PCR detection of the two 'Candidatus' liberobacter species associated with greening disease of citrus

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Greening is a severe and widespread disease of citrus in two main citrus growing areas of the world, Asia and Africa. It is caused by an uncultured phloem restricted bacterium that we have recently characterized from the sequence of its 16S ribosomal DNA. The bacterium is a new 'Candidatus' genus, Liberobacter, in the alpha subdivision of the proteobacteria, and two 'Candidatus species' have been recognized: *Liberobacter asiaticum* and *Liberobacter africanum*. In this paper we describe a PCR method to detect the two liberobacter species in citrus trees by amplification of a 1160 bp fragment of their 16S rDNA. Distinction between the two species has also been achieved by *Xba*I digestion of the amplicons.   © 1996 Academic Press Limited

INTRODUCTION

Greening is a severe and widespread disease of citrus in Eastern Asia (Pakistan to China), South and Eastern Africa, the Arabic Peninsula and the islands of Reunion, Mauritius and Madagascar in the Indian Ocean (for reviews see Refs 1–3). The disease is a threat for other citrus growing areas (Americas and Mediterranean countries) as the insect vectors of the disease, the psyllids *Diaphorina citri* and *Trioza erytreae*, are present respectively in South America and in Madeira Island.

Greening is caused by an uncultured phloem-restricted bacterium that we have recently characterized.[4] The 'Candidatus' generic name liberobacter, as defined by Murray and Schleifer for uncultured organisms,[5] has been given to the bacterium.[4] We have also shown that liberobacters from Asia and Africa belong to two different 'Candidatus' species,[4,6,7] *Liberobacter asiaticum* and *Liberobacter africanum*.

Two DNA probes, In–2.6 and AS–1.7 containing genes for ribosomal proteins (as part of the well known, so-called β operon) have been produced respectively for *L. asiaticum* and *L. africanum*.[7,8] Used in dot-blot hybridization assays, they allow detection of the respective greening liberobacter species in citrus leaf samples collected in infected orchards.[9] The sensitivity of the hybridization assay is similar to that of electron microscopy, the only detection method that was previously available.[9] The necessity of using two different probes for the detection of the two liberobacter species (which occur concomitantly in areas such as the Arabic Peninsula, Reunion and Mauritius), and the fact that DNA extraction for dot-blot hybridization is time consuming, prompted us to look for additional procedures. PCR,[10] known to be simple, quick and sensitive has been envisaged. Indeed, for characterization purposes we have previously cloned and sequenced the 16S rDNA of both an Asian and an African liberobacter strain. From sequence comparisons, primers suitable for amplification of liberobacter ribosomal DNA were defined. In this paper we have investigated the specificity of the primers and defined the conditions required to amplify liberobacter rDNA from citrus extracts. Upon

amplification, characterization of the liberobacter species has been achieved by restriction enzyme digestion of the amplified DNA.

MATERIALS AND METHODS

Plant material

Periwinkle (*Catharantus roseus*) and sweet orange (*Citrus sinensis*) plants infected with different greening liberobacter strains from Asia and Africa were obtained as described previously.[8] The plants were maintained in a greenhouse at 30°C during the day and 25°C at night for Asian strains and 25°C during the day and 20°C at night for African strains. Healthy periwinkle and citrus plants were obtained from seeds and grown at 25/20°C.

Purification of DNA from plants

We have used the CTAB (cetyl trimethyl ammonium bromide) method of Murray and Thompson[11] as previously described.[8]

Preparation of citrus leaf extracts for PCR

Protocol 1

Leaf midribs (0·5 g) were chopped to a fine mince with a razor blade within a disposable Petri dish containing 1 ml of 0·3 M NaCl. The liquid phase was collected with a syringe and centrifuged at 100 *g*. PCR was performed either directly on the supernatant diluted 10- or 100-fold, or on the pellet resulting from centrifugation of the supernatant for 5 min at 16 000 *g* and resuspended in 100 µl of sterile water.

