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## APPLICATION OF IFAS AND PCR FOR *CLAVIBACTER MICHIGANENSIS* SUBSP. *SEPEDONICUS* DETECTION IN POTATO PLANTS DURING THE GROWING SEASON

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### Abstract

An indirect immunofluorescence antibody staining (IFAS) and polymerase chain reaction (PCR) were used to monitor potato stems, stolons and tubers of three cultivars for the presence of the ring rot causal agent, *Clavibacter michiganensis* subsp. *sepedonicus*, during the growing season. Mother tubers were inoculated with *C. michiganensis* subsp. *sepedonicus* and planted on experimental field of Laboratory of Diseases and Quarantine Pests of Potato in Bydgoszcz. The PCR tests conducted according to Schneider et al. (1993) or Firrao and Lozzi (1994) were more suitable for detection of *C. michiganensis* subsp. *sepedonicus* than IFAS test.

**Key words:** bacterial ring rot, *Solanum tuberosum*, immunofluorescence, PCR

### Introduction

The bacterium *Clavibacter michiganensis* subsp. *sepedonicus* (Spieckermann & Kotthoff) Davies et al. (Cms), which causes the ring rot disease of potato (*Solanum tuberosum* L.) is quarantine organism in the European Union and also it is considered as such organism by EPPO.

The bacterial cells of Cms pass from infected seed tuber into vascular system of stems and leaves, where they multiply and dislocate to roots, stolons and progeny tubers, sometimes within eight weeks after planting (De Boer and McCann 1989, De Boer and Hall 1996, De Boer et al. 1992).

Bacterial ring rot of potato is difficult to recognize. It cannot be detected by visual inspection of potato crop in field or in storage, unless disease symptoms became overt. However, symptoms do not appear until late in the growing season

and may be masked by senescence or other pathogens; sometimes symptoms do not develop at all (De Boer et al. 1992). Mild infections in susceptible and tolerant potato cultivars may lead to latent infections (Manzer et al. 1987, Manzer and McKenzie 1988). Quarantine procedure (Council Directive 93/85/EEC... 1993) is based on pre- and postharvest inspections and laboratory screening of latent infection of *C. michiganensis* subsp. *sepedonicus* in tubers using indirect immunofluorescence antibody staining (IFAS), plating of the plants extracts on semi-selective media and bioassay on eggplant.

Our experiments were performed to evaluate the detectability of bacteria during vegetation period in various tissues of potato plants grown from artificially inoculated seeds. Bacteria were detected using IFAS and PCR assays.

## Materials and methods

### Bacterial strains and inoculum

Bacteria of Cms strain isolated from field propagated tubers have been maintained in host potato plants during the winter and in spring time were moved and cultivated on YGM agar medium at 23°C. Bacterial cells were rinsed off from the plates with sterile water and diluted to approximately 10<sup>8</sup> cfu/ml concentration.

### Field experiment

Seed potatoes of susceptible cultivar 'Drop' and tolerant cultivars 'Merrimack' and 'Furore' with two–three sprouts, were inoculated using sterile scalpel dipped into Cms cell suspension. The bacteria were introduced into multiple wounds surrounding each sprout. Inoculated tubers were planted in field plots.

