Quantitative Detection of *Clavibacter michiganensis* subsp. *sepedonicus* by Competitive Polymerase Chain Reaction

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ABSTRACT

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A competitive polymerase chain reaction (PCR) method was developed for detection and quantification of *Clavibacter michiganensis* subsp. *sepedonicus* DNA in infected plant tissues. An internal standard DNA template that served as a control for all PCR tests was generated by amplification of *Arabidopsis* genomic DNA under low annealing temperatures with primers specific for *C. michiganensis* subsp. *sepedonicus*. The 450-bp product amplified from the internal standard DNA template was distinct from the 250-bp product characteristic of *C. michiganensis* subsp. *sepedonicus*. The ratio of the PCR products amplified in the presence of a constant amount of internal standard DNA template increased linearly and competitively with increased amounts of *C. michiganensis* subsp. *sepedonicus* DNA. Cell numbers estimated by immunofluorescence antibody staining (IFAS) were consistent with PCR product ratios obtained from cell cultures and inoculated potato plantlets. Compared to IFAS, competitive PCR was about 10-fold more sensitive and detected as few as 100 cells. Competitive PCR should greatly reduce misdiagnosis from false negatives and provide a quantitative approach for estimating pathogen population sizes in samples.

Additional keywords: bacterial ring rot, *Solanum tuberosum*.

Bacterial ring rot, caused by *Clavibacter michiganensis* subsp. *sepedonicus*, is a significant concern wherever potatoes are grown (5). The potential for great economic losses from bacterial ring rot, such as those suffered in the 1930s, has warranted a zero tolerance for this disease in all seed certification programs in the United States (16) and Canada. Economic losses from bacterial ring rot are incurred through rejection of crops for seed and by yield and storage losses in commercial potato production. Management of bacterial ring rot requires compliance to strict cultural and sanitation practices. One of these practices is the planting of seed pieces from certified, disease-free tubers grown in limited generation programs. Availability and use of disease-free potato seed, however, have not eradicated bacterial ring rot in the United States. Although it is widely accepted that economic losses would be much greater if it were not for the success of current seed-certification programs, considerable emphasis has been placed on improved detection of *C. michiganensis* subsp. *sepedonicus* in the absence or presence of disease (1,7,12,15,17,22,29,31). Rejection of seed for certification in the United States currently relies on visual detection of foliar symptoms and subsequent laboratory detection of the pathogen (34). Accurate diagnosis of bacterial ring rot clearly requires sensitive and reliable detection techniques that preferably give quick results and are conducive to widespread use.

Several techniques are available and have been used as diagnostics for bacterial ring rot (14). Gram staining combined with bioassays of expressed sap in indicator species, such as eggplant, is a simple diagnostic technique suitable for small sample sizes of plants with severe foliar or tuber symptoms (2,23). A relatively high detection limit and potential for false positives from other gram-positive endophytic bacteria are two reasons other diagnostics have been developed. Growth on NCP-88 semiselective medium is diagnostic of *C. michiganensis* subsp. *sepedonicus* in samples containing greater than 10^4 to 10^5 CFU/g of plant tissue (13). Pathogenicity tests and identification of colonies as *C. michiganensis* subsp. *sepedonicus* are compelling diagnostics, if time is unlimited. Immunodetection techniques, such as immunofluorescent antibody staining (IFAS) and enzyme-linked immunosorbent assay (ELISA), also are available (8,9,10). Both immunodetection methods are adaptable to processing large sample sizes, although ELISA is easier than IFAS for this purpose (11,17). The sensitivity of IFAS is equal to (17) or higher than the sensitivity of ELISA (11). Specificity of either immunoassay is defined by the antibody used; monoclonal antibodies provide greater specificity than polyclonals and diminish the potential of false positives from cross-reactions with closely related coryneform bacteria (4,8). Specificity of ELISA and IFAS is similar (17). Inherent antigenic variations among strains of *C. michiganensis* subsp. *sepedonicus*, such as those conferred by mucoid and nonmucoid types and cross-reactivity, can be abated by judicious selection of antibodies (1,3,4,6,7,17). Diagnostic techniques based on DNA hybridization with nucleic acid probes specific for *C. michiganensis* subsp. *sepedonicus* also are available and have detection limits similar to ELISA (21,24,25,35).

