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THE VIRULENCE OF *CLAVIBACTER MICHIGANENSIS* SUBSP. *INSIDIOSUS* STRAINS AND TESTS OF ALFALFA VARIETIES FOR RESISTANCE TO THE WILT PATHOGEN

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Abstract: Bacterial wilt caused by *Clavibacter michiganensis* subsp. *insidiosus* (*Cmi*) is an integral part of chronic vascular wilt of alfalfa. The aim of the presented study was to determine the virulence of *Cmi* strains and later on to evaluate the level of resistance of alfalfa varieties registered in the Czech Republic and of some standard check varieties. Three methods of inoculation were compared at greenhouse conditions. The severity of the disease was determined on the basis of symptoms appearing on the cross section of the roots.
The *Cmi* strain 12/5/98 and its re-isolates showed a high level of virulence. Freeze-dried isolates lost their virulence. The best differentiation of tested varieties according to the level of resistance was obtained by using successive inoculation on cotyledons, stems and roots. Of tested alfalfa varieties, Vernal and Roamer were classified as resistant and Nitranka, Orca and Europe as the most susceptible. Alfalfa varieties (Czech and European) registered in the Czech Republic recently proved lower disease severity in comparison with ones registered formerly ($r = -0.538$, $p\ (0.05) = 0.553$). The above mentioned fact evidences the progress of Cmi resistant alfalfa breeding in Europe.

Key words: bacterial wilt, alfalfa, virulence, resistance, methods of inoculation

INTRODUCTION

In addition to fungi of the genera *Fusarium* and *Verticilium albo-atrum* also *Clavibacter michiganensis* subsp. *insidiosus* (McCulloch) Davis et al. (*Cmi*) bacteria contribute to vascular wilt of alfalfa (*Medicago sativa* L.), which appears as premature thinning of the stand and causes relatively heavy losses in yields. Due to the fact that at the present time no efficient direct chemical control against bacterial wilt is known, to start with, our attention was focused on the breeding and production of resistant varieties. Kůdela (1971) and Kůdela and Řezáč (1972) tested the

resistance of Czech and European alfalfa varieties and two varieties of American provenance. Gubiš (1994; 1996) tested the resistance of an assortment of Czech and Slovakian varieties to *Cmi* in greenhouse conditions. In 1984–1987 Bocsa et al. (1994) focused on yield losses of 4 resistant alfalfa varieties on 11 localities in 6 countries and they discovered that besides fungal pathogens also *Cmi* had a share in yield losses.

In order to test the resistance of alfalfa varieties to bacterial wilt, it is necessary that virulent *Cmi* strains are available. During preservation, the virulence of the pathogen may decrease. Kůdela (1984) compared the virulence of lyophilised *Cmi* isolates preserved on meat-peptone agar containing glucose. In the course of 15 years those ones preserved on nutrient solutions lost their virulence and not even lyophilisation was a reliable guarantee of maintaining virulence. For the cultivation and preservation of *Cmi* strains, Drbal et al. (1987) used callus cultures from segments of stems of alfalfa plants. The advantage of this method compared to the standard procedure (on nutrient agar media) is that the isolates can be preserved for longer periods, at the same time retaining their initial level of virulence.

At present the level of virulence of the new *Cmi* strains is not known, neither is the resistance of alfalfa varieties to these strains. An objective of this study was to determine the virulence of *Cmi* strains from the Czech Republic and other countries and to conduct tests of the resistance of alfalfa varieties to *Cmi* strains which had the highest level of virulence.

