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Chapter 5. PLANT PROTECTION

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BACTERIA ASSOCIATED WITH *CLAVIBACTER MICHIGANENSIS* SUBSP. *SEPEDONICUS* IN POTATO TUBER AND EGGPLANT SAMPLES

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Abstract

Clavibacter michiganensis subsp. *sepedonicus* (Spieckermann & Kotthoff) Davis et al. (1988) (*Cms*), causing potato ring rot, is a quarantine organism. Epiphytic and endophytic bacteria associating with *Cms* in potato samples may complicate *Cms* detection. A total of 150 non-*Cms* strains of bacteria were found at populations of 10^4–10^5 cfu ml^{-1} per one potato tuber or eggplant sample, which were screened in IF (imunofluorescence) and PCR (polymerase chain reaction) tests. The rate of bacteria cross-reacting with monoclonal antibody for *Cms* (Agdia Company, USA) in IF was 2.6%. In contrast, PCR proved to be very specific, because no amplification products were observed with the non-*Cms* strains. Using the Microbial Identification System BIOLOG, ca 51% recovered bacterial strains were presumptively identified. Among the gram-negative bacteria, *Pseudomonas fluorescens* and *Pantoea dispersa* were found most frequently, while the most common gram-positive strains were *Staphylococcus epidermidis, Micrococcus luteus* and *Aureobacterium saperdae.* The potential plant-pathogenic bacteria such as *Agrobacterium tumefaciens / radiobacter* also were recovered.

Key words: BIOLOG, IFAS, PCR, ring rot of potato

Introduction

Clavibacter michiganensis subsp. *sepedonicus* (Spieckermann & Kotthoff) Davis et al. (1988) (*Cms*), the ring rot pathogen on potato, is included among the harmful quarantine organisms in many countries around the world. Potato ring rot is an economically important disease for the potato industry /Van der Wolf et al., 2005/. The main modes of transmission of the bacterium are via infected seed potatoes or contaminated equipment and packaging / storage material /EPPO/CABI, 1997/. For the

control and eradication of the pathogen, phytosanitary regulations depend on the availability of disease-free seed tubers.

Bacteria associating with *Cms* in potato samples might complicate detection of the ring rot pathogen if they cross-react with antibodies or have similar sequences like those that are targets for primers for *Cms.* They might also limit the growth and survival of *Cms* in potato tissues.

The official EU testing method, including disease diagnosis, detection and identification of the ring rot pathogen is based on IFAS (indirect fluorescent antibody stain) and PCR as initial screening tests in order to isolate the pathogen /OEPP/EPPO, 2006/. The bioassay on eggplants (cv. ʻBlack Beautyʼ and other sensitive cultivars) is also presented for isolation and confirmation of *Cms*.

Other immunochemical and also biochemical and molecular-biological tests are used for diagnosis of *Cms* /Gudmestad et al., 1991; Schaad et al., 1999; Pastrik, 2000; Kokošková et al., 2005/. Immunochemical methods are not always reliable because of limited sensitivity and specificity of available antibodies /De Boer et al., 1996; Pastrik, Rainey, 1999; Van der Wolf et al., 2005/. A monoclonal antibody for *Cms* showed close serological relationship to other *Clavibacter michiganensis* subspecies in cross-reacting with *C. m.* subsp. *michiganensis* and *C. m.* subsp. *insidiosus*. Different saprophytic bacteria that have been isolated occasionally from potato tissue cross-reacted with monoclonal antibodies for *Cms* in immunochemical methods /De Boer et al., 1996/.

The aim of the work was to characterise bacteria associating with *Cms* in potato and eggplant samples that might complicate a detection of the ring rot causal agent. In particular, the study deals with bacteria isolated from samples, in which *Cms* has been confirmed.

Material and methods

Material. Three groups of samples were investigated: extracts prepared from potato tubers, extracts prepared from eggplants inoculated with extracts from potato tubers and macerates prepared from eggplants inoculated with pure cultures of *Cms.* The first group (extracts prepared from potato tubers) and the second group (extracts prepared from eggplants inoculated with extracts from potato tubers) were samples analysed by Plant Protection Service (PPS) according to OEPP/EPPO (2006) using IF test and bioassay on eggplants cv. ʻBlack Beautyʼ and after that released for our tests. Samples of extracts were stored at −20 °C. The third group of samples was formed of macerates prepared from eggplant petioles or leaves inoculated by *Cms* at our laboratory.

