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Detection of race 1 strains of *Ralstonia solanacearum* in field samples in Taiwan using a BIO-PCR method

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Abstract Bacterial wilt caused by race 1 strains of *Ralstonia solanacearum* is endemic on tomato produced in diverse agro-ecosystems in Taiwan. Using a new BIO-PCR protocol developed in this study, *R. solanacearum* was detected in soil, weed, and water samples collected from eight fields with different disease histories and cropping systems located in major tomato production areas. The sensitivity of the BIO-PCR was 1.9 CFU ml^{-1} and 17 CFU g^{-1} of soil for pure suspension and infested soil, respectively. The positive detection frequency of the BIO-PCR method was 66.6, 39.6, 23.1, and 31.8% for all tested samples of soil, weed rhizosphere soil, weed root, and water, respectively, and was higher than plating on MSM-1 medium. Detection of *R. solanacearum* from field soil indicated that spatial distribution of the pathogen in the field was not even regardless of the presence or absence of the disease and the different agro-ecosystems where the sampled fields were located, and the degree of unevenness was higher when tomato was absent from the field. Weed rhizosphere soils could be good sampling targets to monitor the pathogen in the field, because a higher positive detection proportion and population of *R. solanacearum* were found in the rhizosphere rather than the root of the collected weed samples. Symptomless weeds and contaminated irrigation, standing, or drainage waters were found to be important for the over-season survival and dissemination of *R. solanacearum*.

Keywords Spatial distribution · Sampling · Selective medium · Soil · Weed · Water

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Introduction

Phylotype I and race 1 strains of *Ralstonia solanacearum* are prevalent in the warm and humid tropics and subtropics. The pathogen causes bacterial wilt on a wide range of crops. Among them, bacterial wilt on tomato is the most widespread and severe (Elphinstone 2005). The pathogen is soil-borne (Kelman 1953), can be transmitted via water (Hong et al. 2005), and is commonly associated with weeds without causing wilting symptoms (Moffett and Hayward 1980; Hong et al. 2005). A sustainable and successful disease management programme

greatly depends on an understanding of the ecology of the pathogen in the environment. However, the survival and dissemination of race 1 strains of *R. solanacearum* in the agro-ecosystem remain poorly understood.

Tomato has been cultivated in diverse agro-ecosystems in Taiwan. The main production areas are located in the lowland, where the crop is commonly rotated with irrigated rice and transplanted in May, July, or October from the north to the south. Tomatoes are also transplanted in late spring in the upland intensive mixed cropping systems aiming to fill the supply gaps in summer and early autumn. Bacterial wilt caused by race 1 strains of *R. solanacearum* is endemic on tomato in all production areas in Taiwan (Hsu 1991). A loss of US$12 million of summer tomato production has been recorded in Taiwan (Hartman et al. 1991). Host resistance and soil amendments have been the two main control approaches in Taiwan (Lin et al. 2008). However, a better understanding of the over-season survival and dissemination of the pathogen among the diverse agro-ecosystems is needed to develop a locally adapted integrated disease management programme. For example, weeds have been reported to harbour the pathogens in Taiwan (Lin et al. 1994; Pan et al. 1996; Lin et al. 1999); however, weed samples were collected randomly in earlier studies and had not focused on tomato production areas. The sources of irrigation water for tomato production in Taiwan include reservoir, natural waterway, or ground water. It is not clear whether water plays a role in pathogen dissemination.

An efficient and simple detection method with good specificity and sensitivity is a prerequisite for conducting ecological surveys of plant pathogens. Several detection methods for *R. solanacearum* have been developed, such as dilution plating on a selective medium, tomato bioassay, pathogen-specific polymerase chain reaction (PCR) methods, immuno-capture-PCR, immunofluorescence colony staining, indirect enzyme-linked immunosorbent assay (ELISA), and double antibody sandwich-ELISA. A review of these methods has been made by Priou et al. (2006). Among the reported detecting methods, direct plating on selective media is still the detection method used in ecological studies of *R. solanacearum* (Wenneker et al. 1999; Pradhanang et al. 2000; Pradhanang and Momol 2001; Hong et al. 2005). Although several PCR-based methods claimed to have higher sensitivity and good specificity, the effectiveness of these methods on field samples is largely unknown, because previous studies were focused on method development and only a few field samples were tested for the proof of concept.

Enrichment of targeted bacteria by incubating samples on general or selective media followed by polymerase chain reactions with specific primers for detection of plant pathogenic bacteria has been termed BIO-PCR (Schaad et al. 1995; Song et al. 2004). The advantages of BIO-PCR include the elimination of false-negatives due to potential PCR inhibitors in sample extracts, elimination of false-positives resulting from dead cells or free DNA, and an increase of detection sensitivity due to an increase in the number of the target cells. Similar approaches have been developed to detect *R. solanacearum* (Ito et al. 1998; Pradhanang et al. 2000). In this study, a BIO-PCR method was developed for detecting *R. solanacearum* using a semi-selective medium named MSM-1 for enrichment and a species-specific primer pair, AU759/760 for detection. MSM-1 medium was developed by Tsai et al. (1985) by modifying the SM-1 medium reported by Granada and Sequeira (1983a). This medium has displayed good selectivity for the pathogen from soil samples collected in Taiwan, and the detection limit is around 100 CFU g^{-1} of soil based on tested artificially or naturally-infested soils (Lin et al. 2008). The primer pair AU759/760 has been tested extensively and shown to be highly specific over a large number of *R. solanacearum* strains (Opina et al. 1997; Ito et al. 1998). The AU759/760 amplified 282-bp fragment is part of the putative *lpxC* gene, the gene encoding UDP-3-*O*-acyl N-acetyl-glucosamine deacetylase in *R. solanacearum* (Villa et al. 2003) which is highly conserved among *R. solanacearum* strains. When blasting the 282-bp sequence in the NCBI database, low homology was detected with only a few bacteria associated with soil and water, such as *Polaromonas* sp. and *Pseudomonas mendocina* (unpublished data).

