A real-time BIO-PCR assay for detection of *Ralstonia solanacearum* race 3, biovar 2, in asymptomatic potato tubers[1]

M. Ozakman and N.W. Schaad

Abstract: *Ralstonia solanacearum*, the causal agent of brown rot of potato, can often be carried latently in seed potato tubers. We designed real-time PCR (polymerase chain reaction) primers and probe and a highly sensitive BIO-PCR assay for specific detection of the strains of race 3, biovar (bv.) 2 of *R. solanacearum* in asymptomatic potato tubers. The biovar-specific primers and probe reacted with all 17 strains of bv. 2 of *R. solanacearum* tested, including 12 from potato and 5 from geranium. None of the other 35 strains of *R. solanacearum* reacted, including 4 strains of bv. 1 from potato or the closely related blood disease bacterium from banana. None of 13 other bacteria reacted, including 5 *Erwinia* and 2 *Clavibacter* species from potato. Using undiluted potato tuber extract, inoculated with *R. solanacearum* bv. 2, as few as 30 cells/mL of extract could be detected. Two of 14 naturally infected potato tubers with no disease symptoms were positive by the newly described real-time BIO-PCR assay, whereas none were positive with a standard real-time PCR assay. This is the first report of the detection of *R. solanacearum* bv. 2 in asymptomatic, latently infected potato tubers by PCR. The real-time assay is very simple and much less time consuming than classical PCR.

Key words: brown rot of potato, molecular identification, bacteria, geranium.

Résumé : Le *Ralstonia solanacearum*, l'agent responsable de la pourriture brune de la pomme de terre, peut souvent être présent sous forme latente dans les tubercules de pomme de terre de semence. Nous avons développé des amorces et une sonde pour une RCP (réaction en chaîne de la polymérase) en temps réel, et un test BIO-RCP très sensible pour la détection spécifique des souches de la race 3, biovar (bv.) 2 du *R. solanacearum* dans les tubercules de pomme de terre asymptomatiques. Les amorces et la sonde spécifiques au biovar ont réagi avec les 17 souches testées du bv. 2 du *R. solanacearum*, comprenant 12 souches de la pomme de terre et 5 du géranium. Aucune des 35 autres souches du *R. solanacearum* n'a réagi, y compris 4 souches de la pomme de terre du bv. 1 et la bactérie apparentée responsable de la maladie du sang du bananier. Aucune de 13 autres bactéries n'a réagi, y compris 5 espèces d'*Erwinia* et 2 de *Clavibacter* provenant de la pomme de terre. Avec un extrait de pomme de terre non dilué, additionné de *R. solanacearum* bv. 2, aussi peu que 30 cellules/mL d'extrait ont pu être détectées. Le nouveau test BIO-RCP en temps réel fut positif sur 2 de 14 tubercules de pomme de terre naturellement infectés et sans symptôme de maladie, alors que le test RCP standard en temps réel fut négatif. C'est la première fois que la détection par la RCP du *R. solanacearum* bv. 2 latent, dans des tubercules de pomme de terre asymptomatiques, est rapportée. Le test en temps réel est très simple et demande beaucoup moins de temps que la RCP classique.

Mots clés : pourriture brune de la pomme de terre, identification moléculaire, bactérie, géranium.

Introduction

Ralstonia solanacearum (Smith) Yabuuchi et al. race 3, biovar (bv.) 2, the causal agent of brown rot of potato, is emerging as a serious threat to potato production in temperate climates (Hayward 2000; Hayward et al. 1998; Stead et al. 1996). The cool climate adapted strain originated in the

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high Andes of Peru and Bolivia and spread to potato-producing countries in infected tubers (Hayward and Fegan 2001). The organism was first reported in Europe in Sweden in 1972 (Olssen 1976). Rapid dissemination in seed tubers throughout Europe led to the adoption of regulations by the European Union (EU) to help prevent further spread (Stead et al. 1996); *R. solanacearum* bv. 2 is listed as a zero-tolerance quarantine organism in the EU (Council of the European Union 1998).

The organism has not been found in potatoes in North America, however, it was discovered in geraniums in Wisconsin (Williamson et al. 2001), Pennsylvania (Kim et al. 2002), Delaware, and Connecticut (Kim et al. 2003) and, most recently, in Indiana in January 2003 (K. Rany, personal communication). *Ralstonia solanacearum* is considered dangerous because it spreads long distances in asymptomatic propagative stocks and can survive for long periods in soil and water, as well as within several weed species (Hayward and Fegan 2001). In those countries affected by brown rot, the costs of disease surveillance and eradication are considerable (Stead et al. 1996). Because of its high threat potential, bv. 2 is included in the select list of plant pathogens of the Animal and Plant Health Inspection Service, US Department of Agriculture (2002).

