CHAPTER 3

DEVELOPMENT OF AN ALTERNATIVE DETECTION METHOD FOR *RALSTONIA SOLANACEARUM* IN SOIL

ABSTRACT

Various methods have been developed for the detection of *Ralstonia solanacearum*. The majority of these were developed to detect *R. solanacearum* in plant material. Applying these methods for detection of the pathogen in soil, presented its own set of problems. An alternative method was therefore developed in this study to detect *R. solanacearum* in soil samples. The technique was based on using a catch crop or indicator plant system. Tissue culture potato plants were used to "entrap" the pathogen from artificially infected soil suspensions. Initially different enrichment broths were used for pathogen enrichment. Buffelspoort cultivar tissue culture potato plants were placed in artificially inoculated soil suspensions, incubated and tissue isolations prepared. The sensitivity of the trapping technique was evaluated using different pathogen concentrations. The efficacy of the trapping technique was compared with selective media and the commercial ELISA kit currently used in South Africa for the isolation of *R. solanacearum*. Although pathogen cell growth was enhanced in sterile conditions, most of the enrichment broths were unsuccessful in terms of enrichment when tested in a soil system. This was mainly due to the proliferation of various soil organisms, causing plants to die before tissue isolations could be made. It was therefore decided to exclude the enrichment step and only use sterile distilled water. Using the trapping technique, the pathogen was successfully re-isolated from concentrations up to 10^1 cfu ml^{-1} in soil suspensions. The technique appears to be practical, not needing specialised equipment and presents an alternative approach to detect *R. solanacearum* in soil samples. However, soil samples taken from a potato field with a well-documented history of bacterial wilt tested negative. None of the other methods used could detect the pathogen, emphasising the difficulties relating to soil testing.

1. Introduction

Throughout the years, various techniques have been developed for the detection of *Ralstonia solanacearum*. Since the pathogen has the ability to spread latently through infected plant parts, many countries have implemented strict quarantine regulations and detection systems to prevent further introduction of the pathogen into new fields (Elphinstone *et al.*, 1996). The majority of detection techniques were primarily developed for the detection of the pathogen in infected plant material. It is however also important to be able to detect the pathogen in infected soil (Nesmith and Jenkins, 1979). Although many of the techniques were originally developed for plant tissue detection it can also be used for soil detection. However, this approach is subject to various difficulties and shortcomings such as low recovery efficacy. Most of the problems encountered can be attributed to the heterogeneous nature of soil and the difficulty ensuring adequate sampling (Jenkins *et al.*, 1967).

One of the techniques most often used for the detection of *R. solanacearum* in soil, is to plant indicator plants (Jenkins *et al.*, 1967; Graham and Lloyd, 1978). These plants show disease symptoms within a short period of time following infection. Tomato and potato seedlings have commonly been used as indicator plants (Graham and Lloyd, 1978). After plant emergence, it took approximately two to three weeks for symptom development and concentrations of 10^4 colony forming units (cfu) ml^{-1} soil sample were detected (Jenkins *et al.*, 1967). According to the authors this method was slow, laborious and provided inconsistent results.

Various different selective and semi-selective media have also been developed. Most of these were based on Kelman's tetrazolium chloride medium (TZC) (Kelman, 1954). One of these media was developed by Graham and Lloyd (1979) and modified by Engelbrecht (1994). This modified version of the traditional TZC medium contains antibiotics such as penicillin G potassium salt, bacitracin, polymixin B sulphate and chloramphenicol. Although there are numerous variations, none of these media gained wide acceptance (Granada and Sequeira, 1983). Most of the media allows growth of too many background bacteria, are appropriate only for certain strains of the pathogen and are difficult and expensive to prepare. Another problem is that the media can only detect the pathogen effectively in concentrations from 10^4 cfu ml^{-1} soil sample (Jenkins *et al.*, 1967; Nesmith and Jenkins, 1979).

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Serological techniques can also be used to detect the pathogen. Jenkins *et al.* (1967) prepared antiserum in rabbits against *R. solanacearum* and was able to detect pathogen concentrations of 10^4 cfu ml^{-1} per soil sample within three days. Although they were able to detect the pathogen, their technique did not distinguish between live and dead bacterial cells. Other immunological techniques have been developed which include immunofluorescent (IF) antibody staining and enzyme linked immunosorbent assays (ELISA) (Seal, 1998). According to Elphinstone *et al.* (1998) the IF technique can detect pathogen concentrations of 10^4 cfu ml^{-1} per sample. The ELISA kit developed by Prof. D.U. Bellstedt, Department of Biochemistry, University of Stellenbosch and used by Potatoes South Africa in their certification program, effectively detects pathogen concentrations of 10^3 cfu ml^{-1} of sample (Bellstedt and van der Merwe, 1989). Although the use of these techniques can result in detection of lower concentrations than selective media, they are expensive and not suitable for frequent use in developing countries where resources are limited (Seal, 1994; Seal and Elphinstone, 1994).

