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Design of division specific primers of *Ralstonia solanacearum* and application to the identification of European isolates

Geneviève Boudazin[1], Anne Claire Le Roux[2], Karine Josi[1], Philippe Labarre[3] and Bernard Jouan[4]
[1]*Laboratoire de Pathologie Végétale INRA BP 57, 49071 Beaucouzé, France (Fax: +33 2 41 22 57 05);* [2]*Laboratoire de Pathologie Végétale FNPPPT/INRA BP 29, 35653 Le Rheu, France;* [3]*Laboratoire National de la Protection des Végétaux, 49000 Angers, France;* [4]*Laboratoire de Pathologie Végétale INRA BP 29, 35653 Le Rheu, France*

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Abstract

A PCR diagnostic test for detection of *Ralstonia solanacearum* at the infraspecific level was developed, based on polymorphisms within the 16S rRNA gene sequence. Partial sequences of this gene were determined for three French isolates which showed the characteristics of *R. solanacearum* subdivision 2a described by Taghavi et al. (1996). Oligonucleotide primers were designed to incorporate the nucleotide triplet (at positions 458–460 of the 16S rRNA gene) which differs between divisions 1 and 2 16S rRNA sequences of *R. solanacearum* isolates. A simple PCR test unambiguously revealed the division of each isolate. The PCR test was useful for identification of isolates of *R. solanacearum* from Europe.

Introduction

Ralstonia solanacearum, ex Burkholderia solanacearum, ex Pseudomonas solanacearum, (Yabuuchi et al., 1992, 1995), is the causal agent of bacterial wilt disease. This soil-borne bacterium is pathogenic for *Musacae* and *Solanacae* in tropical and subtropical climates. Isolates causing brown rot of potato in temperate zones are more adapted to low temperature than other strains (Hayward, 1991), and a great deal of latent infections of tubers are reported (Ciampi et al., 1980; Nyangeri et al., 1984; Smith et al., 1995). Recently, the increasing number of infected sites in several places of Europe (Olsson, 1976; Anonymous, 1992, 1997) worried seed potato producers, thus emphasizing the need for efficient diagnostic tests for material exchanges and epidemiological studies.

Five biovars (based on carbon source utilisation) and five races (based on host range) of *R. solanacearum* have been defined (Hayward, 1991; Buddenhagen, 1986). Isolates adapted to temperate conditions are reported as having a narrow host range and biochemical characteristics of biovar 2 (Hayward, 1994); these two criteria correspond to race 3 of the bacterium.

Cook et al. (1989) described 33 restriction fragment length polymorphism (RFLP) patterns of *R. solanacearum* and showed that the RFLP groups fell into two categories. These were confirmed by Li et al. (1993) by analysis of the 16S rRNA gene sequences. They determined triplets of nucleotides, ACT or TTC at positions 458–460 [in the *Escherichia coli* numbering system (Woese, 1983)] of the gene which were characteristic of divisions 1 and 2 of *R. solanacearum*, respectively. Taghavi et al. (1996) subdivided the second division into two groups (2a and 2b) taking into account other minor variations in the 16S rDNA sequence. Biovar 2 strains from potato used in their studies showed the characteristics of subdivision 2a.

Based on the 16S rDNA sequence, an appropriate PCR test to identify *R. solanacearum* was proposed by Seal et al. (1993). In this test, a 288 bp segment is selectively amplified. One of the primer sequences is specific to *R. solanacearum*, the other hybridising

with a conserved region of the gene. Variations within the 16S rDNA sequence between groups of strains would provide a good target for molecular detection at infraspecific level. For this purpose, we determined the 16S rDNA sequence of three isolates of French origin and confirmed that they belonged to division 2 of *R. solanacearum*. PCR tests were defined to diagnose division 2 isolates and then evaluated for detection of isolates from European countries.

Materials and methods

Bacterial strains

The *R. solanacearum* isolates, the *Pseudomonas syzygii* strain and the banana blood disease bacterium used in this study are described in Table 1. The other bacterial strains tested are listed in Table 2. *R. solanacearum* was routinely cultured for 72 h at 28 °C on YPDA plates containing 0.7% yeast, 0.7% peptone, 0.7% glucose (W/V) and 15 g l^{-1} agar. Other bacteria were grown either on YPDA or King B medium.

