Maintenance In Vitro of the Aster Yellows Mycoplasmalike Organism

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ABSTRACT

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Longevity in vitro of the aster yellows mycoplasmalike organism (AYO) isolated from the leafhopper vector, *Macrosteles fascifrons*, was determined by an infectivity assay after incubation in isolation media in which the factors of pH and osmolarity were varied. Longest in vitro survival, up to 72 hr, occurred at a neutral to slightly alkaline pH (pH 7.0–7.5) and high osmolarity (800–1,000 mOs/kg). At pH greater than 7.5, at any osmolarity tested, survival of the AYO was minimal. The addition of spermine (10 μg/ml) appeared to enhance maintenance at suboptimal osmolarity.

Aster yellows (AY) has been the most widely and intensively studied plant disease of suspected mycoplasmal etiology. Investigations on AY began when Kunkel (17–20) demonstrated that the AY organism (AYO) was transmitted by the six-spotted aster leafhopper, *Macrosteles fascifrons* (Stål), and that the vector was infective only after an incubation period of 9 days, which led him to suggest that the AYO might multiply inside the vector.

Black (1,2) demonstrated that the AYO could be mechanically transmitted by injecting small amounts of hemolymph from infected insects, diluted with 0.85% NaCl (saline), into healthy vectors. By using saline buffered to pH 7.0 with K_2HPO_4, Black (3) was able to detect infectivity for up to 24 hr in extracts stored at 0 C; but longevity was reduced by higher temperatures. Infectivity was detected after 2, but not 3, hr at 25 C, after 10 min at 35 C, but only slightly at 40 C.

Results of Black's (3) experiments involving temperature, pH, freezing, addition of divalent cations, centrifugation, and filtration suggested that the AYO was a "very large unstable virus." However, purification of the AYO eluded both workers. Differential centrifugation (21) and gel filtration (34) yielded ambiguous results with no sharp patterns of concentration.

Some workers have reported maintaining AY infectivity in vitro for brief periods. Hirumi and Maramorosch (15) were able to demonstrate viability of the AYO in Hirumi's leafhopper culture medium supplemented with 20% fetal bovine serum after 6 hr. Davis et al (13) achieved maintenance of the AYO for 3 hr in a simple buffer solution containing 0.3 M glycine, 0.03 M magnesium chloride. They were able to detect viability up to 24 hr at pH 8.0 and 22 C if amino acids, vitamins, inorganic salts, sucrose, cholesterol, and 5% horse serum were added to this buffer.

Caudwell et al (5,6) used the infectivity assay technique to determine physical and nutritional factors beneficial to the in vitro maintenance of the flavescence dorée agent. By successively modifying the media, they were able to increase the longevity of the agent from only a few minutes to more than 44 hr. In addition, they increased the titers of the agent being maintained in their extracts from 0.2 infectious units per milliliter to over 10^5 units per milliliter during the investigation (7).

The successful cultivation in vitro of plant pathogenic spiroplasmas has depended heavily upon augmentation of the osmotic pressure of the media (10,32). Media have been formulated to more closely resemble the osmotic pressure and chemical composition of phloem sap, which contains 12–18% sucrose (27); in fact, phloem sap has been used as a culture medium (26). In addition to the osmotic buffering afforded by the added sugars, a membrane-stabilizing effect of divalent cations has been shown that stabilizes spiroplasmas by preventing leakage of cytoplasmic constituents and loss of viability (33).

Most mycoplasmas prefer a slightly alkaline to neutral pH for growth. Black (3) demonstrated that the AYO was not viable at extreme pH ranges, and required a pH around neutral. Fudl-Allah et al (14) determined that *Spiroplasma citri* also was sensitive to pH extremes; growth did not occur at pH 9.0 and was poor at pH 4.0 and 5.0, but was adequate between pH 6.0 and 8.0.

Since the discovery of the association of mycoplasmalike organisms (MLO) with the yellows diseases, a major objective has been to isolate, culture, and identify the causal organisms. However, to date, Koch's postulates for the proof of pathogenicity have been fulfilled for only two phloem-inhabiting mycoplasmas, *S. citri*, the causal agent of citrus stubborn, and the corn stunt spiroplasma (11,24,38). The characteristic helical morphology of these spiroplasmas was an immense aid to the cultivation of these agents, providing a visual marker that could be closely followed throughout the isolation and subcultivation process. However, this is not the case with the apparently nonhelical mycoplasmas (27), which have not been cultivated and therefore require the use of an infectivity assay to determine viability in vitro.

