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AID Final Scientific Report

Title: The Double-Stranded RNA Viruses of *Rhizoctonia solani* and the Regulation of Plant Pathogens.

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1. Aspects of Research for the Period January 1988 - July 1988.

I. DNA Plasmid of Rhizoctonia solani

A linear plasmid DNA with a mol. wt. of 1.6×10^6 was identified in virulent strains of R. solani (13 & 82), but not in a hypovirulent strain (521). The plasmid was stable at high levels of RNAse but disappeared by DNAse treatment. Restriction mapping of the DNA plasmid was required prior to any attempts to transform R. solani strains which lack the plasmid. Restriction sites of a number of enzymes were mapped - EcoRI, AciI and San3A. Initial attempts to transform puc-13-ligated plasmid DNA were not successful. The experimental procedures were revised in order to remove impurities and raise the efficiency of the transformation process.

II. Curing

Curing virulent strains of the dsRNA viruses will result in conversion of virulent strains to hypovirulent. Curing, therefore, will result in isogenic strains with similar genetic background, but differ in their virulence characters. This is an important step for further studies of virulence factors.

Two virulence-attenuated clones were derived from a virulent strain of R. solani by ethidium bromide treatment. The concentration of EtBr in the culture and the time of incubation was varied, since the viability of cells in EtBr was greatly reduced. EtBr is known to affect curing of plasmids and other extranuclear elements. Only one of the two attenuated strains also lacked dsRNA. However, the cured strain appeared unstable and as it regained the virulence characters, the dsRNA segments also gradually reappeared.

2. Cooperation

During the course of the research project Dr. Ofusu-Asiedu visited twice the laboratory at Tel Aviv University. His stay was used primarily for providing Dr. Asiedu with the opportunity to learn the methodologies used in the study of the dsRNA viruses. In addition, these visits allowed direct interaction and lengthy discussions related to studies performed at Tel Aviv. During this period various proposals related to the studies in Ghana were developed.

During the last year of the research Dr. Koltin traveled to Ghana and spent a week (Feb. 26 to March 7, 1988) in the Forest Products Research Institute at Kumasi. The visit was devoted to:

a. provide a short course (9 hours of lectures) on the dsRNA viruses of the fungi and the significance of these viruses and plasmids to agriculture.

b. to discuss the experimental aspects that were expected from the Ghanian laboratory.

c. to discuss basic problems for which the phenomenon of fungal hypovirulence may be applied.

d. to meet the students in Kumasi Institute of Science and Technology.

In the course 7 staff members and students participated and all displayed considerable enthusiasm to gain understanding of the subject matter. Likewise the students are eager to learn.

Unfortunately, the basic means for simple research are as yet hardly available at this time. The interaction with staff and student was very positive.

3. Publications

The enclosed publications, related to the research on Rhizoctonia solani, were all supported by the AID grant PDC-5544-G-SS-5093, CS-002 for which we are grateful.

a. Isolation of a Virus from Virulent Strains of Rhizoctonia solani. A. Finkler, Y. Koltin, I. Barash, B. Sneh & D. Pozniak. J. Gen. Virol. (1988) 66, 1221-1232.

b. Double-Stranded RNA Viruses of Pathogenic Fungi: Virulence and Plant Protection. Y. Koltin, A. Finkler and B. Ben Zvi. In: Fungal Infection of Plants, Ed. by Pegg & Ayres 1987. Cambridge University Press, New York, pp. 334-348.

c. Transcription and In Vitro Translation of the dsRNA Virus Isolated from Rhizoctonia solani. A. Finkler, B. Ben Zvi, Y. Koltin & I. Barash. Virus Genes (1988) 1, 205-219.

d. Ds-DNA Virus of Rhizoctonia solani. A. Finkler, B. Ben Zvi & Y. Koltin. In: "Viruses of Fungi and Simple Eukaryotes". Ed. by Y. Koltin and M.J. Leibowitz (1988). Marcel Dekker, Inc., New York, pp. 387-410.

J. gen. Virol. (1985), **66**, 1221-1232. *Printed in Great Britain*

Key words: *dsRNA virus*/R. solani/*virulence*

Isolation of a Virus from Virulent Strains of *Rhizoctonia solani*

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SUMMARY

Double-stranded RNA viruses were detected in virulent strains of *Rhizoctonia solani*. The virus particles were 33 nm in diameter, contained two or three major segments of dsRNA (mol. wt. $1{\cdot}6 \times 10^6$, $1{\cdot}45 \times 10^6$ and $1{\cdot}25 \times 10^6$), had a density of 1·34 g/ml and had an estimated sedimentation coefficient of 161S. The major coat protein had a mol. wt. of 46000. An RNA-dependent RNA polymerase activity was associated with the viral capsids. Native hypovirulent strains of *R. solani* were devoid of these viruses and attenuation of virulent strains to hypovirulence appeared to be correlated with the loss of some or all of the segments of dsRNA. Transmission of dsRNA from a virulent to a hypovirulent strain by heterokaryon formation was associated with the transmission of virulence.

INTRODUCTION

Viruses containing dsRNA are not uncommon among fungi. This group of viruses is generally characterized by a lack of symptom expression; in only a few cases have viruses been shown to affect the phenotype of their carrier fungus (Tipper & Bostian, 1984; Day & Dodds, 1979; Hollings, 1978; Ghabrial & Mernaugh, 1983). The lack of apparent expression of these viruses and their detection in an increasing number of plant pathogens has led to the examination of their involvement in the regulation of virulence. The results thus far are inconclusive, since viruses were detected in both virulent and avirulent strains, and in strains that secrete a phytotoxin as well as in those that do not (Rawlinson *et al.*, 1973; Dunkle, 1974).

Indications that the dsRNA of fungi may be associated with the regulation of virulence were reported in *Endothia parasitica*, the causative agent of chestnut blight (Van Alfen, 1982). The available data suggest that the dsRNA segments act as attenuators of virulence and that the expression of virulence is suppressed following the transmission of the dsRNA through cytoduction between virulent and hypovirulent strains. This is the first case in which a role in the aetiology of a disease can be ascribed to dsRNA and this is the only case in which an adaptive value is suggested for the benign relationship between the carrier fungus and the dsRNA.

Three cases have been reported in which a diseased state of the mycelium was related to dsRNA 'plasmids' and dsRNA viruses (Castanho *et al.*, 1978; Hollings, 1978; Ghabrial, 1980). Two of the reports are from the plant pathogenic fungi *Rhizoctonia solani* and *Helminthosporium victoriae*, and the findings suggest that diseased strains that contain dsRNA are less virulent than those which do not. The diseased state was transmitted by cytoduction to healthy strains along with the dsRNA. Since the suppression of virulence might result from incitement of the disease in the fungal cells, this phenomenon is basically different from the regulation of virulence in *E. parasitica* in which the hypovirulent strains remain healthy. Re-examination of the relationship between dsRNA and virulence was therefore undertaken with *R. solani*.

R. solani is a soil-borne pathogen which causes root decay in over 130 species (Baker, 1970). Castanho *et al.* (1978) and Castanho & Butler (1978*a*) showed that dsRNA occurred in the diseased hypovirulent strain and were able to select a healthy virulent strain from this diseased strain. The virulent strain was devoid of dsRNA and was converted into a hypovirulent strain by

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the transmission of dsRNA from the diseased strain to the healthy strain. In this paper we show that the virulence is not related to the diseased state of the strains and that the native hypovirulent strains do not contain dsRNA whereas the virulent strains contain dsRNA viruses.

METHODS

Materials. 5,6-[^{3}H]UTP was purchased from Amersham. The nucleoside triphosphates, polyethylene glycol (mol. wt. 6000), RNase (bovine pancreatic ribonuclease A) and DNase (beef pancreas DNase I) were all obtained from Sigma. Glyoxal (Fisher Scientific, Fairlawn, N.J., U.S.A.) was treated before use with Amberlite Monobed mixed resin MB-3 (BDH). Ultra-pure sucrose was obtained from Bethesda Research Laboratories and CsCl was obtained from Metallgesellschaft (F.R.G.) Restriction endonucleases were obtained from BioLab (St Paul, Min., U.S.A.). The low molecular weight protein standards were from Pharmacia.

Fungal cultures. The strains of *R. solani* used in the study were isolated from soil samples from various locations in Israel according to the procedure described by Boosalis & Scharen (1959). The cultures were maintained and stored both in potato dextrose agar at 24 to 28 °C and in dry soil containing 4% (w/w) wheat bran as described by Butler (1980). The cultures were identified as *R. solani* from their hyphal morphology, by the detection of multinucleate cells following staining with haematoxylin (Parmeter, 1980) and by determination of the anastomosis group (AG) of each isolate using known standard testers (Parmeter *et al.*, 1969) (Table 1).

Pathogenicity tests. The virulence of each strain used in the study was tested by determining its pathogenicity towards six hosts by the plate assay described by Castanho & Butler (1978*b*). The hosts used in these tests were radish (*Raphanus sativus* L.), tomato (*Lycopersicon esculentum* Mill), onion (*Allium cepa* L.), carrot (*Daucus carota* L. var *sativa* DC.), cotton (*Gossypium hirsutum* L.) and bean (*Phaseolus vulgaris* L.). The reliability of the plate assay was tested and confirmed in greenhouse tests and in field experiments.

Extraction of dsRNA. Mycelia grown on agar were blended for 10 to 15 s in a Sorvall Omnimixer in a small volume of distilled water under aseptic conditions and used to inoculate 2 litre flasks, containing 2% glucose, 1% peptone and 1% yeast extract. The cultures were grown for 3 to 5 days at 25 °C on a rotary shaker (New Brunswick Scientific, New Brunswick, N.J., U.S.A.). The mycelium was harvested by filtration, washed with distilled water, squeezed dry and frozen at −20 °C.

Nucleic acids were extracted and characterized essentially as described by Castanho *et al.* (1978), usually from 20 g of mycelium. Each 10 g of frozen mycelium was triturated in 15 ml 0·2 M-glycine, 0·1 M-Na_2HPO_4, 0·6 M-NaCl, 1% SDS (pH 9·5), with 0·1 ml 2-mercaptoethanol and 20 ml water-saturated phenol:chloroform (1:1) at high speed for 5 min in an Omnimixer kept at 4 °C. The homogenate was further processed in a Braun homogenizer for 5 min using 25 g of 0.45 µm glass beads. The homogenate was then returned to the rotary shaker for 90 min. Cell debris was removed by centrifugation at 8000 *g* in a Sorvall SS-34 rotor for 15 min at 4 °C and the aqueous phase was re-extracted with phenol:chloroform (1:1). The nucleic acids were precipitated overnight at −20 °C after addition of 2 vol. ethanol and NaCl to 0·3 M, then pelleted by centrifugation at 12000 *g* for 15 min. This pellet was resuspended in 0·1 M-NaCl, 1 mM-EDTA, 0·05 M-Tris–HCl pH 6·9 (TSE buffer) containing 15% ethanol; dsRNA was separated from the total nucleic acids by chromatography on a CF11 column as described by Franklin (1966). Double-stranded RNA was then precipitated with ethanol (in the presence of 0·3 M-NaCl) and resuspended in the buffer used for electrophoresis (0·04 M-Tris–acetate containing 1 mM-EDTA, pH 7·4).

Characterization of dsRNA. Species of dsRNA were separated by electrophoresis in 4% polyacrylamide slab gels. The gels were stained with 1 µg/ml ethidium bromide for 10 min, viewed with a ChromatoVue Transilluminator (model TM36, UltraViolet Products, San Gabriel, Ca., U.S.A.) and photographed with a Polaroid camera (model MP4) using a Kodak Wratten No. 9 Filter. Nuclease treatments were as described by Castanho *et al.* (1978) except that DNase was used at 4 °C for 10 min.

Isolation and purification of virions. Cultures were grown in 2 litre flasks as described above. In a few experiments a medium containing 2% glucose, 0·2% asparagine and a mixture of salts and trace elements (Weinhold *et al.*, 1969) was used. The mycelium was harvested and washed with lysis buffer containing 50 mM-Tris–HCl, 0·15 M-NaCl, 10 mM-$MgSO_4$, 1 mM-dithiothreitol and 0·1 M-EDTA (pH 7·5) and frozen at −20 °C overnight. Virions were purified essentially as described by Welsh *et al.* (1980). The mycelium was suspended in lysis buffer containing 0·15 M-NaCl and disintegrated as for the extraction of dsRNA. The homogenate was centrifuged for 30 min at 12000 *g* to remove cell debris and the virions were pelleted from the supernatant in a Beckman SW27 rotor for 90 min at 133000 *g*. The pellet was dispersed in lysis buffer containing 0·5 M-NaCl and the virions were precipitated by adding 4% (w/v) polyethylene glycol to the solution. After 1 h at 4 °C the suspension was centrifuged for 10 min at 10000 *g* and the pellet was dispersed in lysis buffer containing 0·6 M-NaCl and 0·5% polyethylene glycol. Following the addition of 0·2% sodium dextran sulphate the virus suspension was incubated for 1 h at 4 °C. The pellet obtained after centrifugation at 4300 *g* for 10 min was resuspended in lysis buffer containing 0.15 M-NaCl and 3 M-KCl (20:3, v/v). After 1 h the dextran sulphate and ribosomes were removed by centrifugation for 10 min at 4300 *g* and the supernatant was dialysed for 2 h against 50 mM-Tris–HCl pH 7·5, 0·1 mM-EDTA, 40 mM-NaCl, 10 mM-$MgSO_4$, 4 mM-2-mercaptoethanol, 10% glycerol (buffer G). The virions were pelleted from the supernatant

by centrifugation at 80000 *g* overnight or at 133000 *g* for 2 h and the pellet was resuspended in buffer G. The virions were further purified by centrifugation in a 10 to 40% sucrose gradient in a SW27 rotor (Beckman) for 4 h at 110000 *g*. Each 1·5 ml fraction was then examined for the presence of virions, for dsRNA and for RNA polymerase activity. The fractions of interest were dialysed for 2 h against a large volume of buffer G and centrifuged. Pellets were resuspended in buffer G lacking glycerol and further purified and characterized by centrifugation in a CsCl density gradient in a SW56 rotor at 18 °C for 45 h at 200000 *g*. The initial density of the CsCl was 1·31 g/ml. Gradients were fractionated (0·3 ml) in an ISCO density gradient fractionator.

Determination of sedimentation coefficient. Virions were resuspended in 0·01 M-Tris–HCl, 1 mM-EDTA, pH 7·5 (TE buffer) and centrifuged in a Beckman Model E ultracentrifuge. The sedimentation coefficient was corrected to $s_{20,w}$ according to Svedberg & Pedersen (1940).

RNA polymerase activity. The conditions for RNA polymerase activity were similar to those defined by Ben-Zvi *et al.* (1984) for *Ustilago maydis* virus. The activities of fractions from the sucrose gradients were tested in the presence of actinomycin D (125 μg/ml) at 30 °C for 2 h.

Characterization of the viral transcripts. Large transcripts were synthesized in reactions incubated for 24 h at 30 °C. Reaction mixtures contained 1 mM of each nucleoside triphosphate and 0·8 mg/ml bentonite to reduce nuclease activity. After incubation, reaction mixtures were extracted with phenol and the nucleic acids were ethanol-precipitated. The products were denatured in glyoxal and characterized by electrophoresis in 1·5% agarose slab gels as described by Carmichael & McMaster (1980).

Characterization of the capsid proteins. Proteins were denatured by heating in 1% SDS, 1% 2-mercaptoethanol, 62·5 mM-Tris–HCl, 2 mM-EDTA, 10% glycerol and 0·05% bromophenol blue (pH 6·8) at 100 °C for 3 min. The proteins were characterized by electrophoresis in 12·5% polyacrylamide slab gels containing 0·05% SDS with a stacking gel of 6% polyacrylamide, at 150 V in Tris–glycine buffer (Laemmli, 1970) containing 0·1% (w/v) SDS. The gels were stained with 0·1% Coomassie Brilliant Blue R in acetic acid:methanol:water (1:5:4, by vol.).

Electron microscopy. Grids were coated with 0·15% Formvar and carbon. Virions were stained with 2% uranyl acetate and examined in a JEOL 100B electron microscope.

RESULTS

Detection of double-stranded RNA in R. solani

Strains of *R. solani* were isolated from a range of diverse ecological niches, in various parts of Israel. Of the eight strains selected for study, four were identified as virulent and four as hypovirulent. The degree of virulence was determined by pathogenicity tests (Table 1). Virulent and hypovirulent strains could not be distinguished morphologically or by differences in their growth rates. The strains selected for the study were healthy in appearance and all grew at the same rate. Examination of the dsRNA content of the fungal mycelia revealed a clear distinction

Table 1. *Host range and disease severity index of strains of* R. solani

		Disease severity index*					
Strain no.	AG	R	T	C	B	Co	O
Virulent							
82	4	5·00 ± 0·00‡	1·00 ± 0·00	5·00 ± 0·00	5·00 ± 0·00	2·00 ± 0·00	4·80 ± 0·09
13	4	5·00 ± 0·00	5·00 ± 0·00	5·00 ± 0·00	3·00 ± 0·00	5·00 ± 0·00	5·00 ± 0·00
56	4	5·00 ± 0·00	5·00 ± 0·00	4·00 ± 0·00	2·00 ± 0·00	5·00 ± 0·00	5·00 ± 0·00
53	4	5·00 ± 0·00	5·00 ± 0·00	5·00 ± 0·00	5·00 ± 0·00	5·00 ± 0·00	0·00
189HT†	1	5·00 ± 0·00	5·00 ± 0·09	4·01 ± 0·09	2·00 ± 0·00	1·00 ± 0·00	0·00
Hypovirulent							
160	2	1·00 ± 0·00	0·00	0·00	0·00	0·00	0·00
521	4	0·70 ± 0·03	0·00	0·00	0·00	0·00	0·00
558	4	1·00 ± 0·00	0·00	2·00 ± 0·00	1·00 ± 0·00	0·00	1·00 ± 0·00
751	1	2·00 ± 0·00	1·09 ± 0·03	1·00 ± 0·00	1·00 ± 0·00	0·00	0·00
189at†	1	0·38 ± 0·01	0·00	0·00	0·00	0·00	0·00

* Virulence ratings were based on the infected area of hypocotyl of seedlings. The results with six hosts are presented (R, radish; T, tomato; C, carrot; Co, cotton; B, bean; O, onion). Tests conducted with five additional hosts gave similar results without any exception. The scale of 1 to 5 refers to the area of infection: 0, no detectable infection; 1, 1 to 10%; 2, 11 to 30%; 3, 31 to 50%; 51 to 80%; 5, over 80%.

† Strains kindly sent by Dr E. E. Butler, Department of Plant Pathology, University of California, Davis, Ca., U.S.A.

‡ Standard error; n = 45.

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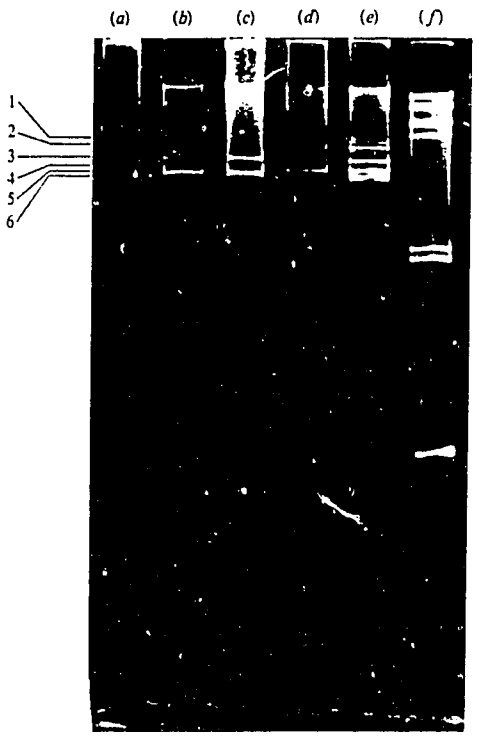

Fig. 1. Polyacrylamide gel electrophoresis of dsRNA isolated from strains of *R. solani*: (*a*) 53 hypovirulent obtained by hyphal tip isolation from 53, (*b*) 53, (*c*) 56, (*d*) 82 hypovirulent obtained by hyphal tip isolation from 82 and (*e*) 82. (*f*) *Ustilago maydis* P4 strain dsRNA (Field *et al.*, 1983). The mol. wt. ($\times 10^6$) of segments marked 1 to 6 are 1·7, 1·6, 1·45, 1·32, 1·25 and 1·225.

between the two groups: dsRNA could not be recovered from those strains which were classified as hypovirulent (no. 160, 521, 558, 751), but was detected by the same procedure in strains that were classified as virulent (no. 13, 53, 56, 82) (Fig. 1).

Since a segmented genome is typical of dsRNA viruses of fungi, it was essential to ascertain precisely the pattern of segmentation obtained in each case and to exclude misleading artefacts. In the course of purification of the dsRNA from the various isolates of *R. solani*, some high molecular weight bands were noticed in polyacrylamide gels even after the recovery of the nucleic acids from CF11 columns, which were specifically designed to separate the replicative and non-replicative forms of ssRNA in viruses. The nucleic acids, recovered from the CF11 column and expected to contain dsRNA, were subjected to various tests to ascertain the nature of these molecules. The samples were exposed to RNase at high and low ionic strength, or treated with DNase or with a series of restriction endonucleases (*Bam*HI, *Hin*dIII and *Sal*I).

The results indicated that the components noticed often in the upper portion of the polyacrylamide gels, with an estimated molecular weight of 3×10^6 to 7×10^6, were not RNA and consisted of a heterogeneous population of molecules. The heterogeneity of this component was readily noticed in 1% agarose gels, in which it did not form a discrete band, and also in the electron microscope this component was seen as a heterogeneous group of molecules. Furthermore, this component was unaffected by RNase at high ionic strength, was slightly sensitive to treatment at low ionic strength and was highly sensitive to digestion with DNase.

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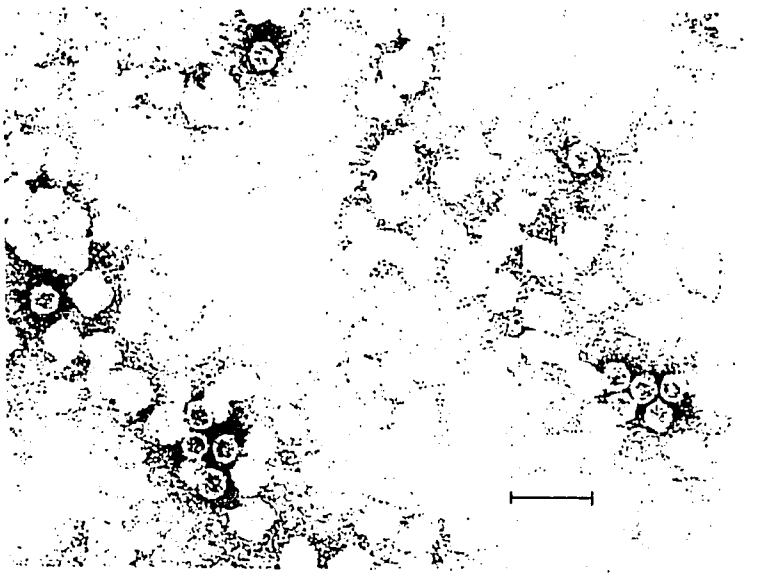


Fig. 2. Electron micrograph of negatively stained *R. solani* strain 13 virus particles purified in a CsCl density gradient. Bar marker represents 100 nm.

Digestion with the restriction enzymes resulted in a non-specific digestion of these molecules; this further confirmed the heterogeneity of this component.

