BIOLOGICAL AND SEROLOGICAL PROPERTIES OF A BACTERIUM ISOLATED FROM GREENING-INFECTED CITRUS IN SOUTH AFRICA

Richard-John Chapman Chippindall

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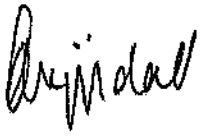
DECLARATION

I declare that this thesis is my own unaided work. It is being submitted for the degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.



Richard-John Chapman Chippindall

17 day of December 1991

DEDICATION

To my father

PREFACE

Some aspects of the work conducted for this thesis have been presented as papers or posters elsewhere:

Chippindall, R-J. and Whitlock, V.H. 1988. Transmission and reisolation of a bacterium associated with greening disease of citrus in South Africa. 26th Annual Congress of the South African Society for Plant Pathology.

Chippindall, R-J. and Whitlock, V.H. 1988. Improvements in the serological detection of greening. Citrus and Subtropical Fruit Research Institute Symposium on Research into Citrus and Subtropical Crops. CSFRI, Nelsprut.

Chippindall, R-J. and Whitlock, V.H. 1988. Transmission of greening disease from citrus to various indicator plants via dodder, and mechanical transmission of an isolate of the suspected causative agent to these plants. Citrus and Subtropical Fruit Research Institute Symposium on Research into Citrus and Subtropical Crops. CSFRI, Nelspruit.

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Chippindall, R-J. and Whitlock, V.H. 1989. Development of an antiserum to detect greening disease of citrus. (Abstract) Phytopathology 79: 1212.

Chippindall, R-J. and Whitlock, V.H. 1989. Transmission and reisolation of a bacterium isolated from greening-infected citrus in South Africa. (Abstract) Phytopathology 79: 1182-1183.

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ABSTRACT

Greening is a severe disease of citrus and is a major cause of crop loss in many parts of Africa and Asia. Numerous attempts have been made by various workers to isolate the aetiological agent of the disease and although reports claiming the successful culture of the organism have appeared, the isolations were never fully confirmed. In one of these reports, a bacterium apparently morphologically identical to the greening organism *in situ* was consistently isolated from greening-infected citrus and the bacterium claimed by the author to be the causative agent of the disease (Garnett, 1985). This study was undertaken principally to investigate the significance of the isolated bacterium in greening disease. The organism was found to induce a diversity of foliar chloroses similar to those observed on naturally-infected plants when mechanically inoculated into healthy citrus seedlings. Reisolation of the bacterium from inoculated plants was achieved and electron microscope examination of leaf midribs showed the presence of bacteria in the phloem of the plants. Dodder transmission of an infective agent from naturally-infected citrus to cucumber was achieved and the same bacterium isolated from symptomatic cucumber plants. The association of this bacterium with greening-infected citrus indicated

by these results was thought to be proof of its aetiological status, however further investigations revealed that i) contrary to the report by Garnett (1985), the morphology of the isolate and the greening bacterium observed in situ differ markedly, ii) vector transmission of the isolate to citus does not occur, iii) mechanically-inoculated citrus plants do not display the gentisic acid marker which is indicative of infection with greening, iv) symptoms displayed by inoculated citrus are transient and the infection does not persist and, v) antisera raised against the cultured isolate do not consistently differentiate between healthy and greening-infected material in serological assys. The results suggest that the bacterium isolated is not the greening organism per se, but probably a mild opportunistic pathogen in citrus which may be a componet of the greening syndrome. Fatty acid profiling of the isolate suggested that the organism is a member of the genus Clavibacter, but further tests are needed to confirm this classification. Because of the doubt cast on the aetiological status of the isolate by the results of this investigation, attempts were made to develop a serological detection system for greening without using the cultured bacterium for the production of a polyclonal anticorum. A number of published, as well as novel bacterial purification schemes were employed in an attempt to extract the greening organism from infected

plant material for use as immunogen. Although the isolation of significant numbers of the organism was not achieved following the different procedures, an antiserum raised against one of the citrus extracts was able to effectively detect disease antigens in greened citrus. However, reaction of the antiserum with citrus affected by a number of other diseases indicated that the antiserum was not specific for greening, even after exhaustive cross-adsorption against preparations of healthy citrus material as well as against material affected by the diseases with which the antiserum reacted positively. Efforts to cultivate the aetiological agent of greening in various artificial media as well as in embryonated hen's eggs and in an invertebrate cell culture system were unsuccessful, although the results of the isolation attempts indicated that both healthy and greening-infected citrus harbour a number of different bacterial species.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Citrus greening is a highly destructive, geographically widespread disease affecting most economically important citrus cultivars, species and hybrids in Southern Africa. The importance of the disease in South Africa was emphasized when approximately 100 000 sweet orange trees were rendered commercially unprofitable (Oberholzer et al., 1965). Greening has caused fruit losses of between 5 and 100% in orchards in different areas of the Transvaal, and farmers in many of these areas have been forced to curtail citrus production (Schwarz, 1967, van den Berg et al., 1990). It is estimated that the current annual loss in South Africa alone due to greening is R35 million (van den Berg et al., 1987).

The disease is not peculiar to Southern Africa, being prevalent in China, Taiwan R.O.C., India, the Philippines, Indonesia, as well as in Southeast Asia. In the eastern countries, the disease is known by various names, viz. citrus yellow shoot or huanglungbin in China, likubin in Taiwan R.O.C., citrus die-back in India, leaf mottling in

the Philippines, and vein-phloem degeneration in Indonesia, the names generally being descriptive of symptoms in infected plants.

Electron microscopic examination of both infected plant material and insect vector tissue collected in South Africa revealed the presence of a bacterium possessing a characteristic cell envelope structure (Moll and Martin, 1974); the various geographical forms of greening are all characterized by organisms with the same envelope system (Garnier et al., 1976, Bové et al., 1980) and this microorganism is now generally considered to be the aetiological agent of greening. Two strains of the disease have been described, namely a heat sensitive African strain which is vectored by the citrus psyllid Trioza erytreae (Del Guercio), and a heat tolerant Asian strain vectored by Diaphorina citri (Kuwayama) (Massonie et al., 1976).

Greening apparently originated in China and was common there by 1925 (Garnsey, 1988). The disease was first observed in South Africa in 1928 at De Wild in the Western Transvaal and during the periods 1932-1937 and 1939-1946 it caused severe losses in parts of the Eastern Transvaal (Oberholzer et al., 1965) and subsequently caused citrus production to cease in the White River, Plaston and Alkmaar areas (Anon., 1968). A survey conducted by Schwarz (1967)

revealed that the main centres of greening occured in the Transvaal, while only a few cases of the disease were reported from orchards in the Eastern and Western Cape. Greening was not, however, detected in orchards in the Northern Cape.

Although the incidence of the disease has increased at an alarming rate in certain parts of South Africa such as the Karino area where it increased from zero to 40% in a ten year period, results show that citrus production in greening-affected areas is possible when the disease is controlled by proper management of the vector and of the aetiological agent of the disease itself (Anon., 1988).

1.1 THE AETIOLOGY OF GREENING DISEASE

It has taken over 60 years to elucidate the aetiology of greening disease and during this period theories regarding the cause of the disease have undergone several changes. The confusion surrounding this aspect of greening is illustrated in a paper by Mali and Chaudhari (1976) in which the authors give an account of the history of the aetiology of citrus die-back (greening) in India. Different workers have attributed the disease there to both non-parasitic causes, viz. wrong selection of site, water

logging, prolonged dry periods, cultural abuses, soil salinity, indiscriminate use of planting material, scionic incompatability, nutritional deficiencies, and pest infestations, and to parasitic agents such as fungi, nematodes, mycoplasma-like organisms, and viruses. The authors deduced that the nature of die-back is complex and involves more than a single factor.

The first detailed account of greening was in a paper by Oberholzer, Von Staden and Basson (1968) cited in McClean and Oberholzer (1965a). They described the disease, gave its distribution in South Africa and reviewed the results of various lines of work done over the years. They concluded that greening is not related to soil conditions, and is not caused by a mineral deficiency or toxicity. They suggested that because greening had been transmitted under certain conditions, the disease might be caused by a virus that was being spread by an insect vector.

That greening might be of viral origin was first suggested in 1948 (Hofmeyer and Oberholzer, 1948). Some evidence of its transmission by grafting was reported by Oberholzer and Hofmeyer (1955), but because of the inconsistency of the results of their experiments, the viral nature of the disease was not proven beyond doubt. In a later publication McClean and Oberholzer (1965a) showed that greening spreads

in the field and can be transmitted through grafts. They concluded from results of their experiments and observations that "greening is a transmissible virus disease that spreads through the air, probably by means of an insect vector". No proof of the viral nature of the causal organism of greening was, however, provided in the report other than the fact that there was no evidence that the pathogen was either a bacterium or a fungus which led the authors to infer that it was a virus.

Greening has peculiar properties which distinguish it from many virus diseases: the infection is seldom complete with the aetiological agent being present in certain sectors of infected trees where it remains confined without spreading while other sectors of the same tree appear to remain free of disease and grow normally. Also the disease does not readily pass to progeny trees propagated by buds from infected parents, even when these parents are extensively and severely diseased, and transmission by grafting cuttings from infected trees onto healthy seedlings is not always successful. This suggests a restricted or localised distribution of the organism within infected plants and was indicated by the inconsistency of results of transmission studies performed by various authors in different geographical localities (McClean and Oberholzer, 1965a, Salibe and Cortez, 1966, McClean, 1970, Huang, 1979).

The hypothesis of a viral aetiology for greening went unchallenged until 1970 when Laflèche and Bové (1970a) demonstrated, with the aid of the electron microscope, the presence of mycoplasma-like organisms (MLO) in the phloem sieve elements of greening-infected sweet orange material from South Africa. These microorganisms were observed not only in association with South African greening, but also with Réunion greening and Indian citrus die-back/decline (Laflèche and Bové, 1970b). Similar structures were found to be present in citrus from the Philippines affected by leaf mottling disease (Saglio et al., 1971), as well as in likubin-infected material from Taiwan (Chen et al., 1971). This common characteristic, together with similarities in transmission patterns and symptoms, suggest that these diseases are analogous.

The MLO aetiology of greening has been further indicated by the therapeutic effect of tetracyclines. In the same year that Laflèche and Bové reported the presence of MLO's in the phloem of infected plants, Martinez et al. (1970) showed that treatment of greening-infected budwood with tetracycline antibiotics prior to bud-inoculation of healthy citrus resulted in the failure of symptom development in the indicator plants. Also, foliar application of antibiotics to infected seedlings lessened the severity of disease symptoms in these plants. The

attenuation of symptoms in sprayed plants and the prevention of disease transmission in the bud inoculation studies suggested that the antibiotic treatments had a suppressive effect on the aetiological agent and led the authors to hypothesise that an MLO was involved and not a virus.

Although initially described as an MLO, investigations carried out by Bové et al. (1980) revealed that the envelope of the greening organism differed structurally from those of other MLO's. The organism was found to be surrounded by double membranes which suggested that it was more like a bacterium than an MLO (Chen et al., 1971). Further ultrastructural studies, carried out on organisms in the phloem sieve elements of infected plants and in the haemolymph of the insect vector, revealed that the envelope surrounding the organism measures approximately 25nm in width and comprises three zones: an electron-dense outer zone, an electron-dense inner zone and an intermediate electron-transparent zone. Each of the two electron-dense zones could be resolved into a triple-layered unit membrane, 5-10nm thick. The width of the separating electron-transparent zone was estimated to be 5-15nm. The inner zone appeared to represent the cytoplasmic membrane and the outer zone the cell wall of the organism (Moll and Martin, 1974, Garnier and Bové, 1977).

The fact that the cell envelope of the greening organism is approximately 25nm thick and appears to include a cell wall, clearly distinguishes it from MLO's which possess a single, triple-layered unit membrane approximately 9-12nm thick (Hull, 1972). Further evidence of the bacterial nature of the organism was provided by studies on the effect of various antibiotics on greening. Miyakawa (1979) reported that treatment of likubin-infected ponkan citrus buds with penicillin resulted in the complete suppression of likubin symptom development when the treated buds were grafted onto indicator seedlings to determine remaining infectivity. Similar results were obtained with tetracycline and tylosine treatments.

Bové et al. (1980) reported that in a greenhouse study greening-infected citrus seedlings which had absorbed penicillin through their roots developed an abundant root system and produced new, vigorous, symptomless shoots. Similar results were obtained under field conditions with penicillin injected into the trunks of trees. The treatment had a marked effect on infected trees, inducing new, symptomless growth and significantly improving fruit quality and yield (Aubert and Bové, 1980). On the basis of these results, as well as on *in situ* ultrastructural studies (Garnier and Bové, 1977), Aubert and Bové (1980) concluded that the greening organism was not a mycoplasma, but "a prokaryote probably having a peptidoglycan-containing Gram negative type of cell wall".

In an earlier investigation, Moll and Martin (1974) compared the cell wall of the greening organism with the wall structure of Gram-negative phytopathogenic bacteria and rickettsia-like organisms. The authors reported that the organism associated with greening displayed a wall structure similar to that of Gram-negative bacteria, although a distinct peptidoglycan or R layer was not observed. The positive effect that penicillin treatment had on likubin-infected budwood (Su and Chang, 1976, Miyakawa, 1979), and on greening-infected citrus (Bové *et al.*, 1980, Aubert and Bové, 1980) provided indirect evidence for the presence of a peptidoglycan layer. Later, Garnier *et al.* (1984) unequivocally demonstrated the presence of a peptidoglycan layer between the inner and outer membranes of the cell envelope using cytochemical treatments. The existence of this layer provided further proof of the Gram-negative nature of the bacterium.

Investigations on likubin-affected citrus revealed pleomorphic bodies in the sieve tubes, some of which were round or oval, others filamentous. These bodies contained an electron-dense ground substance, although spherical bodies with electron lucent central areas were observed as well (Chen *et al.*, 1971). The existence of round or spherical electron-clear bodies (measuring 0.2-1.0 um in diameter) was also reported by Garnier *et al.* (1976),

although the elongated, electron-dense forms (0.15-0.25 um diameter) of the greening organism were most often the only structures present in the sieve tubes of infected plants. The round forms often displayed a distinct separation of the inner and outer cell envelope layers, possibly due to plasmolysis. Both forms of the organism have been observed in the sieve tubes of periwinkle (Catharantus roseus L.) infected with greening disease via dodder (Cuscuta campestris) (Garnier and Bové, 1983). The round and elongated forms probably represent different aspects of the same organism because they occasionally appeared to be connected (Garnier et al., 1976).

Su (undated pamphlet) described three stages in life cycle of the greening organism, namely the mature form of the pathogen (generally a rigid rod measuring 350-550 x 600-1500 nm), flexible elongated rods which grow into new organisms measuring 100-250 x 500-2500 nm, and old spherical bodies 700-800 nm in diameter with a thin cytoplasm. Electron microscopy using serial sections and a three-dimensional configuration was used to confirm the presence of the various stages and the respective dimensions. Su further reported that multiplication of the organism is generally accomplished by budding, althogh binary fission or beading also occurs, albeit less frequently. The investigation by Moll and Martin (1974)

also revealed that the organism appeared to replicate by a form of budding and, unlike mycoplasmas, the daughter cells were of unequal size. The dimensions of the elongated/filamentous forms were given as 350 x 3000 nm in this report.

1.2 SYMPTOMS OF GREENING

Greening was originally thought to be a disease of the fruit, the name "greening" being descriptive of the abnormal fruits produced by affected trees. The disease is, however, not restricted to the fruit, and other parts of the tree, including the leaves and roots, display symptoms of infection as well. Detailed descriptions of the symptoms induced by greening have been published by a number of authors (Matsumoto *et al.*, 1961, McClean and Oberholzer, 1965a, Fraser *et al.*, 1966, Salibe and Cortez, 1966, Schneider, 1966, Schneider, 1968, McClean *et al.*, 1969, Garnsey, 1988).

Although all presently used commercial citrus cultivars in South Africa are susceptible to greening, varietal reactions to the disease have been reported. McClean and Schwarz (1970) observed that certain cultivars and selections display a degree of tolerance to the disease.

Variations in leaf symptoms according to the citrus variety were also reported by Salibe and Cortez (1966). Matsumoto et al. (1961) and Van Vuuren and Moll (1984a) observed that the choice of rootstock affects the tolerance of the stock-scion combination to the disease. Garnsey (1988) noted that most sweet oranges, mandarins, and mandarin hybrids are severely affected, while grapefruit, Rangpur lime, lemons, calamondin and some pummelos show less severe symptoms. Mexican lime, trifoliate orange and trifoliate orange hybrids are the most tolerant and may develop only some leaf mottling.

Greening is often sectorial within a tree. Symptoms may be observed in only one or a few branches, the other limbs remaining apparently healthy. In the absence of the insect vector of the disease, this partially systemic pattern of infection can be maintained for the duration of the affected tree's life. This may be as a result of the relative immobility of the organism within the host phloem, which, as reported by Moll and Van Vuuren (1982), is probably due to the rigidity and proportionally large size of the organism relative to the pore size of the sieve plates. Systemic infection seems to depend on multiple insect inoculations (Schneider, 1968).

A seasonal and geographical variation in the expression of symptoms has been reported: leaf symptoms were reported to be more pronounced in winter than in summer and to show up more clearly in high, cool areas than in low, hot areas (Schwarz, 1968a). McClean et al., (1969) also noted the influer · of temperature and altitude on the severity of greening, adding that the improved appearance of diseased trees in some localities during summer may give the wrong impression of recovery.

McClean and Oberholzer (1965a) reported that affected sectors of a tree display a diversity of foliar chloroses that are similar to evidences of mineral deficiencies in citrus. The chloroses produced are influenced by the age of the leaves, the time of year the leaves unfold, and by the conditions under which the trees are grown. The most distinctive (and specific) symptom is a mottle resulting from an irregular yellow discolouration following the course of the midrib and larger veins of mature leaves. Because of this, the authors suggested that "mottle leaf" would be a more descriptive name for the disease than "greening".

Schneider (1968) divided the leaf symptoms observed on affected trees into two groups on the basis of time of appearance: primary symptoms which appear on leaves after

they mature normally, and secondary symptoms which appear on leafy shoots as they grow from branches with primary symptoms. Histological studies conducted by the author indicated that the yellow mottling and leathery aspect of affected leaves did not arise from immediate anatomical changes caused by the greening organism in the plant, but rather from secondary anatomical changes.

Recognition of the disease in the field is often difficult from symptoms alone, espesially in the case of Asian greening. Very similar leaf symptoms may be induced by a number of factors varying from nutritional disorders to the presence of other diseases such as root rots, gummosis, tristeza and exocortis (Catling, 1970b).

Chronically infected trees are sparsley foliated due to the premature abscission of the small chlorotic leaves borne on weakened twigs and the formation of multiple buds on defoliated branches also characterises severely diseased trees (McClean and Oberholzer, 1965a, McClean et al., 1969). Sparsity of foliage is also associated with dieback of twigs which later extends to whole branches, and the establishment of secondary fungal wood rot in main limbs and tl trunk completes the destruction of the tree (Fraser et al., 1966).

Stunting and out-of-season growth and blossoming are also features of the disease (Calavan, 1968), as are the leathery aspect and tendency of affected leaves to roll (Salibe and Cortez, 1966).

Fruits produced by infected trees are usually undersized and poorly coloured. Although fruits do colour, the colour of some never reaches the rich hue of normal fruits, and the side sheltered from direct sunlight does not mature and often remains green. This assymetrical maturation leads to greened fruit having a lopsided appearance, although some normal-shaped, full-sized specimens are produced (McClean and Oberholzer, 1965a, Schneider, 1968). The juice of affected fruit is low in soluble solids, high in acids and abnormally bitter (Garnsey, 1968), and many of them abort before harvest (Salibe and Cortez, 1966, Schneider, 1968, Frase et al., 1966).

The root system of diseased trees is poorly developed and although the fibrous roots appear normal, they are sparse and seem inadequate to support a tree normally (McClean and Oberholzer, 1965a). This and other symptoms suggest starvation effects caused by faulty translocation of nutrients within the tree, a fact which was confirmed by the investigations of Matsumoto et al. (1961) and Schneider (1968). Matsumoto et al. (1961) also found starch to be

absent from the roots of infected citrus, while in the corresponding parts of healthy control plants, starch had accumulated. In the leaves of diseased plants, large amounts of starch were found to accumulate which the authors suggested was due to blockage of the translocation of carbohydrates caused by phloem necrosis in the foliar vascular elements.

The occurrence of phloem necrosis in diseased tissue was also reported by Schneider (1968). He proposed that this is the first degenerative change induced in leaves upon invasion by the greening organism, and leads to the blockage of the translocation stream. As a result of this impediment, a number of anatomical aberrations occur, including massive accumulation of starch in the plastids. Starch granules within the chloroplasts were observed to become unusually large, stretching the organelle's outer membrane to a thin layer. Schneider suggested that this stretching destroys the grana and chlorophyll, and results in the mottled chlorotic symptoms displayed by leaves. The leathery feel of the leaves is also thought to be as a result of starch accumulation within the chloroplasts. The internal symptoms are thus consistent with the behaviour and the external appearance of infected citrus plants.

1.3 INDEXING OF GREENING

Schwarz (1968, 1968c) reported the presence of a fluorescent substance in the reproductive and vegetative tissues of sweet orange, mandarine and tangelos infected with greening. Chromatographic analysis revealed the presence of a bright violet fluorescent spot in the chromatographic profile at RF 0.08 in extracts of infected tissue, but not in healthy controls. The substance was identified as a monoglucose ester of gentisic acid (gentisoyl-B-D glucose) (Feldman, 1968 cited in Schwarz, 1968b).

The work of Schwarz (1968, 1968c) showed that specific fluorescence of the albedo of sweet orange fruits was strong enough to be observed with the naked eye under an ultraviolet lamp. The fluorescence was not very marked in the albedo of mandarins and tangelos and, consequently, it was not possible to apply this method of analysis to the fruits of these species. The marker substance was, however, found to be present in the bark of these species, and water extraction of the compound followed by chromatographic analysis made it possible to use the bark to index these species.

Schwarz (1968c, 1970) reported that the fluorescent compound could be detected in immature fruits and that the test was specific for greening-infected material. Fruits of trees exhibiting foliage abnormalities caused by mineral deficiencies as well as those of trees affected by exocortis, xyloporosis, tristeza, psorosis and dry root rot did not contain the marker substance. The author concluded that the fluorescence test could be regarded as reliable when sweet orange trees are uniform and grown under optimum conditions. Moreover, the test can be used in commercial indexing and is more sensitive than indexing based on leaf symptoms especially in areas where the symptoms are masked by climatic conditions or other factors.

A second marker compound was identified by Schwarz (1968d) which displayed a RF of 0.40 and was detected in the fruit albedo and bark of diseased sweet orange, mandarin and tangelo and occasionally in grapefruit, but this marker was not present as regularly as the gentisic acid marker.

Catling (1970a) conducted small-scale trials using methods of extraction and detection similar to those used for the gentisic acid marker to determine whether this substance, or a closely-related derivative, was present in the insect vector of greening. The marker was not found in extracts of the vector and no consistent differences were observed in the chromatographic profiles of infected and non-infected adults of T. erytreae.

The fluorescence test is very useful in that it is relatively simple to perform on plant material, can be adapted to large scale testing and gives immediate results. However, it is yet to be proven conclusively that trees testing positive are indeed infected with the greening organism and some workers have questioned the reliability of the test. McClean (1970) reported that results of chromatographic analysis of bark extracts were disappointing with many trees showing no symptoms of infection tested positive. Even after a nine year period of observation, none of these "positive" trees had developed symptoms of greening. Knorr et al. (1970) reported a similar situation in which some healthy-appearing trees showed the marker while some trees with symptoms of greening did not.

Further evidence of the unreliability of the test came from its practical application to the testing of nursery trees. McClean (1970) observed that a number of asymptomatic "negative" and "positive" nursery trees planted in an area apparently free of psylla and monitored over a two-year period showed no signs of infection. The trees were retested after this time in the same laboratory where the preliminary tests were done with the following results: of 19 initially negative trees, five were found to be positive, and of 26 initially positive trees, eight were still positive and 18 negative.

The inconsistent results reported by McClean (1970) and Knorr *et al.* (1970) may be due to the irregular distribution of the greening marker in infected trees. Gupta *et al.* (1972), in an attempt to find the most reliable single site for sampling trees, found that the marker may be present at one sampling site, yet undetectable at another. The authors concluded that in order to compensate for this anomaly, sampling should include roots or bud-union bark, as well as twigs in the canopy. A similar finding was reported by McClean (1970) who observed that when two bark samples from the same tree were tested, the results of the chromatographic analysis were not always in agreement.

1.4 ATTEMPTS AT ISOLATION AND CULTIVATION OF THE GREENING ORGANISM

Despite the problems associated with the fluorescence test, it is a valuable supplementary method for diagnosing greening. However, greater accuracy in the identifying of the disease would be possible with the aid of immunochemical technology.

Reports claiming the successful *in vitro* culture of the greening organism in artificial media have appeared (Ghosh *et al.*, 1971, Sodhi *et al.*, 1973, Garnett, 1985) but the

isolations were never fully confirmed. Numerous unsuccessful attempts have since been made by various researchers to isolate and culture or to purify the greening organism from infected material as the first step in the fulfilment of Koch's postulates and the production of a suitable antiserum for use in serological assays (Moll, 1974, Sibara and Garnett, 1980, Botha et al., 1984, Manicom, 1984, Duncan, 1985, Duncan and Garnett, 1985, Garnett, 1985, Garnier et al., 1987, Jiang, 1988, Mochaba, 1989, Smith, 1988, Sui, 1988, Villechanoux et al., 1990).

Utilising bacterial media, as well as media and conditions successfully developed for the cultivation of Spiroplasma citri, Moll (1974) was unable to isolate the greening organism from citrus tissues or infective insect vectors. Later, Sibara and Garnett (1980) reported the isolation of an organism of similar dimensions to the greening bacterium in situ. The isolate displayed gliding motility, growth at 37 C and possessed a cell envelope of 25-30nm in width. The authors, however, were unable to fulfill Koch's postulates.

Seven different genera of bacteria were isolated from both healthy and greening-infected citrus by Botha et al. (1984). In this study, isolates were obtained from all 283 diseased plants assayed, while 50 of the 53 healthy plants also yielded bacteria. The results clearly indicate that

both healthy and diseased citrus plants have an endogenous bacterial population, a finding supported by the work of Gardner et al. (1982) and Manicom (1984). The mere presence of bacteria in plants is therefore not sufficient proof of their phytopathogenicity, although the organisms could have an important biological effect on the plant.

Duncan (1985) and Garnett (1985) reported the consistent culture of a Gram-negative bacterium, apparently typical of the putative greening bacterium, from greened citrus. The bacterium was reportedly isolated from orchards in most of the major citrus growing areas of South Africa. In addition to the isolates displaying the morphology classically ascribed to the greening organism, they exhibited an antibiotic sensitivity comparable to that described for greening in situ. The morphology, the antibiotic sensitivity and the consistent culture of the bacterium from infected citrus was considered by Garnett (1985) sufficient evidence to justify calling the isolated bacterium "the greening organism". Subsequent electron microscopic studies of a number of these isolates have, however, revealed significant morphological differences between the isolates and the greening organism in situ described by Moll and Martin (1974) and Garnier and Bové (1977) (Chippindall, unpublished report, 1990).

Antisera raised against the cultured isolates were reported to successfully distinguish between healthy and greening-infected material with the ELISA (Duncan, 1985), although Garnier et al.(1987) and Chippindall and Whitlock (1989a, 1989b) were unable to repeat these results using the same antisera.

In an effort to repeat the successful isolation and in vitro cultivation of "the greening organism" reported by Sibara and Garnett (1980) and Garnett (1985), Manicom (1984) made over 75 isolation attempts from a variety of vegetative and reproductive citrus tissues, as well as from insect vector tissues. Although greening-infected and healthy citrus were found to contain a wide variety of microorganisms, no organism similar to the one described by Sibara and Garnett (1980) was isolated. In addition, antisera raised against this organism failed to detect the aetiological agent of greening in infected plant and insect material using standard indirect fluorescent antibody methodology.

Manicom (1984) tested the efficiency of various purification procedures for the extraction of the greening organism from citrus and insect material. Percoll and cesium chloride/sucrose gradients were found to be effective for the density separation of bacteria and plant

material, however the concentration of bacteria obtained by these methods was far too low to be of use. The results indicated that the greening organism has a low titre and erratic distribution in citrus, properties which complicate the purification of the organism. Unsuccessful isolation attempts using similar procedures were also reported by Davis (1989).

In the absence of cultured greening bacterium, Garnier et al. (1987) attempted the production of monoclonal antibodies against the organism from extracts of infected periwinkles (Catharantus roseus L.). Two hybridomas were obtained from mice immunized with preparations of phloem tissue which were found to be specific to greening-infected periwinkle as well as citrus with the ELISA and immunofluorescence assay. The antibodies were reported to react with both the Asian and African strains of the disease, but not with periwinkles individually infected with five other prokaryotic organisms. Subsequent findings have cast doubts on the suitability of these monoclonal antisera in the detection of greening. Jiang (1988) and Garnier et al. (1991) reported that the antisera failed to recognize strains of the the greening organism from other geographical areas. Similarly, Lee (pers. comm.) observed no reaction of the antisera with greening-infected material in his laboratory, although non-specific binding was observed with citrus infected with other phytopathogens.

In a recent report by Villechanoux *et al.* (1990), the monoclonal antibodies of Garnier *et al.* (1987) were used, apparently successfully, in an immunoaffinity chromatographic procedure to purify the greening organism from infected periwinkle plants. The bacteria isolated by the procedure were reported to be identical to the organisms observed in crude plant extracts on the basis of morphology and serological tests. No electron microscopic studies of the purified bacteria were, however, carried out in order to demonstrate the presence of the characteristic greening bacterium cell envelope in the isolated organisms.

Recently, high concentrations of the Asian strain of the bacterium were reported to have been isolated from infected periwinkle material (Sui, 1988). The purification procedure, involving enzymatic digestion of host plant material followed by differential centrifugation, did not affect the integrity of the organisms and appears to be suitable for use in obtaining sufficient bacteria for use as immunogen. The method was, however, unsuitable for extracting the organism from citrus leaf midribs and fruit columella affected by the African strain of the disease (Davis, 1989), possibly due to the low titre and erratic distribution of this strain of the greening organism within infected citrus.

Chippindall and Whitlock (1989b) reported the production of an antiserum against a crude preparation of greening-affected citrus leaf midribs. After exhaustive cross-adsorption against healthy plant material, the antiserum was able to distinguish between healthy and diseased citrus, periwinkle and dodder samples with the ELISA and dot-immunoblot (DIB) assays. The antiserum was, however, later found to be non-specific in that it reacted with citrus infected with a number of other organisms, both viral and prokaryotic (Chippindall, unpublished report, 1990). The results suggested that the antiserum may have been directed against a pathogen-related protein of host origin, rather than against the actual greening organism itself.

Recently, French workers raised monoclonals against several Asian and African isolates, which in combination may have potential universal use (Da Graca, 1991). A preparation of monoclonals prepared in South Africa using extracts of greening-transmitting psylla has been shown to react positively against infected citrus tissue (Da Graca, 1991).

Despite some initial encouraging reports, it is evident from the literature that a suitably sensitive, reliable and specific antiserum has yet to be raised. Little progress has been made in the development of a medium able to

support the *in vitro* growth of the fastidious aetiological agent of greening disease and in the absence of a means whereby this organism can be cultured, purified extracts of the bacterium function as the only source of immunogen.

1.5 THE INSECT VECTOR OF GREENING DISEASE

Oberholzer *et al.* (1963) cited [illegible] an and Oberholzer (1965a) suggested that greeni[illegible] spread by an insect vector, and further sug[illegible] the citrus psyllid *Trioza erytreae* (Del Guerc[illegible]d, either as the vector of a pathogen or beca[illegible] eeding on citrus leaves induced a type of toxicosis.

In an investigation into the spread of aphid-transmissible virus diseases, Schwarz (1964) obtained evidence of the existence of a new insect-transmissible virus in the Nelspruit district which he tentatively called "reticulata" yellows. The author, however, later recognised that the "new" virus and greening were identical. Results of exposing young seedlings in orchards containing differing numbers of greening-infected trees showed that, (i) the spread of the disease was more prevalent in orchards where greening disease was common (indicated by the positive correlation between the number of infected

test seedlings and the percentage of greening in the orchards), (ii) the disease could be transmitted by grafting to both Empress mandarin and sweet orange, (iii) the infected trap plants developed leaf symptoms similar to the symptoms of orchard trees affected by greening, and (iv) the disease was transmitted by a flying insect vector as trap seedlings protected by gauze cages did not develop symptoms. During the exposure the unprotected plants became infected with both citrus psylla and citrus aphid (Toxoptera citricidus (Kirk)), thus implicating these two insect species as possible vectors.

In a later paper by McClean and Oberholzer (1965b), the first positive transmission of greening by adult citrus psylla was reported. Nymphs of the psyllid, however, failed to transmit the disease. As the earlier study by Schwarz (1964) had implicated the citrus aphid as a carrier of greening, this insect species was also assessed for its ability to transmit the disease. The authors found that it was unable to vector greening but was able to transmit tristeza virus. Subsequent reports confirmed the citrus psyllid as a vector of greening (Schwarz et al., 1970, McClean and Schwarz, 1970, Catling, 1970a).

Moll and Martin (1973) provided electron microscope evidence that T. erythreae is a vector of greening. In their study electron microscopy revealed large concentrations of the greening organism in the haemolymph of psylla fed for 30 days on diseased citrus. The organisms were present in large numbers in body cavities, often adjacent to salivary glands, and occasionally within the salivary gland cells. This suggested a circulatory mode of transmission. Moll (1974) also reported the presence of the organism intracellularly within muscle and nerve tissue. The presence of the organism in the haemolymph and within cells is indicative of its ability to penetrate membrane and tissue barriers. The authors also examined psylla from healthy citrus and found that they contained no such organisms. Insects fed for 21 days on infected citrus contained only a few organisms. The dramatic increase in the number of greening organisms in the haemolymph of T. erythreae between the 21st and 30th day led the authors to conclude that the greening organism multiplies in the haemolymph of T. erythreae.

Conflicting reports have appeared regarding the efficiency of T. erythreae as a vector of greening. Based on results of a transmission study, McClean (1974) reported that the number of adult psylla that carry greening is small in relation to the total adult population: 300 adults

collected from infected material in Pretoria failed to transmit the disease individually, but several positives were recorded when test plants were exposed to groups of five or more adults. The percentage of carriers in the population may have been significantly greater, but it is not possible to exclude the fact that the failure of infected adults to transmit greening may have been due to a low efficiency of transmission. Catling (1970a) also reported a low (1.6%) incidence of carriers in tests on single adults collected off trees in Swaziland and noted, from field observations, that fairly high vector populations are needed before appreciable disease transmission occurs (Catling, 1969).

Van Vuuren and Da Graca (1978) obtained results from transmission studies which suggested that the transmission of greening by adult psylla trapped in a greening-infected orchard to healthy sweet orange seedlings is an efficient process and can occur within an exposure time of only 60 minutes. This finding is in contrast to the longer transmission times reported by McClean (1974) and is an important fact to consider when formulating a programme for the control of psylla.

Van Vuuren and co-workers (pers. comm.) recently showed that adult psylla are capable of transmitting greening after an acquisition feeding period of only one day. They also reported transmission rates of between 50 and 100 per cent with as few as 2 adult psylla per test plant, providing further evidence that the insect is able to transmit the disease more efficiently than previously thought (McClean, 1974). Moll (1974) and McClean (1974) maintained that a feeding period of at least 2 to 5 days was required for acquision. Another important fact revealed by the investigation of Van Vuuren and co-workers (pers. comm.) is that the full cycle from acquisition to transmission is completed in 8 days which is far less than the 21 to 30 days reported by Moll and Martin (1973).

The efficiency of spread of many plant diseases is affected by a number of factors including variations in the transmission efficiency of the vector. Investigations by both Catling (1970a) and McClean (1974) suggested a seasonal fluctuation in the transmission efficiency of T. erytreae (as measured in terms of the number of adults that are carriers of the disease). McClean (1974) recorded a high percentage of transmission during late spring and summer months, while adults that emerged during the winter failed to cause any infection. Catling (1970a) found that the highest transmission efficiency occured in spring and

early summer and the lowest efficiency in midsummer. Results recorded by the author at other times of the year were exceedingly variable.

Greening disease has been shown to be sensitive to high temperatures which may explain the seasonal fluctuation in the pathogen content of trees as reported by Schwarz (cited in Catling, 1970a). The author recorded the highest p rcentage of successful graft transmissions in the cooler months and the lowest in the hotter months of summer, suggesting a reduced concentration of organisms within the infected donor plants during the latter period. A temperature-related reduction in the number of organisms in trees during the hot months of summer also offers a possible expl nation for the higher rates of transmission by the insect vector recorded by Catling (1970a) during the cooler months of the year compared to the low rates recorded during summer. During the cooler periods, the titre of the organism within infected trees would be expected to increase leading to a greater probability of acquisition (and hence transmission) by a psyllid feeding on the source. This would affect the success of graft transmission similarly. The failure of adults emerging during winter to transmit greening (as reported by McClean (1974)) could be due to the fact that on the semi-dormant trees of winter, adults are forced to feed on the mature

leaves which contain far lower levels of inoculum than young flush (Moll and Van Vuuren, 1982), thus reducing the probably of the insect acquiring the pathogen during this period.

As the nymph stage of T. erytreae is sedentary, it probably plays no significant part in transmitting greening. Nymphs, however, can acquire greening when feeding on the young growth of diseased trees as indicated by the study of McClean (1974), but there may be some delay, into the adult stage, before such carriers are fully infective and capable of transmitting the disease. Results of serial transfers of adults to indicator plants suggested that the transmission efficiency of psylla that acquired the greening organism as nymphs appear to improve with time. In the same investigation, McClean obtained no evidence to suggest that greening is trans-ovarially transmitted in the vector, and that to become infective, psylla must have fed on diseased tissue as either adults or nymphs.

T. erytreae is sensitive to environmental conditions. The geographical distribution and activity of the insect is largely determined by factors such as altitude, ambient temperature and humidity; psylla are unable to tolerate very hot, dry conditions (Catling and Annecke, 1968) which explains why the insect is almost unknown in the hot

lowlands in the east of the Transvaal and in Swaziland. The effect of environmental conditions on psylla populations probably accounts for the convincing correlation reported between the incidence of greening and activity of the vector (Schwarz, 1967, McClean et al., 1969, Catling, 1970a). McClean et al. (1969) noted that both psylla and greening are plentiful in orchards around Tzaneen where the altitude ranges from 700 to 1370 metres, but not at Letaba and Letsitele just 25 to 30 kilometres to the east (altitude below 600 metres). Differences in temperature, relative humidity and flushing rhythms of the trees were given as reasons for the contrasting incidences of psylla and greening in these two localities.