The ideal diagnostic test required for the detection of *R. solanacearum* should be rapid, specific and able to detect low bacterial concentrations in a soil sample. It should also be easy to apply, affordable and not dependable on specialised equipment. The primary objective of this study was therefore to develop an alternative method for the detection of *R. solanacearum* in soil samples. The efficacy and affordability of this technique were evaluated and compared to selective media and ELISA techniques currently used in South Africa.

2. Materials and Methods

2.1. Plant Material

Tissue culture potato plantlets, cultivar Buffelspoort, were obtained from the Department of Plant Production and Soil Science, University of Pretoria. The plantlets were multiplied and maintained on medium as described by Joerdens-Roettger (1987). Plants were kept in a growth room at 18 to 24°C with a 12 h light/dark cycle for four to maximum six weeks depending on root development.

2.2. Cultures

A.E. Swanepoel of the Vegetable and Ornamental Plant Research Institute, Roodeplaat and Mr. A.N. Hall of the Department of Microbiology and Plant Pathology, University of Pretoria, supplied the original collection of R. solanacearum isolates (Appendix 1). Stock cultures were stored in McCartney bottles at room temperature in sterile distilled water. For this study the South African isolate 111 (biovar 2) was randomly selected. To prepare fresh inoculum, stock suspensions were streaked out on Kelman's TZC agar medium containing 2,3,5-triphenyltetrazolium chloride and incubated at 28°C for 72 h (Kelman, 1954; Appendix 2). After incubation, virulent colonies (irregularly round, white fluidal colonies with pink, typically comma-shaped centres) were picked up with an inoculation loop and suspended in sterile distilled water. The viable total concentration was determined using the spread plate technique. Dilution series were prepared, plated out on TZC medium, incubated for 72 h at 28°C, colonies counted and concentrations adjusted using sterile distilled water. This set of procedures was used throughout all experiments where fresh inoculum was required.

2.3. Suspension Preparation, Inoculation and Evaluation

2.3.1. Evaluation of Pathogen Growth in Different Enrichment Broths

Different enrichment broths were prepared based on Kelman's TZC medium (Kelman, 1954) and those described by Graham and Lloyd (1979) and Granada and Sequeira (1983). The medium of Graham and Lloyd (1979) was modified by replacing 0.00005% (w/v) actidione with 0.01% (w/v) polymixin B sulphate and was labelled SMSA (abbreviation derived from "selective media from South Africa") (Engelbrecht, 1994; Appendix 2). The medium described by Granada and Sequeira (1983) was modified by using 0.00005% (w/v) penicillin G potassium salt, 0.0025% (w/v) bacitracin, 0.0005% (w/v) chloramphenicol and 0.01% (w/v) polymixin B sulphate together with 0.0005% (w/v) crystal violet and was labelled modified TZC (M-TZC) (Appendix 2). The agar component of the media was omitted and either sterile distilled water or sterile standard nutrient solution (Hydro Grow, Ocean Agriculture, Johannesburg) was used to prepare the different enrichment broths. The enrichment broths were autoclaved and allowed to cool down. Sterile tissue culture test tubes, containing 9 ml of each solution were inoculated with 1 ml

inoculum (1×10^6 cfu ml^{-1}) and incubated for two weeks at room temperature. Each treatment (solution) was represented by fifteen replicates. Every three days, three replicates were removed from each treatment and used to calculate the pathogen concentration. Dilution series were prepared from each replicate, plated out on TZC medium and concentrations calculated using the spread plate technique. After a set of tubes was plated out, it was discarded and not used for further evaluation. After two weeks the average cell concentration per treatment was calculated and expressed as $\log_{10}$. The inoculated sterile water treatment acted as control.