Specificity, sensitivity, and ease are three reasons the polymerase chain reaction (PCR) has generated considerable interest as an additional method for diagnosis of *C. michiganensis* subsp. *sepedonicus*. PCR is a rapid and sensitive technique used extensively for diagnosis of animal and plant pathogens (18,19,26,27,28,32). PCR has been used to amplify genomic and plasmid DNA sequences of *C. michiganensis* subsp. *sepedonicus* (15,31,33). Pri-

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mers specific for sequences on a small cryptic plasmid (pCS1), which is autonomous or integrated in nearly all strains of *C. michiganensis* subsp. *sepedonicus* (15,21,25,33), served in the initial development of PCR-based detection of *C. michiganensis* subsp. *sepedonicus*. For example, primers CMS-6 and CMS-7 amplify a 258-bp product from pCS1, and these primers have been used to demonstrate that the pathogen can be detected in infected potato plants (33). Other PCR primers and strategies are being explored to achieve greater specificity (15,31). To our knowledge, none of the PCR procedures for *C. michiganensis* subsp. *sepedonicus* are quantitative in nature; the presence of an amplified product indicates only that the sample contains a detectable amount of *C. michiganensis* subsp. *sepedonicus* DNA. Also, the absence of amplified product in previously described PCR procedures for *C. michiganensis* subsp. *sepedonicus* can be attributed to the absence of *C. michiganensis* subsp. *sepedonicus* DNA or unsuccessful amplification of *C. michiganensis* subsp. *sepedonicus* DNA due to any number of factors, including the presence of endogenous inhibitors of PCR in the sample.

The objective of our research was to develop a competitive PCR technique that would eliminate the risk of false negatives in PCR-based diagnosis of *C. michiganensis* subsp. *sepedonicus* and provide a means of estimating the amount of *C. michiganensis* subsp. *sepedonicus* DNA in a sample. Competitive PCR using an internal standard has been successfully used to monitor false negatives and to quantify template DNA (18,19,26). Competitive PCR has proven to be a simple, rapid, and sensitive technique for detecting *C. michiganensis* subsp. *sepedonicus* in infected plant tissues.

MATERIALS AND METHODS

Bacterial strain. A pathogenic rifampicin-resistant strain (CIC31) of *C. michiganensis* subsp. *sepedonicus* was used throughout this study. Bacterial cultures were grown from glycerol stocks stored at –80°C in NBY medium (36) containing 8 g of nutrient broth (Difco Laboratories, Detroit), 2 g of yeast extract, 2 g of K_2HPO_4, 0.5 g of KH_2PO_4, 2.5 g of glucose, and 15 g of agar per liter supplemented with 50 mg of rifampicin per liter. Cells were incubated at 26°C for 5 to 7 days, and single colonies were subcultured on NBY medium for 5 to 7 days prior to inoculum preparation.

Plant inoculations. Potato plants (*Solanum tuberosum* L. 'Sangre') were micropropagated by nodal stem culture and inoculated as described previously (20). Roots of 3-week-old plants were soaked in a cell suspension of CIC31 (OD_{640} = 0.1) prepared in 20 mM potassium phosphate buffer, pH 7.2. Control plants were treated with phosphate buffer alone. Inoculated plants were transplanted to soil (MetroMix 350, Grace Sierra Horticultural Products Company, Milpitas, CA) supplemented with fertilizer (160 g of Osmocote [13-13-13, Grace Sierra], 75 g of MicroMax [0-4-0, Grace Sierra], and 75 g of gypsum [Montana Limestone Co., Warren, MT] per 25 liters of soil), kept in a mist chamber for 3 days after inoculation, and grown in a greenhouse. Temperatures inside the greenhouse ranged from 25 to 32°C in the summer and from 20 to 28°C during the rest of the year. The greenhouse was covered with a shade cloth during the summer months. Supplemental incandescent and fluorescent lighting was provided to maintain a minimum day length of 12 h throughout the year.

Eggplants *(S. melanogena* L. 'Black Beauty') were grown from seeds under the conditions described above until the two-leaf stage and transplanted into 7.5-cm pots containing MetroMix 350 supplemented with fertilizer as described above. Stems were inoculated by stab inoculation with colonies of CIC31 scraped from 5- to 7-day-old cultures grown on NBY.

