

Ribosome Content of Various Spore Forms of *Phytophthora* spp.

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Supported by California Avocado Advisory Board Grant No. 20649.

We wish to thank S. Bartnicki-Garcia and N. T. Keen for helpful suggestions during the progress of this work; they and J. S. Semancik also critically reviewed the manuscript. We are indebted to D.C. Erwin for providing the initial batches of oospores, and P.H. Tsao for providing the chlamydospores.

Accepted for publication 11 October 1973.

ABSTRACT

Ribosomes and polysomes isolated from swimming zoospores, cysts, and chlamydospores of *Phytophthora* spp. were characterized. No ribonucleoprotein particles typical of 80S ribosomes or ribosomal subunits could be isolated from the dormant oospore under any of the conditions utilized. Subribosomal ribonucleoprotein particles of undetermined

size, low levels of ribosomal RNA, and ribosomal proteins were isolated from oospores, indicating that de novo synthesis of ribosomes would not be required to initiate protein synthesis for germination.

Phytopathology 64:404-408

Basic to any study of the molecular events which occur during morphogenesis is the characterization of the status of the components of the protein synthetic system in the various phases of the life cycle of the organism. The aquatic phycocystete *Phytophthora* is ideally suited for such studies since several different spore types are formed during the course of development (23). These spores are distinct biologically and include the unwalled, free-swimming zoospore which subsequently encysts and germinates, the thick-walled, resistant but not dormant chlamydospore, and the thick-walled, resistant, oospore (1, 2, 4, 5). The oospores are of particular interest since they are the products of the sexual stage (11) and are known to be dormant to the extent that they are generally very difficult to germinate (2, 12, 14, 22).

Earlier studies have shown that nondormant spores, such as the urediospores of rust fungi (16, 17, 18), the conidia of *Erysiphe graminis* (6) and of *Neurospora crassa* (4, 5, 19), and the basidiospores of *Schizophyllum commune* (7) contain functional polysomes. Dormant spores, such as the ascospores of *Neurospora crassa* which require a heat-shock for germination (4), have been shown to contain only monosomes.

These studies were designed to compare the ribosome and polysome content of the motile and encysted zoospores, ungerminated chlamydospores, and ungerminated oospores of *Phytophthora* spp. in order to determine the capacity of these propagules for protein synthesis.

MATERIALS AND METHODS.—*Production of zoospores.*—Encysted zoospores were routinely prepared from *P. palmivora* strain P113 (formerly *P. parasitica*). Up to 100 V-8 agar plate cultures were placed in the dark at 24 C for 5 days and then incubated in continuous light at room temp for an additional 3-5 days. These cultures, bearing abundant mature sporangia were placed at 1 C for 15 min and flooded with sterile distilled water to induce release of the zoospores, which was usually completed within 45 min after flooding. The zoospore suspension was agitated vigorously for 2 min to induce encystment (20), the cysts collected by centrifugation at 12,000 g for 6 min, and immediately lyophilized.

Production of chlamydospores.—The chlamydospores

of *P. parasitica* were generously provided by P. H. Tsao of this department. The methods for the production and harvesting the chlamydospores have been described previously (21). The chlamydospore pellets were lyophilized and stored until use.

Production of oospores.—The homothallic species *P. citricola*, *P. megasperma*, and *P. vignae*, and the heterothallic species *P. cinnamomi* were used in these studies. One hundred petri plate cultures on V-8 juice agar were grown in the dark at 25 C for 3 wk. The oospores were harvested by homogenizing five cultures at a time in a Waring Blendor containing 100 ml of sterile distilled water for 3 min. The process was repeated until a total of 300 ml of water had been added and 9 min of grinding was completed. The homogenized material was dispensed into 250 ml Sorvall centrifuge bottles and additional water was added until each bottle contained a total volume of 200 ml. The homogenate was centrifuged for 6 min at 12,000 g. The oospores formed an orange pellet, easily distinguishable from the layer of white mycelial fragments which sedimented above it. The oospore pellet was removed from each bottle, combined in conical centrifuge tubes, and centrifuged at 1,000 g for 5 min. The remaining mycelial fragments were removed with a pipette. This was repeated several times until the oospores were free of mycelial fragments. The final oospore pellet was immediately lyophilized.

