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Sensitive detection of *Ralstonia solanacearum* in soil: a comparison of different detection techniques

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The sensitivity and specificity of various methods were compared for routine detection of *Ralstonia solanacearum* in a sandy loam soil. Populations fewer than 10^2 CFU per g soil were detected by dilution plating on a modified semiselective medium (SMSA). In comparison, a tomato bioassay was shown consistently to detect populations at or greater than $7{\cdot}5 \times 10^5$ CFU per g soil. An indirect enzyme-linked immunosorbent assay (ELISA) was as sensitive as the tomato bioassay, but detected as few as 10^4 CFU per g soil when the suspension was first incubated in SMSA broth prior to testing. Detection using a nested polymerase chain reaction (PCR) was equally as sensitive as that using culture on SMSA agar, but only when the infested soil sample was first enriched overnight in SMSA broth prior to the nested PCR. Longer incubation periods in SMSA broth also increased the sensitivity of pathogen detection using a conventional PCR method, permitting detection of as few as 10^2 CFU per g soil after 60 h enrichment in SMSA broth. When evaluated using naturally infected field soils in Nepal, isolation of *R. solanacearum* on SMSA was reliable only when pathogen populations were higher than those of saprophytic soilborne bacteria. As few as 5×10^2 CFU of *R. solanacearum* per g were recovered from naturally infested soil, whereas the sensitivity of indirect ELISA was 10^6 CFU g^{-1}.

Keywords: detection, ELISA, PCR, *Ralstonia solanacearum*, selective medium, soil

Introduction

Successful disease management and control practices greatly depend on an understanding of the ecology of the pathogenic organism in the environment. The ability of *Ralstonia solanacearum*, the causal agent of bacterial wilt of many important crops (Kelman, 1953) to survive long-term in soil under natural conditions remains poorly understood. This is largely because of a lack of sensitive detection protocols for studying low residual pathogen populations amongst high numbers of saprophytic bacteria in the soil environment.

Seal & Elphinstone (1994) have reviewed the advances in identification and detection of *R. solanacearum*. Detection of *R. solanacearum* has previously relied on the use of selective media and indicator plants. Several semiselective media were developed, principally for the detection of *R. solanacearum* in soil (Okabe, 1971; Karganilla & Buddenhagen, 1972; Graham & Lloyd, 1979; Nesmith & Jenkins, 1979; Chen & Echandi, 1982; Granada & Sequeira, 1983a). None of these has gained wide acceptance, although detection of as few as 10^2–10^4 CFU per g dry soil was often possible. The major disadvantage was that pathogen growth was often inhibited by overgrowth or competition in the presence of high populations of antagonistic saprophytic bacteria, resulting in false negative diagnoses. Following Granada & Sequeira (1983a, 1983b), an improved semiselective medium from South Africa (SMSA) was developed (Engelbrecht, 1994) and further modified (Elphinstone *et al.*, 1996, 1998) to detect the pathogen in soil, water and potato tuber tissues. The limit of detection in this case was 10^2 CFU per mL infected tuber homogenate (Elphinstone *et al.*, 1996). Colony development and appearance on SMSA was similar to that on triphenyl tetrazolium chloride (TTC) medium (Kelman, 1954) and colonies were countable within 72 h.

An indirect ELISA with polyclonal antiserum to *R. solanacearum* biovar 2 was shown to detect as few as 10^4 CFU per mL infected plant tissue homogenate or soil suspension (Robinson-Smith *et al.*, 1995). Janse (1988), using an immunofluorescent antibody stain, achieved similar levels of sensitivity for detection in

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potato tissue. This method, used in conjunction with a tomato bioassay, is currently recommended by the European Plant Protection Organisation (EPPO) for detection of *R. solanacearum* in potato tubers. The bioassay in tomato seedlings was shown reliably to detect as few as 10^4 cells per mL infected potato tuber extract (Janse, 1988; Elphinstone *et al.*, 1996). Tomato seedlings have also been widely used for both pathogenicity testing and detection of *R. solanacearum* in soil. Typical wilting symptoms are usually apparent within a week of inoculation, depending on the inoculum potential of the bacterium and the availability of optimum environmental conditions (Elphinstone *et al.*, 1996). Although tomato seedlings are easy to grow and are efficient indicator plants, they fail to develop wilt symptoms if night temperatures fall below 21°C (Vaughan, 1944).

In recent years, studies on improved identification and detection of plant pathogens have mostly concentrated on molecular approaches because of their potential advantages of increased specificity and sensitivity. With the development of *R. solanacearum*-specific PCR methods, Seal *et al.* (1993) were able to detect 16S rDNA sequences from a single cell grown in culture. Although highly sensitive *in vitro*, routine application of PCR for pathogen detection is currently limited because of the presence of PCR-inhibitory compounds in plant and soil extracts (Picard *et al.*, 1992; Seal, 1995). Although it is possible to remove PCR inhibitory compounds (Seal, 1995), the procedures can be either time-consuming, hazardous or expensive and are therefore not suitable for routine diagnostic work. As an alternative Elphinstone *et al.* (1996) found that sample dilution and enrichment in SMSA broth, followed by the use of a two-stage nested PCR protocol, was effective during pathogen detection in potato tuber homogenate.