MATERIAL AND METHODS

A. Virulence of *Cmi* strains

We tested the virulence of the following 6 *Cmi* strains in greenhouse conditions: R10 (H), C_2 5 (H), CFBP 1195 (F), CFBP 2404 (F), 239 (USA) and 12/5/98 (CZ), and 6 re-isolates of the collection strain 12/5/98 from the Research Institute for Plant Production in Prague-Ruzyně (1/15/99, 1/21/99, 5/3/01, 9/3/01, N/4/01, T/4/01), where virulence had been tested in preliminary experiments. The re-isolates were obtained from infected alfalfa root tissues. All the tested isolates were pre-cultured on a nutrient medium according to Snieszko and Bonde (1943). Four-day old cultures were used for the inoculation (Kůdela 1978). The weakly susceptible variety Palava was used to determine the virulence of the respective isolates; this variety was used in variety tests as a standard check (Babinec et al. 1997). The plants were inoculated with a bacterial suspension of a 10^6 colony-forming units per milliliter (cfu ml^{-1}) concentration in the stage of cotyledons of 7-day old plants (Kůdela et al. 2002). The experiment was established in three replications, each of 360 plants.

After 8 weeks, based on the histological symptoms on the cross section of the roots, the disease degree was evaluated (Kůdela 1970) and then the index of disease severity was calculated according to Towsend and Heuberger. The results were evaluated statistically using the method of variance analysis and successive Tukey test.

B. Resistance of alfalfa varieties to bacterial wilt

We determined the resistance of alfalfa varieties to the causal agent of bacterial wilt in greenhouse experiments; 11 varieties of Czech origin and 9 varieties from abroad were tested (Tabs. 2–4). The varieties were inoculated with a mixture of isolates (12/5/98, 1/21/99, 5/3/01, 9/3/01, N/4/01 and T/4/01) of a concentration of 10^6 cfu ml^{-1} with following methods:

in the stage of cotyledons of 7-day old plants (method A), in the stage of cotyledons and after 6 weeks on the cut off stems (method B) and in the stage of cotyledons, after 6 weeks on the stems and dipping of shortened roots of 8-week old plants into the bacterial suspension for 17–18 hours (method C).

The pot trials were repeated three times and after 6 months the health condition of the plants was assessed on the basis of macro-symptoms on the cross section of the root. The plants were evaluated according to a 7-point disease rating scale and the index of disease severity was calculated. The respective varieties of all types of inoculation were evaluated in three replications, 28–30 plants in each replication. The results were evaluated statistically using ANOVA by UPAV programme.

RESULTS AND DISCUSSION

A. Virulence of *Cmi* strains

According to the preliminary tests, in which the collection strain *Cmi* (CCM 2684) from the Czech collection of microorganisms was non-virulent after transfer from a lyophilised condition, the above isolates were tested in order to select isolates with the highest level of virulence for successive tests of the resistance of registered alfalfa varieties to bacterial wilt.

The virulence of the respective *Cmi* isolates was expressed as the occurrence of plants in the respective degrees of the 7-point disease rating scale given in percent (Fig. 1). Five out of the 12 tested strains were non-virulent. Alfalfa plants with these strains appeared only under 0 and 1 on the scale. Kůdela (1970) found out, that a sharply demarcated discovered spot, corresponding to class1 of the disease rating scale, could be seen in the center of woody cylinder on the cross section of the roots of the plants inoculated with *Cmi* in early developmental stages. This symptom could be regarded as an expression of either high resistance of plants or as a weak virulence of wilt bacteria. We assumed that in our experiment the class 1 represented a weak virulence of the tested *Cmi* strains. The weak virulent strains originated from France, Hungary and USA and were preserved in a lyophilised condition for a long time, and have lost their virulence. This is in accordance with the findings of Kůdela (1984).