Preparation of crude extracts and purified ribosomes.—

—1) Zoospores.—A suspension of approximately 5×10^9 zoospores was filtered into an equal volume of 0.5% Triton X-100, which we had shown to cause immediate lysis of the zoospore. The cell debris was removed by centrifugation of the liquid at 12,000 g for 15 min. Because of the large volumes involved, no attempts were made to characterize ribosomes from these crude extracts. The supernatants were layered over 2M sucrose in 5 mM Tris-5 mM $MgCl_2$ buffer, pH 7.5 (Tris- Mg^{+2} buffer) and centrifuged at 120,000 g for 2 h. The ribosomal pellet was washed and resuspended in Tris- Mg^{+2} buffer.

—2) Cysts.—Lyophilized cysts were ground with glass beads in a mortar and pestle, resuspended in 10 ml Tris-

Mg²⁺ buffer, and centrifuged at 12,000 g for 15 min. The supernatant fluid was considered the crude extract.

—3) Oospores and chlamydospores.—The lyophilized oospores and chlamydospores were resuspended in 10 ml Tris-Mg²⁺ buffer and ground in a chilled Teflon tissue homogenizer until 95% of the spores were broken (9). This usually required 5-10 min grinding for the 750-1,000 mg dry wt used routinely. The ground spore suspension was centrifuged at 12,000 g for 15 min and the supernatant was considered the crude extract.

Sucrose density-gradient centrifugation.—The ribosomes were characterized by sucrose density-gradient centrifugation as previously described for the characterization of ribosomes from mycelial extracts of *Phytophthora* spp. (8).

RESULTS AND DISCUSSION.—The purified ribosomes of swimming zoospores and crude extracts of cysts and chlamydospores analyzed by sucrose density-gradient centrifugation all showed typical ribosome profiles (Fig. 1, 2, 3). The predominant peaks characteristic of monosomes and absorbancy in the region of the gradient previously shown to contain polysomes (8) were apparent in all profiles. In contrast to the profiles from mycelium, zoospores, cysts, and

chlamydospores, the crude oospore extracts produced density-gradient profiles which lacked any A₂₆₀ peaks corresponding to intact ribosomes. There was also little indication of subunit material since no distinct peaks or shoulders were observed in the upper regions of the gradient. The characteristics of the profile therefore indicated that ribonucleoprotein particles large enough to be sedimented beyond the top of the gradient were not present in extracts from oospores. The oospore profiles indicated some absorbing material near the top of the gradient (Fig. 4, arrows) which apparently represent subribosomal ribonucleoprotein particles of undetermined size. This material was too high in the gradient, however, to be the 60S and 40S ribosomal subunits.

There are several possible explanations of the inability to isolate ribosomes from ungerminated oospores. We have attempted to test them experimentally. First, it was possible that the grinding procedure was insufficient to break the oospores adequately. We feel that this is definitely not the case for several reasons. After 10 min of grinding by the methods previously described, microscopic examination of the extract consistently showed that almost all the spores were broken and empty

