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Biological and serological properties of a bacterium isolated from greening-infected citrus in South Africa

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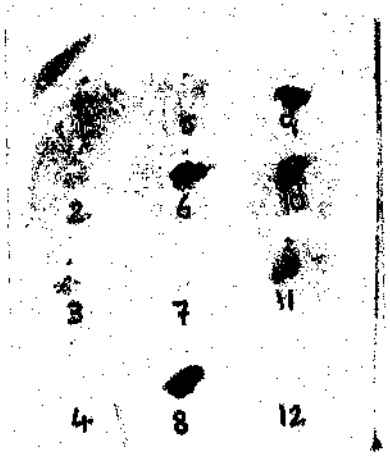


Figure 70

EL-DIB assay. Reaction of bacteria with antiserum directed against C. m. subsp. michiganense.

Spot 1 - GC isolate
2 - NC isolate
3 - CS isolate
4 - LL isolate
5 - LC isolate
6 - C. m. subsp. sepedonicum
7 - TZL isolate
8 - C. m. subsp. michiganense
9 - C. m. subsp. nebraskense
10 - C. m. subsp. insidiosum
11 - C. iranicus
12 - Curtobacterium flaccumfaciens pv. flaccumfaciens

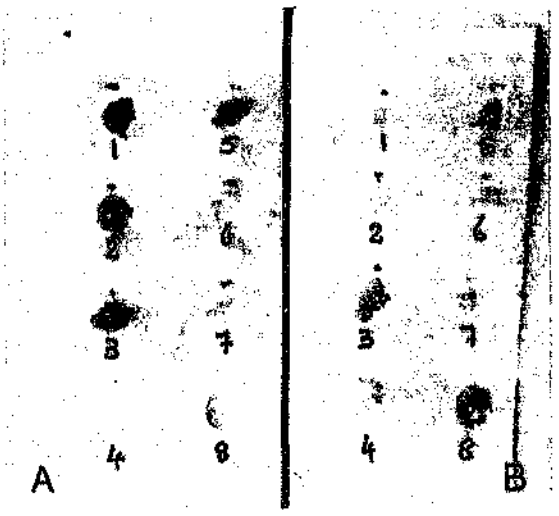


Figure 71

Screening of potential greening isolates in the EL-DIB assay. Immobilized cultured cells were probed with GICE antiserum (B) and, in a partial assay, with antiserum directed against the GC isolate (A).

Spot 1 - GC isolate
2 - NC isolate
3 - CS isolate
4 - BC isolate (Brits district isolates)
5 - GL isolat
6 - PL2 isolate (Pretoria district isolate)
7 - PG1 'solate (Pretoria district isolate)
8 - NL isolate (Nelspruit district isolate)

3.9 PREPARATIVE IMMUNOFLUORESCENCE MICROSCOPY

Analysis of leaf material using this method failed to reveal the presence of microorganisms in samples of greening-affected material probed with the GICE antiserum. Similar results were recorded with material taken from orchards in the Nelspruit, Letaba and Brits areas. Reaction of the antiserum with plant debris was observed, this being expected due to the residual anti-plant activity of the antiserum even after extensive cross-absorption.

When the anti-GC isolate antiserum was used to probe preparations of leaf midribs collected from a tree from which the GC isolate had been cultured, numerous bacteria-like structures were observed (Fig. 72). Some binding of Ig^{G} to plant debris did occur, but a visual distinction could be made between this reaction and the reaction of the antiserum with bacterial cells. Healthy control preparations displayed a minimal amount of fluorescence and the bacteria-like structures observed in the infected samples were not observed in these preparations.

After examination under the fluorescent microscope, the polycarbonate membranes on which the samples had been trapped were prepared for scanning electron microscopy and

viewed with a JSM 840 scanning microscope. This was done in order to confirm that the structures observed under the fluorescent microscope displayed the morphology typical of a rod-shaped bacterium under higher magnification (Fig. 73).

3.10 IMMUNOGOLD LABELLING

Cultured cells of the GC isolate were adsorbed to electron microscope grids and then probed with the antiserum raised against the extract of greening-infected citrus (GICE antiserum). The reaction was visualized by reacting the sample with a gold-labelled anti-rabbit IgG conjugate. Figure 74 shows that binding of the GICE antiserum to the cells did not occur. As a control, the GC isolate was probed with the homologous antiserum under the same conditions. In this case, binding did occur (Fig. 75), indicating a specific reaction.

Organisms displaying a similar morphology to that of the greening organism were extracted from greening-infected citrus following method 5 (section 2.9.2) and the preparation immunogold-labelled using the antiserum raised against the GC isolate. The result is shown in Figure 48. It is evident that specific binding of the antiserum did not occur, suggesting a lack of antigenic homology between the bacterium observed and the cultured GC isolate.

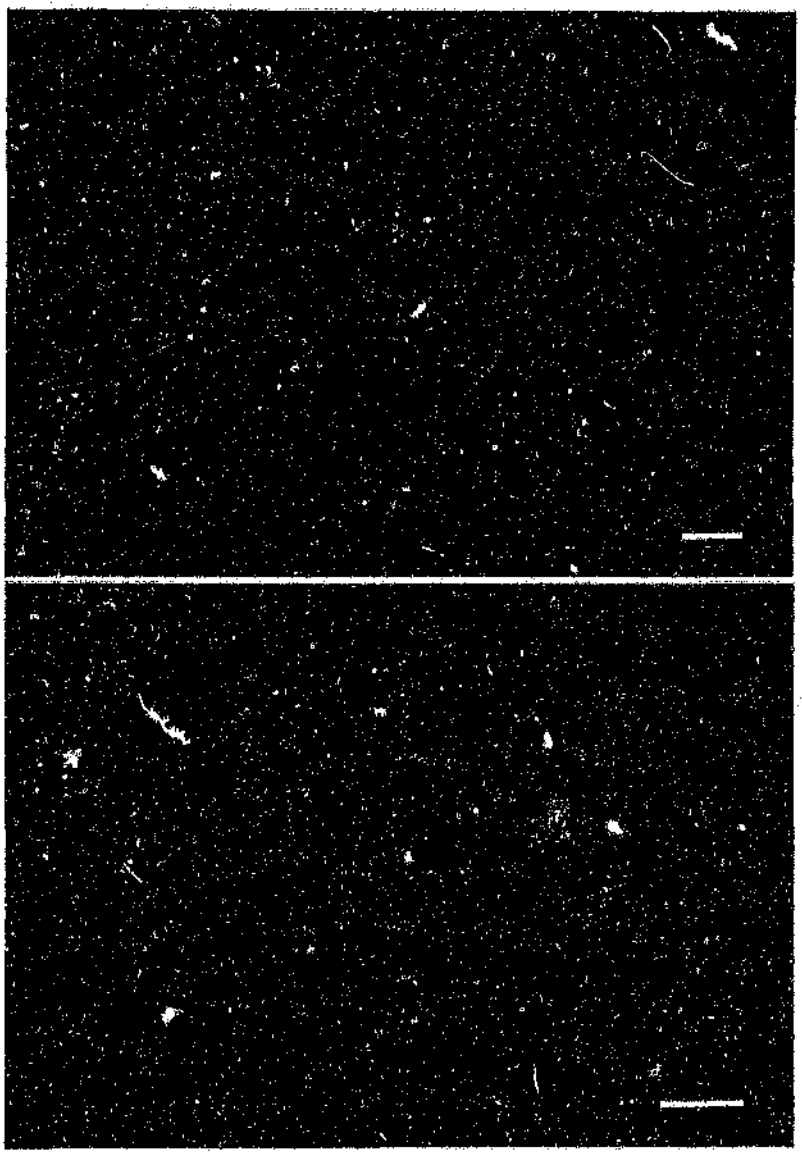


Figure 72

Fluorescence of organisms extracted from infected citrus midrib material. Bars = 5.0 um.

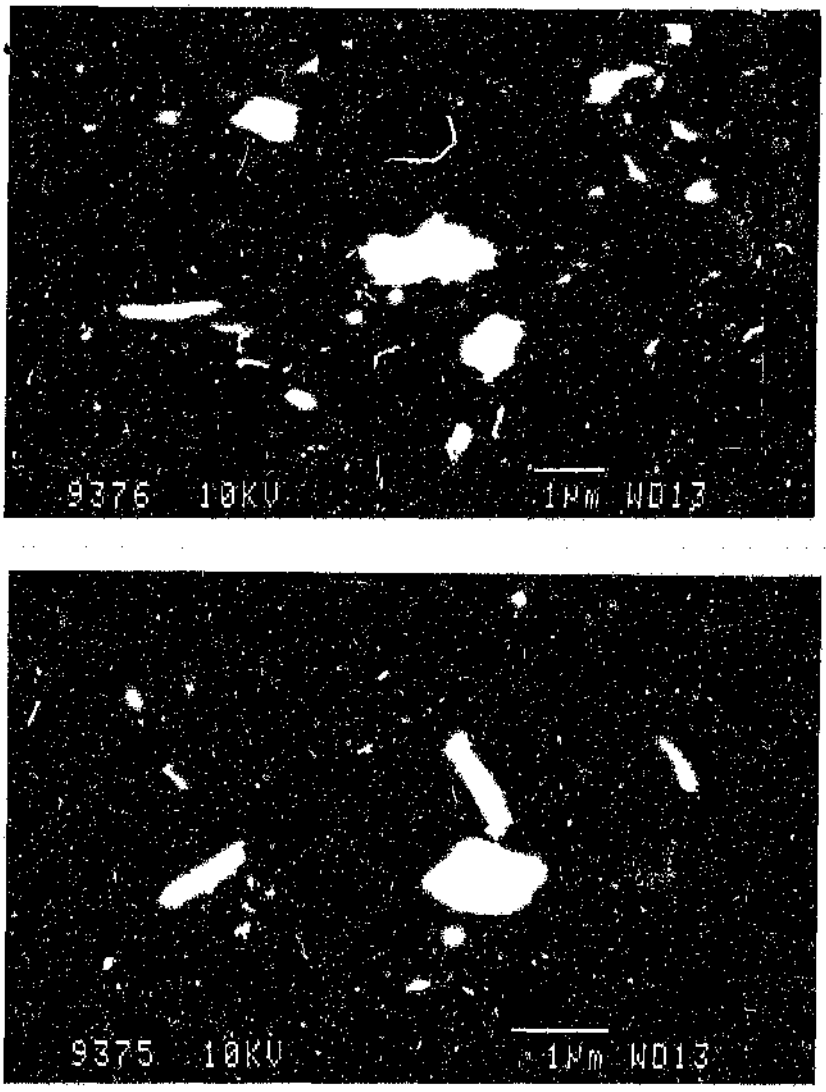


Figure 73

Scanning electron microscopic examination of organisms (arrows) and host plant debris trapped on the polycarbonate membrane from the PIFM investigation. Bars = 1.0 um.



Figure 74

Immunogold labelling of the GC isolate with antiserum raised against an extract of greening-infected citrus (GICE antiserum). Note absence of bound gold conjugate. Bar = 0.25 um.

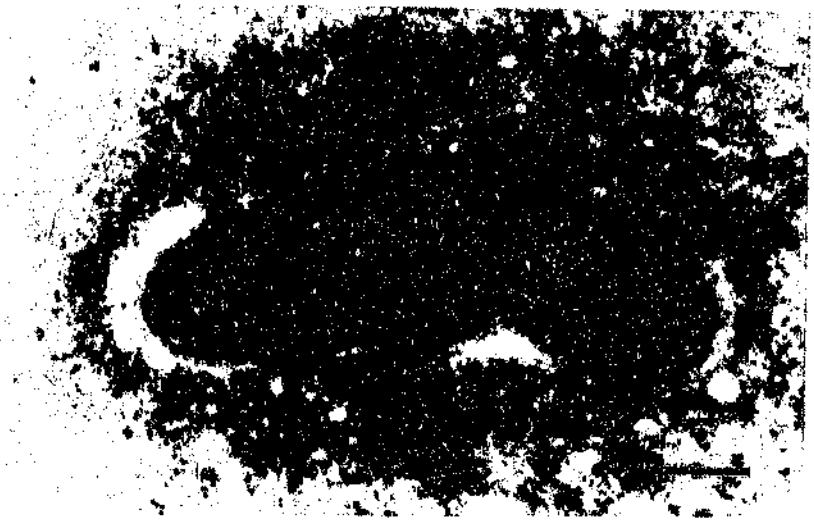


Figure 75

Immunogold labelling of the GC isolate. Cells were reacted with homologous antiserum and probed with gold-labelled anti-rabbit IgG. Note specific binding of label to the cell. Bar = 0.25 um.

3.11 WESTERN BLOTTING

Figure 76 shows the PAGE gel patterns of the Coomassie Blue-stained proteins from the extracts of the cultured GC isolate and the healthy citrus midrib material. After electrophoresis, the proteins on duplicate gels were either stained with Coomassie Blue (as shown in Fig. 76) or transferred to nitrocellulose membrane. The blotted proteins of the citrus extract were probed with antiserum directed against a live, sonicated preparation of the cultured GC isolate, followed by reaction with ^{125}I-labelled Protein A. Three millimetre sections were cut along the length of the NC membrane and the radioactivity of each section determined. The bar graph represents the radioactivity (c.p.m.) along the length of the paper blotted with the citrus proteins.

The gel profiles of the bacterial and plant extracts demonstrate the presence of a 61 kDa and a 67 kDa protein which appear to be common to both samples. When the citrus proteins were probed with the GC isolate antiserum, a positive reaction between the antiserum and the two plant proteins was recorded (Fig. 76, bar graph).

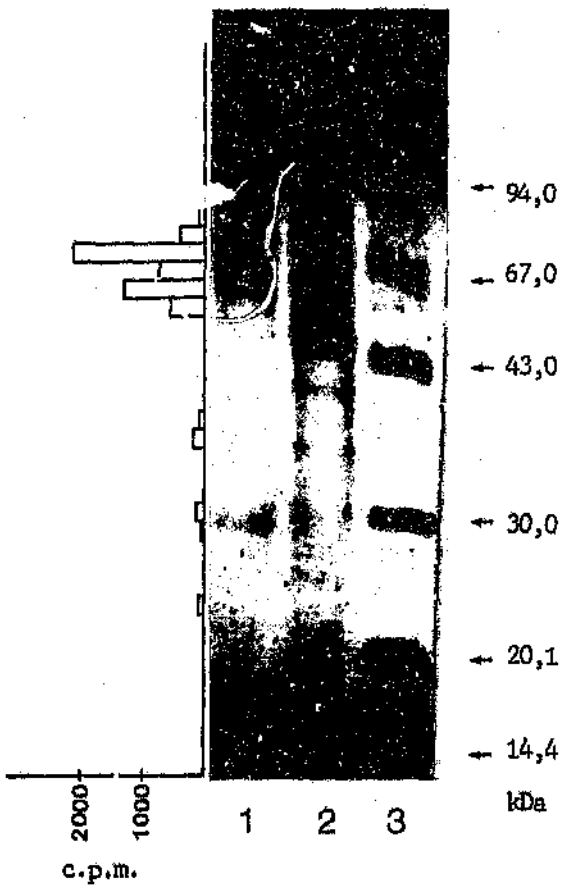


Figure 76

Coomassie blue-stained protein profiles of extracts of healthy citrus midribs (lane 1) and the cultured GC isolate (lane 2). Molecular weight markers (14.4 - 94 kDa; Pharmacia) are shown in lane 3. The bar graph represents the radioactivity (c.p.m.) along the length of the paper for the citrus protein extract. The proteins in this lane were probed with antiserum raised against a live, sonicated preparation of the cultured GC isolate.

3.12 FATTY ACID PROFILING

The fatty acid profiles of the bacterial isolates as determined in the laboratories of the National Collection of Plant Pathogenic Bacteria (NCPPB), England are shown in Appendix L.

The analysis of isolates NC, CS and R-NC (the latter being an organism re-isolated from a citrus seedling inoculated with the NC isolate) conducted in the NCPPB laboratories indicated that these three organisms have the profile of a Gram positive coryneform bacterium and belong to the same taxon. Although the fatty acid profile has affinities with Clavibacter, Micrococcus and Bacillus, the nature of the profile and the Gram stain reaction indicate that the organisms appear to be most closely related to the genus Clavibacter. It is possible that the isolates are members of the Clavibacter michiganense group, their profile being most similar to C.m. subsp. sepedonicum. The similarity indices were found to be fairly low, so great emphasis cannot be placed on the naming of subsp. sepedonicum, although this was the library profile most similar the that of the isolates.

Isolate GC was found to have the same profile as the three aforementioned bacteria, but with 3 small additional peaks which confused the diagnosis. GC was, however, found to be closely related to the C. michiganense group of bacteria.

In the same analysis, isolates TZL and LL displayed profiles which differed from one another and from isolates NC, GC and CS as well.

3.13 PAGE ANALYSIS OF CELLULAR PROTEINS OF THE C. michiganense GROUP OF BACTERIA AND THE GREENING ISOLATES

Fatty acid profiling of isolates GC and NC suggested that the organisms are members of the C. michiganense group (section 3.12). PAGE analysis of proteins was subsequently carried out and the resultant protein profiles of the organisms used as another means of determining a possible relationship between the isolates and representative members of this group of bacteria.

The PAGE protein patterns of the greening isolates and Clavibacter type cultures obtained from the National Collection of Plant Pathogenic Bacteria, U.K. indicate differences in the cellular proteins of isolates GC and NC

and all the members of the C. michiganense group of bacteria investigated (Figs 77 and 78). This finding, as well as the results of the EL-DIB assays (section 3.8.5.3, Figs. 69 and 70), suggest that isolates GC and NC are not closely related to this group of bacteria. Further characterization (e.g. genetic, metabolic and cell wall composition studies) of the isolates is, however, necessary to confirm this.

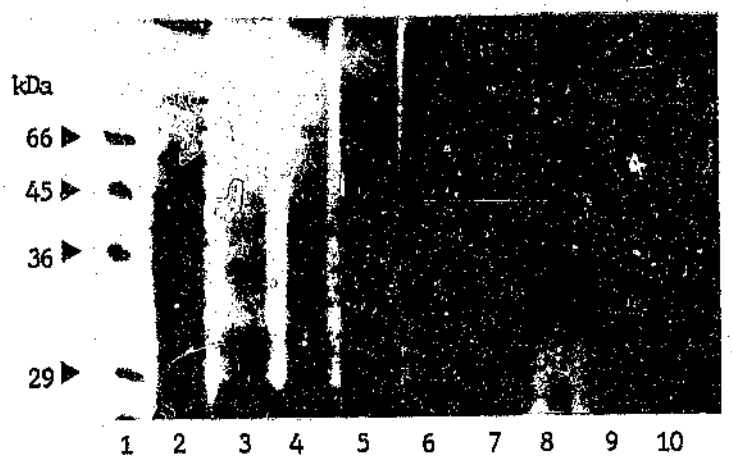


Figure 77

SDS-PAGE protein profiles of greening isolates and members of the genera Clavibacter and Curtobacterium. PAGE samples were prepared by the method of Carlson and Vidaver (1982).