Potato extract preparation

Seventy cones (about 100 mg each), removed from the heel ends of healthy potato tubers (cv. Bintje) were soaked in 8 ml of sterile water for 18 h with gentle shaking. After spinning for 5 min at 300 g, the supernatant was stored for use in sensitivity testing or used directly in PCR.

Conditions for 16S rDNA amplification and sequencing

We amplified 16S rDNA segments from three isolates of *R. solanacearum* described in Table 1: CFBP 3672, 3671 and 4649. The first PCR amplification was performed with the forward primer A: 5′GAGTTTGATCATCGCTCAG3′ (universal primer of the 16S rRNA) and the reverse primer B: 5′GGCGGGACTTAACCCAACATC3′. A second amplification was done with the forward primer D2 (described below) internal to the previous amplified product and the reverse primer E: 5′CCAGTCATGAACCCTACGTG3′. The 16S rRNA sequence of *R. solanacearum* (accession n° U28220, Genbank database) was used to define the primers B (G as the 5′ base was added by error when requesting the primer) and E.

The PCR mixture (100 µl) contained 1.25 U of *Taq* polymerase (Eurogentec, Belgium) with appropriate buffer, 2.5 mM $MgCl_2$, 200 nM of dNTP mix (Eurogentec), 100 ng of each primer and 5 µl of diluted bacteria (10^6 cfu/ml). Amplification was carried out in a MJ Research thermocycler (PTC 100), programmed for the first denaturation step at 95 °C for 3 min, followed by 35 cycles of amplification (92 °C, 1 min; 60 °C, 1 min; 72 °C, 1 min) and a final elongation step of 10 min at 72 °C.

PCR products were purified on resin columns (Amicon). DNA sequencing was performed and analysed at ACTGENE Euro Sequence Gene Services, (EVRY-GENOPOLE, France) on an ABI377 sequencer using ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction kit with Ampli*Taq* DNA Polymerase, FS (Perkin-Elmer/Applied Biosystems division, Foster City, CA, USA).

PCR conditions and primers for diagnostic tests

Primers D1 and D2 were designed to have at the 3′ end, either the sequence ACT (specific for division 1 isolates of *R. solanacearum*), or the sequence TTC (specific for division 2 isolates) at positions 458–460 in the *Escherichia coli* numbering system (Woese, 1983). They were used in PCR tests with the non-specific primer B. Primer OLI1 described by Seal et al. (1993) was used with primer Z, a reverse primer, ending with the triplet of specific nucleotides for division 2 isolates. The sequences of these primers have been presented previously (Boudazin and Leger, Second International Bacterial Wilt Symposium held in Guadeloupe, France, 1997) and are described below.

PCR tests were performed using Perkin Elmer 2400 or MJ Research (PTC 100) thermocyclers, in a final volume of 50 µl containing 1 U of *Taq* polymerase (Perkin Elmer) or 0.5 U *Taq* polymerase (Appligene, France) using the conditions previously described for rRNA gene amplification. Annealing temperature was 60 °C for primer pairs D1-B, D2-B or OLI1-Z, and 68 °C, as described by Seal et al. (1993) for the primer pair OLI1-Y2. The PCR products (10 µl) were run on 2% agarose gel in TBE buffer and revealed after ethidium bromide (10 µl l^{-1}) staining.

Table 1. *Ralstonia solanacearum*, banana blood disease bacterium and *Pseudomonas syzygii* strains tested in PCR using the primer pairs D1-B, D2-B and OLI1-Z

