum*.

RESULTS AND DISCUSSION

Double-stranded RNA was detected by agarose gel electrophoresis in 8 of 10 citrus-pathogenic isolates but was not detected in any of the six nonpathogenic isolates examined (Table 1, Fig. 1). Nucleic acid bands stained with ethidium bromide were shown to be dsRNA by their affinity to CF-11 cellulose column, resistance to RNase in high-salt buffer, susceptibility to RNase in low-salt buffer, and resistance to DNase.

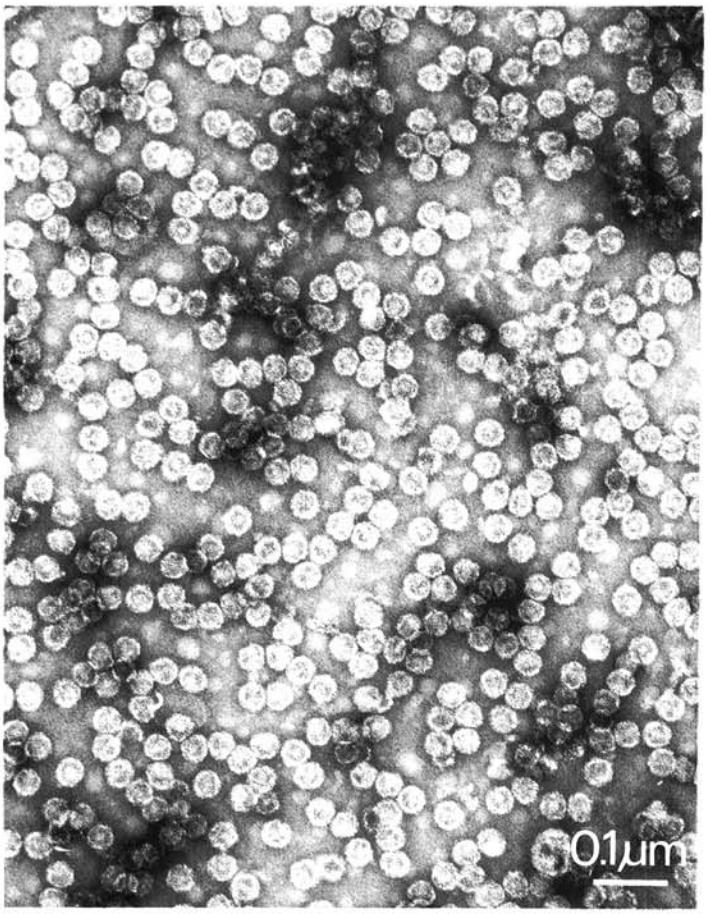

Fig. 2. Electron micrograph of negatively stained (2% uranyl acetate) virus particles from isolate 223 of *Geotrichum candidum* (×36,000).

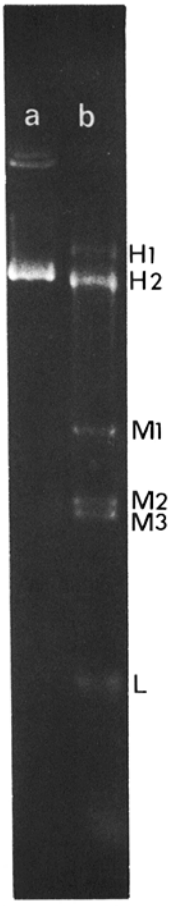

Fig. 3. Agarose gel electrophoresis of dsRNA preparations obtained from (a) VLPs of strain 223 of *Geotrichum candidum*; (b) *Ustilago maydis* strain P-1. H1(4.09×10^6); H2(3.3×10^6); M1(1.12×10^6); M2-1,4(0.72×10^6); M3(0.72×10^6); L(0.23×10^6).