Lane 1 - molecular weight markers (14.2 - 66 kDa; Sigma)
2 - Curtobacterium flaccumfaciens pv. flaccumfaciens (strain NCPPB 1446)
3 - Clavibacter iranicus (strain NCPPB 2253)
4 - C. m. subsp. insidiosum (strain NCPPB 1109)
5 - C. m. subsp. nebraskense (strain NCPPB 2579)
6 - C. m. subsp. michiganense (strain NCPPB 2979)
7 - NC isolate
8 - lysozyme (internal marker)
9 - GC isolate
10 - LL isolate

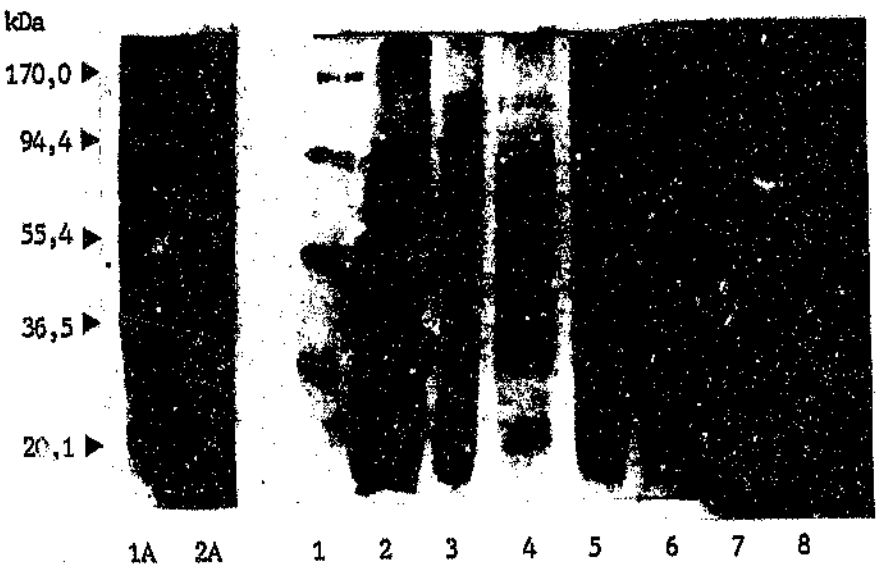


Figure 78

SDS-PAGE protein profiles of C. m. subsp. sepedonicum and greening isolates. The cellular proteins of C. m. subsp. sepedonicum and those of the greening isolates were fractionated on separate gels, but under idential conditions. The same protein standards were used on both gels to allow for comparison of the protein profiles. The protein samples were prepared as described in section 2.9.1.6.

Lane 1A - molecular weight markers (20.1 - 170 kDa: Boehringer Mannheim
2A - C. m. subsp. sepedonicum (strain NCPPB 2137)

1 - molecular weight markers (20.1 - 170 kDa)
2 - healthy citrus midrib extract
3 - GC isolate
4 - NC isolate
5 - CS isolate
6 - LC isolate
7 - LL isolate
8 - TZL isolate

CHAPTER 4

DISCUSSION

4.1 ANALYSIS OF THE CULTURED BACTERIAL ISOLATES

The recognition that phloem-restricted prokaryotic organisms were associated with citrus greening disease (Laflèche and Bové, 1970a and 1970b, Saglio *et al.*, 1971, Chen *et al.*, 1971) raised hopes that the disease agent would be cultured and its aetiological role confirmed. Reports claiming the successful *in vitro* culture of the greening organism in artificial media soon followed (Ghosh *et al.*, 1971, Sodhi *et al.*, 1973). The organisms obtained in culture were mycoplasmas and could therefore not have been the greening organism, known since 1971 to differ from a mycoplasma (Saglio *et al.*, 1971). This fact has since been amply confirmed (Chen *et al.*, 1971, Moll and Martin, 1974, Garnier *et al.*, 1976, Garnier and Bové, 1977, Bové *et al.*, 1980, Garnier *et al.*, 1984).

Some years after these initial isolation attempts, Garnett (1985) reported the isolation of the greening bacterium in a complex medium designated MIG. The evidence presented by the author in support of the claim that the organism

isolated was the aetiological agent of greening disease was largely circumstantial. The fact that i) the morphology of the cultured bacterium was typical of the greening bacterium as viewed *in situ*, ii) the organism was isolated from most of the major citrus growing areas of South Africa where greening exists, and iii) the antibiotic sensitivity displayed by the organism was comparable to that described for greening *in situ*, was considered by the author to be adequate proof of the pathogenicity of the isolate.

In the same journal in which the work of Garnett (1985) is reported, reports by Ariovich and Garnett (1985) and Duncan and Garnett (1985) present further circumstantial evidence to support the claim made that the bacterium is the causative agent of greening disease. Attempts to repeat aspects of the work performed by the above authors in this and other laboratories have met with little success. In the hands of other workers the MIG medium of Garnett (1985) failed to support the growth of the greening organism even when infected periwinkles with high titres of the bacterium were used as inoculum (Manicom, 1984, Garnier *et al.*, 1987). Moreover, an antiserum raised by Garnier *et al.* (1987) against the cultured isolate of Garnett failed to detect greening disease in infected periwinkle and citrus plants in ELISA. The inability of workers to reproduce the

results of Garnett, as well as the fact that the isolations and pathogenicity were never fully confirmed by the fulfilment of Koch's postulates, casts doubt on the aetiological role of the cultured bacteria.

Mochaba (1989), working in the same laboratory as Garnett, also reported the isolation of similar bacteria from greening-infected citrus and attempted to characterize the organisms.

One of the principal aims of this study was to unequivocally determine the pathological significance of the isolates supplied by Garnett and Mochaba through transmission studies. In addition to the attempted fulfilment of Koch's postulates, an analysis of the bacterial isolates was undertaken. This involved Gram staining, PAGE analysis of cellular proteins, electron microscopic examination and serological studies. Early transmission and serological studies conducted during the course of this study suggested that the isolates differed in some respects. Differences in certain properties of the isolates were also observed in the work carried out by Keddy (1989) and Mochaba (1989).

All the citrus greening isolates described by Garnett (1985) and Mochaba (1989) were reportedly Gram-negative. In this study, however, the cultured bacteria (isolates GC and NC) exhibited a Gram-variable stain reaction (Fig. 8) when the staining method detailed in Appendix E was used. The isolates also stained Gram-variable when the the modification of the Gram stain procedure employed by Mochaba (1989) was used. Although the age of a bacteria' culture and the conditions under which the cells are grown are known to affect the staining properties of a particular organism, isolates GC and NC consistently displayed a Gram-variable staining reaction. A culture of C.m. subsp. michiganense grown under the identical conditions was included as a standard. The organism was found to exhibit a Gram-variable stain reaction (Fig. 9) similar to that of isolates GC and NC.

Members of the genus Clavibacter are reported to be Gram-positive (Davis et al., 1984), but isolates decolourize easily and frequently exhibit uneven staining (Moffett et al., 1983, Vidaver and Davis, 1988). As overnight cultures of the bacteria were used for the Gram stains, it is unlikely that the variable stain reaction observed was due to the age of the cultures. It is possible that the decolourization step used in the staining procedure reported here was overly stringent and that the Clavibacter and NC cultures destained easily resulting in the variable stain reaction.

The results of a number of investigations conducted in this study indicate that the GC group of isolates (GC, NC and CS) differ significantly from the other isolates in a number of respects and thus suggest that the groups are unrelated. The comparison by SDS-PAGE of the cellular proteins of isolates GC, NC, CS, LLL, LL, TZL and LC shows significant differences between the GC group and the other organisms analyzed (Figs 10 and 78). The difference is also reflected in the results of the EL-DIB assay in which isolates TZL and LC failed to react with antiserum raised against the GC isolate (Fig. 69). In addition, the inoculation of various herbaceous plant species with either a cell suspension of the different isolates or with the filtered culture medium in which the organisms were grown pointed to differences in the infectivity of the bacteria. Only the plants exposed to isolates GC, NC or CS, or to their metabolites, developed disease symptoms, while isolates LC and TZL failed to induce symptoms in the similarly inoculated plants (Table 7).

4.2 ISOLATION OF THE BACTERIUM FROM CITRUS, PERIWINKLE, DODDER AND THE INSECT VECTOR

Attempts to isolate and culture bacteria identical to the GC group of organisms from citrus and psylla samples collected from various areas of the Transvaal affected by greening met with limited success (section 3.2). The

bacterium was cultured from only a small percentage of the infected citrus branch, leaf and fruit samples assayed (Tables 1 and 2), but not from the infected insect, periwinkle or dodder material. The organism was also isolated in pure culture from apparently healthy citrus material (Tables 1 and 2), although the trees from which these samples were harvested may have been affected by greening but did not display visible symptoms of the disease at the time of collection of the samples.

In addition to the isolation of these organisms, a number of other bacterial species were isolated in pure culture from both healthy and infected citrus, periwinkle and dodder samples using MIG-3 medium (Table 3). The bacteria cultured were most probably residents of the internal plant and insect tissues as the sterilization method employed was stringent and had been found to be effective in neutralizing the surface microflora. Similarly, a number of different bacteria were isolated from plant and insect samples using a number of other culture media (section 3.3.1).

No appreciable differences in the results of the isolation attempts using the two inoculum preparation procedures was observed. The Scholander bomb method was, however, a more convenient means of obtaining sap from citrus material. The

use of the bomb facilitated the extraction of plant sap under aseptic conditions and thus resulted in less contamination of cultures compared to the chopping method. In addition, the Scholander bomb method of extraction yielded an inoculum preparation which was less contaminated with compounds derived from host tissue extracts compared with the chopping method. The presence of such compounds has been found to be inhibitory to the growth of MLO (Eden-Green, 1982) and spiroplasmas (Lee and Davis, 1986) and may similarly affect the growth of bacteria extracted from citrus.

The results of the isolation attempts conducted in this study indicate a low correlation between typical greening symptoms and occurrence of the isolates of Garnett and Mochaba. Nonetheless, the organism does appear to be associated with greening-infected citrus to a limited extent, but not with similarly infected psylla, periwinkle and dodder.

The finding that different bacterial species could be isolated from both diseased and healthy plant material is in agreement with the observations of Gardner et al. (1982) and Botha et al. (1984) who reported that both healthy and diseased citrus plants harbour endogenous bacterial populations. The mere observation of bacteria in plants

does therefore not necessarily imply that the organisms are phytopathogenic. Furthermore, the occurrence of such endogenous microorganisms in citrus plants would obviously complicate the unambiguous isolation of the aetiological agent of greening.

4.3 ATTEMPTED ISOLATION OF THE CAUSAL AGENT OF GREENING

Perhaps the most pertinent area for study of greening disease is the further characterization and identification of the causal agent of the disease, eventually leading to its assignment to a taxon. Advancement in this area would be greatly facilitated by the successful cultivation of the bacterium on artificial media.

Utilization of standard media and culture methods has thus far failed to allow workers to isolate the aetiological agent of greening. The answer to this problem may lie in manipulation of the choice of the inoculum source, existing media and conditions of culture, as well as in the development of novel media and the use of innovatory culture techniques.

Sibara (1982) reported the development of a culture medium, the formulation of which was based on that of an insect tissue culture medium. This medium, designated "MIG".

allowed the author to culture a bacterium with a similar morphology to that of the greening organism. The bacterium was isolated from greening-infected citrus but never from healthy plant material. Using this same medium, Duncan (1985) later isolated a Gram-negative bacterium from infected citrus which reportedly displayed the characteristic cell envelope of the greening organism. As with the isolates of Garnett (1985) and Mochaba (1989), the aetiological role of the bacteria isolated by Sibara (1982) and Duncan (1985) was never confirmed.

Despite the reported success at culturing a Gram-negative bacterium displaying the morphology and ultrastructure of the greening bacterium from infected citrus using MIG medium (Garnett, 1985, Ariovich and Garnett, 1985), a similar bacterium was never isolated during the course of this study from citrus, periwinkle, dodder or insect material affected by greening (section 3.3.1) using MIG or MIG-3 medium (a modification of MIG).

Similarly, isolation of an organism displaying the structural features of the greening bacterium was not achieved using standard bacteriological media, as well as other culture media developed for fastidious plant pathogens (section 3.3.1). Periwinkle, dodder and psyllid tissues have been shown to harbour high titres of the

greening bacterium (Moll and Martin, 1978, Garnier and Bové, 1983, Ghosh et al., 1977) and should therefore be more suitable as sources of the bacterium than citrus. Attempted isolation of the greening organism from these sources was, however, not achieved.

Failure to culture the greening organism can probably be attributed to three factors: i) failure in mechanical extraction of viable organisms from host tissues, ii) inhibition of the bacteria in early passage by compoun derived from host tissue extracts, and iii) inadequacy of media and conditions of culture.

In the case of plant-pathogenic spiroplasmas, the ideal inoculum for primary isolation is one that contains a high titre of viable organisms and a low concentration of potential inhibitors of spiroplasma growth (Lee and Davis, 1986). An inoculum with the same features is likely to be ideal for the culturing of the greening organism.

In the Scholander bomb method of extraction, application of positive pressure on a leafy twig held within the pressure chamber results in the liqu d contents of the plant vascular system being forced up to and extruded from the cut end of the twig. This method is ideally suited to the extraction of xylem-limited as well as phloem-limited

organisms such as the disease agent of greening. The use of the Scholander bomb to obtain sap from infected citrus material was found to facilitate the preparation of inoculua relatively free of host pigments and other contaminants. Visual and light microscopic examination of sap bubbling from the twig (Fig. 2) showed that the sap was minimally contaminated with pigments and particulate matter of host origin. Physical disturbance of vascular-limited organisms is also minimized in this novel extraction procedure.

The relatively "clean" inoculum preparations obtained through the use of the Scholander bomb differed markedly from the plant-contaminated inocula prepared by the chopping method (section 2.2). The latter procedure ruptures host cells releasing plant pigments, tannins, enzymes and other compounds which may inhibit bacterial growth. Procedures such as early serial subculture, dilution of primary culture with fresh medium, or clarification (through use of centrifugation) to remove toxic material, may however be used to minimize the effects of the host-derived inhibitors (Liao and Chen, 1982). In the chopping method of extraction, vortexing of the plant suspension was necessary in order to enhance the release of bacteria from the host tissue. This is a physically disruptive process and may have an adverse effect on any microorganisms present.

Isolation of the greening bacterium from the insect vector may preclude some of the difficulties encountered when using infected plants as sources of inoculum. Haemolymph, for example, could be a useful source of the bacterium for isolation in culture as the haemolymph of T. erytreae collected off greening-infected citrus was reported to contain large numbers of the organism (Moll and Martin, 1973). Collection of psyllid haemolymph is an extremely difficult procedure and whole ground insects had to be used as inoculum in this study rather than the isolated haemolymph. However, when whole insects are ground in a medium or buffer, toxic products may be released (Lee and Davis, 1986) and thus similar precautions such as dilution and clarification may need to be taken with such inocula.

It is unlikely that the inability to culture the causal agent of greening disease in this study was due to the inefficacy of the mechanical extraction procedures employed. Although electron microscopic examination of a number of infected plant extracts prepared using either the Scholander bomb or chopping method failed to reveal the presence of visible numbers of the greening bacteria in the preparations, the nature of the procedures is such that their use should result in the extrication of at least some of the endogenous microorganisms from the host material.

An initial inoculum high in titre of viable organisms is probably as important a goal as a suitable growth medium in culturing the greening organism *in vitro*. The number of bacteria released by either the Scholander bomb or chopping method of extraction may not have been great enough to ensure adequate growth of the culture in the initial stages, and further improvements of these procedures, as well as the investigation of other methods of extraction should thus be investigated.

The presence of potent plant-derived bacterial inhibitors in the inocula is unlikely to be a major factor contributing to the inability to isolate the greening bacterium in pure culture. Although inocula prepared by the chopping method were visibly contaminated with plant components, any inhibitory compounds present would have been diluted considerably upon addition of 0.2 ml of the preparation to 15 ml of culture medium (section 2.2). Clarification and concentration of 1.5 ml volumes of infected extracts by differential centrifugation and their subsequent use as inocula failed to result in the isolation of the greening organism (results not shown). Such treatment of extracts should aid in removing a significant amount of any inhibitory comounds present, as well as concentrating the bacteria present.

The extraction of plant xylem and phloem fluid via the Scholander bomb should yield inocula containing lesser amounts of plant components and higher concentrations of phloem-limited organisms (such as the greening bacterium) than the chopping method of extraction. The use of this apparatus therefore minimizes problems associated with interfering host compounds and maximizes recovery of vascular-limited organisms.

The inability to culture the aetiological agent of greening disease *in vitro* is thus likely to be due to the inadequacy of the culture media and/or conditions of culture. The failure of various media to support the *in vitro* growth of the organism (Moll, 1974, Manicom, 1984, Garnier *et al.*, 1987) suggests that it has limited biosynthetic capabilities and probably requires a number of complex ingredients such as those included in media for the cultivation of plant MLO and spiroplasmas (Lee and Davis, 1986).

Because of the disputed effectiveness of MIG as a culture medium for the greening bacterium, a number of other complex media were used in an attempt to isolate the organism. Three of the media, namely CG3, LD8A3 and LD69, were formulated for primary isolation and routine subculture of plant-pathogenic spiroplasmas. Most media

designed for this purpose are, like media for culture of animal mycoplasmas, enriched with several complex ingredients such as pleuropneumonialike organism (PPLO) broth base, animal serum, and yeast extract or yeastolate (Lee and Davis, 1988).

Medium CG3 (Liao and Chen, 1975) was the first medium formulated for spiroplasmas and is a simple medium commonly used for these microorganisms. Medium LD8A3 'Lee and Davis, 1988) was developed for culture of more fastidious strains and yields superior growth of the plant pathogens. Medium LD59 (Davis at al., 1988) supports good growth of strains that grow poorly if al all in media containing serum. Serum contains a variety of lipids and other ingredients which are essential for growth of some MLO and spiroplasmas, but toxic and inhibitory to others (Lee and Davis, 1988). It is thus essential to use a variety of media formulations when attempting to culture a previously uncultivable microorganism.

