

# Infection of Three Insect Cell Cultures by *Spiroplasma citri* and Other Spiroplasmas

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**ABSTRACT.** *Spiroplasma citri* R8A2, *S. floricola* BNR-1, *S. apis* PPS-1 and the spiroplasma strains B88, CSS, 277F were inoculated into AS-2 (*Aceratagallia sanguinolenta*), AC-20 (*Agallia constricta*) and Dm-1 (*Drosophila melanogaster*) cell cultures and the resulting interactions were studied. Cytopathogenic effects leading to culture death were observed with all strains except CSS. Strains B88, 277F, BNR-1 and PPS-1 grew as well in AS-2 and AC-20 cultures as in fresh LB medium. Strain R8A2 multiplied faster and reached higher titers in AS-2 and AC-20 cultures than in fresh LB medium (on day 3 the titers in AS-2 and fresh LB medium were  $5.0 \times 10^8$  and  $8.1 \times 10^6$  CFU/ml, respectively). This *S. citri* growth stimulating factor was studied by means of conditioned medium.

All 6 strains cytoadsorbed to the cell. Strains R8A2, CSS, BNR-1 and PPS-1 adsorbed slightly, and strains B88 and 277F adsorbed heavily. Fluorescent DNA staining revealed the presence of non-helical forms within the AS-2 and AC-20 cells.

Interactions take place between mycoplasmas and cells, tissues and organs of the insect vectors of mycoplasmal plant diseases. Such interactions can be studied *in vitro* using insect tissue systems. We selected two leafhopper cell lines, AS-2 (*Aceratagallia sanguinolenta*) and AC-20 (*Agallia constricta*) and the Dm-1 *Drosophila melanogaster* line for this purpose.

Spiroplasmas as well as mammalian mycoplasmas and *Acholeplasma laidlawii* multiplied to high titers in Dm-1 cultures (9, 10). Spiroplasma dependent cytopathogenic effects (CPE) and culture death was observed. Attachment of spiroplasmas to the cells but no fusion of membranes was seen by transmission electron microscopy. The 277F spiroplasma was occasionally located intracellularly within vacuoles (11). Intracellular membrane bound spiroplasmas were also found in another insect cell line (7). The spiroplasmas seemed to be filamentous or helical within the vacuoles. In leafhopper tissues, *Spiroplasma citri* and the corn stunt spiroplasma (CSS) seem to be pleomorphic, not helical, with a morphology comparable to mycoplasma-like-organisms (5, 8). *S. citri* was located intracellularly in

clusters which were not surrounded by membranes in the natural vector, *Circulifer tenellus* (5). Our present studies add some new features to these observations and describe some interesting interactions between leafhopper cells and several spiroplasmas which hopefully will contribute to a better understanding of the host-parasite relationship.

## MATERIALS AND METHODS

The agallian cell lines AS-2 and AC-20 were grown in the LB medium (6) at 28°C and passaged every 3 to 5 days. The *Drosophila melanogaster* Dm-1 cell line was maintained at 25°C in Schneider's medium and passaged at weekly intervals. The spiroplasma species and strains used were: *S. citri* R8A2 (group I-1 of Junca *et al.* (4)), *S. floricola* BNR-1 (III), *S. apis* PPS-1 (IV) and the unspecified strains B88 (I-2, from honey bees), the corn stunt spiroplasma CSS E-275 (I-3) and the tick strain 277F (I-4). They were cultured at 30°C in BSR medium with the exception of CSS which was grown in M1A medium.

Culture flasks (T 25) containing  $0.4 \times 10^6$  cells/ml were infected with approximately  $10^6$  CFU/ml

of spiroplasmas a few hours after passage. Infected agallian cell cultures were observed daily with an inverted microscope for gross CPE. At selected days the spiroplasma titers were determined by agar plating and the cells were counted with a hemocytometer using the Trypan blue exclusion method.

Conditioned medium was prepared in the following way. Confluent AS-2 or AC-20 cultures were passaged and incubated at 28°C. The supernatant was collected and filtered through 0.22 or 0.45  $\mu\text{m}$  membrane filters to remove cellular materials after a growth period of normally 3 days. This medium, conditioned by the growth of cells, was then used to study the *S. citri* stimulating factor.

