Effect of Temperature and pH on Growth in vitro of a Mycoplasmalike Organism Associated with Stubborn Disease of Citrus

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Accepted for publication 23 August 1972.

ABSTRACT

A mycoplasmalike organism associated with stubborn-diseased citrus was grown in special cell-free liquid and agar media. The organism grew from pH 4 to 8, optimum pH 7, and from 9 to 39 C, optimum 30 C. Colonies formed on agar media at 9, 12, 15, 18, 21, 36, and 39 C were few and small. Larger colonies appeared at 24 to 33 C, with optimal growth at 30 C. The organism was viable after storage for 2 weeks at 4 C or for 2 months at –20 C.

Phytopathology 63:256-259

A mycoplasmalike organism is frequently cultured from stubborn-diseased, but not from healthy, citrus plants (6, 10). A study of certain factors influencing growth and survival was made to improve in vitro production and maintenance of colonies for use in biological research, including pathogenicity of various isolates. This paper reports the effect of temperature and pH on growth of this organism in special cell-free liquid and agar media.

MATERIALS AND METHODS.–The primary culture of the organism used in this investigation was obtained from aborted seed of field-grown Hinckley seedling sweet orange trees graft-inoculated with California 189 stubborn source (6). Subcultures were grown on media containing 0.1% glucose, 0.1% fructose, 0.5% tryptone, 3% sucrose, 2% PPLO broth, 10% of 25% fresh yeast extract, 20% horse serum, distilled water and, for agar media, 1% Bacto agar. Aliquots of 0.05 ml from the third liquid subculture, 5 days old, were used for inoculation of plates and flasks incubated under different conditions. Inoculated plates were incubated over water in sealed moist chambers for 10 days at temperatures of 9, 12, 15, 18, 21, 24, 27, 30, 33, 36, and 39 C. Unseeded plates were maintained to confirm sterility of the medium. Average diameter and numbers of colonies were obtained from 10 microscopic fields for each treatment.

We studied the effect of pH on amount of growth by adjusting the initial pH of liquid media to values of 4, 5, 6, 7, 8, and 9. We added 0.5 ml inoculum to flasks containing 5 ml sterile liquid media, and incubated them at 30 C for 7 days. Relative amounts of growth were determined by measuring absorbency at 500 mμ in a Beckman DB spectrophotometer and by sedimentation under low-speed centrifugation (10,000 g/20 min) and electron microscopy of pelleted material (6).

RESULTS AND DISCUSSION.–Typical "fried-egg" colonies were obtained only on plates incubated at 27, 30, and 33 C (Fig. 1-G, H, I). Colonies at 30 C had conspicuous, dense, round centers, a finely granulated surface, and an average diameter of 200 μ, but the centers were less clearly defined in most

