Highly sensitive detection of *Ralstonia solanacearum* in latently infected potato tubers by post-enrichment enzyme-linked immunosorbent assay on nitrocellulose membrane

S. Priou, L. Gutarra and P. Aley

International Potato Center (CIP), PO Box 1558, Lima 12 (Peru); e-mail: s.priou@cgiar.org

A post-enrichment enzyme-linked immunosorbent assay (ELISA) on nitrocellulose membrane (NCM-ELISA) is described for the detection of *Ralstonia solanacearum* in latently infected potato tubers. The polyclonal antiserum specificity was significantly improved by adsorption with cross-reacting bacteria. The detection efficiency after enrichment was compared with those of nucleic acid spot hybridization (NASH), double-antibody-sandwich immunoassay (DAS-ELISA) and plating on modified Kelman's medium. After 48 h of incubation of the tuber extracts with modified SMSA broth at 30°C, sensitivities of post-enrichment NCM-ELISA, DAS-ELISA and NASH were similar. As few as 10 cells mL^{-1} were detected in either inoculated or naturally infected tuber extracts. Of 255 field samples, no cross-reactivity of NCM-ELISA was observed. Post-enrichment NCM-ELISA thus provides a reliable and sensitive low-cost method that is rapid and easy to use, making it suitable for assessing susceptibility of breeding lines to bacterial wilt, ecological studies and seed quality control in developing countries.

Introduction

Ralstonia solanacearum is the causal agent of the disease known as bacterial wilt or brown rot, the second major constraint to potato production in tropical and subtropical regions worldwide (Hayward, 1991). Brown rot has recently become a serious threat to potato seed production in the cool-temperate countries of Northern Europe (Janse, 1996). As the pathogen is mainly transmitted through seed-potato tubers, use of healthy planting material is the most effective means of control (Hayward, 1991). Under cool climatic conditions, the plant can be infected without exhibiting visible symptoms, resulting in latent infection in vascular tissues of the progeny tubers (Ciampi *et al.*, 1980; Hayward, 1991). Efficient detection techniques for routine use in quarantine procedures and seed-certification schemes are thus critical. A reliable detection tool would also allow more precise understanding of survival and dissemination of the pathogen, and the assessment of levels of resistance in germplasm.

The standard methods of the European Union for monitoring the occurrence of *R. solanacearum* in seed-potato tubers involve the use of indirect immunofluorescence antibody staining (IFAS) and isolation on selective medium (OEPP/EPPO, 1990). The most commonly used techniques to confirm IFAS-positive samples are bioassay on tomato plants and detection of *R. solanacearum*-specific DNA sequences by polymerase chain reaction from pure bacterial cultures that have been isolated on selective medium (OEPP/EPPO, 1990). Other techniques have become available and have been reviewed by Seal & Elphinstone (1994). However, all these methods are laborious and costly, and lack sensitivity and specificity. There is still no method available for detecting latent infection in tubers that combines the advantages of high sensitivity, low cost and suitability for routine use in developing countries, where laboratory facilities and skilled personnel are often limited (Black & Elphinstone, 1998).

Serological methods have been widely used for virus detection in seed programmes worldwide because they are easier to use and are low-cost. Among them, NCM-ELISA is the cheapest, easiest and quickest technique (Salazar, 1996). In spite of numerous studies on serological methods for detection of *R. solanacearum*, these techniques (except IFAS) have not been widely used in quarantine or certification procedures. This has been due to their insufficient sensitivity and to the high frequency of false positive reactions resulting from lack of specificity of antisera (Seal & Elphinstone, 1994).

In a previous study (Gorris *et al.*, 1997), the existing methods of enrichment of bacteria in tuber extracts have been reviewed and have been refined to increase the sensitivity of serological techniques. The maximum growth rate of *R. solanacearum* during the enrichment procedure was obtained using citrate buffer for sample extraction from tubers and using the modified SMSA broth developed by Elphinstone *et al.* (1996). This paper reports improvements in the immunoassay on nitrocellulose membrane (NCM-ELISA) and in antiserum specificity. A nucleic acid spot hybridization procedure (NASH), using the *R. solanacearum*-specific DNA probe developed by Cook & Sequeira (1991) and isolation on modified Kelman's medium (French *et al.*, 1995), was used to

check the specificity of the serological method. Comparisons of the detection efficiency of the improved NCM-ELISA with NASH and double-antibody-sandwich ELISA (DAS-ELISA) after enrichment are also presented.

Materials and methods

Sample extraction and enrichment

Tubers were washed with tap water and dipped in 1% v/v sodium hypochlorite for 5 min. With a flame-sterilized scalpel, a thin slice of the tuber was removed and discarded from around the stolon end. Strips along the vascular ring (≈ 3 mm wide and 3 mm deep) were removed with a flame-sterilized cuticle remover. The tuber fragments were put in a plastic bag and weighed. The plastic bag containing the tuber fragments was placed vertically on crushed ice to avoid oxidation of the phenols. Sterile citrate buffer (0.1 mol L^{-1} citric acid, 0.1 mol L^{-1} sodium citrate, pH 5.6) was added at 2 mL g^{-1} of tuber tissue, and the tuber fragments were squashed with a rubber mallet. After 30 s to allow sedimentation of tuber debris, 500 µL of the supernatant tuber extract was taken and mixed with 500 µL of the semiselective modified SMSA broth (M-SMSA; Elphinstone *et al.*, 1996) in 1.5-mL Eppendorf tubes. Two tubes were prepared: one was incubated for 24 h, and one for 48 h, at 30°C with constant agitation. The enriched extracts and the remainder of non-enriched extract were stored at −20°C for further use.

