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Detection of *Ralstonia solanacearum* in Soil and Weeds from Commercial Tomato Fields Using Immunocapture and the Polymerase Chain Reaction

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With 2 figures

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Abstract

A detection assay for *Ralstonia solanacearum* in soil and weeds was developed by combining immunocapture and the polymerase chain reaction (IC-PCR). Anti-*R. solanacearum* polyclonal antibodies were produced in a white female rabbit and Dynal® superparamagnetic beads were coated with purified immunoglobulinG (IgG). Using IC-PCR, the 718 bp target DNA was amplified at a detection threshold of approximately 10^4 colony-forming units (CFU) bacteria per millilitre of suspension. DNA was not amplified in soil suspensions derived from autoclaved and non-autoclaved soils, which contained *R. solanacearum* at 1–10^5 CFU/g soil. However, a positive PCR result was obtained when bacteria in the soil suspensions were first enriched in nutrient broth. IC-PCR detected *R. solanacearum* in tomato stems 24 h after inoculation by stem puncture with a suspension containing approximately 10^5 CFU/ml. IC-PCR detected the bacterium in 28 of 55 (51%) weeds and 10 of 32 (31%) soil samples. Of the weeds, *Physalis minima*, *Amaranthus spinosus* and *Euphorbia hirta* had the highest incidence of infection. *R. solanacearum* was not detected in soil taken from fallow fields, but it was discovered in some weed species. Symptomless tomato and pepper plants collected from the fields in which tomato bacterial wilt had previously occurred were found to contain *R. solanacearum*. These discoveries suggest that weeds and latent hosts may play a role in the survival of *R. solanacearum* between cropping cycles.

Introduction

Bacterial wilt of tomato is a major disease inhibiting tomato production in subtropical and warm and humid tropical areas including the northeastern region of Thailand. The symptoms of the disease can be recognized by sudden wilting of the plant during hot and sunny days, which results from blockage of the plant's water conducting vessels. The disease is caused by the soil-borne bacterium *Ralstonia solanacearum* (Hayward, 1991). Controlling tomato bacterial wilt is difficult because the pathogen has an extremely broad host range, and is able to survive in the soil in the absence of the host plant. Moreover, it can colonize host plants like members of the Solanaceae, and non-host plants including many weeds, without producing visible symptoms (Moffett and Hayward, 1980; Hayward, 1986). These carriers could serve as a primary inoculum source for the subsequent tomato crop (Hayward, 1994). *R. solanacearum* can persist at low populations in naturally infested soil for years without a host plant (Akiew, 1986), and the population size could increase to the plant infection threshold within a season after the host plants are returned to the fields. Therefore, early detection of the bacterium in soil, water, weeds, tubers and plant residues could facilitate elimination and certainly reduce the risk of crop loss. A significant reduction in crop loss due to potato bacterial wilt has been demonstrated through early detection of latent infected tubers (Elphinstone et al., 1998).

Several detection methods have been developed for *R. solanacearum*. Among them, the most commonly used is direct plating on selective media and enzyme-linked immunosorbent assay (ELISA). Although polymerase chain reaction (PCR) has great potential as a sensitive and specific detection method, it has not been commonly used for field samples. In the case of *R. solanacearum*, inconsistent PCR detection results from plant and soil samples have been reported (Elphinstone and Stanford, 1998). The inconsistent results could be due to inhibition of the enzymatic PCR reaction by various compounds present in plant and soil samples, such as phenolic compounds or acidic polysaccharides in plants and humic acid in soils

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(Picard et al., 1992; Wilson, 1997). To overcome this problem, anti-target bacteria polyclonal antibodies were applied to separate target microbes from the medium prior to cell rinsing and further processing. This technique is known as immunocapture (IC) (Enroth and Engstrand, 1995) or immunoseparation (Walcott and Gitaitis, 2000). To further enhance separation efficiency, the technique was modified by attaching the anti-bacterium polyclonal antibodies to magnetic beads before mixing them to the samples. This is followed by DNA extraction and PCR using *R. solanacearum* specific primers. This method significantly improves the detection of bacteria in naturally infested samples with low population levels (Rudi et al., 1998).