Asymptomatic, infected seed potato tubers are a major factor in the dissemination of *R. solanacearum* to new production fields (Ciampi et al. 1980). Because pathogen-free seed tubers are very important for controlling disease spread, assays to detect *R. solanacearum* in tubers must be highly sensitive. In the EU, seed potato tubers must be certified as free of *R. solanacearum* bv. 2, on the basis of a serological or classical polymerase chain reaction (PCR)-based technique (Council of the European Union 1998). Detection thresholds for serological and PCR assays are similar, ranging from 10^3–10^5 cfu/mL of water to 10^6 cfu/mL of potato core tissue extracts inoculated with cells of *R. solanacearum* (Elphinstone et al. 1996; Fegan et al. 1998; Janse 1988; Seal et al. 1993). Several classical PCR techniques are available, including protocols with primers designed from the *hrp* gene (Cook et al. 1989; Poussier and Luisetti 2000), 16S rDNA (Seal et al. 1993; Weller et al. 2000a) exo-poly-α-D-galacturonosidase gene (Glick et al. 2002), and a competitive hybridization approach based on a biovar-specific DNA fragment (Fegan et al. 1998). However, classical PCR has not proven to be useful for general diagnostics mainly because of the need to perform a Southern blot analysis to confirm the identity of the PCR product. Also, classical PCR is very open to cross contamination. Weller et al. (2000a) designed primers and probe for a fluorescence-based real-time TaqMan™ PCR assay that was able to detect 10^2 cfu/mL of a pure culture of *R. solanacearum* bv. 2, by employing highly sensitive, broad-host-range *R. solanacearum* probe (RS-P) and primers partially homologous to 16S rDNA primers of Seal et al. (1993). For an assay specific to bv. 2, Weller et al. (2000a) chose sequences of the DNA fragment specific to bv. 2 of Fegan et al. (1998), retaining the original forward primer 630 and selecting a new reverse primer and probe. With a BIO-PCR (Schaad et al. 1995) protocol, Weller et al. (2000b) were able to detect bv. 2 at 10^4 cfu/mL in tuber extracts, 24 h after enrichment, and at 10^1 cfu/mL after a 96-h enrichment of the liquid medium. However, no assays with the probe specific to bv. 2 (B2-P) were included.

We describe, for the first time, a PCR-based assay for the detection of bv. 2 strains of *R. solanacearum* in latently infected potato tubers. The real-time BIO-PCR assay includes a short enrichment period of 24–30 h, on mSMSA agar (Denny and Hayward 2001; Englebrecht 1994), and real-time PCR, with new primers and probe specific to bv. 2, designed from a DNA fragment specific to bv. 2 (Fegan et al. 1998). This real-time BIO-PCR assay is sensitive enough to detect as few as 10^1 cfu of *R. solanacearum* bv. 2 per milliliter of potato tuber extract, without extraction of DNA and without any effect from PCR inhibitors.

Materials and methods

Source and growth of bacterial strains

Strains of *R. solanacearum* and the other bacteria used in this study are listed in Table 1. *Ralstonia solanacearum* was grown and maintained on triphenyltetrazolium chloride (TTC) agar medium (Kelman 1954) at 28°C. Other bacterial strains that served to determine the specificity of the primers and probe were grown on King's medium B or yeast–peptone–glucose–agar (YPGA) medium (Schaad 2001). For the BIO-PCR assay, *R. solanacearum* was enriched on mSMSA (Denny and Hayward 2001; Elphinstone et al. 1996; Englebrecht 1994).

Design and selection of real-time TaqMan PCR primers and probe, and routine PCR

Real-time TaqMan (Perkin Elmer Applied Biosystems, Foster City, Calif.) primers and probe, specific to *R. solanacearum* bv. 2, were designed from published sequences of a 570-bp (base pair) DNA fragment specific to bv. 2 (Fegan et al. 1998), with Primer Express version 1.0 (Perkin Elmer Applied Biosystems, Foster City). The TaqMan probe is labeled at the 5′-terminal nucleotide with the FAM™ (6-carboxyfluorescein) reporter dye and 3′-terminal nucleotide with the TAMRA™ (6-carboxytetramethylrhodamine) quencher dye (Schaad et al. 1999). The PCR mixture for each reaction consisted of the following: 1× PCR buffer; 5 mM $MgCl_2$; deoxyribonucleotide triphosphates (dNTPs), 200 µM each; 1 µM forward primer; 1 µM reverse primer; 400 nM probe; 0.5 U *Taq* DNA polymerase (Perkin Elmer Applied Biosystems, Foster City); 1× additive reagent containing bovine serum albumine at 1 mg/mL, 150 mM trehalose, and Tween 20 at 1% volume fraction (Cepheid, Sunnyvale, Calif.); and 1 or 10 µL of sample or cell suspension in 25-µL Cepheid optical reaction tubes. For 1-µL samples, 6.25 µL of water were used whereas no water was used for 10-µL samples. Polymerase chain reaction was carried out in a portable rapid cycling Smart Cycler® (Cepheid, Sunnyvale). With the help of one set of primers and probe, amplification conditions were optimized for denaturation and annealing times and temperatures. Additional forward primers were ordered and screened for specificity and sensitivity, employing the same reverse primer and probe. The final combination was then optimized. Results were recorded as cycle threshold (C_t) values. The C_t value is defined as the PCR cycle number at which time the signal (fluorescence) of the probe rose above back-

