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An improved enrichment broth for the sensitive detection of *Ralstonia solanacearum* (biovars 1 and 2A) in soil using DAS–ELISA

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A reliable, sensitive, low-cost and easy-to-use technique is described for the detection of *Ralstonia solanacearum* (the causal organism of bacterial wilt, BW) in soil. A total of 273 potato isolates belonging to five different biovars (Bv), originating from 33 countries worldwide, were tested and successfully detected by antibodies produced at the International Potato Center (CIP). Isolates of *R. solanacearum* belonging to Bv1 and Bv2A were successfully detected by double antibody sandwich–enzyme-linked immunosorbent assay (DAS–ELISA) at low population levels after incubation of soil suspensions for 48 h at 30°C in a new semiselective broth containing a potato tuber infusion. Detection thresholds of 20 and 200 CFU g^{-1} inoculated soil were obtained for Bv1 and Bv2A, respectively. Sensitivity of detection of Bv2A was similar or even higher in five different inoculated soil types. No cross-reactions were obtained in DAS–ELISA after enrichment of soil suspensions (i) prepared from 23 different soils sampled in BW-free areas in six departments of Peru; and (ii) inoculated with 10 identified bacteria and 136 unknown isolates of soil microbiota isolated from eight different locations. Only the blood disease bacterium gave a low-level reaction after enrichment. In naturally infested soils, average sensitivities of 97·6 (SE 14·8) and 100·9 (SE 22·6) CFU g^{-1} were obtained for biovars 1 and 2A, respectively. By making serial dilutions of the soil suspension before enrichment, densities of *R. solanacearum* could be determined in a semiquantitative way. Results also showed that composite samples of five soils could be analysed to assess field soil populations without reducing detection sensitivity.

Keywords: bacterial wilt, ELISA, detection, potato, *Ralstonia solanacearum*, selective medium, soil

Introduction

Ralstonia solanacearum is the causal organism of bacterial wilt (BW), a disease that, after late blight, is the second major constraint to potato production in tropical and subtropical regions worldwide (French, 1994). The use of healthy planting material and reduction of soilborne inoculum through crop rotation are the most effective means of controlling this disease (Hayward, 1991; French, 1994). Although several procedures for monitoring seed-tuber infection have been developed and are used routinely worldwide (Janse, 1988; Priou *et al*., 1999, 2001; Anonymous, 2004), understanding of disease epidemiology, and consequently the effectiveness of BW management practices, have been hampered by the absence of a reliable and suitable technique for routine detection and quantification of soil populations of *R. solanacearum*. Detection in soil is complicated by the low densities of the pathogen in soil, and high microbial activity and irregular spatial distribution in the field (van der Wolf *et al*., 1998, 2000). Furthermore, sensitive detection of *R. solanacearum* in field conditions is likely to depend on sampling strategy and extraction methods, as well as on the detection method used (Pradhanang *et al*., 2000).

The most widely used method to detect *R. solanacearum* in soil consists of streaking soil suspension on a specific medium (Granada & Sequeira, 1983; Englebrecht, 1994; Elphinstone *et al*., 1996). However, the sensitivity of the plating technique is variable between soil samples (10^2–10^3 CFU g^{-1}) because antagonistic soil microflora often overgrow or impede growth of *R. solanacearum* (van der Wolf *et al*., 1998; Pradhanang *et al*., 2000). Furthermore, application of this microbiological method requires skilled personnel who can distinguish target colonies from other bacterial saprophytes that grow on the medium, thus limiting extensive use of the method.

The use of indicator plants such as tomato has been reported, but the sensitivity of such bioassays is low: 10^4–10^5 CFU g^{-1} soil (Graham & Lloyd, 1978; Elphinstone

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et al., 1996; Pradhanang *et al*., 2000; van der Wolf *et al*., 2000). Serological techniques have also been developed, such as indirect ELISA (Robinson-Smith *et al*., 1995), but this lacked sensitivity (10^4 CFU g^{-1} soil) and specificity (Pradhanang *et al*., 2000). Using immunofluorescence colony staining, van der Wolf *et al*. (2000) detected lower levels of *R. solanacearum* (100 CFU g^{-1} soil) but, because of the high cross-reaction rate, it was necessary to confirm the identities of fluorescent colonies using PCR techniques. Immunocapture followed by PCR detection was also reported recently, leading to a detection threshold of 10^4 CFU g^{-1} soil (Dittapongpitch & Surat, 2003). The sensitivity of serological methods for the detection of *R. solanacearum* in potato or in soil can be increased by applying an enrichment procedure, for example by incubating extracts in a selective broth (Elphinstone *et al*., 1996; van der Wolf *et al*., 1998; Priou *et al*., 1999; Pradhanang *et al*., 2000; Caruso *et al*., 2002). However, there is a risk that the incubation procedure will increase populations of saprophytic bacteria in soil suspensions, leading to false-positive reactions in ELISA, especially when using polyclonal antibodies (Pradhanang *et al*., 2000; Caruso *et al*., 2002).

Methods based on PCR amplification using *R. solanacearum*-specific primers have recently been widely investigated for detection of soil populations because of their potentially higher specificity, but most procedures combine both PCR amplification and microbiological methods. Pradhanang *et al*. (2000) detected as few as 10^2 CFU g^{-1} soil by performing a two-step nested PCR after overnight enrichment of the soil suspension in semi-selective agar (SMSA) medium (Englebrecht, 1994) as modified by Elphinstone *et al*. (1996). Lee & Wang (2000) obtained a detection sensitivity of 2×10^3 CFU g^{-1} inoculated soil by performing PCR using the DNA extracted from soil as a template. Greater sensitivity (100 CFU g^{-1} soil) could be obtained by incubating inoculated soil suspensions on a specific medium, named PCCG, before conducting PCR on DNA extracted from the isolated colonies (Ito *et al*., 1998). Schönfeld *et al*. (2003) used PCR amplification followed by Southern blot hybridization, allowing them to detect approximately one target DNA molecule per PCR, equivalent to 10^3 CFU g^{-1} bulk soil. However, most of the sensitivities reported may be overestimated, as they were obtained using suspensions from inoculated soils, not naturally infested soils. Moreover, the techniques involving DNA extraction and/or PCR amplification are often not suitable for routine use in seed programmes or for bacterial wilt research in developing countries: such tests would be too costly and labour-intensive considering the number of samples to be tested per field, and the risk of obtaining false positives caused by cross-contamination would be too high under these conditions.

