Contrasting Effects of Horse Serum and Fresh Yeast Extract on Growth of Spiroplasma citri and Corn Stunt Spiroplasma

E. C. K. Igwegbe

Department of Biology, Alabama State University, Montgomery, AL. Present address of author: Department of Crop Science, University of Nigeria, Nsukka, Nigeria.

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ABSTRACT

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Both *Spiroplasma citri* (SS) and corn stunt spiroplasma (CSS) grew faster and attained higher titers in liquid or agar medium supplemented with 5% horse serum than in medium with 20% horse serum. When growth was initiated with a low concentration of inoculum, CSS failed to reproduce but SS achieved excellent growth in a medium containg 20% horse serum. *Spiroplasma citri* grew in more agar medium variations and was less affected by high concentrations of fresh yeast extract than was CSS. These results suggest that CSS and SS differ in their responses to the presence of similar concentrations of horse serum or fresh yeast extract.

Additional key words: mycoplasma, plant yellows diseases.

Recently, several reports have associated mycoplasmalike organisms (MLO) with many yellows diseases of plants (4). With the exception of corn stunt spiroplasma (CSS) and citrus stubborn spiroplasma (SS) which have been cultured (1, 6, 12) the spiroplasmas associated with plant diseases have resisted isolation in cell-free media. The reason for this is unknown, but is possibly due to inadequacies of the isolation media (9).

An earlier paper (8) reported that high concentrations of horse serum inhibited growth of CSS whereas low concentrations stimulated growth. Since several lines of evidence (2, 3, 15) point to a possible relationship between CSS and SS, it appeared of interest to test the effect of horse serum on SS. Furthermore, the effect of fresh yeast extract on growth of CSS and SS was compared because Liao and Chen (10) found that CSS achieved excellent growth in medium lacking fresh yeast extract. The results of this study are presented.

MATERIALS AND METHODS

Organisms and medium.—Corn stunt spiroplasma (ATCC 27954) and the California isolate of SS (ATCC 27665) used in this study were purchased from American Type Culture Collection, Rockville, MD 20852. Initially, CSS and SS were grown for three passages in the medium of Chen and Liao (1) and Fudl-Allah et al. (6), respectively. Thereafter, each organism was transferred to the liquid medium of Liao and Chen (10) as modified by Igwegbe (8). This medium contained: PPLO broth (Difco), 1.5% (w/v); sucrose, 16% (w/v); horse serum (Microbiological Associates, Bethesda, MD 20014), 5% (v/v); phenol red, 0.0015%; and 100 units of penicillin G per ml. Each organism was subjected to four-to-six serial passages in this medium before use.

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Effect of horse serum concentration on growth in liquid medium.—The basal medium of Liao and Chen (10) was supplemented with horse serum (Microbiological Associates, Bethesda, MD 20014) to give final concentrations of 0, 5, 10, or 20% (v/v). Each medium was adjusted to PH 7.5, sterilized by Millipore filtration (0.20 μm, Nalge Sybron Corporation, Rochester, NY 14625), and dispensed in 1.9-ml amounts into 16 × 100 mm tubes. Inoculum consisted of a 10^{-2} dilution (CSS) or a 10^{-3} dilution (SS) of a 4-day-old culture of each organism grown in the medium of Igwegbe (8). This gave approximately 10^5 and 10^4 colony-forming units (CFU) per ml inoculated broths of CSS and SS, respectively. CFU were determined by agar plate counts of 0.1-ml aliquots of serial 10-fold dilutions of the diluted inoculum. Unless stated otherwise the diluent in each case was unsupplemented PPLO (1.5%) broth, pH 7.5. Cultures were incubated aerobically at 29 C (CSS) or 32 C (SS). A 0.1-ml aliquot was removed from each culture for growth assessment on the days indicated (Fig. 1-A, B). Growth was monitored by a dilution plate technique on agar (0.8%, Noble agar, Difco) medium of similar composition as the liquid medium in which the inoculum was prepared. All agar plates were stored at 4 C and used within 2 wk of preparation. Previous studies had indicated that agar media kept well when stored at this temperature for one month provided they were wrapped up in aluminum foil placed inside a paper carton which was in turn placed in a sealed plastic bag. Inoculated agar plates were kept in moist sealed plastic bags and incubated aerobically at the appropriate temperature for each organism. After growth for 14 days (SS) or 21 days (CSS), plates were stained with diluted Diene's stain (13) before colonies were counted under a dissecting microscope (×40). Titer was expressed as CFU/ml.