Therefore, the objectives of this study were to study the survival and dissemination of *R. solanacearum* associated with field soils, weeds, and water in the tomato production areas in Taiwan using a newly developed BIO-PCR protocol. The effectiveness of the BIO-PCR protocol will be compared with a direct plating method.

Materials and methods

Bacterial culture, bacterial suspension, and soil infestation

Ralstonia solanacearum strain Pss4 from the AVRDC—The World Vegetable Centre (AVRDC) collection was used in this study. Strain Pss4 was isolated from tomato and belonged to phylotype I, race 1 and biovar 3. For inoculum preparation, the stored culture was first streaked on 2,3,5-triphenyl tetrazolium chloride (TTC) medium (Kelman 1954) at 30°C for 48 h, then several typical colonies were selected to multiply on 523 medium plates (Kado and Heskett 1970) at 30°C. A bacterial suspension was prepared from the overnight 523 cultures with sterile distilled water (SDW) and adjusted to an optical density of 0.3 at the wavelength of 600 nm. The viable bacterial population in the suspension was about 10^8 CFU ml^{-1}. The artificially infested soil was prepared by thoroughly mixing air-dried AVRDC farm soil with the bacterial suspension in the ratio of 1 to 10 (v/w). The infested soil contained about 10^7 CFU g^{-1} of dry soil.

Bacterial enrichment and PCR

The MSM-1 medium without agar was used to enrich *R. solanacearum* in samples for detection. The MSM-1 basal medium consisted of 10 g peptone, 5 g glucose, 1 g casein hydrolysate, 15 g agar and 50 mg TTC in 1 l SDW with additional antimicrobial compounds [5 mg chloramphenicol, 5 mg crystal violet, 5 mg cycloheximide, 100 mg polymyxin B sulphate (50,000,000 U) and 20 mg tyrothricin] (Tsai et al. 1985). A total of 10 g of each soil sample was added to 90 ml SDW and shaken at 180 rpm at room temperature for 30 min. One milliliter of sample solution was added into 9 ml of MSM-1 broth and incubated at 30°C at 160 rpm for enrichment; 5 µl of each enriched suspension was then transferred in a 200-µl PCR tube, covered with one drop of sterile mineral oil, boiled for 5 min and kept in ice. The PCR was performed as described by Opina et al. (1997) using *R. solanacearum*-specific primer pair AU759f (5′-GTC GCC GTC AAC TCA CTT TCC-3′) and AU760r (5′-GTC GCC GTC AGC AAT GCG GAA TCG-3′). A 25-µl reaction mixture contained 1× PCR buffer [10 mM Tris–HCl (pH 9.0), 50 mM KCl, and 0.1% Triton X-100], 1.5 mM $MgCl_2$, 0.05 mM of each dNTP, 1 pmol of each primer, 2 U of *Taq* DNA polymerase (Promega, Madison, USA), and 5 µl of boiled bacterial suspension or enriched cultures. PCR amplification was performed in a MJ Research PTC-200 thermal cycler (MJ Research, Waltham, MA, USA). Samples were denatured at 94°C for 3 min, annealed at 53°C for 1 min, and extended at 72°C for 1.5 min, followed by 30 cycles of 94°C for 18 s, 60°C for 18 s and 72°C for 18 s and a final extension of 72°C for 5 min. A specific PCR product of 282 bp was visualised under UV light after electrophoresis in 1.5% agarose and staining in ethidium bromide solution (1 µg ml^{-1}).

Detection threshold and enrichment period

For determining the detection threshold of the BIO-PCR and the enrichment period required, Pss4 suspensions and Pss4-infested soils with different densities were prepared. The Pss4 suspension (1.85×10^8 CFU ml^{-1}) described above was diluted with SDW in a tenfold series to generate a total of nine bacterial suspensions with estimated density ranging from 1.85 to 1.85×10^8 CFU ml^{-1} Ten milliliters of each serially diluted suspension was mixed with 100 g of AVRDC soil as described above. The eight infested soil samples prepared had estimated densities ranging from 1.68 to 1.68×10^7 CFU per g^{-1} of dry soil. The samples of the prepared bacterial suspensions and infested soils were enriched following the protocol described above. Viable bacterial counts and PCR detection were conducted for each sample every day for 7 days. Four flasks per sample as four duplicates were collected at each detection time.