The sensitivity of methods based on tomato bioassay, selective medium, serological procedures and PCR for the detection of *R. solanacearum* was compared by Elphinstone *et al.* (1996). The most commonly used method worldwide for detection and identification of *R. solanacearum* has been isolation on TTC medium (Kelman, 1954) because of the relatively low cost, simplicity of use and consistency of results between different laboratories. Increasingly, other methods of detection involving immunofluorescence (IF), SMSA medium and PCR, and identification using fatty acid profiling and rep-PCR are being used in commercial laboratories where speed and accuracy of diagnoses are often more important than cost and ease of use. The objective of this study was to compare the specificity and sensitivity of newly developed techniques for routine detection of *R. solanacearum* in soil.

Materials and methods

Bacterial culture and inoculum preparation

Ralstonia solanacearum (biovar 2), isolate L4 was maintained at −70°C and cultured on casamino acid peptone glucose (CPG) agar (Kelman, 1954) at 28°C for 48 h. Bacteria were then suspended in sterile 0·05 M phosphate buffer (PB, containing 4·26 g Na_2HPO_4 and 2·72 g KH_2PO_4 l^{-1}) and adjusted to a standard optical density of 0·12 at λ_{590}. The viable bacterial population of this suspension was estimated at $7{\cdot}5 \times 10^8$ CFU per mL inoculum suspension following dilution plating on CPG agar.

Soil inoculation and bacterial extraction

Non-sterile sandy loam soil samples (10 g each) collected from a potato field in Oxfordshire, UK were inoculated with 1 mL of suspensions of *R. solanacearum*, previously diluted 10-fold to contain from 10^8–10^2 cells. Inoculated soils were then incubated at 20°C for 1 h prior to testing.

Individual soil samples in heavy gauge polythene bags were suspended in 100 mL PB and shaken vigorously for 2 min. The heavier soil particles were allowed to settle for a further 2 min, then 1 mL of the supernatant was removed and tested using the various detection methods described below. Aliquots of the supernatant were either immediately dilution-plated onto SMSA agar, or used to inoculate tomato seedlings as described below. Further aliquots (500 μL each) were boiled in a heating block for 10 min in screw-capped Eppendorf tubes to inactivate endogenous plant enzyme activity and stored at −20°C to be tested by ELISA. For PCR, 90 μL of the supernatant was mixed with 10 μL 0·5 M NaOH, then boiled for 4 min and stored at −20°C.

Detection techniques

Recovery on modified semiselective medium

Aliquots (100 μL each) from a 10-fold dilution series of each supernatant were spread on replicated plates and incubated at 28°C. *Ralstonia solanacearum* colonies were bold, fluidal and creamy-white in colour on SMSA agar 48 h after incubation at 28°C, and could easily be distinguished from colonies of other bacteria. Presumptive *R. solanacearum* colonies were marked and counted 48 h after incubation, and counts were confirmed on the third day of incubation when typical colonies were fluidal and irregular with a characteristic red centre and whitish periphery as on TTC medium (Kelman, 1954). Identification of presumptive *R. solanacearum* colonies was confirmed by gas chromatographic profiling of whole-cell fatty acid methyl esters, as described by Stead (1992).

Tomato bioassay

Bioassays in tomato seedlings were based on those described by Janse (1988). Two-week-old tomato seedlings (cv. Moneymaker) grown in 9 cm pots were used for inoculation. Five replicate seedlings were inoculated with 5 μL of each supernatant into 1-cm-long longitudinal incisions made with a sterile scalpel in

the stem between the two cotyledons. Wounds were sealed with paraffin wax after inoculation. Three replicates per bacterial suspension were used so that a total of 15 seedlings were inoculated with each bacterial concentration. Seedlings were held at 25°C and the incidence of wilting was recorded weekly. Plants that did not develop wilt symptoms within 21 days of inoculation were tested for latent infection by macerating 1 cm stem sections (cut immediately above the point of inoculation) in 1 mL sterile PB and streaking a 5 μL loopful of the suspension on SMSA agar. Seedlings inoculated with sterile PB with and without addition of a concentrated suspension of *R. solanacearum* (isolate L4) were used as positive and negative controls, respectively.

Enrichment techniques

SMSA broth was prepared as described by Elphinstone *et al.* (1996), by omitting agar and triphenyl tetrazolium chloride from the composition for modified SMSA agar. One mL of supernatant from each inoculated soil suspension was mixed with 9 mL SMSA broth in universal tubes (Sterilin) and incubated overnight at 28°C with the caps loosely fitted for aeration. After enrichment, any presence of the pathogen was detected by dilution plating on SMSA agar as described above. One mL of each broth suspension was then boiled for 10 min for ELISA tests, whereas for PCR 90 μL of broth suspension was mixed with 10 μL of 0·5 M NaOH and then boiled and stored at −20°C as described earlier.

Indirect ELISA

The indirect ELISA protocol described by Robinson-Smith *et al.* (1995), as modified by Elphinstone *et al.* (1996), was followed using polyclonal antiserum produced in rabbit at Rothamsted Experimental Station against strain R 303 of *R. solanacearum* biovar 2. Bound antiserum was detected using antirabbit IgG conjugated to alkaline phosphatase (Sigma). Each replicated suspension was tested in duplicate wells and both nonenriched and enriched soil suspensions were tested. The ELISA was conducted in multiwell plates (Nunc, 'Polysorp') with samples allocated randomly. Positive controls were formaldehyde-fixed (0·01%) suspensions of the homologous bacterial strain containing $7{\cdot}5 \times 10^5$, 10^4 and 10^3 cells mL^{-1} in PB. Negative controls were noninoculated soil suspensions. Control samples were loaded in all plates to check variation between plates. Results were quantified 2 h after addition of substrate by reading the absorbance in each well at 405 nm on a Titretek-Multiscan microtitre plate reader. ELISA readings were considered positive when they significantly exceeded the readings of negative controls.