Despite the routine use of the above-mentioned media for the cultivation of a number of fastidious plant pathogenic microorganisms, the media were unable to support the in vitro growth of the greening bacterium.

In addition to the use of the spiroplasma media, formulations developed for the axenic culture of phytopathogenic coryneform bacteria, including the nutritionally exacting organism causing ratoon stunting disease of sugarcane, were investigated as possible media for the culture of the greening bacterium. These media were found to be unable to support the *in vitro* growth of the organism.

All the media used were, however, able to support the growth of a variety of other bacterial species present in the extracts of both healthy and infected plant and insect samples. The presence of bacteria in the samples assayed was not unexpected: different bacteria were similarly isolated from material during the course of other isolation trials (section 3.2), and the occurrence of endogenous bacterial populations in citrus has been reported by other workers (Gardner *et al.*, 1982, Botha *et al.*, 1984). Insects too have been shown to harbour naturally-occurring bacterial populations (Purcell *et al.*, 1977, Davis *et al.*, 1981).

Because of the failure of the artificial media, alternate methods of culture were applied, namely embryonated hen's eggs and insect tissue culture. The former technique has been used for the *in vitro* culture of animal mycoplasmas

and spiroplasmas (Lee and Davis, 1986). Fastidious plant pathogens have also been cultivated using this method. Multiplication of Spiroplasma citri, honey bee spiroplasmas and flower spiroplasmas in chick embryos has been reported (Rose et al., 1979, T.A. Chen and C.J. Chang, unpublished, H.J. Su, unpublished) and Nienhaus et al. (1978) were similarly able to cultivate a rickettsia-like organism obtained from yellows diseased grapevine roots.

Despite the reported success in the in vitro culture of some phytopathogens displaying limited biosynthetic capabilities, growth of the greening organism in chick embryos was not observed. The failure to stimulate growth of the organism in this culture system under the conditions used here is not, however, surprising. For normal development of the embryos, the eggs had to be incubated at 37 C, a temperature which has been shown to be inhibitory to the African strain of the greening bacterium (Bové et al., 1974). The technique may have been more successful had extracts of material affected by the heat-tollerant Asian strain of the disease been used as inocula.

Because of the absence of an established psyllid cell line, a culture of mosquito larval cells was used in this study. No replication of the greening bacterium was observed to occur in this culture system, a result which was not

totally unexpected since the mosquito is not a known host of the greening organism. The development of a psyllid cell culture system, however, might facilitate the *in vitro* cultivation of the bacterium.

One is left with the conclusion that present artificial media and conditions of culture are simply inadequate for the isolation of the greening bacterium. This may be due to some relatively simple deficiency, and improvements in survival, multiplication and eventual subculture of this agent may come from chance combinations of culture conditions and reagents. Noncultivability, however, may also reflect a truly obligate mode of parasitism, involving a more intimate association with host metabolism such as in the case of the Chlamydiales (Eden-Green, 1982). If this is the case, the development of either plant or insect cell cultures, or other *in vivo* systems, will prove central to further progress in the axenic culture of the greening organism.

4.4 KOCH'S POSTULATES

The history of claimed isolations of microorganisms from greening-infected citrus demonstrates the need for cautious evaluation of the relationship between a newly isolated organism and its plant and/or insect host.

The pathological significance of an organism isolated from an infected source can only be confirmed by the fulfilment of Koch's postulates. As earlier attempts to introduce the cultured isolates back into citrus via mechanical inoculation had not been successful (M. Rota, pers. comm.), an attempt was made in this study to inoculate citrus via the insect vector as well as via stem injection and slashing.

Initial observations suggested that the isolates were in fact able to induce disease symptoms in mechanically inoculated plants (Figs 16 and 17), the symptoms being expressed in successive leaf flushes. Although the foliar chloroses observed were similar to those expressed by naturally infected greened citrus, confirmation of infection with greening by the production of characteristically lopsided fruit was not possible due to the immature nature of the test seedlings. In the absence of fruit, inoculated trees were tested for the presence of the gentisic acid marker of greening disease. The fact that all the symptomatic plants tested were negative for the marker suggests that the symptoms observed were not caused by infection of the plants with greening.

Although expressed in successive leaf flushes, the symptoms observed on inoculated citrus did not persist for longer than 2 years in any of the seedlings and a number of the

plants appeared to be free of disease 18 months after the initial expression of foliar symptoms. The bacterium was reisolated from inoculated seedlings displaying symptoms but attempts to reisolate the bacterium from plants after the apparent disappearance of visible symptoms were not successful, despite the fact that the same plants had previously yielded the bacterium.

Greening-like leaf symptoms have been observed to be induced by a number of factors, both abiotic and biotic. Labuschagne et al. (1984) reported the development of greening-like symptoms in sweet orange inoculated with a bacterium which was later found to be unrelated to the aetiological agent observed in situ. Gardner et al. (1982) and Gardner et al. (1984) also showed that various bacteria isolated from apparently healthy citrus have an effect on plants when inoculated back into citrus. Induction of leaf symptoms in citrus inoculated with various bacteria has also been reported (Labuschagne et al., 1984, Manicom, 1984). It is thus clear that foliar symptoms are an unreliable indication of infection with greening and that evidence should at least include the production of characteristically deformed fruit.

Visualisation of the bacteria in midrib material sampled at sites distant from the point of inoculation suggested that isolates GC and NC were able to spread within mechanically

inoculated plants (Fig. 22). 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. The number of bacteria observed in inoculated plants was small and no dividing cells were encountered, despite an extensive search. Most of the cells observed appeared to be degrading and some atypical forms were seen which resembled apparently degrading cells in late stationary phase cultures of the organism (Fig. 15).

The bacteria were recovered in pure culture from symptomatic citrus plants and displayed identical morphological, metabolic and serological properties to the original inoculum.

Attempts to introduce the isolates into citrus via the insect vector were unsuccessful. Adult psylla and nymphs which had acquired the bacteria through either membrane feeding or micro-injection were unable to infect citrus plants with the organisms. It is unlikely that the reason for the failure of insects to infect target plants was due to the inefficiency of the inoculation procedures used. Inclusion of dye in the feeding solution showed that the insects had in fact imbibed the inoculum and the use of the micro-injection technique ensures the entry of inoculum

into the insect. The failure of insects to transmit the isolates is possibly due to the inability of the psyllid to function as a vector of the isolates.

Although mechanical inoculation of periwinkle was not achieved, it was possible to transmit an infective agent from mechanically inoculated citrus to periwinkle via dodder (Figs 20 and 21). Bacteria were observed in the phloem of symptomatic plants, but not in the control periwinkle (Fig. 23). Dodder transmission of the disease from infected periwinkles to other periwinkle plants was also possible. As in the case of citrus, electron microscopic examination revealed that the titre of bacteria within diseased periwinkles was very low, although disease symptoms were pronounced and developed rapidly.

The low titre of bacteria observed is in contrast to the large numbers of greening bacteria reported by Garnier and Bové (1983) in periwinkle affected with greening. Re-isolation of the bacterium from inoculated symptomatic periwinkle was not achieved and it was therefore not possible to confirm that the bacterium introduced into the citrus was responsible for the symptoms observed in periwinkle.

From the results of the citrus inoculation trials reported here, it would appear that Koch's postulates had been fulfilled. The apparent association of the bacterium with greening disease was further confirmed by the results of the inoculation trials involving cucumber. Cucumber plants were infected with a disease agent from naturally-infected greened citrus via dodder and the same organism isolated in pure culture from symptomatic cucumber as that isolated from naturally-infected citrus (section 3.5).

Extensive TEM examination of infected cucumber plant tissue at various stages of symptom development failed to reveal the presence of bacteria which suggests a low titre of the disease agent within the plant. As no bacteria were visualized in infected material, it is not possible to ascribe the induction of the symptoms observed to the greening organism itself, to the isolate, or to some other biological agent. Considering the severity of the symptoms observed, the apparent absence of bacteria is unusual and may indicate the involvement of a potent bacterial toxin in the disease process.

Dodder parasitizing greening-infected citrus and symptomatic cucumber was not observed to grow as vigorously as that on healthy material. The development of the parasite was greatly retarded on diseased material and

newly-developed shoots rapidly became necrotic and died off. Ghosh *et al.* (1977) reported a high titre of greening organism in dodder parasitizing infected citrus, however, TEM examination of dodder material from diseased citrus and cucumber in this study revealed a low incidence of greening bacteria. The bacteria were erratically distributed within the parasite's vascular tissue and when encountered, were present in relatively large numbers within individual phloem cells (Fig. 29). A large number of bacteria in the infected tissue may account for the marked symptoms observed, but as the titre observed was low, the involvement of a bacterial toxin in this instance may also be possible.

Based on a number findings of this, as well of other investigations, the bacterium (GC group of organisms) isolated by Garnett and Mochaba was initially thought to be the aetiological agent of greening: i) the reported similarity in morphology of the isolates and the greening organism *in situ* (Garnett, 1985, Ariovich and Garnett, 1985), ii) the apparent fulfilment of Koch's postulates, and iii) the isolation of the same bacterium from diseased cucumber.

However, contrary to earlier reports, the electron microscopic investigation of the isolate morphology carried out in this study revealed significant differences between

the morphology of the cultured bacteria and the greening bacterium *in situ*. This study revealed that the isolates do not display the characteristic cell envelope of the greening bacterium (Fig. 29B). The cell envelope of the isolate (Fig. 14) measures 13-19 nm in width, while that of the greening organism *in situ* is reported to be approximately 25 nm (Moll and Martin, 1974, Garnier and Bové, 1977). The isolated bacterium replicates by means of septum formation (Fig. 13) and not by budding as reported for greening (Moll and Martin, 1974). In addition, extensive branching was observed in cultures (Fig. 12), as are club- and boomerang-shaped cells (Fig. 11), these features being atypical of the greening organism *in situ*.

Unless the bacterium has undergone significant morphological changes in culture, the results of the TEM study indicate that the GC group of isolates differ markedly from the greening organism *in situ* and cannot therefore be the causative agent of greening disease. Further results of this investigation also suggest that the isolates differ from the aetiological agent of greening. These include the findings that: i) symptoms on mechanically-inoculated plants were transient and disappeared 18-24 months after inoculation which suggests that the infection does not persist,

ii) antiserum raised against the cultured isolate does not detect the presence of greening in infected citrus and periwinkle material (Figs 63 and 67),

iii) transmission of disease from inoculated plants by grafting does not occur (results not shown), this method of transmission usually being efficient in the case of greening (McClean and Oberholzer, 1965),

iv) mechanically-inoculated symptomatic citrus seedlings do not display the gentisic acid marker (section 3.4.7), the presence of this substance usually being indicative of infection with greening, and

v) transmission of the isolates to citrus via the insect vector of greening did not occur and ELISA, TEM observation and reisolation attempts indicated that the isolates were unable to persist, replicate or spread within injected and membrane-fed insects.

The accumulated results strongly suggest that the bacterium isolated by Garnett and Mochaba is not the greening organism *per se*, although it does appear to be associated with some trees infected with greening disease. The organism may be a mild opportunistic pathogen in citrus, establishing itself in plants already weakened by infection

with other disease agents. As the bacterium has been shown to induce disease symptoms in citrus, it may, to some extent, contribute to symptoms observed in stressed field-infected greened citrus and may be a component of the greening syndrome.

The fact that greening disease may involve more than a single biological agent is suggested by the findings reported here, as well as by the isolation of a number of other bacteria from infected and healthy citrus which induce various symptoms when mechanically inoculated back into healthy plants (Labuschagne et al., 1984, Manicom, 1984, Smith et al., 1988). Additional work is, however, needed to verify the association of these organisms with the disease. Consideration should also be given to the possible involvement of abiotic factors in the disease syndrome.

The findings reported here further illustrate that the induction of disease symptoms by an isolated microorganism in inoculated test plants and the subsequent reisolation of the organism in pure culture does not necessarily imply the fulfilment of Koch's postulates and could merely be the mimicking of the postulates.

4.5 INOCULATION OF HERBACEOUS PLANT SPECIES

The pathogenic effects of the bacterial isolates, as well as the metabolites produced by the cells in culture, were determined by inoculating various herbaceous plant species with the cells or cell products present in the growth medium.

The wilting observed in tomato, green pepper, cucumber and aubergine cuttings after exposure to the spent culture medium of isolates GC, NC and CS suggests the presence of a potent wilt-inducing agent of bacterial origin in the inoculum. Similar wilting was observed in tomato, green pepper and cucumber when leaves of the plants were rubbed with the spent medium. Inoculating aubergine plants in this way failed to result in the same foliar wilting which suggests that the toxin or toxin-like substance was not able to exert its effect through the cuticle of this plant species. Rubbing the leaves in such a way as to cause more damage to the surface may have led to wilting. Aubergine was, however, found to be susceptible to the toxin as seedling cuttings placed in diluted culture supernatant were observed to wilt.

When an aqueous suspension of GC, NC or CS cells was rubbed onto the leaves of tomato, green pepper and cucumber, wilting was also observed. This suggests that the toxin

responsible for this symptom is associated with the glycocalyx of the bacteria. The fact that the toxin is also present in the culture filtrate indicates that the compound is either excreted from the cells or sloughed off during growth *in vitro*.

As the toxin was found to affect a number of unrelated plant species, it appears to be non-specific in its action. The non-specificity of the toxin may indicate that the effect of the compound is physical.

The absence of wilting on citrus exposed to cultured cells or medium indicates that this plant species is unaffected by the toxin under the conditions employed in this investigation. Citrus plants infected with the GC isolate (either naturally or experimentally) were never observed to display wilting symptoms and the wilt-inducing effect of the toxin on citrus thus appears to be minimal.

Wilt-inducing exotoxins have been reported to be produced by the plant pathogenic bacteria *Agrobacterium tumefaciens*, *Xanthomonas phaseoli*, *Erwinia carotovora* and *Corynebacterium michiganense* (Hodgson *et al.*, 1947, 1949, Feder and Ark, 1951, Rai and Strobel, 1969). The phytotoxins associated with these organisms were found to be polysaccharide and non-specific.

When a purification procedure developed for the isolation of polysaccharide phytotoxins was applied to the culture filtrates of isolates GC and NC, the resultant preparation was found to cause similar wilting to that observed in plants rubbed with the unfractionated medium (C. Clarke, pers. comm.). It is thus likely that the toxin produced by isolates GC, NC and CS is a polysaccharide which lends support to the hypothesis that the toxin is associated with the bacterial glycocalyx.

A similar toxin does not appear to be produced by isolates LC and TZL as exposure of the herbaceous plant species to these organisms or to their metabolites failed to result in any visible disease symptoms.

4.6 SEROLOGICAL DETECTION

4.6.1 Pre-treatment of cultured bacterial immunogen

An antiserum raised against a bacterium isolated from greening-infected citrus was reported by Duncan and Garnett (1985) to detect the disease in plant samples. However, when the same antiserum and enzyme conjugates prepared by the aforementioned authors were used in the initial stages of this study, following the same ELISA protocol as that

described by these authors, the antiserum was unable to consistently distinguish between healthy and greening-affected citrus material (results not shown). Because of this finding, renewed attempts were made to develop a suitable antiserum for the detection of the isolates in plant and insect tissues.

Results of preliminary serological detection assays using antisera raised against suspensions of the cultured isolates prepared following the method described by Duncan (1985) were unsatisfactory. The antisera displayed considerable reactivity with healthy plant material and could thus not be used to detect bacterial antigens in the plant extracts (results not shown). Similar results were obtained with antisera were raised against untreated, live bacterial cells (Fig. 34).

Treatment of cultured bacterial cells before injection into rabbits has been shown to influence the quality of the resultant antiserum markedly (Schaad, 1979), and different pre-treatments of bacterial immunogen were therefore investigated in this study in an attempt to develop a suitable antiserum.

Pre-treatment of bacteria with KOH was performed in order to expose alkali-soluble cell surface immunogens. An antiserum raised against cells treated in this way should

be able to detect these antigens in an alkali-extracted preparation of infected plant material using a detection assay similar to that described by Kawarabata and Hayasaka (1987). The alkali extraction method was used here primarily to overcome the problems associated with large particulate bacterial antigens in the indirect ELISA (section 1.9.2.4). The results obtained with this procedure were, however, unsatisfactory (Fig. 34), possibly due to interference of the high concentration of KOH in the sample with the sensitivity of the test, even after neutralization of the alkali with HCl.

An attempt was made to identify and isolate proteins unique to the GC isolate which could be used as immunogen in the production of a mono-specific polyclonal antiserum. An antiserum produced in this way should prove more specific in serological detection assays. Bacterial (GC isolate) and plant proteins were separated in acrylamide gels and the protein profiles of the samples compared. Fractionation under non-denaturing PAGE conditions resulted in inadequate separation of constituent proteins and no bands suitable for use as immunogen could be identified (Fig. 35). SDS-PAGE, however, generated clear protein profiles (Fig. 36) and a 52 kDa MW protein apparently unique to the bacterium was identified and an antiserum raised against the isolated band.

When used in the ELISA, the antiserum raised against the protein band was found to be unsuitable for detecting the bacterium in citrus (Fig. 34). The negative result recorded may be due to the protein isolated being an intercellular bacterial antigen. The water leaching procedure used to prepare plant samples for ELISA does not cause significant disruption of bacteria within the extract and hence internal bacterial proteins would not be exposed. This probably accounts for the comparatively low absorbance reading of the infected sample.

This hypothesis was tested by reacting suspensions of intact and sonicated bacterial cells (GC isolate) separately with the antiserum in the ELISA. The absorbance reading of the disrupted cell suspension was approximately four times greater in magnitude than that of the intact cells (data not shown). These results corroborate the assumption that the isolated protein was an internal cellular antigen. The strong positive reaction observed in this test also indicates that the antibodies elicited against the denatured protein isolated from the SDS-PAGE gel does react with the protein in its native state.

In addition to the 53 kDa MW protein, a number of other protein bands apparently unique to the GC isolate were observed in the profile of the bacterium (Fig. 36). These

proteins were not, however, present in concentrations as high as that of the 58 kDa MW species and were thus not selected for use as immunogens. Had antisera been raised against these proteins, the antisera may have been more suitable for the specific detection of the intact bacterium in the ELISA.

The only cell pre-treatment which yielded antisera suitable for use in a detection assay was that involving heat and formaldehyde treatment of the cells, with or without subsequent sonication. The treatment was found to effectively remove the extracellular matrix of the bacteria and increased the value of the diseased/healthy absorbance ratio from 1.20 (obtained with untreated cells antiserum) to 2.34 in the ELISA (Fig. 34).

