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Simultaneous PCR Detection of the Two Major Bacterial Pathogens of Geranium

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With 4 figures

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Abstract

Xanthomonas campestris pv. *pelargonii* (*Xcp*) and *Ralstonia solanacearum* (*Rs*) are the two most important bacterial pathogens of commercially cultivated geraniums (*Pelargonium* spp.), both causing bacterial wilt and leaf spot. Asymptomatic infections are important reservoirs of infections in commercial growing facilities. Our objective was to design a multiplex PCR (Polymerase Chain Reaction) assay to detect infection by either or both of these pathogens. We used a previously characterized PCR primer pair for *Xcp* that amplifies a region of 200 bp. In addition, we designed a new primer pair specific for *Rs* that amplifies a region of 822 bp. With these two primer pairs, we could detect either or both pathogens. As geranium tissue extracts frequently contain inhibitors of the PCR process, a negative PCR could result from either an accurate indication that the plant was pathogen-free or from a false negative assay. We therefore designed 'amplification competence' primers, targeting a portion of the geranium 18 s rRNA gene, and generating a 494-bp amplification product that confirms amplification competence and validates a negative assay result. Thus, the triple primer pair multiplex PCR screens for the two most important bacterial pathogens of geraniums simultaneously confirms amplification competence for each geranium sample.

Introduction

Xanthomonas campestris pv. *pelargonii* (*Xcp*) and *Ralstonia* (*Pseudomonas*) *solanacearum* (*Rs*) are two types of bacteria that cause widespread disease in geraniums (*Pelargonium* spp.; Daughtrey and Wick, 1993; Jones, 1993; Nameth et al., 1999). In both cases, plants may be infected with the bacteria and not show symptoms when grown under conditions of low temperature and humidity (Daughtrey and Wick, 1993; Jones, 1993). Polymerase chain reaction (PCR) is a

sensitive and rapid method of amplifying the DNA of bacterial pathogens, allowing targeting of species-specific sequences in their genome. Polymerase chain reaction protocols have been designed to detect the presence of *Xcp* and *Rs* in geraniums (Elphinstone et al., 1996; Sulzinski et al., 1996; Ito et al., 1998; Sulzinski et al., 1998; Weller et al., 2000).

Using the published protocols to detect the two pathogens by PCR would require two separate and disparate PCR amplifications, due to similarities in the PCR product size and in the vastly different annealing temperatures needed for primer extension. The ability to amplify and detect both pathogens simultaneously would increase the usefulness of the PCR technique by decreasing the amount of reagents and labor used by one half. With PCR, one can utilize multiple primer sets and detect multiple targets, as long as the amplified DNA species are of different length and the oligonucleotide primer annealing conditions are similar. The multiple DNAs will separate upon agarose gel electrophoresis and can be distinguished by their size. The standard PCR procedure for detection of *Xcp* generates a 200 base-pair (bp) DNA product (Sulzinski et al., 1996). We developed a new set of primers specific for *Rs* that, after PCR, generated an amplified DNA of 822 bp, which was easily distinguished from the 200 bp product. As the published oligonucleotide primers for *Xcp* need to anneal to template DNA at 72°C in order to be specific for this bacterium, the available oligonucleotide primers for *Rs* could not be used as their annealing temperatures were substantially below this temperature. We therefore had to design new oligonucleotide primers specific for *Rs* that annealed to template DNA at 72°C. Another problem common in the PCR detection of plant pathogens is the presence of amplification inhibitors in crude extracts (Sulzinski et al., 1997). This makes it impossible to determine if a negative result is accurate, or the result of inhibitors of the amplification

process. As part of our diagnostic test for the two bacterial pathogens, we included primers for a plant nucleic acid sequence that will always be present in geranium extracts. The presence of these primers will always result in amplified DNA if the plant extract is free of inhibitors. The existence of this amplified DNA demonstrates the amplification competence of the extract and indicates that a negative result for the bacterial pathogens most likely reflects the absence of those pathogens. We therefore designed an additional set of PCR primers that would amplify a 494-bp sequence of the geranium 18 s rRNA gene. This DNA band should always be present in any PCR of plant tissue. Its presence provides assurances that PCR was not inhibited and establishes amplification competence of all geranium samples analyzed by PCR.