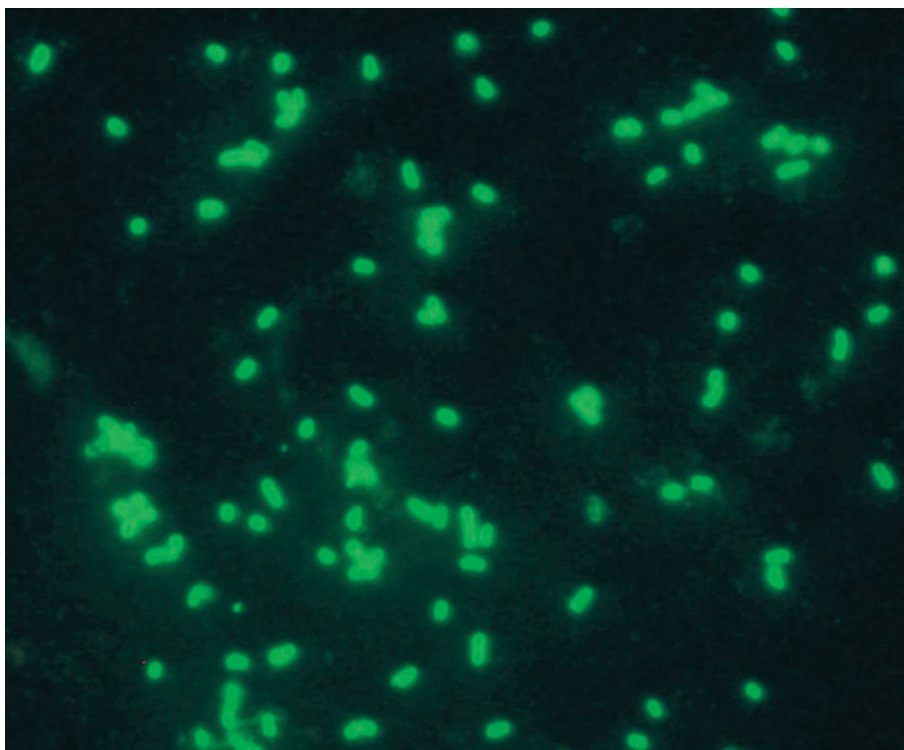
### Potato tissue samples

Plant tissues were collected on 10 terms once a week from the beginning of eight weeks after planting. Stems (10 cm segments up the soil level) and stolons were cut into 9–10 pieces.

### Laboratory testing

Stems, stolons and tubers tissues were tested for the presence of Cms using IFAS (Council Directive 93/85/EEC... 1993). The IFAS test (Phot. 1) was performed with polyclonal anti- *C. michiganensis* subsp. *sepedonicus* antiserum (IPO-DLO, The Netherlands).

Detectability of various domestic strains and most representative foreign strains of *C. michiganensis* subsp. *sepedonicus* from The Netherlands, USA, Russia and Finland was compared by PCR reaction (Phot. 2). 56 samples taken from bulk sample were tested using PCR (Schneider et al. 1993, Firrao and Lozzi 1994). DNA was isolated according to Davies et al. (1986) protocol.



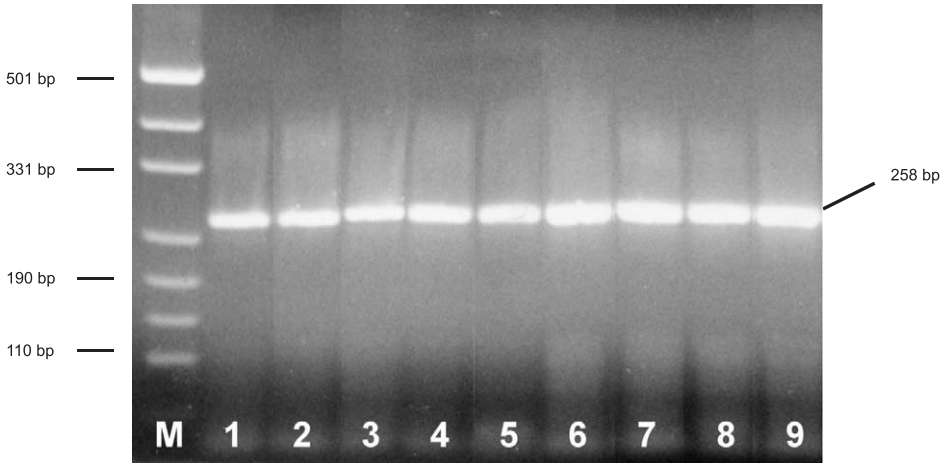
Phot. 1. Image of *Clavibacter michiganensis* subsp. *sepedonicus* cells in fluorescence microscope (magnification 650 ×) (photo by P. Gontarek)

An amplification was carried out in UNO-Thermoblock Biometra DNA thermocycler using the following PCR conditions, for primers CMS 6/7: initial denaturation at 95°C for 2 min, followed by 35 reaction cycles of 95°C for 1 min 30 s and 55°C for 1 min 30 s and 72°C for 1 min and 72°C for 10 min – final elongation (Schneider et al. 1993); for primers A 47 a/b: initial denaturation at 96°C for 2 min followed by 30 cycles of 96°C for 20 s and 70°C for 40 s and 72°C for 5 min – final elongation (Firrao and Lozzi 1994).