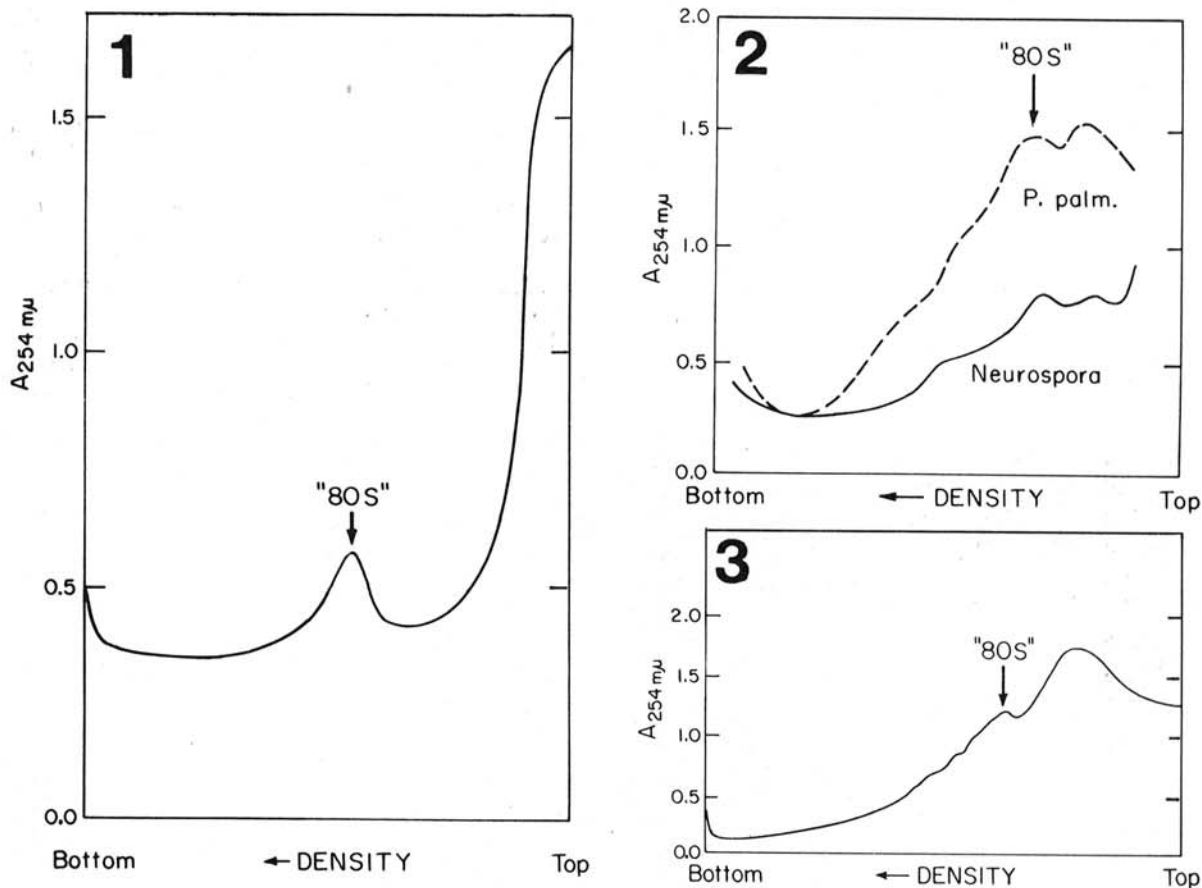


Fig. 1-3. 1) Sucrose density-gradient profile (5-20%) of purified ribosomes from zoospores of *Phytophthora palmivora*. 2) Sucrose density-gradient profile (5-20%) of crude extracts from cysts of *Phytophthora palmivora* (— — — —) and mycelium of *Neurospora crassa* (————). 3) Sucrose density-gradient profile (5-20%) of crude extracts from chlamydospores of *Phytophthora parasitica* (————).

(Fig. 5). Crude extracts of mycelium and oospores from two sources were prepared and total protein determined by the Lowry procedure (10) (Table 1). These extracts were then analyzed by sucrose density-gradient centrifugation (Fig. 6). It is clear that even though the relative protein content is similar, only the mycelial extract contained intact ribosomes.

Another possibility was that the extracted oospores were either too young and therefore so immature that the ribosome content was so low it could not be detected, or too old and the cell contents had degenerated. Sucrose density-gradient analysis of oospores produced by *P. vignae* after 7 days and *P. cinnamomi* 4 wks after mating again revealed no ribosomes.

