BRIEF REPORT

Folia Microbiol. **48** (5), 682–686 (2003) **http://www.biomed.cas.cz/mbu/folia**

Determination of Genetic Differences between Fluid and Nonfluid Variants of *Clavibacter michiganensis* subsp. *sepedonicus* Using rep-PCR Technique

J. FOUSEK[a,b], I. MRÁZ[a]

[a]*Department of Plant Virology, Institute of Plant Molecular Biology, Academy of Sciences of the Czech Republic, 370 05 České Budějovice, Czechia*

fax +420 385 310 363
e-mail janfousek@umbr.cas.cz

[b]*University of South Bohemia, Faculty of Biological Sciences, 370 05 České Budějovice, Czechia*

Received 10 February 2003
Revised version 30 June 2003

ABSTRACT. Testing of 23 isolates of *Clavibacter michiganensis* subsp. s*epedonicus* for analysis by rep-PCR (using BOX, ERIC, REP primer sets) was used for the purpose of localization of genetic markers for fluid and/or nonfluid strains. None of the primer sets was successful in detecting genetic differences between the isolates and no polymorphism was generated.

The Gram-positive coryneform bacterium *Clavibacter michiganensis* subsp. *sepedonicus* (SPICKERMAN et KOTHOFF 1914) DAVIS, GILLASPIE, VIDAVER et HARRIS 1984 (*Cms*) is a causal agent of a serious disease called bacterial ring rot of potato (BRR). This has occurred in major potato-growing areas on all continents except Australia (Hocker 1981). The name of BRR originates from the characteristic "ring rot" symptom (destruction of vascular ring) visible after cutting of infected tuber. *Cms* and other subspecies of *Clavibacter michiganensis* are highly biotrophic pathogens preferring colonization of the vascular system, particularly the xylem vessels (Rothwell 1968). Colonization of these tissues leads to blocking of the natural transport of water and nutrients followed by wilting of infected leaves and stems. Environmental conditions, cultivar susceptibility, inoculum dose and virulence of infecting bacterial strain are the main factors significantly influencing the severity and character of the expressed symptoms (Bishop and Slack 1987; Westra and Slack 1994). Latent and asymptomatic infections are frequent and the pathogen could persist undetected in field or tissue culture for three generations (Schuld *et al*. 1992; De Boer and McCann 1990). These facts make routinely done diagnosis by visual inspection and eggplant bioassay (Lelliot and Sellar 1976; Olsson 1976) unreliable and another confirmation method is necessary (Mills *et al.* 1997; Pastrik 2000).

Reduced virulence of some *Cms* isolates could be caused by the absence of several known factors. Genes coding the protein(s) that elicit a hypersensitive response in non-host plant were discovered to be necessary for efficient multiplication of bacteria in the host (Nissinen *et al*. 1997). Cellulase encoded by the native plasmid pCS1 is important for symptom expression (Laine *et al.* 2000). The amount and composition of the extracellular polysaccharides (EPS) affects antigenic properties (Bishop *et al.* 1988), colony morphology (fluid and/or nonfluid) (Henningson and Gudmestad 1991) and influences the ability to form occlusions on the membrane pits and to induce wilting (Van Alfen *et al*. 1983).

The rep-PCR technique used here is based on three specific conserved repetitive sequences (BOX, ERIC, REP) distributed in diverse bacterial genomes. Three primer sets are commonly used for genomic fingerprinting analysis and correspond to repetitive sequences. The primers are designed to amplify intervening DNA between two adjacent repetitive elements. Detected fingerprint polymorphism between closely related samples is caused either by different localization of a repetitive sequence in the genome or by changes in the region between repetitive sequences. In the second case we could accept the hypothesis in which the phenotypic change could be affected by the detected polymorphism.

Despite the BRR importance, very little is known about the mechanisms by which the *Cms* induces the disease and the genetic background of these processes. This study is focused on the determination of genetic differences between so-called fluid and nonfluid isolates of *Cms*. The main purpose was to discover a polymorphism that could occur after analysis used as a genetic marker for recognition of fluid or nonfluid strains

or starting point for genetic analysis of the EPS synthesis metabolism. Detailed knowledge of this factor will help to discover the ways leading to the change of the cell-wall composition and antigenic character.

