Characteristics of Polyclonal Antisera for Detection and Determination of *Clavibacter michiganensis* subsp. *insidiosus*

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Abstract

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Three polyclonal antisera for detection and determination of *Clavibacter michiganensis* subsp. *insidiosus*, the causal agent of bacterial wilt of lucerne, were prepared. The original titers of these antisera CI 12, CI 13 and CI 14 were 1:32, 1:256 and 1:64, respectively. Their specificity was increased after cross-absorption with *Ralstonia solanacearum* and *Pseudomonas fluorescens*, after which the titers of antisera CI 12, CI 13 and CI 14 decreased to 1:16, 1:128 and 1:8, respectively. Polyclonal antisera CI 12 and CI 13 could be used for determination and detection of *C. m. insidiosus* by the ELISA method. Using PTA ELISA, a homologous antigen was identified at a concentration of 10^6–10^5 cfu/ml, while with DAS ELISA only at one of 10^7–10^6 cfu/ml. Highly fluidal strains of *C. m. insidiosus* were not reliably identified by the polyclonal antisera. The PTA ELISA test was suitable for rapid negative diagnosis.

Key words: bacterial wilt of lucerne; *Clavibacter michiganensis* subsp. *insidiosus*; slide agglutination, ELISA

Bacterial wilt of lucerne is widespread in many countries over the world (OEPP/EPPO 1997). Significant losses are frequent in the 2nd a 3rd years of planting in areas with a colder climate (MOFFETT *et al.* 1983). The disease is caused by *Clavibacter michiganensis* subsp. *insidiosus* (McCulloch) Davis, Gillaspie, Vidaver et Harris, which is an important quarantine organism (OEPP/EPPO 1997). In the Czech Republic, the pathogen was first recorded in the 1960-s (KŮDELA 1969).

Bacteria living in roots and crowns of infected plants commonly survive in infected plant debris (CORMACK 1961) and seed (CORMACK & MOFFATT 1956). They are transmitted by nematodes (HAWN 1963) and also by *Sitona lineatus* (KŮDELA *et al.* 1984). The pathogen is dangerous because it survives in seed for many years, and can thus be disseminated over large distances, complicating international trade. This is practically the only way to introduce the pathogen to an area where it did not occur before (OEPP/EPPO 1997). The only reliable protection is to use seed from areas free of the agent of bacterial wilt.

Testing the health state of seed is performed according to EPPO methods (OEPP/EPPO 1982, 1990, 1997). Surveys in lucerne stands during the growing season are also recommended, but are insufficient to assure healthy seed. As no reliable method for detection of the pathogen in seed exists, various diagnostic methods are combined. Neither an antiserum for immunochemical tests nor primers for methods of molecular biology are available on the market. Common physiological tests and biochemical tests, such as cultivation on selective media, O/F (oxidative-fermentative) test, and Gram staining are used. Slide agglutination (HALE 1972) and IF (immunofluorescence) staining (NÉMETH *et al.* 1991) with antisera, which are prepared in laboratories conducting diagnostics of *C. m. insidiosus*, and tests of the pathogenicity on lucerne are carried out (KŮDELA 1970). These tests are very labor- and time-consuming and not reliable enough. The results of tests influence the decision whether to allow or deny permission of export or import of seed lots.

Bacterial strains of *C. m. insidiosus* include not only fluidal, but also nonfluidal strains. Sometimes nonfluidal strains cannot be easily identified by serological methods. Fluidal strains are observed more frequently and usually are more virulent than nonfluidal strains. These difficulties with fluidal strains are due to the amount of extracellular polysaccharides in cell walls of the bacteria (VAN ALFEN *et al.* 1987).

We prepared polyclonal antisera for determination of *C. m. insidiosus* bacteria by slide agglutination test and ELISA (enzyme-linked immunosorbent assay) with the aim to verify their specificity and to optimize serological methods for the detection of *C. m. insidiosus* in lucerne seed.

