Detection of Citrus Greening-Infected Citrus in South Africa Using a DNA Probe and Monoclonal Antibodies

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ABSTRACT. Monoclonal antibodies directed against Indian, Chinese and South African greening originally developed in France, were used to detect the greening organism in South African citrus plants by indirect Enzyme-Linked Immunosorbent Assay and immunofluorescence. In addition, a Taiwanese DNA probe was used to evaluate cross reaction with South African greening by dot blot hybridization. Monoclonal antibody results were variable, but most positive reactions were obtained using the Indian monoclonal 2D12 (Poona strain) which cross-reacted with greening infected material obtained from hotter citrus growing areas in South Africa. No reaction was found between the Taiwanese DNA probe and South African greening.

Citrus greening is an economically important disease, not only in South Africa (17), but also internationally (2). Two forms of greening have been recognised viz. Asian and African greening (8). The former is more widespread and occurs at lower altitudes where ambient average temperatures vary between 30-35C (3), as compared to the more localized African greening which occurs at higher altitudes with average temperatures between 20-23C (16). Asian greening is transmitted by *Diaphorina citri* (Kuwayama) and African greening by *Trioza erytreae* (Del Guercio) (2). It is generally accepted that the two forms of greening are caused by different strains of the same pathogen (23). Currently, control in South Africa focuses mainly on reduction of inoculum by management practices such as vector control, pruning of infected branches, removal of infected trees and using healthy nursery material (4). Early detection of greening in the nursery or in newly planted orchards is therefore essential. Due to the non-specific nature of leaf symptoms, greening can often be confused with mineral deficiency, root rot or other stress-related leaf symptoms.

Hitherto, only unsuccessful attempts at isolating and culturing the greening organism (GO) on artificial media (5), have been made. However, numerous indirect molecular approaches to detect the GO *in situ* have been developed (8, 12, 13, 19, 22, 23). With these indirect approaches, greening-infected material was identified and used, either as antigen to raise monoclonal antibodies (mAbs) (8, 12, 13, 19), or to extract greening-specific DNA for development of DNA probes (20, 23). In 1987 two mAbs were produced in France against Indian greening (8) and later three against South African and five against Chinese greening (7).

This paper reports on the preliminary testing of South African greening material by Enzyme Linked Immunosorbent Assay (ELISA) and immunofluorescence (IF) using mAbs produced in France against Indian, Chinese and South African greening. In addition, a Taiwanese DNA probe was also tested with South African greening material collected at various citrus producing areas by dot blot hybridization.

MATERIALS AND METHODS

Evaluation of Indian greening monoclonal antibody 2D12 by means of ELISA. A total of 70 greening-infected citrus leaf and twig samples were collected from several citrus producing areas in South Africa. This included samples from the severely affected areas of Pretoria (central Transvaal) and Mooinooi (western Transvaal), and from a moderately affected area, Letaba (northern Transvaal) (14). Asymptomatic leaves were collected from Delta Valencia on rough lemon rootstock (DV/RL) maintained in the greenhouse, University of Pretoria which

were originally bud-inoculated with greening-infected material obtained from the Philippines (Table 1). Bark was stripped from twigs, midribs excised from leaves and samples cut into small pieces before 2 g of material per sample were powdered in liquid nitrogen. Ten ml sterile phosphate buffered saline, pH 7.0 (PBS) was added, mixed thoroughly on a vortex and incubated for 1 hr at 37C. The slurry was centrifuged for 1 min at 11 000 *g* at 4C in a Sorvall superspeed cooled centrifuge. Supernatants were collected and frozen at -20C until required. Extracts were also made from the bark of a symptomatic greening-infected branch collected at Mooinooi from at 5 cm and 10 cm below a fruit with symptoms, and from the fruit columella and the midrib from the leaves above the fruit. Samples were tested three times to verify results.

An indirect ELISA (21) was used to evaluate the efficacy of monoclonal 2D12 (Indian greening) obtained from M. Garnier and J. M. Bové (INRA, Bordeaux, France) with above mentioned extracts as solid phase antigens. Samples were considered positive if ELISA signals were greater than the sum of three times background and three times standard deviation with a corresponding coefficient of variation of less than 15%.

