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Detection of *Ralstonia solanacearum* (biovar 2A) in stems of symptomless plants before harvest of the potato crop using post-enrichment DAS-ELISA

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The detection of *Ralstonia solanacearum* (biovar 2A) in stems of symptomless plants before harvest of the potato crop, instead of tubers, would not only save highly valued planting material but would be less time-consuming and would also enhance farmers' market decisions. Although pathogen detection in stems has been proven efficient for ring rot, this has never been investigated for bacterial wilt (BW). Therefore the possibility of detecting BW latent infection in stem pieces about three weeks before harvest was assessed in 57 fields of the Andean highlands of Peru. Two sensitive, specific and user-friendly serological methods were used to detect the pathogen in latently infected tubers and stems: double-antibody sandwich (DAS)-ELISA and indirect ELISA on nitrocellulose membrane (NCM) after enrichment of the plant extracts in a semi-specific broth. Optimum sample sizes of stems and tubers were evaluated for 37 potato crops showing between 0 and 0·1% BW incidence using a binomial distribution model to calculate the detection probabilities. Although results of detection using the two serological techniques had 100% concordance, detection probabilities were higher using DAS-ELISA, whatever the plant part tested. BW detection probabilities were higher for tubers than for stems; a 99% detection probability was obtained by analysing 400 stems sections or 250 tubers using DAS-ELISA. Detection of BW infection in symptomless plants 20 days before harvest using post-enrichment DAS-ELISA is a reliable and user-friendly technique that can easily be used by national plant protection services and seed programmes in developing countries.

Keywords: bacterial wilt, brown rot, seed testing, serological techniques

Introduction

Ralstonia solanacearum (biovar 2A) is the causal organism of bacterial wilt (BW), a disease that is a major constraint to potato production in tropical and sub-tropical regions worldwide and a threat to the potato seed industry in developed countries (French, 1994; Elphinstone, 2005). In cool conditions, such as those found in the tropics at altitudes above 2500 m or under cool temperate regions, infected but symptomless plants may harbour the bacterium and transmit it to progeny tubers as latent (symptomless) infection. This may lead to severe disease outbreaks when the tubers are grown at warmer sites and spread to BW-free areas. Therefore, the use of healthy planting material is the most effective means of controlling the disease (Hayward, 1991). In most countries as in Peru, BW is considered as a quarantine disease, thus seed certification programmes have a zero tolerance for the disease. Original planting material must be visually inspected during the growing season for BW symptoms and progeny tubers sampled at harvest or during storage must be tested to detect latent infections in symptomless seed lots.

For both visual and serological detection, the chances of sampling a diseased plant or tuber are a function of disease incidence and sample size. Moreover, the necessary sample size to achieve the desired probability of detection of low levels of disease contamination also has to take into account practicality and cost effectiveness (De Boer & Hall, 1996). As assay sensitivity ceases to be a limiting factor, the accuracy and reproducibility of test results may become more dependent upon appropriate sample selection and processing than upon any inherent limitation of the assay itself (Slack *et al.*, 1996). In the European Union, the standard sample size to monitor the occurrence of *R. solanacearum* in potato seed tubers is 200 tubers out of 25 t (Anonymous, 2004). This number is based on a 95% probability of detecting at least one infected tuber in a seed lot if 1·5% of the tubers are infected, according to the Poisson distribution. Clayton & Slack (1988) mentioned that in a zero-tolerance situation it is appropriate to take advantage of a stepwise sampling scheme. Therefore, the theoretical sample size of 4605 could be divided into three samples: 3005 plants for the first visual inspection (generally 40–60 days after

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planting), 1200 plants for the second visit (generally 75–100 days after planting), and 400 tubers for seed testing. In the Andes, using a binomial distribution model, Priou *et al.* (2001) determined the optimum sample size of 350 tubers per seed lot harvested from a field of 0·5 to 2 ha potatoes by assessing latent infection in composite samples of symptomless seed lots produced at high elevations. In the case of potato ring rot caused by *Clavibacter michiganensis* subsp. *sepedonicus*, another zero-tolerance situation, De Boer & Hall (1996) calculated the sample size using a binomial distribution model. The authors concluded that 400 tubers is a feasible sample size for testing seed potato lots from a 10 ha field, planted with seed tubers of which 1% were infected with the ring rot pathogen.