Strain	Origin	Biovar	Location	Host	Primer pairs		
Ralstonia solanacearum					D1-B	D2-B	OLI1-Z
CFBP4646		2	France	Tomato	−	+	+
CFBP4649		2	France	Tomato	−	+	+
CFBP3579		2	France	Potato	−	+	+
CFBP3580		2	France	Potato	−	+	+
CFBP3581		2	France	Potato	−	+	+
CFBP3671		2	France	Tomato	−	+	+
CFBP3672		2	France	Potato	−	+	+
CFBP3673		2	France	Potato	−	+	+
CFBP3784		2	Portugal	Potato	−	+	+
CFBP3785		2	Portugal	Potato	−	+	+
CFBP3857	PD2762	2	The Netherlands	Potato	−	+	+
CFBP3858	PD2763	2	The Netherlands	Potato	−	+	+
CFBP3886		2	Belgium	Potato	−	+	+
CFBP3927	UW72	2	Greece	Potato	−	+	+
CFBP3937		2	France	Bittersweet	−	+	+
CFBP4650		2	France	Tomato	−	+	+
CFBP4651		2	France	Tomato	−	+	+
CFBP4652		2	France	Tomato	−	+	+
CFBP3884	PD441.2	2	Sweden	Potato	−	+	+
CFBP4788		2	France	Tomato	−	+	+
CFBP3525		2	Morocco	Potato	−	+	+
CFBP3582		2	Egypt	Potato	−	+	+
CFBP3583		2	Egypt	Potato	−	+	+
CFBP3864		2	Egypt	Potato	−	+	+
CFBP1411	NCPPB326	2	Costa Rica	Potato	−	+	+
CFBP1810	Hingand 264	2	Haiti	Potato	−	+	+
CFBP3103	CIP 108	N2	Peru	Potato	−	+	+
CFBP1410	Kelman A 164	N2	Colombia	*Musa* sp.	−	+	+
CFBP1414	Kelman A 81	N2	Colombia	Potato	−	+	+
CFBP1420	Kelman A 80	N2	Colombia	*Solanum phureja*	−	+	+
CFBP766	Perez JE.PR 80	nd	Porto Rico	Tomato	−	+	+
CFBP1180	Monllor PR212	1	Porto Rico	Tomato	−	+	+
CFBP1184	Buddenhagen SFR	1	Honduras	*Musa* sp.	−	+	+
CFBP1183	Buddenhagen D	1	Costa Rica	*Heliconia* sp.	−	+	+
CFBP3880		1	French West Indies	Potato	−	+	+
CFBP2957		1	French West Indies	Tomato	−	+	+
CFBP3934	UW275	1	Costa Rica	*Melampodium*	−	+	+
CFBP2047	NCPPB325 (K60)	1	USA	Tomato	−	+	+
CFBP2144	IW1509	1	Colombia	*Musa* sp.	−	+	+
CFBP1412	NCPPB2314	1	Colombia	*Musa* sp.	−	+	+
CFBP3058		1	Burkina Faso	Eggplant	+	−	−
CFBP3936	UW373	5	China	Mulberry	+	−	−
CFBP734		1	Madagascar	Potato	+	−	−
CFBP4647		3	Cape Verde Islands	*Anthurium*	+	−	−
CFBP1185	Perez JE., J2	3	Japan	Tomato	+	−	−
GMI 1000		3	French Guyana	Tomato	+	−	−
CFBP1038		3	French West Indies	Tomato	+	−	−
CFBP2965		3	French West Indies	Eggplant	+	−	−
15.63*		3	French West Indies	Tomato	+	−	−
CFBP1960		3	Algeria	Pepper	+	−	−
CFBP1418	Kelman A 6	3	Costa Rica	*Heliconia* sp.	+	−	−
CFBP1813		3	French Guyana	Eggplant	+	−	−

Table 1. Continued

Strain	Origin	Biovar	Location	Host	Primer pairs		
Ralstonia solanacearum					D1-B	D2-B	OLI1-Z
CFBP3935		4	Australia	Ginger	+	−	−
CFBP3930	UW356	4	China	Eggplant	+	−	−
CFBP765	Perez JE J4	3/4	Japan	Tobacco	+	−	−
Pseudomonas syzygii							
CFBP4311					−	+	+
Banana blood disease bacterium							
CFBP3568					−	+	+

CFBP: Collection Française de Bactéries Phytopathogènes. INRA 49071 Beaucouzé Cedex, France.
*Isolate referenced in the LNPV collection (Laboratoire National de la Protection des Végétaux, 49000 Angers, France).

Evaluation of the specificity and sensitivity of the PCR diagnostic tests

The specificity of the primer pairs D1-B, D2-B and OLI1-Z was evaluated with pure cultures of European *R. solanacearum* isolates and strains from other countries as listed in Table 1, as well as with other bacteria from potato and tomato rhizospheres or bacteria closely related to *R. solanacearum*.

To estimate test sensitivity, bacterial colonies of CFBP 4646 grown for 72 h on YPDA plates were suspended in sterile distilled water. The turbidity of the solution was adjusted to 5.5 with sterile distilled water (turbidimetre HACH, RATIO/XR), and a series of 10-fold dilutions either in sterile distilled water or in potato extracts were carried out. Aliquots of 50 µl of each dilution in water were spotted on YPDA plates for enumeration while 5 µl of potato extract dilutions were subjected to PCR tests.