Preparation of inoculated tuber extracts

A healthy tuber extract (HTE) was prepared with *R. solanacearum*-free tubers (cv. Revolución) from the International Potato Center (CIP) seed-multiplication station at Huancayo (PE). A suspension of *R. solanacearum* in citrate was prepared by culturing strain CIP 204 (biovar 2A) for 48 h at 30°C on modified Kelman's medium (MKM; French *et al.*, 1995), but without tetrazolium chloride. The cells were harvested in citrate buffer and the bacterial concentration was evaluated by measuring the absorbance at 600 nm of a diluted aliquot. An OD_{600} of 0.1 was equivalent to 2×10^8 cells mL^{-1}. The concentrated suspension was then adjusted to 2×10^9 cells mL^{-1}. The healthy tuber extract (HTE) was mixed with the suspension of *R. solanacearum* to obtain a final bacterial concentration of 2×10^8 cells mL^{-1}. Nine 10-fold dilution series in HTE were prepared to reach the theoretical concentration of 0.2 cells mL^{-1}. Each dilution was mixed with the enrichment broth as previously described and incubated for 24 h and for 48 h at 30°C with constant agitation. Another set of tubes was incubated without agitation, and another set agitated manually twice a day to evaluate the effect of agitation on enrichment efficiency. The healthy tuber extract and the M-SMSA broth were incubated for 48 h at 30°C as controls. The remaining non-enriched dilutions were stored at −20°C. The population of *R. solanacearum* in the initial inoculated tuber extract was verified by spreading 50 µL of a 10^{-5} dilution in sterile distilled water on MKM. After 48 h of incubation at 30°C, the average number of typically fluid and red-tinted *R. solanacearum* colonies was recorded from five plates. The whole experiment was repeated three times.

Preparation of naturally infected tuber extracts

Eight single-tuber extracts were prepared from infected tubers obtained by field inoculation of 1-month-old plants of cv. Yungay with *R. solanacearum* strain CIP 311 (biovar 2A). Ten 10-fold dilution series of the eight infected extracts in HTE of cv. Yungay were prepared. Populations of *R. solanacearum* in the original infected extracts were estimated by spreading 50 µL of each of the dilutions on MKM (three plates each). The average *R. solanacearum* colony numbers from three plates was counted after 48 h incubation at 30°C, from the dilution allowing the counting of well-separated colonies, and the original concentration was estimated by multiplying this number by the dilution factor. Each dilution was mixed with the M-SMSA broth and incubated for 24 h and 48 h at 30°C. The healthy tuber extracts and the M-SMSA broth were incubated for 48 h at 30°C as controls. Aliquots of the non-enriched dilutions and the enriched extracts were kept at −20°C prior to use.

In total, 255 extracts were prepared from tubers of 122 different potato clones and from cvs Revolución (susceptible), Yungay (susceptible), Canchan (susceptible), Molinera (tolerant) and Cruza 148 (resistant), which were harvested from inoculated plants in the same germplasm evaluation trial. The tuber extracts were prepared by mixing the tuber fragments removed from 20 tubers per genotype taken at random from the harvest of five plants. Isolations of *R. solanacearum* were performed on MKM from the non-enriched extracts. The tuber extracts were incubated in the M-SMSA broth for 24 h and 48 h at 30°C. Enriched extracts and the remainder non-enriched extracts were kept at −20°C.

Dot blotting of samples

An 8 × 12 cm nitrocellulose membrane (NCM) 0.45-µm pore size (Biorad) was immersed for 5 min in 30 mL of TBS buffer (0.02 mol L^{-1} Tris-HCl, pH 7.5, 0.05 mol L^{-1} NaCl, 0.01% NaN_3) for NCM-ELISA and for 15 min in 5 × standard saline citrate (SSC) for NASH (1 × SSC contains 0.15 mol L^{-1} NaCl, 0.015 mol L^{-1} trisodium citrate, pH 7.0). One slightly larger sheet of filter paper (Whatman 3 MM) soaked with TBS was put on top of the perforated plate of a dot-blot apparatus. The wet membrane was then placed on top of the wet filter paper avoiding the formation of air bubbles. Samples (20 µL) were put on the membrane by applying vacuum (≈125 mm of mercury vacuum). The non-enriched tuber extract was diluted 1 : 5 in citrate buffer before placing it on the membrane to avoid the high quantity of starch in the crude extract, which can impair ELISA. The NCM was transferred to a dry filter paper and air-dried for at least 60 min.

Polyclonal antisera production

Antigen preparation

Strains CIP 204 (biovar 2A) and CIP 104 (biovar 2A) of *R. solanacearum* were used for the rabbit immunizations. They were cultured on MKM, but without tetrazolium chloride, for 48 h at 30°C. The cells were harvested in 0.01 mol L^{-1} phosphate-buffered saline pH 7.4 (0.5× PBS), centrifuged for 10 min at 10 000 *g* and washed three times in 0.5× PBS. They were resuspended in 0.5× PBS; their number was estimated by absorbance at 600 nm and the concentration adjusted to 2×10^9 cells mL^{-1}. The cells were then fixed with 2% glutaraldehyde according to the method of Allan & Kelman (1977). Aliquots of 0.5 mL were prepared and stored at −20°C until required.

