Greening Disease in South Africa - A Multi-Pathogen Syndrome?

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ABSTRACT. Bacteria isolated from greening-affected citrus and reportedly morphologically identical to the greening organism *in situ* were found to cause a diversity of foliar chloroses similar to those observed on naturally infected cultivars when inoculated into healthy citrus seedlings. Re-isolation of the bacterium from these plants was achieved, and electron microscopic observation of leaf midribs revealed the presence of bacteria in the phloem of the inoculated plants. Dodder transmission of an infective agent from naturally-infected citrus to cucumber was achieved and the same bacterium was isolated from symptomatic cucumber plants. The association of this bacterium with infected citrus was taken as proof of its etiological status, however, further investigations revealed that *i*) the isolated bacterium previously reported by other workers to be the causative agent differed morphologically from the greening bacterium observed *in situ*, *ii*) vector transmission of the isolated bacterium to citrus was not accomplished, *iii*) mechanically inoculated citrus plants did not display the gentisic acid marker which is indicative of infection by the greening agent, *iv*) symptoms displayed by citrus inoculated with the isolated bacterium are transient and the infection does not persist and, *v*) antiserum raised against the bacterium does not consistently differentiate between healthy and greening-infected material. The results strongly suggest that the bacterium isolated is not the greening organism *per se*, but probably a mild opportunistic pathogen in citrus which may be a component of the greening syndrome.

The etiology of greening disease of citrus in South Africa has been the subject of a protracted debate. Initial reports suggested a virus as the causative agent (9). Mycoplasma-like organisms (14) were next thought to be involved until Hull (10) claimed the pathogen was a bacterium, and Garnier *et al.*, (8) produced proof of the Gram negative nature of the greening organism. Garnett (6) described the isolation of a bacterium with a similar morphology to the organism of Garnier *et al.*, (8), and claimed to have finally determined the etiology of greening in South Africa. Attempts to repeat this work in various laboratories, including our own, were unsuccessful, and an antiserum raised in rabbits against this isolate was found to be non-reactive with greening-infected plant extracts. Discrepancies in the morphological characteristics of this isolate (1, 2) and the greening organism observed *in situ* during the course of this work could, at least in part, account for the unsuccessful serological tests (Chippindall and Whitlock, unpublished data).

In the present study we re-examine all the isolates and antisera of previous workers in this laboratory and compare them to our own.

MATERIALS AND METHODS

Bacterial isolates. Isolates (coded NC) obtained from infected trees in the Randburg and Nelspruit areas of the Transvaal were found to be identical and were a gift from H. Garnett (6) and F. Mochaba (16). The bacteria were maintained on slants of solidified (1.5% agar) MIG-3 medium: potassium-D-gluconate 15.0 g; yeast extract 0.5 g; L-proline 2.5 g; L-histidine 2.5 g; L-alanine 2.5 g; $(NH_4)_2S0_4$ 2.0 g; NaCl 5.0 g; $FeS0_4.7H_20$ 262.5 mg; $ZnS0_4.7H_20$ 0,62 g; $CuS0_45H_20$ 1.925 mg; Hutner's Mineral Base 10 ml.

Components were dissolved in glass distilled water, pH adjusted to 7.2 with KOH and the volume made up to one liter before filter sterilization (0.22 μm). Bacterial cultures were maintained at room temperature or frozen at -70 C in 15% aqueous glycerol for long term storage. The bacteria used in all inoculation trials had been passaged less than six times to avoid attenuation of pathogenicity.

Isolations from infected plants. To investigate the association of the reported bacterium with greening disease, samples of greening-infected leaf midrib and fruit columella material

were collected during the cool winter months of May to August from the following areas of the Transvaal province of South Africa: Brits, Pretoria, Nelspruit, Letaba, Letsitele, Zebediela and Tzaneen. Material was taken from five different sites in the canopy of each of the trees sampled and included material displaying symptoms ranging from mild to severe. Trees were identified as being greening infected by the presence of typical disease symptoms, as well as by the presence of the fluorescent greening marker (19). Symptomless trees testing negative for the fluorescent marker were considered to be free of the disease and served as sources of healthy control samples.

