Purification of Mycoplasma-like Organisms from Lettuce with Aster Yellows Disease

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We thank P. Pogers for manuscript typing and X. F. Jiang for graphics preparations. This research was supported in part by the New Jersey Agricultural Experiment Station, Publication D-11160-2-86.

Accepted for publication 17 December 1986 (submitted for electronic processing).

ABSTRACT

Jiang, Y. P., and Chen, T. A. 1987. Purification of mycoplasma-like organisms from lettuce with aster yellows disease. Phytopathology 77:949-953.

A technique was developed to partially purify the mycoplasma-like organisms (MLOs) associated with the aster yellows (AY) disease. Young lettuce plants were inoculated with the AY agent by leafhopper transmission and kept in a greenhouse. Leaf veins from symptomatic lettuce were isolated and homogenized in an isolation medium consisting of D-mannitol, 3-(N-morpholino)-propane sulfonic acid (MOPS), polyvinylpyrrolidone, L-cysteine, and ethylenediamine tetraacetic acid, before low- (8 min at 1,500 *g*) and high- (30 min at 35,000 *g*) speed centrifugation. The pellet was resuspended in suspending medium containing mannitol and MOPS and layered on a discontinuous Percoll density gradient of 15, 30, and 50% (v/v). After centrifugation at 20,000 *g* for 20 min, the fraction formed at the interface of the 30 and 50% solutions was collected and diluted with suspending medium. Gradient material was removed by centrifuging the diluted fraction at 100,000 *g* for 2 hr. Electron microscopy of thin sections made from purified preparation showed a high concentration of MLOs with well-preserved cellular structures identical to those in the phloem of diseased plants. These MLO cells exhibited a trilaminar limiting membrane, cytoplasmic ribosome granules, and a network of DNA.

Aster yellows (AY), one of the most studied yellows diseases, was considered of viral etiology for more than 70 yr but is associated with mycoplasma-like organisms (MLOs) (3,11). In 1967, Doi, Ishiie, and co-workers (5,9) first demonstrated MLOs in the sieve elements of plants with several yellows diseases, including aster yellows (AY). Their evidence was based on electron microscopy of diseased tissues and a therapeutic effect of tetracycline antibiotics. Since then, MLOs have been associated with more than 200 plant diseases (2).

A major obstacle limiting research on the AY disease is that the MLOs have not so far been isolated and cultivated in vitro. The pleomorphic MLOs are fragile and occupy relatively small areas within their plant host (sieve tubes). Attempts to purify the disease agents have met with limited success (15). Viability of the AY-associated MLO (AY-MLO) in vitro has been maintained for only 6–72 hr (4,7,21). Chiykowski (1) showed that AY-MLO could remain viable for over 12 mo only by storing the infective vector leafhopper (*Macrosteles fascifrons* Stal) at −20 C. These facts have hampered efforts to identify and characterize this microorganism.

Purification of yellows viruses had been attempted long before MLOs were recognized to be associated with the yellows diseases (12). Recently, Sinha (17–20) used activated carbon, a Celite (diatomaceous earth) pad filter, and a Sepharose 2B column to remove plant materials before subjecting the diseased plant extracts to sucrose density gradient centrifugation. Preparations of purified organisms from plants infected with clover phyllody and AY appear to contain mostly membrane-bound empty bodies in electron micrographs (15,18). Nevertheless, Sinha demonstrated a very low level of infectivity and produced antisera against the MLOs.

Mitochondria in plants are membrane-bound bodies that, to some extent, are similar to the membrane-bound bodies of MLOs. Douce et al (6) used mannitol in a medium to isolate mitochondria from spinach leaves. The medium kept the mitochondrial membrane intact during the isolation process. Jackson et al (10) modified the method with discontinuous density gradients to obtain highly purified mitochondria preparations from potato tubers and spinach and lettuce leaves. Most of the oxidative enzyme properties of intact mitochondria were recovered in the preparation.

We report the development of a new technique for purifying AY-MLOs that we assessed by electron microscopy and serology using monoclonal antibodies specific against AY-MLO.