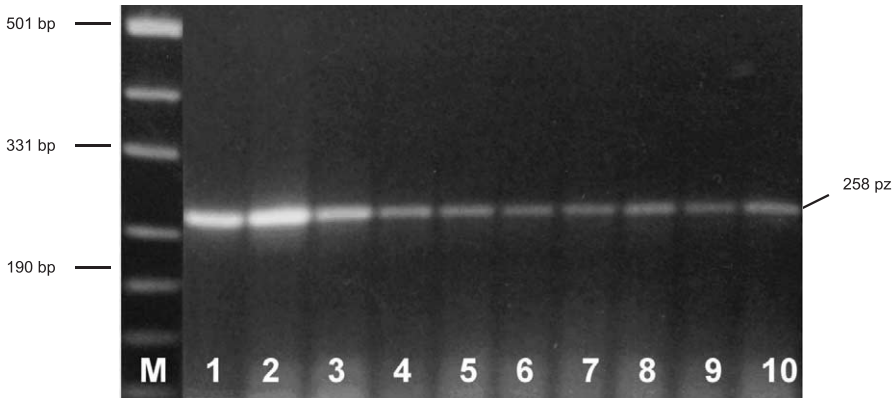
DNA isolated from domestic and representative strains of Cms isolated in Finland, Norway, The Netherlands, Russia and USA has been used for PCR reaction under the same conditions.

### Sensitivity of PCR

To determine the detection limit of the PCR, a culture of *C. michiganensis* subsp. *sepedonicus* strain BPR IOR 528 suspended to approximately  $10^8$  cfu/ml was serially diluted by 10-fold increments in sterile water. Number of viable bacterial cells was estimated by plating 100  $\mu$ l of the serial dilutions on YGM medium and expressed as colony forming units per 1 ml. DNA was extracted from the serial dilutions.



Phot. 2. PCR amplification of DNA from pure culture of *Clavibacter michiganensis* subsp. *sepedonicus* strains using primers according to Schneider et al. (1993) (CMS 6/7). Lanes 1–3 – BPR IOR (Pol): 527, 528, 529, lanes 4–9 – PD: 221 (Hol), 406 (Fin), 1488 (Rus), 1093 (USA), 330 (Nor), 323 (Hol), lane M – DNA size marker (100 bp ladder, Fermentas MBI, Lithuania) (photo by Z. Sadoch)



Phot. 3. Sensitivity of the PCR detection of *Clavibacter michiganensis* subsp. *sepedonicus* (strain BPR IOR 528) using CMS 6/7 primers (Schneider et al. 1993). Lanes 1–10 correspond to bacterial suspension:  $4 \times 10^8$ ,  $2 \times 10^8$ ,  $1 \times 10^8$ ,  $4 \times 10^7$ ,  $4 \times 10^6$ ,  $4 \times 10^5$ ,  $4 \times 10^4$ ,  $4 \times 10^3$ ,  $4 \times 10^2$ ,  $4 \times 10$  cfu/ml, lane M – DNA size marker (100 bp ladder, Fermentas MBI, Lithuania) (photo by Z. Sadoch)

## Results

Highest sensitivity detection of *C. michiganensis* subsp. *sepedonicus* in pure culture (40 cfu/ml) has been obtained by PCR procedure when specific primers (Schneider et al. 1993) were employed (Phot. 3).