The extraction procedure was a modification of that reported by Mochaba (16). Excised columella and midrib samples were immersed in ethanol for 2 min, transferred to a 1% (v/v) solution of sodium hypochlorite containing 0.1% (v/v) Tween 20 for 20 min and finally rinsed in three changes of sterile distilled water. Half a gram of plant material was chopped finely in a small volume of sterile quarter-strength Ringer's solution and then added to a further 2 ml of the Ringer's solution. After standing for 15 min the suspension was vortexed for 2 min, the plant debris allowed to settle and 0.2 ml of the supernatant used to inoculate 15 ml of MIG3 medium in each of five, 100-ml Erlenmeyer flasks. The cultures were incubated at 25 C on a gyratory shaker (150 rpm) and monitored daily for bacterial growth.

Antiserum production. The first antiserum produced was against the isolate of Garnett (6), designated NC. The organism was grown in MIG-3 medium and a suspension of washed, heat-killed cells was used as the antigen. The second antiserum was prepared against an extract of greening-infected plant material (designated GICE antiserum) (3).

Inoculation of plants. Two groups each of 24 healthy tangelo seedlings with a lower stem diameter of approximately 8 mm were inoculated by stem injection with the isolates in the following way: the area to be injected was swabbed with alcohol and a pilot hole made in the centre of the stem using a sterile needle (25G x 1.9 cm). A Venisystems Butterfly-19 (Abbott Ireland Ltd., Sligo, Ireland) connected to a 5-ml syringe containing 2 ml of bacterial suspension was used to introduce the inoculum into the plants over an 8-24 hr period. Positive pressure was maintained within the syringe with the aid of a perspex syringe holder designed specifically for this study. Eighteen plants received only sterile water as a control. An additional two groups of ten seedlings each were inoculated with the isolates by stem injection coupled with stem slashing (approximately 25, 2-4 mm deep bark slashes per plant were made, the scalpel blade being dipped into a suspension of the cells (10^9 cells/ml) between each slash).

All inoculated and control plants were maintained under a 14-hr photoperiod with a day temperature of 23 C and a night temperature of 15 C. Plants were treated fortnightly with a fertilizer containing trace-elements to prevent mineral deficiency.

ELISA sample preparation. To obtain antigen from the infected plants, midribs of young symptomatic leaves were washed in a large volume of distilled water. The samples were then treated in six different ways to determine the most effective method of obtaining the antigens as indicated by the results of the heparin-enhanced plate trapped antigen ELISA (HEPTA-ELISA) (Coreejes, Chippindall and Whitlock, unpublished data). The treatments were:

1) Finely chopping the plant material in a small volume of sterile quarter- strength Ringer's solution by hand with a sterile scalpel blade, and then adding a further 2 ml of the Ringer's solution. After standing at room temperature for 15 min, the suspension was vortexed for 2 min, the plant debris allowed to settle, and the supernatant used.

2) Leaf press extract was prepared by expressing sap from midribs

of young, symptomatic leaves with an Erich Polane leaf press.

3) Polytron homogenization was carried out by placing washed, excised midribs in a test tube with 10 ml chilled 1/4 strength Ringer's solution per gram of leaf material and thoroughly homogenising over ice using a Polytron. The homogenate was filtered through a double layer of miracloth and clarified by centrifugation (200 *g* for 5 min) followed by filtration through Whatman's No 4 filter paper. The filtrate was then centrifuged (12,000*g* for 30 min) to concentrate bacteria present and the pellet resuspended in 4 ml coating buffer or sample buffer per gram original leaf material.

4) Enzymatic treatment of midribs was carried out by thoroughly washing them in running water and swabbing with alcohol before being chopped as finely as possible in a small amount of enzyme solution (2% w/v) Cellulase TC (Serva, FRG), 1.5% (w/v) Rhoment P5 (Serva), 0.5% (v/v) 2-mercaptoethancl, 0.02% (w/v) NaN_3, in PBS, pH 6.0). The solution was prepared just prior to use.

The suspension was then finely ground using a pestle and mortar and additional enzyme solution was added so that a total of 10 ml enzyme solution was added per gram leaf material. The suspension was then incubated in a shaking incubator for 24 hr at 28 C. EDTA (0.1% w/v final concentration) was added and shaken for another hour. The slurry was then filtered and clarified as described for the polytron method.

5) The KOH extraction of alkali-soluble antigens was essentially the same as the method of Kawarabata and Hayasaka (11).

6) The water leaching method simply entailed the chopping of sterile midribs and resuspending the material in just enough sterile distilled water to cover it, and agitating the suspension overnight at room temperature. The leachate was collected by allowing the plant material to settle and then diluted 100-fold in ELISA coating buffer for HE-PTA-ELISA.