Cells were seeded in Leighton tubes with glass coverslips for fluorescent microscopy. Cultures were infected by approximately  $10^6$  CFU spiroplasmas after cell attachment, were incubated for varying periods, and processed. Fluorescent DNA staining with Hoechst 33258 was as described elsewhere (9). Coverslips were fixed in acetone, washed 3 times in water and incubated at room temperature for 30 minutes with the rabbit anti R8A2 antiserum for indirect immunofluorescence. Fluorescein conjugated goat anti-rabbit antiserum was added after additional washings and the coverslips were re-washed 30 minutes later and mounted.

## RESULTS

*Spiroplasma citri* R8A2, *Spiroplasma floricola* BNR-1, *Spiroplasma apis* PPS-1 and the strains B88 and 277F produced CPE in AS-2 and AC-20 cell cultures. Strains R8A2, BNR-1 and PPS-1, induced the formation of intracellular vacuoles and globules. In AS-2 cultures, more vacuoles than globules were observed. In AC-20 cultures, more globules than vacu-

oles were observed. The dominant CPE in strains B88 and 277F was rounding of cells. The CPE always lead to the detachment of the cell monolayer with all 5 spiroplasma strains. Definite CPE was recognizable with strain B88 after 1-2 days, with strains BNR-1 and PPS-1 after 3 days and with strains R8A2 and 277F after 4-5 days. The cell counts performed with infected AS-2 cultures showed a time dependent decrease of viable cells. Only strain CSS had no influence on cell multiplication. This strain did not grow and its titer remained at the inoculation level without any change in morphology. In both cell cultures strains B88, 277F, BNR-1 and PPS-1 reached the same titers as in acellular LB medium on day 3. Titers ranged from  $2 \times 10^8$  to  $4 \times 10^9$  CFU/ml and are comparable to those measured in spiroplasma media. However, within the same 3-day period strain R8A2 grew to higher titers in AS-2 and AC-20 cultures than in fresh LB medium. The titers were  $5.0 \times 10^8$  and  $8.1 \times 10^6$  CFU/ml for AS-2 and LB medium respectively. Analysis of the growth kinetics in AS-2 cultures and LB medium confirmed this result. Strain R8A2 grew faster and reached a higher titer in the AS-2 cell line.

The following experiments were performed to further study this *S. citri* stimulating effect. Supernatant of 3-day-old cultures was collected and filtered through a 0.45  $\mu\text{m}$  membrane. Such conditioned medium, fresh LB medium, and AS-2 and AC-20 cultures were inoculated with appropriately  $10^6$  CFU of strain R8A2 and 3 days later the titers were determined by agar plating (table 1). The titers were more than one log unit higher in both cell cultures and in conditioned AS-2 and AC-20 media than in fresh LB medium. Conditioning of the 2 leafhopper media CB (2) and HM (3) in which

TABLE 1  
SEPARATION OF *SPIROPLASMA CITRI* GROWTH STIMULATING FACTOR FROM AS-2 OR AC-20 CELLS\*

Medium	CFU/ml after 3 days of culture
LB fresh	9.9x10 <sup>6</sup>
LB + AS-2	5.5x10 <sup>8</sup>
LB conditioned with AS-2	1.6x10 <sup>8</sup>
LB fresh	9.9x10 <sup>6</sup>
LB + AC-20	1.7x10 <sup>8</sup>
LB conditioned with AC-20	1.1x10 <sup>8</sup>

\**S. citri* inoculum = 1.24x10<sup>6</sup> CFU/ml.

strain R8A2 does not grow (10<sup>2</sup> CCU compared to 10<sup>9</sup> CCU in BSR and M1A) also resulted in clearly increased titers on day 3 after infection. In one representative experiment 5.8x10<sup>6</sup> and 1.7x10<sup>8</sup> CFU/ml were obtained for fresh HM and conditioned HM, respectively and 5.2x10<sup>6</sup> and 1.6x10<sup>8</sup> CFU/ml for fresh CB and conditioned CB, respectively after infection with 5.4x10<sup>5</sup> CFU/ml. The slight growth in fresh HM and CB media is very probably due to carry over substances in the inoculum. This *S. citri* stimulating factor is now being characterized by means of conditioned medium. Preliminary results indicate filterability through 0.22 and 0.1 μm mem-

branes and heat stability at 60°C for an hour.

Primary isolations of *S. citri* from diseased Washington navel sweet oranges collected in Syria were attempted. The oranges were cut into 2 halves, and the columellas were dissected and chopped with a razor blade in 5-8 ml M1A medium. The homogenate was then filtered through 0.45 μm membranes and 0.1 ml was inoculated in parallel into M1A, LB, conditioned LB and AS-2 cultures. After an incubation period of 7 days at 28°C the titers were determined. In all experiments AS-2 cultures yielded the highest titers. Other titers were in the following order: AS-2 > M1A > conditioned AS-2 medium > LB.

