STUBBORN, GREENING, and RELATED DISEASES

Stubborn and Greening: A Review, 1969–1972

J. M. Bové and P. Saglio

Stubborn disease has been increasingly studied in the last 10 years, but it still remains one of the major threats to citrus (*2, 33*). Most studies have dealt with transmission and indexing of the disease, culminating in the demonstration that very young leaves from affected seedlings were good sources of the pathogen that was causing high percentages of infection. This finding suggested that the stubborn pathogen was present in the functioning phloem of young, growing leaves (*3*), and opened the way for further studies on phloem as a favorable site for the causal agent.

With the discovery of mycoplasmalike bodies in the phloem tissues of plants affected by diseases hitherto considered to be virus maladies (*10*), the possibility emerged that these microorganisms, rather than virus particles or infectious nucleic acid molecules, represented a new class of causal agents of yellows-type diseases of plants.

It is known that plants affected by stubborn disease show a number of symptoms characteristic of both yellows and greening disease. Similarities between stubborn and greening diseases have often been stressed (*2*). Transmission patterns of the pathogens of the two diseases are also similar. Thus several lines of evidence, including symptomatology, phloem tissues as the site of the pathogen, and failure to find virus particles in the affected plants, prompted the search for mycoplasmalike bodies (MLB) in plants affected by stubborn and greening.

MYCOPLASMALIKE BODIES IN SIEVE TUBES OF PLANTS AFFECTED BY STUBBORN AND GREENING

Mycoplasmalike bodies were first described in the sieve tubes of greening-affected plants (*21*). It was soon realized that these structures were more filamentous than were ordinary MLB (*22*) and later their envelope was found to be 150 to 250 Å wide—too large to be a simple-unit membrane (*30*). These structures were observed not only with South Africa greening disease, but also in association with Reunion greening and India citrus decline (*22*), Philippines leaf mottling (*30*) (fig. 1), and Taiwan likubin (*5*).

The appearance of their envelope system, which resembles a wall (*31*), makes it difficult to consider the greening structures as representative of the Mycoplasmatales. They might very well represent a new type of phytopathogen (*23*).

The association of MLB with greening immediately suggested treatment of diseased trees with tetracyclines. Injections of antibiotics in the trunks of greening-affected trees were started with apparently beneficial effects (*34*).

Mycoplasmalike bodies were also observed in the phloem elements of citrus seedlings affected with stubborn disease

Fig. 1. Mycoplasmalike structures in the sieve tubes of Madam Vinous sweet orange seedlings infected with stubborn strain C 189, compared with structures observed in phloem elements of sweet orange seedlings infected with greening (South Africa), citrus decline (India), leaf mottling (Philippines), and Reunion greening (Reunion).

(*19, 20*). These findings were confirmed by Laflèche and Bové (*22*). In 1971, Zelcer *et al.* (*35*) showed the association of MLB with little-leaf disease of citrus in Israel. In doing so they added one more argument in favor of the common identity of stubborn disease and little leaf.

A clear-cut difference exists between the MLB associated with stubborn and the structures observed in greening; the former are surrounded by a true-unit membrane approximately 100 Å thick, similar to that of true mycoplasmas, whereas the latter have an envelope system twice as thick, suggesting the presence of a cell wall in addition to a cytoplasmic unit membrane (*31*) (fig. 1).

By the end of 1970 the first experimental evidence was obtained showing that the causal agents of stubborn and greening were not viruses but MLB or phloem-confined microorganisms. That the etiological agent in both diseases appeared to be a microorganism could easily explain the long-noted similarities between the two diseases. It may also explain the differences, since the respective causal microorganisms also differ from one another. To fully ascertain the role of microorganisms in the etiology of stubborn and greening, it will be necessary to cultivate them.

ISOLATION AND CULTIVATION OF MLB ASSOCIATED WITH STUBBORN DISEASE

By 1970, survival and perhaps even multiplication of plant mycoplasmas in liquid medium had been inferred from indirect evidence (*4, 14*). Claims for isolation and cultivation of these organisms, however, were not sufficiently substantiated by the published evidence (*7, 18, 24, 26*).

In attempts to isolate and cultivate the agent associated with stubborn disease, great care was given to the choice of plant material. Very young, but not mature, citrus leaves, from greenhouse-grown, stubborn-affected sweet orange seedlings were the favored tissues for transmitting the stubborn pathogen (*3*). The number of MLB in the sieve tubes of such leaves was also much higher when seedlings were grown at 32°C than when grown at 24°C (*22*). The specific effect of high temperatures on expression of stubborn symptoms was noted earlier (*1, 27*).