Plant tissue culture. *Protoplasts.* Mesophyll protoplasts were prepared from young, healthy leaves of the tangelo plants by a method adapted from Kunitake *et al.*, (12). Infection of the protoplast cultures with NC cultures was affected by introducing a loopful of the NC culture (107 cells/ml into 2 ml aliquots of the protoplast suspension. Control protoplast suspensions were included with a loopful of sterile NC culture medium, while negative controls were similarly treated with a suspension of *Shigella* grown on nutrient agar.

Callus. Callus was produced from surface sterilized tangelo seeds. The embryo and endosperm were aseptically removed and placed on callus inducing medium consisting of full MS salts (17), and allowed to grow into callus at 25 C in the dark. Infection of the callus was achieved by pipetting 2 ml of the NC suspension (10^7 cells/ml) onto five calli on solid medium in individual mini petri dishes. Three additional calli were similarly treated with sterile fresh NC medium, and another three with *Shigella* in nutrient broth. The experiment was terminated after 10 days, and the infected callus cells removed and washed twice in liquid callus embryonic medium by centrifugation at 500 *g* for 5 min. This was done to remove all unattached bacteria.

RESULTS AND DISCUSSION

Isolation from infected plants. Attempts to culture bacteria identical to those supplied to us (isolate NC) from citrus samples collected from various areas of the Transvaal Province of South Africa met with limited success. The bacterium was isolated from 17 of the 153 (11.1%) infected citrus leaf and fruit samples assayed. The results of our isolation attempts suggest the association of the bacterium isolated by Garnett (6) and Mochaba (16) with greening-affected citrus is limited.

The low incidence of the bacterium in greening infected citrus indicated by

our isolation attempts may be only a reflection of the difficulties associated with isolating the organism from plant material. A low concentration and/or an erratic distribution of the organism in infected leaf material may account for the low number of successful isolations.

Inoculation of plants. The report by Garnett (6) describing the consistent culture of a bacterium from greening-infected citrus alludes to the fact that the organism described is the etiological agent of greening disease. Reports by Ariovich and Garnett (1, 2) and Duncan and Garnett (4) present further circumstantial evidence to support the claim made by Garnett (6) that the isolated bacterium is the causal agent of the disease. Garnett (6) and Mochaba (16) reported that the bacterium was Gram-negative, but using the identical staining procedure we found it to be Gram-variable, the staining characteristics of the organism varying with changes in the culture conditions.

The pathological significance of any organism isolated from an infected source can only be confirmed by the fulfilment of Koch's postulates. Attempts to demonstrate the pathogenicity of the isolated bacterium were not, however, reported by any of the abovementioned authors.

In our attempts to fulfil Koch's postulates, mechanical inoculation of citrus with the NC isolate resulted in 44 of the 68 plants displaying disease symptoms approximately 6 months after inoculation, the symptoms being similar to those expressed by naturally infected field material. Leaf symptoms were observed on some of the inoculated plants eight weeks after inoculation, but it was usually between 10 and 12 weeks after inoculation that greening-like symptoms became clearly visible. A diversity of chloroses was produced by inoculated plants, the most common symptom being a blotchy-mottle chlorosis. Yellowing of the leaf tissue immediately adjacent to the leaf veins was also observed and in some cases the interveinal tissue became yellow as well.

Although the foliar symptoms were observed on several successive leaf flushes, the infection did not appear to persist, as symptoms on inoculated citrus were transient, disappearing 18-24 months after inoculation. Sweet orange plants infected with greening via psylla and maintained under identical conditions continued to display greening symptoms, even after a period of 36 months.

Visualization of the bacteria in midrib material sampled at sites distant from the point of inoculation suggests that the bacteria were able to spread within mechanically inoculated plants (Fig. 2). The bacteria were also cultured from this material, further suggesting the organism's ability to migrate within the plant tissue. There was, however, no evidence of active cell multiplication *in planta*. The number of bacteria observed in inoculated plants was low and no dividing cells were encountered, despite an extensive search. Most of the cells observed appeared to be degrading; some atypical forms were seen which resembled apparently degrading cells in late stationary phase cultures of the organism (Fig. 3).

The bacterium was recovered in pure culture from symptomatic plants and displayed identical morphological, metabolic and serological properties to the original inoculum. Attempts to reisolate the bacterium from inoculated plants after the disappearance of visible symptoms were, however, not successful, despite the fact that the same plants had previously yielded the bacterium.