The presence of *C. michiganensis* subsp. *sepedonicus* in different tissues of plants grown from artificially infected seed potatoes 'Drop' cv. was examined several times during growing season. PCR assay with CMS 6/7 primers allowed for Cms detection in eight weeks old stems. However, IFAS allowed to detect bacteria in one week older stems, stolons and tubers of susceptible 'Drop' cv. (Table 1), while positive IFAS results were obtained much later for tolerant 'Furore' cv. than for susceptible 'Drop' cv. (Table 2). Only doubtful IFAS results have been obtained for resistant 'Merrimack' cv. (Table 2).

The PCR tests with primers according to Schneider et al. (1993) and Firrao and Lozzi (1994) are more suitable for detection of *C. michiganensis* subsp. *sepedonicus* than IFAS test in susceptible 'Drop' cv. (Phot. 4, Fig. 1). Bacteria were detected in 44.6% samples by IFAS and in 46.5% samples by PCR with A 47 a/b primers and in 60.7% samples by PCR with CMS 6/7 primers.

Table 1

Detection of *Clavibacter michiganensis* subsp. *sepedonicus* in potato plants 'Drop' cv. at one week intervals during the growing season by IFAS and PCR

Cultivar	Part of plant	Weeks after planting																										
		8			9			10			11			12			13			14			15			16		
		A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
'Drop'	stem	-	+	+	+	+	+	-	+	+	+	+	+	?	+	+	+	-	+	+	+	+	/	/	/	/	/	/
	stolon	?	-	-	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+	/	/	/	/	/	/
	tuber	-	-	-	+	+	+	-	/	-	+	+	+	-	/	-	+	+	+	+	+	+	+	+	+	+	+	+

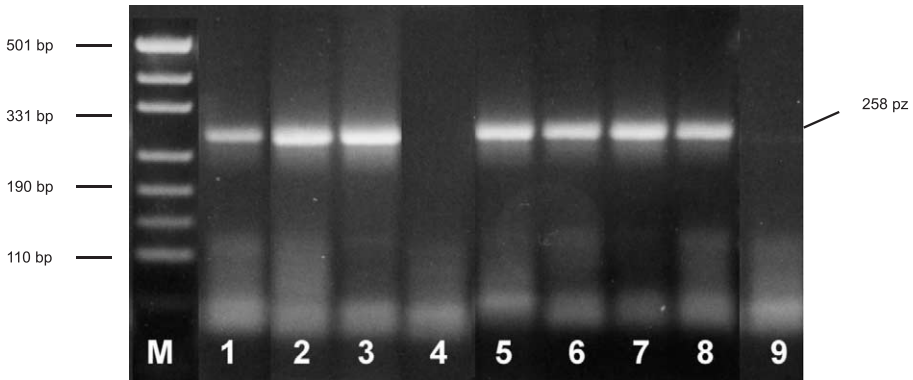
+ - positive test result, -- negative test result, ? - doubtful test result, / - not tested.  
A - IFAS, B - PCR with primers A 47 a/b, C - PCR with primers CMS 6/7.

Table 2

Detection of *Clavibacter michiganensis* subsp. *sepedonicus* in potato plants 'Furore' and 'Merrimack' cvs at one week intervals during the growing season by IFAS

Cultivar	Part of plant	Weeks after planting									
		8	9	10	11	12	13	14	15	16	
'Furore'	stem	-	-	-	-	-	-	?	/	/	
	stolon	-	?	-	+	+	?	+	/	/	
	tuber	-	-	-	-	-	-	-	-	-	
'Merrimack'	stem	-	-	-	-	?	?	-	-	/	
	stolon	-	-	-	-	-	-	-	-	/	
	tuber	-	-	-	-	-	-	-	-	-	

+ - positive test result, -- negative test result, ? - doubtful test result, / - not tested.