MATERIALS AND METHODS

Source of diseased plants. Lettuce plants (*Lactuca sativa* L. 'Mesa') infected with AY-MLO were originally collected from an experimental vegetable farm in New Jersey. *M. fascifrons*, the vector leafhopper of the AY agent, was reared on rye plants in an insect room kept at 25 C. Adult leafhoppers were allowed to lay eggs in the leaves of young seedlings. After the eggs were hatched, the second- or third-stage nymphs were first transferred to AY-diseased lettuce for an acquisition feeding for 2 or 3 days and then to fresh young rye plants for 14 days. After this incubation, the insects were distributed (3–5 insects per plant) onto young lettuce seedlings. Usually a 1-day inoculation was enough, and leafhoppers could be transferred daily to fresh lettuce plants until the leafhoppers died. Inoculated lettuce plants were kept in the greenhouse with temperatures ranging from 25 to 30 C.

Isolation medium. Isolation medium contained 0.3 M D-mannitol, 4 mM L-cysteine, 30 mM (N-morpholino)-propanesulfonic acid (MOPS), 1 mM ethylenediamine tetraacetic acid (EDTA), and 0.6% polyvinylpyrrolidone (PVP), adjusted to pH 7.2, and was stored at 4 C until use.

Suspending medium. This medium consisted of 0.3 M mannitol and 20 mM MOPS adjusted to pH 7.0 and was stored at 4 C.

Discontinuous density gradients. The density medium was made iso-osmotic by adding nine parts (v/v) of Percoll to one part (v/v) of 2.5 M sucrose in H_2O. Solutions of lower density, but still iso-osmotic, were made by dilution with 0.25 M sucrose and 10 mM MOPS (pH 7). Step gradients were prepared in (v/v) so that final concentrations of iso-osmotic media were as follows: group 1—10% (11 ml), 15% (11 ml), and 30% (6 ml); group 2—12.5% (11 ml), 30% (11 ml), and 40% (6 ml); group 3—15% (11 ml), 30% (11 ml), and 50% (6 ml); group 4—15% (11 ml), 30% (11 ml), and 60% (6 ml); group 5—20% (11 ml), 35% (11 ml), and 60% (6 ml); and group 6—15% (9 ml), 30% (9 ml), 50% (5 ml), and 60% (5 ml). The solutions were layered into 25- × 89-mm Ultra-Clear centrifuge tubes (Beckman Instrument Inc., Palo Alto, CA) immediately before use.

Preparation of plant materials. Two hundred grams of stems and leaf tissues of AY-infected lettuce were cut into small pieces in

200 ml of chilled isolation medium and were disrupted two or three times with a Sorvall Omni-mixer (Sorvall-Dupont, Wilmington, DE) at low speed in 3-sec intervals. The disrupted tissues were ground with a mortar and pestle until no vascular fibers were discernible. The sap was squeezed through three layers of cheesecloth, and the volume was adjusted to 800 ml with isolation medium, then centrifuged at 1,500 *g* for 8 min (low speed). The supernatant solution was saved and centrifuged at 35,000 *g* for 30 min (high speed). The pellets were resuspended in suspending medium, pooled, and centrifuged at low and high speed. The final pellet was resuspended in approximately 10 ml of chilled suspending medium. Healthy plant tissues were treated similarly and used as controls.

Discontinuous gradient centrifugation. About 0.8 ml of preparation was added to the top of each discontinuous gradient. Centrifugations at 4 C for 20, 40, and 60 min were at 14,000, 20,000, and 30,000 *g* in an angle rotor (Beckman, type 30). After centrifugation the fractions were collected and diluted immediately with suspending medium and centrifuged at 100,000 *g* for 2 hr to remove the Percoll from the solution. The pellet that aggregated above the surface of the hard cushion of Percoll was carefully collected and suspended in phosphate-buffered saline (PBS, pH 7.0). This suspension was used to prepare antigen for immunizing mice, used as a sample for electrophoresis, or concentrated and prepared for electron microscopy.