Darkfield microscopy and fluorescent DNA staining with Hoechst 33258 revealed different degrees of cytoadsorption to AS-2, AC-20 and Dm-1 cells (table 2). Strains B88 and 277F adsorbed heavily. Strains CSS, BNR-1 and PPS-1 adsorbed only slightly to all 3 cell types. Strain R8A2 also attached slightly at the beginning of the infection but in older AS-2 and AC-20 cultures an increase was found due to aggregates.

DNA staining of all 6 spiroplasmas showed that non-helical forms were present within AS-2

TABLE 2  
SPIROPLASMA ATTACHMENT TO CELLS FROM 3 INSECT TISSUE CULTURES\*

Group		LB +	LB +	Schneider's +
Junca et al.	Spiroplasma strain or species	AS-2	AC-20	Dm-1
I-1	<i>Spiroplasma citri</i> R8A2	+†‡	+‡	+
I-2	B88	++	++	++
I-3	E275	+	+	+
I-4	277F	++	++	++
III	<i>Spiroplasma floricola</i> BNR-1	+	+	+
IV	<i>Spiroplasma apis</i> PPS-1	+	+	+

\*Darkfield microscopy and DNA stain Hoechst 33258.

†+ = Many individual spiroplasmas attached per cell. ++ = Less than 1 spiroplasma attached per cell.

‡Increased attachment in older cultures due to aggregates.

and AC-20 cells, whereas only helical forms were found on the glass surface of the coverslips (figure 1). These nonhelical forms consisted dominantly of ring-like structures, but pleomorphic and comma-like forms could also be seen. Further studies with R8A2 infected agallian cultures showed a time dependent increase in the number of nonhelical forms per cell and in the number of cells with such forms. Even after 4 days not all cells showed such non-helical forms although many possessed a large number of them. A quantitative difference was found between the two cell types. In AC-20 cells there were always fewer non-helical forms than in AS-2 cells. These fluorescent DNA particles were confirmed to be R8A2 by indirect immunofluorescence.

Several *S. citri* strains or isolates differing in their geographical origin (Morocco, France, Algeria, Iran, Israel), number of passages (from primary isolates to hundreds of passages) and the

number of extrachromosomal DNA bands on PAGE were inoculated into both leafhopper cell cultures which then were screened for the presence of non-helical forms. No difference could be detected.

## DISCUSSION

A leafhopper transmitted spiroplasmal plant pathogen has to follow a complex route within the insect. It has to pass through the gut wall, reach the salivary glands and penetrate into the salivary duct. During all these events interactions between spiroplasmas and host cells are possible and probably will take place. We chose to study some of these interactions by means of *in vitro* cell culture systems and we used the two agallian leafhopper cell lines AS-2 and AC-20 as well as the *Drosophila melanogaster* Dm-1 line.

Infection of the AS-2 and AC-20 lines by *S. citri* R8A2, *S. floridicola* BNR-1, *S. apis* PPS-1 and the strains B88 and 277F resulted in a clear CPE and decreased cell vi-