Greening-like symptoms have been observed to be induced by a number of factors, both abiotic and biotic. Labuschagne *et al.* (13) and Gardner *et al.* (5) reported that various bacteria isolated from healthy citrus produced disease-like symptoms on reinoculation. Foliar symptoms are an unreliable indication of infection with greening and evidence should at least include the production of characteristic fruit symptoms. Due to the immature nature of the test seedlings used in this investigation, the observation of fruit symptoms was not possible.

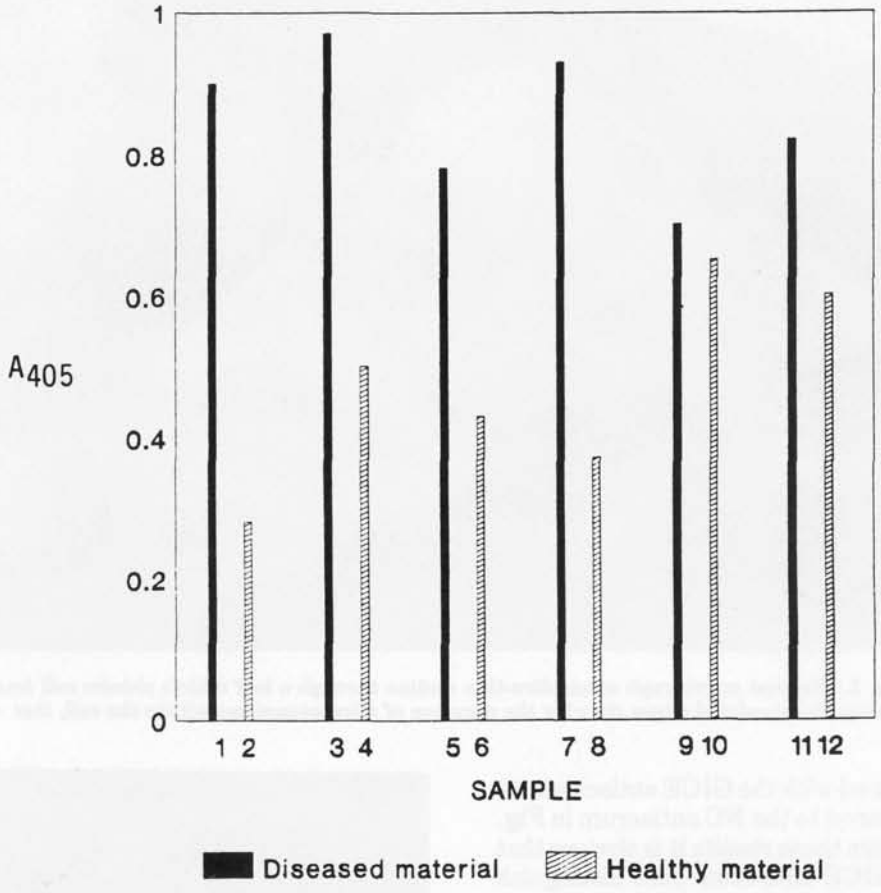

Fig. 1. Results of the HE-PTA-ELISA in which greening-affected citrus leaf midrib material was prepared following different sample preparation procedures. The diseased and healthy control samples were probed with the GICE antiserum cross-adsorbed against a preparation of healthy citrus proteins.

Bar No.	*Sample Preparation Procedure*	*Diseased/Healthy Ratio*
1 2	Water leaching	3.14
3 4	Polytron homogenization	1.93
5 6	Leaf press extract	1.80
7 8	"Chopping" method	2.50
9 10	Enzymatic treatment	1.05
11 12	KOH treatment	1.37

Serological assays. Results of the comparative assays of the six different antigen preparation methods showed that the water leaching method, while being quick and easy, also gave very good results in the HE-PTA-ELISA (Fig. 1).

Antisera raised against the cultured NC isolate failed to distinguish between healthy and greening-infected citrus or periwinkle midrib preparations in HE-PTA-ELISA (Fig. 4). The infected citrus samples were from Nelspruit (South Africa) and Reunion. The periwinkle material was collected from plants infected with greening via dodder as described by Garnier and Bovè (7). Antisera raised against other isolates of Garnett (6) and Mochaba (16) also failed to react with greening infected material (results not shown). The HE-PTA-ELISA results