For the detection of ring rot, testing of stems 80 to 90 days after planting rather than tubers after harvest has been recommended because high bacterial population levels develop in stems (De Boer & Hall, 1996; Slack *et al.*, 1996). Moreover, detection sensitivity and specificity in ELISA using stems were higher than those obtained using tubers, probably due to more saprophytic bacteria and soil debris associated with tubers than with stems (De Boer *et al.*, 1994). However, stem detection tests have never been investigated for bacterial wilt seed indexing purposes. Testing of stems prior to harvesting of the potato crop has some advantages. Stems can be tested earlier than tubers, enhancing the usefulness of the results for growers' market decisions, i.e. whether to sell the lot as seed or ware potatoes. It is also simpler to randomly sample stems in the field than tubers from storage. Transportation of stem pieces to the laboratory is easier and they are more easily processed than tubers, which requires individual tuber ring fragments for crushing (Priou's protocol; Priou *et al.*, 1999, 2001) or stolon end cones (EU protocol; Anonymous, 2004).

An improved serological test with a high sensitivity and specificity is available at the International Potato Center (CIP) to detect *R. solanacearum* in latently infected tubers (Priou *et al.*, 1999, 2001). The method, post-enrichment enzyme-linked immunosorbent assay (ELISA) on nitrocellulose membrane (NCM), is suitable for seed testing in resource-poor countries and has been used by seed programmes or certification agencies in a dozen developing countries worldwide. Another highly sensitive and specific serological test, post-enrichment double-antibody sandwich (DAS) ELISA, was also optimized at CIP for the detection and semi-quantification of *R. solanacearum* in soil (Priou *et al.*, 2006). Efficiencies of the two serological detection techniques to detect *R. solanacearum* in latently infected stems and tubers were compared in Peru; random composite stem and tuber samples were tested from 57 potato crops exhibiting between 0 and 2% BW incidence. Optimum sample sizes of stems and tubers were evaluated for 37 potato crops showing between 0 and 0·1% BW incidence using a binomial distribution model to calculate the detection probabilities.

Materials and methods

Sample collection

The best sample time for stems was determined in a preliminary experiment during the main cropping season in 2004. Three potato fields with low BW incidence were selected in Cajamarca Department, two in Namora district (2750 m above sea level [m.a.s.l.]), one planted with cv. Yungay and the other with cv. Amarillis, and one in San Marcos district (2530 m.a.s.l.) planted with cv. Amarillis. From each field, 350 stems were randomly sampled following a zig-zag pattern in the field at initiation of flowering stage (70 days after planting), at full flowering stage (85 days after planting) and 20 days before harvest (100 days after planting). These fields had 0·15, 0·25 and 0·5% BW incidence at the first sampling date and 1·5, 2·5 and 2% BW incidence at the last evaluation. A fragment of one principal stem cut at the stem base (*ca.* 10 cm above the ground) was collected from each of the 350 plants. It is well known that higher populations of the pathogen occur in the plant base of the potato, and for this reason the 'stem vascular flow test' used for bacterial wilt field diagnosis is performed using a stem base fragment (first reported by Kelman in 1953; Martin & French, 1996); this has also been verified in some experiments at CIP on potato in the greenhouse (unpublished data). Similarly, Grimault *et al.* (1994) on tomato and Swanson *et al.* (2005) on geranium showed that *R. solanacearum* biovar 2A is present more often and in higher populations in the lower stem. The scissors were disinfected between the cutting of each composite sample (25 stem sections) by dipping them in a 1% solution of NaOCl. The 25-stem pieces of one composite sample were wrapped in paper towel and put inside a paper bag. The 14 bags per plot were sent in a carton box to CIP's laboratory within 24 h. Samples were kept at temperatures of 10–15°C while in the highlands and 15–20°C during transportation and at Lima. Samples were processed the next day and analysed using post-enrichment DAS-ELISA as described below.

Tubers were randomly selected from heaps at harvest or from stores within 4 weeks of harvest and sent in seed bags to CIP. Upon receipt at the laboratory, tubers were randomly divided into 14 composite samples of 25 tubers each and were kept in paper bags stored at 15–20°C before being analysed within 2 weeks of collection using post-enrichment NCM-ELISA as described below.

For the validation study, 57 farmers' fields of 0·2–1 ha located at altitudes ranging 1620–3100 m.a.s.l. in Ancash and Cajamarca departments in Peru (31 and 26 fields, respectively), planted with potato cultivars Amarilis, Canchán, Perricholi or Yungay and exhibiting between 0 and 1% BW incidence were selected during field surveys over five cropping seasons in 2005, 2006 and 2007. Four additional fields showing 2% BW incidence were also sampled as positive controls. Percentage wilted plants were estimated at one single visit (at the sample collection time) 20–30 days before harvest depending on earliness

