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Optimisation of sample size for the detection of latent infection by *Ralstonia solanacearum* in potato seed tubers in the highlands of Peru

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Summary

A sampling strategy was evaluated in the Andean highlands of Peru to optimise the detection of *Ralstonia solanacearum* in seed tubers harvested from symptomless crops. A sensitive and specific serological method developed at CIP was used to detect the pathogen in latently infected tubers. Optimum sample size was evaluated for symptomless crops after analysing various numbers of composite samples and using a binomial distribution model to calculate the detection probabilities. *R. solanacearum* was detected in all lots from fields with visible symptoms, so validating the detection technique. About half of the seed lots from apparently healthy fields at altitudes of up to 3,100 m were found positive for the pathogen. *R. solanacearum* was detected with 99% probability in samples of 350 tubers from seed lots from symptomless crops. This number of seed tubers could feasibly be processed in a seed-health test without incurring too high a cost for labour and materials.

Introduction

Bacterial wilt (BW) or brown rot of potato is a devastating disease in both the developed and developing world. In cool conditions, such as are found in the tropics at altitudes above 2,500 m, infected but symptomless plants may harbour the bacterium and transmit it to progeny tubers as latent infection. This may lead to severe disease outbreaks when the tubers are grown at warmer sites (Ciampi et al., 1980; Hayward, 1991). Therefore, the use of healthy planting material is the most effective means of controlling the disease (Hayward, 1991). Original planting material used in formal or traditional multiplication schemes must be tested for latent infection by *Ralstonia solanacearum* (Smith) Yabuuchi et al. (1992, 1995), the causal agent of bacterial wilt in potato.

In Australia and in many countries of Europe, North America and Latin America, BW is considered a quarantine disease with zero tolerance in certified tubers. In a certification scheme, visual inspection is complemented by laboratory tests to detect latent infections. However, the principles underlying the probability of visual and serological detection remain the same, i.e. the chances of sampling a diseased plant or tuber are a function of disease incidence and sample size (De Boer & Hall, 1996). Sample size necessary to achieve the desired probability of detection of low levels of

disease contamination also has to take into account the aspects of practicality and cost effectiveness (Geng et al., 1983; De Boer & Hall, 1996).

In the European Union, the standard sample size for monitoring the occurrence of *R. solanacearum* in potato seed tubers is 200 tubers out of 25 t (Janse, 1988; Anon., 1990). 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 (De Boer, personal communication).

In South Africa, in G2 to G8 seed multiplication crops, 4,605 tubers/planting unit are analysed by enzyme-linked immunosorbent assay (ELISA). This sample size is based on a statistical model developed by Clayton & Slack (1988) to give a 99% probability of detection if 0.1% of tubers are infected and the planting unit is 10 ha or more (Swanepoel & Theron, 1999). However, Clayton & Slack (1988) mentioned that in a zero-tolerance situation it is appropriate to take advantage of a stepwise sampling scheme. This is because potato inspection schemes involve multiple inspections and generally comprise two field visits and one tuber test. If disease is not found in a particular sample, then subsequent sampling will take place. There is a potential reduction in costs and labour when inspections take place in such a stepwise manner. Therefore, the theoretical sample size of 4,605 could be divided into three samples: 3,005 plants for the first visual inspection (generally 40–60 days after planting), 1,200 plants for the second visit (generally 75–100 days after planting) and 400 tubers for seed testing (Clayton & Slack, 1988).

Potato ring rot disease, caused by *Clavibacter michiganensis* subsp. *sepedonicus*, is also a 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.

Certification agencies in several southern states of the USA (e.g. Florida and California) have a set sample size for virus testing in winter-planted seed tubers. Regardless of field size under 16 ha, the sample size is 400 tubers per seed lot (Anon., 1985).

Most of the reported sampling strategies mentioned above are based on statistical models. There are no data on in situ assessment of the optimum sample size for BW, nor on the incidence of latent BW infection in tubers produced at high elevations. Therefore, a zero-tolerance sampling strategy was evaluated in the Andean highlands of Peru to optimise the detection of *R. solanacearum* in seed tubers harvested from symptomless crops. A sensitive and specific serological method to detect *R. solanacearum* in latently infected tubers is now available at CIP (Priou et al., 1999a, 1999b). The method, called post enrichment enzyme-linked immunosorbent assay on nitrocellulose membrane (NCM-ELISA), is suitable for seed testing in resource-poor countries. Effectiveness of the detection technique was validated in crops exhibiting low BW incidence. Optimum sample size was evaluated for symptomless crops after analysing various numbers of composite samples and using a binomial distribution model to calculate the detection probabilities. When the percentage of diseased seed is very low, the probability of diseased seed occurring in a sample follows a Poisson

distribution (Lund & Sun, 1985). However, the binomial distribution is applicable to our system because several composite samples are analysed instead of a single sample, and the seed lot is considered as positive if at least one composite sample is found positive.