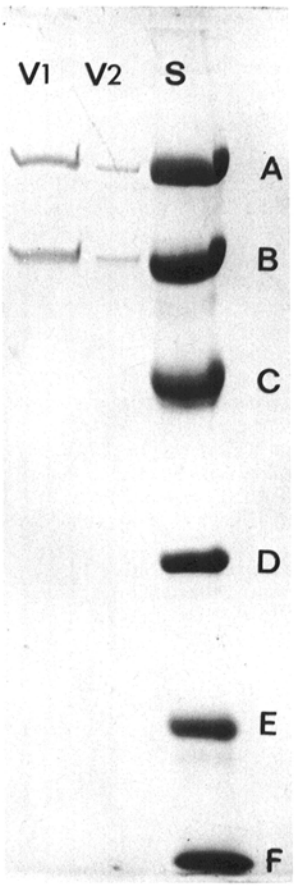

Fig. 4. SDS polyacrylamide gel electrophoresis of virus associated proteins. Electrophoresis was performed with 10% acrylamide. V = virus particles (V_1 50 μg, V_2 5 μg of protein), S = standard proteins, A = phosphorylase b (subunit MW 94,000), B = albumin (67,000), C = ovalbumin (43,000), D = carbonic anhydrase (30,000), E = soybean trypsin inhibitor (20,000), and F = α-lactalbumin (14,400).

The pathogenic isolate 223 was used for extraction and purification of VLPs, as described in Materials and Methods. The UV (254 nm) absorbance profile, following the sucrose density gradient centrifugation step during purification, yielded a single band. This indicated the presence of a homogenous virion population. The virions were separated from the band and reconcentrated by centrifugation for 16 hr at 75,000 *g* and subjected to electron microscopy. Negatively stained isometric VLPs with an average diameter of 40 nm were observed by electron microscopy (Fig. 2). Extraction of dsRNA from the purified VLP preparation resulted in a single type of dsRNA molecule with identical electrophoretic mobility as the dsRNA obtained directly from cells. This result may indicate that VLPs are present in all other isolates in which a dsRNA molecule was detected. The approximate molecular weight of the dsRNA, as estimated by agarose gel electrophoresis, was 3.6×10^6 daltons (Fig. 3).

The coat-associated proteins of VLPs from isolate 223 were characterized by SDS polyacrylamide gel electrophoresis (Fig. 4). Two major protein bands were identified (by staining with Coomassie blue) with estimated molecular weights of ~94,000 and 71,000 daltons. Since it codes for at least two different proteins, the single type of viral dsRNA molecule does not appear to be monocistronic. Assuming that 270 base pairs code for a 10,000-dalton protein (10), a coding capacity of 4,455 base pairs should be sufficient to code for the two viral proteins. The viral genome has a MW of 3.6×10^6 daltons and contains more than 5,000 base pairs (12). Therefore, it may code for additional proteins not associated with the virus particles.

The foregoing results may lead to the conclusion that some isolates of the sour rot pathogen from different parts of the world contain nucleocapsids of mycoviruses at high frequency and concentration. In contrast, VLPs are absent in nonpathogenic isolates from citrus. It is, however, obvious that a larger sample of *G. candidum* should be examined for the presence of dsRNA and VLPs before this conclusion can be validated.

The absence of detectable dsRNA in two highly virulent isolates of the sour rot pathogen indicates that viral infection may play little or no part in determining pathogenicity of *G. candidum* on citrus fruits. Similar results were obtained with other pathogenic fungi (14). However, dsRNA segments were implicated in hypovirulence (9,13,14) or low aggressiveness (17), with some plant pathogens. In nearly all instances the comparisons were made between naturally occurring isolates with or without the viruses. Conclusive evidence depends on experimental infection or transformation of a defined fungal strain with an in vitro preparation of VLPs or dsRNA. This goal has not yet been achieved with phytopathogenic fungi.

As mentioned earlier, the "citrus race" of *G. candidum* is characterized by some physiological and pathological properties that distinguish it from the rest of the complex (7). It is therefore possible that high susceptibility to the mycovirus described here is an additional unique property of the "citrus race" of *G. candidum*. The nonpathogenic isolates may be characterized by resistance to this virus. It is possible that the "citrus race" represents an imperfect stage of a new *Geotrichum* species which is different from the *Endomyces* sp. to which the nonpathogenic isolates belong (Table 1). This might better explain the differential host specificity of the virus we have described here.