The only collection isolate with higher virulence was the *Cmi* 12/5/98 strain from the Research Institute for Plant Production in Prague-Ruzynì preserved in deep-frozen condition (–70°C) (Jeřábková, personal communication). Of the plants inoculated with this strain, 24% were found under point 2 of the scale, and 8% under point 3. A very surprising discovery was that after the inoculation of re-isolates of the virulent strain 12/5/98 the attack was milder than after inoculation with the original strain. No statistically highly significant difference was observed between the strain

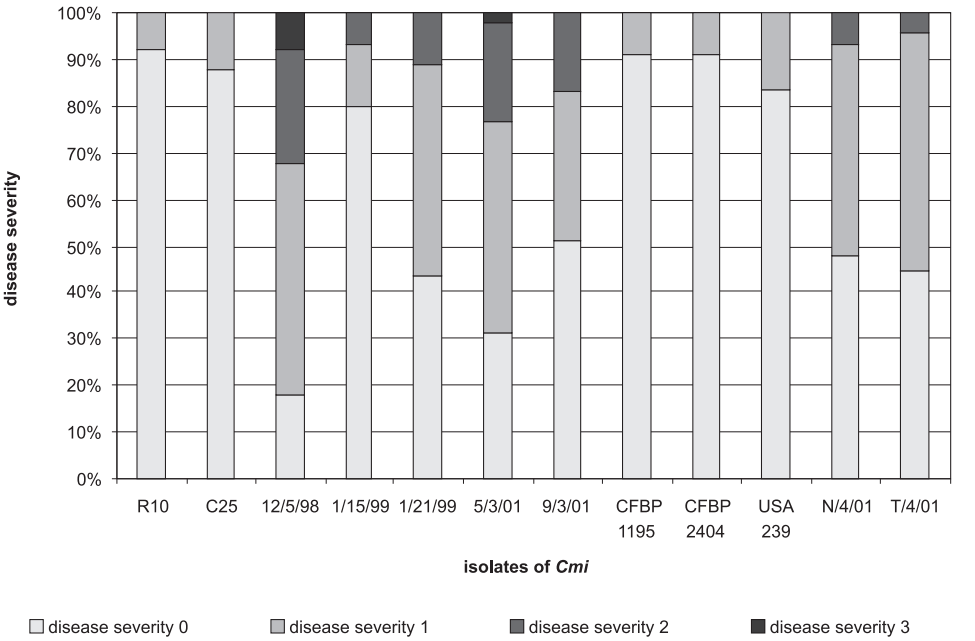

Fig. 1. Virulence of *Clavibacter michiganensis* subsp. *insidiosus* (*Cmi*) strains

Cmi 12/5/98 and its re-isolate 5/3/01 (Tab. 1). Compared to other isolates, the index of disease severity of these two isolates was the highest and the difference between them and the other re-isolates was statistically highly significant. However, we have to admit that the virulence of *Cmi* isolates tested in our experiments was deeply under the level of virulence of isolates used by Kůdela (1971; 1978).

Table 1. Virulence of *Clavibacter michiganensis* subsp. *insidiosus* (Tukey test)

Number	Strain		Index	p > 99%
3	12/5/98	CZ	30.55	A
6	5/3/01	CZ	24.16	A
5	1/21/99	CZ	16.94	B
7	9/3/01	CZ	16.38	B
11	N/4/01	CZ	14.72	B
12	T/4/01	CZ	14.44	B
4	1/15/99	CZ	6.66	C
10	239	USA	4.18	C
2	$C_2$5	H	3.05	C
8	CFBP 1195	F	2.24	C
9	CFBP 2404	F	2.22	C
1	R10	H	1.66	C

B. Resistance of alfalfa varieties to bacterial wilt

Tables 2–4 have shown the index of disease severity of the respective varieties in dependence on the method of inoculation. If we imply all the methods of inoculation

Table 2. Reaction of alfalfa varieties to inoculations with *Clavibacter michiganensis* subsp. *insidiosus* on the cotyledons