Results

Sequence of the rDNAs

Near complete 16S rDNA sequence information was determined for each of the three isolates. The sequences showed the characteristics of the division 2 as described by Taghavi et al. (1996) with the nucleotide triplet TTC at positions 458–460 in the *Escherichia coli* numbering system (Woese, 1983). The nucleotides A and T at the positions 669 and 737 respectively, indicated that the strains did not belong to the division 2b described by Taghavi et al. (1996). On the basis of the nucleotides A, A, C, A, A, T, T and G at the positions 167, 264, 269, 474, 649, 737, 1424 and 1428 respectively, the strains were the same as the other strains of subdivision 2a. However, additional nucleotides G, G and C were present at positions 94 (CCT**G**CCG), 156 (CTA**G**CTG) and 1026 (GTG**C**TCG), respectively, in the *Escherichia coli* numbering system (Woese, 1983).

Division specific PCR tests

The program PileUp, (Genetic Computer Group, Madison, WI) was used to multi-align the 16S rRNA sequences of *R. solanacearum* from the Genbank database. The following primers were designed from the variable regions of the gene: D1: 5′GTCCGGAAAGAAATCGCACT3′, D2: 5′GTCCGGAAAGAAATCGCTTC3′ and Z: 5′CCACTCCAGGTATTAACCGAA3′. When D1 or D2 were used with non-specific primer B, a 650 bp DNA segment was expected and would indicate the division of *R. solanacearum* isolates (Figure 1). Primer pair, OLI1 and Z amplified a 403 bp DNA PCR product with isolates of *R. solanacearum* division 2. Isolates listed in Table 1 were checked with the primer pairs D1-B, D2-B and OLI1-Z.

Evaluation of PCR tests

Specificity

When isolates from European regions were subjected to PCR with the primers D1-B or D2-B, the target DNA was amplified with the primer pair D2-B. No amplification of template DNA occurred when the primer pair

Table 2. List of bacterial isolates from potato and tomato rhizospheres giving negative results when tested by PCR with the primer sets D1-B, D2-B and OLI1-Z

Bacillus sp. (S344[£]);
Bacillus polymyxa (CFBP1954);
Bacillus pumilus (S300[£]);
Bacillus sp. (cereus complex) (S338[£]);
Burkholderia andropogonis (CFBP2421 and 3576, NCPPB1127[¤]);
Burkholderia caryophylli (CFBP2429 and 3572, NCPPB353[¤] and 2151[¤]);
Burkholderia cepacia (CFBP2227 and 3571, NCPPB945[¤]);
Burkholderia gladioli (3.46[*]);
Burkholderia gladioli pv. *alliicola* (CFBP2422 and 2427);
Burkholderia glumae (12.69[*], NCPPB3708[¤]);
Burkholderia plantarii (NCPPB3590[¤]);
Clavibacter michiganensis subsp. *michiganensis* (CFBP5 and 1460);
Clavibacter michiganensis subsp. *sepedonicus* (CFBP1027, 1598, 2049, 2050 and 3559);
Corynebacterium (R8);
Coryneform (IPO1690[£], 1691[£], 1692[£], S346[£]);
Cytophagaceae (R1c-3a[¤]);
Enterobacteriaceae (IPO1695, IPO1696, S339[£], S342[£], S343[£], S345[£]);
Erwinia carotovora subsp. *atroseptica* (CFBP1329 and 1453, 86.20[*]);
Erwinia carotovora subsp. *carotovora* (CFBP194, 1349 and 2046, 2.33[*]);
Erwinia chrysanthemi pv. *chrysanthemi* (CFBP2048);
Erwinia chrysanthemi (0.62[*]);
Erwinia herbicola (3.37[*]);
Ochrobacterium anthropi (T3B7[¤], IPO1689[£] S306[£] S307[£]);
Pseudomonas sp. (R6);
Pseudomonas corrugata (CFBP1432);
Pseudomonas marginalis pv. *marginalis* (CFBP1538 and 2037);
Pseudomonas mendocina (CFBP2434);
Pseudomonas putida (3.41[*] R9, CFBP2066);
Pseudomonas stutzerii (R7);
Pseudomonas syringae pv. *tomato* (CFBP1318);
Ralstonia pickettii (CFBP2459, 3567, 3786 and 3788);
Rhodococcus sp. (S340[£], S347[£]);
Sphingobacterium multivorum (CFBP3577);
Xanthomonas sp. (S341[£]);
Xanthomonas campestris pv. *vesicatoria* (CFBP2537);
Unidentified bacteria: S348[£], R3, R4, R5, R10, 2697D.