2.3.2. Evaluation of Host-Pathogen Interactions in Different Enrichment Broths

Tissue culture plants were removed from their tubes and rinsed in sterile distilled water to dislodge remaining pieces of medium. A few roots were cut to facilitate pathogen entry and plants were placed into tissue culture test tubes containing 9 ml of one of the enrichment broths and inoculated with 1 ml inoculum (2×10^6 cfu ml^{-1}). These test tubes were incubated at 20 $\pm$ 2°C with a 12 h light/dark cycle for three weeks. Each treatment consisted of twelve replicates. Every week, four plants were removed and surfaced sterilised, using a 1% sodium hypochlorite solution. After 30 sec, plants were rinsed three times in sterile distilled water and placed in sterile Petri dishes containing 9 ml sterile Ringers solution. Plants were cut into small pieces, allowed to diffuse and after 15 min a dilution series was prepared from each Petri dish. Samples were plated out on TZC medium, incubated at 28°C for 72 h and evaluated for presence of *R. solanacearum* colonies. Each treatment also included three control plants, placed in the different enrichment broths without adding the pathogen. The experiment was repeated to confirm results.

2.3.3. Evaluation of Host-Pathogen Interactions in Different Enrichment Broths Containing Soil

The procedures described in 2.3.2 were repeated but this time soil was added to the different enrichment broths. Soil solutions were prepared by adding 100 g of red, clay-loam soil, collected at the University of Pretoria's experimental farm (previously tested negative for *R. solanacearum* by L. Stander), to 900 ml of each growth medium described under 2.3.1. The solutions were agitated vigorously on a mechanical shaker for 30 min, allowed to settle for 2 min and filtered through a Whatman no.1 filter to remove insoluble material. The prepared soil solutions were then used with tissue culture plants as described in 2.3.2.

2.4. Comparison Between the Trapping Technique and Procedures Used in South Africa for the Detection of *R. solanacearum* in Soil Samples

2.4.1. Lowest Pathogen Concentration Detectable

Soil suspensions were prepared by adding 100 g pathogen free soil to 900 ml sterile distilled water (Refer to 2.3.3). Inoculum was prepared and the concentration calculated at 3×10^6 cfu ml^{-1} using the spread plate technique. A dilution series for dilution factors 10^{-1} to 10^5 was prepared using sterile distilled water. Each treatment-concentration tested consisted of five replicates.

Three different approaches were selected to determine the detectibility of *R. solanacearum* at various concentrations. The different techniques were repeated to validate results.

a) Trapping Technique

The procedures described in 2.3.2 and 2.3.3 were repeated, except that only sterile distilled water was used to prepare soil solutions and four to five plants were placed together in sterile tissue culture bottles. Each bottle contained 45 ml soil solution and 5 ml inoculum from each concentration. Final concentrations were between 3×10^5 and 3×10^{-1} cfu ml^{-1} and each concentration was represented by four replicates (bottles). Due to the large number of individual plants, plants from each replicate were pooled together and tissue isolations made after seven days incubation.

b) Selective Media

Standard TZC, M-TZC and SMSA agar media were prepared (Appendix 2). Dilution series were prepared from each inoculum concentration and 100μl plated out in duplicate on the different media and incubated at 28°C. After 72 h cultures were evaluated for presence of *R. solanacearum* colonies.

c) ELISA

Aliquots from each inoculum concentration were sent to Potatoes South Africa, Seekoegat-laboratory for analyses, using the commercial ELISA kit to detect *R. solanacearum* (Bellstedt and van der Merwe, 1989). A multi scan spectrophotometer was used to analyse the samples at 405 nm. The cut-off value for positive results was an absorbance of 0.15 and lower readings were regarded as negative results (Pers. Comm. N.J.J. Mienie, Potatoes South Africa, Seekoegat).

2.4.2. Economic Comparison

Costs of the chemicals required for the different techniques were obtained and the average cost per treatment calculated in order to give a relative indication of the affordability of each technique. Quotes were obtained from Sigma-Aldrich, Merck Laboratory Supplies and Labretoria. Costs of glassware, general appliances and specialised equipment such as the multi scan spectrophotometer were not taken into account.