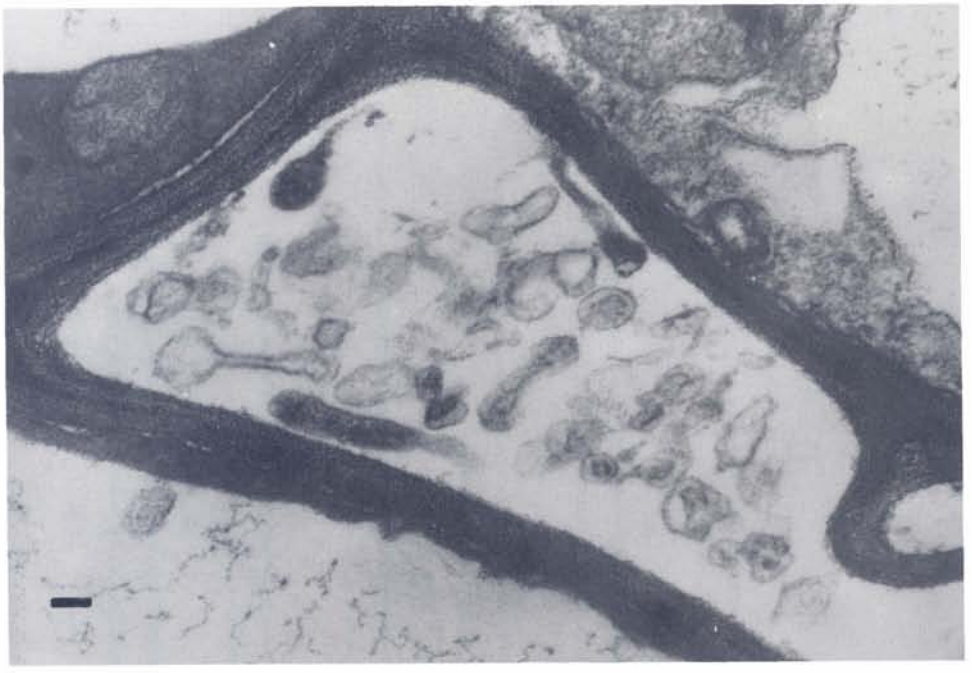

Fig. 2. Electron micrograph of an ultra-thin section through a leaf midrib phloem cell from mechanically-inoculated citrus showing the presence of microorganisms within the cell. Bar = 0.5μm

obtained with the GICE antiserum are compared to the NC antiserum in Fig. 4. From these results it is obvious that the GICE antiserum does distinguish between healthy and infected material while the NC antiserum does not. However, when the GICE antiserum was used against water leachates of plants infected with a number of other citrus diseases, all the assays were positive (Fig. 5), despite the fact that the antiserum had been cross adsorbed against extracts of citrus affected with these diseases. The NC antiserum failed to react with any of these samples (results not shown). A possible explanation for these seemingly anomolous results can be that the GICE antiserum reacted with a disease shock protein common to all the diseases.

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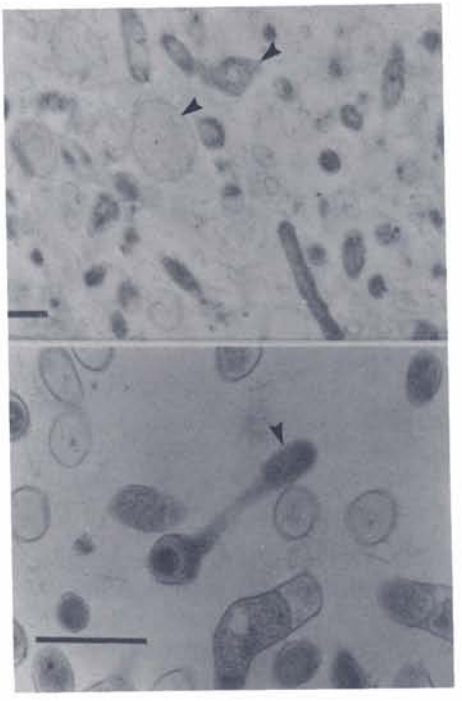

Fig. 3. Electron micrographs of ultra-thin sections through late stationary phase cultures of the isolate showing apparently degrading cells (arrows). Bar = 1.0μm.