Protocol 2

Leaf midribs (0·1 to 0·3 g) were chopped to a fine mince with a razor blade in a disposable Petri dish containing 1 ml of TE buffer (10 mM Tris pH 8·0, 400 mM EDTA, 1%SDS) to which 0·25 mg of proteinase K was added. The homogenate was transferred to an Eppendorf tube and incubated for 2 h at 65°C. The suspension was centrifuged for 15 min at 12 000 *g* and the supernatant mixed with 1 ml of Wizard miniprep DNA purification resin (Promega). The resin was transferred to a minicolumn and washed twice with 2 ml of 80% isopropanol. Next, 50 µl of hot water (80°C) were added, and after 1 min, the column was

centrifuged for 30 s at 16 000 *g* in an Eppendorf tube. This step was repeated, yielding 100 µl of extract (wizard extract). Two to 10 µl of wizard extract were used for PCR.

PCR

Universal primers fD1 and rP1[12] were used for general amplification of procaryotic 16S rDNA. Primers OI2c and OI1, defined from the 16S rDNA sequence of *L. asiaticum* (strain Poona, India)[4] and primer OA1 defined from the 16S rDNA sequence of *L. africanum* (strain Nelspruit, South Africa)[7] and identical to OI1 except for three base changes, were evaluated for specific amplification of liberobacter 16S rDNA. The PCR reaction was performed in 50 µl of reaction mixture containing 0·5 µM of each of the primers, 200 µM of each of the four dNTP, 78 mM Tris-HCl pH 8·8, 2 mM $MgCl_2$, 17 mM $(NH_4)_2SO_4$, 10 mM β mercaptoethanol, 0·05% W1 detergent (Gibco BRL), 200 µg ml^{-1} BSA, and 2·5 U of *Taq* polymerase (Gibco BRL). A thermocycler (Perkin Elmer Cetus) with the following programs was used for DNA amplification: 35 cycles each at 92°C for 30 s, 54°C for 30 s, and 72°C for 60 s when fD1/rP1 primers were used; 35 cycles each at 92°C for 40 s (denaturation step) and 72°C for 90 s (annealing of primers and elongation) with primers OI2c/OI1, OI2c/OA1 or OI2c/OI1/OA1. Following amplification, 8 µl aliquots of each reaction mixture were analysed by electrophoresis on 0·7% agarose gels.

Restriction enzyme analysis of amplified DNA

Eight microlitres of amplified DNA were digested with 20 U of *Xba*1 restriction enzyme (Eurogentec) in a final volume of 35 µl (according to the manufacturer) at 37°C overnight. The digested DNA was analysed by electrophoresis on 4% agarose gels.