Bacterial strains. Each of 23 bacterial strains (Table I) was a well-characterized strain coming from collections of microorganisms. Colony morphology characterization of strains was done at the *Research Institute of Crop Production* in Prague by Dr. B. Kokošková. Strains were randomly chosen to make two groups displaying different colony morphology.

Culture media and growth conditions. The isolates were grown on Nutrient Broth Yeast extract (NBY) (Vidaver 1967) at 23 °C for 3–5 d.

Isolation of DNA. Genomic DNA was isolated using a combination of the alkaline lysis method and boiling (Rademarker and de Brujin 1997). The DNA recovered was not further purified and it was directly added to the PCR reaction mixture.

rep-PCR analysis and electrophoresis. PCR was performed on a Mini Cycler (*MJ Research*, USA). BOX, ERIC and REP primers (Versalovic *et al.* 1994) were synthesized by *Generi Biotech* (Czechia). The reaction mixture contained 12.5 μL of 2× PPP Master mix (75 mmol/L Tris-HCl, pH 8.8; 20 mmol/L $(NH_4)_2SO_4$; 100 ppm Tween 20; 2.5 mmol/L $MgCl_2$; dNTP 200 μmol/L each; 2.5 U Taq purple DNA polymerase and stabilization additives) obtained from *TopBio* (Czechia); 2.5 μL Me_2SO; 2 μL of each primer (20 pmol); 1 μL of DNA template and 5 μL of double-distilled H_2O (7 μL for the BOX variant). Amplification protocol followed rep-PCR procedure of Louws *et al.* (1998). Amplified PCR products were separated on 1.5 % agarose gel for 3 h. DNA was stained with Sybr Green (*Sigma–Aldrich*).

Table I. Bacterial strains (fluid and nonfluid) of *Clavibacter michiganensis* subsp. *sepedonicus*[a]

No.	Fluid	No.	Nonfluid
1	OFF-1	*14*	INM-1
2	As-1	*15*	SD-1
3	PD 54	*16*	PD 1487
4	PD 56	*17*	PD 1488
5	PD 59	*18*	PD 1849
6	PD 60	*19*	PD 2637
7	PD 328	*20*	PD 3799
8	PD 681	*21*	PD 3800
9	PD 1306	*22*	PD 3802
10	PD 1664	*23*	NCPPB 3279
11	PD 3538		
12	PD 3559		
13	NCPPB 3467		

[a]Origin of strains:
PD – Dr. J. Janse (*Plantenziektenkundige Dienst*, Wageningen, The Netherlands);
OFF-1, As-1, INM-1, SD-1 – Dr. K. Pernezny (*University of Florida*, Gainesville, USA);
NCPPB – *National Collection of Plant Pathogenic Bacteria*, Harpenden (UK).

Selection and characterization of fluid and nonfluid isolates. Cms naturally occurs in two morphological variants. The phenomenon of fluid and nonfluid morphology is common in other bacterial species (Bodman and Farrand 1995; Martín *et al*. 2000). There are also other terms used for description of this phenomenon: mucoid *vs.* nonmucoid, smooth *vs.* rough. The main origin of colony morphology dissimilarities is unequal production of EPS. Nonfluid variants differ in both the amount of produced EPS and participation of individual sugar fractions. Previous studies allowed detailed characterization of these chemical compounds and separate them into four fractions by their molar mass (Henningson and Gudmestad 1992; Westra and Slack 1992).

The tested strains were divided into two groups: (*1*) isolates with fluid morphology only, and (*2*) isolates with intermediate and nonfluid morphology. The separated bacterial strains were analyzed by the rep-PCR method. Changes of colony morphology could be induced artificially under laboratory conditions by temperatures above 28 °C and low contents of sugar in the cultivation medium (Kokošková and Kůdela 2002). However, these changes are not permanent and the colony returns to its original stage after cultivation under natural conditions. The bacterial isolates used in this work are of natural origin and no induction protocol was applied to them.