Materials and Methods

PCR primers

The *Rs* primer pair (RS3 and RS4) was designed to amplify a portion of the exo-poly- α -D-galacturonosidase gene (PehB; Huang and Allen, 1997). The DNA sequence of PehB (GenBank accession no. U60106) was obtained from GenBank (<http://www.ncbi.nlm.nih.gov/GenBank/>, National Center for Biotechnology Information) and cut and pasted into an internet accessible PCR primer design program known as GENE FISHER (Giegerich, University of Bielefeld, Germany). The possible primer choices were then tested against other known DNA sequences using the National Center for Biotechnology Information (BLAST). Primers showing homology with many other known sequences were discarded. The resulting primer pair is known as RS3/RS4 and targets the PehB gene of *Rs* (Huang and Allen, 1997).

The geranium primer pair (DG1/DG2) were designed to target a portion of geranium DNA that codes for 18 s rRNA (GenBank accession no. U42541). The primer pair was designed using GenBank, Gene Fisher and BLAST as described above. The primer pair that amplifies a portion of the DNA of *Xcp* (*XcpM1*/*XcpM2*) has previously been described by Sulzinski et al. (1996). The nucleotide sequences of all three primer pairs are listed in Table 1.

PCR amplification

Amplification was recorded in a volume of 25 μ l that contained the appropriate primers (2.0 μ M), dNTPs

Table 1
Oligonucleotide primers used for amplification

¹ XcpM1	(5'-ACGCGCTACCAAAAGGCAAAGAG-3')
XcpM2	(5'-GATCTGCGGTTGTCTGAAGATTGG-3')
² DG1	(5'-TCGACGCGGGCTTTGCCCGTTG-3')
DG2	(5'-GGAGCGCATCGCCGCGAGAAGG-3')
³ RS3	(5'-AGCACGACCGGTGCGACCTGCT-3')
RS4	(5'-CACCCGCGCGTGTCTCGTAG-3')

¹200 bp amplicon targeted to *Xc*. ²494 bp amplicon targeted to geranium rRNA gene. ³822 bp amplicon targeted to *Rs*.

(80 μ M each), MgCl₂ (1.5 mM), 1.6X MasterAmp PCR Enhancer (Epicentre Technologies, Madison, WI, USA), *rTth* reverse transcriptase buffer (2 mM Tris-HCl, pH 8.3, 18 mM KCl), chelating buffer [4% (v/v) glycerol, 8 mM Tris-HCl, pH 8.3, 80 mM KCl, 0.6 mM EGTA, 0.04% (v/v) Tween-20], 0.5 units of *Tth* DNA polymerase (Epicentre Technologies), and template. Reaction tubes, which were kept on ice, received 20 μ l of a 1.25X chilled master mix and 5.0 μ l of the template. The PCR was performed with a 'hot start' in which sample tubes were moved directly from ice to the 85°C block in the thermal cycler [Perkin Elmer GeneAmp PCR system 2400 (Perkin Elmer Corp., Norwalk, CT, USA)]. The amplification program consisted of 85°C for 1 min, 94°C for 3 min and 15 s, followed by 40 cycles of 94°C for 30 s and 72°C for 30 s. Amplification products were examined by agarose gel electrophoresis [2% (w/v) agarose] and ethidium bromide staining (Sulzinski et al., 1995). When all three primer pairs were utilized together, the dNTP concentration was raised to 160 μ M while the MgCl₂ concentration was raised to 2 mM.

Bacterial isolates, culture and nucleic acid extraction

Bacteria were obtained from the sources shown in Table 2. Isolates were cultured in yeast dextrose calcium carbonate (YDC) broth (10 g l⁻¹ yeast extract, 20 g l⁻¹ dextrose, and 10 g l⁻¹ CaCO₃) by shaking at 200 r.p.m. at 28°C for 2 days. At this time, serial dilutions demonstrated that the viable bacteria concentration was usually $\sim 1 \times 10^9$ CFU ml⁻¹ broth. All nucleic acid extraction was performed according to standard methods (Sulzinski et al., 1995).

