Application of ELISA to the Detection of *Spiroplasma citri* in Plants and Insects

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There is an obvious need for quick detection of *Spiroplasma citri*, not only in citrus and the many nonrutaceous hosts known to harbor the pathogen (Calavan and Oldfield, 1979), but also in known or presumptive leafhopper vectors. Techniques are also required to identify and characterize the various plant or insect spiroplasmas which, in addition to *S. citri*, have been isolated and cultured in recent years. Protein analysis on polyacrylamide gels can be conveniently used to characterize the spiroplasma organisms once they have been obtained in pure culture (Mouches *et al.*, 1980). The enzyme-linked immunosorbent assay (ELISA) can be used for identifying cultured spiroplasmas and for detection of the organism directly in plant or insect material. The first two reports on the application of ELISA to the detection of *S. citri* appeared independently in 1978 (Clark *et al.*, 1978; Saillard *et al.*, 1978). Since then we have used ELISA for the detection of *S. citri* in citrus, periwinkles, and leafhoppers from Morocco and Corsica, as well as for the identification of spiroplasmas cultured from such material (Bové *et al.*, 1978; Bové *et al.*, 1979*a* and *b*). The experience gained and some of the results obtained in the course of these studies are presented in this paper.

MATERIALS AND METHODS

ELISA was carried out essentially as described previously (Saillard *et al.*, 1978). Specific rabbit immunoglobulins G (IgG) were prepared against *S. citri* strain R8A2 (ATCC 27556) grown in BSR medium prepared with horse serum (Bové *et al.*, 1978). For antigen production, all spiroplasmas were grown in BSRH medium (BSR medium containing 0.06 M HEPES, pH 7.6), except the suckling mouse cataract agent (SMCA) which was cultured in SP4 medium (Tully *et al.*, 1977); both BSRH and SP4 media contained foetal calf serum instead of horse serum.

Polystyrene M29AR microtiter plates (Cooke system) were coated with IgGs (6 μg/ml in coating buffer; 1.59 g Na_2CO_3. 2.93 g $NaHCO_3$, 0.2 g NaN_3 per liter). All washings were done with phosphate-buffered saline (PBS) containing 0.05 per cent Tween 20 from Merck-Schuchardt (PBS-Tween). The stock solution of alkaline phosphatase (Sigma) linked IgG (enzyme conjugate) was used at a 400-fold dilution (in enzyme-IgG buffer; PBS-Tween containing 2 per cent polyvinyl-pyrrolidone (PVP, Fluka AG MW 25,000 kollidon 25) and 0.2 per cent ovalbumin (Sigma). Unless indicated otherwise, samples were prepared in 0.1 M sodium phosphate, pH 7.4, containing 0.33 M NaCl. Five-gram leaf samples, essentially midveins and secondary veins, were homogenized in 5 ml of sample buffer with a mortar and pestle. The homogenate was gently squeezed through four layers of cheesecloth, and 250 μl of filtered homogenate was placed in each well of the microtiter plate. In certain experiments, the filtered homogenate was diluted with sample buffer before addition to the wells. After comparison with the 3-step technique, the 4-step method (table 1) was used throughout. The substrate was 1.0 mg/ml of *p*-nitrophenyl phosphate (Sigma) in substrate buffer (10 per cent diethanolamine, 0.02 per cent NaN_3, pH 9.8). The reaction was stopped after 30 minutes with 3M NaOH. The contents of duplicate wells (2 x 300 μl)

were mixed and the absorbance determined spectrophotometrically at 405 nm (OD_{405}). Samples were considered positive when the OD_{405} of the sample was 0.1 OD_{405} units greater than that of the sample buffer. Optical density of the blank was always below 0.050 OD_{405}.