The possibilities that the extraction procedures were inadequate or resulted in the breakdown of ribosomes were investigated. Grinding in Tris buffer containing 2-, 5-, and 10-times the normal $MgCl_2$ concn, or in buffer containing ribonuclease inhibitors (i.e., diethyloxydiformate), did not change the density gradient profiles. Considering that the ribosomes were perhaps bound to the cell wall debris, and therefore not suspended

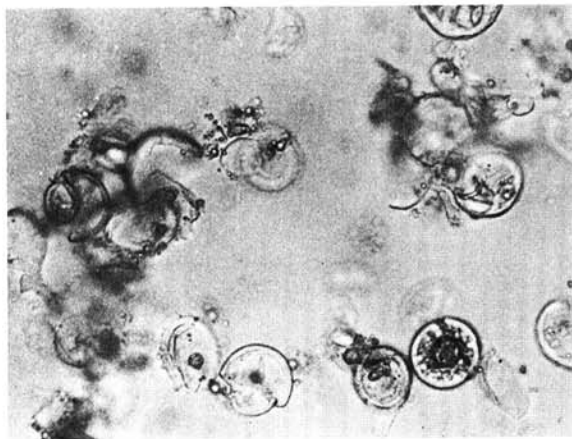
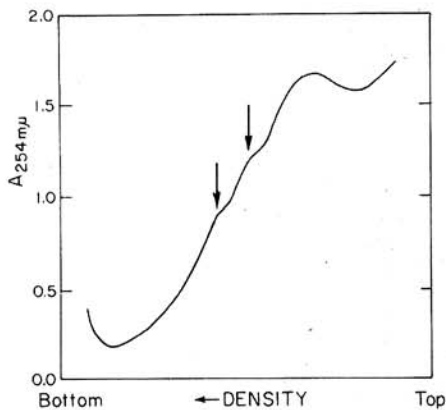


Fig. 4-5. 4) Sucrose density-gradient profile (5-20%) of crude oospore extracts from *Phytophthora vignae* (—). 5) Oospores of *P. vignae* after 10 min grinding. Note that almost all spores are broken and empty.

TABLE 1. Yield of soluble protein from mycelia or oospores of *Phytophthora* spp.

Source	Dry wt starting material (mg)	Protein concn ($\mu\text{g}/\text{ml}$)	μg Protein per mg dry wt
<i>P. palmivora</i> 24-h mycelium	65	120	9.2
<i>P. palmivora</i> 36-h mycelium	200	345	17.3
<i>P. cinnamomi</i> oospores	100	310	10.5
<i>P. vignae</i> oospores	100	210	11.0

during extraction, modifications of the extraction procedure were employed:

(i) The extractions were made in Tris- Mg^{+2} buffer containing 1% sodium desoxycholate (DOC).

(ii) The cell debris was resuspended in buffer containing 1% DOC, and recentrifuged.

(iii) The cell debris was resuspended in buffer containing 1% DOC, ground again for 10 min, and recentrifuged.

(iv) The cell debris was resuspended in buffer containing 1% DOC, glass beads were added, and extraction attempted with a Bronwill homogenizer. The extract was recentrifuged as above.

(v) The cell debris was resuspended in buffer, sonicated at 4 C at maximum frequency for two 10 min periods, and centrifuged.

Sucrose density-gradient analysis of these supernatants, either separately or combined with the original crude extracts, again resulted in profiles which demonstrated no absorbancy peaks in the region of the gradient corresponding to intact ribosomes.

These same procedures were repeated, the oospore extracts layered over 2M sucrose and centrifuged at 120,000 g for 3 h. In no instance was an opalescent ribosomal pellet obtained, and the material which did pellet had little absorbancy in the ultraviolet.

The possibility that the oospore contained an unusual ribonuclease and/or that the ribosomes of the oospore are more sensitive to ribonuclease was considered to be a possible explanation for these results. This, also, does not appear to be the case for several reasons. Firstly, total breakdown of the ribosomes during the short extraction procedure does not appear likely. Secondly, if total breakdown of the ribosomes did occur, the amount of A_{260} absorbing material at the top of the gradient should be far in excess of that observed, especially when compared to the profiles from other spore types. Thirdly, the use of ribonuclease inhibitors should have restricted breakdown and yet no ribosomes were present in extracts prepared in this way. Finally, if the extracts contained only breakdown material, the free ribosomal RNA content should be high. Therefore, 100 mg of oospores were extracted by the standard procedure, and subsequently the total RNA extracted by phenol-SDS procedure (3). When compared to the sucrose density-gradient profile of the RNA extracted from 60 mg of

