Culture of a Mycoplasmalike Organism Associated with Stubborn Disease of Citrus

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ABSTRACT

A pleomorphic organism was cultured from young leafy shoots and seeds of stubborn-diseased citrus in cell-free media. Colonies of the "fried-egg" type were obtained on agar media, and electron micrographs of pelleted material from liquid cultures showed slender filaments and round, ovoid, or irregular mycoplasmalike bodies similar to those present in sieve tubes of infected citrus. Filaments and bodies had unit membranes about 10 nm thick.

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Recent anatomical and cytological investigations (1, 7, 8) revealed mycoplasmalike bodies in the sieve tubes of stubborn-diseased Madam Vinous sweet orange seedlings, suggesting that stubborn of citrus, a yellows and stunt disease, may be caused by a mycoplasmalike pathogen rather than by a virus. In recent years, mycoplasmalike organisms have been reported as the probable etiological agents of many similar plant diseases (4, 6, 13). This paper describes the culturing and the in vitro appearance of a pleomorphic organism associated with stubborn disease in citrus plants, and confirms the findings of concurrent work by Saglio et al. (12). Preliminary reports of portions of our work have been published (1, 5).

The principal source of the stubborn pathogen used here and by Saglio et al. (12) was California 189, obtained from a Washington Navel orange tree near Riverside, Calif., in 1957 and determined to be free of all known diseases except stubborn.

Young leaves and shoots of stubborn-affected greenhouse-grown Madam Vinous sweet orange seedlings, infected with California 189 stubborn, were surface-sterilized in 0.5-1.0% sodium hypochlorite, rinsed in sterile water or in 70% ethanol, and finely chopped on a sterile surface or triturated in a small amount of special liquid medium in a mortar or blender. Washing the shoots in a solution of penicillin G (3,000-5,000 units/ml of sterile water) prior to chopping or trituration had no apparent effect.

We first obtained cultures in May 1970 in a liquid medium modified from that used by Razin & Tully (10). Subsequently, we grew cultures of the mycoplasmalike organism in nine other formulations of liquid media. All media used successfully contained either horse serum or ascitic fluid. The organism grew well in the medium used by Chen & Granados (2) and in liquid media containing either 20 ml ascitic fluid or 2 mg cholesterol and 1 mg palmitic acid instead of horse serum. Excellent results were obtained with a medium modified from that used by Saglio et al. (12), containing 2.1 g Difco PPLO broth, 0.1 g fructose, 0.1 g glucose, 0.1 g sucrose, 0.1 g tryptone, 5.0 g sorbitol, 20 ml horse serum, 10 ml 25% fresh yeast extract, and 60 ml distilled water. The ingredients, excepting horse serum and yeast extract which were passed through a 0.2-μ filter, were adjusted to pH 7.5-7.8 and autoclaved at 121 C (15 psi) for 20 min. The complete medium was distributed in sterile flasks, 5 ml in each, and kept at 4 C until used. A similar solid medium (3.4 g Difco PPLO agar replaced the PPLO broth) was used for most subcultures, but was less suitable than liquid media for primary cultures. Chopped tissue was seeded directly to the media.

Triturated diseased tissue was drawn through a 0.45-μ filter; then small aliquots were added to liquid media or seeded on the agar medium and incubated at 33 C. Controls were prepared in the same manner from healthy seedlings. Blank controls were incubated from each batch of medium to detect possible contaminants.

After 7-14 days' incubation, 0.1-ml aliquots from most liquid cultures and controls were transferred to agar media and incubated in moist sealed containers at 30 C. On agar, the organism formed classical "fried-egg" colonies (3), each with a dense, dark center surrounded by a lighter-colored, thin, broad, and circular zone (Fig. 1-A). These colonies developed slowly, reaching a maximum diameter of 0.1-0.2 mm in about 15 days. No colonies were obtained from the controls.

Some liquid cultures remained clear and others became very slightly turbid or changed color slightly within 12 days, after which they and their controls were routinely centrifuged individually at 7,000 *g* for 20 min and examined by electron microscopy.

Electron micrographs of pelleted cultures, which had been resuspended and negatively stained with 2% neutral phosphotungstic acid (PTA), revealed round, ovoid, or irregular-shaped main bodies, 0.5-2.0 μ across, connected to slender filaments 60-100 nm wide and up to 7 μ long (Fig. 1-B, C), similar to the mycoplasmalike organism isolated by Chen & Granados (2) from stunted corn. Identical results were obtained from the clear and from the slightly turbid cultures, but no pleomorphic bodies were present in the 12 controls or in the eight unseeded sterile flasks of liquid media. Twenty-one of 25 attempts yielded cultures from leafy shoots of stubborn seedlings.

