strains (Fegan *et al.* 1998). The other 3 exceptional strains (CIP042, 428, and 432) were also amplified using these biovar 2A-specific primers (M. Fegan, personal communication). These 6 strains may be biovar-2A strains mutated to biovar 1, because there are few genes responsible for these biochemical traits. Assuming this hypothesis, the method failed to characterize only 2.5% of the strains; but race-1 strains (biovars 1, 3 and 4) were still successfully differentiated from race-3 (biovar 2A) strains.

Thus, the method described above could be used for rapid presumptive identification of the pathogen approximately 7 h after its isolation. Since *R. solanacearum*-specific primers are used, PCR could be performed directly from infected potato-plant extract or vascular oozing.

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Sensitive detection of *Ralstonia solanacearum* (race 3) in soil by post-enrichment DAS-ELISA

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ABSTRACT

A post-enrichment double antibody sandwich (DAS) ELISA is described for the sensitive detection of *Ralstonia solanacearum* (race 3) in soil. *R. solanacearum* was successfully detected by ELISA after 48 h incubation at 30°C of soil extracts in selective broth (i.e., enrichment) at low population levels. As few as 20 and 144 cells/g soil were detected in DAS-ELISA from inoculated and naturally infested soils, respectively. Serial dilutions of the soil extract before enrichment allowed semi-quantification of soil populations of *R. solanacearum*. No cross-reaction in DAS-ELISA was obtained after enrichment of saprophytic bacteria isolated from soil. Post-enrichment DAS-ELISA thus provides a reliable and sensitive detection technique suitable for development of control components and epidemiological studies of bacterial wilt.

INTRODUCTION

Ralstonia solanacearum (Smith) Yabuuchi *et al.* is the causal organism of bacterial wilt or brown rot, a disease that is the second major constraint to potato production in tropical and sub-tropical regions worldwide (Hayward 1991). Since the pathogen is mainly transmitted through tuber seed, the use of healthy planting material is the most effective means to control this disease. *R. solanacearum* can also survive in plant debris and in the rhizosphere of potato, many weeds and other crops (Hayward 1991). Thus another major component of BW management is the planting of potato in *R. solanacearum*-free soils. However, studies on the epidemiology and control of *R. solanacearum* have been limited by the inability to detect and quantify the pathogen in low densities in soil. The most widely used methods to detect *R. solanacearum* in soil consist of streaking soil suspension on specific medium (Granada and Sequeira 1983; Englebrecht 1994). The plating technique requires skilled personnel to distinguish colonies of the pathogen from other bacterial saprophytes, limiting its extensive use. Sensitivity of the method is also variable among soil samples because the soil antagonistic microflora can impair the growth of *R. solanacearum*. The use of indicator plants such as tomato has been reported, but the sensitivity of the bioassay is low (10^4–10^5 cells/g soil; Graham and Lloyd 1978). Indirect ELISA has also been developed (Robinson-Smith *et al.* 1995) but it lacks sensitivity (10^4 cells/g soil) and specificity. Using immunofluorescence colony-staining (IFCS), Van der Wolf *et al.* (1998) could detect slightly lower levels of *R. solanacearum* (5×10^3 cells/g soil) but this technique requires

CORRIGENDUM: In Priou *et al.* "Sensitive detection of *Ralstonia solanacearum* (race 3) in soil by post-enrichment DAS-ELISA" the order of authors should be S.Priou, L.Gutarra, H.Fernandez and P.Aley and not as given. In Table 3 the three rows of data under "Potato plants without symptoms" should be replaced with the following:

3.0×10^3	3.477	0.213	-	10^{-1}	300
3.3×10^4	4.518	0.213	-	10^{-2}	330
3.7×10^4	4.568	0.016	-	10^{-2}	370

The rest of the Table is unchanged. My apologies to authors and readers (Editor).

costly equipment. The sensitivity of the serological methods for the detection of *R. solanacearum* in potato or in soil has been increased by applying an enrichment procedure, e. g. incubating the extracts in a selective broth (Elphinstone *et al.* 1996; Van der Wolf *et al.* 1998; Priou *et al.* 1999).

This paper reports the detection of *R. solanacearum* in soil after selective enrichment using double antibody sandwich immunoassay (DAS-ELISA) in microtitration plates. The detection effectiveness of post-enrichment DAS-ELISA has been compared with plating on a selective medium.