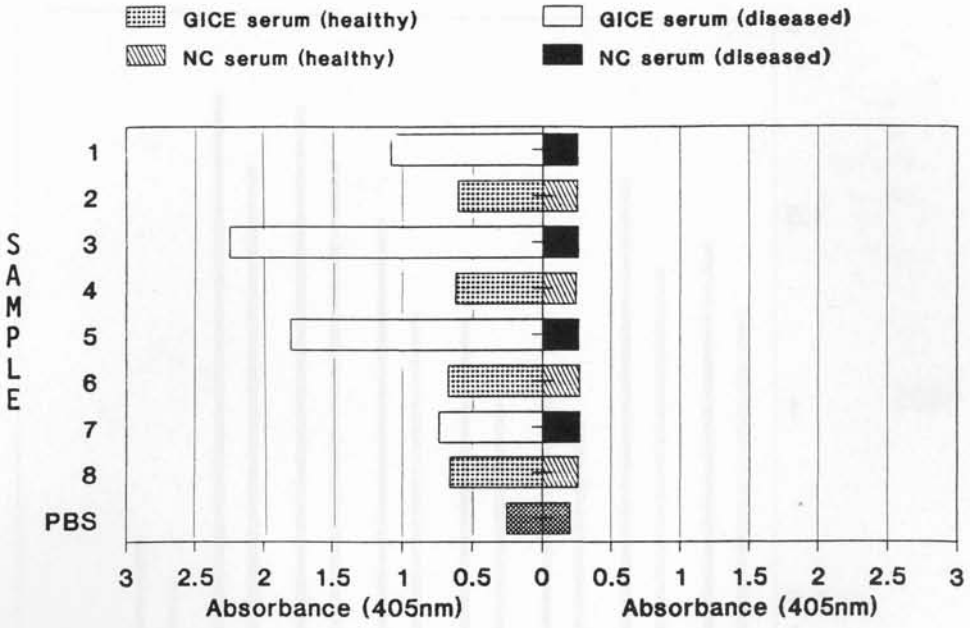

Fig. 4. Comparison of the NC and GICE antisera in the HE-PTA-ELISA.

Bar No.	*Sample*	*Diseased/Healthy Ratio* *GICE*	*NC*
1 2	Periwinkle midrib	1.80	1.05
3 4	Citrus midrib (SA)	3.63	1.09
5 6	Citrus midrib (Reunion)	2.69	0.93
7 8	Dodder strand	1.14	1.07

rate within the plant tissue. There was, however, no evidence of active cell multiplication *in planta*. The number of bacteria observed in inoculated plants was low and no dividing cells were encountered, despite an extensive search. Most of the cells observed appeared to be degrading; some atypical forms were seen which resembled apparently degrading cells in late stationary phase cultures of the organism (Fig. 3).

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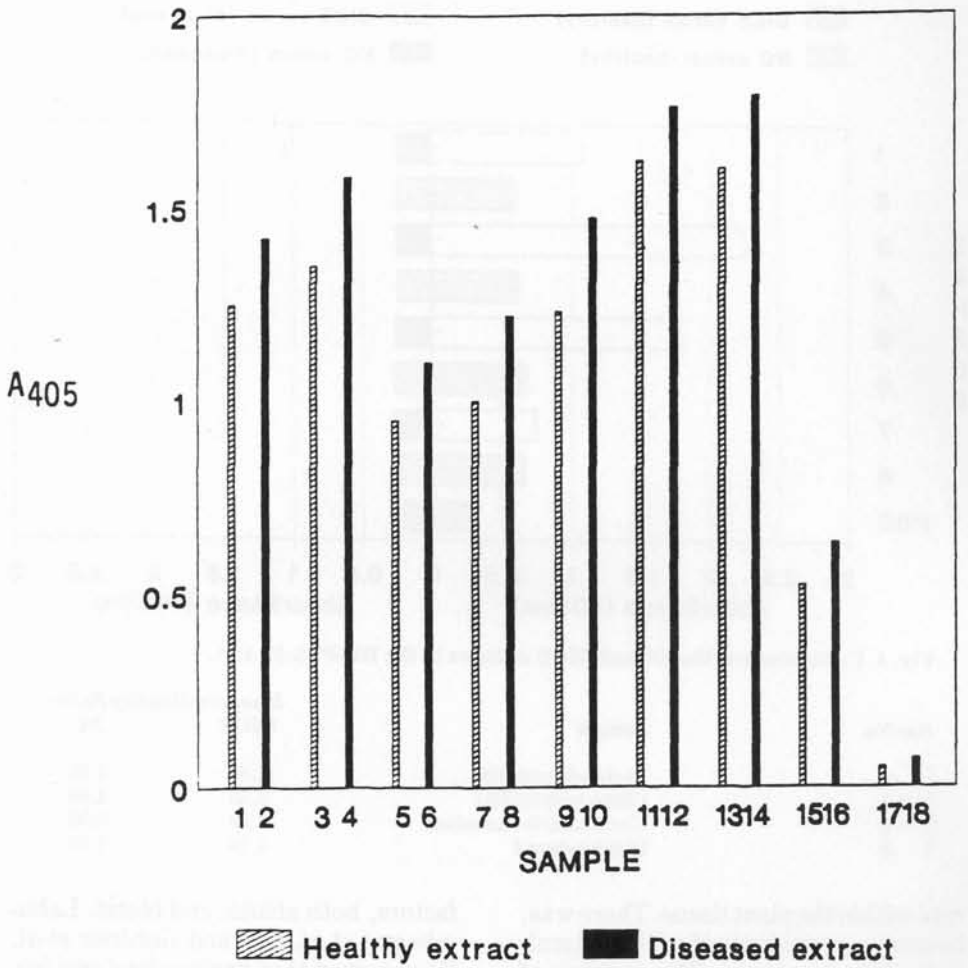