Isolation of bacterial DNA. Bacterial colonies grown on NBY plates were scraped and resuspended in 1 ml of sterile water. The cells were harvested by centrifugation at 10,000 rpm for 2 min, washed in 1 ml of washing buffer (50 mM Tris, pH 8.0, 5 mM EDTA, 50 mM NaCl), and resuspended in 100 µl of the washing buffer containing 5 mg of lysozyme per ml. After a 2-h incubation at 37°C, proteinase K was added to a final concentration of 0.35 mg/ml, and the mixture was incubated at 37°C for 30 min. The mixture was extracted with an equal volume of phenol saturated with 50 mM Tris (pH 7.4) and 1 mM EDTA (TE) for 30 min by shaking at 150 rpm and finally was extracted with chloroform/isoamyl alcohol (24:1). The phases were separated by centrifugation at 14,000 rpm for 5 min. The aqueous phase was recovered, adjusted to 0.3 M NaOAc (pH 7.5), and precipitated with 2 volumes of 95% ethanol. The pellet was washed with 75% ethanol, dried, and dissolved in 25 µl of TE. The concentration of bacterial DNA and plasmid DNA was determined by comparing the intensity of the ethidium bromide-stained band with a DNA mass ladder (GIBCO BRL, Gaithersburg, MD).

Isolation of DNA from plant tissues. DNA from inoculated and mock-inoculated eggplant and potato plant tissues was extracted with hexadecyltrimethylammoniumbromide (CTAB) buffer as described previously, with modifications (19). Plant tissue (150 to 250 mg fresh weight) was cut into 1-mm segments, ground with a mortar and pestle in liquid nitrogen, suspended in 500 µl of 2× CTAB buffer (1.4 M NaCl, 20 mM EDTA, 0.1 M Tris-HCl, pH 8.0, 1% polyvinylpyrrolidone-40, and 2% CTAB), and incubated for 5 min at 65°C. Lysozyme was added to a final concentration of 7.5 mg/ml, and the sample was incubated at 37°C for 1 h with shaking (150 rpm). The mixture was extracted with an equal volume of TE-saturated phenol and then with chloroform/isoamyl alcohol. The aqueous phase was collected, mixed with 0.1 volume of 10× CTAB buffer (10% CTAB, 0.7 M NaCl), and extracted with chloroform/isoamyl alcohol. DNA was precipitated with ethanol and dissolved in 500 µl of TE. DNA was further purified by extracting with phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol, precipitated with ethanol, and dissolved in 25 µl of sterile water.

Amplification of *C. michiganensis* subsp. *sepedonicus* DNA. PCR was conducted in 50 µl of 1× PCR buffer (20 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$) containing 0.2 mM of each deoxyribonucleotide triphosphate, 12.5 pmol of each oligonucleotide primer, and 2 units of *Taq* DNA polymerase (GIBCO BRL). A set of previously described (33) synthetic oligonucleotide primers CMS-6 (5′-CGCTCTCCCTCACCAGACTC) and CMS-7 (5′-TCCCGTGCTTGCCTGCGTTG) was used to amplify *C. michiganensis* subsp. *sepedonicus* DNA. Amplification was performed in a GeneAmp PCR System 9600 (Perkin-Elmer, Norwalk, CT) for 35 reaction cycles, each cycle consisting of a 1-min denaturation step at 95°C, a 1-min annealing step at 60°C, and a 1-min extension step at 72°C. A 5-µl aliquot of the PCR reaction was fractionated on 1% agarose gel, and the DNA was visualized by ethidium bromide staining.

Generation of an internal standard for quantification of *C. michiganensis* subsp. *sepedonicus*. An internal DNA template was prepared by amplifying *Arabidopsis thaliana* genomic DNA with CMS-6 and CMS-7 primers at a low annealing temperature (37°C for 2 min) as described previously (19). A PCR product of about 450 bp was subsequently cloned into pBluescript vector (Stratagene, La Jolla, CA) to create the plasmid pIS. pIS was introduced and maintained in *Escherichia coli* XL1-BLUE cells. The plasmid DNA was isolated using a plasmid spin kit (QIAGEN, Chatsworth, CA) and was included in all PCR assays as an internal standard. For quantitative analyses, competitive PCR was performed with increasing concentrations of *C. michiganensis* subsp. *sepedonicus* DNA in the presence of a constant amount of internal standard DNA (0.2 pg of pIS) and a trace amount of [α-^{32}P]dATP (0.5 µCi). The PCR products were separated in agarose or polyacrylamide gels, which were dried on Whatman 3 MM paper (Whatman, Clifton, NJ), and exposed to X-ray film. Subsequently, the amount of product generated by *C. michiganensis*

subsp. *sepedonicus* DNA and internal DNA was quantified by scintillation counting of radioactivity in amplified bands excised from gels. A PCR product ratio was calculated by dividing the radioactivity in the PCR product generated from *C. michiganensis* subsp. *sepedonicus* DNA by the radioactivity in the PCR product generated from the internal standard DNA.

