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# Research Note

## A rapid and sensitive detection of *Clavibacter michiganensis* subsp. *michiganensis* in tomato seeds by polymerase chain reaction

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### Summary

A polymerase chain reaction-based method was applied for the detection and identification of *Clavibacter michiganensis* subsp. *michiganensis* in tomato seed. Two primers, $CM_3$ and $CM_4$, were used to amplify a specific 645bp DNA fragment when the target bacterium was present in the amplification step. This fragment was produced with DNA from all *C. m.* subsp. *michiganensis* strains and contaminated seed extracts tested from as less as 40 cells $ml^{-1}$, but not from DNA of other plant-pathogenic bacteria or tomato saprophytes. The results were confirmed by isolation, IF, pathogenicity tests and RFLP analysis of the amplified fragments by the endonuclease *Sal*I.

### Experimental and discussion

*Clavibacter michiganensis* subsp. *michiganensis* (Smith) Davis *et al.* (CMM), the causal agent of bacterial canker of tomato, is a quarantine organism under the European Union Plant Health legislation (European Union, 1995) causing severe losses all over the world (Strider, 1969). Infected seed is often considered as the primary inoculum source (Thyr, 1969). The population levels of the pathogen in/on the seed or during periods of latency may be very low, affecting its detection by means of conventional methods. The availability of sensitive, reliable and rapid detection methods such as PCR would be extremely useful for detection of low infection levels of the pathogen in seed lots.

Isolates of *C. michiganensis* subsp. *michiganensis, C. m.* subsp. *sepedonicus, C. m.* subsp. *insidiosus, Erwinia chrysanthemi, Pseudomonas solanacearum, P. syringae* pv. *syringae, Arthrobacter* sp. and three saprophytes isolated from tomato seeds have been tested. They were grown on YPGA for 24–48 hrs at 28 °C, except for the *C. m.* subsp. *sepedonicus* strain which was grown at 21 °C. A volume of 5 µl of cell suspensions in sterile double distilled water (SDDW) plus 50 µl of NaOH 0.05M were lysed by immersion in a boiling water bath for 15 min.

Extracts of tomato seeds cv. 'Moneymaker' were obtained according to Fatmi and Schaad (1989), contaminated with suspensions of CMM, decimal diluted in SDDW and 1 ml from each suspension centrifuged at 1,000 rpm with 2.5% polyvinylpolypyrrolidone (PVPP). The supernatant was centrifuged for 10 min at 14,000 rpm and the pellet resuspended in 150 µl of 0.05M NaOH and boiled for 10 min for bacterial cell lysis. Following the same protocol, eight coded samples of tomato seed from the same cultivar, five of which inoculated under vacuum with a suspension of CMM were also tested.

For the PCR reaction, 5 µl of the lysates were added to 45 µl of the reaction mix [1× reaction buffer (Perkin Elmer Cetus); 1.5 mM $MgCl_2$; dNTP's at 200 µM each (Boehringer Mannheim) primers at 1 µM each and 2.5U of AmpliTaq DNA polymerase (Perkin Elmer Cetus)]. The primers $CM_3$–5'CCT CGT GAG TGC CGG GAA CGT ATC C 3' and $CM_4$–5'CCA CGG TGG TTG ATG CTC GCG AGA T 3' were obtained from a DNA fragment that was selected from a shot-gun cloning of total DNA from a pathogenic Hungarian isolate, no. 3613, supplied by Ib Dinesen, Stattens Planteavesforsog, Lyngby, Denmark. The PCR conditions were 35 cycles of 94°C–1.5 min; 60°C–1 min and 72°C–1.5 min, with a final elongation of 72°C–10 min. Analysis was performed by running 12 µl of amplified products plus 3 µl of loading buffer on 1.5% agarose TAE gel (Sambrook *et al.*, 1989), gel staining with ethidium bromide at 0.5 µg $ml^{-1}$ (w/v) and visualisation under UV light.