Table 1. Strains of *Ralstonia solanacearum* and other bacteria studied and results of real-time PCR, using a Smart Cycler®.

Strain (laboratory No.)	Source*	Origin	Host	Race	Biovar	PCR
Ralstonia solanacearum						
UW-139 (FC-6)	1	Costa Rica	*Musa* sp.	2	1	–
UW-275 (FC-7)	1	Costa Rica	*Melampodium* spp.	1	1	–
JT-526 (FC-325)	2	Réunion Island	*Pelargonium* sp.	ND	1	–
JR-659 (FC-326)	2	U.S.A.	Tomato	ND	1	–
JS-740 (FC-327)	2	Colombia	Potato	ND	1	–
JS-768 (FC-328)	2	Guadeloupe	Potato	ND	1	–
JS-775 (FC-329)	2	Honduras	*Musa* sp.	ND	1	–
Rso 81-2 (FC-230)	3	U.S.A.	Tomato	ND	1	–
Rso 81-5 (FC-231)	3	U.S.A.	Tomato	ND	1	–
Rso 84-1 (FC-232)	3	U.S.A.	Tomato	ND	1	–
Rso 87-105 (FC-234)	3	U.S.A.	Tomato	ND	1	–
Rso 96-41 (FC-235)	3	U.S.A.	Tomato	ND	1	–
PS-102 (ATCC-9910)	4	U.S.A.	Tobacco	ND	1	–
PS-119	4	U.S.A.	Potato	ND	1	–
PS-120	4	U.S.A.	Peanut	ND	1	–
PS-121	4	U.S.A.	Potato	ND	1	–
PS-123	4	U.S.A.	Tomato	ND	1	–
PS-124	4	U.S.A.	Tobacco	ND	1	–
UW-72 (FC-530)	1	Greece	Potato	3	2	+
NL-pot. (FC-510)	5	Netherlands	Potato	3	2	+
TR-105 (FC-529)	6	Turkey	Potato	3	2	+
UW-276 (FC-533)	1	Mexico	Potato	3	2	+
UW-257 (FC-535)	1	Costa Rica	Potato	3	2	+
JT-516	2	Réunion Island	Potato	3	2	+
MB-12 (FC-311)	7	Nepal	Potato	3	2	+
MB-9 (FC-310)	7	Nepal	Potato	3	2	+
NA-5 (FC-305)	7	Nepal	Potato	3	2	+
NF-5 (FC-306)	7	Nepal	Potato	3	2	+
BA-4 (FC-309)	7	Nepal	Potato	3	2	+
UW-145 (FC-53)	1	Australia	Potato	3	2	+
FC-396	8	Guatemala	*Pelargonium* sp.	3	2	+
FC-400	8	Guatemala	*Pelargonium* sp.	3	2	+
FC-410	8	Guatemala	*Pelargonium* sp.	3	2	+
FC-410	8	Guatemala	*Pelargonium* sp.	3	2	+
FC-417	8	Guetamela	*Pelargonium* sp.	3	2	+
UW-457 (FC-17)	1	Peru	Potato	ND	N2	–
UW-416 (FC-11)	1	Australia	*Solanum nigrum* L.	1	3	–
UW-432 (FC-140)	1	Australia	*Zinnia* sp.	1	3	–
UW-434 (FC-15)	1	Australia	*Solanum nigrum*	1	3	–
UW-440 (FC-16)	1	Australia	*Streltzia reginae*	1	3	–
P-1 (FC-254)	7	Thailand	Pepper	1	3	–
P-2 (FC-255)	7	Thailand	Pepper	1	3	–
Pe-UD (FC-256)	7	Thailand	Pepper	1	3	–
Pe-BK (FC-257)	7	Thailand	Pepper	1	3	–
To-4 (FC-290)	7	Thailand	Tomato	1	3	–
Po-1155	7	Thailand	Pepper	1	3	–
Supp-1875 (B2-1)	2	Japan	Tobacco	1	3	–
PB 41-2 (FC-296)	7	Thailand	*Zingiber officinale* Roscoe	1	4	–
PB 41-3 (FC-297)	7	Thailand	*Zingiber officinale*	1	4	–
PB 41-1 (FC-295)	7	Thailand	*Zingiber officinale*	1	4	–
Cu-1290 (FC-274)	7	Thailand	*Cucuma alismatifolia*	1	4	–
Cu-1291 (FC-275)	7	Thailand	*Cucuma alismatifolia*	1	4	–
Cu-1351 (FC-276)	7	Thailand	*Cucuma alismatifolia*	1	4	–
Cu-1352 (FC-277)	7	Thailand	*Cucuma alismatifolia*	1	4	–
UW-357	1	China	Olive	1	4	–
UW-74	1	Ceylon	Potato	1	4	–