Electron microscopy. The fractions collected from the density gradients were placed in separate centrifuge tubes and fixed with 1.5% glutaraldehyde in the suspending medium for 1 hr at 4 C. The suspensions were centrifuged at 15,000 *g* for 30 min with a microcentrifuge. The supernatants were carefully aspirated and the pellets were rinsed twice with suspending medium at 5-min intervals and postfixed with 1% osmium tetroxide (OsO_4) in suspending medium at 4 C. After being washed twice with suspending medium, the pellets were dehydrated in a cold graded ethanol series and then in propylene oxide. The pellets were embedded in Spurr's medium (Ladd Research Industries, Inc., Burlington, VT) and polymerized in an oven at 70 C for 8 hr.

Ultrathin sections (60–80 nm) were cut with a diamond knife with a Sorvall MT 2-B ultramicrotome (Sorvall-Dupont, Wilmington, DE). Specimens were stained with 4% uranyl acetate in 50% ethanol for 15 min, washed by dipping the grids 10 times in 50% ethanol, rinsed with distilled water for 3 min, dried, and stained with 2% lead citrate for 6 min. Electron microscopy was performed using a Siemens IA microscope.

The collections of AY-diseased and healthy lettuce veins were treated as described.

Indirect enzyme-linked immunosorbent assay (ELISA). After centrifugation at 20,000 *g* for 20 min, 42 fractions (0.45 ml) of group 3 gradients were collected. Each fraction was homogenized by ultrasonication (microtip set at 35%, Fisher, Sonic Dismembrator, model 300) for 2 min at 4 C. A healthy lettuce preparation was treated similarly. Each fraction was diluted with carbonate-bicarbonate coating buffer (pH 9.6, 1:9 v/v) and was used to coat two wells in a 96-well microtiter plate (Vangard International, Neptune, NJ) with 50 μl per well.

Monoclonal antibodies against AY agent (13) were diluted with PBS to make 70 μg of protein per milliliter, and 50 μl was added per well. Biotinylated antimouse IgG and IgM was used as the second antibody followed by avidin-biotin complex (8). The color reaction was detected with an ELISA spectrophotometer (Titertek Multiskan MC, Flow Lab, MD) at 492 nm.

RESULTS

Discontinuous density gradient centrifugation. Slight or no difference could be detected between healthy and diseased lettuce preparations from density gradients of groups 1 (10, 15, and 30%) or 2 (12.5, 30, and 40%). When centrifuged at 14,000 *g* for 20 min, most of the material layered on top of gradients was at the bottom of the tubes. A slight difference in absorption profiles between the healthy and diseased preparations could be detected in group 5 gradients (20, 35, and 60%), but differences were not as clear as those in groups 4 and 6. In groups 4 and 6, a marked difference in absorbance profiles (254 nm) between healthy and diseased plant