Conventional PCR

A conventional PCR was performed as described by Seal *et al.* (1993). A 50 μL reaction mixture contained 37·3 μL molecular grade water, 5 μL 10× PCR buffer (PE Applied Biosystems), 0·2 mM of each dNTP, 1 μM each of specific primer OLI-1 and nonspecific primer Y-2, 1 U AmpliTaq DNA polymerase (PE Applied Biosystems), and 2 μL soil suspension (previously boiled for 4 min). After initial heating to 94°C for 2 min, 35 amplification cycles were performed in a DNA thermal cycler (Perkin Elmer 9600) through phases of denaturation (94°C for 20 s), annealing (68°C for 20 s) and extension (72°C for 20 s), with a final period of 10 min at 72°C. An *R. solanacearum*-specific PCR product of 288 bp was generated and visualized under UV transillumination after electrophoresis in 2% agarose and staining in ethidium bromide (0·5 $\mu g\ mL^{-1}$).

Nested PCR

A two-stage nested PCR was performed. For the first stage the 50 μL reaction mixture contained 37·3 μL molecular grade water, 5 μL 10× PCR buffer (PE Applied Biosystems), 0·2 mM of each dNTP, 1 μM each of specific primers OLI-1 (5′-GGGGGTAGCTTGCTA CCTGCC-3′) (Seal *et al.*, 1993) and OLI-2 (5′-CGTC ATCCACTCCAGGT-ATTAACCGAA-3′) (Elphinstone *et al.*, 1996), 1 U AmpliTaq DNA polymerase (PE Applied Biosystems), and 2 μL soil suspension (previously boiled for 4 min). After initial heating to 94°C for 2 min, 35 amplification cycles were performed in a DNA thermal cycler (Perkin Elmer 9600) through phases of denaturation (94°C for 20 s), annealing (68°C for 20 s), and extension (72°C for 20 s), with a final period of 10 min at 72°C.

For the second PCR stage the same 50 μL reaction mixture was used, but with nested primers JE2 (5′-GTGGGGGATAACTAGTCGAAAGAC-3′) (Elphinstone *et al.*, 1996) and Y2 (5′-CCCACTGCTGCCTC CCGTAGGAGT-3′) (Seal *et al.*, 1993), and 2 μL of the product from the first reaction. In the second stage, 30 cycles of denaturation (94°C for 20 s), annealing (55°C for 20 s), and extension (72°C for 20 s), with a final period of 10 min at 72°C, were used. A specific PCR product of 172 bp was generated after the second PCR stage and visualized under UV transillumination after electrophoresis in 2% agarose and staining in ethidium bromide (0·5 $\mu g\ mL^{-1}$).

Multiplication of *R. solanacearum* in SMSA broth

An additional experiment was conducted to determine the degree of pathogen multiplication during incubation in SMSA broth. Phosphate buffer in 1 mL amounts containing from $6{\cdot}0 \times 10^8$ to $6{\cdot}0 \times 10^2$ CFU mL^{-1} were inoculated into 10 g soil. The inoculated soil was then suspended by shaking in 100 mL PB. Aliquots

(1 mL each) of the suspensions were then diluted 1 : 10 in sterile tubes containing 9 mL SMSA broth as described above, and *R. solanacearum* populations were monitored after 0, 20, 40 and 60 h incubation at 28°C. Aliquots (100 μL each) from the broth suspensions were spread on replicated SMSA agar plates and incubated at 28°C. *Ralstonia solanacearum* colonies were identified and counted as described earlier. To check for antagonistic effects of saprophytic bacteria in the soil on multiplication of the pathogen during enrichment, growth of *R. solanacearum* was also quantified after inoculation of tubes of SMSA broth with suspensions of a pure culture initially containing an estimated 10^5, 10^4 and 10^3 CFU mL^{-1} in sterile PB. The effect of incubation on detection efficiency was determined using the nested and conventional PCR techniques described earlier.

Comparative efficiency of detection of *R. solanacearum* using SMSA agar and indirect ELISA in Nepal

A total of 60 rhizosphere soil samples (sandy loam) from bacterial wilt-affected potato plants at Ghandruk village (2000 m asl) in the western hills of Nepal were tested to compare selective culture on SMSA agar and ELISA for detection of *R. solanacearum*. This experiment was repeated over two growing seasons (1995 and 1996). Rhizosphere soil samples were collected from around roots of wilted and nonwilted plants, and mixed together to obtain a range of *R. solanacearum* populations. Subsamples of soil (2 g) removed from 10 plants were mixed well in a thick plastic bag. The resulting 10 g composite soil samples were then suspended in 100 mL PB for bacterial extraction. The methods of extraction and detection using SMSA and ELISA were as described previously.