Psylla populations are also controlled by factors such as flush quality of citrus, natural enemies, interspecific competition with citrus aphids and in some seasons intraspecific competition for breeding sites (Catling, 1970a).

While T. erytreae is the vector of African greening, Diaphorina citri has been shown to be the vector of the Asian strain of the disease (Capoor et al., 1967). T. erytreae is restricted to Africa south of the Sahara, and its presence in Madagascar, Mauritius, Réunion and Saint Helena has also been recorded (Catling, 1970b). It is

the only known vector of greening disease in Southern Africa, while in China, India and south-east Asia the vector of the disease is the oriental psylla, D. citri (Capoor et al., 1967, Martinez and Wallace, 1967, Catling, 1970b, Massonie et al., 1976). D. citri is widely distributed in the Orient and is present in Brazil, as well as in Réunion, but not in Africa (Catling, 1970b, Aubert et al., 1980). Greening-like diseases have been reported from 11 out of the 16 countries where this insect is known to occur on citrus (Catling, 1970b).

While T. erytreae does not tolerate hot and dry climates, D. citri resists high temperatures and low humidity. Consequently the Asian form of greening occurs not only in cool, elevated areas, but also in hot low-lying areas (Aubert, 1984). The two psyllid vectors as well as the two forms of greening are present on the islands of Mauritius and Réunion (Aubert, 1984). In Réunion the distribution of D. citri is from sea-level up to an elevation of 600 metres and that of T. erytreae generally above 600 metres, so greening disease extends up to an elevation of 1000 metres on the island (Aubert et al., 1980).

1.8 STRAINS OF GREENING DISEASE

The two forms of the disease were characterised by Bové et al. (1974) on the basis of temperature sensitivity studies. The African form is heat-sensitive and no symptoms are expressed when infected plants are kept between 27 and 32 C, while the Asian form is heat-tolerant and good symptom expression is observed in this temperature range. In addition, the Asian form is far more destructive than the heat-sensitive African form.

Although South Africa appears to be affected only by the less-destructive African strain of greening, Massonie et al. (1976) demonstrated that T. erytreae is capable of transmitting the Asian strain of the disease. Similarily, D. citri has been shown to be a potential vector of the African strain of greening. Transmission of the African strain by the Asian psyllid was obtained experimentally by Lallemand (1984) cited in Aubert (1984). These observations indicate that each type of psyllid is not necessarily bound to the greening organism of its geographical zone. This is of particular importance in the South African context as the introduction into this country of the more virulent Asian strain would represent a serious threat to the citrus industry as it could be spread by the local vector.

1.7 ALTERNATE HOSTS OF THE GREENING ORGANISM

In addition to being transmitted by tissue grafts, vegetative propagation and by psylla, greening disease has been shown to be transmitted by dodder (Cuscuta campestris), although this route of transmission is of little importance as far as the spread of the disease in the field is concerned. Garnier and Bové (1983) reported the development of peculiar yellowing symptoms on a periwinkle (C. roseus) plant connected to a sweet orange seedling infected with the Asian strain of greening. Subsequent electron microscope observations of thin sections of midribs from the symptomatic periwinkle revealed the presence in sieve tubes of prokaryotic microorganisms possessing the characteristic cell envelope of the greening organism. Transmission of the disease from periwinkle to periwinkle was subsequently achieved by graft inoculation. Similar results were obtained when an orange seedling infected with African greening was used in transmission experiments. The authors did not report on the relationship between the greening organism and dodder itself, but in an earlier investigation Ghosh et al. (1977) observed that the bacterium multiplied to high titres within two species of the parasite, C. campestris and C. subinclusa. The organism was found to multiply more favourably in both species of dodder than in its citrus host and the authors speculated on the possibility of using dodder as a potential alternate host.

A variety of herbaceous plant species were investigated as possible alternate hosts of African greening by Schwarz (1968b). Mechanical transmission from infected sweet orange to the herbaceous species resulted in only cucumber (Cucumis sativus cv. National Pickling) displaying a reaction after inoculation. The symptoms consisted of yellow lesions on the cotyledons which later collapsed. This was followed by yellowing and subsequent necrosis of the veins of the first true leaves, coupled with a reduction in vigour of the plants. A low percentage of positive mechanical transmission of the disease from cucumber back to citrus was reported, although this was not confirmed.

Capoor et al. (1975) endeavoured to mechanically inoculate cucumber cv. Long Green with extracts of citrus infected with the Asian strain of greening. A higher percentage of positive transmissions was reported in this study compared to that reported by Schwarz (1968b), and symptoms in the inoculated cucumber differed in some respects and were more severe. Also, leaves developing subsequently failed to show symptoms and back-transmission of the disease from symptomatic cucumber to both citrus and cucumber was not achieved. Incubating extracted sap of diseased citrus at 95 C for 10 minutes was found not to inactivate the agent inducing symptoms in cucumber. The evidence led the authors

to conclude that a prokaryotic organism such as a MLO was not involved in inducing the symptoms in cucumber, but rather a specific and active principle such as a vivotoxin produced by the aetiological agent of the disease in citrus.

1.8 CONTROL AND TREATMENT OF GREENING DISEASE

Control of greening disease can be attempted by effectively controlling the vector of the disease, or by controlling the greening disease organism itself. However, it is probably only through a combination of both these control measures that really effective reduction in the incidence of the disease can occur. The ultimate solution to the greening problem, however, appears to lie in breeding for resistance in citrus.

1.8.1 Control of the vector

Effective control of the vector is crucial to the containment of greening, especially during periods when enviromental and host plant conditions favour high psylla populations.

1.8.1.1 Biological control

At present, biological control of the citrus psyllid is not widely used as a means of control, although a number of biological agents effective against psylla have been identified and their potential as control agents in the field investigated.

Aubert (1984) reported a drastic reduction in the number of psylla on Réunion Island following the release of two parasitic chaloid Hymenoptera into citrus orchards on the island. Active ectoparasitism by Tetrastichus radiatus (Waterston) shrank the D. citri population from large colonies to small localised aggregations, while parasitism on T. erytreae by Tetrastichus dryi (Waterston) led to the complete disappearance of the psyllid from the orchards. As a result of the action of the two eulophid parasites, new orchards planted on Réunion were kept either free of greening, or the rate of disease progression in the orchards was extremely low. This method of vector control, although more specific and less costly than other practices, is difficult to implement and the outcome remains less predictable (Aubert, 1987). Phyllaephagus pulvinatus has also been reported to be a good endoparasite of T. erytreae in Réunion, although it does not thrive as well as the eulophid T. dryi (Aubert and Quilici, 1984).

In an ealier study carried out at Malkerns, Swaziland, Catling (1970a) found that naturally occuring parasites of T. erytreae were not an important regulatory factor. Two parastites were found to consistently attack the third, fourth and fifth instars of T. erytreae. Tetrastichus radiatus was the main parasite of the psyllid but was at times challenged in importance by P. pulvinatus. The degree of parasitism was found to be variable and depended on the presence of a favourable synchrony between the parasites and the host. Effective parasitism rarely exceeded 50%, and under conditions of poor synchrony it dropped below 10%; these levels of parasitism were found not to prevent the insect from regularly surging to high population densities. Regular applications of insecticides had no marked effect on the parasite complex. Predators of T. erytreae (mairly the nymphal stages) were also reported. These included green lacewings, Chrysopa spp., a brown lacewing, Micromus sjoestedti (Weele), syrphids, a coccinellid, and several spiders and mites. The incidence of predation by these species was irregular, assuming greater importance from midsummer to midwinter in the area under observation. No microorganisms appeared to be associated with the psyllid.

Van Den Berg (1984) reported that predators, especially spiders, play an important role in the reduction of citrus psylla populations in orchards under integrated spray programmes, yet are unable to maintain an acceptably low level of the pest. This suggests that predatory control on natural host plants of the vector not exposed to insecticides may be even better and probably keeps the number of psylla that move between these natural host plants and orchard trees at lower levels.

The fungus Cladosporium sp. nr oxysporum has been reported as a secondary fungus on certain insect cadavers and also as a primary cause of death of certain Homoptera and was assessed as a biological control agent of T. erytreae by Grech and Samways (1984). The authors reported that applications in the field and in the laboratory of the pathogen grown axenically caused substantial mortality in populations of the insect, death of infected insects probably being due to a toxin produced by the fungus, rather than by direct hyphal penetration. Despite the definite results obtained in the study, the authors concluded that Cladosporium sp. nr oxysporum has severe limitations against citrus psylla in Southern Africa, the reason being that the pathogen exhibits a density-dependent response in its parasitism of the insect and is therefore unsuitable for the control of low-level insect populations.

The insect is an efficient vector of greening disease, and normal control therefore aims at keeping orchards completely free of psylla. Also, the fungus is sensitive to dessication which limits its use under normal field conditions.

1.8.1.2 Chemical control

Because biological control is usually only effective when large numbers of the host are present, chemical control of the citrus psylla is generally the preferred method. Many chemicals can be used to eliminate the citrus psylla at various stages of its life cycle. When frequent foliar applications of a chemical are used, care has to be taken to ensure that this does not affect the level of control exerted on the target pest by naturally-occuring or introduced biological agents in the field, especially in an integrated control programme. Pesticides which are relatively specific and which have a short residual effect are therefore favoured for foliar sprays. Also used are systemic compounds which have a negligible effect on benefical phyllosphere organisms. These are applied either to the soil or introduced into the trunks of trees.

Catling (1970a) tested the efficacy of a number of compounds against all stages of T. erytreae under insectary conditions. Temik, parathion, malathion, endosulphan, dimethoate, mineral oil and lime sulphur all gave some degree of control, but dimethoate (Rogor) was considered to be the most promising as it was effective at low concentrations against eggs and nymphs (0.0025% a.i. [active ingredient]) and adults (0.01% a.i.). Such low concentrations are inexpensive and were found not to disturb integrated control programmes. In addition, dimethoate possesses both contact and systemic action and can be applied either as a foliar spray or as a soil-applied systemic insecticide, although Catling found foliar sprays to be the more effective method of application.

Another formulation of dimethoate, Perfekthion, has also been shown to be effective against psylla. Wortmann and Schafer (1977) reported excellent control of psylla populations with soil applications of this compound. This route of application eliminates the destruction of beneficial foliar organisms and also requires less effort and labour. It is possible, however, that infective psylla may survive long enough on trees treated with Perfekthion to transmit greening (Van Vuuren and Da Graca, 1978). In the same publication, the authors reported the effects of

foliar applications of monocrotophos, an insecticide registered for use against psylla in South Africa, on the transmission of greening by psylla. The results indicated that monocrotophos (0,01% a.i.) remained effective in controlling psylla throughout the 29 day period of the study, but after four days its action was too slow to prevent transmission.

In order to be effective in preventing transmission of greening, an insecticide must be able to kill psylla alighting on treated plants within at least 30 minutes as an exposure time of this duration has been shown to be sufficient for transmission of the disease to occur (Van Vuuren and Da Graca, 1978).

Milne *et al.* (1978) reported that timing of pesticide applications is an important factor. In the nursery, applications of dimethoate should be made every 6 to 7 weeks, while in the field, treatment should be applied as soon as the first signs of psyllid adults or eggs are seen on the new flush with follow-up treatments being applied 6 to 7 weeks later if psyllid pressure is still high. This treatment not only suppresses psyllid population numbers, but is also successful in controlling the black citrus aphid (*Toxoptera citricidus*) and red scale, two important pests of citrus in South Africa. Milne *et al.* (1978) advocate the concurrent application of tetracyclines to reduce the risk of spread of greening disease to a minimum.

Control of the vector of Asian greening, D. citri, can be achieved by four weekly sprays of 0.03% dimethoate, 0.012% methyl demeton, 0.085% phosphamidon, or 0.02% monocrotophos (Nariani and Bhagabati, 1980). Effective control of the insect has also been obtained with a 2% spray of China berry seed oil, a strong feeding inhibitor of the psyllid (Huang and Liu, 1985, cited in Wu and Faan, 1988).

It is unlikely that the spread of greening disease will be attained simply by the eradication of the vector in orchards. T. erytreae has been reported to complete its development on at least 18 different plant species, including various indigenous rutaceous plants, which can serve as natural reservoirs of the insect (Begeman, 1984, Aubert, 1987). The possibility that these species serve as reservoirs of the greening organism itself appears not to have been investigated. It is possible that the citrus psyllid may also utilise non-rutaceous plant species as a food source (Begeman and Botha, 1984). Thus, a disease management scheme consisting only of vector control in the orchard is unlikely to be effective.

Begeman (1984) reported that progress in the control of greening at Zebediela Estates has been made by adopting the following measures: establishing a nursery and new orchards away from natural reservoirs of the insect, planting only

greening-free seedlings in the orchards, removing greening-infected trees from orchards, effectively controlling the vector with applications of dimethoate and endosulfan, and injecting affected trees with antibiotics in an attempt to rid affected trees of the disease.

1.8.2 The response of greening to heat therapy

Greening-infected budwood can serve as an important source of inoculum, however shoot tip grafting and thermotherapy have also been shown to be efficient means of freeing material of greening. Su and Leu (1972) showed that the likubin pathogen was easily inactivated by dipping infected scions in water at 48 to 54 C for 10 minutes. Su and Chang (1976) reported recovery from disease when likubin-infected seedlings were heated at 40 C in a hot-air chamber for 10 days. No remission of symptoms occured with exposure times of between three and seven days, and treatments of longer than 13 days were reported to injure plants. Treatment of likubin-infected seedlings with a day/night temperature cycle of 40/30 C for four weeks or longer was shown to render seedlings free of disease symptoms (Su and Chang, 1976, Huang, 1978).

Symptoms induced by the African strain of greening have also been shown to be suppressed by thermotherapy. Plants infected with the disease failed to develop symptoms when kept at 32 C for 6 months (Garnier and Bové, 1983). A similar suppression of symptoms was observed when greening-infected sweet orange plants were kept at a temperature of between 27 and 32 C for 18 weeks (Bové et al., 1974).

1.8.3 Control of greening with antibiotics

Since the report of Lafleche and Bové (1970) in which it was suggested that greening is caused by a MLO, the use of antibiotics such as tetracycline and other chemotherapeutants has been investigated as a means of controlling the disease. Antibiotics of the tetracycline group have been tested as control agents against phytopathogens affecting a number of different plant species (Davis et al., 1968, Nyland and Moller, 1973, Hunt et al., 1974, Rosenberger and Jones, 1977) including citrus (Schwarz and Van Vuuren, 1971, Igwegbe and Calavan, 1973, Capoor and Thirumalachar, 1973).

Treatment of greening-infected citrus trees with tetracycline HCl was shown by Schwarz and Van Vuuren (1971) and Schwarz et al. (1974) to significantly lower the

percentage of greened fruit on the trees and the positive effects of the treatment were observed for several years after a single application of the antibiotic. The trees were not, however, completely cured of the disease. Similar results were obtained in a later investigation by Van Vuuren (1977).

In tests carried out by Schwarz and Van Vuuren (1971) the antibiotic was administered to the trunk of affected trees by gravity feeding from plastic containers, while Schwarz et al. (1974) and Van Vuuren (1977) used pressure from modified brass blowlamps to administer the antibiotic. The results obtained in the different trials indicated that the treatment was less effective when tetracycline was applied under pressure. The differences observed may be due to the fact that excessive pressure severely damages the conductive tissue of trees, reducing the xylem to a brown spongy consistency. This obviously affects the translocation of the antibiotic, nutrients and water within the vascular bundles of the plant (Goodwin, pers. comm., Van Vuuren, 1977). As a method of application, however, pressure transfusion does have advantages over gravity feeding in that it promotes a better intake and longer retention of the antibiotic (Chiu et al., 1979a).

Despite the amelioration of greening symptoms reported with tetracycline treatments, a number of serious problems are associated with it. Because tetracycline is poorly soluble in water, an effective dose of the antibiotic (up to 18g per tree) requires between one and six litres of water for solubilisation. Adsorption of this large volume requires drilling a number of 9mm diameter holes in the trunk of the tree and the application of positive pressure to the solution. Depending on the time of the year (which affects the strength of the sap flow in the trees) greater or lesser pressures are required to introduce the solution into the trunk (Goodwin, pers. comm.). The drilled holes may take many months to heal during which time the trees may become infected with various diseases (Begeman, pers. comm.).

In an attempt to overcome some of these problems, a micro-injection system was developed. This technique is, however, not without its own problems. Because only a small volume of solution can be administered, a highly concentrated solution of antibiotic is required which is absorbed very slowly because of its viscosity and because the tetracycline HCl starts precipitating within four minutes under pressure (Buitendag and Bronkhorst, 1983)

Foliar application of tetracycline is a non-invasive method of application. Martinez et al. (1970) and Capoor and Thirumalachar (1973) showed that solutions of the antibiotic applied via this route suppressed symptoms in greening-affected trees. However, Capoor and Thirumalachar (1973) found stem injection treatment to be far superior to foliar sprays in suppressing symptoms. Leaf sprays have been reported to be very inefficient as a way of introducing antibiotics (McCoy, 1976, Rosenberger and Jones, 1977) and this method has little value as a field operation in disease control.

Antibiotics can be absorbed by plant roots when applied as soil drenches, but this method generally requires a high dosage and, in the case of oxytetracycline HCl, much of the antibiotic activity is immobilised by clay and organic substances in the soil, rendering the activity uptake by plants rather inadequate (Gonsalves and Tucker cited in Chiu et al., 1979b). Root application is therefore impractical in a field programme for disease control. As the greening organism is restricted to the phloem of infected plants, the only effective method of application would seem to be by stem injection (Milne et al., 1978).

Although trunk applications of tetracycline HCl have been shown to suppress disease symptoms, treated trees fail to recover completely (Van Vuuren, 1977) and symptoms generally reappear sometime after the initial treatment (Capoor and Thiramalachar, 1973). Resurgence of disease symptoms may be due to the lack of persistence of antibiotic activity in fruit and leaves of treated plants as shown by Chiu et al. (1979b), or due to the lack of translocation of the antibiotic to all parts of the plant (Chiu et al., 1979b). The authors detected no antibiotic activity in either the roots or the bark of plants that were transfused with the compound. The failure of tetracycline to reach these parts would allow the greening organism to survive there and later function as a possible source of reinfection once the level of antibiotic activity had dropped below an inhibitory level in the other parts of the plant. Reinfection of treated plants by infective psylla cannot be discounted as reason for the reappearance of disease symptoms.

Time of application of tetracycline has been reported to influence the recovery index, with greater successes being recorded when the sap flow in trees is strong (Goodwin, pers. comm.). A strong sap flow probably results in more efficient translocation of the antibiotic within the plant. Pruning off diseased twigs soon after transfusion was found

by Su and Chang (1978) to improve the effectiveness of trunk injections with tetracycline HCl. The authors speculate that the transfused antibiotic was unable to diffuse to all the tips of diseased twigs due to low transpiration caused by the small diseased leaves on such twigs. Pruning the diseased terminal twigs after transfusion would eliminate any untreated inoculum in these parts of the plant.

More recently, research has shown that a member of the tetracycline group of antibiotics, rolitetracycline, has a highly beneficial effect on greening-infected citrus trees and will bring them back into production during the same season in which it is administered (Gaenssler, Goodwin and Marshall, unpublished report). Moreover, certain properties of the antibiotic make it an attractive alternative to tetracycline HCl and other members of this group of antibiotics, the application of which suffers from numerous practical problems.

Rolitetracycline (PMT) is highly soluble at neutral pH, it does not form insoluble residues, and distributes better in trees (Gaenssler, Goodwin and Marshall, unpublished report). These characteristics make it ideal for use with the micro-injection application technique. Buitendag and Bronkhorst (1983) reported that PMT applied at half the

dosage required for tetracycline HCl gave the same degree of greening control. PMT had the added advantage that discolouration of the xylem was considerably reduced, adsorption took half as long as in the case of tetracycline HCl, and, unlike tetracycline HCl, PMT could easily be absorbed by the tree in winter when the level of transpiration was reduced.

Despite the beneficial effects of antibiotic treatments, this method of greening control suffers from the major drawback that resistance to the antibiotics can occur rapidly because at the relatively low concentrations of tetracyclines used their action is bacteriostatic rather than bactericidal (Van Vuuren and Moll, 1984b). Also, reinfection of successfully treated trees may still occur, even with the use of insecticides (Van Vuuren and Da Graca, 1978). Future control of the disease should therefore be aimed at an efficient vector control programme and tolerant or resistant citrus cultivars.

Van Lolyveld and Van Vuuren (1988) found a positive correlation between peroxidase activity and tolerance exhibited by citrus cultivars to greening. Levels of peroxidase activity were found to be highest in leaves of the greening tolerant cultivar Tahiti lime and decreased with increased susceptibility, the highly susceptible sweet

orange and Mineola exhibiting the lowest levels of activity. The researchers suggested that peroxidase activity in mature leaves can be used as a means of assessing the susceptibility of citrus cultivars to greening disease, thereby providing an alternative to the long-term testing of hybrids bred for greening resistance.

1.9 METHODOLOGIES

1.9.1 Koch's postulates

1.9.1.1 Isolation of the greening organism from infected material

The isolation and culture of the aetiological agent of greening disease *in vitro* would be of great significance as it would facilitate not only the characterization of the organism, but also the development of a sensitive, specific serological or nucleic acid-based detection system for the disease. In addition, it would be the first step in confirming, by the fulfilment of Koch's postulates, that the prokaryotic organism observed by various workers in infected plant and insect material is the causative agent of the disease.

The recognition that greening disease was associated with structures resembling prokaryotic microorganisms (Laflèche and Bové, 1970b) raised early hopes that the disease agent would be cultured and its aetiological role confirmed. Many attempts have since been made to isolate the causal agent utilizing methodologies developed for a variety of fastidious microbial pathogens. Early reports claiming the successful *in vitro* culture of the greening organism (Ghosh *et al.*, 1971, Sodhi *et al.*, 1973) were inaccurate as the organisms isolated were mycoplasmas and were therefore probably contaminants. A later report by Garnett (1985) claiming the isolation and culture of the aetiological agent of the disease was never confirmed and must therefore remain highly questionable. The report, however, alludes to the fact that the organism described is the causative agent of greening disease, despite the lack of conclusive experimental evidence in support of the author's claim. In the same journal, reports by Ariovich and Garnett (1985) and Duncan and Garnett (1985) present further circumstantial evidence to support the claim made by Garnett that the bacterium is the aetiological agent of greening disease. Attempts to repeat aspects of this work performed by these authors in this laboratory and by other workers have met with little success. In a later publication, Ariovich and Garnett (1989) reported the recognition of bacteria in greening-infected plant material

by an antiserum raised against one of the bacterial isolates from infected citrus. The authors felt that this finding further supported the contention that the cultured organism was in fact the causal agent of citrus greening disease.

The note of caution sounded by Maramorosch (1972) in the early stages of research into the isolation of the aetiological agent of the yellows diseases probably applies just as much to the work carried out on the isolation of the citrus greening organism.

Plant pathogenic bacteria can conveniently be divided into 1) those that grow on stardard bacteriological media and 2) those that do not. When attempting to isolate a bacterium about which little is known, it is usual practice to employ several different common broth and agar media. If growth of the organism is not supported by standard media it becomes necessary for the researcher to investigate the more complex media developed for organisms such as mycoplasmas and spiroplasmas which are difficult to culture axenically. There is obviously also scope for developing new culture media and investigating innovatory techniques such as cultivation in embryonated hen's eggs and insect cell cultures (reviewed by Liao and Chen (1982) and Eden-Green (1982)). Nienhaus et al. (1978) reported the successful use

of chick embryos to cultivate Rickettsia-like organisms (RLO) from yellows-diseased grapevines. The authors observed a high concentration of the RLO in the chorioallantoic fluid and chorioallantoic membrane of eggs 10 days after inoculation with extracts of root material from diseased grapevine.

In order to verify the hypothesis proposed by Garnett (1985) and Ariovich and Garnett (1989) that the cultured bacterium is the causal agent of greening, it is necessary to fulfil the criteria laid down as logically essential to establish the causal relationship between a specific microorganism and a specific disease, viz. Koch's postulates. The fulfilment of these postulates would unequivocally demonstrate the pathological significance of the isolate in greening disease.

1.9.1.2 Inoculation of plants and insects with cultured isolates

In addition to the fulfilment of Koch's postulates, a knowledge of the relationship between a putative greening isolate and the insect vector of the disease would be an aid in determining the aetiological role of the isolated organism. Moll and Martin (1973) and Moll (1974) reported

that after ingestion of the organism, the insect becomes capable of transmitting the disease and that the greening bacterium replicates within the insect and is capable of penetrating various host membrane and tissue barriers. The demonstration of an isolate's ability to multiply and spread within T. erytreae would therefore serve to further substantiate the pathological significance of the cultured bacterium and its causal relationship with greening disease.

The inoculation of a susceptible plant species with a particular insect-vectored phytopathogen via the vector is a feasible alternative to mechanical inoculation of the plant host with the cultured organism. Moreover, in the case of a number of phloem-limited pathogens, inoculation of plants via this route may be the only means of effectively introducing the organism into a plant.

Initial attempts at inoculating healthy citrus by immersing excised leaf discs in a suspension of the cultured isolate and then grafting the discs back into the same leaves was unsuccessful (M. Rota, pers. comm.). Mechanical inoculation of citrus by stem injection as well as inoculation of plants via T. erytreae (the isolate having been introduced into the vector via either membrane feeding and micro-injection) are alternative methods which may be used

in attempt to fulfil Koch's postulates. Furthermore, demonstration of the insect vector's ability to transmit the isolate may serve as additional evidence of the aetiological relationship between the bacterium and greening disease.

1.9.1.3 Alternate host plants

Periwinkle (Catharantus roseus L.) has been shown to be an alternate host of the greening organism (Garnier and Bové, 1983) while cucumber was reported to develop various symptoms when mechanically inoculated with extracts of greening-infected citrus (Schwarz, 1968b, Capoor et al., 1975). The inclusion of these two plant species in transmission trials with a putative greening isolate is therefore relevant. Dodder has been shown to be an alternate host plant of the greening organism (Ghosh et al., 1977) as well as functioning as a vector for the transmission of the greening organism between susceptible plant species (Garnier and Bové, 1983).

1.9.2 Detection of greening disease

1.9.2.1 The need for a detection assay for greening

Infected propagative material is a major source of primary inoculum for most of the important phytobacterial diseases of food crops, including citrus greening. If introduced into an established orchard to replace mature trees uprooted because of disease, or if used in the planting of new orchards, greening-infected citrus material can serve as a potential source of the disease for dissemination by the insect vector of the disease. Thus, the production, testing and subsequent planting of pathogen-free material is an important means of control of this and other crop diseases.

The use of symptom observation alone as a criterion for infection with greening is unreliable as the expression of "greening-like" foliar chloroses may be due to abiotic factors such as micro-element deficiency, as well as to the presence of pathogens other than greening (Catling, 1970b).

Although the fluorescense test of Schwarz was reported as being specific for greening disease and of use in the commercial indexing of citrus (Schwarz, 1968c, 1970), the test has been found by some authors to be unreliable

(McClean, 1970, Knorr et al., 1970). Also, the test is not applicable to the insect vector of the disease (Catling, 1970a).

In the absence of a definitive detection system for greening, such as a serological test, certification programmes are based primarily on visual inspections of the growing crop for the detection of the disease. Plants showing no visible signs of infection are generally presumed free of the pathogen and hence any propagating material harvested from symptomless plants is generally considered "clean". This practice may lead to the collection of infected material from susceptible cultivars which appear symptom free.

1.9.2.2 Development of a serological detection system

Diagnostic serological assays for plant pathogens other than viruses are being developed with increasing frequency (Schaad, 1979, Johnson et al., 1982, Malin et al., 1985, Moyer and Echandi, 1986, Anderson and Nameth, 1990). The vast array of antigens contained in organisms such as bacteria and fungi (compared with viruses), together with the likelihood that closely related epiphytic or endophytic saprophytic organisms may be present in or on the same plant tissues, has complicated the development of

serological detection systems for these organisms (Schaad, 1979). For an antiserum to be useful it should, ideally, recognize the fewest antigens common to both the pathogenic and non-pathogenic strains of a particular bacterium (Moyer and Echandi, 1986). In addition to antigens shared by pathogenic and non-pathogenic bacteria, the possible existence of antigens common to both the target organism and the host plant may further complicate the development of an effective diagnostic serological assay.

Serology is perhaps the most useful method available for rapid detection and presumptive identification of bacteria. Serological tests do, however, have limitations, the major disadvantage of *any* serological assay being that a positive result is not absolute, but supplemental in many cases.

The production of an effective greening-specific antiserum and its use in a rapid, reliable and sensitive serological assay for the detection of *greening* in both plant and insect material is clearly of vital importance. A means whereby the disease can be detected in the early stages of infection, prior to the development of visible symptoms, is necessary for the effective management of greening.

Attempts have been made to develop serological detection systems for the disease. Using extracts of either cultured bacterial isolates or infected plant material as immunogen, both monoclonal and polyclonal antisera have been raised in a number of laboratories and employed in different detection assays with varying success (Duncan, 1985, Garnier *et al.*, 1987, Smith *et al.*, 1988, Koreten *et al.*, 1991, Garnier *et al.*, 1991, Chippindall and Whitlock, 1989a, 1989b).

Although a polyclonal antiserum raised against the cultured bacterium was reported to detect greening in the ELISA assay (Duncan, 1985) and in electron microscopic studies using colloidal gold as an immunohistochemical marker (Ariovich and Garnett, 1989), the antiserum has been reported to be unreliable by other workers (Garnier *et al.*, 1987, Chippindall and Whitlock, 1989a, 1989b). The antiserum did not always distinguish between healthy and greening-infected samples and showed inadequate specificity, possibly due to cross-reactivity with either host plant components or epiphytes on or in susceptible plant tissue. Problems such as these may be overcom with the use of the monoclonal antibodies as these are more specific than conventional polyclonal antisera.

Initial results obtained with monoclonal antibodies have been promising but thus far none of the seroreagents developed have been shown to be suitable for universal application. Monoclonals raised against Indian and African greening recognized greening isolates from Reunion, India and the Philippines (Garnier et al., 1987), but not from China (Jiang, 1988) and other geographical areas (Garnier et al., 1991) which suggests that the antibodies were too specific. Taking this into account, as well as the high cost, technology required and time involved in the production of monoclonal antibodies, the development of a high quality polyclonal antiserum is clearly desirable.

1.9.2.3 Improvement of detection assay results through increased antiserum specificity

The diagnostic sensitivity of enzyme immunoassays varies with the antiserum and the type of procedure used and, ultimately, the efficacy of a particular serological detection assay is determined by the quality of the antiserum used. Ideally a polyclonal antiserum should exhibit a combination of high titre and high specificity if it is to prove effective in a diagnostic assay (Schaad, 1979). The specificity of an antiserum is usually determined by the nature and relative purity of the immunogen preparation used for injection. The multitude of

antigens present in bacterial pathogens, together with the increased probability that antigenically related pathogenic and non-pathogenic organisms may occupy the same host tissues, has made the development of diagnostic assays for bacteria more complex (Schaad, 1979). Various preparations of the modified bacterial immunogen can, however, be used for the immunization of laboratory animals which may lead to the improvement of the quality of the resultant antiserum.

Where an antiserum is produced against a phytopathogenic bacterium, the use of whole, live bacterial cells has been found to be adequate (Nambiar and Anjaiah, 1985, Anderson and Nameth, 1990). However, by using preparations of heat-killed cells for immunization, workers have observed a resultant improvement in the specificity of the antiserum (Schaad, 1979). A number of other procedures have been used to pre-treat immunogen for the production of highly specific antisera. LiCl-extracted membrane protein complexes from various microorganisms have been used as immunogens in order to raise specific sera for use in identifying plant-pathogenic bacteria (Yakrus and Schaad, 1979, Chang and Schaad, 1983, Azad and Schaad, 1988). A lipopolysaccharide preparation from Agrobacterium tumefaciens was shown to induce the production of strain-specific antibodies (Bouzar et al., 1988).

Polyacrylamide gel electrophoresis (PAGE) (with or without sodium dodecyl sulfate [SDS]) is another technique which can be used to identify and isolate specific immunogens from a complex mixture of substances. The technique has been used by a number of workers to isolate specific peptides from mixtures of proteins for use as immunogen the production of specific antisera (McMillen and Consigli, 1977, Renart et al., 1979, Tjian et al., 1974, Boulard and Lecroisey, 1982, Teesmer and Derrick, 1989). Mausher (1990) isolated specific proteins from blighted citrus trees by SDS-PAGE and used these as immunogens in the production of a polyclonal antiserum. The antiserum was found to be specific for blighted citrus. Using the Western blotting procedure, the author showed that the IgG fraction of the antiserum reacted with extracts of blighted citrus, while a negative antiserum response was observed with citrus material infected with tristeza virus and with material from trees stressed by water deprivation and zinc deficiency.

The denaturing conditions of SDS-PAGE may be a disadvantage in the preparation of immunogen as an antiserum raised against a denatured protein may not be able to recognize exposed antigenic determinants in folded proteins (McMillen and Consigli, 1977). Denaturation of immunogen, however, may not affect the recognition of a particular protein in

its native state in all instances. Tjian et al. (1974) reported that antiserum raised against denatured Bacillus subtilis σ Factor was able to precipitate the native protein in diffusion assays. Boulard and Lecroisey (1982) reported the use of SDS-PAGE to purify insect proteins for use as immunogen in the production of specific antisera. The authors observed that neither staining of the protein with Coomassie Blue nor denaturation of the protein with SDS was found to modify the immunogenicity of the protein trapped within the acrylamide gel. The preparative separation of viral polypeptides by SDS-PAGE for use in the production of monospecific antisera has also been reported (Teasmer and Dernick, 1989).

Antisera raised against crude preparations of immunogen which display unacceptable levels of cross-reactivity may be rendered more specific by procedures such as adsorption. The procedure generally renders an antiserum specific for a given antigen (Lommel et al., 1982, Moyer and Echandi, 1986). Exhaustive cross-adsorption against healthy citrus material and various non-related bacteria may thus significantly improve the specificity of a polyclonal antiserum raised against either a cultured bacterial isolate or an extract of greening-infected citrus.

If lack of antiserum specificity is an unavoidable problem in a procedure such as ELISA, a method such as preparative immunofluorescence microscopy (PIFM) may prove useful. This technique should also be useful in the detection, as well as in the enumeration, of phytopathogens which occur in plant and insect vector tissues in low numbers. The procedure was adapted by the author from the direct epifluorescent filter technique (DEFT) developed by Pettipher and Rodrigues (1982) for the enumeration of microorganisms in foods. PIFM combines membrane filter techniques with immunfluorescent microscopy. The technique involves the enzymatic digestion and removal of debris from homogenates of infected plant material followed by the concentration of microorganisms by membrane filtration. Trapped microorganisms are reacted with a specific antiserum followed by reaction with a secondary antibody conjugated to a fluorochrome. Microscopic observation of membranes reveal the presence or absence of particular organisms by visualizing fluorescing bacterial cells. Thus, rather than simply observing a precipitin band or measuring an enzyme reaction, PIFM allows the worker to discriminate morphology of the stained target organism vs. non-specific reactions from host components and contaminating cells. Until optimal antisera are developed for a particular antigen, this feature makes PIFM particularly useful as the additional information is valuble for assessing the validity of serological reactions.

The major disadvantage of the PIFM is the subjectivity of analysis, the need for specific equipment such as a fluorescent microscope, and the time and expense involved in sample preparation and examination

In addition to increasing the specificity of an antiserum as a means of improving the efficacy of a serological detection assay, simple modifications of the basic assay procedure such as altering incubation times and temperatures and including various blocking steps may also lead to improvement of test results.

1.9.2.4 Improvement of assay results through the alteration of sample preparation procedures

The successful detection of bacteria in plant material is, to a large extent, dependent upon the development of appropriate procedures to efficiently extract and isolate the desired target pathogen. Diagnostic assays necessarily must be designed to detect and identify the least number of cells possible and the primary intent of an extraction scheme should thus be to optimize the percent target recovery. Plant material serves as a reservoir for a wide variety of saprophytic organisms and effective target recovery depends upon minimizing the competitive influence of these saprophytes on the target, as well as upon the minimization of interference by host tissue debris.

The procedure used for the preparation of samples for analysis in detection assays may significantly affect the efficacy of a particular test. A simple step such as the vigorous pre-washing of leaf tissue reduces the surface microflora and debris carried on the sample and minimizes interference and potential cross-reactions of saprophytes. Tissue maceration is a commonly used method employed to release target organisms from plant material, however, maceration can also increase the release of interfering substances and toxic materials, thereby decreasing the sensitivity of the assay.

Certain applications of ELISA are limited by steric problems but these may be overcome by adopting different approaches to the method of sample preparation. Large particulate-type antigens such as bacterial cells do not bind strongly to the assay plates and are easily dislodged during washing. In an attempt to overcome this problem, Kawarabata and Hayasaka (1987) developed an indirect ELISA assay for the detection of soluble surface antigens of a microsporidian spore extracted with alkaline solutions. The authors reported that the preparation of alkali-soluble surface antigens was simple and that these antigens were more suitable for ELISA than the intact spores. It is possible that steric problems associated with the particulate nature of bacterial cells may be overcome through the use of similar extracts in the assay.

The double antibody sandwich ELISA (DAS-ELISA) (Voller et al., 1976) is a widely used assay for the detection of phytopathogens. The disadvantage of this method, however, is that the immunoglobulin of each test serum must be purified and coupled to an enzyme. In addition, the specificity of the test can preclude detection of even closely related strains of the same antigen (Koenig, 1978). The indirect ELISA (Lommel et al., 1982) avoids the necessity of making specific enzyme conjugates for each antigen to be tested and eliminates the extreme specificity. However, a problem associated with the use of the indirect ELISA for detection of plant pathogens in crude plant extracts is the interference by host proteins on the detection capabilities of the test (Lommel et al., 1982). This interference has been attributed in part to competition between target antigen and host protein for a finite number of binding sites on the solid phase (Lommel et al., 1982). The same authors reported that quantitative detection of target antigen was unaffected by the presence of contaminating host proteins in the DAS-ELISA.

Similar interference phenomena were reported by Leach et al. (1987) who described a dot-immunobinding assay (DIA) for the detection of a xanthomonad in sorghum. The authors reported that the abundance of plant proteins released by the grinding of samples saturated membrane binding sites

and plant pigments stained the membrane, making accurate detection difficult. An alternative method of sample preparation involving the elution of bacteria from leaf material by soaking the excised tissue in distilled water was found by the same authors to be more satisfactory. This method allowed for the adequate release of bacteria into the suspending medium without heavy contamination of the sample by host plant components. A similar leaching procedure was used by Malin et al. (1985) for the preparation of sample for various serological assays. Because of the obvious advantages of this water leaching method, it was investigated as an alternative to sample homogenation and other extraction procedures in this study.