The determination and elimination of the primary inoculum source is one of our strategies for managing tomato bacterial wilt in northeastern Thailand. Currently, specific primers to *R. solanacearum* strains endemic to the region have been developed from cloned common randomly amplified polymorphic DNA (RAPD) fragments. Their highly specific relationship to the strains of pathogen prevalent in the region indicate that these primers may effectively detect *R. solanacearum* in northeastern Thailand (V. Dittapongpitch and T. Wangsomboondee, unpublished data). In this study, we have developed an IC-PCR protocol by using polyclonal antibodies against *R. solanacearum* developed in-house, and evaluated its sensitivity by examining soil and weed samples collected in the major commercial tomato fields of northeastern Thailand.

Materials and Methods

Bacterial strains and culture

R. solanacearum strains were isolated on tetrazolium chloride medium (TZC) from wilted tomato plants collected from tomato fields in the northeast of Thailand during 2000-2001. The bacteria were stored under 20% glycerol at −80°C. For studies, the bacteria were subcultured on TZC and incubated at 32°C for 48 h. Other bacteria were isolated on nutrient agar (NA) from suspensions prepared from 100 g of tomato rhizosphere soil in 100 ml sterile distilled water. For liquid cultures, the bacteria were grown in nutrient broth (NB) for 16–18 h at 32°C (a mid log-phase culture) and shaken at 120 r.p.m. in shaking water bath (model 1092, Gesellschaft für Labortechnik mbH, Burgwedel, Germany).

Soil, weed and symptomless host plant samples

All samples were collected from three northeast provinces, Sakhon Nakhon (SK), Nakhon Phanom (NP) and Nong Khai (NK), of Thailand in November 2001. Thirty-two soil samples were collected from 14 tomato production fields. Four soil samples were randomly collected from two weed fallow fields where tomatoes were cultivated in the previous year, and 28 soil samples were taken from tomato rhizosphere soils at 10-cm intervals from the tomato plant and 20 cm beneath the soil surface. The samples were maintained at 4°C in plastic bags during transportation and storage before testing. Weeds with attached roots were collected from the same fields. Identification of their genus and species was accomplished according to The National Weed Science Research Project, Thailand (Noda et al., 1994). Non-wilting tomato and pepper (*Capsicum annuum*) stems with roots attached were also collected from production fields where 80% of the previous tomato crop was lost due to bacterial wilt.

Polyclonal antibody preparation

Antiserum for *R. solanacearum* was developed in a 5-month-old New Zealand white female rabbit using a race 1 biovar 3 of *R. solanacearum* strain P60 isolated from tomato. To prepare the antigen, the bacteria were harvested from a mid log-phase liquid culture by centrifugation at $20\,800 \times g$ (Centrifuge 5417 C, Eppendrof, Hamburg, Germany) for 5 min and the cell pellets were washed twice and resuspended in 0.01 M phosphate-buffered saline (PBS) pH 7.4 (0.13 M NaCl, 0.01 M KH_2PO_4, 0.008 M Na_2HPO_4) before whole cells were sonicated (100 amplitude %, cycle 1 with type U50 Control, IKA Labortechnik, Staufen, Germany). Concentration of the antigen was determined at 595 nm using a Spectronic Genesys 5 spectrophotometer (Milton Roy, Rochester, NY, USA) and adjusted to 0.5 mg/ml with 0.01 M PBS. Intramuscular and intravenous immunization were performed every 7 days over a period of 4 weeks. Peripheral blood was collected from the posterior auricular vein with a sterile syringe. The antibodies were obtained from the blood plasma after the blood was set at 4°C overnight.

Sensitivity of the antibodies (anti-P60) was determined by indirect ELISA methods (Crowther, 1995) against *R. solanacearum* strain P60 in the bacterial suspension containing 10^2–10^7 colony-forming units (CFU)/ml. Specificity of anti-P60 was verified by indirect ELISA methods against tomato strains of *R. solanacearum*, other plant pathogenic bacteria, and soil bacteria isolated from the tomato rhizosphere. The positive ELISA results were determined based on average absorbance of the product of the enzyme reaction from three repetitions at 405 nm with an ELISA reader (Fin-00811, Labsystems, Helsinki, Finland) using *R. solanacearum* strain P60 as a positive control.