Here is described a sensitive and reliable procedure for qualitative or semiquantitative detection of *R. solanacearum* (biovars 1 and 2A) in soil after selective enrichment in an improved semispecific broth developed at the International Potato Center (CIP). Enriched soil suspensions were analysed in double-antibody sandwich enzyme-linked immunosorbent assay (DAS–ELISA) using polyclonal antibodies produced at CIP. The detection specificity of postenrichment DAS–ELISA was assessed using identified bacteria, a large collection of unknown soil saprophytes and soils sampled in BW-free areas. The sensitivity of the procedure was compared with growing on modified SMSA medium and with DAS–ELISA after enrichment in modified SMSA broth (Elphinstone *et al*., 1996) using samples from both inoculated and naturally infested soils. Sensitivity of detection of *R. solanacearum* (Bv2A) in different soil types and in composite samples of infested and noninfested soils was also assessed.

Materials and methods

Preparation of soil suspensions

Soil suspensions were prepared by mixing 10 g soil at around field capacity with 90 mL phosphate-buffered saline (PBS). The soil suspension was agitated for 30 min at room temperature (24–28°C) and allowed to settle for 40 s. The supernatant was used for detection either directly, or after enrichment as described below.

Selective enrichment

Enriched soil suspensions were prepared by mixing, in an Erlenmeyer flask, 1 mL of the original soil suspension with 19 mL of the new CIP enrichment broth (CIPEB) or 19 mL of modified SMSA broth (M-SMSA; Elphinstone *et al*., 1996) and by incubating the mixture for 48 h at 30°C with constant agitation (170 r.p.m.). Tenfold dilutions of the original soil suspension in sodium citrate buffer (0·1 M citric acid, 0·1 M sodium citrate pH 5·6) were also prepared and 1 mL of each diluted soil suspension was mixed with 9 mL enrichment broth and incubated as described above. Dilutions of soil suspensions in sodium citrate buffer improved the detection efficiency in DAS–ELISA compared with dilutions in water (data not shown). CIPEB was prepared by adding filter-sterilized solutions of 100 mg L^{-1} polymyxin B sulphate, 25 mg L^{-1} bacitracin, 0·5 mg L^{-1} penicillin-G, 5 mg L^{-1} chloramphenicol, 5 mg L^{-1} crystal violet, 50 mg L^{-1} 2,3,5-triphenyl tetrazolium chloride (TTC), 100 mg L^{-1} cycloheximide, and 62·5 mg L^{-1} vitamin C to 1 L of the basal broth (previously sterilized and cooled to 50°C). One litre of the basal broth contained 1 L potato-tuber infusion (prepared by boiling 200 g unpeeled potato cv. Perricholi tubers, susceptible to bacterial wilt, for 10 min in 1 L distilled water and filtering the potato juice), 10 g Bacto peptone (Difco), 1 g casamino acids (Difco) and 2·5 g dextrose.

DAS–ELISA

Polyclonal antibodies were produced at CIP, applying a long immunization schedule as reported by Priou *et al*. (1999). DAS–ELISA was performed as described by Clark & Adams (1977) using the immunoglobulins (IgG) purified from the *R. solanacearum*-specific polyclonal rabbit antiserum produced at CIP. The IgG were conjugated to

alkaline phosphatase and diluted 1 : 1500 for detection. Microtitre plates (Bioreba AG) were coated and incubated with the IgG for 4 h at 37°C, and with the samples overnight at 4°C. Absorbance at 405 nm was determined using a Bio-Rad Model 2550 plate reader following 1 h incubation at 24–28°C. As suggested by Clark (1981) and Crowther (2001), the upper negative limit was considered to be the mean of the negative (BW-free soils) values plus three standard deviations. As a standard deviation of 0·016 was obtained on 99 negative values obtained from three BW-free soils (mean OD_{405} = 0·104; SE = 0·002), ELISA readings were considered positive when OD_{405} exceeded the negative control readings by >0·05.

Specificity of antiserum

A total of 273 potato isolates of *R. solanacearum* from the CIP collection were used to check the specificity of the antibodies. Isolates of five different biovars (50 of Bv1, 177 of Bv2A, 26 of Bv2T, 14 of Bv3, six of Bv4), originating from 33 countries worldwide (33 from Africa, 42 from Asia, 17 from Australia, two from Europe, 176 from Latin America and three from USA) were tested. Suspensions of the 10 identified non-*R. solanacearum* bacterial strains listed in Table 1, and 136 unknown bacterial saprophytes, isolated before enrichment (49 isolates) and after enrichment in CIPEB (87 isolates) from soil suspensions prepared from eight soils from different parts of Peru, were tested in DAS–ELISA in sodium citrate buffer suspensions containing 10^8 CFU mL^{-1}. To assess the specificity of DAS–ELISA after enrichment, these 146 isolates were inoculated into soil suspensions at a final concentration of 10^6 CFU mL^{-1} and enriched as described previously. Bacterial suspensions were prepared by culturing for 48 h at 30°C on modified Kelman's medium (French *et al.*, 1995), but without TTC. The cells were harvested in sterile water and the bacterial concentration was evaluated by measuring the optical density (OD) at 600 nm of a diluted aliquot. An OD_{600} of 0·1 was equivalent to 2×10^8 CFU mL^{-1}.

Twenty-three different soils sampled from BW-free areas in six departments in Peru (Table 2) were also analysed to confirm the absence of cross-reactions in DAS–ELISA. Enriched soil solutions were prepared as described above.