Effect of soilborne saprophytic bacteria on detection efficiency

This experiment was conducted to verify whether saprophytic bacteria could adversely affect recovery of *R. solanacearum* on SMSA agar. Individual 10 g sandy loam field soil samples were autoclaved for 15 min at 121°C to eliminate saprophytic bacteria. Four treatments were composed of a factorial 2 × 2 combination of known concentrations of *R. solanacearum* and a common saprophytic bacterium (unidentified) that had frequently been observed to grow on SMSA agar medium (see Table 4). Suspensions (1 mL) of *R. solanacearum* and the saprophyte in phosphate buffer were inoculated into the sterile soil sample. The bacteria were then allowed to bind to soil colloids for 1 h, after which they were recovered by extraction and isolation on SMSA agar as described previously.

Table 1 Recovery of *R. solanacearum* from an artificially inoculated sandy loam soil on semiselective SMSA agar medium

Mean[a] log CFU of *R. solanacearum* inoculated per mL soil suspension	Mean[a] log CFU of *R. solanacearum* recovered per mL soil suspension	Percentage recovery
6·87	6·73	97·9
5·87	5·71	97·3
4·87	4·64	95·3
3·87	3·42	88·4
2·87	2·62	91·3
1·87	1·67	89·3
0·87	0·76	87·3

[a]Mean of two replicates.

Results

Detection on semiselective SMSA agar medium

Ralstonia solanacearum was recovered on SMSA medium from each of the inoculated soil suspensions, indicating that original populations as low as 75 CFU per g artificially inoculated soil could be detected (Table 1).

Detection by tomato bioassay

On average only one of every five tomato seedlings developed typical bacterial wilt symptoms, even when the original inoculum density was as high as 10^7 CFU per g soil (Table 2). When the seedlings were tested for latent infection, the bacterium could be reliably detected provided the populations in the soil suspension were at least $7{\cdot}5 \times 10^4$ CFU mL^{-1} (10^5 CFU per g soil).

Indirect ELISA

The lowest population of *R. solanacearum* detected in soil by the indirect ELISA method was 10^5 CFU g^{-1}. The sensitivity of detection was increased when the suspension was incubated in SMSA broth prior to testing, whereafter populations of 10^4 CFU g^{-1} could be detected (Fig. 1). This procedure, termed, bio-ELISA,

Table 2 Detection of *R. solanacearum* populations in an artificially inoculated sandy loam soil by bioassay in tomato (cv. Moneymaker)

Mean CFU of *R. solanacearum* inoculated per g soil	Mean[a] number of plants with:	
	Wilt symptoms	Latent infection
$7{\cdot}5 \times 10^7$	1·0	4·0
$7{\cdot}5 \times 10^6$	1·3	3·7
$7{\cdot}5 \times 10^5$	0·3	3·3
$7{\cdot}5 \times 10^4$	0·3	0·3
$7{\cdot}5 \times 10^3$	0·0	0·0
$7{\cdot}5 \times 10^2$	0·0	0·0
$7{\cdot}5 \times 10^1$	0·0	0·0

[a]Three replicates of five tomato plants inoculated per treatment with 5 μL of each 10% soil suspension.

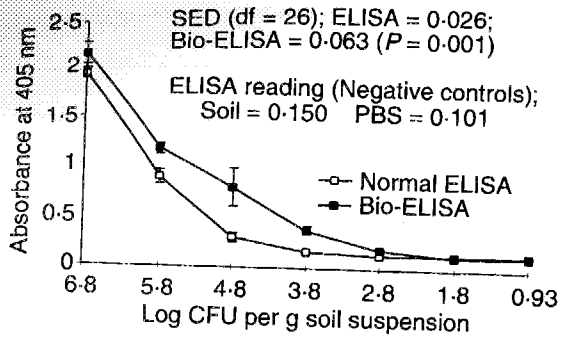

Figure 1 Sensitivity of detection of *R. solanacearum* populations in artificially inoculated sandy loam soil suspensions by indirect-ELISA and bio-ELISA; SED, standard error of difference between any two means; vertical bars show standard error of mean.

resulted in higher absorbance values than those obtained with nonenriched soil suspensions containing equivalent pathogen populations. The mean bio-ELISA reading obtained with soil suspensions containing 10^3 CFU mL^{-1} was significantly higher than that of readings obtained with noninoculated soil suspension.

Conventional PCR

With the conventional PCR method of Seal *et al.* (1993), *R. solanacearum* was not detected in any of the inoculated soil suspensions prior to enrichment. However, after overnight enrichment positive PCR results were obtained on broth that had been inoculated with suspensions of soil that originally contained at least 10^6 CFU g^{-1} (data not shown). Furthermore, if the broth cultures were enriched for 60 h and then 10-fold diluted prior to PCR, positive results were obtained with soil samples originally containing as few as 10^2 CFU per g soil (Fig. 2).

Nested PCR

The nested PCR method was sufficiently sensitive to detect as few as $7{\cdot}5 \times 10^1$ CFU *R. solanacearum* per mL soil suspension ($7{\cdot}5 \times 10^2$ CFU per g soil), but only if first incubated overnight in SMSA broth (Fig. 3). Before dilution and enrichment in the broth only the highest bacterial concentration ($7{\cdot}5 \times 10^6$ CFU per g soil) could be detected.