Among the segments that clearly behaved as dsRNA, two distinct and consistent patterns were identified. Pattern A, found in strains 53 and 82, was of three distinct dsRNA segments with molecular weights of $1{\cdot}6 \times 10^6$, $1{\cdot}45 \times 10^6$ and $1{\cdot}25 \times 10^6$. Pattern B, found in strains 13 and 56, was of two distinct segments of dsRNA with molecular weights of $1{\cdot}45 \times 10^6$ and $1{\cdot}25 \times 10^6$. These patterns were seen both in polyacrylamide and agarose gels. However, when tested in polyacrylamide gels, pattern A dsRNA also contained minor bands with molecular weights of $1{\cdot}7 \times 10^6$, $1{\cdot}325 \times 10^6$ and $1{\cdot}225 \times 10^6$.

Characterization of virus particles

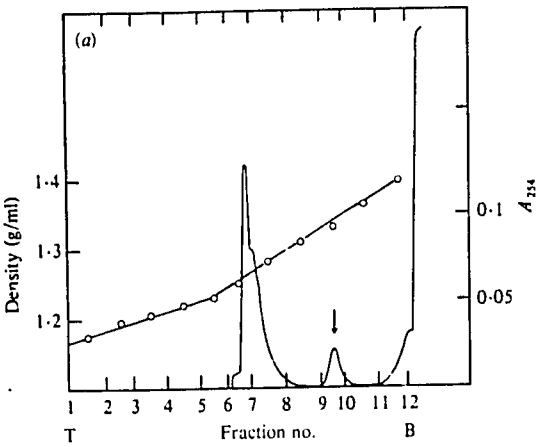
Sedimentation in sucrose gradients

Using the procedure for the isolation of virions, sucrose gradient fractions of virulent strains, but not hypovirulent strains, were found to contain virus-like isometric particles with a diameter of 30 to 35 nm (Fig. 2). Most of the dsRNA extractable from fractions from the sucrose gradients was in the fractions that contained virions (Fig. 5*b*). The dsRNA was resolved by polyacrylamide gel electrophoresis into segments having the same molecular weights, those of dsRNA extracted directly from the mycelium. However, in most preparations only two segments of dsRNA (mol. wt. $1{\cdot}45 \times 10^6$ and $1{\cdot}25 \times 10^6$) were extracted from virions purified from isolates giving dsRNA of pattern A.

The sedimentation coefficient was determined using a virus preparation which had been centrifuged in two successive sucrose gradients. A value of 161S $\pm$ 4S was obtained.

Centrifugation in CsCl density gradients

Further purification of the virions was obtained by centrifugation in CsCl density gradients. Two components were obtained which had densities of 1·34 g/ml and 1·29 g/ml (Fig. 3*a*). Virus particles were detected by electron microscopy in both peaks but RNA extraction yielded



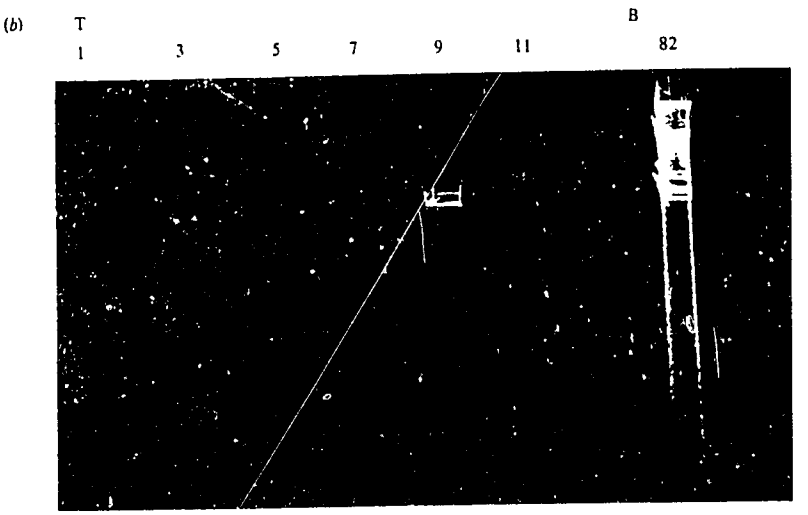


Fig. 3. (*a*) CsCl density profile (T, top; B, bottom) of *R. solani* virus from strain 13. Centrifugation was for 45 h at 18 °C and 200000 *g*. (*b*) dsRNA distribution along CsCl density gradient: 0·05 ml samples of each fraction were extracted with phenol:chloroform (1:1), precipitated with ethanol at −20 °C and electrophoresed in a 4% polyacrylamide gel. 82 is RNA as in Fig. 1(*e*).

dsRNA only from the more dense component (Fig. 3*b*). It is quite likely that the less dense peak consists of empty virions, and that as reported for many fungal viruses (Bozarth, 1979), these comprise a large proportion of the purified virions.

Particle protein

The protein content of virions purified by centrifugation in a CsCl density gradient was examined by SDS-PAGE (Fig. 4). A single protein with mol. wt. 46000 was detected in both density components. These results suggest that the lower density component consisted of empty capsids. It contained, in addition to the major protein, two other minor proteins. The results of translation experiments *in vitro* (A. Finkler, B. Ben-Zvi & Y. Koltin, unpublished results) suggest that these additional proteins are contaminants.

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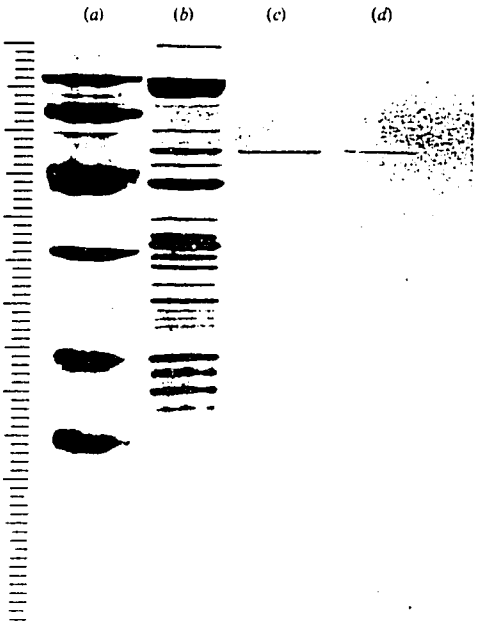

Fig. 4. SDS-PAGE of viral proteins purified from *R. solani* strain 56. (*a*) Low mol. wt. marker proteins of 94000, 67000, 43000, 30000, 20000 and 14000. (*b*) Protein from virus purified by sedimentation in a sucrose gradient. (*c*) Protein from virus forming the light component (Fig. 3) after centrifugation in a CsCl density gradient. (*d*) As (*c*), but material from the heavy component.

RNA polymerase activity

Virus-associated RNA-dependent RNA polymerase activity has been found in most of the fungal viruses in which this enzyme activity has been sought (Ben-Zvi *et al.*, 1984; Buck, 1975, 1979; Buck *et al.*, 1981; Ratti & Buck, 1979). Therefore, this polymerase activity was used to test for the presence of virions among the fractions of a 10 to 40% sucrose gradient. Maximum activity of the RNA polymerase coincided with the position of those fractions containing dsRNA (Fig. 5*a*, *b*) which, as shown earlier, were those containing virus particles. Some dsRNA was extracted from the bottom fraction of the gradient, presumably because some virions had aggregated and pelleted, but that fraction lacked polymerase activity. Similar results were obtained with virions purified in a second sucrose gradient. No polymerase activity was found in similar tests with extracts from hypovirulent strains. This result, as well as the absence of dsRNA and virus particles, suggests that these strains lack virions. A linear relationship was observed between RNA polymerase activity and virus concentration up to 2 mg/ml (Fig. 6). Thus, the virion-associated RNA-dependent RNA polymerase activity that is characteristic of many fungal viruses is also found in the virus particles isolated from *R. solani* and can be used to monitor the isolation of these viruses.

During the examination of the fractionated sucrose gradients it became clear that the high molecular weight components, shown earlier to be heterogeneous DNAs, were not associated in any way with the polymerase activity. This component did not band clearly in the sucrose gradients and appeared in many fractions along the gradient in which there was no polymerase activity. Furthermore, in the CsCl density gradient this fraction did not band with the dsRNA.

When RNA polymerase reactions were incubated for 24 h in the presence of bentonite, the reaction did not reach a plateau. The products of the long reaction were characterized initially

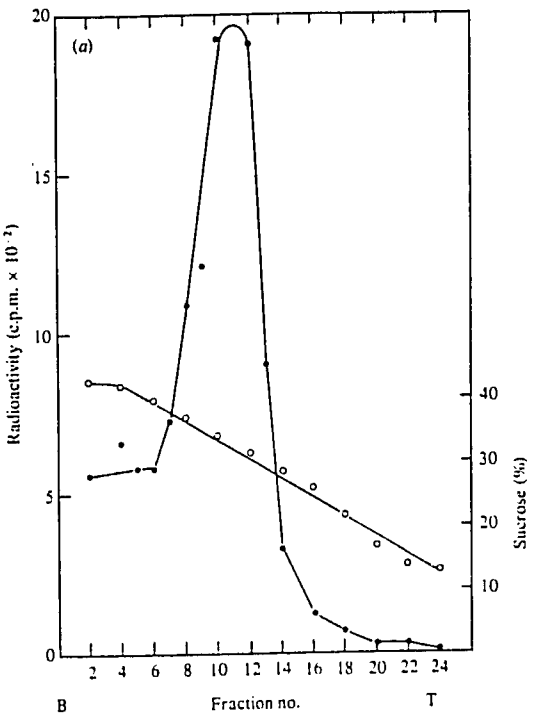

Fig. 5. (*a*) Distribution of RNA polymerase activity of *R. solani* strain 56 virus in fractions of a 10 to 40% sucrose gradient after centrifugation for 4 h at 110000 *g* at 4°C. ●, Polymerase activity; ○, sucrose concentration. (*b*) Electrophoresis of virion dsRNA extracted from fractions of the sucrose gradient. Samples (0·3 ml) were extracted with phenol and analysed on 4% polyacrylamide gels. 56 is RNA as in Fig. 1(*c*).

by CF11 column chromatography, used for separation of ssRNA from dsRNA (Franklin, 1966). It appeared that 87% of the labelled UTP was incorporated into ssRNA that was eluted from the column with 15% ethanol. The dsRNA that was eluted with TSE buffer without ethanol contained approximately 13% of the labelled UTP incorporated during the polymerase reaction.

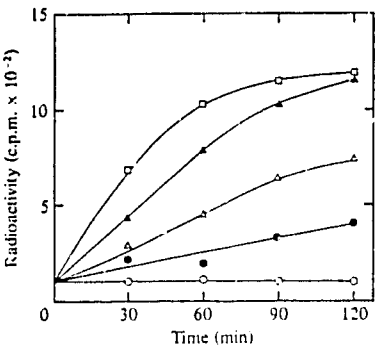

Fig. 6

Fig. 7

Fig. 6. Dependence of the RNA polymerase reaction on the concentration of virions. At the indicated times, 5 μl samples were tested for TCA-precipitable [^{3}H]UTP (sp. act. 190 c.p.m./pmol). A virus preparation was purified from *R. solani* strain 12 by sedimentation in a sucrose gradient and used at the following concentrations: ○, 225 μg/ml; ●, 450 μg/ml; △, 1120 μg/ml; ▲, 2230 μg/ml; □, 2800 μg/ml.

Fig. 7. Polyacrylamide gel containing dsRNA isolated from the virulent donor strain (82) and the recipient strain (521) 'transformed' to virulence. (*a*, *b*) dsRNA from the two strains 'transformed' to virulence. (*c*) dsRNA from the virulent donor strain. (*d*) dsRNA mol. wt. markers (*U. maydis* P4 virus).

These results suggest that the major product of the polymerase activity is ssRNA and that the RNA-dependent RNA polymerase activity associated with the virions represents transcription activity. The radioactivity associated with the dsRNA may result from incomplete transcripts synthesized on a dsRNA template.

The molecular weight of the newly synthesized transcripts was estimated by gel electrophoresis in agarose after treatment of the RNA with glyoxal. One band of mol. wt. 0·75 × 10^6 was obtained, although it is possible that more than one transcript was present but difficult to resolve.

Conversion of virulent strains to hypovirulence

Attenuation of virulence was obtained by hyphal tip isolation from virulent strains (no. 82 and 53). A hypovirulent strain was recovered from each of the virulent strains. The frequency of recovery of the hypovirulent cultures from both strains was about 1%. No virulent strains were isolated from hypovirulent strains. To determine whether there is any correlation between virulence and an alteration of the dsRNA content of these strains, the dsRNA was extracted and analysed in polyacrylamide gels (Fig. 1*d*, *a*). The analysis clearly indicated a change in the hypovirulent clones derived from the virulent strains. The clone derived from strain 82 had lost two dsRNA segments (mol. wt. 1·25 × 10^6 and 1·225 × 10^6) which the virulent strain 82 initially possessed, whereas the clone derived from strain 53 had lost all the major segments of dsRNA. The faint bands seen in Fig. 1(*b*) were not observed in agarose gels.

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Co-transmission of virulence and dsRNA

To determine whether the cytoplasmic transmission of dsRNA coincides with the transmission of virulence, a classical heterokaryon transfer experiment was performed (Jinks, 1964). A virulent strain (no. 82) and a hypovirulent strain (no. 521), of the same incompatibility group (anastomosis group, AG 4), were selected based on their differential sensitivity to different fungicides. The virulent strain was resistant to Benomyl at a concentration of 2·5 μg/ml, but sensitive to BTN (BAY NTN 19701, Mobay Chemicals, U.S.A.). The hypovirulent strain (521) was resistant to BTN at a concentration of 75 μg/ml but sensitive to Benomyl. The differential sensitivity permits identification of the two strains and selection of the parental strains. Both agents affect the microtubules, and nuclear resistance to Benomyl has been reported in a number of fungi (Sheir-Ness *et al.*, 1978; Neff *et al.*, 1983). In addition to the resistance to the fungicides, the strains differed in the synthesis of melanin and production of sclerotia. The virulent strain contained dsRNA viruses whereas the hypovirulent strain did not.

Two methods were used for sampling hyphae after interaction between the two strains. In the first method the strains were confronted in a non-selective medium and allowed to interact for 48 h. Samples were withdrawn from the line of confrontation and plated on selective medium in which only the hypovirulent strain could grow. In the second method the hypovirulent strain was inoculated in non-selective medium in the centre of the plate and allowed to grow in optimum conditions until two-thirds of the plate area was covered by this strain. The virulent strain was then inoculated into the centre of the plate, thus establishing immediate contact with the mycelium of the hypovirulent strain. Hyphal tips were sampled from the periphery of the hypovirulent mycelium after 48 h, grown in non-selective medium and then tested for resistance to the fungicides and for virulence. The advantage of this method is that the area from which the hyphal tips are isolated is at a distance from the growing cells of the virulent strain, as can be determined by measuring the radial growth in control plates. Thus, the probability of recovering only the hypovirulent strain is very high using this method.

Of 25 colonies, obtained in both methods, two virulent colonies were identified with all the markers typical of the hypovirulent strain (no. 521). These cultures derived from hyphal tips were resistant to BTN, sensitive to Benomyl, did not secrete melanin and produced tiny hyaline sclerotia typical of the hypovirulent strain. Yet, in tests of virulence both were virulent in the plate assay. When the new 'transformed' virulent strains were tested for the presence of dsRNA (the parental hypovirulent strain was devoid of dsRNA), they were found to contain it. The pattern of segmentation recovered was typical of the dsRNA segments contained in viruses recovered from the virulent strain (no. 82) (Fig. 7).

DISCUSSION

The results indicate the presence of viruses in virulent strains of *R. solani*. These particles were 30 to 35 nm in diameter and, like many other fungal viruses, contain a dsRNA genome. Segments of mol. wt. $1{\cdot}45 \times 10^6$ and $1{\cdot}25 \times 10^6$ were extracted from virion preparations. It is impossible as yet to determine whether these are separately encapsidated. A RNA-dependent RNA polymerase is also associated with the virus particles. Hypovirulent strains, however, are devoid of dsRNA and virus particles.

In the course of this study a dsRNA segment with a molecular weight of $1{\cdot}6 \times 10^6$ has been extracted from the mycelia of a number of virulent strains. This segment has not been detected in the virions. It is unclear whether the $1{\cdot}6 \times 10^6$ mol. wt. segment is either not encapsidated or is much less abundant in particles than are the $1{\cdot}45 \times 10^6$ and $1{\cdot}25 \times 10^6$ mol. wt. species. The involvement of naked dsRNA in suppressing virulence in *Endothia parasitica* has been described by Van Alten & Hansen (1983). It is also possible that the $1{\cdot}6 \times 10^6$ mol. wt. segment belongs to a different virus lost during the isolation procedure. Mixed infections by two or more viruses are common in fungi.

The results of the attenuation experiments, altering virulent strains to hypovirulence, in which a loss of specific dsRNA segments (mol. wt. $1{\cdot}25 \times 10^6$ and $1{\cdot}225 \times 10^6$) from strain no. 82, and the loss of the major segments of dsRNA in strain no. 53 were detected, suggest that the

dsRNA viruses code for the information which produces or regulates the virulence. This idea is further supported by the simultaneous cytoplasmic transmission of virulence and dsRNA from a virulent to a hypovirulent strain. Thus, corrective evidence that a genetic element determining virulence is transmissible with dsRNA is provided. A recent report (Hashiba *et al.*, 1984) correlates the presence of a small DNA plasmid with hypovirulence in *R. solani*. However, the characterization of the nucleic acid as DNA is incomplete and the strains studied were debilitated and very different from the hypovirulent strains used in the present study. Another report (Martini *et al.*, 1978) claims that determinants of virulence in *R. solani* lie on a large DNA plasmid. We have also found a DNA component (mol. wt. 3×10^6 to 7×10^6) but it was present in both virulent and hypovirulent strains and was not associated with viral particles. Whereas more than one factor may be involved in regulation of virulence, final proof of the role of each will only be obtained by infection experiments with purified virus and by transformation experiments in the case of a DNA plasmid.

The results presented are contradictory to those published earlier by Castanho *et al.* (1978). The differences may stem from the fact that in the present study the virulent and hypovirulent strains used were healthy whereas in the earlier study the hypovirulent strain was diseased. Castanho *et al.* (1978) have claimed the transmission of the diseased state which they referred to as hypovirulence. It is clear from the present study that the diseased state cannot be equated with hypovirulence and that the degree of virulence of the so-called hypovirulent strain may have been masked by the diseased state. This is strengthened by experiments in our laboratory in which virulent cultures were derived from Castanho's hypovirulent strain, yet we were unable to detect virulent cultures among hundreds of hyphal tip isolates from a number of hypovirulent strains used in our study.

Castanho *et al.* (1978) identified three segments of dsRNA in the diseased strain and only occasionally were traces found in the healthy virulent strain derived from the diseased strain. Re-examination of these strains in our laboratory confirmed the findings with respect to the diseased strain. However, dsRNA was also detected in the virulent strain. The molecular weights of the segments were identical to those found in the virulent strains in the present study. The occurrence of dsRNA in the mycelia of virulent strains is also supported by recent findings of Zanzinger *et al.* (1984). However, these authors reported the isolation of dsRNA from a hypovirulent strain but their criteria are similar to those of Castanho *et al.* (1978), namely slow growth.

A more relevant comparison is between our findings in *R. solani* and those reported in *E. parasitica* (Van Alfen, 1982). The dsRNA found in hypovirulent strains of this pathogenic fungus is thought to suppress virulence. However, viewing the dsRNA viruses as adaptive factors, it is conceivable that in some forms the information may be suppressive whereas in others it may induce virulence. Available information from other dsRNA viruses of fungi (Tipper & Bostian, 1984; Wigderson & Koltin, 1982) suggests that even within one species some molecules may act in the expression of functions encoded by the dsRNA and other molecules may act as eliminators of dsRNA segments encoding these functions. The nature of the dsRNA-encoded information in pathogenic fungi should provide an insight on the regulation of virulence.

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Double-stranded RNA viruses of pathogenic fungi: virulence and plant protection

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Introduction

Two decades have passed since viruses were first isolated from a fungus (Kleinschmidt & Ellis, 1967). Viruses are now known to occur in many fungi including some 40 different species that are plant pathogens (Day & Dodds, 1979). The genetics, molecular biology and physical properties of these viruses have been described in detail by Lemke (1979) and more recently by Buck (1986). The information to date suggests that only in a very few cases do the carriers of these viruses display a unique phenotype that can be related to the viruses (Bevan, Herring & Mitchell, 1973; Wood & Bozarth, 1973; Castanho, Butler & Shepherd, 1978; Hollings, 1978; Ghabrial & Mernaugh, 1983). Rarely do phenotypes conferred by the viruses contribute a selective advantage to the carrier. The complexity of their organisation and maintenance (Bruenn & Brennan, 1980; Buck *et al.*, 1981*a*, 1981*c*; Welsh & Leibowitz, 1982; Field *et al.*, 1983) suggests that some selective forces must operate to maintain them.

A segmented genome of double-stranded RNA typifies the fungal viruses (Bozarth, 1979; Bozarth *et al.*, 1981). The segments are separately encapsidated, each with its unique information but with an antigenically identical coat, and an RNA-dependent RNA polymerase is associated with the viral coats. The general features of the system are thus similar to those of a major virus with a number of satellite viruses. No proviral state has yet been detected (Bruenn, 1980; Ben-Zvi *et al.*, 1984). Transmission of the viruses is through cytoplasmic exchange between compatible mating types of one species. Artificial infection methods have been devised very recently (Stanway & Buck, 1984).

Genetic instability of various characters in fungi, at a rate exceeding

that attributable to gene mutations, has been reported for many years. Some of the unstable characters are related to sporulation, morphology, synthesis of secondary metabolites and virulence (Jinks, 1959; Grindle, 1964). As the factors related to genetic instability in prokaryotes and eukaryotes are resolved, and the role of plasmids and transposable elements is recognised (Shapiro, 1983), one overriding fact that emerges in consideration of fungal instability is that DNA plasmids are not abundant. In fungi the extent of instability related to transpositions is as yet unclear (Esser *et al.*, 1983; Roeder & Fink, 1983) but the abundance of dsRNA viruses suggests these factors are a much more significant source of instability. Genetic drift in RNA viruses has been documented both in molecular terms and in epidemiological outbreaks (Ortino *et al.*, 1980; Sugiyama, Bishop & Roy, 1981; Holland *et al.*, 1982). It seems that in the absence of an RNA repair mechanism, and lacking a DNA proviral state, such viruses show a high frequency of genetic alterations and a strong genetic drift which may manifest itself as instability in the host.

Phenotype expression in the fungal host resulting from viral infection includes the secretion of a toxin (Puhalla, 1968; Day & Dodds, 1979), degeneration of fungal cells (Castanho, Butler & Shepherd, 1978; Hollings, 1978; Ghabrial & Mernaugh, 1983) and, in one instance, the attenuation of virulence (Van Alfen, 1982). The latter findings are the first in which a positive selection is indicated and an adaptive advantage to the fungal host is conferred by the dsRNA virus. They suggest an explanation for the rapid establishment of equilibrium between the host and pathogen that was recorded for the epidemic of the chestnut blight caused by the pathogen *Endothia parasitica* (Grente & Sauret, 1969; Van Alfen *et al.*, 1975).

Although dsRNA viruses have been detected in many plant pathogens, in only a few has there been any examination of the relationship between virulence and the virus. Results have been often confusing. In some, the complexity of the system resulting from the occurrence of multiple viral infections, such as in the causative agent of the take-all disease, precluded a clear analysis (Rawlinson *et al.*, 1973). In others, incomplete characterisation of the viruses and their genome organisation made the analysis premature. For example, both diseased and normal strains of *Helminthosporium victoriae*, the causal agent of Victoria blight of oats, contain virus-like particles (Ghabrial, Sanderlin & Calvert, 1979), as do toxin and nontoxin producing strains of *Periconia circinata* (Dunkle, 1974). In addition, the multicomponent problem, i.e. the involvement of the fungal

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pathogen, viruses of the fungal pathogen and the host plant, all possibly interacting genetically, has led to premature conclusions in various studies. Also, since the definition of virulence may be subjective unless well defined criteria are used, interpretation of the data can be very confusing.