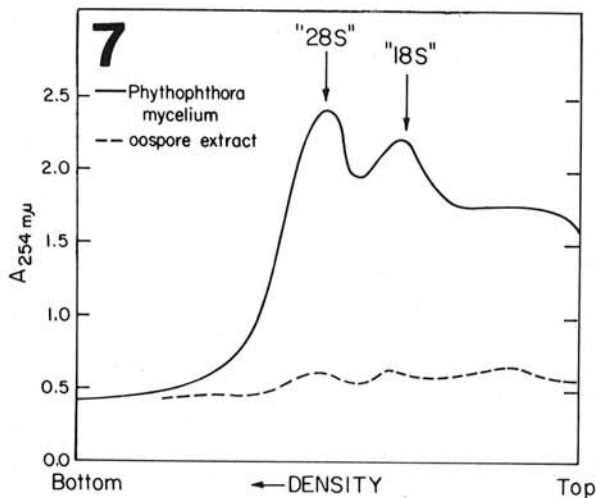
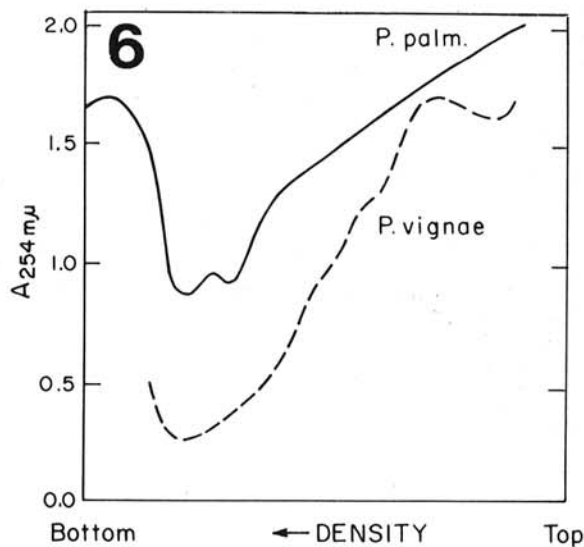


Fig. 6-7. 6) Sucrose density-gradient profile (5-20%) of crude mycelial extracts from *Phytophthora palmivora* (—) and crude oospore extracts from *Phytophthora vignae* (---). 7) Sucrose density-gradient profile (5-20%) of RNA isolated from 48-h-old mycelium of *Phytophthora palmivora* (—) and RNA isolated from oospores of *Phytophthora vignae* (---).

Phytophthora palmivora mycelium (Fig. 7), it is immediately obvious that far less RNA was isolated from the oospores. Even more significant is the fact that the rRNA species are demonstrable and very little ultraviolet-absorbing material remained at the top of the gradient. If ribonuclease activity was very high in the oospore, this should not have been the case.

We therefore conclude that the dormant oospore of *Phytophthora* spp. lacks intact, functional ribosomes. We are confident that the possibilities that experimental procedures are responsible for the failure to demonstrate ribosomes have been adequately eliminated. The absence of this basic component of the protein synthesis system

could explain the strict dormancy of these spores. The presence of a low level of ribosomal RNA (Fig. 7) and our preliminary serological demonstration of ribosomal protein in the oospore (J. V. Leary et al., unpublished) argue that the precursors of ribosomes are present in the oospore in some undetermined form. Subribosomal ribonucleoprotein particles similar to those which we feel exist in *Phytophthora* oospores have been described previously (13, 15). These particles sediment more slowly in sucrose gradients, have lower buoyant densities than ribosomes, and contain messenger-like RNA (15). The presence of such particles in the oospore would mean that de novo synthesis of ribosomes would not be required upon the onset of spore germination but only their final assembly. Therefore, the oospores of *Phytophthora*, if induced to germinate, would represent an ideal system for studying the synthesis of ribosomal subunits and/or the assembly of intact, functional ribosomes.

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