of the potato variety. In each field 600 stems and 600 tubers were collected. Stems were sampled from 24 rows of 25 plants each selected at random following a zig-zag pattern in the field. One principal stem was collected from each of the 600 plants as described above. Samples were processed at CIP's laboratory the same day of arrival (1 or 2 days later) or kept no more than one day in a cool place (around 15°C) and analysed using post-enrichment NCM-ELISA and DAS-ELISA as described below. Tubers were randomly selected from heaps at harvest or from stores within 4 weeks of harvest and sent in seed bags to CIP. Upon reception at the laboratory, tubers were randomly divided into 24 composite samples of 25 tubers each and were kept in paper bags stored at 15–20°C before being processed within 2 weeks of collection. In Cajamarca Department samples were collected by seed inspectors of the National Agrarian Health Service (SENASA) in charge of potato seed certification and sent to SENASA seed testing laboratory in Cajamarca. Stems were sent to the CIP laboratory for processing but tuber extracts were prepared in the SENASA laboratory and enriched extracts were sent to CIP by express courier where they were analysed using both serological techniques as described below.

Sample testing

Stem detection

Stem pieces of each composite sample were washed individually with tap water and allowed to dry on clean paper towel. From each of the original 10-cm pieces, a section of approximately 5 cm in length was cut using horticultural scissors at 1–2 cm either side of an internode (thus including the two nodes). Scissors were flame sterilized between each composite sample. The 25 sections of a composite sample were dipped in a solution of 0·5% NaOCl for 3 min, this solution was then discarded and stem sections were rinsed in the same flask, first with boiled tap water (at least two times), then with alcohol at 70%, and finally with boiled tap water. Samples were allowed to dry on clean paper towel. From each of the 5-cm stem pieces, a 0·5–1·0-cm long fragment was cut within the internode using horticultural scissors. Again the scissors were flame sterilized before they were used on the next 25-stem composite sample. The 25 fragments from each composite sample were placed in a plastic bag, weighed and crushed with a wooden rolling pin. Then 3 mL of sterile citrate extraction buffer (0·1 M citric acid, 0·1 M sodium citrate, pH 5·6) per gram of stem tissue were added and the sample homogenized. The plastic bags were allowed to stand vertically on crushed ice for no more than 1 h, in order to avoid oxidation of the phenols. Stem extracts were enriched by incubating a mixture of 500 µL of stem extract with the same volume of modified Selective Medium from South Africa (SMSA) modified by Elphinstone *et al.* (1996) into sterile 1·5-mL Eppendorf tubes for 48 h at 30°C with constant agitation. The modified SMSA broth was supplemented with 780 µg L^{-1} thimerosal to increase specificity of the test. Thimerosal has been shown to eliminate cross-reactions caused by three saprophytes after enrichment of tuber extracts isolated in 2000 from native potatoes from two Peruvian departments, without losing sensitivity and specificity of the test toward the *R. solanacearum* strains previously tested (unpublished data). Two replicates for each composite sample were incubated. At the end of the incubation time, enriched stem extracts were analysed in DAS-ELISA as described in Priou *et al.* (2006). Positive results in NCM- and/or DAS-ELISA were confirmed by either isolating *R. solanacearum* on modified Kelman's medium (2·5 g L^{-1} glucose was used instead of 10 g L^{-1} to allow faster growth of biovar 2A strains; French *et al.*, 1995) or by polymerase chain reaction (PCR) if the pathogen could not be isolated from the sample as described by Priou *et al.* (2001).

Tuber detection

Tubers were processed and enriched tuber extracts tested in post-enrichment NCM-ELISA as described by Priou *et al.* (1999, 2001). The same enrichment broth was used as for the stems.

Antibodies used in ELISA

Polyclonal antibodies were produced at CIP by applying a long immunization schedule of a rabbit cross Rex × New Zealander with strains CIP 204 and CIP 104 (both of biovar 2A) of *R. solanacearum* as described in Priou *et al.* (1999). Antiserum adsorption was performed by mixing the antibodies to an equal mixture of five strains: *Pectobacterium caratovorum* subsp. *atroseptica* (CIP 421), *P.c.* subsp. *carotovorum* (CIP 400), *Dickeya dadantii* (previously *Pectobacterium chrysanthemi*; CIP 367), *Ralstonia syzygii* (NCPPB 3792) and *R. pickettii* (NCPPB 3899). Immunoglobulins (IgG) purified from the *R. solanacearum*-specific polyclonal rabbit antiserum P359 were used for both NCM- and DAS-ELISA and conjugated IgG were produced at CIP as described in Priou *et al.* (2006). IgG specificity was tested using water suspensions at 10^8 cells mL^{-1} of 273 potato isolates of *R. solanacearum* from CIP's collection, belonging to five different biovars and originating from 33 countries, and after enrichment in the broth inoculated with each strain at 10^6 cells mL^{-1}. These 273 strains were all successfully detected in NCM- and DAS-ELISA. Without enrichment of tuber extracts, sensitivity of NCM-ELISA is 10^6–10^7 cells mL^{-1} and DAS-ELISA 10^5 cells mL^{-1}, and both techniques have similar sensitivities of 2–10 cells mL^{-1} after enrichment of inoculated tuber extracts (Priou *et al.*, 1999, 2006). Similar sensitivities were obtained from naturally latently infected tuber extracts estimated by counting colonies of *R. solanacearum* on Kelman's medium before enrichment (Priou *et al.*, 1999). Sensitivity in NCM- and DAS-ELISA with strain CIP 308 belonging to biovar 1 (the most common biovar on potato) was similar to that obtained with CIP 204, the biovar 2A strain (unpublished data). The IgG of the selected antiserum P359 were also highly specific as no cross-reactions were obtained in NCM- and DAS-ELISA after enrichment of