Generalisations cannot be made about the relationship of fungal dsRNA viruses and virulence and the range of effects expressed. The adaptation by attenuation of virulence in *E. parasitica*, described above which led to the restoration of host-pathogen equilibrium and permitted new planting of the chestnut in Italy, is only one form of expression. In addition, the viruses may act also as inducers of virulence, in a way similar to that of bacterial plasmids (Elwell & Shipley, 1980). Other forms of expression not recognised as relevant to the regulation of virulence may be applied to combat various plant pathogens. To illustrate these possibilities, studies of the regulation of virulence by a dsRNA virus, and the control of plant pathogens by a virus-encoded product, this chapter describes relationships between two pathogenic fungi *Rhizoctonia solani* and *Ustilago maydis*, and their viruses.

Rhizoctonia solani

Early studies and the occurrence of hypovirulent strains

Rhizoctonia solani causes damping off in some 130 different plant species (Baker, 1970; Castanho & Butler, 1978) and is found in most agricultural soils. Using debilitated strains assumed to be hypovirulent, Castanho, Butler & Shepherd (1978) reported the occurrence of attenuation, similar to that in *E. parasitica*. A survey of a natural population in Israel to determine the degree of virulence, defined by the effect on 11 host species, revealed that the population consisted of a mixture of virulent strains causing damage of varying degree of severity to one or more hosts and of avirulent strains that caused no discernible damage to any host (Ichielevich-Auster *et al.*, 1985*b*). Of 107 isolates, 32 were avirulent and confirmed as *R. solani* by compatibility tests with anastomosis group tester strains. These strains are referred to as hypovirulent strains.

Double-stranded RNA was detected in mycelia of the hypovirulent strains but was not found in any of virulent strains. Virus particles were not found in either the virulent or the hypovirulent strains. Debilitated growth was transmitted to a virulent strain via cytoplasmic exchange along with the dsRNA found in the hypovirulent strain. These early studies led to the conclusion that the properties of the hypovirulent strains of *R. solani* were similar to those of hypovirulent strains of *E. parasitica*.

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However, the debilitated strains persisted in the soil for less than one month and the precise relation between debilitated growth and dsRNA, or any other cytoplasmic factor, was unclear. The reason was that, as shown in later studies, growth rate and hypovirulence are independent factors. Furthermore, if hypovirulence represents a state of adaptation with a positive selection value it should be anticipated that hypovirulent strains would not be debilitated. Also, one of the strains originally claimed to be virulent and devoid of dsRNA (Castanho, Butler & Shepherd, 1978) was re-examined recently and found to contain dsRNA (A. Finkler and Y. Koltin, unpublished).

A later study (Zanzinger, Bandy & Tavantzis, 1984) reported the occurrence of dsRNA in both virulent and hypovirulent strains but virulence was defined by the use of one host, thus nonstringent criteria were adopted that precluded precise characterisation of the level of virulence of each of the strains used. A DNA plasmid was also reported recently in *R. solani* with the implication that this plasmid is related to virulence (Hashiba *et al.*, 1984). However, the latter study also failed to define stringent criteria to distinguish between virulent and hypovirulent strains. In both studies the cytoplasmic transmission of virulence or attenuation of virulence was not tested and the effect of curing strains of the cytoplasmic factors on the degree of virulence of the fungal host was not examined.

Virulence and the dsRNA viruses

To approach the question of whether phenotypic expression of the dsRNA viruses affects virulence in *R. solani*, highly virulent and avirulent strains were examined for the presence of viruses. The strains examined were isolated from various locations representing a range of ecological niches and seven anastomosis groups. The relation of growth rate to the degree of virulence was also examined, since it is possible that slow-growing strains may be less virulent if their virulence is only effective over a short range, for example, if it depends on enzymatic degradation rather than toxic effect. The range in growth rate of all the strains was found to be similar. None of the strains was debilitated and those identified as avirulent caused no discernible damage such as damping off or growth inhibition to the 11 different species used as indicators of virulence. The virulent strains caused severe damage to all 11 hosts.

Nine strains were used to examine the relation of the dsRNA to virulence (Finkler *et al.*, 1985). Only the virulent strains contained viruses; neither viruses nor dsRNA were recovered from the avirulent strains.

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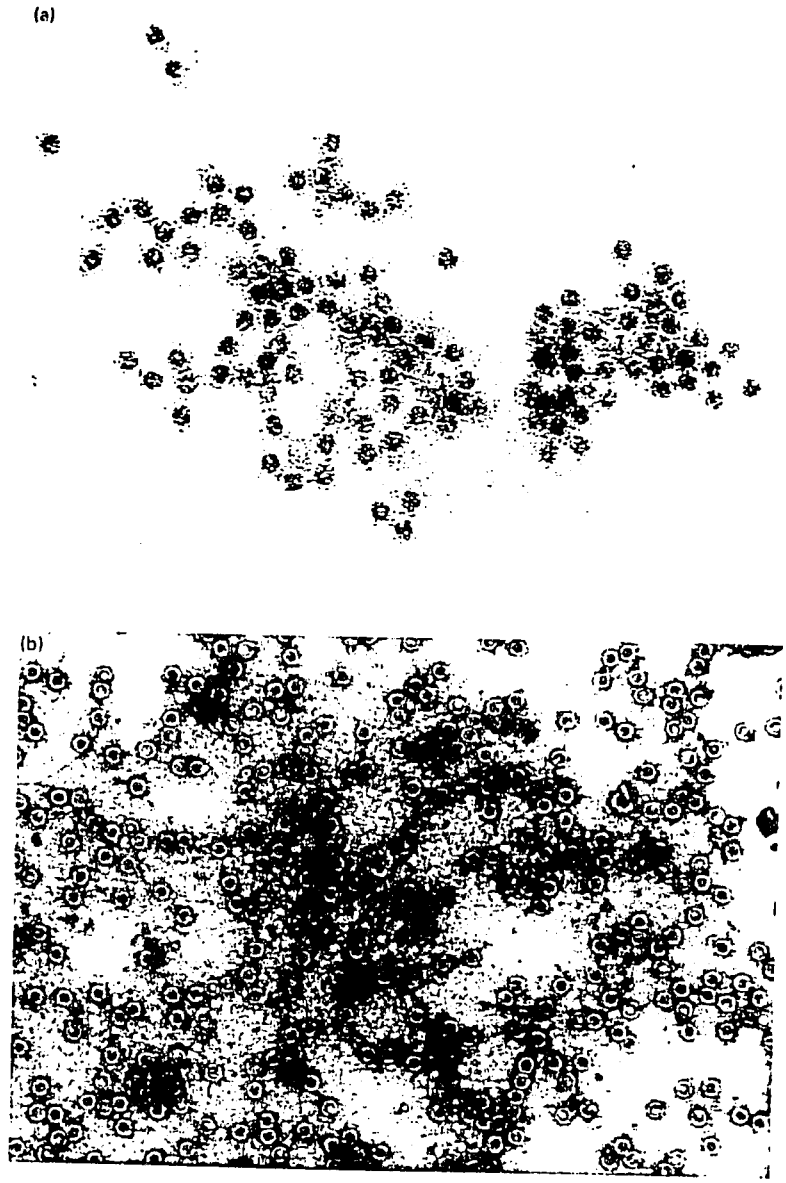

Fig. 17.1. Electron micrograph of negatively stained virus particles: (a) 30–35 nm in diameter, purified on sucrose density gradients, from a virulent strain of *Rhizoctonia solani*, (b) 41nm in diameter recovered from a killer strain of *Ustilago maydis*.

The viruses were 30–35nm in diameter (Fig. 17.1a) and contained two segments of dsRNA with molecular weights of 1.45 and 1.32 × 10^6 (Fig. 17.2). The virus particles from different strains seemed to be interrelated. Their coat protein was of a similar size, 55K, and viruses from different strains were immunoprecipitated by antibodies derived against one of the isolates. Furthermore, as in all other dsRNA viruses, an RNA polymerase acting as a transcriptase was coat-associated. *In vitro* transcription reactions have been defined for the *R. solani* virus and for a number of other fungal viruses (Buck, 1979; Bruenn *et al.*, 1980; Buck *et al.*, 1981*b*; Ben-Zvi *et al.*, 1984).

Viral transcripts were obtained *in vitro* using virus particles supplemented with the four ribonucleotide triphosphates. The RNA-directed

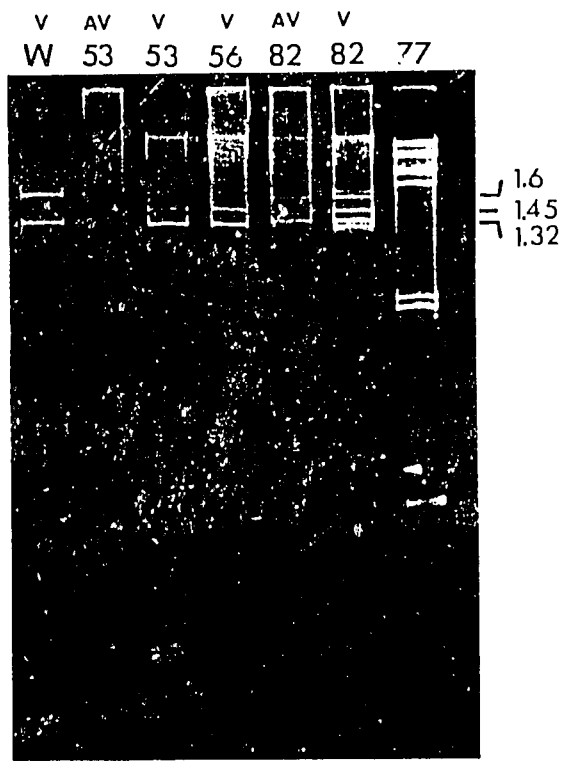

Fig. 17.2. Polyacrylamide gel electrophoresis of unique segments of dsRNA isolated from an *Ustilago maydis* killer strain (right lane) and from virulent strains of *R. solani* (other lanes). The dsRNA was stained with ethidium bromide and viewed with UV. All extracted strains contained virus particles.

RNA polymerase activity, which is currently a routine assay to monitor the viruses in purification procedures, was tested in hypovirulent strains but no traces were detected. The transcripts obtained were used in various studies with the fungal viruses for the resolution of viral-encoded proteins and also as a probe to resolve the relatedness of the viruses, since such probes avoid artefacts introduced by impurities of ribosomal RNA adhered to the dsRNA. Using such probes under stringent conditions (65°C, 0.6M NaCl, 60mM trisodium citrate) cross hybridisation between these probes and the viral dsRNA of various isolates revealed that all the viruses isolated from the virulent strains are interrelated. In addition, no variation of coat proteins from the viruses or in the products of *in vitro* translated viral transcript products could be resolved by fingerprinting using *Staphylococcus aureus* V8 protease (A. Finkler, B-S. Ben-Zvi & Y. Koltin, unpublished).

Virus curing and virulence

Since only virulent strains contained viruses, the question that emerged was how will curing the strains of dsRNA viruses affect the expression of virulence? However, since there are no practical, direct experimental approaches to distinguish between carriers and virus-free descendants, the emphasis was placed on the recovery of avirulent subcultures and the examination of these for the occurrence of a specific pattern of dsRNA.

Among subcultures of hyphal tips from virulent strains, about 1% of the new cultures were avirulent. Virulence was never recovered in subcultures from avirulent strains. Therefore, a clear unidirectional relationship was established and the high incidence of phenotypic alteration suggested that a loss of a cytoplasmic factor in the virulent strains led to a reduced level of virulence. In one avirulent subculture a loss of the dsRNA was noticed, while in another the loss of a specific segment of the viral information was noticed. To confirm further that dsRNA may be associated with virulence, additional evidence was sought.

Conversion of hypovirulent strains

The conversion of a hypovirulent strain to a virulent strain by the introduction of dsRNA viruses was attempted. The major difficulty was that infective methods are available for only one fungal virus and methods have not yet been devised for *R. solani*. Therefore, the natural mode of transmission of the fungal viruses was adopted, that is via cytoplasmic exchange in heterokaryons. The drawback of such a method

is the lack of control over the cytoplasmic transmission of various cytoplasmic elements. Techniques might be refined if restriction polymorphism could be found in the mitochondrial DNA of the interacting strains. However, with the need to use genetically identifiable strains it is difficult to meet all the required conditions and so our experiments were based on cytoplasmic exchange using strains that could be identified and selected in defined conditions.

To test for the cytoplasmic nature of the agents associated with virulence, the classical heterokaryon transfer experiment was performed (Jinks, 1969). A virulent strain and an avirulent strain of the same compatibility group (Parmeter, Sherwood & Platt, 1969) were selected on the basis of their differential sensitivity to the fungicides Benomyl and Bay NTN, 19701 (N-[(4-chlorophenyl)methyl]-N-cyclopentyl-N′-phenylurea, Mobay Chemicals, USA). Differential sensitivity permits the identification of each of the parental strains and the dissociation of the heterokaryon. In addition to the differential resistance to two fungicides, the strains differed in a number of other morphological characteristics, such as a distinguishable difference in growth rate and the synthesis of melanin in culture. The virulent strain contained dsRNA viruses (Finkler *et al.*, 1985) whereas the avirulent strain contained neither the viruses nor dsRNA.

Two methods were used for sampling cells after conjugation between the two strains. In the first, strains were confronted while growing on nonselective medium and were allowed to interact 24 or 48 h. Mycelium was taken from the line of confrontation and plated on medium that selected for the avirulent strain. In the second, the hypovirulent strain was inoculated on nonselective medium and allowed to grow in optimal conditions until two-thirds of the plate area was colonised. The virulent strain was then inoculated in the centre of the plate, thus establishing immediate contact with the mycelium of the avirulent strain. Hyphal tips were sampled from the periphery of the avirulent mycelium after 24 and 48h, grown in nonselective medium and then tested for resistance to the fungicides, for morphological characteristics and for virulence. The advantage of this method is that the area from which the hyphal tips were isolated was at a distance from the growing points of the virulent strains and thus the probability of recovering only the avirulent strain was very high.

Of 25 colonies isolated in both methods, two virulent colonies were identified which possessed all the markers typical of the hypovirulent strain. These cultures were resistant to Bay NTN, sensitive to Benomyl, did not secrete melanin and displayed a growth rate typical of the aviru-

lent strain. Both were among those isolated after 48h of interaction. Both strains contained dsRNA, which was absent from the parental avirulent strain, and the pattern of segmentation was typical of the dsRNA pattern characterised in viruses recovered from the virulent strain that served as the donor.

These results, together with the earlier indications from curing experiments and the occurrence of the viruses in virulent strains and their absence in avirulent strains, provide good reason to suggest that in *Rhizoctonia solani* the dsRNA viruses are associated with the regulation of virulence. However, contrary to the situation in *Endothia parasitica*, the dsRNA viruses induce virulence and do not suppress virulence.

Viral information and virulence

To determine whether the content of viral information extends beyond the capacity to regulate the synthesis of structural proteins, efforts have been made to reveal its full coding potential. Characterisation of the proteins encoded by the viral dsRNA indicates a coding capacity extending two to three times beyond that of the dsRNA segments, if one assumes no overlapping genes. The viral coat protein is the major translation product, but clearly other proteins are produced. Their reaction to polyclonal antiviral antibodies suggests they are nonstructural. The nature of these proteins is unknown and neither enzymatic activities thought to be related to virulence, nor their indirect involvement in the induction of virulence, have yet been examined.

One of the major difficulties in the elucidation of the role of the viral encoded proteins is the absence of sufficient biological characterisation of virulent and hypovirulent strains. The pectolytic enzymes of a number of virulent and hypovirulent strains used in our studies were examined as markers for virulence. The role of pectolytic enzymes in the pathogenesis of *R. solani* has been well established (Weinhold & Motta, 1973). Four enzymes, known as endopolygalacturonase I (endo PG-I) and II, pectinesterase (PE) and endopectinolyase (endo PL), were purified to homogeneity from a number of virulent strains (Marcus *et al.*, 1986). Endo PL was found in all virulent strains examined but was not detected in the five hypovirulent strains used in this study. It is not yet known whether the 'converted' hypovirulent strains derived from the heterokaryon transfer experiment also display endo PL activity but this enzyme is clearly a good marker to follow since the potential for its production may be introduced directly to the fungus by the viruses or induced in the fungus by the viruses.

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Hypovirulence and biological control

It became clear from a series of experiments including field trials that hypovirulent strains can be used for biological control of root rot caused by *R. solani* (Ichielevich *et al.*, 1985*a*). Seedlings infected by the hypovirulent strain were protected against superinfection by virulent strains of *R. solani* (Fig. 17.3). In a series of experiments with one of the hypovirulent strains the damage was reduced by 75–94% in various experiments with different crop plants including cotton, radish, wheat, lettuce and carrots. The protection was effective against a challenge by virulent strains and only occurred in the presence of the hypovirulent strain. However, the protection by one hypovirulent strain was effective in a number of very different ecological niches differing in climatic conditions and soil type. The only limitation was the specificity displayed since the protection was effective against *Rhizoctonia* but not against other soil-borne pathogens. Effective protection was displayed using a hypovirulent strain of one anastomosis group and individually against a number of virulent strains of different anastomosis groups. It is unclear whether the hypovirulent strain induces a resistance mechanism in the host plant or creates a barrier against superinfection merely by efficient superficial invasion of the plant tissue, i.e. exploitation of a limited resource. Nonetheless, the protective mechanism operates irrespective of the incompatibility system and there is no need for the selection of hypovirulent strains from the different anastomosis groups. The hypovirulent strains

Fig. 17.3. Protection of cotton seedlings by hypovirulent isolate of *Rhizoctonia solani* (521). From left to right, uninfected control, infected with virulent strain 82, infected with hypovirulent strain 521, infected with strain 521 and challenged after 3 d with the virulent strain 82.

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in addition to, or as a part of, their protective effect appear to promote host growth; thus, in all experiments the protected plants clearly displayed vigorous growth that was expressed in higher yields than in controls.

It is unclear why hypovirulent strains are common in nature in spite of the fact that the mere acquisition of the viral information can convert a hypovirulent strain to a virulent strain. The hypovirulent strains studied were not limited to a unique or rare anastomosis group and, unless vegetative incompatibility occurs within anastomosis groups, they are widely accessible to cytoplasmic exchange and viral transmission. Information available from a better-characterised system, the viruses of *Saccharomyces cerevisiae*, suggests that many nuclear genes are involved in the maintenance of such viruses (see review by Tipper & Bostian, 1984). In that system as many as 30 genes play some part in the maintenance and replication of the fungal viruses. In the viruses of the corn smut pathogen, *Ustilago maydis*, another well characterised system, exchange of viral information is dependent on a nuclear gene that confers resistance to a viral-encoded system (Finkler, Peery & Koltin, 1984). Furthermore, other cytoplasmic factors may impose constraints on the exchange of viral information. Mechanisms of this nature may play a role in the persistence of hypovirulent strains of *R. solani*.

Ustilago maydis (Corn Smut)

Virus-related interstrain inhibition

Ustilago maydis represents a class of fungal pathogens in which the expression of a virus harboured by the pathogen inhibits growth of sensitive strains of the fungus. This phenotypic expression has stimulated consideration of the use of this phenomenon for plant protection.

In the course of studies on the sexual interaction among haploid lines of *U. maydis* growing on solid medium the occurrence of interstrain inhibition was reported (Puhalla, 1968) and referred to as the killer phenomenon. We now know that the factors associated with this phenomenon are polypeptides secreted by some strains of *U. maydis*. Such strains constitute c. 1% or less of the natural isolates examined. The polypeptides are highly specific and affect other members of the Ustilaginales including the plant pathogens of graminaceous plants *U. tritici*, *U. hordei* and *U. nuda*. The phenotype associated with the secretion of these polypeptides is related to a group of dsRNA viruses found in *U. maydis* (Fig. 17.1b) (Wood & Bozarth, 1973; Kiltin & Day, 1976). Almost all strains of *U. maydis* contain dsRNA viruses but very few secrete the toxic polypeptides. The gentic organisation of these viruses is complex: a major viral component provides the genetic information coding the coat protein and also the

replicative machinery that provides for the maintenance of a number of satellite viruses (Koltin, Levine & Perry, 1980). Some of the satellite viruses contain the information that encodes the inhibitory polypeptides. A detailed account of the *Ustilago* killer system was recently given by Koltin (see Buck, 1986).

Use of viral information for plant protection

The broad spectrum of the inhibitory phenomenon among the Ustilaginales merits some consideration for future plant protection. Resistance to the *U. maydis* virus-encoded toxin was not detected in six other members of the Ustilaginales that were tested. The species barriers, and the lack of common hosts in this order, suggest that many species have not been evolving under the selective pressure of similar killer systems. (A killer system has not been reported in other Ustilaginales.) Therefore, the toxin may be effective against these species. The use of the toxic polypeptides as fungicides is impractical but the introduction of the viral sequences that encode these polypeptides as cDNA, cloned in a plant expression vector, appears as a feasible approach. The dsRNA encoding the toxin is comprised of c. 1000 base pairs (Field *et al.*, 1983). If plant cells of wheat, barley, oats and other hosts of the various Ustilaginales can process the information and express the virus-encoded toxin, the plant tissues should be protected against infection by their respective smut fungus. It is proposed that the information derived from studies of viruses of *U. maydis* could be applied to programmes for the control of infections of other members of the Ustilaginales in other Graminaceous hosts.

The feasibility of this nonconventional approach in plant protection is difficult to assess at this stage in the development of plant molecular genetics and genetic engineering. Notwithstanding the lack of the proper vectors it is clear, however, that certain prerequisites must be met for the expression of the viral information by the plant. Specific processing by the plant tissue will be required and, as yet, it is unclear whether the processing signals recognised by the fungus will be also recognised by the plant.

Conclusions

Although they are abundant in many different species and within each species, dsRNA viruses of fungi have been largely unexplored beyond the morphological description of many and physical characterisation of a few. Elements with such an adaptive potential should have drawn much attention, as did the bacterial plasmids with their drug

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resistance factors and virulence factors but, possibly because of their indirect impact via their fungal host cell, viruses have attracted much less attention than the human pathogens. At present, information relating to the ecological implications of fungal viruses is scarce and even those cases that have been reported here require further elucidation. However, agents affecting at least four forms of phenotypic expression, debilitation of growth, supression of virulence, induction of virulence and secretion of species-specific inhibitory polypeptides, have been reported. Of these phenotypes, clearly the aspects related to virulence are the most complicated to study. Yet with heterokaryosis and parasexuality so prominent among plant pathogens, it is imperative that we understand better the role of cytoplasmic factors as adaptive agents which can play a role in each of the above-mentioned phenotypes. Molecular and genetic studies of viruses found in plant pathogens could resolve their specific role in virulence and explain the persistence of various forms *in vivo*.

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Transcription and In Vitro Translation of the dsRNA Virus Isolated from Rhizoctonia solani.