Rabbit immunization

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 0.5× PBS, which was emulsified in an equal volume of Freund's incomplete adjuvant (Difco). The rabbits were immunized again, 32 days later, intramuscularly in both legs with a total of 10^8 glutaraldehyde-fixed whole cells (same strain mixture) in each leg, in 1 mL of 0.5× PBS, which was emulsified in an equal volume of Freund's incomplete adjuvant. This last injection was repeated weekly, nine times. Blood was collected from the lateral ear vein 52 days 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.

Antiserum adsorption

One millilitre of each antiserum was mixed with 9 mL of a bacterial suspension in 0.5× PBS, containing a total of 1.5×10^{10} cells of an equal mixture of five strains: *Erwinia carotovora* subsp. *atroseptica* (CIP 421), *E. c. carotovora* (CIP 400), *E. chrysanthemi* (CIP 367), *Ralstonia syzygii* (CNPPB 3792) and *R. pickettii* (NCPPB 3899). This mixture was incubated for 1 h at 32°C with constant agitation (50 rev min^{-1}) and centrifuged for 10 min at 10 000 *g*. The supernatant was collected and the antibody level determined by NCM-ELISA.

NCM-ELISA

The NCM dotted with the samples was incubated for 1 h in 30 mL of the blocking solution (2% non-fat powdered milk in TBS buffer) on a Petri dish 15-cm diameter, with gentle rotary agitation (50 rev min^{-1}). The blocking solution was discarded and the membrane was incubated for 2 h with gentle agitation in 30 mL of the antibody solution (100 µL of *R. solanacearum*-specific antiserum diluted 1 : 1000 was added to another 30 mL of the same blocking solution). The antibody solution was then discarded and the membrane was washed to remove the unbound *R. solanacearum* antibodies with 30 mL of T-TBS (Tris-buffered saline with 0.05% Tween-20) three times for 3 min each with constant agitation at 100 rev min^{-1}. After discarding the last washing buffer, the membrane was incubated for 1 h with gentle agitation with 30 mL of the conjugated solution, containing goat anti-rabbit antibodies conjugated to alkaline phosphatase (Biorad) diluted 1 : 4000 in 30 mL of the blocking solution. Before the washing steps, the NBT solution was prepared in Eppendorf tubes by dissolving 30 mg of NBT (*p*-nitroblue tetrazolium) in 800 µL of the solvent (70% dimethylformamide in sterile distilled water). The BCIP solution was prepared by dissolving 15 mg of BCIP (*p*-toluidine salt of 5-bromo, 4-chloro, 3-indolyl phosphate) in 800 µL of the solvent (100% dimethylformamide). The membrane was rinsed three times for 3 min each with T-TBS, with constant agitation (100 rev min^{-1}) to remove the unbound conjugate. During the last washing step, the colour development (substrate) solution was prepared by adding, drop by drop while agitating, first 100 µL of NBT solution and then 100 µL of BCIP solution in a dark flask containing 25 mL of substrate buffer (0.1 mol L^{-1} Tris base, 0.1 mol L^{-1} NaCl, 0.005 mol L^{-1} $MgCl_2.6H_2O$, pH 9.6). The membrane was then incubated with 25 mL of the substrate solution with gentle agitation for 5–30 min, the time necessary for the reaction to take place depending on room temperature (higher temperatures produce a faster reaction). The reaction was stopped by discarding the substrate solution and by rinsing the membrane thoroughly with tap water. The membrane was then placed on filter paper to dry.

The effect of water quality and pH on ELISA was determined by making all the buffers and solutions with either distilled sterile water (pH 5.0), boiled water from rain (pH 6.0), boiled well water (pH 7.6) or commercially distilled water for car batteries (pH 5.9).

DAS-ELISA

Double-antibody-sandwich ELISA was performed to compare with the NCM-ELISA sensitivity, as described by Clark & Adams (1977), using the immunoglobulins (diluted 1 : 500) purified from the adsorbed polyclonal rabbit antiserum P-359. The immunoglobulins conjugated to alkaline phosphatase and diluted 1 : 500 were used for detection. Nunc (Polysorp) microtitre plates were coated overnight with 180 µL of tuber extracts in duplicate wells. Incubation times with the immunoglobulins were 3 h each at 37°C. Absorbance at 405 nm was determined using a Biorad Model 2550 plate reader after 30 min, 1 h and 1.5 h of incubation at room temperature. ELISA readings were considered positive when they exceeded twice the mean of the negative controls.

NASH

Nucleic-acid spot-hybridization assay (NASH) was used to check the specificity of the serological methods. Bacterial

DNA from tuber extracts on the NCM was released with alkali, neutralized and fixed by UV cross-linking. Plasmid pBS2338 developed by Cook & Sequeira (1991), containing a 2-kbp DNA fragment specific for *R. solanacearum* race 3, was used for hybridization of *R. solanacearum* in tuber extracts after linearization by digestion with *Eco*RV. The ^{32}P-labelled probe was prepared by transcription as described by Querci *et al.* (1993). After overnight hybridization at 55°C as described by Salazar & Querci (1992), membranes were washed in 0.02 mol L^{-1} Tris-HCl (pH 7.5), 0.36 mol L^{-1} NaCl, SDS 0.1% twice for 20 min at room temperature; membranes were washed once in 0.1× SSC, 0.1% SDS for 30 min at 65°C, and twice in 2× SSC for 10 min at room temperature before autoradiography at −70°C using Kodak X-OMAT AR film and an intensifying screen. Genomic DNAs of *R. solanacearum* (strains CIP 204 and CIP 311, biovar 2A) and *E.c.* subsp. *carotovora* (CIP 400) were used as positive and negative controls and were prepared according to Cook *et al.* (1989).