Evaluation of Asian and African greening monoclonal antibodies using immunofluorescence. Greening-infected citrus leaf samples were collected from the Pretoria, Mooinooi and Letaba areas, as well as Zebediela (central Transvaal), Nelspruit (moderately affected areas), and Malelane (eastern Transvaal; no natural spread area) (Table 2). Asymptomatic leaves were also collected from greenhouse maintained DV/RL trees inoculated with greening-infected material collected from Mooinooi, Nelspruit, Letaba, Zebediela and Pretoria. Healthy citrus leaves were also collected from DV/RL certified disease-free trees, maintained in the greenhouse, University of Pretoria. These trees were originally obtained from the South African citrus plant improvement program, Uitenhage. Symptomatic leaves with either yellow vein, mottling, green islands or secondary "Zn-deficiency-type" symptoms, were selected for sectioning (Table 2). Eight sections of 15-20 μm were cut with a Reichert-Jung cryomicrotome and mounted on a 8-well multitest slide (Sterilin). One well per slide was used as a negative control and was treated with Sp2/0 supernatant instead of monoclonal supernatant. Sections were treated with 70% methanol for 5 min,

TABLE 1
EVALUATION OF ANTIBODY 2D12 (INDIA) FOR CROSS-REACTION WITH SOUTH AFRICAN GREENING BY MEANS OF ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Area[a]	Cultivar	No. of samples tested	Symptoms[b]	Percentage positive[c]
Letaba (moderate area)	Valencia	25	Yv	16(4/25)
Mooinooi (severe area)	Valencia	28	Yv	18(5/28)
Pretoria (severe area)	Eureka	7	Yv	0(0/7)
Phillipines[d]	Valencia	0	As	60(6/10)

[a]Citrus producing areas in South Africa were categorised according to greening severity in the area (13).
[b]Greening symptoms were categorised according to: Yv = yellow vein; As = asymptomatic.
[c]Sample was designated positive if ELISA signal was greater than the sum of three times background and three times standard deviation with a corresponding coefficient of variation of less than 15%.
[d]Greening infected material from the Philippines was bud inoculated into Delta Valencia on rough lemon rootstocks and were maintained in the greenhouse, University of Pretoria.

and the methanol subsequently sucked off and replaced with each of the different monoclonal antibody supernatants, which included 2D12, 10A6, 1A5 and 2B6 (Indian strain), MG8, 10H8, 14A1 (South African strain) and 5H10 and 10F4 (Chinese strain) (obtained from M. Garnier and J. M. Bové). Slides were placed in a moist chamber and incubated for 40 min on a drying oven at 50C. Sections were washed once with 0.01% Tween 20 in PBS. Prepared slides were incubated at room temperature for 5 min. Sections were washed as described and treated with a 1:500 dilution fluorescein isothiocyanate conjugated goat-anti-

TABLE 2
EVALUATION OF GREENING-INFECTED AND HEALTHY SAMPLES OBTAINED FROM VARIOUS CITRUS PRODUCING AREAS IN SOUTH AFRICA FOR CROSS-REACTION WITH INDIAN, CHINESE AND SOUTH AFRICAN MONOCLONAL ANTIBODIES BY IMMUNOFLUORESCENCE

Area[a]	Cultivar	Monoclonal antibody		No. of samples tested	Symptoms[b]	Percentage positive
Zebediela	Valencia	India-	1A5	8	Yv	0
	Valencia		10A6	8	Yv	0
	Valencia		2D12	8	Yv	15
	Valencia	China-	10F4	8	Yv	0
	Valencia		5H10	16	Yv;m	42
	Valencia	SA-	14A1	8	Yv	0
	Valencia		10H8	8	Yv;m	48
	Valencia		MG8	8	Yv	75
Mooinooi	Valencia	India-	1A5	16	m	13
	Valencia		10A6	16	m	38
	Valencia		2D12	16	m	100
	Valencia	China-	10F4	8	m	0
	Valencia		5H10	16	m	13
	Valencia	SA-	14A1	16	m	0
	Valencia		10H8	24	m	21
	Valencia		MG8	8	m	0
Nelspruit	Navel	India-	2D12	24	Yv	0
	Navel		10A6	21	Yv	0
	Navel		1A5	16	Yv	0
	Navel	China-	5H10	16	Yv	0
	Navel		10F4	16	Yv	0
	Navel	SA-	MG8	16	Yv	0
	Navel		10H8	16	Yv	0
	Navel		14A1	16	Yv	0
Malelane	Valencia	India-	2D12	3	As	0
	Valencia		10A6	3	As	0
Pretoria	Eureka lemon	India-	2D12	8	m	40
	Eureka lemon		1A5	16	m	0
	Eureka lemon		10A6	16	m	0
	Eureka lemon	SA-	14A1	8	m	0
	Eureka lemon		MG8	8	m	15
	Eureka lemon	China-	5H10	16	m	0
	Eureka lemon		10F4	16	m	0
Cape[c]	Delta Valencia	India-	2D12	8	Healthy	0
	Delta Valencia		1A5	16	Healthy	0
	Delta Valencia		10A6	16	Healthy	0
	Delta Valencia	SA-	MG8	8	Healthy	0
	Delta Valencia		14A1	8	Healthy	0
	Delta Valencia		10H8	16	Healthy	0
	Delta Valencia	China-	5H10	16	Healthy	0
	Delta Valencia		10F4	16	Healthy	0