Fig. 5. Reaction of the GICE antiserum with healthy citrus and with citrus infected with various phytopathogens. Two different preparations of the antiserum were used: GICE cross-adsorbed against an extract of healthy citrus ("healthy extract") and a similar extract of citrus leaf samples infected with different phytopathogens ("diseased extract").

Bar No.	*Citrus Samples Affected By:*
1 2	Vein enation
3 4	Citrus tristeza virus (CTV) (7K6 isolate)
5 6	Multiple Sprouting + CTV
7 8	Psorosis + CTV
9 10	Impieratura + CTV
11 12	Greening (Nelspruit area)
13 14	Greening (Reunion Island)
15 16	Healthy Citrus
17 18	Buffer (PBS) control

preparations in HE-PTA-ELISA (Fig. 4). The infected citrus samples were from Nelspruit (South Africa) and Reunion. The periwinkle material was collected from plants infected with greening via dodder as described by Garnier and Bove (7). Antisera raised against other isolates of Garnett (6) and Mochaba (16) also failed to react with greening infected material (results not shown). The HE-PTAELISA results obtained with the GICE antiserum are

compared to the NC antiserum in Fig. 4. From these results it is obvious that the GICE antiserum does distinguish between healthy and infected material while the NC antiserum does not. However, when the GICE antiserum was used against water leachates of plants infected with a number of other citrus diseases, all the assays were positive (Fig. 5), despite the fact that the antiserum had been cross adsorbed against extracts of citrus affected with these diseases. The NC antiserum failed to react with any of these samples (results not shown). A possible explanation for these seemingly anomolous results can be that the GICE antiserum reacted with a disease shock protein common to all the diseases.

Plant tissue culture. Protoplast cultures infected with the cultured NC organism are shown in Fig. 6. Adhesion of the organisms to the protoplasts seemed to be irreversible, since they could not be dislodged by quite vigorous flushing with the culture medium, while the controls infected with *Shigella* showed no adhesion, even before flushing the protoplasts (Fig. 7). These results indicate a definite relationship between the organism and citrus protoplasts, but to extrapolate these findings directly to whole plants would be inadvisable without further investigation because the possible effects of the cell wall are not considered.

Callus culture. Electron microscopy of callus five days post-infection with NC showed extensive adhesion of the organisms to the callus even after flushing. No adhesion was observed in the *Shigella* inoculated callus. If the exposure of the callus to the organism was allowed to continue for 10 days, the callus started disintegrating and died. Whether this is a direct result of the physical effects of the attachment, or whether an extracellular metabolite is produced by the organisms which causes callus death, has not yet been established. It is, however, clear that as in the case of the protoplasts, there is a definite association between the organism and citrus callus.

CONCLUSIONS

The fact that the symptoms in mechanically-inoculated plants disappeared after 18-24 months and that the