tuber extracts inoculated with 10 identified non-*R. solanacearum* bacterial strains (CIP 400 of *P.c* subsp. *carotovorum,* CIP 421 of *P. c.* subsp. *atrosepticum,* CIP 367 of *D. dadantii,* NCPPB 1806 and 1808 of *Pseudomonas putida,* NCPPB 1965 of *Ps. aeruginosa*, NCPPB 2993 of *Burkolderia cepacia,* NCPPB 3792 of *Ralstonia syzygii,* NCPPB 3899 of *R. pickettii* and UW443 of the Blood Disease Bacterium), 20 unknown saprophytes isolated from enriched tuber extracts and 136 unknown different soil isolates. For the present study, this same antiserum batch P359 was used. The adsorbed antiserum was kept in aliquots in 50% glycerol at −20°C. After each purification of the IgG (about once a year), sensitivity and specificity were checked using suspensions of strain CIP 204 of biovar 2A at 10^5 to 10^8 cells mL^{-1}, of the 10 identified non-*R. solanacearum* bacterial strains listed above and of three non-identified saprophytes that grew in the enriched tuber extracts (cross-reactions were suppressed by supplementing the broth with thimerosal).

Probability of detection

For both techniques, the number of positive composite samples for each plot was recorded. Only one aliquot of the two analysed needed to be positive to rate the composite sample positive. Raw data shown in Table 2 were used to calculate the probabilities of detection by both serological techniques and plant part tested using a binomial distribution model. Data of the four positive control fields with 2% BW incidence were not included in the analyses. When the percentage of infected seed is very low, the probability of infected seed occurring in a sample follows a Poisson distribution (Lund & Sun, 1985). However, the binomial distribution is applicable to this system because several composite samples are analysed instead of a single sample, and the seed lot is considered positive if at least one composite sample is found positive. The average number M of positive composite samples per lot could be calculated as M = number of positive composite samples/number of positive lots. This average gives M = 24 p where p is the probability that the composite sample in a lot is positive. Thus p = M/24 and from p one can obtain probability q of obtaining no infected sample (q = 1 – p). By applying the binomial distribution, the probability P of obtaining at least one infected composite sample (i.e. enough to consider the lot infected) is $P = 1 - q^X$ where X is the number of 25-stem or 25-tuber samples to be analysed. Different values of X are tested and the optimum sample size is the number X that allows a probability (P) of detection of 99% (Snedecor & Cochran, 1980).

Results

The preliminary experiment to determine the optimum sampling date for stems showed that BW incidence increased two weeks after the initiation of flowering of the potato crop (i.e. at full flowering), and were higher 20 days before harvest (Table 1). The number of positive composite samples also increased with the sampling time and was highest three weeks before harvest in all three fields. Tubers harvested from these lots were also positive for BW latent infection with a relatively similar number of positive composite tuber samples (Table 1). Thus for the validation experiment, all stem samples were taken about 3 weeks before harvest to avoid the risk of missing late infections.

In the validation study, 45 of the 57 farmers' fields randomly sampled were positive (79%). Positive samples were all confirmed by isolation on modified Kelman's medium (French *et al.*, 1995). *Ralstonia solanacearum* was detected in 17 out of 27 symptomless fields (63%) and in all lots with visible wilt incidence even from those with very low incidence (Table 2). The stems and tubers sampled from the four fields with 2% wilt incidence (lots 10, 14, 16 and 57) were positive with a high number of infected composite samples: NCM- and DAS-ELISA analysis showed an average of 13 and 18 positive for stems and 23 and 24 positive for tubers among the 24 sub-samples analysed, respectively. The number of positive tuber sub-samples was higher than the number of