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Key words: *Rhizoctonia solani*, dsRNA, fungal virus, transcription, translation

Abstract

A segmented double-stranded dsRNA virus has been isolated from virulent strains of *Rhizoctonia solani*. The dsRNA genome has mol. wts. of 1.45 and 1.32 × 10^6. Two full-size transcripts with mol. wts. of 0.74 and 0.66 × 10^6 (2.2 kb and 2 kb, respectively) were synthesized by the virus-associated RNA-dependent RNA polymerase and resolved by denaturing polyacrylamide gel electrophoresis. The transcripts cross-hybridized to the viral dsRNA isolated from a number of strains. The transcripts did not hybridize with the genomic DNA. An uneneapsidated species of dsRNA with mol. wt. of 1.6 × 10^6 did not hybridize with the viral transcripts. No cross-hybridization between the two viral dsRNA segments was obtained. The viral-encoded proteins were studied by *in vitro* translation using the rabbit reticulocyte lysate system. The transcripts served as mRNA for the synthesis of the major capsid protein of 55 kD, and a number of other products. The viral coat protein was immunoprecipitated with antibodies against purified virus particles. Partial proteolysis of the major *in vitro* product and the authentic capsid protein using *Staphylococcus aureus* V8 protease produced similar peptide patterns. Denatured viral dsRNA also directed the synthesis of proteins identical to those translated from the transcripts *in vitro*.

Introduction

The fungal plant pathogen *Rhizoctonia solani* causes severe damage to a large number of hosts (1) and is a severe pathogen throughout the world. By screening many isolates of *R. solani* for virulence, a number of studies indicated the occurence of both virulent and avirulent strains in the natural population (2–4). A double-stranded dsRNA virus has been recently isolated from strains of *R. solani* (5, 6). The viral genome is composed of two segments of dsRNA with mol. wts. of 1.45 and 1.32 × 10^6 (2.2 kbp and 2 kbp, respectively). An additional dsRNA

molecule of 1.6×10^6 (2.4 kbp) is found in many strains by extraction of mycelia but has not been recovered from virions. The major capsid protein is 55 kD. An RNA-dependent RNA polymerase is associated with the viral capsids and is clearly a transcriptase (5).

Although initial studies reported that strains of the pathogen containing dsRNA are debilitated and hypovirulent (7), later studies indicated that a correlation between the presence of dsRNA and virulence cannot be established (2). Only recently, by stringent tests to clearly distinguish between virulent and hypovirulent strains of the pathogen, a clear correlation was established between the presence of viral information and virulence (4). Hypovirulent strains were found to be devoid of viruses and all the virulent strains examined contained dsRNA viruses. The relationship between the dsRNA viruses and virulence was further examined in attenuated strains and a correlation was established between the loss of virulence and either the total loss of dsRNA segments or the loss of specific segments (5). Furthermore, transmission of dsRNA from a virulent to an avirulent compatible strain by cytoplasmic exchange resulted in the transmission of virulence. These results suggested that dsRNA viruses may be associated with the regulation or production of virulence factors. The analysis of the virus-encoded proteins may therefore be significant for the elucidation of nonstructural proteins associated with the regulation of virulence.

In vitro translation of virus-specific mRNA has been extensively used in other dsRNA viral systems to determine the coding potential of the viral genome (8–12). *In vitro* translation of denatured dsRNA has also been reported in a number of dsRNA viruses (9, 13–16).

In this report we describe the proteins encoded by the *R. solani* viral genome. We have used both the viral-specific mRNA, synthesized *in vitro* by the virus-associated RNA-dependent RNA polymerase, and unfractionated dsRNA to direct the synthesis of proteins in rabbit reticulocyte lysate systems. In addition, the relationship between encapsidated and unencapsidated dsRNAs and the relatedeness of the viruses isolated from different strains is described.

Methods

Strains

R. solani cultures (No. 13, 82, 53, 56, and W) were isolated from various locations in Israel (4), and maintained on potato-dextrose agar at 27°C. The virulence of each strain was determined in a plate assay as described by Castanho & Bulter (17).

Purification of virus particles and dsRNA extraction

The techniques to purify virus particles and to extract dsRNA described by Finkler et al. (5) were followed.

Purification of dsRNA segments

Individual dsRNA segments were purified by at least 2 cycles of electrophoresis in 1.5% agarose and were finally electroeluted from the agarose using BND-cellulose columns as described by Silhavy et al. (18).

3' End-labelling

Segments of dsRNA were end-labelled with 5'-^{32}P-cytidine-5'-3'-diphosphate using T4 RNA ligase (Pharmacia) as described by Bruenn and Brennan (19). The specific activity of the [^{32}P]pCp was 3000 Ci/mmole. The labelled dsRNA was purified by phenol extraction, ethanol precipitation, and desalting was performed on Sephadex G100.

DNA isolation

Mycelia grown overnight at 27°C were incubated with 10 mg/ml Novozyme (Novo Industries, Sweden) in 0.6 M mannitol (pH 5.8) on a shaker for 1-2 hr at 30°C. The mycelia were centrifuged and treated with 50 µg/ml proteinase K and 1% $NaDodSO_4$ for 1 hr at 37°C in 10 mM Tris-HCl buffer (pH 7.5). The nucleic acids were extracted with phenol:chloroform (1:1).

Synthesis of viral transcripts

Full-size transcripts were synthesized basically as described by Ben-Zvi et al. (20) with few modifications. The reaction mixtures, containing 1 mM of each of the 4 nucleoside triphosphates and 1.6 mg/ml bentonite to inhibit RNase activity, were incubated with 3 mM Mg^{+2} at a pH optimum of 7 for 24 hr at 30°C. Labelled transcripts were synthesized in the presence of 10^7 cpm/ml of [^{32}P] UTP. After incubation, the reaction mixture was extracted with phenol-chlorophorm (1:1) and the nucleic acids were ethanol precipitated. The sRNA transcripts were purified on a CF11 column (21), ethanol precipitated, and washed with 70% cold ethanol several times to remove salts.

Analysis of viral transcripts

Size determination of viral transcripts by electrophoresis was performed in 3% polyacrylamide gel in the presence of 7M urea as described by Marzluff and Huang (22) with few modifications: ^{32}P-labelled transcripts with specific activity of 5×10^5 cpm/µg RNA were denatured by heating at 65°C for 6 min in sample buffer

containing 50 mM Tris, 50 mM boric acid, 50 uM EDTA (TBE buffer) to which 5M urea, 10% glycerol, 0.05% bromophenol blue and 0.05% xylene cyanol were added. The samples were quickly cooled and then electrophoresed for 24 hr in a vertical 12 × 23 cm gel at 110 volts using TBE buffer. The transcripts were detected by autoradiography of the dried gel.

RNA blot hybridization analysis

Double-stranded RNA samples were electrophoresed in horizontal 1% agarose gel in MOPS buffer containing 1mM EDTA, 5 mM sodium acetate and 20 mM Na-MOPS (pH 7.5) for 3 hr at 110 volts. Treatment of the gel prior to the transfer to nitrocellulose followed the procedure provided by J. Bruenn (personnal communication). The gel was incubated in 50% formamide and 6% formaldehyde in MOPS running buffer at 55°C. The RNA was denatured by treatment with 50 mM NaOH and 100 mM NaCl, and transferred from the gel to nitrocellulose filters in 20 × SSC (1 SSC = 0.15 mM NaCl, 0.015 mM sodium citrate as described by Southern (23). The filter was air dried and then baked at 80°C for 2 hr under vacuum.

The filters were prehybridized for 2–4 hr at 65°C in a hybridization mix containing 10 × Denhardt, 4 × SSC, 0.1% $NaDodSo_4$, 0.2% Ficoll, 0.2% polyvinyl pyrrolidone and 100 μg/ml denatured salmon sperm DNA.

Hybridization was performed at 65°C for 12–24 hr in hybridization mix with 1 × Denhardt. Transcripts with a specific activity of 5×10^5 cpm/μg were added. When ^{32}P-end-labelled dsRNA was used as a probe, it was denatured for 90 sec at 100°C. After hybridization, the blot was washed as described by Thomas (24).

Southern blot analysis

Hybridization of ^{32}P-labelled transcripts to DNA followed the procedure described by Southern (23).

In vitro translation

Double-stranded RNA (1 μg) was precipitated overnight with ethanol at −20°C and washed twice with 70% ethanol. To denature the dsRNA, the dried sample was resuspended in 2 μl distilled H_2O containing 1 mM EDTA, incubated 90 sec at 100°C and immediately cooled in ice water and subjected to *in vitro* translation. Dried samples of dsRNA (1 μg) were also denatured with 2 μl of 10 mM methyl mercury hydroxide for 10 min at room temperature prior to *in vitro* translation.

Purified transcripts or denatured dsRNA were translated in rabbit reticulocyte *in vitro* translation systems (Amersham, U.K. and Orgenics Ltd, Israel). Translation was carried out in proportions of 8 and 6 volumes of lysate, respectively, 1–2

volumes of RNA and 0.8–1.5 volume of [^{35}S]methionine (0.8–1.5 μCi/10 μl), and incubated at 30°C for 60–80 min.

Incorporation of the label into total proteins synthesized was determind by spotting 2 μl aliquots on GF filters and assaying for trichloroacetic acid precipitable radioactivity. The in vitro-synthesized proteins were electrophoresed in 10% $NaDodSO_4$-polyacrylamide gels using the buffer system of Laemmli (25). The proteins were detected by fluorography using an intensifying solution, Amplify (Amersham, U.K.), for 30 min. The gel was dried and autoradiographed.

Preparations of immune serum

Virus particles were purified on three successive 10–40% sucrose gradients, according to Finkler et al. (5) and fractions containing the viruses were identified by the polymerase activity. These fractions were pooled and dialyzed against buffer containing 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 40 mM NaCl, 10 mM $MgSO_4$, 4 mM 2-mercapthoethanol and 10% glycerol, and pelleted at 133000g for 4 hr. New Zealand white rabbits were immunized intradermally with 120 μg of purified virus preparation emulsified with an equal volume of Freund's complete adjuvant. Injections were repeated 21 days and 42 days after the initial immunization, with 60-100 μg of virus preparation. The rabbits were bled from the ear vein 10 days after each boost. Gammaglobulins were purified from the serum by ammonium sulfate precipitation and by dialysis against buffer containing 2 mM NaH_2PO_4, 10 mM Na_2HPO_4 and 150 mM NaCl (PBS), as described by Palmiter et al. (26). Finally, the gammaglobulin preparation was tested by double-diffusion Ouchterlony assays (27) against purified virus particles containing 1% $NaDodSO_4$.

Immunoprecipitation assay

The in vitro translation products of *R. solani* transcripts and denatured dsRNA were analyzed by immunoprecipitation as described by Segev et al. (28), using the rabbit antiserum and immunogammaglobulins prepared against purified viral particles.

Peptide mapping

Peptide mapping of proteins isolated from $NaDodSO_4$-polyacrylamide gels followed the procedure described by Cleveland (29). Both hydrated and dried gel slices were digested with *Staphylococcus aureus* V8 protease (1μg) and fractioned on 15% $NaDodSO_4$-polyacrylamide gels.

Results

Relatedness of dsRNA segments

The genome of the *R. solani* virus was shown to consist of two segments of dsRNA with mol. wts. of 1.45 and 1.32 × 10^6 (5). No additional dsRNA segments were obtained from viruses, as confirmed by 3′ end-labelling of the encapsidated dsRNA molecules. An additional nonencapsidated dsRNA segment with mol. wt. of 1.6 × 10^6 was purified from the mycelia of a number of virulent strains of *R. solani*. The relationship between the dsRNA segments was studied by hybridization of the total dsRNA isolated from the fungal mycelium to each purified ^{32}P-end-labelled dsRNA segment. The results in Fig. 1 indicate that the two encapsidated segments are unique since they do not cross-hybridize. The minor band in Fig. 1c is a result of cross-contamination between the dsRNA segment with mol. wt. of 1.45 × 10^6, used as a probe, with the 1.32 × 10^6 dalton segment. Purification of the heavier segment was hindered due to the proximity of the two bands. However, the dsRNA segment with mol. wt. of 1.6 × 10^6 did not hybridize to any viral dsRNA molecule. This further supports the earlier results suggesting the nonencapsidation of the latter dsRNA segment.

Analysis of viral transcripts

RNA-dependent RNA polymerase activity has been found in virus particles isolated from *R. solani*. The major product of the polymerase reaction is ssRNA and represents transcription activity (5).

In earlier studies of the virus-associated RNA polymerase activity, the viral transcripts were examined in 1.5% agarose gels after treatment with glyoxal. Only one transcript of 0.75 × 10^6 daltons was identified (5). In the current study, two viral transcripts were resolved using denaturing (7 M urea) polyacrylamide gel (Fig. 2). The second transcript is 0.66 × 10^6 daltons. Each of these transcripts corresponds to one half the molecular weight of one of the viral dsRNA segments. A transcript corresponding to the 1.6 × 10^6 dalton dsRNA segment, recovered from the mycelium, was not detected among the viral transcripts.

Relatedness of viral isolates

To determine the relatedness of the viruses obtained from different virulent strains of *R. solani*, the viral transcripts were used as probes in hybridization experiments with dsRNA extracted from the mycelia of a number of fungal isolates. All *Rhizoctonia* isolates contained two dsRNA segments with mol. wts. of 1.45 and 1.32 × 10^6 that are contained in virus particles. The mycelia of a number of strains (53 and 82) contained an additional dsRNA segment with mol. wt. of 1.6 × 10^6. The

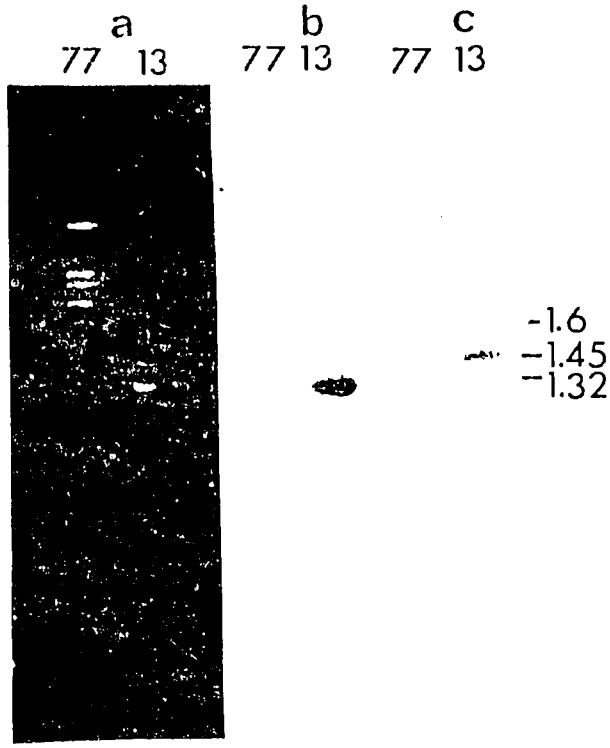

Fig. 1. Cross hybridization analysis of dsRNA segments. dsRNA isolated from *U. maydis* 77 and *R. solani* 13 were subjected to electrophoreis on a 1.5% agarose gel, stained with ethidium bromide (a), trasnferred to nitrocellulose as described in Methods and hybridized with pCp-labelled 1.32×10^6 dsRNA segment (b), pCp-labelled 1.45×10^6 dsRNA segment (c). Hybridizing bands were detected by autoradiography (b, c).

latter dsRNA band was never recovered from virions and appears to be an unencapsidated species. The viral transcripts, used as probes, served also to resolve the relationship of the heavy segment of dsRNA to that found encapsidated in viral particles.

As shown in Fig. 3a, the transcripts synthesized from strain 82 hybridized to the dsRNA segments with mol. wts. of 1.45 and 1.32×10^6, isolated from the *Rhizoctonia* strains W, 56, 82, 13, and 53. An identical hybridization pattern was obtained with the transcripts synthesized from strain 13 (Fig. 3b). No hybridization was obtained between either *Rhizoctonia* viral transcripts or the *U. maydis* dsRNA molecules. These results indicate that in every case the transcripts hybridized only to the segments that are known to be encapsidated in virions. Therefore, the

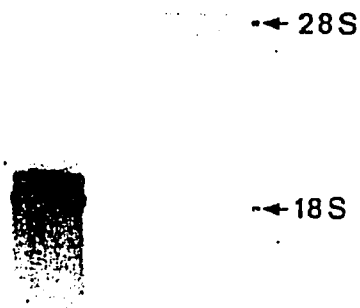

Fig. 2. Autoradiography of ^{32}P-labelled viral transcripts. ^{32}P-labelled transcripts were denatured as described in Methods and run on 3% polyacrylamide gel containing 7 M urea. Rabbit reticulocyte rRNA (28S and 18S) molecules, used as molecular weight markers, were treated as above and visualized by ethidium bromide staining.

viruses found in the differnt strains of *Rhizoctonia*, originating from different locations, are interrelated. The nonencapsidated dsRNA segment with a mol. wt. of 1.6×10^6 appears to be unrelated to the viral genome since there is no homology between this segment and the viral transcripts.

The viral transcripts were used also to test for the presence of a proviral state in the genome of the *Rhizoctonia* host. No homology was detected between genomic DNA of the host (strain 13) and the viral transcripts as determined by Southern blot hybridization of the restricted genomic DNA with the viral transcripts used as probes. Therefore, similarily to all other dsRNA viruses of fungi, the *R. solani* virus

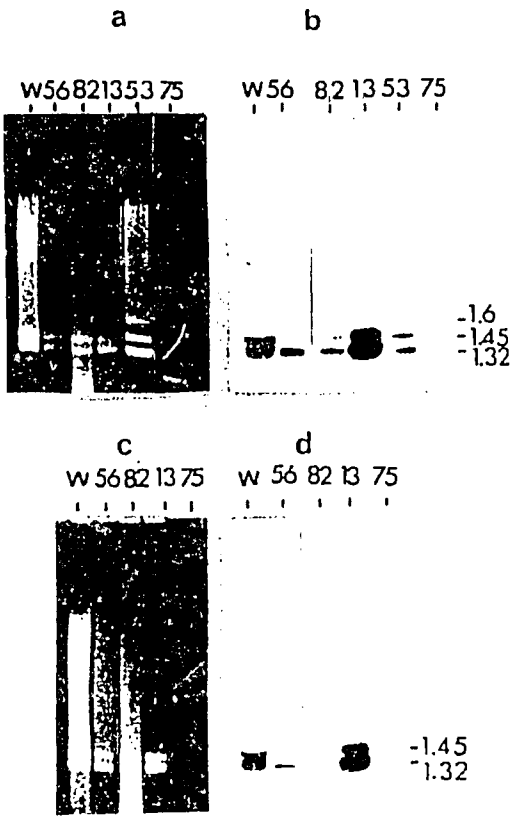

Fig. 3. Northern blot hybridization of dsRNAs. dsRNAs were separated on 1% agarose gels, stained with ethidium bromide (a, c) and transferred onto nitrocellulose. ^{32}P-labelled transcript from strain 82 was used as probe in hybridization with dsRNAs isolated from *R. solani* strains W, 56, 82, 13, and 53 and *U. maydis* 75 (b). ^{32}P-labelled transcript from strain 13 was used as probe in hybridization with dsRNAs isolated from *R. solani* strains W, 56, 82, and 13 and *U. maydis* 75 (d).

In vitro translation of viral transcripts

To study the coding potential of the viral genome, in vitro translation experiments were performed with purified ssRNA transcripts. Maximum incorporation of [^{35}S]methionine into proteins was obtained with 100 μg/ml of purified transcripts obtained from viruses isolated from two strains, 82 and 13. The products of the in vitro translation assays were analyzed on $NaDodSO_4$-polyacrylamide gels (Fig.4). Identical patterns of in vitro translation products were obtained from the transcripts of the two virus preparations. A major protein of about 55 kD was synthesized in vitro. Occasionally, a closely migrating submajor product of 53 kD was observed in addition to a number of "minor" proteins of 47, 45, 43, 40, 38, and 35 kD. In an effort to determine whether some of these minor components are incomplete products, the time of the reaction was extended to 4 hr but the pattern

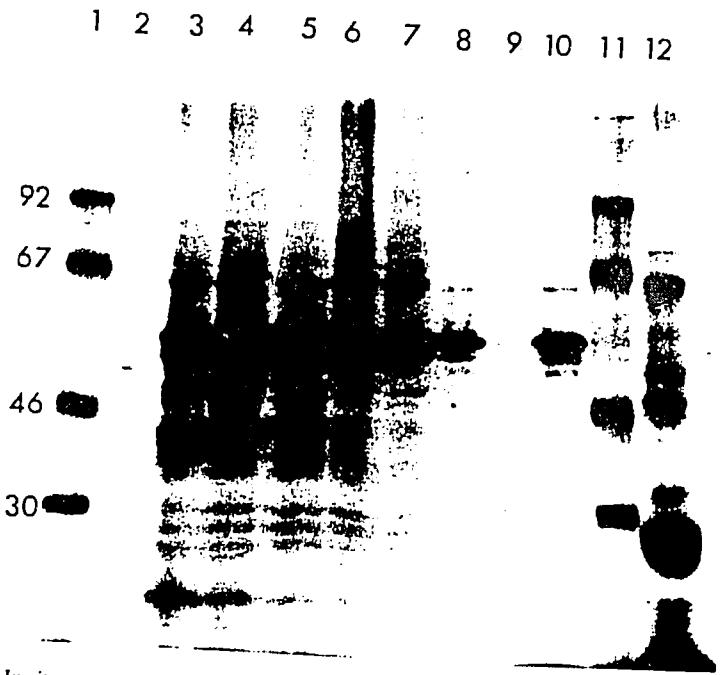

Fig. 4. In vitro translation of *R. solani* viral transcripts synthesized from strain 82. Each reaction mixture (10 μl) was diluted with 2 × Laemmli sample buffer, incubated 3 min at 100°C and analyzed on 10% $NaDodSO_4$-polyacrylamide gel. Lane 1, 11: [^{14}C]methyl-labelled protein molecular weight standards; lane 2, reticulocyte lysate reaction without added RNA; lanes 3–10, translation products of transcript RNA (50, 75, 100, 150, 200, 250, 300, 400 μg/ml respectively); lane 12, products of globin mRNA.

of these minor proteins remained unchanged. Whether these are pause products found often in in vitro translation or authentic nonstructural proteins is unclear and requires further characterization of virion coat proteins. A number of additional proteins of 10–30 kD, found among the products of the in vitro translation, were not virus encoded. Some of these proteins were found in control assays lacking RNA and others may represent short peptides wtill bound to tRNA.

Double-diffusion Outchterlony assays were used to test the identity of the major viral coat protein using rabbit antiserum and partially purified gammaglobulins prepared against purified virions. A single precipitin band was obtained between antiviral antibodies and the virus particles. These antibodies were used to immunoprecipitate the [^{35}S]methionine-labelled in vitro translation products derived from the viral transcripts (Fig. 5). It appears that the proteins of the viral capsids isolated from two different strains (13 and 82) are similar since both have the

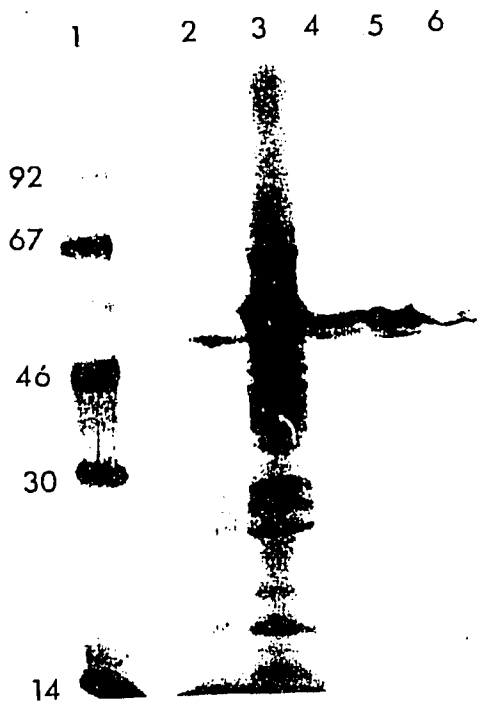

Fig. 5. Immunoprecipitation of in vitro translation products with antibodies against virions isolated from strain 13. Lane 1, [^{14}C] methyl-labelled protein standards; lane 2, reticulocyte lysate reaction without added tRNA; lane 3, in vitro translation products of viral transcript; lanes 4–6, translation products immunoprecipitated with 20, 15 and 10 μg/ml gammaglobulins, respectively. All reactions were previously treated with 5 μl normal rabbit serum and a 10% fixed suspension of *Staphylococcus aureus* to remove nonspecific binding antigens.

same mol. wts. and both are immunoprecipitated by antibodies prepared against the virus isolated from strain 13. A major protein of 55 kD was precipitated in all the experiments. Occasionally a closely migrating submajor product of 53 kD was synthesized. This protein was also precipitated by anticoat antibodies. Two other minor products of 47 and 45 kD were immunoprecipitated and are seen as faint bands on longer exposure; these may be due to nonspecific immunoprecipitation.