Table 2
Bacterial Isolates and their sources

Isolate	Isolate Number	Description
Rs2	22054-99 ^a	<i>Rs</i>
Rs4	54054-99 ^a	<i>Rs</i>
Rs5	62068-99 ^a	<i>Rs</i>
Rs6	62069-99 ^a	<i>Rs</i>
Rs7	62070-99 ^a	<i>Rs</i>
Rs8	62071-99 ^a	<i>Rs</i>
Rs9	62075-99 ^a	<i>Rs</i>
Rs11	62077-99 ^a	<i>Rs</i>
Rs12	62079-99 ^a	<i>Rs</i>
Rs13	62080-99 ^a	<i>Rs</i>
Rs14	62081-99 ^a	<i>Rs</i>
Rs15	62083-99 ^a	<i>Rs</i>
Rs16	62084-99 ^a	<i>Rs</i>
Rs17	80869-95 ^a	<i>Rs</i>
Rs18	81849-99 ^a	<i>Rs</i>
Rs19	81862-99 ^a	<i>Rs</i>
2	Ohio ^b	<i>Xcp</i>
3	80266 ^a	<i>Xcp</i>
23	54166-91 ^a	<i>Xcp</i>
24	42101-91 ^a	<i>Xcp</i>
G2	32179-88 ^c	<i>C. fascians</i>
G4	082-39 ^c	<i>P. cichorii</i>

^aSource: Seong Hwan Kim, Pennsylvania Department of Agriculture, Harrisburg, PA, USA. ^bSource: Penn State University Plant Disease Clinic, University Park, PA, USA. ^cSource: Jeff Jones, University of Florida, Gainesville, FL, USA.

Plants

Geraniums (*Pelargonium X hortorum*) 'Red Elite' (Park Seed Co., Greenwood, SC, USA) were grown from seed under greenhouse conditions, and utilized at 5–6 weeks of age. Other geranium varieties were obtained as cuttings (Oglevee Ltd., Connellsville, PA, USA). These included *P. domesticum* cv. Maiden Orange, *P. graveolens*, *P. peltatum* cv. White Nicole, *P. peltatum* cv. Nicole, and *Pelargonium* sp. cvs. Jessica, Elizabeth, Frank Headly, Wilhelm Langeth, Evening Glow, Northstar, and Red Satisfaction.

Plant Inoculation

Inoculation was performed by removing the leaf from a petiole with a sterile razor, and by adding 5 μ l of a bacterial suspension ($\sim 1 \times 10^9$ CFU/ml) to the freshly cut surface. Within 10 min, the petiole imbibed the inoculum. The mock inoculated plants received 5 μ l sterile YDC broth on the freshly cut petiole surface.

Preparation of plant samples

The PCR testing of infected plant material was initiated as soon as the plants developed the first visible symptoms of infection such as wilting and water soaked leaves.

A 1 cm length of petiole from infected or non-infected plants was macerated with a hand-held roller ball homogenizer and universal extraction bags (Bioreba Inc., Carrboro, NC, USA) in 7.5 ml of Agdia general extraction buffer with Tween (Agdia Inc., Elkhart, IN, USA). Five microlitres of the macerate were added to 20 μ l of GeneReleaserTM (BioVentures, Inc., Murfreesboro, TN, USA) in 500 μ l thin-walled microcentrifuge tubes, vortexed for 5 s and then heated for 6 min at high power (900 W) in a Hotpoint model RE144 0001 (Hotpoint, Louisville, KY, USA) microwave oven. Heated tubes were centrifuged at full speed for 30 s in an Eppendorf model 5415C microcentrifuge (Eppendorf Scientific Inc., Westbury, NY, USA). The upper 5 μ l of the supernatant was used as template for PCR amplification.

In those experiments where plants were infected with bacteria, the number of bacteria in the infected plant was determined by viability counting. In this case, sterile YDC broth was used as the homogenization buffer. The number of viable bacteria was determined by serial dilution and plating on YDC agar plates.

Results

Specificity of *R. solanacearum* primers

To determine the specificity of the RS3/RS4 primer pair for *Rs*, the primers were used for performing amplification of total nucleic acid of laboratory isolates of bacterial pathogens *Xcp*, *Pseudomonas cichorii* and *Rhodococcus (Corynebacterium) fascians*. Nucleic acid templates were either from crude bacterial lysates or from partially purified bacterial nucleic acids containing, in either case, 10–200 ng DNA in 5 μ l.

Sixteen *Rs* isolates were tested and each produced the predicted 822 bp amplified product which can be observed in Fig. 1, lanes 2–17. [In addition to the 822 bp product, another lower molecular weight product (~ 700 bp) was consistently observed; the identity and significance of this minor product is unknown.] The lanes (18–20) containing *Xcp*, *P. cichorii*, and *C. fascians*, respectively, show no amplification product. The PCR assay for *Rs* is thus both robust and specific, as it detected all *Rs* strains tested and provided a negative result for the other three tested bacterial pathogens of geraniums (*Xcp*, *P. cichorii*, and *C. fascians*).