Table 1 (*concluded*).

Strain (laboratory No.)	Source*	Origin	Host	Race	Biovar	PCR
UW-359	1	China	*Zingiber officinale*	1	4	–
FC-338	7	Japan	*Solanum melongena* L.	ND	4	–
UW-360	1	China	Mulberry	1	4	–
UW-151	1	Australia	*Zingiber officinale*	1	4	–
UW-373	1	China	Mulberry	1	5	–
Blood disease bacterium						
Supp-1723	2	Indonesia	Banana	NA	NA	
Erwinia atroseptica						
Eca-602	6	Turkey	Potato	NA	NA	
Eca-504	6	Turkey	Potato	NA	NA	
***Erwinia carotovora* (Jones) Bergey et al.**						
Ecc-Tub	6	Turkey	Potato	NA	NA	
Ecc-604	6	Turkey	Potato	NA	NA	
Ecc-301	6	Turkey	Potato	NA	NA	
***Pseudomonas fluorescens* Migula**						
ATCC-17559 (FC-122)	9	U.S.A.	Unknown	NA	NA	–
ATCC-9446 (FC-123)	9	U.S.A.	Unknown	NA	NA	–
ATCC-12985 (FC-124)	9	U.S.A.	Unknown	NA	NA	–
***Pseudomonas marginalis* (Brown) Stevens**						
PM-174 (FC-85)	4	U.S.A.	*Dahlia* spp.	NA	NA	
***Clavibacter michiganensis* subsp. *sepedonicus* (Spieckermann & Kotthoff) Davis, Gillaspie, Vidaver & Harris**						
CMS-INM (FH-20)	10	U.S.A.	Potato	NA	NA	–
CMS-OFF (FH-22	10	U.S.A.	Potato	NA	NA	–
***Xanthomonas campestris* (Pammel) Dowson**						
XC-125 (FB-1018)	4	U.S.A.	Cauliflower	NA	NA	–
LMG-523 (FB-1021)	11	Burundi	*Brassica* spp.	NA	NA	–

Note: ND, not determined; NA, not appropriate; ATCC, American Type Culture Collection; –, fluorescence remained below background after 40 cycles; +, fluorescence rose above background.

*1, C. Allen, Wisconsin, U.S.A.; 2, Y. Takikawa, Japan; 3, R. Gitaitis, Georgia, U.S.A.; 4, N.W. Schaad, International Collection of Phytopathogenic Bacteria, Maryland, U.S.A.; 5, J.D. Janse (diseased tuber), Netherlands; 6, M. Ozakman, Turkey; 7, N. Thaveechai, Thailand; 8, S. Kim, Pennsylvania, U.S.A.; 9, J. Loper, Oregon, U.S.A.; 10, T. German, Wisconsin, U.S.A.; 11, M. Lemattre, France.

ground. All tests were run in duplicate and each run contained a negative (water) and positive (*R. solanacearum* bv. 2, strain TR-105) control.

Specificity and sensitivity of primers

To test primer specificity, 17 strains of *R. solanacearum* bv. 2, 1 of bv. N2, 18 of bv. 1, 11 of bv. 3, 13 of bv. 4, 1 of bv. 5, the closely related blood disease bacterium (Seal et al. 1993), 7 other bacteria associated with potatoes, 4 pseudomonads, and 2 xanthomonads (Table 1) were grown on agar media for 48 h. After washing the cells from the plates and diluting 1:100 in sterile, milliQ-purified (MQ) water, 1.0-mL samples were stored in microfuge tubes at –20°C.

To determine cell sensitivity, strain TR-105 was grown on TTC agar medium at 28°C for 24 h. The cells were washed from the plate in sterile MQ water and the suspension was adjusted to a 0.1 optical density at 600 nm (OD_{600}), under a Smartspec 3000 spectrophotometer (Bio-Rad Laboratories, Hercules, Calif). Such suspensions contained approximately 10^8 cfu/mL. Actual cell concentrations were determined by preparing 10-fold serial dilutions to 10^{-9}. Dilutions at 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} were then plated with 100 µL each onto four plates of TTC agar medium. After 48 h, the colonies were counted and recorded. One milliliter of each of the four dilutions was boiled for 10 min in a sealed microfuge tube and stored at –20°C for direct PCR (no DNA extraction).