Bacterial isolates

In total, 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. Other bacteria were also used and are listed in Table 1. All bacterial strains were cultured for 48 h at 30°C on MKM without tetrazolium chloride, and the cells were harvested in citrate buffer. The suspensions were adjusted to 10^9 cells mL^{-1} for NASH, and 10^8 cells mL^{-1} for ELISA, as previously described. Suspensions of the 11 identified non-*R. solanacearum* bacterial strains listed in Table 1 and of 20 unknown saprophytes isolated from enriched tuber and soil extracts were also diluted in healthy tuber extract to a final concentration of 10^6 cells mL^{-1}, mixed with M-SMSA broth (1 : 1) and incubated for 48 h at 30°C to check the cross-reactivity of the ELISA after enrichment. Few bacterial species can grow in the M-SMSA broth and the 20 isolated from enriched tuber and soil extracts were a representative sample of the variability of colony morphology found in MKM and SMSA agar plates.

Results

Improvement of the specificity of the antiserum by adsorption

Several antisera had previously been obtained by immunizing rabbits according to various protocols, including those described by Robinson-Smith *et al.* (1995) and Digat & Cambra (1976). The immunization schedule reported in this paper was found to produce more specific antibodies and a higher titre. But when immunization was carried out with only strain CIP 204, the antiserum reacted with all *R. solanacearum* potato strains of biovars 1, 2T, 3 and 4

Table 1 Cross-reactions obtained with non-*Ralstonia solanacearum* bacterial strains in NCM-ELISA using the crude or the adsorbed antiserum P-359 when in pure culture in citrate buffer at 10^8 cells mL^{-1} (without enrichment) and after 48-h enrichment in tuber extract from an original concentration of 10^6 cells mL^{-1}

		Without enrichment		After 48 h of enrichment	
	Strain no.	non-adsorbed	adsorbed	non-adsorbed	adsorbed
Erwinia carotovora subsp. *carotovora*	CIP 400	+	−	−	−
Erwinia carotovora subsp. *atroseptica*	CIP 421	+/−	−	−	−
Erwinia chrysanthemi	CIP 367	+/−	−	−	−
Ralstonia 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 from the potato saprophytic flora	68 isolates	+ (16/68) +/− (34/68)	+ (12/68) +/− (27/68)	Not done	Not done
Unknown bacterial isolates from enriched tuber extracts	11 different isolates	+ (6/11)	+/− (6/11)	+/− (7/11)	− (11/11)
Unknown bacterial isolates from enriched soil extracts	9 different isolates	+ (7/9)	+/− (2/9)	+/− (4/9)	− (9/9)

+/−, + and ++, coloration intensities equivalent to those obtained with *R. solanacearum* in citrate buffer at concentrations of 10^6 cells mL^{-1}, 10^7 cells mL^{-1} and 10^8 cells mL^{-1} respectively; −, not detected.

tested, but did not recognize a group of strains of biovar 2A. Therefore, two strains, CIP 204 and CIP 104 (biovar 2A), the latter belonging to the non-recognized group, were used for immunization. From two antisera produced, antiserum P-359 was chosen because it recognized all strains of *R. solanacearum* tested at 10^8 cells mL^{-1} of citrate buffer. Its titre against the homologous strains was high (32 000), and the working dilutions 1 : 8000 for NCM-ELISA and 1 : 500 for DAS-ELISA were established.

Before adsorption, 33% of the saprophytic isolates tested, *Erwinia* spp., *R. pickettii*, *R. syzygii* and *Pseudomonas celebensis* (when in pure culture in citrate buffer at 10^8 cells mL^{-1}), were detected in NCM-ELISA, with a coloration intensity corresponding to that obtained with *R. solanacearum* in citrate buffer at concentrations of at least 10^7 cells mL^{-1} (Table 1). After adsorption of the antiserum, the titre decreased to 1 : 8000 and the working dilution of 1 : 1000 for NCM-ELISA was selected. All 259 *R. solanacearum* strains tested remained recognized by the adsorbed antiserum. The cross-reactions with non-identified saprophytes were reduced by 18.2%, and *E.c.* subsp. *carotovora and R. pickettii* were not recognized by the adsorbed antibodies (Table 1). The bacteria that slightly cross-reacted with the crude antiserum did not cross-react with the adsorbed antiserum. *R. syzygii*, the Sumatra clove disease agent, and *P. celebensis*, the banana blood disease bacterium, still reacted strongly (Table 1). After 48 h of enrichment in tuber extract from an original concentration of 10^6 cells mL^{-1}, there was no cross-reaction with *Erwinia* spp. because these bacteria did not grow in the M-SMSA. *R. pickettii*, *R. syzygii* and *Burkholderia cepacia* gave slight cross-reactions after enrichment, as did 11 out of the 20 isolates from enriched tuber and soil extracts. However, all these cross-reactions (except for *R. syzygii*) were eliminated by adsorption of the antiserum (Table 1). *P. celebensis* grew well in M-SMSA and strongly cross-reacted after enrichment, even with the adsorbed antiserum.

All *R. solanacearum* biovar 2A strains tested were hybridized with probe pBS2338, but none of the non-*R. solanacearum* bacterial strains tested was detected by NASH at concentrations of 10^9 cells mL^{-1} in citrate, or after 48 h of enrichment with an original concentration of 10^6 cells mL^{-1} in tuber extract. NASH thus provided a specific tool for assessing the risk of cross-reactions in tuber extracts naturally infected with *R. solanacearum* biovar 2A and for evaluating the specificity of the serological methods.