Prior to enrichment, a minimum population of 10^6 cells per mL soil suspension (10^7 CFU per g soil) could be successfully detected by the nested PCR method (data not shown). As few as 10^3 cells per mL soil suspension (10^4 CFU per g soil) were detected by the same technique after dilution of the suspension in SMSA broth, even before incubation of the broth culture (Fig. 4). The sensitivity of detection increased to 10^1 CFU per mL inoculated soil suspension (10^2 CFU per g soil) as a result of multiplication of the target bacterium after 20 or 40 h incubation.

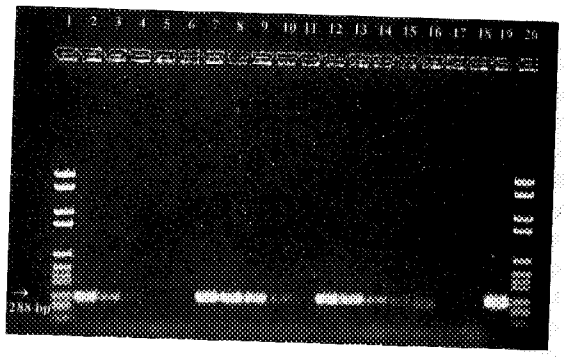

Figure 2 Detection of *Ralstonia solanacearum* using a conventional PCR method (Seal *et al.*, 1993) following enrichment of the target bacterium by incubating the soil samples in SMSA broth (1:10 dilution) for different periods. The 288-bp PCR product amplified using primer pairs OLI-1/Y-2 is visible on the ethidium bromide stained agarose gel.Lane 1 & 20 DNA Marker (Boehringer Mannheim Marker VI).Lane 2 20 hours enrichment; inoculum concentration 7.5×10^6 CFU *R. solanacearum* per g soil.Lane 3 20 hours enrichment; inoculum concentration 7.5×10^5 CFU *R. solanacearum* per g soil.Lane 4 20 hours enrichment; inoculum concentration 7.5×10^4 CFU *R. solanacearum* per g soil.Lane 5 20 hours enrichment; inoculum concentration 7.5×10^3 CFU *R. solanacearum* per g soil.Lane 6 20 hours enrichment; inoculum concentration 7.5×10^2 CFU *R. solanacearum* per g soil.Lane 7 40 hours enrichment; inoculum concentration 7.5×10^6 CFU *R. solanacearum* per g soil.Lane 8 40 hours enrichment; inoculum concentration 7.5×10^5 CFU *R. solanacearum* per g soil.Lane 9 40 hours enrichment; inoculum concentration 7.5×10^4 CFU *R. solanacearum* per g soil.Lane 10 40 hours enrichment; inoculum concentration 7.5×10^3 CFU *R. solanacearum* per g soil.Lane 11 40 hours enrichment; inoculum concentration 7.5×10^2 CFU *R. solanacearum* per g soil.Lane 12 60 hours enrichment; inoculum concentration 7.5×10^6 CFU *R. solanacearum* per g soil.Lane 13 60 hours enrichment; inoculum concentration 7.5×10^5 CFU *R. solanacearum* per g soil.Lane 14 60 hours enrichment; inoculum concentration 7.5×10^4 CFU *R. solanacearum* per g soil.Lane 15 60 hours enrichment; inoculum concentration 7.5×10^3 CFU *R. solanacearum* per g soil.Lane 16 60 hours enrichment; inoculum concentration 7.5×10^2 CFU *R. solanacearum* per g soil.Lane 17 Negative control (non-inoculated soil suspension).Lane 18 PCR reaction mix only.Lane 19 Positive control (pure suspension of 10^5 CFU per mL *R. solanacearum*, biovar 2, isolate L4).

Multiplication of *R. solanacearum* in SMSA broth

An estimated 87–95% of *R. solanacearum* populations were recovered on SMSA agar plates after 0 h incubation in the SMSA broth. Bacterial growth was approximately 100-fold after 20 h incubation irrespective of the suspension enriched. This represented a 10-fold increase compared to the populations in the original inoculated soil suspensions (Fig. 5). Saprophytic populations in the broth within this period did not inhibit growth of *R. solanacearum*. After 40 h incubation, *R. solanacearum* was recovered on SMSA plates only from broth which had been inoculated with soil suspensions containing the highest concentration of the pathogen, whereas the bacterium was not detected in other treatments, caused by excessive overgrowth by saprophytic bacteria on the