Number	Variety		Index	Year of registration	p >99%
12	Nitranka	SK	0.85	NR	A
15	Palava	CZ	0.73	1967	AB
1	Bobrava	CZ	0.72	NR	AB
9	Morava	CZ	0.62	1990	ABC
3	Gloria	RO	0.56	NR	ABCD
2	Europe	F	0.55	1972	BCD
8	Magda	CZ	0.53	1986	BCD
20	Zuzana	CZ	0.51	1990	BCD
14	Orca	F	0.49	1973	BCD
4	Hana	CZ	0.48	NR	BCDE
19	Vanda	SK	0.44	1995	BCDE
11	Oslava	CZ	0.42	2003	CDE
6	Jitka	CZ	0.41	1995	CDE
18	Vlasta	CZ	0.40	1995	CDE
10	Narragansett	USA	0.35	NR	CDEF
13	Niva	CZ	0.35	1995	CDEF
5	Jarka	CZ	0.32	1995	DEF
7	Lucia	SK	0.19	1990	EF
16	Roamer	CAN	0.07	NR	F
17	Vernal	USA	0.07	NR	F

NR – not registrated in the Czech Republic

Table 3. Reaction of alfalfa varieties to inoculations with *Clavibacter michiganensis* subsp. *insidiosus* on the cotyledons and stems

Number	Variety		Index	Year of registration	p > 99%
3	Gloria	RO	1.02	NR	A
10	Narragansett	USA	0.98	NR	A
15	Palava	CZ	0.98	1967	A
14	Orca	F	0.91	1973	AB
9	Morava	CZ	0.89	1990	ABC
12	Nitranka	SK	0.88	NR	ABC
1	Bobrava	CZ	0.85	NR	ABC
19	Vanda	SK	0.83	1995	ABC
8	Magda	CZ	0.78	1986	ABC
11	Oslava	CZ	0.77	2003	ABC
20	Zuzana	CZ	0.76	1990	ABC
4	Hana	CZ	0.76	NR	ABC
18	Vlasta	CZ	0.75	1995	ABCD
5	Jarka	CZ	0.70	1995	ABCD
2	Europe	F	0.66	1972	ABCDE
7	Lucia	SK	0.56	1990	BCDEF
6	Jitka	CZ	0.53	1995	CDEF
13	Niva	CZ	0.37	1995	DEF
16	Roamer	CAN	0.30	NR	EF
17	Vernal	USA	0.19	NR	F

NR – see table 2

Table 4. Reaction of alfalfa varieties to inoculation with *Clavibacter michiganensis* subsp. *insidiosus* on the cotyledons, stems and roots

Number	Variety		Index	Year of registration	p > 99%
2	Europe	F	3.08	1972	A
14	Orca	F	2.97	1973	AB
12	Nitranka	SK	2.50	NR	ABC
19	Vanda	CZ	2.35	1995	ABCD
7	Lucia	SK	2.34	1990	ABCD
18	Vlasta	CZ	1.98	1995	ABCDE
8	Magda	CZ	1.74	1986	BCDEF
4	Hana	CZ	1.69	NR	BCDEF
15	Palava	CZ	1.64	1967	CDEF
5	Jarka	CZ	1.54	1995	CDEFG
1	Bobrava	CZ	1.50	NR	CDEFG
11	Oslava	CZ	1.40	2003	CDEFG
6	Jitka	CZ	1.20	1995	DEFG
20	Zuzana	CZ	1.20	1990	DEFG
10	Narragansett	USA	1.19	NR	DEFG
3	Gloria	RO	1.17	NR	DEFG
9	Morava	CZ	1.04	1990	EFG
13	Niva	CZ	1.03	1995	EFG
16	Roamer	CAN	0.52	NR	FG
17	Vernal	USA	0.32	NR	G

NR – see table 2

and all the investigated varieties we see that the most resistant varieties were Vernal and Roamer: the results correspond to the characteristics of the varieties in terms of resistance to diseases.Variety Vernal (USA) is rated as a resistant one and Roamer (CAN) as medium-resistant to *Cmi* (Anonymous 2000). In conditions of the Czech Republic the variety Vernal had already been tested for resistance in the early 1970's (Kůdela 1971) and up to now is used as a resistant standard check variety.