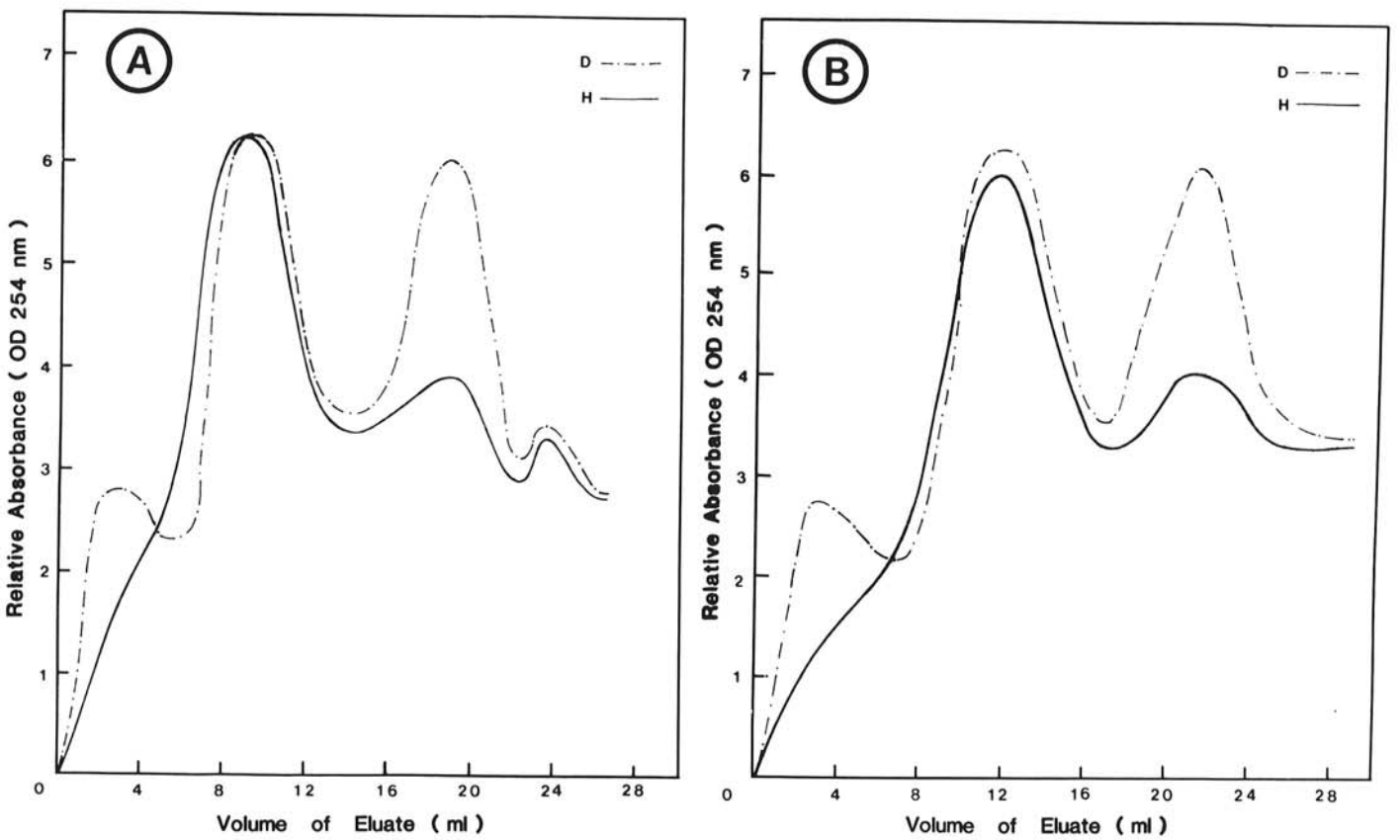

Fig. 1. Relative absorbance (254 nm) of eluates after centrifugation at 20,000 *g* for 20 min, showing two peaks that differentiate lettuce with aster yellows (D) from healthy lettuce (H) preparations. **A,** 25, 30, 50, and 60% density gradients. **B,** 15, 30, and 50% gradients. Top of gradient on left.

preparations was noted. Several peaks could be seen in the group 6 gradients (Fig. 1A) with differences between healthy and diseased preparations in eluates 2 to 4 and in eluates 16 to 21. A similar trend could be seen in group 3 tubes (Fig. 1B), but differences between eluates from healthy and diseased preparations were more pronounced and the absorption peaks were sharper. The turbidity of the regions could be detected with the naked eye, particularly at or near the interface between 30 and 50% layers (Fig. 2).

When the various forces and lengths of centrifugation were compared, the best result was obtained at 20,000 *g* for 20 min. With centrifugation at 20,000 *g*, if the time was more than 20 min (40 or 60 min), the peak of absorption occurred relatively close to the bottom of the tube, and the difference between healthy and diseased preparations was greatly reduced; higher centrifugal force (30,000 *g*) disturbed the discontinuous gradients, and a great deal of unwanted plant materials accumulated in the region with AY-MLOs.

Morphological studies of partially purified AY-MLOs. Electron micrographs of AY-MLOs in the tissue sections from infected lettuce clearly showed the typical ultrastructure of MLOs with various morphotypes (Fig. 3A). No AY-MLO was observed in sections from healthy lettuce.