The glycocalyx of the citrus greening isolate was shown by Keddy (1989) to consist of glycoprotein. Removal of the bacterial glycocalyx by heat and formaldehyde treatment prior to injection resulted in an antiserum which exhibited considerably lower levels of anti-plant activity than the antiserum directed against live, untreated cells (Fig. 34). The cross-reactivity observed with the anti-whole cell antiserum may have been due to recognition of plant glycoprotein antigens by antibodies directed against bacterial glycoproteins. This is suggested by the decrease

in the level of anti-plant activity observed with the antiserum produced against unencapsulated cells as opposed to whole cells (Fig. 34).

The existence of antigens common to plant hosts and parasites is widespread (DeVay and Adler, 1976), and it does therefore not seem unusual that the bacterial isolates and citrus might possess common [illegible]genic determinants. The possibility of such shared [illegible] was investigated using the Western blotting procedure.

In this assay, SDS-PAGE frac[illegible] plant and bacterial (GC isolate) proteins demonst[illegible]he presence of two proteins which appear to be common to both samples (Fig. 76). When the plant protein profile was blotted onto nitrocellulose membrane and probed with antiserum specific for the cultured bacterial isolate, the antiserum reacted positively with the two plant proteins (Fig. 76), indicating a similarity in the antigenicity of the proteins in the plant and the bacterium. The presence of those proteins offers a possible explanation for the significant anti-plant activity observed with the antiserum raised against the cultured isolate in the ELISA (Fig. 34).

4.8.2 Extraction of putatively disease-specific immunogens from infected plant material

An area of research with great potential lies in the development of more effective techniques for extraction and purification of the greening organism from the plant and insect hosts. Significant progress in this area has been made by the development of an immunoaffinity chromatographic procedure for the purification of the greening organism (Villechanoux *et al.*, 1990). The development of an effective procedure for the purification of the bacterium would facilitate study of the biochemical and serological properties of the organism, and could lead to elucidation of its relationship to known groups of prokaryotes, as well as to the possible development of new diagnostic techniques.

In the absence of culture media able to support the *in vitro* growth of the greening organism, the only source of greening-specific antigens remains affected plant or insect material. The development of a purification procedure which can be used to isolate sufficient quantities of greening antigens for use in the production of an antiserum is therefore essential.

The need for a greening-specific antiserum was highlighted by the poor results obtained using antisera raised against laboratory cultures of an organism claimed by Garnett (1986) to be the aetiological agent of greening disease. The antisera directed against the isolate were found in preliminary trials to be unsuitable for the detection of greening in various detection assays. Negative results were frequently obtained with material collected from plants known to be affected with greening.

In order to avoid using the cultured isolate in the preparation of greening-specific antisera, attempts were made to extract greening-associated antigens from naturally-infected plant material using a variety of methods. A number of purification protocols were investigated for this purpose, the majority having been successfully used by various authors to isolate bacteria, spiroplasmas and MLO from infected plant tissue (section 3.7.1.3).

Assessment of the purification procedures using artificially contaminated leaf homogenates indicated that method was the most effective for extracting bacteria (with physical characteristics similar to those of C.m. subsp. michiganense) from leaf homogenates (Table 8). However, electron microscopic examination of the extracts

of infected citrus material obtained following this method indicated that only a small number of bacteria were present in the preparations. Bacteria displaying a morphology similar to that of the greening organism were observed in infected extracts (Fig. 46), but never in preparations of healthy tissue.

A feature of the extracts of infected citrus was the presence of large numbers of amorphous crystalline structures (Fig. 47). The structures were never found associated with healthy citrus and were also isolated from greening-free citrus infected with other pathogens. The fact that the crystals were present in citrus affected by a number of different pathogens, both viral and bacterial, but not in healthy material, suggests that these structures may be of host origin and produced on infection of the plant by a pathogen.

It is improbable that the crystals observed were associated with the uranyl acetate stain used on the grids, this being suggested by the fact that the structures were never observed in stained preparations of healthy citrus extracts, as well as the failure of thorough washing to remove the crystals from the grids.

Manicom (1984) reported the presence of large amounts of hesperidin crystals in citrus which co-migrated with endogenous bacteria on density gradients and thus interfered with extraction of the organisms. It is unlikely that the crystalline structures observed in this study were hesperidin crystals as it was not possible to remove them by dissolving at ph 8.5, this treatment being reportedly adequate for their solubilization (Manicom, 1984).

The nature of the structures was not determined, although when aliquots of the extracts were prepared for transmission electron microscopy, the crystals were not observed in the ultra-thin sections. This finding suggests that they were probably solubilized during the process of fixation and embedding.

The crystals were further purified by repeated cycles of differential centrifugation and the preparation analyzed spectrophotometrically. The absorbance spectrum of the sample exhibited a peak at 282 nm which suggests that the crystals are proteinaceous. A sample of healthy material prepared in the same way failed to exhibit a similar absorbance peak (data not shown).

As method 5 was found to be the most effective method for extracting bacteria from artificially contaminated tissue homogenates (Table 8), and was the only procedure which

appeared to isolate bacteria resembling the greening organism, extracts prepared in this way were used for the production of an antiserum against plant-derived disease antigens.

A significant amount of host plant material was, however, co-purified with the endogenous bacteria following method 5 and attempts to further purify the bacteria on sucrose and Percoll gradients were largely unsuccessful. Because Percoll is a polydisperse colloid, its component particles sediment at different rates under centrifugal force, creating a smooth gradient. Such a continuous gradient was found not to be sufficient to remove plant materials from any bacteria present in the sample because the components of plant tissue vary in density and were distributed throughout the gradient (Fig. 44). Moreover, the density of the greening organism is not expected to be very uniform because of its pleomorphic nature. As a result, host plant material with density similar to the greening organism would be mixed with the bacteria in the continuous density gradients.

Because of the presence of contaminating host components, extensive cross-adsorption of the resultant antiserum was necessary to remove the anti-plant activity before the antiserum could be of any use in a serological detection system.

Although bacteria were isolated from greening-infected citrus using the other purification procedures, the organisms did not display the morphology of the greening bacterium and were probably part of the natural endophytic microflora of the plants (Figs 37, 42 and 43). Similar bacteria were sometimes found in extracts of healthy citrus material (Fig. 38). The occurrence of bacteria in both diseased and healthy preparations corroborate the results of earlier isolation attempts (sections 3.2 amd 3.3.1), as well as the work of Gardner et al. (1982) and Botha et al. (1984), which showed that citrus harbours naturally-occurring endophytic bacterial populations.

It is unlikely that significant amounts of disease-specific antigens were isolated from infected plant material following methods 1 - 4. When aliquots of the concentrated extracts were probed with the antiserum raised against the extract obtained following method 5 (GICE antiserum - this antiserm had been shown to detect disease antigens in citrus, cucumber and periwinkle samples [Figs 62 and 66]), no difference between the absorbance readings of the infected citrus and healthy controls was observed.

The percentage recovery of the C.m. subsp. michiganense added to the leaf homogenate in method 2 was extremely low (33%). Visual examination of the artificially contaminated

homogenate at various stages of the procedure suggested that passage of the preparation through the celite pad resulted in a significant loss of bacteria. This was indicated by a marked decrease in the turbidity of the preparation after this treatment. Sinha (unpublished data cited in Eden Green, 1982) found that Celite irreversibly adsorbed a large proportion of Acholeplasma laidlawii suspended in the same Mg-glycine buffer as that used in purification method 2, which suggests that this technique should be used with caution.

These findings suggest that method 2 is not ideal for extracting phytopathogenic bacteria in sufficient quantities for use as immunogen in antiserum production, especially in the case of a disease agent such as the bacterium causing African greening which is present in the host plant at a low titre.

In addition to the bacteria observed, numerous vesicle-like structures were present in the extracts of infected citrus obtained using methods 2 and 4 (Figs 37 and 39). Similar structures were not observed in similar preparations of healthy material. Treatment of the infected extracts with the enzymes cellulase and pectinase appeared to degrade the vesicles which suggested that they were of host plant origin.

Another type of vesicle- or envelope-like structure was also consistently isolated from greening-infected citrus following method 2 (Figs 40 and 41). The vesicles displayed a different morphology to that of the other vesicles (Figs 37 and 39) and appeared to be resistant to cellulase and pectinase treatment.

Similar structures have been observed in preparations of Rickettsia prowazeki obtained from embryonated hen's eggs. When stained with uranyl acetate, the purified rickettsiae were found to rapidly undergo morphological alterations resembling plasmolysis (Palmer et al., 1974). This resulted in the organisms assuming a number of different morphological shapes, including round empty envelope ghosts resembling the vesicles observed in greening-infected citrus preparations (Fig. 41). The envelope structures described by Palmer et al. (1974) displayed holes from which the internal components of the cells appeared to have been expelled. Perforations were similarly observed in the envelope of the structures isolated from citrus and it is through these that the contents may have been extruded, giving rise to these forms.

In addition to the perforations observed in the spherical structures isolated from greening-infected citrus and from hen's eggs infected with R. prowazeki, the morphology and

size of the structures from both sources were also found to be similar. The diameter of the structures isolated from citrus ranged from 0.7 to 1.17 um, while that of the vesicle described by Palmer et al. (1974) was found to be 0.9 um.

Although R. prowazeki is a pathogen of certain vertebrate and invertebrate species and is not reported to infect plant hosts, the empty vesicles observed in the citrus preparations may have been the empty envelope ghosts of plant pathogenic rickettsiae or rickettsia-like organisms which were present in the citrus material sampled and which form similar envelope-like structures to those formed by R. prowazeki.

The extraction procedure described by Sui (1988) (method 4) was developed by the author specifically for the isolation of the greening bacterium from plant material. When applied in this study to both periwinkle and citrus affected with greening disease, the method failed to yield the large numbers of greening bacteria reportedly obtained by Sui (1988). No bacteria resembling the greening organism were isolated, even when periwinkle displaying severe symptoms of greening was used as a source of the disease agent.

Periwinkle affected by greening has been reported to harbour significantly greater numbers of the greening organism than similarly infected citrus material (Garnier and Bové, 1983) and the extracts of periwinkle would therefore be expected to contain far greater numbers of the disease agent. Although bacteria were were isolated from infected periwinkle following this protocol, there was no discernable difference between the periwinkle and citrus preparations.

It should be borne in mind that Sui (1988) used periwinkle plants affected with the Asian strain of greening disease from which to purify the aetiological agent. Plants affected by this strain of the disease are known to harbour a higher titre of the bacterium than those affected by the African strain of the disease (Whitlock, pers. comm.). Use of plants affected by the Asian strain in this study may have resulted in the recovery of similar numbers of the greening bacterium as those reportedly recovered by Sui (1988).

The use of PAGE to identify and purify pathogenesis-related proteins from diseased citrus material was unsuccessful. Analysis of extracts of affected leaf midrib material by both denaturing and non-denaturing PAGE was not sufficiently sensitive to reveal disease-related protein

bands unique to the greening-infected samples (Figs 49 and 50). If present in sufficient quantities, such proteins would be suitable for use as immunogen in the production of a mono-specific antiserum.

Proteins specific to greening disease may well have been present within the protein profile of the infected material, but at a concentration too low to be detected by Coomassie Blue staining. In an attempt to determine whether additional bands could be visualized, the Coomassie-stained gels were re-stained using the silver staining method. Silver staining is reported to be 100 times more sensitive than staining with Coomassie Blue, but applying the silver stain after a preliminary staining with Coomassie Blue has been shown to lead to even greater sensitivity and reliability of the silver stain (Andrews, 1986).

Despite the greater sensitivity of double staining, application of the technique did not expose additional protein bands which might serve as immunogen (results not shown).

In addition to analysis of citrus tissue affected by the African strain of greening, a.. analysis of leaf midribs from citrus affected by the Asian strain of the disease was undertaken. The latter strain is reported to be far more

destructive and to elicit symptoms which are more severe than those induced by the African strain of the disease (Bové et al., 1974). This may be due to the Asian strain replicating to a higher titre in the citrus plants. Significantly greater numbers of the greening bacterium have been observed in dodder infected with the Asian strain of the organism compared to similar material affected by the Asian strain, and thus a similar situation may occur in affected citrus. If so, this material should serve as a better source of bacterial protein.

Due to the limited amount of time available for analysis of the Asian samples, as well as the small quantity of infected material provided, the PAGE fractionation of Asian greening samples could not be repeated in order to optimise the conditions of extraction and fractionation.

Preparation of sample following the phenol extraction method gave the most satisfactory results (Fig. 51). The resolution of the high molecular weight proteins was good (Fig. 51, lanes 3 and 4), although streaking of proteins in the low molecular weight range did occur. Examination of the profiles of the infected and healthy samples did not reveal bands unique to the infected material.

The results obtained with the phenol extraction technique did not justify the length of time required for the procedure. Comparison of the protein profiles of samples prepared following the lengthy phenol method (Fig. 51) and the simpler method detailed in section 2.9.1.6 (Fig. 49) indicate that the latter method is adequate for sample preparation.

The other sample preparation procedures investigated were largely unsatisfactory. TCA extraction yielded a sample that exhibited heavy streaking and made detection of specific protein bands impossible (Fig. 51, lanes 1 and 2). Sample preparation using SDS and high temperature to solubilize proteins was inadequate and yielded preparations containing quantities of protein too low to give suitable profiles (Fig. 51, lanes 5 and 6).

Adequate resolution of proteins extracted from material of both African and Asian origin was not achieved under non-denaturing PAGE conditions (Figs 50 and 52, respectively). Because of the inadequate separation of proteins obtained with this technique, only proteins forming prominent bands can be accurately excised and used as immunogens. As with profiles generated under denaturing conditions, no such bands unique to infected citrus could be identified.

4.6.3 Cross-adsorption of antisera raised against plant-derived immunogens

In a report by Sinha and Chiykowski (1984), MLOs associated with peach eastern X-disease were purified from infected plants and an antiserum raised against the isolated antigens. The isolation method reportedly yielded pure preparations of MLOs and the resultant antiserum was specific for disease antigens in plant extracts, without the need for prior removal of anti-plant activity by cross-adsorption.

In contrast to this, the procedure used by Clark et al. (1983) to obtain S. citri- and clover phyllody-associated antigens from infected plants yielded extracts which were still deep green and clearly impure in not consisting solely of MLO or S. citri derived components. Inevitably, with such heterogeneous preparations used for injection, the resulting antisera exhibited considerable anti-plant2 activity. In order to reduce this unwanted activity, it was necessary for the authors to cross-adsorb the antisera and to dilute the antibody in extracts of healthy plants. After this treatment the antisera were able to clearly detect disease-specific antigen in infected plants and, with the appropriate controls, showed high specificity for the antigen under test.

The procedure used in this study to isolate disease-specific antigens from greened citrus (method 5, section 2.9.2) yielded extracts which were similarly contaminated with plant material and the resultant antiserum thus exhibited an appreciable amount of anti-plant activity (Fig 53). In order to remove this activity, various methods of cross-adsorption were investigated.

The use of the ammonium sulphate precipitation technique gave the most promising results when used to prepare plant material for the purpose of antiserum cross-adsorption, this being indicated by a diseased/healthy (d/h) absorbance ratio of 2.65 recorded in this assay (Fig. 53). Antiserum from which anti-plant activity was removed using either acetone-fixed midrib tissue or affinity column chromatography also gave d/h ratios of 2.0 or greater (this being considered a positive result). Ammonium sulphate precipitation was, however, the method of choice as cross-adsorption with plant material prepared in this way was reliable and efficient, as well as being simple to perform. Aliquots of the precipitated plant proteins could be also be stored for long periods at -20 C without any apparent deleterious effects on the proteins. In addition, purification of plant proteins by this method excludes a significant amount of the plant-derived pigments and

enzymes from the preparation and should therefore minimize the deleterious effect these compounds have on the antiserum.

In addition to the use of healthy plant material, preparations of citrus affected by pathogens other than greening were used for the purpose of cross-adsorbing the GICE antiserum. Results of diagnostic assays showed that antiserum from which plant-specific antibodies had been adsorbed using extracts of uninfected plant material exhibited a considerable amount of reactivity with preparations of citrus affected by diseases other than greening (Fig. 54). This phenomenon might be ascribed to serum antibodies directed against pathogenesis-related proteins (or other antigens) of plant origin. Such proteins are usually produced by the plant in response to infection or other conditions of stress and are thus not disease specific.

Cross-adsorbing the antiserum against an extract of material collected from several citrus plants infected with different pathogens was not effective in removing the non-specific activity (Fig. 54). There was no appreciable difference between absorbance values recorded using the serum treated in this way and that cross-adsorbed against an extract of healthy citrus. Repeating the

cross-adsorption procedure up to three times failed to improve the results, as did diluting the cross-adsorbed antiserum in sample conjugate buffer to which had been added a buffer extract of the infected citrus (data not shown). Removal of non-specific activity by either the affinity column chromatographic or acetone fixation techniques was also proved to be ineffectual (data not shown).

The results shown in Figure 64 are unusual in that an antiserum from which specific antibodies have been removed by cross-adsorption is expected to exhibit a reduced level of reactivity with the material against which the antiserum was cross-adsorbed. Despite the application of a number of adsorption techniques which had proven to be effective in removing specific antibodies, significant reactivity against the infected plant material with which the antiserum had been adsorbed was still evident.

It is evident that the antigens responsible for the non-specific activity were not removed by the adsorption procedures employed and that additional methods of removing specific antibodies from an antiserum will have to be investigated. Alteration of the diagnostic assay procedure used may also aid in improving the specificity of the GICE

antiserum. Until such modifications prove successful, it will not be possible to use an antiserum raised in this way to specifically detect greening disease in plant material.

Although the cross-adsorbed GICE antiserum was of no use in detecting greening-specific antigens in affected plant material using ELISA, an immunofluorescence technique (such as PIFM) in which it is possible to distinguish between organisms reacting with a particular antiserum on the basis of their morphology, may be more suitable for use with a non-specific antiserum such as GICE. This antiserum may also prove useful in detecting greening-specific antigens in insect vector tissue as the presence in this tissue of plant-derived compounds would probably be minimal, thus reducing the possibility of cross-reactivity. As a source of greening organism-free psylla was not available at the time of this aspect of the study, it was not possible to test insect material.