Field sampling and sample preparation

Field samples, including field soil, weed rhizosphere soil, weed root, and water, were collected from fields in tomato production areas. The codes and information for each sampled field are presented in Table 1. Fields in Hsinchu and Tainan counties belong to the lowland rice system, while fields in Taichung and Nantou are in the upland intensive mixed cropping system. Field soil samples were collected from eight fields where tomato was cultivated at the sampling time or in the past. The area of each field was ca. 1,000 m^2. Each field was divided into 20 plots with equal size, ca. 50 m^2. A total of nine sub-samples

Table 1 Information on tomato production fields where samples were collected for detection

Sampling Field[a]	Soil Texture[b]	Sampling Date (dd/mm/yr)	Location	Previous crop	Present crop[c] or status	BW[d]
$WL1_a$	SiL	04/11/02	Chunglin, Hsinchu	Rice	Tomato	+/M
$WL1_b$	SiL	04/03/03	Chunglin, Hsinchu	Rice	Tomato	+/M
$WL1_c$	SiL	06/05/03	Chunglin, Hsinchu	Tomato	Mixed crops	+/L
$WL1_d$	SiL	14/07/03	Chunglin, Hsinchu	Mixed crops	Ploughed	+
$WL1_e$	SiL	20/10/03	Chunglin, Hsinchu	Mixed crops	Tomato	+/H
SF1	SL	05/11/02	Chunglin, Hsinchu	Rice	Tomato	−/H
SS1	L	04/11/02	Chunglin, Hsinchu	Corn	Fallow	−
$TC1_a$	SiL	15/03/02	Kuantien, Tainan	Rice	Tomato	−/M
$TC1_b$	SiL	22/10/02	Kuantien, Tainan	Cucumber	Tomato	+/L
$TC1_c$	SiL	28/11/02	Kuantien, Tainan	Cucumber	Tomato	+/L
TC2	L	17/12/02	Kuantien, Tainan	Cucumber	Tomato	+/L
$TC3_a$	SiL	16/06/03	Kuantien, Tainan	Tomato	Yard-long bean	+
$TC3_b$	SiL	15/08/03	Kuantien, Tainan	Yard-long bean	Ploughed	+
$TC3_c$	SiL	12/09/03	Kuantien, Tainan	Yard-long bean	Empty beds	+
HS1	CL	02/04/02	Hsinshe, Taichung	Sesbania	Melon	+
YC1	SL	23/07/02	Yuchyr, Nantou	Tomato	Ploughed	+

[a] Each code represents a specific field. When collecting samples in the same field repeatedly over time, the codes were marked with a subscripted small letter

[b] *SiL* silt loam, *SL* sand loam, *L* loam and *CL* clay loam

[c] Mixed crops consist of bitter gourd, cherry tomato, corn, cucumber, and yard-long bean. 'Ploughed' means fields were ploughed recently without presence of beds and weeds. "Fallow" means fields have been abandoned for a period of time and covered by weeds.

[d] History of bacterial wilt (BW) incidence in sampling field: + (incidence) and − (no incidence). Degree of severity of tomato BW during the sampling: *H* (high; incidence >50%), *M* (moderate; incidence <50%), and *L* (low; incidence <20%)

following a zigzag pattern were collected within each plot at 5 to 20 cm depth with a 3-cm diam soil auger. The sub-samples were combined and mixed well in a bag to form a composite sample per plot. Ten grams of soil from each composite sample were taken to prepare the soil suspension to be used for detection. Weed samples of the predominant species were collected randomly from three fields, i.e. SS1, WL1, and TC3. Each weed plant was dug out with soil attached on the roots. To collect rhizosphere soil, each weed plant was shaken to remove most soil on the roots, then placed in a 250-ml Erlenmeyer flask with 20 to 50 ml SDW depending on the root biomass, and shaken vigorously. The soil suspensions were then used for detection. Afterwards, the roots of each weed plant were washed with tap water, soaked in 70% alcohol for 5 min, rinsed in SDW twice, and blotted dry on paper towels. Each root sample was weighed, placed in a plastic bag containing SDW nine times its weight, and then macerated. The liquid extract from the roots was used for detection. Water samples were collected from four fields, i.e. SS1, SF1, WL1, and TC3. Five water samples of 50 ml each were randomly collected at each sampling point with autoclaved screw-cap plastic bottles. Water samples collected in each field included the irrigation water at the entrance point, standing water accumulation in the field, and water at the drainage exit. The water samples were used directly for detection. All field samples were stored under low temperature before detection and processed within 3 days. Four duplicates per sample were conducted during the detection process.

Results

Multiplication of *R. solanacearum* in MSM-1 broth and sensitivity of the BIO-PCR method

Ralstonia solanacearum in pure culture was able to grow and multiply in MSM-1 broth for 7 days