IFAS of *C. michiganensis* subsp. *sepedonicus*. Half of the tissue collected from each plant was used for PCR-based detection of *C. michiganensis* subsp. *sepedonicus*. The other half was used for detection of *C. michiganensis* subsp. *sepedonicus* by IFAS. Potato tissue samples (200 mg) from stems or roots were harvested and stored at –80°C until analysis. Tissue was homogenized with a mortar and pestle and incubated for 30 min in 1 ml of PBS buffer (12.5 mM K_2HPO_4, 5 mM KH_2PO_4, 150 mM NaCl, 3 mM sodium azide, 0.25% dry skim milk, pH 7.2). The extract was transferred to a microfuge tube and centrifuged briefly (5 s at 14,000 rpm) to remove plant debris. Cells of *C. michiganensis* subsp. *sepedonicus* were pelleted by centrifugation for 15 min at 14,000 rpm at 20°C and resuspended in 40 µl of PBS buffer. Serial 10-fold dilutions were made in PBS buffer. Aliquots (10 µl) from each dilution were treated with a monoclonal 9A1 mouse anti-*C. michiganensis* subsp. *sepedonicus* (purchased from Agdia, Elkhart, IN) and stained with fluorescein isothiocyanate goat anti-mouse immunoglobulin (Ig) G plus IgM according to the manufacturer's recommendations. Slides were viewed on a microscope equipped with a mercury short-arc lamp. Viewing was done in the dark with nondrying, low-fluorescence immersion oil under a 100× oil immersion lens and 10× ocular. *C. michiganensis* subsp. *sepedonicus* cells were identified as bright yellow-green rods with round ends. Bacterial population sizes were calculated from an average of 10 microscopic views per sample and presented as the number of IFAS units/cells per gram of tissue.

To quantify the number of *C. michiganensis* subsp. *sepedonicus* in samples used for standardization of the competitive PCR assay, a cell suspension containing about 10^8 CFU/ml (OD_{640} = 0.1), as determined by dilution plating on NBY medium, was prepared in 20 mM potassium phosphate buffer, pH 7.2, and serial 10-fold dilutions were made in the same buffer. A 10-µl aliquot of each dilution was used for IFAS and to prepare DNA for PCR as described above. The average number of IFAS units from 10 microscopic fields was used to calculate the number of cells per 10-µl sample.

RESULTS

Competitive PCR with purified *C. michiganensis* subsp. *sepedonicus* DNA. Nonspecific amplification of *Arabidopsis* DNA by primers CMS-6 and CMS-7 resulted in several bands. A 450-