1.9.2.5 Detection of disease-specific antigens using various immunodiagnostic assays

Since the first report of the use of serology to identify a plant pathogenic bacterium in 1918, the detection and identification of phytopathogenic bacteria has been carried out using a variety of serological techniques including tube agglutination and precipitation, agar precipitin and immunofluorescence (Schaad, 1979). The enzyme-linked immunosorbent assay was first applied to virus detection by Avrameas (1969) and after the report in 1971 by Engvall and

Perlmann (1971), ELISA became recognised for its potential in the detection of phytopathogens. It is, however, only since the work of Clark and Adams (1977) on ELISA protocols for use with plant viruses that the assay has been widely used for the detection and quantitative estimation of a large number of antigens from plants. Several variations of this procedure have been developed.

ELISA is a routine method for detecting and assaying plant pathogens because of its sensitivity. Despite its relative simplicity, the technique is rather laborious and time-consuming and requires some special equipment. Also, bacterial applications of the technique are often limited by steric problems, but these can, to a certain extent, be overcome by altering the sample extraction procedure and employing a variation of the conventional ELISA procedure, namely the dot-immunobinding assay (DIA) (Leach et al., 1987).

The principles of DIA are almost the same as those of ELISA, differing only in that antigen is bound to a nylon membrane or to nitrocellulose (NC), rather than to a well in a polystyrene microtiter plate, and that the product of the enzyme reaction is insoluble. DIA is the most simple, rapid and sensitive assay for immunological characterization of phytopathogenic bacteria and fastidious

prokaryotes, obtained either from culture or from diseased tissue (Lazarovits, 1990). Additional advantages of this technique over ELISA are a large number of samples can be processed within 2 to 3 hr., rather than the day usually required with conventional ELISA, and both the antibody and the enzyme conjugate solutions can be re-used several times. Also, DIA does not require highly trained personnel for interpretation of results.

Solid-phase radioimmunosorbent assay (RIA) is in principle the same as the DIA. In RIA, however, a radio-labelled secondary antibody is used in place of an enzyme-labelled molecule. RIA is preferred in instances where plant enzymes such as peroxidases interfere with the ELISA enzyme reaction. The assay is particularly useful when plant pigments interfere with the interpretation of DIA results because of staining of the solid phase. RIA is considered slightly more sensitive (2- to 4-fold) and less subject to variation than conventional ELISA (Benedict and Alvarez 1990), although ELISA is usually preferable as it avoids the handling and storing of radioactive materials.

Numerous immunodiagnostic assays have been developed for bacteria and important considerations in choosing a specific assay system are pathogen variability, assay sensitivity requirements, complexity, cost and time.

1.9.2.8 Western blotting

The transfer of proteins separated by gel electrophoresis from the gel matrix to paper and the subsequent probing of the immobilized proteins with specific antisera (Western blotting) is a valuble teonique for the detection of specific proteins in crude extracts. The technique is extremely sensitive and has allowed the detection of very low amounts (1 pg - 1 ng) of antigens in cells or natural fluids. In this way, for instance, Newcastle disease virus has been detected in chick embryo cells (Bolen et al., 1982), hepatitis B virus in human sera (McMichael et al., 1981) and plant viruses in plant sap (O'Donnell et al., 1982).

A further applicatio of the Western blotting technique is its use in selecting appropriate preparations of antigen against which polyclonal antisera may be reacted in the production of mono-specific antisera. Moyer and Echandi (1986) reported that Western blot comparisons of electrophoretic protein profiles of different strains of Streptomyces ipomoea indicated that quantitative differences among the strains revealed by ELISA were due to differences in the numbers of antigens recognized by the antiserum in extracts from pathogenic strains and from

contaminating and closely related strains. The authors used the technique to select strains against which their antiserum could be absorbed to yield an antiserum specific for a pathogenic strain of the organism.

Protein blotting is a simple technique, convenient to perform and economical with respect to reagents and time. Its high sensitivity and innate versatility clearly allow it to be applied to more than just the routine analysis of complex mixtures of proteins.

1.7.3 Production of antisera against host plant-derived bacterial immunogens

A major obstacle limiting research on many fastidious phytopathogenic prokaryotes is the inability to isolate and culture these organisms *in vitro*. In the absence of suitable media to support the growth of these organisms outside their plant hosts and insect vectors, numerous attempts have been made to obtain the organisms directly from extracts of infected plants and insects using various purification methods (Sinha, 1974, French *et al.*, 1977, Archer *et al.*, 1982, Clark *et al.*, 1983, Sinha and Chiykowski, 1984, Jiang and Chen, 1987, Martin-Gros *et al.*, 1987, Chen and Jiang, 1988, Jiang *et al.*, 1988, Sui, 1988, Chippindall and Whitlock, 1988b, Garnier *et al.*, 1991, Koroton *et al.*, 1991).

As in the case of sample preparation for detection assays, the primary intent of any extraction procedure is to optimize the percent target recovery. The percentage recovery of target bacteria from plant tissue is largely dependent upon the inoculum concentration, as well as on the optimal release of the target from tissue, the minimization of contamination by saprophytic microflora and the minimization of interference and contamination by plant components. When very low target inoculum densities are involved, a concentration step following extraction may be advantageous. This can be done by filtration or centrifugation, however, drawbacks with these methods include concomitant concentration of saprophytes and host plant debris.

Extraction and purification procedures for a particular microorganism have to be determined empirically if little is known about the physical nature of the target organism. Artificially contaminated samples may be used in preliminary assays but the final evaluation of any procedure must be done using naturally-infected material.

Methods reported in the literature for the extraction of microorganisms from infected material range from simple vacuum extraction of rickettsia-like bacteria from peach tissue affected with phony disease (French et al., 1977) to

more complex and lengthy techniques such as that developed by Sinha (1974) and Sinha and Chiykowski (1984) to extract different mycoplasma-like organisms from plant material. The purification procedure reported by these authors involve the following steps: (i) vacuum infiltration of leaf tissue with $MgCl_2$-glycine buffer, (ii) homogenization, (iii) treatment of clarified sap with activated charcoal and Celite 545 and adsorption of MLOs on a Celite pad, (iv) high-speed centrifugation of the eluted opalescent fractions, (v) further purification by column chromatography, and finally, (vi) sucrose gradient centrifugation and high-speed centrifugation to obtain a pellet of 'purified" MLOs.

Most of the other reported purification procedures simply involve homogenization of material in extraction buffer followed by two cycles of differential centrifugation. Chippindall and Whitlock (1988b) and Sui (1988) included enzymatic degradation steps in order to maximize the release of bacteria from greening-infected citrus leaf material. Jiang et al. (1988) utilized a monoclonal antibody specific against aster yellows MLO in an affinity column chromatographic technique to obtain highly-purified preparations of the MLO from disease lettuce.

Utilizing preparations of organisms from infected material, both monoclonal and polyclonal antisera have been raised against the partially purified target organisms. Monoclonal antibodies against a preparation of maize bushy stunt MLO were found to react specifically with MLO-associated antigens in ELISA and immunofluorescent staining (Chen and Jiang, 1988). Similarly, monoclonal antibodies raised against the aetiological agent of greening disease extracted from infected periwinkle plants by Garnier et al. (1987) reacted specifically with disease-associated antigens in infected plant material.

Clark et al. (1983) produced a polyclonal antiserum against clover phyllody-associated antigens using partially purified preparations from infected plants. The antiserum exhibited a relatively low specific precipitin titre which precluded the use of the standard DAS-ELISA and instead the $F(ab')_2$ indirect sandwich procedure had to be used. Also, the antiserum exhibited very high anti-plant activity and it was necessary to cross-absorb the antiserum to plant-derived material. Although the cross-absorption did not remove all the background reaction, a differential in ELISA values was obtained between the partially-purified preparations from infected and healthy plants. The antiserum was specific for its homologous antigen and no cross-reactions were observed between the antiserum and

extracts of plant material infected with various unrelated MLOs. The procedure employed by Sinha and Chiykowski (1984) to purify the peach eastern X-MLO yielded preparations of the organism which were relatively free of host plant contaminants. The resulting polyclonal antiserum could therefore be used in the DAS-ELISA without the need to cross-adsorb it to healthy material.

It is evident from the reports cited above that the development of an antiserum exhibiting a high binding affinity and specificity against a phytopathogen which cannot be cultivated *in vitro* is possible through the use of extracts of infected material as immunogens.

1.10 OBJECTIVES

A variety of microorganisms have been isolated from greening-affected citrus (section 1.5), but as the isolations have never been fully confirmed by the fulfilment of Koch's postulates, the aetiological status of the organisms is highly questionable.

Because of the severity and economic importance of citrus greening disease, there is an urgent need to isolate and culture the aetiological agent of the disease. The *in vitro*

propagation of the disease agent would allow for the characterization of the organism which may lead to a better understanding of the disease process and possibly to a cure for greening as well. Furthermore, the isolation of the organism would greatly facilitate the development of a sensitive and reliable detection system for the disease.

This study was undertaken to:

i) Determine the pathological significance of the bacterial isolates of Garnett and Mochaba by attempting to fulfil Koch's postlates,

ii) Investigate the frequency of association of the GC and NC isolates with citrus affected by greening,

iii) Investigate the relationship between the isolates and *L. exitrase*, as well as between the isolates and various plant species,

iv) Attempt to isolate and culture the aetiological agent of greening using various artificial media as well as embryonated hen's eggs and invertebrate cell culture,

v) Develop a suitable serological assay for the detection of the isolates of Garnett and Mochaba,

vi) Investigate the feasibility of extracting greening-specific antigens from naturally-infected citrus and periwinkle for use as immunogen in the production of a greening-specific polyclonal antiserum, and

vii) Develop an assay for the detection of greening disease utilizing the antiserum raised against the extract from greened citrus.

CHAPTER 2

MATERIALS AND METHODS

2.1 BACTERIAL ISOLATES FROM GREENING-INFECTED CITRUS

Cultures of bacteria isolated from greening-infected citrus were a gift from H.M. Garnett and F.M. Mochaba. The isolates were coded according to both the geographical location of the infected source material and the section of the plant from which the bacteria were originally cultuted. The following isolates were used in this study:

NC - Nelspruit, columella
GC - Randburg, columella
LC - Letaba, columella
LL - Letaba, leaf (midrib)
TZL - Tzaneen, leaf (midrib)

In addition to the above isolates, a bacterium coded CS was later isolated in pure culture from the stem of a symptomatic cucumber plant inoculated via dodder with a phytopathogen from naturally-infected greened citrus.

Unless otherwise stated, isolates were cultured using either MIG-1 (Mochaba, 1989) or MIG-3 medium (Appendix A).

For short-term storage, isolates could be maintained on sealed MIG-1 or MIG-3 slants or plates at room temperature for 7 days without showing an appreciable decrease in viability. For long-term storage, bacteria in the exponential phase of growth were harvested from liquid culture, resuspended in 15% (v/v) glycerol in distilled water and frozen at -70 C.

2.2 ISOLATION OF THE BACTERIUM FROM VARIOUS SOURCES

Garnett (1985) and Mochaba (1989) suggested that the bacteria cultured by them from greening-infected citrus (and made available to the author) were isolates of the aetiological agent of greening disease. In an attempt to investigate the frequency of association of the isolated bacterium with greening disease, isolations were carried out from citrus material collected in different areas of the Transvaal.

Samples of greening-infected leaf midrib and fruit columella were collected during the cool winter months of May to August in the following areas: Brits, Pretoria,

Nelspruit, Letaba, Letsitile, Zebediela and Tzaneen. In every isolation attempt, leaf and fruit material was collected at 5 different sites on the canopy of each tree sampled. Samples from infected trees included material displaying symptoms ranging from mild to severe.

For the preparation of inoculum from infected plant material, two extraction procedures were used. The first was a modification of that reported by Mochaba (1989). Excised columella and midrib samples were immersed in ethanol for 2 min., transferred to a 1% solution of sodium hypochlorite containing 0.1% (v/v) Tween 20 for 20 min. and finally rinsed in 3 changes of sterile distilled water. Half a gram of plant material was chopped finely in a small volume of sterile 1/4-strength Ringer's solution (Appendix K) and then 2 ml of the same solution added. After standing for 2 min. the suspension was vortexed for 2 min., the plant debris allowed to settle out and 0.2 ml of the supernatant used to inoculate 15 ml of MIG-3 medium in each of five 100 ml Erlenmeyer flasks. The cultures were incubated at 25 C on a gyratory shaker (150 rpm) and monitored daily for bacterial growth.

Attempts to isolate and culture the bacteria from periwinkle (Catharantus roseus L.) infected with greening via dodder (Cuscuta campestris), as well as from the

parasitizing dodder were performed in the same way with the exception of the surface-sterilization step which was carried out for 5 min. only because of the delicate nature of the leaf tissue of these plant species. Strands of dodder emerging from haustoria within the host plant served as the source material for isolation from the parasite.

The second inoculum preparation procedure (used for the extraction of sap from cit[illegible]olved the use of a pressure chamber (Scholander [illegible]arder et al., 1965) (Fig. 1). In this meth[illegible]xcised, leafy shoot was placed within the pressu[illegible] with the cut end of the stem protruding through a gastight seal. The cut end, as well as approximately 3 cm of the adjacent stem, was then sterilized by swabbing with alcohol followed by immersion in a 1% solution of sodium hypochlorite for 5 min. After thorough rinsing in dH_2O, 1 cm of the sterilized stem was cut off using a scalpel blade in order to expose an area of the vascular tissue which had not been exposed to the alcohol and sodium hypochlorite. An inert gas (N_2) was then admitted into the chamber from a compression tank and the pressure in the chamber increased until the plant sap bubbled from the cut surface of the stem (Fig. 2). The sap was collected using a sterile glass capillary tube and aliquots (approximately 50 ul) used to inoculate 10 ml volumes of MIG-3 medium contained within 100 ml flasks. The flasks were incubated as described above.

Figure 1

Pressure chamber (Scholander bomb) used to obtain sap from leafy twigs.

A - pressure guage
B - air inlet
C - pressure chamber

Figure 2

Sap (arrow) bubbling from the cut end of a citrus twig held within the Scholander bomb. The sap was collected aseptically and used to inoculate various culture media.

Attempts were also made to isolate the bacteria from adult psylla of various ages collected from infected trees in the Nelspruit, Randburg and Brits areas. Surface-sterilized insects were individually ground with 2 drops of 1/4-strength Ringer's solution in a watch-glass using the rounded end of a test tube and the homogenate used to inoculate 15 ml volumes of MIG-3 medium and incubated as for isolation from plant material.

In all isolation attempts, uninfected material (plant and insect) was included which served as a control.

An isolation was considered positive if the bacterium cultured was identical with respect to morphology, Gram stain reaction and analytical profile indices (API 20NE and API 20B Micromethods, API Systems, France) to the NC and GC isolates supplied by Garnett and Mochaba, and if the bacterium reacted positively in the heparin-enhanced plate-trapped antigen ELISA (HE-PTA-ELISA) (section 2.10.2.3).

2.3 ATTEMPTED ISOLATION AND CULTURE OF THE CAUSAL AGENT OF GREENING

The discovery of prokaryotic organisms in greening-infected plants stimulated many laboratories to attempt to cultivate these microorganisms in artificial media. Attempts made at isolating the aetiological agent utilizing different methodologies have been unsuccessful (Ghosh *et al.*, 1971, Sodhi *et al.*, 1973, Moll, 1974, Botha *et al.*, 1984, Manicom, 1984), and renewed efforts were made in this study to culture the greening organism from various sources using a variety of media and methods.

2.3.1 Artificial media

The following artificial media developed originally for the *in vitro* cultivation of phytopathogenic bacteria, mycoplasmas and spiroplasmas were used in this study: MIG-1, MIG-3, Tryptone soy broth (Oxoid Ltd., U.K.), Nutrient broth yeast extract medium (NBY) (Gross and Vidaver, 1979), SC medium (Davis *et al.*, 1980), S8 medium (Davis *et al.*, 1980), RSD medium (Davis *et al.*, 1984), C3G medium (Davis *et al.*, 1988), LD8A3 medium (Davis *et al.*, 1988), LD59 medium (Davis *et al.*, 1988), and a medium supplemented with fresh orange juice (OJ medium) (Appendix A).

Using the above media, attempts were made to isolate and culture the greening organism from infected citrus leaf

midrib and fruit columella, from periwinkle, dodder and from infective psylla, as described above (section 2.2). Samples were collected during the cool winter months of May to August, as well as during the warmer months of November and December.

In addition to the preparation of inoculum from leaf midrib and citrus fruit columella, inoculum was also prepared from freshly excised citrus and periwinkle stems using the pressure chamber method detailed above. In addition to inoculating the various media with extracts of infected material, similar extracts of healthy material were used which served as controls.

2.3.2 Embryonated hen's eggs

For ection into embryonated hen's eggs, extracts of diseased and, for the controls, f healthy citrus and periwinkle cuttings obtained ase.tically via both the pressure chamber method and the modified method of Moohaba (1989) (section 2.2) (chopping method) were used.

Fertile eggs were obtained from a flock free of infection with known viral or other microbial agents. The eggs were incubated at 37 C in an atmosphere of approximately 50% humidity. To prevent adhesions of the embryonic membranes and to keep the embryos more or less centralized, the eggs were turned four times a day.

Two routes of inoculation were used, namely the yolk-sac and allantoic routes.

The yolk-sac route of inoculation is the route of choice for isolation and propagation of rickettsiae and chlamydiae, these organisms growing readily in the entodermal cells lining the yolk sac. In this procedure, eggs were incubated for 6 days after fertilization at which stage the yolk sac was large and could readily be entered without concern for appropriate orientation. The air-sac end of the egg was swabbed with alcohol, and the shell over the air sac punctured with a needle. A 2 ml syringe fitted with a 4.5 cm 23-guage needle was used to introduce the inoculum into the yolk sac. Five hundred microlitres of inoculum prepared according to the chopping method of Moohaba was injected into each egg. When the inoculum was prepared following the pressure chamber method, a 50 ul volume of plant sap per egg was used. After inoculation, the punctured area of the shell was painted with tincture of iodine and sealed with candle wax. The inoculated eggs were incubated at 35 C and candled daily to monitor growth of the embryo. Embryos dying before 5 days post-infection were usually killed by infection with saprophytic bacteria or other microbial contaminants, and therefore disregarded.

Using the yolk-sac route of inoculation, 6 eggs were injected with inoculum prepared following the chopping method and 6 with inoculum prepared following the pressure chamber method. As controls, eggs were inoculated with extracts of healthy plant material prepared following the same two methods (2 eggs per preparation). Two eggs were each inoculated with 0.5 ml of 1/4-strength Ringer's solution as an additional control.

The yolk was harvested between 10 and 12 days after inoculation. The egg was chilled to induce contraction of the blood vessels, and the shell over the air sac removed aseptically. The contents of the egg were decanted into a sterile Petri dish, the yolk sac cut away from the embryo and the sac ruptured to release the yolk. The sac membrane was washed free of yolk, stained by the Giemsa method (Appendix B) and then examined microscopically to determine the presence or absence of lesions in the membrane. In order to visualize any microorganisms present in the yolk, a smear of the yolk was made on a glass microscope slide and stained by the Giemsa method.

For the isolation of microorganisms from the yolk, the yolk was diluted by the addition of 2 volumes of sterile PBS and then clarified by centrifugation at 200 g for 10 min. The supernatant was then centrifuged at 35 000 g for 30 min.

Following resuspension of the pellet in a large volume of PBS and centrifugation at 200 g for 10 min., the clarified supernatant was centrifuged at 35 000 g for 30 min. to sediment any microorganisms present. The pellet was resuspended in a minimal volume of PBS and negatively stained preparations of the suspension examined under the electron microscope. The presence of any greening-specific antigens within the resuspended sediment was determined in the enzyme-linked immunosorbent assay (ELISA) (section 2.10.2.3) in which immobilized egg yolk-derived antigen was reacted with antiserum raised against an extract of greening-infected citrus (section 2.8.2, method 8).

In the allantoic route of inoculation, 10 day-old embryos were used. The eggs were candled and a pencil used to demarcate an area over the air sac near the membrane boundary opposite the embryo where there are few or no major blood vessels. The marked area was painted with tincture of iodine and the shell punctured. The egg was then inoculated with a tuberculin syringe fitted with a 1.5 cm 26-guage needle. The needle was inserted approximately 1.0 cm into the egg in a direction parallel to the side. Approximately 0.2 ml of inoculum (prepared following the chopping method) was introduced into the allantoic cavity in this way. Fifty microlitres of plant sap obtained using

the pressure chamber method was injected into eggs in a similar way. Inoculated eggs were incubated at 35 C for 12 days and the development of the embryos monitored daily as described above.

For the harvest of infected materials, eggs were chilled, swabbed with alcohol and the shell over the air sac broken away. The shell membrane was removed aseptically with forceps and the allantoic fluid aspirated with a pasteur pipette. The chorioallantoic membrane was collected, stained using the Giemsa method and examined microscopically. A smear of the fluid was prepared and examined as described above. Attempts to isolate bacteria from the fluid were made following the method described for the isolation of bacteria from egg yolk and the pellet obtained after the final centrifugation step examined by transmission electron microscopy (TEM) and ELISA.

The number of eggs inoculated with each inoculum preparation via the allantoic route was the same as that inoculated via the yolk sac route. The number of healthy and buffer controls used was also the same as that used for the yolk sac route.

2.3.3 Invertebrate cell culture

In the absence of an established T. erytreae cell line, an attempt was made to cultivate the aetiological agent of greening in a cell culture system established from larvae of the mosquito Aedes albopictus (clone C6/36). The cells were grown in HEPES-buffered Dulbecco's modified Eagle's medium with non-essential amino acids in Earle's BSS supplemented with 10% (v/v) foetal bovine serum. The cells were grown in plastic Leighton tubes # 3393 (Costar, USA) and were maintained at room temperature (approximately 23 C) in the dark.

Cell layers were infected when at 70-80% confluence. Prior to infection with diseased plant sap extract, the maintenance medium was decanted off and the cells washed briefly with serum-free medium. Basic medium supplemented with 2% serum was then added to the tubes (2 ml per tube) and the inoculum added. The inoculum consisted of sap aseptically extruded from leafy twigs of infected citrus using the Scholander bomb. Twigs bearing leaves displaying both mild and severe greening symptoms were collected from three trees growing in the Brits area and the sap obtained from the twigs pooled. Sap from healthy citrus served as a control. Approximately 20 ul of sap was added to each Leighton tube and the tubes inoubated at room temperature.

The cultures were monitored daily for changes in the appearance of the insect cells, as well as for bacterial growth. Bacterial growth occuring within a period of 2 days following inoculation was usually due to contamination of the medium by saprophytic bacteria or other contaminants and the affected vials were discarded. The medium was replaced with fresh medium + 2% serum when a drop in the pH of the medium was indicated by a change in the colour of the medium. Spent culture medium was collected and centrifuged at 35 000 g for 30 min. in order to sediment any microorganisms present in the medium. The resulting pellet was resuspended in a minimal amount of PBS and examined electron microscopically for the presence of bacteria.

Fourteen days after inoculation with plant sap, the plastic cover slips of the Leighton tubes on which the cells were growing were removed and stained by the Giemsa method. The stained cell layers were then examined microscopically for changes in cell morphology, cellular degeneration (cytopathic effects [CPE]) and plaque formation.

In the experiment, 12 tubes were inoculated with greening-infected citrus sap and 6 with sap from healthy citrus. An additional 4 tubes were left uninoculated.

2.4 ESTABLISHMENT OF LABORATORY COLONIES OF T. erytreae

For conducting transmission experiments and determining the relationship between the bacterial isolates and T. erytreae, a laboratory colony of uninfected psylla was established in order to ensure a continuous supply of the insects.

Psylla free of the greening organism were a obtained from a "clean" colony established by G.J. Begeman of Zebediela Citrus, Zebediela. Adult psylla, as well as nymphs and eggs, were transported to the laboratory on healthy citrus seedlings contained in gauze cages. Plants infested with psylla were removed from the cages and placed among healthy, vigorously flushing Minneola tangelo (Citrus sinensis x C. reticulata) seedlings and the insects allowed to spread to these plants naturally.

Optimal conditions for rearing T. erytreae in the laboratory were established empirically. Plants were housed in an indoor area kept free from other insect pests. Psylla were cultured under a 16-hour photoperiod, and lighting was provided by a bank of 4 x 115W VHO Gro-Lux fluorescent tubes (Sylvania, USA) and 2 x 100W tungsten filament bulbs (Philips, RSA) positioned 20 cm above the plants.

An ambient temperature of 20-23 C was maintained by means of a standard air-conditioning unit during summer and a fan heater during winter.

Humidity of 60-65% RH was maintained with the use of a Sunbeam ultrasonic humidifier (Model 2600).

Insects were handled as little as possible to avoid injury but when it was necessary to move adults and nymphs between plants, this was achieved with the use of an aspirator in the case of adults while nymphs were moved with the aid of a fine sable-hair artist's paint brush.

Plants were pruned regularly and fertilized every two weeks via soil application of a fertilizer/trace element solution (Multifeed P, Plaaskem, RSA) in order to ensure the continuous availability of new flush on the seedlings.

A colony of infected psylla was established and maintained under similar conditions. Insects from this colony served as a source of the greening organism in isolation attempts and for inoculating healthy citrus seedlings with greening disease.

The psylla were collected from an orchard located in Nelspruit which exhibited a high incidence of greening. The insects were maintained on both healthy and greening-infected seedlings in the laboratory. The subsequent development of typical greening symptoms on healthy laboratory seedlings which were exposed to the psylla confirmed the carrier status of the field-collected insects.

2.5 KOCH'S POSTULATES

In an attempt to fulfill Koch's postulates, healthy citrus seedlings were inoculated by various means with isolates NC, GC, LL, LC and TZL.

2.5.1 Laboratory inoculation of citrus with bacteria via the insect vector of greening

2.5.1.1 Inoculation of T. erytreae with the bacterial isolates by membrane feeding

2.5.1.1.1 Feeding chamber design

The suitability of a feeding chamber design was assessed for use in the laboratory inoculation of nymphs and adult psylla by membrane feeding. The design of the chamber is based on that used by Storey (Storey, 1932, cited in Mitsuhashi, 1979) and is illustrated in Fig. 3.

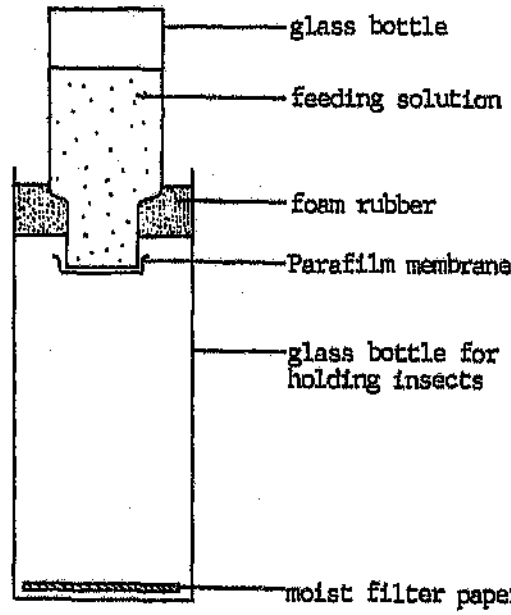


Figure 3

Apparatus for feeding Trioza erytreae adults and nymphs. Adapted from Storey (1932), cited in Mitsuhashi (1979).

A preliminary investigation established that the design of the chamber was suitable for confining groups of 15 nymphs or adults for up to 5 days without an adverse effect on the insects. In order to determine this, the feeding solution in the chamber was replaced by a sweet orange leaf held firmly in place over the mouth of the chamber with adhesive tape. The chamber was kept under conditions of light, temperature and humidity as specified in section 2.4 and the insects observed over a 5-day period for any negative effects that the cage environment may have on the nymphs and adults.

2.5.1.1.2 Feeding solutions

The solution in which a bacterium is suspended for the membrane feeding route of insect inoculation must act as a feeding stimulant as well as be able to sustain the insect during the period of exposure to the suspension. Also, the constituents of the feeding solution should in no way adversely affect the viability of the bacterium.

A number of formulations were assessed for their suitability as both feeding stimulants and suspending media for the bacterial isolates.

a) Sap extracts

Sap was extracted from midribs excised from young sweet orange leaves using an Erich Polane leaf press. The extract was used undiluted, as well as diluted to concentrations of 50%, 10% and 1% (v/v) in double distilled water.

The effect of adding sucrose to the sap preparations was also investigated. Sucrose was added to the undiluted and diluted sap to a final concentration of 5% (w/v).

b) Sucrose solutions

1%, 5% and 10% (w/v) sucrose in distilled water.

c) Sucrose/serum solution

5% (w/v) sucrose in distilled water containing 1% (v/v) foetal calf serum. This sucrose/serum solution was included in the trial as a similar solution is reportedly used for most of the work done on leafhopper acquisition and transmission of mycoplasmas (Markham, 1982).

All feeding solutions initially contained 1% (v/v) commercial green food colouring (Robertsons, RSA) which functioned as a marker substance and facilitated the

identification of adults and nymphs which had fed on the solution (insects imbibing the solution displayed clearly visible green alimentary tracts). Solutions were sterilized by filtration through 0.2 um nitrocellulose membranes (Millipore Corp., USA).

The preference of the nymphs and adults was determined by a multiple choice experiment. The solutions were placed in sterile glass test tubes and Parafilm M (American Can Comp., USA) (sterilized by immersion in 70% alcohol for 2 minutes) stretched over the mouths of the tubes. Groups of 30 3rd and 4th instar nymphs and 30 4-day old adult psylla were placed separately in beakers into which the inverted feed tubes were then inserted. Nymphs and adults were exposed to the solutions for a period of 48 hours. The number of insects observed on the feeding solutions at intervals of 0.5, 1, 2, 5, 10, 14, 24, 34 and 48 hours was recorded.

In order to determine that the solution on which the psylla were most frequently observed was in fact serving as a feeding stimulant, insects were confined in feeding chambers with the particular solution for 24 hr. After this period of exposure, the gut of each insect was dissected out and the contents examined for the presence of the green marker substance.

2.5.1.1.3 Inoculation of psylla

Isolates NC, GC, LC, TZL and LL from fresh MIG-3 plates were used to inoculate 50 ml aliquots of MIG-3 in 250 ml Erlenmeyer flasks. The cultures were incubated on a rotary shaker (150 rpm) at 25 C and the cells harvested when in the exponential phase of growth by centrifugation (12 000 g for 15 minutes), washed once in a large volume of phosphate-buffered saline pH 7.4 (PBS, Appendix K) containing 0.1% (w/v) ethylenediaminetetra-acetic acid (EDTA), and suspended in 5% (w/v) sucrose in distilled water at a concentration of 10^7 cells/ml.

Bacterial isolates used in insect inoculations had been passaged less than 6 times to avoid attenuation of pathogenicity due to prolonged subcultivation in vitro.

For the laboratory inoculation of psylla via membrane feeding, 3 groups each consisting of 16 3rd and 4th instar nymphs and 16 4-day old adults were confined separately in feeding chambers and allowed access to each isolate over a 24 hr period. In this way, 48 adult psylla and 48 nymphs were exposed to each of the 5 isolates. As a control, 20 adults and 20 nymphs from the same colony were exposed to the sterile sucrose solution without added bacteria.

The chambers housing the insects were darkened with a covering of brown paper while the bottle containing the feeding solution was covered with yellow cellophane and placed under a light source in order to encourage the insects to congregate on the Parafilm membrane.

After exposure to the isolates, the insects from each group were divided among 2 healthy Minneola seedlings and confined in micro-cages to the young flush of the test plants. The cages were constructed from clear plastic film formed into a cylinder and sealed at either end with a foam rubber disc (Fig. 4).

2.5.1.2 Inoculation of psylla by micro-injection

Fine capillary needles were drawn from 0.5 mm diameter glass tubing with the aid of a micro-forge. The tip of the needle used to inject adult psylla did not exceed 5 mm in length to reduce the possibility of breakage during use. The point of the capillary was broken with fine forceps under a dissecting microscope to give a sharp, slanted needle with an orifice 10-20 um in diameter. Needles were sterilized in the autoclave prior to use.

The pressure required to eject the inoculum from the needle came from a laboratory air supply controlled via a mouth-operated T-joint valve.

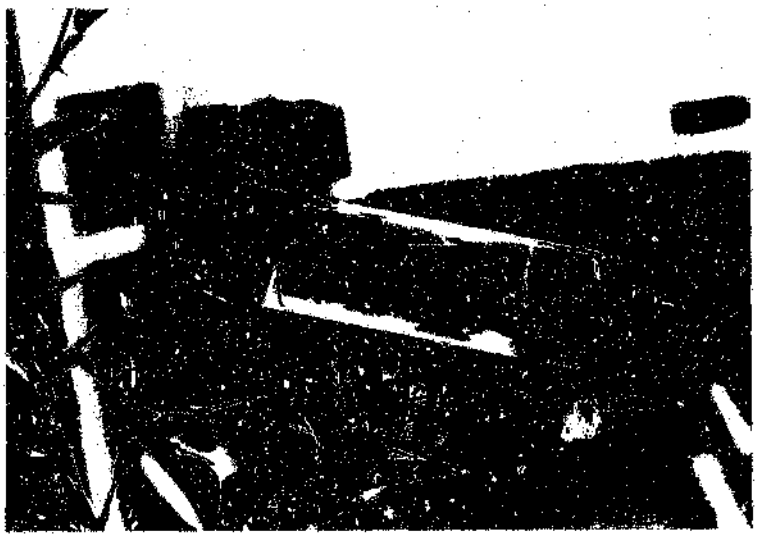


Figure 4

Plastic cage for confining *T. erytreae* to flushing citrus leaves.

For the inoculation of psylla by micro-injection, the isolates were cultured as described for membrane feeding and, after washing, resuspended in sterile, 1/2-strength normal saline at a concentration of 10^9 cells/ml.

Three- to four-day-old greening-free adult psylla were inoculated in this way. Psylla were anaesthetized with CO_2 for a few seconds, positioned on a petri dish lid with their ventral surface exposed and immobilized under a Parafilm membrane. Insects were injected ventrally either through the conjunctiva at the junction of the thorax and abdomen, or in an anteroposterior direction through the conjunctiva between adjacent abdominal sternites.

One-tenth of a microlitre of the freshly-prepared cell suspension was injected into adult psylla which were free of the greening organism, thereby introducing an inoculum of 10^5 into the insects. Two groups of 20 insects were inoculated with each isolate. As a control, 20 psylla were similarly injected with 0.1 ul of sterile saline.

The insects in each group were divided between 2 healthy sweet orange seedlings and confined in micro-cages to the young flush of the test plants.

The plants from both the membrane feeding and micro-injection inoculation experiments were maintained under the conditions described in section 2.4. The adult psylla emerging from nymphs which had been inoculated via membrane-feeding were allowed to feed on the leaves of the test plants until the insects died. Psylla inoculated in the adult stage of their life cycle by either membrane feeding or micro-injection were left to feed on the test plants until they had completed that stage of their life cycle. Any eggs and nymphs present on the plants after the death of all the adults were eradicated by spraying the plants with dimethoate (0.01% a.i.).

Psylla were monitored daily to determine the effect, if any, of the bacteria on the insects' longevity. In order to observe any replication and spread of the inoculum within membrane-fed and injected insects, the psylla were collected as soon as they died and fixed and embedded for electron-microscopy (section 2.6). The multiplication and spread of the bacteria within the insects was also monitored by means of the indirect enzyme-linked immunosorbent assay (section 2.10.2.3)

All test and control plants were monitored over a period of 2 years for the development of typical greening or other disease symptoms.

The transmission experiments were carried out during May 1986 and repeated during November of the same year.

2.5.2 Mechanical inoculation of citrus

Isolates were cultured in MIG-3 medium and harvested in the exponential phase of growth. Cells were washed twice in 10 x volume of sterile normal saline containing 0.1% EDTA and finally resuspended in sterile dH_2O. The cell concentration was adjusted to 10^9 /ml.

Healthy Minneola tangelo seedlings with a lower stem diameter of between 7 and 10 mm were selected for inoculation by stem injection. Plants were drought stressed for 24 hours prior to inoculation to reduce turgor pressure as this was found to result in more efficient uptake of inoculum.

The area to be injected was swabbed with alcohol and a pilot hole made into the centre of the stem using a sterile needle with an internal diameter of 0.6 mm. A Venisystems Butterfly (Abbott Ireland Ltd., Sligo, Ireland) connected to a 5 ml syringe, containing 1 ml of the bacterial suspension, was used to introduce the inoculum into the plants over an 8-24 hour period (Fig. 5). Positive pressure was maintained within the syringe by using a perspex syringe holder designed specifically for this study (Fig. 6).

Figure 5

Injecting citrus seedlings with bacterial isolates.

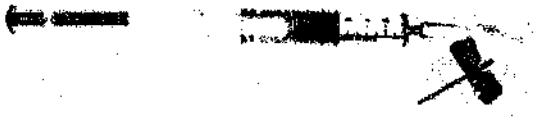


Figure 6

Inoculation apparatus used to introduce the bacterial isolates into the citrus seedlings.

An isolate was inoculated into 12 seedlings in this way while 10 control plants received only sterile dH_2O.

An additional 25 healthy seedlings were inoculated by stem injection coupled with stem slashing. For this experiment, groups of five plants were inoculated with a different isolate. The plants were injected as described above and then additional inoculum was introduced into the seedlings by dipping a sterile scalpel blade into the prepared inoculum and making approximately 25, 2-4 mm deep slashes into the bark of the stem. The blade was dipped into the inoculum between each slash. The seedling stems were swabbed with alcohol prior to inoculation. Four seedlings which received only sterile water served as controls.

All inoculated and control plants were maintained under a 14 hour photoperiod with a day temperature of 23 C and a night temperature of 16 C. Plants were sprayed every two weeks with a solution of "Multifeed P" (prepared according to the manufacturer's instructions) to prevent mineral deficiency.

2.5.3 Mechanical inoculation of periwinkle

Isolates were cultured and prepared as for mechanical inoculation of citrus. Ten healthy 16-week-old periwinkle plants were mechanically inoculated in the following way: A

needle with an internal diameter of 0.8 mm connected to a syringe containing the bacterial suspension was inserted through a fleshy part of the stem which had been swabbed with alcohol. A drop of the inoculum was gently expelled onto the tip of the needle and the needle drawn back through the stem, thereby introducing the suspension into the stem. Approximately 10 such inoculations were made at various sites on each plant. Five control periwinkle plants were inoculated in this way with only sterile distilled water. Plants were maintained under the conditions described above.