In plants, which were inoculated only on the cotyledons (method A), the index of disease severity was very low (0.07 Vernal – 0.85 Nitranka). After inoculation on both, the cotyledons and stems (method B), the index of disease severity was higher (0.19 Vernal – 1.02 Gloria). A highly significant correlation was found out between methods A and B ($r = 0.7520$, $p > 0.5487$).

In inoculation method C, the *Cmi* suspension was applied successively on the cotyledons and root system. In this case the index of disease severity compared to the previous methods was higher (0.02 Vernal – 3.08 Europe) and the differences in resistance between the respective varieties were more distinctive. Likewise, Cormack et al. (1957) achieved better results after inoculation on the roots than on the stems. Method of inoculation *Cmi* on roots is recommended in "Standard tests to characterize alfalfa cultivars" (Fox et al. 1995) in the USA. When inoculation method C was applied, the level of attack of the susceptible varieties Europe, Orca and Nitranka was the highest, according to findings of Kůdela and Řezáč (1972). No correlation was found between methods A and B (inoculation of the above-ground organs), and method C where also the roots were inoculated ($r = 0.4244$, $p < 0.5487$ and $r = 0.4204$, $p < 0.5487$, respectively). That being so, the order of

some varieties in the tested set markedly changed: e.g. in the A and B methods of inoculation the variety Gloria ranked among the varieties with the highest level of infecton, while in method C the index of disease severity was relatively low.

It is true that when applying inoculation method C (i.e. infection on cotyledons, stems and roots) the differences in the resistance between the varieties to *Cmi* were more marked, all the same, in our experiments these varieties did not achieve such a high level of attack as they did 30 years ago (Kůdela and Řezáč 1972). This was obviously caused by the changed virulence of the *Cmi* strains. In our experiments the Narragansett variety, registered in the USA in 1946, repeatedly appeared as medium-resistant, although it was used as a susceptible standard variety in USA. That might be caused by other *Cmi* strains used for assessing the resistance.

Breeding for resistance to bacterial wilt has been practiced in the Czech Republic since 1970s. We have found significant correlation between date of registration of 13 alfalfa varieties grown in the Czech Republic (Czech and foreign altogether) and index of disease severity ($r = –0.538$, $p\ (0.05) = 0.553$). Although this correlation was not found for Czech varieties ($r = –0.235$, $p\ (0.05) = 0.631$), newly registered Czech varieties Oslava, Jitka, Niva, Morava and Zuzana appeared as more resistant than older ones (Palava, Bobrava) in our tests. The achieved results correspond with the findings of Chloupek et al. (1996) who defined the Jitka variety as resistant to *Cmi* in comparison to standard variety Palava. Also Gubiš (1994) rated the Morava variety as significantly resistant to bacterial wilt.

Our results correspond with the situation in the fields. Bacterial wilt was very important disease of alfalfa in seventies. Nowadays thanks to the achievements of plant breeding and using of new varieties this disease is of very low importance in practice. Despite of that its incidence has to be surveyed and resistance breeding continue for that incidence and spreading of new virulent pathotypes can not be excluded.

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REFERENCES

Anonymus 2000. Alfalfa disease resistance ratings. Ministry of Agriculture and Food, British Columbia http://www.agf.gov.bc.ca/croplive/croppprot/alfalfaratings.htm

Babinec J., Fikesová E., Kozová Z. 1997. Breeding for resistance to vascular wilt and to stem nematodes. Seed Production of Lucerne. Proc. 12th EUCARPIA meeting of the Group *Medicago*: 120–121.

Bosca I., Pummer L., Horompoli T. 1994. Importance of main diseases in Eastern Europe as determined by the use of specifically resistant varieties. Acta Phytopath. Entom. Hung., 29 (1–2): 39–48.

Cormack M.W., Peake R.W., Downey R.K. 1957. Studies on methods and materials for testing alfalfa for resistance to bacterial wilt. Can. J. Plant Sci., 37: 1–11.