Collection of field samples

Leaves were collected from trees with or without symptoms of greening in various orchards in Vietnam and Mauritius. The leaves were kept in plastic bags in cool boxes, then in the refrigerator until they were processed or sent by express mail to our laboratory in Bordeaux (48 to 72 h delivery time). Some of the samples from Vietnam were kept for up to 10 days before they were sent to France. Upon arrival, midribs were excised; batches of 2 g of midribs were frozen at −80°C for further DNA extraction and dot–blot
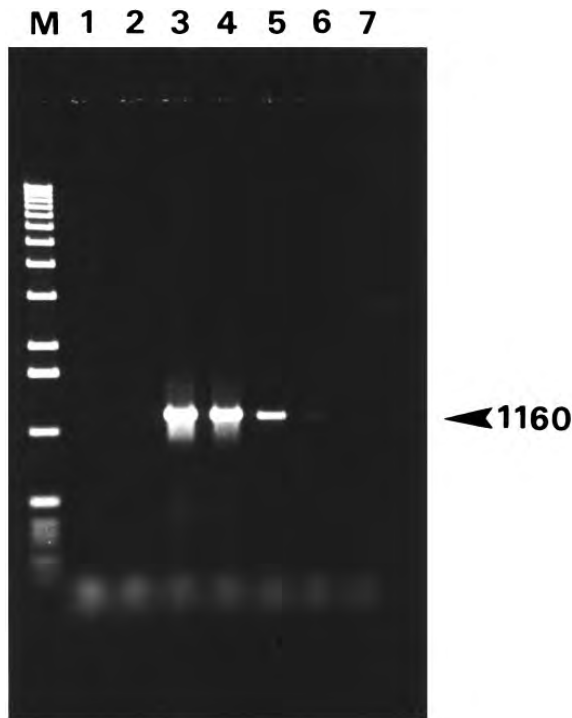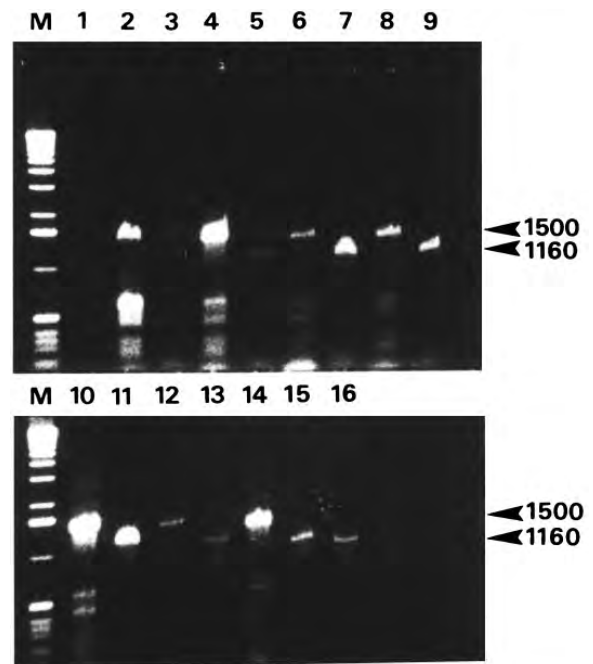
Fig. 1. Electrophoresis on 0·7% agarose gel of DNA amplified with primers OI2c/OI1 from water (lane 1) or DNA extracted from healthy (lane 2) or *Liberobacter asiaticum* infected sweet orange seedlings (lanes 3 to 7). The amount of DNA used as template is 20 ng in lanes 2 and 3; 10 ng in lane 4; 1 ng in lane 5; 0·1 ng in lane 6; 0·01 ng in lane 7. M, 1 kb ladder (Gibco, BRL).

Fig. 2. Electrophoresis on 0·7% agarose gel of DNA amplified with 16S rDNA universal primers (lanes 1, 2, 4, 6, 8, 10, 12 and 14) or liberobacter 16S rDNA specific primers OI2c/OI1 (lanes 3, 5, 7, 9, 11, 13, 15 and 16) from water (lane 1), plant extracts following protocol 1 from healthy sweet orange seedlings (lanes 2, 3) or from sweet orange seedlings infected with the following liberobacter strains: South Africa (lanes 4, 5), China (lanes 6, 7), India (lanes 8, 9), Indonesia (lanes 10, 11), The Philippines (lanes 12, 13), Taïwan (lanes 14, 15) and Thailand (lane 16). M, 1 kb ladder (Gibco, BRL).

hybridization; wizard extracts were prepared from 0·1 to 0·3 g of midribs for PCR analysis.

RESULTS

PCR amplification of liberobacter 16S rDNA with purified DNA from citrus plants

We had shown previously that primers OI2c and OI1 were able to amplify a 1160 bp fragment of the greening liberobacter 16S rDNA from purified periwinkle DNA whether an African or an Asian strain was present in the plant.[4] Figure 1 shows that amplification of a 1160 bp fragment also occurred when DNA was purified from a sweet orange seedling infected with *L. asiaticum* (lanes 3 to 7) and that a positive amplification was still visible when the reaction mixture contained as little as 0·1 ng of purified DNA (lane 6). No amplification was observed when water (lane 1) or DNA extracted from a healthy sweet orange seedling (lane 2) was used in the reaction mixture.