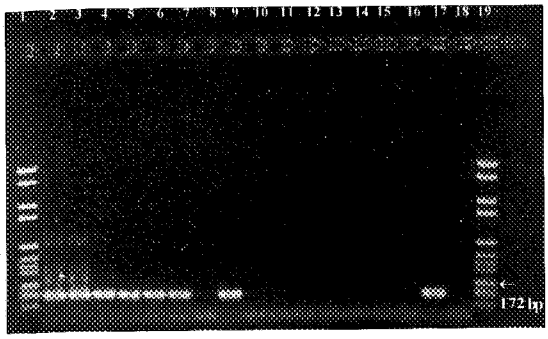

Figure 3 Detection of *R. solanacearum* by nested-PCR from enriched and non-enriched soil suspensions. The 172-bp PCR product amplified by second stage primer pairs JE-2/Y-2 is visible on the ethidium bromide stained gel.Lanes 1 & 19 DNA Marker (Boehringer Mannheim Marker VI).Lane 2 20 hours enrichment; inoculum concentration 7.5 x 10^7 CFU *R. solanacearum* per g soil.Lane 3 20 hours enrichment; inoculum concentration 7.5 x 10^6 CFU *R. solanacearum* per g soil.Lane 4 20 hours enrichment; inoculum concentration 7.5 x 10^5 CFU *R. solanacearum* per g soil.Lane 5 20 hours enrichment; inoculum concentration 7.5 x 10^4 CFU *R. solanacearum* per g soil.Lane 6 20 hours enrichment; inoculum concentration 7.5 x 10^3 CFU *R. solanacearum* per g soil.Lane 7 20 hours enrichment; inoculum concentration 7.5 x 10^2 CFU *R. solanacearum* per g soil.Lane 8 20 hours enrichment; inoculum concentration 7.5 x 10^1 CFU *R. solanacearum* per g soil.Lane 9 No enrichment; inoculum concentration 7.5 x 10^7 CFU *R. solanacearum* per g soil.Lane 10 No enrichment; inoculum concentration 7.5 x 10^6 CFU *R. solanacearum* per g soil.Lane 11 No enrichment; inoculum concentration 7.5 x 10^5 CFU *R. solanacearum* per g soil.Lane 12 No enrichment; inoculum concentration 7.5 x 10^4 CFU *R. solanacearum* per g soil.Lane 13 No enrichment; inoculum concentration 7.5 x 10^3 CFU *R. solanacearum* per g soil.Lane 14 No enrichment; inoculum concentration 7.5 x 10^2 CFU *R. solanacearum* per g soil.Lane 15 No enrichment; inoculum concentration 7.5 x 10^1 CFU *R. solanacearum* per g soil.Lane 16 Negative control (non-inoculated soil).Lane 17 Positive control (pure suspension of 10^5 CFU per mL *R. solanacearum*, biovar 2, isolate L4).Lane 18 PCR reaction mix only.

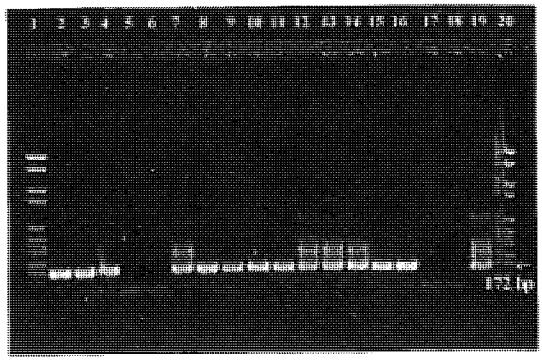

Figure 4 Detection of *R. solanacearum* by nested-PCR following enrichment by incubation of inoculated soil suspensions in SMSA broth (1:10 dilution) for different time periods. The 172-bp PCR product amplified by second stage primer pairs JE-2/Y-2 is visible on the ethidium bromide stained agarose gel.Lanes 1 & 20 DNA Marker (Boehringer Mannheim Marker VI).Lane 2 No enrichment; inoculum concentration 7.5 x 10^6 CFU *R. solanacearum* per g soil.Lane 3 No enrichment; inoculum concentration 7.5 x 10^5 CFU *R. solanacearum* per g soil.Lane 4 No enrichment; inoculum concentration 7.5 x 10^4 CFU *R. solanacearum* per g soil.Lane 5 No enrichment; inoculum concentration 7.5 x 10^3 CFU *R. solanacearum* per g soil.Lane 6 No enrichment; inoculum concentration 7.5 x 10^2 CFU *R. solanacearum* per g soil.Lane 7 20 hours enrichment; inoculum concentration 7.5 x 10^6 CFU *R. solanacearum* per g soil.Lane 8 20 hours enrichment; inoculum concentration 7.5 x 10^5 CFU *R. solanacearum* per g soil.Lane 9 20 hours enrichment; inoculum concentration 7.5 x 10^4 CFU *R. solanacearum* per g soil.Lane 10 20 hours enrichment; inoculum concentration 7.5 x 10^3 CFU *R. solanacearum* per g soil.Lane 11 20 hours enrichment; inoculum concentration 7.5 x 10^2 CFU *R. solanacearum* per g soil.Lane 12 40 hours enrichment; inoculum concentration 7.5 x 10^6 CFU *R. solanacearum* per g soil.Lane 13 40 hours enrichment; inoculum concentration 7.5 x 10^5 CFU *R. solanacearum* per g soil.Lane 14 40 hours enrichment; inoculum concentration 7.5 x 10^4 CFU *R. solanacearum* per g soil.Lane 15 40 hours enrichment; inoculum concentration 7.5 x 10^3 CFU *R. solanacearum* per g soil.Lane 16 40 hours enrichment; inoculum concentration 7.5 x 10^2 CFU *R. solanacearum* per g soil.Lane 17 Negative control (non-inoculated soil suspension enriched in SMSA broth).Lane 18 Negative control (SMSA broth only).Lane 19 Positive control (6.0 x 10^5 CFU per mL CFU per mL *R. solanacearum*, biovar 2, isolate L4).

agar surface. After 60 h incubation the bacterium could not be detected in any of the broth cultures after dilution plating on SMSA. However, the pathogen continued to multiply, even after 60 h incubation, in broth inoculated with pure suspensions of the bacterium in sterile buffer.