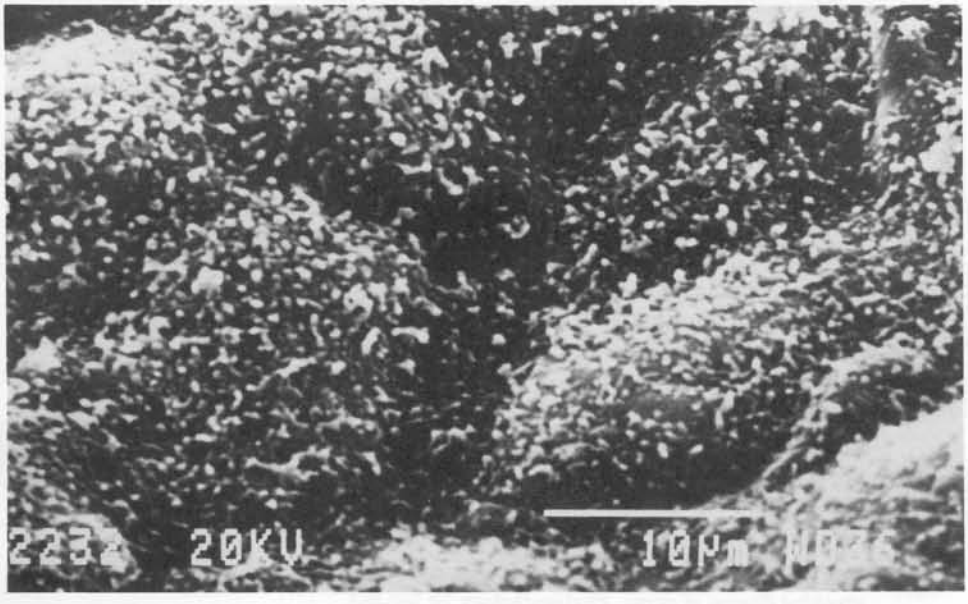

Fig. 6. Scanning electron micrograph showing adherence of the cultured NC isolate to citrus protoplasts.

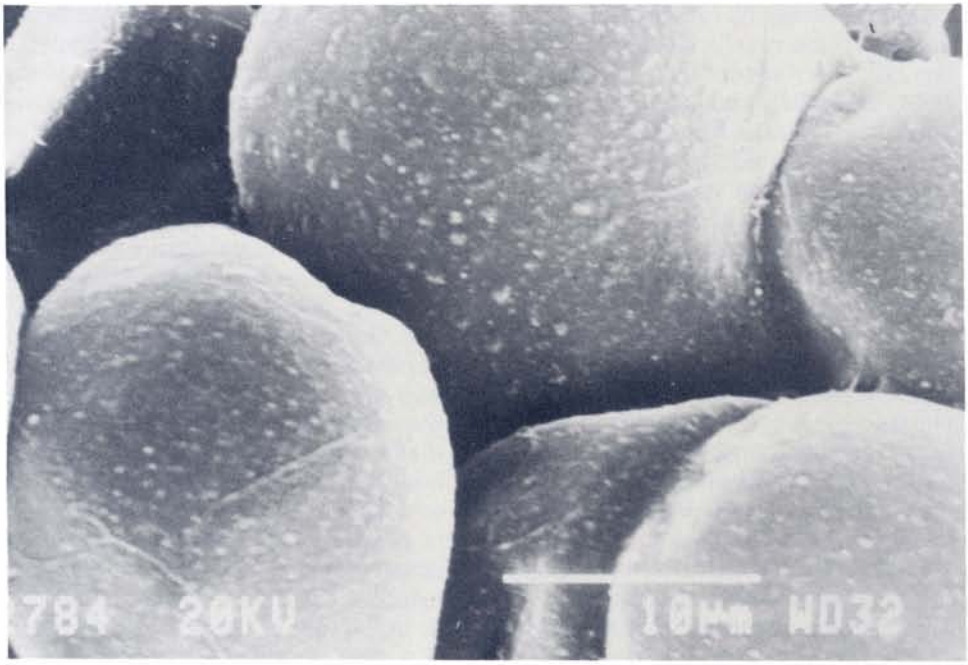

Fig. 7. Control protoplast culture inoculated with *Shigella*. No adherence of the bacteria to the protoplasts is evident.

antiserum against the cultured isolate failed to give consistent results with greening-infected citrus or periwinkle suggest that the isolate of Garnett (6) is not the sole casual agent of greening in South Africa. This is supported by the results of a subsequent investigation which showed that *i*) disease transmission from mechanically-inoculated plants to healthy tangelo via both grafting and the insect vector of greening was unsuccessful; *ii*) mechanically-inoculated, symptomatic plants failed to display the gentisic acid marker; and *iii*) the ultrastructure and mode of division of laboratory cultures of the isolate differ from that of the greening organism *in situ* (Chippindall and Whitlock, unpublished data).

However, the isolation from different citrus samples of a bacterium identical (on the basis of morphology, antigenicity and fatty acid profiles) to the NC isolates, as well as the relationship of the NC isolates with citrus protoplasts and callus, suggests that this organism may play a role in the greening syndrome.

In addition, the fact that another agent(s) may be involved is suggested by the isolation of a number of other bacteria from both diseased and healthy citrus which can cause a variety of symptoms when mechanically inoculated back into healthy plants (13, 15, 18).

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