Effect of fresh yeast extract concentration on growth in liquid medium.—Fresh yeast extract (Microbiological Associates, Bethesda, MD 20014) was added to the basal

medium of Liao and Chen (10) to give final yeast extract concentrations of 0, 2.5, 5.0, or 10% (v/v). Horse serum was present at a final concentration of 5% (v/v). All other techniques and procedures employed in this test were similar to those described above, except that aliquots for growth assessment for SS and CSS were removed on days 4 and 8, respectively.

Effect of horse serum and fresh yeast extract concentration on growth on agar.—Horse serum and fresh yeast extract concentrations and agar used in this test are indicated in Table 1. All media were prepared and sterilized at 121 C for 15 min before fresh yeast extract and horse serum were aseptically added. Each medium contained 0.8% (w/v) Noble agar (Difco). The inoculum was the same as that used for liquid-medium studies. Plates were dried for 2-3 hr at 32 C before each was inoculated with 0.1 ml of a 10^{-3} or a 10^{-6} dilution of CSS, or a 10^{-4} or a 10^{-6} dilution of SS, made in unsupplemented PPLO (1.5%) broth, pH 7.5. Incubation and procedure for growth assessment were as described above.

RESULTS

Effect of horse serum concentration on growth in liquid medium.—Growth of both CSS and SS was more rapid and higher titers were achieved in media with 5% or 10% horse serum than at 20% horser serum (Fig. 1-A, B). The inhibitory effect of high concentrations of horse serum was more noticeable on CSS than SS although both organisms experienced long lag periods in medium containing 20% horser serum. The results in Table 2 indicate that when the final inoculum concentration of CSS was 10^4 CFU/ml instead of 10^5, the organism achieved excellent growth in media with 5% horse serum, moderate growth in 10% horse serum, but apparently did not grow in the presence of 20% horse serum. In contrast, reduction of the final inoculum concentration of SS from 10^4 to 10^3 CFU/ml did not significantly affect the ability of this organism to grow at all three concentrations of horse serum tested. However, higher titer was obtained in a medium with 5% horse serum compared with 20% horse serum (Table 2). Neither organism grew in the absence of horse serum, regardless of final inoculum concentration.

Effect of yeast extract concentration.—Growth of SS in liquid medium increased with increasing concentrations of fresh yeast extract up to 5%. Above this value there was a slight decrease in growth. With CSS, growth was best in yeast-extract-free medium and decreased with increasing fresh yeast extract concentrations (Table 3). In fact, at 10% fresh yeast extract concentration the organism failed to reproduce.

Effect of horse serum and yeast extract concentrations on growth on agar.—Colonies of SS were obtained on all agar media tested except agar H. Typical "fried-egg" colonies were observed on agars A, B, and E (Table 1). These colonies had dense centers and a thick extensive periphery (Fig. 2). Conversely, SS colonies on agars F and I possessed conspicuously large centers with a very small and ill-defined periphery. Largest SS colonies (avg diam = 1 mm) were on agars F, I, and E. Smallest SS colonies were on agars D, C, and G.

Colonies of CSS were present on agars A, B, E, F, and I. No colonies of CSS were observed on other agars tested. Typical "fried-egg" colonies of CSS were noted on agars A and E only. These two agar media, in addition, contained the largest colonies (avg diam = 0.5 mm) of CSS. Colonies of CSS on all other agar media were small and granular (Fig. 2).