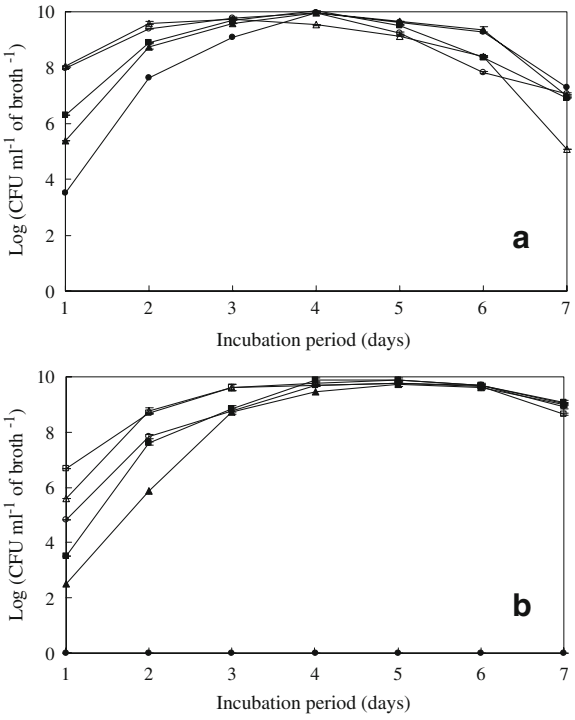

Fig. 1 Recovery of *R. solanacearum* Pss4 strain on MSM-1 agar following enrichment in MSM-1 broth at 30°C with different incubation periods from **a** bacterial suspensions with estimated initial log population of 0.27 (●), 1.27 (▲), 2.27 (■), 3.27 (○), 4.27 (△) CFU ml^{-1} and from **b** artificially infested AVRDC soils with estimated initial log population of 0.23 (●), 1.23 (▲), 2.23 (■), 3.23 (○), 4.23 (△), 5.23 (□) CFU g^{-1} of soil. *Y-bar* represents standard error of mean

(Fig. 1a) with maximum growth observed after 3 to 4 days of incubation, after which growth gradually declined. Similar growth trends were observed when MSM-1 broth was inoculated with infested soil suspensions (Fig. 1b), except a minor decline of growth after 5 days of incubation. The lower the initial bacterial population, the more obvious the enrichment effect resulted. The exception was the treatment with initial 0.23 log population g^{-1} of soil, which failed to multiply. Based on the results, incubation of three days was used as a standard enrichment period in our BIO-PCR protocol. *Ralstonia solanacearum* could be detected from template samples prepared from pure bacterial suspensions and infested AVRDC soils following the BIO-PCR protocol developed in this study (Fig. 2). With a 3-day enrichment period in MSM-1 broth followed by PCR detection with AU759/760 primers, this method could detect as few as 1.9 CFU ml^{-1} from pure suspension or 17 CFU g^{-1} of soil from infested AVRDC soil.

Detection of *R. solanacearum* from field soils

A total of 320 soil samples were collected from eight fields. The positive detection frequencies by growth on MSM-1 and BIO-PCR methods were 39.7% and 66.6%, respectively. Except for fields $WL1_d$ and SF1, the number of positive detections by the BIO-PCR method was higher than that by MSM-1 among the 20 samples collected from each field (Table 2). In fields SS1, $TC1_b$, and $TC3_b$, the pathogen was not detectable by the MSM-1 method, but was detected by the BIO-PCR method. No clear relationship was observed between the proportion of positive detection and the bacterial population present in the field. For example, similar ranges of *R. solanacearum* populations were present in fields $WL1_a$ and $TC1_c$ or HS1 and YC1, but the difference of positive detection frequency of the pathogen was quite large. While fields $TC1_c$ and $TC3_c$ had the same proportion of positive detection, the bacterial population ranges were much wider in $TC1_c$.

Spatial distribution and monitoring of *R. solanacearum* within a single field

Spatial distribution of *R. solanacearum* was determined in eight tomato production fields. Results clearly showed that the distribution of the pathogen was not even regardless of the presence or absence of the disease and the differences in the agro-ecosystems where the sampled fields were located (Fig. 3). In general, the distribution of the pathogen was more even, when the disease occurred in the field during sampling time (field $TC1_{a–c}$, $WL1_{a–e}$, and SF1). Field SS1 had no history of bacterial wilt and the pathogen could be detected only with BIO-PCR in a few plots. Distribution of the pathogen was monitored over seasons in fields WL1, TC1 and TC3. The pathogen was distributed rather uniformly in field WL1 throughout the 1-year monitoring period (Fig. 3a). Despite the presence of the disease in the field, *R. solanacearum* was detected in fewer plots in field TC1 over the monitoring periods compared with field WL1 (Fig. 3b). TC3 is a field with a history of tomato bacterial wilt but without the presence of tomato and the disease during the monitoring period. However a few spots harboured the pathogen persistently (Fig. 3c).