Bacteria. Numbers of bacteria in plant samples were found by colony counting after their growth on plates where 10 µl aliquots from serial dilutions (neat, diluted 1:10, 1:100, 1:1000) were plated on C medium /Snieszko, Bonde, 1943/ or NBY medium /EPPO/CABI, 1997/. Isolates were distinguished according to morphological characteristics and several common tests (Gram stain, O/F test, oxidase test, catalase test, malachite test) and placed in several groups.

Apart from isolates recovered from plant samples, reference strains originating from two Czech and three foreign collections of microorganisms were tested (data not shown). Strains isolated from potato and eggplant samples were compared to reference strains for morphology and biochemical tests.

Biochemical tests. Subsequently, strains were identified using the *Biolog MicroPlate System*™, version 4.01 B. Bacterial suspensions were prepared in sterile buffer adjusted to an optical density at 590 nm as recommended by manufacturer's recipe (Biolog Inc., USA). Evaluation was performed by naked eye after 4, 24 and 48 h of incubation. Cultures were identified using the *MicroLog*™ 2 database for gram-positive and gram-negative bacteria. Identification of bacteria is described by similarity index (SIM) = 0.000–1.000, which is included in manual of Microbial Identification System BIOLOG. Reliability of identification was evaluated as very good (SIM = 0.750–1.000), good (SIM = 0.500–0.749) or none (SIM = 0.000–0.499).

Tests of FAA (fatty acid analysis) with selected bacterial isolates were conducted in diagnostic laboratory of the State Phytosanitary Administration, Olomouc, Czech Republic. Whole cell fatty acids were extracted and analysed as methyl esters derivatives using the Microbial Identification System MIDI (Newark, USA) according to Janse (1991) and Stead et al. (1992).

Immunochemical tests. The strains investigated were evaluated by IFAS to identify potential cross-reactions with monoclonal antibodies for *Cms* (Agdia Company, USA) carried out according to manufacturer's recipe, but preparation and evaluation of slides was conducted according to OEPP/EPPO (2006). Isolates were tested at optical density 0.1, 0.01, 0.001 and 0.0001 (620 nm). Immunofluorescent slides were observed under a light microscope fitted for epifluorescence at 1000x magnification using a mercury lamp and suitable filter system.

Molecular tests. The strains investigated were also tested by PCR to identify any similar sequences that are target for primers for diagnosis of *Cms*. PCR was performed on a Mini Cycler (MJ Research, Watertown, MA, USA). For the specific amplification of *Cms* strains, the pathogen – specific primer set PSA-1/PSA-R was used /Pastrik, Rainey, 1999/. Reaction mix and amplification protocol were performed according to Pastrik (2000). After the PCR, aliquots of the reaction mixture were resolved by electrophoresis on a 1% agarose gel. DNA fragments were visualized by staining with SYBR GREEN solution or ethidium bromide.

Biological tests. Test of pathogenicity on carrot discs was used for strains presumptively identified using *Biolog Bacteria* and FAA methods as potential plant pathogenic bacteria /Klement et al., 1990/.

Results and discussion

The frequency of recovered bacteria in potato tuber and eggplant samples usually ranged from 10^4–10^5 cfu ml^{-1} per sample (Table 1). The highest populations of associated bacteria were found for the group of strains from eggplants inoculated with *Cms.* In groups of extracts stored before testing at −20 °C, the number of associated bacteria per one extract was lower (Table 1). Bacteria probably died during the time of low storage temperature. Janse and Vaerenbergh (1987) published, that freezing of potato extracts resulted in a significant reduction in the number of bacteria present. The difference could be also caused by the lower competitive ability of some bacteria, which did not survive long-term at −20 °C.

Some of recovered strains might behave as potential antagonists of ring rot pathogen. De la Cruz et al. (1992) published data about *Pseudomonas fluorescens* strains

significantly reducing *Cms* population, ring rot infection, and symptom expression when inoculated into potato seedlings.