Detection of amplification competence in geraniums

The DG1/DG2 primer pair was developed to consistently produce a 494-bp amplification product from geranium plant extracts under the same high stringency conditions utilized for the primer pair XcpM1/XcpM2. It was initially tested in *Pelargonium X hortorum* 'Red Elite.' Extracts from 12 genetically diverse geranium varieties were obtained and the primer pair was used to amplify the target DNA. In all cases (Fig. 2, lanes 2–13), the expected 494 bp amplification product was produced, indicating the general robustness of this primer

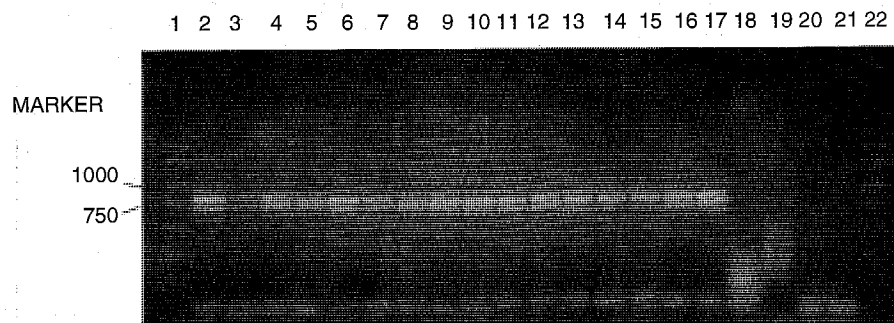


Fig. 1 Gel electrophoretic analysis of the PCR products obtained from amplifying nucleic acid from purified laboratory isolates of *Rs* and other bacterial geranium pathogens using primer pair RS3/RS4 (targeting *Rs*). The photograph depicts ethidium bromide stained (2% w/v) agarose Tris-acetate-EDTA (TAE) gel. Lanes 1 and 22, DNA molecular weight markers; lanes 2 through 17, *Rs* isolates [lane 2, isolate *Rs*2; lane 3, isolate *Rs*4; lane 4, isolate *Rs*5; lane 5, isolate *Rs*6; lane 6, isolate *Rs*7; lane 7, isolate *Rs*8; lane 8, isolate *Rs*9; lane 9, isolate *Rs*11; lane 10, isolate *Rs*12; lane 11, isolate *Rs*13; lane 12, isolate *Rs*14; lane 13, isolate *Rs*15; lane 14, isolate *Rs*16; lane 15, isolate *Rs*17; lane 16, isolate *Rs*18; lane 17, isolate *Rs*19]; lane 18, *Xcp* (isolate 3); lane 19, *P. cichorii* (isolate G4); lane 20, *C. fascians* (isolate G2); lane 21, amplification negative control (water substituted for nucleic acid template)

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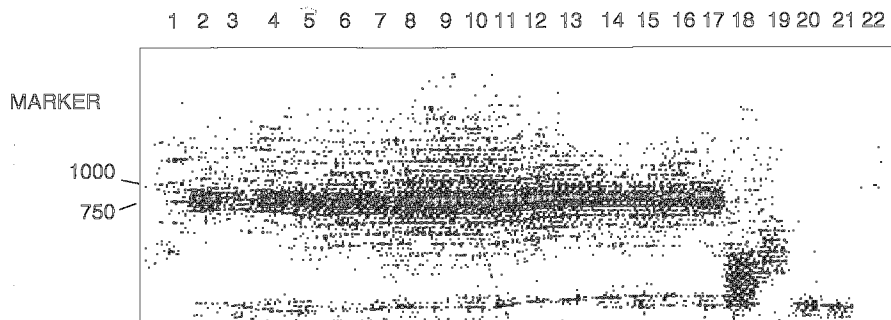