Comparative efficiency of detection of *R. solanacearum* by selective culture on SMSA agar and ELISA in samples of field soil from Nepal

The ELISA method used was less effective than culture on the semiselective SMSA medium in detecting soilborne *R. solanacearum*, particularly when viable counts estimated by the latter method were less than 10^6 CFU mL^{-1} (Table 3). Furthermore, positive ELISA results obtained from samples from which it was not possible to culture the organism indicated either nonspecificity of the antisera used, or detection of nonviable or nonculturable cells of *R. solanacearum*.

Table 3 Comparative detection of soilborne *R. solanacearum* in 60 samples of naturally infested soil tested in 1995 and 1996 by indirect ELISA and culture on semiselective SMSA medium

R. solanacearum population estimate in soil samples[a]	No. samples in which given population observed		No. samples positive for *R. solanacearum* by indirect ELISA	
	1995	1996	1995	1996
0	32	24	1	9
10^4	1	3	0	0
10^5	3	5	0	2
10^6	18	21	14	19
10^7	6	7	6	7

[a]Determined by dilution plating on SMSA medium (CFU per g dry soil).

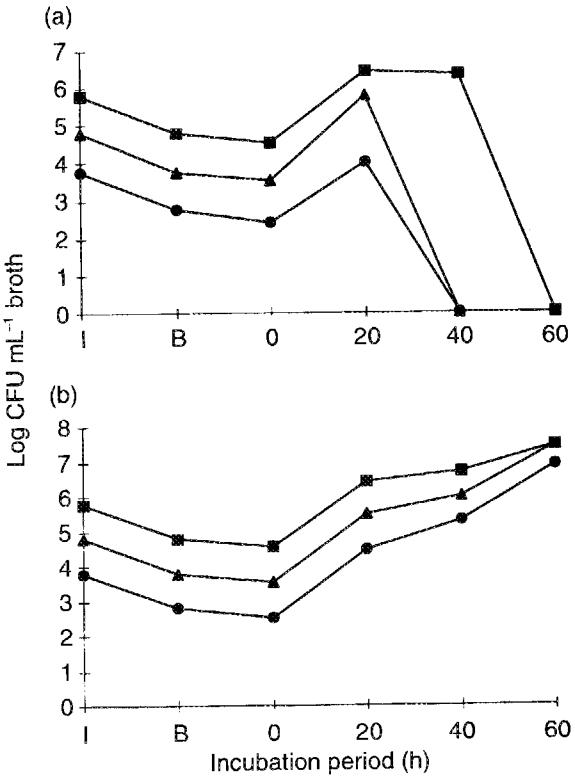

Figure 5 Recovery on SMSA agar of *Ralstonia solanacearum* from (a) inoculated soil suspensions and (b) bacterial suspensions in phosphate buffer, following enrichment in SMSA broth with different incubation periods.I, Initial log populations: ■, 5.69, ▲, 4.69; ●, 3.69 CFU per mL suspension; B, Populations following addition of 1 mL of each suspension to 9 mL of SMSA broth. Note 10-fold decrease of populations after dilution in SMSA broth and increase thereafter during incubation.

Effect of the presence of a saprophytic bacterium on the efficiency of recovery of *R. solanacearum* from sterilized soil.

Although *R. solanacearum* and an unknown saprophytic bacterium were co-inoculated into sterilized soil in high numbers, the former could be recovered on SMSA agar only when it outnumbered the saprophyte, which grew preferentially on the medium (Table 4). The failure of *R. solanacearum* to compete with the common saprophyte resulted in false negative results, indicating a need for the development of a more specific selective medium.

Discussion

The results presented above show that it was possible to detect less than 10 viable CFU of *R. solanacearum* per mL inoculated soil suspension (or $<10^2$ CFU per g soil) on the modified SMSA medium, suggesting that the sensitivity of pathogen detection in soil could be improved using this medium. However, a number of saprophytic soilborne bacteria were observed to grow on SMSA agar, which could adversely affect detection under natural conditions due to competition or antagonism. The results indicated that *R. solanacearum* could not be recovered when the natural population of soilborne saprophytic bacteria that grew on the SMSA medium exceeded that of the target pathogen by at least 10-fold. Furthermore, *R. solanacearum* population estimates based on colony counts on SMSA medium could be underestimated if unrecognizable, nonfluidal, avirulent colony-forming mutants or nonculturable forms of *R. solanacearum* prove to be important in long-term survival.

Soilborne *R. solanacearum* populations have been recovered on SMSA agar up to 18 months after harvest of an infected potato crop (Pradhanang, 1998b), indicating that residual populations can remain viable over this period. Under natural conditions the lowest detectable population of *R. solanacearum* on SMSA was found to be 5×10^2 cells per g soil (Pradhanang, 1998a), although a low frequency of detection in multiple soil samples suggested an uneven distribution of the pathogen in the soil. Future optimization of testing methods for *R. solanacearum* in soil will therefore need to consider sampling error and efficiency of extraction methods, as well as the method used for final detection.