Materials and methods

Sample collection. Six hundred tubers/seed lot were collected from fields of 0.25 to 1.5 ha during two cropping seasons, 1999 and 2000. A seed lot is defined as comprising tubers of one cultivar harvested from one field site and originating from the same seed source. Tubers were randomly selected from heaps at harvest or from stores within 4 weeks of harvest. Sixteen fields in Cajamarca and La Libertad departments in Peru exhibiting low incidence of BW (0.1–3%) were selected. BW incidence was scored according to the number of wilted plants observed among a total of 400 plants per hectare by examining 20 rows of 20 plants following a cross-pattern design.

Samples were also collected from harvested tubers of symptomless crops in BW-endemic areas or in areas at higher elevations suspected of having been planted with infected seed produced in endemic areas at lower elevations. After one or two visual field inspections to confirm the absence of BW symptoms, 34 samples were collected in seed lots in seven provinces (Cajamarca, Celendin, Chota, Contumaza, San Marcos, San Miguel y San Pablo) in Cajamarca Department and Huamachuco Province of La Libertad in Peru. Two fields were chosen as tentative negative controls because they were obtained from reliable in vitro propagation (lots 8 and 10, Table 1). Samples were taken from seed lots of various commercial and native cultivars (Table 1). Altitudes for symptomless fields ranged from 2200 to 3400 m. Tubers were kept in paper bags and stored at room temperature before being processed within 2 weeks of collection.

Sample testing. Tubers were randomly divided into 24 composite samples of 25 tubers each. For the detection of *R. solanacearum*, tubers were processed as described by Priou et al. (1999a, 1999b) in the laboratory of the Peruvian Agrarian Health Service (SENASA) in Cajamarca, Peru. After tuber disinfection, strips along the vascular ring were removed from the stolon end of each tuber with a flame-sterilized cuticle remover; fragments from a composite sample of 25 tubers were put together in one plastic bag and weighed. Sterile citrate buffer (0.1 M citric acid, 0.1 M sodium citrate, pH 5.6) was added at 2 ml g^{-1} of tuber tissue and the tuber fragments were squashed with a wooden roll. Two 0.5 ml aliquots of tuber extract were taken from each bag and mixed with an equal volume of modified SMSA broth (Elphinstone et al., 1996) for enrichment; these were incubated for 48 h at 30 °C with manual agitation at least twice a day. For each seed lot, 20 µl each of the resulting 48 enriched tuber-extract aliquots were dotted on the 8×12 cm-nitrocellulose membrane (NCM) 0.45 µm pore size (Biorad) previously immersed for 5 min in 30 ml TBS (Tris-buffered saline: 0.02 M Tris-HCl (pH 7.5), 0.05 M NaCl, 0.01% NaN_3) using a dot-blot apparatus and analysed by 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) on a Petri dish

15-cm diameter, with gentle rotary agitation (50 rev min^{-1}). The blocking solution was discarded and the membrane incubated for 2 h with gentle agitation in 30 ml of the antibody solution; 100 µl of CIP *R. solanacearum* specific antiserum diluted 1:1000 were added to another 30 ml of the same blocking solution. The antibody solution was then discarded and the membrane washed with 30 ml T-TBS (TBS with 0.05% Tween-20) three times for 3 min each with constant agitation at 100 rev min^{-1} to remove unbound *R. solanacearum*-antibodies. After discarding the last washing

Table 1. Detection of *Ralstonia solanacearum* in seed lots from fields with various bacterial wilt (BW) incidences in Cajamarca and La Libertad departments, Peru.