Evaluation of PCR on plant extracts

Protocol 1

When amplification was performed on the low speed supernatant diluted 10 times or on the resuspended pellet obtained after high speed centrifugation, no amplified DNA could be seen on the gel whether universal (rp1/fD1) or liberobacter specific primers, OI2c/OI1 were used (results not shown). When universal (Fig. 2, lanes 1, 2, 4, 6, 8, 10, 12 and 14) or specific primers (Fig. 2, lanes 3, 5, 7, 9, 11, 13, 15 and 16) were used for amplification with the 100 fold diluted supernatant, DNA bands of respectively 1500 bp and 1160 bp were observed with infected sweet orange seedlings containing liberobacter strains from South Africa (lanes 4, 5), China (lanes 6, 7), India (lanes 8, 9), Indonesia (lanes 10, 11), the Philippines (lanes 12, 13), Taïwan (lanes 14, 15) and Thailand (lane 16, specific primers only). With extracts from healthy sweet orange seedlings, amplification of 16S
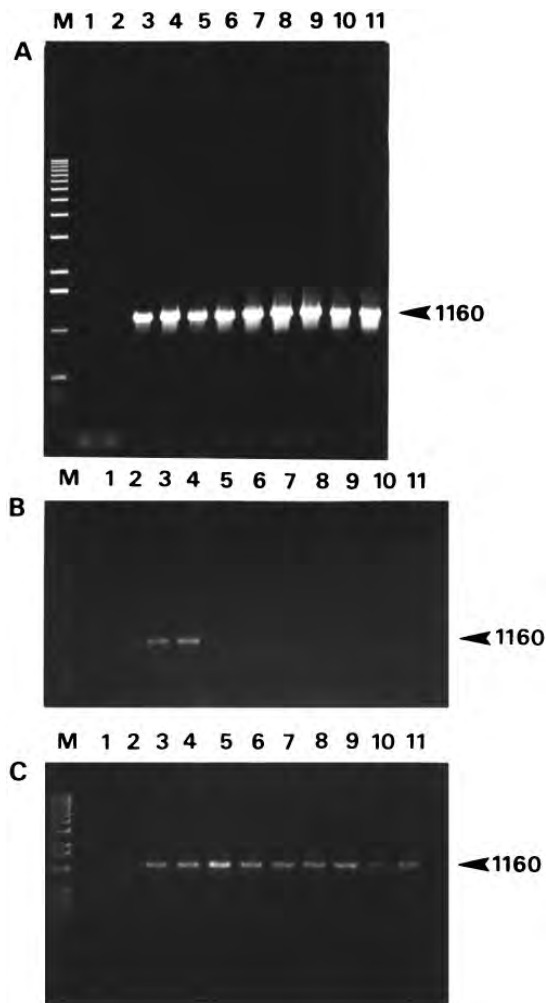
Fig. 3. Electrophoresis on 0·7% agarose gel of DNA amplified with primers OI2c/OI1 (A); OI2c/OA1 (B) and OI2c/OI1/OA1 from water (lane 1) or wizard extracts from healthy sweet orange seedlings (lane 2) or sweet orange seedlings infected with *L. africanum* from South Africa (lane 3), Zimbabwe (lane 4), or *L. asiaticum* from China (lane 5), India (lane 6), Indonesia (lane 7), Nepal (lane 8), Philippines (lane 9), Taïwan (lane 10) and Thailand (lane 11). M, 1 kb ladder (Gibco, BRL).

rDNA occurred with universal primers (lane 2), but not with liberobacter-specific primers (lane 3).

However, when different extracts from the same plants were used, results were not consistant, as false negatives were obtained. Higher dilutions of the supernatant did not improve the results. Therefore, second protocol in which a quick partial DNA purification was performed, has been evaluated (protocol 2, material and methods).