MATERIAL AND METHODS

Bacterial Cultures and Culture Media: Isolates of *C. m. insidiosus* obtained from naturally infected lucerne plants and seed were used in our tests. Most of them were isolated from infected plant tissues of varieties susceptible to bacterial wilt planted at the Plant Breeding Station Želešice.

The isolates were confirmed as *C. m. insidiosus* by their morphological, physiological and biochemical properties (ERWIN 1990), and some of them also through tests of pathogenicity on lucerne (KŮDELA *et al.* 1984). Bacterial strains were cultivated 4–6 days on medium C (SNIESZKO & BLONDE 1943) or on nutrient glucose agar (NGA) at 21–23°C.

Strain *C. m. insidiosus* CCM $C_2$5 from the Czech Collection of Microorganisms (Brno) and strain 37 from our own collection at the Bacteriology Department were chosen to prepare the antigen (Table 1).

Some of the tested isolates were highly fluidal, others were nonfluidal according to their morphological characteristics, but most of them did not belong definitely to one or the other of these two groups (Table 1).

Table 1. Determination of *Clavibacter michiganensis* subsp. *insidiosus* by slide agglutination with polyconal antisera CI 12 and CI 14

Strain	Origin	Antiserum	
		CI 12	CI 14
Cmi $C_2$5 (f)	CCM	+++	–
Cmi 37/96 (nf)	RICP	++	+++
Cmi 1589	CCM	(+)	++
Cmi 2684 (f)	CCM	–	–
Cmi 9/96	RICP	+	++
Cmi 12/5/96 (f)	RICP	–	–
Cmi 12/6/96 (nf)	RICP	+	++
Cmi 6/96	RICP	+	++
Cmi 3/2 96	RICP	++	+++
Cmi 3/4/96	RICP	+++	+++
Cmi 13/96	RICP	(+)	+
Cmi 15/96	RICP	+	+
Cmi 18/96	RICP	–	+
Cmi 21/96	RICP	+++	+++
Cmi 35/96 (nf)	RICP	+++	+++
Cmi 40/96	RICP	+	+
Cmi 61/96 (nf)	RICP	++	++
Cmi 50/1/96	RICP	++	+++
Cmi 8/1/98	RICP	++	+
Cmi 8/2/98	RICP	+	+

CCM – Czech Collection of Microorganisms
RICP – collection of plant pathogenic bacteria, Department of Bacteriology, Research Institute of Crop Production
f – fluidal; nf – nonfluidal
negative reaction = –; intensity of positive reaction = +, ++, +++; between negative and weak positive reaction = (+)

Antiserum Production: Antigens were prepared from bacterial isolates that were grown on YDC (yeast extract-dextrose-calcium carbonate) medium for 5 days (LELLIOT & STEAD 1987). The cultures were maintained in heavy suspensions in sterile saline (8.5 g NaCl, 1 l destilled water, pH 7.2) and centrifuged for 10 min and 10 000 g. The formaldehyde-fixed antigens were prepared by treating the suspensions with formaldehyde at a final concentration of 1% in a thermostat of 21°C overnight. The suspensions were centrifuged for 10 min at 10 000 g next day. The cells were resuspended in sterile saline, adjusted to a concentration of 10^5cfu/ml (colony forming units) and used for immunization (EDWARDS & EWING 1972).

The antigens were injected into each of two rabbits per antigen at weekly intervals according to an immune schema (HALE 1972). On the 10th day after the last injection, the rabbits were bled at the marginal ear vein. The blood was clotted for 1 h at 37°C, refrigerated overnight at 4°C and next day centrifuged at 3000 g for 20 min. Antisera were evaluated in a slide agglutination test (CLAFLIN & SHEPARD 1977). They were titred with homologous antigens of *C. m. insidiosus* and with heterologous antigens of related subspecies of *Clavibacter michiganensis* (subsp. *michiganensis*, subsp. *sepedonicus*, subsp. *nebraskensis*) and antigens of the genera *Agrobacterium, Curthobacterium, Erwinia, Pantoea, Pseudomonas, Ralstonia and Xanthomonas* (Table 2).