Sections taken from density gradient preparations of group 3 and group 6 collections near the interface between 30 and 50% layers or group 4 collection near interface between 30 and 60% layers showed high concentrations of well-preserved cells of AY-MLOs. Cell structures were identical to those in diseased lettuce sections. The highest AY-MLO concentration with the least contamination of host organelles was observed in the sections from group 3 preparations (Fig. 3B). Very few AY-MLO bodies were observed in the sections from other zones in group 3 gradients or from other preparations of other density gradient groups, i.e., groups 1 and 2. No AY-MLO was observed in the sections from any of the healthy density gradient preparations.

High resolution electron micrographs clearly showed a trilaminar unit membrane, cytoplasmic ribosome granules, and a nuclear area with DNA fibrils in the purified AY-MLO section (Fig. 4). Bud- and bead-like structures were also frequently observed extending from the cell surface. Binary shaped cells showing possible fission have also been observed in sections of purified AY-MLOs. Moniliform and many filament cells were visible in most photographs taken from the purified AY-MLO sections.

Indirect ELISA method. ELISA readings of fractions from group 3 gradients are shown in Figure 5. There was a gradual increase in ELISA readings until fraction 36, at which the highest reading (0.565) was recorded. In comparison, none of the fractions from healthy plant preparations contained AY-MLOs, as indicated by ELISA readings less than 0.1. Fractions 33, 34, 35, 36, and 37 corresponded to the 17–23 ml region from the top of the gradient where the concentration of MLOs was highest (Fig. 1B).

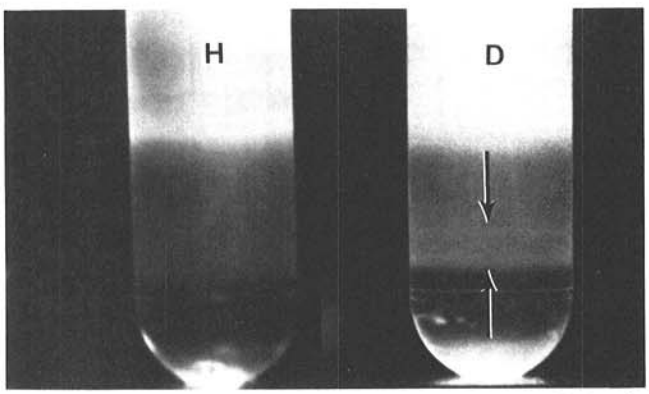

Fig. 2. Discontinuous density gradients (15, 30, and 50%) after centrifugation at 20,000 *g* for 20 min. Distribution of fraction of mycoplasma-like organisms associated with aster yellows from diseased lettuce preparation is indicated in tube D with arrows. A healthy lettuce preparation is shown in tube H.

DISCUSSION

In the 1950s attempts to isolate and purify the yellows agents met with failure (15). Because the causal agents were assumed to be viruses, all purification procedures followed those for virus isolation. Although sucrose density gradient centrifugation is an excellent technique that is used routinely to isolate and purify