As with the inoculation of citrus via the insect vector, the mechanical inoculation trials were performed twice, the first being during the month of May and the second during November of the same year.

2.5.4 Dodder transmission of the bacterial isolates from mechanically-inoculated citrus to periwinkle

Surface-sterilized seeds of dodder (Cuscuta campestris) were germinated on sterile, moist filter paper and when the shoots were approximately 10 mm in length, they were transferred to a Minneola tangelo plant which was free of greening. The dodder was allowed to establish itself on the plant and once its strands were between 5 and 10 cm long,

connections were established between the plant and a symptomatic, mechanically-inoculated (GC isolate) citrus seedling. Once the dodder had formed haustoria within the infected seedling, the connecting strands between the two plants were severed. Ten days after the establishment of haustoria within the inoculated citrus, newly-formed dodder strands less than 2 cm in length originating from the plant were allowed to parasitize 4 16-week-old healthy periwinkle plants. The dodder bridges were maintained for 3 weeks after which time the parasite was removed from the periwinkle. Plants were monitored for the appearance of symptoms over a period of 8 months.

As a control, dodder connections were established as described above between 2 healthy periwinkle plants and a citrus seedling inoculated with only sterile dH_2O.

2.5.5 Reisolation of bacteria from inoculated plants and insects

The extraction procedure was adapted from that reported by Mochaba (1989).

Leaves collected from inoculated and control citrus and periwinkle plants were washed in tap water and then swabbed with alcohol. The midribs were excised and immersed in

absolute ethanol for 2 min., drained briefly and transferred to a 1% (v/v) solution of sodium hypochlorite containing 0.1% (v/v) Tween 20 for 20 min. Surface-sterilized midribs were finally rinsed in 3 changes of sterile dH_2O.

Under aseptic conditions, 0.5 g of midrib material was chopped finely in a small volume of sterile 1/4-strength Ringer's solution and the suspension made up to 2 ml. The suspension was left to stand for 15 min. at room temperature after which it was vortexed for 2 min. and again left to stand to allow the plant debris to settle out. Approximately 0.2 ml of the supernatant fluid was used to inoculate 15 ml MIG-3 medium in each of five, 100 ml Erlenmeyer flasks. Flasks were incubated at 25 C on a gyratory shaker (150 rpm) and monitored daily for bacterial growth.

Inoculated psylla were killed by exposure to ether fumes and their external appendages carefully removed and discarded. The insects were surface-sterilized by immersion in absolute ethanol for 1 min., transferred to a 1% (v/v) solution of sodium hypochlorite containing 0.1% Tween 20 for 15 min., and finally rinsed in 3 changes of sterile dH_2O. Individual insects were ground together with 2 drops 1/4-strength Ringer's solution in a sterile

watch-glass using the rounded end of a test-tube. Each homogenized insect was used to inoculate a 15 ml volume of MIG-3 in a 100 ml Erlenmeyer flask and the flasks incubated as described above.

Inoculum from control psylla was prepared in the same way.

2.5.6 Detection of the fluorescent greening marker by thin layer chromatography (TLC)

Approximately 500 mg bark tissue was cut from the stems and twigs of inoculated and control seedlings. The bark was cut into small pieces and suspended in a small volume (just enough to cover the bark pieces) of dH_2O in a glass vial. The samples were agitated for 30 min. after which the extract was spotted 6 times on a 20 x 20 cm TLC plate coated with Kieselguhr G (Merck, FRG). Plates were developed in a water saturated butanol solvent and then dried in a fume cupboard, before being sprayed with a sodium borate buffer solution, pH 8.7 and viewed under an ultraviolet lamp (Portable Ultraviolet Lamp, type II, Hanovia, England; 95% rad. at 365 nm). Healthy and greening-infected bark samples were included as negative and positive controls, respectively, in all tests.

An extract exhibiting a chromatographic profile containing a bright violet coloured spot with an approximate Rf value of 0.12 - 0.15 was considered positive for greening.

2.6 PREPARATION OF MATERIAL FOR TRANSMISSION ELECTRON MICROSCOPY (TEM)

In the preparation of plant material, excised midribs from citrus and periwinkle leaves were cut into 1-2 mm blocks, fixed in 2.5% glutaraldehyde under vacuum for 4-6 hr, washed 3 x in 0.1M sodium cacodylate buffer pH 7.2, post-fixed in 2% OsO_4 for 1 hr and then washed 3 x in buffer. Dehydration was carried out in a series of acetone solutions and the material then embedded in Spurr's resin gradually while rotating continuously for 5-7 days with daily resin changes before polymerization at 70 C overnight. Details of the dehydration and embedding are given in Appendix C.

For the preparation of insect samples for transmission electron microscopy, the legs and wings of adult psylla were removed and the abdomen separated from the head and thorax. The abdomen and head and thorax regions were then fixed and embedded as for plant material.

The external appendages of psyllid nymphs were removed and the intact body then processed for electron microscopic examination.

For the preparation of bacterial cultures for TEM, cells from a liquid culture were washed twice in 0.1M sodium cacodylate buffer, fixed in 2.5% (v/v) glutaraldehyde for 1 hr and washed 3 x in buffer. The cells were sedimented by centrifugation (Eppendorf microfuge - 2 min.) and the pellet gently resuspended in a minimal amount of molten (45 C) highly-purified agar. The solidified agar was cut into 1-2 mm blocks, post-fixed in 2% (v/v) OsO_4 for 1 hr, washed, and then stained for 8 hr in a 1% (w/v) aqueous solution of uranyl acetate. The samples were dehydrated in a series of alcohols and embedded in Spurr's resin.

Dehydration and embedding were carried out in less than 1 hr to prevent excessive leaching out of the uranyl acetate. Ultra-thin sections were cut and stained with uranyl acetate followed by lead citrate.

2.7 DODDER TRANSMISSION OF GREENING FROM NATURALLY-INFECTED CITRUS TO CUCUMBER

Dodder strands from a parasitized healthy citrus seedling were allowed to establish themselves on 2 Minneola seedlings which had been infected with greening via infective psylla. Citrus infected with greening in this way (as opposed to bud transmission) was used as the source of the disease as this route reduces the possibility of simultaneous infection of the plant with other pathogens such as tristeza virus (S.P. van Vuuren, pers. comm.). Ten days after the establishment of haustoria within the infected plants, newly-formed dodder shoots (less than 2 cm in length) originating from these plants were allowed to parasitize 6 4-week-old cucumber (Cucumis sativus cv. Fletcher) plants. The dodder connections between the infected citrus and the cucumber were maintained for 3 weeks after which time the dodder was removed and the cucumber monitored for the appearance of disease symptoms.

Three similar cucumber plants connected to healthy Minneola seedlings via dodder served as controls. The dodder on one of the control cucumber plants was not removed after 3 weeks and was allowed to infest the host plant heavily in order to determine what effect, if any, the parasite had on cucumber.

Strands of dodder adjacent to the haustoria in cucumber were collected, cut into 1 mm-long sections and processed for TEM as described for citrus (section 2.6). Sections of cucumber stem and petiole were also collected and similarly processed for TEM.

2.7.1 Extraction and culture of bacteria from dodder and cucumber

The procedure for the isolation of bacteria from dodder and cucumber was essentially the same as that described for citrus (section 2.5.5). The source material for isolation from dodder consisted of 2-3 cm long shoots collected from parasitized greening-infected citrus and healthy (control) plants. Young stems and sections of leaf veinal material were used when isolating from cucumber plants.

2.8 INOCULATION OF HERBACEOUS PLANT SPECIES

In order to determine the effect of the bacterial isolates on other commonly-used herbaceous indicator plants, the following species were mechanically inoculated with isolates NC, GC, CS, TZL and LC; Cucumber cv. Fletcher, Tomato (Lycopersicom esculentum L. cv. Marglobe), Green

Pepper (Capsicum annuum cv. Californian Wonder) and Aubergine (Solanum melongena L. cv. Black Beauty). The plants were germinated from surface-sterilized seeds in sterile potting soil and inoculated when approximately 3 weeks old.

For the preparation of inoculum, a colony from a fresh MIG-3 plate was used to inoculate 50 ml of liquid MIG-3 in a 250 ml Erlenmeyer flask. Cultures were incubated overnight on a rotary shaker (150 rpm) at 25 C. One millilitre of the suspension was removed and added to a fresh flask of broth medium as before and placed on a rotary shaker. The cultures were incubated until they reached a turbidity reading of 0.08-0.1 optical units at 640 nm. The bacteria were harvested by centrifugation and resuspended in sterile dH_2O at a concentration of 10^9 cells/ml.

Test plants were maintained under the conditions described in 2.5.2 prior to and following inoculation.

Five specimens of each of the test plants were inoculated by rubbing the fully-expanded leaves gently with an aliquot of the cell suspension. Another 5 plants were inoculated by injecting approximately 50 ul of sample directly into the stem immediately above the cotyledons with a fine hypodermic needle.

As a control, 5 plants of each species were rubbed with only sterile dH_2O.

To determine the effect of bacterial metabolites produced in culture on test plants, the isolates were grown in liquid MIG-3 until in the stationary phase. The cells were pelleted by centrifugation and the spent culture supernatant sterilized by filtration through a 0.2 um nitrocellulose membrane.

Five plants of each species were exposed to the metabolites by rubbing fully-expanded leaves with the sterile culture supernatant, without the use of abrasive. In an alternative method, stem cuttings of each plant species were allowed to imbibe the spent medium (diluted 1:1 with sterile distilled water) by submerging the freshly cut end of the stems in the fluid for 30 min. The cuttings were then transferred to sterile dH_2O and monitored for any reaction. Control seedlings were exposed to diluted (1:1) sterile fresh medium.

Immediately after application of the cell suspension or supernatant, plants were transferred to a humid chamber where they were left for 4 hr in order to prevent dehydration of the inoculum. Thereafter, plants were maintained under normal temperature, humidity and day-light conditions.

The effect of the spent culture supernatant on healthy Minneola seedlings and stem cuttings was determined in a similar way.

2.9 PRODUCTION OF ANTISERA AGAINST CULTURED BACTERIA

2.9.1 Preparation of bacterial immunogen

In an attempt to determine whether the efficacy of antisera raised against the cultured isolates from citrus could be improved, treatments were carried out on the bacterial immunogens prior to the immunization of rabbits. Pre-treatments of the bacterial cells included removal of capsular material, exposure of internal antigens by sonication of the cells, extraction of alkali-soluble fractions, and fractionation of total cellular protein by SDS-PAGE and selection of certain protein bands as immunogens.

The effect of the different treatments on the quality of the resultant antiserum was assessed using the GC isolate. In all the procedures, the bacterium was grown in liquid MIG-3 and the cells harvested in the exponential phase. The serum was prepared following the procedure detailed in section 2.9.3 and tested in the HE-PTA-ELISA (section 2.10.2.3). The unfractionated serum was reacted against

citrus leaf midrib material harvested from greenhouse plants from which bacteria identical (with respect to Gram stain reaction, morphology and PAGE protein profiles) to the GC isolate had previously been isolated. Healthy midrib material served as a control. Plant samples were prepared following the water leaching method (section 2.10.1.6), except in the case of the antiserum prepared against KOH-treated bacteria where the serum was reacted against an alkali extract of the plant material (section 2.10.1.5).

2.9.1.1 Preparation of live cells

Cells were harvested by centrifugation and washed 3 x in a large volume of sterile physiological saline. After the final wash the cells were resuspended in saline at a concentration of 10^7/ml and 0.75 ml aliquots of the suspension frozen at -20 C until required for immunization.

2.9.1.2 Preparation of heat-killed cells

Cells were prepared as described above and 0.75 ml aliquots of the suspension heated in a boiling water bath for 30 min. prior to the immunization of rabbits.

2.9.1.3 Removal of capsular material and treatment with formaldehyde

Cells were harvested by centrifugation and the pellet resuspended in 50 ml of normal saline containing 0.5% formaldehyde. The suspension was stirred continuously in a boiling water bath for 15 min., allowed to cool and then centrifuged at 22 000g for 50 min. at 4 ℃ (Keddy 1989). The supernatant containing the capsular material was discarded and the cells washed and aliquoted as described above.

2.9.1.4 Disruption of cells by sonication

The concentration of a suspension of heat-killed, unencapsulated cells was adjusted to 10^7 cells/ml and 3 ml aliquots pulse sonicated (60% duty cycle) for 5 x 5 min. periods with an Ultrasonics Inc. (USA) Cell Disruptor (Model W-225R) at 43% power output using a micro-tip probe. The suspension was kept on ice to prevent excessive heating during treatment and after each 5 min. period of sonication the sample was allowed to chill thoroughly before resumption of treatment.

2.9.1.5 Treatment of bacteria with KOH

Alkali treatment of antigens was carried out to expose bacterial cell surface antigens using a modification of the method of Kawarabata and Hayasaka (1987). A pellet of heat-killed, unencapsulated cells was resuspended in 0.1N KOH in normal saline and the cell concentration adjusted to 10^7 cells/ml. The suspension was incubated at 27 C for 40 min., the cells pelleted by centrifugation (22 000g for 15 min. at 4 C) and then resuspended in an equal volume of cold PBS containing 1% formaldehyde and fixed at 4 C overnight. The cells were then washed 2 x in PBS and finally suspended in PBS at a concentration of 10^7 cells/ml. The preparation was aliquoted into 0.75 ml volumes and stored at -20 C until used.

2.9.1.6 Fractionation and isolation of bacterial and plant proteins by PAGE

Initial results of ELISA tests using antiserum raised against cultured isolates showed a significant amount of cross-reactivity between the antiserum and preparations of healthy plant material (Fig. 55). The Western blotting technique was later used to demonstrate the presence of proteins common to both the bacterial isolates and healthy plant material (Fig. 76). The existence of such common

antigenic determinants may offer a possible explanation for the high background readings recorded. By comparing the profiles of plant and bacterial proteins fractionated by PAGE and selecting protein bands unique to the bacteria for use as immunogens, it is theoretically possible to obtain an antiserum which exhibits little or no cross-reactivity with plant components.

For the preparation of bacterial proteins, a pellet of washed, live cells (harvested in the exponential phase) was resuspended in 0.125 M Tris-HCl, pH 6.8, the concentration adjusted to 10^7 cells/ml and the cells disrupted by sonication as described above. The suspension was centrifuged at 22 000g for 10 min. to remove insoluble debris and the supernatant lyophilized. The concentrated proteins were resuspended in dH_2O at a final concentration of approximately 10 mg/ml, as determined by the Bio-Rad protein assay (Bio-Rad Laboratories, FRG).

Buffer-soluble plant proteins were extracted from mid-ribs excised from healthy citrus leaves. Half a gram of mid-rib material was chopped very finely in a small volume of 0.125 M Tris-HCl, pH 6.8. Additional buffer was added to make a liquid suspension and the preparation incubated on ice for 15 min. The suspension was then shaken vigorously

for 10 min. and left to stand for the large particulate material to settle out. The supernatant was collected and clarified by centrifugation at 200 g for 5 min. The protein concentration was determined using the Bio-Rad protein assay and the sample diluted by the addition of buffer to a final protein concentration of 10 mg/ml. If the protein content was found to be too low, the samples could be concentrated by lyophylization.

The composition of all PAGE buffers is detailed in Appendix D. Prepared samples were mixed with an equal volume of splitting solution and denatured by heating at 100 C for 4 min. Samples containing approximately 200 ug of protein were fractionated under denaturing conditions in 10% acrylamide gels (100 x 160 mm 1.5 mm) using a modification of the discontinuous buffer system of Laemmli (1970) (Appendix D).

For the fractionation of protein samples under non-denaturing conditions, samples were prepared in the same way, the lyophylized preparation resuspended in non-denaturing sample buffer and then fractionated in non-denaturing acrylamide gels (Appendix D).

Samples were electrophoresed in the cold (4 C) at 60V until the marker dye had migrated into the stacking gel and was visible as a compacted front. The voltage was then increased to 150V and the samples electrophoresed until the dye reached the bottom of the separating gel.

Gels were stained for 1-2 hr with Coomassie brilliant blue G-250 (BDH, London) (0.2% in dH_2O) and passively destained in water until protein bands were visible against the background. Desired bands containing between 100 and 200 ug of protein were carefully excised from the gel and transferred to a 5 ml plastic syringe fitted with a large-gauge needle. The gel containing the protein sample was homogenized by being extruded through the needle twice and the gel homogenate then mixed with an equal volume of Freund's complete adjuvant (for the initial immunization) or incomplete adjuvant (for subsequent immunizations). The mixture was then extruded through a small-gauge needle 5 times or until thoroughly emulsified. Rabbits were immunized with the gel homogenate as described in section 2.9.3.

If gel electrophoresis was carried out in order to merely compare the protein profiles of the different samples and not to isolate bands for use as immunogen the gels were stained in the following manner: After electrophoresis,

gels were soaked 2 x in an excess of 50% methanol, 10% acetic acid and 40% dH_2O (wash solution) for 30 min. each time in order to remove excess SDS from the gels. The gels were then stained for 4 hr or more in 0.2% (w/v) Coomassie brillant blue R-250 (Sigma) dissolved in wash solution with gentle agitation. Destaining was carried out in a large volume of wash solution until protein bands were visible against a clear background.

Seven hundred and fifty microlitre volumes of prepared antigen emulsified with an equal volume of Freund's adjuvant were injected into rabbits intramuscularly (2.9.3). The emulsion was prepared just prior to immunization, although excised protein bands could be stored at -20 C sealed in plastic cling film until needed.

2.9.2 Extraction of putatively disease-specific immunogens from naturally-infected plant material

Antisera raised against the cultured bacterial isolates were unable to consistently detect the presence of greening in material from plants known to be affected by the disease (Figs. 63 and 67). In order to avoid using these isolates as immunogen, attempts were made to raise polyclonal antisera against putatively disease-specific antigens extracted from greening-affected citrus by various methods.

The efficacy of each of the extraction procedures employed was assessed using freshly-harvested infected citrus leaf midribs, as well as bark from young, fleshy stems as the source material. The immature bark from stems of infected plants has been observed to harbour large numbers of the greening bacterium (Ariovich, pers. comm.) and should therefore serve as a suitable source of the organism. When extraction from midribs was carried out, the midribs were excised from young as well as from mature leaves displaying disease symptoms ranging from mild to severe.

Citrus plants used as a source of greening-affected material were tested for the presence of the disease by TLC (section 2.5.6) prior to use. Specimens exhibiting the characteristic gentisic acid marker were considered to be affected by the disease. Healthy control plants tested negative for greening by the same method.

In addition to the use of infected citrus material, infected periwinkle bark and leaf midrib tissue was used as additional source material to evaluate the efficacy of the extraction procedure reported by Sui (1988). In the assessment of the extraction procedures, healthy plant material was treated in the same way as infected material and served as controls.

After the final high-speed centrifugation of each procedure, the resultant pellet was either resuspended in a minimal volume of PBS and then examined under the electron microscope, or resuspended in ELISA coating buffer and the amount of disease-specific antigen present determined in the direct ELISA using the antiserum raised against an extract from infected citrus (GICE antiserum) (section 2.9.2, method 5).

For TEM examination of preparations, the sample was adsorbed onto an electron microscope grid and then negatively stained by dipping the grid briefly into a drop of 0.1% (w/v) aqueous solution of uranyl acetate. Excess stain was removed by touching the edge of the grid with filter paper. Samples were viewed in a 100S Jeol electron microscope.

In addition to the use of naturally-infected plant material, extraction procedures 2 - 5 were evaluated using healthy leaf midrib homogenates artifically contaminated with cultured Clavibacter michiganense subsp. michiganense (C.m. subsp. michiganense) cells. The bacteria were cultured in tryptone soy broth, harvested when in the exponential phase and washed 2 x in PBS. The bacteria were added to the plant preparation immediately after the initial homogenization step at a final concentration of

10^6 cells/ml of homogenate. After the final centrifugation, the resultant cell pellet was resuspended in PBS and the number of bacteria recovered determined using a haemocytometer.

a) Method 1

This is a variation of the KOH extraction method reported by French (1974). Twenty grams of either leaf midrib or bark tissue were washed thoroughly in a 0.1% (v/v) solution of Tween 20 and rinsed in a large volume of dH_2O. The plant material was then chopped into small pieces and suspended in 1 M KOH. The mixture was incubated at 25 C for 15 min. with gentle agitation. The suspension was then filtered through 2 layers of miracloth and a single layer of Whatman No. 4 paper and the filtrate centrifuged at 35 000g for 40 min. in order to sediment any bacteria present. The resuspended pellet was washed 3 x in a large volume of Ringer's solution before final resuspension in a minimal volume of PBS.

b) Method 2

This procedure is a modification of that reported by Sinha (1974). Fifty grams of leaf midrib or bark tissue were washed in tap water and then soaked in a large volume of 1% sodium hypochlorite and 0.1% Tween 20 for 15 min. The

material was thoroughly rinsed in dH_2O, blotted dry, chopped into small pieces and then infiltrated with a solution of 0.1 M $MgCl_2$ (Mg-solution) and 0.3 M glycine, pH 8.0 (Mg-glycine solution) under vacuum (450 mm Hg) for 30 min. The leaf or bark material was homogenized with a polytron, filtred through 2 layers of miracloth, the volume adjusted to 150 ml with Mg-solution and the sap centrifuged at 200g for 10 min. (low-speed centrifugation). The supernatant was adjusted to pH 8.0 and centrifuged again at low speed. The volume of the supernatant was measured and to it was added 5% (w/v) activated charcoal powder. The solution was stirred for 1 min. and then 5% (w/v) Celite 545 was added and the solution stirred for another minute before being passed through Celite pads in order to adsord any microorganisms from the mixture. The pads consisted of a 15 mm layer of Celite 545 sandwiched between 2 circles (..0 mm diam.) of Whatman No. 4 filter paper held in a Buchner funnel. A maximum of 75 ml of the mixture was passed through a single pad in order to effectively remove contaminating plant pigments. The mixture measuring 150 ml was divided into 2 equal lots and passed through 2 separate pads. Organisms were eluted from the pads with Mg-solution under gentle vacuum and 20 ml fraction collected. No opalescent fractions were observed so all the fractions were pooled and centrifuged at 25 000g for 30 min. and the resulting pellet soaked in a minimal amount of Mg-solution

overnight on ice. The volume of the suspension was adjusted to 5 ml and the pellet thoroughly resuspended by gently agitating the tube. The suspension was then clarified by centrifuging at 100g for 10 min.

In order to determine whether any bacteria were present in the preparation before further purification by column chromatography and density gradient centrifugation (as described by Sinha (1974)), 1.5 ml of the supernatant was centrifuged at 25 000g for 30 min. and the pellet resuspended in a single drop of PBS and viewed under the electron microscope.

A variation of this extraction method which included an enzymatic degradation step was also used. The very finely chopped plant tissues were infiltrated with an enzyme-buffer solution consisting of 0.1 M $MgCl_2$, 0.6 M glycine, 0.5 M mannitol, 2% (w/v) Cellulase R-10, 1% (w/v) Macerozyme R-10, pH 6.0 in place of the Mg-glycine solution used above (both enzymes were purchased from Yakult Honsha Co. Ltd., Japan). Ten millilitres of enzyme solution per gram of leaf material were used and the suspension incubated at 30 C for 3 hr with gentle agitation. After degradation, the material was treated in the same way as detailed above.

c) Method 3

This procedure was adapted from the technique developed by Jiang and Chen (1987) for the purification of the MLO associated with the Aster Yellows disease from lettuce. The procedure was essentially the same as that reported by the authors but with minor modifications.

One hundred grams of midrib or bark tissue were washed as described in method 2, chopped finely in 100 ml of chilled isolation medium (0.3 M D-mannitol, 4 mM L-cysteine, 30 mM (N-morpholino)-propanesulfonic acid (MOPS), 1 mM EDTA, 0.8% (w/v) PVP, pH 7.2) and disrupted with a polytron. The tissue was then ground with a mortar and pestle until no vascular fibres were discernable. The sap was squeezed through 3 layers of cheesecloth, the volume adjusted to 400 ml with isolation medium, and then centrifuged at 1 500g for 8 min. The supernatant was filtered through a layer of Whatman No. 4 filter paper and centrifuged at 35 000g for 30 min. at 4 C. The pellet was resuspended in approximately 200 ml of chilled suspending medium (0.3 M mannitol, 20 mM MOPS, pH 7.0) and differentially centrifuged at the low and high speeds. The final pellet was resuspended in approximately 5 ml of chilled suspending medium. Before attempts to further purify the preparation by discontinuous gradient centrifugation, the presence of bacteria was ascertained as described in method 2.

d) Method 4

Infected and healthy citrus, as well as periwinkle midrib and bark tissue was processed according to the method developed by Sui (1988) for the purification of the Asian greening organism.

Fifty grams of plant material was used in each extraction attempt. The material was weighed, rinsed 3 times in a large volume of dH_2O and to it added three-fold (v/w) (150 ml) isolation medium (0.1 M $MgCl_2$, 0.5 M mannitol, 0.6 M glycine, pH 7.4). The tissue was homogenized with the aid of a polytron as described above and cellulase added to a final concentration of 1% (w/v). Enzymolysis of the tissue was allowed to occur for 3 hr at 35 C with gentle agitation. The slurry was filtered (2 layers of cheesecloth followed by a single layer of miracloth) and the filtrate centrifuged at 1 500g for 20 min. at 4 C. The supernatant was then centrifuged at 35 000g for 40 min. at 4 C, the resulting pellet resuspended in 150 ml isolation medium and the suspension subjected to another cycle of differential centrifugation at the low and high speeds. The pellet was resuspended in 150 ml of suspending solution (0.5 M mannitol, 30 mM cysteine, pH 7.2) and another two cycles of differential centrifugation were carried out. The final pellet was resuspended in a minimal amount of suspending solution, centrifuged at low speed and the supernatant collected.

e) Method 5

This procedure was used to obtain a crude preparation of antigen from greening-infected citrus and periwinkle material.

A 25 g sample of leaf or bark material was surface-sterilized as in method 2, chopped finely nd then suspended in enzyme solution (1 mM $CaCl_2$, 0.6 M mannitol containing 1% (w/v) macerozyme and 2% (w/v) cellulase, pH 5.5). The material was placed in the bowl of a mini food processor (Salton Le Whizz, Teltron, RSA) fitted with a steel blade, and 6 ml enzyme solution added per gram plant tissue. The material was pulse-homogenized until fine and the resultant slurry incubated in an Erlenmeyer flask for 3 hr at 30 C on an orbital shaker (150 rpm). The material was homogenized again with the aid of a polytron and filtered through 2 layers of miracloth. The solids were rinsed with chilled PBS containing 0.6 M mannitol (PBS-mannitol) and squeezed firmly to extract all the sap. The filtrate was clarified by centrifugation at 200g for 5 min. at 4 C (low-speed centrifugation). The supernatant was then filtered through a single layer of Whatmans No. 4 filter paper and the filtrate subjected to high-speed centrifugation at 4 C (20 000g for 30 min.). The pellet was gently resuspended in a large volume of PBS-mannitol, the

particulate matter sedimented by high-speed centrifugation and the pellet washed a second time by resuspension in PBS-mannitol. After high-speed centrifugation, the pellet was resuspended overnight on ice in a small volume (approximately 8 ml) of PBS-mannitol containing 0.1% (w/v) EDTA (PBS-mannitol-EDTA) with the aid of a magnetic stirrer. The suspension was clarified by low-speed centrifugation and the supernatant divided into 2 equal volumes. Each volume of supernatant was layered onto to a discontinuous density gradient consisting of 4 ml of 30% (w/v) sucrose layered on 2 ml of 70% (w/v) sucrose. The sucrose was made up in PBS-mannitol-EDTA and the gradient centrifuged in a Beckman SW 28 swing-out rotor at 70 000g for 60 min. The band at the interface of the two sucrose solutions was harvested and washed 2 x in a large volume of PBS-mannitol-EDTA before final resuspension in a minimal volume of PBS.

As an alternative to the use of sucrose density centrifugation, self generating Percoll gradients were used in an attempt to optimize the purification of the bacteria from plant preparations. Two concentrations of Percoll were used, namely 30% and 70% (v/v). Sterile Percoll (Pharmacia, Uppsala, Sweden) was diluted in the isolation medium of Sui (1988) (0.1 M $MgCl_2$, 0.5 M mannitol, 0.6 M glycine, pH 7.4). Thirty five millilitres of Percoll solution was added

to a Beckman Ultra-Clear tube (25 x 89 mm) and 5 ml of sample layered on top of the Percoll. The preparations were centrifuged at 30 000g for 30 min. in a Beckman SW 55 angle-head rotor. When only small volumes of sample were available, gradient centrifugation was performed using 11 x 34 mm polycarbonate tubes (1.5 ml volume) and a Beckman TLV 100K rotor.

f) Method 6

Both denaturing and non-denaturing PAGE were used in an attempt to identify pathogenesis-related proteins unique to greening-infected citrus. Such proteins would serve as an ideal source of immunogen for the production of a mono-specific antiserum for the disease.

The following plant leaf midrib samples were analyzed by SDS-PAGE: i) healthy sweet orange (certified greening-free), ii) greening-infected sweet orange (greenhouse-grown seedlings infected with the disease via T. erytreae and provided by the C.S.F.R.I., Nelspruit, iii) troyer citrange infected with tatter leaf virus and citrus tristeza virus (CTV), iv) rough lemon infected with vein enation virus and CTV, v) mandarin affected by CTV and "multiple sprouting" (a condition of citrus thought to be caused by a mycoplasma or MLO (Da Graca, pers. comm.)),

vi) sweet orange affected by psorosis and CTV, and vii) grapefruit infected with CTV as well as with a severe strain of CTV (strain 7K6). The citrus infected with agents other than greening were supposedly free of greening disease and were kindly supplied by Prof. J. Da Graca of the University of Natal, Pietermaritzburg.

Citrus infected with diseases other than greening was analysed in order to identify any pathogen-induced proteins of host origin which may be produced by plants in response to either stress or infection with various phytopathogens. Antisera raised against such proteins would obviously not specifically detect greening disease.

Sample preparation for denaturing as well as non-denaturing PAGE was carried out as described in section 2.9.1.6 with the addition of a tissue homogenization step. In this modification of the protein extraction procedure, the chopped midribs were thoroughly homogenized with the aid of a polytron after being allowed to stand on ice for 15 min. and thereafter treated as described in section 2.9.1.6. Samples were suspended in splitting solution and then heated, or suspended in non-denaturing sample buffer. The preparations were layered onto 10% acrylamide gels and fractionated and stained as described in section 2.9.1.6.

Citrus affected by the Asian strain of greening disease was also analysed in this study by PAGE under denaturing, as well as non-denaturing conditions. Samples of infected as well as healthy material were supplied by Prof. Hong-Ji Su of the National Taiwan University, Taiwan, R.O.C. and this aspect of the work carried out at the Development Centre for Biotechnology in Tz'pei. R.O.C.

For the analysis of samples under non-denaturing conditions, midribs were excised from washed leaves, frozen in liquid N_2 and ground to a fine powder using a pestle and mortar. The material was then suspended in extraction buffer (30 mM Tris-HCl, pH 8.7 containing 1 mM dithiothreitol (DTT), 10 mM ascorbic acid, 10 mM EDTA, 5 mM $MgCl_2$ and 0.5% (w/v) insoluble PVP). The phenol complexing agent (PVP) and reducing agents (DTT and ascorbic acid) were added to the Tris buffer in order to prevent peroxidase and phenoloxidase activity which can result in artefacts as well as in sample streaking and poor resolution (Granier 1988).

After the addition of extraction buffer, the sample was ground further and then shaken vigorously for 15 min. The suspension was centrifuged in a microfuge for 10 min. and the buffer-soluble proteins in the supernatant concentrated by acetone precipitation. In addition to concentrating the

protein, acetone precipitation allows for the removal of plant pigments, phenols and lipids, all of which may also cause sample streaking (Granier 1988).

In the acetone precipitation step, sucrose was added to a 2 ml volume of the supernatant to a final concentration of 5% (w/v) followed by the addition of 8 ml of ice-cold acetone containing 0.07% (v/v) 2-mercaptoethanol. The solution was mixed well and incubated at -20 C for 1 hr. The precipitated proteins were pelleted by centrifugation (36 000g for 10 min.), the pellet dried under vacuum and then solubilized in PBS. To this preparation was added an equal volume of non-denaturing PAGE sample buffer and samples containing 100 - 250 ug of protein were fractionated on 10% acrylamide gels using a SE250 Mighty Small II Slab Gel Electrophoresis Unit (Hoefer Scientific Instruments, USA).

Three different sample preparation procedures, namely phenol, SDS and trichloroacetic acid-acetone (TCA-acetone) extraction, were employed in the analysis of the Asian citrus samples by PAGE under denaturing conditions. Denaturing extraction in the presence of SDS at high temperature allows the solubilization of membrane-bound proteins as well as the recovery of soluble proteins while

at the same time inactivating plant proteases and phenoloxidases, both of which cause poor gel resolution (Granier 1988).

The solubilization of membrane proteins with phenol is reportedly as good as that obtained with SDS but phenol extraction has the added advantage of eliminating most polysaccharides and glycoproteins in addition to desalting samples, thus ensuring reproducible separation of protein (Meyer et al., 1988). Although time consuming, phenol extraction allows for the preparation of highly concentrated protein samples from tissues with a low protein content (Meyer et al., 1988).

Direct precipitation of proteins using a combination of the two denaturing and precipitating agents, namely TCA and acetone, allows visualization of total proteins without artefacts caused by plant enzyme action. The addition of TCA and acetone results in the immediate inactivation of plant proteases and phenoloxidases (Granier, 1988). The extraction procedure, however, does not effectively recover membrane proteins as these usually require high temperature for solubilization.

For SDS extraction of proteins, leaf midrib samples were homogenized in a solution comprising of 4% (w/v) SDS,

5% (v/v) 2-mercaptoethanol, 5% (w/v) sucrose and 0.5% (w/v) insoluble PVP. The resulting slurry was heated in a boiling water bath for 5 min., cooled rapidly and then centrifuged to remove insoluble material. An equal volume of non-denaturing PAGE sample buffer was added to the sample prior to fractionation by PAGE.

In the phenol extraction procedure, an emulsion of 50% (w/v) high-grade phenol in 0.1 M Tris-HCl, pH 8.0, containing 5% (v/v) 2-mercaptoethanol and 5% (w/v) sucrose was prepared. Approximately 2 g of frozen leaf material was homogenized in 10 ml of the chilled emulsion and the suspension stirred over ice for 30 min. The phenol and aqueous phases were separated by centrifugation (5 000g for 10 min.), the phenol phase collected and re-extracted for 15 min. with an equal volume of phenol-saturated 0.1 M Tris-HCl, pH 8.0, containing 5% 2-mercaptoethanol. The phenol phase was collected and proteins precipitated by adding 4 volumes of ice-cold methanol containing 0.1 M ammonium acetate (methanol-ammonium acetate) and incubating the mixture at -20 C for 4 hr. The precipitate was collected by centrifugation, washed 6 x with chilled methanol-ammonium acetate and dried under vacuum. The extract was resuspended in a solubilization mixture consisting of 9.5 M urea, 5 mM K_2CO_3, 1.25% (w/v) SDS, 0.5% (w/v) DTT, and 6% (v/v) Triton X-100, vortexed well and mixed with an equal volume of non-denaturing PAGE sample buffer.

For TCA-acetone precipitation of proteins, leaf midribs were frozen in liquid N_2 and crushed to a fine powder. Ice-cold extraction solution (10% (w/v) TCA and 0.07% (v/v) 2-mercaptoethanol in acetone) was added to the cold powder and the suspension vortexed. After standing at -20 C for 1 hr, the precipitate was recovered by centrifugation (35 000g for 15 min.) and the pellet soaked in a large volume of chilled acetone containing 0.07% 2-mercaptoethanol for 1 hr at -20 C. The soaking solution was decanted off, the pellet dried under reduced pressure for 1 hr, and resuspended in solubilization mixture detailed above. After vortexing, the suspension was centrifuged briefly (15 000g for 5 min.) to remove insoluble material and the supernatant mixed with an equal volume of non-denaturing PAGE sample buffer.

2.9.3 Immunization of rabbits

Antisera to preparations of cultured cells, as well as to extracts from naturally-infected plant material, were raised in 1.5-2 kg female New Zealand white rabbits. Animals were immunized via either the intravenous or intramuscular routes. Pre-immune serum was taken from each rabbit and tested using the microprecipitation test (Hill, 1984) for the presence of any pre-existing

antibodies to the bacterial isolates, plant extracts and healthy plant material. The microprecipitin test was also used to determine the antibody titre of the test bleeds as well as that of the final antisera.

Immunization via the intravenous (IV) route generally yielded antisera against cultured isolates in a short period of time (approximately 28 days) but with a relatively low titre (usually between 1/256 and 1/512, as determined in the microprecipitin test). In this procedure, 750 ul of immunogen suspended in sterile normal saline was injected into the marginal ear vein of a rabbit every alternate day over a period of 14 days. A test bleed was performed 7 days after the last injection and a single booster inoculation administered if necessary.

Because of the large amount of contaminating plant compounds present in the extracts obtained from infected plant tissue, the IV route of immunization could not be used in these instances.

Although requiring a longer period of time for production of antiserum, the intramuscular route was the prefered route of immunization as preliminary studies indicated that resultant antisera against isolates were of a higher titre (between 1/1024 and 1/4096).

In this procedure, a 750 ul volume of immunogen (either a preparation of cultured bacterial cells or a plant extract) was emulsified with an equal volume of Freund's adjuvant (complete Freund's was used for the initial immunization and incomplete Freund's for subsequent boosters). Each rabbit received a total of 5 inoculations administered at weekly intervals into the thigh muscle of the animal's hind leg. Two weeks after the last inoculation, a test bleed was performed, the titre of the antiserum determined, and a booster given if necessary.

At the end of the immunization schedule, the rabbits were anaesthetized and exsanguinated by cardiac puncture. Collected blood was allowed to clot at 37 C for 1 hr and then incubated on ice for 2 hr in order to shrink the clot. The serum was collected, centrifuged (5000 g for 5 min.) to remove any remaining blood cells and aliquoted into 5 ml volumes for storage at -20 C.

2.9.4 Cross-adsorption of antisera

Cross-adsorption of the putatively greening-specific antiserum which was raised against the extract from infected plant material was essential in order to reduce the high background readings recorded in the ELISA

(Fig. 53). The antiserum was cross-adsorbed against extracts of healthy citrus material, as well as against extracts of citrus material collected from plants ostensibly free of greening but affected by other phytopathogens. This infected material was used for cross-adsorption in order to remove antibodies directed against pathogenesis-related proteins of plant origin which would affect the specificity of the antiserum.