Detection sensitivity with extracts from inoculated soils

Suspensions of *R. solanacearum* were prepared by culturing strains CIP204 (Bv2A) and CIP308 (Bv1) for 48 h at 30°C on modified Kelman's medium (French *et al.*, 1995), but without TTC, and inoculum was prepared as described above. For assessing detection sensitivity, a nonsterile *R. solanacearum*-free clay loam soil from Chinchao (3070 m a.s.l., Huánuco Department, Peru) was inoculated by mixing 10 mL diluted water suspensions of the *R. solanacearum* strain with 90 g soil to obtain a moisture content around field capacity and final concentrations of 2×10^7, 2×10^6, 2×10^5, 2×10^4, 2×10^3, 2×10^2, 20, 2 and 0·2 CFU g^{-1} wet soil. Three replicates of each concentration of soil

Table 1 Mean absorbance at 405 nm obtained in DAS–ELISA with *Ralstonia solanacearum* and other bacterial strains in pure culture in sodium citrate buffer at 10^8 CFU mL^{-1} and after incubation for 48 h at 30°C in CIP enrichment broth (CIPEB), from an original concentration of 10^6 CFU mL^{-1} soil suspension

		Without enrichment		After enrichment in CIPEB	
Species	Strain	OD[a]	SE[b]	OD	SE
Pectobacterium carotovorum ssp. *carotovorum*	CIP 400	0·087	0·002	0·081	0·001
Pectobacterium atrosepticum	CIP 421	0·082	0·000	0·085	0·002
Pectobacterium chrysanthemi	CIP 367	0·093	0·011	0·088	0·003
Ralstonia syzygii	NCPPB 3792	2·903	0·017	0·111	0·001
Ralstonia pickettii	NCPPB 3899	0·084	0·002	0·090	0·003
Burkholderia cepacia	NCPPB 2993	0·092	0·003	0·091	0·002
Pseudomonas aeruginosa	NCPPB 1965	0·129	0·030	0·102	0·004
Pseudomonas putida	NCPPB 1806,	0·119	0·017	0·119	0·020
	NCPPB 1808	0·090	0·002	0·099	0·003
Blood disease bacterium	UW 443	0·131	0·004	0·218	0·006
Unknown bacteria isolated from five different soils before enrichment	49 isolates	0·083	0·031	0·088	0·002
Unknown bacteria isolated from eight different soils after enrichment	87 isolates	0·093	0·001	0·096	0·001
Ralstonia solanacearum	CIP 204[c]	2·325	0·051	1·746	0·106
Noninfested soil		0·111	0·003	0·099	0·003
Sodium citrate buffer		0·098	0·001	0·090	0·002

[a]ELISA readings ($OD_{405\,nm}$) 1 h after addition of substrate at room temperature. Data are means of three replications, except for unknown bacteria for which data are means of OD_{405} obtained with each of the 49 and 87 isolates. Results were considered positive when OD_{405} exceeded negative control readings by >0·05.

[b]Standard error of mean.

[c]Although the absorbance obtained with only one strain of *R. solanacearum* is shown, similar values were obtained with the other 272 potato isolates tested.

Table 2 Mean absorbance at 405 nm obtained in DAS–ELISA with 23 different soils sampled from bacterial wilt-free areas in Peru without (–E) and with (+E) enrichment in CIP enrichment broth (CIPEB) for 48 h at 30°C

Department/locality	Altitude (m a.s.l.)	Soil type[a]	Soil pH	Without enrichment		After enrichment in CIPEB	
				OD[b]	SE[c]	OD	SE
Cerro de Pasco/Ninacaca	3920	SCL	7·4	0·127	0·004	0·132	0·001
Cerro de Pasco/Cerro de Pasco	3933	L	6·7	0·151	0·008	0·131	0·006
Cerro de Pasco/Villa de Pasco	3980	L	5·9	0·156	0·006	0·125	0·001
Junín/Carhuamayo	3885	SL	4·8	0·143	0·006	0·130	0·008
Junín/Huaripampa	3245	C	7·6	0·121	0·002	0·120	0·003
Junín/Muquiyauyos	3250	CL	7·9	0·139	0·004	0·132	0·002
Junín/Orcotuna	3195	L	7·9	0·122	0·003	0·115	0·004
Junín/Muquiyauyo	3225	C	7·8	0·126	0·003	0·130	0·005
Junín/Jauja	3210	L	7·4	0·120	0·002	0·113	0·001
Junín/Jauja	3210	L	7·9	0·111	0·002	0·108	0·000
Junín/Yanachacra	3310	SiL	7·8	0·120	0·002	0·119	0·006
Junín/Sincos	3200	L	7·8	0·136	0·005	0·130	0·008
Junín/Casapalca	4200	SL	7·9	0·131	0·003	0·131	0·003
Junín/Tambo	3100	CL	7·6	0·134	0·004	0·129	0·001
Cajamarca/Namora	2980	LS	4·4	0·122	0·003	0·123	0·004
Cajamarca/Chucmar	2675	SL	4·0	0·108	0·004	0·102	0·005
Ancash/Carhuaz	2810	SL	4·2	0·130	0·004	0·116	0·001
Ancash/Carhuaz	2810	SL	5·9	0·126	0·006	0·102	0·001
Huánuco/Chinchao	2345	SL	4·6	0·124	0·005	0·126	0·006
Huánuco/San Marcos	2551	L	5·1	0·099	0·001	0·095	0·004
Lima/La Molina	500	SL	7·7	0·091	0·005	0·076	0·000
Lima/Quilmaná	151	SL	6·9	0·127	0·003	0·152	0·025
Lima/San Benito	151	SL	7·2	0·083	0·002	0·088	0·006
Ralstonia solanacearum		10^8		2·286	0·003		
CIP204 (Bv2A) in PBS buffer		10^7		2·253	0·021		
(CFU mL^{-1})		10^6		1·745	0·102		
		10^5		0·284	0·025		
Average negative controls[d]				0·104	0·002	0·098	0·002
PBS buffer				0·089	0·001	0·085	0·002