An alternative to enrichment in or on selective growth media is to inoculate an indicator host plant with sample soil, followed by incubation and assessment for expression of wilt symptoms or latent infection in the roots (Graham & Lloyd, 1978; Saumtally *et al.*, 1993). In this study, the low relative sensitivity of detection observed using the tomato bioassay (Table 2) may have been caused by the presence of antagonistic bacteria in

Table 4 Recovery from soil on semiselective SMSA agar medium of *R. solanacearum* and a saprophytic bacterium following inoculation of the soil with both bacteria in differing populations

Mean[a] CFU inoculated per mL soil suspension		Mean[a] CFU recovered per mL soil suspension	
R. solanacearum	Saprophytic bacterium	R. solanacearum	Saprophytic bacterium
$1{\cdot}3 \times 10^6$	$4{\cdot}8 \times 10^7$	0	$2{\cdot}4 \times 10^7$ (50%)
$1{\cdot}3 \times 10^6$	$4{\cdot}8 \times 10^5$	$1{\cdot}2 \times 10^6$ (92%)	$2{\cdot}5 \times 10^5$ (52%)
$1{\cdot}3 \times 10^4$	$4{\cdot}8 \times 10^7$	0	$5{\cdot}2 \times 10^6$ (11%)
$1{\cdot}3 \times 10^4$	$4{\cdot}8 \times 10^5$	0	$2{\cdot}5 \times 10^5$ (52%)

[a]Mean of two replicates.

the soil suspensions which prevented further growth of *R. solanacearum* and colonization of the host. Detection in soil suspensions was reliable only when they contained $>10^4$ CFU mL^{-1}. Janse (1988), using a similar technique, detected a range of 10^2–10^4 cells per mL infected potato tuber homogenate, but it is expected that fewer saprophytic bacteria would be present in tissue than in soil.

The detection limit by indirect ELISA was 10^4 cells per mL soil suspension, confirming the findings of Robinson-Smith *et al.* (1995). This also compares with the sensitivity of the currently recommended immunofluorescent antibody staining (IFAS) technique for detection in potato extracts (Janse, 1988). Jenkins *et al.* (1967) were also able to detect similar populations of *R. solanacearum* in soil using agar immunodiffusion plates. However, when the bio-ELISA technique was used in this study, populations of 10^3 CFU per mL soil suspension were reliably detected after enrichment of the target bacterium in SMSA broth. Testing of naturally infested field soils in Nepal showed the lowest detection limit by ELISA to be 10^6 cells per mL soil suspension. Sensitivity of detection by ELISA may therefore be lower in naturally infested than in inoculated soils. Interestingly, Priou *et al.* (1999) were able to detect as few as 20 cells per g naturally infested soil using double antibody-sandwich (DAS) ELISA in post-enriched samples. The enrichment efficiency in SMSA broth was improved by adding potato broth (1 : 1). However, the fact that polyclonal antisera to *R. solanacearum* can cross-react with a range of commonly occurring soilborne bacteria (Caruso *et al.*, 1998) suggests that serological methods will require careful evaluation to determine the potential for false-positive reactions.

This is the first report of the use of nested PCR for the successful detection of *R. solanacearum* in soil. The combination of primer sets readily amplified nested PCR products despite the presence of substances inhibitory to PCR, which always occur in soil (Picard *et al.*, 1992; Seal *et al.*, 1993). The sensitivity of detection by the nested PCR method was high, equalling that obtained by culture on SMSA medium. As there are two amplification stages, dilution of soil components in the second PCR stage may account for the increased sensitivity compared with the conventional PCR method. In the nested PCR protocol used here, only the first primer set specifically amplifies *R. solanacearum* 16S rDNA sequences. Amplification with the second primer pair can generate the same nested PCR product from related bacteria such as *Ralstonia pickettii* and *Burkholderia cepacia* (Elphinstone *et al.*, 1998) which, in this case, were not found in the noninoculated control soil used. Further development of more appropriate primer sets will be required if nested PCR is to be used as a single detection test. Practical application may also be limited by the risks of cross-contamination associated with nested PCR, and by the high cost of reagents. These limitations have been addressed to some extent by the development of single-tube nested PCR methods (Yourno, 1992).

In this study, improved effectiveness of conventional PCR could be attributed to dilution in the SMSA broth of soil components inhibitory to the activity of DNA polymerase, as well as to the increase in number of the target bacterium during enrichment. The above results indicated that overnight enrichment for 20 h was sufficient to increase the sensitivity of detection by nested PCR. There was no additional advantage of longer incubation periods. Using conventional PCR, as few as 10^1 cells per mL soil suspension could be detected, but only after incubation overnight in SMSA broth. In this case, the longer incubation periods (60 h) enabled improved detection of low populations of the bacterium. More research is required to exploit the full potential of this PCR reaction which is *R. solanacearum*-specific and therefore less prone to false positive reactions than the nested PCR technique.

In summary, PCR has potential as a sensitive and specific detection technique for studying the survival of *R. solanacearum* in soil. Indirect ELISA, although useful in low-cost screening of large numbers of samples where populations exceed 10^4 CFU mL^{-1}, lacked the sensitivity required for epidemiological studies and was potentially prone to giving false-positive reactions. Culture on modified semiselective medium combined sensitive and specific detection with reasonable cost of application (approximately £0·30 per plate) and provided accurate quantification of viable populations of *R. solanacearum* in soil. The latter would therefore be the method of choice for use in epidemiological studies. Nevertheless, it should be stressed that although the level of sensitivity achieved during this study should be sufficient to monitor occurrence and survival of the bacterium in soil, sensitive detection in field soil is likely to depend largely on sampling strategy and extraction methods, as well as on the detection method employed (Pradhanang, 1999).

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