The identity of the major in vitro translation protein band was further studied by comparing the peptide map pattern of the authentic viral capsid and the 55 kD in vitro translation protein. The major in vitro translated protein band of strain 13, located by autoradiography of the $NaDodSO_4$-polyacrylamide gels, was excised and hydrolyzed with *Staphylococcus aureus* V8 protease. The authentic viral capsid was similarly treated. The resulting peptides were resolved on 15% $NaDodSO_4$-polyacrylamide gels and gave similar patterns (Fig. 6). Identical peptide map patterns were also obtained with the major in vitro translation protein of strain 82. The major protein of 55 kD is therefore the capsid protein. These results suggest that two proteins of 55 and 53 kD are associated with the viral capsids, and the 53

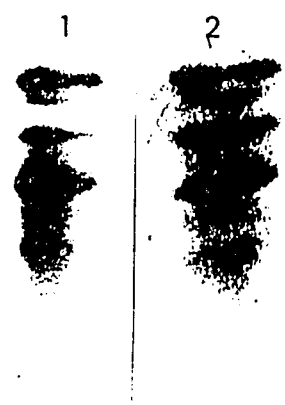

Fig. 6. Comparison of peptide map patterns of the major in vitro translation product and capsid protein. Gel slices were treated with 1 μg *Staphylococcus aureus* V8 protease and electrophoresed on 15% $NaDodSO_4$-polyacrylamide gel. Lane 1, autoradiograph representing peptide mapping of the major in vitro translation product of strain 13; lane 2, peptide mapping of authentic capsid protein (strain 13) visualized by Coomassie blue staining.

kD protein appears to be an incomplete product. The identity of the other products as nonstructural proteins will be discussed later.

In vitro translation of dsRNA

Total dsRNA extracted from the mycelium and purified on CF11 columns was used for in vitro translation. These samples contained the viral dsRNA segments (1.45 and 1.32 × 10^6 daltons) and in addition the unencapsidated dsRNA (1.6 × 10^6 daltons). The products of in vitro translation of the total dsRNA shown in Fig. 7 appear similar to those synthesized by the viral transcripts, but included an

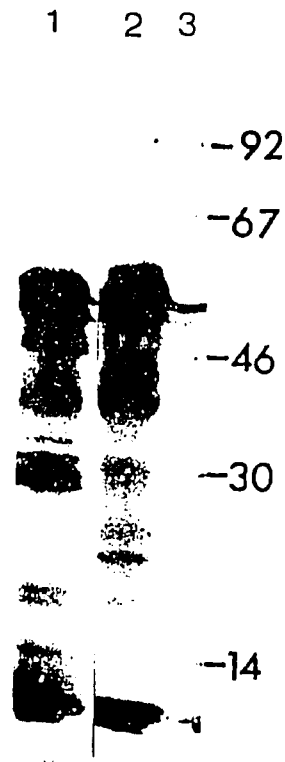

Fig. 7. Comparison between in vitro translation products of total denatured dsRNA and viral transcripts. Reaction mixtures (10μl) were run on 10% $NaDodSO_4$-polyacrylamide gels and analyzed by fluorography. Lane 1, in vitro translation of viral transcripts (strain 13); lane 2, translation of total dsRNA extracted from strain 13; lane 3, immunoprecipitations of protein, synthesized by total dsRNA.

additional protein of 60 kD. This protein was not immunoprecipitated by the antiviral antibodies. It is probably the product of the 1.6×10^6 dalton dsRNA segment that is not encapsidated.

Discussion

Based on purification of viral dsRNA and end-labelling of the segments it appears that the *R. solani* virus consists of two dsRNA segments. The two segments do not cross hybridize and they appear to be unique. Two unique transcripts are synthesized in vitro by the virus-associated RNA-dependent RNA polymerase. The full-size transcripts have a coding capacity of ca. 1300 amino acids assuming there are no overlapping open reading frames and unresolved overlapping segments.

The viral transcripts bear no homology to the host genomic DNA. Thus, as in all other dsRNA fungal viruses, the *R. solani* virus does not integrate into the host chromosomes and has no proviral state.

The viral isolates are interrelated, as indicated by hybridization of dsRNA extracted from a number of isolates with a probe of a viral transcript synthesized by one of the viral isolates. The relatedness of the viruses recovered from different sources is reflected also at the level of the proteins. In vitro translation of viral transcripts synthesized by two virulent strains resulted in the formation of a major protein of identical size, identical antigenicity and peptide maps. The major in vitro translation product, a 55 kD protein, corresponds clearly to the viral capsid protein by the following criteria: a) the viral capsid protein comigrated with the major in vitro translation product; b) this product was immunoprecipitated by antibodies prepared against virus particles; and c) identical peptide mapping patterns were obtained following limited proteolysis.

The polypeptides identified so far in the in vitro translation exceed the coding capacity of the viral information. Therefore, verification of the minor in vitro translation products must be further resolved. The 53 kD product that is occasionally synthesized closely with the major coat protein and immunoprecipitated by the antiviral antibodies may be an incomplete coat product. Other in vitro translation products are not immunoprecipitated with anticoat antibodies and therefore may be among the nonstructural proteins of the viral information. The identity of these proteins will have to be resolved by immunological tests and fingerprinting.

The coding assignments for the purified genome segments of the *Rhizoctonia* virus is under investigation and should distinguish the nonstructural components.

Acknowledgments

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dsRNA Virus of *Rhizoctonia solani*

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INTRODUCTION

Rhizoctonia solani, a soil-borne fungal plant pathogen, causes damping-off in some 130 plant species, and is found in most agricultural areas throughout the world (Baker, 1970). The characteristic features of *R. solani* Kuhn [*Thanatephorus cucumeris* (Frank) Donk] include multinucleate hyphal cells that do not generally sporulate, and the formation of sclerotia by vegetative cells. Vegetative incompatibility is determined by six anastomosis groups, and different strains can form hyphal fusions and exchange cytoplasm only if both mates are of the same anastomosis group. Sexuality is very rare and had been reviewed by Sherwood (1970). Strains investigated displayed either homothallism or bipolar incompatibility (Whitney and Parmeter, 1963).

While dsRNA viruses of fungi are abundant and have been under investigation for some time (reviewed by Lemke, 1979; Buck, 1986), the role of these viruses in fungal ecology has not yet been elucidated. Transmissible hypovirulence in *Endothia parasitica* is the only case in which an effect on the etiology of a disease can be ascribed to dsRNA molecules. Attempts to correlate the presence of viruses with unique phenotypes or biological properties of fungal carriers is feasible in few cases and is quite confusing in most others.

The majority of fungal infections with viruses are symptomless (Hollings, 1978), and in only few cases do the carriers exhibit a unique phenotype that can be ascribed to viral infection: toxin production in *Saccharomyces cerevisiae* and *Ustilago maydis* is related to viral infection (Bevan et al., 1973; Wood and Bozarth, 1973); conditional lysis of certain strains of the genus *Penicillium* (Borre et al., 1971); the disease of the cultivated mushroom *Agaricus bisporus* (Marino et al., 1976), and diseased mycelium in *Helminthosporium victoriae* (Ghabrial and Mernaugh, 1983; see Chapter 16) are among the few such cases. Other effects, such as reduced growth rate and poor sporulation, have been reported, but these correlations with viral infections are complex. A disease of *H. victoriae*, the causative agent of Victoria blight, appeared to be transmitted to normal strains by cytoplasmic exchange (Ghabrial and Mernaugh, 1983). Diseased strains were characterized by reduced growth, poor sporulation, and lysis. Both normal and diseased strains contain 190S virus particles, but only the diseased strain contains an additional group of 145S virus particles. It is suggested that the 145S virus is the causal agent of the disease of *H. victoriae*.

The correlation between virus-infected isolates of the wheat take-all fungus *Gaeumannomyces graminis*, characterized by poor sporulation and instability in culture, is quite confusing due to the complexity of viruses reported to infect individual isolates of this species (Buck et al., 1981). It is possible that take-all decline depends upon an unstable balance of dsRNA components in the fungal population in soil adjacent to host roots (Day and Dodds, 1979).

The relation between virulence and dsRNA virus particles in *R. solani* was initially examined by Castanho et al. (1973) following a study of *Endothia parasitica*, the causative agent of the chestnut blight (Van Alfen et al., 1975), in which the attenuation of virulence by cytoplasmic factors was suggested. It appeared that dsRNA molecules act as attenuators of virulence in *E. parasitica* and that transmission of dsRNA by cytoduction from hypovirulent to virulent strains suppresses virulence (see Chapters 13 and 17, this volume). The early studies with *R. solani* revealed the occurrence of a similar phenomenon. The attenuation of virulence was transmissible but the hypovirulent strains were debilitated and it was this debilitated state that was transmitted. In these studies, dsRNA, but not virus particles, was detected in the mycelia of hypovirulent strains. Virulent strains were reported devoid of dsRNA. The transmission of the debilitated growth to a virulent strain of *R. solani* through cytoplasmic exchange along with dsRNA suggested that the phenomena of hypovirulence in *E. parasitica* and *R. solani* were similar. However, since hypovirulence and debilitation of growth are two separable phenotypes it is clear that rigorous criteria must be set in defining hypovirulence to avoid confusion between growth retardation and hypovirulence. Therefore,

in studies by Finkler et al. (1985) to reexamine the relation of dsRNA viruses to virulence in *R. solani*, hypovirulent strains were defined as those strains that infect a wide variety of hosts but incur almost no discernible damage to the host. Such strains should have no growth disadvantage, and their growth rate should be similar to that of the virulent strains. Virulent strains were those that affect all the tested hosts and incur damage of high severity. Furthermore, since *R. solani* has a very wide host range, a distinction between virulent and hypovirulent strains must represent this potential. An examination of the response of a variety of hosts to each strain is a minimal requirement in the definition of such a complex parameter as virulence. Therefore, results reported by Zanzinger et al. (1984), in which dsRNA occurred in both virulent and hypovirulent strains, may be misleading by the use of nonstringent criteria for the distinction between virulent and hypovirulent strains. It appears that the data reported thus far in a number of systems is subject to various interpretations due to the inaccurate definition of hypovirulence. We have, therefore, undertaken the study of the relationship of dsRNA viruses to the virulence of *R. solani*, by examination of a local population and determining those elements that appear as most critical in the phenomenon of hypovirulence. Furthermore, in an effort to resolve whether the dsRNA viruses play a role in the regulation of virulence, we addressed the major question of whether virulence or hypovirulence is cytoplasmically transmitted.

VIRULENCE AND OCCURRENCE OF dsRNA VIRUSES IN *Rhizoctonia solani*

Isolation

Strains of *R. solani* were isolated from soil samples in Israel. The isolates were from diverse ecological niches (Ichielevich-Auster et al., 1985a) and varied in host range and disease severity when tested on as many as 11 different host plants (Table 1). Approximately 30% of the isolates were nonpathogenic to all the host plants tested, and these are considered to be hypovirulent. Strains selected for the study displayed normal growth rate and with no significant difference in growth rate between the virulent and hypovirulent strains. Similarly, no other morphological differences were distinguished, except that hypovirulent strains produced fewer sclerotia and their mycelia appeared to contain less melanin. Formation of sclerotia and melanin may be useful markers for identification of virulent strains, but the number of strains examined was insufficient to establish a correlation. In a sample of 109 strains tested, most anastomosis groups were represented and both virulent and hypovirulent strains were found within each group. In addition, no special geographic distribution was noticed among the virulent and the hypovirulent strains or for the anastomosis groups.

Table 1 Host Range and Disease Severity Index of Strains of *Rhizoctonia solani*

Strain No.	AG[b]	Disease severity index[a]					
		R	T	C	B	Co	O
Virulent							
82	4	5.00 ± 0.00[c]	1.00 ± 0.00	5.00 ± 0.00	5.00 ± 0.00	2.00 ± 0.00	4.80 ± 0.00
13	4	5.00 ± 0.00	5.00 ± 0.00	5.00 ± 0.00	3.00 ± 0.00	5.00 ± 0.00	5.00± 0.00
56	4	5.00 ± 0.00	5.00 ± 0.00	4.00 ± 0.00	3.00 ± 0.00	5.00 ± 0.00	5.00 ± 0.00
53	4	5.00 ± 0.00	5.00 ± 0.00	5.00 ± 0.00	5.00 ± 0.00	5.00 ± 0.00	0.00
189HT[c]	1	5.00 ± 0.00	5.00 ± 0.00	4.01 ± 0.09	2.00 ± 0.00	1.00 ± 0.00	0.00
Hypovirulent							
160	2	1.00 ± 0.00	0.00	0.00	0.00	0.00	0.00
521	4	0.70 ± 0.03	0.00	0.00	0.00	0.00	0.00
558	4	1.00 1 0.00	0.00	2.00 1 0.00	1.00 ± 0.00	0.00	1.00 ± 0.00
751	1	2.00 ± 0.00	1.09 ± 0.03	1.00 ± 0.00	1.00 ± 0.00	0.00	0.00
189a[c]	1	0.38 ± 0.01	0.00	0.00	0.00	0.00	0.00

[a]Virulence ratings were based on the infected area of hypocotyl of seedlings. The results with six hosts are presented (R, radish; T, tomato; C, carrot; B, bean; Co, cotton; O, onion). Tests conducted with five additional hosts gave similar results without any exception. The scale of 1 to 5 refers to the area of infection: 0, no detectable infection; 1, 1 to 10%; 2, 11 to 30%; 3, 31 to 50%; 4, 51 to 80%; 5, over 80%.
[b]Anastomosis group.
[c]Strains kindly sent by Dr. E. E. Butler, Department of Plant Pathology, University of California, Davis, California.
Source: Finkler et al. (1985).

Detection of dsRNA Viruses

Of the nine strains selected for the study, five were virulent and four hypovirulent as determined by pathogenicity tests. A unique nucleic acid was extractable only from the virulent strains. The unique nucleic acids extracted from the mycelia of each of the virulent strains were shown to be dsRNA by resistance to RNase at high ionic strength and to DNase treatments, and sensitivity to RNase at low ionic strength. Also, binding properties to a cellulose column (CF11) routinely used to separate between single-stranded and double-stranded RNA (Franklin, 1966) was indicative of the dsRNA nature of this material. The high-molecular-weight material seen in gels of extracts consisted of a heterogenous population of DNA molecules, as determined by digestion with DNase and with restriction enzymes as well as by electron microscopy.

The results indicated that only virulent strains contained dsRNA (Figure 1); hypovirulent strains were devoid of dsRNA (Finkler et al., 1985). By electrophoresis of the dsRNA, a number of segments were identified in polyacrylamide gel and their pattern of segmentation varied slightly. Three distinct patterns were identified: class I included strains 13 and 56, which consisted of two segments with M.W. of 1.45 and 1.32 × 10^6; class II included strains 82 and 53 which contained an additional segment with a M.W. of 1.6 × 10^6, and class III, represented by strain W, contained two dsRNA segments with M.W.s of 1.6 and 1.32 × 10^6, but lacked the 1.45 × 10^6 species. Additional segments with M.W.s of 1.7, 1.25, and 1.225 × 10^6 were identified only in strain 82. Repeated extractions of dsRNA from the mycelia of *R. solani* maintained on potato-dextrose agar plates and in soil for over a year revealed some differences in the pattern of dsRNA segments. For example, strain W displayed an additional segment with M.W. of 1.45 × 10^6 in later extractions; strain 13 was found lately to contain two additional segments with M.W.s of 1.6 and 1.25 × 10^6. The variation did not yield totally unknown segments, and looking at the population of isolates, it appears that four segments are predominant: 1.6, 1.45, 1.32, and 1.25 × 10^6. These fluctuations in dsRNA segments are not uncommon among dsRNA fungal viruses and a change in titer was also shown in *G. graminis* by Buck et al. (1981) and noticed in *U. maydis* (Koltin, 1986).

Virus particles were sought in virulent and hypovirulent strains but were detected only in virulent strains (Figure 2). Repeated extractions of dsRNA from the viruses indicated only two segments, M.W. 1.45 and 1.32 × 10^6, are recovered from the particles. Thus, it appears that the additional segments detected in the mycelia of a number of isolates are probably not encapsidated.

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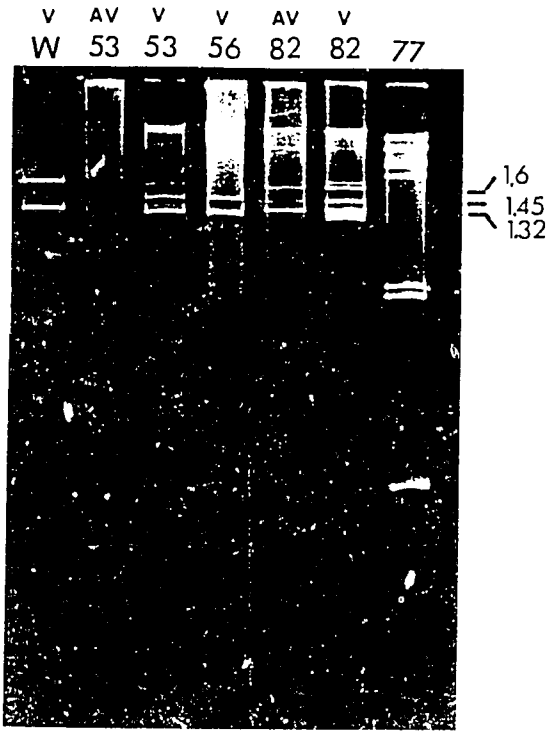


Figure 1 Polyacrylamide gel electrophoresis of dsRNA isolated from virulent strains of *R. solani* (V) and from virulent strains transformed into hypovirulence (AV). *Ustilago maydis* P4 viral (77) dsRNA was used as the molecular weight marker. *Source*: Finkler et al., 1985.

Characterization of *R. solani* Viruses

The viruses isolated from virulent strains of *R. solani*, as described by Finkler et al. (1985), are isometric, with a diameter of 33 nm (Figure 2). The virus particles were purified on a 10 to 40% sucrose gradient and identified in specific fractions by electron microscopy. Virus-associated RNA-dependent RNA polymerase has been detected in most fungal viruses (Buck, 1979; Ratti and Buck, 1979; Ben-Zvi et al., 1984). Virus-associated RNA-dependent RNA polymerase activity was tested in each fraction from the gradient and maximum activity of this enzyme coincided with those fractions containing dsRNA (Figure 3).

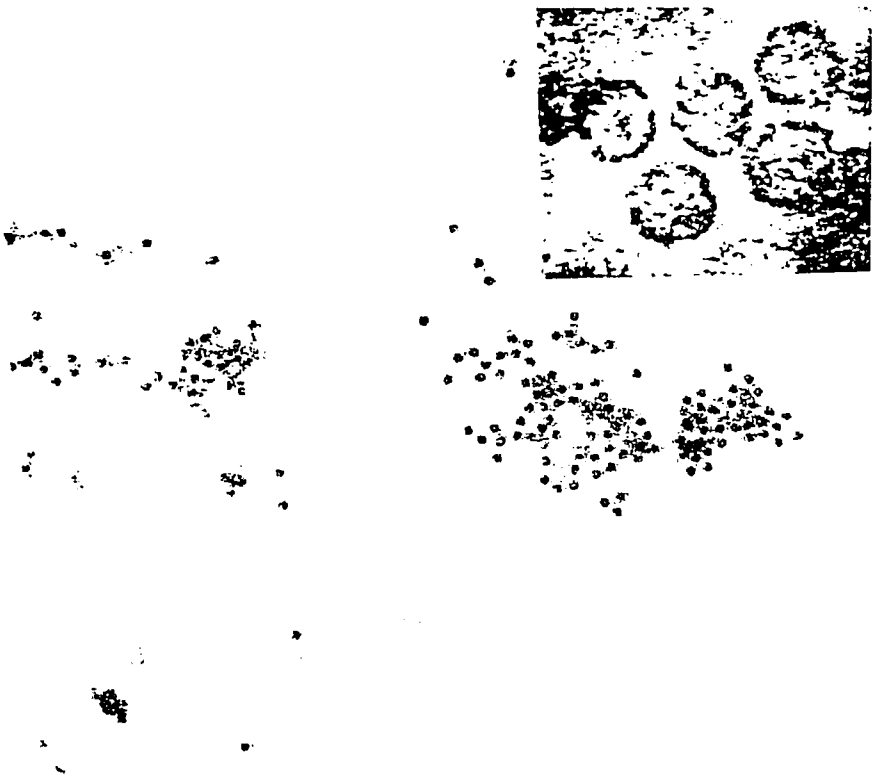
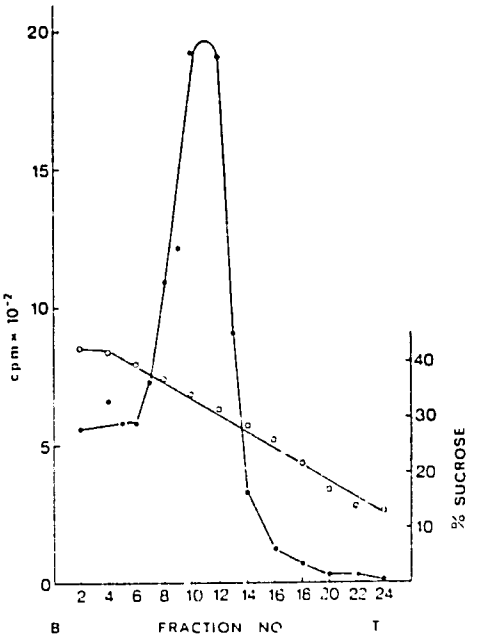


Figure 2 Electron micrograph of negatively stained virus particles purified from a virulent strain of *R. solani*.

Examination of the viral structural proteins on SDS-polyacrylamide gels revealed a single major capsid protein, 54 kDa (Figure 4). The results of in vitro translation of viral transcripts suggested that the additional minor proteins observed in the gel were not encoded by the viral nucleic acids.

Analysis of Viral Transcripts

Full-size transcripts were synthesized by the viral-associated RNA polymerase in reaction mixtures containing [^{32}P]UTP as described by Ben-Zvi et al. (1984). The reaction products were analyzed on a denaturing polyacrylamide gel (Figure 5). The transcripts were 0.74



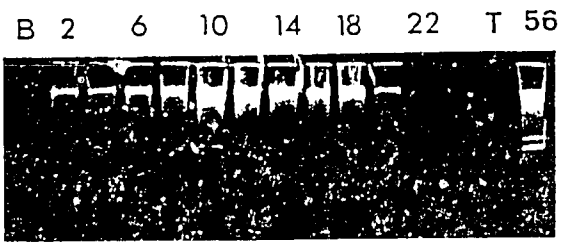


Figure 3 Distribution of RNA polymerase activity of *R. solani* virus and dsRNA content in fractions of a 10 to 40% sucrose gradient. The graph indicates RNA polymerase activity (•) and sucrose density (○) of the fractions. The photograph is an ethidium bromide-stained polyacrylamide gel of dsRNA extracted from each gradient fraction. B and T indicated bottom and top of gradient. The dsRNA extracted from virus particles isolated from strain 56 are shown in the right lane. *Source*: Finkler et al., 1985.