The specificity of antisera was increased by cross-absorption (AZAD & SCHAAD 1988). For short-term storage, sodium azide (0.1%) was added as a preservative and the antisera were stored at 2°C. Antisera with good specificity were used for IgG preparation, those with lower specificity in a slide agglutination test.

Slide Agglutination (SA): Slide agglutination tests were conducted by mixing equal amounts (20 µl) of diluted antiserum and bacterial suspension on a slide. Whole untreated bacterial cells suspended in sterile saline were used as antigens in concentrations of 10^8 to 10^9 cfu/ml. Antisera were diluted in sterile saline. The slides were incubated in a humid chamber, and precipitation was evaluated after 15 min and 30 min with a stereoscope Meopta. Reaction and intensity were classified according to the following scale: +++ = strong positive reaction, distinct agglutination and clearing of the suspension background in 15 min; ++ = positive reaction, distinct agglutination and clearing of background in 30 min; + = slight agglutination without clearing of background in 30 min; – = negative reaction, the suspension remains uniformly milky.

Enzyme-linked Immunosorbent Assay (ELISA): The immunoglobulins were precipitated with a saturated solution of ammonium sulfate. The IgG fraction was dialyzed overnight against 100mM Tris with 100mM NaCl, pH 7.8, and then purified using DEAE-cellulose (Serva), following the manufacturer's instructions. Purified IgG was adjusted to 1 mg/ml and conjugated with alkaline phosphatase (Boehringer Mannheim) using a one-step glutaraldehyde method (CLARCK & ADAMS 1977).

Table 2. Determination of cross-reactions of polyclonal antisera CI 12 and CI 14

Bacteria	Strain	Age of bacterial culture (days)	Reaction before cross-absorption	Reaction after cross-absorption	
			CI 12/CI 14	A1 CI 12/CI 14	A2 CI 12/CI 14
Clavibacter michiganensis subsp. *insidiosus*	$C_2$5	4	+++/+++	+++/+++	+++/+++
Clavibacter michiganensis subsp. *insidiosus*	37	4	++/+++	++/+++	++/+++
Clavibacter michiganensis subsp. *insidiosus*	9	4	+++/+++	+++/+++	+++/+++
Clavibacter michiganensis subsp. *insidiosus*	12	4	–/–	–/–	–/–
Agrobacterium tumefaciens	2835	2	(+)/–	–/–	–/–
Clavibacter michiganensis subsp. *sepedonicus*	3467	4	–/–	–/–	–/–
Clavibacter michiganensis subsp. *sepedonicus*	3279	4	–/–	–/–	–/–
Clavibacter michiganensis subsp. *michiganensis*	109	4	–/–	–/–	–/–
Clavibacter michiganensis subsp. *michiganensis*	202	4	–/–	–/–	–/–
Clavibacter michiganensis subsp. *nebraskensis*	2749	4	–/–	–/–	–/–
Curthobacterium flaccumfaciens	2403	4	(+)/–	–/–	–/–
Erwinia amylovora	1617	1	–/(+)	–/–	–/–
Erwinia carotovora subsp. *carotovora*	1008	1	–/–	–/–	–/–
Erwinia chrysanthemi	989	1	–/–	–/–	–/–
Pantoea agglomerans	2406	1	–/–	–/–	–/–
Pseudomonas syringae pv. *syringae*	4073	2	(+)/(+)	–/–	–/–
Pseudomonas fluorescens	2115	2	+++/+++	+++A2/+++A2	–/–
Ralstonia solanacearum race 1	1579	2	–/+	–/–	–/–
Ralstonia solanacearum race 2	5712	2	+/+(+)	–/–	–/–
Ralstonia solanacearum race 3	2505	2	++A1/++A1	–/–	–/–
Xanthomonas vesicatoria	2102	2	–/–	–/–	–/–