2.4.3. Detection from Soil Samples

a) Soil Sampling

Soil samples were taken from a potato field (20 m x 3 m) with a well-documented history of bacterial wilt from the experimental farm at the University of Pretoria (Stander, 2001). Soil sampling was done based on the method described by Pradhanang (1999). A soil auger was used and 14 samples were taken randomly. The top 10 cm soil was discarded and the 10 cm below that was collected and mixed together. A 4 mm mesh screen was used to remove large soil particles and insoluble material. Five, 100 g sub-samples were taken from the sieved soil and dissolved in 900 ml sterile distilled water. The samples were agitated for 30 min on a mechanical shaker and allowed to settle for 2 min and filtered through a Whatman no.1 filter to remove insoluble material.

b) Comparison Between Different Techniques

The techniques described in 2.4.1 were repeated, using suspensions prepared from the soil samples collected in 2.4.3a. Five replicates were made from each sub-sample resulting in 25 isolations per treatment.

3. Results

3.1. Suspension Preparation, Inoculation and Evaluation

3.1.1. Evaluation of Pathogen Growth in Different Enrichment Broths

Ralstonia solanacearum could effectively grow in all media tested for enrichment (Fig.1). Inoculation in the two TZC solutions, both the SMSA solutions and the M-TZC solution prepared with sterile distilled water, resulted in an increase in pathogen concentrations between 10^{10} and 10^{13} cfu ml^{-1} after two weeks. The cell concentrations in the distilled water, nutrient solution and the M-TZC solution prepared with distilled water, remained in the range of 10^6 to 10^7 cfu ml^{-1}.
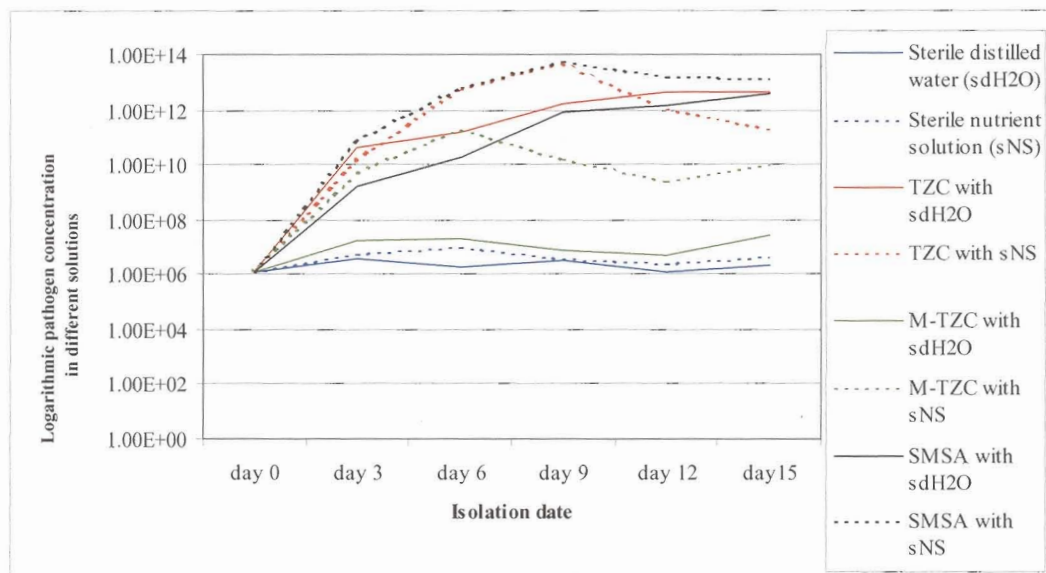

Figure 1. Evaluation of *Ralstonia solanacearum* growth in different enrichment broths expressed as Log$_{10}$ cfu ml^{-1}

3.1.2. Evaluation of Host-Pathogen Interactions in Different Enrichment Broths and Different Enrichment Broths Containing Soil

Positive isolations were made from all plants maintained in distilled water, nutrient solution and TZC solution prepared with distilled water (Table 1). These plants did not disintegrate and only started to turn yellow after three weeks. The majority of plants in the TZC solution prepared with sterile nutrient solution, both the SMSA solutions and the M-TZC solutions prepared with sterile distilled water and sterile nutrient solution disintegrated after two to three weeks and it was difficult to make tissue isolations. None of the plants showed typical wilt symptoms, but the majority of leaves turned light yellow after a week or two and older leaves started to drop, especially in the modified TZC, M-TZC and SMSA solutions.

When soil was added to each solution, positive isolations of *R. solanacearum* could only be made from plantlets maintained in distilled water and nutrient solution. One to three positive isolations could be made from plants in the TZC solution prepared with distilled water and both of the SMSA solutions but no positive isolations could be made from plants in the TZC solution prepared with nutrient solution and the M-TZC solutions. Disintegration of plants in the modified enrichment broths started after one or two weeks and it was difficult to make tissue isolations (Table 2).