Comparison of the detection sensitivity of NCM-ELISA, DAS-ELISA and NASH

Inoculated tuber extracts

The positive samples appeared as bluish-purple spots, the coloration ranging in intensity from light-pink to dark-purple depending on the concentration of *R. solanacearum* in the samples. The lowest concentration of *R. solanacearum* detectable with NCM-ELISA was 2×10^6 cells mL^{-1} when in pure culture in citrate buffer. The sensitivity of NCM-ELISA in tuber extracts was slightly lower, 2×10^7 cells mL^{-1}, because the crude extract had to be diluted 1 : 5 before blotting owing to its high starch content, which impairs ELISA. In the present work only the purple coloration obtained with at least *R. solanacearum* at 2×10^7 cells mL^{-1} was rated positive. Unacceptable coloration included a light-purple (equal to or lighter than that for the concentration of 10^6 cells mL^{-1}), similar to that caused by some saprophytic bacteria, and brown (colour of the tuber extract). A higher sensitivity was obtained in DAS-ELISA: suspensions containing at least 2×10^5 cells mL^{-1} of *R. solanacearum* in citrate or in tuber extract gave readings significantly higher (OD 0.760 and 0.685 respectively) than the buffer control (OD 0.202) and the healthy tuber extract (OD 0.126). In NASH, not less than 2×10^8 cells mL^{-1} of *R. solanacearum* in citrate could be detected without enrichment.

Sensitivities of DAS-ELISA and NASH were similar after 48 h of enrichment (Table 2). By using two enrichment times, *R. solanacearum* populations in the extracts could be semi-quantified and four classes of tuber infection could be characterized. With the dilution plating technique on MKM, *R. solanacearum* could be detected in the inoculated non-enriched tuber extracts up to a concentration of 100–200 cells mL^{-1}. Elphinstone *et al.* (1996) and Caruso *et al.* (1998) reported the same sensitivity for plating on M-SMSA. However, the efficiency of isolation on MKM after enrichment was variable among the samples because of the presence of bacterial saprophytes overgrowing *R. solanacearum* colonies. Thus the plating on MKM was further used to correlate with the results obtained in ELISA before enrichment of the extracts. NASH was used as the tool to

Table 2 Sensitivity of NCM-ELISA for the detection of *Ralstonia solanacearum* without (−E) and with enrichment (+E) of the tuber extracts and according to the enrichment time (24 h and 48 h) in modified SMSA broth at 30°C

	Enrichment time			Estimated *R. solanacearum* population in the tuber extracts (C in cells mL^{-1})	
	−E	+E 24 h	+E 48 h	Inoculated	Naturally infected
Results in NCM-ELISA	−	−	−	$C < 2$–20	$C < 10$
	−	−	+	2–$20 \leq C < 2 \times 10^3$	$10 \leq C < 2.5 \times 10^4$
	−	+	+	$2 \times 10^3 \leq C < 2 \times 10^7$	$2.5 \times 10^4 \leq C < 6 \times 10^7$
	+	+	+	$C \geq 2 \times 10^7$	$C \geq 6 \times 10^7$

evaluate the cross-reactivity of the serological methods after enrichment.

The effect of agitation on enrichment efficiency was investigated in NCM-ELISA because vibrator incubators may not be available in some laboratories. Without agitation, the sensitivity in NCM-ELISA was decreased by a factor of 100, but the same detection limits were obtained if the Eppendorf tubes were manually agitated twice a day. Furthermore, as the quality of the water used for the buffer preparation is a variable factor in different laboratories where distilled water is not always available, we assessed the effect of water quality and pH on the sensitivity of NCM-ELISA. The detection limit was determined by using the same extracts previously obtained from dilutions of inoculated tuber extract. The same sensitivity was obtained, with similar colour intensity, when using distilled water, boiled rain or well water, but no reaction was obtained using commercially distilled water for car batteries. Some additional reagent may have been added to this water that impaired the ELISA.

Naturally infected tuber extracts

Log_{10} viable counts in the eight infected extracts were estimated from three plates containing MKM at the dilution that allowed counting (Table 3). A uniform 10-fold decrease in pathogen population at each original dilution step was assumed. Colony morphology on MKM was typical of virulent *R. solanacearum*. The sensitivity in NCM-ELISA, without enrichment and after 48 h of incubation in M-SMSA broth, averaged 6×10^7 cells mL^{-1} and 6.5 cells mL^{-1} respectively (Table 3), which was close to the sensitivity obtained with inoculated extracts (Table 2). However, after 24 h of enrichment, the sensitivity was lower than that obtained with inoculated extracts and there was some variation among the samples (Table 3). The same variability was observed for DAS-ELISA and NASH, but their sensitivity without enrichment and after 48 h of enrichment was similar to NCM-ELISA, and to that obtained with inoculated extracts.

Efficiency of NCM-ELISA for the detection of *Ralstonia solanacearum* in tuber samples from a germplasm evaluation trial

Of 255 tuber samples tested, 56.5% were found to be negative in NCM-ELISA after 48 h of enrichment. The same result was obtained with NASH and isolation on MKM, demonstrating that the NCM-ELISA did not lack sensitivity. In all the samples found positive in NCM-ELISA (42.4%), the presence of *R. solanacearum* was confirmed either by isolation on MKM or by DNA hybridization (or with both techniques), revealing no cross-reactivity of ELISA. Among positive samples, 11.4% were found to be positive without enrichment of the tuber extract: 18.8% after 24 h of enrichment and 12.2% after 48 h of enrichment, demonstrating various levels of tuber infection. However, this could not be related to the potato genotype, for the same clone could harbour different *R. solanacearum* population levels among samples. In 1.1% of the samples analysed, the bacteria were isolated in MKM plates but were not detected with either ELISA or NASH. This may be the result of either a contamination of the loop with previously streaked highly infected tuber extracts, or a lack of sensitivity of the methods. Some clone extracts may harbour inhibitory compounds or excessive antagonistic flora that impair *R. solanacearum* growth in M-SMSA broth. This did not occur with any extract of the potato cultivars. In 18% of the samples, NASH was less sensitive than NCM-ELISA since the pathogen population after enrichment may have been exactly or slightly above the limit detectable with ELISA (2×10^7 cells mL^{-1}) and below the one for NASH (2×10^8 cells mL^{-1}).