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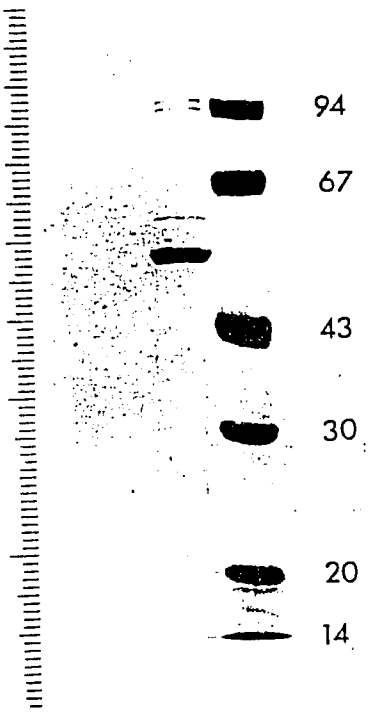

Figure 4 SDS-PAGE of viral proteins.

and 0.66 × 10^6 M.W., corresponding to the encapsidated dsRNA templates with M.W.s of 1.45 and 1.32 × 10^6, respectively. While dsRNA extracted from virus particles contained two segments of 1.45 and 1.32 × 10^6 M.W., the dsRNA segment, 1.6 × 10^6 M.W., detected in the mycelia of a number of virulent strains, was never extracted from the viruses. The relatedness of the 1.6 × 10^6 segment to those encapsidated was examined by hybridization experiments. Labeled transcripts obtained from the viruses were used as probes in hybridization experiments. The results presented in Figure 6 indicate that the transcripts synthesized from strain 82 did not hybridize with the 1.6 × 10^6 segment, thus providing further support to the contention that this segment is not encapsidated.

To determine the relatedness between the different viral isolates, the viruses of one isolate were used for hybridization with other dsRNA

Figure 5 Autoradiography of ^{32}P-labeled virus transcripts subjected to electrophoresis in a denaturing polyacrylamide gel. Transcripts were denatured at 65°C with 5 M urea and electrophoresed in 3% polyacrylamide gel containing 7 M urea at 110 V for 24 hr. *Source*: Finkler et al., 1988.

segments obtained from different isolates. The results indicate that the viruses found among different virulent isolates of *R. solani* that are collected in different geographical locations are all interrelated since they cross-hybridize. In addition, these transcripts were used for hybridization studies with fungal host DNA. The results obtained indicated that no homology exists between host DNA and the viral genome. Therefore, similarly to other fungal dsRNA viruses, no "provirus" state occurs in *R. solani* (Bruenn, 1980; McFadden et al., 1983; Ben-Zvi et al., 1984).

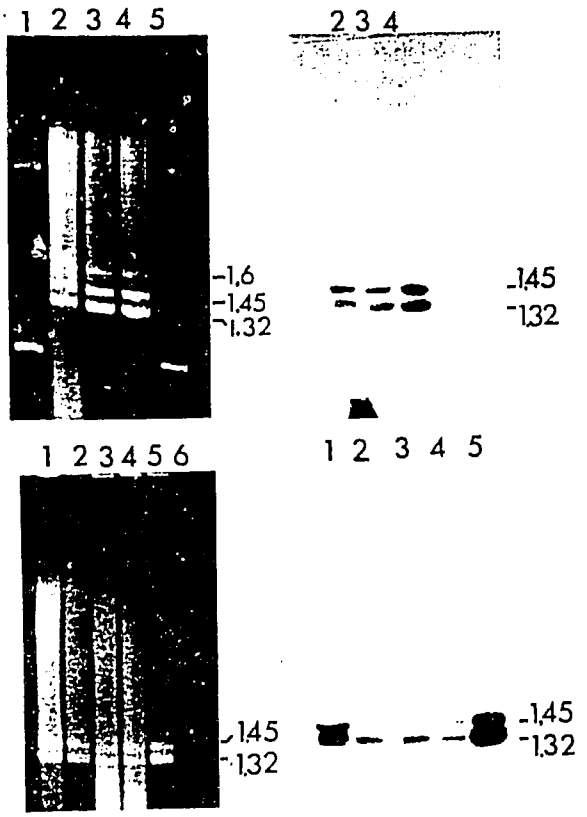

Figure 6 Hybridization experiments: Top, left panel: ethidium bromide staining of dsRNA extracted from strains 82 (lane 2) and 53 (lanes 3, 4) and *U. maydis* P6 (lanes 1, 5). Right panel: autoradiography of ^{32}P-labeled transcript isolated from strain 82 and used as a probe in hybridization with the dsRNA shown in the left panel. Bottom, left panel: ethidium bromide staining of dsRNA isolated from *R. solani* 13 (lane 1), 82 (lane 2), 56 (lanes 3, 4), W (lane 5) and *U. maydis* P6 (lane 6). Right panel: autoradiography of ^{32}P-labeled transcript synthesized from strain 82 used as a probe in hybridization with the dsRNAs shown in the left panel.

Translation Studies of the Viral Genome

To study the coding potential of the viral genome, purified viral transcripts were used for in vitro translation experiments using the rabbit reticulocyte lysate system. The incorporation of [^{35}S]methionine into proteins was analyzed on SDS-polyacrylamide gels. Identical patterns of in vitro translation products were obtained from the transcripts of two virus preparations from strains 13 and 82. Maximum incorporation of label was obtained with 100 μg/ml of purified ssRNA. A major protein, about 55 kDA and a close submajor product of 53 kDa were synthesized in vitro in addition to a number of other "minor" proteins: 47, 45, 43, 40, 38, and 35 kDa (Figure 7). It is not known whether these smaller proteins are the result of premature termination. Extension of the incubation period of translation fourfold did not alter the pattern of these minor proteins. Some of the additional bands de-

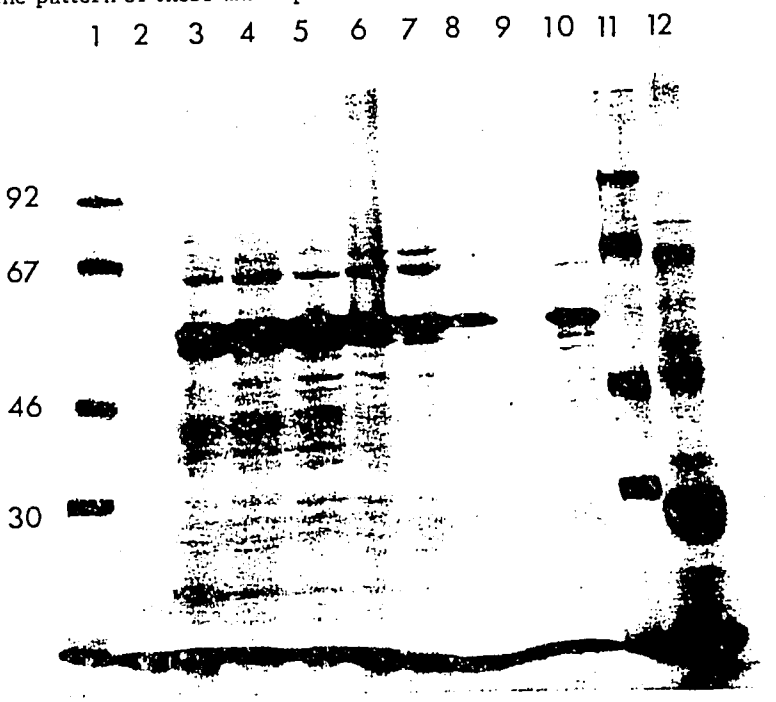

Figure 7 Translation of *R. solani* viral transcript in vitro. Lanes: (1 and 11) [^{14}C]methyl protein molecular weight standards; (2 and 9) in vitro translation products with no added RNA; (3-8) reticulocyte lysate reaction products of transcript RNA (50, 100, 150, 200, 250, 3 μg/ml, respectively); synthesized from strain 13; (10) translation products of transcript synthesized from strain 82 (100 μg/ml); (12) translation of globin mRNA. *Source*: Finkler et al., 1988.

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tected among the products of in vitro translation were also found in control assays lacking RNA and many represent a low level of endogenous translation, nonspecific binding of [^{35}S]methionine to lysate proteins, or short peptides bound to tRNA.

Viral structural proteins were identified by immunoprecipitating the in vitro translation products with rabbit antiserum and with partially purified γ-globulins prepared against purified virus particles. The major protein, 55 kDa, was precipitated in all experiments along with a submajor product, 53 kDa (Figure 8). These results suggest that at least two proteins (55 kDa and 53 kDa) are associated with viral capsids.

The identity of the major product synthesized by in vitro translation of viral RNA was further elucidated by peptide mapping. The patterns of the peptide map of the authentic viral capsid and the major in vitro protein product, analyzed by digestion with *Staphylococcus aureus* V8 protease and examined by electrophoresis on 15% SDS-polyacrylamide gel, were identical (Figure 9). This result indicates that the 55 kDa major protein obtained by in vitro translation is indeed the major capsid protein.

REGULATION OF VIRULENCE

dsRNA Viruses and Virulence

If pathogenicity of *R. solani* is controlled by dsRNA viruses, it should be possible to convert virulent strains to hypovirulence. This conversion should occur simultaneously with the loss of dsRNA. Indeed, hypovirulent strains were recovered by hyphal tip isolation from virulent strains. While the frequency of recovery of hypovirulent strains was about 1%, virulence was never recovered from hypovirulent strains. Loss of total or specific dsRNA segments was associated with the recovery of hypovirulent strains from virulent strains (Figure 1). No direct correlation was made between a specific segment of dsRNA and virulence. Since elimination of the dsRNA segments with M.W.s of 1.25 and 1.225 × 10^6 in strain 82 was also associated with diminishing levels of the 1.45 × 10^6 segment, it may be speculated that a balanced interaction between the various segments is required to regulate virulence.

Further evidence supporting the relation between dsRNA viruses and virulence was provided by cytoplasmic transmission of virulence (Finker et al., 1985). A heterokaryon was formed between compatible strains, one of which (82) was virulent and the other (521) hypovirulent. The two strains differed in their dsRNA content and in their resistance to the fungicides Benomyl and Bay NTN. The virulent strain was Benomyl-resistant but sensitive to Bay NTN, while the hypovirulent strain was resistant to Bay NTN but sensitive to Benomyl. While

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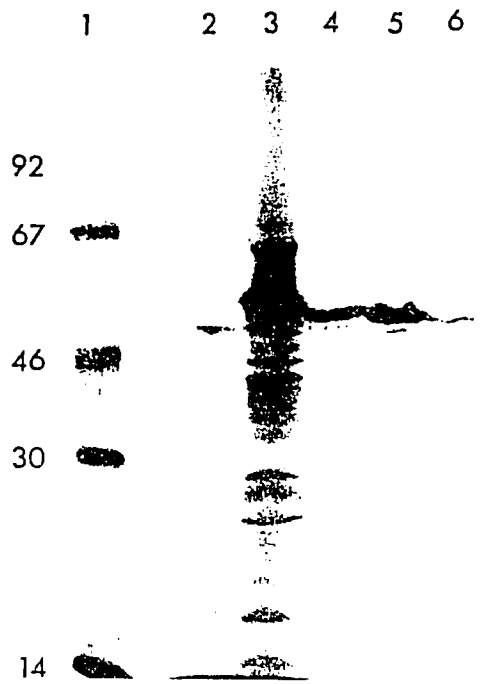

Figure 8 Immunoprecipitation of in vitro translation products with anti-virus antibodies. Lanes: 1: [^{14}C]methyl protein standards; 2: reticulocyte lysate reaction without added RNA; 3: in vitro translation products; 4-6: translation products immunoprecipitated with varying amounts of γ-globulins prepared against virus particles. *Source*: Finkler et al., 1988.

strain 82 contained a number of dsRNA segments, the hypovirulent 521 strain totally lacked dsRNA molecules. Using the differential sensitivities as genetic markers, selection of hypovirulent strains transformed to virulence was performed (Figure 10). The new virulent cultures, Benomyl-sensitive, Bay NTN-resistant, had the morphological characters of the hypovirulent strain 521 (less melanin and producing fewer sclerotia). To determine whether the transformation of the hypovirulent strain to virulence occurred simultaneously with the transmission of the dsRNA viruses, the dsRNA of the transformed strains was extracted. Two segments of M.W.s of 1.45 and 1.32 × 10^6 were recovered from the transformed strains (Figure 11). This pattern of segmentation was identical to the viral dsRNA content of the donor

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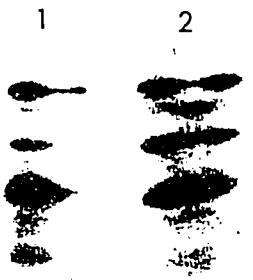

Figure 9 Comparison of peptide map patterns of the major 55 kDa in vitro translation product (1) and authentic capsid protein (2). *Source*: Finkler et al., 1988.

virulent strain. The additional segments, present in the mycelium of the donor virulent strain but not encapsidated, were not detected in the recipient strain. Therefore, unlike the transmissible hypovirulence of *E. parasitica*, in which dsRNA molecules suppress virulence, the dsRNA viruses in *R. solani* appear to be associated with induction of virulence.

Pectolytic Enzymes of *R. solani* and Virulence

Enzymatic activities associated with virulence of *R. solani* are unknown. It is therefore difficult to relate viral proteins to a specific enzymatic activity or to the induction of specific enzymes in the host. However, due to lack of sufficient biological characterization of virulent and hypovirulent strains, a study of pectolytic enzymes in a number of isolates of *R. solani*, as possible markers for virulence, was pursued. The role of pectolytic enzymes in the pathogenesis of *R. solani* has been well established (Weinhold and Motta, 1973). Four enzymes, known as endopolygalacturonase I (endo PG-I) and II, pectinesterase (PE) and endopectinolyase (endo-PL), were purified to homogeneity from a number of *R. solani* isolates (Marcus et al., 1986). The endo-PL was found in all the virulent strains used in this study, but was not detected in any of the five hypovirulent strains examined. Although

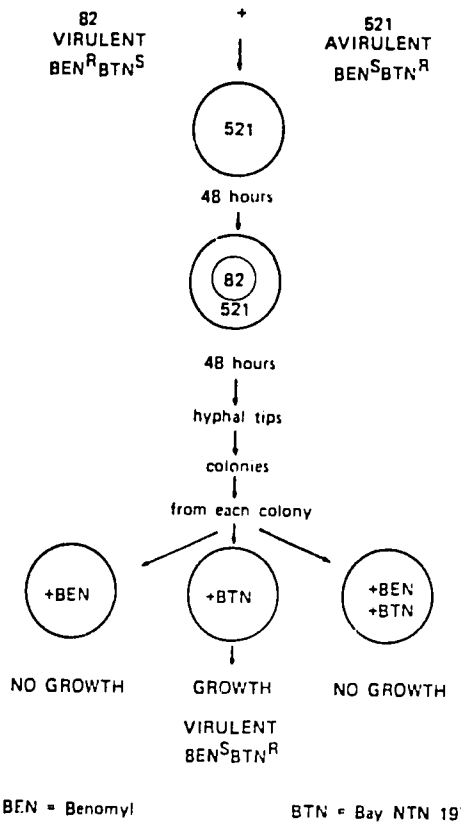

Figure 10 Transmission of virulence by heterokaryon from a virulent to a hypovirulent strain.

the role of endo-PL in the virulence of *R. solani* is supported by other reports in which detection of the enzyme was associated with tissue degradation (Sherwood, 1966), the late production of endo-PL in culture suggested that this activity is essential only in the later stages of infection. The association between endo-PL and transmission of virulence will be studied by cytoplasmic exchange between virulent and hypovirulent isolates. The occurrence of endo-PL activity after transformation of the hypovirulent strains into virulence may indicate a viral-encoded or -induced activity.

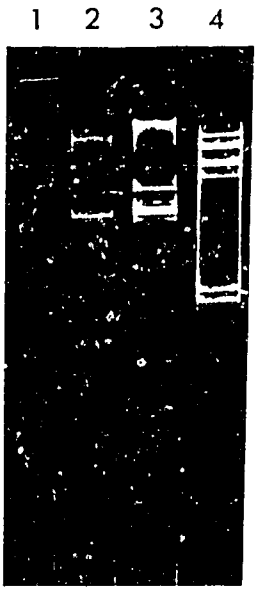

Figure 11 Polyacrylamide gel containing dsRNA isolated from the virulent donor strain (82) and the recipient strain (521) transformed to virulence. Lanes 1 and 2: dsRNA from the two strains "transformed" to virulence. Lane 3: dsRNA from the virulent donor strains. Lane 4: dsRNA molecular weight marker (*U. maydis* P4 virus). *Source*: Finkler et al., 1985.

Biological Control of *R. solani*

Bernard (1909) reported the protection of orchid bulbs against a virulent strain of *Rhizoctonia repens* following inoculation of orchid roots with a weak isolate of the same organism. Similar studies were conducted by Ichielevich-Auster et al. (1985b) to verify whether a similar phenomenon existed in *Rhizoctonia solani* isolates. Indeed, a high degree of protection against damping-off caused by virulent strains of *R. solani* was obtained using hypovirulent isolates. The protection of seedlings of diverse plant species such as cotton, wheat, radish, carrot, and lettuce was effective when the seedlings were infected with a hypovirulent strain prior to a challenge with a virulent strain (Figure 12). Apparently, in an experiment with one hypovirulent strain (521), the damage was reduced by 75-94%. The protection phenomenon was also observed in plants grown in soil infested by the hypovirulent strain and then transferred into soil infested with the virulent isolates. This suggested that the hypovirulent strain was acting

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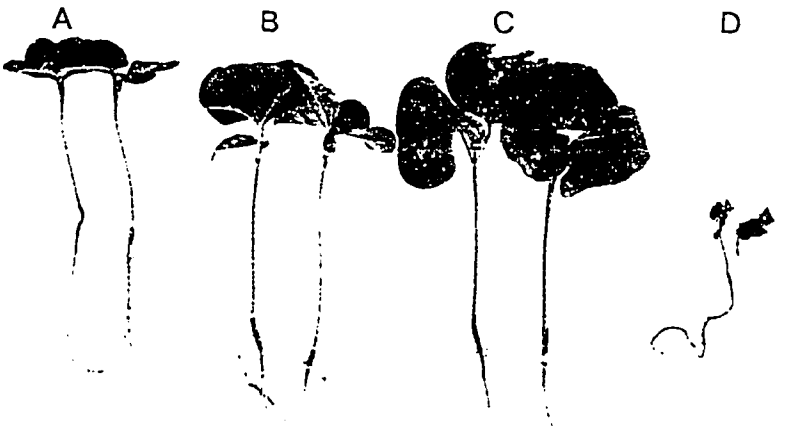

Figure 12 Protection of cotton seedlings by a hypovirulent strain of *R. solani*. A, control, uninoculated; B, inoculated with the hypovirulent strain; C, as B but challenged 3 days later with the virulent isolate, and D, inoculated with the virulent isolate.

in or on the plant. Moreover, cytoplasmic compatibility was probably not essential for protection since a similar effect could be obtained between strains of *R. solani* and *Rhizoctonia zeae*. Therefore, biological control of *R. solani* does not depend only on cytoplasmic exchange, since it can be induced by an isolate from one anastomosis group against virulent strains of different anastomosis groups. Biological control of *E. parasitica* and *R. solani* by hypovirulent strains are probably achieved via different mechanisms. While the cytoplasmic transmissible dsRNA molecules contain some virulence-suppressive information in *E. parasitica*, the dsRNA viruses in *R. solani* may be associated with induction of virulence. The protection of plants by hypovirulent strains of *Rhizoctonia* appears to be in part by induction of a specific defense mechanism. Thus, hypovirulence is not directly related to the dsRNA viruses.

It should be pointed out that the hypovirulent strain used in this study was not diseased and exhibited a normal growth rate which did not differ significantly from virulent isolates, unlike the diseased hypovirulent isolates used by Castanho and Butler (1978). Biological control of *R. solani* by hypovirulent strains was attributed by these authors to transmission of dsRNA molecules, presumed to contain some

suppressive factors. However, our results are different, and the mechanism by which protection is obtained is as yet unresolved. Protection, however, is not induced by competition for nutrients, since repeated addition of nutrients to the host's hypocotyl did not reduce the protective effect. It is unclear whether the hypovirulent strain elicits a natural resistance response, or it creates a barrier against superinfection merely by efficient superficial invasion of the plant tissue. Since hypovirulent strains are known to lack dsRNA viruses, protection could be conferred in addition by prevention of viral transmission. Various mechanisms are known from other systems that prevent this type of transmission. As many as 30 nuclear genes in *Saccharomyces cerevisiae* are involved in the maintenance and expression of dsRNA viruses in yeast (reviewed by Tipper and Bostian, 1984). A single gene mutation in one of the nuclear genes affecting maintenance could be sufficient to prevent viral transmission (Wickner, 1986). Other mechanisms may involve vegetative incompatibility (Burnett, 1975), viral exclusion mechanisms by cytoplasmic factors (Wigderson and Koltin, 1982), defective viruses and specific dsRNA plasmids (Sommer and Wickner, 1982; Field et al., 1982).

Another phenomenon related to the hypovirulent strain was its capability to promote plant growth, which was also expressed in increased yields (Sneh et al., 1986). There is no evidence for stimulation of growth via hormonal regulation by the hypovirulent strain, and enhancement may result from increased area for absorption of nutrients and minerals.

CONCLUDING REMARKS

The occurrence of dsRNA viruses in virulent strains of *R. solani* and their absence from hypovirulent strains are described in this chapter. The results presented are contradictory to those published by Castanho et al. (1978). These authors reported the occurrence of dsRNA molecules in the mycelia of hypovirulent strains only. However, the hypovirulent strain used by Castanho et al. (1978) was debilitated, and therefore the degree of virulence of this strain may have been masked by its diseased state. Indeed, while virulent strains were derived from Castanho's hypovirulent strains in their studies, virulence was never detected among hyphal tip cultures from a number of hypovirulent strains used in our study. Moreover, reexamination of Castanho's strains in our laboratory indicated the occurrence of dsRNA molecules in both hypovirulent and virulent cultures. The differences between these results and those reported in our study may therefore stem from differences in definition of hypovirulence. Hypovirulence as defined in our studies refers to a low level of virulence resulting from the regulation of virulence and not from secondary effects as reduced growth

rate of the debilitated state of such strains. Evidence is also presented correlating induction or regulation of virulence with dsRNA viruses in *R. solani*. With the present state of the art one cannot exclude transmission of other cytoplasmic organelles or plasmids. More direct support should be provided by infection of protoplasts derived from hypovirulent strains with purified virus particles and the conversion of hypovirulent strains to virulent. Infection experiments will exclude other cytoplasmic factors that may confuse the issue since clearly *R. solani* contains a number of cytoplasmic elements in addition to the dsRNA viruses: unencapsidated dsRNA and a DNA plasmid (Hashiba et al., 1984), and perhaps others. The identity of the viral-induced virulence-promoting activity has not yet been elucidated. Indeed, activity of pectinolyase is absent from hypovirulent strains, but it is most probably not the primary and only virulence factor. Further studies will be directed toward resolution of the mechanism that regulates or induces virulence by dsRNA viruses in *R. solani*. Identification of virulence-specific mRNA from the host may permit us to determine the alteration of transcription and translation in hypovirulent strains transformed to virulence.

It appears that fungal pathogens display an array of interactions with the host, ranging from a high degree of virulence to hypovirulence. The former may be a virus-mediated effect and the latter as a form of suppression of viral replication, as in *R. solani*, or an independent fungal effect attenuated by a virus as in *E. parasitica*. Equilibrium in the fungal-plant interaction is maintained by the fungal genome in *R. solani* and by the viral genome in *E. parasitica*.

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Title: The Double-Stranded RNA Viruses of *Rhizoctonia solani* and the Regulation of Plant Pathogens.