Using very young leaves from stubborn-affected Madam Vinous sweet orange seedlings grown at 32°C for 16 hrs and 27°C for 8 hrs, Saglio *et al.* (*29, 30*) were able to isolate and grow a mycoplasmalike organism in liquid broth as well as on solid medium. Their conclusion that the cultured mycoplasma was not the result of contamination by an animal or human mycoplasma was based on the following: (1) the organism was obtained only from stubborn-affected citrus leaves, never from similar healthy control leaves growing next to diseased material; (2) the organism was consistently isolated and grown in 17 independent experiments with stubborn-affected leaves, but not in 11 experiments with healthy material (*30*) (22 out of 28 experiments finally gave positive results (*31*)); (3) uninoculated liquid or solid media controls never showed any signs of microbial growth; and (4) the optimal growth of the isolated organisms was close to 32°C, clearly different from that of animal mycoplasmas.

Concurrently, workers in California isolated and grew a MLB from stubborn-affected young leaves of Madam Vinous sweet orange seedlings (*11, 12*). They reported the same small, fried egg-type colonies (diam 0.1–0.2 mm) similar to those described by Saglio *et al.* (*29, 30*). In California, however, cultures also contained spherical cells of undetermined relationship, averaging 0.6μ diam and enclosed temporarily in wall-like coatings 55–90 nm thick

(*11*). A pure culture of the MLB was also obtained from aborted seeds of diseased fruits of various citrus cultivars (*12*).

The MLB grown by Saglio *et al.* (*29*) were isolated from Madam Vinous sweet orange seedlings affected by a California isolate of stubborn (C 189) supplied by Dr. E. C. Calavan, and was the same isolate used by the California group (*12*). A MLB was also isolated from stubborn-affected, field-grown Washington navel trees in the Tadla area of Morocco (strain R8 A2) (*30, 31*).

The belief that both the California and Morocco isolates were mycoplasmas was based on the fried egg colony morphology on agar, ultrastructure of the organisms, their resistance to penicillin and sensitivity to tetracyclines, the absence of reversion to walled forms after 10 passages in penicillin-free medium, and isolation and cultivation in the total absence of any antibiotic (*12, 30, 31*).

CHARACTERIZATION OF THE MICROORGANISM ASSOCIATED WITH STUBBORN

It was found necessary to further characterize the microorganisms isolated from stubborn-affected plants serologically, biochemically, biophysically, and ultrastructurally. This characterization revealed that the stubborn organism possesses unique properties which justify its designation as *Spiroplasma citri,* a new genus and species.

So far we have not been able to cultivate the structures associated with greening disease by using the same techniques and media as those used for stubborn. Ghosh *et al.* (*13*) report having succeeded in growing the greening organism. Published evidence does not yet support this claim.

Morphology. One of the most striking properties of the stubborn organism is its morphology in liquid culture. The structures are essentially filamentous, with a beaded appearance at various places (*12, 31*). Filaments were sometimes seen to be connected to irregularly shaped main bodies (*12*). Branching of filaments was also observed (*12, 31*). Recent studies show the filaments to be helical in form, and motile (*6*). The helices can be seen by dark-field microscopy of living preparations (fig. 2A) and