Attention should be drawn to the fact that authors who used a similar approach to that used in this study for the production of antisera against non-cultivable phytopathogens failed to determine the specificity of the antisera (Sinha, 1979, Sinha and Benhamou, 1983, Sinha and Chiykowski, 1984). Although the authors reported that the antisera raised against plant-derived immunogens were

useful in detecting the disease in affected host material, no attempt was made to determine whether the antisera reacted with disease-associated antigens in plants affected by other unrelated diseases. If found to cross-react, these antisera would be of little value.

4.6.4 Cross-adsorption of antiserum raised against cultured bacteria

Initial attempts to differentiate between symptomatic and healthy plant samples in the ELISA using antisera raised against cultured bacterial isolates met with limited success due to high background readings associated with healthy plant material. This made detection of low concentrations of the target organism in host plant material difficult. Western blotting later demonstrated the existence of proteins with similar molecular weights and common antigenic determinants in both healthy citrus material and the bacterium (GC isolate) (section 3.11). The presence of such proteins may account for the high background observed.

In an attempt to reduce this anti-plant activity and thus improve the sensitivity of the assay, antisera were reacted with aliquots of healthy citrus extracts prepared following

the ammonium sulphate precipitation technique. Treatment of the antiserum in this way resulted in a small decrease in the absorbance readings of both the healthy and infected samples (Fig. 55). This suggests that some antibodies directed against the apparently common antigenic determinants were neutralized by the treatment, although the cross-adsorption did not improve the results of the assay markedly (the value of the d/ ratio increased from 1.9 to 2.1 (an increment of only 0.2) with the use of the cross-adsorbed antiserum).

4.6.5 Comparison of different ELISA sample preparation methods

A number of different sample preparation procedures were investigated in an attempt to find a method which was both simple and rapid to perform, as well as giving acceptable results in the HE-PTA-ELISA.

A sample preparation scheme should ideally extract the desired organism efficiently while minimizing the competitive influence of host tissue debris and endogenous microorganisms which may interfere with detection of the target pathogen.

ELISA sample preparation following the polytron homogenization and leaf press extraction methods yielded extracts which were heavily contaminated with host plant components. The use of samples prepared in these ways in the indirect ELISA was found to give less satisfactory results than the water leaching method which yielded extracts containing a minimal amount of plant material (Fig. 56). Similar interference of host plant compounds on the detection capabilities of the indirect test was reported by Lommel et al. (1982) and Leach et al. (1987). Lommel et al. (1982) attributed the interference in part to competition between target antigen and host protein for a finite number of binding sites on the solid phase. Saturation of membrane binding sites by plant proteins in the dot-immunobinding assay was found by Leach et al. (1987) to make accurate detection of target pathogens in samples prepared by grinding difficult.

While the presence of host plant proteins interferes with the detection of specific antigens in the indirect ELISA, quantitative detection of target antigens is reportedly unaffected by contaminating proteins in the DAS-ELISA (Lommel et al., 1982). The latter assay is, however, not as convenient as the indirect ELISA because of its inherent specificity and the need for specific enzyme conjugates for each antigen to be tested (Koenig, 1978). The attributes of

the indirect ELISA justify the time spent in the development of a sample preparation procedure suitable for use with this assay.

Sample preparation by homogenization of plant material followed by enzymatic degradation of the plant tissues in order to enhance the release of endogenous bacteria was found to result in poor detection of the target antigens in the indirect ELISA (Fig. 56, bars 9 and 10). The resultant extract was heavily contaminated with plant compounds and the interference of these compounds on the detection capabilities of the assay was exacerbated by the presence of cellulase and pectinase in the sample. The host plant proteins, as well as the enzymes present in the sample, represent a significant amount of non-target protein against which any disease-specific antigens present would have to compete for the solid phase binding sites. The poor results achieved with this extraction method, as well as the cost of the reagents employed and the length of time required (over 24 hr), make it an unattractive alternative to the more simple method of water leaching.

The extraction of alkali-soluble antigens with KOH is a method of ELISA sample preparation developed by Kawarabata and Hayasaka (1987) in an attempt to overcome the steric problems associated with intact microsporidian spores in

the indirect ELISA. These authors reported that the preparation of alkali-soluble surface antigens was simple and the antigens were more suitable for ELISA than the intact spores. Because of their similar particulate nature, antigens such as bacterial cells bind weakly to ELISA plates and can thus easily be dislodged during the assay. The use of soluble surface antigens in place of whole cells may overcome this problem.

The use of this preparation method, however, yielded poor results (Fig. 56, bars 11 and 12), possibly due to interference of the high salt concentration in the sample after neutralization of the KOH with HCl.

The "chopping" and water leaching methods of sample preparation undoubtedly gave the best results in the indirect ELISA, this being shown by the d/h absorbance ratios of 2.50 and 3.14, respectively. Examination of the extracts prepared by these methods indicated that the preparations were minimally contaminated with host plant compounds which makes the procedures ideal for use in the indirect ELISA. The "chopping" method has the advantage of including a concentrating centrifugation step which may be important in the detection of bacteria present in host material at low concentrations. However, d/h ratios recorded for the water leaching method suggested that this

method was ideal for use with greening-affected midrib material and concentration of target antigens by centrifugation was not necessary.

Compared to the "chopping" method, water leaching of target antigens was more useful as a preparation procedure as it was simple to perform and far less time-consuming. Although the method detailed for this procedure (section 2.10.1.6) calls for overnight incubation of the leaf sections in water, adequate detection assay results were recorded with samples incubated for as little as 2 hr (data not shown).

Another advantage of the leaching procedure is that small samples of plant material such as individual leaf midribs may be assayed for the presence of target antigens. Because of its obvious qualities, water leaching was routinely used as a method of sample preparation for use with the indirect ELISA.

It is pertinent to mention that the usefulness of all of the above-mentioned extraction procedures was determined only in the indirect HE-PTA-ELISA using the GICE antiserum and that the results may well have been different using another ELISA procedure, as well as another antiserum.

4.6.6 Comparison of different enzyme and radio-isotope based serological detection assays

In the development of a detection assay for the routine screening of plant material, factors such as the limited skill of workers available to perform the assays, the time required and the cost of equipment and reagents needed are important considerations. To be widely applicable, such an assay must be rapid and easy to perform, and not require sophisticated equipment.

In the comparison of the Fab'-, $F(ab')_2$-, DAS- and HE-PTA-ELISAs, no marked difference in the results obtained using the four different assay procedures was observed. Detection of disease antigens was achieved in all the assays, this being indicated by d/h ratios of greater than 2.0 for the sample used (Fig. 57). Of the enzyme-based assays, however, the HE-PTA-ELISA was the most simple to perform. This assay is less time consuming and does not require the use of fractionated serum. In addition, commercially-available enzyme conjugate can be used.

The use of a radioactive label in a detection assay is undesirable as the material is hazardous and certain precautions need to be taken while working with it. Furthermore, the reaction can only be quantified using

sophisticated equipment. A radio-isotope has a limited half-life and cannot be stored for indefinite periods without loss of activity as can an enzyme-labelled compound. These features make assays such as Dot- and Bead-RIA unsuitable for general use as detection methods for greening disease.

Although able to detect disease antigens in infected samples, Bead-RIA was a lengthy procedure (results could only be obtained the day after the test was performed) and handling of the polystyrene beads was difficult. The d/h ratio calculated for the samples represented by bars 3 and 4 in Figure 58B was 2.69, while the ratio of the identical sample analyzed in the HE-PTA-ELISA was found to be 3.40 (Fig. 58A). The latter assay was thus more sensitive than Bead-RIA, as well as being rapid and more convenient to perform.

Another problem associated with the the Bead-RIA was the large sample standard deviations observed. This was also a feature of the Dot-RIA which proved to be the least sensitive of the three assays. In this assay, the value of the d/h ratio of the samples represented by bars 3 and 4 in Figure 58C was 1.94, compared to 2.69 for the same samples in the Bead-RIA and 3.40 in the HE-PTA-ELISA. It is possible that the concentration of target and non-target

proteins in the samples spotted onto the NCM was too high resulting in inefficient binding of the sample to the membrane. Dilution of the samples prior to spotting, as well as more effective fixation of the sample material to the membrane, may have led to better results.

From the results of this investigation, the HE-PTA-ELISA appears to be the most effective detection assay under the conditions applied. In addition to its sensitivity, it is quick and easy to perform and is not wasteful as small volumes of reagents are required for each test. The absorbance reading of sample replicates in the assay was observed to generally be consistent and the large sample standard deviations associated with the radio-isotope based assays was not a problem associated with this assay.

4.6.7 Detection of disease antigens in different citrus tissues and in alternate host plants

In an attempt to determine the distribution of disease antigens in greening-affected citrus, various tissues of the plant were assayed for the presence of the antigens in the HF-PTA-ELISA using the GICE antiserum.

The results presented in Figure 61 suggest that citrus seed components and fruit columella are not suitable for use as sample material in the indirect ELISA, possibly due to low levels of disease antigens in these tissues, or to interference of host components with the detection of the antigens. Samples had, however, been prepared by the water leaching method and did not appear to be too heavily contaminated with plant material. Despite the apparent low levels of host contamination, high absorbance readings were recorded for the healthy control material.

The GICE antiserum had been cross-adsorbed against a preparation of healthy leaf midribs, but the use of an extract of uninfected columella or seed material to cross-adsorb the antiserum may have rendered it more specific for disease antigens present in these tissues by reducing the anti-plant activity in the serum.

The antiserum was able to effectively detect the presence of disease antigens in the citrus bark and midrib material included in this assay (Fig. 61). In contrast to the small d/h absorbance ratios calculated for the seed and columella samples, the ratios for the bark and leaf midrib samples were high, being 2.32 and 3.83, respectively.

When the same antiserum preparation was used to probe extracts of periwinkle and cucumber (infected with a disease agent from greening-infected citrus via dodder), as well as extracts of dodder collected off an infected citrus seedling, only the cucumber sample tested positive for the presence of disease antigens (Fig. 82). Although the periwinkle leaves assayed displayed symptoms of greening infection, the d/h absorbance ratio of the sample (1.8) fell below the threshold value of 2.0. The absorbance of the infected periwinkle sample was, however, greater than that of the healthy control so it is possible that disease antigens were present in the material but not at a concentration high enough to yield a ratio of greater than 2.0.

Dodder has been shown to be an alternate host of the greening organism and was expected to test positive in this assay. The dodder sampled here, however, did not appear to contain detectable levels of disease antigens. As the dodder had only been established on the greening-infected citrus for 4 days before it was assayed, it is possible that the greening organism had not yet migrated into the parasite from the citrus plant, or the organism had not replicated within the dodder tissues to a titre high enough to be detected in the assay.

Greening-infected citrus leaf material collected from Réunion Island was found to contain disease antigens detectable by the GJCE antiserum. As this antiserum has been shown not to specifically detect greening-associated antigens in citrus, but to react with proteins which appear to be produced by the citrus host in response to infection with a variety of phytopathogens (Fig. 54), one cannot say with any degree of accuracy that the greening bacteria present in material from South Africa and Réunion Island are antigenically similar.

When the identical periwinkle, cucumber, citrus and dodder extracts were probed with antiserum directed against the cultured GC isolate, positive reactions were not observed with any of the samples (Fig. 63). The results suggest that the plant tissues assayed either did not contain the bacterium, or harboured levels of the organism too low to be detected in the assay.

A bacterium identical to the GC isolate had, however, been cultured from the cucumber plant during the dodder transmission study (section 3.5) which indicates that the organism must have been present in the cucumber leaves, but probably at a very low concentration.

4.6.8 Analysis of individual citrus leaf midribs

Using the water leaching method of sample preparation, it was possible to analyze individual leaf midribs for the presence of specific antigens. In an attempt to determine whether or not there was a correlation between the severity of leaf symptoms and the level of disease-specific antigens, sweet orange leaves displaying varying degrees of foliar chloroses were assayed individually in the HE-PTA-ELISA using the GICE antiserum.

Seventeen leaves (Fig. 64) collected off a greening-infected tree were assayed in this way and found to contain similar amounts of disease-specific antigens (Fig. 66). This finding suggested that there was no apparent relationship between the degree of chlorosis exhibited by an infected citrus leaf and the amount of disease-specific antigen present within the leaf tissue.

Analysis of the same leaves for the presence of the GC organism indicated that they did not contain detectable levels of this, or an antigenically similar organism (Fig. 67).

The analysis of individual leaves with the GICE and GC antisera was performed on material collected from other areas of the Transvaal and the results found to be similar to those obtained with the Pietermaritzburg samples.

4.6.9 Periodate oxidation of ELISA samples

Woodward et al. (1985) reported a general method that permits the identification of antibodies recognizing carbohydrate-containing epitopes associated with glycoproteins and glycolipids. The method involves periodate oxidation (cleavage) of vicinal hydroxyl groups on sugars to dialdehydes at acid pH and is applicable to Western blots and ELISA in the case of glycoproteins and ELISA and thin layer chromatograms in the case of glycolipids. The treatment specifically destroys carbohydrate epitopes without altering amino acids involved in peptide epitopes. The conditions used for periodate oxidation determine the extent and types of cleavage achieved.

In this study, mild periodate oxidation of plant samples prior to reaction with the GICE antiserum was found to have a marked effect on the results of the HE-PTA-ELISA (Fig. 59). The significant decrease in the A_{405} values of both the healthy and infected samples after treatment suggests that oxidation inhibited binding of the antiserum to a large proportion of the sample epitopes. It is likely that these epitopes were carbohydrate in nature as studies have shown that mild periodate oxidation (under the same conditions as those applied in this investigation)

specifically destroys carbohydrate epitopes (thus preventing the binding of antibodies) without altering amino acids involved in peptide epitopes (Woodward et al., 1985, Laine and Faye, 1988). It is thus probable that the GICE antiserum is directed primarily against carbohydrate epitopes of the immunogen preparation.

Laine and Faye (1988) reported the widespread occurrence of highly immunogenic carbohydrate moieties in glycoproteins which have been found to be common to most seed plants. The authors suggested that this may be the basis of antigenic cross-reactivity observed among glycoproteins from different plant species. Plant and invertebrate (mollusc and insect) cells were reported by the same authors to contain immunogenic glycoproteins with identical structures, a fact which may result in cells from these organisms being antigenically cross-reactive. The problem of producing specific antibodies to glycoprotein-containing organisms is thus not restricted to plants.

Antisera raised against live (whole) bacteria have been shown to cross-react with other species of bacteria, but removal of the bacterial glycocalyx prior to immunization has been shown to increase antiserum specificity (Schaad, 1979). This antigenic cross-reactivity observed among the different species may be due to the presence of common immunogenic carbohydrate epitopes in the extracellular glycoproteins of these organisms.

Similar cross-reactivity was observed between healthy plant material and the bacterium cultured from greening-infected citrus (Fig. 34, bars 1 and 2 and Fig. 60). The antiserum in this instance had been raised against a preparation of cultured live bacterial cells (GC isolate). Removal of the bacterial glycocalyx was found to reduce the level of reactivity against plant constituents (Fig. 34, bars 7 and 8). These findings suggest the existence of common antigenic determinants in the host plant and in the extracellular matrix of the bacterium.

This hypothesis of shared antigens was further supported by results of Western blotting (section 3.11). The analysis indicated the presence of two proteins in citrus and in the GC isolate with similar molecular weights and which displayed significant antigenic cross-reactivity (Fig. 76). The nature of these proteins was not established, although staining of the PAGE gel with the appropriate stain would indicate whether or not the bands visualized were glycoproteins. The nature of the proteins could be further determined by periodate oxidation of the blot prior to reaction with the bacterial antiserum. Failure of the antiserum to react positively with the treated proteins would indicate that antibodies binding to the untreated bands are directed against carbohydrate epitopes of the bacterial glycoprotein.

Although the blotted proteins were not subjected to periodate oxidation in this study, treatment of plant extracts adsorbed to microtitre plates followed by probing with the GC isolate antiserum indicated that the cross-reactivity initially observed between the antiserum and healthy plant extracts may be due to the presence of identical or related immunogenic carbohydrate moieties in the bacterium and in the plant (Fig. 60).

Because of this cross-reactivity, it was speculated that exclusion of anti-carbohydrate reactivity from the detection system (employing the GICE antiserum) by periodate oxidation of carbohydrate epitopes might enhance the specificity and sensitivity of the assay. This treatment should preclude recognition of carbohydrate determinants by serum antibodies and any reactivity observed would thus be due to binding of antibodies directed against non-carbohydrate antigens, such as peptide epitopes, or against periodate-resistant carbohydrate moieties.

Periodate oxidation of midrib and columella extracts, however, failed to improve the results of the HE-PTA-ELISA. Treatment of samples lead to a dramatic decrease in the A_{405} readings of both the healthy and infected plant extracts as well as to a decrease in the d/h absorbance ratios (Fig. 59).

4.6.10 Enzyme-linked dot-immunoblot assay

Although extraction of alkali soluble antigens from plant material could not be used as a method of sample preparation for the ELISA (section 3.8.1, Fig. 56), KOH extraction of citrus columella antigens could be used in conjunction with the EL-DIB assay. A 1N solution of KOH was found to effectively leach out antigens from columella sections without significant deterioration of plant tissue after 30 min. This method of extraction was adapted from that developed by French (1974) for the observation of rickettsia-like bacteria associated with phoney peach disease.

Using the Bio-Dot microfiltration apparatus, up to 50 ul of prepared columella sample could be conveniently concentrated in a spot on the NCM without blocking or significant staining of the membrane. Blocking of the membrane usually occurred when samples were prepared by chopping or homogenization, as did colouration which interfered with interpretation of the final enzyme colour reaction.

In a preliminary assay, the columella from greening-infected fruits were subjected to KOH leaching and varying amounts of the leachate applied to NCM. When 50 ul

of sample was spotted, healthy and infected samples could be clearly differentiated. However, no difference between the samples was discernible when 25 ul or less of the leachate was spotted, indicating the relative insensitivity of this assay.

In addition to the detection of disease-specific antigens in plant extracts, the EL-DIB assay was also used to investigate the serological relatedness of various bacterial isolates. Determination of relatedness using this assay was both simple and rapid.

Probing cultures of isolates GC, GL (an isolate from a leaf midrib from the same tree which yielded GC), NC, LL, CS, LC and TZL with antiserum raised against the GC isolate indicated that while GC, GL, NC and CS appeared to be similar with respect to their antigenicity, isolates LC and TZL were not recognized by this antiserum (Fig. 89). This apparent unrelatedness of the isolates is also reflected in the PAGE protein profiles of the cellular proteins (section 3.1.2).

