Detection of Bacterial Wilt Pathogen of Lucerne by PCR

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Abstract

A polymerase chain reaction (PCR) based assay was compared with assay involving traditional microbiological tests to detect and identify *Clavibacter michiganensis* subsp. *insidiosus* (Cmi) inside plant tissues following artificial inoculations. Primers for Cmi detection were selected from the Cmi insertion sequence IS 1122. External disease symptoms on the tops and histological symptoms on the cross-sections of the roots were evaluated visually and microscopically during two years after inoculation. Extracts were prepared from samples collected from the tops and roots of lucerne plants for both PCR and microbiological analyses. Colonies which morphologically resemble to be Cmi were tested using PCR. Cmi bacteria were detected in stem and root tissues using both methods. The PCR can rapidly detect Cmi in lucerne plants showing histological disease symptoms on the cross-sections of the roots. Further work is needed to detect reliably Cmi in lucerne plants with latent infections and in infected seeds or contaminated seed lots.

Keywords: *Clavibacter michiganensis* subsp. *insidiosus*; bacterial wilt; detection; polymerase chain reaction; PCR; insertion sequence

INTRODUCTION

Clavibacter michiganensis subsp. *insidiosus* (Cmi) is a causal agent of bacterial wilt of lucerne (MCCULLOCH 1925). The disease causes severe losses in North America and it occurs only in some European countries including the Czech Republic (KŮDELA 1969). Therefore, Cmi is considered of quarantine significance (SMITH *et al*. 1996). The pathogen is transmitted by seeds at a long distance. High sensitive, rapid and reliable techniques for screening of great numbers of plant samples and seed lots are clearly essential for control of the disease, but such techniques have been unavailable until recently. A rapid serological identification method was developed by HALE (1972). A seed-testing method using immunofluorescence staining was proposed by NÉMETH *et al*. (1991).

MATERIALS AND METHODS

Specific primer for detection Cmi by PCR. The DNA sequences of the Cmi insertion sequence IS 1122 of bacteria *Clavibacter michiganensis* subsp. *insidiosus,* and of *Cms* insertion sequence IS 1121 *Clavibacter michiganensis* subsp. *sepedonicus* (published in Genbank under accession numbers AF079818 and AF079817), were aligned using a program Multalin version 5.3.3. (I.N.R.A. France). Comparisons were also made with published sequence data of the same region in other bacteria retrieved from Genbank. A primer analysis software Primer 3 (ROZEN & SKALETSKY (1998) was used to select PCR primers targeting the spacer region of Cmi avoiding possible formation of primer dimers, self-priming, primer hairpin, and primer multiple binding sites. Specific primers IL2 and IR2 were proposed. The primers were custom synthesised by SIGMA GENOSYS (UK).

Upstream primer

IL2 5'-AGGACCCGAGATTCCGATCA-3'

Downstream primer

IR2 5'-CTTCTCGTCCGGGTGGAT-3'

Isolation of bacterial DNA. The bacterial strains tested in the experiment are listed in Table 1. All strains were grown in nutrient glucose broth or on

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nutrient glucose agar plates. Template for PCR amplification was prepared from whole bacterial cells. Lysis was performed by boiling a loopful of cells from a colony resuspended in 100 μl of sterile distilled water for 5 min at 100°C in thermal cycler PTC-200 (MJ Research). After cooling for 5 min at 0°C 5 μl of the prepared culture was used per reaction without further treatment.

Isolation of bacterial DNA from plants. Inoculated lucerne plants were tested for presence of Cmi in their stems and roots by PCR. Thin cross sections were made by sharp blade, crushed with mortar and pestle and boiled with sterile water for 5 min at 100°C in thermal cycler PTC-200 (MJ Research). After cooling for 5 min at 0°C 5 μl of the prepared culture was used per reaction without further treatment.