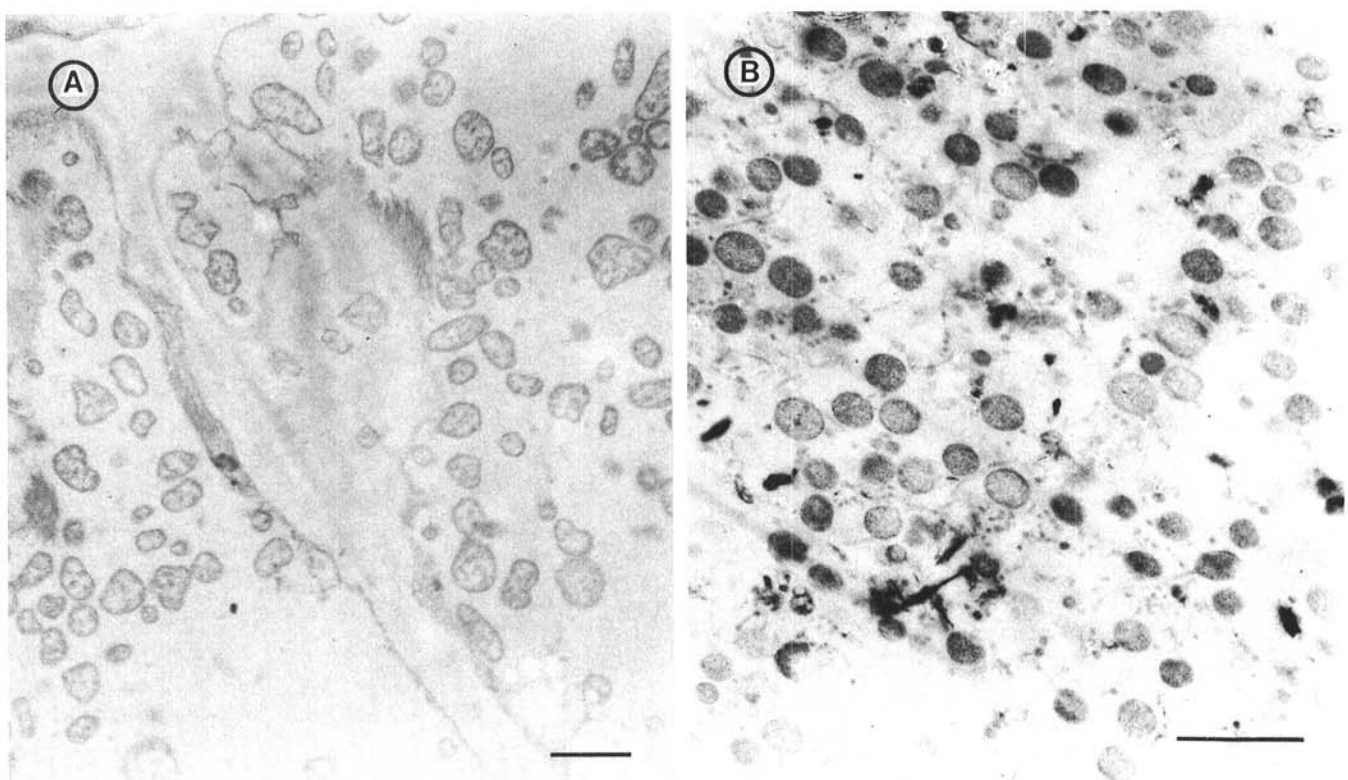

Fig. 3. Electron micrograph of mycoplasma-like organisms from aster-yellows infected lettuce phloem **(A)** and a purified preparation **(B)** showing pleomorphic cells in vivo and spherical to oval cells in vitro. Bar = 1.85 μm.

viruses, we found that adapting this method for MLOs is not suitable. The purpose in sucrose density gradient centrifugation is to produce a continuous gradient of sucrose concentrations throughout the tube. Materials of different density are thus separated in different regions of the solution by centrifugal force. Theoretically this should work equally well with MLOs. However, in the study of MLOs several factors must be considered. The sieve tube elements where MLOs reside have particularly high hydrostatic pressure. A sudden change of the pressure during isolation could be detrimental to the organisms. Probably the most important factor is osmolarity of the environment since MLOs are cell wall-less membrane-bound procaryotes that are sensitive to external changes in osmolarity. If discontinuous sucrose density gradient was used, MLOs would pass through areas of unequal osmotic pressure through the gradient. On the other hand, a continuous sucrose density gradient method fails to remove much of the contaminating subcellular plant materials including the chlorophyll. Thus, a rapid procedure allowing the removal of contaminating material under iso-osmotic conditions has advantages over more conventional sucrose gradients.

MLOs have not been cultivated and apparently cannot survive for any length of time in artificial media. Thus, maintenance of MLOs in vitro is the first important obstacle to be overcome before these microorganisms can be better studied. For this reason, we used an isolation medium that provides the optimal conditions to preserve MLOs during extraction. Mannitol protects the plasma membrane. MOPS, with pH 7.2, keeps a stable pH buffer range between 6.5 and 7.9. PVP regulates the osmolarity of the medium to possibly prolong MLO survival.