[a]SCL = sandy clay loam; SL = sandy loam; L = loamy; LS = loamy sand; C = clay; CL = clay loam; SiL = silty loam.
[b]ELISA readings ($OD_{405\ nm}$) 1 h after addition of substrate at room temperature. Data are means of three replications. Results were considered positive when OD_{405} exceeded negative control readings by >0·05.
[c]Standard error of mean.
[d]Averages of 99 different OD_{405} values obtained with three BW-free soils used as standard negative controls in the laboratory.

inoculum were prepared. Inoculated soils were kept at room temperature (20–28°C) for 24 h before use. Soil suspensions were prepared and used either directly for detection in DAS–ELISA, or after enrichment as described previously. Fifty μL of each soil suspension were cultured before enrichment on M-SMSA medium. Three Petri dishes per concentration were incubated for 48 h at 30°C, and colonies of *R. solanacearum* were counted to estimate the original soil population.

Sensitivity of detection of strain CIP204 (Bv2A) was also assessed following the same protocol for four BW-susceptible cultivars (Canchan, Désirée, Perricholi and Yungay) used to prepare the potato-tuber infusion included in the CIPEB.

Furthermore, the detection threshold was determined for six different *R. solanacearum*-free nonsaline and nonsterile soils from Peru with different textures, pH and organic matter contents (see Table 5), each inoculated with strain CIP204 (Bv2A), enriched and analysed following the protocol described previously.

Finally, to assess detection sensitivity in composite soil samples, inoculated soil samples containing strain CIP204 (Bv2A) at concentrations of 2×10^6, 2×10^5, 2×10^4, 2×10^3, 2×10^2 and 20 CFU g^{-1} soil were mixed with noninfested clay loam soil in proportions of 1 : 1 to 1 : 4 (w/w). Suspensions of each soil mixture were prepared, and the original soil suspension was enriched and analysed in DAS–ELISA for qualitative detection of *R. solanacearum*.

For the latter three experiments, covariance analysis of absorbance data at 405 nm obtained in DAS–ELISA was performed using the GLM procedure of SAS software (SAS Institute Inc., USA), the covariate being the population density of *R. solanacearum* expressed as log CFU g^{-1} inoculated soil.

Detection sensitivity with naturally infested soils

To assess the sensitivity of detection in field conditions, a total of 21 soil samples were taken from two

Bv2A-infested clay loam and loamy soils (Carhuaz, 2640 m a.s.l., Ancash Department; Malcas, 2120 m a.s.l., Cajamarca Department, Peru) and 21 soil samples from two fields with Bv1-infested sandy loam soil (San Ramon, 960 m a.s.l., Junin Department, Peru) in the rhizosphere of wilted and neighbouring symptomless plants of different potato genotypes. Soil samples *c*. 100 g were taken at *c*. 0–20 cm deep using a small shovel that was disinfected with 1% sodium hypochlorite solution and rinsed with tap water between samples. Soil samples were collected from the four borders of the hole, and for each sample *c*. 100 g soil were brought to the laboratory within 15 h and kept in open plastic bags at 15°C in a ventilated place. Soil moisture content was maintained at around field capacity by adding sterile water when necessary. Soil suspensions were prepared within 2–3 days after sampling, as previously described, and eight tenfold serial dilutions were made using sodium citrate buffer. Nondiluted and diluted soil suspensions were enriched as described previously and analysed in DAS–ELISA for the semiquantitative detection of *R. solanacearum*: the highest dilution at which the pathogen was detected in DAS–ELISA after enrichment in CIPEB or M-SMSA broth was recorded. Fifty μL of the 10^{-1} to 10^{-3} dilutions of soil suspensions were transferred to three Petri dishes containing M-SMSA medium before enrichment, and incubated for 48 h at 30°C. Colonies of *R. solanacearum* were counted to estimate the original soil population.

Results

Specificity of antiserum

All 273 isolates of *R. solanacearum* tested (of five different biovars, Bv1, 2A, 2T, 3 and 4, and originating from 33 countries worldwide) were recognized by the polyclonal antibodies (data not shown). From the 10 strains tested identified as not *R. solanacearum*, only the blood disease bacterium gave a low-level reaction in DAS–ELISA after enrichment. *Pseudomonas syzygii*, the agent of Sumatra disease of clove, also present only in Indonesian soils, cross-reacted with the antibodies when in pure culture, but not after enrichment of the inoculated soil suspension in CIPEB (Table 1). No cross-reaction was observed in DAS–ELISA without or after enrichment of soil suspensions inoculated with the 136 soil saprophytes tested (Table 1). Negative reactions were also obtained in DAS–ELISA with enriched soil solutions prepared from 23 different soils sampled in BW-free areas in six departments in Peru (Table 2). Although the values obtained with some soil solutions were higher than those obtained with the soils generally used as negative controls, no OD_{405} value exceeded the negative control readings by >0·05 to be considered as positive. Moreover, the absorbance at 405 nm of all soil samples did not increase significantly after enrichment (P = 0·05; Table 2).