Protocol 2

Figure 3 shows the results of PCR amplification with liberobacter specific primers OI2c/OI1 (Fig. 3A),

OI2c/OIA (Fig. 3B) and OI2c/OI1/OIA (Fig. 3C) with 2 µl of water (lane 1), healthy sweet orange extract (lane 2), leaf extracts from sweet orange seedlings infected with liberobacters from South Africa (lane 3), Zimbabwe (lane 4), China (lane 5), India (lane 6), Indonesia (lane 7), Nepal (lane 8), Philippines (lane 9), Taïwan (lane 10) and Thailand (lane 11). No amplification occurred with water or extracts from healthy sweet orange midribs. Characteristic 1160 bp bands were observed only with extracts from infected sweet orange leaves regardless of the liberobacter strains present when primers OI2c/OI1 (Fig. 3A) or OI2c/OI1/OA1 (Fig. 3C) were used. On the contrary, when PCR was carried out with primers OI2c/O1A (Fig. 3B), the 1160 bp band was obtained only with the African strains, the Asian strains being poorly or not amplified. This procedure could be performed repeatedly on independent extracts from the same plants, and has always given consistant results. In addition, the amplified DNA bands were more intense than in the first protocol (Fig. 3 compared with Fig. 2).

### Conditions for plant sample collection and preservation

Amplification was obtained equally well with fresh leaf midribs or when they were dried on the bench at room temperature (20 to 25°C), over silicagel for several days, or when the whole leaves were kept for up to 3 weeks in plastic bags in the refrigerator, provided that no fermentation occurred. Freezing the midribs for up to 3 weeks seemed to reduce the sensitivity of PCR. Extracts from decayed or fermented leaves could not be amplified either with universal primers for 16S rDNA amplification or with liberobacter specific primers.

Positive wizard extracts still have a positive amplification when they were tested after up to 6 months at 4°C in the refrigerator or in the freezer at −20°C.

The experiments described above were performed with 0·1 to 0·3 g of leaf midribs, this amount can be raised up to 1 g provided that the volume of the 'chopping' buffer is adjusted in the proportion of 2 ml for 1 g.

Incubation time of plant extract with proteinase K and amount of wizard extract used in PCR

PCR amplification with primers OI2c/OI1 was tested on various amounts of wizard extracts (2, 5, 10 or 25 µl) prepared from healthy or infected citrus midribs
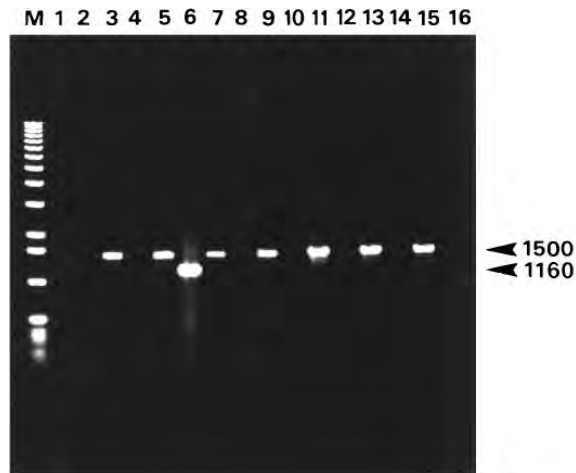
Fig. 4. Electrophoresis on 0·7% agarose gel of DNA amplified with rDNA universal primers (lanes 1, 3, 5, 7, 9, 11, 13 and 15) or liberobacter 16S rDNA specific primers OI2c/OI1 (lanes 2, 4, 6, 8, 10, 12, 14 and 16) with water (lanes 1, 2), or wizard extracts from healthy (lane 3, 4) *L. asiaticum* (lanes 5, 6) or tristeza virus (lanes 7, 8) infected sweet orange seedlings or DNA from *Spiroplasma citri* (lanes 9, 10) *Xanthomonas campestris* pv citri (lanes 11, 12), *Acinetobacter lwoffii* (lanes 13, 14) and *Agrobacterium tumefaciens* (lanes 15, 16). M, 1 kb ladder (Gibco, BRL).

incubated with proteinase K during 30 min, 1, 2, 4 or 12 h. A positive amplification was obtained after a minimum of 2 h of incubation of the homogenate with proteinase K. Incubation could be conveniently carried out overnight. Under these conditions, amplification was obtained with 2 to 10 µl of wizard extracts, but larger quantities of extract (25 µl) had an inhibitory effect on the PCR reaction.