TABLE 2 (CONTINUED)

Area[a]	Cultivar	Monoclonal antibody	No. of samples tested	Symptoms[b]	Percentage positive
Greenhouse[d]	Eureka lemon	India- 1A5	16	As	0
	Eureka lemon	10A6	16	As	0
	Eureka lemon	2D12	8	As	0
	Eureka lemon	SA- MG8	8	As	15
	Eureka lemon	14A1	8	As	0
	Eureka lemon	10H8	16	As	0
	Eureka lemon	China- 5H10	16	As	0
	Eureka lemon	10F4	16	As	0

[a]Citrus producing areas in South Africa were categorised according to general greening severity in the area (13).
[b]Greening symptoms were categorised according to: Yv = yellow vein; As = asymptomatic; m = mottling.
[c]Cape trees maintained in the greenhouse, University of Pretoria are from the South African plant improvement program, Uitenhage.
[d]Greenhouse samples maintained at the University of Pretoria were bud inoculated on Delta Valencia on rough lemon rootstock with greening material from Mooinooi, Nelspruit, Letaba, Zebediela and Pretoria.

mouse immunoglobulins (Dakopatts, Denmark) and Evans blue (BDH, Poole, England) in PBS. After incubation in darkness for 30 min, sections were washed twice with Tween/PBS and mounted with mounting solution (0.7% $NaHCO_3$ and 0.16% Na_2CO_3 in 10 ml PBS and 90 ml glycerol). Slide preparations were observed using a Zeiss epifluorescence microscope with filter combination BP450 - 490 / FT 510 / LP 520. Five microscopic fields under a 100 x magnification were viewed per section and presence or absence of fluorescent cells noted.

Evaluation of a nucleic acid probe prepared in Taiwan against Asian greening for detection of greening in South Africa. A DNA probe developed by Su, *et al.* (19, 20) in Taiwan, was tested using a total of 80 symptomatic greening samples collected from Pretoria, Mooinooi, Letaba, Zebediela, Nelspruit and Malelane (Table 3). The disease-free and greening-inoculated greenhouse material previously mentioned was also included.

The DNA extraction protocol (20) includes the following basic steps. Air dried bark strips or midveins were powdered in liquid nitrogen. Two volumes of extraction buffer (0.1 M Tris-HCl, 0.1 M EDTA, 0.25 M NaCl) were added, the sample stirred and debris removed by gauze filtering and slow speed centrifugation (10 000 *g*, 10 min at 4C). After high speed centrifugation (16 000 *g*, 10 min at 4C) the pellet was treated with 1 % n-lauroyl-sarcosine (in extraction buffer) for 2 hr at 55C followed by centrifugation (12 500 *g*; 10 min at 4C). After isopropanol nucleic acid precipitation and high speed centrifugation, proteins were denatured in 20% SDS and Proteinase K (5 mg/ml buffer) for 1 hr at 37C. Polysaccharides were subsequently adsorbed with CTAB/NaCl (10% hexadecyltrimethylammonium bromide in 0.7 M NaCl) at 65C for 10 min. Samples were clarified by adding equal volumes of chloroform/iso-amyl alcohol (24:1) followed by centrifugation (12 500 *g*; 5 min at 4C). Phenol/chloroform/isoamyl alcohol (25:24:1) was added in equal volumes to supernatant before centrifugation (12 500 *g*; 5 min at 4C). DNA was precipitated with isopropanol at -20C for 20 min, centrifuged (16 000 *g*; 15 min at 4C) and pellets washed with 70% ethanol to remove residual CTAB. After drying, pellets were resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored at -20C. Three microliters of each sample was spotted onto nitrocellulose membranes (Amersham, Hybond-C Extra 0.45 μm)