Lot N°	Bacterial wilt in the field (%)	ELISA-positive among 24 composite samples	Cultivar	Site	Altitude (m)
1	0	5	Canchan	La Encañada	2800
2	0	7	Canchan	La Encañada	3000
3	0	8	Canchan	Huamachuco[a]	3000
4	0	5	Amarilis	San Juan	2200
5	0	3	Amarilis	Cajamarca	2750
6	0	14	Canchan	La Encañada	3000
7	0	6	Amarilis	Huamachuco[a]	3000
8	0	0	Amarilis	Contumaza	2700
9	0	0	Yungay	La Encañada	3000
10	0	0	Perricholi	Porcon cooperative	3150
11	0	4	Tumbay	Chota	2900
12	0	1	Atahualpa	San Miguel	2800
13	0	0	Huagalina	Sucre	2650
14	0	0	Huagalina	Sucre	2900
15	0	0	Canchan	Huamachuco[a]	3200
16	0	0	Canchan	Huamachuco[a]	3350
17	0	0	Yungay	Huamachuco[a]	3400
18	0	0	Amarilis	Huamachuco[a]	3200
19	0	0	Yungay	Huamachuco[a]	3150
20	0	0	Canchan	Huamachuco[a]	3200
21	0	0	Canchan	Huamachuco[a]	3200
22	0	8	Yungay	La Encañada	2900
23	0	11	Yungay	Baños del Inca	3000
24	0	4	Amarilis	La Encañada	3000
25	0	6	Amarilis	La Encañada	3100
26	0	0	Amarilis	Baños del Inca	2800
27	0	0	Amarilis	Baños del Inca	2900
28	0	13	Yungay	Baños del Inca	2950
29	0	10	Amarilis	La Encañada	3100
30	0	7	Amarilis	La Encañada	3000
31	0	0	Amarilis	Sucre	2650
32	0	9	Amarilis	Sucre	2700
33	0	0	Canchan	Sucre	2900
34	0	5	Yungay	Baños del Inca	3000
Subtotal		126			

Table 1. (continued).

Lot N°	Bacterial wilt in the field (%)	ELISA-positive among 24 composite samples	Cultivar	Site	Altitude (m)
35	0.1–1	1	Amarilis	Llacanora	2900
36	0.1–1	15	Amarilis	Sucre	2850
37	0.1–1	19	Canchan	Sucre	2600
38	0.1–1	18	Canchan	Eduardo Villanueva	2200
39	0.1–1	17	Canchan	Sucre	2750
40	0.1–1	3	Canchan	Eduardo Villanueva	2240
41	0.1–1	8	Canchan	Sucre	2650
42	0.1–1	8	Mixture	Sucre	2700
43	0.1–1	5	Chaucha	San Pablo	2700
44	1.1–3	11	Canchan	José Galvez	2600
45	1.1–3	13	Canchan	Sucre	2650
46	1.1–3	18	Mixture	Sucre	2650
47	1.1–3	13	Mixture	Sucre	2700
48	1.1–3	21	Amarilis	Sucre	2600
49	1.1–3	3	Amarilis	San Juan	2200
50	1.1–3	15	Amarilis	Llacanora	2900
Subtotal		188			

[a] designates those sites in La Libertad.

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, 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). BCIP solution was prepared by dissolving 15 mg of BCIP (*p*-toluidine salt of 5-bromo, 4-cloro, 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 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 M Tris base, 0.1 M NaCl, 5 mM $MgCl_2$, $6H_2O$, 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 number of positive composite (25-tuber) samples for each seed lot was recorded. Only one aliquot of two needed to be positive to rate the composite sample positive.

Positive results in ELISA were confirmed by either isolating *R. solanacearum* on modified Kelman's medium (French et al., 1995) in SENASA, or at CIP by

polymerase chain reaction (PCR) if the pathogen could not be isolated from the sample. One ml of enriched tuber extract was centrifuged at 12,000 rpm for 5 min, then the pellet was washed three times with nuclease-free water. It was then resuspended in 20 µl nuclease-free water and boiled for 15 min to denature DNA. Samples were kept at -20 °C until use. Two µl of this suspension was used as a template for PCR using primers 759 (5'GTCGCCGTCAACTCACTTTCC3') and 760 (5'GTCGCCGTCAGCAATGCGGAATCG3') of Opina et al. (1997) at the final concentration of 0.5 pmol μl^{-1}. PCR reactions were conducted in 25 µl of 1×PCR buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton), 0.05 mM of each dNTP, 2 µl of the template, and 0.01 U/µl of *Taq* DNA polymerase. Amplifications were performed with a PTC 200 programmable Thermo Cycler (MJ Research, USA) using the following temperature sequence: samples were denatured at 94 °C for 3 min, annealed at 53 °C for 1 min and extended at 72 °C for 1.5 min, followed by 30 cycles of 94 °C for 15 sec, 62 °C for 15 sec and 72 °C for 15 sec and a final extension at 72 °C for 5 min (Opina et al., 1997). An aliquot of 15 µl of the PCR product was separated by electrophoresis on a 1.5% agarose gel, and the 281 bp-fragment was visualized after staining with 0.5 µg ml^{-1} ethidium bromide. Pathogenicity of the isolates of *R. solanacearum* was confirmed by inoculating tomato plantlets as described by Janse (1988).