Table 1 Detection of *Ralstonia solanacearum* in 14 composite samples of 25 stems (post-enrichment DAS-ELISA) or 25 tubers (post-enrichment NCM-ELISA) according to the sampling date in three fields in Cajamarca Department, Peru

Field location	Sampling time (days after planting)	BW[a] incidence (% wilted plants)	Number positive samples/ 14 composite stem samples	Number positive samples/ 14 composite tuber samples
Namora 1	Initial flowering (70)	0·15	1	
	Full flowering (85)	0·5	1	
	20 days before harvest (100)	1·5	5	
	Harvest			4
Namora 2	Initial flowering (70)	0·15	1	
	Full flowering (85)	1·5	3	
	20 days before harvest (100)	2·5	7	
	Harvest			5
San Marcos	Initial flowering (70)	0·5	1	
	Full flowering (85)	1	2	
	20 days before harvest (100)	2	5	
	Harvest			5

[a]BW, bacterial wilt.

Table 2 Results of detection of *Ralstonia solanacearum* using post-enrichment NCM- or DAS-ELISA in 24 composite samples of 25 stems or 25 tubers each collected from 57 potato fields with 0 to 2% bacterial wilt incidence in two departments in Peru

Field lot[a]	BW incidence[b]	Province – Locality	Altitude (m)	Composite samples positive in NCM-ELISA		Composite samples positive in DAS-ELISA	
				Stems	Tubers	Stems	Tubers
1	0·5	Carhuaz - El Puente	2480	3	2	7	11
2	0·1	Carhuaz - El Tambo	2100	5	1	8	8
3	0	Yungay – Mitocucho	2250	3	14	17	19
4	1	Yungay – Mancos	2320	9	1	15	1
5	0	Yungay – Huancapapa	2315	0	0	0	0
6	0·1	Yungay – Pampachacra	2375	1	1	7	6
7	0·1	Yungay - Ranrahirca	2300	9	21	17	22
8	0·1	Yungay – Ranrahirca	2315	5	22	8	22
9	0	Caraz – Llocllasaca	2125	0	0	0	0
10	2	Carhuaz - Carhuaz	2515	8	24	17	24
11	0·1	Caraz – Cañasbamba	2350	1	3	1	5
12	0	Caraz – Cañasbamba	2350	2	0	2	0
13	0	Yungay – Mancos	2300	0	0	0	0
14	2	Caraz - Caraz	2300	22	24	24	24
15	1	Yungay – Campo Santo	2350	18	12	22	17
16	2	Caraz - Cañasbamba	2300	4	23	10	24
17	0	Yungay - Ranrahirca	2350	1	1	1	1
18	0	Yungay - Ranrahirca	2350	0	0	0	0
19	0	Yungay - Huarascucho	2300	0	0	0	0
20	0	Caraz - Cañasbamba	2250	3	3	2	2
21	0[c]	Yungay - Aura	2300	21	21	17	19
22	0·5	Yungay – Cañasbamba	2500	16	23	16	24
23	0	Yungay - Ancoraca	3100	3	7	4	8
24	0·8	Yungay - Yungay	2350	7	21	7	23
25	0·5	Yungay - Huarascucho	2350	5	7	8	7
26	0·6	Yungay - Yungay	2450	4	16	4	19
27	0·4	Carhuaz - Carhuaz	2550	6	12	9	16
28	0	Caraz - Pueblo Libre	2320	2	4	4	7
29	0	Caraz - Santa Cruz	2500	2	4	3	7
30	0	Carhuaz - Hualcan	2400	3	4	4	4
31	0	Yungay - Mancos	2350	1	3	2	3
32	0	Cajabamba – Cajabamba	2900	0	0	0	0
33	0	Cajamarca - Sulluscocha	2800	0	0	0	0
34	0	San Marcos - Cochamarca	2600	0	0	0	0
35	0·1	Chota - Chuyubamba Bajo	2283	1	2	1	2
36	1	Celendín - Cruz Verde	2736	19	20	21	20
37	0	Chota - Yacuchingana	2916	0	0	0	0
38	0·02	Cajabamba - Nuñumabamba	1620	9	9	15	12
39	0	San Miguel - Catilluc	2850	1	8	2	13
40	0·24	Contumaza - La Travesía	2820	20	11	24	17
41	0·65	Celendín - Bellavista	2721	1	0	1	0
42	0	San Marcos - La Colmena	2590	0	0	0	0
43	0·03	Cajabamba - El Porvenir	1975	0	1	0	2
44	0·39	Contumaza – La Cocha	2550	11	8	11	10
45	0	Chota - Cadmalca Alto	2966	4	3	4	3
46	0·1	Hualgayoc - Maygasbamba	2601	10	12	12	20
47	1	Cajamarca - Pampa Larga	2530	1	8	1	10
48	0·1	Hualgayoc - Maraypampa	2568	2	3	4	4
49	0	Cajamarca - Sulluscocha baja	2720	5	1	5	1
50	0	Cajamarca - Sulluscocha baja	2720	3	5	5	5
51	0·29	Celendin - Anchorco	2937	5	17	5	23
52	0	San Marcos - Chuquipuquio	2900	2	1	3	6
53	0	San Marcos - Chuquipuquio	2900	1	16	5	20
54	0·2	Celendín - Shigua	2846	7	20	13	23
55	0	Contumaza - Cocha	2550	0	3	0	4
56	0·21	San Miguel - La Huanchilla	2780	1	1	1	3