Discussion

R. solanacearum was successfully detected by post-enrichment NCM-ELISA, even at low population levels. The specificity of NCM-ELISA using the adsorbed antiserum seemed satisfactory, no cross-reaction was detected after enrichment, without resulting in a failure to detect all strains of *R. solanacearum* as reported by Robinson *et al.* (1995). The banana blood disease bacterium, *P. celebensis*, strongly cross-reacted after enrichment, but it is unlikely to be present in tuber extracts. After 48 h of enrichment, sensitivity of post-enrichment NCM-ELISA was found to be similar to those for DAS-ELISA and NASH, with both inoculated and naturally infected extracts. Sensitivity and non-cross-reactivity of post-enrichment NCM-ELISA was demonstrated in 255 field samples. The lower sensitivity of post-enrichment ELISA obtained by Elphinstone *et al.* (1996) may be due to the use of PBS buffer for the sample extraction, since the same enrichment broth was used in the present study. Indeed, the growth of *R. solanacearum* was much lower in this buffer than in citrate (Gorris *et al.*, 1997). The semiquantification of *R. solanacearum* populations in the extracts, which can be useful for epidemiological studies and varietal comparisons for susceptibility to bacterial wilt, cannot be done by applying two enrichment times since many variations were found in NCM-ELISA sensitivity after 24 h of enrichment of naturally infected tuber samples. Dilutions of the extract before enrichment might be a more reliable quantification method, as reported for *Erwinia* spp. (López *et al.*, 1998), but this method may be more laborious and costly.

The procedure approved by EPPO for testing seed potatoes involves the detection of *R. solanacearum* in bulk samples of 200 tubers per 25 t of seed potatoes using IFAS (OEPP/EPPO, 1990). Elphinstone *et al.* (1996) reported a positive detection using IFAS, indirect ELISA, and PCR in up to 1000-fold dilutions of the infected extract because the concentrations of *R. solanacearum* in the extracts

Table 3 Sensitivity of NCM-ELISA, DAS-ELISA and NASH for the detection of *Ralstonia solanacearum* in 10-fold dilutions of eight naturally infected tuber extracts without and with enrichment in modified SMSA broth for 24 and 48 h at 30°C

Extract number	Enrichment time (h)	1	2	3	4	5	6	7	8
Original population in the extract* (cells mL^{-1})		8.66×10^5	6.73×10^5	6.33×10^4	2.30×10^9	8.40×10^7	10^8	1.66×10^9	7.60×10^8
Original population in the extract, Log_{10} (cells mL^{-1})		5.938 (±0.015)†	5.827 (±0.046)	4.801 (±0.021)	9.360 (±0.055)	7.924 (±0.021)	8.000 (±0.005)	9.220 (±0.015	8.880 (±0.023)
Last dilution where *R. solanacearum* was detected by NCM-ELISA	0	ND‡	ND	ND	10^{-2}	10^{0}	10^{0} §	10^{-2}	10^{-1}
	24	10^{-1}	10^{-1}	10^{-1}	10^{-5}	10^{-5}	10^{-5}	10^{-5}	10^{-6}
	48	10^{-5}	10^{-5}	10^{-4}	10^{-9}	10^{-7}	10^{-7}	10^{-9}	10^{-8}
Estimated detection limit by NCM-ELISA after 48 h enrichment (cells mL^{-1})		8.6	6.7	6.3	2.3	8.4	10	1.7	7.6
Last dilution where *R. solanacearum* was detected by DAS-ELISA	0	10^{-0}	10^{-0}	10^{-0}	10^{-4}	10^{-2}	10^{-2}	10^{-4}	10^{-4}
	24	10^{-2}	10^{-2}	10^{-2}	10^{-7}	10^{-6}	10^{-6}	10^{-6}	10^{-7}
	48	10^{-5}	10^{-5}	10^{-4}	10^{-9}	10^{-7}	10^{-7}	10^{-9}	10^{-8}
Estimated detection limit by DAS-ELISA after 48 h enrichment (cells mL^{-1})		8.6	6.7	6.3	2.3	8.4	10	1.7	7.6
Last dilution where *R. solanacearum* was detected by NASH	0	ND	ND	ND	10^{-1}	ND	ND	10^{-1}	10^{0}
	24	ND	10^{-1}	ND	10^{-5}	10^{-4}	10^{-4}	10^{-4}	10^{-5}
	48	10^{-5}	10^{-5}	10^{-4}	10^{-9}	10^{-7}	10^{-7}	10^{-9}	10^{-8}
Estimated detection limit by NASH after 48 h enrichment (cells mL^{-1})		8.6	6.7	6.3	2.3	8.4	10	1.7	7.6

* Estimated from colony counts after plating on three plates containing modified Kelman's medium incubated for 48 h at 30°C.

† SD.

‡ ND, not detected.

§ Bacteria detected only in the non-diluted extract.