Table 1. Number of bacteria associating with *Clavibacter michiganensis* subsp. *Sepedonicus* in potato tuber and eggplant samples, their cross-reactivity in serological tests and identification using *Biolog Bacteria* method
1. lentelė. *Bakterijų, paplitusių kartu su Clavibacter michiganensis subsp. sepedonicus, kryžminė sąveika serologiniuose testuose ir identifikavimas taikant Biolog Bacteria metodą bulvių gumbų ir baklažanų bandiniuose*

Origin of isolates *Izoliatų kilmė*	Number of investigated samples / recovered isolates *Tirtų bandinių / nustatytų izoliatų kiekis*	Assessment of frequency of bacteria per sample (cfu ml^{-1}) *Bakterijų dažnumo bandinyje įvertinimas (ksv ml^{-1})*	Number of cross-reacting isolates *Kryžmiškai reaguojančių izoliatų skaičius*		Isolates identifed in *Biolog Bacteria* of all 146 tested % *Biolog Bacteria metodu identifikuoti izoliatai (tirta 146) %*
			IFAS	PCR/PGR	
Potato tuber extracts *Bulvių gumbų ekstraktai*	31/5	6.30×10^4	2	0	48.4
Eggplants inoculated with potato tuber extracts *Baklažanai, inokuliuoti bulvių gumbų ekstraktais*	16/23	4.65×10^4	0	0	56.5
Eggplants inoculated with *Cms*[a] *Baklažanai, išokuliuoti Cms*	7/32	7.91×10^4	2	0	46.9
Total number *Bendras skaičius*	54/150	/[b]	4	0	50.6
Total % *Iš viso %*	100	/	2.6	0	-

[a] *Cms – Clavibacter michiganensis* subsp. *Sepedonicus.*
[b] – not applicable / *netaikytinas.*
IFAS – indirect fluorescent antibody stain/ *netiesioginis fluorescencinis antikūno metodas.*
PCR – polymerase chain reaction / *PGR – polimerazės grandininė reakcija.*

A lot of saprophytes were found in tissue of potato and eggplants. A total of 150 bacterial strains were randomly selected for screening tests. Gram-negative bacteria occurred more frequently than gram-positive bacteria. Among the gram-negative strains,

the most frequently isolated bacteria were *Pseudomonas fluorescens* and *Pantoea dispersa,* further *Flavobacterium marinotypicum, Sphingomonas paucimobilis* and others (Table 2). Among the gram-positive strains, the most frequently found were bacteria *Staphylococcus epidermidis*, *Micrococcus luteus*, *Aureobacterium saperdae*, *Curtobacterium albidum*, *Kocuria rosea* and others (Table 2). Sturz et al. (1999) during a study of periderm of healthy potato tubers also often isolated *Staphylococcus* sp. and *Pseudomonas* sp. We suggest these bacteria are commonly occurring saprophytes in tissue of potato.

Table 2. Saprophytic bacteria associating with *Clavibacter michiganensis* subsp. *Sepedonicus* in potato tuber and eggplant samples identified presumptively using the Microbial Identification System BIOLOG

2 lentelė. *Saprofitinės bakterijos, paplitusios kartu su Clavibacter michiganensis subsp. Sepedonicus, identifikuotos bulvių gumbų ir baklažanų bandiniuose naudojant mikrobų identifikavimo sistemą BIOLOG*

Bacteria *Bakterijos*	GN / GP[a]	N[b]	SIM[c] (average) *Panašumo indeksas (vidutinis)*	
			0.750–1.000 very reliable *labai patikimas*	0.500–0.749 reliable *patikimas*
Paenibacillus sp.	GP	3	0.847	
Aureobacterium flavescens	GP	3	0.832	
Staphylococcus epidermidis	GP	10	0.758	
Aureobacterium saperdae	GP	5	0.756	
Flavobacterium marinotypicum	GN	3	0.757	
Sphingomonas paucimobilis	GN	3	0.760	
Pseudomonas fluorescens	GN	7		0.678
Rhodococcus rhodochorus	GP	1		0.673
Micrococcus luteus	GP	9		0.661
Pasteurella betyae	GN	1		0.640
Cellulomonas cellulans	GP	3		0.613
Actinobacillus suis	GN	2		0.607
Curtobacterium albidum	GP	4		0.602
Rhodococcus australis	GP	3		0.591
Phyllobacterium myrsinacearum	GN	1		0.563
Clavibacter michiganensis	GP	3		0.561
Staphylococcus pasteuri	GP	3		0.555
Bacillus sp.	GP	2		0.519
Kocuria rosea	GP	4		0.514
Pantoea dispersa	GN	4		0.504