Percoll (Pharmacia, Piscataway, NJ), a medium for isolating cells, viruses, and subcellar particles by density gradient centrifugation, is composed of collodial silica, coated with PVP to render the material nontoxic. It produces a very low osmotic pressure (20 mOs/kg of H_2O) and is compatible with living cells, allowing separation and recovery of intact, fully active systems. Biological membranes are impermeable to Percoll, and, thus, no change occurs in the buoyant density of cells during centrifugation.

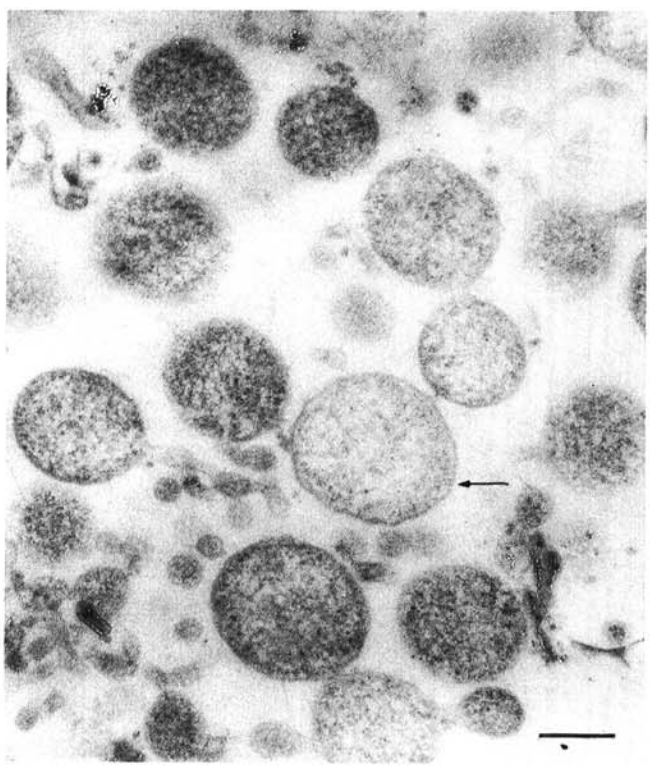

Fig. 4. Electron micrograph of cells of mycoplasma-like organisms associated with aster yellows from a purified preparation showing budlike structure and binary shaped or branched cells. Note the ribosome granules and DNA fibrils in the cells. Arrow indicates a trilaminar unit membrane. Bar = 0.2 μm.

Because it has a low viscosity (10 ± 5 cP), density gradients form spontaneously because of the heterogeneity of particle sizes in the medium (16). Low osmotic pressure gives discontinuous density gradients that are virtually iso-osmotic throughout. This permits precise adjustment to physiologic conditions without significant interference from the medium, and thus damage to MLOs due to changes in osmolarity can be minimized. We also noticed that the low viscosity of medium resulted in rapid separation of MLOs from plant materials. The short time in density gradient centrifugation might considerably reduce lysis of AY-MLO in vitro.

Optimal resolution of AY-MLO depends on the concentration of density gradients, centrifugal force, and centrifugation time. First, it is critical to determine the concentration of layers, because no previous studies have determined AY-MLO density. During this experiment, we chose 12.5, 15, 20, 30, 35, 40, 50, and 60% layers in various combinations to make the three-step density gradients. A four-step density gradient (group 6) was chosen to determine whether the density of AY-MLOs could be higher than that of the 60% layer. Materials collected at the interface of 50 or 60% layers of gradients from either diseased or healthy preparations showed little difference in absorbance at 254 nm, and electron micrographs confirmed that this region contained few AY-MLOs but many host materials. The three-step gradient, 15, 30, and 50%, yielded the highest AY-MLO concentration with the least host material contamination. Second, increased centrifugal force and prolonged centrifugation time resulted in increased contamination with plant subcellular materials in the AY-MLO fractions. Most of the contaminating particles were mitochondria, disrupted host cell membranes, and other host cell organelles with densities similar to AY-MLOs, but different in sizes. Because Percoll is a polydisperse colloid, its component particles will sediment at different rates, creating a smooth gradient when