TABLE 3
EVALUATION OF GREENING-INFECTED AND HEALTHY SAMPLES OBTAINED FROM VARIOUS CITRUS PRODUCING AREAS IN SOUTH AFRICA WERE TESTED WITH A DNA PROBE DEVELOPED AGAINST TAIWANESE GREENING USING THE DOT BLOT HYBRIDIZATION TECHNIQUE

Area	Cultivars	No. of samples	Symptoms[a]	Dot blot hybridisation
Pretoria:				
field	Delta Valencia	4	Yv	-
field	Eureka lemon	2	Yv	-
greenhouse[c]	Eureka lemon	16	As	-
Cape[d]	Delta Valencia	10	Healthy	-
Mooinooi	Valencia	6	Yv	-
	Navel	3	As	-
	Mandarin	6	As	-
Zebediela	Valencia	3	Yv;Zn-type	-
Letaba	Valencia	3	Yv;m	-
Nelspruit area:				
Crocodile Valley	Navel	5	As;Yv	-
Friedenheim	Eureka lemon	13	Yv;m;Zn-type	-
Haffenden	Valencia	19	Yv;m;Zn-type	-
Halls & Sons	Valencia	6	Yv;m	-
Karino	Valencia	6	Yv;m;Zn-type	-
Malelane	Valencia	3	Only on fruit	-
Positive control[d]	Valencia	10	Likubin	+
Negative control[e]	Valencia	10	Healthy	-

[a]Greening symptoms were categorised according to: Yv = yellow vein; m = mottling; As = asymptomatic and Zn-type = secondary Zn-type deficiency symptoms.
[b]Greenhouse samples maintained at the University of Pretoria were bud inoculated on Delta Valencia on rough lemon rootstock with greening material from Mooinooi, Nelspruit, Letaba, Zebediela and Pretoria.
[c]Cape trees maintained in the greenhouse, University of Pretoria are from the South African plant improvement program, Uitenhage.
[d]DNA extract from likubin greening.
[e]DNA extract from healthy Taiwanese citrus material.

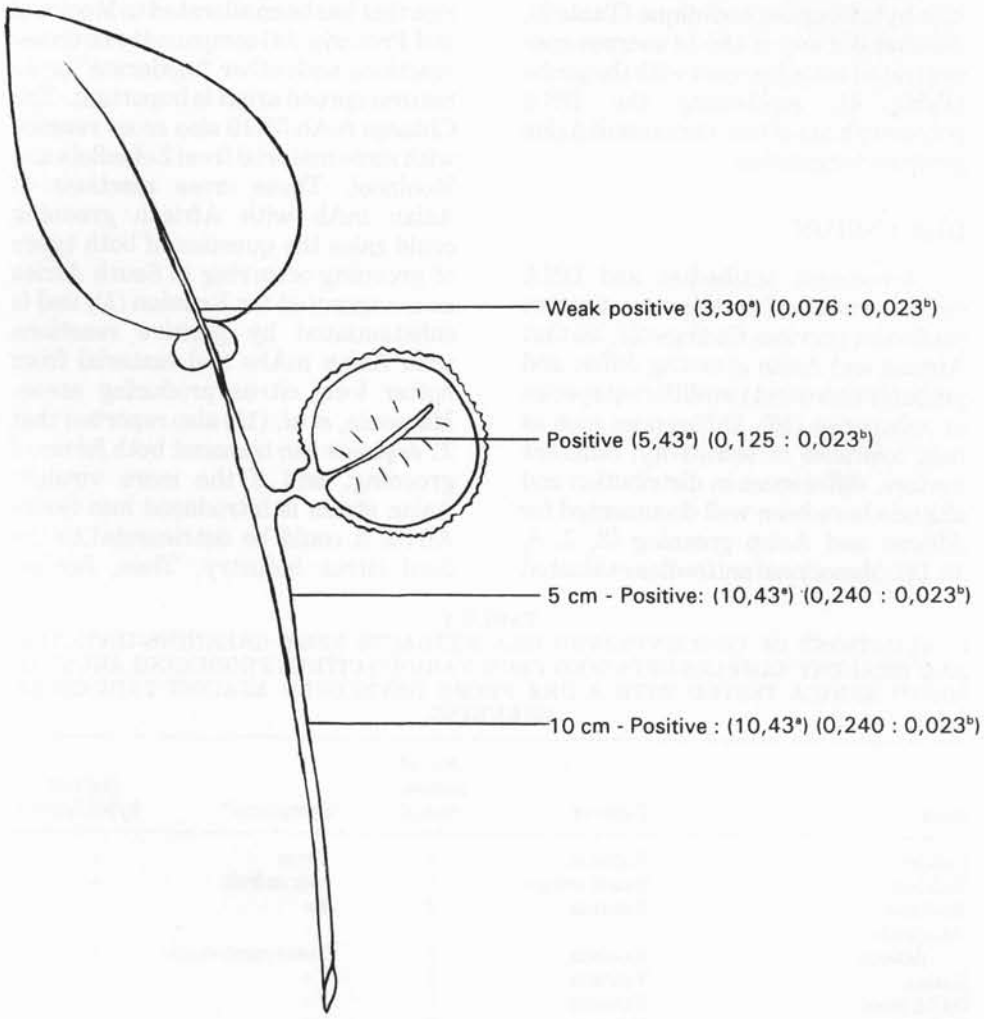
after equal volumes of 6 X SSC (20 X SSC stock solution: 175.3 g NaCl, 88.2 g Na citrate, pH 7.0) was added and the aliquots were denatured. On each membrane a positive Asian greening DNA extract and negative healthy citrus DNA extract were included. Hybridization was carried out with a biotinylated DNA probe and coloring (nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate in diethanolamine buffer) was achieved with the Blue Gene system (20). Due to the lower titre of the greening organism in phloem and uneven distribution in the tree (20), 14 samples from Letaba, Malelane, Mooinooi and Nelspruit were concentrated by including a 40% sucrose gradient centrifugation step prior to sarcosine treatment.