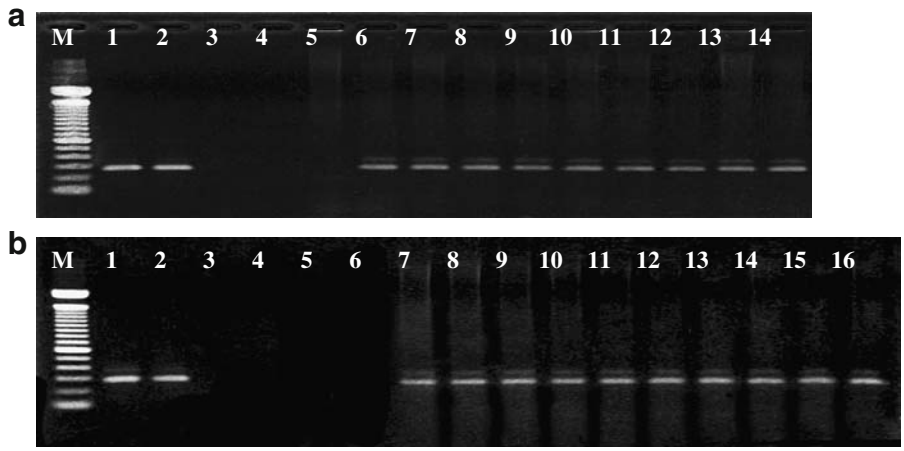

Fig. 2 Detection of *R. solanacearum* using a PCR method following enrichment of the target bacterium by incubating the bacterial suspensions (Gel *a*) and infested AVRDC soil suspensions (Gel *b*) in MSM-1 broth (1:10 dilution) for 3 days. The 282-bp PCR product amplified using primer pairs of AU759/AU760 was visible on the ethidium bromide-stained agarose gel. Gels *a* and *b*, *lane M*: 100-bp DNA ladder (Invitrogen, Carlsbad, CA, USA); *lanes 1 and 2*: positive control (pure suspension of Pss4, 10^8 CFU ml^{-1}); *lanes 3 and 4*: negative control (water); initial log population (CFU ml^{-1}) in samples of Gel *a* are 0.27 (*lanes 5 and 6*), 1.27 (*lanes 7 and 8*), 2.27 (*lanes 9 and 10*), 3.27 (*lanes 11 and 12*), 4.27 (*lanes 13 and 14*); initial log population (CFU g^{-1} of soil) in samples of Gel *b* are 0.23 (*lanes 5 and 6*), 1.23 (*lanes 7 and 8*), 2.23 (*lanes 9 and 10*), 3.23 (*lanes 11 and 12*), 4.23 (*lanes 13 and 14*), 5.23 (*lanes 15 and 16*)

Table 2 Detection of *R. solanacearum* in field soils collected from tomato production fields using selective MSM-1 medium and BIO-PCR method

Field	Population[a]	MSM-1	BIO-PCR
$WL1_a$	0.8–5.1	13/20[b]	20/20
$WL1_b$	0.9–4.3	15/20	19/20
$WL1_c$	0.8–3.7	13/20	17/20
$WL1_d$	0.8–4.3	17/20	16/20
$WL1_e$	1.0–5.1	17/20	18/20
SF1	1.9–5.8	17/20	17/20
SS1	n.d.	0/20	8/20
$TC1_a$	0.8–5.0	13/20	18/20
$TC1_b$	n.d.	0/20	11/20
$TC1_c$	0.8–5.2	2/20	12/20
TC2	0.8–4.6	4/20	11/20
$TC3_a$	2.0–4.5	4/20	10/20
$TC3_b$	n.d.	0/20	4/20
$TC3_c$	0.8–2.0	2/20	12/20
HS1	1.4–1.5	7/20	12/20
YC1	1.1–2.1	3/20	8/20

[a] Range of means of four duplicate determinations of *R. solanacearum* population (logarithm of CFU g^{-1} of dry soil) measured by dilution plating on selective MSM-1 medium. *n.d.* not detected

[b] Number of positive samples/number of tested soil samples

Detection of *R. solanacearum* from weed samples

Symptomless plants of 13 weed species were collected from tomato production fields WL1, SS1, and TC3 during 2002 and 2003. The BIO-PCR method resulted in a similar or higher positive detection frequency than MSM-1 from rhizosphere soil or root samples except in rhizosphere soil samples of *Ageratum conyzoides*, *Polygonum lapathifolium*, *Solanum nigrum*, and *Cyperus difformis* (Table 3). A higher positive detection frequency and population of *R. solanacearum* were found in rhizosphere soils compared to the roots of weeds. *R. solanacearum* could be detected by the BIO-PCR method from the rhizosphere soils in 11 out of 13 tested species with a positive detection frequency of 39.6%, compared with nine species by the MSM-1 medium with a positive detection frequency of 31.9%. *R. solanacearum* was detected by the BIO-PCR method from the roots of seven weed species compared to six species by the MSM-1 medium. BIO-PCR and MSM-1 plating methods had a 23.1% and 13.2% positive detection frequency from root samples, respectively. Root samples from *L. prostrata* had the highest positive detection frequency (85.7% and 57.1%).