To test for DNA sensitivity, strain TR-105 was grown in 5 mL of nutrient broth (NB) medium (Difco, Detroit, Mich.) at 28°C for 24 h and cells were harvested by centrifugation at 14 000 r/min (1 r = 2π rad) for 3 min. After washing the cells three times in sterile saline (0.85% NaCl) solution, DNA was extracted with a Puregene Easy DNA extraction kit (Gentra Systems, Minneapolis, Minn.). The concentration of DNA was measured with a Smartspec 3000, adjusted to 10 ng/µL with sterile MQ water, and 10-fold serial dilutions were made down to 100 fg/µL in sterile MQ water.

Production of naturally infected tubers

Potato plants, *Solanum tuberosum* L. 'Norchip', were grown to the flowering stage in "sterile" potting soil, in sterile 20-cm pots, in a growth chamber within a BSL-3P (biosafety level 3 for whole plants) containment facility, un-

der a 12-h photoperiod cycle, at 23°C (day) and 12°C (night). A 24-h liquid NB culture (10 mL) of bv. 2, strain TR-105, adjusted to 0.1 OD_{600} and diluted to 10^{-3}, was poured onto the soil surface of each pot. After growing for 30 to 40 days as above, plants with slight wilting symptoms were removed and all resulting tubers harvested, washed, dried, and stored in paper bags at 10°C.

Potato tubers extracts

Potato tuber extracts were obtained according to official EU methods (Council of the European Union 1998). Briefly, core tissue was removed aseptically from the stem end of each tuber and placed into a flask containing 25 mL of 50 mM phosphate buffer, pH 7.2. After shaking for 4 h at room temperature, the suspension was centrifuged at 10 000 *g* for 10 min at 4°C and suspended in 1 mL of 10 mM phosphate buffer, pH 7.2.

Assaying inoculated tuber extracts

To inoculate extracts with *R. solanacearum*, strain TR-105 was grown and diluted 10 fold to 10^{-8}, as above. Bacterial suspension, 100 µL of each dilution (10^{-8}–10^{-1}), was then added to 900 µL of potato core tissue extracts. To determine the actual cfu of *R. solanacearum* per milliliter, 100 µL of dilutions 10^{-5}, 10^{-6}, and 10^{-7} were spread onto each of three plates of mSMSA with an L-shape glass rod and incubated at 28°C. At the same time, 100 µL of each dilution was spread onto each of five plates of mSMSA for BIO-PCR assay. The remaining 200 µL of each dilution were boiled for 10 min, as above, and stored at –20°C for direct PCR (no DNA extraction). As a positive control to recognize colonies of *R. solanacearum*, a culture was streaked onto mSMSA and incubated at 28°C. After 24–30 h of incubation, pinpoint-size colonies of *R. solanacearum*, plus any other bacterial colonies occurring on each of three plates of mSMSA, were washed from the plates with 1 mL of sterile water, pooled into one sample, and boiled for 10 min. The remaining original potato extract was boiled for 10 min in a microfuge tube and immediately put on ice for real-time direct PCR. The other two plates were maintained at 28°C for 5 days for visual recovery of *R. solanacearum*.

Assaying naturally infected potato tubers

A total of 14 asymptomatic tubers were tested. The stem-end core of each tuber was removed, added to 199 healthy tubers, and extracted, as above. As a control, 200 healthy tubers, purchased at a local grocery store, were assayed similarly. Real-time direct PCR and BIO-PCR assays were carried out as above.

Results

Design and selection of real-time PCR primers and probe

Of the different forward primers tested, RSC-F (5′-TTCACCGCAAACAGCG-3′) gave the best results with reverse primer RSC-R (5′-TACGCCCCAGCAGATG-3′) and probe RSC-P (5′-TTCGCCGATGCTTCCCA-3′) (Table 2). The selected primers and probe were only one nucleotide apart (Fig. 1). The following amplification conditions were chosen: 2 min of denaturation at 95°C, followed by 40 cycles of 5 s of denaturation at 95°C, and 30 s of annealing at 58°C. Of the four forward primers tested in combination with reverse primers RSC-R and probe RSC-P, primer RSC-F had the lowest C_t value (Table 2).

Table 2. Comparison of real-time PCR, using reverse primer RSC-R and probe RSC-P with four different forward primers and genomic DNA of *Ralstonia solanacearum* biovar 2, race 3, strain TR-105.