Separation of immunoglobulin G (IgG) of the anti-P60 polyclonal antiserum was accomplished by fractionation using Protein A Sepharose™ CL-4B (Amersham Pharmacia Biotech AB, Uppsala, Sweden) based on the method described by Sambrook et al. (1989). The purified IgG concentration was determined at 280 nm using a Spectronic Genesys 5 spectrophotometer. To coat immunomagnetic beads, 200–400 μg IgG were incubated with 200 μl (1.2×10^8 beads) super-paramagnetic beads pre-coated with sheep anti-rabbit IgG (Dynabeads® M280, Dynal, Oslo, Norway) based on the method described by Walcott and Gitaitis (2000). To determine a successful coating of Immunomagnetic beads (IMBs), approximately 10^6 IgG-MB

were incubated with 10^8 CFU/ml of *R. solanacearum* strain P60 suspension prior to PCR amplification.

Detection threshold of IC-PCR

To determine the detection threshold of IC-PCR, bacterial suspensions of P60 were prepared by making a 10-fold dilution series from a mid-log phase liquid bacterial culture. Soil samples were spiked with the pathogen by mixing each 100 g autoclaved (121°C for 30 min) and non-autoclaved soil (60% clay and 30% sand) with 10 ml of bacterial suspensions each containing 10–10^6 CFU/ml. The inoculated soil samples (1–10^5 CFU/g of soil) were incubated at 25°C for 24 h prior to testing. To detect *R. solanacearum* in plant tissue, tomato cultivar L390 was artificially inoculated by piercing the leaf axil with a micropipette tip containing 200 μl of 10–10^7 CFU/ml (2–2 x 10^6 cells). Three plants were inoculated with each dilution of the cell suspension and the plants were kept at 25°C and 83% relative humidity. The experiments were repeated three times.

Sample preparation and immunocapture of *R. solanacearum*

Soil extractions were prepared by adding 100 ml of sterile distilled water into 100 g of soil, shaking at 120 r.p.m. for 30 min, and stationary incubation for another 30 min at 25°C. Forty millilitres of each soil suspension was centrifuged twice at $1800 \times g$ (Sorval GLC-1, Ivan Sorval Inc., Newton, Connecticut, USA) for 5 and 10 min to remove the large and fine soil particles, respectively. To harvest the bacteria, the suspension was further centrifuged for 20 min at $3800 \times g$ and the pellets were resuspended in 100 μl Tris-EDTA buffer (TE) (10 mM Tris-Cl and 0.1 mM EDTA, pH 8.0). For detecting *R. solanacearum* from the plants, 4 cm of the inoculated tomato stem was cut from 2 cm above and below the inoculated leaf axil. For weed and symptomless tomato and pepper samples, both the entire roots and 5 cm of the stem were washed with tap water and air-dried. All samples were macerated in 3 ml 0.01 M PBS. Two millilitres of the plant suspension was centrifuged at $20\,800 \times g$ for 5 min and the pellets were resuspended in 30 μl TE. To separate *R. solanacearum* from the sample, approximately 10^6 IgG-MB was added to the suspension and the mixture was incubated at 4–8°C for 2 h with gentle agitation using a low speed shaker (50 r.p.m.). The bacteria-beads mixture was washed twice with 1 ml of PBS and a magnetic particle concentrator (Dynal) was used to trap the IgG-MB in the tube during washing. The trapped beads were suspended in 30 μl TE prior to PCR amplification or enrichment of the trapped bacteria in nutrient broth.

Bacterial enrichment

To increase the number of *R. solanacearum* cells, the bacteria–IgG-MB complex was incubated in 10 ml NB for 16 h at 32°C and shaken at 120 r.p.m. for aeration. The bacteria were harvested by centrifugation at $20\,800 \times g$ for 10 min and resuspended in 100 μl TE prior to DNA extraction.

Primer specificity

Primers BF and BR which are specific to tomato strains of *R. solanacearum* prevalent in the northeast of Thailand (V. Dittapongpitch et al., unpublished data) were used in PCR reaction. To ensure specificity the primers were tested against 118 strains of *R. solanacearum* using conventional PCR.