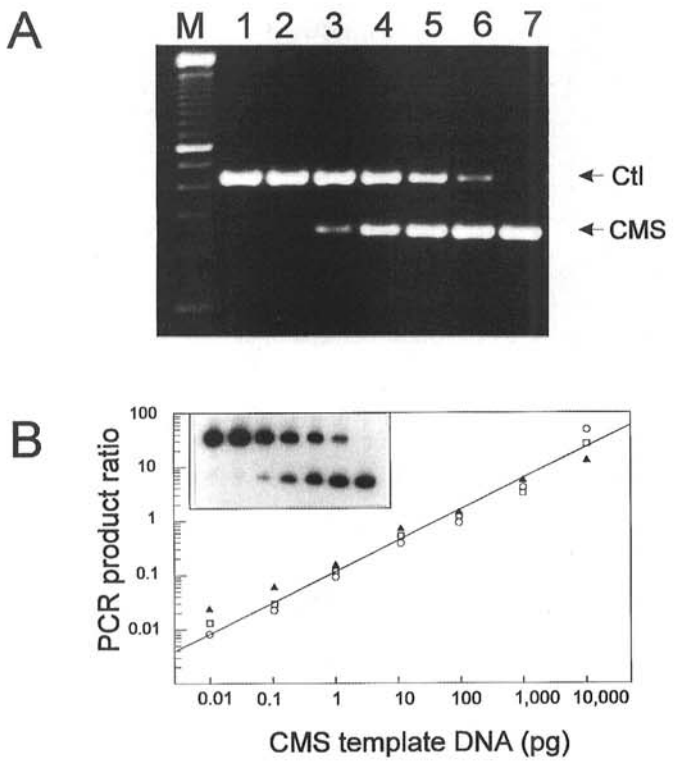

Fig. 1. Relationship between the amount of *Clavibacter michiganensis* subsp. *sepedonicus* (CIC31) DNA and the resulting polymerase chain reaction (PCR) product ratio. Varying amounts of CIC31 DNA were PCR amplified in the presence of 0.2 pg of internal standard DNA. **A,** The PCR products were fractionated on a 1% agarose gel and stained with ethidium bromide. Lanes 1–7 contain increasing amounts in 10-fold increments (0.01 pg to 10 ng) of CIC31 DNA. The upper band of about 450-bp is a PCR product from the internal standard (Ctl), and the lower band is the PCR product (258-bp) amplified from CIC31 DNA (CMS). Lane M, 100-bp ladder. **B,** The PCR product ratio (amount of bacterial product/internal standard product) was determined for each DNA concentration and plotted against the concentration of bacterial DNA. The radioactivity in PCR products was quantified by excising the region of the gel that corresponded to bands on an autoradiogram (inset) and counting in a liquid scintillation counter. Results for three experiments (open circles, open squares, and solid triangles) are presented together with a regression line.

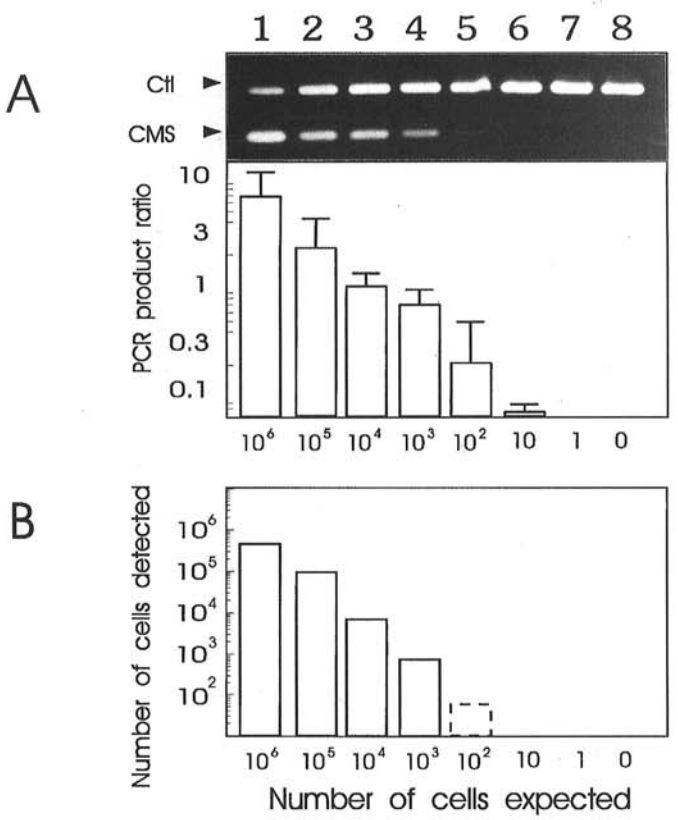

Fig. 2. Quantification of *Clavibacter michiganensis* subsp. *sepedonicus* (CIC31) by polymerase chain reaction (PCR)-based method and immunofluorescence antibody staining (IFAS) analysis. Serial 10-fold dilutions of CIC31 cells were made, and a 10-µl sample from each dilution was used for IFAS analysis and to prepare DNA for PCR. **A,** Top: gel showing the PCR product amplified from the DNA isolated from lanes 1–7, 10^6, 10^5, 10^4, 10^3, 10^2, 10^1, and 1 CIC31 cells and 0.2 pg of internal standard, respectively. Lane 8, PCR product from a control containing only internal standard DNA (0.2 pg) and no *C. michiganensis* subsp. *sepedonicus* DNA. The upper band of about 450-bp is a PCR product from the internal standard (Ctl), and the lower band is the PCR product (258-bp) amplified from CIC31 DNA (CMS). Bottom: PCR product ratio for each dilution. **B,** Quantification of number of cells by IFAS. The average number of IFAS units from 10 views was used to calculate the number of cells per 10 µl. Results were pooled from two independent sets of experiments, and PCR analysis was repeated twice. The dashed bar denotes inconsistency in detecting cells in this range by IFAS.