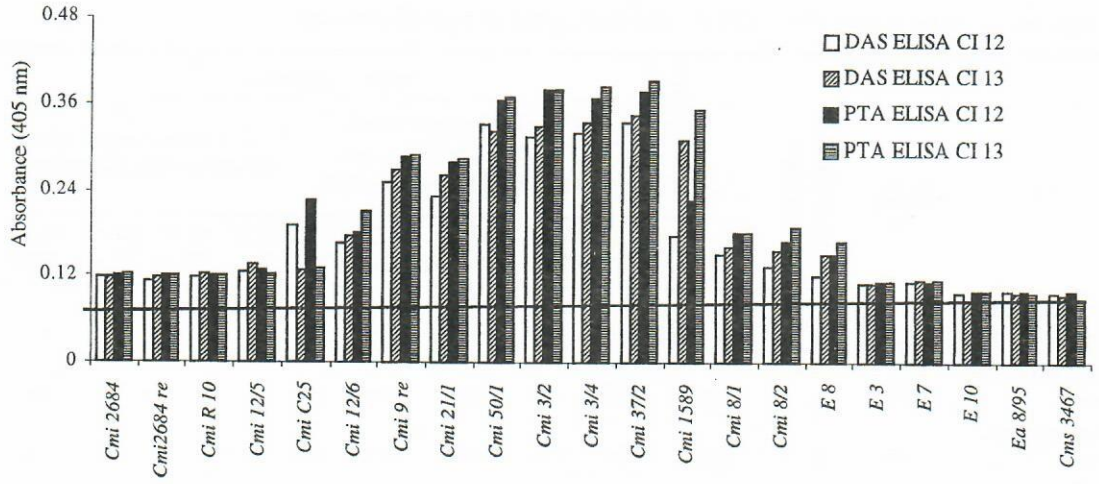
A1 – absorption with *Ralstonia solanacearum*; A2 – absorption with *Pseudomonas fluorescens*
negative reaction = –; intensity of positive reaction = +, ++, +++; between negative and weak positive reaction = (+)

ELISA tests were optimized with various concentrations of antigens IgG, IgG-AP and anti-rabbit IgG-AP. We used untreated, sonicated and heat treated (EDWARDS & EWING 1972) antigens. These concentrations were tested with the 15 strains of *C. m. insidiosus* and four extracts prepared from seed of lucerne according to the Quarantine Procedure prescibed by EPPO (OEPP/EPPO 1992), and by employing the methods DAS (double sandwich), ELISA (CLARK & ADAMS 1977) and PTA (plate trapped antigen) ELISA (HARLOW & LANE 1988). Antigens were diluted in sample buffer for DAS ELISA and in covered buffer for PTA ELISA to concentrations 10^8 to 10^3 cfu/ml. The conjugate for DAS ELISA test was used in a dilution of 1:100 to 1:500. Anti-rabbit IgG-AP (Boehringer Mannheim Biochemica) was used as conjugate for PTA ELISA in a concentration of 200–400 mU/ml following the manufacturer's instructions. The substrate was *p*-nitrophenyl phosphate $Na_2 \times 6H_2O$. The reaction was stopped by 3M NaOH after 60–90 min, and the absorbance was measured at 405 nm. The reaction was considered positive if the mean absorbance value was at least twice the value of the negative control, while the blank was always clear.

***Detection and Determination of** C. m. insidiosus:* Polyclonal antisera prepared for slide agglutination, DAS and PTA ELISA were used with the strains of *C. m. insidiosus* and extracts (resuspended pellets in phosphate buffer) obtained from seed of lucerne (Table 1). Because of a shortage of naturally infected plant material (infected plants, seed), most of the tests were conducted with pure cultures of *C. m. insidiosus*. Isolates of *C. m. insidiosus* were used in concentrations of 10^8–10^3 cfu/ml; the extracts were diluted 10-fold.