DNA fingerprints were visually evaluated for the presence of distinct PCR products (Fig. 1).

Reproducibility of rep-PCR fingerprints was comparable with the values published by Smith *et al.* (2001). Differences between single reactions were caused rather by reactions condition than by genetically based variability. We compared all fingerprints but no stable polymorphism for any isolate was discovered (*data not shown*).

Determination of genetic dissimilarities by the rep-PCR method has been successfully used for taxonomic determination and genetic variability analysis of important plant-associated bacteria, such an *Erwinia amylovora*, *Burkholderia solanacearum* and *Xanthomonas campestris* pv. *vesicatoria* (Louws *et al.* 1995; McMannus and Jones 1995; Frey *et al*. 1996). The rep-PCR method was used for its ability to detect dissimilarities between closely related specimens; its sensitivity allowed detection of genetic variability even between single bacterial strains in a population of *Clavibacter michiganensis* subsp. *michiganensis* (Louws *et al.* 1998). We used three sets of primers (BOX, ERIC, REP) to obtain complex analysis of the genome of 13

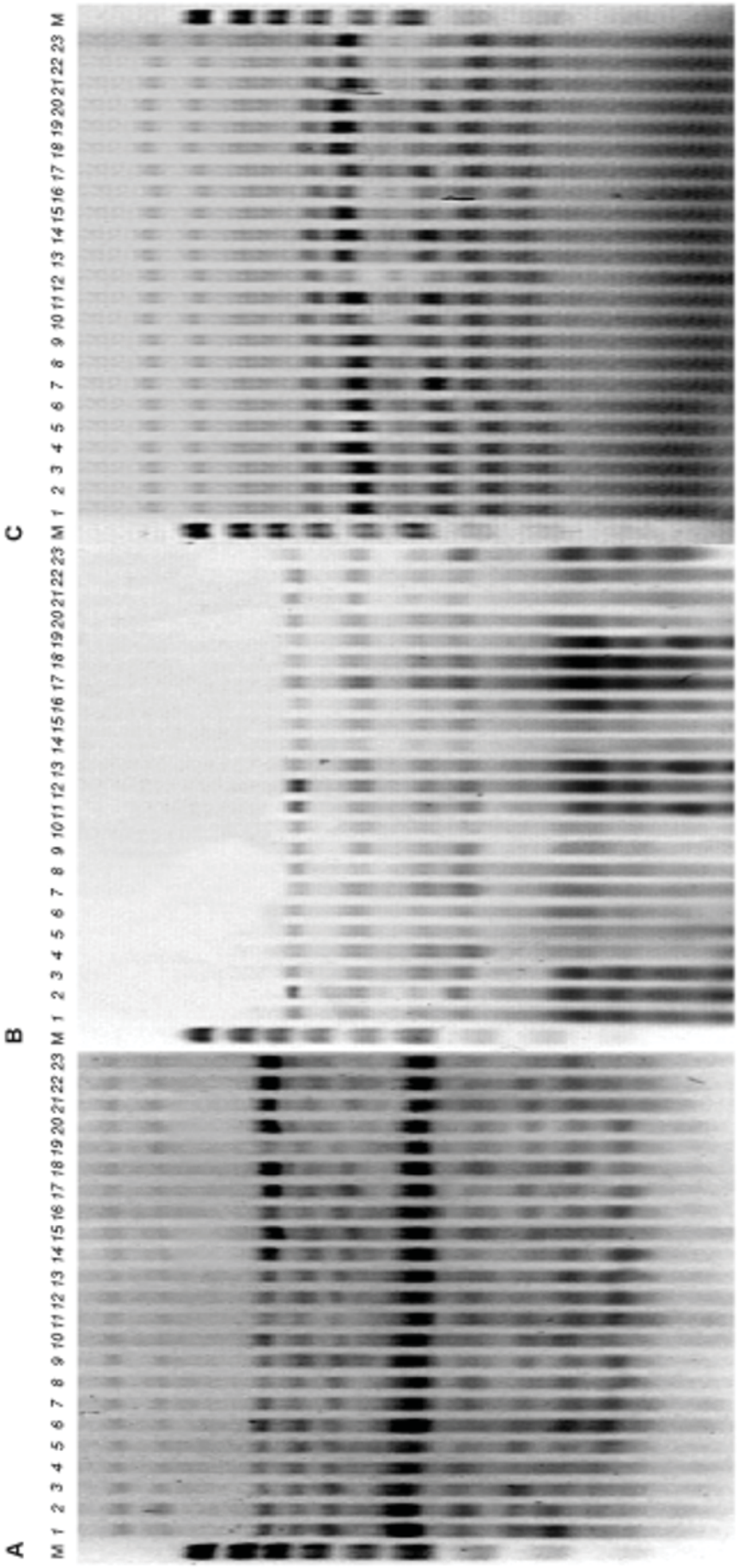

Fig. 1. BOX-PCR (**A**), ERIC-PCR (**B**), REP-PCR (**C**) fingerprints of fluid and nonfluid strains of *Cms*; M – 100-bp ladder (*New England Biolabs*); numbers of lanes *1–23* correspond to those in Table I.

fluid and 10 nonfluid strains. Although we selected a relatively wide testing file and used different primers we could not localize any size polymorphism of the generated PCR products that could set nonfluid variants apart from the fluid ones. This result corroborates previous rep-PCR work, which detected the population of *Cms* to be very uniform (Smith *et al.* 2001; Fousek *et al.* 2002). This opinion was also confirmed by some other techniques including RFLP analysis of the repetitive region IS1121 (Mogen *et al*. 1990), low-molar-mass RNA and fatty-acid profiling (Henningson and Gudmestad 1991; Palomo *et al*. 2000).