DISCUSSION

The results show that both CSS and SS achieved higher titers in liquid media with 5% or 10% horse serum than with 20% horse serum. However, there was evidence that the growth of SS in presence of 20% horse serum was less affected than that of CSS (Fig. 1). The sensitivity of CSS to high concentrations of horse serum was previously reported (8). The more rapid growth of these two plant mycoplasmas in medium with 5% horse serum compared with medium containing 20% horse serum is of interest because most mycoplasma media contain 20% horse serum (7) and only in special tests have lower concentrations been recommended (14). A titer of 10^8 CFU/ml achieved by both CSS and SS in the yeast extract-free medium supplemented with 5% horse serum is comparable to the titer attained by SS in other media (6, 12) and slightly better than the value of 10^7 CFU/ml reported by Chen and Liao in medium with 20% horse serum. Since both CSS and SS achieved excellent growth in the medium of Chen and Liao (1) as modified by

TABLE 1. Recovery and colony size of corn stunt (CSS) and citrus stubborn (SS) spiroplasmas on agar medium variations

Agar medium	Horse serum concn (%)	Yeast extract concn (%)	Growth (CFU/ml)		Colony size[a]	
			CSS	SS	CSS	SS
A	5	0	3.8×10^8	7.2×10^8	Medium	Large
B	5	2.5	9.0×10^6	7.0×10^8	Small	Large
C	5	5.0	None	7.0×10^8	No growth	Medium
D	5	10.0	None	7.2×10^8	No growth	Medium
E	10	0	3.6×10^8	7.4×10^8	Medium	Large
F	10	2.5	1.0×10^7	7.0×10^8	Small	Large
G	10	5.0	None	7.2×10^8	No growth	Medium
H	10	10.0	None	None	No growth	No growth
I	20	0	2.8×10^8	7.0×10^8	Small	Large

[a]Large = >1.0 mm, medium = > 0.5 mm, and small = < 0.5 mm diameter.

Igwegbe (8), this medium is strongly recommended for cultivation of these two plant spiroplasmas.

The failure of CSS but not of SS to grow in medium with 20% serum, using small inoculum concentration, is possibly of practical importance. Some plant mycoplasmas occur in low concentrations in their hosts (4) and most media used for their isolation contained 20% horse serum. Although the failure to obtain primary cultures of certain plant mycoplasmas may not have been entirely attributable to the high concentration of horse serum in the media, it is important that a range of horse serum concentrations be tried since the results of this study show that CSS and SS behave differently in presence of the same concentration of horse serum.

The experiments on the effect of the fresh yeast extract concentration of the liquid medium indicate that CSS and SS also differ in their growth on fresh yeast extract. Thus, CSS grew best in medium free of fresh yeast extract and actually failed to multiply in medium containing 10% fresh yeast extract (Table 3). In contrast, SS grew fairly well at all concentrations of fresh yeast extract tested, although best growth was in a medium with 5% fresh

TABLE 2. Relationship between horse serum concentration in liquid media and growth of corn stunt spiroplasma (CSS) and *Spiroplasma citri* (SS) using low concentration of inoculum[a]

Horse serum concentration (%)	Growth[b]	
	CSS (CFU/ml)	SS (CFU/ml)
5	1.2×10^8	3.7×10^8
10	4.8×10^6	2.6×10^8
20	4.0×10^3	1.4×10^7

[a]Final inoculum concentrations of CSS and SS at day zero were 1.2×10^4 and 5.5×10^3 colony-forming units (CFU)/ml, respectively.

[b]Number of colony-forming units (CFU) of SS and CSS per ml of medium was determined after days 5 and 13, respectively.

TABLE 3. Effect of fresh yeast extract concentration on growth of corn stunt (CSS) and citrus stubborn (SS) spiroplasmas in liquid media[a]

Yeast extract concn (%)	Growth[b]	
	CSS (CFU/ml)	SS (CFU/ml)
0	2.3×10^8	2.7×10^8
2.5	6.0×10^7	5.7×10^8
5.0	6.0×10^5	7.0×10^8
10.0	5.0×10^2	4.0×10^7

[a]Final inoculum concentrations for CSS and SS were 1.2×10^5 and 5.5×10^4 colony-forming units (CFU)/ml of medium, respectively.

[b]Number of colony-forming units was determined after day 4 (SS) and day 8 (CSS).