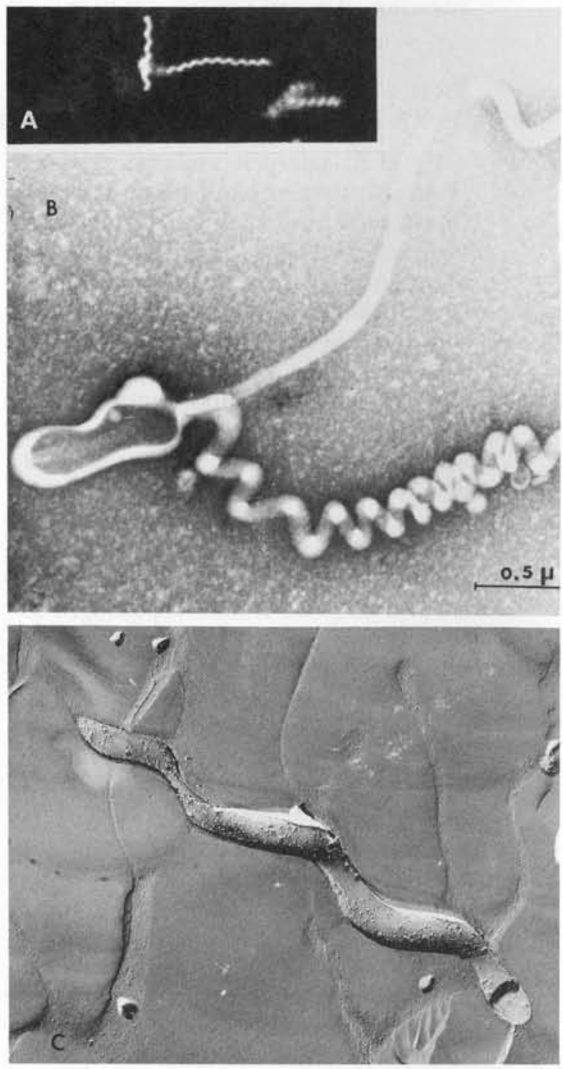

Fig. 2. Helical morphology of the stubborn organism in liquid culture as seen by: (A) dark-field microscopy; (B) preparations negatively stained with ammonium molybdate; (C) electron microscopy of freeze-dried preparations.