Detection sensitivity with inoculated soil extracts

The minimum population of *R. solanacearum* (Bv2A and Bv1) detected in DAS–ELISA was 10^5 CFU m mL^{-1} water or sodium citrate buffer (data not shown). In soil suspensions, the detection threshold decreased to 2×10^6 CFU g^{-1} soil for both biovars (Table 3). The sensitivity of DAS–ELISA was increased significantly by the enrichment procedure in CIPEB: as few as 20 and 200 CFU g^{-1} soil could be detected from soils inoculated with Bv1 and Bv2A strains, respectively, whereas the detection threshold obtained using the M-SMSA broth for enrichment was

Table 3 Sensitivity of detection of *Ralstonia solanacearum* (*Rs*) CIP 308 (Bv1) and CIP 204 (Bv2A) transferred modified SMSA (M-SMSA) medium and by DAS–ELISA, without and with incubation of suspensions prepared with inoculated clay loam soil in M-SMSA broth or in CIP enrichment broth (CIPEB) for 48 h at 30°C

Test[a]	Biovar of *Rs*	CFU of *Rs* inoculated g^{-1} soil									
		NI[b]	0·2	2	20	2×10^2	2×10^3	2×10^4	2×10^5	2×10^6	2×10^7
Percentage recovery of *Rs* after transfer to M-SMSA without enrichment[c]	1	0	0	0	0	0	32·51 (3·25)	45·52 (8·60)	56·26 (1·17)	73·17 (2·82)	78·05 (5·63)
	2A	0	0	0	0	0	34·39 (14·73)	60·84 (9·54)	66·13 (7·00)	67·46 (3·97)	79·63 (4·11)
DAS–ELISA without enrichment[d]	1	–	–	–	–	–	–	–	–	+	+
	2A	–	–	–	–	–	–	–	–	+	+
Cultured on M-SMSA with enrichment in M-SMSA broth	1	–	–	–	–	–	–	+	+	+	+
	2A	–	–	–	–	–	–	+	+	+	+
DAS–ELISA with enrichment in M-SMSA broth[d]	1	–	–	–	–	–	–	+	+	+	+
	2A	–	–	–	–	–	–	+	+	+	+
Cultured on M-SMSA with enrichment in CIPEB	1	–	–	–	–	–	+	+	+	+	+
	2A	–	–	–	–	–	+	+	+	+	+
DAS–ELISA with enrichment in CIPEB[d]	1	–	–	–	+	+	+	+	+	+	+
	2A	–	–	–	–	+	+	+	+	+	+

[a]Results obtained from three replications of inoculated soil.
[b]Noninoculated soil.
[c]Standard error of mean shown in parentheses.
[d]–, Negative; +, positive in DAS–ELISA.

2×10^4 CFU g^{-1} soil for both biovars (Table 3). Sensitivity of culturing on M-SMSA without enrichment was 2×10^3 CFU g^{-1} soil for both biovars. However, percentage recovery of *R. solanacearum* on M-SMSA medium averaged 79·6 and 78% for Bv2A and Bv1, respectively, with bacterial populations of 2×10^7 CFU g^{-1} soil, but decreased to *c.* 30% for both biovars with an inoculum density of 2×10^3 CFU g^{-1} soil, close to the detection limit (Table 3). After enrichment in CIPEB, sensitivity of culturing was improved tenfold compared with enrichment in M-SMSA broth (Table 3). However, the colonies of *R. solanacearum* were hardly visible at the threshold concentration because many saprophytic bacteria overgrew them on the agar medium. Thus it is recommended that soil suspensions should be incubated on agar plates before enrichment.

Sensitivity of detection of *R. solanacearum* (Bv2A) was similar (200 CFU g^{-1} soil) when cvs Perricholi, Désirée and Canchan were used to prepare the potato-tuber infusion included in the CIPEB, but sensitivity was lower (2×10^3 CFU g^{-1} soil) with cv. Yungay (Table 4). When comparing six different nonsterile soils, detection thresholds ranged between 20 and 2×10^3 CFU g^{-1} soil (Table 5).

Ralstonia solanacearum (Bv2A) was detected after enrichment of original soil suspensions prepared from mixtures of infested and noninfested clay loam soil in proportions up to 1 : 4 (w/w) when the pathogen concentration in the inoculated soil ranged from 2×10^6 to 2×10^2 CFU g^{-1} (Table 6). Moreover, the absorbance obtained after enrichment of soil mixtures up to 1 : 4 did

Table 4 Sensitivity of detection of *Ralstonia solanacearum* (Bv2A) by DAS–ELISA after incubation of artificially inoculated clay loam soil solutions in CIP enrichment broth (CIPEB) for 48 h at 30°C, according to the potato cultivar used for broth preparation

Log CFU *R. solanacearum* inoculated g^{-1} soil	Cultivar			
	Perricholi	Désirée	Yungay	Canchan
6·301	2·453[a]	2·181	2·284	2·410
	(0·014)	(0·047)	(0·046)	(0·007)
5·301	2·454	2·060	2·362	2·414
	(0·008)	(0·019)	(0·013)	(0·007)
4·301	2·446	2·342	0·783	2·392
	(0·001)	(0·022)	(0·031)	(0·020)
3·301	1·095	2·041	**0·174**	0·833
	(0·398)	(0·038)	**(0·014)**	(0·120)
2·301	**0·389**	**0·550**	0·113	**0·209**
	(0·047)	**(0·032)**	(0·002)	**(0·026)**
1·301	0·145	0·134	0·103	0·170
	(0·035)	(0·008)	(0·003)	(0·026)
0·301	0·102	0·111	0·102	0·112
	(0·008)	(0·018)	(0·002)	(0·019)
Noninoculated soil	0·112	0·120	0·100	0·110
	(0·002)	(0·015)	(0·003)	(0·010)

[a]ELISA reading ($OD_{405\,nm}$) 1 h after addition of substrate at room temperature; means of three replications of inoculated soils. Results were considered positive (bold type) when OD_{405} exceeded negative control readings by >0·05. Standard error of mean shown in parentheses. Potato variety factor was highly significant ($P = 0·0036$), MSE = 0·1972 (df = 91).