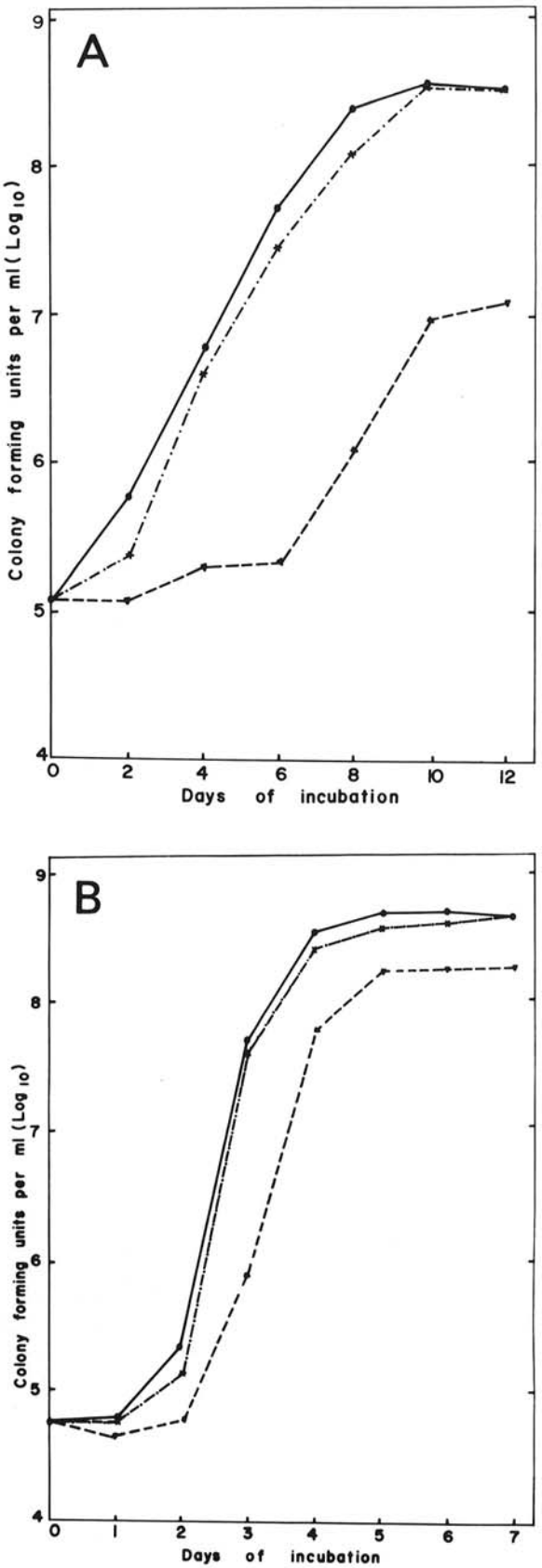

Fig. 1-(A, B). Growth curves of **A)** corn stunt spiroplasma and **B)** citrus stubborn spiroplasma in liquid medium containing: 5% horse serum •——•; 10% horse serum ×– · – · – · ×; 20% horse serum ▽ - - - - ▽.