The objective of this study was to determine the physical and chemical environmental requirements for the maintenance in vitro of the AYO by means of an infectivity assay. Ultimately this information will be used in development of a medium foundation for the culture of this fastidious prokaryote.

MATERIALS AND METHODS

Insect rearing. The leafhopper vector of the AYO, *M. fascifrons*, was reared in growth chambers at 25–28 C day temperature and 20–25 C night temperature or in large cages housed in an air-conditioned room under constant fluorescent lighting and held at approximately 25 C. The insects were cultured on a variety of plants, including China aster (*Callistephus chinensis* (L.) Nees, 'Ball Florist Mix'), oats (*Avena sativa* L.), rye (*Secale cereale* L.), corn (*Zea mays* L.), and periwinkle (*Catharanthus roseus* (L.) G. Don). Healthy colonies were monitored for contamination by frequently checking the asters for symptom development.

The AYO-infected insects were reared on asters or periwinkle with a strain of the AYO originally obtained from Canada. Healthy insects placed on AY-diseased plants were held for a minimum of 7 days to allow multiplication of the AYO within the insect before extraction was attempted.

Extraction of AYO. Infected insects were collected and ground in a tissue grinder in a 0.3 M glycine and 0.03 M magnesium chloride buffer at a 1:100 dilution of insect fresh weight to volume.

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The buffered extract was then passed through a filter (0.45-μm pore diameter) by using a syringe and a Swinney filter holder, topped with a layer of mineral oil to prevent oxidation, and incubated at 27 C. Small amounts were drawn off at 0, 2, 4, 8, 12, 24, 48, 72, and 96 hr after the initial extraction to be assayed for pathogenicity.

Effects of the chemical and physical environments on the viability in vitro of the AYO were tested by varying the factors of pH and osmolarity of the isolation medium. Osmolarity was varied within the range 400–1,200 mOs/kg by addition of sucrose at a constant pH of 7.5. Osmolarity was determined using a Wescor, Inc. (Logan, UT 84321) vapor pressure osmometer. The effect of pH was tested by holding osmolarity constant at 600 mOs/kg while pH was varied between 6.0 and 8.0 at 0.25 intervals by adding NaOH. The effect of spermine, a membrane-stabilizing polyamine, was tested at 10 μg/ml at pH 7.5 and 600–1,000 mOs/kg. Buffer solutions without the insect extracts were used as controls.

Infectivity assay. The AYO preparations were assayed for pathogenicity by observing the ability of leafhoppers injected with the preparations to transmit the AYO to aster, as evidenced by development of typical AY symptoms. The injection technique used in this study is a modification of that of Caudwell et al (8). Insects were immobilized under stretched Parafilm and injections were made through the Parafilm into the abdomen of the insects. Injection needles were made from glass capillary tubes, pulled on an electrode puller and calibrated to deliver approximately 0.25–0.50 μl per injection. Approximately 21,000 insects were injected by this method during this study.

Transmission to plants. Injected insects were collected with an aspirator and caged on an immune host, either oats or rye, for one to two weeks at 25 C. This allowed sufficient time to complete the incubation period in the insect. Insects were then transferred in groups of three or more to young China aster seedlings at the 4- to 6-leaf stage. Thereafter, the insects were transferred biweekly to new seedlings to prevent stunting of the plants by prolonged confinement in the plastic cages or insect feeding damage. Plants were grown under supplemental incandescent lighting for a 16-hr daily photoperiod to prevent premature flowering.

At 15–30 days after injection, symptoms began to appear on the plants to which the insects had been confined. The first symptom to appear on aster was vein clearing in the immature leaves, followed by stunting associated with decreased internode length, reduced leaf size, and chlorosis. In mature or flowering asters, virescence and phyllody were the predominant symptom types (Fig. 1). In periwinkle, the older leaves became yellow and, as new growth developed, hyperplastic shoots grew from lateral buds that normally remained dormant, flowers were reduced in size and became leaflike, and witches' brooms were formed.

RESULTS

Results are presented as a summation of three experiments. In the first experiment, injections were made 0, 2, 4, 8, 12, 24, and 48 hr after the MLOs were extracted. Injections were not made beyond 48 hr because the AYO was not expected to remain viable in the buffer solution this length of time. However, since transmissions were readily achieved after 48-hr incubation in several treatments, the second and third experiments consisted of daily injections at 0, 24, 48, 72, and 96 hr after extraction of the AYO. These experiments were done in the months of October through April. After that time (from May through September, when temperatures in Florida are constantly in the 25–35 C range) transmission was erratic and growth of the asters was poor and slow.