Forward primer	Reverse primer	Probe	C_t*
RSC-F	RSC-R	RSC-P	18.84
RSM1-F	RSC-R	RSC-P	26.15
RSM2-F	RSC-R	RSC-P	32.19
RSM3-F	RSC-R	RSC-P	27.44

Note: DNA was extracted from cells of TR-105, using Puregene Easy DNA extraction kit. The concentration of DNA was adjusted using a Smartspec 3000 to 10 ng/mL.

*C_t value is the PCR cycle number at which time the fluorescence value rises above background. Samples run on a Smart Cycler for 40 cycles.

Fig 1. Nucleotide sequences within a cloned 570-bp DNA fragment (Fegan et al. 1998) of *Ralstonia solanacearum* race 3, biovar 2, strain TR-105. Primers RSC-F (forward) and RSC-R (reverse) and probe RSC-P are shown in bold and underlined.

CAGCAGGTCG CCATTCCCAT ACAGAATTCG ACCGGCACGC

CGAGCCTGAA CCTTGCGCGC GGTGGCCAAA CTCATCTGGG

CCATTCTTGC GAAACGACTT TCCACTTCGT ACCATCCGGC

GCCACGGGTT TGTCATGGCG CTCCTGA**TTC**

RSC-F.....> RSC-P.....> <.....

ACCGCAAACA GCGATTCGCC GATGCTTCCC AGCATCTGCT

....RSC-R

GGGGCGTAAT CACTTCCTGG CGCACTGCAC TCAACGCTTC

CAGCAGGTGT TCGCTTGAA ATTCGTAGGC GAATTGCATG

TGATTGCCCC GTGGTGATGG AGATGCGCCA GCGAGGCCGC

CCCACCTATT TCTTGTAGAC CAACCGCCCC ATACGCTGTT

Specificity and sensitivity of primers and probe

Results of optimization showed that 1 µM primer concentration provided the lowest C_t value and highest end-point fluorescence (data not presented). All strains of bv. 2 tested were positive and resulted in C_t values of 26 or less (Table 1). None of the 43 strains of bvs. 1, 3, 4, and 5 or the strain of bv. N2 produced any fluorescence after 40 cycles. Furthermore, none of the other bacteria, including the closely related blood disease bacterium, produced any fluorescence (Table 1). The maximum sensitivity of primers RSC-F and RSC-R and probe RSC-P was 100 fg/µL (C_t = 35.29) based on DNA and direct PCR. For boiled cells and direct PCR, the threshold was 3.0×10^3 cfu/mL (C_t = 38.25; Table 3).

Table 3. Sensitivities of real-time direct PCR and BIO-PCR assays for detection of *Ralstonia solanacearum* biovar 2, race 3, in inoculated water and potato extracts, using a Smart Cycler® for 40 cycles.

		Potato extract (4 h)		
			BIO-PCR§	
Cfu/mL*	Water, direct PCR†	Direct PCR, 1-µL sample‡	1-µL sample	10-µL sample
3.0×10^{7}	23.67	25.31	20.27	ND
3.0×10^{6}	26.72	29.28	23.89	22.48
3.0×10^{5}	29.60	32.34	27.77	24.35
3.0×10^{4}	32.85	35.27	31.14	27.91
3.0×10^{3}	38.25	38.37	33.95	30.56
3.0×10^{2}	–	–	36.38	33.29
3.0×10^{1}	–	–	–	36.03

Note: Results recorded as cycle threshold (C_t) values. C_t is defined as the cycle at which time the fluorescence rises above background. –, no fluorescence detected.

*A cell suspension prepared from a 24-h-old culture of strain TR-105 grown on TTC agar medium (Kelman 1954) was adjusted to 0.1 OD_{600} in sterile water and diluted 10 fold in sterile water to 10^{-7}. Samples of 100 µL were plated onto each of three plates of TTC agar, and the number of colonies was counted after 3 days at 28°C.

†Aqueous bacterial cell suspensions (10 µL) were used for direct real-time PCR.

‡Inoculated potato extracts (10 µL) were boiled for 10 min, and 1 µL was used directly for direct real-time PCR.

§Inoculated potato extracts (100 µL) were spread onto each of three mSMSA (Denny and Hayward 2001) plates and incubated at 28°C for 24 h. Each plate was washed with 1 mL of sterile water, pooled, boiled for 10 min, and used directly for real-time PCR.

Table 4. Comparison between real-time, direct, and BIO-PCR for detecting *Ralstonia solanacearum* biovar 2, race 3, in 14 asymptomatic tubers, using a Smart Cycler® for 40 cycles.