MATERIALS AND METHODS

Inoculated soil extracts

A non-sterile *R. solanacearum*-free loamy-clay soil from Huancayo, Peru, was inoculated by mixing 10 ml of a water suspension of *R. solanacearum* strain 204 (Bv 2A) with 90 g of soil to obtain final concentrations of 10^7, 10^4, 10^2, 20, 2 and 0.2 cells/g soil. Three replications of each concentration of soil inoculum were prepared. Inoculated soils, in which the moisture content was at field capacity, were kept at room temperature for 24 h before use. Soil suspensions were prepared by mixing 10 g of soil with 90 ml of PBS buffer. The soil suspension was agitated for 30 min. and allowed to settle for 40 sec. The supernatant was either directly used for detection or enriched. Fifty µl of each soil suspension were plated on M-SMSA medium. Three plates per concentration were incubated for 48 h at 30°C and colonies of *R. solanacearum* were counted to estimate the original soil population.

Naturally infested soil extracts

Twenty-five soil samples (loamy-clay soil) were taken from a race-3 infested field (germplasm evaluation trial in Carhuaz, Peru), in the rhizosphere (at approximately 20–30 cm depth) of wilted and symptomless plants of different potato genotypes. Soil extracts were prepared two days after sampling as previously described for inoculated soils, and 8 tenfold serial dilutions were done in sterile distilled water. Non-diluted and diluted soil suspensions were enriched. Fifty µl of the three first dilutions of the soil suspensions were plated on M-SMSA medium (3 plates each) and incubated for 48 h at 30°C. Colonies of *R. solanacearum* were counted to estimate the original soil population.

Selective enrichment

The enriched soil extracts were prepared by mixing, in an Erlenmeyer, 2 ml of soil suspension with 38 ml of modified SMSA broth (M-SMSA, Elphinstone *et al.* 1996) supplemented with potato broth (1:1), and incubating the mixture for 48 h at 30°C with constant agitation (170 rpm).

Polyclonal antiserum production

Strains CIP 204 (biovar 2A) and CIP 104 (biovar 2A) of *R. solanacearum* were used for the rabbit immunizations. They were cultured on Kelman's medium (Kelman 1954) without tetrazolium chloride for 48 h at 30°C. The cells were harvested in 0.01 M phosphate buffered saline pH 7.4 (0.5X PBS), centrifuged for 10 min at 10,000 × g and washed three times in 0.5X PBS. They were resuspended in 0.5X PBS, their number was estimated by absorbance at 600 nm and the concentration adjusted to 2×10^9 cells/ml. The cells were then fixed with 2% glutaraldehyde following the method of Allan and Kelman (1977).

Female Rex × New Zealander rabbits were immunized by intradermic injection of 2×10^9 glutaraldehyde-fixed whole cells of an equal mixture of strains CIP 204 and CIP 104 in 1 ml of 0.5X PBS, which was emulsified in an equal volume of Freund's incomplete adjuvant (Difco). The rabbits were immunized again 32 d later intra-muscularly in both legs with a total of 10^8 glutaraldehyde-fixed whole cells (same strain mixture) in each leg, in one ml of 0.5X PBS, which was emulsified in an equal volume of Freund's incomplete adjuvant. This last injection was repeated weekly, for nine times. Blood was collected from the lateral ear vein 52 d after the first immunization, and then weekly. The blood was allowed to clot at 4°C, separated by centrifugation (15 min at 10,000 × g) and the serum fraction collected. Antibody levels in the serum were determined by NCM-ELISA (Priou and Gutarra, 1998).

DAS-ELISA

Double antibody sandwich ELISA was performed as described by Clark and Adams (1977) using the immunoglobulins (IgG; diluted 1:1500) purified from the *R. solanacearum*-specific polyclonal rabbit antiserum produced as described above. The IgG was

TABLE 1. Cross-reactions obtained in DAS-ELISA with non-*Ralstonia solanacearum* bacterial strains when in pure culture in citrate buffer at 10^8 cells/ml (– E) and after 48 h enrichment (+ E) from an original concentration of 10^6 cells/ml soil suspension.