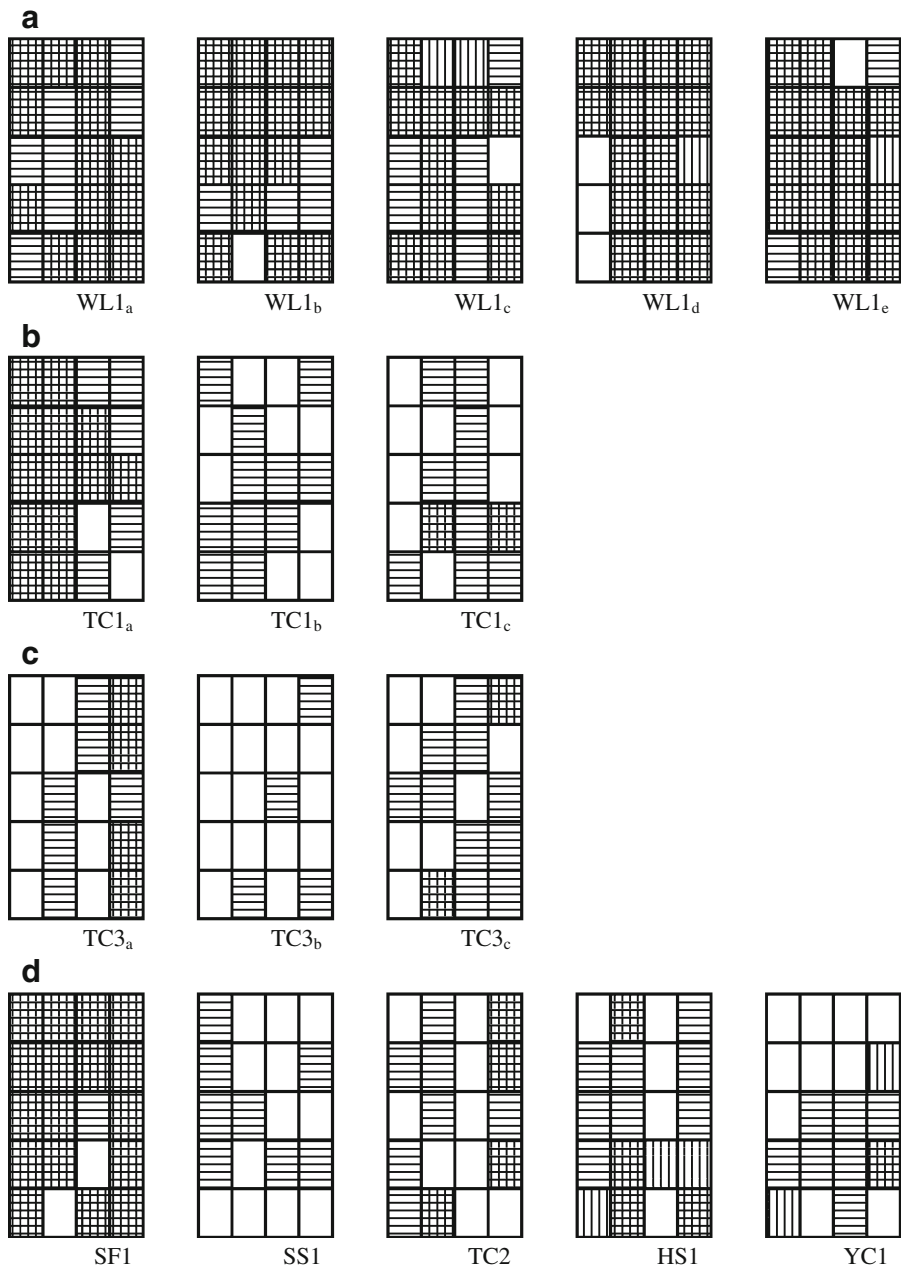

Fig. 3 Spatial distribution of *R. solanacearum* in tomato fields WL1 (**a**), TC1 (**b**), and TC3 (**c**) and in fields SF1, SS1, TC2, HS1, and YC1 (**d**). Each field was divided into 20 plots with equal size (5 m×10 m). Field soil samples were collected from each plot for detection by direct plating on MSM-1 and BIO-PCR. Detection results were marked with vertical (+ by MSM-1 plates), horizontal (+ by BIO-PCR), grid (+ by both methods), or blank (− by both methods)

Detection of *R. solanacearum* from water samples

Ralstonia solanacearum was detected in irrigation water, standing water in the field, or drainage water at the exit point of three tomato fields (Table 4). When monitoring *R. solanacearum* in water samples collected from field WL1 over several seasons, frequency of positive detection by BIO-PCR was the highest in water samples collected from drainage water (nine out of 20), followed by standing water sample (five out of 15), and the lowest from irrigation water (one out of 25). The pathogen populations detected by MSM-1 plating from water samples of field WL1 ranged from 10 to 20 CFU ml^{-1} in the irrigation water, 1.5×10^{3} to 1.1×10^{4} CFU ml^{-1} in the standing water, and 55 to 5.0×10^{2} CFU ml^{-1} in drainage water at the exit point. The pathogen was also positively detected in irrigation water and drainage water collected from field SF1 and SS1, but not TC3. The number of positive detections among the 85 water

Table 3 Detection of *R. solanacearum* in rhizosphere soil and weed roots collected from tomato fields using selective MSM-1 medium and BIO-PCR methods