Leaf midribs from plants infected with the following pathogens were used for cross-adsorption: i) rough lemon infected with vein enation virus and CTV, ii) rough lemon infected with vein enation virus only, iii) grapefruit infected with CTV as well as with the severe 7K6 strain of CTV, iv) mandarin affected by CTV as well as by "multiple sprouting", v) sweet orange infected with psorosis and CTV, and vi) troyer citrange infected with citrus tatter leaf virus and CTV. Equal quantities of each sample were combined for the preparation a pooled extract of infected material for the cross-adsorption of antisera.

All the infected leaf material was obtained from the University of Natal, Pietermaritzburg while healthy citrus material was collected from sweet orange seedlings provided by the C.S.F.R.I. in Nelspruit. Leaf material known to be affected by greening was also obtained from the latter source.

Three different cross-adsorption techniques were used to remove anti-plant activity from the antiserum raised against the infected citrus extract prepared following method 5 (sucrose and not Percoll was used in the gradient centrifugation). The efficacy of the three adsorption techniques was determined and compared.

In an attempt to reduce the reactivity observed between the antiserum raised against the cultured isolate (GC) and healthy plant material, the antiserum was cross-adsorbed against plant proteins prepared following the ammonium sulphate precipitation technique (2.9.4.2).

2.9.4.1 Cross-adsorption against acetone-fixed plant material

A modification of the method of Lin and Chen (1986) was used. Five grams of midrib material was cut into very fine sections using a scalpel blade and the sections fixed in acetone for 30 min. and then air-dried. The fixed material was ground to a fine powder and the antisera cross-adsorbed by mixing 1 ml of whole serum with 1 ml PBS and the acetone-fixed powder prepared from 5 g of leaf midribs. The mixture was incubated at 37 C for 1 hr and then at 4 C overnight. The immunoprecipitates were removed by

centrifugation at 13 000g for 15 min. and the serum stored at 4 C (with 0.02% (w/v) NaN_3 added) or at -20 C (without NaN_3). However, cross-adsorbed serum was generally prepared freshly prior to use as it was found that prolonged storage at either 4 C or -20 C resulted in poor detection assay results.

2.9.4.2 Cross-adsorption against ammonium sulphate-precipitated plant proteins

The method used was a modification of that described by Lommel et al. (1982). Five grams of healthy citrus midrib tissue were homogenized in 10 ml PBS over ice, the suspension filtered through a layer of miracloth and then clarified by low-speed centrifugation (3 800g for 15 min.). Protein in the supernatant was precipitated by the addition of an equal volume of saturated ammonium sulphate followed by incubation on ice for 30 min. The precipitate was pelleted by centrifugation at 15 000g for 15 min., resuspended in 1 ml PBS and then dialyzed exhaustively against PBS (12 000 - 14 000 MW cut-off dialysis tubing was used). The preparation was clarified by centrifugation (8 000g - 10 min.) and stored at -20 C if not used immediately.

Antisera were cross-adsorbed by mixing 1 ml of the concentrated plant protein with 0.1 ml of whole serum. The mixture was incubated for 1 hr at 37 C and then overnight at 4 C. The precipitate was removed by centrifugation in an Eppendorf microfuge for 10 min. For short-term storage, NaN_3 was added to the serum to a final concentration of 0.02% (w/v) and the serum kept at 4 C. Generally, cross-adsorbed antisera were not kept for more than 2 days.

2.9.4.3 Cross-adsorption by affinity column chromatography

Midribs were excised from both young and mature healthy sweet orange leaves, thoroughly rinsed with dH_2O and cut into small sections. The material was frozen by the addition of liquid N_2 and ground to a fine powder in a mortar. Coupling buffer (0.1 M $NaHCO_3$, pH 8.3 containing 0.5 M NaCl) was added and the material ground further. The suspension was transferred to a centrifuge tube and shaken vigorously for 1 min. and then centrifuged at 12 000 g for 5 min. The supernatant was filtered through Whatmans No. 4 filter paper, its protein content determined spectrophotometrically, and the solution diluted by the addition of coupling buffer to a final protein concentration of approximately 5 mg/ml. The prepared ligand was coupled to CNBr-Activated Sepharose 4B (Pharmacia,

Uppsala, Sweden) overnight at 4 C according to the manufacturer's instructions. After washing away excess ligand, any remaining active groups were blocked by treatment with 1 M ethanolamine, pH 9 for 2 hr. The protein-Sepharose 4B conjugate was packed in a 150 x 10 mm column and equilibrated with PBS prior to use.

For removal of plant-specific antibodies from antiserum, the serum was diluted 1:10 in PBS and then passed through the packed column at a flow rate of approximately 0.25 ml/min. Two millilitre volumes of diluted antiserum were used each time, between which bound antibodies were removed by washing the column with acetate buffer (0.1 M, pH 4), followed by re-equilibration with PBS. One millilitre fractions of eluate were collected and those displaying an OD_{280} of 1.0 or more were pooled and used in the ELISA.

2.9.5 Fractionation of immunoglobulin

In the various serological detection assays, antiserum was used either whole or fractioned. The immunoglobulin fraction (IgG) was purified from whole serum by the Rivanol/ammonium sulphate method as described by Horejsi and Smetana (1966).

2.9.6 Production of Fab' and $F(ab')_2$ fragments

Fab' and $F(ab')_2$ fragments of IgG were prepared by pepsin digestion and cysteine reduction from purified immunoglobulin as described by Barbara and Clark (1982). The fragments were isolated from the reaction mixture by affinity chromatography through 5 ml of wet Protein A-Sepharose CL-4B (Pharmacia Fine Chemicals, Uppsala) packed in a column consisting of a 10 ml disposable plastic syringe. The purity of the Fab' and $F(ab')_2$ preparations was confirmed by immunoelectrophoresis.

2.9.7 Conjugation of enzyme label to IgG

Alkaline phosphatase was conjugated to purified IgG as described by Clark and Adams (1977).

2.10 SEROLOGICAL DETECTION ASSAYS

2.10.1 reparation of plant samples

Ideally, a sample preparation procedure should yield a suspension containing the antigen of interest with a minimal amount of contaminating host plant material. In an attempt to optimize the extraction procedure, a variety of methods were examined.

Plant material used in this investigation was collected from greening-affected citrus seedlings provided by the C.S.F.R.I. The presence of the disease was confirmed by the TLC assay (section 2.5.8). Healthy material was from the same source and tested negative for the presence of the gentisic acid marker of greening disease. Leaf midribs were excised from the material and mixed together in a large container and the material required for each procedure randomly taken from this pooled sample. This method of selecting leaf material ensured a relative degree of uniformity among the different samples.

For the comparison of the extraction procedures, the prepared samples were reacted with the GICE antiserum in the HE-PTA-ELISA (section 2.10.2.3).

2.10.1.1 Leaf Press extraction

An Erich Polane leaf press was used to extract the sap from washed, excised leaf midribs as described by Duncan (1985), omitting the sonication step.

2.10.1.2 "Chopping" method

Washed midribs were weighed and then chopped very finely with a scalpel blade in a small volume of either sample conjugate buffer PBS (Boehringer Mannheim (BM), FRG) or coating buffer (BM, FRG). Sample conjugate buffer was used for the preparation of samples for DAS-ELISA, Fab-ELISA and $F(ab')_2$-ELISA, while coating buffer was used when samples were prepared for PTA-ELISA. The suspension was transferred to a 20 ml Erlenmeyer flask and additional buffer added so that the leaf material was adequately suspended in the liquid. After standing for 10 min. the suspension was vortexed for 5 min., the plant debris allowed to settle out, and the supernatant collected. Particulate matter in the suspension was sedimented by centrifugation (12 000g for 30 min.) and the resulting pellet thoroughly resuspended in 4 ml of the appropriate buffer per gram original leaf material.

2.10.1.3 Polytron homogenization

Washed midribs were placed in a large test tube with 10 ml chilled 1/4-strength Ringer's solution per gram leaf material and thoroughly homogenised over ice using a polytron (Kinematica, Switzerland) (speed setting 3). The

homogenate was filtered through a double layer of miracloth and clarified by centrifugation (200g for 5 min.) followed by filtration through Whatmans No.4 filter paper. The filtrate was then centrifuged (12 000g for 30 min.) to concentrate bacteria present and the pellet resuspended in 4 ml coating buffer or sample buffer per gram original leaf material.

2.10.1.4 Enzymatic treatment of plant material

In an attempt to maximise the release of phloem-limited bacteria from diseased tissue, midrib tissue was subjected to enzymatic digestion.

Midribs were washed thoroughly in running water and swabbed with alcohol before being chopped as finely as possible in a small amount of enzyme solution (2% (w/v) Cellulase TC (Serva, FRG), 1.5% (w/v) Rhoment P5 (Serva), 0.5% (v/v) 2-mercaptoethanol, 0.02% (w/v) NaN_3, in PBS, pH 6.0). The solution was prepared just prior to use.

The suspension was then finely ground using a pestle and mortar and additional enzyme solution was added so that a total of 10 ml enzyme solution was added per gram leaf material. The suspension was transferred to a small conical

flask and incubated on a gyratory shaker (80 rpm) at 28 C for 24 hr. EDTA was then added to a final concentration of 0.1% (w/v) and the suspension shaken for a further hour. The resulting slurry was filtered through 2 layers of miracloth and further clarified and concentrated as described for polytron homogenization.

2.10.1.5 Extraction of alkali-soluble antigens

Alkali-soluble antigens were extracted from plant material using a modification of the method of Kawarabata and Hayasaka (1987).

Samples were prepared using a polytron as described above and the pellet obtained after high-speed centrifugation resuspended in 2 ml 0.1 N KOH per gram original leaf material. The suspension was incubated in a 27 C water bath for 30 min. with gentle agitation evey 5 min. The suspension was then centrifuged (12 000g for 30 min.) at 4 C to sediment the particulate matter and the supernatant containing the alkali-soluble antigens collected. An equal volume of 0.1 N HCl was added to neutralize the solution. The sample was diluted in coating buffer for the indirect ELISA assay.

2.10.1.6 Water leaching of antigens

A single leaf midrib was cut into very fine sections using a scalpel blade and the sections transferred to a 1 ml Eppendorf tube. A volume of sterile dH_2O just large enough to suspend the sections (usually 200-300 ul) was added to the tube and the suspension agitated vigorously at room temperature overnight. The leachate was collected and diluted in either sample or coating buffer, depending on the detection assay to be employed.

2.10.2 Enzyme-linked immunosorbent assays

A number of variations of the conventional ELISA assay were investigated in an attempt to determine the most satisfactory method of detecting the organism in infected plant material.

The following ELISA buffers used were obtained from Boehringer Mannheim as either powders or concentrated stock solutions and were made up according to the manufacturer's instructions: coating buffer, sample conjugate buffer (PBS), wash buffer and substrate buffer. 4-nitrophenyl phosphate was added to the appropriately diluted substrate buffer to a final concentration of 0.1% (w/v) just prior to use.

Sample conjugate buffer was used as diluent for all the serореagents. The blocking buffer used in all assays consisted of a solution of 1% (w/v) bovine serum albumin (BSA), fraction V in PBS. Protein A - alkaline phosphatase conjugate was prepared according to the method of Engvall (1978). Goat anti-rabbit IgG (whole antibody) alkaline phosphatase conjugate (GAR-AP) was purchased from Sigma (St. Louis, USA). Nunc (Denmark) Microwell Module F-16 immuno quality high binding capacity plates were used in all the ELISA's. In direct ELISA, the IgG-alkaline phosphatase conjugate used was prepared according to the method of Clark and Adams (1977).

Before practical application of the ELISA, the optimal dilution of the sample, each preparation of immunoglobulin, Fab', $F(ab')_2$, as well as the enzyme conjugate preparations was determined experimentally using a modified version of the chequerboard scheme similar to that described by Hill (1984).

Preliminary tests were carried out in order to determine the optimal incubation times and temperatures for the reactions in all the detection assays employed. All reactions were carried out at 37 C unless otherwise stated. Immobilized enzyme was visualized by the addition of enzyme substrate and incubating the plates at 25 C. The reactions were stopped by the addition of 3 M KOH (50 ul/well).

Although the use of crude plant extracts was found to give satisfactory results in the DAS-, Fab'-, and $F(ab')_2$-ELISA, the water leaching method was used in preference to all other sample preparation procedures because of the simplicity of this technique and the fact that it allowed for the assay of target antigen in individual leaf midribs.

In all the preliminary tests, the final working dilutions selected for the various reagents were those that gave the greatest colour reaction in the test wells combined with the least reaction in control wells.

In addition to a buffer control well, a sample of healthy host plant material as well as one known to be infected with greening were included in all detection assays as negative and positive controls, respectively.

Assays were made in wells containing 200 ul of reagent. Microtiter plates were subjected to 3 x 5 min. washes between each step.

Absorbance readings were measured in a Titertek Multiskan 8-channel photometer, blanked against unused wells of the microtitre plate.

Reactions were judged positive for the target antigen when A_{405} values were equal to or greater than twice the mean for the healthy control samples within the same microtitre plate. A set of the latter controls was included within each microtitre plate because, previously, when such controls were included in several plates tested at the same time, the controls for different plates were sometimes significantly different (data not shown).

2.10.2.1 Double-antibody sandwich ELISA (DAS-ELISA)

Wells were coated with trapping IgG diluted in coating buffer for 2 hr and the unoccupied binding sites blocked by the addition of blocking buffer for 1 hr. Prepared sample was added to the wells and incubated at 4 C overnight. Trapped antigen was reacted with antigen-specific IgG-alkaline phosphatase conjugate in sample conjugate buffer for 1 hr and the amount of bound enzyme visualised by the addition of substrate.

2.10.2.2 Fab'- and $F(ab')_2$-ELISA

Wells were coated with purified Fab' or $F(ab')_2$ in coating buffer for 2 hr and the unoccupied binding sites blocked for 1 hr. as described above. Prepared sample was

allowed to react with the immobilized Fab' or $F(ab')_2$ overnight at 4 C after which detecting IgG was added and incubated for 2 hr at 30 C. The bound IgG was reacted with Protein A-alkaline phosphatase diluted in PBS for 2 hr. at 30 C and the reaction quantified by the addition of enzyme substrate.

2.10.2.3 Heparin-enhanced plate-trapped antigen ELISA (HE-PTA-ELISA)

The addition of the polyanion heparin to incubation buffers containing the primary and secondary antibodies in Western immunoblots and ELISA assays has been reported to reduce non-specific reactions in these tests (Dietzgen and Francki, 1987, Coreejes, 1989). In an attempt to reduce the high background readings initially recorded in ELISA tests using antiserum raised against cultured bacterial isolates, varying concentrations of heparin were added to the incubation buffers used in the indirect PTA-ELISA (Appendix F).

In the preliminary study, heparin was added at concentrations of 2, 5, 10, 500 and 1000 U/ml to the sample conjugate buffer in an attempt to determine the optimal concentration of this reagent in the HE-PTA-ELISA. Results

of this test indicated that the addition of heparin at 5 U/ml yielded the greatest difference in colour reaction between test and control wells (Appendix F). Heparin was added to the sample conjugate buffer in which all seroreagents were diluted.

In the HE-PTA-ELISA, samples were prepared by the water leaching method and diluted in coating buffer. All reactions were carried out at 37 C. The standard procedure adopted for HE-PTA-ELISA was as follows: Microtitre plates were coated with test sample diluted in coating buffer for 2 hr. Unoccupied binding sites were blocked with the addition of blocking buffer for 1 hr. Whole antiserum diluted in the supplemented sample conjugate buffer was then incubated in the wells for 90 min. and the bound IgG reacted with GAR-AP diluted in the same buffer for 60 min. The reaction was quantified by the addition of enzyme substrate.

To test for nonspecific hydrolysis of the substrate (which may be caused by endogenous plant phosphatases), some of the microtitre plate wells were coated and blocked as described above and the enzyme substrate added without prior addition of the IgG or enzyme conjugate.

2.10.3 Periodate oxidation of ELISA samples

In an investigation into the immunogenicity of plant glycans, Laine and Faye (1988) observed a widespread occurrence of highly immunogenic carbohydrate moieties and anti-glycan antibodies in antisera raised against plant glycoproteins. The authors suggested that this may be the reason for the antigenic cross-reactivity observed among glycoproteins. In the same publication it was reported that antibodies prepared against a single protein, purified from a plant extract by SDS-PAGE, reacted with other proteins in extracts from the same or different plant species. Mild periodate oxidation of plant samples was found to specifically destroy the carbohydrate epitopes (thus preventing the binding of anti-carbohydrate antibodies to plant glycoproteins) and allowed for the distinction between antibodies directed against carbohydrate and against peptide antigenic determinants in the Western blot procedure.

Evidence for the presence of xylose-containing glycans in molluscs and insects (Faye and Chrispeels, cited in Laine and Faye, 1988) indicates that the difficulties in producing specific antibodies, due to the high immunogenicity of the carbohydrate constituents of glycoproteins present, are probably not restricted to plants.

In an attempt to determine the nature of the immunogens present in the preparation of infected citrus used to raise an anti-greening antiserum (section 2.9.2, method 5), a modification of the periodate oxidation treatment reported by Laine and Faye (1988) was used in the HE-PTA-ELISA. ELISA plates were coated with sample diluted in coating buffer as described in section 2.10.2.3. After washing, the wells were briefly rinsed with 50 mM sodium acetate buffer, pH 4.5 (acetate buffer) and a solution of 10 mM sodium metaperiodate in acetate buffer then added to each well. Plates were incubated in the dark at room temperature for 2 hr after which the wells were briefly rinsed with acetate buffer. A solution of 50 mM sodium borohydride in PBS was added to each well and allowed to stand for 30 min. The wells were then washed 3 x with ELISA wash buffer and the treated sample probed with primary and secondary antibody and the reaction visualized as described in section 2.10.2.3. The metaperiodate and borohydride solutions were prepared just prior to use.

2.10. Enzyme-linked dot-immunoblot assay (EL-DIB)

The constituents of the buffers used in the EL-DIB assay are listed in Appendix G.

The EL-DIB assay could not be used to analyze leaf midrib tissue as samples were found to stain the membrane and thus interfere with interpretation of the results. Even sample preparation procedures such as the water leaching method which yields preparations relatively free of unwanted plant material did not adequately prevent contamination of the sample with plant pigments. Soaking membranes in 70% ethanol or treatment with chloroform or carbon tetrachloride was not effective in removing plant pigments from the membrane.

Citrus fruit columella material could, however, be analysed using this assay if the samples were prepared using a variation of the KOH extraction method of French (1973). Other extraction methods yielded samples which either blocked or stained the membrane.

To prepare samples, columella were excised and cut in half longitudinally. Each half was then cut laterally into thin sections and transferred to a 1 ml Eppendorf tube. Approximately 750 ul of 1 N KOH (sufficient to adequately suspend the sample) was added to the tubes each containing a single sectioned columella. The suspension was incubated at room temperature for 45 min. with occasional agitation. The suspending liquid was then drawn off and 50 ul amounts spotted onto nitrocellulose membrane (NCM) (0.2um pore

size, Schleicher and Schuell, Dassel, FRG) held in a Bio-Dot Microfiltration Apparatus (Bio-Rad Laboratories). The NCM was thoroughly wetted with Tris-buffered saline (TBS) before use. Fifty microlitres was the maximum volume of leachate that could be applied without significant colouration or blocking of the NCM.

Residual KOH was removed from the membrane by drawing 500 ul of TBS containing Tween 20 (TTBS) through each well of the Bio-Dot apparatus under vacuum. This washing step was repeated twice. Unoccupied binding sites on the NCM were blocked by adding 200 ul of blocking buffer to each well and allowing the solution to pass through the membrane by gravity filtration. The wells were washed with 2 x 500ul volumes of TTBS and 100 ul of whole antiserum diluted in AB buffer added to each well. The solution was allowed to drain through by gravity filtration and the wells washed 3 times. Bound primary antibody was detected with GAR-AP diluted 1:2000 in AB buffer. Wells were washed 3 times with TTBS, once with TBS and then with AP buffer. The NCM was removed from the apparatus and placed on a glass plate in a humid chamber and 25 ul of NBT-BCIP colour reagent applied to each sample spot. The membrane was incubated in the dark at room temperature for 2-4 hours, or until a colour reaction was clearly visible. The reaction was stopped by washing the NCM in 10 mM Tris-HCl, 5 mM EDTA pH 7.5 for 10 min.

Wet membranes were observed in transmitted light. The membranes could be stored dry in the dark and re-wetted in dH_2O to restore the colour intensity.

Samples of both infected and healthy fruit collected from orchards of the C.S.F.R.I. in Nelspruit were analysed using the EL-DIB assay.

The EL-DIB assay was also used to determine the serological relatedness of the bacterial isolates (GC, NC, LC, LL, TZL and CS). In addition, the assay was found useful for the initial screening of isolates for their reactivity with the GICE antiserum. As this antiserum was raised against an extract of greening-infected citrus, it was speculated that any isolates reacting positively with the antiserum might be associated with the disease. Such cultures were investigated further with respect to their ultrastructure and pathogenicity.

For the analysis of cultured bacteria, cells were harvested from exponential phase cultures by centrifugation and washed in a large volume of PBS. The re-pelleted cells were then spotted onto NCM which had first been soaked in TBS and allowed to air dry briefly. The membrane was allowed to dry thoroughly before fixing the cells to the membrane by immersion in a 10% (v/v) acetic acid / 25% (v/v) ethanol

solution for 15 min. The NCM was rinsed in a large volume of dH_2O followed by a 5 min. wash in TBS. The membrane was then blocked in TBS containing 2% (w/v) BSA for 1 hr at 37 C. This, as well as all subsequent incubation steps were carried out with gentle agitation. Excess blocking solution was removed by 3 x 15 min. washes in TTBS.

Trapped antigen was reacted with primary antibody (whole antiserum) diluted in TTBS containing 1% BSA (antibody buffer) for 1 hr at 37 C. The NCM was then washed (3 x 15 min. washes) and the membrane transferred to a solution of GAR diluted 1:2 000 and incubated at 37 C for 1 hr. Excess enzyme conjugate was removed by 2 x 15 min. washes in TTBS followed by 2 x 5 min. washes in TBS and finally 2 x 5 min. washes in AP buffer. The reaction was visualized by the application to each sample spot of 25ul volumes of the colour development solution for alkaline phosphatase (NBT/BCIP - Appendix G) prepared just before use. The membrane was placed on a glass sheet and incubated in a humid chamber in the dark. Positive reactions began to appear as purple spots after 5 to 10 min., but the sheet was incubated for 2 to 4 hr to complete the reaction and then washed for 10 min. in 10 mM Tris-HCl, 5 mM EDTA, pH 7.5. The wet sheets were observed in transmitted light and a well-defined purple spot was regarded as a positive reaction.

2.10.5 Radio-immunosorbent assay (RIA)

In establishing the positive-negative threshold for RIA, reactions were judged positive when the Gamma counts were equal to or greater than twice the mean of the healthy control samples within the same test.

As with the ELISA, preliminary chequerboard titration assays were performed in order to determine the optimal sample and reagent dilutions, as well as the optimal incubation times and temperatures for each assay.

2.10.5.1 Dot-RIA

Unlike the EL-DIB assay, Dot-RIA could be used to analyze both leaf and columella samples prepared by either water leaching, homogenization or a method involving KOH extraction of antigen as results of this assay were found to be unaffected by plant enzymes and staining of the membrane by plant pigments. Results of preliminary tests indicated that the water leaching method gave adequate results and this method was therefore employed in all subsequent tests.

Leaf midrib samples were prepared following the water leaching method detailed in section 2.10.1.6. A sheet of NCM was divided into 10 x 10 mm squares using a soft pencil

and then soaked in TBS for 15 min. After air drying briefly, 5 ul volumes of undiluted sample were applied to the centre of the squares and allowed to dry before application of an additional 5 ul of sample. The NCM was dried thoroughly and the sample fixed to the NCM by immersing the membrane in a 10% (v/v) acetic acid / 25% (v/v) ethanol solution for 15 min. The NCM was rinsed in a large volume of dH_2O followed by a 5 min. wash in TTBS. Unoccupied membrane binding sites were blocked in TTBS containing 2% (w/v) BSA for 1 hr at 37 C. This, as well as all subsequent incubation steps were carried out with gentle agitation. Excess blocking solution was removed with 3 x 5 min. washes in TTBS. Trapped antigen was reacted with primary antibody (whole antiserum) diluted in TTBS containing 1% BSA (antibody buffer) for 1 hr at 37 C. The NCM was washed 4 times and the membrane transferred to a solution of ^{125}I-labelled Protein A (Amersham, UK) diluted 1:1000 in antibody buffer for 1 hr at 37 C. After 4 washes in TTBS and a single wash in TBS, the NCM was allowed to dry thoroughly and the individual squares cut out and transferred to scintillation vials. Five millilitres of Filter-Count (Packard Instrument Co. Inc., Illinois, USA) was added to each vial, the vials refrigerated in the dark overnight and then shaken vigorously before counting in order to dissolve the NCM.

2.10.5.2 Bead-RIA

As an alternative to using NCM as the solid phase in RIA, polystyrene beads can be used. The method used in this study was developed by Lee for the detection of xylem-limited bacteria (R. Lee, pers. comm.). In this procedure, 6.35 mm (diameter) polystyrene beads (Precision Plastic Ball Co., Illinois, USA) were coated with purified IgG diluted in ELISA coating buffer at 37 C for 2 hr. Coated beads were then washed twice (each wash lasting 10 min.) in a large volume of PBS containing 0.1% (v/v) Tween 20 (wash buffer). The beads could be stored in this buffer at 4 C for at least 2 weeks without any decrease in binding efficiency. To a small plastic tube (70 x 9 mm), 250 ul of sample was added followed by a coated plastic bead. Samples were prepared using the water leaching method and diluted 1:100 in ELISA sample conjugate buffer. The beads were incubated with the sample overnight at 4 C and then the sample aspirated off and the beads washed 3 times in wash buffer. Each wash step involved adding at least 1.5 ml of wash buffer to each tube and leaving the beads to soak for 5 min. after which the buffer was aspirated off.

^{125}I-labelled IgG conjugate (prepared according to the method detailed in Appendix H) was added to each tube (conjugate was diluted in sample conjugate buffer such that the 250 ul aliquot added to each tube exhibited an activity

of 40 000 - 50 000 CPM). Binding of the conjugate to trapped antigen was allowed to take place overnight at 4 C after which the beads were washed 3 times as described earlier. The beads were then transferred to scintillation vials, 5 ml of Filter-Count added and the vials allowed to stand at 4 C in the dark overnight before counting.

2.11 PREPARATIVE IMMUNOFLUORESCENCE MICROSCOPY (PIFM)

The composition of all PIFM buffers is given in Appendix I.

One gram of washed leaf midrib material was chopped finely in 3 ml of 1/4-strength Ringer's solution and then thoroughly homogenised using a pestle and mortar. The homogenate was transferred to a test tube and 3 ml 3% (w/v) trypsin solution and 3 ml 1.5% (v/v) Triton X-100 added (both the enzyme and Triton X-100 were made up in Ringer's solution). A preliminary study carried out to compare the efficacy of two different enzymatic treatments indicated that degradation of host plant material by trypsin and Triton X-100 was more effective than degradation by an enzyme "cocktail" consisting of pectinase, cellulase and protease.

The suspension was incubated in a 50 C water bath for 10 min. and the undigested particulate matter removed by filtration through a double layer of miracloth and a single layer of Whatmans No.4 filter paper. The filtrate was divided into 2 equal volumes and each passed through a filter series consisting of a 25 mm cellulose pre-filter (Millipore AP-100, Millipore Corp., USA) and 0.2 um Nuclepore black polycarbonate membrane (Nuclepore Corp., USA) on which microorganisms were finally trapped. The pr filter was positioned above the trapping membrane in a ' clepore Swin-Lok multiple membrane holder.

After filtration, the pre-filter was removed from the upper support grid of the membrane holder and all subsequent incubations carried out with the trapping membrane still held on the lower support grid of the apparatus. The Swin-Lok multiple holder adaptor conveniently formed a chamber above the trapping membrane into which the reaction mixtures could be placed and incubated. To remove spent reagents from the chamber and to wash the membrane without loss of trapped microorganisms, the apparatus was re-assembled and the reagent or wash buffer forced through the membrane under gentle pressure applied with a syringe.

After the initial filtration, the trapping membrane was washed with 5 ml of warm (37 C) wash buffer. In order to minimise non-specific binding of immunoglobulin to the

membrane, 0.5 ml blocking buffer was added to the chamber and incubated at 37 C for 1 hr. During the incubation steps, the outlet of the apparatus was closed in order to prevent gravity filtration of the buffer through the membrane. The blocking buffer was removed and the membrane washed with 5 ml warm wash buffer. Trapped antigen was then reacted with 0.5 ml of IgG in incubation buffer for 1 hr at 37 C. The filter was washed and the primary antibodies detected with 0.5 ml anti-rabbit IgG (whole molecule) fluoroscein isothiocyanate (FITC) conjugate (Sigma) diluted 1:50 in incubation buffer. After 30 min. at room temperature the conjugate was discarded and the membrane washed 2 x with 5 ml warm washing buffer and once with warm dH_2O. The membrane was transferred to a microscope slide and a coverslip mounted using distilled water as the mounting fluid. The membrane was viewed under a Leitz Wetzlar Dialux 20 fluorescent microscope with epi-fluorescence. Flooding the membrane with 96% ethanol just prior to viewing was found to enhance fluorescence of the FITC.

Greening-affected leaf samples were collected from Nelspruit, Letaba and Brits orchards during August 1987. Five symptomatic trees in each orchard were sampled, whilst leaves and fruit from 3 trees showing no disease symptoms were collected from the same orchards to serve as controls.

Leaf material from a tree from which the GC isolate had been cultured was included in the assay. The sample prepared from this material was reacted with IgG purified from an antiserum raised against the cultured GC isolate.

2.12 IMMUNOGOLD LABELLING

Immunogold labelling was used as an alternative to the EL-DIB assay in determining the serological relatedness of the bacterial isolates, as well as the relationship between the GICE antiserum and bacteria either cultured or purified from greening-infected citrus.

Bacteria cultured in liquid media were harvested by centrifugation, washed in a large volume of 0.1 M phosphate-citrate buffer (pH 7.2-7.4), re-pelleted and then suspended in buffer. Formvar-coated carbon-backed grids were floated on drops of the cell suspensions for 15 min. and then drained by touching to a filter paper strip. When the bacterial content of a plant preparation was analysed using this technique, grids were floated on the extract diluted in buffer. All steps were carried out at room temperature. After adsorption of sample, the grids were floated on rabbit primary antiserum diluted in 0.1 M phosphate-citrate buffer containing 1% (w/v) BSA and 0.05%

NaN_3 (antibody buffer) for 15 min. Grids were then washed on 8 drops of 0.1 M phosphate-citrate buffer by flotation for several seconds and drained by touching to a filter paper strip. The grids were transferred to drops of goat anti-rabbit IgG (whole molecule) gold conjugate (10 nm) (Sigma, U.S.A.) diluted 1:50 in antibody buffer and incubated for 15 min. Grids were washed in buffer as described followed by washes on 3 drops of ddH_2O before staining with 0.1% (w/v) uranyl acetate in ddH_2O for 5 min.

Normal (pre-immune) rabbit serum served as a control in all cases. Optimal sample and reagent dilutions were established in preliminary trials.

2.13 WESTERN BLOTTING

In order to investigate the possible existence of proteins common to both cultured bacteria (isolate GC) and healthy citrus, extracts of the two materials were prepared and fractionated on acrylamide gels following the SDS-PAGE method detailed in section 2.9.1.6. Proteins were electrophoretically transferred to nitrocellulose paper and probed with whole antiserum directed against a live, sonicated preparation of the cultured GC isolate following the procedure detailed in Appendix J.

2.14 FATTY ACID PROFILING OF BACTERIAL ISOLATES

Bacterial isolates were cultured on Tryptone Soy agar and the fatty acid profiles of the isolates determined in the laboratories of the National Collection of Plant Pathogenic Bacteria (NCPPB), Ministry of Agriculture, Fisheries and Food, Hatching Green Harpenden, United Kingdom.

CHAPTER 3

RESULTS

3.1 ANALYSIS OF THE CULTURED BACTERIAL ISOLATES

3.1.1 Gram stain reaction

A smear was made of a NC colony picked from a 24 hour-old TSA plate (Fig. 7) and the preparation air-dried and then heat-fixed. The cells were stained following the Gram stain method detailed in Appendix E and examined under the light microscope. Cells from a colony of C.m. subsp. michiganense were similarly stained and used as a standard with which to compare the NC isolate. The C.m. subsp. michiganense culture was included as a comparison because results of fatty acid profiling suggested that the NC isolate was a member of this group of bacteria (section 3.12).

Examination of the stained NC preparation indicated that the bacteria were essentially Gram variable (Fig. 8). Isolate GC exhibited a similar Gram stain reaction. The C.m. subsp. michiganense culture also exhibited a variable Gram stain reaction (Fig. 9).

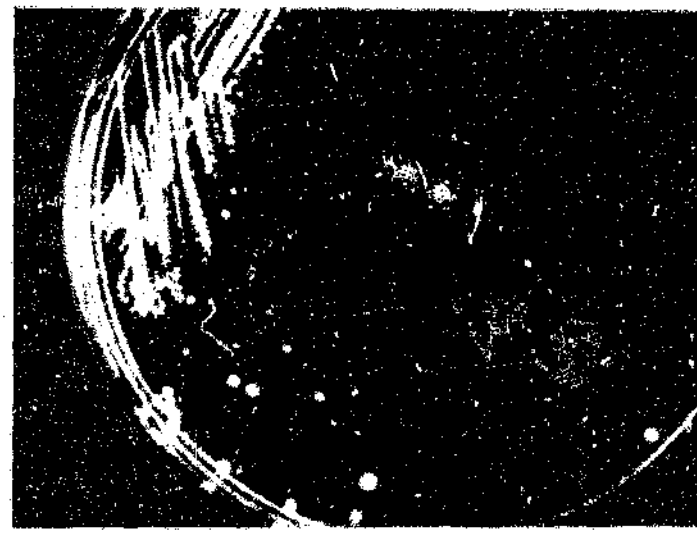


Figure 7

Colonies of isolate NC cultured on Tryptone Soy Agar.

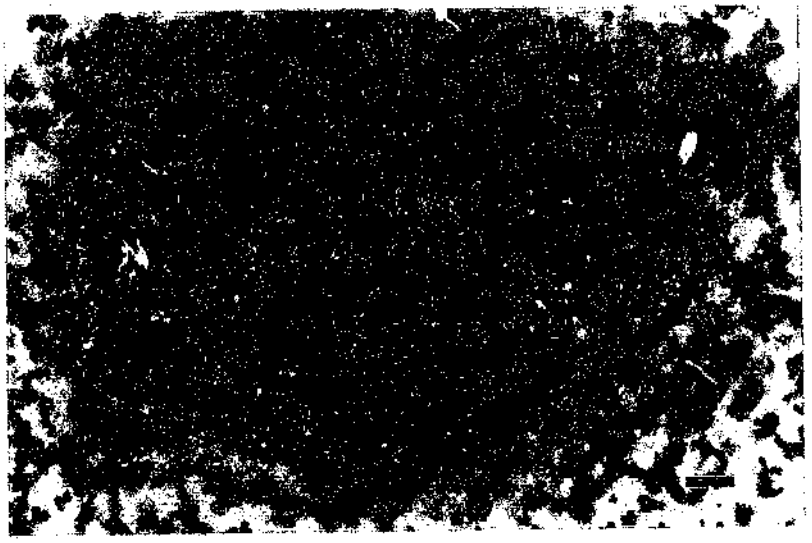


Figure 8

Gram stain reaction of isolate NC. Bar = 2 um.

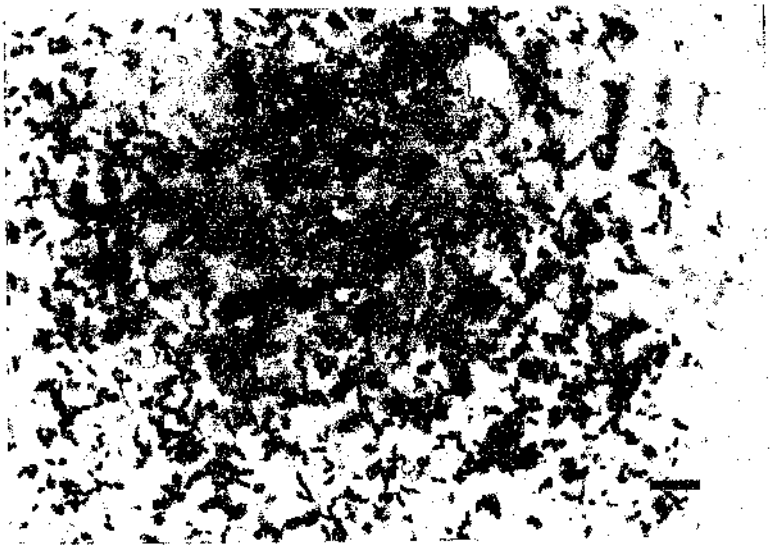


Figure 9

Gram stain reaction of C. m. subsp. michiganense. Bar = 2 um.

3.1.2 Comparison of SDS-PAGE protein profiles of the isolates

Polyacrylamide gel electrophoresis was employed to compare the patterns of SDS dissociated proteins obtained from preparations of isolates GC, NC, CS, LLL, LL, TZL and LC. The protein profiles generated are shown in Fig. 10. The result indicate that isolates GC, CS and NC (lanes 3, 4 and 5) generate similar protein patterns, while the other isolates analysed differ markedly in their constituent proteins (lanes 6, 7, 8 and 9) and are thus unlikely to be related to the GC group of organisms.

Extracts of citrus midribs were fractionated in the same gel (lanes 2 and 10), but the profiles generated were not required for this aspect of the investigation.

3.1.3 Transmission electron microscopic examination of cultured cells

Negative staining of an exponential phase GC culture revealed the presence of club- and boomerang-shaped cells (Fig. 11). Extensive branching of cells was observed in an ultra-thin section of the same culture (Fig. 12). The organism appeared to replicate by means of septum formation (Fig. 13). The cell envelope structure is shown in Fig. 14. Negative staining of a late stationary phase culture of the organism showed apparently degrading cells (Fig. 15).

Figure 10

SDS-PAGE protein profiles of bacterial isolates. Samples were prepared and described in section 2.9.1.6.