[a]GN / GP – gram-negative / gram-positive bacteria / *gramneigiamos / gramteigiamos bakterijos.*
[b]N – number of tested isolates / *tirtų izoliatų skaičius.*
[c]SIM – index similarity (0.000–1.000) / *panašumo indeksas*.

Using the Microbial Identification System BIOLOG, ca 51% of all tested bacterial strains were presumptively identified (Table 1). The 4-hour incubation interval did not facilitate reliable identification of any strain, which agrees with the experience of Krejzar and Kokošková (2000), when using the *Biolog* system. The 24-hour incubation interval was used most frequently for evaluation of *Biolog* microplates.

Reliability of species identification for rhizosphere bacteria using the Microbial Identification System BIOLOG was assumed according to identification of individual tested strains. According to the evaluation criterion of the manufacturer, a strain tested is identified when the index of similarity (SIM) is higher than value 0.5. In Table 2, species were sorted in descending order according to value of index similarity. Species placed at the top of the table (SIM = 0.750–1.000) were identified more reliably, while species at the bottom of the table were identified less reliably (SIM = 0.500–0.749). Bacteria with lower SIM than 0.500 were not included in Table 2.

The reliable identification of some bacteria was impossible. Some strains were not able to survive during subculturing on agar media. Several rough (non-mucoid) strains were probably not identified because of decreased metabolic activity compared with smooth strains /Kokošková, Kůdela, 2002/. Pathovar metabolic patterns of some bacterial species are so similar in the BIOLOG databases that it was impossible to identify some strains tested to pathovar / subspecies level, which agrees with Jones et al. (1993) results. The decreased metabolic activity of gram-positive spore-forming bacteria such as *Bacillus* sp. resulted in difficult identification to the species level confirming the conclusions in the product manual. In this study, identification of all spore-forming isolates was considered reliable only to the genus level, because reference strains of *Bacillus cereus*, *B. megaterium* and *B. subtilis* behaved similarly. In addition, databases of some bacterial species in the Microbial Identification System BIOLOG are insufficient and need to be improved /Jones et al., 1993; Kokošková, Kůdela, 2002/.

Biochemical tests (*Biolog Bacteria* and FAA) and tests of pathogenicity showned that potential plant pathogenic bacteria *Agrobacterium tumefaciens / radiobacter* occurred also among potato rhizosphere. Two isolates were confirmed as *A. tumefaciens / radiobacter* using BIOLOG system and FAA. The symptoms on carrot discs were comparable with reference strain (CCM 2835), but at isolate no. 7 were very weak (Table 3).

Of all 150 strains tested in IFAS, the four of them cross-reacted with monoclonal antibody from Agdia Co. (USA) for *Cms* (Table 1). The cross-reacting strains were not reliably identified using *Biolog Bacteria* system. In all cases, much lower frequency of fluorescent cells was observed in microscope compared to the positive control. In three cases there were typical and in one case atypical shaped cells. One of the causes of cross-reactions could be common antigenic determinants content in cells of cross-reacting bacteria published by Crowley and Boer (1982) who during screening of bacteria isolated from potato stems found cross-reacting coryneform and non-coryneform bacteria when using the IF test. The rate of bacterial cross-reactions with monoclonal antibody for *Cms* (Agdia Company, USA) was 2.6% in our IF tests. This value is comparable to results reported previously /De Boer et al., 1996; Kokošková, Jeřábková, 2001/. Monoclonal antibody from "Agdia" used in our study is considered of high quality. Excellent results were obtained in comparable experiments conducted in

eight diagnostic laboratories parallel within four years, where specificity of IFAS was 100%, because none false positives occurred /De Boer, Hall, 2000/.