Template preparation and DNA amplification

PCR template was prepared by the direct boiling method as described by Maes et al. (1996) with the slight modification of boiling the bacterial suspension for 10 min. After centrifugation at $20\,800 \times g$ for 5 min, 5 μl of the solution was used for DNA amplification. PCR was conducted in each 50 μl aliquot of the reaction mixture which contained 200 μM of each dNTP, 1.5 mM $MgCl_2$, 2 U *Taq* DNA polymerase (Promega, Madison, WI, USA), 25 pmol of each primer (BF, 5'-GGTGAGGCAGGTGCTTTTCTTG and BR, 5'-ACCCGACTTGCGGCACGTGGTCTAT: the oligonucleotides were synthesized by Bioservice Unit, Bangkok, Thailand) and 5 μl of template DNA from boiled bacterial suspension. The mixture was covered with 50 μl of light mineral oil (Sigma, St Louis, MO, USA). To test the effect of PCR inhibitor in PCR reaction mixture, 1 μl of the boiled *R. solanacearum* P60 suspension was included as a positive control using the same master mix. Sterile reverse osmosis water was substituted for DNA template as the negative control. DNA amplification was performed in a thermal cycler Omn-E (Hybaid, Middlesex, UK) with denaturation at 95°C for 5 min and the remaining 30 cycles consisted of denaturation, 95°C for 30 s; annealing, 60°C for 30 s; and extension, 72°C for 45 s. The final extension was continued at 72°C for 5 min. Presence of the 718 bp specific PCR product (V. Dittapongpitch et al., unpublished data) was determined on 1% agarose gel electrophoresis (Seakem® LE, FMC BioProducts, Maine, USA) in Tris-acetate/EDTA (TAE) buffer. Photo documentation was done using Polaroid film after staining the gel in 0.5 μg/ml ethidium bromide and destaining for 10 min.

Results

Specificity and sensitivity of the anti-P60

The anti-P60 polyclonal antibodies showed positive reactions to all tested *R. solanacearum* strains based on indirect ELISA. The anti-P60 antibodies did not yield a positive ELISA reaction with other plant pathogenic bacteria and the bacteria isolated from tomato rhizosphere (Table 1). The minimal cells of *R. solanacearum* strain P60 required for the positive ELISA reaction were at 10^4 CFU/ml (data not shown).

Specificity of PCR primers

The 718 bp amplicon was produced in the reactions in which the template was prepared from *R. solanacearum* strains only (Table 1).

Table 1
Bacteria used in polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA) and specificity tests with primers BF and BR and polyclonal anti-*Ralstonia solanacearum* strain P60 antiserum (anti-P60)

Bacteria	No. of isolate	PCR[1]	ELISA[2]
R. solanacearum[3]			
Strain P60[4]	1	1	1
Strains isolated in 2000	7	7	7
Strains isolated in 2001 from			
Sakhon Nakhon (SK)	30	30	30
Nakhon Phanom (NP)	45	45	45
Nong Khai (NK)	43	43	43
Erwinia carotovora subsp. *carotovora*	10	0	0
Xanthomonas campestris pv. *vesicatoria*	7	0	0
Other bacteria[5]	15	0	0

[1]Number of bacterial strains yielding 718 bp DNA fragment with primers BF and BR. The test was carried out with three repetitions;
[2]Number of bacterial strains giving positive indirect ELISA results which were determined from an average absorbance of the product of enzyme reaction (from three repetitions) at 405 nm;
[3]Bacteria isolated from wilted tomatoes;
[4]Positive control;
[5]Bacteria isolated from tomato rhizosphere.

Coating of IMBs and detection threshold of IC-PCR

IC-PCR amplified the 718 bp amplicon in the suspension of strain P60 containing 10^8 CFU/ml (data not shown). The detection threshold of IC-PCR was 10^4 CFU/ml. IC-PCR failed to amplify the target DNA in soil suspensions prepared from both autoclaved and non-autoclaved soils containing 0–10^5 CFU *R. solanacearum* per gram soil (Table 2). However, a 718 bp amplicon was produced in all soil suspensions containing additional template DNA prepared from boiled *R. solanacearum* strain P60. Twenty-four hours after inoculation, none of the inoculated tomato plants displayed bacterial wilt symptoms; however, the plants inoculated with 10^4 CFU level (10^5 CFU/ml) yielded a positive PCR reaction. No amplicon was produced when IC-PCR was conducted on plants inoculated with 0–10^3 CFU (0–10^4 CFU/ml) unless the DNA template prepared from boiled *R. solanacearum* strain P60 was added (Table 2).