3.2. Comparison Between the Trapping Technique and Procedures Used in South Africa for the Detection of *R. solanacearum* in Soil Samples

3.2.1. Lowest Pathogen Concentration Detectable and Economic Comparison Between Different Techniques

Results of the lowest concentration detectable as well as the time needed for results to develop are presented in Table 3 and the general costs involved are presented in Table 4. The trapping technique could detect the lowest pathogen concentration (10^1 cfu ml^{-1}) but needed ten days to produce results. It was also more expensive than the selective media, but was more affordable than the ELISA. Although the other techniques only needed three days to produce results, it

could only detect the pathogen from concentrations higher than 10^3 (M-TZC and SMSA) and 10^4 cfu ml^{-1} (TZC).

3.2.2. Detection from Soil Samples

Although the trapping technique, selective media and the ELISA kit were used for the detection of *R. solanacearum*, no positive detection could be made.

Table 1 Comparison of different enrichment broths to enable successful entrapment of *Ralstonia solanacearum* in potato tissue culture plantlets

| Enrichment broth | Tissue isolations[a] | | | | | | | | | | | |
| | Week 1 | | | | Week 2 | | | | Week 3 | | | |
	1	2	3	4	1	2	3	4	1	2	3	4
Sterile distilled water (sdH$_2$O)	+	+	+	+	+	+	+	+	+	+	+	+
Sterile nutrient solution (sNS)	+	+	+	+	+	+	+	+	+	+	+	+
TZC prepared with sdH$_2$O	+	+	+	+	+	+	+	+	+	+	+	+
TZC prepared with sNS	+	+	+	+	+	-	-	-	+	+	-	-
M-TZC prepared with sdH$_2$O	+	+	+	-	+	-	-	-	+	-	-	-
M-TZC prepared with sNS	+	+	+	-	+	-	-	-	+	-	-	-
SMSA prepared with sdH$_2$O	+	+	+	-	+	+	-	-	+	-	-	-
SMSA prepared with sNS	+	+	+	+	+	-	-	-	+	-	-	-

[a] Positive detection of *R. solanacearum* indicated with (+) and absence with (-)

Table 2 Comparison of different enrichment broths added to soil solutions to enable successful entrapment of *Ralstonia solanacearum* in potato tissue culture plantlets

Enrichment broth	Tissue isolations[a]											
	Week 1				Week 2				Week 3			
	1	2	3	4	1	2	3	4	1	2	3	4
Sterile distilled water (sdH$_2$O)	-	+	+	+	+	+	+	+	+	+	+	+
Sterile nutrient solution (sNS)	-	+	+	+	-	+	+	+	+	+	+	+
TZC prepared with sdH$_2$O	-	-	-	-	-	-	-	-	-	-	-	+
TZC prepared with sNS	-	-	-	-	-	-	-	-	-	-	-	-
M-TZC prepared with sdH$_2$O	-	-	-	-	-	-	-	-	-	-	-	-
M-TZC prepared with sNS	-	-	-	-	-	-	-	-	-	-	-	-
SMSA prepared with sdH$_2$O	-	-	-	+	-	-	-	-	-	-	-	+
SMSA prepared with sNS	-	-	+	+	-	-	-	+	-	-	-	-

[a] Positive detection of *R. solanacearum* indicated with (+) and absence with (-)

Table 3 Comparison of different isolation techniques to evaluate the successful detection of *Ralstonia solanacearum* concentrations in soil solutions and the rapidity of each technique

Technique	Inoculum concentration (cfu ml^{-1})[a]							Days required for results
	10^5	10^4	10^3	10^2	10^1	10^0	10^{-1}	
Tissue culture plants	+	+	+	+	+	-	-	10
TZC medium	+	+	-	-	-	-	-	3
M-TZC medium	+	+	+	-	-	-	-	3
SMSA medium	+	+	+	-	-	-	-	3
ELISA	+	+	-	-	-	-	-	3

[a] Positive detection of *R. solanacearum* indicated with (+) and absence with (-)

Table 4 Comparison of general costs involved with the different techniques used for the

detection of *Ralstonia solanacearum* in soil solutions

Technique	Cost (Rand) in 2001	General remarks
Tissue culture plants	R 352.94	Cost include purchase price of 100 Buffelspoort tissue culture plants, one 24 cm, no.1 Whatman filter paper and 1 *l* TZC medium
TZC medium	R 51.82	Costs calculated for 1 *l* medium
M-TZC medium	R 106. 82	Costs calculated for 1 *l* medium
SMSA medium	R106.79	Costs calculated for 1 *l* medium
ELISA	R 750.00	Purchase price of one ELISA kit. A multi scan spectrophotometer is also needed