Some pellets were fixed in 2-3% glutaraldehyde in

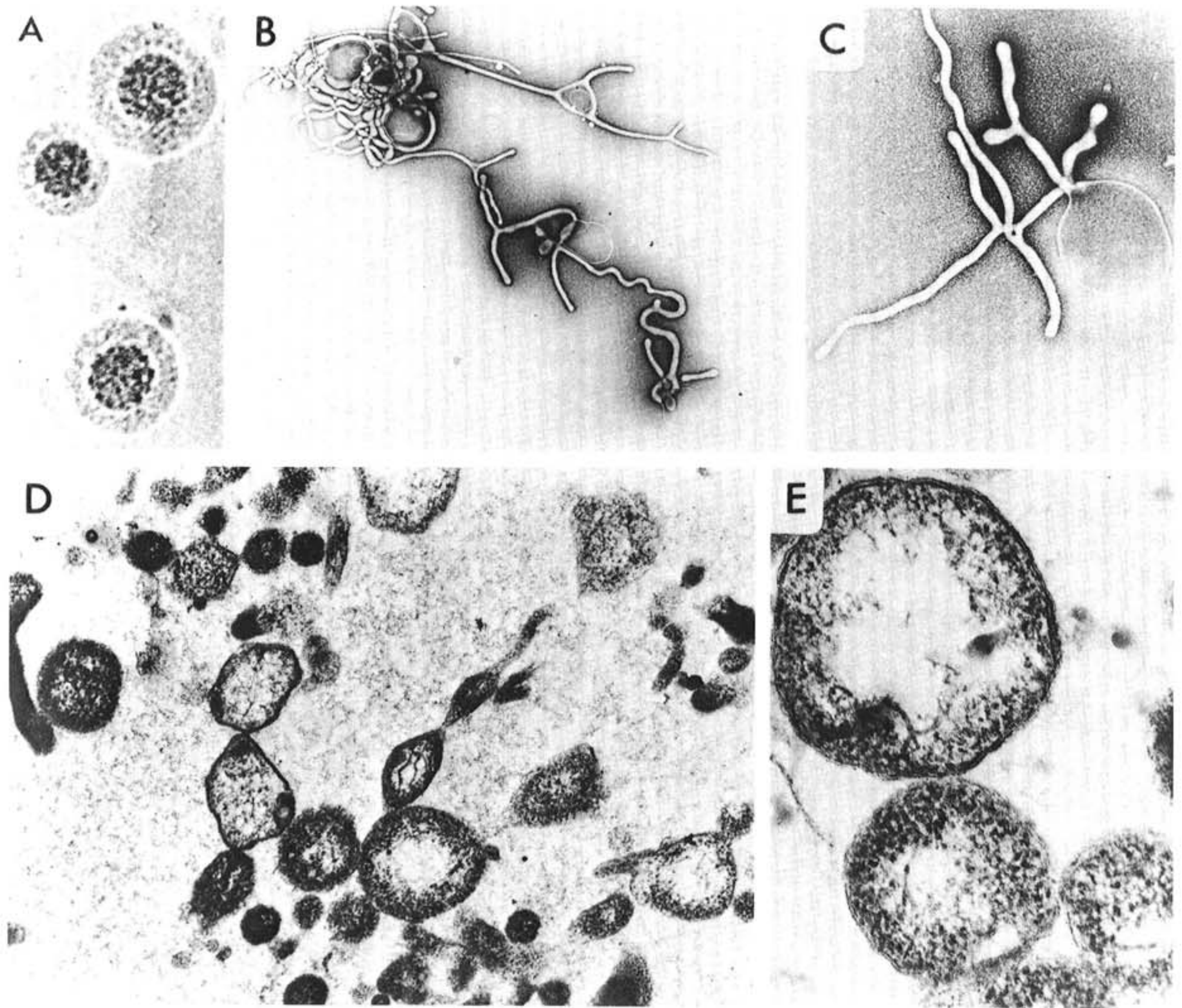

Fig. 1. A) Mycoplasmalike colonies on agar medium (× 120). **B)** Negatively stained (2% PTA) preparation, showing long filaments, from pellets from 12-day-old liquid culture (× 6,500). **C)** Round body with attached filaments, negatively stained (× 13,000). **D)** Ultrathin section of pelleted material showing different stages of growth of mycoplasmalike bodies, some with netlike strands (× 25,000). **E)** Round bodies with unit membranes from section of pellet (× 51,000).

0.1 M phosphate buffer, pH 7.4, for 1 hr at 4 C, rinsed, and postfixed in 1% osmium tetroxide in the same buffer for 3 hr at 4 C. The fixed material was dehydrated in a graded alcohol series, then passed through two final changes of propylene oxide, embedded in Epon (9), and cut with a diamond knife on a Porter-Blum MT-2 ultramicrotome. Sections were collected on Formvar-coated grids and stained with uranyl acetate and lead citrate (11). Examination under a Hitachi HU-12 electron microscope revealed filaments and irregular-shaped bodies that had unit membranes about 10 nm thick (Fig. 1-D, E). Ribosomelike particles and a network of fine strands, presumably nucleic acids, were also observed within the cells. Bodies from cultures were morphologically indistinguishable from the pleomorphic bodies observed in sieve tubes of stubborn-diseased plants (1, 7, 8) and those cultured by Saglio et al. (12).

Whole seeds, chopped seeds, chopped seed coats, and seeds with coats removed were aseptically removed from fruits, placed in flasks of liquid media, and incubated at 30 C. An organism, apparently identical to that cultured from diseased young sweet orange shoots, was obtained from aborted seeds of diseased fruits of field-grown trees of Valencia orange in May 1971, and later from aborted seeds of diseased Marsh pink grapefruit, Chandler pomelo, and Hinkley and Pineapple sweet orange. The pomelo and Pineapple orange trees were naturally infected within the last few years. Ten primary cultures were grown, in 14 attempts, from aborted seeds; no cultures were obtained in 10 attempts with seeds from normal trees, in three attempts with seeds removed from

their coats, nor in four unseeded flasks. Aliquots from all flasks were centrifuged and examined by electron microscopy. Infected seeds seem to be a good source for culturing the mycoplasmalike organism associated with stubborn disease and, being encased in the fruit, are freer from contaminants than leaves and shoots. None of the cultures from shoots or seeds has been proven pathogenic.

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