	Number of colonies on SMSA*	Direct PCR†	BIO-PCR†
Tuber No. 1			
Direct extract	204.0		31.6
1/10 dilution	19.0		33.8
1/100 dilution	2.0		37.3
Tuber No. 2			
Direct extract	377.0	38.3	31.0
1/10 dilution	39.0	–	33.1
1/100 dilution	3.6	–	36.5
Tubers Nos. 3–14	–	–	–

Note: Extracts from asymptomatic potato tubers were diluted 10 fold to 10^{-2}. For BIO-PCR, 100 µL of undiluted extract and of each dilution were plated onto each of five mSMSA (Denny and Hayward 2001) plates; three of those plates were incubated for visual counts of colonies of *R. solanacearum* after 3 days and two plates were washed for BIO-PCR after 24 h. Ten microliters of the undiluted extract and of each dilution were used for both direct real-time PCR and BIO-PCR.

*Mean number per plate after 3 days at 28°C.

†Results recorded as cycle threshold (C_t) values. –, no amplification after 40 cycles. Water control was negative and positive control (pure culture of *R. solanacearum*) had a C_t value of 22.5. C_t is defined as the cycle at which time the fluorescence rises above the background.

Assaying inoculated potato extracts

The threshold for real-time direct PCR, using 1 µL of potato extract, was 3.0×10^3 cfu/mL (C_t = 38.37; Table 3). In contrast, similar samples containing as few as 3.0×10^2 cfu were positive (C_t = 36.38) with BIO-PCR. When the amount of sample in the BIO-PCR reaction mixture was increased to 10 µL, the sensitivity increased to as few as 3.0×10^1 cfu (C_t = 36.03; Table 3). With a 10-µL sample and BIO-PCR, there was no significant difference between boiled and nonboiled; typical C_t values for boiled and nonboiled were 36.03 and 36.09, respectively, for plates containing 10 cfu.

Assaying naturally infected tubers

BIO-PCR detected *R. solanacearum* in 2 out of 14 tubers (Table 4). The extract of the two tubers gave C_t values of 31.57 and 30.99, respectively. The same two tubers were positive by isolation techniques. Concentrations of *R. solanacearum* for the same two samples were 2040 and 3770 cfu/mL, respectively, (Table 4). Real-time direct PCR detected *R. solanacearum* in the tuber containing 3770 cfu/mL (C_t = 38.3) but not in the one containing 2040 cfu/mL (Table 4). All assays were negative for the remaining 12 tubers.

Discussion

Serological tests, such as immunofluorescent antibody staining, are available for *R. solanacearum*, but the level of sensitivity is not higher than 10^4 cells/mL (Elphinstone et al. 1996; Janse 1988; van der Wolf et al. 2000). To increase the sensitivity of a serological assay, Priou et al. (1999) combined enrichment with ELISA to detect *R. solanacearum* in soil. Serological tests have a disadvantage of detecting dead cells and often result in false positives (Elphinstone et al. 1996; Janse 1988). For the detection of bacteria in seeds (Schaad et al. 1995) and soil (Ito et al. 1998), BIO-PCR techniques have several advantages over standard PCR, including: (i) elimination of PCR inhibitors, (ii) reduction of the chance of a false positive due to dead cells or free DNA, (iii) significant increase in sensitivity due to enrichment of the target cells (Elphinstone et al. 1996; Schaad et al. 1995, 1999; Weller et al. 2000b), and (iv) results based on viability.

A conventional real-time PCR assay has been described for *R. solanacearum*. Weller et al. (2000a) were able to detect 10^2 cfu/mL, in pure culture, with both the bv.-2-specific B2-P probe and species-specific RS-P probe. However, when undiluted inoculated potato extracts were employed, the limit of detection was only 10^7 cfu/mL with either set of primers and probe. Only by diluting the inoculated extracts to eliminate PCR inhibitors were they able to lower the detection thresholds. At a 1:100 dilution, the limits of detection were between 10^4 and 10^5 cfu/mL for the RS primers and probe, and 10^6 cfu/mL with the B2 primers and probe. With BIO-PCR and the highly sensitive 16S primers and probe, Weller et al. (2000b) were able to detect as few as 10^4 cfu/mL after a 24-h enrichment and 10^1 cfu/mL after a 96-h enrichment. However, the B2 primers and probe specific to bv. 2 were not tested. Weller et al. (2000b) and

Elphinstone et al. (1996) concluded that the increase in sensitivity was partly due to the elimination of PCR inhibitors in potato extracts. To eliminate PCR inhibition from samples of tomato seeds, plant samples, water, and soil, Poussier et al. (2002) evaluated several DNA extraction procedures for use in a classical PCR assay for *R. solanacearum* and concluded that a commercial extraction kit was highly effective for all samples except soil. In contrast, Ito et al. (1998) developed a BIO-PCR assay that eliminated PCR inhibition and was highly effective in detecting *R. solanacearum* in soil.