RESULTS

Evaluation of Indian greening monoclonal antibody 2D12 by means of ELISA. Sixteen percent of the 25 samples from Letaba, 18% from Mooinooi and 60% from the Philippines samples tested positive with the Indian greening mAb (2D12) (Table 1). The other area, viz. Pretoria, tested negative (Table 1). Material from Mooinooi was re-tested three times in order to verify these findings, giving similar results every time. Greening-infected branches from Mooinooi tested positive

10 cm down from symptomatic fruit but tested only borderline positive higher up in the branch above the fruit. Columella as such tested positive (Fig. 1). An increased signal was obtained lower down the branch (Fig.1). These results were confirmed in three repeats.

Evaluation of Asian and African greening monoclonal antibodies using immunofluorescence. Of the total of 635 sections viewed, greening-infected material from Zebediela was most effectively detected with clone MG8 (SA), followed by clone 10H8 (SA) and 5H10 (China) and least effectively with clone 2D12 (India), and 10A6 (India). Clone 10F4 (China) could not detect greening in Zebediela material (Table 2). Material from Mooinooi which tested positive with the ELISA consistently tested positive with IF using the same 2D12 mAb (Table 2). Clones 10H6 (India), 10H8 (SA), and to a lesser extent 1A5 (India) and 5H10 (China), reacted with Mooinooi greening. No reaction was obtained with clones 14A1



[a]Values expressed as signal:background ratios.
[b]Actual signal and background values.

Fig. 1. Greening symptomatic branch obtained from Mooinooi, South Africa tested at various intervals with monoclonal antibody 2D12 (Indian) using the Enzyme-Linked Immunosorbent Assay (ELISA).

(SA), MG8 (SA) and 10F4 (China). No reaction was found with Nelspruit, Malelane or Cape material. Monoclonal 2D12 (India) also reacted with material from Pretoria and greenhouse material, while clone MG8 (SA) reacted with Pretoria greening.

Evaluation of a nucleic acid probe prepared in Taiwan against Asian greening for detection of greening in South Africa. None of the 95 symptomatic greening nor the ten healthy samples reacted positively with the Taiwanese probe when tested with the dot blot hybridization technique (Table 3). Neither did any of the 14 sucrose-concentrated samples react with the probe (Table 4), confirming the DNA polymorphism of the African and Asian greening organisms.