Table 4 Comparison of methods for the detection of *Ralstonia solanacearum* in latently infected potato tubers without and with enrichment in modified SMSA broth for 48 h at 30°C

	Enrichment	Plating on MKM*	NCM-ELISA	DAS-ELISA	NASH
Sensitivity (cells mL^{-1})	+	2×10^2	2×10^7	2×10^5	2×10^8
	−	10	10	10	10
Test duration after 48 h enrichment (h)		48	8	48	72
Ease of use		Moderate	High	Moderate	Low
Equipment needs		Moderate	Low	Moderate	High
Cost (EUR per sample)†	+	0.60‡	0.24	0.40	0.60
	−	0.62	0.26	0.42	0.62

* Modified Kelman's medium (French, *et al.*, 1995).
† Estimates based only on the cost of supplies at CIP, Lima (PE).

were around 10^7-10^8 cells mL^{-1}. However, the present work demonstrates that tubers can be latently infected with lower population levels (6.3×10^4 cells mL^{-1}); thus the pathogen would not be detected by most techniques after mixing a single, infected tuber core with 199 healthy tuber cores. But *R. solanacearum* would be detected with post-enrichment NCM-ELISA, because it could be detected in the 10^{-4} dilution of the infected single-tuber extract.

Table 4 summarizes the relative sensitivities and some characteristics of the methods used in the present work. Post-enrichment NCM-ELISA combines the advantages of high sensitivity, low cost, ease of use and speed, and it does not require extensive laboratory equipment. The sensitivity of NCM-ELISA after 48 h of enrichment was significantly higher than that obtained with IFAS, indirect ELISA and PCR (Janse, 1988; Elphinstone *et al.*, 1996; Caruso *et al.*, 1998; Seal, 1998) and corresponded to that obtained with post-enrichment nested PCR (Elphinstone *et al.*, 1996). However, this last technique is costly (≈0.70 EUR per sample; Seal, 1998) and requires extensive laboratory facilities and resources, and highly skilled personnel. It is therefore not suitable for seed testing in resource-poor countries. Moreover, post-enrichment ELISA detects only viable cells of *R. solanacearum*, whereas PCR may detect dead cells.

Post-enrichment NCM-ELISA will be further evaluated for the detection of *R. solanacearum* in root and stem extracts, and water for epidemiological studies of *R. solanacearum*. This technique may also be applicable to the detection of latent infection in other propagating materials, because bacterial wilt is also an important disease of other major crops including tomato, capsicum, aubergine, banana, groundnut and ginger. An NCM-ELISA kit including the M-SMSA enrichment broth has been developed at CIP. It is currently being distributed to countries for routine screening of potato stocks by national seed programmes, and for assessing susceptibility of breeding lines to bacterial wilt. Adequate sampling strategies will be further investigated to detect latent infection by *R. solanacearum* in seed lots for quality control in seed trade and certification schemes, and for germplasm evaluation.

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Post-enrichissement ELISA sur membrane de nitrocellulose (NCM-ELISA): une méthode très sensible de détection de *Ralstonia solanacearum* dans les tubercules de pomme de terre infectés de manière latente

Un test post-enrichissement ELISA sur membrane de nitrocellulose (NCM-ELISA) est décrit pour la détection de *Ralstonia solanacearum* dans les tubercules de pomme de terre infectés de manière latente. La spécificité du sérum polyclonal a été améliorée par immuno-adsorption avec des souches bactériennes qui donnaient lieu à des réactions croisées avec le sérum. Les performances de la méthode après enrichissement ont été comparées avec celles de l'hybridation moléculaire, DAS-ELISA et de l'isolement sur milieu Kelman modifié. Après incubation des extraits de tubercules avec le milieu spécifique SMSA modifié durant 48 h à 30°C, les sensibilités obtenues furent similaires. Un minimum de 10 bactéries par mL a pu être détecté dans les extraits de tubercules inoculés ou naturellement infectés. Aucune réaction croisée n'a été obtenue en testant 255 échantillons prélevés au champ. Par conséquent, le post-enrichissement NCM-ELISA est une méthode fiable, sensible, rapide, économique et facile à utiliser, ce qui en fait un outil idéal pour les comparaisons variétales pour la sensibilité au flétrissement bactérien, les études épidémiologiques et le contrôle sanitaire des semences de pomme de terre dans les pays en voie de développement.

Высокочувствительный метод обнаружения *Ralstonia solanacearum* в латентно инфицированных клубнях картофеля после обогащения ELISA на нитроцеллюлозной мембране

Для обнаружения Ralstonia solanacearum в латентно инфицированных клубнях картофеля описан анализ ELISA на нитроцеллюлозной мембране (NCM-ELISA) с вторичным обогащением (пост-обогащение). Специфичность поликлональной антисыворотки была существенно улучшена за счет адсорбции с перекрестно реагирующими бактериями. Эффективность обнаружения после обогащения сравнивалась с таковым для спот-гибридизации нуклеиновой кислоты (NASH), иммунологического анализа двойного сэндвича антитела (DAS - ELISA) и культивирования разбавленных препаратов на модифицированной среде Келмана. После 48 часов инкубации экстрактов клубня с модифицированным бульоном SMSA при 30°C чувствительности пост-обогащения NCM-ELISA, DAS-ELISA и NASH были одинаковыми. Только 10 клеток/мл были обнаружены либо в инокулированных, либо в естественно инфицированных экстрактах клубня. Из 255 полевых образцов не наблюдалось никакой перекрестной реактивности NCM-ELISA. Метод NCM-ELISA(пост-обогащение), таким образом, обеспечивает надежный и чувствительный сравнительно дешевый метод, который является быстрым и удобным и подходит для оценки восприимчивости селекционных линий к бактериальному вилту, экологическим исследованиям и контролю за качеством семян в развивающихся странах.