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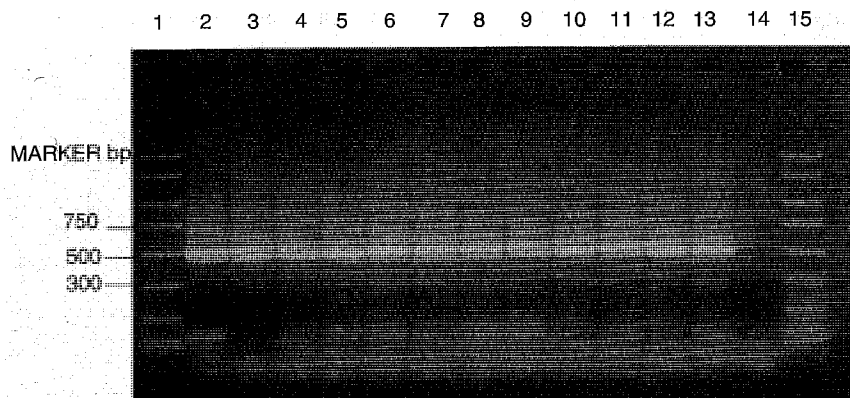


Fig. 2 Gel electrophoretic analysis of the PCR products obtained from amplifying extracts of 12 varieties of healthy geraniums with primer pair DG1/DG2 (targeting gene of geranium rRNA). The photograph depicts an ethidium bromide stained agarose TAE gel. Lanes 1 and 15: DNA molecular weight markers; lane 2, *Pelargonium X hortorum* cv. Red Elite; lane 3, *P. domesticum* cv. Maiden Orange; lane 4, *P. graveolens*; lane 5, *P. peltatum* cv. White Nicole; lane 6, *P. peltatum* cv. Nicole; lane 7, *Pelargonium* sp. cv. Jessica; lane 8, *Pelargonium* sp. cv. Elizabeth; lane 9, *Pelargonium* sp. cv. Frank Headly; lane 10, *Pelargonium* sp. cv. Wilhelm Langeth; lane 11, *Pelargonium* sp. cv. Evening Glow; lane 12, *Pelargonium* sp. cv. Northstar; lane 13, *Pelargonium* sp. cv. Red Satisfaction; lane 14, amplification negative control (water substituted for nucleic acid template)

pair for demonstrating the amplification competence of geranium extracts.

Multiplex PCR amplification: simultaneous detection of *R. solanacearum* infection and confirmation of amplification competence in geraniums

After establishing the feasibility of using primer pairs RS3/RS4 and DG1/DG2 separately to detect *Rs* and amplifiable host DNA respectively, we attempted simultaneous detection in a two primer pair multiplex PCR. The two primer pairs worked effectively independently of each other (Fig. 3). In geraniums that had been artificially inoculated with any of the 12 different isolates of *Rs*, both the 822 bp and 494 bp bands were detected (Fig. 3, lanes 2–13). Extracts from a geranium artificially inoculated with *Xcp* (lane 14) and a mock-inoculated geranium (lane 15) each generated only the

494 bp product targeted to the geranium rRNA gene. These results demonstrate that these two newly-designed primer pairs function simultaneously without any major interference of one primer pair on another.

The infected plants had approximately 7.5×10^8 CFU *Rs* per 1 cm length of petiole, as determined by plating out samples on YDC agar for viability counting.

Triple primer pair multiplex PCR

Amplification with three primer pairs was attempted to demonstrate the simultaneous detection of three discrete targets: *Rs*, *Xcp* and geranium rRNA gene using primers RS3/RS4, XcpM1/XcpM2 and DG1/DG2, respectively. Each of the three primer pairs detected its target with no obvious interference from the other primers. Extracts from plants inoculated with *Rs* were combined with extracts from plants inoculated with *Xcp*. The solutions,