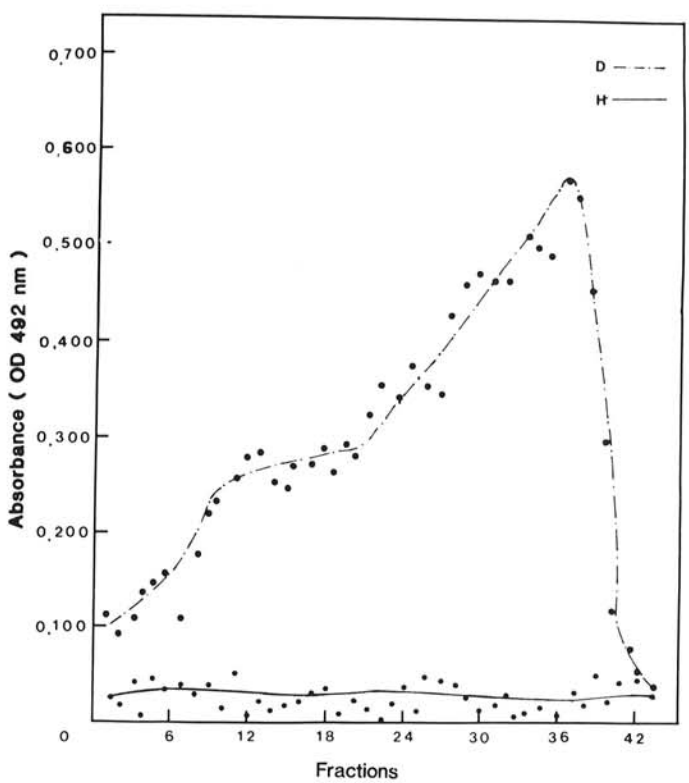

Fig. 5. Determination of mycoplasma-like organisms associated with aster yellows by enzyme-linked immunosorbent assay in a density gradient (15, 30, and 50%) after centrifugation at 20,000 *g* for 20 min. The 42 fractions were collected from top to bottom in equal volumes. A monoclonal antibody specific for AY-MLO was used to measure the titer of the AY-MLO by indirect ELISA. D represents a diseased preparation showing AY-MLO distribution throughout the gradient solution with the highest concentration of AY-MLO in fraction 36; H represents healthy plant control showing no trace of AY-MLO antigen.

centrifugal force is more than 25,000 *g* for 20 min. Such a continuous gradient is not sufficient to remove plant materials from AY-MLOs because materials from plant tissues vary in density and can be distributed throughout the gradient. Moreover, the density of MLOs is not very uniform because of their pleomorphic nature. As a result, a great deal of host material with density similar to AY-MLOs would be mixed with AY-MLOs in the continuous density gradients. These contaminations were easily observed in sections prepared from materials after gradient centrifugation at 30,000 *g* for 40 or 60 min.

Upon fractionation, three absorbance peaks were seen from the diseased lettuce preparation. The 2-ml fraction collected near the interface of the 30 and 50% layers contains the most AY-MLO, as confirmed by electron micrographs and a highest ELISA reading. The first peak that occurred in the beginning of collection (Fig. 1) may reflect the release of DNA from disrupted AY-MLO cells. The majority of materials collected from the second peak were mitochondria. Indirect ELISA, which revealed the distribution of AY-MLO in the density gradient, also indicated that fractions near the 30 and 50% interface contained the highest titer of AY-MLOs.

Purified AY-MLOs are less pleomorphic than those in situ. Micrographs from purified preparations showed that the shape of AY-MLOs changed to more or less spherical, and the cells were smaller than those in lettuce tissue sections. This could be explained by the high centrifugal force (100,000 *g* for 2 hr) that drove the cells into a compact structure. This suggests that the fluidity of plasma membrane of AY-MLOs is high.

Some of the monoclonal antibodies against AY-MLOs in this study were produced using purified AY-MLOs from infected lettuce as antigen (13). The AY-MLOs purified from the infected lettuce had high antigenicity when immunizing the balb/c mice. This was shown by the high ratio of hybridoma cells producing specific antibodies against AY-MLO (13,14) after fusing myeloma cells (NS-1) with primed spleen cells.

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