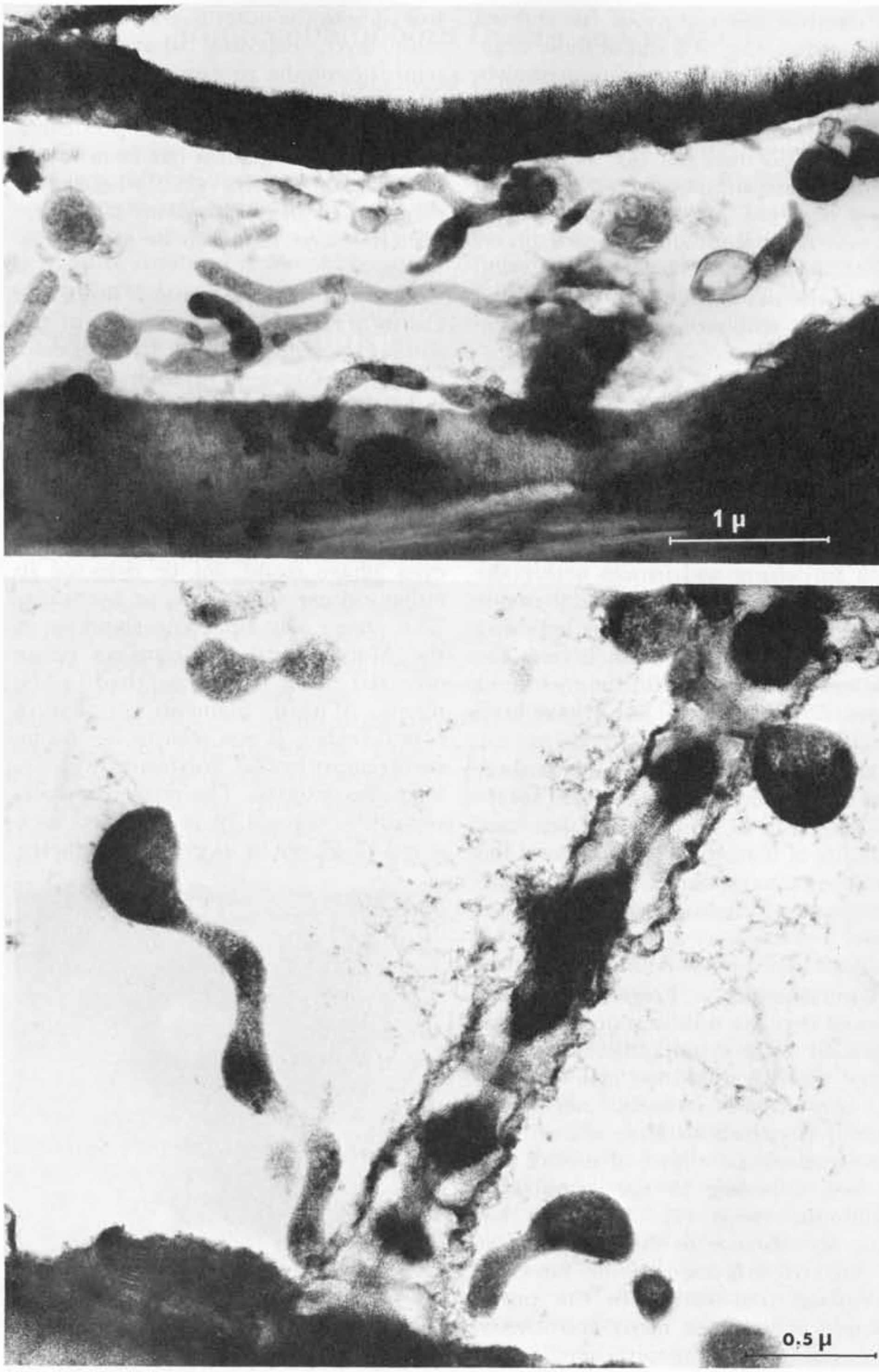

Fig. 3. Helical morphology of the stubborn organism in sieve tubes of an affected sweet orange seedling.

by electron microscopy of freeze-dried preparations (fig. 2C) and of those negatively stained with ammonium molybdate (fig. 2B). Ammonium molybdate preserves the helicity, while phosphotungstic acid does not (*6*). The helical filaments are similar in appearance to those observed previously in juice expressed from stunt-affected corn plants (*8*). Helical filaments have been detected within the sieve tubes of thick ultrathin sections of stubborn-affected citrus material (fig. 3).

Some indication of helicity can be seen in previous electron micrographs. Comparing the stubborn structures with those present in greening-affected phloem tissues, the former were described as being "sinusoidal" (*32*, plate II B). The presence of helical filaments both in culture and *in situ* within the sieve tubes of diseased material is one of the strongest arguments for believing that the cultured organism is from the diseased plant. Except for the corn stunt agent, no other helical MLB have been described.

Motility. The helical filaments show two types of motility (*6*), a rapid rotary motion and a slow undulation and bending of filaments. The organism has no flagella, axial filaments, or other organelles that might account for its motility. The capacity of the organism for independent motion is unexplained.

Ultrastructure. Previous studies showed that the stubborn organism was bounded by a cytoplasmic unit membrane without a definite cell wall (*12, 29, 30*). More recently, negatively-stained preparations have shown that the organism has a layer of surface projections adhering to the cytoplasmic membrane (nap) (*6*). This nap has some resemblance to the outer surface of the triple-layered outer envelope (periplast) that surrounds the protoplasmic cylinder of many spirochetes. The analogy with the spirochetes can be extended further. Filaments have been observed on which certain regions were free of both the outer nap and an innermost layer, exposing what could be remnants of the protoplasmic cylinder associated with spirochetes.

It should be added at this point that the stubborn organism has been found to be gram-positive (*32*), whereas all members of the Mycoplasmatales have hitherto been found to be gram-negative.

Bacteriophage infection. Both the California and Morocco strains of the stubborn agent isolated in Bordeaux have been shown to be infected with a tailed bacteriophage of type B morphology (fig. 4) (*6*). The phage was seen in the cultures after 21 serial passages. Subcultures cultured in Bethesda continued to show abundant phage for about 10 passages. After 50 more passages, however, phage could not be detected in either culture at Bethesda or Bordeaux. The phage was especially common in the Morocco strain. Complete phage occurred either free or attached to filaments. Within filaments or within round bodies, it was seen to be incomplete (empty heads). Polyheaded tubules were also detected. The phage head was usually hexagonal in profile, and measured 47–50 nm in its greatest diameter.

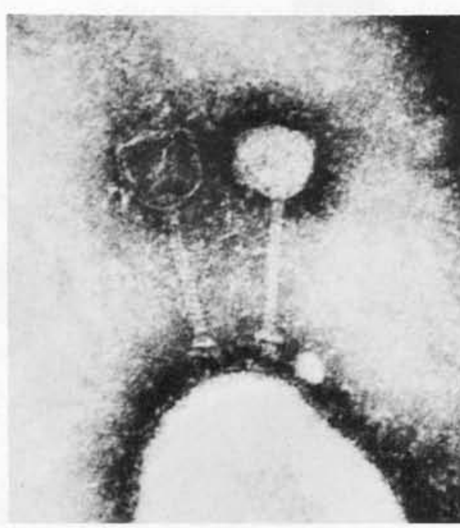

Fig. 4. Features of the bacteriophage associated with *Spiroplasma citri*.