bp PCR product was chosen to develop an internal standard because it was easily distinguished from the 258-bp product amplified with DNA from *C. michiganensis* subsp. *sepedonicus*. The *Arabidopsis* DNA in pIS was consistently amplified at the higher stringencies used for amplification of *C. michiganensis* subsp. *sepedonicus* DNA. Amplification of pIS was observed in the absence or presence of *C. michiganensis* subsp. *sepedonicus* DNA (Fig. 1, 2, and 3).

Competitive PCR mixtures contained varied amounts (0.01 pg to 10 ng) of DNA purified from *C. michiganensis* subsp. *sepedonicus* and a constant amount (0.2 pg) of pIS as an internal DNA standard (Fig. 1A). The amount of 258-bp product from *C. michiganensis* subsp. *sepedonicus* increased, whereas the amount of 450-bp product from the internal standard decreased with increasing levels of pathogen DNA in the reaction (Fig. 1A). The PCR product ratio also increased proportionally with increasing levels of pathogen DNA over the range tested (0.01 pg to 10 ng) (Fig. 1B). As shown in Figure 1, the PCR conditions used detected as little as 0.1 pg and routinely detected 1 pg of purified *C. michiganensis* subsp. *sepedonicus* DNA.

Competitive PCR detected *C. michiganensis* subsp. *sepdonicus* DNA purified from as few as 10 to 100 cells (Fig. 2A). IFAS consistently detected 1,000 cells and inconsistently detected 100 cells, as would be expected given the physical detection limit for IFAS is about 1,000 cells. The number of cells observed by IFAS and the intensity of the PCR signal from the corresponding sample increased with the number of input cells. Sensitivity of competitive PCR was at least 10-fold greater than IFAS (Fig. 2).

Competitive PCR with *C. michiganensis* subsp. *sepedonicus* DNA isolated from eggplant. Eggplant is used routinely in our lab as an index host for study of *C. michiganensis* subsp. *sepedonicus* (22,30). Inoculated plants typically develop symptoms within 7 days. Pathogen populations in leaf, stem, and root tissues of eggplants were monitored 0, 2, 7, 14, 21, and 28 days after stem inoculation. Immediately following inoculation, a high amount of *C. michiganensis* subsp. *sepedonicus* DNA was detected in stems, but this amount gradually declined over the 28-day postinoculation period (Fig. 3). DNA of *C. michiganensis* subsp. *sepedonicus* was detected in leaves 2 days after inoculation and in roots 7 days after inoculation (Fig. 3) . The largest amount of *C. michiganensis* subsp. *sepedonicus* DNA detected was from leaves collected 21 to 28 days postinoculation. PCR products indicative of *C. michiganensis* subsp. *sepedonicus* were not obtained from noninoculated plants (Fig. 3).

Competitive PCR with *C. michiganensis* subsp. *sepedonicus* DNA isolated from potato. Competitive PCR and IFAS were used to monitor DNA levels and bacterial population sizes in inoculated potato plants. Stems and roots were collected 21, 35, and 49 days after inoculation; stems were divided into upper, middle, and lower sections. The results obtained from PCR and IFAS analyses are presented in Figure 4. Extrapolation from a standard curve of *C. michiganensis* subsp. *sepedonicus* DNA and the PCR product ratio (Fig. 1B) provided estimates of pathogen DNA of 197.5, 60, 40, and 12.5 pg/g fresh weight in the root, lower, middle, and upper stem, respectively, 21 days after inoculation. Foliar symptoms of bacterial ring rot were observed about 28 days postinoculation, which was at least 1 week later than successful detection