Effect of pH. In vitro viability of the AYO was detected over the entire pH range tested at a constant osmolarity of 600 mOs/kg (Fig. 2). Maintenance to 24 hr was obtained at pH 6.0–6.25 but maintenance was increased to 48 hr between pH 6.5 and 7.5. However, very poor AYO survival occurred at pH values greater than 7.5; at pH 7.75 and 8.0 the longevity was 4 hr or less.

Effect of osmolarity. Maintenance was achieved to 24 hr at 400 mOs/kg, but the longest viability recorded for this series, 72 hr, occurred at 800 and 1,000 mOs/kg (Fig. 3). Transmission experiments at 1,200 mOs/kg were inconclusive due to the high mortality of the insects.

Effect of spermine. During the second and third replications, spermine was added at a concentration of 10 μg/ml to solutions of pH 7.5 and osmolarity of 600 or 800 mOs/kg. The addition of spermine increased longevity of the AYO from 48 to 72 hr at the suboptimal osmolarity of 600 mOs/kg. Longevity beyond 72 hr was not achieved at 800 mOs/kg (Fig. 3).

DISCUSSION

Verification of viability, pathogenicity, and ultimately of cultivation of any of the nonhelical MLOs is dependent upon use of the infectivity assay. Development of typical AY symptoms of vein clearing, stunting, reduced foliar size, chlorosis, virescence, and phyllody in these plants demonstrated that viable organisms were present in the injected extracts. The assay, although laborious and

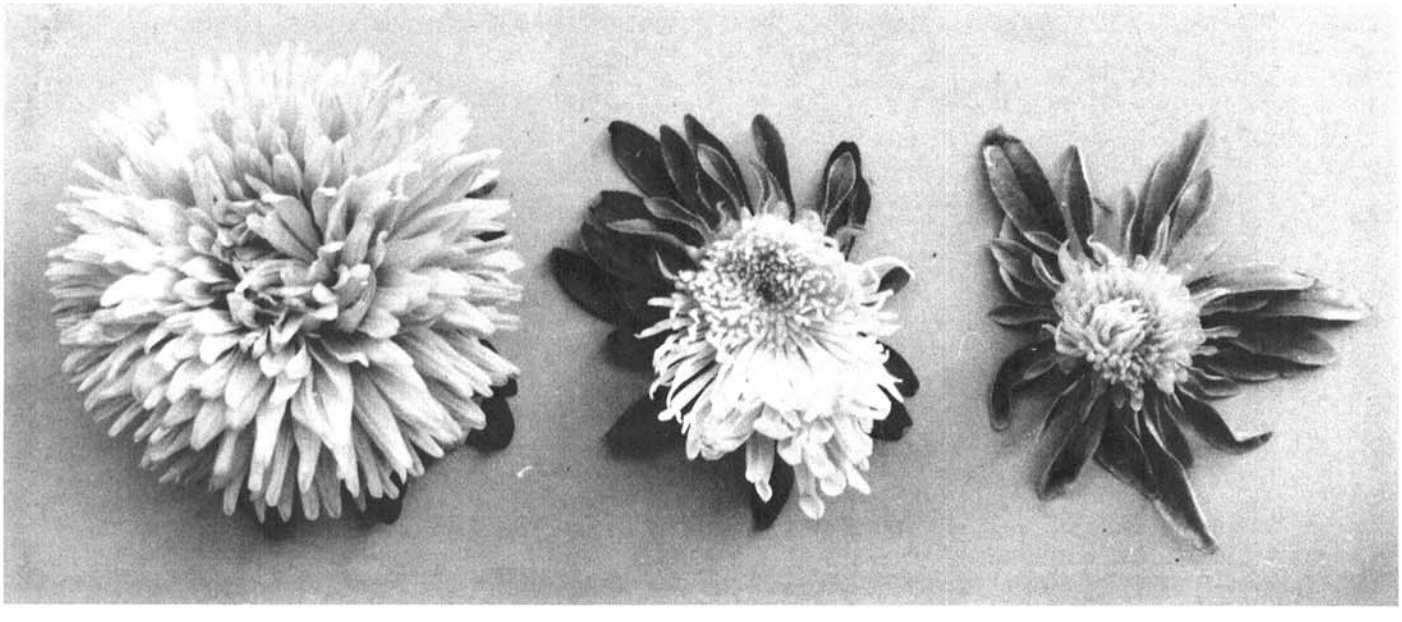

Fig. 1. Floral symptoms of aster yellows in China aster: **A,** healthy; **B,** unilateral development of virescence in a plant infected at a late stage of growth; **C,** typical virescence symptoms in a mature plant infected at an earlier stage of growth. All flowers are from plants of the same age.

time consuming, is a necessity for proving viability and is a step in Koch's postulates for proving pathogenicity of insect-vectored plant diseases.