Specificity and sensitivity of PCR

We have shown previously that primers OI2c and OI1 were specific for greening liberobacter 16S rDNA amplification as all liberobacters strains could be amplified and no amplification was obtained with *E. coli*, *Xylella fastidiosa* and the phytoplasmas of witche′s broom disease of lime and of tomato stolbur.[4] We have further investigated the specificity of the primers for the liberobacters on other citrus pathogens such as *Spiroplasma citri*, the mollicute responsible for citrus stubborn disease, *Xanthomonas campestris* the bacterial agent of citrus canker, bacteria that have been recovered from citrus (*Acinetobacter lwoffii*)[13] and the plant pathogenic bacterium, *Agrobacterium tumefaciens*, belonging to the same phylogenetic cluster than liberobacter. We have also tested amplification on DNA extracts from tristeza virus infected citrus leaves as this virus (RNA virus) is endemic in Asia and Africa, and coinfections with greening and tristeza are frequent. As shown in Fig. 4 lanes 7 to

16, amplification with DNA extracted from these organisms or infected plants was obtained only with universal primers for procaryotic 16S rDNA (lanes 7, 9, 11, 13, 15), but not with liberobacter specific primers (lanes 8, 10, 12, 14, 16). The DNA amplified with universal primers in the case of tristeza virus-infected plant DNA (lane 7) is chloroplastic 16S rDNA.[4] Lanes 5 and 6 show the amplifications obtained with DNA extracted from citrus infected with *L. asiaticum* (Poona, India strain), respectively with universal and specific primers. Controls in which water replaced DNA gave no amplification (lanes 1, 2) and DNA extracted from healthy citrus plants could, as expected, be amplified with universal primers (lane 3) and not with liberobacter specific primers (lane 4).

As the greening bacterium cannot be cultured, it is difficult to determine the sensitivity of PCR. In order to evaluate the detection limit of the method, known amounts of infected midribs (from 10 mg to 0·5 g) were mixed with increasing amounts of healthy midribs (0 to 1 g) and treated according to protocol 2. Amplification was still obtained when 20 mg, but not when lesser amounts of infected midribs were mixed with 1 g of healthy midribs.

Characterization of the amplified DNA

The sequences of the liberobacter 16S rDNAs indicate that restriction enzyme *Xba*I should hydrolyse the 16S rDNA of *L. africanum* into three fragments (520 bp, 506 bp and 130 bp) and that of *L. asiaticum* into two fragments (640 bp and 520 bp). When amplified DNAs from *L. africanum* strain Nelspruit (South Africa) (Fig. 5, lane 1), strain Letaba (South Africa) (lane 2) or strain Harare (Zimbabwe) (lane 3), and *L. asiaticum* from China (lane 4), India (lane 5), Indonesia (line 6), Philippines (lane 7), Taïwan (lane 8) and Thailand (lane 9) were submitted to *Xba*I digestion and analysed on 4% agarose gels, bands of the expected number and size were obtained and the two species could be easily identified from their restriction profiles.