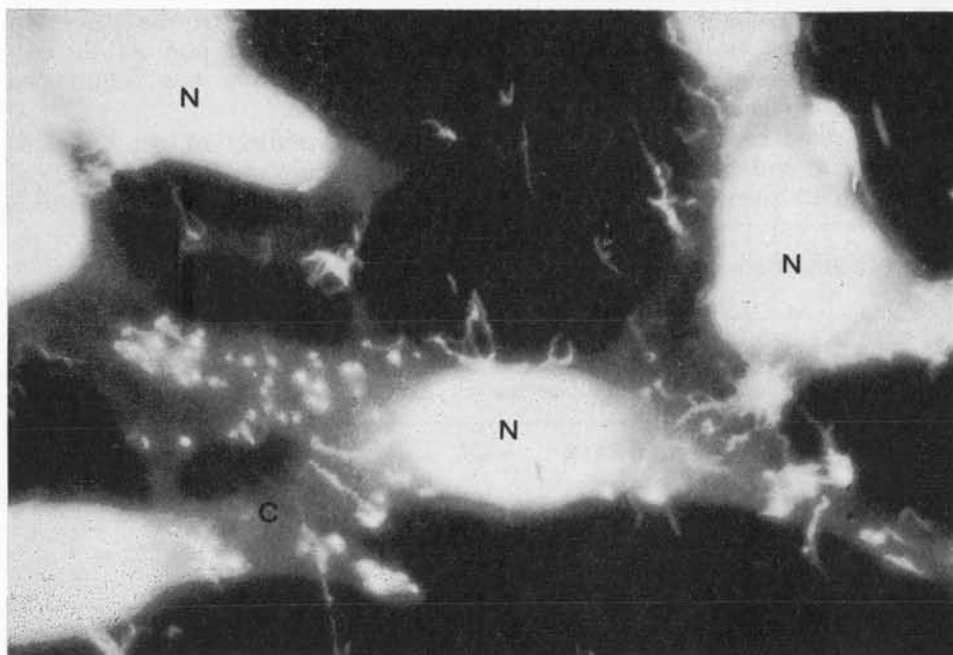


Fig. 1. A two day *S. citri* infected AS-2 culture stained with the fluorescent DNA stain Hoechst 33258. N = nucleus; C = cytoplasm.



ability followed by death of leaf-hopper cell cultures. The CPE was clearly visible after 4-5 days with strains R8A2 and 277F, after 3 days with strains BNR-1 and PPS-1 and after only one day with strain B88. Since the 3 latter strains grow more quickly than the former two as judged by color change, it might be argued that decreased pH is responsible for the CPE. However, two facts contradict this idea, at least for strains B88 and 277F. Strains B88, BNR-1 and PPS-1 produce a color change within one day but the CPE is already visible within a day with strain B88 compared to 3 days with strains BNR-1 and PPS-1. The survival rate with strain 277F had decreased to 20% (control = 89%) on day 6 but the pH had dropped only about 0.2 of a unit to 6.31 (control = 6.51), which is very close to the optimal pH for the AS-2 culture (1).

No CPE was observed with CSS, and no growth was recorded after 9 days of observation. Strains B88, 277F, BNR-1 and PPS-1 grew to high titers in AS-2, AC-20 as well as in fresh acellular LB medium. These titers are comparable to those found in conventional spiroplasma media. With strain R8A2 a clear difference was found between the titers measured in the two cell lines and the one in fresh LB medium. The titer was nearly 2 log units higher in AS-2 and AC-20 cultures than in LB on day 3 (table 1). The growth kinetics showed that titers were lower and multiplication rate was reduced in LB medium. The estimated doubling times are in the range of 6 and 27 hours for AS-2 cultures and LB medium, respectively.

This *S. citri* stimulating effect is separable from the cells by means of conditioned medium and is transferable to CB and HM media in which *S. citri* does not grow. It is heat stable at 60°C and filterable

through 0.1  $\mu\text{m}$  membranes. The *S. citri* stimulating factor is not limited to laboratory adapted strains since it was also seen in the primary isolation experiments. In these experiments the isolates grew quicker in AS-2 than in M1A, but M1A was superior to conditioned and fresh LB medium. However, this factor is not effective for CSS even though the LB medium is quite appropriate to maintain its morphology for at least 10 days. Nevertheless this first report indicating that insect cells in culture contribute to the growth of spiroplasmas is encouraging. It may be possible to cultivate MLOs in an appropriate insect cell culture system.

Studies with AS-2, AC-20 and Dm-1 cell cultures showed that the spiroplasmas can be divided in two groups with respect to adsorption (table 2). Strains B88 and 277F adsorbed heavily to all 3 types of cells and strains R8A2, CSS, BNR-1 and PPS-1 adsorbed poorly. The higher adsorption observed with strain R8A2 in older agallian cultures is attributable to attached spiroplasma aggregates. This strong adsorption of strains B88 and 277F might explain why strain B88 produces CPE much faster than BNR-1 and PPS-1, and why CPE was found with 277F although no pH change occurred.

Fluorescent DNA staining also revealed another interesting feature, namely the formation of non-helical spiroplasma forms within the cells. These forms were found with all 6 strains included in these studies as well as a number of *S. citri* strains or isolates differing in geographical origin, number of *in vitro* passages and number of PAGE extrachromosomal DNA bands. Immunofluorescence confirmed that non-helical R8A2 forms were not artifacts or cellular DNA particles. With both fluorescent methods these non-helical forms

are normally clearly separable one from the other indicating that they are probably not packed in clusters within membranes as has been shown for Dm-1 cells (11) and cells from *Lymantria dispar* (7). However transmission electron microscopy should be performed to confirm intracellular location of the spiroplasmas and to determine

whether they are membrane bound or not. These studies are now being performed.

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