LITERATURE CITED

1. Barash, I., and Mor, H. 1973. Regulation of nicotinamide adenine dinucleotide phosphate-specific glutamate dehydrogenase in germinated spores of *Geotrichum candidum*. Plant Physiol.

51:852-858.
2. Bozarth, R. F. 1977. Biophysical and biochemical characterization of virus-like particles containing a high molecular weight dsRNA from *Helminthosporium maydis*. Virology 80:149-157.
3. Bozarth, R. F., Wood, H. A., and Nelson, R. R. 1972. Viruslike particles in virulent strains of *Helminthosporium maydis*. Phytopathology 62:748-753.
4. Bozarth, R. F., Koltin, Y., Weissman, M. B., Parker, R. L., Dalton, R. E., and Steinlauf, R. 1981. The molecular weight and packaging of dsRNA in the mycovirus from *Ustilago maydis* killer strains. Virology 113:492-502.
5. Buck, K. W. 1980. Viruses and killer factors of fungi. Pages 329-375 in: The Eukaryotic Microbial Cell. G. W. Gooday, D. Lloyd, and A. P. J. Trinci, eds. Cambridge University Press, New York.
6. Butler, E. E. 1960. Pathogenicity and taxonomy of *Geotrichum candidum*. Phytopathology 50:665-672.
7. Butler, E. E., Webster, R. K., and Eckert, J. W. 1965. Taxonomy, pathogenicity and physiological properties of the fungus causing sour rot of citrus. Phytopathology 55:1262-1268.
8. Butler, E. E., and Petersen, L. J. 1972. Sexual reproduction in *Geotrichum candidum*. Science 169:481-482.
9. Castanho, B., Butler, E. E., and Shepherd, R. J. 1978. The association of double-stranded RNA with Rhizoctonia decline. Phytopathology 68:1515-1519.
10. Davis, R. W., Botstein, D., and Roth, J. R. 1980. A Manual for Genetic Engineering. Advanced Bacterial Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
11. Dodds, J. A. 1980. Double-stranded RNA and virus-like particles in *Endothia parasitica*. Association of type I viral-like dsRNA with club-shaped particles in hypovirulent strains of *Endothia parasitica*. Virology 107:1-12.
12. Field, L. J., Bruenen, J. A., Chang, T., Pinhasi, O., and Koltin, Y. 1983. Two *Ustilago maydis* viral dsRNAs of different size code for the same product. Nucleic Acid Res. 9:2765-2778.
13. Grente, J., and Savret, S. 1969. L'hypovirulence exclusive est-elle controllée par des déterminants cytoplasmiques. C. R. Acad. Sci. Paris 268:3173-3176.
14. Hollings, M. 1978. Mycoviruses: Viruses that infect fungi. Adv. Virus Res. 22:3-53.
15. Koltin, Y., and Steinlauf, R. 1980. The killer phenomenon in *Ustilago*: Electron microscopy and the dsRNA encapsidated in individual virus particles. Arch. Microbiol. 128:45-52.
16. Morris, T. J., and Dodds, J. A. 1979. Isolation and analysis of double-stranded RNA from virus-infected plant and fungal tissue. Phytopathology 69:854-858.
17. Pusey, P. L., and Wilson, C. L. 1982. Detection of double-stranded RNA in *Ceratocystis ulmi*. Phytopathology 72:423-428.
18. Rawlinson, C. J., Hornby, D., Pearson, V., and Carpenter, J. M. 1973. Virus-like particles in the take-all fungus *Gaeumannomyces graminis*. Ann. Appl. Biol. 74:197-209.
19. Schachnai, A., and Barash, I. 1982. Evaluation of fungicides for sour-rot control in lemon fruits. Plant Dis. 66:733-735.
20. van der Walt, J. P. 1959. *Endomyces reessii* nov. sp. Antonie van Leeuwenhoek Ned. Tijdschr. Hyg. Microbiol. Serol. 25:458-464.