Polymerase chain reaction. The PCR reaction contained the primers (0.5 mM each), four dNTPs (0.2mM each), 1× PCR buffer (Promega), $MgCl_2$ (1.5mM), 2 units of *Taq* DNA polymerase (Promega), and 1 ng/ml of template DNA in a total volume 25 μl. PCR was conducted in a thermal cycler (MJ Research, PTC-200) for 1 cycle hot start (denaturation at 94°C, 3 min; annealing at 61°C, 30 s; elongation at 72°C, 2 min); for 40 cycles with following parameters: denaturation at 94°C, 30 s; annealing at 61°C, 30 s; elongation at

Table 1. *Clavibacter michiganensis* subsp. *insidiosus* strains and other bacteria strains used in this study

Samples	Species	Strain code	Collection
1	*Clavibacter michiganensis* subsp. *insidiosus*	Cmi CCM 2684	CCM
2	*Clavibacter michiganensis* subsp. *insidiosus*	Cmi $C_7$5	RICP
3	*Clavibacter michiganensis* subsp. *sepedonicus*	Cms NCPPB 3279	NCPPB 3279
4	*Clavibacter michiganensis* subsp. *sepedonicus*	Cms NCPPB 3467	NCPPB 3467
5	*Clavibacter michiganensis* subsp. *michiganensis*	Cmm 202/95	RICP
6	*Clavibacter michiganensis* subsp. *nebraskensis*	Cmn CCM 2749	CCM 2749
7	*Clavibacter accumfaciens* var. *violaceum*	Cfv CCM 2403	CCM 2403
8	*Ralstonia solanacearum*	Rs NCPPB 325	NCPPB 325
9	*Xanthomonas arboricola* pv. *juglandis*	Xaj CCM 1449	PDDCC 49
10	*Xanthomonas arboricola* pv. *pruni*	Xap CCM 889	NCPPB 416
11	*Xanthomonas campestris* pv. *campestris*	Xcc CCM 22	NCPPB 45
12	*Xanthomonas arboricola* pv. *citri*	Xac CCM 1439	CCM 1439
13	*Xanthomonas hortorum* pv. *pelargonii*	Xhp CCM612	NCPPB 400
14	*Xanthomonas vesicatoria*	Xv CCM 2102	NCPPB 1423
15	*Pseudomonas syringae* pv. *pisi*	Psp NCPPB 1434	NCPPB 1434
16	*Pseudomonas syringae* pv. *syringae*	Pss 4073	RICP
17	*Pseudomonas viridi ava*	LMG Pv 2352	LMG 2352
18	*Pseudomonas corrugata*	Psc 8892-85	Italy
19	*Pseudomonas fluorescens*	Pf 2115	RICP
23	*Flavobacterium* sp.	F CCM 2847	CCM 2847
24	*Micrococcus luteus*	Ml CCM 2847	CCM 169
25	*Pantoea agglomerans*	Pa CCM 2406	CCM 2406
26	*Erwinia amylovora*	Ea 55/91	RICP
27	*Erwinia carotovora* subsp. *atroseptica*	Eca CCM 322	CCM 322
28	*Erwinia carotovora* subsp *carotovora*	Ecc CCM 989	CCM 989
20	*	5/1/98	RICP
21	*	6/1/98	RICP
22	*	6/2/98	RICP
29	*	8/4/98	RICP
30	negative control (–)	H_2O	–

* Unidentified bacteria (isolate from lucerne)