Viability of the AYO was maintained in vitro up to 72 hr by adjusting the physiochemical environment of a simple unsupplemented glycine-magnesium chloride buffer solution. Longevity of the AYO in vitro was greatly influenced by the pH and osmolarity of the buffer solution. Both of these factors appear to be critical to survival of the AYO. Maximum longevity was achieved when the buffer was adjusted to a high osmotic pressure (800–1,000 mOs/kg) and a neutral to slightly alkaline pH (pH 7.0–7.5).

The addition of a low concentration of spermine, a membrane protectant, increased the longevity of the AYO in vitro by 24 hr at 600 mOs/kg and pH 7.5 as demonstrated in Fig. 3. Spermine at the concentration of 0.001 M has been reported to have the same effect as a 0.5 M sucrose solution in preventing cellular lysis (35). This compound is known to exert stabilizing effects in vitro on various biological membranes and it is comparable in effect to a high concentration of cations. The stabilizing effect of spermine on mycoplasmal membranes has been documented (33).

Multiplication of the AYO probably did not occur in the extraction solutions used in this study since the basic nutritional requirements of the mycoplasmas were not met. However, values of osmolarity and pH were determined that were not antagonistic to AYO survival. Transmission occurred when organisms were drawn up from the buffered extracts and injected into the insect vector. Since classical AY symptoms of vein clearing, yellowing, stunting, virescence, and phyllody were produced, it is clear that the AYO was present in the injected extracts.

Since the discovery of the cultivable plant pathogenic spiroplasmas, many definitive studies have described various media for cultivation of these agents (10,36). Workers in several laboratories recently have isolated spiroplasmas from plants purportedly affected by aster yellows. However, claims of cultivation of this organism and description of some of its physical properties are at odds with established properties of the AYO. One major factor is temperature. Growth of possible AY spiroplasmas at 32 (9) and 36 C (31) is inconsistent with the temperature inactivation studies of Kunkel (19,20) and the fact that heat therapy of AYO-infected insects was accomplished temporarily at temperatures of 31–32 C for 3 days. Transmission ability was permanently lost when infectious insects were heat treated for 12 days at 32 C. Maramorosch (23) and Kondo et al (16) reported culture of an AY spiroplasma from diseased lettuce. Although 3 yr have elapsed since the initial report, there has been no published documentation of the fulfillment of Koch's postulates. A comparative study of the morphology in vitro of an aster yellows spiroplasma and a pear decline spiroplasma by negative staining also was reported (22). However, failure to consider the adverse effects of phosphotungstic acid on the morphology of spiroplasmas (12) compromised the results; only artifacts were compared. Polyacrylamide gel electrophoresis, and the serological growth inhibition and deformation tests have demonstrated the identity of these "AY spiroplasmas" to *S. citri* (28,30,31,37). Electron microscopy of AY-diseased plants and insects has never revealed the presence of helical filaments in these hosts, although helices are readily observed in sieve elements and vector insects infected with *S. citri* or the corn stunt spiroplasma (27).

The many contradictions in the properties of the new "AY spiroplasmas" to the established properties of the AYO indicate that the cultured organisms and the AYO are completely different. Oldfield (29) recently reported natural dual infections of periwinkle and brassicaceous weeds with *S. citri* and MLO in California. Also demonstrated was the ability of groups of field-collected citrus stubborn vectors, *Circulifer tenellus* Anth., to transmit simultaneously a spiroplasma, *S. citri,* and an MLO to periwinkle, and the subsequent development of virescence-type symptoms instead of the symptoms typically produced by single infections of *S. citri* (4).