Lane 1 - molecular weight protein markers (14.2 - 66.0 kDa; Sigma, Appendix D).
2 - diseased citrus midrib extract
3 - isolate GC
4 - isolate CS
5 - isolate NC
6 - isolate LLL (isolated from midrib of a lemon tree from the Letsitile valley)
7 - isolate LL
8 - isolate TZL
9 - isolate LC
10 - healthy citrus midrib extract

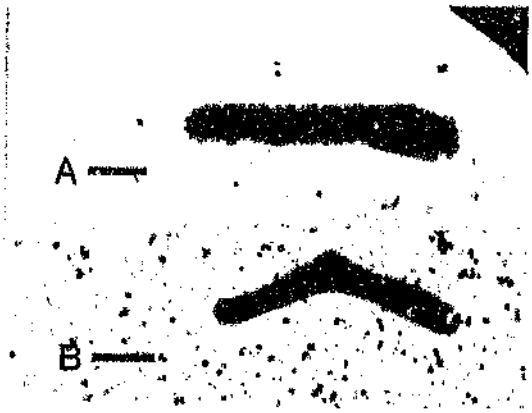


Figure 11

Club-shaped (A) and boomerang-shaped (B) cells typical of bacterial isolates NC and GC. Cells were negatively stained with 1% (w/v) uranyl acetate. Bar = 0.5 um.

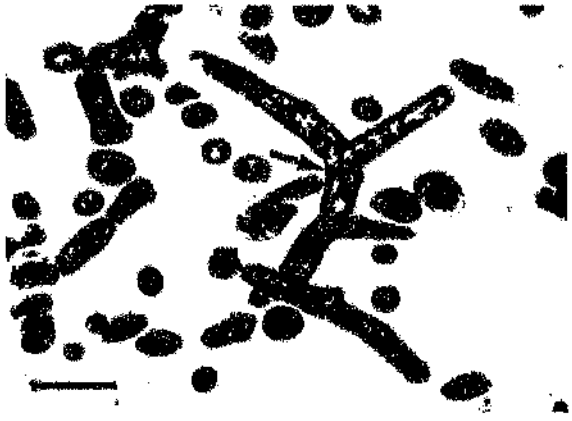


Figure 12

Ultra-thin section through a liquid culture of isolate NC showing branching (arrow) typical of this group of isolates. Bar = 1.0um.

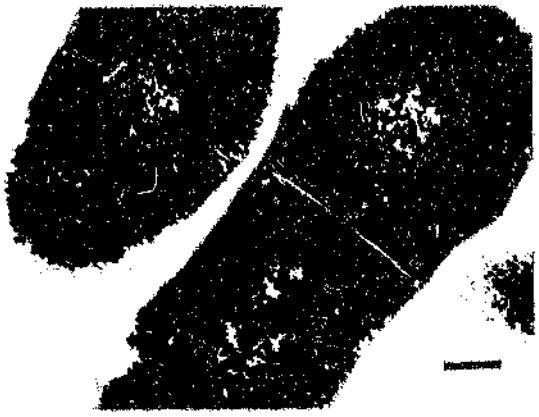


Figure 13

Replication of cultured bacteria (NC isolate) occurring by means of septum formation. Bar = 0.1 um.

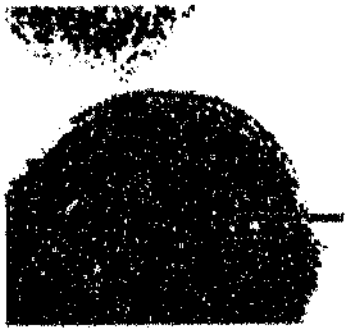


Figure 14

Enlargement of NC isolate showing cell envelope. Bar = 0.1 um.

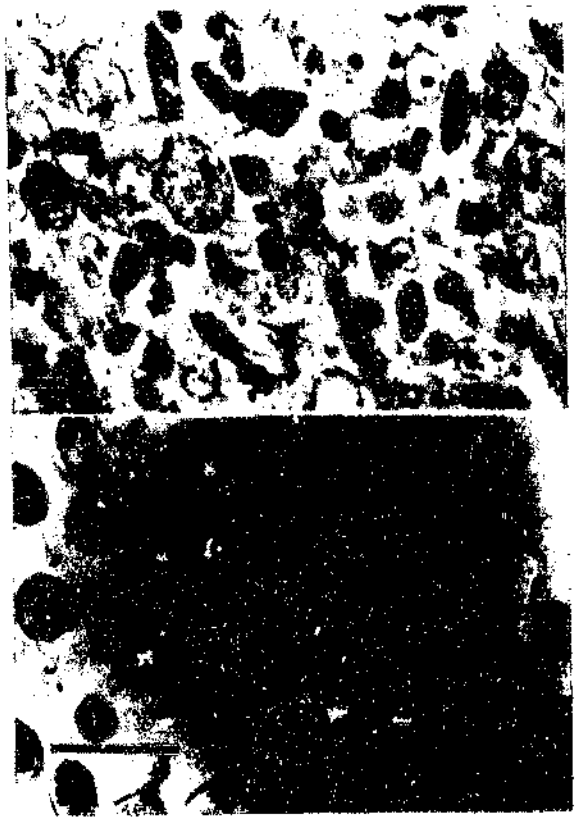


Figure 15

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

3.2 INVESTIGATION OF THE FREQUENCY OF ASSOCIATION OF THE ISOLATES OF GARNETT AND MOCHABA WITH GREENING-AFFECTED PLANT AND INSECT MATERIAL

Attempts to consistently isolate and culture bacteria identical to those isolated by Garnett and Mochaba (isolates GC and NC) from citrus collected in various areas of the Transvaal met with limited success. The bacterium was isolated from only 17 of the 153 (11.1%) infected fruit and leaf samples prepared by the chopping method (Table 1). The organism was also isolated from 3 of the samples collected from symptomless plants. Similar results were obtained when the inoculum was prepared by the Scholander bomb method (Table 2). The bacteria isolated from the samples were considered to be identical to the GC group of isolates on the basis of morphology, Gram stain reaction, antigenicity and API 20NE and API 20B profiles (the profile indices of isolates GC and NC are shown in Appendix M).

The isolation attempts from leaf midrib material of 13 greening-infected periwinkle plants did not yield bacteria similar to those isolated by Garnett and Mochaba (isolates GC and NC). Similarly, the bacterium was not isolated from dodder strands collected from infected citrus and periwinkle plants. Attempts to isolate the bacterium from 68 adult psylla of various ages collected on infected citrus from the different geographical locations were also negative.

A number of bacterial species (dissimilar to isolates GC and NC) were cultured from both greening-infected and healthy citrus, from periwinkle, as well as from insect samples. The results are summarized in Table 3. Both Gram-negative and Gram-positive organisms were isolated from these samples. Bacteria were isolated more frequently from infected citrus than from corresponding healthy material. Approximately 62% of the infected samples yielded bacteria compared to 46% of the healthy samples. No attempt was made to identify or classify the bacteria further.

TABLE 1

Isolation of the bacterium from naturally-infected citrus leaf midribs and columella. Inoculum prepared using the chopping method.

sampling site	no. of positive isolations / no. of infected plants sampled		no. of positive isolations / no. of symptomless plants sampled	
	leaf midrib	columella	leaf midrib	columella
Brits	3/18	4/14	1/11	1/7
Pretoria	1/8	2/4	0/2	0/2
Nelspruit	4/23	3/19	1/20	0/13
Letaba	0/16	0/16	0/6	0/5
Letsitile	0/3	0/1	ND	ND
Zebediela	0/10	0/12	0/10	0/4
Tzaneen	0/6	0/3	0/4	0/4

ND = not done

TABLE 2

Isolation of the bacterium from naturally-infected citrus twigs. Inoculum prepared using the Scholander bomb.

Sampling site	No. of positive isolations / No. of infected plants sampled	No. of positive isolations / No. of symptomless plants sampled
Brits	2/16	0/5
Pretoria	0/6	0/1
Nelspruit	4/18	1/5
Zebediela	0/3	ND

ND = not done

TABLE 3

Isolation of other bacterial species from greening-infected and healthy plant and insect samples.

Source material	Citrus		Periwinkle		Dodder		Psylla	
	Infected	Healthy	Infected	Healthy	Infected	Healthy	Infected	Healthy
No. of different bacterial species isolated	6	3	3	2	0	0	0	1

3.3 ATTEMPTED ISOLATION OF THE CAUSAL AGENT OF GREENING

Because of the morphological differences observed between the isolates of Garnett and Mochaba and the aetiological agent of greening disease (section 3.1.3), renewed attempts were made to isolate the disease agent using various methodologies.

3.3.1 Artificial media

Attempts to isolate an organism possessing the same morphological features as those described for the aetiological agent of greening (Laflèche and Bové, 1970a, 1970b, Chen et al., 1971, Moll and Martin, 1974, Garnier and Bové, 1977, 1983, Su, undated pamphlet) were unsuccessful in all the media used. Although a number of bacterial species were isolated from both diseased and healthy specimens of the citrus, periwinkle and dodder material, as well as from adult psylla collected on greening-infected citrus in the field and in the laboratory, none of the isolates were found to possess the characteristic cell envelope structure of the greening organism when ultra-thin sections of the bacteria were observed electron microscopicallly. No apparent differences

between the isolations carried out during the cool and warm months of the year were observed. The cultured bacteria were not identified and no attempt was made to evaluate the suitability of the different media employed for the isolation and culture of bacteria from plant and insect samples.

3.3.2 Embryonated hen's eggs

Of the 32 eggs inoculated with plant sap via either the yolk-sac or allantoic route, only 3 were found to be affected by saprophytic or other microbial contaminants, these dying within 3 to 5 days after inoculation. The embryos of the remaining eggs appeared to develop normally during the 12 day incubation period. No difference between eggs injected with inoculum prepared using either the Scholander bomb or the chopping method was observed.

Microscopic examination of the Giemsa-stained yolk-sac and chorioallantoic membranes from eggs inoculated with sap from infected citrus plants failed to reveal the presence of membrane lesions or other histological abnormalities, such features usually being associated with microbial proliferation within eggs.

Bacteria were not observed in smears of either the yolk or allantoic fluid from inoculated eggs. Similarly, the presence of microorganisms could not be detected in pellets obtained after high-speed centrifugation of both fluids.

The absence of plant disease agents in egg tissue was further confirmed by results of the enzyme-linked immunosorbent assay. In these assays, no appreciable difference was noted between absorbance values of extracts of eggs inoculated with infected sap and those inoculated with healthy sap.

3.3.3 Insect tissue culture

The mosquito cell line used failed to support the growth of the greening organism or any other microorganism of plant origin in this study. Neither cellular degeneration nor plaque formation was evident in any of the inoculated or control culture tubes.

Electron microscopic examination of the spent culture medium after high-speed centrifugation failed to reveal the presence of bacteria in the concentrated sample.

3.4 KOCH'S POSTULATES

3.4.1 Laboratory inoculation of T. erytreae with bacterial isolates

The results of the multiple choice feeding experiment indicated that the solution of 5% (w/v) sucrose in distilled water served as the most effective attractant for both nymphs and adult psylla (Table 4). Examination of the gut contents of insects after a 24 hr period of exposure to the solution confirmed that the psylla had been stimulated to feed on the sucrose and this solution was therefore used in all subsequent membrane feeding experiments.

Preliminary tests wer performed to determine the effect of the sucrose on the viability of the bacterial isolates, as well as the influence of the bacteria themselves on the effectiveness of the sucrose solution as a feeding stimulant. It was found that suspending the bacterial isolates in 5% sucrose for 24 hr did not affect the viability of the cells significantly within this time period. This fact was determined by enumerating (using the plate count method) the number of viable cells present at the time of suspension in the solution and again after 24 hr (results not shown).

TABLE 4

Results of multiple choice feeding experiments. Figures represent the number of nymphs and adult psylla observed on the feeding solutions at different time intervals.

Time period		30min.		1h		2h		5h		10h		14h		24h		34h		46h	
		n	a	n	a	n	a	n	a	n	a	n	a	n	a	n	a	n	a
Feeding Solution		3	4	4	3	3	2	2	0	0	0	0	2	0	1	0	0	0	0
Sap	1%	3	4	4	3	3	2	2	0	0	0	0	2	0	1	0	0	0	0
	10%	2	1	2	0	2	1	0	0	0	0	1	1	0	1	0	0	1	0
	50%	0	2	0	0	0	2	0	1	0	0	0	0	0	0	0	0	0	0
Sap + sucrose	1%	4	4	5	6	2	3	0	0	3	1	1	0	0	2	1	0	0	0
	10%	2	0	3	4	3	2	1	0	0	0	0	1	0	0	0	0	0	0
	50%	0	2	2	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Sucrose	1%	4	3	2	0	3	3	4	4	2	3	4	1	3	4	3	3	4	3
	5%	1	3	4	4	5	4	3	7	4	5	2	6	6	6	7	5	7	6
	10%	2	1	3	3	2	1	1	2	2	2	3	4	3	2	2	3	2	2
Sucrose + Serum		0	2	2	1	2	0	3	2	3	4	1	2	2	0	0	3	2	0

n = nymph
a = adult

The presence of the cultured bacteria within the feeding solution did not appear to deter either nymphs or adults from feeding on the suspension. Examination of the gut contents of a number of the insects in the feeding chambers confirmed that psylla had imbibed the solution.

Careful monitoring of nymphs and adults after inoculation (by either micro-injection or membrane feeding) revealed that neither the method of inoculation nor the introduced bacterial isolates had any marked effect on the longevity or fecundity of the insects. Female adults continued to lay viable eggs and nymphs developed into apparently normal adults.

Extensive electron microscopic examination of adults and nymphs at different times after inoculation suggested that none of the isolates were able to either persist or multiply and spread within the insect body. ELISA's of the insects (employing the homologous antisera) also indicated the inability of the organisms to persist or multiply within psylla, as did fruitless attempts to reisolate the bacteria from insects at periods of 7 and 21 days after inoculation.

3.4.2 Inoculation of citrus via psylla

Transmission of the isolates to citrus via the insect vector of greening disease was not achieved (Table 5). The feeding on young citrus leaves of adult psylla and nymphs inoculated with the 5 different isolates by either membrane feeding or micro-injection failed to result in development of disease symptoms in the test plants within a 2 year period after inoculation.

3.4.3 Mechanical inoculation of citrus

Approximately six months after mechanical inoculation of citrus seedlings with the cultured isolates, 90 of the 170 inoculated plants displayed disease symptoms (Table 5). No significant difference between the efficacies of the two mechanical inoculation techniques was observed: 52.5% of plants inoculated via stem injection developed disease symptoms compared to 54% of plants inoculated by a combination of stem injection and stem slashing.

Leaf symptoms were observed on some of the inoculated plants 8 weeks after inoculation, but it was usually between 10 and 20 weeks after inoculation that greening-like symptoms became clearly visible. A diversity

TABLE 5

Mechanical and vector inoculation of citrus with bacterial isolates

Inoculation route	No. of trees inoculated						No. of trees developing symptoms					
	GC	NC	LL	T2L	LC	Control	GC	NC	LL	T2L	LC	Control
Mechanical												
Stem injection	24	24	24	24	24	20	13	17	15	8	10	0
Stem injection + slashing	10	10	10	10	10	8	8	6	6	2	5	0
Insect												
Membrane-feeding	24	24	24	24	24	8	0	0	0	0	0	0
Micro-injection	8	8	8	8	8	4	0	0	0	0	0	0

of foliar chloroses was produced by plants inoculated with isolates GC and NC. The most frequently observed symptom was a blotchy-mottle chlorosis, superficially suggestive of micro-element deficiency, which appeared after the leaves matured normally (Figs 16C and 17). Yellowing of the leaf tissue immediately adjacent to the mid-vein and lateral veins was also observed (Fig. 16A) and in some cases the interveinal tissue became yellow as well (Fig. 16B). Stunted growth was another feature of the inoculated seedlings, as was the leathery aspect of leaves displaying chlorotic symptoms. The establishment of possible secondary infection in the main stem and twigs lead to die-back in some of the seedlings (Fig. 18).

Seedlings inoculated with isolates LC and TZL displayed similar foliar symptoms but the chlorosis was not as marked and usually did not progress beyond a mottled yellowing of the leaf edge (Fig. 19). Stunting and die-back of plants inoculated with these isolates were not observed.

Although the foliar symptoms described above were observed on several successive leaf flushes, the infection did not appear to persist as symptoms on inoculated citrus were transient and disappeared 18-24 months after inoculation.

Control plants inoculated with sterile water only failed to develop symptoms.

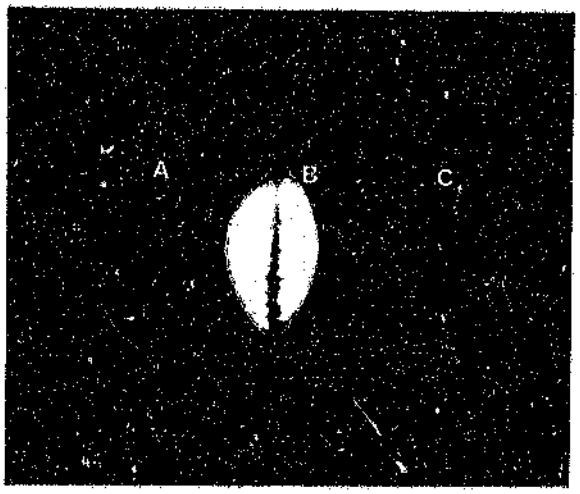


Figure 16

Chlorotic leaf symptoms induced in citrus seedling inoculated with isolates GC and NC:

A - yellowing of laminar tissue adjacent to the veins
B - yellowing of interveinal tissue
C - "blotchy-mottle" chlorosis

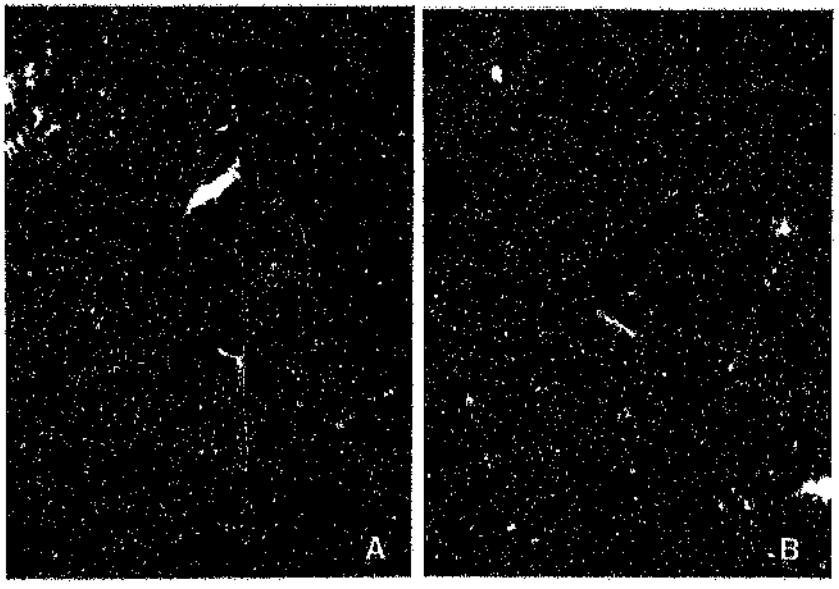


Figure 17

Chlorotic symptoms induced by inoculation of citrus with isolates GC and NC as viewed with lighting from above the leaf (A) and from below (B).

Figure 18

Citrus die-back observed in some of the citrus seedlings inoculated with isolates GC and NC.

Figure 19

Foliar chlorosis typically induced by mechanical inoculation of citrus seedlings with isolate LC. A control plant inoculated with only sterile water is shown on the right.

3.4.4 Mechanical inoculation of periwinkle

Periwinkle plants mechanically inoculated with either the bacterial isolates or with sterile water failed to develop disease symptoms.

3.4.5 Dodder transmission from mechanically-inoculated citrus to periwinkle

Establishment of dodder connections between periwinkle and a symptomatic citrus seedling which had been mechanically inoculated with the GC isolate 12 weeks prior to the transmission experiment appeared to result in the passage of an infective agent from the citrus to periwinkle. Nine weeks after the establishment of the dodder bridge between these two plant species, 2 of the 4 periwinkle plants displayed yellowing symptoms (Fig. 20). The yellowing was first observed on the leaves immediately adjacent to the site of haustoria formation within the periwinkle but later spread to other leaves along the shoot and eventually to the rest of the plant.

The first symptom observed was the yellowing of the leaf apex which rapidly spread along the margin of the leaf and culminated in the entire leaf yellowing and wilting (Fig. 21). The shoot on which the dodder was initially

Figure 20

Periwinkle inoculated via dodder with an infective agent from citrus mechanically inoculated with the GC isolate (right). A control plant is shown on the left. The inoculated plant displays leaf chlorosis. Leaves have fallen from the terminal shoot on which the dodder was initially established (arrow).

Figure 21

Leaves from inoculated and control (C) periwinkle plants.

established was soon devoid of leaves (Fig. 20, arrow) and eventually died off. Flowers produced by these plants were greatly reduced in size and lacked the pigment of the flowers produced by healthy plants.

Establishment of dodder connections between the symptomatic periwinkle plants and other healthy periwinkles resulted in the transmission of the same disease to these plants.

Periwinkles developing symptoms died within six months. Control plants connected to citrus inoculated with only sterile water failed to develop any disease symptoms.

3.4.6 Reisolation of bacteria from inoculated plants and insects

3.4.6.1 Reisolation from mechanically-inoculated citrus

Reisolation of bacterial isolates in pure culture from citrus seedlings was attempted six months after mechanical inoculation. Plants used in the reisolation attempts were randomly selected from among the inoculated plants displaying disease symptoms, as well as from among the symptomless controls. The results are summarized in Table 8.

TABLE 6

Results of reisolation attempts from citrus seedlings mechanically inoculated with bacterial isolates 6 months earlier.

Isolate	No. of symptomatic plants sampled	No. of positive isolations
GC	18	12
NC	18	8
LL	11	8
T2L	8	4
LC	11	7

The reisolates cultured from inoculated citrus were found to be identical to the bacteria inoculated into the citrus plants six months earlier on the basis of Gram stain reaction, morphology, fatty acid profiles and analytical profile indices (API 20NE and API 20B, Appendix M). The reisolates were also found to react positively in the PTA-ELISA with antiserum raised against the original inoculum.

Attempts at reisolating the bacteria from the same plants after the disappearance of disease symptoms were unsuccessful. These plants had earlier tested positive on culture in MIG-3 medium.

All 10 control citrus seedlings tested were negative on culture in MIG-3 medium.

3.4.6.2 Reisolation from periwinkle

Reisolation of the bacteria from mechanically inoculated periwinkle, as well as from symptomatic periwinkle inoculated via dodder, was not achieved.

3.4.6.3 Reisolation from psylla

Efforts made to culture the isolates from nymphs as well as from adult psylla which had been inoculated via either membrane feeding or micro-injection were unsuccessful. Attempts to reisolate the bacteria were made at periods of 7 and 21 days after inoculation.

3.4.7 Thin-layer chromatographic analysis of inoculated citrus

All insect and mechanically inoculated plants, as well as the control plants failed to display the fluorescent gentisic glucoside marker, the presence of which is reported to be indicative of infection with greening (Schwarz 1965, 1968a).

3.4.8 Transmission electron microscopy of inoculated plant and insect samples

TEM observation of leaf midribs from citrus mechanically inoculated with isolates GC or NC, as well as of midribs from periwinkles inoculated via dodder, revealed the presence of bacteria within the phloem cells of both the citrus (Fig. 22) and periwinkle plants displaying symptoms (Fig. 23).

Figure 22

Electron micrograph of an ultra-thin section through a leaf midrib phloem cell from mechanically-inoculated (GC isolate) citrus showing the presence of microorganisms within the cell. PC = phloem cell; Bar = 0.5 um.

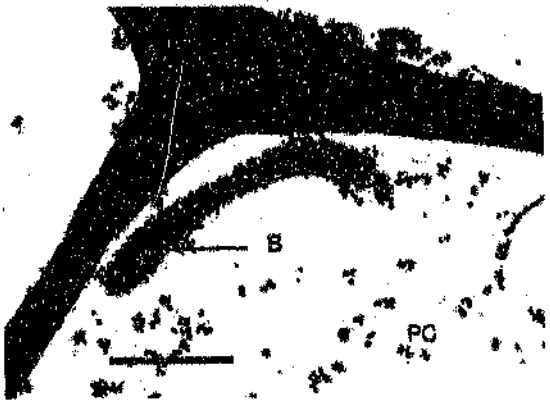


Figure 23

Ultra-thin section of a leaf midrib from periwinkle inoculated with an infective agent via dodder showing a bacterium within a phloem cell. B = bacterium; PC = phloem cell; Bar = 0.5 um.

Organisms were not observed in tissues of citrus plants inoculated with isolates LL, TZL or LC. Similarly, bacteria were not observed in any of the control plants examined.

Extensive TEM examination of nymphs and adult psylla at periods of seven and 21 days after inoculation did not reveal the presence of bacteria within the insect bodies.

3.5 DODDER TRANSMISSION OF GREENING TO CUCUMBER

Approximately two weeks after the formation of haustoria within the cucumber plants, cotyledon as well as leaf and stem symptoms were observed in five of the six cucumber plants connected to infected citrus via dodder. Cotyledon and leaf symptoms consisted of a diffuse chlorosis (Fig. 24) accompanied by wilting (Fig. 25), while stem symptoms consisted of necrotic lesions, initially associated with the sites of haustoria formation (Fig. 26A), but which later spread along the stem (Fig. 26B). Stem splitting was also observed (Fig. 27) and symptom expression terminated with the collapse of the base of the stem (Fig. 28) and the subsequent death of the plants.

Three control cucumber plants connected to healthy citrus displayed no symptoms. The dodder on one of the control plants was allowed to infest the plant heavily to determine the effect the parasite itself had on the cucumber, but the plant appeared to grow normally and developed no symptoms (Fig. 25).

An organism was isolated in pure culture from each of the 5 symptomatic test plants and was shown to be identical to bacteria isolated from greening-infected citrus (isolates GC and NC) on the basis of morphology, API tests, fatty acid profiling and serological properties. The isolate obtained from affected cucumber was coded CS. The single asymptomatic test plant, as well as the control plants were negative on culture in MIG-3 medium.

Attempted isolation of the same bacteria from the dodder bridging the infected citrus and cucumber was not successful.

TEM revealed the presence of greening bacteria within the phloem tissue of the dodder connecting the cucumber to the greening-infected citrus (Fig. 29). Extensive examination of symptomatic cucumber stem, petiole and leaf midrib samples, however, did not reveal the presence of prokaryotic organisms within these tissues.

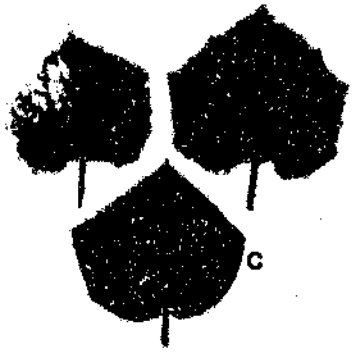


Figure 24

Diffuse chlorosis observed in leaves of cucumber plants inoculated with an infective agent from greening-infected citrus via dodder. Leaves from control plants (C) remained healthy.

Figure 25

Wilting observed in cucumber plants parasitized by dodder originating from citrus infected with greening (centre and right). Control plants (left) failed to develop disease symptoms, despite heavy dodder infestation.

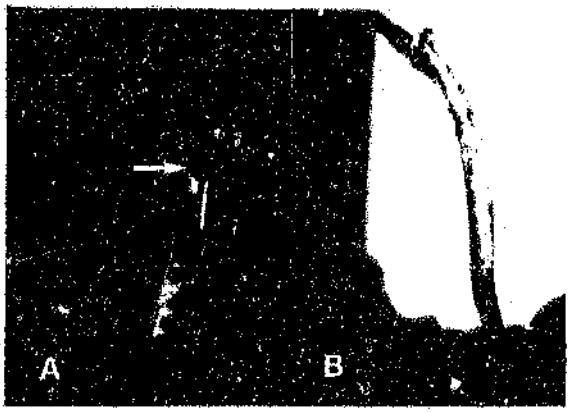


Figure 26

A. Cucumber stem necrosis initially associated with the site of dodder haustorium formation (arrow), but which later spread along the length of the stem B.

Figure 27

Stem splitting observed in affected cucumber seedlings. Sites of haustorium penetration are visible (arrow).

Figure 28

Collapse of affected cucumber stem base.

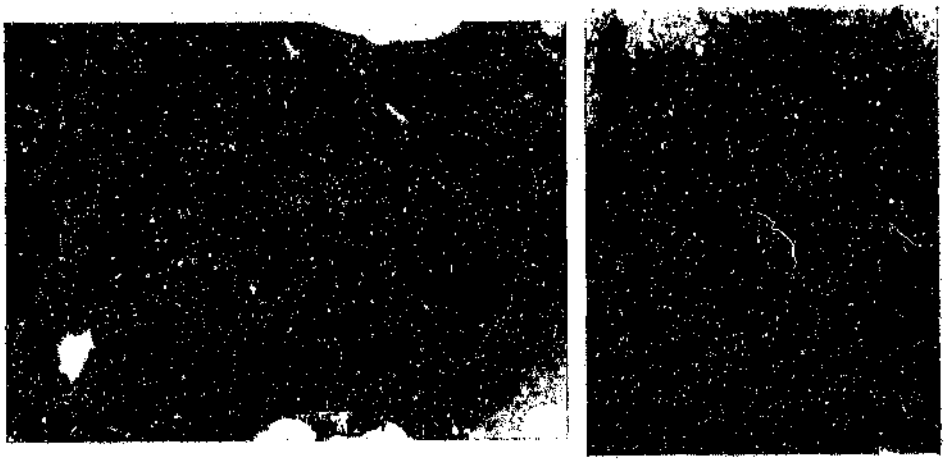


Figure 29

A. Transverse section through a phloem cell of dodder bridging greening-infected citrus and symptomatic cucumber showing greening bacteria. Bar = 1.0 um.

B. Enlargement of greening bacterium showing cell envelope. Bar = 0.1 um.

3.6 INOCULATION OF HERBACEOUS PLANT SPECIES

Results of inoculation experiments are summarized in table 7. Leaves of cucumber, tomato, aubergine and green pepper plants rubbed with a cell suspension of isolates GC, NC or CS, or with the filtered culture medium in which these isolates were grown, displayed various symptoms. The most marked symptom was a rapid wilting of the treated leaves of cucumber, tomato and green pepper plants (wilting was recorded when the margins of leaves became flaccid). Similar inoculation of seedlings with cell suspensions or culture filtrates of isolates LC and TZL failed to result in symptom expression by any of the plants.

Wilting displayed by tomato plants was spectacular; leaves rubbed with culture filtrates wilted withi· 10 min. (Fig. 30), and 24 hr later had shrivelled up and died. This wilting was also observed in cucumber, tomato and pepper stem cuttings placed in the diluted filtrates and suggests the presence of a potent wilt-inducing agent of bacterial origin in the spent medium. Control plants exposed to sterile fresh culture medium failed to develop symptoms.

TABLE 7

Results of mechanical isolation of herbaceous plants with bacterial isolates and spent culture supernatant. Figures represent the number of plants developing symptoms. A total of 5 plants were inoculated in each case.

Inoculum	Bacterial cells												Culture supernatant					
Inoculation route	Leaf rubbing						Stem injection						Leaf rubbing					
Isolate	GC	NC	CS	LC	T2L	Control	GC	NC	CS	LC	T2L	Control	GC	NC	CS	LC	T2L	Control
Plant species																		
Cucumber	5	5	5	0	0	0	5	5	4	0	0	0	5	5	5	0	0	0
Tomato	5	5	5	0	0	0	5	3	5	0	0	0	5	5	5	0	0	0
Green pepper	5	5	5	0	0	0	3	2	4	0	0	0	5	5	5	0	0	0
Aubergine	3	5	4	0	0	0	1	0	2	0	0	0	3	4	5	0	0	0
Citrus	0	0	0	0	0	0	ND	ND	ND	ND	ND	ND	0	0	0	0	0	0

ND = Not done

Leaves of tomato plants rubbed with an aqueous suspension of the cultured cells also displayed this severe, almost immediate, wilting. Necrosis of the vascular system of the treated leaves and petioles was also observed in these plants. The necrosis later spread to the main stem of the affected plants. Sap extracted from the stems was found to contain bacteria morphologically identical to the original inoculum when examined electron microscopically. The stems of inoculated plants eventually collapsed and the plants died. The findings suggest that the bacteria were able to spread and possibly multiply within tomato.

Tomato plants injected with the bacteria also exhibited wilting, but this was only evident after approximately 1 hr. Plants inoculated in this way also displayed vascular necrosis.

Leaves of green pepper rubbed with spent medium or with the cell suspensions displayed wilting (Fig. 31), although this only became evident after approximately 30 min. Wilting was observed in cuttings placed in culture filtrates after 15 min. In addition to wilting, symptoms of leaf curling and vein necrosis were observed to develop in plants treated with cell suspensions between one and two days after inoculation. Wilting and vascular necrosis were also

Figure 30

Wilting of tomato leaves rubbed with NC culture filtrate. Similar wilting was observed when leaves were rubbed with the filtered spent medium of GC and CS cultures.

Figure 31

Leaves of a green pepper seedling displaying wilting symptoms after rubbing with GC culture filtrate. Uninoculated leaves remained erect.

displayed by plants injected with cells, but these symptoms became apparent only four days after inoculation. Infection with the bacteria led ultimately to the death of the plants.

Cucumber leaves were observed to wilt a short while after rubbing with culture filtrates or with cell suspensions, or after placing cuttings in the diluted medium. Plants inoculated with bacteria displayed foliar chlorosis similar to that exhibited by cucumber seedlings infected with an agent from greened citrus via dodder (section 3.5). Necrosis of cucumber vascular tissue was not observed. The symptoms of wilt and chlorosis spread to uninoculated parts of the plant and diseased plants eventually died.

Symptoms induced by isolates CC, NC and CS, when rubbed onto leaves of aubergine seedlings, were blistering of the leaves (Fig. 32) and necrosis of leaf veins and the petioles (Fig. 33). The infection appeared to be restricted to inoculated leaves as the symptoms did not spread to other parts of the plant. Wilting of the rubbed leaves did not occur although wilting was observed in stem cuttings placed in the diluted culture filtrates. The wilting was not, however, as severe as that observed in similarly inoculated tomato and green pepper plants. Inoculation of

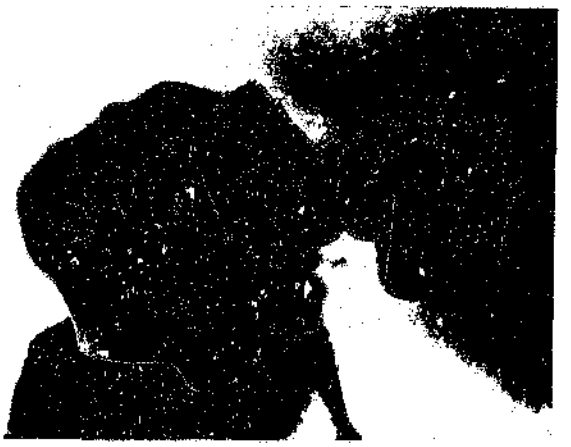


Figure 32

Blistering of aubergine leaves caused by rubbing with a suspension of GC cells.

Figure 33

Necrosis of aubergine leaf and petiole tissue after inoculation with the GC isolate.

aubergine with the isolates did not result in the death of the plants. Similar symptoms were observed on plants inoculated via stem injection.

Minneola seedlings exposed to either culture filtrates or to live cells failed to develop disease symptoms. Citrus stem cuttings did not wilt within a period of 1 hr after being placed in diluted culture filtrates.

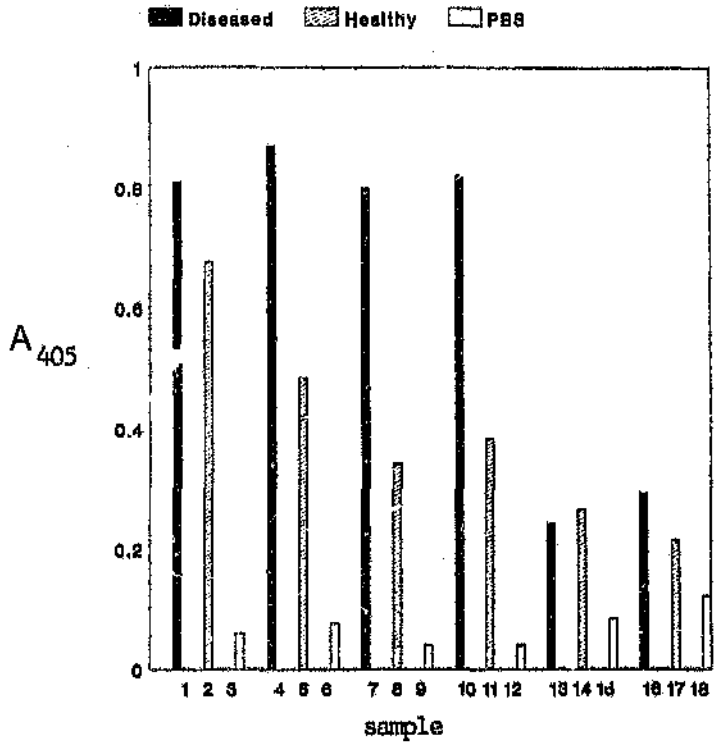
Wilting of control seedlings or cuttings exposed to water only was not observed during the course of this study.

3.7 ANTISERUM PRODUCTION

3.7.1 Immunogen preparation

3.7.1.1 Pre-treatment of whole cells

The influence of the various pre-treatments of bacterial cells (GC isolate) on antiserum quality is shown in Fig. 34. Antisera raised against untreated live cells (bars 1-3) proved to be unsuitable for effectively detecting the homologous organism in citrus leaf material in the ME-PTA-ELISA. Similar results were recorded in numerous other serological detection assays using the same antiserum as well as antisera raised against similar preparations of the NC and CS isolates (results not shown).



Bar No.	Immunogen	diseased/healthy ratio
1 2 3	live cells	1.20
4 5 6	heat-killed cells	1.80
7 8 9	unencapsulated cells	2.34
10 11 12	sonicated cells	2.14
13 14 15	KOH-treated cells	0.91
16 17 18	PAGE protein band	1.37

Figure 34

The effect of pre-treatment of bacterial immunogens on the quality of the resultant antiserum as determined by the HE-PTA-ELISA.

Antisera were cross-adsorbed against a preparation of healthy citrus (section 2.9.4.2) prior to use. Diseased material was collected from a greening-affected citrus seedling known to harbour the CC isolate. Samples of both diseased citrus (solid bars) and healthy citrus (shaded bars) were prepared following the water leaching method and probed with the different antisera in the HE-PTA-ELISA. For the antiserum raised against KOH-treated cells, particulate matter in the water leachate of the sample material was sedimented by centrifugation and alkali-soluble antigens extracted from the resultant pellet by the alkali extraction method detailed in section 2.10.1.5.

The standard deviations (SD) did not exceed 0.04 absorbance units.