Table 5 Sensitivity of detection of *Ralstonia solanacearum* (*Rs*) (Bv2A) by DAS–ELISA after incubation of artificially inoculated soil solutions in CIP enrichment broth (CIPEB) for 48 h at 30°C according to soil type

		Soil type					
		Clay loam	Sandy clay loam	Loamy	Sandy loam	Sandy loam	Loamy sand
	pH	7·6	7·4	7·4	7·9	4·8	4·4
Log CFU *Rs*	OM[a]	2·9	1·2	4·6	1·1	5·1	2·4
inoculated g^{-1} soil	EC[b]	0·86	0·30	0·54	0·25	0·43	0·43
6·301		2·453[c]	2·292	2·440	2·465	2·251	2·437
		(0·014)	(0·019)	(0·018)	(0·020)	(0·025)	(0·003)
5·301		2·454	2·305	2·436	2·389	2·163	2·433
		(0·008)	(0·187)	(0·019)	(0·070)	(0·043)	(0·002)
4·301		2·446	2·323	2·438	1·590	2·202	2·391
		(0·001)	(0·025)	(0·014)	(0·061)	(0·023)	(0·008)
3·301		1·095	2·291	2·082	**0·204**	2·318	0·480
		(0·398)	(0·020)	(0·185)	**(0·003)**	(0·022)	(0·063)
2·301		**0·389**	2·292	**0·381**	0·149	1·566	**0·215**
		(0·047)	(0·018)	**(0·048)**	(0·029)	(0·188)	**(0·005)**
1·301		0·145	**2·271**	0·130	0·115	**0·387**	0·156
		(0·035)	**(0·009)**	(0·014)	(0·009)	**(0·052)**	(0·013)
0·301		0·102	0·156	0·095	0·119	0·141	0·148
		(0·008)	(0·014)	(0·003)	(0·002)	(0·006)	(0·013)
Non-inoculated soil		0·112	0·132	0·096	0·129	0·143	0·108
		(0·002)	(0·001)	(0·009)	(0·004)	(0·007)	(0·009)

[a]Organic matter content (% weight).

[b]Electrical conductivity = salinity (dS m^{-1}).

[c]ELISA reading ($OD_{405\,nm}$) 1 h after addition of substrate at room temperature, means of three replications of inoculated soils. Standard error of mean shown in parentheses. Results were considered positive (bold type) when OD_{405} exceeded negative control readings by >0·05. The soil-type factor was highly significant ($P < 0·0001$), MSE = 0·2505 (df = 137).

Table 6 Detection of *Ralstonia solanacearum* (Bv2A) by postenrichment DAS–ELISA in bulk samples containing mixtures of inoculated with noninfested clay loam soil in proportions up to 1 : 4

Log CFU *R. solanacearum* inoculated g^{-1} soil	Ratio inoculated : noninfested soil (w/w)	OD[a]	SE[b]
6·301	1 : 0	**2·079**	0·226
	1 : 1	**1·617**	0·080
	1 : 2	**2·027**	0·075
	1 : 3	**2·282**	0·047
	1 : 4	**2·128**	0·079
5·301	1 : 0	**1·670**	0·202
	1 : 1	**1·479**	0·245
	1 : 2	**1·309**	0·218
	1 : 3	**1·382**	0·080
	1 : 4	**2·100**	0·181
4·301	1 : 0	**2·091**	0·239
	1 : 1	**2·444**	0·041
	1 : 2	**2·505**	0·136
	1 : 3	**2·555**	0·126
	1 : 4	**2·664**	0·003
3·301	1 : 0	**2·361**	0·418
	1 : 1	**1·898**	0·484
	1 : 2	**1·931**	0·419
	1 : 3	**1·421**	0·605
	1 : 4	**1·468**	0·505
2·301	1 : 0	**1·944**	0·645
	1 : 1	**0·964**	0·682
	1 : 2	**0·371**	0·082
	1 : 3	**0·973**	0·368
	1 : 4	**0·414**	0·124
1·301	1 : 0	**0·881**	0·341
	1 : 1	**0·254**	0·029
	1 : 2	0·159	0·013
	1 : 3	0·132	0·003
	1 : 4	0·157	0·021
Non-infested soil		0·124	0·007

[a]ELISA readings ($OD_{405\,nm}$) 1 h after addition of substrate at room temperature; values are means of three replications. Results were considered positive (bold type) when OD_{405} exceeded negative control readings by >0·05. ELISA readings in bold were not significantly different at $P = 0{\cdot}05$; the covariate (log CFU g^{-1} soil) was highly significant ($P < 0{\cdot}0001$), MSE = 0·5213 (df = 82).
[b]Standard error of mean.

not decrease significantly ($P = 0{\cdot}3509$) compared with that obtained after enrichment of the inoculated soil. However, when the pathogen concentration in soil was 20 CFU g^{-1} it was detected only up to the 1 : 1 mixture.

Detection sensitivity with naturally infested soil

In naturally infested soils, the minimum population sizes detected averaged 101 and 98 CFU g^{-1} soil after enrichment in CIPEB for Bv2A and Bv1, respectively (Table 7). The pathogen was undetected in many samples when M-SMSA broth was used for enrichment, especially in Bv1-infested soil; the detection sensitivity with positive samples was 10- to 100-fold lower than when CIPEB was used for enrichment.

Discussion

Ralstonia solanacearum was successfully detected by postenrichment DAS–ELISA at low population levels in inoculated as well as naturally infested soils. As little as 20 and 200 CFU g^{-1} soil could be detected from inoculated soils for Bv1 and Bv2A, respectively. Sensitivity of detection of *R. solanacearum* Bv2A was similar when three BW-susceptible potato cultivars, the Peruvian cvs Perricholi and Canchan and the more widespread cv. Désirée, were used to prepare the enrichment broth. However, when the Peruvian cv. Yungay was used, which is less susceptible than the other three to BW, detection sensitivity decreased. This demonstrates that, although the method could be applied in other countries using different potato varieties, it is preferable to assess the detection sensitivity in local conditions to select the best cultivars for broth preparation. Perricholi was chosen for these experiments because it allowed a high detection sensitivity and is available all year round in Peru. Unfortunately, attempts to replace the potato broth by chemicals, to standardize the enrichment broth, were unsuccessful as sensitivity was always higher with the potato broth (data not shown).