Evaluation of PCR on field samples collected in Vietnam and Mauritius

PCR was evaluated in comparison with DNA–DNA hybridization on 54 field samples showing greening symptoms collected in various orchards in North, Central and South Vietnam in January–February 1995, and on 32 samples showing greening symptoms collected in Mauritius in April 1995. Hybridizations were
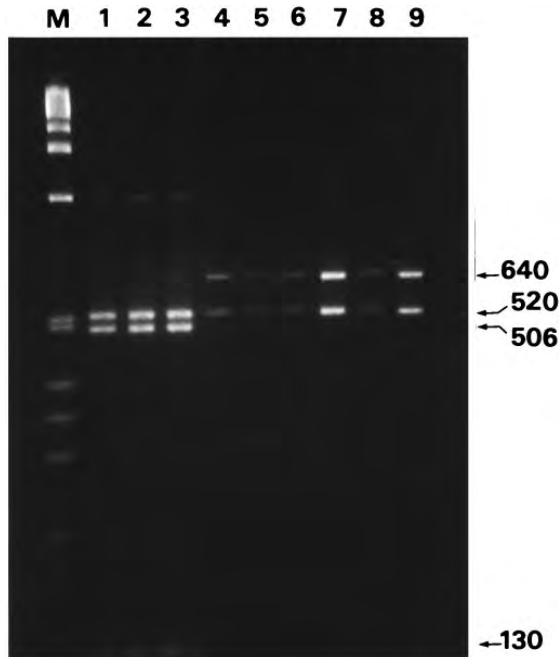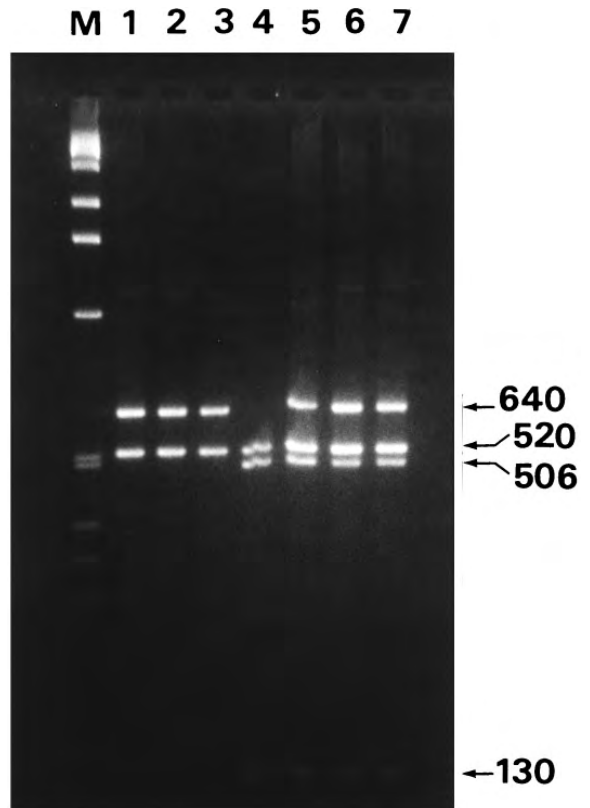
Fig. 5. Electrophoresis on 4% agarose gel of *Xba*I digested DNA amplified from *L. africanum*, strain Nelspruit (South Africa) (lane 1) strain Letaba (South Africa) (lane 2) and strain Harare (Zimbabwe) (lane 3) or *L. asiaticum* from China (lane 4), India (lane 5), Nepal (lane 6), Philippines (lane 7), Taïwan (lane 8) and Thailand (lane 9). M, 1 kb ladder (Gibco, BRL),

Fig. 6. Electrophoresis on 4% agarose gel of *Xba*I digested DNA amplified from field samples collected in Mauritius. *L. asiaticum* profile of three samples which gave positive hybridization with probe In–2.6 (lanes 1, 2 and 3); *L. africanum* profile of a sample which gave positive hybridization with probe AS–1.7 (lane 4); Combination of *L. asiaticum* and *L. africanum* profiles for the three samples which gave a positive hybridization with the two probes (lanes 5, 6 and 7). M, 1 kb ladder (Gibco, BRL).

carried out with In-2.6 and AS-1.7 specific for *L. asiaticum* and *L. africanum*, respectively.