Phot. 4. PCR amplification of DNA isolated from stems, stolons and tubers of the plants, which were grown from artificially infected seeds. Primers CMS 6/7 were employed for PCR reaction (Schneider et al. 1993). Lanes 1–8 – DNA from different extracts of potato, lanes 1, 4 – tubers, lanes 2, 5, 7 – stolons, lanes 3, 6, 8 – stems, lane 9 – negative control, lane M – DNA size marker (100 bp ladder, Fermentas MBI, Lithuania) (photo by Z. Sadoch)

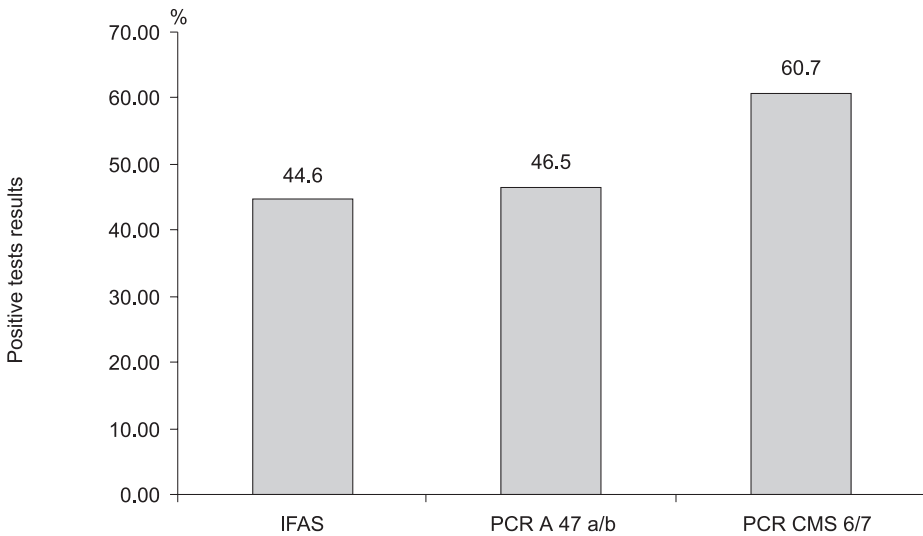


Fig. 1. Comparison of *Clavibacter michiganensis* subsp. *sepedonicus* detection in potato plants by eight to 16 weeks after planting using IFAS and PCR

## Discussion

*Clavibacter michiganensis* subsp. *sepedonicus* cells move by passive transport throughout the vascular tissue, and then migrate back to newly formed stolons and tubers. Important for crop indexation is an early detection of bacterial infection. Presented results indicate, that lower part of the stems of susceptible cultivars like

'Drop' develops sufficient numerous bacterial population to be detected already eight weeks after planting, when PCR assay has been employed for examination. Detection of bacteria has been possible by IFAS assay one week later, when bacteria were already present in all underground parts of the plant. Spreading of the bacteria within tissues of tolerant cultivar – 'Furore' – was much less dynamic, while the presence of bacteria in the tissues of resistant cv. 'Merrimack' was barely detectable by IFAS assay.

Similar results were obtained by De Boer et al. (1992), who were able to detect *C. michiganensis* subsp. *sepedonicus* in the stems of susceptible cultivar 'Red Pontiac' 55 days after planting of infected seed potatoes. However, when seed pieces of the susceptible cultivar 'Red Pontiac' were given low inoculum doses, *C. michiganensis* subsp. *sepedonicus* was not detected until in the next season. Sometimes, Cms-contaminated tubers produce plants free of the pathogen (De Boer and McNaughton 1986, De Boer and McCann 1989). There is not enough knowledge, which factors affect the rate of bacteria multiplication and migration within the vascular tissues of various potato cultivars. Movement may be delayed or prevented by competition with other bacteria (De Boer et al. 1992). Low level of inoculum might trigger defence reactions, which can arrest pathogen growth. Romanenko et al. (1997, 1999) reported specific response of various cultivars on exopolysaccharidic bacterial toxin.

Results suggests, that successful indexation of potato crops might be dependent on the behaviour of bacterial cells in plant tissues. Some cultivars are prone to prolonged latent phase of infection. Therefore recognition of those cultivars belonging to this category, is reasonable step for ring rot eradication. Tolerant cultivars are suspected to be the source of ring rot bacteria dissemination. Symptomless appearance of these plants makes them difficult to trace (Slack 1987).

## Literature

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