Bacterial enrichment and detection threshold

Enrichment of immunocaptured *R. solanacearum* cells from autoclaved soil inoculated with 1–10^5 CFU/g soil in NB yielded positive PCR results. Soil suspensions of non-autoclaved soils initially containing bacteria at least 10^4 CFU/g soil yielded a 718 bp amplicon after 16 h enrichment in NB. Likewise, the tomato strains inoculated with approximately 10^3 CFU level (10^4 CFU/ml) yielded 718 bp amplicon after incubating

Table 2
Threshold of IC-PCR detection of *Ralstonia solanacearum* in artificially contaminated water, soil and tomato plants

Bacterial cells	Non-enrichment[1]	Added template DNA[2]	Enrichment[3]	Added template DNA[2]
Bacterial suspension (CFU/ml)				
10^5	+[4]	+	+	+
10^4	+	+	+	+
10^3	–[5]	+	+	+
10^2	–	+	+	+
10	–	+	+	+
0	–	+	–	+
Soil suspension of non-autoclaved soil (CFU/g soil)				
10^5	–	+	+	+
10^4	–	+	+	+
10^3	–	+	–	+
10^2	–	+	–	+
10	–	+	–	+
1	–	+	–	+
0	–	+	–	+
Autoclaved soil (CFU/g soil)				
10^5	–	+	+	+
10^4	–	+	+	+
10^3	–	+	+	+
10^2	–	+	+	+
10	–	+	+	+
1	–	+	+	+
0	–	+	–	+
Tomato plant (CFU/ml)				
10^7	+	+	+	+
10^6	+	+	+	+
10^5	+	+	+	+
10^4	–	+	+	+
10^3	–	+	–	+
10^2	–	+	–	+
10	–	+	–	+
0	–	+	–	+

[1]Bacteria-IgG-MB was direct prepared template DNA;
[2]1 μl of 10^8 CFU *R. solanacearum* strain P60 per millilitre was added to the PCR reaction mixture;
[3]Bacteria-IgG-MB was enriched in nutrient broth for 16 h prior to template DNA preparation;
[4,5]Presence (+) and absence (–) of specific 718 bp fragment based on three repetitions.

the bacteria beads in the medium. All PCR reaction tubes with additional template DNA yielded positive PCR results (Table 2).

Detection of *R. solanacearum* from weeds, symptomless host and soils

Fifty-five weed plants collected from both weed fallow fields and tomato cropping fields were classified into 15 families with 21 genera and 23 species (Table 3). Using enrichment and IC-PCR, the 718 bp amplicon was produced (Fig. 1) in 28 weed plants which comprised approximately 51% of all samples. The 28 IC-PCR-positive weed samples belong to 13 families with 17 genera and 18 species (Table 3). All weeds except *Cyperus rotundus* are dicotyledon. *Physalis minima*, *Amaranthus spinosus* and *Euphorbia hirta* were the most common weeds in the fields with over 50% of each weed species yielding IC-PCR positive results. *Cleome viscosa* and *Portulaca oleracea* were other common weeds in the fields but detection of *R. solanacearum* in these weed species was rare (Table 3). The pathogen was detected in *P. minima* from the samples collected from fields in all three provinces (Table 3). Likewise, after the enrichment treatment, the target DNA fragment was amplified in 10 of 32 soil samples

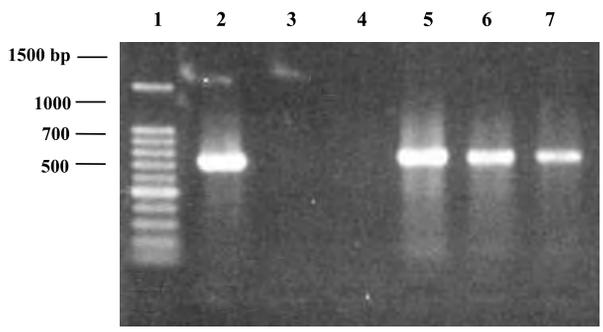

Fig. 1 Agarose gel electrophoresis (1% in TAE buffer) of the results of IC-PCR on weeds collected from tomato fields. Lane 1, 100 bp DNA Ladder (Promega, St Louis, MO, USA); lane 2, PCR positive control (template DNA was from *R. solanacearum* P60); lane 3, results of PCR which water was replaced template DNA; lanes 4–7, results of PCR on weed species including *Chenopodium murale, Physalis minima, Amaranthus spinosus*, and *Euphorbia hirta*, respectively