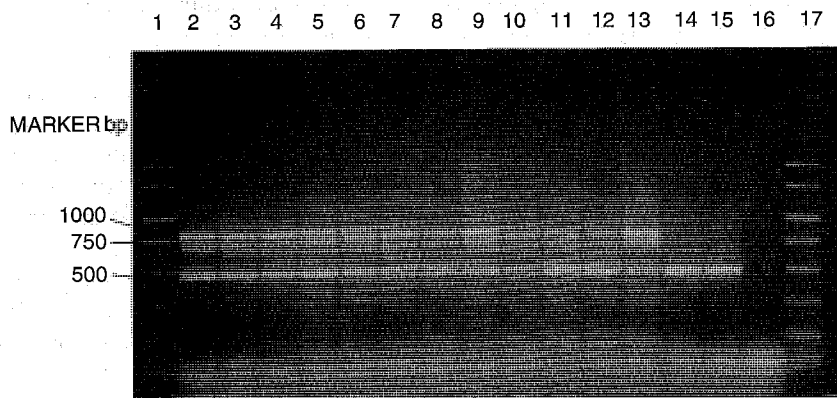


Fig. 3 Gel electrophoretic analysis of the PCR products obtained from amplifying extracts of plants inoculated individually with 12 different isolates of *Rs*. Simultaneous amplification was performed with primer pairs DG1/DG2 (targeting gene of geranium rRNA) and RS3/RS4 (targeting *Rs*). The photograph depicts an ethidium bromide stained (2% w/v) agarose TAE gel. Lanes 1 and 17, DNA molecular weight markers; lanes 2–13: extracts of geraniums infected with one of 12 different isolates of *Rs*. [lane 2, isolate Rs2; lane 3, isolate Rs4; lane 4, isolate Rs5; lane 5, isolate Rs6; lane 6, isolate Rs7; lane 7, isolate Rs8; lane 8, isolate Rs9; lane 9, isolate Rs12; lane 10, isolate Rs13; lane 11, isolate Rs15; lane 12, isolate Rs16; lane 13, isolate Rs19]; lane 14, extract from geranium inoculated with *Xcp* isolate 2; lane 15, extract from mock-inoculated healthy geranium; lane 16, amplification negative control (water substituted for nucleic acid template)

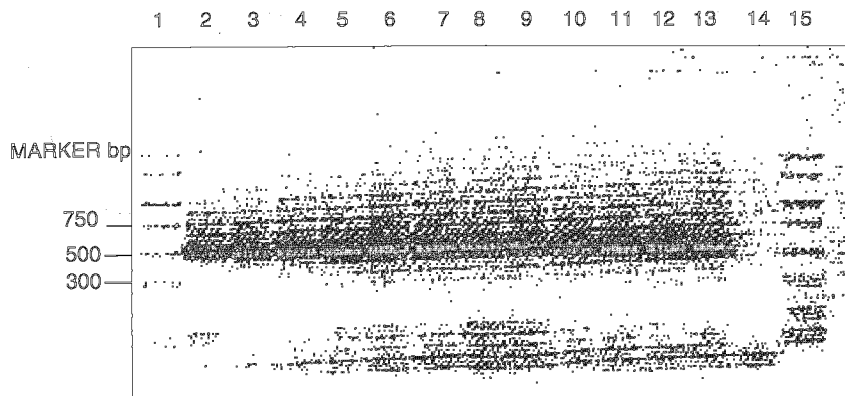


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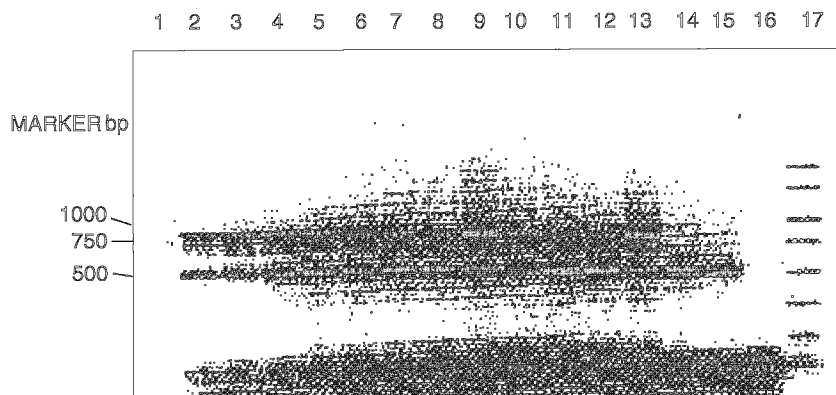


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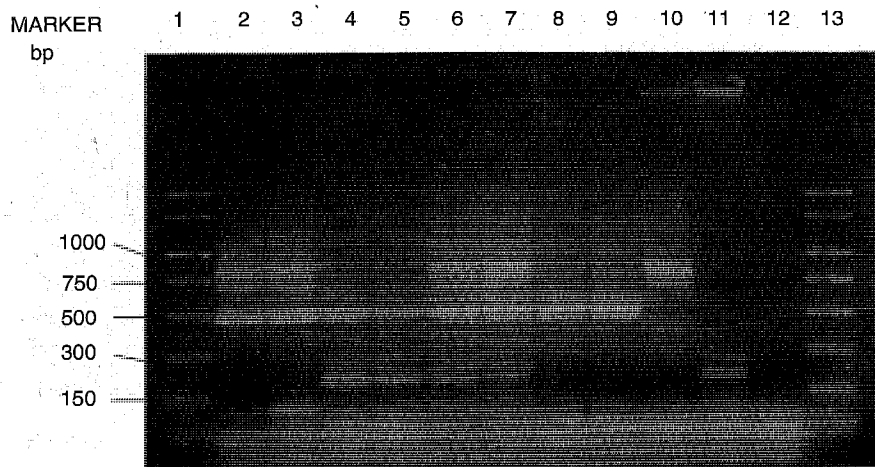