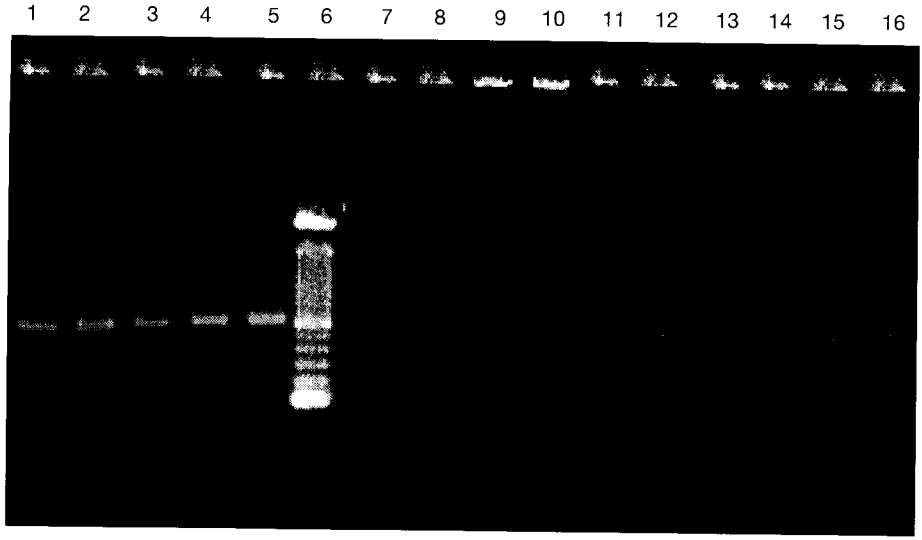


Figure 1. Evaluation of specificity of primers $CM_3$ and $CM_4$. Lanes 1–5: *Clavibacter michiganensis* subsp. *michiganensis* strains PD 223, PD 1386, PD 1675, PD 1962 and LMG 5602; Lane 6: Molecular weight 100bp ladder (Gibco-BRL); Lane 7: *C. m.* subsp. *sepedonicus*-PD 37; Lane 8: *C. m.* subsp. *insidiosus*-PD 239; Lane 9: *Pseudomonas solanacearum*-PD 511; Lane 10: *P. syringae* pv. *syringae*-PD 1704; Lane 11: *Erwinia chrysanthemi*-PD 483; Lane 12: *Arthrobacter* sp.-PD1804; Lanes 13-15: Saprophytes from tomato seed-CNPPA 15, 16 and 17; Lane 16: Healthy seed extract.

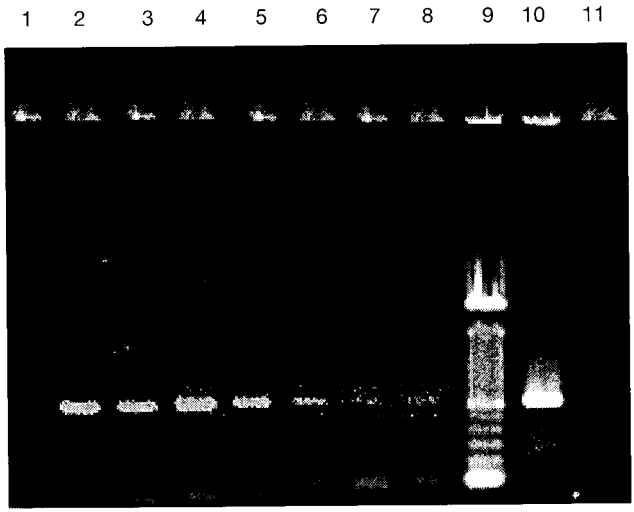


Figure 2. Evaluation of sensitivity of primers $CM_3$ and $CM_4$. Lanes 1-8: Dilution series of tomato seed extract contaminated with *C. m.* subsp. *michiganensis* ($4x10^8$ to $4x10^1$ cells $ml^{-1}$); Lane 9: Molecular weight 100bp ladder (Gibco-BRL); Lane 10: *C. m.* subsp. *michiganensis* ($4\times10^{10}$ cells $ml^{-1}$); Lane 11: Healthy seed extract.