RESULTS

Application of ELISA to *in vitro* grown spiroplasmas using anti *S. citri* immunoglobulins (IgG). In our first experiment, 100 ml of cultures of various spiroplasmas were centrifuged at 20,000 *g* for 15 minutes. The pellets were resuspended in sample buffer and the suspension recentrifuged. This washing step was repeated three times. The protein concentration of the final suspension was determined (Lowry *et al.*, 1951), and adjusted to 50.0 μg/ml before various dilutions were prepared with sample buffer. Aliquots of 250 μl were tested in individual wells of microtiter plates. Figure 1 shows that under these conditions, the smallest amount of *S. citri* protein that could be detected was 12.5 ng. A positive reaction was observed to the corn stunt and the honey bee spiroplasmas, however, at a 100 times higher antigen concentration. These heterologous reactions confirm previous reports that these two spiroplasmas and *S. citri* are serologically related (Tully *et al.*, 1973; Chen and Liao, 1975; Williamson and Whitcomb, 1975; Davis *et al.*, 1979; Williamson *et al.*, 1979).

ELISA can be conveniently used to identify and characterize a newly isolated spiroplasma. For instance, Bové *et at.* (1978) have cultured a spiroplasma (strain M4) from an ornamental periwinkle growing in Rabat, Morocco. Figure 2 shows that the ELISA titration curve for strain M4 closely fits the curve of *S. citri*, but not that of the serologically related corn stunt spiroplasma. This indicates that strain M4 is serologically identical to *S. citri.* Protein analysis on polyacrylamide gels (Mouches *et al.*, 1979) has confirmed that strain M4 is a strain of *S. citri* (Bové and Saillard, 1979).

Detection of *S. citri* in periwinkles. We first investigated the effect of a periwinkle homogenate on the ELISA reaction given by cultured *S. citri.* Various dilutions of *S. citri* (R8A2), honey bee spiroplasma (BC3), and corn stunt spiroplasma (E275) were prepared with either sample buffer or filtered plant homogenate prepared from healthy periwinkle leaves.

The presence of periwinkle material had little effect on the ELISA readings in this experiment. However, in other experiments, there was approximately a 50 per cent inhibition at high *S. citri* concentrations (0.125 and 1.25 μg proteins/well) but only a small effect at low *S. citri* concentrations.

We tested healthy *S. citri*-infected and MLO-infected periwinkles. The assay was performed in three or four steps (table 1). Both techniques gave positive reactions with *S. citri*-infected periwinkles even at 2.5 mg tissue/ml, and negative reactions with healthy or MLO-infected periwinkles. The *S. citri*-infected periwinkles used in the experiments were grown in a glasshouse cooled to approximately 25°C, which is considerably below the optimum growth temperature (32°C) for *S. citri*, and still gave a strong positive reaction. The 4-step assay yielded somewhat higher OD_{405} readings with higher *S. citri* concentrations or undiluted plant homogenate, but both techniques are equally sensitive at the lower *S. citri* concentrations.

We have also applied ELISA to the detection of *S. citri* in field-grown periwinkles exposed to natural infection from June to October 1978, in the Tadla area of Morocco (Bové *et al.*, 1979*b*; Nhami *et al.*, 1980). Of a total of 165 periwinkles, 16 per cent showed, at the beginning of October, chlorosis and varying wilt symptoms very similar to those induced by *S. citri* in periwinkles grown at 32°C. Other periwinkles of the experiment showed phyllody-virescence (19 per cent) or flower drawfing-stolbur (26 per cent) symptoms. All wilted plants, but none of the other periwinkles gave positive ELISA reactions with

OD_{405} readings ranging from 0.212 to 0.875 (table 2). Electron microscopy has revealed the presence of helical mycoplasmas in the wilted periwinkles and a spiroplasma, characterized as *S. citri* (Nhami *et al.*, 1980), could be cultured from their leaves.

Healthy periwinkle leaves gave OD_{405} values close to zero, whatever the buffers used to grind the leaf samples (table 3). With *S. citri*-infected periwinkles, the presence of 1 or 2 per cent polyvinylpyrrolidone (PVP) in the sample buffer increased OD_{405} values, and 10 per cent PVP decreased values significantly.