Symptoms induced by spiroplasmas include chlorosis, stunting of vegetative organs and reduction in flower size (4,25). The nonhelical MLOs not only produce yellowing and stunting, but are uniquely associated with virescence, phyllody of floral organs, and vegetative proliferation or witches' broom. Neither *S. citri* nor the

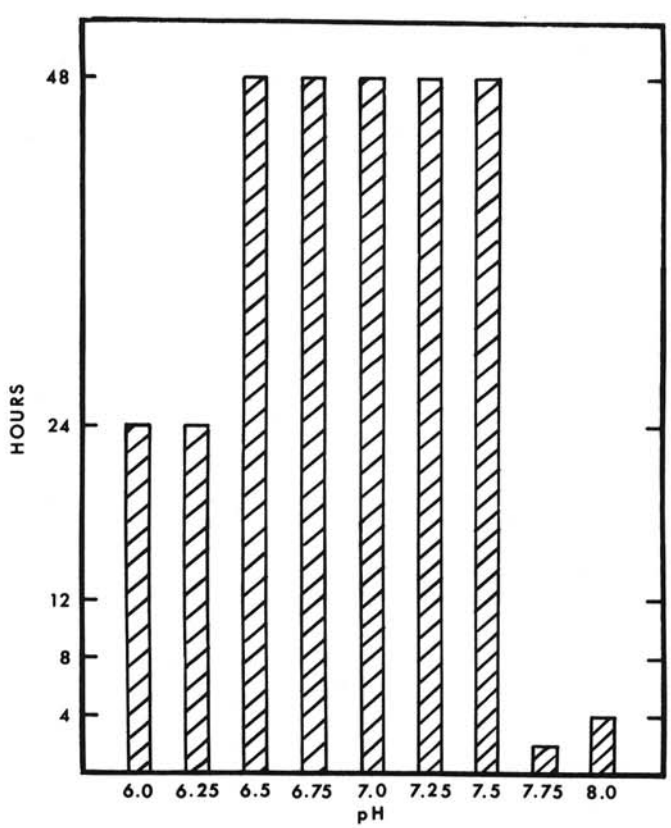

Fig. 2. Effect of pH on longevity in vitro of the aster yellows organism at 600 mOs/kg.

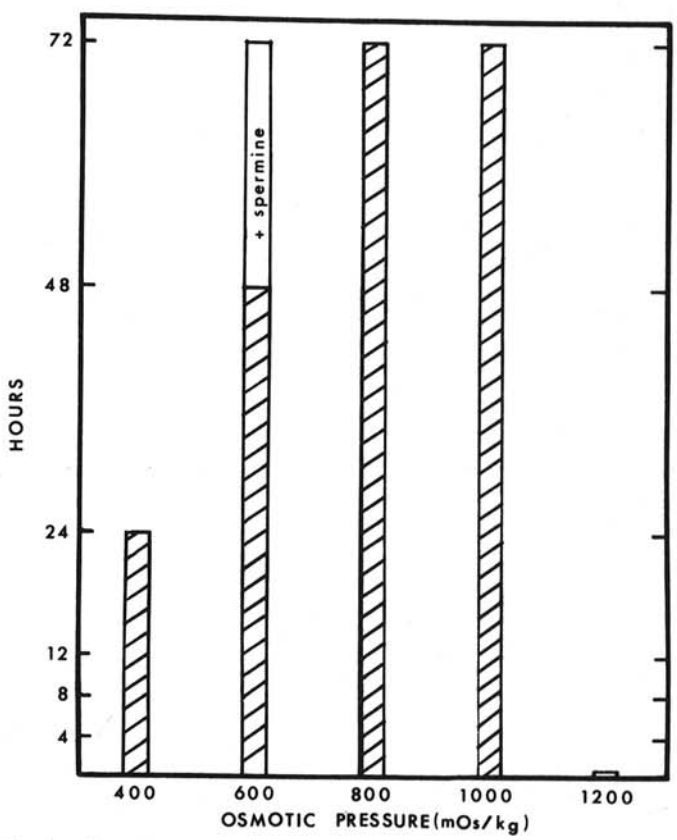

Fig. 3. Effect of osmolarity and spermine on the longevity in vitro of the aster yellows organism at pH 7.5.

corn stunt agent induce virescence (4).

In conclusion, the physical values of osmolarity and pH that are conducive to in vitro survival of the AYO have been determined. This information will be of direct value in future attempts to cultivate the MLOs associated with the yellows diseases. The differences in properties of the newly isolated "AY spiroplasmas" associated with the AY disease and the serological identity of these organisms to *S. citri* argue against coidentity of these agents. This information together with the known potential for dual infections of *S. citri* and a virescence agent in the same plant may readily explain the occasional isolation of *S. citri* from AY-diseased plants. Consequently, the cultivation of the AYO is still an open question.

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