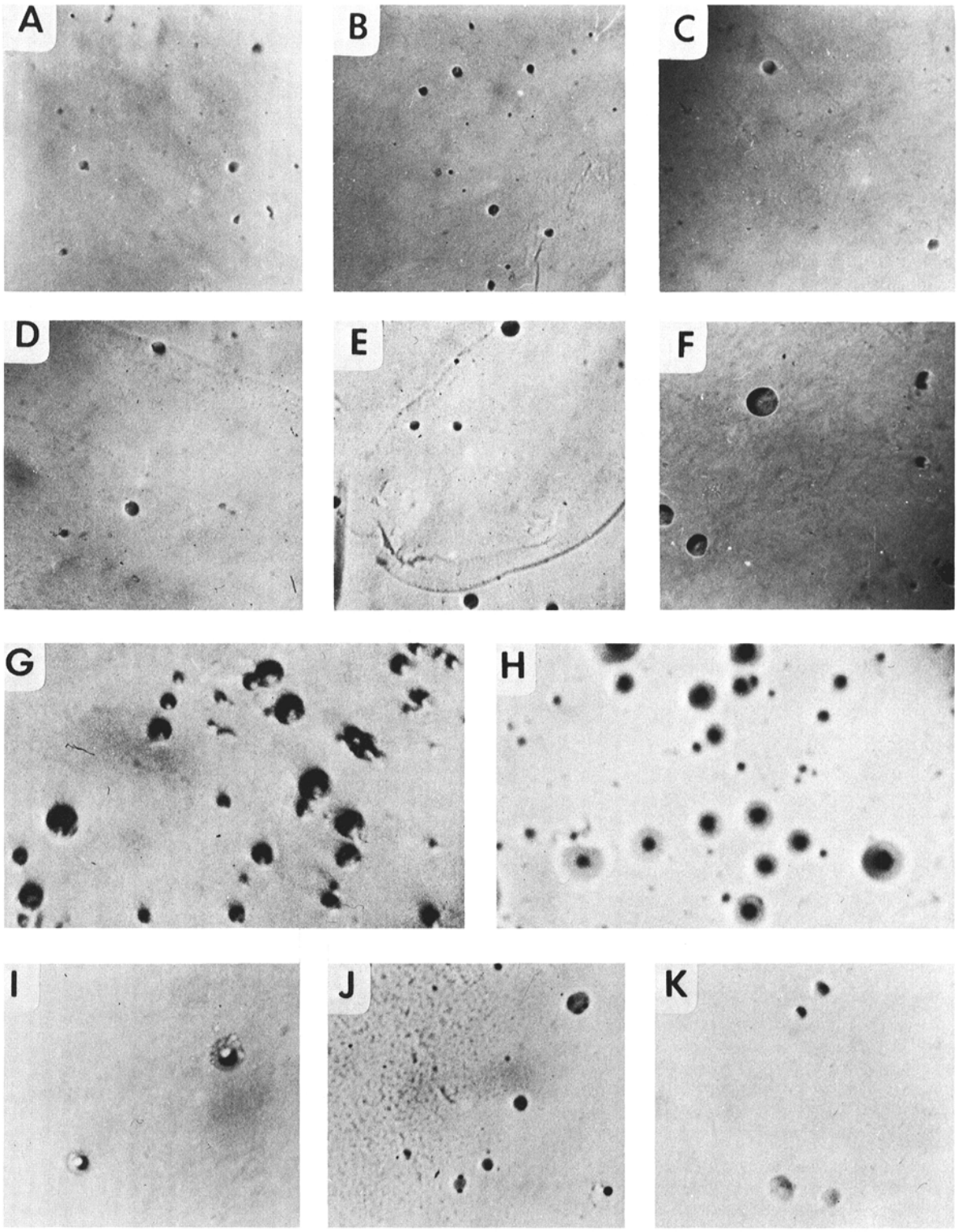

Fig. 1. Effect of temperature on growth, in vitro, of the mycoplasmalike organism associated with stubborn disease of citrus. Agar plates were incubated 10 days at **A)** 9 C; **B)** 12 C; **C)** 15 C; **D)** 18 C; **E)** 21 C; **F)** 24 C; **G)** 27 C; **H)** 30 C; **I)** 33 C; **J)** 36 C; **K)** 39 C. Growth was minimal at 9, 12, 15, 18, 21, 36, and 39 C. Larger and more colonies were obtained at temperature range of 24 to 33 C with optimal growth at 30 C.

colonies at 27 C (Fig. 1-G, H). Number and average diameter of colonies were largest at 30 C, and were larger at 27 than at 33 C (Fig. 2).

Colonies at 9, 12, 15, 18, 21, 36, and 39 C were few, less than 50 μ in average diam, translucent, and irregular in shape with margins and central spot poorly defined (Fig. 1, 2). Similar results were obtained at 24 C, except that colonies were denser and about 60 μ in diam (Fig. 1-F, 2).