Table 3
Weed species collected from commercial tomato fields in three northeast provinces in Thailand during 2001 and IC-PCR results

Plant species	Common name[1]	Total plants	IC-PCR[2]
Aizoaceae			
Trianthema portulacastrum	Horse purslane (NK)	1	1 (O)
Amaranthaceae			
Amaranthus gracilis	Slender amaranth (SK)	1	0
A. spinosus L.	Spiny amaranth (NP, NK)	7	3 (J, L, N)
Cleosia argentea	(NP)	1	0
Asteraceae			
Ageratum conyzoides	Goatweed (SK)	1	0
Eupatorium odoratum L.	Bitter bush (SK)	1	1 (A)
Capparidaceae			
Cleome viscosa L.	Wild caia (NP)	6	2 (G, H)
Chenopodiaceae			
Chenopodium murale	Chenopodium (NK)	1	0
Commelinaceae			
Commelina benghalensis L.	Dayflower (SK, NP)	2	0
Cyperaceae			
Cyperus rotundus L.	Purple nutsedge (NP)	1	1 (G)
Euphorbiaceae			
Euphorbia hirta	Garden spurge (NP, NK)	7	4 (I, J, M, N)
E. geniculata	Painted spurge (NK)	2	1 (M)
Phyllanthus niruri	Niruri (SK)	1	1 (E)
Leguminosae			
Cassia tora	Sicklepod (NP)	1	1(G)
Mimosa invisa	Giant sensitive plant (SK,NP)	2	1 (F)
Phaseolus lathyroides L.	Phasey bean (SK, NP, NK)	3	2 (A, F)
Onagraceae			
Ludwigia hyssopifolia	Water primrose (SK)	1	1 (E)
Passifloraceae			
Passiflora foetida L.	Redfruit passion flower (NP)	1	1 (H)
Portulacaceae			
Portulaca oleracea L.	Pig-weed or purslane (NK)	5	2 (K, L)
Rubiaceae			
Richardia scabra L.	Pursley (NP, NK)	2	1 (F)
Scrophulariaceae			
Limnophila laotica	– (SK)	1	1 (A)
Solanaceae			
Physalis minima	Chinese lantern plant (SK, NP, NK)	6	3 (E, I, K)
Solanum sp.	Wild egg plant (SK)	1	1 (A)

[1]Alphabets after common names are initials of three northeastern provinces (Sakhon Nakhon, SK; Nakhon Phanom, NP; and Nong Khai, NK) where the plants were collected;
[2]Alphabets in parenthesis refer to fields where the samples were positive (*R. solanacearum* was detected) in IC-PCR detection.

Table 4
Plant and soil samples collected from commercial tomato fields in three northeast provinces in Thailand during 2001. Positive means *Ralstonia solanacearum* was detected based on IC-PCR reactions

	No. of positive samples\no. of total samples			
			Symptomless plants	
Province and field code	Weed	Soil	Tomato	Pepper
Sakhon Nakhon				
A[1]	4\5	0\1	nd[2]	nd
B	nd	1\1	5\5	nd
C	0\1	1\1	5\5	nd
D	0\2	1\1	nd	nd
E	3\4	1\1	5\5	nd
Nakhon Phanom				
F	3\6	0\4	nd	nd
G	3\5	0\4	5\5	nd
H	2\3	3\4	nd	nd
I	2\5	2\4	nd	nd
J	2\4	0\4	nd	nd
Nong Khai				
K	2\6	0\1	nd	5\5
L	2\3	nd	nd	nd
M	2\4	0\2	nd	nd
N	2\4	1\1	nd	nd
O[1]	1\3	0\3	nd	nd

[1]Weed fallow field in which a previous crop was tomato;
[2]nd, not determined as no sample was collected.