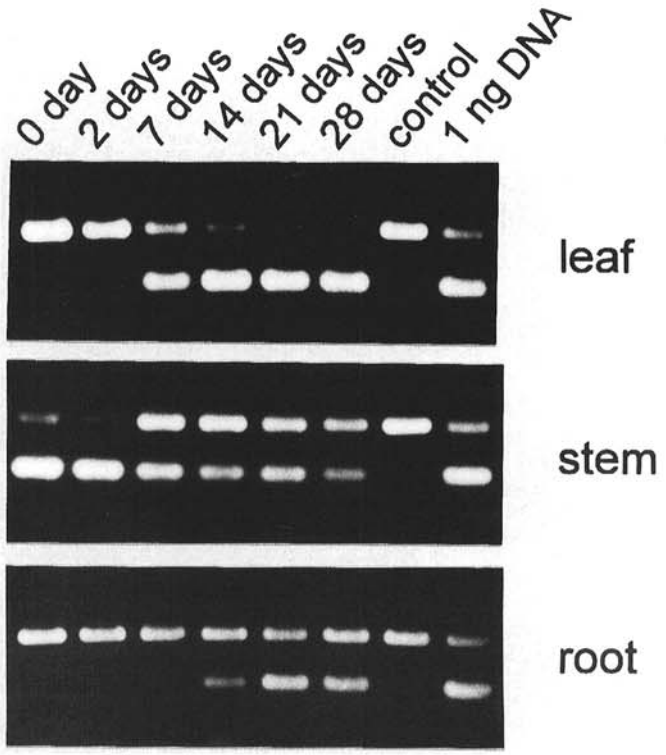

Fig. 3. Polymerase chain reaction (PCR) detection of *Clavibacter michiganensis* subsp. *sepedonicus* (CIC31) in eggplant. Leaf, stem, and root tissues were collected at 0, 2, 7, 14, 21, and 28 days after stem inoculation. DNA from 0.2 g of tissue was isolated as described in text, dissolved in 25 µl of water, and a 5-µl sample was used for amplification by PCR. One-tenth of the PCR reaction was analyzed on 1% agarose gel. The upper band is a product of the internal standard DNA, and the lower band represents the amplified product from CIC31 DNA. Control lane shows the PCR product from a sample containing only internal standard DNA. The far right lane (1 ng DNA) shows the PCR products obtained with 1 ng of CIC31 DNA and 0.2 pg of internal standard DNA template in the reaction. Similar results were obtained in two independent experiments.

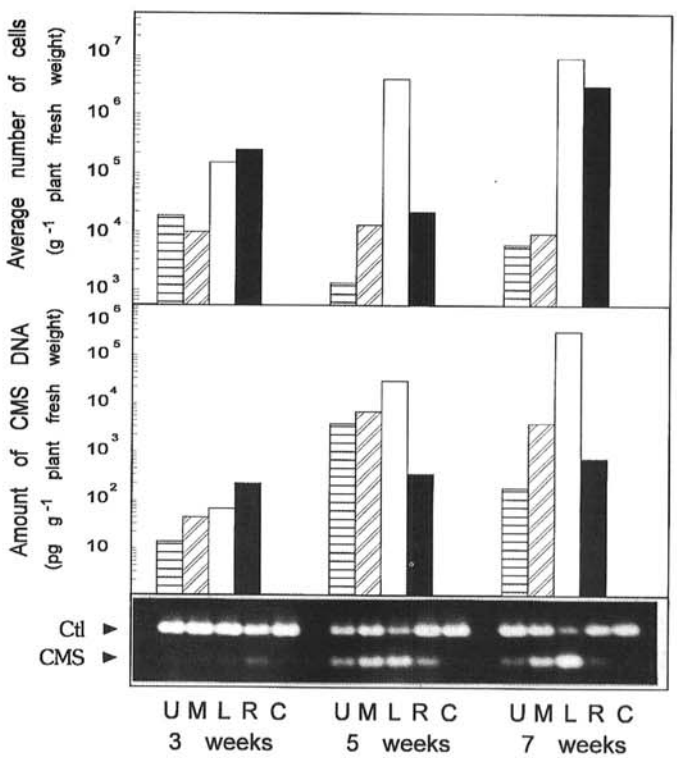

Fig. 4. Comparison of quantification of *Clavibacter michiganensis* subsp. *sepedonicus* (CIC31) in potato stems and roots by top: immunofluorescence antibody staining and bottom: competitive polymerase chain reaction (PCR). Top panel shows the number of cells per g fresh weight. The bottom panel shows the amount of pathogen DNA estimated by determining the PCR product ratio and extrapolation from a standard curve shown in Figure 1. Each DNA sample was tested by PCR at least three times with similar results. Roots (R), upper (U), middle (M), and lower (L) stem sections of root-inoculated plants were used for analyses. Control sample (C) was collected from stem section of a mock-treated plant. The upper band of about 450-bp is a PCR product from the internal standard (Ctl), and the lower band in each panel is the PCR product (258-bp) amplified from CIC31 DNA (CMS).