RESULTS

Three polyclonal antisera for serological diagnostics of *C. m. insidiosus*, CI 12 (as antigen used strain C25), CI 13 and CI 14 (as antigens used strain 37) were prepared. Antiserum CI 12, CI 13 and CI 14 had titer 1:32, 1:256 and 1:64, respectively. The cross-reactions with non-target bacteria were eliminated, however the titer of polyclonal antisera significantly decreased (Table 2). After absorption with cross-reacting *P. fluorescens* and *R. solanacearum* bacteria, titers of antisera CI 12 and CI 14 decreased to ratio 1:16 and 1:8, respectively. Antiserum CI 13 was repeatedly cross-absorbed only with cross reacting *P. fluorescens* bacterium. After absorption, false positive reaction with *P. fluorescens* was eliminated, however titer of antiserum CI13 decreased to ration 1:128. Due to the low titer, polyclonal antiserum CI 14 was suitable only



DAS – double sandwich ELISA test; PTA – plate trapped antigen ELISA test
A_{405} 0–0.12: negative reaction = –; A_{405} 0.13–0.19: slightly positive reaction = +; A_{405} 0.20–0.29: medially positive reaction = ++; A_{405} > 0.30 – strongly positive reaction = +++
Cmi – Clavibacter michiganensis subsp. *insidiosus*; *Ea – Erwinia amylovora*, *Cms – Clavibacter michiganensis* subsp. *sepedonicus* – negative controls; E – extracts of seed; re – reizolate of strain 2684

Fig. 1. Detection and determination of *Clavibacter michiganensis* subsp. *insidiosus* by DAS and PTA ELISA tests

for a use in slide agglutination tests. When polyclonal antisera CI 12 and CI 14 were assayed with 20 pure cultures of *C. m. insidiosus* by slide agglutination test, sufficient sensitivity of both antisera to homologous antigen was proved (Table 1).

DAS and PTA ELISA methods were used for detection of pure bacterial cultures and pathogen in extracts from seed samples, too. PTA ELISA appeared to be more sensitive for bacterial cultures and also for seed extracts (Fig. 1). Before absorption, ELISA test was optimized for IgG in ratio 1.0 to 2.0 mg/ml, IgG-AP in ratio 1:100 to 1:200, and anti-rabbit IgG-AP in ratio 300–400 mU/ml. After cross-absorption, optimal concentrations for the first antibody (IgG) moved to 2.0–3.0 mg/ml, for IgG-AP in ratio 1:50 to 1:100, and for anti-rabbit IgG-AP to 400 mU/ml.

The mean absorbance values of positive reactions in DAS and PTA ELISA methods for pure cultures of *C. m. insidiosus* ranged from 0.13 to 0.40. The threshold absorbance value of A_{405} was set on 0.12. The mean absorbance values for the isolates from seed of lucerne reached close up to the threshold level. Fluidic strains of *C. m. insidiosus* tended to give lower absorbance values than nonfluidal strains. The differences ranged from 0.05 to 0.15. Seed extracts free of the pathogen gave ELISA values almost zero (Fig. 1).

Bacterial strains included fluidic and nonfluidic strains of *C. m. insidiosus* tested differed by morphology. Highly fluidic strains were not enough reliably determined by prepared antisera.

Polyclonal antiserum CI 13 was more sensitive than polyclonal antiserum CI 12 (Fig. 1). PTA ELISA was more sensitive (Fig. 1) and more specific (Table 3) than DAS ELISA test.

DISCUSSION

Three polyclonal antisera prepared for serological diagnostics of *C. m. insidiosus* had to be repeatedly cross-absorbed with *R. solanacearum* and *P. fluorescens* because of low specificity of the antisera. After cross-absorption, the titers of antisera CI 12, CI 13 and CI 14 with homologous antigen significantly decreased. Therefore, polyclonal antiserum CI 14 was suitable only for the slide agglutination method, while antisera CI 12 and CI 13 could be used in the ELISA test. It is known that cross-reacting bacteria always complicate the specificity of antisera, and that after cross-absorption the sensitivity of antisera usually decreases (MILLER 1984). According to MILLER (1984) and also our own experience, *P. fluorescens* is the most frequent cross-reacting bacterium in serological tests (PÁNKOVÁ & MATYÁŠOVÁ 1994; KOKOŠKOVÁ & PÁNKOVÁ 1998).