Our newly described real-time primers and probe, designed from sequences of the 570-bp DNA fragment specific to bv. 2 described by Fegan et al. (1998), were highly specific to *R. solanacearum* bv. 2. No strains of any other biovar of *R. solanacearum*, or any other bacteria tested, reacted with the primers. These results and the results of Weller et al. (2000a) agree that the 570-bp DNA fragment, originally identified by Fegan et al. (1998), is unique to *R. solanacearum*. We found real-time BIO-PCR and a portable Smart Cycler protocol to be suitable for detecting bv. 2 strains of *R. solanacearum* in potato tubers that showed no disease symptoms of any kind. Detecting as few as 20 cells/mL in potato extract diluted by 1:100, indicates that the real-time BIO-PCR technique is highly sensitive and useful for quarantine and certification seed assays. Like Weller et al. (2000a), we also observed PCR inhibitors in potato tuber extracts with a real-time direct PCR assay. However, with the agar-based BIO-PCR protocol, no PCR inhibition was observed. Our real-time BIO-PCR assay, with RSC primers and probe and enrichment on mSMSA, is somewhat less sensitive than the liquid BIO-PCR technique of Weller et al. (2000b), which uses the highly sensitive 16S rDNA primers and probe. However, the 16S primers and probe are not specific to bv. 2 strains. Furthermore, that technique was only tested with inoculated tuber extracts; no naturally infected tubers were evaluated. Liquid BIO-PCR has an advantage of being easier and less open to possible contamination than an agar-based protocol, however, the latter is normally found to be more sensitive (N.W. Schaad, personal observations). The reason for the higher sensitivity is, most likely, the greater efficiency of the agar medium in absorbing any PCR inhibitors that may be present. The 24- to 30-h incubation period for *R. solanacearum*, needed to produce pinpoint-size colonies on mSMSA, is much faster than a BIO-PCR technique for the slow-growing potato ring rot organism (Schaad et al. 1999). However, we find that the geranium bv. 2 strains tend to grow somewhat slower and that 36 to 40 h of incubation works better (data not presented). The real-time BIO-PCR assay, described here, is capable of detecting *R. solanacearum* bv. 2 in asymptomatic potato tubers and should be especially suitable for quarantine and seed certification programs. Since our results showed no difference between boiled and nonboiled samples, our final assay does not include boiled samples prior to PCR.

This is the first time that the brown rot organism is detected in naturally infected asymptomatic tubers with PCR. The portable Smart Cycler has several advantages, including an extremely fast run time, 16 separate reaction chambers for quick optimization of protocols, portability, and reasonable costs. With direct real-time PCR, a run time of only 20 min is needed for on-site diagnosis of Pierce's disease of grape caused by *Xylella fastidiosa* Wells, Raju, Hung, Weisburg, Mandelco-Paul & Brenner (Schaad et al. 2002). Watermelon fruit blotch can be diagnosed in 1 h or less, including sampling time (Schaad et al. 2000). A direct real-time PCR assay for brown rot could be completed in 1 to 2 h, whereas BIO-PCR requires an extra 24–30 h. However, direct PCR is considerably less sensitive then BIO-PCR (Elphinstone et al. 1996; Schaad et al. 1995, 1999; Weller et al. 2000b). With a highly regulated organism such as *R. solanacearum* bv. 2, both sensitivity and specificity are of great importance. Although the available RS primers and probe are more sensitive than the RSC and B2 primers and probes, they cross-react with several other bacteria, including the blood disease bacterium (Seal et al. 1993; Weller et al. 2000a).

An assay protocol for seed potatoes should be quick, but even so, sensitivity and specificity are probably more important than speed when we consider the role of latent tuber infection in the dissemination of such an highly regulated organism as *R. solanacearum*. If speed is critical, direct PCR could be conducted first and, if results are positive, the BIO-PCR assay would not be needed unless a viable culture was desired. If so, mSMSA is very reliable for the isolation of *R. solanacearum* from asymptomatic tubers, however, 4 to 5 days are required for incubation. Preliminary data shows that the BIO-PCR assay works well for detection in geranium as well as potato (Nikolaeva et al. 2003).

The following simplified real-time BIO-PCR assay protocol is recommended for assaying potato tubers:

(i) Extract core tissue from 200 tubers by soaking in 25 mL of buffer for 4 h; retain 1 mL of sample.
(ii) Plate 100 µL of extract onto each of five plates of mSMSA and incubate at 28°C.
(iii) Do direct real-time PCR, using RSC primers and probe with duplicate 10-µL samples.
(iv) If direct PCR is negative, wash each of three plates with 1 mL of water after 24–30 h of incubation and use 2 × 10 µL of each plate for PCR.
(v) After 5 days, observe the other two plates of mSMSA for growth of colonies of *R. solanacearum*.
(vi) Clone the suspect colonies by streaking onto mSMSA and conduct pathogenicity tests.

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