The only effective method able to distinguish between strains with different virulence is genomic fingerprinting based on restriction analysis of high-molar DNA, and CHEF gel electrophoresis. Thus detected differences correlate with the occurrence of genes for hypersensitive response and cellulase but not with different colony morphology (Brown *et al*. 2001). There are many studies where the rep-PCR technique was able to differentiate between very closely related organisms, but it failed in our case. The explanation could be that genes belonging to the EPS synthesis pathway are necessary for the bacterial life cycle. And that is why we cannot detect any deviation at the DNA level by such a method as rep-PCR. The changes should be searched for on the RNA level where different regulation steps occur, but this hypothesis needs to be confirmed.

This work was supported by the *Grant Agency of the Czech Republic* (grant no. GA 522/00/0887) and by the *Grant Agency of the Academy of Sciences of the Czech Republic* (grant no. S 5051014). The authors would also like to thank to Dr. Kokošková, Dr. Janse and Dr. Pernezny for providing the bacterial strains.

REFERENCES

BECK VON BODMAN S., FARRAND S.K.: Capsular polysaccharide biosynthesis and pathogenicity in *Erwinia stewartii* require induction by an *N*-acylhomoserine lactone autoinducer. *J.Bacteriol.* **177**, 5000–5008 (1995).

BISHOP A.L., SLACK S.A.: Effect of cultivar, inoculum dose and strain *Clavibacter michiganensis* subsp. *sepedonicus* on symptom development in potatoes. *Phytopathology* **77**, 1085–1089 (1987).

BISHOP A.L., CLARKE R.G., SLACK S.A.: Antigenic anomaly in a naturally occurring nonfluidal strain of *Corynebacterium sepedonicum. Am.Potato J.* **65**, 237–246 (1988).

BROWN S., REILLEY A.A., KNUDSON D.L., ISHIMARU C.A.: Genomic fingerprinting of virulent and avirulent strains of *Clavibacter michiganensis* subsp. *sepedonicus*. *Curr.Microbiol.* **44**, 112–119 (2002).

DAVIS M.J., GILLESPIE A.G. Jr., VIDAVER A.K., HARRIS R.W.: *Clavibacter*, a new genus containing some phytopathogenic coryneform bacteria, including *Clavibacter xyli* subsp. *xyli* and *Clavibacter xyli* subsp. *cynodontis*, pathogens that cause ratoon stunting disease of sugarcane and Bermudagrass stunting disease. *Internat.J.Syst.Bacteriol*. **34**, 107–117 (1984).