Drbal J., Nedbálková B., Konečná D. 1987. Využití kalusových kultur vojtěšky při studiu *Corynebacterium michiganense* pv. *insidiosum* (McCulloch) Dye & Kemp. Sb. vìd. prací OSEVA, VŠÚP Troubsko u Brna 10: 137–142.

Fox Ch.C., Berberet R., Gray F.A., Grau C.R., Jessen D.L., Peterson M.A. 1995. Standard tests to characterize alfalfa cultivars. North American Alfalfa Improvement Conference. Third edition, 110 pp.

Gubiš V. 1994. Náchylnost' odrod lucerny siatej (*Medicago sativa* L.) na bakteriove vadnutie a had'atko zhubne. Ročenka Genetické zdroje rastlin: 35–37.

Gubiš V. 1996. Resistance of selected alfalfa (*Medicago sativa* L.) to stem nematode and bacterial wilt. Plant Gen. Resour.: 34–38.

Chloupek O., Babinec J., Holubář J. 1996. Development and evalution of new synthetic varieties of lucerne Jitka and Niva. Acta Univ. Agric. Silvic. Mendel. Brunensis 44: 19–23

Kůdela V. 1970. Způsob hodnocení odolnosti odrůd vojtěšky seté vůči cévnímu vadnutí. Rostl. výroba 16: 1041–1045.

Kůdela V. 1971. Odolnost československých odrůd vojtěšky proti bakteriálnímu vadnutí. Ochr. rostl., *7* (3): 169–177.

Kůdela V., Řezáč A. 1972. Testování evropských odrůd vojtěšky na odolnost proti bakteriálnímu a verticiliovému vadnutí. Ochr. rostl., 8 (4): 269–176.

Kůdela V. 1978. Soustava ochranných opatření proti cévnímu vadnutí vojtěšky. Metodiky pro zavádění výsledků výzkumu do praxe 2, p. 26.

Kůdela V. 1984. Vliv lyofilizace na virulenci izolátů *Corynebacterium michiganase* pv. *insidiosum*. Sborník ÚVTIZ – Ochr. rostl. 20 (4): 241–244.

Kůdela V., Novacky A., Fucikovsky L. 2002. Rostlinolékařská bakteriologie. Academia Praha, p. 347.

Snieszko S.F., Bonde R. 1943. Studies on the morphology, physiology, serology, longevity, and pathogenicity of *Corynebacterium sepedonicus*. Phythopathology 33: 1032–1044.

POLISH SUMMARY

WIRULENCJA IZOLATÓW *CLAVIBACTER MICHIGANENSIS* SUBSP. *INSIDIOSUS* ORAZ ODPORNOŚĆ ODMIAN LUCERNY NA PATOGENA

Bakteryjne więdnięcie powodowane przez *Clavibacter michiganensis* subsp. *insidiosus* (*Cmi*) jest integralną częścią trwałych więdnięć lucerny.

Celem niniejszej pracy było określenie wirulencji izolatów *Cmi* oraz poziomu odporności odmian lucerny zarejestrowanych w Republice Czeskiej na *Cmi*. Dla tych celów porównano w warunkach szklarniowych trzy metody inokulacji. Nasilenie choroby określano na podstawie objawów występujących na przekrojach korzeni.

Najwyższy poziom wirulencji wykazał szczep 12/5/98. Izolaty *Cmi* przechowywane w postaci liofilizatów traciły swoją wirulencję. Najlepsze zróżnicowanie odmian pod względem odporności uzyskano przy sukcesywnym zakażaniu *Cmi* liścieni, pędów oraz korzeni. Z testowanych odmian za odporne uznano odmiany Vernal i Roamer, a Nitranka, Orca i Europe były najbardziej podatne. Odmiany lucerny (czeskie i europejskie) zarejestrowane w Republice Czeskiej wykazały niższą intensywność objawów chorobowych w porównaniu z odmianami zarejestrowanymi wcześniej. Fakt ten potwierdza postęp w hodowli odmian lucerny odpornych na *Cmi* w Europie.