Detection of *S. citri* in sweet orange leaves. ELISA gave positive, but relatively weak reactions (OD_{405} of 0.1 to 0.4) with young, 1- to 2-cm-long leaves from *S. citri*-infected sweet orange seedlings maintained in a growth chamber (table 3). Mature or middle-size sweet orange leaves, as well as healthy or greening-infected young, sweet orange leaves, gave negative reactions (Saillard *et al.*, 1978).

With leaf samples collected early in October 1978 on field trees grown in the Tadla area of Morocco, positive ELISA reactions were obtained from stubborn-affected trees or symptomless trees in affected orchards, but the reactions were inconsistent. Trees with rather severe symptoms of stubborn sometimes gave negative ELISA reactions (table 4). Negative results were also given occasionally with *S. citri*-infected seedlings grown at 32°C in a growth chamber. More experiments need to be performed, and a more-sensitive assay must be obtained, before ELISA can be used reliably to index field-grown trees.

Certain buffers used to grind leaves gave positive ELISA reactions with healthy leaves (table 3). With *S. citri*-infected leaves, the presence of 2 per cent PVP in the grinding buffers increased OD_{405} values.

Detection of *S. citri* in leafhoppers. Positive ELISA reactions were obtained with 7 out of 41 species of leafhoppers collected in stubborn-affected sweet orange orchards in Morocco (Bové *et al.*, 1979*a*). Twenty-nine of 63 samples in which these 7 species were found gave positive ELISA reactions. None of the leafhoppers from 16 samples collected far from citrus orchards were positive.

We placed individual *Fieberiella florii* leafhoppers on *S. citri*-infected periwinkles. Every 6 hours leafhoppers were collected and tested by ELISA. The OD_{405} remained close to zero during the first 54 hours; but after 68 hours, values above 0.400 were reached. While ELISA can be used to follow the increase of *S. citri* in leafhoppers, certain buffers give positive ELISA values with uninfected *Fieberiella florii* leafhoppers (table 5). We have settled on buffer VII (PBS + Tween + 2 per cent PVP), which gave OD_{405} values close to zero with uninfected material and some of the highest values with *S. citri*-infected leaves or leafhoppers.

DISCUSSION

ELISA can be conveniently used to characterize newly cultured spiroplasmas, and it can also be used to detect *S. citri* in infected material. The technique gives consistent results with *S. citri*-infected periwinkles even when the plants are grown at temperatures lower than 32°C, the optimum growth temperature of *S. citri*. With *S. citri* infected sweet orange seedlings or trees, less consistent results have been obtained even with very young leaves grown at temperatures close to 32°C. The technique, as used in these experiments, is able to detect 10 ng of *S. citri* proteins, but a sensitivity to about 1 ng is needed for the rather low concentration of *S. citri* in citrus leaves. Plant materials such as flowers or fruit should also be tried. The detection of *S. citri* in leafhoppers by ELISA is useful for screening leafhoppers as presumptive vectors of *S. citri*, but choice of the grinding buffers is important to avoid positive ELISA reactions with uninfected material. Finally, whenever possible, a positive ELISA reaction should be confirmed by culturing the organism from an aliquot of the same specimen.

TABLE 1
OUTLINE OF 3-STEP AND 4-STEP ELISA

3-step ELISA	4-step ELISA
1) Coat with IgG at 37°C for 4 hours — wash 3 times.	1) Coat with IgG at 37°C for 4 hours — wash 3 times.
2) Add sample (antigen) and alkaline phosphatase linked IgG, both in PBS-Tween-PVP-Ovalbumin buffer. Leave overnight at 6°C — wash 3 times.	2) Add sample (antigen) in phosphate buffer. Leave overnight at 6°C — wash 3 times.
3) Add *p*-nitrophenyl phosphate. After 30 min stop reaction with NaOH. Read OD_{405}.	3) Add alkaline phosphatase linked IgG in PBS-Tween-PVP-Ovalbumin buffer. Leave at 37°C for 4 hours — wash 3 times.
	4) Add *p*-nitrophenyl phosphate. After 30 min stop reaction with NaOH. Read OD_{405}.