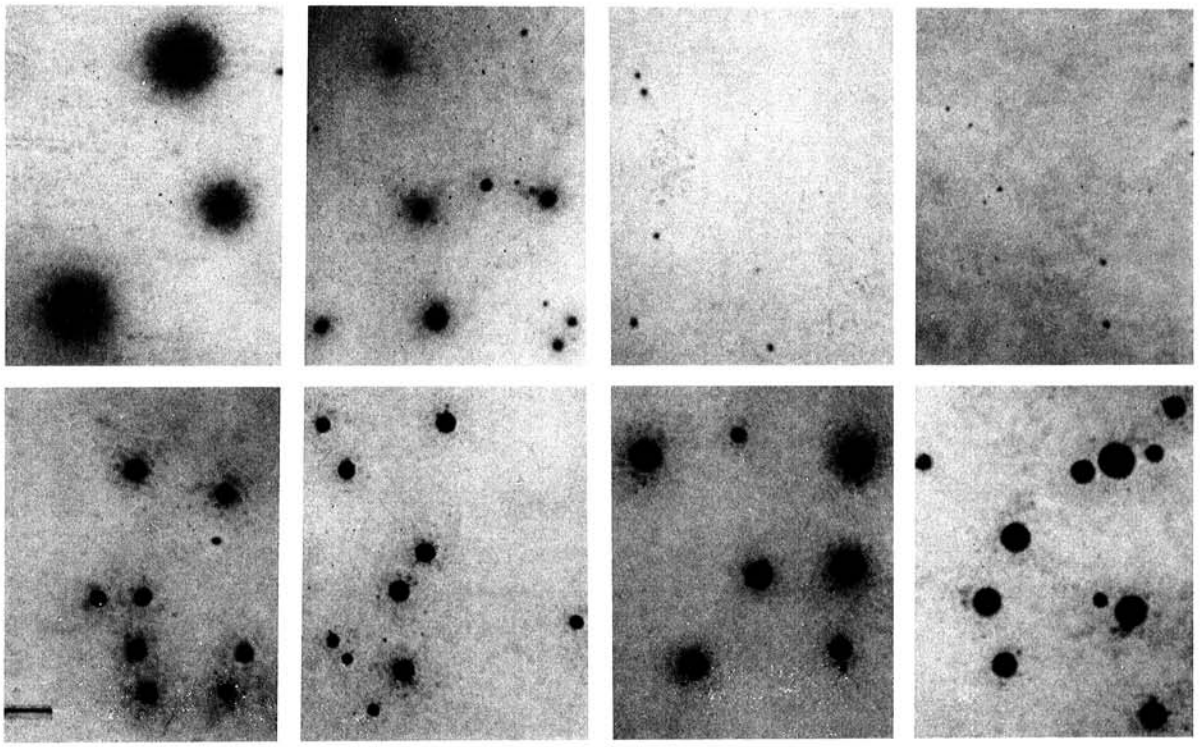

Fig. 2. Colonies of corn stunt spiroplasma (upper row), and citrus stubborn spiroplasma (lower row) on agar media containing different combinations of horse serum (HS) and fresh yeast extract (YE). From left to right: 5% HS plus 0% YE; 10% HS plus 0% YE; 10% HS plus 2.5% YE; and 20% HS plus 0% YE. The bar represents 0.5 mm.

yeast extract. The need to include different concentrations of fresh yeast extract in media for primary culture of plant mycoplasmas is evident. The growth-promoting effect of fresh yeast extract on SS has been reported by others (5, 9).

The data in Table 1 show that the ability of CSS and SS to grow in solid media varied considerably according to the medium employed. The growth of SS in more agar than CSS may be due to the fact that SS grew better in the presence of high concentrations of horse serum and fresh yeast extract than did CSS. Agar H was judged unsuitable for both CSS and SS since no colonies were obtained on it. This agar contained equal amounts (10%) of horse serum and yeast extract. Since both organisms grew quite well in liquid medium with 10% horse serum and poorly in media with 10% fresh yeast extract it may be concluded that lack of colonies on agar H was due to a growth-inhibitory effect caused by the high concentration of fresh yeast extract in this medium.

Although SS produced colonies on agars C and D, and CSS formed colonies on B, F, and I, these agars were considered inferior for each organism because of colony morphology and small colony size which made accurate counting difficult. Conversely, agars A, B, E, F, and I for SS, and A and E for CSS were judged superior to all other agars with regard to recovery, colony morphology, and stainability. The ability of both CSS and SS to form typical "fried-egg" colonies on agars A and E made colony counting easy. Since recovery of CSS and SS on these two agar media was excellent, both are recommended for growth of plant spiroplasmas and will henceforth be called plant spiroplasma agars.

These results indicate that CSS can produce typical "fried-egg" colonies on certain agar media and that the failure of some workers (11) to obtain classical "fried-egg" colonies of CSS on solid media may be partly due to high concentrations of horse serum present in the media used. The development of good solid media (plant spiroplasma agars A and E) for CSS should facilitate growth studies on this organism.

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