Calculations. For a given bacterial wilt incidence (%), the average number of positive composite (25-tuber) samples per lot, M, 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 we obtain q=1-p (probability of obtaining no infected sample). By applying the binomial distribution, the probability P to obtain at least one infected composite sample (i.e. enough to consider the lot infected) is $P=1-q^X$ where X (here 24) is the number of 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 95% (Snedecor & Cochran, 1980).

In studying sampling schemes for a zero-tolerance situation, we focus on the probability of erroneously accepting a field in which BW is present (PEA). This probability is given by PEA=1-P. To determine this probability we assume (1) that disease occurs randomly in the field (generally the case when the main inoculum source is infected seed), (2) that a sample size n is taken at random and (3) that each plant has the same probability of being diseased (Clayton & Slack, 1988).

Results

R. solanacearum was detected in several composite samples of all lots that came from fields with visible symptoms, even from those with very low levels of wilt incidence (Table 1). For the lots coming from fields with 0.1–1% wilt incidence, a probability of 95% to detect one positive sample was reached by analysing 150 tubers (Table 2). For fields with between 1.1 and 3% wilt, 100 tubers needed to be analysed to obtain

Table 2. Probability (P) of detecting *Ralstonia solanacearum* in seed lots from fields with various BW incidences according to the sample size analysed in Peru.

Composite samples (X)	Tubers	P for 0% BW [a] 2200–3350 m	P for 0.1–1% BW [b] 2200–2900 m	P for 1.1–3% BW [c] 2200–2900 m
1	25	0.292	0.435	0.560
2	50	0.499	0.681	0.806
3	75	0.645	0.820	0.915
4	100	0.749	0.898	**0.963**
5	125	0.822	0.942	0.984
6	150	0.874	**0.967**	0.993
7	175	0.911	0.982	0.997
8	200	0.937	0.990	0.999
9	225	**0.955**	0.994	0.999
10	250	0.968	0.997	1.000
11	275	0.978	0.998	1.000
12	300	0.984	0.999	1.000
13	325	0.989	0.999	1.000
14	350	0.992	1.000	1.000
15	375	0.994	1.000	1.000
16	400	0.996	1.000	1.000
17	425	0.997	1.000	1.000
18	450	0.998	1.000	1.000
19	475	0.999	1.000	1.000
20	500	0.999	1.000	1.000
21	525	0.999	1.000	1.000
22	550	0.999	1.000	1.000
23	575	1.000	1.000	1.000
24	600	1.000	1.000	1.000

[a] $P = 1 - 0.708^X$
[b] $P = 1 - 0.565^X$
[c] $P = 1 - 0.440^X$

P=95% (Table 2).

Eighteen seed lots (53.3%) coming from apparently healthy fields were found positive in ELISA (Table 1). Five of these positive lots were from recognized seed growers. This result indicates that infected seed coming from the contaminated inter-Andean valleys of Cajamarca is responsible for contamination of seed-producing areas. Lots 8 and 10, which served as tentative negative controls, were found negative. These negative results confirm the specificity of the test (absence of cross-reaction in ELISA). All positive results in ELISA were confirmed by isolating the pathogen in Cajamarca or by PCR at CIP; pathogenicity tests on tomato were also positive (data not shown).

The analysis of 225 tubers gives a probability of 95% to detect at least one positive sample in lots harvested from symptomless fields (Table 2). When data were separately analysed by year (14 lots analysed in 1999 and 20 lots in 2000), approximately the same results were obtained (275 tubers in 1999 and 200 in 2000, data not shown).

Discussion

The serological detection technique has been validated because the pathogen could be detected in all fields with visible BW symptoms. No cross-reaction was observed (all positive ELISA results were confirmed), and expected negative seed lots were found negative. Under our conditions, sensitivity of NCM-ELISA is high: 2–10 cells ml^{-1} (Priou et al., 1999a, 1999b) which is the level of sensitivity reported only for nested PCR (Elphinstone et al., 1996), but NCM-ELISA is faster, cheaper and easier to use than PCR-based methods, making it a suitable technique for seed testing in developing countries.