Table 3. Potential plant pathogenic bacteria associating with *Clavibacter michiganensis* subsp. *sepedonicus* in potato tuber samples identified using biochemical and pathogenicity tests
3 lentelė. *Potencialiai augalams patogeniškos bakterijos, paplitusios kartu su Clavibacter michiganensis subsp. sepedonicus, bulvių gumbų bandiniuose identifikuotos, naudojant biocheminius ir patogeniškumo testus*

Strain *Kamienas*	Name *Pavadinimas*	Reliability of methods *Metodų patikimumas*		Test of pathogenicity on carrot discs *Patogeniškumo tyrimas ant bulvių diskų*
		Biolog Bacteria	Fatty acid analysis *Riebiųjų rūgščių analizė*	
CCM 2835	*Agrobacterium radiobacter / tumefaciens*	>0.5	>0.5	+
1	*Agrobacterium radiobacter / tumefaciens*	>0.5	>0.5	+
7	*Agrobacterium radiobacter / tumefaciens*	>0.5	>0.5	(+)

Note. CCM – Czech Collection of Microorganisms, Brno, CR.
Pastaba. CCM – Čekijos mikroorganizmų kolekcija, Brno, ČR.
Identification: reliable (>0.5) / *Identifikavimas: patikimas* (*>0.5*).
+ – positive reaction / *teigiama reakcija*, (+) – very week positive reaction / *labai silpna teigiama reakcija.*

Conclusions

The official EU testing protocol, including detection and identification of the ring rot pathogen is based on IFAS and PCR as initial screening tests and a bioassay with eggplant for isolation and confirmation of *Cms*. Our results showed the rate of bacteria cross-reacting with monoclonal antibody for *Cms* (Agdia Company, USA) in IFAS to be less than 3%. PCR test showed the high specificity of the primers developed by Pastrik and Rainey (1999), because none of the non-*Cms* strain showed the same or similar amplification products as *Cms*. If IF test was combined with a highly specific PCR test, the results of *Cms* detection might be more reliable, as has recently been recognised and is reflected in a recent update of the EU official testing method /OEPP/EPPO, 2006/.

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BAKTERIJOS, PAPLITUSIOS KARTU SU *CLAVIBACTER MICHIGANENSIS* SUBSP. *SEPEDONICUS* BULVIŲ GUMBŲ IR BAKLAŽANŲ BANDINIUOSE

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S a n t r a u k a

Bakterija *Clavibacter michiganensis* subsp. *sepedonicus* (*Cms*), sukelianti bulvių žiedinį puvinį, yra karantininis organizmas. Epifitinės ir endofitinės bakterijos, susijusios su *Cms* bulvių bandiniuose, gali komplikuoti *Cms* nustatymą. Bulvių gumbų ir baklažanų bandinius patikrinus IF (imunofluorescencija) ir PGR (polimerazės grandininė reakcija) metodais, viename bandinyje buvo nustatyta 10^4–10^5 ksv ml^{-1} bakterijų populiacijų, tarp jų 150 – ne *Cms* bakterijų kamienai. Bakterijų, kryžmiškai reaguojančių su monokloniniu antikūnu dėl *Cms* („Agdia Company“, USA), kiekis IF buvo 2,6 %. Ir atvirkščiai, PGR pasirodė labai specifiška, nes nebuvo pastebėta amplifikacijos produktų su ne *Cms* kamienais. Naudojant mikrobų identifikavimo sistemą BIOLOG, buvo nustatyta apie 51 % atkurtų bakterinių kamienų. Tarp gramneigiamų bakterijų *Pseudomonas fluorescens* ir *Pantoea dispersa* buvo randama dažniausiai, o patys dažniausi gramteigiami kamienai buvo *Staphylococcus epidermidis*, *Micrococcus luteus* ir *Aureobacterium saperdae*. Augalams potencialiai patogeniškos bakterijos – *Agrobacterium tumefaciens / radiobacter* – taip pat buvo nustatytos.

Reikšminiai žodžiai: BIOLOG, IFAS, PGR, bulvių žiedinis puvinys.