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I. Introduction

The fungal plant pathogen *Rhizoctonia solani* causes severe damage to a large number of hosts and is a severe pathogen throughout the world. By screening many isolates of *R. solani* for virulence a number of studies indicated the occurence of both virulent and avirulent strains in the natural population. A double-stranded RNA virus has been recently isolated from strains of *R. solani*. The viral genome is composed of two segments of dsRNA with mol. wts. of 1.45 and 1.32 x 10^6 (2.2 kb and 2 kb, respectively). An additional dsRNA molecule of 1.6 x 10^6 (2.4 kb) is found in many strains by extraction of mycelium but is not recovered from virions. The major capsid protein is 55,000 daltons. A RNA-dependent RNA polymerase is associated with the viral capsids and is clearly a transcriptase.

Two full-size transcripts with mol. wts. of 0.74 & 0.66 x 10^6 (2.2 kb and 2 kb, respectively) were synthesized by the virus-associated RNA-dependent RNA polymerase and resolved by denaturing polyacrylamide gel electrophoresis. The transcripts cross-hybridized to the viral dsRNA isolated from a number of strains. The transcripts do not hybridize with the genomic DNA. An uncapsidated species of dsRNA with molecular weight of 1.6 x 10 did not hybridize with the viral transcripts. No cross-hybridization between the two viral dsRNA segments was obtained. The viral encoded proteins were studies by *in vitro* translation using the rabbit reticulocyte lysate system. The

transcripts served as mRNA for the synthesis of the major capsid protein with a mol. wt. of 55,000 daltons, and a number of other products. The viral-coat protein was immunoprecipitated with antibodies against purified virus particles. Partial proteolysis on the major *in vitro* product and the authentic capsid protein using *Staphylococcus aureus* V8 protease produced similar peptide patterns. Denatured viral dsRNA also directed *in vitro* the synthesis of identical proteins to those translated from the transcripts. By stringent tests to clearly distinguish between virulent and hypovirulent strains of the pathogen, a clear correlation was established between the presence of viral information and virulence (Finkler *et al.* 1985). Hypovirulent strains were found to be devoid of viruses and all the virulent strains examined contained dsRNA viruses. The relation between the dsRNA viruses and virulence was further examined in attenuated strains and a correlation was established between the loss of virulence and either the total loss of dsRNA segments or the loss of specific segments. Furthermore, transmission of dsRNA from a virulent to an avirulent compatible strain by cytoplasmic exchange resulted in the transmission of virulence. These results suggested that dsRNA viruses may be associated with the regulation or production of virulence factors. The analysis of the virus-encoded proteins is therefore significant for the elucidation of nonstructual proteins associated with the regulation of virulence.

II. Isolation of a plasmid DNA in virulent strains of R. solani.

Rhizoctonia solani is a soil-borne plant pathogenic fungus with a broad spectrum of virulence. A number of reports attempted to correlate the relation between phytopathogenicity and presence of dsRNA viruses (Castanho et al., 1984, Finkler et al., 1985 and Zanzinger et al., 1985) and DNA plasmid (Hashiba et al., 1984). We have reported the presence of dsRNA viruses in virulent strains of R. solani, but complete absence of these viruses in hypovirulent strains. Both attenuation and transmission of virulence were correlated with loss and transmission of dsRNA viruses, respectively (Finkler et al., 1985). However, following the report of Hashiba et al. (1984) in which a linear DNA plasmid with a mol. wt. of 1.68×10^6 was detected in weakly pathogenic strains of R. solani, we have started, too, a search for a DNA plasmid in virulent strains of R. solani. The role of such a plasmid may extend beyond the regulation of virulence. The DNA plasmid may also serve as a vector in transformation of R. solani. Characterization of the plasmid DNA by restriction analysis is required to asses the options with respect to the insertion of selected sequences that may serve as markers for transformation.

Isolation of DNA plasmid

DNA plasmid was extracted from mycelia of strain 13 with Novozyme (10 mg/ml in 0.9 M sorbitol, 0.1 M EDTA, 0.1 M sodium citrate pH 5-6). The mycelia was shaken 1-2 hrs at 30 C. The extract was treated with 50 mg/ml proteinase K and 1% SDS for 1 hr at 37 C and was kept on ice for 1 hr after the addition of potasium-acetate to 1 M. Following centrifugation the supernatant was ethanol-meci-precipitated.

Purification of plasmid nucleic acids: By phenol:chloroform extraction and ethanol precipitation. The plasmid nucleic acids was subjected to 1% agarose gel electrophoresis. The gel was stained with ethidium bromide and visualized under uv light. The plasmid bands were cut and elutted from gel by phenol treatment at -70 C and ethanol precipitation.

Characterization of plasmid nucleic acids

a) Treatment with RNase and DNase. The plasmid disappeared when treated with DNase (10 mg/ml, 15 min at 0 C) but was resistant to the RNase treatment (10 mg/ml, 15 min at 37 C).

b) Structure and size of plasmid DNA. In electron microscopy the DNA plasmid isolated from *Rhizoctonia* strain 13 and purified from agarose gel, were found to consist of homogeneous populations of linear DNA. No circular molecules were detected.

The size of the linear molecules of plasmids was determined by measuring their length relative to the length of open circular PBR-322, which was used as an internal standard in the same spreading. The length of the DNA was ca. 2,700 bp.

c) The molecular weight of the plasmid DNA was also estimated on the basis of mobility by electrophoresis in comparison with linear DNA fragments obtained from Hind III digestion of DNA. The molecular weight of the plasmid was 1.58×10^6 .

d) Digestion of the DNA with restriction enzymes Hind III and Eco RI resulted in 2 bands on agarose gel electrophoresis, suggesting only one restriction site existed on the linear DNA for each of the enzymes. No digestion was obtained with BAM HI and PSTI enzymes. A restriction analysis is currently performed.

e) No plasmid DNA was detected in the hypovirulent strain (521).

III. In vitro translation of purified ds-RNA segments.

In vitro translation of virl ss-RNA transcripts was reported by Finkler *et al* (1985). The proteins synthesized *in vitro* by total dsRNA extracted from the mycelia of a virulent strain (*R. solani* 13) has been also reported by Finkler *et al* (1988). We have been lately engaged with the separation and purification of dsRNA segments (1.32 & 1.45 x 10^6 dalton), in order to direct the synthesis of proteins *in vitro*.

A number of methods have been used to extract dsRNA from agarose gels: the BND-cellulose-technique (Sedat *et al*., 1967), electro-elution in dislysis tubes and phenol extraction, electro-elution with DEAE-cellulose paper (Dretzer *et al*., 1981) and rapid phenol extraction at -70 C (Benson and Zagursky, Per. Comm.). The latter technique produced the highest yields of recovery. Moreover, this method avoided the high-salt elution step which greatly hindered translasion unless great care was taken in washing and removal of salts (on G-100 columns).

Denaturation of dsRNA strands is the most critical step in translation *in vitro*, since undenatured dsRNA significantly inhibits protein synthesis. We have tried both heating at 100 C for 90 sec with 1 mM EDTA and incubation with 10 mM Methyl Mercury Hydroxide for 20 min at room temperature as the denaturation procedures; the latter produced best results. We have used the rabbit reticulocyte lysate *in vitro* translation

system (provided by New England Nuclear and Promega) using ^{35}S-methionine as the amino-acid label. *In vitro* translation products were run 10% PAGE-SDS gels and the proteins were identified by autoradiography. The results indicated that the viral capsid protein (55kd) is encoded by the dsRNA segment with mol. wt. of 1.32×10^6. It is yet unclear what is the coding role of the 1.45×10^6 dsRNA segment.

IV. Research plan

We will concentrate for the next few months on further characterization of the DNA plasmid, restriction mapping and screening of additional virulent and hypovirulent strains of *R. solani* for presence of plasmid DNA. The relation between virulence and plasmid DNA, if any, will be analyzed.

Purified dsRNA segments will also be used to direct synthesis of proteins *in vitro* using the wheat germ system and other labelled amino acids. It is possible that labelled methionine is not incorporated extensively in the viral encoded proteins. The wheat germ, system may also overcome problems caused by high level of endogenous protein synthesis obtained in the reticulocyte lysate.

We are, at present, engaged in experiments of curing the virulent strains of the dsRNA viruses. If curing will result in conversion of virulent strains to hypovirulent, as we anticipate, we will be able to obtain isogenic strains with similar genetic background, but differ in their virulence characters. This is an important step for future studies of the virulence factors, which will enable us to pick up virulence-associated mRNA sequences.

Construction of a genomic DNA library of R. solani is also planned. The libraries of R. solani will serve for studies on the biology and virulence of this pathogen, as it will allow the selection of genes of interest as those relate the expression of virulence by the use of virulent and hypovirulent strains.

V. Description of cooperation

Prof. Ofuso-Asiedu visited once again our laboratory in October 1987 to further discuss and communicate our views regarding the work which has been initiated in Ghana. In the Forest Research Institute in Kumasi, Ghana, a system for distinguishing virulent and hypovirulent strains of the wood-rotting fungus, *Pseudophaeolus bandorii*, is being developed. The strains are being classified with respect to their level of virulence. These strains will be used to test for biclogical control of the disease caused by *Pseudophaeolus* affecting the forest trees in Ghana. At a later stage these isolates will be examined for the presence of dsRNA and virions in an effort to test the relations between virulence and these viruses. Prof. Koltin from Tel-Aviv University has visited Ghana to asist Prof. Asiedu group with the molecular biology aspect of the study.

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I. Summary

Rhizoctonia solani is a plant pathogenic fungus which causes damping off to a large number of hosts. Virulent strains, but not hypovirulent, contain double-stranded RNA viruses. We have already reported some evidence suggesting a correlation between virulence and the dsRNA viruses in *R. solani*. Elucidation of the viral-encoded proteins which may have a role in the regulation or induction of virulence was undertaken.

Translation *in vitro* of full-size transcripts is described. The viral capsid protein was identified by immunoprecipitation with anti-viral antibodies and peptide mapping using *Staphylococcus aureus* V8 protease. In addition to the four viral structural proteins, 2-4 non-structural proteins were synthesized, but their role has not yet been resolved. The relatedness of viruses from in different strains of *R. solani* was studied by hybridization of ^{32}P-labelled transcripts to dsRNA extracted from the mycelia of different isolates. The viruses appeared interrelated.

The relation between virulence and dsRNA viruses should be further substantiated by cytoplasmic exchange between genetically marked virulent and hypovirulent strains, and transformation of hypovirulent cells into virulence by direct infection with a viral suspension. The preparation of protoplasts, reported here, is required for both induction of auxotrophic mutants and viral

infection. Protoplasts were prepared by digestion of young mycelia with Novozyme at 27 C for 3-4 hrs. The concentration of both enzyme and osmotic stabilizer were optimized.

The cooperation with Dr. A. Ofuso-Asiedu from the Forest Research Institute, Kumasi, Ghana, is described. Application of techniques Dr. Ofuso-Asiedu has aquired durring his visit to our laboratory, to the study of virulence of tropical fungal pathogens may provide a solution for eradication of tree diseases of utmost economical importance in Ghana.

II Transcription and *In Vitro* Translation Studies of the Virus Isolated from *Rhizoctonia solani*.

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ABSTRACT

A segmented double-stranded RNA virus, with mol. wts. of 1.45 & 1.25 x 10^6, has been isolated from virulent strains of *Rhizoctonia solani*. Full-size transcripts with mol. wts. of 0.74 & 0.66 x 10^6 were synthesized by the virus-associated RNA-dependent RNA polymerase. The two transcription products, resolved by denaturing polyacrylamide gel electrophoresis, appear to be complete transcripts of one strand of the respective templates. The transcripts cross-hybridized to the viral dsRNA isolated from a number of strains. The viral-encoded proteins were studied by *in vitro* translation using the rabbit reticulocyte lysate systems. The transcripts served as mRNA for the synthesis of the major capsid proteins with a mol. wt. of 55000 daltons, and a number of other products, also synthesized on denatured dsRNA. The viral-coat proteins were immunoprecipitated with antibodies against purified virus particles. Partial proteolysis of the major *in vitro* product and the authentic capsid protein using *Staphylococcus aureus* V8 protease produced similar peptide patterns.

INTRODUCTION

Rhizoctonia solani is a plant pathogenic fungus which causes severe damage to a large number of hosts (Baker, 1970). Screening of a large number of isolates of *R. solani* for virulence indicated the occurence of both virulent and avirulent strains in the natural population (Ichielevich *et al.*, 1985). A double-stranded RNA virus has been recently isolated from virulent strains of *R. solani* (Finkler *et al.*, 1985). Avirulent strains tested were devoid of viral particles. The viral genome is composed of two segments of dsRNA with mol. wts. of 1.45 and 1.25 x 10 , and the major capsid protein is 55000 daltons. A RNA-dependent RNA polymerase is associated with the viral capsids.

The relation between the dsRNA viruses and virulence was examined in attenuated strains and a correlation was established between the loss of virulence and the total loss of dsRNA segments or the loss of specific segments. Furthermore, transmission of dsRNA from a virulent to an avirulent compatible strain by heterokaryon, resulted in the transmission of virulence. These results suggested that dsRNA viruses may be associated with the regulation or production of virulence factors. The analysis of the virus-encoded proteins may therefore be important for the elucidation of nonstructual proteins associated with the regulation of virulence.

In vitro translation of virus-specific mRNA has been extensively used in other dsRNA viral systems to elucidate the coding potential of the viral genome (Inglis *et al.*, 1977; McCrae & Jokliks, 1978; Paucha *et al.*, 1978; Bruenn *et al.*, 1980 and McCrae & Mertens, 1983). *In vitro* translation of denatured dsRNA has also been reported in a number of dsRNA viruses (Hopper *et al.*, 1977; McCrae & Jokliks, 1978; Bostian *et al.*, 1980; Mertens & Sangar, 1984; Dalton *et al.*, 1985). It has been shown that the positive strand of the dsRNA genome can be translated *in vitro* provided that the two strands are separated.

In this report we have applied these techniques to the virus of *R. solani* to obtain some information on the proteins encoded by the viral genome. We have used the viral-specific mRNA, synthesized *in vitro* by the virus-associated RNA-dependent RNA polymerase, and unfractionated dsRNA, to direct the synthesis of proteins in rabbit reticulocyte lysate systems.

METHODS

Strains: *R. solani* cultures were maintained on potato-dextrose agar at 27 C. The virulence of each strain was determined by the plate assay described by Castanho & Butler (1978b).

Purification of virus particles and dsRNA extraction: The techniques described by Finkler *et al.* (1985) were followed to purify virus particles and to extract dsRNA.

Elution of dsRNA: Purified dsRNA was eluted from 1% agarose gels with BND-cellulose following the procedure described by Silhavy et al. (1984).

DNA isolation: Mycelia grown overnight at 27 C was treated with 10 mg/ml Novozyme in 0.6M mannitol (pH 5.8) by shaking 1-2 hrs at 30 C. Undigested mycelia were removed by centrifugation and the extract was treated with 100 μg/ml proteinase K and 1% SDS for 1-2 hrs at 37 C followed by phenol-extraction and ethanol precipitation.

Synthesis of viral transcripts: Full size transcripts were synthesized in 500 μl reaction mixtures containing 1 mM of each of the 4 nucleoside triphosphate and 1.6 mg/ml bentonite to inhibit RNase activity. The reaction mixture was incubated for 24 hrs at 30 C, essentially as described by Ben-Zvi et al. (1984). Labelled transcripts were synthesized in the presence of $2 \times 10^{5}-10^{6}$ cpm/ml of [^{32}P]-UTP. The conditions for the RNA-dependent RNA polymerase activity were optimized for the Rhizoctonia virus. A pH optimum of 7 and a Mg concentration of 3 mM were found to improve transcription. After incubation, the reaction mixtures were extracted with phenol-chlorophorm (1:1) and the nucleic acids were ethanol-precipitated. Purification of the ssRNA transcripts on CF11 columns (Franklin, 1966) was followed by ethanol precipitation and several washes with 70% cold ethanol.

Analysis of viral transcripts; Size determination of viral transcripts by electrophoresis was performed in 3% polyacrylamide gel in the presence of 7M Urea as described by Marzluff & Huang

(1984) with few modifications: ^{32}P-labelled transcripts with a specific activity of 5 x 10^5 cpm/μg RNA were denatured by heating at 65 C for 6 min in TBE buffer containing 50 mM-Tris, 50 mM-boric acid, 50μM-EDTA to which 5M Urea, 10% glycerol, 0.05% bromophenol blue and 0.05% xylene cyanol were added. The samples were immediately electrophoresed for 24 hrs in a vertical 12x 23 cm gel at 110 volts using TBE buffer and analyzed by autoradiography of the dried gel.

RNA blot hybridization analysis: Ds-RNA samples were electrophoresed on horizontal 1% agarose gel in MOPS buffer containing 1mM-EDTA, 5 mM-sodium acetate and 20 mM-Na-MOPS (pH 7.5) for 3 hrs at 110 volts. Treatment of the gel prior to the transfer to nitrocellulose followed the procedure provided by J. Bruenn (personnal communication). The blot was prepared as described by Southern (1975). ^{32}P-labelled transcripts were used as probes for hybridization analysis following the procedure provided by J. Bruenn (personnal communications). Blots were washed with as described by Thomas (1980).

Southern blot analysis: Hybridization of P-labelled transcripts to DNA followed the procedure described by Southern (1975).

Denaturing of DsRNA: DsRNA (1 μg) was precipitated overnight with ethanol at -20 C and washed twice with 70% ethanol. The dried sample was resuspendend in 2 μl distilled H_2O containing 1 mM-EDTA, incubated 90 sec at 100 C and immediately cooled in ice water and subjected to *in vitro* translation.

In vitro translation: Purified transcripts or denatured dsRNA were translated in a rabbit reticulocyte *in vitro* translation system (Amersham, U.K. and Orgenics Ltd, Israel). Translation was carried out in proportions of 8 and 6 volumes of lysate respectively, 1-2 volumes of RNA and 0.8-1.5 volume of [^{35}S]-methionine (0.8-1.5 μci/10μl), and incubated at 30 C for 60-80 min.

Incorporation of the label into total proteins synthesized was determined by spotting 2 μl aliquots on GF filter discs and assaying for trichloroacetic acid precipitable radioactivity. The *in vitro* synthesized proteins were electrophoresed in 10% SDS-polyacrylamide gels using the buffer system of Laemmli (1970). The proteins were detected by fluorography using Amplify (Amersham Ltd. U.K.).

Preparations of immune serum: Virus particles isolated from strain 13 were purified on 10-40% sucrose gradients, dialyzed against buffer G containing 50 mM-Tris-HCl pH 7.5, 0.1 mM-EDTA, 40 mM-NaCl, 10 mM-$MgSO_4$, 4 mM-2-mercapthoethanol and 10% glycerol, and pelleted at 133000g for 4 hrs. The resuspendend pellet was loaded on a second sucrose gradient and the procedure was repeated two more times. New-Zealand white rabbits were immunized intradermally with 120 μg of purified virus preparation emulsified with an equal volume. of Freund's complete adjuvant. Injections were repeated 21 days and 42 days later, with 60-100μg virus preparation. The rabbits were bled from the ear vein 10 days after each boost. The gammaglobulins were purified from the serum by ammonuim sulfate precipitation and by dialysis against

PBS buffer containing 2 mM-NaH_2PO_4, 10 mM-Na_2HPO_4 and 150 mM-NaCl, as described by Palmiter et al. (1971). Finally, the gammaglobulin preparation was tested by double-diffusion Ochterlony assays (Ochterlony, 1962) against purified virus particles containing 1% SDS.

Immunoprecipitation assay: The in vitro translation products of R. solani transcripts and denatured dsRNA were analyzed by immunoprecipitation as described by Segev et al. (1985), using the rabbit antiseum and immunogammaglobulins prepared against purified viral particles.

Peptide mapping: Peptide mapping of proteins isolated from SDS-polyacrylamyde gels followed the procedure described by Cleveland (1983). Both hydrated and dried gel slices were digested with Staphylococus aureus V8 protease (1μg) and fractionated on 15% SDS-polyacrylamide gels.

RESULTS

Analysis of viral transcripts

One viral transcript was resolved in earlier studies(Finkler et al., 1985). The size of that transcript was estimated as 0.75 x 10^6 daltons. Determination of the number and size of the viral transcripts was essential in order to asses the viral informational content and in vitro translation experiments. Full-size transcripts were synthesized in the presence of [^{32}P]-UTP and the products were analyzed on denaturing polyacrylamide gel (Fig. 1). An additional transcript was

detected. The mol. wts. of the transcripts were 0.74 and 0.66 x 10^6 dalton corresponding precisely to the viral dsRNA templates of 1.45 and 1.25 x 10^6, respectively. Each transcript appears, based on its size, to be a complete transcript of one strand of the corresponding template.

Relatedness of viral isolates

The transcripts were used as probes in hybridization experiments with dsRNA extracted from the mycelia of a number of virulent strains from various localities. All isolates contained two dsRNA segments with mol. wt. of 1.45 and 1.25 x 10^6. Some contained an additional segment with mol. wt. of 1.6 x 10^6 (strains 53 and 82). However, the dsRNA purified from virus particles contained only the segments with mol. wts. 1.45 & 1.25 x 10^6, and from such preparations the heavy dsRNA band (1.6 x 10^6) was never recovered. The viral transcripts were used as probes to resolve the relation of the heavy segment of dsRNA to those found encapsidated in viral particles and to determine the relatedness of the various viral isolates.

As shown in Fig. 2, the transcripts hybridized to the dsRNA segments with mol. wt. of 1.45 & 1.25 x 10^6 extracted from mycelia of four isolates. Transcripts of one viral isolate hybridize to segments of all isolates. These results confirm that the viruses found in the different strains of Rhizoctonia are interrelated.

The non-encapsidated dsRNA segment with mol. wt. of 1.6×10^6 appears to be not related to the viral genome since there is no homology between this segment to the transcripts. Moreover, no homology was detected between the genomic DNA of the host and viral transcripts, as seen by a Southern blot hybridization. Therefore, similarly to other dsRNA viruses of fungi the *R. solani* virus has no 'provirus' state.

In vitro translation of viral transcripts

To study the coding potential of the viral genome, purified ssRNA transcripts were used for *in vitro* translation experiments. Maximum incorporation of [^{35}S]-methionine into proteins was obtained with 100 μg/ml purified ssRNA obtained from viruses isolated from two strains, 82 and 13. The products of the *in vitro* translation assays were analyzed on SDS-polyacrylamide gels (Fig. 3). Identical patterns of *in vitro* translation products were obtained from the transcripts of the two virus preparations. A major protein with mol. wt. of about 55K was synthesized *in vitro* in addition to a number of 'minor' proteins with mol. wts. of 47K, 45K, 43K, 40K, 38K and 35K daltons. Extension of incubation period of translation to 4-fold did not alter the pattern of these minor proteins. A number of additional proteins found among the products of the *in vitro* translation were not virus-encoded. These proteins were either found in control assays lacking RNA or may represent short peptides still bound to tRNA.

Rabbit antiserum and partially purified gammaglobulins prepared against purified virus particles were tested in double-diffusion Ochterlony assays. A single precipitin band was obtained between antiviral antibodies and the virus particles. These antibodies were used to immunoprecipitate the [^{35}S]-methionine labelled *in vitro* translation products of the viral transcripts (Fig. 4). The major protein with a mol. wt. of 55000 dalton was precipitated in all experiments along with a close sub-major product with a mol. wt. of 53000. Two other proteins with mol. wts. of 47K & 45K were also faintly immunoprecipitated. These results suggest that two proteins, with mol. wts. of 55K and 53K daltons, are associated with the viral capsids. The identity of the other products as non-structural proteins will be discussed later. It appears also that the capsids of the viruses isolated from two different strains are similar since both have the same mol. wts. and are immunoprecipitated by antibodies prepared against one of the viruses.