In naturally infested soils, the detection limit was also greatly improved (10- to 100-fold) by incubating soil suspensions in CIPEB rather than M-SMSA broth, and in many cases *R. solanacearum* could not be detected after enrichment in M-SMSA broth, whereas it was successfully detected using CIPEB. The sensitivity of detection of soil populations of *R. solanacearum* in DAS–ELISA was considerably increased by the enrichment procedure, relative to that reported by Robinson-Smith *et al.* (1995), who obtained a sensitivity of 10^4 CFU g^{-1} soil using indirect ELISA. The sensitivity obtained in DAS–ELISA after enrichment in CIPEB was much higher than that reported by Pradhanang *et al.* (2000), who reported a detection level of 10^4 CFU g^{-1} for inoculated soil and 10^6 CFU g^{-1} for naturally infested soil using indirect ELISA after enrichment of soil suspensions in M-SMSA broth. The sensitivity reported in the present study is similar that obtained by van der Wolf *et al.* (2000), who reported a detection threshold of 10^2 CFU g^{-1} inoculated soil using immunofluorescence colony staining after growth of soil samples in an improved agar medium. However, the high rate of cross-reactions with saprophytic bacteria in immunofluorescence colony staining makes it necessary to perform PCR amplification to confirm the identities of fluorescent colonies. In contrast, in DAS–ELISA no nonspecific reaction was obtained after enrichment of a large collection (136 isolates) of bacterial saprophytes from eight soils from different parts of Peru, including 87 bacterial isolates that grew in CIPEB. Moreover, no cross-reaction was obtained with 23 different soils from BW-free areas in six departments in Peru. These results contradict many authors' expectation of a low specificity of serological detection of *R. solanacearum* in tubers and soil when using polyclonal antibodies, especially after enrichment (Pradhanang *et al.*, 2000; Caruso *et al.*, 2002). However, detection specificity is often tested by analysing pure

Table 7 Comparative detection of *Ralstonia solanacearum* (*Rs*) in suspensions of naturally infested sandy loam and loamy soils by DAS–ELISA with enrichment in modified SMSA (M-SMSA) broth or CIP enrichment broth (CIPEB) for 48 h at 30°C

Original population in soil isolated on M-SMSA medium[a]		Estimated original population in soil[c]	Highest dilution	Highest	Estimated min. population
(log CFU g^{-1})	SE[b]	(log CFU g^{-1})	M-SMSA[e]	dilution CIPEB[f]	CIPEB[g] (CFU g^{-1})
Biovar 1-infested soils					
3·358	0·046	3·846	ND[d]	10^{-2}	70·2
4·094	0·043	4·436	ND	10^{-3}	27·3
4·491	0·012	4·833	ND	10^{-3}	68·1
4·739	0·087	5·081	10^{-1}	10^{-3}	120·5
3·991	0·072	4·479	10^{-1}	10^{-2}	301·4
4·341	0·035	4·683	ND	10^{-3}	48·2
4·039	0·035	4·381	ND	10^{-3}	24·0
4·524	0·064	4·866	ND	10^{-3}	73·5
4·588	0·092	4·930	ND	10^{-3}	85·1
4·753	0·123	5·095	ND	10^{-3}	124·5
4·755	0·018	5·097	ND	10^{-3}	125·0
3·886	0·014	4·374	ND	10^{-2}	236·7
4·451	0·123	4·793	ND	10^{-3}	62·1
4·508	0·076	4·850	ND	10^{-3}	70·8
4·301	0·000	4·643	10^{-1}	10^{-3}	44·0
3·539	0·196	4·027	ND	10^{-2}	106·4
3·650	0·285	4·138	ND	10^{-2}	137·4
4·627	0·306	4·969	10^{-1}	10^{-3}	93·1
3·301	0·000	3·789	ND	10^{-2}	61·5
3·452	0·123	3·794	ND	10^{-2}	62·2
3·690	0·318	3·846	ND	10^{-2}	107·6
Mean (SE)[b]					97·6 (14·8)
Biovar 2A-infested soils					
4·113	0·027	4·329	ND[d]	10^{-2}	213·4
3·452	0·122	3·915	ND	10^{-3}	8·2
4·142	0·051	4·358	10^{-2}	10^{-3}	22·8
5·064	0·131	5·244	10^{-3}	10^{-3}	175·3
5·267	0·010	5·447	10^{-3}	10^{-4}	28·0
5·352	0·008	5·532	10^{-2}	10^{-4}	34·0
4·717	0·094	4·933	10^{-1}	10^{-3}	85·7
6·085	0·029	6·256	10^{-3}	10^{-5}	18·0
3·778	0·000	4·241	10^{-2}	10^{-3}	17·4
4·841	0·195	5·057	10^{-2}	10^{-4}	11·4
3·602	0·000	4·065	10^{-1}	10^{-2}	116·3
6·293	0·025	6·464	10^{-2}	10^{-4}	290·9
6·367	0·087	6·538	10^{-3}	10^{-4}	344·9
3·301	0·000	3·764	10^{-1}	10^{-2}	58·1
6·367	0·033	6·538	10^{-2}	10^{-5}	34·5
2·823	0·000	3·286	ND	10^{-1}	193·4
5·990	0·044	6·170	10^{-3}	10^{-4}	147·8
6·486	0·050	6·657	10^{-2}	10^{-5}	45·4
6·017	0·050	6·188	10^{-2}	10^{-5}	15·4
5·467	0·101	5·647	10^{-3}	10^{-4}	44·3
3·865	0·139	4·328	10^{-1}	10^{-2}	213·0
Mean (SE)[b]					100·9 (22·6)

[a]Estimated from colony counts after plating on three plates containing M-SMSA medium, incubated for 48 h at 30°C.
[b]Standard error of mean.
[c]Colony counts on M-SMSA divided by percentage recovery at each population level according to data in Table 3.
[d]No *R. solanacearum* detected in any dilution of the soil suspension.
[e]Highest dilution in which *R. solanacearum* was detected after enrichment in M-SMSA.
[f]Highest dilution in which *R. solanacearum* was detected after enrichment in CIPEB.
[g]Estimated minimum population detected after enrichment in CIPEB.

cultures of isolates from potato or soil microbiota and other plant pathogenic bacteria at concentrations $\geq 10^8$ CFU mL^{-1}, which are unlikely to occur in soil suspensions incubated in semispecific broth where the occurrence of multiple bacteria and competition for nutrients may limit the multiplication of a particular isolate at a high rate. Therefore soil suspensions were inoculated at a final concentration of 10^6 CFU mL^{-1} and tested after incubation in CIPEB. Although some of these bacteria did grow in the specific enrichment broth, the concentrations reached at the end of the 48-h incubation time were below the detection limit of 10^8 CFU mL^{-1} for cross-reacting bacteria. Similar specificity results were obtained with saprophytes present in tuber extracts in indirect ELISA on nitrocellulose membrane using the same antibodies (Priou *et al*., 1999). However, for soil samples DAS–ELISA was preferred to avoid background effects caused by soil particles and to increase detection specificity, as a more diverse saprophytic microflora occurs in soil than in the plant vascular system.