When plates incubated 10 days at 9, 12, 15, 18, 21, and 24 C were transferred to 30 C, colonies grew normally and increased in size and number in similar fashion to those previously obtained at 30-C constant temperature. However, colonies in plates incubated at 36 or 39 C did not recover when transferred to 30 C, possibly due to desiccation at the higher temperatures.

Broth cultures derived from 5-day-old liquid culture produced at 30 C usually remained viable at 4 C for 2-3 weeks; and at −20 C for over 2 months.

It seems that 30 C is the approximate optimal temperature for growth of the mycoplasmalike organism associated with stubborn. This temperature falls within the 28-33 C range found favorable for symptoms of stubborn (E. C. Calavan, *unpublished data*). Similarly, the avian *Mycoplasma gallisepticum* has an optimal temperature of 38 C (7), which correlates to its natural occurrence in birds having relatively high body temperatures. Also, the T strains of mycoplasma from the human genitourinary tract multiply satisfactorily between 30 and 36 C, but 36 C is optimal for their growth (5) and is close to human body temperatures.

One saphrophytic species, *M. laidlawii,* has an optimum temperature of 30 C, although it also grows at 22 to 37 C (4). Unlike mycoplasma associated with stubborn, *M. laidlawii* does not require sterols (1). However, no growth will be obtained with mycoplasma associated with stubborn in absence of horse serum, cholesterol, or ascitic fluid (6).

Insofar as we know, *Mycoplasma* spp. pathogenic to warm-blooded animals have optimum temperatures in culture near those of the host's body temperature. This is considerably higher than the optimum for the mycoplasmalike organism consistently isolated from stubborn-diseased plants.

Since Dienes & Edsall's report (2), most investigators have considered the optimal pH of culture media for the isolation and cultivation of mycoplasma to be pH 7.8 to 8.0. Our results indicate that no growth was obtained at pH 9, and growth was poor at pH 4 and 5. Good growth occurred at pH 6 to 8, with optimal growth at pH 7. For routine use, however, a medium should be adjusted to about pH 7.5, to offset the decrease in pH resulting from growth of the organism. Shepard & Lunceford (11) suggested that an initial pH of 7.8 to 8 (2) may not be optimal for the cultivation of all species of human mycoplasma. Edward (3) studied in detail the effect of pH on growth of a number of mycoplasmas and recommended media at pH 8.0. However, Edward & Freundt (4) indicated that Edward's results might be criticized, as he was using laboratory strains already adapted to an alkaline medium.

Our findings indicate that the mycoplasmalike organism associated with stubborn disease of citrus (8, 9) can be cultured in cell-free media adjusted to pH 7.5 and incubated at 27 to 33 C.

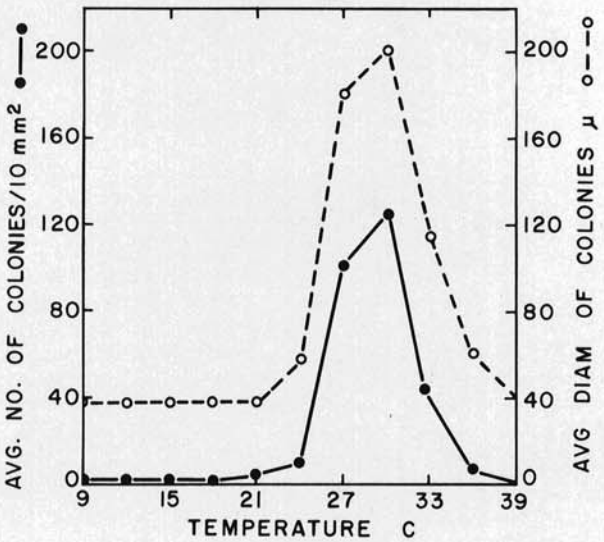

Fig. 2. Effect of temperature on growth of the mycoplasmalike organism associated with stubborn disease of citrus. Average number and diameter were obtained from 10-day-old colonies on agar media.

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