In the same test, isolates were reacted with an antiserum raised against C.m. subsp. michiganense in order to test the serological relatedness of the isolates with this bacterium. Fatty acid profiling of the GC, NC and CS

isolates had shown that the organisms possess the profile of a Gram-positive coryneform and are possibly members of the Clavibacter michiganense group (Appendix L). The EL-DIB assay, as well as analysis of cellular proteins by PAGE, was carried out in order to investigate this tentative identification further. The results of the diagnostic assay indicate that isolates NC, GC, CS and GL are serologically similar, but do not react with the antiserum directed against C.m. subsp. michiganense (Fig. 70) and are thus unlikely to be related to this group of bacteria. This finding is consistent with the PAGE analysis of the cellular proteins (section 3.13).

The analysis of bacteria cultured from greening-infected citrus using the EL-DIB assay was convenient for the initial screening of potential greening isolates. The immunological probe used for this purpose was the GICE antiserum. Reactions of eight different isolates are shown in Fig. 71B. Probing the spotted cultures with the GICE antiserum failed to result in a positive reaction with any of the isolates. This suggested that none of the bacteria had been present in the infected citrus material from which immunogen for the GICE antiserum had been prepared and are thus unlikely to be associated with greening disease. As a control, the anti-GC antiserum was used in a parallel experiment. The antiserum was observed to bind only to homologous antigen (Fig. 71A).

4.6.11 Preparative immunofluorescence microscopy

The PIFM technique was adapted from the direct epifluorescent filter technique of Pettipher and Rodrigues (1982) developed for the enumeration of bacteria in foods. PIFM combines membrane filter techniques with immunofluorescent microscopy. In theory, the technique should be useful in the diagnosis of diseases caused by pathogens which are present in the affected host tissues at low concentrations as microorganisms in the sample are concentrated onto a membrane filter, thereby facilitating their detection.

PIFM was unable to detect bacteria-like structures in extracts of greening-infected leaf midrib tissue using the GICE antiserum. This may have been due to the presence of greening bacteria in the tissue at a concentration too low to be detected by this assay. The highest magnification attained by the fluorescent microscope available in the laboratory in which this work was performed was 500 X and unless large numbers of bacteria clumped on the trapping membrane, visualization of the cells was difficult. The use of material (such as insect vector tissue) which contains greater numbers of the bacterium may be more suitable for use in this assay.

PIFM analysis of leaf material known to harbour the GC isolate using the anti-GC antiserum allowed adequate detection of homologous antigen in the sample. Large numbers of fluorescing bacteria were observed on the filter when viewed under the fluorescent microscope (Fig. 72).

Contamination of samples with host plant material, resulting in membrane blocking and interference with the interpretation of results, was kept to a minimum by the efficient enzymolysis of the host material. Because PIFM allows one to discriminate morphology of the stained target organism vs. non-specific reactions from host components, interference of host material should never be serious problem.

The observation of bacteria on the trapping membranes prepared for SEM viewing (Fig. 73) confirmed that the fluorescing structures seen on the stained membranes were bacteria isolated from the plant tissues.

4.8.12 Immunogold labelling

Probing antigens with colloidal gold-labelled antibodies was used in this study as an alternative method to the EL-DIB assay for investigating the antigenicity of various bacterial isolates.

When cells from a culture of the GC isolate were probed with the GICE antiserum followed by gold-labelled secondary antibody, binding of the label did not occur (Fig. 74) indicating a lack of homology of bacterial antigen and primary antiserum. The failure of the GICE antiserum to recognize the bacterial antigens suggests that the GC isolate was probably not present in the greening-infected citrus extract against which the GICE was raised and is thus unlikely to be associated with greening disease. Analogous results were obtained in the EL-DIB assay.

A similar lack of reactivity was observed when the anti-GC antiserum was used to probe bacteria isolated from greening-infected citrus (Fig. 46). The organisms in the extract exhibited the morphology of the agent of greening disease and were thus thought to be greening bacteria. If this is the case, then failure of the anti-GC antiserum to recognize the organisms suggests that the GC isolate is not related to the aetiological agent of greening.

As the extractions from greening-infected citrus were carried out before the development of the GICE antiserum, the reaction of this antiserum with the isolated bacteria (Fig. 46) could not be determined. Positive reaction of this antiserum with the cells would confirm the association of the bacteria with greening-infected citrus. Attempts to

repeat the isolation of the bacteria from citrus met with limited success due to the low titre of greening bacteria in citrus material of African origin. The situation was further complicated by the fact that it was necessary to collect plant material during summer and high temperatures are known to inhibit the greening organism.

4.7 WESTERN BLOTTING

The use of the Western blotting technique was useful in determining the reason for the cross-reactivity of the antiserum raised against the cultured isolate and host plant material.

SDS-PAGE fractionation of buffer-soluble bacterial and citrus proteins revealed the presence of two proteins with similar molecular weights in the profiles of these two samples (Fig. 76). When transferred to NCM and probed with antiserum raised against the bacterium, a positive reaction between the antiserum and the two plant proteins was observed (Fig. 76, bar graph). This finding suggests that the proteins from both samples share a significant number of antigenic determinants and this offers a possible explanation for the anti-plant activity of the bacterial antiserum.

Removal of some of this non-specific reactivity by cross-adsorption of the antiserum with an extract of healthy citrus failed to render the antiserum appreciably more specific for the bacterium, compared with the untreated serum (section 3.7.2.2). The observation that mild periodate oxidation of bacterial sample in the indirect ELISA resulted in a significant decrease in the binding of the homologous antiserum (Fig. 60), suggests that the cross-reactivity may be due to common antigenic carbohydrate epitopes in the bacterium and in the plant. Periodate oxidation of such epitopes is known to inhibit their recognition by anti-carbohydrate antisera (Laine and Faye, 1988).

It is probable that the capsular material of the bacterium is responsible for the cross-reactivity of the antiserum with plant material. Removal of the extracellular matrix of the bacterial immunogen was found to render the antiserum more specific by reducing its reactivity with plant material (Fig. 84).

4.8 FATTY ACID PROFILING AND PAGE ANALYSIS OF BACTERIAL ISOLATES

Information regarding the cellular fatty acid composition has proved of considerable value in the classification and identification of bacteria (Collins and Jones, 1980, Suzuki and Komagata, 1983). It must be noted, however, that a fatty acid profile is a single diagnostic test and it must not be assumed that the first choice of a taxon for an unidentified bacterium is the correct determination. Rather it should be used as an aid in determination or diagnosis. In order to assign an unknown bacterium to a particular taxon, the fatty acid profile of the bacterium must be compared with the profiles derived from a wide selection of bacterial taxa. If the unidentified bacterium displays a profile which differs significantly from those of the bacteria in a library of known organisms, assignment to a particular taxon is not possible.

Using analysis of cellular fatty acids as a diagnostic tool in the identification of the bacterial isolates of Garnett and Mochaba, it was not possible to assign the organisms to a particular taxon with any certainty. However, on the basis of cellular morphology, Gram stain reaction and the degree of profile similarity to bacteria in the library of the NCPPB, it was suggested that the GC group of isolates

are most closely related to the genus Clavibacter (D. Stead, pers. comm.). The fatty acid profile of this group of isolates was found to be most similar to the C. michiganense group of organisms (Appendix L).

Profiling of isolates TZL and LL indicated that these organisms are unrelated to one another as well as to the GC group of bacteria. This differentiation of the isolates is supported by the cellular protein patterns which indicates significant differences in the profiles (Fig. 78).

Another method which has been widely used in the identification and classification of microorganisms is the analysis of their electrophoretic protein patterns (Kersters and De Ley, 1975, Carlson and Vidaver, 1982, Davis et al., 1984, Bielenin et al., 1988). This method of analysis is particularly useful in the differentiation of bacteria because, in contrast to conventional tests, several gene products from more than one bacterium can be compared simultaneously in a PAGE slab gel.

The marked similarity in the electrophoretic protein patterns of isolates GC, NC and CS (Figs 10 and 78) suggest that they are isolates of the same bacterium. This finding corroborates the results of the EL-DIB assay which demonstrated the antigenic homology of these organisms (Fig. 69).

The protein patterns of isolates TZL, LC and LL differed from one another, as well as from those of members of the GC group of isolates (Figs 10 and 78). The apparent unrelatedness of the isolates was also indicated by results of the EL-DIB assay in which an antiserum directed against the GC bacterium failed to recognize the TZL and LC isolates (Fig. 69).

Similar observations were made in SDS-PAGE analyses conducted by Mochaba (1989). The author reported that isolates GC and NC were identical with respect to their protein profiles but different from the LC isolate.

Because analysis of the cellular fatty acid composition of the GC and NC isolates had suggested that the organisms were similar to members of the C. michiganense group of bacteria, a comparison between the protein patterns of representative members of this group and the greening isolates was made.

PAGE analysis demonstrated differences in the ce. proteins of the Clavibacter type cultures and the GC group of isolates (Figs 77 and 78). The differences suggest that the bacteria from greened citrus are not related to the C. michiganense group, a hypothesis also given credence by the results of the EL-DIB assay (Figs 69 and 70).

Extensive metabolic and biochemical testing, as well as analysis of cell wall composition and the G + C content of the bacterial DNA will, however, have to be conducted in order to either confirm or refute this tentative identification of the isolates.

4.9 CONCLUSION

The accumulated results of this investigation suggest that the bacterium (GC group of isolates) cultured from infected citrus by Garnett and Mochaba is not the aetiological agent of greening disease. The organism does, however, appear to be associated with some trees affected by greening and may be a mild opportunistic pathogen in citrus, establishing itself and inducing symptoms only in plants weakened by infection and/or other factors. In this way, the organism may contribute to the symptoms observed in greened citrus. The fact that the full greening syndrome may be caused by more than one organism is suggested by the findings reported here. The hypothesis that the disease involves more than a single factor has also been proposed by other workers (Mali and Chaudhari, 1976, Da Graca, 1991).

Results of inoculation trials, serological assays, PAGE analysis and fatty acid profiling conducted here indicate that the GC group of isolates differ significantly from the

other isolates of Garnett and Mochaba (isolates LC, LL and TZL). Differences between the PAGE protein profiles of the GC group and the LC isolate were also reported by Mochaba (1989). It is thus unlikely that these bacteria are related, despite the recent claim made by Hortelano Hap and Garnett (1991) that the isolates are strains of the same bacterium.

A number of different artificial media developed for the in vitro culture of fastidious phytopathogens failed to support the growth of the greening organism, as did embryonated hen's eggs and an invertebrate (mosquito) cell culture system. The development of a cell line from the insect vector of greening may facilitate the isolation of the organism.

Attempts to develop a reliable serological detection system for greening disease were largely unsuccessful. Although the GICE antiserum which was raised against an extract from infected citrus was able to differentiate between healthy and greening-infected citrus, the antiserum proved to be unsuitable as it was found to react positively with citrus material affected by a number of other diseases. The production of monoclonal antibodies against similar extracts may prove to be of more use in a serological assay.

APPENDIX A

CULTURE MEDIA

MIG-3 medium

potassium-D-gluconate	15.0g
yeast extract	0.5g
L-proline	2.5g
L-histidine	2.5g
L-alanine	2.5g
$(NH_4)_2SO_4$	2.0g
NaCl	5.0g
$FeSO_4.7H_2O$	282.5mg
$ZnSO_4.7H_2O$	820.0mg
$CuSO_4.5H_2O$	1.925mg
Hunters mineral base (Cohen-Bazire *et al.* 1957)	10.0ml

The components were dissolved in glass distilled water, the pH adjusted to 7.2 with KOH and the volume made up to 1000ml before filter sterilization through a 0.22um nitrocellulose membrane.

OJ medium

	g/l	
Papaic digest of soy meal	8.0	
Bovine hemin chloride	15.0ml	of a 0.1% (w/v) solution in 0.05M NaOH
Trisodium citrate dihydrate	27.06	
Citric acid monohydrate	1.68	
$MgSO_4.7H_2O$	0.2	
Corn meal agar	17.0	
Glucose	1.0ml	of a 50% (w/v) aqueous solution
L-Cysteine (free base)	10.0ml	of a 10% (w/v) aqueous solution
BSA Fraction V	10.0ml	of a 20% (w/v) aqueous solution
*Fresh orange juice	300.0ml	

The ingredients are added and dissolved in deionized water in the order given. The medium components, except the glucose, cysteine, BSA and orange juice, are autoclaved. Glucose, cysteine and BSA stock solutions are filter sterilized and added with the prepared orange juice to the rest of the medium at 50 C.

*The orange juice is squeezed from healthy sweet oranges, filtered through two layers of miracloth and then centrifuged for 30 min. at 23 000g at 4 C. The supernatant is then passed through Whatman's No. 4 filter paper before being filter sterilized.

APPENDIX B

GIEMSA STAIN

Prepare the following 2 solutions:

1) M/15 Na_2HPO_4 using 9.5g of the anhydrous salt in 1 litre of distilled water

2) M/15 NaH_2PO_4 by dissolving 9.2g of the salt in 1 litre of distilled water

To make buffered water of pH 7.2, mix 72ml of solution 1 with 28ml of solution 2 and 900ml of distilled water.

Giemsa stain is prepared by dissolving 0.5g of powder (Giemsa's stain, BDH, U.K) in 33ml of acetone-free absolute methanol. The solution is mixed thoroughly, allowed to sediment, and stored at room temperature for use as a stock. Dilutions of this stock stain are made with buffered water, in a ratio of 1 part of stock Giemsa solution to 50 parts diluent.

When the material being investigated is a fluid such as egg yolk or amniotic fluid, a smear of the fluid is made, the smear is air-dried, fixed with absolute methanol for 7-10 min., and dried again. It is then covered with the diluted Giemsa stain (freshly prepared the same day) for 1 hr and the slide then rinsed rapidly in 95% ethyl alcohol to remove excess dye and to enhace differentiation. The slide is then dried and examined microscopically.

APPENDIX C

DEHYDRATION AND EMBEDDING OF TEM SAMPLES

Fixed sample material is dehydrated in the following series of aqueous acetone solutions:

50%, 70%, 80%, 90%, 2 x 100%

The sample is suspended in each of the the acetone solutions (in the order given) for 15 min. and then taken through the following series of acetone:propylene oxide (PO) solutions:

2:1, 1:1, 1:2, 0:1 (acetone:PO)

The sample is suspended in each solution for 15 min. and then taken through the following series of PO:Spurr's resin solutions:

2:1, 1:1 (PO:Spurr's resin)

The sample is suspended in the first solution for 30 min. and for 14 hr in the second. The sample is then impregnated

with neat resin for 2 days with a change of resin after the first day. The impregnation step is carried out in a vial attached to a vertical rotating platform.

The sample is finally placed in a suitable mould, covered with fresh resin and the resin polymerized overnight at 65 C.

APPENDIX D

POLYACRYLAMIDE GEL ELECTROPHORESIS

1. DENATURING SDS-PAGE (after Laemmli, 1970)

STOCK SOLUTIONS

High grade reagents were used throughout

Acrylamide-bisacrylamide stock

150.0g acrylamide
4.0g bisacrylamide
dissolved in 500ml (final volume) ddH_2O
filter through Whatman's No. 4 paper

4x Lower Tris (1.5 M Tris-HCl, pH 8.8; 0.4% SDS)

181.7g Trisma base (Sigma) dissolved in 700ml ddH_2O
pH to 8.8 with conc. HCl
add 40ml of 10% SDS and ddH_2O to a final volume of 1000ml.

4x Upper Tris (0.5 M Tris-HCl, pH 6.8; 0.4% SDS)

60.6g Trisma base dissolved in 700ml ddH_2O

pH to 6.8 with HCl

add 40ml of 10% SDS and ddH_2O to a final volume of 1000ml.

10x Running buffer (0.025 M Tris-glycine, pH 8.9; 0 % SDS)

60.0g Trisma base

288.0g glycine

60.0g SDS

make up to 2000ml with ddH_2O

3x Sample buffer

3.0ml glycerol

1.6ml B-mercaptoethanol

0.9g SDS

3.75ml 4x upper Tris

50ul of 0.1% (w/v) bromophenol blue in water

volume brought to 10ml with ddH_2O

Upper (stacking) gel

dd water	9.4ml
acrylamide stock	2.6ml
4x upper Tris	4.0ml
TEMED	16.0ul
Ammonium persulphate (10%)	48.0ul

Lower (separating) gel

	% acrylamide		
	7.5%	10%	15%
dd water	18.1	14.8	8.2 ml
50% glycerol	2.0	2.0	2.0 ml
acrylamide stock	9.9	13.2	19.8 ml
4x lower Tris	10.0	10.0	10.0 ml
TEMED	20.0	20.0	20.0 ul
Ammonium persulphate	200.0	200.0	200.0 ul

To the prepared sample add an equal volume of sample buffer, mix and heat in a boiling water bath for 4 min. Cool rapidly and centrifuge briefly in a microfuge before layering onto the gel.

Gels are run at 4 C at 60 V until the sample has migrated into the stacking gel after which the voltage is increased to 150 V. Gels are run until the dye front reaches the bottom of the gel or, if interested only in the high molecular weight proteins, the gel may be run for a further hour thereafter.

Staining solution

0.2% (w/v) Coomassie brilliant blue R-250 in 50% methanol, 10% acetic acid and 40% dH_2O

Destaining solution

50% methanol, 10% acetic acid and 40% dH_2O

After electrophoresis, gels were soaked twice in an excess of destaining solution to remove SDS from the gel. The gels were then stained for 4 hr or more in staining solution with gentle agitation. Destaining was carried out in a large volume of destaining solution until protein bands were visible against a clear background.

2. NON-DENATURING PAGE

STOCK SOLUTIONS

Acrylamide-bisacrylamide stock

As for denaturing PAGE

4x Lower Tris (1.5 M Tris-HCl, pH 8.8)

181.7g Trisma base dissolved in 700ml ddH_2O
pH to 8.8 with conc. HCl
make up to 1000ml with ddH_2O

4x Upper Tris (0.5 M Tris-HCl, pH 6.8)

60.6g Trisma base dissolved in 700ml ddH_2O
pH to 6.8 with HCl
make up to 1000ml with ddH_2O

10x Running buffer (0.025 M Tris-glycine, pH 8.9)

60.0g Tris
288.0g glycine
make up to 2000ml with ddH_2O

Sample buffer

10ml glycerol

12.5 ml 4x upper Tris

50ul of 0.1% (w/v) bromophenol blue in water

make up to 100ml with ddH 0
2

The quantities of buffers and reagents used in the non-denaturing upper and lower gels are the same as those listed for the denaturing gels.

For the preparation of samples, an equal volume of sample buffer to the protein solution is added layer it directly onto the gel. Gels are run under the same conditions as those specified for denaturing PAGE.