The variability of detection sensitivity, ranging from 8 to 345 CFU g^{-1} soil among all 42 naturally infested samples tested (Table 7), could be caused by antagonistic soil microflora that impaired optimal growth of *R. solanacearum* in the enrichment broth. Detection sensitivity was more variable for Bv2A-infested soils, probably because samples from the Bv1-infested field were taken only from nonwilted plants (wilted plants were too decomposed in the tropical humid lowland conditions where Bv1 occurs in Peru), whereas those from the Bv2A-infested fields came from rhizosphere soil of both wilted and symptomless plants. On the other hand, the sensitivity of detection was similar (200 CFU g^{-1} soil) or even higher (20 CFU g^{-1} soil) when five different, nonsterile soil types were inoculated with *R. solanacearum* Bv2A, but lower with a sixth soil with the highest pH (Table 5). This suggests that detection sensitivity is likely to depend on both soil biotic and abiotic characteristics. The effect of soil physicochemical characteristics and soil microbial community on *R. solanacearum* survival, and hence on detection efficiency, is being investigated further.

Sensitivity of plating on M-SMSA medium corresponded to that reported by other authors (van der Wolf *et al*., 1998; Pradhanang *et al*., 2000) with inoculated soils, but was variable and often lower with naturally infested soils. Moreover, at low concentrations in soil, the percentage recovery of *R. solanacearum* on M-SMSA averaged only *c*. 30% (Table 3). Granada & Sequeira (1983) also reported lower recovery efficiency with low pathogen densities. However, Pradhanang *et al*. (2000) obtained high recovery rates on M-SMSA (*c*. 90%) regardless of inoculum concentration in the soil. It is possible that soil texture and humidity may have resulted in this difference, as Pradhanang *et al*. (2000) used sandy or sandy loam soils, whereas the artificially inoculated soil used in the present study was clay loam. Moreover, Pradhanang *et al*. (2000) plated freshly inoculated soil suspensions, whereas in the present study soil was inoculated 1 day before soil suspension preparation. There were also fewer saprophytic bacteria in SMSA plates if dry soils were used, so it is possible that Pradhanang *et al*. (2000) used dry soil samples leading to less growth competition with soil saprophytes and thus higher recovery on SMSA plates. However, as rapid reduction of pathogen populations in dry soils (data not shown) was observed in the present study, it is recommended that fresh soil samples, with humidity around field capacity, are used and that they are analysed within a week.

Quantification of soil populations is important in research in order to assess the effectiveness of control measures to reduce soil inoculum, which need to be validated locally, such as soil treatments or amendments, planting less-susceptible varieties and/or various rotation crops. For that purpose, soil inoculum can be determined in a semiquantitative way by diluting the soil suspension before enrichment to find the last dilution at which *R. solanacearum* is detectable. For qualitative assays (presence or absence of the pathogen), only the first two dilutions need be analysed in DAS–ELISA, but for quantitative assays up to seven dilutions could be tested.

The results of this study show that composite samples of five soils could be analysed to assess field soil populations without significantly decreasing detection sensitivity. By analysing composite soil samples, the probability of detecting widely dispersed pathogen populations will increase without increasing the workload.

Post-enrichment DAS–ELISA thus provides a reliable and sensitive detection technique suitable for the development of control components and epidemiological studies of potato bacterial wilt, at a reasonable cost (estimated at CIP as US$0·4 for each dilution of soil suspension analysed in duplicated wells). This technique is less costly and easier to use than PCR, and does not require highly skilled personnel or sophisticated laboratory facilities and equipment, with the exception of an incubator agitator for the enrichment procedure in Erlenmeyer flasks. Although the incubation of soil suspensions in smaller volumes, in Eppendorf tubes, led to the same sensitivity levels with inoculated soils (results not shown), analysing 1 mL soil suspension would ensure a greater probability of detecting the pathogen than analysing only 100 μL.

This technique is being applied successfully for the assessment of various rotation crops and schedules in different agroecological conditions in the Peruvian and Bolivian Andes; for studies on disease epidemiology; and to predict the occurrence of *R. solanacearum* in soil to determine the suitability of these areas for seed-potato production. Positive samples in ELISA from which *R. solanacearum* cannot be isolated on M-SMSA plates are analysed in nested PCR according to Pradhanang *et al*. (2000) to assess possibilities of cross-reactions. Studies to determine the optimum sample size for each detection purpose are also ongoing. However, pathogen population estimates, based on colony counts or on bacterial multiplication in enrichment broth, could be underestimated if viable but nonculturable forms of the pathogen prove to be important in long-term survival in soil (van Elsas *et al*., 2000; Grey & Steck, 2001). No detection technique can

fully exclude the presence of *R. solanacearum* in soil, as results are dependent on the sampling strategy, extraction method and detection assay, especially if the pathogen survives in soil in the absence of hosts in viable but non-culturable forms. The most reliable test will almost certainly remain planting a susceptible potato cultivar in the field, followed by detection of latent infection in plants and tubers if the crop remains symptomless.

DAS–ELISA kits, including the sterilized, concentrated (10×) enrichment broth, are available at CIP for international distribution.

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