Fig. 4 Gel electrophoretic analysis of the PCR products obtained from amplifying extracts of plants inoculated individually with either or both *Rs* and *Xcp*. Simultaneous amplification was performed with primer pairs DG1/DG2 (targeting gene of geranium rRNA), RS3/RS4 (targeting *Rs*), and *Xcp*M1/*Xcp*M2 (targeting *Xcp*). The photograph depicts an ethidium bromide stained (2% w/v) agarose TAE gel. Lanes 1 and 13, DNA molecular weight markers; lanes 2 and 3, extracts from geraniums inoculated with two different isolates of *Rs* (Rs13 and Rs8); lanes 4 and 5, extracts from geraniums inoculated with two different isolates of *Xcp* (isolates 3 and 24); lane 6, extract from geranium inoculated with *Rs* (isolate Rs13) combined with extract from geranium inoculated with *Xcp* (isolate 3); lane 7, extract from geranium inoculated with *Rs* (isolate Rs8) extract from geranium inoculated with *Xcp* (isolate 24); lanes 8 and 9, extracts from uninoculated 'Red Elite' geraniums; lane 10, nucleic acid from laboratory isolate 8 of *Rs*; lane 11, nucleic acid from laboratory isolate of isolate 24 of *Xcp*; lane 12, amplification negative control (water substituted for nucleic acid template)

which mimicked a doubly infected plant, generated three amplification products: the 200 bp product specific for *Xcp*; the 822 bp product specific for *Rs* and the 494 bp geranium control DNA (Fig. 4, lanes 6 and 7). Plants infected with only *Rs* generated the 822 bp and 494 bp amplicons, but not the 200 bp amplicon (Fig. 4, lanes 2 and 3). Plants infected with only *Xcp* generated the 200 bp and 494 bp amplicons, but not the 822 bp amplicon (Fig. 4, lanes 4 and 5). Mock-inoculated plants generated only the 494 bp amplicon (Fig. 4, lanes 8 and 9). These data demonstrate the capacity of each primer to recognize and amplify its target without significant interference from the other two primer pairs under the conditions tested.

The plants infected with *Rs* had roughly 5×10^8 CFU *Rs* per 1 cm length of petiole, as determined by plating out samples on YDC agar for viability counting. The plants infected with *Xcp* had about 1×10^9 *Xcp* per 1 cm length of petiole.

Discussion

As both *Rs* and *Xcp* are serious bacterial pathogens of geraniums it would be useful to have an assay that detected both of these pathogens simultaneously. Our lab had previously developed a specific PCR-based assay for *Xcp* with a 72°C annealing temperature, which results in an amplification product of 200 bp (Sulzinski et al., 1996). Previously published PCR procedures for detection of *Rs* utilize annealing temperatures in the 55° to 60°C range (Elphinstone et al., 1996; Ito et al., 1998). These procedures could not be used in a multiplex system because the primer pair for *Xcp* requires an annealing temperature of 72°C to maintain pathovar specificity. We developed a PCR-based assay for *Rs* that produced an 822-bp amplification product at an

annealing temperature of 72°C, that is easily distinguishable from the product of 200 bp for *Xcp*.

Occasionally, geranium tissue extracts contain inhibitors of the amplification process. The use of Gene-Releaser™ with the tissue extracts usually eliminates the inhibitors (Sulzinski et al., 1998), but there is still the possibility that a plant infected with one or both of the bacterial pathogens might not generate a 200- or 822-bp amplification product because of one or more amplification inhibitors present even in the treated geranium extract. In order to increase our confidence in the accuracy of a negative result, we developed a third primer pair which amplifies a 494-bp region of geranium 18 s rRNA gene. As DNA from geranium will be present in every geranium extract, all geranium samples should demonstrate an amplification product of this size. In our PCR-based assay for the two bacterial pathogens, if a plant does not have a 200 or 822-bp amplification product while it continues to exhibit the 494 bp product, then there is more confidence in this negative result being obtained. On the other hand, if a given geranium sample fails to generate the 494 bp product, the result should be considered inconclusive, and a retest is indicated.