Gels are stained in a 0.2% (w/v) aqueous solution of Coomassie brilliant blue G-250 for 1-2 hr and then passively destained in distilled water.

3. MOLECULAR WEIGHT PROTEIN MARKERS

Sigma SDS molecular weight markers

	MW
β-lactalbumin	14 200
Trypsin inhibitor	20 100
Trypsinogen	24 000
Carbonic anhydrase	29 000
Glyceraldehyde-3-phosphate dehydrogenase	36 000
Egg albumin	45 000
Bovine albumin	66 000

Pharmacia low molecular weight markers

	MW
β-lactalbumin	14 400
Sybean trypsin inhibitor	20 100
Carbonic anhydrase	30 000
Ovalbumin	43 000
Bovine serum albumin	67 000
Phosphorylase b	94 000

Boehringer Mannheim molecular weight markers

	MW
Soybean trypsin inhibitor	20 100
Lactate dehydrogenase	36 500
Glutamate dehydrogenase	55 400
Phosphorylase b	97 400
α_2-macroglobulin	170 000

All molecular weights are given in Daltons.

APPENDIX E

GRAM STAIN

Crystal violet

Crystal violet	0.5g
Distilled water	100ml

Iodine mordant

Iodine	1.0g
Potassium iodide	2.0g
Distilled water	100ml

Acetone-iodine decolourizer

*Strong iodine solution	3.5ml
Acetone	96.5ml

*Strong iodine solution:

Iodine	10.0g
Potassium iodide	6.0g
Distilled water	10ml
90% ethanol to	100ml

Dissolve iodine and potassium iodide in the water and then adjust the volume with the ethanol

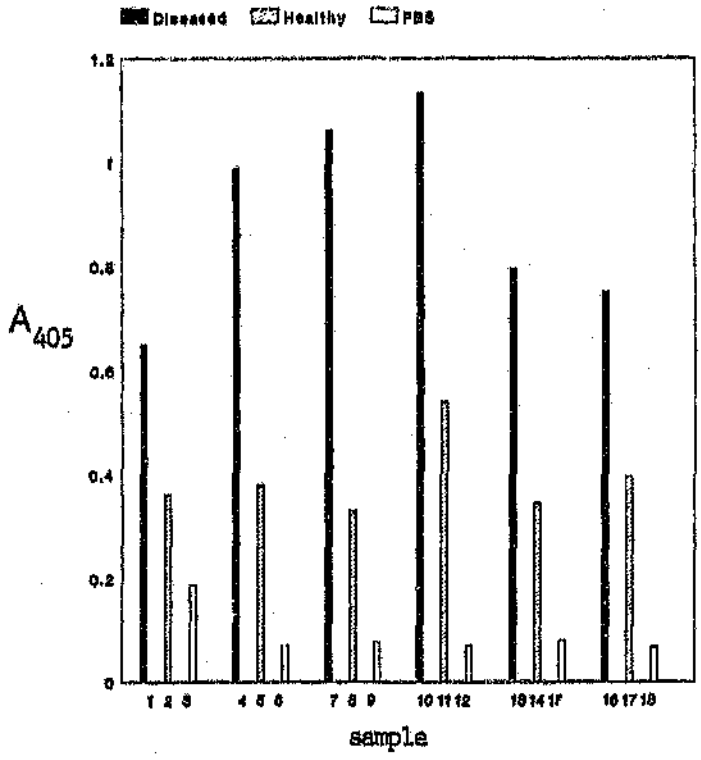
Counter stain

Safranin O	1.0g
Distilled water	100ml

Immerse an air-dried, heat-fixed film of bacteria in crystal violet stain for 1 min. Rinse the stain from the film with iodine mordant and then immerse the film in fresh iodine mordant for 1 min. Wash the film with a gentle and indirect stream of tap water for 2 seconds, blot around the film with absorbant paper but do not allow the film to dry. Add the acetone-iodine decolourizer dropwise to the film, with the slide slanted, until no colour appears in the drippings (less than 10 seconds). Allow the film to air dry. Immerse the film in the counter stain for 30-60 seconds and then wash the film in a gentle and indirect stream of tap water until no colour appears in the run-off. Blot the film dry with absorbent paper.

APPENDIX F

THE EFFECT OF HEPARIN ON THE INDIRECT ELISA



Bar no.	Heparin concentration (U/mℓ)	diseased/healthy ratio
1 2 3	0	1.80
4 5 6	2.0	2.60
7 8 9	5.0	3.21
10 11 12	10.0	2.10
13 14 15	50.0	2.30
16 17 18	100.0	1.90

Figure legend on the following page

Addition of heparin to seroreagent diluting buffer

The effect of adding different concentrations of heparin to the seroreagent diluting buffer in the HE-PTA-ELISA. Heparin was added to the sample conjugate buffer at concentrations of 2, 5, 10, 50 and 100 U/ml and the effect of the polyanion on the binding of the GICE antiserum immunoglobulin to immobilized antigen was determined.

Extracts of leaf midribs from greening-infected and healthy citrus seedlings were prepared by the water leaching method and probed with the GICE antiserum. The antiserum was cross-adsorbed against a preparation of ammonium sulphate-precipitated healthy citrus proteins prior to use in the assay. The antiserum, as well as the enzyme conjugate, were diluted in the supplemented sample conjugate buffer.

The standard deviation did not exceed 0.05 absorbance units.

The following sample and reagent dilutions were used in the assay:
* sample - 1:100
* antiserum - 1:1000
* enzyme conjugate - 1:1000

APPENDIX G

EL-DIB ASSAY BUFFERS

Tris-buffered saline (TBS):

20mM Tris-HCl containing 0.5M NaCl, pH 7.5

Tween Tris-buffered saline (TTBS):

TTBS containing 0.05% (v/v) Tween-20

Blocking buffer:

TTBS containing 1% (w/v) bovine serum albumin (BSA) and 2% (w/v) polyvinylpyrrolidone (PVP, mol. wt. 40 000, Sigma)

Antibody buffer (AB):

TTBS containing 0.5% (w/v) BSA and 2% PVP

AP buffer:

0.1M Tris-HCl containing 0.1M NaCl and 5mM $MgCl_2$, pH 9.5

All buffers contained 0.02% (w/v) sodium azide as a preservative.

Colour development solution (NBT-BCIP reagent):

AP buffer containing 0.33 mg/ml nitro blue tetrazolium (NBT, grade III, Sigma) and 0.17 mg/ml 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (BCIP, Sigma), prepared just before use as follows:
One mg NBT was dissolved in 3ml AP buffer by mixing vigorously for 1-2 min., then centrifuged for 5 min. in an Eppendorf microfuge. One-half mg BCIP was dissolved in 10ul N,N-dimethyl-formamide by mixing with a vortex mixture for 1 min. and added dropwise with gentle mixing into the NBT solution.

APPENDIX H

CONJUGATION OF RADIOACTIVE LABEL TO IMMUNOGLOBULIN G

(R. Lee, pers. comm)

1. Dissolve IODO-GEN (Pierce Chemical Co., Rockford, IL, USA) in methylene chloride (150 ug/ml).

2. Place 20 ul aliquot in 10 x 75 mm test tube (reaction tube).

3. Evaporate methylene chloride in the reaction tube with N_2 gas at room temperature.

4. To a separate tube, add 0.5 mg IgG in 0.5 ml PBS (0.015 M sodium phosphate, pH 7.4, plus 0.15 M NaCl) + 0.02% (w/v) sodium azide. Cool this tube and the reaction tube to 0 C in an ice bath.

5. Initiate conjugation reaction by transferring IgG to the reaction tube followed by adding 500 ui Ci of $Na^{125}I$. Parafilm the reaction tube.

6. Allow iodination to run for 1+-2 hr. at 0 C.

7. Terminate the reaction by removing the mixture from the reaction tube, add 5 mg/ml of BSA to the mixture, place mixture in a dialysis membrane and excessively dialyze at 4xC against PBS with 0.02% sodium azide. Monitor the CPM in dialysis fluid, usually by the fourth buffer change all unbound ^{125}I has been removed.

Specific activity of ^{125}I IgG is usually 2.0 x 10^{5} to 1 x 10^{6} CPM per ug IgG. Store at 4 C in sealed plastic tubes.

APPENDIX I

PIEM BUFFERS

Wash buffer:

1/4-strength Ringer's solution containing 0.1% (v/v) Triton X-100

Blocking buffer:

1/4-strength Ringer's solution containing 0.1% (v/v) Triton X-100, 2% (w/v) BSA, and 2% (w/v) PVP (MW 40 000)

Incubation buffer:

1/4-strength Ringer's solution containing 0.1% (v/v) Triton X-100, 0.2% (w/v) BSA, and 2% (w/v) PVP

All buffers contained 0.02% (w/v) sodium azide as a preservative.

APPENDIX J

WESTERN BLOTTING PROTOCOL

1. REAGENTS

Transfer buffer:

25mM Tris-HCl containing 192mM glycine and 20% (v/v) methanol, pH 8.3

Blocking buffer:

10mM Tris-HCl, pH 7.4 containing 200mM NaCl and 2% (w/v) BSA

Wash buffer:

10mM Tris-HCl, pH 7.4 containing 0.05% (v/v) Tween-20

Antibody buffer:

10mM Tris-HCl, pH 7.4 containing 1% (w/v) BSA

4. PROCEDURE

After electrophoretic separation of proteins, the PAGE gel was soaked in an excess of transfer buffer for 30 min. A sheet of nitrocellulose membrane (NCM) was cut to roughly the same size as the gel and wetted with transfer buffer. The NCM was placed against the gel on a flat surface and the NCM smoothed over the gel by rolling with a glass pipette to remove all air bubbles. A double layer of Whatman's No. 4 filter paper, prewetted with transfer buffer, was wrapped around the gel/NCM to make a sandwich. The paper/gel/NCM/paper sandwich was placed in an electrophoretic transfer apparatus (Hoefer Transphor Electrophoresis Unit, Hoefer Scientific Instruments, USA) with the gel facing the cathode. After placement in the buffer tank, transfer buffer was added to cover the gel and electrophoretic transfer of proteins carried out for 4 hr at 0.5 Amp.

The NCM was removed from the sandwich and washed briefly in a large volume of dH_2O. The NCM was immersed in blocking buffer and incubated at 37 C for 1 hr in order to saturate all unbound NCM binding sites. Excess blocking buffer was removed by washing the NCM in wash buffer for 1 hr at room temperature. The blotted proteins were probed with whole antiserum diluted in antibody buffer (a 1:1000 dilution of the GC antiserum was used in this study) overnight at 4 C. The NCM was then subjected to four 5 min. washes in wash

buffer and transferred to a solution of ^{125}I-labelled Protein A (Amersham, U.K.) diluted 1:1000 in antibody buffer for 1 hr at 37 C. After four washes in washing buffer and a single wash in 10mM Tris-HCl, pH 7.4, the NCM was allowed to dry thoroughly and then cut into 3 mm sections along the length of the membrane. The sections of NCM were transferred to scintillation vials and 5ml of Filter-Count (Packard Instrument Co. Inc., USA) added to each vial. After overnight refrigeration in the dark, the radioactivity of each section was determined.

All incubation and washing steps were carried out with gentle agitation.

APPENDIX K

PHOSPHATE-BUFFERED SALINE AND RINGER'S SOLUTION

Phosphate-buffered saline (PBS)

	g/litre
NaCl	8.0
KCl	0.2
$Na_2HPO_4.7H_2O$	1.15
KH_2PO_4	0.2

1/4-strength Ringer's solution

	g/litre
NaCl	2.25
KCl	0.105
$CaCl_2.2H_2O$	0.12
$NaHCO_3$	0.05

Dissolve in distilled water and pH to 7.0

APPENDIX L

FATTY ACID PROFILING

INTERPRETATION OF PROFILES

Each profile report includes ie GC run, a profile identifying and quantifying the fatty acids as percentages of the total peak area, a library search report with similarity index and a histoplot comparing your values with the library entry for the taxon with the closest fit.

GC run. This gives the date and time of the run, the bottle number, sequential analysis ID number, the bacteriology section file number prefixed with an A, a brief sample description with your reference number or code and the file number for profile retrieval should you require a spare copy.

The profile runs between the letters S and IF and only peaks with the correct area/height ratios are analysed. The large initial peak is the solvent front. The values above the peaks are the retention times (rt, in minutes.

Profile. This again gives the date and time of run and the date and time of regeneration if obtained later from the files. The bottle number, ID number and sample description are also given. This will include the method of sample cultivation e.g. Aerobe.

The profile lists for each peak the retention time (RT), the area, the area/height ratio, the response, the equivalent chain length (ECL), the identity based on ECL, the relative amount (%) and one or two comments (e.g. ECL deviation from expected value). Where two or more acids are known to co-elute such that discrimination is difficult, the identity is given only as a summed feature. For each of these the most likely identity is given in the comment sections. Approximately 120 acids can be identified (list attached).

The profile also gives the solvent area, sums the total peak area, named peak area, % peaks named, gives the number of selected reference calibration peaks in the profile and also gives some indication of the reproducibility of the chromatography by comparison with these reference acids. This is referred to in the ECL deviation and reference ECL shift values. Few of these data will be of use to you, but they are of use to the operator who can assess the quality of profiling. Standardisation is essential in work of this type and the software is programmed to detect abnormalities which are based upon the values desribed in this paragraph.

Library Search Report. The profile of named acids and their relative amounts derived from acceptable chromatogaphic parameters discussed above is compared with the profiles derived from a wide selection of bacterial taxa.

A list is given of the generic, specific and infraspecific taxa with the most similar profiles to each test profile. The degree of profile and similarity is assessed in the form of a similarity index (0 - 1.0) for each of the listed taxa. This is calculated using a pattern recognition algorithm.

It must be noted that a fatty acid profile is a single diagnostic test and it must not be assumed that the first choice is the correct determination. Rather it should be used as an aid in determination or diagnosis. The library is not complete. It represents a selection of the major taxa held in a variety of reputable culture collections (list attached). Each taxon is represented by a comprehensive range of strains.

Interpretation of the library search report data varies with the group of bacteria and it is impossible to apply hard and fast rules. In general, values greater than 0.7 are excellent matches. An index value of 0.5 is approximately three standard deviations away from libary entry mean value.

Classification reports giving a single match greater than 0.5 have a strong likelihood of being correct.

Single match determinations with indices below 0.5 may indicate a closely related organism not in the library. Some groups of bacteria comprise bacteria with very similar profiles for which there is overlap. This can occur at generic level within for example the Enterobacteriaceae or at specific and infraspecific levels within for example the fluorescent pseudomonads. In such cases it is possible to have a library search resulting in three matches all with similarity indices greater than 0.5.

In such cases a few conventional tests may easily differentiate between them. Even values at the 0.1 level quite often represent closely related species. Some taxa are heterogenous and if the library does not include a particular sub group within the strains in the library, then similarity index values may be lower than expected. Where such differences are known the taxa in the library are subdivided into different Gas chromatographic sub groups to aid determination.

Histoplot chart. This is a visual representation of the results of the library search. The chart represents the library entry for each acid in the profile of the closest match. The + value is the mean library entry (% of total acids) with a window of two standard deviations around this mean. The x value is the value for that acid in the sample extract. This simple statistical analysis can be very useful but the calculation of the similarity index involves the use of a complex algorithm that takes into account various other parameters such as relationship between acids, non-Gaussian distributions of percentages etc, and is of greater accuracy. Even when a match is not given in the library search report, a histoplot of closest profile is given. The distance value is indicative of the number of standard deviations distance from the library mean values.

REFERENCE ACIDS

1	9:0	-2*	15:1 ISO H/13:0 30H	82	17:1 CIS 10
2	8:0 30H	-2*	13:0 30H/15:1 i I/H	83	17:1 C
3	unknown 9.521	-2*	15:1 ISO I/13:0 30H	84	17:0 CYCLO
4	10:0 ISO	44	unknown 14.503	85	unknown 16.918
5	10:0	45	15:1 ANTEISO A	86	17:0
6	9:0 30H	46	15:0 ISO	87	16:1 20H
7	11:0 ISO	47	15:0 ANTEISO	88	16:0 ISO 30H
8	11:0 ANTEISO	48	15:1 A	89	16:0 20H
-3*	12:0 ALDE .?	49	15:1 B	90	18:1 ISO F
-3*	unknown 10.928	50	15:1 CIS 10	91	18:1 ISO G
9	11:0	51	unknown 14.966	92	18:1 ISO H
10	unknown 11.097	52	15:0	93	16:0 30H
11	10:0 20H	53	14:0 ISO 30H	94	18:3 CIS 6,12,14
12	10:0 30H	54	14:0 20H	95	18:0 ISO
13	unknown 11.541	55	16:1 ISO E	-6*	18:2 CIS 9,12/18:0a
14	12:0 ISO	56	16:1 ISO F	-6*	18:0 ANTEISO/18:2 c
15	12:0 ANTEISO	57	16:1 ISO G	96	18:1 CIS 9
16	unknown 11.798	58	16:1 ISO H	-7*	18:1 CIS 11/t 9/t6
17	12:1 AT 11-12	-3*	16:1 ISO I/14:0 30H	-7*	18:1 TRANS 9/t6/c11
18	12:0	-3*	14:0 30H/16:1 ISO I	-7*	18:1 TRANS 6/t6/c11
19	11:0 ISO 30H	59	unknown 15.549	97	18:1 TRANS 11
20	unknown 12.112	60	16:0 ISO	98	18:1 B
21	11:0 20H	61	unknown 15.665	99	18:0
22	11:0 30H	62	16:0 ANTEISO	100	17:0 ISO 30H
23	unknown 12.486	63	16:1 A	101	17:0 20H
24	13:0 ISO	64	16:1 B	102	TBSA
25	13:0 ANTEISO	65	16:1 CIS 9	103	19:1 ISO I
26	13:1 AT 12-13	-4*	15:0 ISO 20H/16:1t9	104	17:0 30H
27	13:0	-4*	16:1 TRANS 9/15:20H	105	19:0 ISO
28	12:0 ISO 30H	66	16:1 C	106	19:0 ANTEISO
29	12:0 20H	67	16:0	-8*	unknown 18.756/19:1
30	12:1 30H	68	15:0 ISO 30H	-8*	19:1 CIS 10/18.576
31	14:1 ISO E	69	15:0 20H	107	19:1 TRANS 7
32	12:0 30H	70	17:1 ISO E	-9*	un 18.846/18.858
33	unknown 13.566	71	17:1 ISO F	-9*	un 18.858/.846/19cy
34	14:0 ISO	72	17:1 ISO G	-9*	19:0 CYCLO C9-10/un
35	14:0 ANTEISO	73	17:1 ISO H	108	19:0 CYCLO C11-12
-1*	14:1 TRANS 9/CIS 9	-5*	17:1 ISO I/ANTEISO B	109	19:0
-1*	14:1 CIS 9/TRANS 9	-5*	17:1 ANTEISO B/i I	110	18:1 20H
36	unknown 13.961	74	15:0 30H	111	18:0 20H
37	14:0	75	17:1 ANTEISO C	112	unknown 19.368
38	13:0 ISO 30H	76	17:1 ANTEISO A	113	20:4 CIS 5,8,11,14
39	13:0 20H	77	unknown 16.580	114	18:0 30H
40	unknown 14.258	78	17:0 ISO	115	20:0 ISO
41	15:1 ISO E	79	17:0 ANTEISO	116	unknown 19.735
42	15:1 ISO F	80	17:1 A	117	20:1 CIS 11
43	15:1 ISO G	81	17:1 B	118	20:1 TRANS 11
				119	20:0

c = CIS, t = TRANS, i = ISO, a = ANTEISO
* two or more peaks coelute; peaks are part of a summed group.