Table 2 *Continued.*

Field lot[a]	BW incidence[b]	Province – Locality	Altitude (m)	Composite samples positive in NCM-ELISA		Composite samples positive in DAS-ELISA	
				Stems	Tubers	Stems	Tubers
57	2	San Miguel – La Huaylla	2105	17	23	21	24
All lots except four lots (in bold) showing 2% BW incidence	Total positive composite samples		233	352	318	449	
	Number positive lots		41	41	41	41	
	Number lots tested		53	53	53	53	
	M[d]		5·68	8·59	7·76	10·95	
	p[d]		0·24	0·36	0·32	0·46	
	q[d]		0·76	0·64	0·68	0·54	
Only lots showing ≤ 0·1% BW incidence	Total positive composite samples		100	173	153	225	
	Number positive lots		25	26	25	26	
	Number lots tested		37	37	37	37	
	M[d]		4·00	6·65	6·12	8·65	
	p[d]		0·17	0·28	0·26	0·36	
	q[d]		0·83	0·72	0·75	0·64	

[a]1 to 31 from Ancash Department, 32 to 57 from Cajamarca Department. Four lots showing 2% BW incidence and omitted for the probability calculation are in bold.
[b]Percentage wilted plants estimated at one single visit 20–30 days of harvest depending on earliness of the potato variety.
[c]Adjacent to a highly infected field.
[d]M = [number of positive composite samples / number of positive lots] and M = 24 p, where 24 is the number of composite samples tested per lot in the field validation study and p the probability that a composite sample in a lot is positive; thus, the probability of obtaining no infected sample is q = 1 – p = 1 – M/24.

positive stem sub-samples in all four lots. In the other 41 positive lots the number of positive sub-samples ranged from 1 to 24 with averages of 5·7 and 7·8 for stems, and 8·6 and 10·9 for tubers analysed by NCM- and DAS-ELISA, respectively (Table 2). If only lots showing between 0 and 0·1% BW incidence are considered, these averages decreased to 4 and 6·1 for stems, and 6·7 and 8·7 for tubers, respectively.

When comparing serological techniques, there was 100% concordance between the two ELISA types whatever the plant part tested. However, the number of positive samples was higher when using DAS- than NCM-ELISA for both tubers and stems as shown by M values (i.e. number of positive composite samples / number of positive lots) presented in Table 2.

When comparing stem and tuber detection results, 93% of lots gave the same results whatever the ELISA technique used (Table 2). There were only four lots that showed non-concordance of results between stem and tuber tests: two lots were positive with stems but negative with tubers (lots 12 and 41), and two lots were negative with stems but positive with tubers (lots 43 and 55).

Probabilities of detection obtained with both serological techniques according to the plant part tested for the 37 lots with ≤ 0·1% incidence are shown in Table 3 (the lots with higher BW incidences are unlikely to be analysed for latent infection because of the presence of visible BW symptoms at harvest). When analysing data from Cajamarca and Ancash departments separately, very similar probabilities of detection were obtained (data not shown). The probability of detection was higher with DAS-ELISA than with NCM-ELISA whatever the plant part tested, leading to a lower sample size (Table 3). A 95% probability of detection was obtained by analysing 250 stems or 175 tubers using DAS-ELISA, or by analysing 400 stems or 225 tubers using NCM-ELISA (Table 3). The optimum sample size (i.e. to get a 99% detection probability) was 375 stems and 250 tubers using DAS-ELISA, and 600 stems and 325 tubers using NCM-ELISA.