The development of the multiplex assay required extensive optimization. The reliable appearance of all three bands only occurred when the dNTP concentration was 160 μM and the magnesium concentration was 2.0 mM during amplification. In addition, the appearance of all three amplification bands required the use of a hot start PCR procedure. This procedure, previously described in Materials and Methods, prevented the formation of spurious amplification products.

The technique described herein will allow for the simultaneous detection and differentiation of *Rs* and

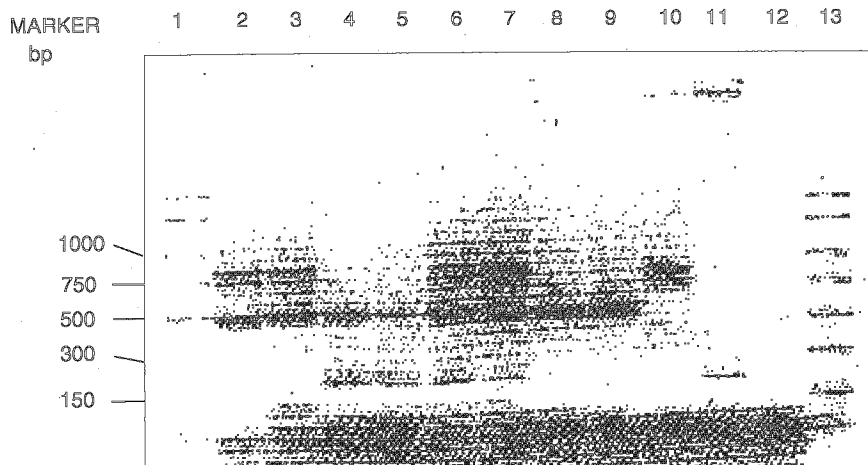


Fig. 4 Gel electrophoretic analysis of the PCR products obtained from amplifying extracts of plants inoculated individually with either or both *Rs* and *Xcp*. Simultaneous amplification was performed with primer pairs DG1/DG2 (targeting gene of geranium rRNA), RS3/RS4 (targeting *Rs*), and *Xcp*M1/*Xcp*M2 (targeting *Xcp*). The photograph depicts an ethidium bromide stained (2% w/v) agarose TAE gel. Lanes 1 and 13, DNA molecular weight markers; lanes 2 and 3, extracts from geraniums inoculated with two different isolates of *Xcp* (isolates 3 and 24); lanes 4 and 5, extracts from geraniums inoculated with two different isolates of *Xcp* (isolates 3 and 24); lane 6, extract from geranium inoculated with *Rs* (isolate Rs13) combined with extract from geranium inoculated with *Xcp* (isolate 3); lane 7, extract from geranium inoculated with *Rs* (isolate Rs8) extract from geranium inoculated with *Xcp* (isolate 24); lanes 8 and 9, extracts from uninoculated 'Red Elite' geraniums; lane 10, nucleic acid from laboratory isolate 8 of *Rs*; lane 11, nucleic acid from laboratory isolate of isolate 24 of *Xcp*; lane 12, amplification negative control (water substituted for nucleic acid template)

which mimicked a doubly infected plant, generated three amplification products: the 200 bp product specific for *Xcp*; the 822 bp product specific for *Rs* and the 494 bp geranium control DNA (Fig. 4, lanes 6 and 7). Plants infected with only *Rs* generated the 822 bp and 494 bp amplicons, but not the 200 bp amplicon (Fig. 4, lanes 2 and 3). Plants infected with only *Xcp* generated the 200 bp and 494 bp amplicons, but not the 822 bp amplicon (Fig. 4, lanes 4 and 5). Mock-inoculated plants generated only the 494 bp amplicon (Fig. 4, lanes 8 and 9). These data demonstrate the capacity of each primer to recognize and amplify its target without significant interference from the other two primer pairs under the conditions tested.

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The technique described herein will allow for the simultaneous detection and differentiation of *Rs* and

Xcp in symptomatic geraniums, whilst confirming the amplification competence of negative samples. Current work is underway to adapt this amplification scheme to a detection format that can handle many samples in a rapid throughput design.

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