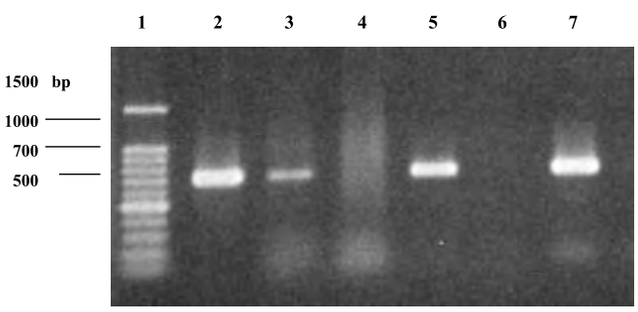

Fig. 2 Detection of *Ralstonia solanacearum* in natural soil by IC-PCR following enrichment of the bacteria in nutrient broth. The 718 bp DNA produced by IC-PCR of soil suspensions of naturally infested soils (lanes 2, 3, 5 and 7). No DNA was detected in weed fallow soils (lanes 4 and 6). Lane 1 is 100 bp DNA Ladder (Promega, St Louis, MO, USA) used as the size marker

using IC-PCR (Table 4). No positive reaction was obtained in the soil from the weed fallow fields (Table 4 and Fig. 2). Furthermore, IC-PCR detected *R. solanacearum* from non-wilted tomato and pepper plants taken from fields (Table 4) in which 80% of the previous tomato crop had been lost due to heavy infection by the bacteria.

Discussion

Several approaches have been used to detect *R. solanacearum* in naturally infested samples (Jenkins et al., 1966; Engelbrecht, 1994; Hartung et al., 1998; Pradhanang et al., 2000b); however, different factors restricted their ability to detect low numbers of the bacterium (Wilson, 1997; Elphinstone et al., 1998). Our previous attempt to detect *R. solanacearum* in the northeastern naturally infested soils using the bacterial PCR-specific primers (Seal et al., 1999) was inadequate in terms of sensitivity and specificity to the bacterium strains endemic in the northeastern region, Thailand (V. Dittapongpitch, unpublished data). In 2000, primers BF and BR were designed and tested against *R. solanacearum* strains endemic in Thai commercial tomato fields. Unfortunately, the conventional PCR using the primers was neither reliable nor precise enough for routine use due to PCR inhibitors in soil that reduce the assay sensitivity (V. Dittapongpitch et al., unpublished data). Therefore, in this study, IC was used to separate and concentrate *R. solanacearum* from samples prior to PCR amplification.

Specificity of the anti-P60 was indubitably based on indirect ELISA; however, sensitivity of the anti-P60 determined by indirect ELISA was restricted to 10^4 CFU/ml (data not shown). A similar finding regarding ELISA sensitivity has been reported (Elphinstone and Stanford, 1998).

The amount of IgG required for optimal coating of IMB was not determined in our study; however, according to the manufacturer, 0.8–3.0 μg IgG/10^7 beads is recommended and we applied excess IgG in the bead coating. In addition, the affinity of the purified IgG to the sheep anti-rabbit IgG precoated on the beads was not verified in this test. However, IC-PCR yielded a positive result in the suspension of P60 containing 10^8 CFU/ml. Thus, the bead suspension may possibly contain both the coated IgG-MB and the non-coated IMB. Moreover, sensitivity of the anti-P60 was restricted at 10^4 CFU/ml. Therefore, these reasons may explain the high detection threshold of IC-PCR in the samples. Detection thresholds of IC-PCR in detection of other bacteria contaminate in natural substrates are varied between laboratories depend on quality of polyclonal antibodies and species of target cells (Walcott and Gitaitis, 2000; Peng et al., 2002). Nevertheless, similar detection threshold of the technique to other bacteria are reported (Blake and Weimer, 1997; Osaki et al., 1998; Liu and Li, 2002). This is the first attempt of using IC-PCR to detect *R. solanacearum* in soil and plants in order to overcome PCR inhibitors and increase sensitivity. The technique requires an efficacy of coating IgG on the beads and an enhancement of association between IgG and *R. solanacearum* because numerous factors affect disassociation of complexes of antibodies and antigen on a bead surface (Liu and Li, 2002).

PCR amplicons were not produced without additional template DNA in the PCR reaction of inoculated soil suspensions, possibly because none of the bacteria was trapped on the IgG-MB, or insufficient template DNA was available for PCR amplification. However, the positive results of PCR in the reaction tubes after incubation in NB and the tubes with additional template DNA confirmed that *R. solanacearum* was on the beads. However, the target may have been below the detection threshold. In addition, PCR amplification

might be less sensitive in the presence of low template DNA concentration and contain inhibitory soil components such as humic acid (Wilson, 1997). Successful attempts to amplify the 718 bp fragment in the soil suspension spiked with template DNA supported our point.