Discussion

The best sampling time for stem detection seemed to be at the end of the cropping season (i.e. 20–30 days before harvest) since the number of positive samples in three different BW-infected plots increased with time. Similarly, De Boer & Hall (1996) reported probabilities of detection of ring rot stem infection higher 80 days than 60 days after planting. First symptoms of BW generally appear at the initiation of flowering stage; however, early detection could miss some later infections due to disease spread through soil, water or farmers' tools. It was found that the lignification of stems of senescent plants was not a problem for processing stem sections considering that the pathogen is located in xylem vessels and not in the lignified pith. Thus the bacteria are removed from the vessels while crushing even partially highly lignified stem

Table 3 Probability (P) of detection of *Ralstonia solanacearum* according to the number of tubers or stems analysed using post-enrichment NCM- or DAS-ELISA for the 37 lots showing equal to or less than 0·1% wilt incidence

Number of stems or tubers analyzed	Number of 25-tuber or 25-stem composite samples	P[a] using NCM-ELISA		P[a] using DAS-ELISA	
		Stems	Tubers	Stems	Tubers
		q = 0·833[b]	q = 0·723	q = 0·745	q = 0·639
25	1	0·17	0·28	0·26	0·36
50	2	0·31	0·48	0·44	0·59
75	3	0·42	0·62	0·59	0·74
100	4	0·52	0·73	0·69	0·83
125	5	0·60	0·80	0·77	0·89
150	6	0·67	0·86	0·83	0·93
175	7	0·72	0·90	0·87	**0·96**
200	8	0·77	0·93	0·91	0·97
225	9	0·81	**0·95**	0·93	0·98
250	10	0·84	0·96	**0·95**	**0·99**
275	11	0·87	0·97	0·96	0·99
300	12	0·89	0·98	0·97	1·00
325	13	0·91	**0·99**	0·98	1·00
350	14	0·92	0·99	0·98	1·00
375	15	0·94	0·99	**0·99**	1·00
400	16	**0·95**	0·99	0·99	1·00
425	17	0·95	1·00	0·99	1·00
450	18	0·96	1·00	1·00	1·00
475	19	0·97	1·00	1·00	1·00
500	20	0·97	1·00	1·00	1·00
525	21	0·98	1·00	1·00	1·00
550	22	0·98	1·00	1·00	1·00
575	23	0·98	1·00	1·00	1·00
600	24	**0·99**	1·00	1·00	1·00

[a]Probability P of obtaining at least one infected composite sample following a binomial distribution is $P = 1 - q^X$ where X is the number of composite 25-stem or 25-tuber samples to be analysed and q is the probability of obtaining no infected sample ($q = 1 - p$ where p is the probability that a composite sample in a lot is positive). Different values of X are tested and the optimum sample size is the number X that allows a probability P of detection of 95% or 99% (in bold).
[b]As obtained in Table 2.

sections using a hammer or wooden roll. However, special care should be taken with stems with empty pith to avoid cross-contamination during the disinfection process; this is why the stem sections were cut at each side of an internode, so that the final fragment to be analysed was cut within the internode.

There is no apparent correlation between the estimated wilt incidence and the number of stem or tuber sub-samples positive in ELISA, thus the field evaluation of BW symptoms is not a good estimation of the crop health status. However, the wilt incidences are only rough estimations since they were scored during one single visit 20–30 days before harvest when the crop was sometimes already senescent, and in some cases later field infections could have occurred. Trace incidence of BW can also escape field inspection if diseased plants dry up partially and recover or if seed growers remove them before the technician's visit.

The high percentage (63%) of symptomless fields positive for BW latent infection is in concordance with the results from 2000 where 53% of symptomless fields tested from Cajamarca Department were latently infected (Priou *et al.*, 2001). This confirms that infected seed tubers coming from the contaminated inter-Andean valleys of Ancash and Cajamarca departments are responsible for contamination of seed-producing areas in the highlands of Peru. Surprisingly, BW infection was found at high elevations (2900 to 3100 m.a.s.l.) where normally the BW disease does not occur. Although low temperatures prevent development of wilt, the pathogen remains in the plant and is transmitted to progeny tubers. These results provide data to local authorities and plant protection services for mapping latently infested areas and applying certification regulations for the production of BW-free seed. Moreover, detection of BW infection in areas where BW was previously not reported is a consequence of the flow of contaminated seed from lower endemic areas to higher areas. Farmers often move the degenerated seed to higher areas to improve its physiological condition and reduce virus infection, but this leads to contamination of disease-free areas.

The number of positive sub-samples was higher when using DAS- than NCM-ELISA whatever the plant part tested. Although both techniques have sensitivities of 2–10 cells mL^{-1} after enrichment of inoculated tuber extracts, sensitivity of DAS is ten to a hundredfold higher than NCM with bacterial suspensions without enrichment (Priou *et al.* 1999, 2006). Thus in naturally infected

samples enrichment of plant extracts with very low populations of *R. solanacearum* may lead to bacterial concentrations below the detection threshold for NCM but sufficient to rate positive in DAS.