of the pathogen DNA. The amount of amplified DNA from all tissue types increased between 3 and 5 weeks after inoculation. The greatest amount of *C. michiganensis* subsp. *sepedonicus* DNA was detected in the lower stem sections of potato plants. Population sizes estimated by IFAS were consistent with the intensities of the PCR signals obtained with matched samples (Fig. 4). The number of cells of *C. michiganensis* subsp. *sepedonicus* detected by the intensity of PCR signals and IFAS was greater in lower-stem sections than in roots, upper-, and middle-stem sections 35 and 49 days postinoculation.

DISCUSSION

Availability of sensitive and accurate methods for diagnosis of bacterial ring rot, including potential applications of PCR technology, has increased dramatically over the past 10 years. As with other technologies, PCR has its limitations, some of which are specificity of primers for amplification of pathogen DNA, sensitivity, and the potential for false negatives due to inherent variation in plant extracts. PCR primers and reaction conditions can be manipulated to enhance specificity (18). Reduction of inhibition of PCR by plant extracts can be improved by attention to reaction conditions and purity of pathogen template (19). Current PCR-based methods to detect *C. michiganensis* subsp. *sepedonicus* do not address false negatives or quantification of the pathogen (15,33).

In the present study, we developed a competitive PCR assay for detection and quantification of *C. michiganensis* subsp. *sepedonicus* DNA in infected plants. The strength of competitive PCR is the presence of an internal standard DNA that uses the same primers as the pathogen DNA and, thus, serves as an indicator of successful amplification and the absence of endogenous inhibitors in the extract or occasional reaction failure. An additional advantage is that the relative intensities of the PCR product from an internal DNA standard and the pathogen estimates the quantity of *C. michiganensis* subsp. *sepedonicus* DNA in the sample.

Competitive PCR permitted detection of pathogen DNA prior to symptom appearance and was more sensitive than other PCR methods for *C. michiganensis* subsp. *sepedonicus.* (15,33). The detection limit was reported to be 0.5 ng of DNA with DNA hybridization, which corresponds to 2×10^5 bacteria (21) and 4×10^3 cells with PCR (15). Under the conditions reported here, we could detect 1 pg of DNA (Fig. 1A) or less than 100 cells (Fig. 2A). A possible reason for the increased sensitivity with our method could be the use of purified DNA rather than the direct use of cells for PCR amplification (15). When cells are used for PCR, inefficient lysis or endogenous inhibitors may affect the sensitivity of amplification.

Results obtained with competitive PCR compare favorably with IFAS and were consistent with patterns of symptom development in eggplant and potato. As with IFAS, detection of pathogen DNA does not indicate the presence of viable cells. Nonetheless, another potential application of competitive PCR is the estimation of bacterial population sizes. Competitive PCR provides an advantage over direct colony counts in that it is much faster and sensitive. The direct colony counting method requires more than 10^6 CFU, whereas competitive PCR allows the detection of less than 100 cells. Naturally occurring strains of *C. michiganensis* subsp. *sepedonicus* grow slowly on agar medium and can be antagonized by other microorganisms growing in the medium. Thus, colonies of *C. michiganensis* subsp. *sepedonicus* are typically evident only when the population of this pathogen is greater than that of the background microflora ($>10^5$ to 10^6 CFU/g) (13). Assuming a genome size of 2,500 kb (21), 0.1-pg amplified product corresponds to about 40 bacterial cells. Although such estimates depend on the ideal extraction of DNA from tissues, these estimates of population sizes may be useful as a means of corroborating IFAS data and providing relative measurements of contamination when other methods of diagnosis are unavailable. Furthermore, competitive PCR can be integrated into other PCR-based detection systems as improved primers for specific amplification of all genotypic and phenotypic variants of *C. michiganensis* subsp. *sepedonicus* become available.

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