The major labelled protein band obtained by *in vitro* translation on SDS-polyacrylamide gels and located by autoradiography, was excised and hydrolyzed with *Staphylococcus aureus* V8 protease. The authentic virus capsid was similarly treated. The fractionated peptides, obtained by electrophoresis on 15% SDS-polyacrylamide gels were similar (Fig. 5). The major protein with a mol. wt. of 55K daltons is therefore the capsid protein.

In vitro translation of dsRNA

Total dsRNA, purified on CF11 columns, and dsRNA eluted from agarose gels, containing either 2 segments (1.45 & 1.25 x 10^6) or 3 segments (1.6, 1.45 & 1.25 x 10^6) were used for *in vitro* translation (Fig. 6). Identical products were obtained by the translation of the different dsRNA preparations. This may result from contamination of the dsRNA preparation containing 2 segments with the 1.6 x 10^6 segment during elution. It could also be argued that the total dsRNA preparation contains some proportion of unterminated strands which would lead to generation of non terminated products. These would interfere with the identification of viral-encoded non-structural proteins. The eluted dsRNA preparation is most probable composed of terminated strands and thus allow a better analysis of the results. The fact that no differences were found between total and eluted dsRNA suggests that the proteins are synthesized from full-sized strands. The 'minor' proteins with mol. wts. of 43K, 40K & 38K daltons appear therefore to be non-structural proteins. An additional protein with mol. wt. of 60K daltons was not found in the translation of transcript but was synthesized by total dsRNA. Moreover, it was not immunoprecipitated by the anti-virus antibodies. It could be the product of the 1.6 x 10^6 dsRNA segment, which is not virally encapsidated.

DISCUSSION

R. solani viral transcript was found to act as an efficient messenger for in vitro translation. Similar studies could be made with unfractionated dsRNA provided that the strands were first separated by denaturation in H_2O containing 1 mM-EDTA at 100 C and followed by rapid cooling. Analysis of the ssRNA products of the RNA-dependent RNA polymerase reaction on denaturing polyacrylamide gels indicated the existence of two full-size transcripts, as anticipated from the size of the dsRNA segments.

The major in vitro translation product of the viral messenger or denatured dsRNA, with a mol. wt. of 55000 daltons, has been shown to correspond to the viral capsid protein by a number of criteria: (a) the capsid protein co-migrated with the major in vitro translation product, (b) this product was immunoprecipitated by antibodies prepared against virus particles and (c) identical peptide mapping patterns were obtained following limited proteolysis. The identity and activity associated with the other proteins is yet unresolved.

The total coding potential of the dsRNA genome is estimated at 150,000 daltons (80K for the 1.45 x 10^6 segment and 70K for the 1.25 x 10^6 segment). However, the total mol. wts. of the proteins obtained by in vitro translation exceeded the coding capacity of the genome. If this is true, the discrepancy could be explained as follows: (a) some products may represent non-terminated proteins, (b) genes may overlap (c) additional non-resolved dsRNA segments may exist. The latter possibility is strengthened by latest unpublished results in which prolonged

polyacrylamide electrophoretic runs enabled the resolution of an additional dsRNA segment with mol. wt. of 1.3×10^6. It is possible that longer runs are required , too, to resolve additional transcripts.

The coding assignments for the purified genome segments of the Rhizoctonia virus will be worked out in the following studies.

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LEGENDS TO FIGURES

Figure 1 Autoradiography of P-labelled transcripts. Full-size ^{32}P-labelled transcripts were denatured as described in the Methods and run on 3% polyacrylamide gel containing 7M Urea. Rabbit reticulocyte rRNA(28S & 18S) molecules, used as molecular weight markers, were treated as above and vizualized by ethidium bromide staining.

Figure 2 Northern blot hybridization of dsRNAs. The Northerns were performed by transfers onto nitrocellulose of dsRNAs separated on 1% agarose gels and stained with ethidium bromide (a). ^{32}P-labelled transcript isolated from strain 13 was used as probe in hybridization with dsRNAs isolated from R. solani strains 13, 82, 56 and W and U. maydis 75 (b). A transcript isolated from strain 82 was used as probe for hybridization with R. solani strains 53 and 82 and U. maydis 75 (c).

Figure 3 Translation of R. solani viral transcripts in vitro. Each reaction mixture (10 µl) was diluted with 2 x Laemmli sample buffer, incubated 3 min at 100 C and analyzed on 10% SDS-polyacrylamide gel and fluorography. Lanes: 1, [^{14}C]-methyl labelled protein molecular weight standards; 2 reticulocyte lyase reaction without added RNA., 3-10, translation products of transcript RNA (50, 100, 150, 200, 250, 300, 400, µg/ml respectively), 12, products of globin mRNA.

Figure 4 Immunoprecipitation of in vitro translation products with anti-virus antibodies.
Lanes: 1, [^{14}C]-methyl-labelled protein standards, 2, reticulocyte lysate reaction without added RNA, 3, in vitro translation products of viral transcript (10µl reaction), 4-6, translation products immunoprecipitated with 10 µg gammaglobulins prepared against virus particles. All reactions were previously treated with 5 ul normal rabbit serum and a

10% fixed suspension of *Staphylococcus aureus* to remove non-specific binding antigens.

Figure 5 Comparison of peptide map patterns of the major *in vitro* translation product and capsid protein. Gel slices were treated with 1 μg *Staphylococcus aureus* V8 protease and electrophoresed on 15% SDS-polyacrylamide gel. 1, autoradiograph describing peptide mapping of the major *in vitro* translation product; 2, peptide mapping of authentic capsid protein virualized by Coomassie Blue staining.

Figure 6 (a) *In vitro* translation products of unfractionated denatured dsRNA. Reaction mixtures (10 μl) were run on 10% polyacrylamide gel and analyzed by fluorography.

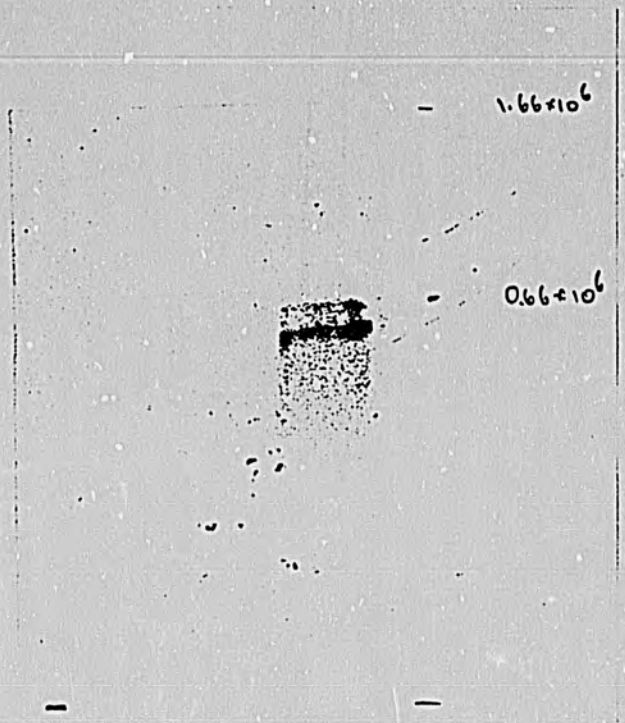

Fig. 1

(a)

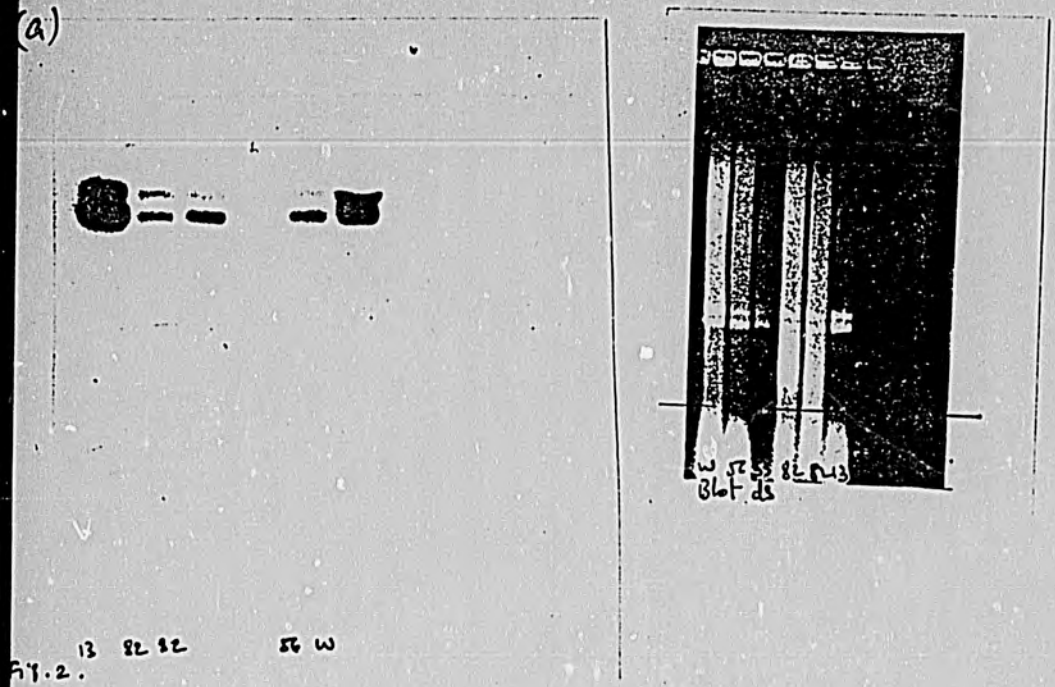

Fig. 2. 13 S2 S2 S6 W

(b)

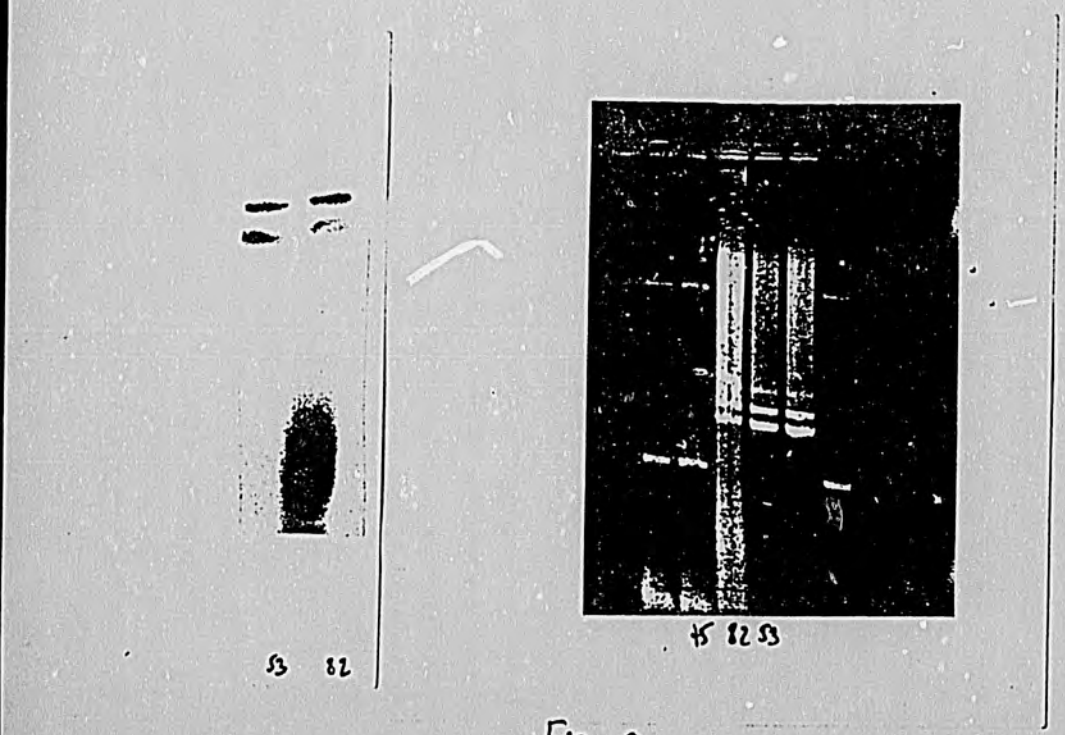

Fig 2

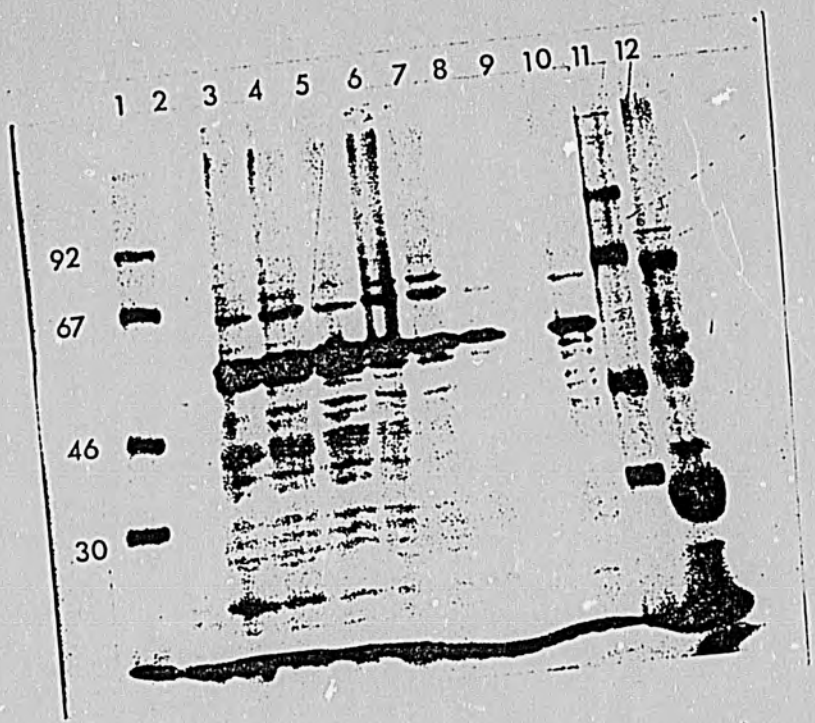

Fig 3

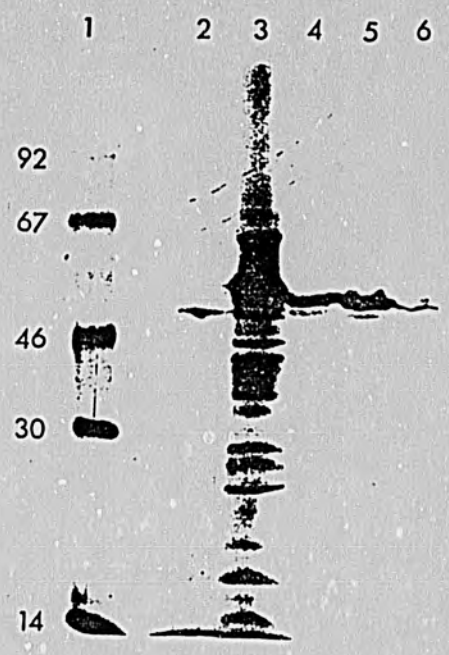

Fig 4.

Fig. 5.

Fig 6

III. Preparation of Protoplasts

The need to isolate protoplasts emerged from the need to develop techniques required to overcome the difficulties encountered with genetic manipulations in *R. solani*. These difficulties have arisen because of certain properties of *R. solani*, such as a tough cell wall, lack of a sporulation phase on artificial media and multinucleate hyphal cells. Recently an increasing number of research workers have become interested in using protoplasts obtained from fungal cells for physiological, biochemical and genetic studies of fungi (Anne & Peberdy, 1976; Kevi & Peberdy, 1977).

The introduction of an effective method for obtaining protoplasts from *R. solani* may be of a great advantage for further studies. protoplasts can be used for distinguishing between multinucleate hyphal cells and cells containing a single nucleus. These protoplasts can be subsequently exposed to mutagenes for induction of auxotrophic mutants (Hashiba & Yamada, 1984). Genetically marked strains can be used for cytoplasmic exchange experiments between virulent and hypovirulent isolates of *R. solani*. The genetic markers will facilitate the identification of "transformed" hypovirulent strain into virulence with the simoultanous aquisition of dsRNA viruses. The correlation between virulence and viruses will be further strengthened by infection of fungal protoplasts with purified virus preparations.

The method used for preparation of protoplasts followed basically the procedure described by Hashiba & Yamada (1982). The method included digestion of young meclia (1g) with Novozyme (Nova Industries, Sweden) which is a Trichoderma lytic enzyme, at 27 C by shaking slowly in 0.6M mannitol, used on an osmotic stabilizer. Comparison between the yields of protoplast release from R. solani mycelium at 34 C, 30 C, 25 and 27 C was made. Different concentrations of Novozyme (7.5, 5, 3 and 1.5 mg/ml) and osmotic stabilizer (0.6, 0.8, 1M) were tested too (Fig. 1). It appeared that maximum production and release of protoplasts occured at 27 C with 1.5 mg/ml Novozyme in 0.6M mannitol. The techniques required for regeneration have to be refined, in order to ensure removal of all mycelial fragments from the protoplast suspension.

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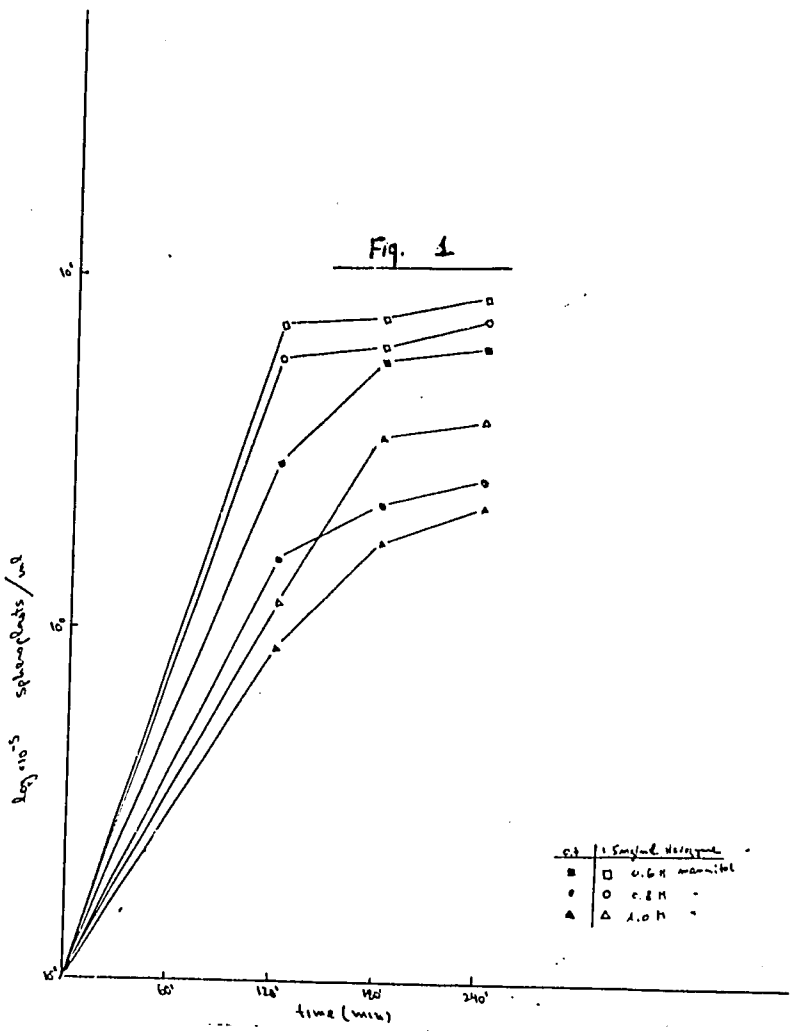

IV. Description of Cooperation

Fungal-induced tree diseases cause severe trouble to Ghana's economy, since wood is Ghana's major export product. Infection of trees by *Pseudophylla* leads to eradication of large areas of woods. The disease is spread rapidly and the spores are maintained in soil long after the trees are eradicated.

The attenuation of the chestnut blight disease induced by hypovirulent strains of *Endothia parasitica* containing dsRNA, suggested that dsRNA molecules are involved in the regulation of virulence (Van Alfen & Hansen, 1982). Moreover, the study in *Rhizoctonia solani* suggested a correlation between dsRNA viruses and inoduction of virulence (Finkler *et al.*, 1985). Biological control of both chestnut blight and damping-off were implemented successfully (Van Alfen *et al.*, 1975; Ichielevich *et al.*, 1985).

We have attempted to assist Dr. Ofuso-Asiedu to develop biological techniques to control the fungal-induced diseases affecting trees in Ghana. His recent visit to our laboratory in May-June 1986 has enabled him to aquire techniques and ideas in the methodology of dsRNA extraction and virus purification from fungal mycelia. A number of fungal strains from various localities in Ghana will be tested for virulence and dsRNA content. Assistance will be provided at any step which requires more sophisticated instruments not yet obtainable in the laboratories of Dr. Ofuso-Asiedu, in the Forest Research Institute of Ghana.

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CS-002

AID Scientific Report.

Report from September 1985 - December 1985.

Title: The Ds-RNA Viruses of Fungi and the Regulation of Plant Pathogens.

Principal Investigators Yigal Koltin
Microbiology Dept.

Tel Aviv University
Ramat Aviv
Israel

Issac Barash
Botany Dept.

A. The pectolytic enzymes of R. solani.

The results accumulated thus far suggest that in Rhizoctonia solani the double-stranded RNA viruses contain genetic information which either regulate or produce virulence factors (Finkler et al., 1985). As part of an effort in this direction the pectolytic enzymes of a number of virulent and hypovirulent strains of R. solani were examined. The role of pectolytic enzymes in the pathogenicity of R. solani has been clearly demonstrated by Weinhold & Motta (1973). Four enzymes designated as endopolygalacturonase I (endo PG-I) and II, pectin esterase (PE) and endopectinolyase (endo PL) were purified to homogeneity by a single chromatographic step on a cross-linked polypectate column. These enzymes were detected in a number of virulent strains of R. solani. However, the endo PG-I, endo PG-II and PE

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but not endo-PL were identified in a number of hypovirulent strains. Various biophysical characteristics of these enzymes were determined. The molecular weight (MW), pH optimum, isoelectric point (PI) and optimal temperature (T) for each enzyme were as follows: endo PG-I (MW 34K, pH 6.8, PI 6.8, T 50° C), endo PG-II (MW 37K, pH 5.4, PI 7.4, T 42 C), PE (MW 26K, pH 4.4, PI 6.2, T 48° C) and endo PL (MW 45K, pH 8.4, PI 8.1, T 53 C). The study of endo PL as a virulence marker can provide a handle to resolve if this factor is introduced by the viruses or is induced by the viruses. The preparation of anti-PL antibodies for immunoprecipitation of viral *in vitro* translation products is underway.

B. Preparation of anti-viral antibodies.

To evaluate the coding potential of the viral dsRNA and to characterize the viral-encoded proteins by *in vitro* translation, the effort has been directed towards preparation of anti-viral antibodies. These will be used to identify the synthesis of viral-transcribed proteins.

C. Northern blot hybridizations.

The relatedness of the various viruses isolated from strains of *R. solani* from different locations was studied by nucleic acid hybridization using the viral transcript as a probe in Northern blots. The synthesis of the viral transcripts is directed by the virus-associated RNA-dependent RNA polymerase, an enzyme found in most fungal viruses. The transcripts were used

as a probe to resolve the relatedness of the viruses since such probes avoid artifacts introduced by impurities of rRNA adhered to dsRNA. Using such probes under stringent conditions at 65°C and 4 x SSC, cross hybridizations between these probes and the viral dsRNA of various isolates revealed that all the viruses isolated from virulent strains are interrelated.

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