PTA ELISA appeared to be more sensitive in our tests than the DAS ELISA assay (Fig. 1). After cross-absorption, the optimal concentrations for IgG, IgG-AP and also anti-rabbit IgG-AP (the first antibody IgG moved to 2.0–3.0 mg/ml, IgG-AP in ratio 1:50 to 1:100, and anti-rabbit IgG-AP to 400 mU/ml) were significantly higher than are commonly used in ELISA tests with other plant pathogenic bacteria from the genera *Erwinia, Pseudomonas* and *Xanthomonas* (LELLIOTT & STEAD 1987; PÁNKOVÁ & MATYÁŠOVÁ 1994).

Pure cultures of *C. m. insidiosus* reacted in DAS ELISA only to the concentrations 10^7–10^6 cfu/ml, but in PTA ELISA to the concentrations 10^6–10^5 cfu/ml. Identification of pure cultures of *C. m. insidiosus* was easiest at the concentrations 10^8 to 10^7 cfu/ml. At this high concentra-

Table 3. Determination of cross-reactions of polyclonal antisera CI 12 and CI 13 in ELISA tests

Bacteria	Strain	Age of bacterial culture (days)	Reaction before cross-absorption		Reaction after the 1st cross-absorption with *Pseudomonas fluorescens* and *Ralstonia solanacearum*				Reaction after the 2nd cross-absorption with *Pseudomonas fluorescens*			
			CI 12	CI 13	CI 12 (DAS)	CI 12 (PTA)	CI 13 (DAS)	CI 13 (PTA)	CI 12 (DAS)	CI 12 (PTA)	CI 13 (DAS)	CI 13 (PTA)
Clavibacter michiganensis subsp. *insidiosus*	$C_2$5	4	+++	+++	+++	+++	+++	+++	++	++	++	++
Clavibacter michiganensis subsp. *insidiosus*	37	4	+++	+++	+++	+++	+++	+++	++	++	++	++
Clavibacter michiganensis subsp. *insidiosus*	9	4	+++	+++	+++	+++	+++	+++	++	++	++	++
Clavibacter michiganensis subsp. *insidiosus*	12	4	+++	+++	+++	+++	+++	+++	++	++	++	++
Agrobacterium tumefaciens	2835	2	–	–	–	–	–	–	–	–	–	–
Clavibacter michiganensis subsp. *sepedonicus*	3467	4	+	+	+	–	+	–	–	–	–	–
Clavibacter michiganensis subsp. *sepedonicus*	3279	4	+	+	–	–	–	–	–	–	–	–
Clavibacter michiganensis subsp. *michiganensis*	109	4	+	+	+	+	+	+	–	–	–	–
Clavibacter michiganensis subsp. *michiganensis*	202	4	+	+	+	+	+	+	–	–	–	–
Clavibacter michiganensis subsp. *nebraskensis*	2749	4	+	+	+	+	+	–	–	–	–	–
Curthobacterium flaccumfaciens	2403	4	+	–	–	–	–	–	–	–	–	–
Erwinia amylovora	1617	1	–	–	–	–	–	–	–	–	–	–
Pantoea agglomerans	2406	1	+	+	+	–	–	–	–	–	–	–
Pseudomonas syringae pv. *syringae*	4073	2	+	+	+	–	–	–	–	–	–	–
Pseudomonas fluorescens	2115	2	++	+++	+	+	+	+	+	–	+	–
Ralstonia solanacearum race 3	2505	2	++	+++	++	+	+	–	–	–	–	–
Xanthomonas vesicatoria	2102	2	–	–	–	–	–	–	–	–	–	–