In the presence of low bacterial populations, the target bacteria need to be cultivated in an appropriate or a selective medium to enhance detection efficiency (Enroth and Engstrand, 1995; Priou et al., 1999). We enriched *R. solanacearum* in NB prior to DNA amplification and it enhanced detection sensitivity which might have resulted from increased bacterial cells as well as the dilution of natural PCR inhibitors in the samples. Without enrichment, we were unable to detect *R. solanacearum* in the soil suspensions; however, the poor sensitivity in non-autoclaved soil suggested that other soil micro-organisms, or perhaps some soil components interfered with the binding of the IgG and the bacteria. To overcome the problem, non-target bacterial populations should be reduced by using a selective or semi-selective medium (Engelbrecht, 1994).

Unlike the soil suspensions, positive IC-PCR results were obtained without enrichment from tomato plants which were inoculated with 10^5–10^7 CFU/ml *R. solanacearum* 1 day after inoculation. This result suggested that the natural plant compounds in tomato plants have a minor effect on PCR. As the inoculated tomato plants were symptomless, this result suggests that enrichment and IC-PCR could be used to detect latent infection in tomato plants. Furthermore, IC-PCR could detect *R. solanacearum* in non-wilting tomato and pepper plants grown in fields where tomato had been the previous crop and the fields were infested with the pathogen in earlier years. As bacterial wilt was not observed in those fields when the samples were taken and no information regarding the tomato or pepper cultivars was available, this suggests that latent infection might have occurred in those plants.

Survival of *R. solanacearum* either in soil or non-host plants has been pointed out by many researchers (Moffett and Hayward, 1980; Pradhanang et al., 2000a; Pradhanang and Momol, 2001). In the fallow fields, the pathogen was not detected from natural soils but it was found in some weed plants. This suggests that the weeds are important reservoirs for the pathogen after the season. However, not every weed plant collected from the same field was infested, suggesting that pathogenicity may depend on some factor(s) (Smith and Goodman, 1999). Titatarn (1986) reported that in a number of weeds collected from tomato fields in the north of Thailand *R. solanacearum* biovar 3 was endemic. It is interesting that the major weed species belonging to Asteraceae and *Ageratum conyzoides* and *Eupatorium odoratum* were among the host lists. Both weed species are very common in Thailand and they always remain around tomato fields after cultivation. We detected *R. solanacearum* in *E. odoratum* but not in *A. conyzoides* and the bacteria prevalent in the northeast fields were biovars 3 and 4 (V. Dittapongpitch, unpublished data). This suggests that *E. odoratum* might serve as the major alternate host for various strains of *R. solanacearum* in Thailand. Both *E. odoratum* and *A. conyzoides* are also the major sources for some virus insect vectors including whitefly (*Bemisia* sp.) (a tomato yellow leaf curl disease) and thrips (Jamjanya et al., 2002). Therefore, eradication of both weed species should not only reduce crop loss due to viral diseases but also eliminate an inoculum source for the bacterium. Most of the *A. spinosus, E. hirta* and *P. minima* were common in tomato fields with poor weeding during the cropping season and the weed plants showed evidence of *R. solanacearum*. Thus, we speculate that those weeds may be possible hosts for the bacterium in those fields. In addition, we were able to detect the bacterium in *P. minima* samples collected from the production fields located in three major tomato production provinces and the weed shares the same family with the symptomless tomato and pepper samples. Therefore, some other unexamined *Solanaceous* weeds in Thailand may have a high potential as an inoculum source for *R. solanacearum*. It was interesting to find the bacterium in *C. rotundus*, which is the only monocotyledonous sample. This explains the tendency of other narrow leaf weed plants towards bacterial wilt incidence, as many grass species are dominant in tomato fields. This study demonstrated that IC-PCR with prior enrichment could enhance detection sensitivity of *R. solanacearum*. We will continue to apply this detection method in bacterial wilt detection. One possible future direction is to relate the presence of *R. solanacearum* in soil and weed samples with the disease incidence in tomato production fields to verify the importance of various inoculum sources.

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