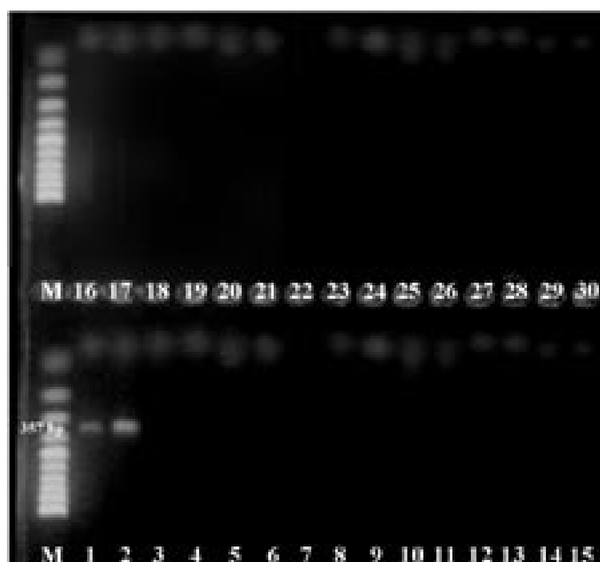
72°C, 45 s and final incubation at 72°C for 5 min. PCR products were analysed by agarose (1.5% w/v) gel electrophoresis, at 4–5 V/cm during 2h.

Evaluation of symptoms. Disease severity of inoculated lucerne plants was evaluated according to histological symptoms in cross sections of the roots by means of the following 7 point scale:

0 – no apparent discoloration;
1 – small reddish brown or dark brown spots in the centre of the xylem;
2 – yellowing in the central part of the xylem;
3 – half (incomplete) yellowish ring on the circumference of the xylem cylinder;
4 – extensive yellowing of the circumferential xylem appearing as a continuous ring;
5 – a part of the yellow ring of the circumferential xylem is darkened;
6 – deep discoloration of the xylem cylinder or the whole plant is dead.

RESULTS AND DISCUSSION

To determine the specificity of oligonucleotide primers IL2 and IR2, polymerase chain reactions were carried out with DNA of all the strains listed in Table 1. The primers permitted the amplification of a single DNA fragment of 357 bp of two Cmi strains only samples No. 1 and 2 (Table 1, Figure 1). The fragment was reproducible from one experiment to the other. No amplification was observed with Cms, Cmm, Cmn and Cf DNA, with any other strains of plant pathogenic and saprophytic bacteria tested samples 3–30 (Table 1, Figure 1).



Lane M – molecular marker 100 bp (MBI Fermentas)
Lane 1 to Lane 30 – for description see Table 1

Figure 1. Specificity of oligonucleotide primers IL2 and IR2

Cmi was detected by PCR in roots and stems in plants inoculated with the bacteria showing symptoms the disease (Table 2). Inoculated plants without symptoms were negative in PCR probably because the inoculations were unsuccessful or concentrations of wilt bacteria in asymptomatic plants were under detectable levels (Table 2).

Table 2. Results of *Clavibacter michiganensis* subsp. *insidiosus* detection in infected lucerne plants

Plants with histological symptoms	PCR		Microbiological analysis	
	stems	roots	stems	roots
0	–	–	–	–
1	–	+	–	+
2	+	+	+	+
3	+	+	+	+
4	+	+	+	+
5	+	+	+	+
6	0	0	0	0

This PCR technique is suitable for rapid identification of Cmi in epidemiological studies, in breeding of lucerne for resistance to Cmi and in the diagnostic phytosanitary laboratories.

References

HALE C.N. (1972): Rapid identification methods for *Corynebacterium insidiosum* (McCulloch, 1925) Jensen, 1934. N.Z. J. Agric. Res., **15**: 149–154.

KŮDELA V. (1969): Bakteriální vadnutí vojtěšky v Československu. Ochr. Rostl., **5**: 193–200.

MCCULLOCH L. (1925): *Aplanobacter insidiosum* n.sp., the cause of alfalfa disease. Phytopathology, **15**: 496–497.

NÉMETH J., LASZLO E., EMODY L. (1991): *Clavibacter michiganensis* subsp. *insidiosus* in lucerne seeds. OEPP/EPPO Bull., **21**: 713–718.

SMITH L.M. *et al.* (eds) (1996): Quarantine Pests for Europe. 2nd ed. CAB International, Wallingbord.

ROZEN S., SKALETSKY H.J. (1998): Whitehead Institute for Biomedical Research, Wi, USA – http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cg