References

Allan E & Kelman A (1977) Immunofluorescent stain procedures for detection and identification of *Erwinia carotovora* var. *atroseptica*. *Phytopathology* **67**, 1305–1312.

Black R & Elphinstone JG (1998) Developing appropriate detection methods for developing countries. In *Bacterial Wilt Disease: Molecular and Ecological Aspects* (eds Prior P *et al.*), pp. 123–127. Springer-Verlag, Berlin (DE).

Caruso P, Llop P, Palomo JL, Garcia P, Morente C & López MM (1998) Evaluation of methods for detection of potato seed contamination by *Ralstonia solanacearum*. In *Bacterial Wilt Disease: Molecular and Ecological Aspects* (eds Prior P *et al.*), pp. 128–132. Springer-Verlag, Berlin (DE).

Ciampi L, Sequeira L & French ER (1980) Latent infection of potato tubers by *Pseudomonas solanacearum*. *American Potato Journal* **57**, 377–386.

Clark MF & Adams AN (1977) Characteristics of the microplate method enzyme-linked immunosorbent assay for the detection of plant viruses. *Journal of General Virology* **34**, 475–483.

Cook D, Barlow L & Sequeira L (1989) Genetic diversity of *Pseudomonas solanacearum*: detection of restriction fragment length polymorphisms with DNA probes that specify virulence and the hypersensitive response. *Molecular Plant–Microbe Interactions* **2**, 113–121.

Cook D & Sequeira L (1991) The use of subtractive hybridization to obtain a DNA probe specific for *Pseudomonas solanacearum* race 3. *Molecular and General Genetics* **227**, 401–410.

Digat B & Cambra M (1976) Specificity of antigens in *Pseudomonas solanacearum*. In *Proceedings of the First International Planning Conference on the Ecology and Control of Bacterial Wilt Disease caused by Pseudomonas solanacearum* (eds Sequeira L & Kelman A), pp. 38–57. North Carolina State University, Raleigh (US).

Elphinstone JG, Hennessy J, Wilson JK & Stead DE (1996) Sensitivity of different methods for the detection of *Pseudomonas solanacearum* in potato tuber extracts. *Bulletin EPPO/OEPP Bulletin* **26**, 663–678.

French ER, Gutarra L, Aley P & Elphinstone J (1995) Culture media for *Pseudomonas solanacearum*: isolation, identification and maintenance. *Fitopatologia* **30**, 126–130.

Gorris MT, Priou S, Gutarra L, French ER, Cambra M & López MM (1997) [Improvement in the sensitivity of serological and molecular techniques for detection of *Ralstonia solanacearum* by selective enrichment media.] *Fitopatologia* **33**, 32 (abstr.) (in Spanish).

Hayward AC (1991) Biology and epidemiology of bacterial wilt caused by *Pseudomonas solanacearum*. *Annual Review of Phytopathology* **29**, 65–87.

Janse JD (1988) A detection method for *Pseudomonas solanacearum* in symptomless potato tubers and some data on its sensitivity and specificity. *Bulletin OEPP/EPPO Bulletin* **18**, 343–351.

Janse JD (1996) Potato brown rot in western Europe – history, present occurrence and some remarks on possible origin, epidemiology and control strategies. *Bulletin OEPP/EPPO Bulletin* **26**, 679–695.

López MM, Cambra M, Gorris MT & Pérombelon MCM (1998) Enrichment ELISA. In *Methods for the Detection and Quantification of Erwinia carotovora subsp. atroseptica on Potatoes. Laboratory Manual.* (eds Pérombelon MCM & van der Wolf JM), pp. 28–35. Scottish Crop Research Institute Occasional Publication no. 10, Dundee (GB).

OEPP/EPPO (1990) EPPO Standards PM 3/26. Phytosanitary procedures. *Pseudomonas solanacearum*. *Bulletin OEPP/EPPO Bulletin* **20**, 255–262.

Querci M, Salazar LF & Fernandez-Northcote EN (1993) Detection of Andean potato virus X isolates by radioactive and nonradioactive nucleic acid spot hybridization tests. *Phytopathology* **83**, 171–176.

Robinson-Smith A, Jones P, Elphinstone JG & Forde SMD (1995) Production of antibodies to *Pseudomonas solanacearum*, the causative agent of bacterial wilt. *Food and Agricultural Immunology* **7**, 67–79.

Salazar LF (1996) Serological methods for virus detection. In *Potato Viruses and their Control*, pp. 113–132. International Potato Center, Lima (PE).

Salazar LF & Querci M (1992) Detection of viroids and viruses by nucleic acid and probes. In *Techniques for the Rapid Detection of Plant Pathogens* (eds Duncan J & Torrance L), pp. 129–144. Blackwell Scientific Publications, Oxford (GB).

Seal SE (1998) Molecular methods for detection and discrimination of *Ralstonia solanacearum*. In *Bacterial Wilt Disease: Molecular and Ecological Aspects* (ed Prior P *et al.*), pp. 103–109. Springer-Verlag, Berlin (DE).

Seal SE & Elphinstone JG (1994) Advances in identification and detection of *Pseudomonas solanacearum*. In *Bacterial Wilt: the Disease and its Causative Agent Pseudomonas solanacearum* (eds Hayward AC & Hartman GL), pp. 35–58. CAB International, Wallingford (GB).