For RFLP analysis, 26 µl of the amplified products plus 3 µl of 10 × H(igh) buffer and 1 µl of the enzyme *Sal*I (Boehringer Mannheim) were incubated for 45 min at 37 °C

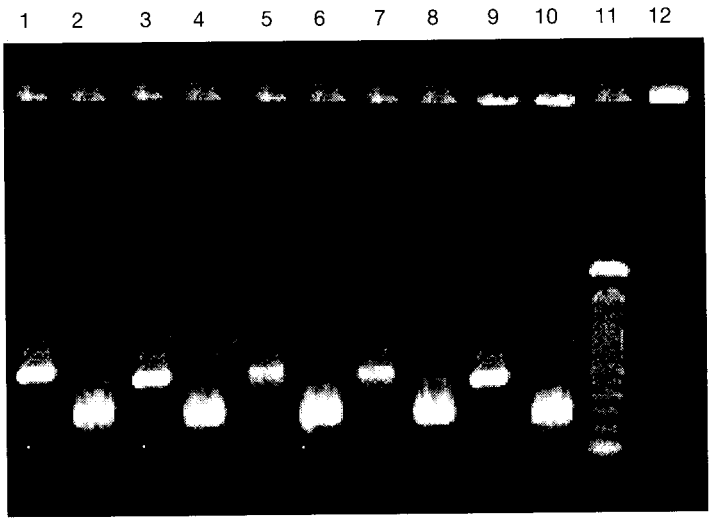


Figure 3. RFLP analysis of amplified products from extracts of vacuum contaminated tomato seeds. Lanes 1, 3, 5, 7 and 9: Amplified products from samples 2, 4, 6, 8 and 10; Lanes 2, 4, 6, 8 and 10: Digests from the corresponding amplified products; Lane 11: Molecular weight 100bp ladder (Gibco-BRL); Lane 12: Healthy seed extract.

and 15 µl from each sample plus 3 µl of 5× loading buffer were analysed by electrophoresis as before.

Immunofluorescence staining of pure cultures and contaminated seed extracts was performed according to Franken and van Vuurde (1988) and dilution plating and pathogenicity tests were done following Fatmi and Schaad (1989).

Amplification of a specific 645bp DNA fragment occurred from the five CMM strains tested but not from DNA from other pathogenic or saprophytic bacteria (Figure 1). These results showed the high specificity of primers $CM_3$ and $CM_4$. The specific 645bp DNA fragment was also produced with DNA from contaminated seed extract and from the five vacuum infiltrated seed samples, where DNA from as little as 40 CMM cells $ml^{-1}$ of seed extracts could still be detected. This confirmed the high sensitivity of the PCR technique (Figure 2). These results were obtained when samples were diluted in SDDW and treated with PVPP before being added to the PCR mix, otherwise no amplification occurred, possibly due to the presence of inhibiting compounds in the extracts.

RFLP analysis of the amplified products showed the presence of two DNA bands of approximately 300 and 345bp, thus confirming the identification of the pathogen (Figure 3).

In all cases, the results from isolation, IF and pathogenicity tests agreed with those from PCR analysis showing the reliability of this technique. As the method presented is both rapid, sensitive and specific, it has very high potentialities and would be a useful tool for sanitary selection of seed-lots.

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## References

European Union (1995). Commission Directive 95/4/EC amendment of 21 February 1995 to the European Community Plant Health Directive (77/93/EEC). *Official Journal of the European Communities* **L 44**, 56–60.

Fatmi, M. and Schaad, N. W. (1989). Detection of *Clavibacter michiganensis* subsp. *michiganensis* in tomato seed. In: *Detection of bacteria in seed and other planting material*, (eds. A. W. Saettler, N. W. Schaad and D. D. Roth), pp. 45–49. APS Press, St. Paul, Minnesota.

Franken, A. A. J. and Van Vuurde, J. W. L. (1988). Standardisation of immunofluorescence microscopy. In: *Report of the 1st International Serology Workshop*, pp. 24–28, ISTA, Zurich.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular cloning: a laboratory manual*, 2nd ed. 6.9–6.22, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Thyr, B. D. (1969). Assaying tomato seed for *Corynebacterium michiganensis*. *Plant Disease Reporter*, **5**, 858–860.