	Strain #	DAS-ELISA	
		– E	+ E
Erwinia carotovora subsp. *carotovora*	CIP 400	– †	–
Erwinia carotovora subsp. *atroseptica*	CIP 421	–	–
Erwinia chrysanthemi	CIP 367	–	–
Pseudomonas syzygii	NCPPB 3792	++ *	–
Ralstonia pickettii	NCPPB 3899	–	–
Burkholderia cepacia	NCPPB 2993	–	–
Pseudomonas aeruginosa	NCPPB 1965	–	–
Pseudomonas putida	NCPPB 1806	–	–
	NCPPB 1808	–	–
"Pseudomonas celebensis"	UW 443	++	+ *
	UW 446	++	+
Unknown bacteria isolated from three different soil extracts	29 isolates	– (29/29)	– (29/29)
Unknown bacteria isolated from three different soil extracts after enrichment	37 isolates	– (37/37)	– (37/37)

† Not detected.

* + and ++: coloration intensities equivalent to those obtained in DAS-ELISA with *R. solanacearum* in citrate buffer at concentrations of 10^5 cells/ml and above 10^7cells/ml, respectively.

TABLE 2. Minimum populations of *Ralstonia solanacearum* (Rs) detected by plating on modified SMSA medium (M-SMSA) and in DAS-ELISA without (–E) and with incubation of the inoculated soil extracts in M-SMSA supplemented with potato broth (1:1) for 48 h at 30°C (enrichment, +E).

Density of Rs added to soil Cells/g soil	– E		+ E	
	Plating on M-SMSA % recovery *	DAS-ELISA O.D. **	Plating on M-SMSA	DAS-ELISA O.D.**
10^7	70.3 (7)	0.417 (0.098)	+ ‡	> 2.000
10^4	60.0 (10)	0.143 (0.001)	+	> 2.000
10^3	20.0 (0)	0.142 (0.007)	+	1.505 (0.467)
10^2	0	0.157 (0.001)	+	0.460 (0.064)
20	0	0.152 (0.002)	-	0.565 (0.372)
2	0	0.124 (0.004)	-	0.214 (0.009)
0.2	0	0.147 (0.009)	-	0.171 (0.009)
SE †	0	0.151 (0.013)	-	0.178 (0.016)

* Means of three replications of inoculated soils (standard deviations are in parenthesis).

** ELISA readings ($A_{405\ nm}$) 1 h after addition of the substrate at room temperature (standard deviations are in parenthesis).

† SE = non-inoculated soil extract.

‡ Isolated on M-SMSA medium but colonies not counted after enrichment.

conjugated to alkaline phosphatase and diluted 1:1500 for detection. Bioreba microtitre plates 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 Biorad Model 2550 plate reader following 1 h incubation at room temperature. ELISA readings were considered positive when they exceeded two times the mean of the negative controls.

Bacterial isolates and cross-reaction tests

A total of 259 potato isolates of *R. solanacearum* from CIP's international collection including different biovars (170 biovar 2A, 20 biovar 2T, 48 biovar 1, 15 biovar 3, and 6 biovar 4) originating from several countries, were used to check the antiserum. Suspensions of the 11 identified non-*R. solanacearum* bacterial strains listed in Table 1 and 66 unknown saprophytes isolated from soil extracts of 3 different soils (Huancayo, Carhuaz and San Ramon, Peru) were tested in DAS-ELISA when in pure culture in citrate buffer at 10^8 cells/ml. To assess the specificity of DAS-ELISA after enrichment, these 77 isolates were diluted in soil extract to a final concentration of 10^6 cells/ml soil suspension and enriched as previously described.

RESULTS

Specificity of the antiserum

Several antisera had previously been obtained by immunizing rabbits following various protocols but the immunization schedule reported in this paper was found to produce more specific antibodies and a higher titre. When immunizing with these two strains of biovar 2A (CIP 204 and CIP 104), all 259 strains of *R. solanacearum* tested were recognized by the antiserum (Priou *et al.* 1999). No cross-reaction was observed in DAS-ELISA after enrichment of soil extracts inoculated with the 66 soil saprophytes (Table 1). *P. celebensis* (the Blood Disease Bacterium) was only weakly detected in DAS-ELISA after enrichment, but not *P. syzygii*, the agent of the Sumatra disease of clove, also present in Asian soils.