TABLE 2
ELISA ON NATURALLY INFECTED PERIWINKLES IN THE TADLA AREA OF MOROCCO (ORCHARD UP 3006)

Periwinkle number	Wilting	Stolbur symptoms	ELISA OD_{405}
2	+*		0.875
3	++†		0.212
4	++		0.312
5		++	0.000
6		++	0.000
7	+		0.311
8	++		0.674
9	+		0.820
10	+		0.771
11		++	0.000

* + = clear cut symptoms.
† ++ = severe symptoms.

TABLE 3
EFFECT OF DIFFERENT BUFFERS ON ELISA READINGS FOR HEALTHY AND *S. CITRI*-INFECTED LEAF SAMPLES

Buffers	OD_{405}			
	Sweet orange seedlings*		Periwinkles*	
	Healthy	Infected	Healthy	Infected
Experiment I				
I: 0.1 M phosphate pH 7.4 + 0.33 M NaC1	0.000	N.D.‡	0.050	0.780
II: I + 1% PVP†	0.002	N.D.	0.000	1.167
III: I + 2% PVP	0.027	N.D.	0.000	0.795
IV: I + 10% PVP	0.000	N.D.	0.047	0.580
Experiment II				
V: PBS + Tween	0.024	N.D.	0.033	0.687
VI: V + 1% PVP	0.000	N.D.	L§	0.802
VII: V + 2% PVP	0.020	N.D.	0.000	0.787
VIII: V + 10% PVP	L	N.D.	0.000	0.453
Experiment III				
I	0.000	0.179	0.000	0.324
III	0.000	0.281	N.D.	N.D.
VII	0.030	0.388	0.000	0.689
IX: VII + Ovalbumin (0.2%)	0.104	0.152	0.000	0.698
X: IX + Na diethyldithio-carbamate (0.2%)	0.128	0.153	0.000	0.730
XI: VII + nicotine (2%)	0.183	0.190	0.000	0.742

* The sweet orange seedlings were grown at 32° C during the day (16 hrs of artificial light) and at 27° C during the 8-hour night. The periwinkles were grown in the glasshouse at 20-26° C.
† PVP = polyvinylpyrrolidone.
‡ N.D. = not determined.
§ L = Lost.

TABLE 4
ELISA TESTS ON SWEET ORANGE LEAVES FROM FIELD TREES GROWN IN THE TADLA AREA OF MOROCCO

Orchard	Tree number	Severity of stubborn*	ELISA (eye reading)
UP 1809			
(WN)†	1	0	+
	2	3	++
	3	3	++‡
	3	3	-§
	4	5	++
UP 3003			
(WN)	1	0	-
	2	0	-
	3	1	-
	4	1	++
UP 3003			
(Valencia)	1	0	-
	2	1	+
	3	3	++
	4	4	-

* o = no apparent symtoms; 5 = very severe symptoms.
† WN = Washington navel.
‡ Young leaves were used.
§ Mature leaves were used.

TABLE 5
EFFECT OF DIFFERENT BUFFERS ON ELISA READINGS OF HEALTHY LEAFHOPPER (*FIEBERIELLA FLORII*) EXTRACTS

Buffers	OD_{405}	
	Experiment I	Experiment II
I: 0.1 M phosphate pH 7.4 + 0.33 M NaC1	0.124	0.280
II: I + 1% PVP*	0.134	L†
III: I + 2% PVP	0.160	0.065
IV: I + 10% PVP	0.088	0.000
V: PBS + Tween	0.030	0.079
VI: V + 1% PVP	0.087	0.000
VII: V + 2% PVP	0.000	0.000
VIII: V + 10% PVP	0.015	0.000

* PVP = polyvinylpyrrolidone.
† L = lost.