Gas chromatograph profile, library search report and histoplot chart of isolate NC

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HP 5898A: MICROBIAL IDENTIFICATION SYSTEM (Rev: 2.1) (Sw s/n: 2711A10116) (Hw s/n: 2350012          20-JUL-88 18:13:49

Id:    3248      CITRUS GREENING BACT.BSNC1(REPEAT)                    Date of run: 20-JUL-88 21:47:34
Bottle: 59       STRT SAMPLE CAERO&..

  RT      Area     Ar/Ht Respon     ECL        Name              %      Comment 1              Comment 2
------- --------- ------ ------ ------ ---------------- ------ ---------------------- ----------------------
  1.586  41832000  0.093  . . .  7.041 SOLVENT PEAK . . . . . . < min rt
  1.782      1827  0.049  . . .  7.491 . . . . . . . . . . . . < min rt
  1.879       759  0.026  . . .  7.710 . . . . . . . . . . . . < min rt
  6.189      3362  0.036  0.982 13.617 14:0 ISO . . . . . .  1.01 ECL deviates -0.001 Reference -0.003
  7.589      9537  0.049  0.966 14.621 15:0 ISO . . . . . .  3.60 ECL deviates  0.000 Reference -0.001
  7.723    124220  0.040  0.965 14.713 15:0 ANTEISO . . . . 52.32 ECL deviates  0.002 Reference  0.001
  9.145     52351  0.041  0.957 15.625 16:0 ISO . . . . . . 21.86 ECL deviates -0.001 Reference -0.002
  9.745      5199  0.042  0.955 15.999 16:0 . . . . . . . .  2.17 ECL deviates -0.001 Reference -0.002
 10.803      2209  0.044  0.954 16.629 17:0 ISO . . . . . .  0.92 ECL deviates  0.000 Reference -0.002
 10.950     43587  0.044  0.954 16.723 17:0 ANTEISO . . . . 18.13 ECL deviates  0.001 Reference -0.001

Solvent Ar  Total Area  Named Area  % Named  Total Unnd  Nbr Ref  ECL Deviation  Ref ECL Shift
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  41832000      238520      238520   100.00      229502        7          0.001          0.002

        TSBA (Rev 2.01    Clavibacter . . . . . . . . . . . . . . . . . . . . 0.103 (Corynebact. sepedon.)
                            C. michiganense . . . . . . . . . . . . . . . . . 0.103 (Corynebact. sepedon.)
                              C. m. sepedonicum . . . . . . . . . . . . . . . 0.103 (Corynebact. sepedon.)
                          Micrococcus . . . . . . . . . . . . . . . . . . . . 0.064
                            M. kristinae . . . . . . . . . . . . . . . . . . 0.064

Comparison with TSBA (Rev 2.01): Clavibacter-michiganense-sepedonicum(Corynebact. sepedon.)          Distance: 6.329

                    0    5   10   15   20   25   30   35   40   45   50   55   60   65   70   75   80   85   90   95  100
                    '    '    '    '    '    '    '    '    '    '    '    '    '    '    '    '    '    '    '    '    '
14:0 ISO. . . . . . *x   .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .
15:1 ANTEISO A. . . x----*----.    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .
15:0 ISO. . . . . . -*- x.    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .
15:0 ANTEISO. . . . .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .
16:0 ISO. . . . . .  .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .
16:0. . . . . . . .  .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .
17:0 ISO. . . . . .  .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .
17:0 ANTEISO. . . .  .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .
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Gas chromatograph profile, library search report and histoplot chart of reisolate NC (RNC)

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MP 5898A: MICROBIAL IDENTIFICATION SYSTEM (Rev: 2.1) (Sw s/n: 2711A10116) (Hw s/n: 2350612          19-JUL-88 16:54

ID:     3240        CITRUS GREENING BACT, EX S. AFRICA BGRNC1          Date of run: 20-JUL-88 16:30:47
Bottle: 51          STAF SAMPLE [AEROBIC]

  RT      Area     Ar/Ht Respon    ECL          Name              %      Comment 1          Comment 2
------- -------- ------ ------ ------ ---------------------- ------ ------------------- ---------------------
 1.573 42361000  0.084  . . .  7.043 SOLVENT PEAK . . . . . .        < min rt
 1.868      676  0.029  . . .  7.712 . . . . . . . . . . . .        < min rt
 6.161     2763  0.036  0.981 13.618 14:0 ISO . . . . . .   1.00 ECL deviates -0.000 Reference  0.000
 7.557     9459  0.038  0.966 14.621 15:0 ISO . . . . . .   3.36 ECL deviates -0.000 Reference  0.002
 7.691   145670  0.040  0.965 14.713 15:0 ANTEISO . . . .  51.67 ECL deviates  0.002 Reference  0.003
 9.112    63253  0.042  0.957 15.625 16:0 ISO . . . . . .  22.25 ECL deviates -0.001 Reference  0.002
 9.710     6980  0.045  0.955 15.999 16:0 . . . . . . . .   2.45 ECL deviates -0.001 Reference  0.002
10.768     2427  0.044  0.954 16.629 17:0 ISO . . . . . .   0.85 ECL deviates  0.000 Reference  0.003
10.925    52531  0.041  0.954 16.723 17:0 ANTEISO . . . .  18.12 ECL deviates  0.001 Reference  0.003

Solvent Ar Total Area Named Area % Named Total Amnt Nbr Ref ECL Deviation Ref ECL Shift
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  42361000     283083     283083  100.00     272050       7         0.001         0.002

        TSBA [Rev 2.01]  Clavibacter . . . . . . . . . . . . . . . . . . . . 0.118 (Corynebact. sepedon.)
                         C. michiganense . . . . . . . . . . . . . . . . . . 0.118 (Corynebact. sepedon.)
                         C. m. sepedonicum . . . . . . . . . . . . . . . . . 0.118 (Corynebact. sepedon.)

Comparison with TSBA [Rev 2.01]: Clavibacter-michiganense-sepedonicum(Corynebact. sepedon.)          Distance: 6.133

                    0   5   10  15  20  25  30  35  40  45  50  55  60  65  70  75  80  85  90  95  100
                    .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .
14:0 ISO. . . . . +x  .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .
15:1 ANTEISO A. . . x------+------,   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .
15:0 ISO. . . . . --+-x   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .
15:0 ANTEISO. . . .   .   .   .   .   .   .   .   .   .   .------+---- x   .   .   .   .   .   .   .
16:0 ISO. . . . . .   .---.---+------ .   x   .   .   .   .   .   .   .   .   .   .   .   .   .   .
16:0. . . . . . . . --x--+------.   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .
17:0 ISO. . . . . +x  .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .
17:0 ANTEISO. . . .   .   .   .   . x .---------+-------.   .   .   .   .   .   .   .   .   .   .   .
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Gas chromatograph profile, library search report and histoplot chart of isolate GC

HP 5898A: MICROBIAL IDENTIFICATION SYSTEM (Rev: 2.1) (Sw s/n: 2211A10116) (Hw s/n: 2350G12DATA1:F08728530 29-JUL-88 09:42:07

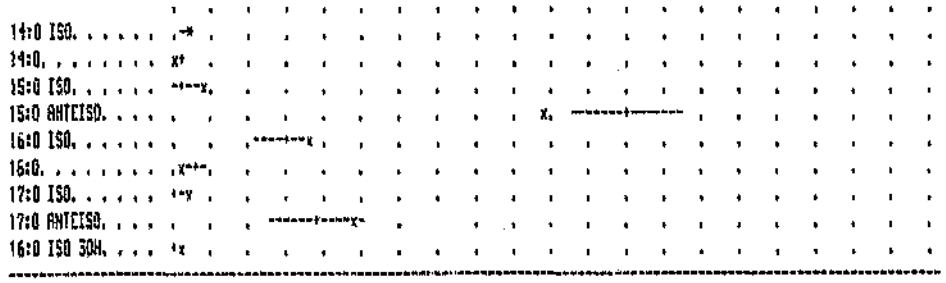
ID: 3311 CITRUS GREENING BACT. EX S. AFRICA 856C, Date of run: 28-JUL-88 15:16:18
Bottle: 2 SAMPLE [AERODC]

RT	Area	Ar/Ht	Region	ECL	Name	%	Comment 1	Comment 2
1.586	42441000	0.085	...	7.045	SOLVENT PEAK		< min rt	
6.189	3740	0.036	0.979	13.618	14:0 ISO	1.78	ECL deviates -0.000	Reference -0.003
7.588	7563	0.040	0.965	14.621	15:0 ISO	3.54	ECL deviates 0.000	Reference -0.001
7.721	104729	0.039	0.964	14.712	15:0 ANTEISO	48.95	ECL deviates 0.001	Reference -0.000
9.144	38735	0.042	0.957	15.625	16:0 ISO	17.98	ECL deviates -0.001	Reference -0.002
9.744	2489	0.042	0.956	16.000	16:0	1.15	ECL deviates 0.000	Reference -0.001
10.801	3526	0.045	0.955	16.629	17:0 ISO	1.63	ECL deviates -0.000	Reference -0.003
10.959	52193	0.044	0.955	16.723	17:0 ANTEISO	24.18	ECL deviates 0.001	Reference -0.002
11.689	1699	0.053	0.956	17.153	16:0 ISO 3OH	0.79	ECL deviates 0.000	
13.603	762	0.048	...	18.261	.....			

Solvent Ar	Total Area	Named Area	% Named	Total Amnt	Nbr Ref	ECL Deviation	Ref ECL Shift
42441000	215433	214671	99.65	206234	7	0.003	0.002

ISBA [Rev 2.01 Micrococcus 0.134
M. kristinae 0.134
Clavibacter ..... 0.133 (Corynebact. sepedon.)
C. michiganense ..... 0.133 (Corynebact. sepedon.)
C. m. sepedonicum ..... 0.133 (Corynebact. sepedon.)

Comparison with ISBA [Rev 2.0]: Micrococcus-kristinae Distance: 5.951



Gas chromatograph profile, library search report and histoplot chart of isolate CS

HP 5898A: MICROBIAL IDENTIFICATION SYSTEM (Rev: 2.1) (Sw s/n: 2211A10116) (Hw s/n: 2350612 20-JUL-88 19:13:19

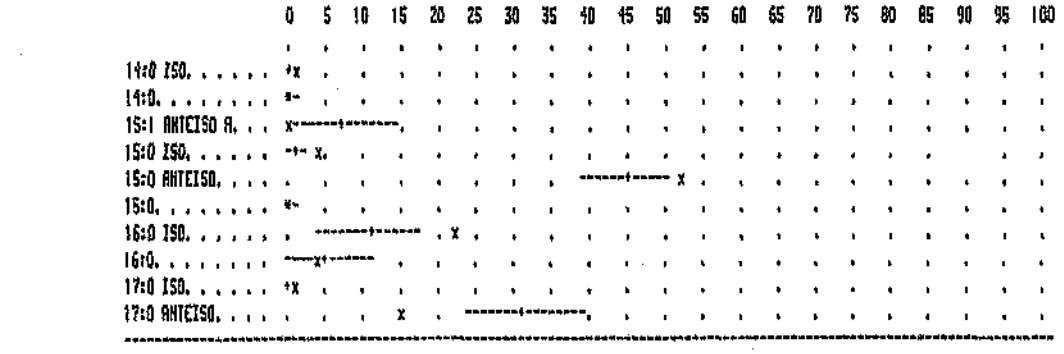
ID: 3243 CITRUS GREENING BACT. EX S. AFRICA 88CS Date of run: 20-JUL-88 19:17:19
Bottle: 51 STAT SAMPLE [AEROBE]

RT	Area	Ar/Ht	Respon	ECL	Name	%	Comment 1	Comment 2
1.583	41940000	0.084	. . .	7.041	SOLVENT PEAK		< min rt	
4.449	511	0.033	. . .	12.101	. . . . .			
6.190	3964	0.036	0.982	13.617	14:0 ISO	1.22	ECL deviates -0.001	Reference -0.002
6.680	759	0.036	0.975	14.080	14:0	0.23	ECL deviates 0.000	Reference -0.001
7.589	13477	0.039	0.966	14.621	15:0 ISO	4.08	ECL deviates -0.000	Reference -0.001
7.724	170770	0.039	0.965	14.713	15:0 ANTEISO	51.64	ECL deviates 0.002	Reference 0.001
8.144	1017	0.043	0.962	15.000	15:0	0.31	ECL deviates -0.000	Reference -0.001
9.146	74696	0.042	0.957	15.625	16:0 ISO	22.40	ECL deviates -0.001	Reference -0.002
9.745	14292	0.042	0.955	15.999	16:0	4.28	ECL deviates -0.001	Reference -0.002
10.804	2855	0.044	0.954	16.629	17:0 ISO	0.85	ECL deviates 0.000	Reference -0.001
10.961	50168	0.044	0.954	16.723	17:0 ANTEISO	14.99	ECL deviates 0.001	Reference -0.001
14.165	988	0.059	. . .	18.587	. . . . .			
15.002	717	0.051	. . .	19.073	. . . . .			
17.562	1113	0.048	. . .	20.571	. . . . .		> max rt	
19.360	2124	0.111	. . .	21.622	. . . . .		> max rt	
19.911	11719	0.071	. . .	21.944	. . . . .		> max rt	

Solvent Ar	Total Area	Named Area	% Named	Total Amnt	Nbr Ref	ECL Deviation	Ref ECL Shift
41946000	334206	331998	99.34	319223	9	0.001	0.001

TSBA [Rev 2.01 Clavibacter . . . . . 0.06] (Corynebact. sepedon.)
C. michiganense . . . . . 0.06] (Corynebact. sepedon.)
C. m. sepedonicum . . . . . 0.06] (Corynebact. sepedon.)

Comparison with TSBA [Rev 2.01: Clavibacter-michiganense-sepedonicum(Corynebact. sepedon.) Distance: 7.014



All information and data supplied by the NCPPB, Hatching Green Harpenden, England.

APPENDIX M

ANALYTICAL PROFILE INDICES

API 20E analytical profile index of isolates and reisolates

CHARACTER	ISOLATE 1	ISOLATE 2	REISOLATE 1	REISOLATE 2
Gelatin hydrolysis	+	+	+	+
Nitrate reduction	-	-	-	-
β-Galactosidase	+	+	+	+
Saccharose	+	+	+	+
Arabinose	+	+	+	+
Mannitol	+	+	+	+
Fructose	+	+	+	+
Glucose	+	+	+	+
Maltose	+	+	+	+
Starch (Amidose)	-	-	-	-
Rhamnose	-	-	-	-
Galactose	-	-	-	-
Mannose	+	+	+	+
Sorbitol	-	-	-	-
Glycerol	+	+	+	+
Urease	-	-	-	-
H_2S production	-	-	-	-
Acetoin	-	-	-	-
Simmons citrate	-	-	-	-
Cytochrome oxidase	+	+	+	+
Catalase	+	+	+	+
Oxidation/Fermentation	O	O	O	O
Moltility	+	+	+	+
Spore	-	-	-	-

API 20NE analytical profile index of isolates and reisolates

CHARACTER	ISOLATE 1	ISOLATE 2	REISOLATE 1	REISOLATE 2
Nitrate reduction	-	-	-	-
Indole from Tryptohan	-	-	-	-
Glucose (acidification)	-	-	-	-
Arginine dihydrolase	-	-	-	-
Urease	-	-	-	-
Aesculin	+	+	+	+
Gelatin	+	+	+	+
PNPG	+	+	+	+
Assimilation				
Glucose	+	+	+	+
Arabinose	+	+	+	+
Mannose	+	+	+	+
Mannitol	+	+	+	+
N-acetyl Glucosamine	+	+	+	+
Maltose	+	+	+	+
Gluconate	+	+	+	+
Carpate	-	-	-	-
Adipate	-	-	-	-
Malate	-	-	-	-
Citrate	+	+	+	+
Phenylacetate	-	-	-	-
Oxidase	+	+	+	+

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UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

URC'S ANIMAL ETHICS COMMITTEE

448

CLEARANCE CERTIFICATE

PROTOCOL NO. 87/96

APPLICANT : R J Chippindall

DEPARTMENT : Microbiology

PROJECT TITLE : Use of antibodies in the detection of the putative greening organism in plant tissue samples

DATE OF APPLICATION : 22 June 1987

DATE CONSIDERED : 7 July 1987

RECOMMENDATION OF COMMITTEE :

APPROVED

Signed : G. Nelson Date : 15.7.87

Chairman: Animal Ethics Committee

DECLARATION BY INVESTIGATOR

(a) I am fully aware of, and understand the terms and conditions under which I am authorised to conduct the abovementioned research.

(b) I undertake to carry out only those experimental procedures which have been specifically authorised by the Animal Ethics Committee.

(c) I undertake, in carrying out any authorised experimental procedure, to exercise an acceptable degree of competence and skill.

(d) I declare that the experiment will be carried out by the people listed in the protocol.

(e) I understand that in the event of any breach of these undertakings, the Animal Ethics Committee may, in its sole discretion, withdraw this clearance immediately.

THIS CLEARANCE CERTIFICATE IS VALID FOR A MAXIMUM PERIOD OF TWO YEARS ONLY.

Signed : RJ Chippindall Date : 1.8.1987



Author: Chippindall Richard-John Chapman.
Name of thesis: Biological and serological properties of a bacterium isolated from greening-infected citrus in South Africa.

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