Detection sensitivity with inoculated soil

The minimum concentration of *R. solanacearum* detected in DAS-ELISA was 10^5 cells/ml, when in pure culture (data not shown). In soil extracts, the detection threshold decreased to 10^7 cells/g soil (Table 2). Sensitivities of DAS-ELISA and plating on M-SMSA were greatly increased by the enrichment procedure: as few as 20 and 100 cells/g soil could be detected from inoculated soils, respectively (Table 2). According to the ELISA readings, the populations of 20 and 10^2 cells/g multiplied to 10^7 and above 10^8 cells/g soil, respectively. The enrichment efficiency has been improved by adding potato broth to the M-SMSA broth (1:1) because the bacteria did not grow well in the mixture of M-SMSA broth and soil suspension. The potato broth aimed at replacing the tuber extract since the pathogen growth is very high in the mixture of tuber extract and M-SMSA (Priou *et al.* 1999). Percentage recovery of *R. solanacearum* on M-SMSA medium averaged 65% with high bacterial concentrations but was only 20% with low inoculum density close to the detection limit (10^3 cells/g soil). Moreover, the colonies of *R. solanacearum* were hardly visible at this concentration because of many saprophytic bacteria overgrowing them in the plate.

Detection sensitivity with naturally infested soil

In preliminary assays to detect *R. solanaceraum* in a naturally infested soil, minimum populations detected averaged 144 cells/g soil (Table 3). However, the sensitivity varied from 26 to 530 cells/g soil among samples, that could be due to the occurrence of a different antagonistic soil microflora impairing optimal growth of *R. solanacearum* in the enrichment broth. The inoculum levels detected in M-SMSA plates were high and quite homogeneous among the samples for wilted potato plants. In the rhizosphere of asymptomless plants, the soil populations were either high and similar to those for wilted plants or no *R. solanacearum* could be detected in M-SMSA as well as in DAS-ELISA.

Discussion

R. solanacearum (race 3) was successfully detected by post-enrichment DAS-ELISA at low population levels in inoculated soil. The sensitivity of detection was considerably increased by the enrichment procedure as compared to that reported by Robinson-Smith *et al.* (1995) for indirect ELISA. Initial populations of 10^2 cells/g soil were increased within 48 h to higher than 10^8 cells/g, that correspond to results of Van der Wolf *et al.* (1998) using the same enrichment broth. In naturally infected extracts, sensitivity after enrichment was more variable but similar to that reported for plating on specific media (Granada and Sequeira 1983; Englebrecht 1994). However, the serological technique is easier to use because it does not require skilled personnel to differentiate between saprophytic and pathogenic colonies. Granada and Sequeira (1983) obtained a slightly higher detection limit (around 10^2 cells/g soil) by plating on selective medium without enrichment, however, the medium and the inoculation method were different in the present

TABLE 3. Sensitivity of DAS-ELISA for the detection of *Ralstonia solanacearum* (Rs) in tenfold dilutions of naturally infested soil extracts from a race 3-infested field without (– E) and with enrichment (+ E) in modified SMSA supplemented with potato broth (1:1) for 48 h at 30°C.

Original population in soil Cells/g *	Log_{10} (cells/g)	S.D. †	Last dilution in which Rs could be detected In DAS-ELISA – E	Last dilution in which Rs could be detected In DAS-ELISA + E	Estimated minimal population detected after enrichment Cells/g
Potato plants with symptoms					
3.3×10^6	6.518	0.124	—	10^{-4}	330.0
1.0×10^6	6.000	0.000	—	10^{-4}	100.0
2.6×10^5	5.414	0.269	—	10^{-4}	26.0
1.2×10^6	6.079	0.061	—	10^{-4}	120.0
2.8×10^6	6.447	0.017	—	10^{-5}	28.0
5.3×10^4	4.724	0.174	—	10^{-2}	530.0
1.4×10^6	6.146	0.088	—	10^{-4}	140.0
5.4×10^6	6.732	0.045	—	10^{-5}	50.0
3.9×10^5	5.591	0.047	—	10^{-3}	390.0
1.0×10^6	6.017	0.023	—	10^{-4}	100.0
2.9×10^5	5.462	0.064	—	10^{-4}	29.0
4.5×10^6	6.653	0.097	—	10^{-5}	45.0
2.9×10^6	6.462	0.151	—	10^{-5}	29.0
1.0×10^6	6.000	0.124	—	10^{-4}	100.0
6.2×10^5	5.792	0.100	—	10^{-4}	62.0
Potato plants without symptoms					
4.5×10^6	6.653	0.013	—	10^{-4}	450.0
3.4×10^5	5.531	0.109	—	10^{-4}	34.0
2.8×10^6	6.447	0.045	—	10^{-5}	28.0
0			—	—	
0			—	—	
0			—	—	
0			—	—	
0			—	—	
0			—	—	
0			—	-	

* Estimated from colony counts after plating on three plates containing modified SMSA medium incubated for 48 h at 30°C.
† S. D. = standard deviation.

study. They also reported lower recovery efficiency with low pathogen densities.