Field	Weed family	Species	Population[a]		MSM-1		BIO-PCR	
			Rhizosphere Soil	Root	Rhizosphere Soil	Root	Rhizosphere Soil	Root
$WL1_{c-e}$	Amaranthaceae	*Amaranthus viridis*	6.0	2.6	1/2[b]	1/2	1/2	1/2
	Compositae	*Ageratum conyzoides*	2.5–5.1	1.8	4/5	1/5	2/5	1/5
		Ageratum houstonianum	4.6–6.2	6.0	3/11	1/11	4/11	4/11
		Eclipta prostrate	n.d.	n.d.	0/3	0/3	0/3	0/3
	Cyperaceae	*Cyperus iria*	5.1	n.d.	1/6	0/6	1/6	0/6
	Gramineae	*Eleusine indica*	4.2–6.4	2.4–4.9	4/9	3/9	5/9	4/9
	Polygonaceae	*Polygonum lapathifolium*	4.8–5.7	3.2–5.6	4/6	2/6	3/6	2/6
	Portulacaceae	*Portulaca oleracea*	n.d.	n.d.	0/3	0/3	0/3	0/3
	Solanaceae	*Solanum nigrum*	2.6–3.8	n.d.	2/6	0/6	1/6	2/6
SS1	Compositae	*Crassocephalum crepidioides*	n.d.	n.d.	0/3	0/3	2/3	0/3
		Eclipta prostrate	n.d.	n.d.	0/5	0/5	1/5	0/5
		Erigeron bonariensis	n.d.	n.d.	0/5	0/5	4/5	0/5
	Cyperaceae	*Cyperus difformis*	3.6	n.d.	1/4	0/4	0/4	0/4
	Solanaceae	*Solanum nigrum*	n.d.	n.d.	0/3	0/3	1/3	0/3
$TC3_a$	Compositae	*Ageratum houstonianum*	4.7	n.d.	1/2	0/2	1/2	0/2
	Cyperaceae	*Cyperus iria*	4.8	n.d.	1/9	0/9	2/9	0/9
	Gramineae	*Eleusine indica*	5.6	n.d.	1/2	0/2	1/2	1/2
	Onagraceae	*Ludwigia prostrate*	5.4–6.8	3.5–6.7	6/7	4/7	7/7	6/7

[a] Ranges of means of four duplicate determinations of *R. solanacearum* population (logarithm of CFU g^{-1} of rhizosphere soil or CFU g^{-1} of root tissue) measured by dilution plating on selective MSM-1 medium. *n.d.* not detected

[b] Number of positive samples/number of tested weed samples

samples was 18 (21.2%) and 27 (31.8%) by MSM-1 and BIO-PCR, respectively.

Discussion

Race 1 strains of *R. solanacearum* cause tomato bacterial wilt in the tropics and subtropics and can result in severe yield loss. However, the survival and dissemination of this soil-borne pathogen in agro-ecosystems remain poorly understood. In Taiwan, the disease is endemic on tomato and occurs under diverse agro-ecosystems. In this study, a BIO-PCR method was used to monitor the pathogen in field soils, weeds, and waters present in the main tomato production areas in Taiwan.

A new BIO-PCR method was developed and used to detect the pathogen in soil, weed, and water samples in this study. Although several PCR-based detection methods have been developed for *R. solanacearum*, this is the first report that demonstrates the effectiveness of a PCR-based method for detecting the pathogen from diverse kinds of field samples. The sensitivity of detecting *R. solanacearum* by prior enrichment with proper selective media observed in this study agrees with earlier reports (Elphinstone et al. 1996; Van der Wolf et al. 1998; Pradhanang et al. 2000; Priou et al. 2006). The enrichment step increased the sensitivity of PCR detection of the primer pair AU759/760 from 10^7 CFU g^{-1} of dry soil (without enrichment; unpublished data) to 17 CFU g^{-1} of dry soil (with enrichment) from artificially-infested soil samples. Such levels of sensitivity were better than other reported protocols with a similar approach. Ito et al. (1998) reported a sensitivity of ca. 100 CFU g^{-1} of soil using a protocol of enriching on PCCG plates followed by DNA extraction of the isolated colonies. And similar levels of sensitivity were achieved by the protocol reported by Pradhanang et al. (2000), in which the pathogen in soil was enriched in SMSA broth for 60 h followed by a PCR detection with specific primer OLI-1 and non-specific primer Y-2.

Detection sensitivity of the BIO-PCR method was compared with direct plating on MSM-1 plates. Overall,

Table 4 Detection of *R. solanacearum* in water samples collected from tomato production fields

Field	Entrance	Plot area	Exit
MSM-1			
$WL1_a$	0/5[a]	0/5	1/5
$WL1_b$	0/5	3/5	2/5
$WL1_c$	0/5	n.t.[b]	2/5
$WL1_d$	0/5	n.t.	n.t.
$WL1_e$	2/5	4/5	4/5
SF1	0/5	n.t.	0/5
SS1	0/5	n.t.	0/5
$TC3_c$	0/5	n.t.	n.t.
BIO-PCR			
$WL1_a$	0/5	0/5	1/5
$WL1_b$	0/5	3/5	3/5
$WL1_c$	0/5	n.t.	2/5
$WL1_d$	0/5	n.t.	n.t.
$WL1_e$	1/5	2/5	3/5
SF1	4/5	n.t.	2/5
SS1	2/5	n.t.	4/5
$TC3_c$	0/5	n.t.	n.t.

Sampling points included water entrance point beside the field, standing water accumulated in the field, and exit point of water from the field using selective MSM-1 medium and BIO-PCR method

[a] Number of positive detection/number of tested water samples

[b] *n.t.* not tested due to lack of water accumulation inside the field or at exit point during sampling

BIO-PCR showed a higher positive detection frequency from field soil samples (66.6% vs. 39.7%), weed rhizosphere soil (39.6% vs. 31.9%), weed roots (23.1% vs. 13.2%), and water samples (31.8% vs. 21.2%). However, there were cases when the pathogen could only be detected by plating on MSM-1 but not by the BIP-PCR method. The negative detection by BIO-PCR in these samples could be related to the failure to enrich targeted bacteria to a high enough population. This could be due to the presence of bacterial phages (Yamada et al. 2007), fast-growing microorganisms competing for nutrients, or antagonistic bacteria in the enrichment cultures (Pradhanang et al. 2000; Priou et al. 2006).