Among the 54 samples collected inVietnam, 8 were decayed on arrival in Bordeaux; they yielded positive hybridization signals with In-2.6 but not with AS-1.7 and no PCR bands were obtained whether universal or liberobacter specific primers were used, indicating that the DNA could not be amplified even though it was still acceptable for hybridization. The remaining 46 samples gave positive PCRs but 4 of them were hybridization negative. Electron microscopy was performed on two of these four samples, and was able to confirm the presence of the greening bacterium in the samples. *Xba*I digestion of the DNA amplified from the 46 PCR positive samples gave, as expected, upon agarose gel electrophoresis, the profile corresponding to Liberobacter asiaticum.

The 32 samples from Mauritius were also tested using the two DNA probes, and PCR was evaluated with the three different mixture of primers as we have found previoulsy that in this island both *L. asiaticum* and *L. africanum* are present (unpublished). Twenty seven samples were positive with the two techniques, five were positive by PCR and negative by hybridization. Among the 27 samples positive by hybridization, 23 hybridized with the probe for *L.*

asiaticum, one with that for *L. africanum*, and three with the two probes. The samples giving positive hybridization with the probe for *L. asiaticum* (In-2.6) gave positive PCR with primers OI2c/OI1, and primers OI2c/OI1/OA1 but not with primers OI2c/OA1. After digestion of the amplified DNA with *Xba*I, a profile characteristic of Asian greening liberobacter was obtained as illustrated on Fig. 6, lanes 1–3, for three of these samples. Samples reacting with the probe for *L. africanum* or with the two probes gave amplification with the three different combinations of primers. Digestion of the amplicons with *Xba*I, gave a profile characteristic of African greening liberobacter for the sample hybridizing with the *L. africanum* specific probe (AS-1.7) (Fig. 6, lane 4) and a combination of *L. asiaticum* and *L. africanum* profiles for the three samples reacting with the two probes (Fig. 6, lanes 5–7). Leaves from trees showing no symptoms of greening collected in Mauritius and

Vietnam as well as from areas where greening is not present (Corsica, Martinique) were included in the experiment, they gave negative results with the two techniques.

DISCUSSION AND CONCLUSIONS

Using three different primer combinations, we have been able to specifically amplify 16Sr DNA from the two 'Candidatus' Liberobacter species known today: *L. asiaticum* and *L. africanum*. The primer pair, OI1/OI2c is able to amplify the two species, while the pair OI2c/OA1 amplifies preferentially *L. africanum*. Thus, in countries where the two species are known or suspected to be present, we recommend the use of the three primers OI2c/OI1/OA1, in the same PCR mixture, especially if further identification of the liberobacter species involved is envisaged. Indeed, it is likely that if the two liberobacter species are present in the same tree, the use of the primer pair OI1/OI2c might favour amplification of *L. asiaticum* over that of *L. africanum* as OI1 is 100% homologous to the *L. asiaticum* 16S rDNA sequence but has three mismatches with that of *L. africanum*.

Detection of plant pathogens by polymerase chain reaction in crude plant extracts is known to be difficult because of the presence of inhibitors of the PCR reaction. To avoid this, capture of pathogens by antibodies[14] prior to amplification has been largely used. However, in the case of the uncultured liberobacters of citrus greening disease, this method cannot be applied, as many serotypes of the bacterium have been shown to occur, and none of the monoclonal antibodies that have been produced so far, is able to react with all liberobacter serotypes.[15,16] This is why a procedure had to be developed to eliminate PCR inhibitors in the plant extracts. A protocol based on differential centrifugation and dilution of the extract gave amplification of liberobacter DNA but not consistently enough to allow reliable diagnosis. A second protocol in which total DNA was extracted from the plant and trapped on a column has been worked out. It gave consistent results for the detection of both *L. asiaticum* and *L. africanum* species in greenhouse and field grown citrus trees with a sensitivity at least equivalent to that of DNA–DNA hybridization. The length of the procedure is much shorter than that for DNA–DNA hybridization, the longest step being midrib excision and chopping (around 10 min per sample).

For epidemiological studies, it is now possible, upon *Xba*I digestion of the amplified DNA, to identify the liberobacter species present in a given tree.

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