The following sample and reagent dilutions were used:

* sample 1:100
* antiserum 1:1000 (except in the case of the antiserum raised against the PAGE protein band which was diluted 1:200, and the antiserum raised against the preparation of sonicated cells which was diluted 1:500).
* enzyme conjugate 1:1000

Antisera directed against heat-killed (bars 4-6), as well as alkali-treated (bars 13-15) cell preparations were also found to be unsatisfactory. The only cell preparations which yielded antisera giving diseased/healthy ratios of greater than 2.0 were the formaldehyde-treated, unencapsulated whole bacteria and the sonicated preparation of the unencapsulated cells. Disruption of bacteria by sonication was a tedious procedure and thus the pre-treatment of choice was removal of capsular material and fixation by the method detailed in section 2.9.1.3. This method of cell preparation was used to produce antisera against all isolates used in this study.

3.7.1.2 Isolation of bacterial proteins by PAGE

Polyacrylamide gel electrophoresis of bacterial and plant samples under non-denaturing conditions did not adequately resolve constituent proteins. Under these conditions, it was not possible to obtain a clear profile of bacterial proteins from which specific bands could be selected for use as immunogen (Fig. 35).

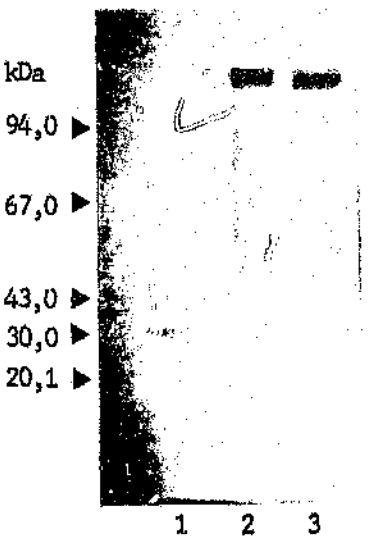


Figure 35

Non-denaturing PAGE separation of proteins extracted from the GC isolate (lane 2) and from healthy citrus midrib tissue (lane 3). Molecular weight protein markers (14.4 - 94 kDa: Pharmacia, Appendix D) are in lane 1. Midrib sample was prepared following the water leaching method. Bacterial sample was prepared as described in section 2.9.1.6.

Unlike non-denaturing PAGE, fractionation of samples by SDS-PAGE resulted in a clear separation of the proteins. The protein profiles of the GC isolate and the extract of healthy citrus midrib material are shown in Fig. 36. In the preparation of the leaf midrib sample, the water leaching procedure was employed which yielded an extract containing relatively low concentrations of soluble protein. Thus only proteins present in citrus midrib tissue in large amounts were detected by this method.

A major protein band (52 kDa) apparently unique to the bacterium was detected using PAGE (Fig. 36, arrow) and sufficient quantities of the protein isolated by this method for the immunization of rabbits. The resulting antiserum was reacted against extracts of citrus in the HE-PTA-ELISA. The extracts were prepared from material collected from a symptomatic citrus plant from which the bacterium (GC isolate) had previously been isolated, as well as from healthy (control) citrus. Results of the assay are shown in Fig. 34 (bars 16-18). Although the mean absorbance value of the diseased sample (0.293) is slightly higher than that of the healthy sample (0.214), the test result is considered to be negative as the value of the diseased/healthy ratio is less than 2.0.

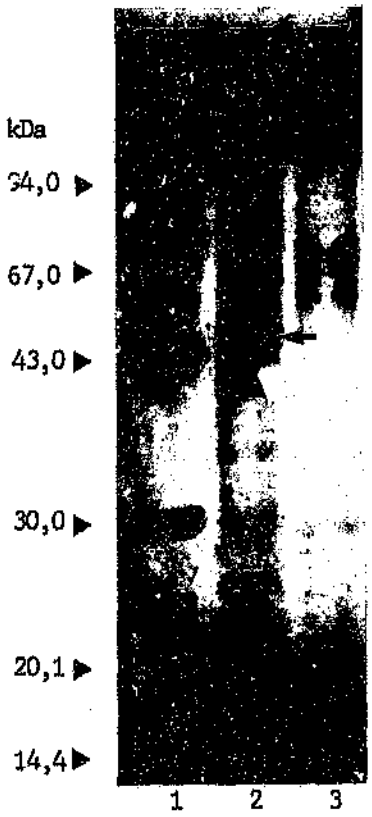


Figure 36

SDS-PAGE protein profiles of *GC* isolate (lane 2) and healthy citrus midrib proteins (lane 3). Molecular weight markers are shown in lane 1 (14.4 - 94 kDa; Pharmacia, Appendix D). The arrow indicates a prominent bacterial protein band (52 kDa) unique to the organism. Samples were prepared as for Figure 35.

3.7.1.3 Extraction of potentially disease-specific immunogen from infected plant material

Method 1

The KOH extraction procedure adapted from that reported by French (1974) was found to be an unsuitable method for the extraction of bacteria from either infected bark or midrib material. Both light and electron microscopic examination of the concentrated leachates failed to reveal the presence of bacteria or bacteria-like structures in the preparations. Samples of both infected and healthy control material were, however, contaminated with particulate matter of plant origin.

Method 2

Application of the modified purification procedure of Sinha (1974) to greening-infected bark and leaf midrib tissue resulted in the release of only a small number of bacteria from these tissues (Fig. 37). As the concentration of organisms in the partially-purified preparations was low, further purification of the extract by column chromatography and density gradient centrifugation was not carried out.

Figure 37

Representative bacteria extracted from greening-infected citrus material following method 2. Numerous vesicle-like structures co-purified with the bacteria are visible in the preparation. Bar = 0.5 um.

Figure 38

A bacterium observed in an extract of healthy citrus prepared following method 2. Note absence of the vesicle-like structures observed Figure 37. Bar = 0.5 um.

The enzymes cellulase and macerozyme (pectinase) were employed in an attempt to maximize the release of organisms present within the host plant tissues. The degradation of host cell constituents by the action of these enzymes should facilitate the liberation of endogenous bacteria. Enzymolysis of host tissue, however, did not appear to increase the yield of microorganisms in this instance, although visual inspection of extracts suggested that the treatment resulted in less contamination of the preparations with plant debris.

The morphology of the microorganisms extracted from infected material by the purification procedure was unlike that of the greening organism *in situ* (as described by Moll and Martin, 1973 and Garnier and Bové, 1983) or in extracts of infected periwinkle leaf midribs (Villechanoux *et al.*, 1990). When the concentrated samples were reacted with the antiserum raised against an extract of greening-infected citrus (GICE antiserum) in the HE-PTA-ELISA, no appreciable difference between the absorbance values of the test sample and a healthy control sample was observed (data not shown). Similar negative results were recorded with the immunogold labelling technique. The antiserum used had previously been shown to distinguish between greening-infected and healthy plant material (section 3.8.5). It is thus unlikely that the bacteria observed were related to the causal agent of greening disease.

Cells displaying a similar morphology to that of the organisms from infected samples were extracted from healthy citrus tissue (Fig. 38), although a significantly smaller number were recovered from this material.

In addition to the bacteria observed in the preparations, a large number of amorphous vesicle-like structures were isolated from diseased plants (Figs 37 and 39). The structures varied in size and shape and were not observed in extracts of healthy leaf and bark material prepared in the same way (Fig. 38). The nature of the particles was not established, although they appeared to be associated with diseased material only and may be of host plant origin as treatment of the extracts with cellulase and pectinase rendered the preparations free of these structures.

Another type of vesicle or envelope-like structure was consistently isolated from greening-infected citrus material by following method 2 (Figs 40 and 41). The size of the vesicles was greater than those of the aforementioned structures and their shape more regular. The vesicles appeared to be empty and were unaffected by treatment with cellulase and pectinase. They were associated with infected material only and were not observed in healthy extracts.

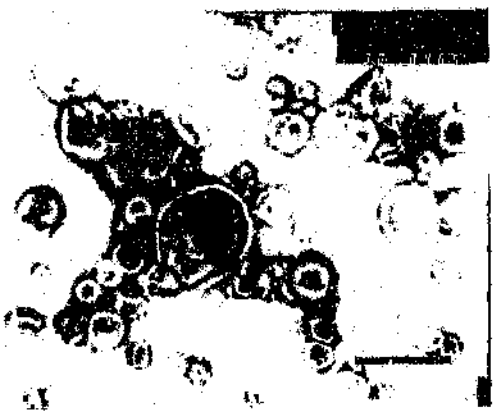


Figure 39

Enlargement of vesicle-like structures co-purified with bacteria from greening-infected citrus tissue. Bar = 0.5 um.

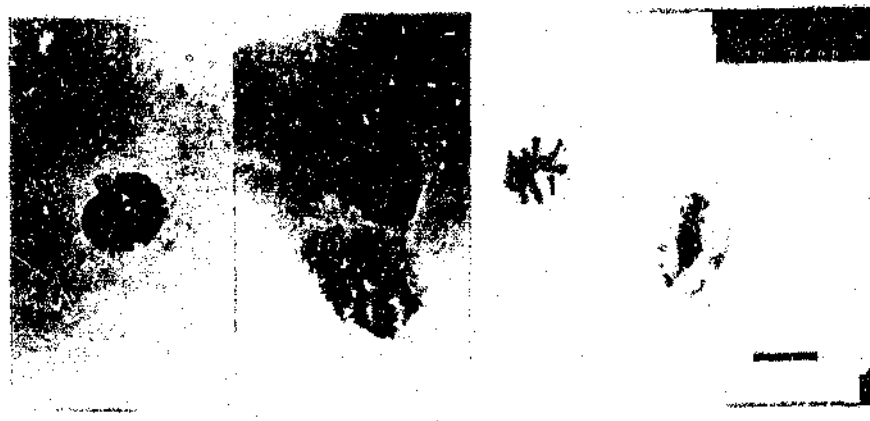


Figure 40

Collapsed vesicle- or envelope-like structures isolated from greening-infected citrus material following method 2. Bar = 0,5 um.

Figure 41

Enlargement of an empty vesicle- or envelope-like structure purified from greening-infected citrus. Bar = 0.5 um.

Method 3

Using this purification procedure, a small number of organisms displaying a morphology similar to that of the bacteria isolated following method 2 (Fig. 42) were observed in preparations of infected citrus midrib and bark material. Similar organisms were not observed in corresponding healthy preparations.

Reacting the diseased citrus preparation (the sample was concentrated by means of high-speed centrifugation prior to analysis) with the GICE antiserum in the HE-PTA-ELISA suggested that the organisms isolated were serologically unrelated to the causal agent of greening (results not shown).

The vesicle-like structures found associated with preparations of diseased tissue following the previous protocol were not observed in the samples of infected citrus obtained with method 3.

The percentage cell recovery recorded (Table 8) suggests that this technique is relatively efficient for the isolation of bacteria (with physical properties similar to those of C. m. subsp. michiganensa) from homogenized citrus material.

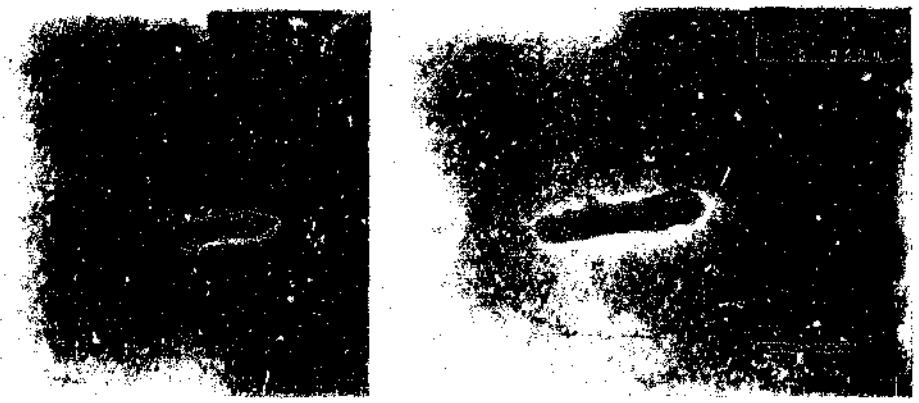


Figure 42

Bacteria isolated from greening-infected citrus following method 3. Bar = 1.0 um.

TABLE 8

Assessment of purification procedures using citrus leaf homogenates artificially contaminated with C. m. subsp. michiganense.

Purification procedure	Cell count (cells/ml)		% cell recovery
	Before purification	After purification	
Method 1	-	-	-
Method 2	10^6	[a] $3,3x10^5$	33
Method 3	10^6	[b] $6,8x10^5$	68
Method 4	10^6	$5,5x10^5$	55
Method 5	10^6	[c] $8,7x10^5$	87
Method 6	-	-	-

[a] Final cell count made before purification by column chromatography and density gradient centrifugation.

[b] Final cell count made before gradient centrifugation.

[c] Final cell count made after discontinuous sucrose gradient centrifugation. The efficacy of Percoll gradient centrifugation was not assessed.

Method 4

The purification protocol described by Sui (1988) was developed specifically for the isolation of the greening bacterium and was reported to yield large numbers of the organism from periwinkle affected by the Asian strain of the disease (Sui 1988).

The attempts made in this study to isolate the bacterium from both periwinkle and citrus affected by the African strain of the disease did not yield preparations of the bacterium similar to those described by Sui (1988). A very small number of bacteria-like organisms were isolated following this protocol, but they did not appear to be morphologically similar to the greening organism *in situ* or in preparations of the bacterium obtained from infected plant material (Villechanoux *et al.*, 1990) (Fig. 43). Similar organisms were never isolated from healthy control material.

In addition to the bacteria observed, structures resembling vesicles were isolated from the infected citrus material (Fig. 43). The vesicles appeared to be similar to those isolated from diseased citrus using method 2 (Fig. 37) and were not observed in preparations of healthy material. These structures were not isolated from affected periwinkle plants.

Figure 43

Bacteria and vesicle-like structures isolated from greening-infected citrus following method 4. Bar = 0.5 um.

When concentrated preparations of the diseased citrus and periwinkle material were reacted with the GICE antiserum in the HE-PTA-ELISA, the similar absorbance readings recorded for these test samples and the healthy control samples indicated that the organisms observed were serologically unrelated to the greening organism (ELISA data not shown).

The use of citrus midrib homogenates artificially contaminated with bacterial cells to assess the efficacy of the isolation technique indicated that almost half the added cells were lost during the procedure (Table 8). A significant loss of bacteria occurred during the initial clarifying steps of the three cycles of differential centrifugation. This was indicated by microscopic examination of the pellets formed after each period of low-speed centrifugation which revealed the presence of a large number of cells. Reduction of the centrifugal force during the clarifying step, or shortening the length of the centrifugation run may reduce the number of organisms lost during purification.

Method 5

This extraction protocol was determined largely empirically using artificially contaminated homogenates. The principal object of the technique was the optimization of cell recovery and little emphasis was placed on the removal of host plant debris. Preliminary studies indicated that the procedure was effective, this being shown by the 87% cell recovery recorded (Table 8). The efficacy of the protocol was further demonstrated by the purification of large numbers of MLO's from leaves of citrus affected by "multiple sprouting".

Extracts of greening-infected citrus midrib tissue prepared using this purification procedure were used for the immunization of rabbits (the resultant antiserum was designated "GICE"). The plant preparations obtained were heavily contaminated with plant material, this being indicated by the intense green colouration of the extracts. Electron microscopic examination revealed that samples contained very little plant material of a particulate nature (Fig. 48) and the contamination was probably due to the presence of soluble host plant pigments. Passage of the sample through a 30% sucrose solution failed to exclude the pigmented contaminants, although a significant amount of the particulate plant contaminants was removed.

The use of self-generating Percoll gradients as an alternative to the sucrose gradient proved to be unsatisfactory. After centrifugation, the samples were found to have been fractionated in such a way as to make accurate collection and analysis of each band impossible (Fig. 44). Sucrose gradients were thus used to prepare sufficient quantities of infected material for use as immunogen.

Because of the difficulty in locating specific bands within the Percoll gradients, a preparation of cultured C.m. subsp. michiganense cells were layered onto a 70% (v/v) Percoll solution and centrifuged under the same conditions as the plant extracts in order to determine the location of the bacteria after centrifugation. Figure 45 indicates that the organisms do not sediment in a single discreet band but are spread over a broad region of the gradient. A similar situation may occur with the greening bacteria in infected plant extracts, thus making recovery of the organisms even more complicated. Microscopic examination of 0.1 ml fractions of the Percoll gradient in which the diseased plant extract was fractionated (Fig. 45, centre tube) failed to reveal the presence of significant numbers of bacteria in any one particular area of the gradient.

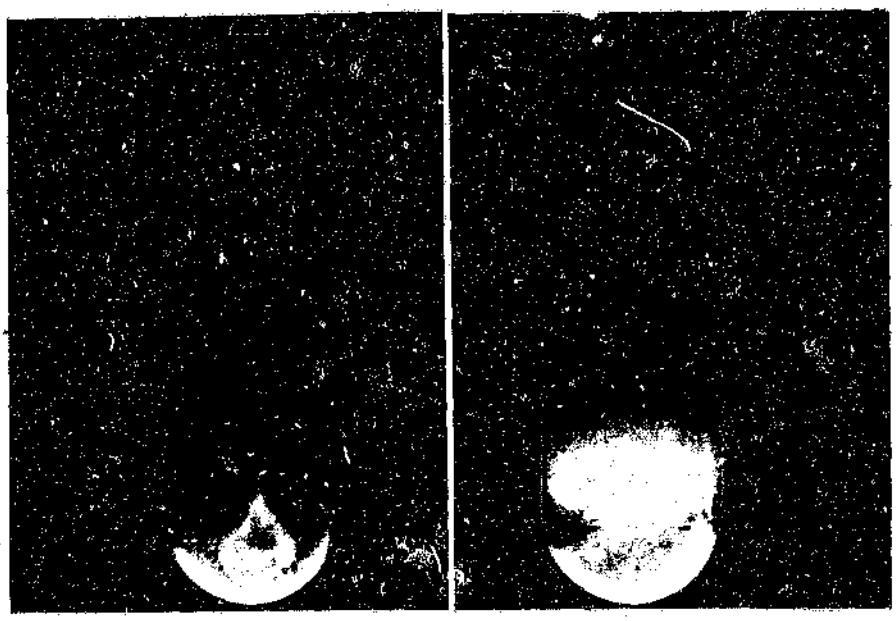


Figure 44

Fractionation of greening-infected citrus extracts (method 5) in self-generating Percoll gradients. The Percoll was held within Beckman Ultra-Clear tubes. The fractionation of sample in a 70% (v/v) Percoll solution is shown on the left, while on the right is shown the band pattern generated in a 30% (v/v) Percoll solution.

Figure 45

Fractionation of healthy (left) and greening-infected (centre) citrus extracts, as well as a suspension of *C. m. subsp. michiganense* cells in 70% Percoll self-generating gradients. The samples were fractionated in 1.5 ml volume Beckman polycarbonate tubes.

Despite the effectiveness of this bacterial purification method (as determined by the preliminary studies), very few bacteria or bacteria-like structures were isolated from citrus tissue. A small number of bacteria displaying the morphology and dimensions of the greening organism were consistently isolated (Fig. 46) from diseased bark and midrib material. Similar organisms were never observed in extracts of healthy samples.

Preparations of infected material also contained large numbers of amorphous crystalline structures (Fig. 47). These structures were present in all infected extracts but not in any of the healthy samples (Fig. 48) (the healthy control samples (bark as well as midrib material) were collected from eight different seedlings which were certified greening-free and provided by the C.S.F.R.I., Nelspruit).

Leaf midribs collected from citrus seedlings supposedly free of greening disease but infected with a variety of other phytopathogens were subjected to this same purification procedure in order to determine whether these crystalline structures were associated with greening-infected material only. The following samples were

Figure 46

A bacterium extracted from greening-infected citrus following method 5. The morphology of the organism is similar to that of the greening organism *in situ*. The preparation was reacted with an antiserum raised against the GC isolate in the immunogold labelling assay (sections 2.12 and 3.10) to determine the serological relationship of the two organisms. Note the absence of bound gold label. Bar = 0.25 um.

Figure 47

Amorphous crystalline structures extracted from infected citrus following method 5. Bars = 1.0 um.

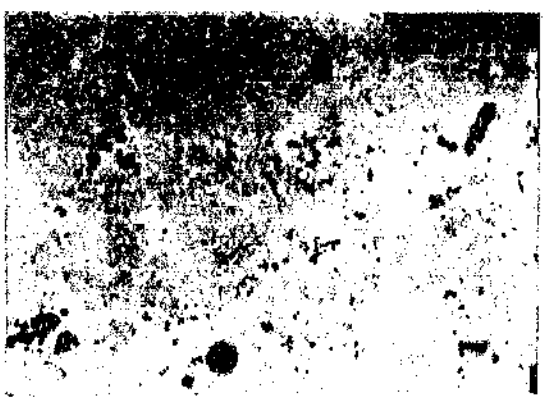


Figure 48

Negative staining of extract of healthy citrus prepared following method 5. Bar = 1.0 um.

analysed in this way: i) troyer citrange infected with both tatter leaf virus and citrus tristeza virus (CTV), ii) rough lemon infected with vein enation virus and CTV, iii) mandarin infected with CTV and "multiple sprouting", iv) sweet orange infected with psorosis and CTV, and v) grapefruit infected with CTV as well as with a severe strain of CTV (strain 7K6). The samples were provided by Prof. J. Da Graca of the University of Natal, Pietermaritzburg.

All the diseased samples analysed were found to contain the same crystalline structures as those observed in extracts of greening-infected citrus. This finding suggests that the crystals are not specifically associated with greening-infected citrus and are probably produced by the plant as a consequence of infection by a variety of phytopathogens.

Crystals purified by repeated cycles of differential centrifugation were found to exhibit an absorbance peak at 282 nm when analysed spectrophotometrically. This finding suggests that the structures are proteinaceous, a fact which may have been confirmed by treatment of the preparation with protease or by the application of a simple assay for protein.

Method 6

The attempt to identify and isolate disease-specific antigens from infected plant material by SDS-PAGE fractionation of protein extracts was not successful.

Figure 49 shows the profiles of proteins extracted from citrus affected by different pathogens. No bands unique to the greening-infected sample are visible.

Non-denaturing PAGE fractionation of plant samples failed to generate clear profiles of constituent proteins in which specific bands could be identified (Fig. 50, top). Fractionation under denaturing conditions, however, resulted in better separation of the proteins (Fig. 50, bottom), although no bands suitable for use as immunogen were visualized in the resultant profiles.

Attempts to isolate greening-specific proteins from material affected by the Asian strain of greening was also unsuccessful (Figs 51 and 52). Of the three sample preparation procedures used, phenol extraction of proteins appeared to yield the most satisfactory results (Fig. 51, lanes 3 and 4). TCA extraction resulted in heavy streaking of the samples (lanes 1 and 2), while the method employing SDS failed to extract quantities of protein great enough to generate a clear profile (lanes 5 and 6).

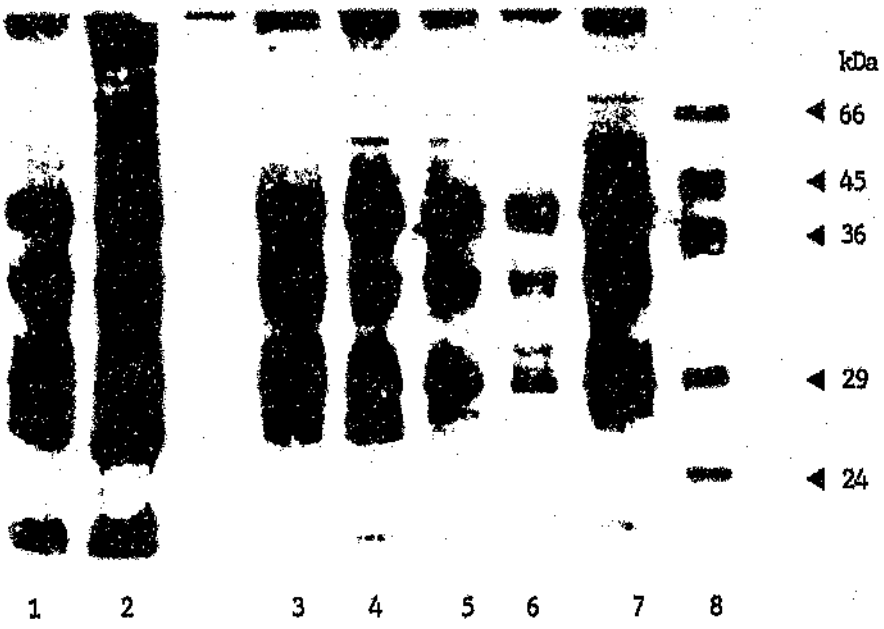


Figure 49

SDS-PAGE fractionation of proteins extracted from citrus leaf midribs.

Lane 1 - African greening-infected citrus (Pietermaritzburg district)
2 - African greening-infected citrus (Nelspruit district)
3 - healthy citrus
4 - citrus affected by tatter leaf virus and CTV
5 - citrus affected by vein enation virus and CTV
6 - citrus affected by CTV and CTV strain 7K6
7 - citrus affected by psorosis and CTV
8 - molecular weight protein markers (14.2 - 66 kDa; Sigma)

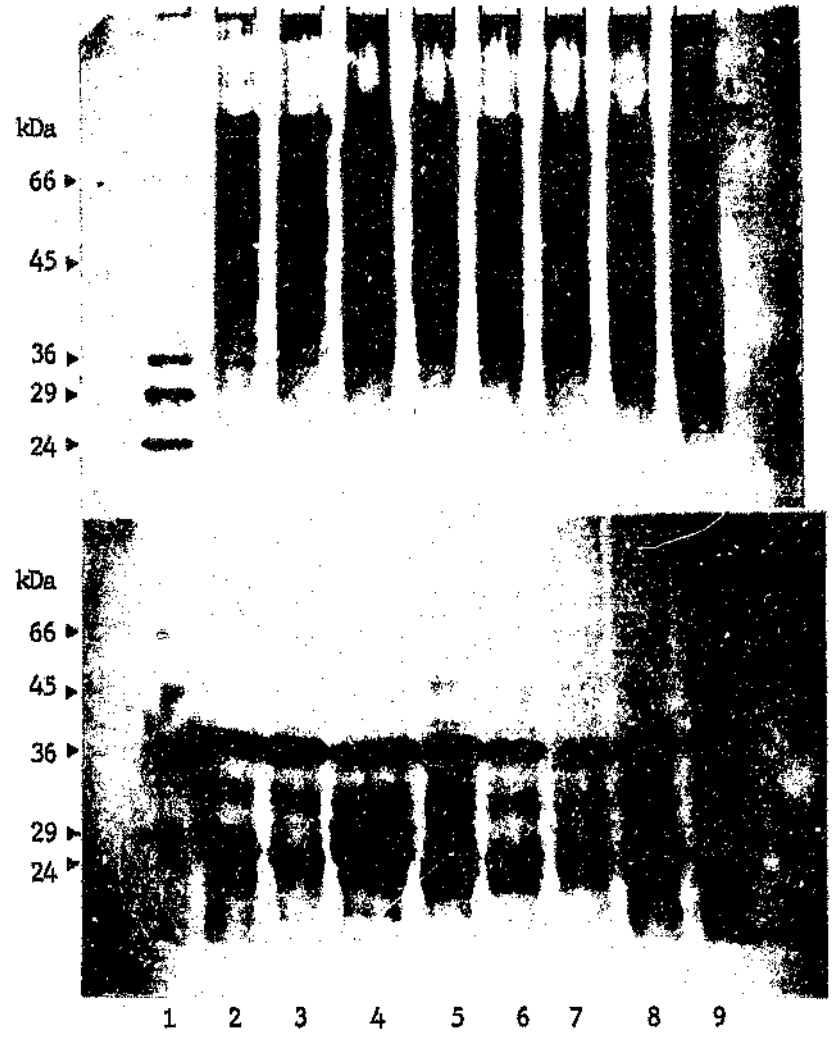


Figure 50

Fractionation of citrus leaf midrib protein extracts by non-denaturing PAGE (top) and SDS-PAGE (below).

Lane 1 - molecular weight protein markers (14.2-66 kDa: Sigma)
2 - healthy citrus
3 - African greening-infected citrus (Pietermaritzburg district)
4 - African greening-infected citrus (Brits district)
5 - citrus affected by tatter leaf virus and CTV
6 - citrus affected by vein enation virus and CTV
7 - citrus affected by "multiple sprouting" and CTV
8 - citrus affected by psorosis and CTV
9 - citrus affected by CTV and CTV strain 7K6

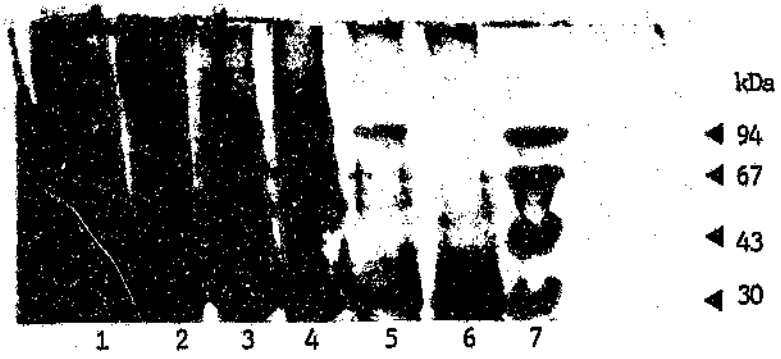


Figure 51

SDS-PAGE fractionation of citrus leaf midrib proteins extracted from material affected by the Asian strain of the disease. PAGE samples were prepared using differed methods.

Lane 1 - infected citrus prepared by the TCA extraction method
2 - healthy citrus prepared by the TCA extraction method
3 - infected citrus prepared by the phenol extraction method
4 - healthy citrus prepared by the phenol extraction method
5 - infected citrus prepared by the SDS extraction method
6 - healthy citrus prepared by the SDS extraction method
7 - molecular weight protein markers (14.4 - 94 kDa: Pharmacia)

1 2 3 4 5 6

Figure 52

Non-denaturing PAGE fractionation of citrus leaf midrib proteins extracted from material affected by the Asian strain of the disease. Varying concentrations of protein were loaded onto the different lanes. Molecular weight protein markers were not loaded onto the gel.

Lane 1 - infected citrus : 100 ug protein
2 - healthy citrus : 100 ug protein
3 - infected citrus : 200 ug protein
4 - healthy citrus : 200 ug protein
5 - infected citrus : 250 ug protein
6 - healthy citrus : 250 ug protein

Application of the silver staining method of Amersham (Amersham, U.S.A.) to the Coomassie Blue-stained gels failed to reveal additional proteins which may have been present, but at concentrations too low to be detected by Coomassie staining (results not shown). Double-staining of PAGE gels has been reported to be far more sensitive than the use of Coomassie Blue alone (Andrews, 1986).

3.7.2 Cross-adsorption of antisera

3.7.2.1 Cross-adsorption of antiserum raised against an extract of diseased citrus

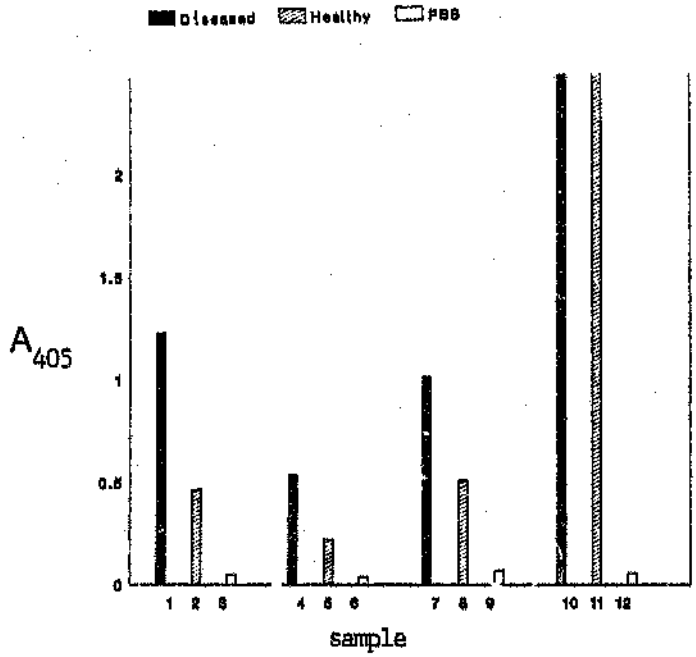
The extraction procedure (section 2.9.2, method 5) used to prepare the immunogen contained a significant amount of plant contamination. It was necessary to use an extraction procedure yielding as much of the bacterial content of the infected material as possible, although such a procedure would naturally contain a substantial amount of contaminating host plant material as well.

Because of the presence of citrus material in the immunogen preparation, the antiserum raised was not suitable for use in a serological detection assay without prior

cross-adsorption against host plant antigens. This is clearly indicated by high absorbance reading recorded for uninfected material in the HE-PTA-ELISA (Fig. 53).

Preliminary studies showed that cross-adsorption of antiserum with a crude sap extract of healthy citrus leaf tissue was not suitable for the efficient removal of antibodies directed against plant components. Even after extensive treatment, the antiserum displayed considerable cross-reactivity against healthy control samples and, in addition to this, the presence of plant pigments and enzymes appeared to have a deleterious effect on the antiserum by reducing its sensitivity (data not shown). It was therefore necessary to investigate other means of removing unwanted antibodies from the antiserum.

All three cross-adsorption techniques employed were found to efficiently remove a significant proportion of the plant-specific antibodies from the antiserum. Although cross-adsorption did not remove all the background activity, a differential in absorbance values was obtained between the extracts from healthy and infected plants in the HE-PTA-ELISA (Fig. 53).



Bar no.	Cross-adsorbed method	diseased/healthy ratio
1 2 3	Ammonium sulphate precipitated proteins	2.65
4 5 6	affinity column cromatography	2.40
7 8 9	acetone-fixed material	2.00
10 11 12	untreated antiserum	-

Figure 53

Removal of plant-specific reactivity from the GICE antiserum by cross-adsorption against different plant preparations.

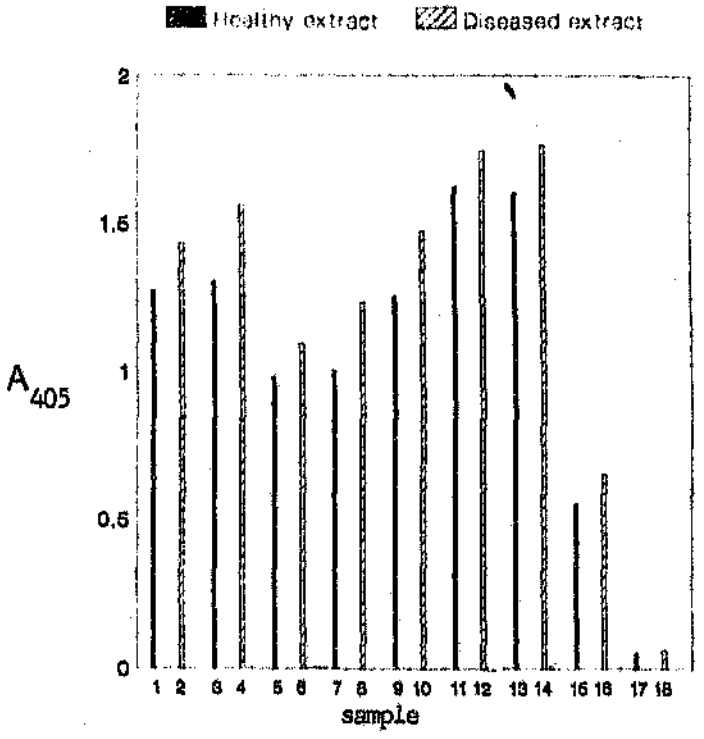
Samples of greening-infected and healthy citrus midrib material were prepared following the water leaching method and probed with the cross-adsorbed antisera in the HE-PTA-ELISA.

The SD did not exceed 0.05 absorbance units.

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

Attempts to reduce the anti-plant activity further by diluting the cross-adsorbed antiserum in an extract of healthy citrus made in ELISA sample conjugate buffer (10 g fresh weight/litre) were largely unsuccessful. The presence of healthy plant antigens in the antiserum diluting buffer has been reported to improve detection assay results considerably (Clark et al., 1983, Rybicki, 1984). No significant difference was, however, observed between results obtained in this study using the modified buffer as diluent and those obtained using unadulterated sample conjugate buffer (data not shown).

Host plant material affected by diseases other than greening were also used for serum cross-adsorption as diagnostic assays showed that the GICE antiserum displayed a considerable amount of reactivity with citrus material infected with a number of other pathogens (Fig. 54). The treatment, however, failed to remove this unwanted activity (Fig. 54).



Bar no.	Citrus sample affected by:
1 2	vein enation + CTV
3 4	CTV + 7K6
5 6	multiple sprouting
7 8	psorosis
9 10	impersatura
11 12	greening (Nelspruit area)
13 14	greening (Réunion Island)
15 16	healthy citrus
17 18	buffer (PBS) control

Figure 54

Reaction of GICE antiserum with healthy citrus and with citrus infected with various phytopathogens. Two different preparations of the antiserum were used: GICE cross-adsorbed against an ammonium sulphate precipitated extract of healthy citrus ("Healthy extract") and a similar extract of various citrus leaf samples infected with different phytopathogens (section 2.9.4) ("Diseased extract").

Plant samples were prepared by the water leaching method and probed with the cross-adsorbed antiserum in the HE-PTA-ELISA.

The SD did not exceed 0.06 absorbance units.

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

3.7.2.2 Cross-adsorption of antiserum raised against cultured bacteria

Because of the anti-plant activity observed with the antiserum raised against the cultured GC isolate, differentiation between symptomatic and healthy plant samples in the ELISA was difficult. Cross-adsorption of this antiserum with healthy citrus plant proteins prepared following the ammonium sulphate precipitation technique was carried out in an attempt to reduce the high background readings.

The results of the HE-PTA-ELISA in which the untreated and cross-adsorbed antiserum was compared are represented graphically in Figure 55. Although cross-adsorption of the antiserum reduced the anti-plant activity, a significant difference between the treated and untreated antiserum was not observed, this being indicated by the similar d/h absorbance ratios recorded with the two antisera (a ratio of 1.9 with the untreated antiserum and a ratio of 2.1 with the cross-adsorbed antiserum).