DE BOER S.H., MCCANN M.: Detection of *Corynebacterium sepedonicum* in potato cultivars with different propensities to express ring rot symptoms. *Am.Potato J.* **67**, 685–694 (1990).

FOUSEK J., MRÁZ I., PETRZIK K.: Comparison of genetic variability between Czech and foreign isolates of phytopathogenic bacteria *Clavibacter michiganensis* subsp. *sepedonicus* by rep-PCR technique. *Folia Microbiol.* **47**, 450–454 (2002).

FREY P., SMITH J.J., ALBAR L., PRIOR P., SADDLER G.S., TRIGALET-DEMERY D., TRIGALET A.: Bacteriocin typing of *Burkholderia* (*Pseudomonas*) *solanacearum* race 1 of the French West Indies and correlation with genomic variation of the pathogen. *Appl. Environ.Microbiol*. **62**, 473–479 (1996).

HENNINGSON P.J., GUDMESTAD N.C.: Fatty acid analysis of phytopathogenic coryneform bacteria. *J.Gen.Microbiol.* **137**, 427–442 (1991).

HENNINGSON P.J., GUDMESTAD N.C.: Comparison of exopolysaccharides from mucoid and nonmucoid strains of *Clavibacter michiganensis* subsp. *sepedonicus*. *Can.J.Microbiol.* **39**, 291–296 (1992).

HOCKER W.J.: *Compendium of Potato Diseases*. APS, St.Paul, MN (USA) 1981.

KOKOŠKOVÁ B., KŮDELA V.: Induction of nonmucoid variant of *Clavibacter michiganensis* subsp. *sepedonicus* and comparison their immunochemical and biochemical characteristics. *J.Plant Dis.Protect.* **109**, 630–638 (2002).

LAINE M.J., HAAPALAINEN M., WAHLLOOS T., KANKARE K., NISSINEN R., KASSUWI S., METZLER M.C.: The cellulase encoded by the native plasmid of *Clavibacter michiganensis* subsp. *sepedonicus* plays a role in virulence and contains an expansin-like domain. *Physiol.Mol.Plant Pathol.* **57**, 221–233 (2000).

LELLIOT R.A., SELLAR P.W.: The detection of latent ring rot (*Corynebacterium sepedonicum* (SPIEK et KOTTH.) SKAPT et BURKH) in potato stocks. *EPPO Bull.* **6**, 101–106 (1976).

LOUWS F.J., FULBRIGHT D.W., STEPHENS C.T., DE BRUIJN F.J.: Differentiation of genomic structure by rep-PCR fingerprinting to rapidly classify *Xanthomonas campestris* pv. *vesicatoria. Phytopathology* **85**, 528–536 (1995).

LOUWS F.J., BELL J., MEDINA-MORA C.M., SMART C.D., OPGENORTH D., ISHIMARU C.A., HAUSBECK M.K., DE BRUIJN F.J., FULBRIGHT D.W.: rep-PCR-mediated genomic fingerprinting: a rapid and effective method to identify *Clavibacter michiganensis*. *Phytopathology* **88**, 862–868 (1998).

LUPSKI J.R., WEINSTOCK G.M.: Short, interspersed repetitive DNA sequences in prokaryotic genomes. *J.Bacteriol.* **174**, 4525–4529 (1992).

MARTÍN M., LORETO J., SANCHEZ-CONTRERAS M., BONILLA I., REVILE R.: Muck is necessary for galactoglucan production in *Sinorhizobium meliloti* EFB1. *Molec.Plant Microbe Interact.* **13**, 129–135 (2000).

MC MANNUS P.S., JONES A.L.: Genetic fingerprinting of *Erwinia amylovora* strains isolated tree-fruit crops and *Rubus* spp. *Phytopathology* **85**, 1547–1553 (1995).

MILLS D., RUSELL B.W., HANUS J.W.: Specific detection of *Clavibacter michiganensis* subsp. *sepedonicus* by amplification of three unique DNA sequences isolated by subtraction hybridization. *Phytopathology* **87**, 853–861 (1997).