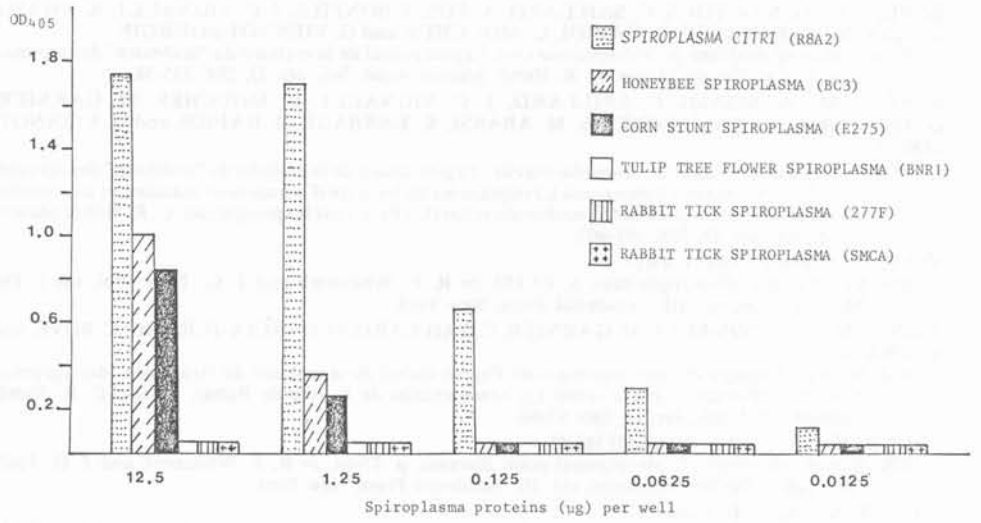

Fig. 1. Application of ELISA to *S. citri* and other cultured spiroplasmas using rabbit IgGs against *S. citri* (R8A2).

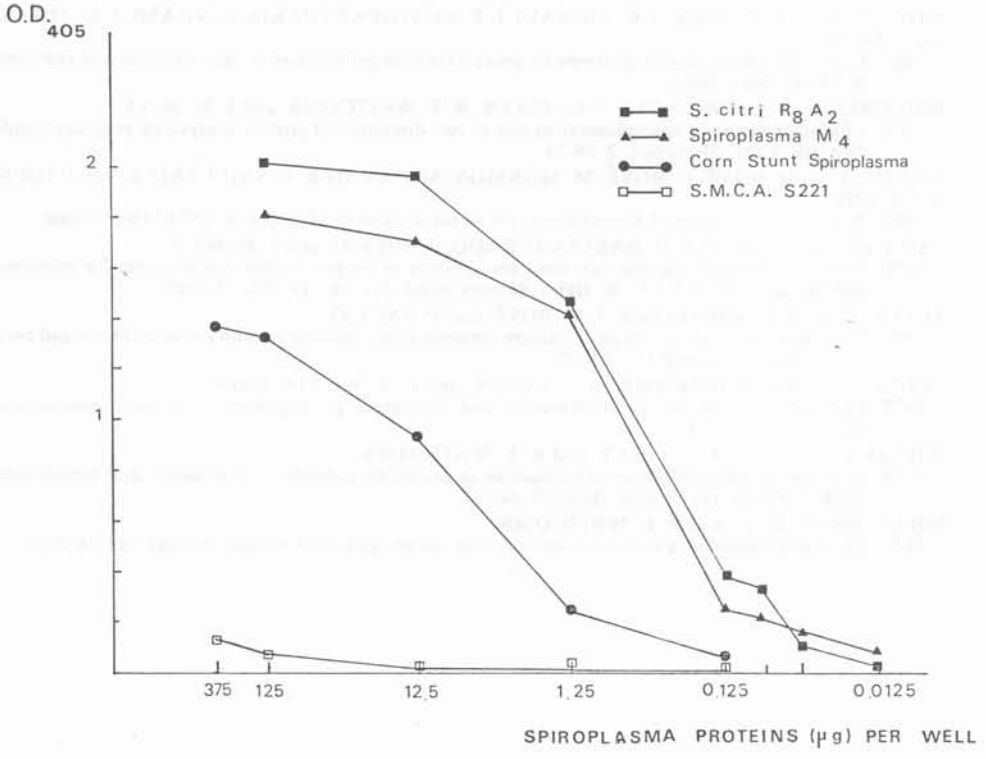

Fig. 2. Serological relationship among various spiroplasmas by ELISA.

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