Surprisingly, BW infection was found at elevations up to 3100 m where the disease does not normally 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 demonstrates 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 (Thiele, 1998).

Results in Peru allow us to set the sample size at 350 tubers/seed lot to detect latent infection by *R. solanacearum* (at least a 99% confidence level) from a field of 0.25 to 2 ha with no or only trace BW incidence. Trace incidence of BW can escape field inspection if diseased plants dry up or if unscrupulous seed growers remove them before the technician's visit. Analysing 250 tubers would allow a detection probability of 95%. These are numbers of seed that feasibly can be processed in a seed-health test without incurring too high a cost of labour and material.

The number of 400 tubers has been selected by many seed certification agencies. According to our results in Peru (Table 2), the European standard of 200 tubers would provide a PEA of 6.3% (P=0.937) with symptomless crops. This is slightly higher than the theoretical probability of 5%. Considering that moderately to highly susceptible potato cultivars can harbour latent infection in their progeny tubers at frequencies ranging from 20 to 70% (Priou et al., 2002), field incidences of 1% and 0.1% would lead to percentages of infected tubers of 0.2–0.7% and 0.02–0.07%, respectively. According to the binomial distribution, numbers of tubers theoretically needed for testing to detect these levels of tuber infection at the 95% level of confidence would be much higher than that necessary in Peru to detect the same tuber infection rates (Table 3).

To be certain that a crop is free from BW, all the tubers would need to be tested, and this is impractical. A finite number of samples can be tested, within the limits of practical constraints and cost effectiveness, to obtain an estimate of the likelihood that a seed lot is infected. The probability of detecting an infected sample (PD) is equal to the probability of one infected sample in the samples analysed (PS) with 100% sensitivity (SE) and specificity (SP) of the test (De Boer, 1991). If efficiency is not

Table 3. Number of tubers (n) to be analysed to detect *Ralstonia solanacearum* in seed lots with various tuber infection levels (p) at confidence levels P of 95% and 99%.

p' = wilt incidence	p = infected tubers rate	1-p	P	n theoretical [a]	n observed
0.011	0.0022	0.9978	0.95	1360	100
0.011	0.0077	0.9923	0.95	388	100
0.001	0.0002	0.9998	0.95	14977	150
0.001	0.0007	0.9993	0.95	4278	150
0.011	0.0022	0.9978	0.99	2091	150
0.011	0.0077	0.9923	0.99	596	150
0.001	0.0002	0.9998	0.99	23024	200
0.001	0.0007	0.9993	0.99	6577	200

[a] n theoretical was calculated following the binomial distribution ($P = 1 - [1-p]^n$) and compared with the sample sizes (n observed) that were effective to detect these levels of infection in Peru, according to Table 2.

100%, then PD=PS(SE)+(1-SE)(1-SP). In CIP laboratory conditions, *R. solanacearum* can be detected in 10^{-4} dilutions in healthy tuber extract obtained from one latently infected tuber, demonstrating the very high sensitivity of the technique. This ensures that a single infected tuber in a composite sample of 25 tubers would be detected (Priou et al., 1999a). Thus, we could assume that for this serological test, PD=PS, but we cannot ensure that this would be valid under all laboratory conditions.

The results of laboratory tests can provide valid information about the disease status of a potato crop. Zero tolerance can be applied in the sense that when one lot tests positive it cannot be certified. Nor could it even be planted if the crop protection service establishes drastic quarantine regulations to avoid contamination of BW-free areas. But a negative test does not imply certainty that the lot is BW-free. The number of samples to analyse is a result of a compromise between cost, labour, and physical limitations for conducting the seed testing, and the level of confidence required according to the purpose of the detection. For instance, when infection of a lot with *R. solanacearum* is suspected in a BW-free seed-producing area, a higher probability should be applied to calculate the sample number to avoid disease spread to the entire area.

If the incidence of BW can be considered uniform throughout the field, the samples required would be the same regardless of the size of the field because calculations are based on sampling from an infinite population of tubers. This requirement that diseased plants occur randomly at a uniform rate across the field may be hard to maintain as fields become larger. In the Andes, however, the great majority of growers are small-scale farmers with less than 1 ha of potato (Thiele, 1998). Fields with variability in disease rate or where plants originate from different seed sources should be handled as two or more separate units for sampling purposes (Lund & Sun, 1985). Otherwise a stratified sampling should be applied, but sampling should be at random in each strata since disease rate should be constant within each strata (De Boer, 1991; Clayton & Slack, 1988).

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