DAS – double sandwich ELISA test; PTA – plate trapped antigen ELISA test; A_{405} 0–0.12 – negative reaction
A_{405} 0–0.12: negative reaction = –; A_{405} 0.13–0.19: slightly positive reaction = +; A_{405} 0.20–0.29: medially positive reaction = ++; A_{405} > 0.30 – strongly positive reaction = +++

tion of antigens the differences between positive and negative reactions were most distinct. Such results by ELISA would be considered as insufficient for other phytopathogenic bacteria, except for the genus *Clavibacter* (LELLIOTT & STEAD 1987). The mean absorbance ratio of positive reactions in DAS and PTA ELISA methods for pure cultures of *C. m. insidiosus* with values of 0.13 to 0.4, and for isolates from seed of lucerne which came close to the identification level, also agreed with the literature (KRÄMER & GRIESBACH 1995). Absorbance values for seed extracts (Fig. 1) in DAS and PTA ELISA tests reached only the bottom of the identification level. This could be improved by using monoclonal antibodies (DE BOER *et al.* 1988). These values are in ELISA tests for the other plant pathogenic bacteria mentioned as a negative (KOKOŠKOVÁ & PÁNKOVÁ 1998).

The bacterial strains included fluidal and nonfluidal strains of *C. m. insidiosus* and that differed by morphology and virulence (KŮDELA – personal communication). Highly fluidal strains could not be determined reliably enough by the antisera; they were below or just reached the identification level. Medium or low fluidal strains were well identified. Because of difficulties with the determination of highly fluidal strains we prepared antiserum CI 12 from highly fluidal strain $C_2$5 during 1999. We prepared a diagnostic set for ELISA tests and verified its properties (Table 2).

Polyclonal antiserum CI 13 was more sensitive than antiserum CI 12 (Fig. 1). PTA ELISA was more sensitive than DAS ELISA. Therefore, we recommend PTA ELISA for routine detection and determination of *C. m. insidiosus*; it is completely satisfactory for determination of pure

cultures and also of extracts from seed samples of lucerne. A low concentration of the causal agent of bacterial wilt of lucerne could not be determined by this assay in diluted plant extracts and symptomless parts of plants. In these cases, the concentration of pathogen was probably under 10^5 cfu/ml, and thus below the limit for detection of bacteria in plants that for PTA ELISA had been set at 10^5 CFU/ml. We tried without success to improve the detection limits. Better antibodies and prior treatment of the antigen did not result in a stronger ELISA reaction. We also found it impossible to improve the reaction in the ELISA test if the antigen was heat treated or sonicated prior to absorption to microtiter plate wells. DE BOER *et al.* (1988) tried to use extracellular polysaccharide and protein as antigens for a new monoclonal antibody against *Clavibacter michiganensis* subsp. *sepedonicus,* and succeeded with extracellular polysaccharide. The sensitivity of the new monoclonal antibody reached 10^4 CFU/ml (DE BOER *et al.* 1988).

Preparation of polyclonal antisera for serological diagnostics of *C. m. insidiosus* is very difficult, because the bacterial cells produce rather large amounts of extracellular polysaccharides and the immune system of rabbits forms antibodies to them very slowly (KWAPINSKI 1972; VIDAVER 1980). The sensitivity of polyclonal antisera prepared against bacteria of the genus *Clavibacter,* including *C. m. insidiosus,* is lower than of polyclonal antisera against members of other genera of plant pathogenic bacteria. A higher concentration, e.g., up to 10^8 cfu/ml, is toxic for rabbits and the animals often die of toxicosis. If lower concentrations of antigen are used, to which rabbits are more tolerant, the immune schedule has to be longer to obtain antisera with an at least average titer. Cross-reactions mostly occur if the immune schedule is extended. They must be subsequently eliminated by the absorption with cross-reacting bacteria (EDWARDS & EWING 1972; YAKRUS & SCHAAD 1979), but this decreases the sensitivity of the polyclonal antisera. An assurance of sufficient sensitivity and specificity of polyclonal antisera against plant pathogenic bacteria of the genus *Clavibacter* is more difficult than of such antisera prepared against bacterial species of *Erwinia, Pseudomonas, Ralstonia* or *Xanthomonas* (MILLER 1984; PÁNKOVÁ & MATYÁŠOVÁ 1994). We think that it is not feasible to make the determination and detection of *C. m. insidiosus* by polyclonal antibodies more reliable, because all known ways of their preparation were tried. Commercial companies do not produce antisera against *C. m. insidiosus* bacteria at all, probably because of those difficulties with their preparation (CLAFLIN & SHEPARD 1977; SLACK *et al.* 1979; WESTRA *et al.* 1994). Also, there is much less information in the literature about the diagnostics of this pathogen than about other plant pathogenic bacteria.