4. Discussion

Initially modified enrichment broths, based on commercial selective media were used to enrich *R. solanacearum* concentrations. Although these enrichment broths were able to increase pathogen concentrations under sterile conditions, it was unsuccessful when soil was added to the system. This was most likely due to the proliferation of other soil microorganisms. These organisms infected the plants and caused them to disintegrate before successful tissue isolations could be made. It was therefore decided to only use sterile distilled water for the preparation of soil suspensions. Since this solution was unable to increase pathogen concentrations, only concentrations from 10^1 cfu ml^{-1} and higher could be detected. With the selective media we were able to detect concentrations of 10^3 cfu ml^{-1} (M-TZC and SMSA) and 10^4 cfu ml^{-1} (TZC) while the ELISA proved effective from 10^4 cfu ml^{-1}. Although the selective media and ELISA methods produced results within three days, the ELISA technique was expensive and required specialised equipment such as the multi scan spectrophotometer. The selective media were the most affordable and although less sensitive for the detection of low pathogen concentrations, could be the best method to use in developing countries with limited resources. The trapping technique could effectively be used as an alternative method for the detection of *R. solanacearum* in artificially inoculated soil samples but should not replace the traditional selective media. Another possible application of the tissue culture technique could be the revival of old cultures. One of the characteristics of *R. solanacearum* is the rapid loss of pathogenicity once it is maintained in culture (Kelman, 1953). Previously scientists had to stem-inoculate mature susceptible host

plants with cell suspensions and re-isolate the pathogen after symptom development to maintain virulence (Kelman and Sequeira, 1965). The tissue culture plants were able to take up the pathogen within a week and based on colony morphology, virulent colonies could successfully be re-isolated (Kelman, 1953). One of the disadvantages of this technique is that it requires a continuous supply of tissue culture potato plants. Where plants are not readily available or facilities do not permit own *in vitro* multiplication of potato plants, scientists will have to use traditional methods for detection and revival of old cultures.

Ralstonia solanacearum could not be detected in the soil samples from an infected potato field. This could probably be due to incorrect sampling since Jenkins *et al.* (1967), Elphinstone (1993) and Pradhanang (1999) reported similar problems with soil sampling. According to them, it was difficult to obtain adequate dispersal of soil and bacteria. Smaller samples (2 g) should have been taken and more sub-samples prepared (Pradhanang, 1999). Bacteria do not to exist as individual cells and colonies are enveloped in mucilage (Jenkins *et al.*, 1967). According to Lloyd (1978) these enveloped colonies could act as a sheltered site where the bacteria survive between successive plantings of a susceptible crop. Since a 4 mm mesh screen was used to sieve the soil samples, many of the aggregates could have been removed, resulting in a lower pathogen concentration. Graham and Lloyd (1979) reported that the pathogen could be detected at all soil depths between 15 and 75 cm but not in the 0 to 15 cm zone, most probably due to desiccation during dry weather prior to soil sampling. This could explain why *R. solanacearum* was not detected. Since the soil was extremely dry and was undisturbed for six months since the last potato crop, the pathogen could have been present in deeper soil layers and samples should have been taken deeper. According to Elphinstone (1993) sampling depths should be between 20 and 30 cm. Before collecting soil samples, field plots should be ploughed to distribute any inoculum present and samples taken from a depth of 25 cm (Pradhanang, 1999). Due to practical limitations, this could not be done at the time of sampling.

Although many different methods exist for the detection of *R. solanacearum*, and the trapping technique could possibly be used as an alternative method for the detection of the pathogen in artificially inoculated soil suspensions, soil sampling remains a problem. Another way to overcome the difficulty of soil sampling is to use indicator plants in suspected fields (Jenkins *et al.*, 1967; Graham and Lloyd, 1978). Although this method takes longer to obtain results, it is cheaper and easier to apply and could possibly be used to give an indication of the presence of *R. solanacearum* in a field.

This study showed the successful development of an alternative approach to isolate *R. solanacearum* from artificially inoculated soil samples. The trapping technique proved highly sensitive but not rapid enough for commercial use and cannot replace selective media and the ELISA technique.

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