Early studies on the spatial distribution of *R. solanacearum* in the field were conducted based on the observed disease incidence (Smith 1943). This is the first report that demonstrates the uneven spatial distribution of *R. solanacearum* in tomato production fields by actual pathogen detection and a defined sampling method. The results indicate that the presence of the host crop and the disease contributed to the more uniform pathogen distribution in the field. Due to the uneven distribution of the pathogen in the field, sampling method is important in determining the presence of *R. solanacearum* in a specific plot. Pradhanang (1999) reported that mixing 20 randomly collected sub-samples in a 100 m^2 plot of potato fields could achieve the highest probability of a positive detection by SMSA plating. The sampling density of one sub-sample per 5 m^2 is similar to our one sub-sample per 5.5 m^2.

R. solanacearum was detected from rhizosphere soils and/or roots of symptomless weed plants present in tomato production fields with or without the presence of the disease, which implies that the pathogen could survive in association with weeds for a long period of time. This is in agreement with the results of Pradhanang and Momol (2001). Our results showed that the pathogen could be detected with higher frequency from rhizosphere soil than roots of the weed samples, which indicates that *R. solanacearum* survives more frequently in the rhizosphere rather than invading the roots of weeds. Thus weed rhizosphere could be a good target to determine the presence of the pathogen in a particular field. Our results are contrary of those of Granada and Sequeira (1983b). They monitored a race 1 strain on the root system of non-host crops such as bean and corn in the greenhouse and found the race 1 strain could be detected for a longer period in root rather than in rhizosphere soil, and considered root infection capacity is important for the pathogen to survive on non-host crops. Whether the confined space in pots resulted in poor survival of the pathogen in the rhizosphere or the pathogen interactions with bean and corn are different with weeds remains to be determined. The colonisation of the pathogen in the weed rhizosphere seems to be a random effect with no clear preference. For example, *Portulaca oleracea* has been reported to shelter the pathogen by Lin et al. (1994), but *R. solanacearum* was not isolated from this species in field WL1, where the pathogen density was high.

The transmission of *R. solanacearum* race 3, biovar 2, through water has been well documented (Farag et al. 1999; Wenneker et al. 1999). For race 1 strains, Hong et al. (2005) reported that *R. solanacearum*-contaminated irrigation pond water could be an important inoculum source for tomato bacterial

wilt in Florida. Our results suggest that the regular occurrence of high incidence of tomato bacterial wilt in Chunglin area could be due to the contaminated irrigation water. The pathogen was detected in irrigation water entering fields as well as drainage water exiting WL1, SF1, and SS1 in Chunglin. These fields share a common irrigation water source from a nearby water reservoir and the water is distributed through canals. Moreover, irrigation and drainage water travel along the same canals around the fields. It is not surprising to detect the pathogen in the irrigation water, which could have been contaminated from water drained from infested fields. This was supported by the positive detection of the pathogen in the standing water of a neighbouring paddy field, into which water drained from field WL1 (unpublished data). The regular practice of paddy rice rotation in Chunglin could suppress the pathogen population, but not complete eradication as reported by Michel et al. (1996). Therefore, possible contamination of irrigation water should be taken into consideration when designing local disease management programmes. Although the ground water source used to irrigate fields TC3 had a negative detection of the pathogen, more samples are needed to conclude the clearness of the water source and the role of water dissemination in the local endemic.

Monitoring *R. solanacearum* in field WL1, TC1 and TC3 provided important information on the over-season survival of the pathogen in tomato production fields. The consistently uniform distribution of the bacterium over almost one year of monitoring in field WL1 was attributed to the presence of tomato crops and the disease throughout almost the entire monitoring period, as well as the use of contaminated irrigation water and the presence of weeds harbouring the pathogen. Rotation with one crop of cucumber or yardlong bean did not eradicate *R. solanacearum* under the conditions of field TC1 and TC3. Similar results have been reported by Arthy and Akiew (1999). The low pathogen incidence in field TC1, particularly in October and November, could be due to the low rainfall during the season and less frequent irrigation practiced by the local farmers. After ploughing, the pathogen could only be detected in four scatter plots in field TC3. However, after about one month, the pathogen re-appeared in more plots. Such a resurgence of the pathogen could be due to the growth of more weeds observed in the field.

Using the BIO-PCR method, this study demonstrated the uneven spatial distribution of race 1 strains of *R. solanacearum* in tomato production fields, the importance of contaminated water in local disease epiphytotics, and the common presence of the pathogen in weed rhizosphere over the diverse agro-ecosystems in Taiwan. The BIO-PCR method developed in this study can be used for future ecological and epidemiological studies of the pathogen. Furthermore, the BIO-PCR detection results could be applied to estimate the potential risk of bacterial wilt in a particular field after the relationship with inoculum potential was investigated.

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