Chicken, goats and other animals had been preferred before rabbits were used to prepare polyclonal antisera against species of the genus *Clavibacter*. The advantages of preparing monoclonal antibodies against plant pathogenic bacteria of *Clavibacter* has been definitely confirmed recently (ERWIN 1990; WESTRA *et al.* 1994). Isolation of the causal agent of bacterial wilt of lucerne is difficult and labour-intensive. Therefore, it is preferable to detect the pathogen straight in extracts prepared from host plants or seed. The monoclonal antibodies should ensure a higher quality of detection and determination of the pathogen in latent form in host plants and lots of seed.

One of the most important phytosanitary requirements for seed of lucerne is that it is free from *C. m. insidiosus*. The applied methods for detection and determination of *C. m. insidiosus* bacteria used until now are time-consuming. Positive results take several months from the start of analyses, because pathogenicity tests are necessary. PTA ELISA can offer rapid negative diagnoses within a few days. A negative result would make other tests superfluous and a phytosanitary certificate could be issued. Tests of pathogenicity have to be carried out only on those samples that were suspicious in previous assays. The PTA ELISA method is particularly suitable for testing seed from areas declared as free from the agent of bacterial wilt.

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Souhrn

KOKOŠKOVÁ B., PÁNKOVÁ I., KREJZAR V. (2000): **Charakteristiky polyklonálních antisér pro detekci a determinaci bakterií** *Clavibacter michiganensis* subsp. *insidiosus*. Plant Protect. Sci., **36**: 46–52.

Pro detekci a determinaci bakterií *Clavibacter michiganensis* subsp. *insidiosus*, původce bakteriálního vadnutí vojtěšky, byla připravena tři polyklonální antiséra, označená jako CI 12 (titr 1 : 32), CI 13 (1 : 256) a CI 14 (1 : 64). Specifičnost antisér byla zvýšena odstraněním křížových reakcí s bakteriemi *Ralstonia solanacearum* a *Pseudomonas fluorescens*. Po vysycení poklesl titr antisér u CI 12 (titr 1 : 16), CI 13 (1 : 156) a CI 14 (1 : 8). Polyklonální antisérum CI 14 bylo využito pouze pro sklíčkovou aglutinaci. Z polyklonálních antisér CI 12 a CI 13 byly získány IgG a připraveno též IgG-AP, které byly využity při determinaci a detekci bakterií *C. m. insidiosus* v čisté kultuře i v homogenátech připravených z osiva vojtěšky metodou ELISA. Lépe se osvědčila metoda PTA-ELISA, která umožnila identifikovat homologní antigen v koncentraci 10^6–10^5 cfu/ml, zatímco metoda DAS- ELISA v koncentraci 10^7 –10^6 cfu/ml. Po vysycení surových antisér se téměř odstranila falešně pozitivní reakce s *P. fluorescens*, ale vzhledem k poklesu celkové koncentrace IgG bylo nutné upravit konečná ředění IgG na 2,0–3,0 mg/ml, IgG-AP v rozsahu 1 : 50 až 1 : 100 a anti-rabbit IgG-AP na hodnotu 400 mU/ml. Výrazně fluidní kmeny byly připravenými séry obtížně identifikovatelné. Připravená polyklonální antiséra je možné použít pro rutinní stanovení *C. m. insidiosus* z čistých kultur i ze vzorků osiva vojtěšky. Doporučujeme metodu PTA ELISA, kterou může být provedena rychlá negativní diagnóza.

Klíčová slova: bakteriální vadnutí vojtěšky; *Clavibacter michiganensis* subsp. *insidiosus*; sklíčková aglutinace; ELISA

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