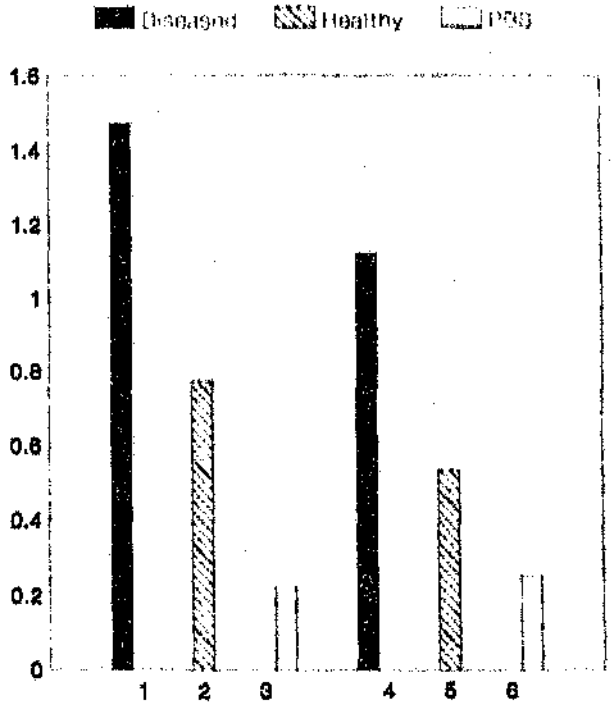


Figure 55

Results of the HE-PTA-ELISA in which plant material known to harbour the GC isolate, as well as uninfected material, was probed with cross-adsorbed (bars 4 and 5) and untreated (bars 1 and 2) antiserum raised against a suspension of live cultured GC cells. A preparation of ammonium sulphateprecipitated citrus midrib proteins was used for the cross-adsorption of the antiserum.

The SD did not exceed 0.03 absorbance units.

Plant samples were prepared by the water leaching method. The following sample and reagent dilutions were used:
* sample - 1:10C
* antiserum - 1:1000
* enzyme conjugate - 1:1000

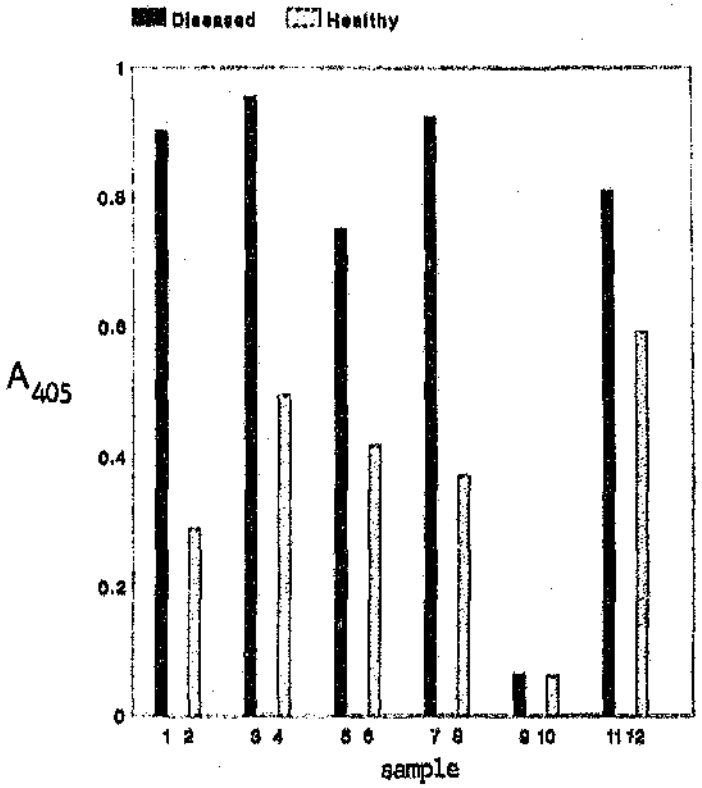
3.8 ENZYME AND RADIO-ISOTOPE BASED SEROLOGICAL DETECTION ASSAYS

3.8.1 ELISA sample preparation

The efficacy of the various sample preparation procedures was determined in the HE-PTA-ELISA and the results obtained represented graphically in Figure 56. Positive results (d/h absorbance ratio of 2.0 or more was considered positive) were recorded only with the samples prepared following the water leaching and the "chopping" methods. It is evident from the absorbance ratios that preparation of leaf midrib material following the water leaching method yielded the best results.

3.8.2 Comparison of the Fab', $F(ab')_2$, DAS and HE-PTA enzyme assays

The results of the four ELISA variations are summarized in Figure 57. The water leaching method was used in the preparation of sample for all the assays. Three distinct lots of excised leaf midribs were used to prepare three samples of infected and healthy plant material. The different samples are represented in Figure 57 by the figures 1, 2 and 3.



Bar no.	Sample preparation procedure	diseased/healthy ratio
1 2	water leaching	2.14
3 4	polytron homogenization	1.93
5 6	leaf press extraction	1.80
7 8	"chopping" method	2.50
9 10	enzymatic treatment	1.05
11 12	KOH treatment	1.37

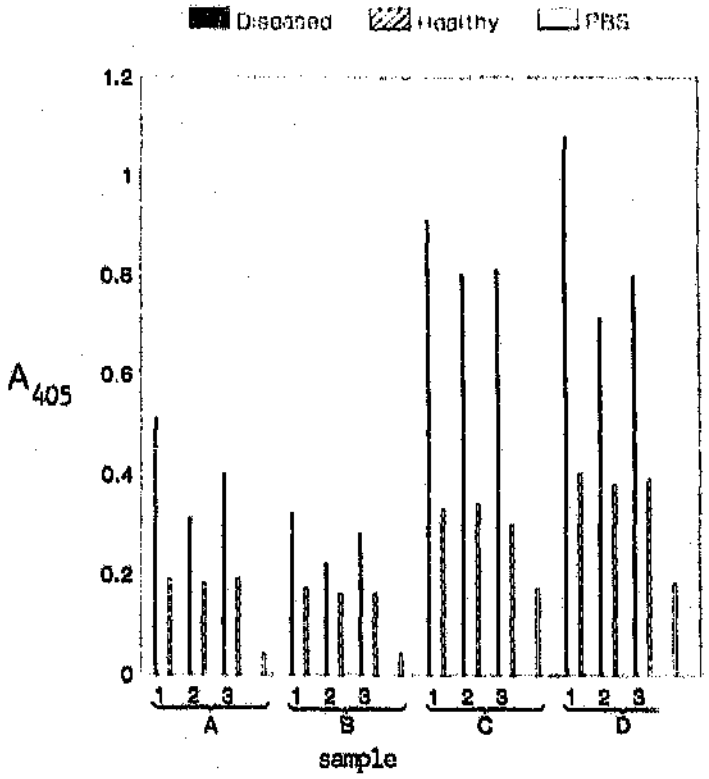
Figure 56

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

The SD did not exceed 0.04 absorbance units.

The following sample and reagent dilutions were used. Optimal dilutions were determined using a chequerboard scheme similar to that described by Hill (1984).

Preparation procedure	Sample	Antiserum	Enzyme Conjugate
water leaching	1:100	1:1000	1:1000
polytron	1:2	1:1000	1:1000
leaf press	1:4	1:1500	1:1000
"chopping"	1:10	1:1000	1:1000
enzyme	1:2	1:1000	1:1000
KOH	1:10	1:1000	1:1000



Bar group	Enzyme assay	Largest SD recorded
A	$F(ab')_2$	0.009 abs. units
B	Fab'	0.011 abs. units
C	DAS-ELISA	0.030 abs. units
D	ME-PTA-ELISA	0.040 abs. units

Figure 57

Comparison of the efficacy of different enzyme immunoassays in detecting disease-specific antigens in citrus leaf midrib tissue.

The numbers 1, 2 and 3 in each bar group represent three distinct leaf midrib samples. The same sample preparations were used in the different detection assays. Samples of greening-infected and healthy citrus midrib material were prepared by the water leaching method and probed with GICE antiserum (or the IgG fraction thereof) cross-adsorbed against a preparation of healthy citrus proteins. Infected and control material was collected from seedlings provided by the C.S.F.R.I.

The following sample and reagent dilutions were used. Optimal dilutions were determined in preliminary chequerboard titration assays.

Reagent	Detection assays			
	$F(ab')_2$	Fab'	DAS-ELISA	ME-PTA-ELISA
* trapping antibody	1 ug/ml	1 ug/ml	10 ug/ml	"
* sample	1:2	1:2	1:4	1:100
* detecting antibody	10 ug/ml	10 ug/ml	"	1:1000
* enzyme conjugate	1:5000	1:3000	1:5000	1:1000

All the detection assays used were able to detect the presence of disease antigens in the three preparations of infected sample material. There appeared to be no significant difference between the results obtained using the different assays, this being indicated by the similar d/h absorbance ratio calculated for the four tests. The respective d/h ratios for samples 1, 2 and 3 assayed in the four tests were: $F(ab')_2$ - 2.76, 2.21 and 2.60

Fab' - 2.41, 1.81 and 2.40

DAS-ELISA - 2.90, 2.39 and 2.86

HE-PTA-ELISA - 2.83, 2.10 and 2.48

No hydrolysis of the substrate p-nitrophenyl phosphate directly by absorbed components of plant extracts was observed in the HE-PTA-ELISA.

3.8.3 Comparison of the HE-PTA-ELISA, Bead-RIA and Dot-RIA

It was not possible to differentiate between the healthy and greening-affected leaf extracts with the Dot-RIA (Fig. 58C). The d/h ratios for the three samples assayed in this test all fell below the cut off value of 2.0. Both the HE-PTA-ELISA and Bead-RIA were, however, able to detect disease antigens in the three different infected preparations (bars 1, 3 and 5, Figs 58A and B). The enzyme-based assay proved to be more sensitive than the radio-immunoassay, this being indicated by the greater d/h ratios generated by the former technique.

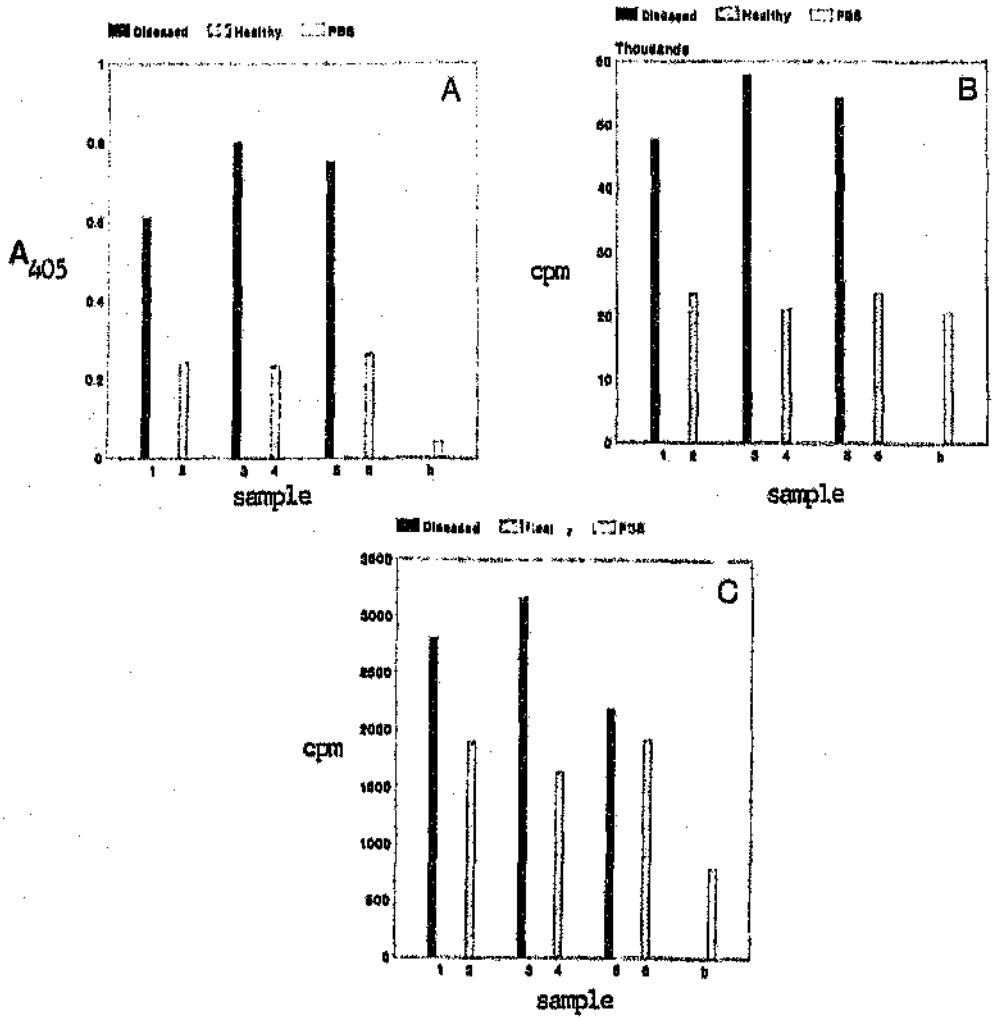


Figure 58

Detection of disease-specific antigen in citrus leaf midrib samples by ME-PTA-ELISA (A), Bead-RIA (B) and Dot-RIA (C).

Bars 1, 3 and 5 represent three distinct samples of greening-infected leaf midrib material, while bars 2, 4 and 6 represent three distinct healthy midrib samples. The same sample preparations were used in the three detection assays. Samples were prepared by the water leaching method and probed with the CICR anti-serum cross-adsorbed against healthy citrus plant proteins. Infected and control samples were collected from seedlings provided by the C.S.P.A.I.

The largest standard deviation recorded for each assay was:
ME-PTA-ELISA = 0.03 abs. units
Bead-RIA = 6300 c.p.m.
Dot-RIA = 493 c.p.m.

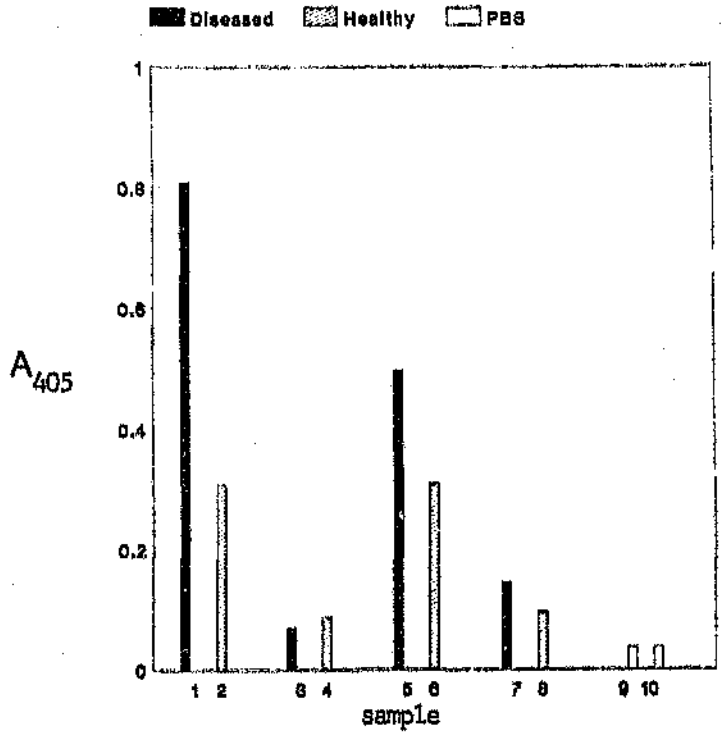
Optimal sample and reagent dilutions were determined in preliminary chequerboard titration assays. The following dilutions were used in the assays:

Reagent	Detection assay		
	ME-PTA-ELISA	Bead-RIA	Dot-RIA
* trapping antibody	-	10 ug/ml	-
* sample	1:100	1:10	undiluted
* detecting antibody	1:1000	-	1:200
* enzyme/ isotope conjugate	1:1000	150-200 c.p.m./ul	1:1000

3.8.4 Periodate oxidation of samples

The effect of periodate treatment on ELISA samples reacted with the GICE antiserum is illustrated in Fig. 59. Leaf midrib leachates were adsorbed directly onto the microtitre plates and analysed in the HE-PTA-ELISA. Mild oxidation of the plant samples (midrib and columella) had a marked effect on the results of the diagnostic assay, a significant decrease in the A_{405} readings for both the diseased and healthy extracts being observed.

A decrease in absorbance was also observed when similarly treated samples were reacted with an antiserum raised against a preparation of live bacterial cells (GC isolate) (Fig. 60).



Sample	Treatment	diseased/healthy ratio
midrib	untreated (bars 1 and 2)	2.63
	periodate oxidation (bars 3 and 4)	0.80
columella	untreated (bars 5 and 6)	1.61
	periodate oxidation (bars 7 and 8)	1.52

Figure 59

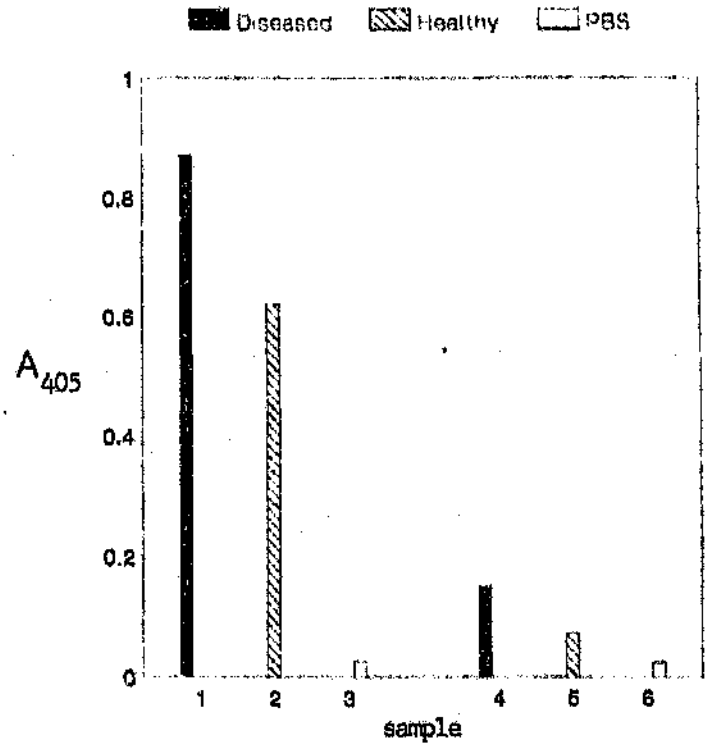
The effect of periodate oxidation of midrib and columella extracts on the binding of immunoglobulin to immobilized antigens in the HE-PTA-ELISA.

Infected samples were collected from a tree known to be affected with greening disease. Samples were prepared by the water leaching method and probed with the GICE antiserum cross-adsorbed against healthy citrus proteins. After adsorption of sample antigens to the microtitre plate, wells were either subjected to mild periodate oxidation (bars 3, 4, 7, 8 and 10) or left untreated (bars 1, 2, 5, 6 and 9).

The SD did not exceed 0.04 absorbance units.

The following sample and reagent dilutions were used:

* sample	- midrib	-	1:100
	columella	-	1:200
* antiserum	- midrib	-	1:1000
	columella	-	1:1500
* enzyme conjugate		-	1:1000



Treatment	diseased/healthy ratio
untreated (bars 1-3)	1.40
periodate oxidation (bars 4-6)	2.11

Figure 60

The effect of periodate oxidation of midrib extracts on the binding of immunoglobulin to immoblized antigens in the HE-PTA-ELISA.

Infected midrib samples were collected from a citrus seedling known to be infected with the GC isolate. Samples were prepared by the water leaching method and probed with an antiserum raised against a preparation of cultured, live GC cells. After adsorption of sample antigens to the microtitre plate, wells were either subjected to mild periodate oxidation (bars 4, 5 and 6), or left untreated (bars 1, 2 and 3).

The SD did not exceed 0.03 absorbance units.

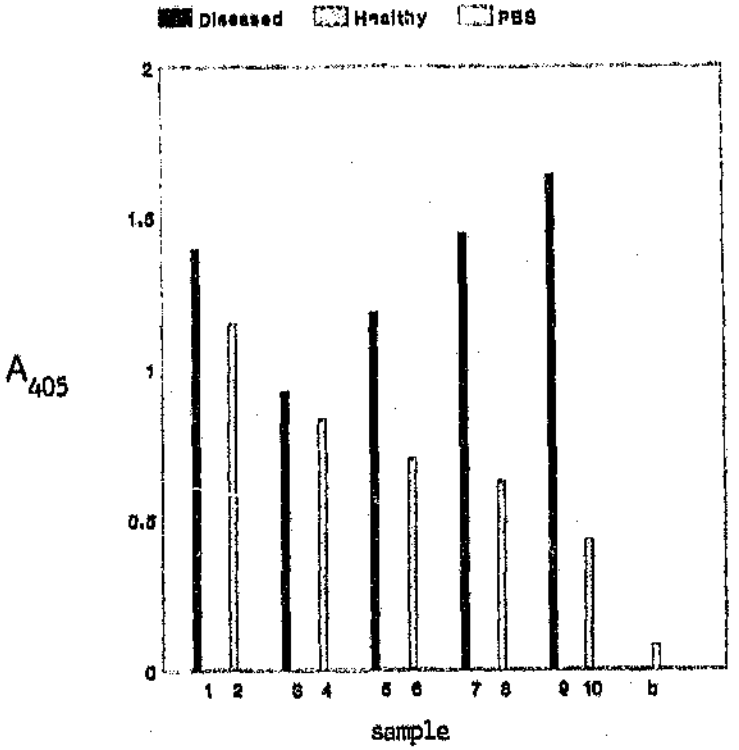
The following sample and reagent dilutions were used in the assay:
* sample - 1:100
* antiserum - 1:1000
* enzyme conjugate - 1:1000

3.8.5 Detection of disease antigen with the antiserum raised against an extract of greening-infected citrus (GICE antiserum)

3.8.5.1 Detection of disease antigen in different citrus tissues and in alternate host plants

In order to determine whether various citrus tissues differed in the amount of disease antigen that they harboured, samples of bark, seed endosperm, fruit columella and leaf midrib were prepared following the water leaching technique and tested for the presence of disease antigens in the HE-PTA-ELISA using the GICE antiserum.

The results presented in Figure 61 suggest that seed coat, endosperm and fruit columella did not serve as good sources of disease antigen, this being indicated by the small d/h ratio calculated for these tissues. In contrast, samples prepared from bark and leaf midrib material tested positive for the presence of antigen and thus probably contained higher levels of disease antigen than the other tissues assayed.



Bar no.	sample	diseased/healthy ratio
1 2	seed coat	1.22
3 4	endosperm	1.11
5 6	columella	1.50
7 8	bark	2.32
9 10	midrib	3.83

Figure 61

Detection of disease antigens in different citrus tissues using the ME-PTA-ELISA.

All samples were prepared by the water leaching method and probed with the GICE antiserum cross-adsorbed against healthy citrus midrib proteins.

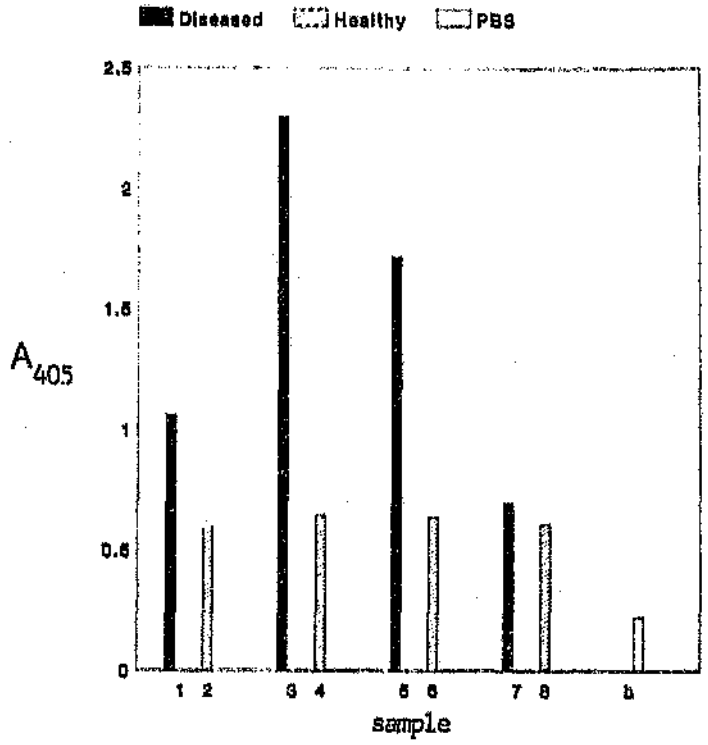
The SD did not exceed 0.05 absorbance units.

The following sample and reagent dilutions were used. Optimal dilutions were determined in a preliminary chequerboard titration assay.

Reagent	Samples				
	Coat	endosperm	columella	bark	midrib
* sample	1:100	1:200	1:200	1:100	1:100
* antiserum	1:1000	1:1500	1:1500	1:1000	1:1000
* enzyme conjugate	1:1000	1:1000	1:1000	1:1000	1:1000

In an attempt to test alternate host plant species for the presence of disease antigen, samples of the infected plants (periwinkle, cucumber and dodder) were reacted with the GICE antiserum in the HE-PTA-ELISA. To determine whether the same plants harboured the GC isolate, aliquots of the samples were reacted with the antiserum directed against an unencapsulated, formaldehyde-treated preparation of the cultured bacterium (GC isolate) in the same system. Greening-affected leaf midrib material collected from the Indian Ocean island of Réunion was included in the same analysis to test for the presence of disease antigen in citrus material from this geographical location.

The results of the assays are shown in Figures 62 and 63. Probing samples with GICE antiserum indicated that the cucumber and Réunion citrus material contained detectable levels of disease-specific antigen (Fig. 62). The d/h ratio calculated for the periwinkle sample, although being almost 2.0, was less than the negative-positive threshold value of 2.0 and must therefore be considered negative. It is possible, however, that disease antigen was present in the sample. The d/h ratio for the dodder leachate fell well below the 2.0 cut-off value and it is thus unlikely that it harboured detectable levels of GICE-specific antigen.



Bar no.	Sample	diseased/healthy ratio
1 2	Periwinkel midrib	1.80
3 4	Cucumber midrib	3.59
5 6	Citrus midrib (Réunion)	2.69
7 8	Dodder strand	1.14

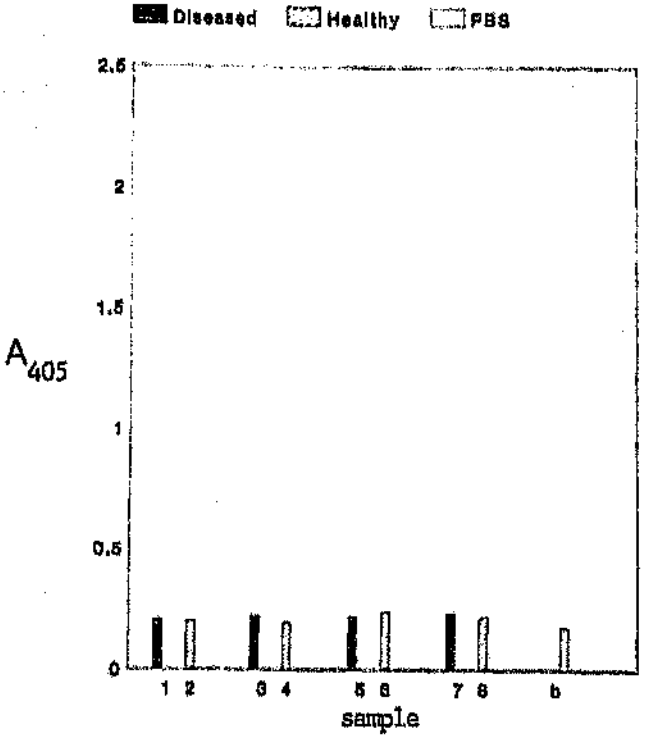
Figure 62

Detection of disease antigens in citrus and other plant species by the GICE antiserum.

Periwinkle sample material was taken from a symptomatic seedling infected with greening disease via dodder. Cucumber midribs were excised from leaves collected off a symptomatic plant infected with a disease agent from greening-infected citrus via dodder. The dodder material assayed was collected from a parasitized, greening-infected citrus seedling. Samples were prepared by the water leaching method and probed with the GICE antiserum (cross-adsorbed against healthy citrus proteins) in the ME-PTA-ELISA.

The SD did not exceed 0.06 absorbance units.

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



Bar no.	Sample	diseased/healthy ratio
1 2	Periwinkle midrib	1.05
3 4	Cucumber midrib	1.16
5 6	Citrus midrib (Réunion)	0.93
7 8	Dodder strand	1.07

Figure 63

HE-PTA-ELISA absorbance values recorded when the same plant preparations as those probed with the GICE antiserum (Figure 62) were probed with antiserum raised against the GC isolate. The antiserum was raised against an unencapsulated preparation of GC cells and cross-adsorbed against a preparation of healthy citrus proteins before use.

The SD did not exceed 0.01 absorbance units.

The following sample and reagent dilutions were used in the assay:
* sample - 1:100
* antiserum - 1:1500
* enzyme conjugate - 1:1000

Analysis of the identical plant leachates within the same microtitre plate using the antiserum directed against the GC isolate indicated that none of the samples contained detectable levels of organisms related serologically to the GC isolate (Fig. 63).

3.8.5.2 Analysis of individual leaf midribs

The water leaching method of sample preparation was found in this study to be suitable for analysing single leaf midribs in various serological detection assays.

Using this preparation procedure, midribs from sweet orange leaves displaying varying degrees of greening symptoms were assayed individually for the presence of disease-specific antigen. The infected leaves used (Fig. 64) were collected from an orchard in the Pietermaritzburg district of Natal. The tree sampled had been found to test positive for the gentisic acid marker of greening disease and displayed typical leaf and fruit symptoms. Healthy control leaves (Fig. 65) were collected off greening-free seedlings from the C.S.F.R.I. The midrib leachates were reacted with the GICE antiserum as well as antiserum raised against the GC isolate in the HE-PTA-ELISA (Figs 66 and 67).

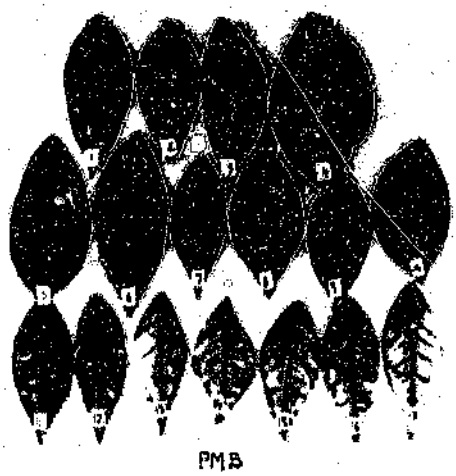


Figure 64

The greening-infected leaves from which midribs were excised for the HE-PTA-ELISA (Figs 66 and 67).

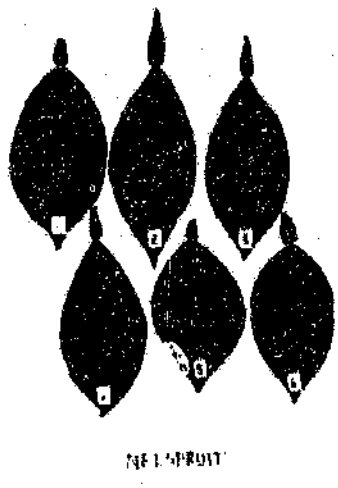


Figure 65

The healthy citrus leaves from which midribs were excised for the HE-PTA-ELISA (Figs 66 and 67).

The 17 infected leaf midrib· were positive for disease-specific antigens (as determined using the GICE antiserum) in this assay (Fig. 66), the d/h ratio calculated for all the samples being above 2.0. There appeared to be no significant difference in absorbance readings between leaves displaying severe symptoms (leaves 11-17) and those displaying mild chlorotic symptoms (leaves 1-4). All 6 healthy leaves sampled tested negative.

It is apparent from the results shown in Figure 67 that none of the midribs from the same symptomatic leaves contained antigen detectable with the anti-GC serum.

Similar results were recorded with leaves collected off greening-affected trees in the Brits and Nelspruit areas (results not shown).

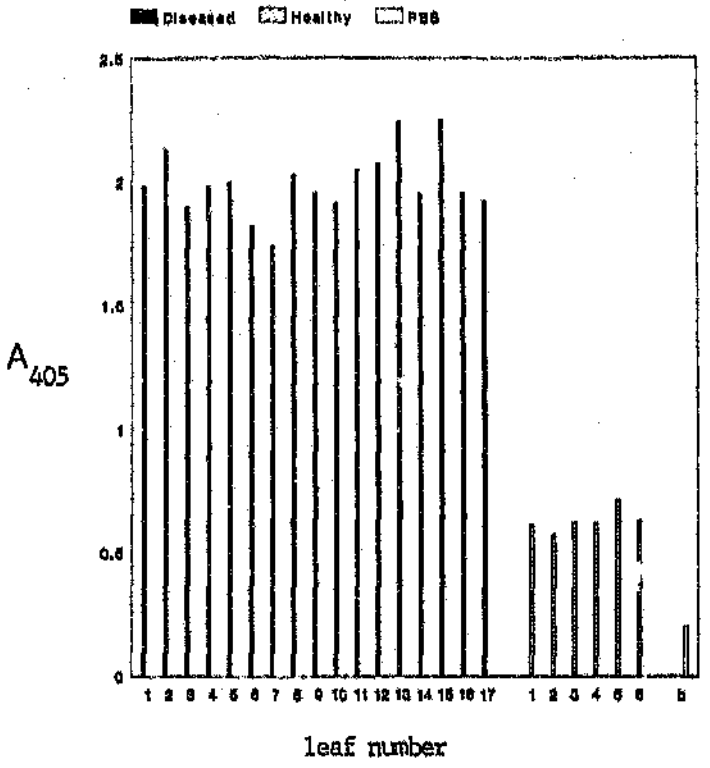


Figure 66

ELISA absorbance values for GICE antiserum-specific antigens in midribs from the greening-infected and healthy citrus leaves shown in Figures 64 and 65. The SD did not exceed 0.06 absorbance units.

Water leachates of each midrib were prepared and then probed with the GICE antiserum (cross-adsorbed against healthy citrus proteins) in the HE-PTA-ELISA.

The sample and reagent dilutions used were as follows:

* sample - 1:100
* antiseru - 1:1000
* enzyme c ijugate - 1:1000

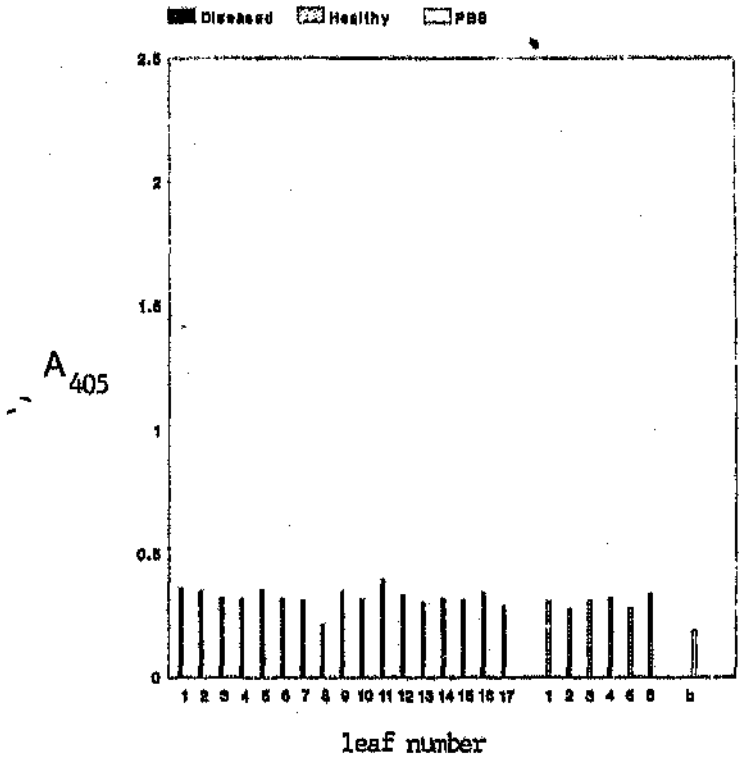


Figure 67

ELISA absorbance values obtained when the same citrus midrib samples were probed with antiserum raised against cultured GC cells. The SD did not exceed 0.02 absorbance units.

The GC antiserum was cross-adsorbed against a preparation of healthy citrus proteins before use.

The sample and reagent dilutions used were as follows:
* sample - 1:100
* antiserum - 1:1000
* enzyme conjugate - 1:1000

3.8.6.3 Enzyme-linked dot-immunoblot assay

Preparation of citrus columella samples for blotting onto NCM by alkali leaching of antigens was found to be an effective method. Extracts of columella material collected from orchards of the C.S.F.R.I. were prepared in this way and reacted with the GICE antiserum in the EL-DIB assay. Results of the assay are presented in Figure 68. It was not possible to quantify the reaction by determining the reflectance of the spots as the necessary equipment (a chromatoscanner) was not available. There was, however, a visible difference in the intensity of different sample spots which was great enough to allow for a qualitative assessment of the results.

The serological relatedness of various bacterial isolates was determined using the EL-DIB assay. The reaction of different isolates with antisera directed against the GC isolate and against C.m. subsp. michiganense is shown in Figures 69 and 70.

The assay was also used to screen various bacteria cultured from greening-infected material and thought to be putative greening isolates. The reaction of 8 different bacteria cultured from greening-infected citrus material with the GICE antiserum is shown in Figure 71.

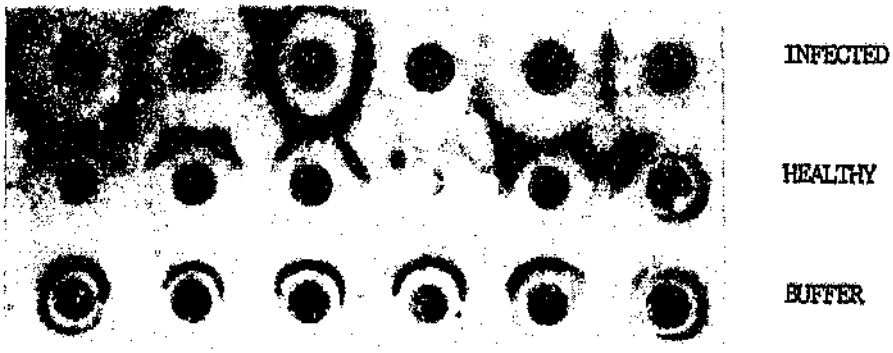


Figure 68

Enzyme-linked dot-immunoblot assay of antigens in greening-infected and healthy columella tissue. Samples prepared by KOH leaching.

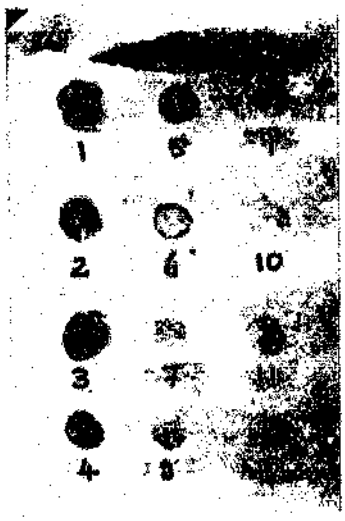


Figure 69

EL-DIB assay. Reaction of the citrus bacterial isolates, members of the C. michiganense group of bacteria, and other organisms with antiserum directed against the cultured isolate.

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