The specificity of DAS-ELISA was satisfactory since no cross-reaction was detected after enrichment of soil extracts inoculated with soil saprophytes. Non cross-reactivity of post-enrichment DAS-ELISA was also demonstrated in soil samples from an infested field.

Quantification of soil populations is important in research for the development of control components effective in reducing soil inoculum. Relative semi-quantification of soil inoculum potential could be achieved by diluting the soil extract before enrichment and recording the last dilution in which *R. solanacearum* was detected.

Post-enrichment DAS-ELISA thus provides a reliable and sensitive detection technique suitable for management and epidemiological studies of bacterial wilt. A DAS-ELISA kit is being developed at CIP. The replacement of the potato broth by an effective chemical is being investigated to be able to produce a synthetic enrichment medium to be included in the kit. The effectiveness of this technique to detect soil populations of race-1 strains of *R. solanacearum* is also being evaluated.

ACKNOWLEDGEMENTS

The authors thank Christian Delgado and Violeta Flores from CIP Virology Laboratory for technical assistance.

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Effect of Short Term Rotation on *Ralstonia solanacearum* populations in soil

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INTRODUCTION AND METHODS

Rotation crops such as forage sorghum, signal grass and Rhodes grass were planted into 90 microplots that had been previously inoculated with a rifampicin resistant strain of *Ralstonia solanacearum*. Potatoes were planted into the microplots three months prior to sowing the rotation crops to increase the inoculum level in the soil. In addition, the microplots were drenched with a suspension of both wild type and rifampicin resistant strains of *R. solanacearum* after sowing. The rotation crops included in the trial were:

- Forage sorghum — var. Bully Beef (*Sorghum* spp.)
- Forage sorghum — var. Superdan (*Sorghum* spp.)
- Signal grass (*Brachiaria decumbens*)
- Rhodes grass cv. Nemcat (*Chloris gayana*)
- Weed fallow (Control)

After sowing in November 1997, soil samples were taken monthly to determine the population of *R. solanacearum* and the soil moisture content. The dilution plating technique was used to measure population levels. The growth period of the rotation crops was from November 1997 to June 1998. Initially a watering schedule was implemented to carry the crops over until the wet season began. After the wet season the crops were left unattended and without regular watering. In June 1998, the rotation crops were pulled out, the soil turned over and fertilised and potatoes (Sebago variety) were planted. Three seeds were planted into each microplot and a regular watering schedule was resumed. The potatoes were monitored weekly for symptoms of bacterial wilt infection and those showing symptoms were sampled and taken back to the laboratory for confirmation of infection. The potatoes were grown for 4 months after which the tubers were dug up and a final soil sampling was carried out.

As expected initial population levels were high, however in February the bacterium could not be detected in any plots which corresponds to very low soil moisture at the time of sampling. Following good rain and high temperatures, the level increased in March and dropped slightly in April. (Fig. 1) The following graph shows the soil moisture content during the sampling period. (Fig. 2)

Ten weeks after planting a total of 53% of the microplots had plants infected with bacterial wilt. This 53% can be broken down into 27% control, 20% Bully Beef, 18% Signal Grass, 16% Superdan and 16% Rhodes Grass. In all cases with the exception of Superdan and Rhodes Grass, over half the plots in each treatment were infected with bacterial wilt.

CONCLUSIONS

These results have not been statistically analysed however preliminary conclusions can be drawn based on trends. It is evident that soil moisture content has a direct effect on population levels of *R. solanacearum*. The sampling in February demonstrates this phenomenon. In addition, both treatments with lowest soil moisture contents in April had undetectable levels of the bacterium and other treatment population levels reflect soil moisture content in the same month.

Signal grass (*Brachiaria decumbens*) survived the best during dry conditions which explains the lower soil moisture content in these microplots.

The final infection results suggest that having a rotation crop is better than having no rotation as the highest level of infection was found in the control plots. However over 50% infection in all treatments except Superdan and Rhodes grass suggests that a short term rotation (7 months) would not be long enough to control *R. solanacearum* under these conditions.