The result discrepancies between stem and tuber detection in four lots could not be due to a higher rate of cross-reactions in NCM- than in DAS-ELISA since all positive samples were confirmed by isolation of *R. solanacearum* from the enriched extracts and 100% concordance of results between the two ELISA techniques was obtained whatever the plant part analysed. Several hypotheses can explain these discrepancies: (i) the most probable one is a different origin (e.g. from a different field) of tubers sampled by farmers at harvest or in storage than the mother plants sampled during the growing season by the technician (it was not feasible to be present at all harvest dates to sample tubers); (ii) sampling differences in the field and at harvest or in storage; and/or (iii) different tuber infection pathways, e.g. infection of tubers directly through the soil without prior infection of stems vessels. It is generally admitted that the bacterium must move from infected stems via xylem vessels in the stolons to infect progeny tubers. Negative results in stems and positive in tubers may reveal that tubers can be directly infected by soil inoculum through stolon roots without translocation of the pathogen through the stem vascular system. Another study at CIP, where all stems and tubers from individual plants were analysed from four different fields in two departments in Peru, showed an average 83% concordance of detection between stems and tubers that seems to confirm this hypothesis of direct tuber infection from the soil (unpublished results). So the stem detection technique may not be highly reliable for indexing of individual plants, e.g. for positive selection purposes in developing countries where sometimes BW-free potato crops are not available, and hence the zero-tolerance is not applicable for local seed production.

Alternatively, discrepancies between positive stem samples and negative tuber samples might be due to slow translocation of the pathogen within the plant, resulting in failure of the bacterium to reach some of the tubers prior to harvest, or only a portion of the xylem vessels in each stem may be infected so that only very few tubers whose vascular tissue is continuous to infected stem vessels become infected.

With both serological techniques probabilities of BW detection were higher for tubers than for stems. This is not in agreement with De Boer & Hall (1996) who reported that probabilities of ring rot detection were lower for tubers than for stems. With a sample size of 250, a 99% probability of detection of *R. solanacearum* in tubers was achieved using DAS-ELISA (Table 3). This is much lower than the optimum sample size recommended to analyse tubers from symptomless fields using NCM-ELISA, where 350 tubers were necessary to obtain a probability of 99% to detect at least one positive sample (Priou *et al.* 2001). However, if stem sections are analysed using DAS-ELISA, 400 would be the optimum sample size. This sample size is recommended to ensure high detection efficiency in symptomless crops in areas where environmental conditions are not favourable to disease development. De Boer & Hall (1996), using a binomial distribution model for ring rot detection, concluded that 400 tubers is a feasible sample size for testing seed potato lots from a 10 ha field, planted with seed tubers of which 1% were infected with the ring rot pathogen. However, in BW-endemic areas, to increase practicability and reduce the cost of the test, the sample size could be reduced to 250 stem sections or 200 tubers collected from a field of 0·5–2 ha, which according to FAOSTAT (2009) produces an average of 12 t harvested potatoes per ha in the Andes. This number of tubers is closer to the standard sample size used in the European Union to monitor the occurrence of *R. solanacearum* in potato seed tubers (i.e. 200 tubers out of 25 t of potatoes; Anonymous, 2004). Since laboratory indexing is applied to seed lots that have passed visual field inspections, the added chances to detect the disease by indexing helps to identify seed lots that could have inadvertently served as sources of inoculum.

In conclusion, detection of BW infection in symptomless plants before harvest of symptomless crops using post-enrichment DAS-ELISA is a sensitive and user-friendly technique that national plant protection services and seed programmes in developing countries can use for mapping BW latent infection in potato growing areas and for seed certification, without incurring too high a cost of labour and material. This method is even more suitable to Andean highland areas where sloping land and limited means of transportation make sampling and movement of tubers difficult for field inspectors. This procedure has one disadvantage in that stems have to be processed within 2–3 days of sampling, whereas tubers can be kept for several weeks under cool conditions until processing. In some cases the use of NCM-ELISA would be preferred by users because after dotting membranes with enriched extracts they can be kept until further ELISA analyses can be performed with several membranes at once, or can be sent to another laboratory for performing the serological test. Moreover, DAS-ELISA could give unreliable results in unstable temperature conditions during the incubation of the conjugated antibodies if there are excessively warm conditions in the laboratory. Therefore, users should choose the technique that suits best their laboratory conditions and adjust the optimum sample to be analysed according to the technique used and the detection purposes. Since the antibodies produced at CIP can detect a wide range of strain diversity from five different biovars of *R. solanacearum*, this technique could also be used in other countries where several biovars may occur in the potato fields.

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