Bacterial strains came from the French collection of phytopathogenic bacteria (CFBP) INRA 49071 Beaucouzé Cedex, France, or were kindly supplied by D. Caffier (indicated by superscript [*]), J. van der Wolf (superscript £), and J. Elphinstone (superscript ¤). Bacteria named R were isolated from rotted potato tubers by A.C. Le Roux.

D1-B was employed. Other isolates of biovar 2 or N2 were also detected with the primer pair D2-B but not by D1-B. Isolates belonging to biovars 3 or 4 were detected by D1-B but not by D2-B. Nine *R. solanacearum* isolates of biovar 1 (including the reference strain CFBP 2047 or K60) were detected by D2-B but not by D1-B. In contrast, the CFBP 734 and 3058 isolates of biovar 1, were detected by D1-B but not by D2-B. Likewise, a 403 bp fragment was obtained for isolates of biovar 2 or most of biovar 1 when tested in PCR with the primers OLI1-Z, while other isolates and biovar 1 isolates CFBP 734 and 3058 were negative (Table 1).

Pure cultures of bacteria listed in Table 2 were tested with the three sets of primers D1-B, D2-B and OLI1-Z. No PCR product of the size expected appeared with primers D2-B and OLI1-Z for any of the tested bacteria, except *P. syzygii* (CFBP 4311) and banana blood disease bacterium (CFBP 3568), which reacted as *R. solanacearum* of division 2. Template DNA from all *B. andropogonis*, *B. caryophylli*, *B. cepacia*,

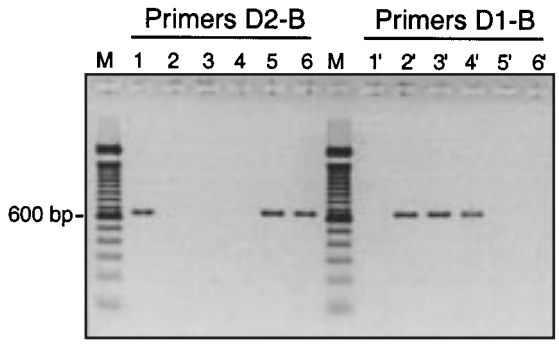

Figure 1. Agarose gel electrophoresis of PCR amplified DNA from isolates CFBP 4646 (biovar 2), CFBP 3058 (biovar 1), CFBP 4647 (biovar 3), GMI 1000 (biovar 3), CFBP 3886 (biovar 2), CFBP 3673 (biovar 2) using primers D1-B (lanes 1–6 successively) and primers D2-B (lanes 1′–6′ respectively). Lanes marked M are a 100 bp ladder (Life Sciences).

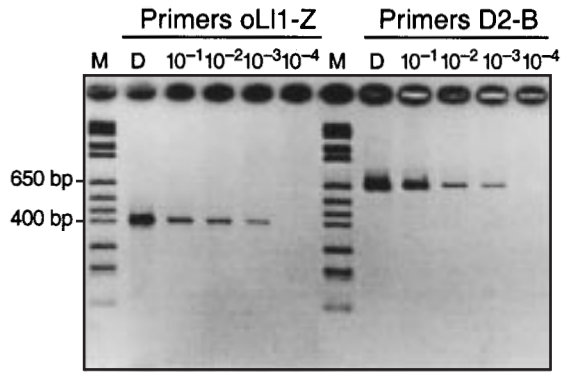

Figure 2. Detection limit of the PCR tests using primers OLI1-Z (left side) and D2-B (right side) for isolate CFBP 4646. The PCR mixtures contained 2 µl of the initial dilution (D) in distilled water or from a 10-fold dilution series in potato tuber extract (cv. Bintje). At the limit of detection (10^{-3} dilution) the concentration of the bacteria was 4×10^4 cfu/ml.