DISCUSSION

Monoclonal antibodies and DNA probes evaluated in this investigation confirmed previous findings (22, 23) that African and Asian greening differ, and probably represent two different species or subspecies (10). Differences such as heat tolerance or sensitivity, different vectors, differences in distribution and altitude have been well documented for African and Asian greening (2, 3, 8, 16,18). Monoclonal antibodies evaluated in this study highlighted the strain specificity previously reported (23), rendering them ineffective for diagnostic purposes (22). Interesting results with IF were observed with Indian greening mAb 2D12, which consistently reacted with greening from Mooinooi, reacted moderately with greening from Pretoria and less so with greening from Zebediela. No cross-reaction was found with healthy material or material from Malelane or Nelspruit. Asian greening mAbs recognised the "more severe" greening category in South Africa that has been allocated to Mooinooi and Pretoria (14) compared to no cross-reactions with other "moderate" or no natural spread areas is important. The Chinese mAb 5H10 also cross reacted with some material from Zebediela and Mooinooi. These cross reactions of Asian mAb with African greening could raise the question of both types of greening occurring in South Africa as is suggested for Reunion (1), and is substantiated by positive reactions with Asian mAbs and material from hotter local citrus producing areas. Massonie, *et al.* (15) also reported that *T. erytreae* can transmit both forms of greening, and if the more virulent Asian strain is introduced into South Africa it could be detrimental to the local citrus industry. Thus, further

TABLE 4
EVALUATIONS OF CONCENTRATED DNA EXTRACTS FROM GREENING-INFECTED AND HEALTHY SAMPLES OBTAINED FROM VARIOUS CITRUS PRODUCING AREAS IN SOUTH AFRICA TESTED WITH A DNA PROBE DEVELOPED AGAINST TAIWANESE GREENING

Area	Cultivar	No. of samples tested	Symptoms[a]	Dot blot hybridization
Letaba	Valencia	2	Yv;m	-
Malelane	Sweet orange	1	only on fruit	-
Mooinooi	Valencia	2	Yv	-
Nelspruit:				
-flowers	Valencia	1	Tree symptomatic	-
Karino	Valencia	1	Yv	-
Hall & Sons	Valencia	1	Yv	-
Haffenden	Valencia	3	Yv	-
Friedenheim	Eureka lemon	3	Yv, m	-
DNA extract from likubin greening		2	Likubin	+
DNA extract from healthy Taiwanese citrus material		2	Healthy	-

[a]Greening symptoms were categorised according to: Yv = yellow vein, m = mottling.

monitoring is of importance to elucidate the presence of Asian greening in South Africa.

The ELISA used in this investigation with the Indian mAb 2D12 confirmed the Mooinooi results obtained with the IF but not the Pretoria greening results. Similarly, Hsu *et al.* (11) reported that five mAb tested with IF reacted with greening-infected plants in China but only clones 5H10 and 10F4 reacted positively in the ELISA. Plant background antigens could interfere with the ELISA signal and thereby influence the results. According to M. Garnier (pers. comm.), IF is the preferred detection system since it is more sensitive, accurate and easier to apply than ELISA. High percentage positive reactions of Indian mAb 2D12 with asymptomatic Philippines greening material maintained in our greenhouse were observed especially when compared to the "no cross-reaction" report of Garnier, *et al.* (9). Monoclonal antibody results found in this investigation confirm the presence of serotypes of the greening organism (23). Downward movement of the organism was found in this investigation, which confirms a report that the disease can spread down from the site of infection (S. P. Van Vuuren, pers. comm.).

DNA probes developed against likubin in Taiwan did not cross-hybridize with any greening symptomatic material collected from different citrus producing areas in South Africa, while it cross-hybridized with greening positive controls from Taiwan. Concentration of DNA by means of sucrose-density gradient centrifugation before DNA purification and hybridization, did not result in any cross-hybridization. This may suggest a DNA polymorphism between Asian and African greening. Such a homology difference has indeed been demonstrated when DNA probes ln 2.6 and ln 1.9 cross hybridized with Asian greening from India, Thailand, Philippines, Indonesia, China and Taiwan, but not from South Africa (21). At intermediate stringencies In 2.6 was able to detect South African greening (24). It is generally accepted that the highest possible stringency conditions should be used in order to minimize the hybridization of the probe to related but non identical sequences (25). Since the Asian and African greening are related, but with different DNA homologies, it would be interesting to see if the Taiwanese DNA probe will cross-hybridize with South African greening under lower stringency conditions, similar to that described for In 2.6.

From this investigation it seems that some African strains have epitopes with Asian strains and has probably no DNA homology with the Taiwan probe. Monoclonal antibodies are currently believed to be less promising for field diagnostics due to irregular epitope expression (20) and high strain specificities (7, 22) even when used in mixtures (9). However, we feel that mAb can be a useful "tool" in etiological studies such as detecting spread of the greening organism in situ, and therefore has a place in future greening research alongside the more effective, wider specificity range DNA probes (23).

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