MOGEN B.D., OLSON H.R., SPARKS R.B., GUDMESTAD N.C, OLESON A.E.: Genetic variation in strains of *Clavibacter michiganense* subsp. *sepedonicum*: polymorphism in restriction fragments containing a highly repeated sequence. *Phytopathology* **80**, 90–96 (1990).

NISSINEN R., LAI F.M., LAINE M.J., BAUER P., REILLEY A.A., LI X., DE BOER S.H., ISHIMARU C.A., METZLER M.C.: *Clavibacter michiganensis* subsp. *sepedonicus* elicits the hypersensitive response in tobacco and secrets hypersensitive response-inducing protein(s). *Phytopathology* **87**, 678–684 (1997).

OLSSON K.: Experience of ring rot caused by *Corynebacterium sepedonicum* (SPIECK. et KOTTH.) SKAPT et BURKH. in Sweden. Particulary detection of the disease in its latent form. *EPPO Bull.* **6**, 209–219 (1976).

PALOMO J.L., VELASQUEZ E., MATEOS P.F., GARCIA-BENAVIDES P., MARTINEZ-MOLINA E.: Rapid identification of *Clavibacter michiganensis* subsp. *sepedonicus* based on the stable low molecular weight RNA (LMW RNA) profiles. *Europ.J.Plant Pathol.* **106**, 789–793 (2000).

PASTRIK K.-H.: Detection of *Clavibacter michiganensis* subsp. *sepedonicus* in potato tubers by multiplex PCR with coamplification of host DNA. *Europ.J.Plant Pathol.* **106**, 155–165 (2000).

RADEMARKER J.L.W., DE BRUJIN F.J.: Characterization and classification of microbes by rep-PCR genomic fingerprinting and computer-assisted pattern analysis, pp. 1–20 in *DNA Markers: Protocols, Applications and Overviews* (G. Gaetano-Anolles, P.M. Greshoff, Eds). J. Wiley & Sons, New York 1997.

ROTHWELL A.: Bacterial canker of tomato caused by *Corynebacterium michiganense* (SMITH) JENSEN. *Rhod.Agric.J.* **1871**, 75–78 (1968).

SCHULD B.A., CRANE J., HARRISON M.D.: Symptomless infection with *Clavibacter michiganensis* subsp. *sepedonicus* during tissue culture propagation potato. *Can.J.Plant Sci*. **72**, 943–953 (1992).

SMITH N.C., HENNESEY J., STEAD D.E.: Repetitive sequence-derived PCR profiling using the BOX-A1R primer for rapid identification of the plant pathogen *Clavibacter michiganensis* subspecies *sepedonicus*. *Eur.J.Plant Pathol.* **107**, 739–748 (2001).

SPIECKERMANN A., KOTTHOFF P.: Untersuchungen über die Kartoffelpflanze und ihre Krankheiten. Die Bakterienringfäule der Kartoffelpflanze. *Landwirtschft.Jahrsb*. **46**, 659–728 (1914).

VAN ALFEN N.K., MCMILLAN B.D., TURNER V., HESS W.M.: Role of pit membranes in macromolecule-induced wilt of plants. *Plant Physiol.* **73**, 1020–1023 (1983).

VERSALOVIC J., SCHNEIDER M., LUPSKI J.R.: Genomic fingerprinting of bacteria using repetitive sequence based PCR (rep-PCR). *Meth. Cell.Mol.Biol*. **5**, 25–40 (1994).

VIDAVER A.K.: Synthetic and complex media for the rapid detection of fluorescence of phytopathogenic *Pseudomonas*: effect of the carbon source. *Appl.Microbiol*. **15**, 1523–1524 (1967).

WESTRA A.A.G., SLACK S.A.: Isolation and characterization of extracelullar polysaccharide of *Clavibacter michiganensis* subsp. *sepedonicus*. *Phytopathology* **82**, 1193–1199 (1992).

WESTRA A.A.G., SLACK S.A.: Effect of interaction of inoculum dose, cultivar and geographic location on the magnitude of bacterial ring rot symptom expression in potato. *Phytopathology* **84**, 228–235 (1994).