B. gladioli, *B. glumae*, *B. plantarii* did not give an amplification product when subjected to PCR with D1-B, D2-B or OLI1-Z primer pairs. Some unidentified coryneform bacteria (IPO 1690$^{£}$, 1692$^{£}$, S346$^{£}$), and some *enterobacteriaceae* (IPO1695, S343$^{£}$, S345$^{£}$) did produce amplification products with D1-B primer pair, but these were either of larger or smaller sizes than the expected amplification product. PCR products were not amplified by any primer set from healthy potato extracts.

Detection limit

The level of detection with the primers D2-B and OLI1-Z was examined by amplification from serially-diluted bacterial cells. For all primer pairs, the test was positive for the 10^{-3} dilution while results were variable for the 10^{-4} dilution (Figure 2). Accordingly, detection limit was 4.10^4 cfu/ml equal to about 200 cultivable bacteria per PCR reaction. The detection limit was the same for primer pair OLI1-Y2.

Discussion

As expected, the 16S rDNA sequence of the isolates that were tested corresponded with other strains of subdivision 2a sequenced by Taghavi et al. (1996). However, the sequences of our isolates varied from the published sequences at positions 94, 156 and 1026, in the *Escherichia coli* numbering system (Woese, 1983), where nucleotides G, G and C were present in our sequences but missing in the sequences given in Genbank by Taghavi et al. (1996) (Genbank accession n° U28221–U28233). Similar nucleotides were previously found by Li et al. (1993) for three isolates of *R. solanacearum* including the reference strain K60 (Genbank accession n° X67040, X67036, X67035) but were not confirmed on the same strains by Taghavi et al. (1996). However, these additional nucleotides were not considered characteristic of the isolates in this study.

The PCR results obtained here showed that the division of isolates based on the 16S rRNA sequence could be unambiguously identified using oligonucleotide primers D1-B (to determine division 1) and D2-B or OLI1-Z (to specify division 2). Cook et al. (1989) proposed that division 1 contains *R. solanacearum* biovars 3, 4 and 5, and division 2, biovars 1, 2 and N2. Gillings and Fahy (1993) and Taghavi et al. (1996) mentioned similar observations, although the latter authors found that some biovar-2 phenotypes clustered with division 1 isolates (Fegan et al., 1998a). Interestingly, two of our isolates of biovar 1 (CFBP 734 and CFBP 3058) which did not fall into division 2 showed other exceptions to this rule, indicating that there is not a strict correspondence between the biovar and the 16S rRNA division of isolates.

In the reports of the Second International Bacterial Wilt Symposium, Fegan et al. (1998a,b) also described PCR primers capable of distinguishing the divisions of *R. solanacearum* or detecting selectively the isolates of biovar 2. Although they targeted the same differential triplet of the 16S rRNA gene, their sequences differed from the primers described here which were

constructed with the differential nucleotide triplet at the 3′ end. This ensures that DNA binding of the polymerase is possible only if the division-specific nucleotides are recognised.

As expected from the 16S rDNA sequences of *P. syzygii* and the banana blood disease bacterium by Taghavi et al. (1996) (Genbank accession n° U28234–U28236 and U28237 or U28238, respectively), both were identified by primers D2-B and OLI1-Z as the division 2 isolates of *R. solanacearum*. These bacteria are known to be closely related to *R. solanacearum* and have a similar serological pattern (Roberts et al., 1990; Baharuddin et al., 1994). We are currently evaluating PCR tests which avoid this problem by using primers targeting the *R. solanacearum* race 3 specific segment identified by Cook et al. (1991).

The two primer pairs D2-B and OLI1-Z which are specific for the division 2 isolates could be useful complements to the primers OLI1-Y2 described by Seal et al. (1993), which detect a larger spectrum of isolates. All these primers are targeted to the same gene and their detection limit is equivalent in this study. The specificity of the primer Z, added to that of the primer OLI1, give a double guarantee for PCR reaction specificity.

All the European isolates tested, from both potato or tomato crops, were detected by primer pairs D2-B and OLI1-Z, and are proposed as useful tools for detection of division-2 isolates. Moreover, used in parallel, the pairs D1-B and D2-B, could be of interest in epidemiological studies, as would be the tRNA targeted primers of Seal et al. (1992), in particular to determine whether the bacterial population in weeds in Europe is limited to division-2 isolates.

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