Occurrence of *Clavibacter michiganensis* subsp. *sepedonicus* in Lithuania

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Bacterial ring rot caused by *Clavibacter michiganensis* subsp. *sepedonicus* (Cms) is considered as one of the most important bacterial diseases of potatoes and is a constant challenge for potato growers. The disease is distributed in nearly all the countries that grow potatoes and causes high yield losses. Due to its latent stage the bacterium is difficult to detect and spreads quickly.

The symptoms such as plant wilting and tuber rotting characteristic of bacterial ring rot were observed in different localities of Lithuania during 1998–2001. The disease incidence reached on average 5% in the field and 5 to 21% in the storage places. Immunofluorescence staining (IF) and enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) were used for Cms detection. Pathogenicity tests were conducted on eggplants.

Key words: potato, ring rot, *Clavibacter michiganensis* subsp. *sepedonicus*

INTRODUCTION

Bacterial ring rot caused by *Clavibacter michiganensis* subsp. *sepedonicus* is widespread and infects potatoes in various places of their growth, causing plant wilting and tuber rotting. The latent stage of the pathogen in potato tubers can last for several generations of potato production [14, 15]. It is a vascular pathogen readily transmitted from seed to daughter tubers. It is particularly difficult to diagnose, especially when symptoms are advanced, and in latent stage. Because of these factors it can spread quickly and undetected. Cms can easily spread via contaminated surfaces of farm equipment. Due to export trade the risk for further dissemination is increasing. Once the disease is induced, the pathogen can be very difficult to eliminate. Cms is listed as an A2 quarantine pathogen by European and Mediterranean Plant Protection Organization. It is important, especially within the EU, due to the acceptance of new states with ring rot history.

Bacterial ring rot of potatoes has not been studied extensively in Lithuania. Only some references were reported [18, 20, 21] on the possible distribution of the disease. Unfortunately, special investigations on the disease and its causal agent to confirm the distribution of ring rot in Lithuania have not been carried out so far [1].

Regarding the economic importance of potato production and a severe degree of tuber infection, investigation of the disease distribution and identification of the causal agent were undertaken.

MATERIALS AND METHODS

Isolation and identification of the pathogen. The samples were regularly collected in separate potato growth localities and storage places during the period 1998–2001. The percentage of injured potatoes in storage was calculated from the amount of check potatoes on 81 potato growth farms. On 11 farms potatoes were found to be contaminated with Cms. The disease incidence was determined in two contaminated fields from the ten tested during the 1998 vegetation period. The study followed a standard scheme for Cms detection [17]. The presence of Cms was detected by ELISA assay using polyclonal antibodies and indirect immunofluorescence cell staining (IF) with monoclonal antibodies in the suspended pellet prepared from the cores of 200 potato tubers (one sample). The cores were macerated and placed in 300 ml Erlenmeyer flasks filled with 0.05 M phosphate buffer saline. The flasks were placed on a rotary shaker at 100 and 110 g for 4 h at room temperature or incubated at 4 °C overnight. The homogenate of 50 ml (cm^3) was centri-

fuged at 10000 g for 10 min. Then the supernatant was discared, the pellet was suspended in sterile 0.01 M phosphate buffer pH 7.2 to give a total volume of 1 ml. Half of the sample was used for IF and ELISA and the other for pathogenicity test.

Pathogenicity test. Eggplant is usually used as an index host for the study of Cms [11, 17]. The test was conducted on eggplants at the third leaf stage. The stem of the plant was inoculated with a pellet obtained from each tuber sample. The inoculated plants were incubated for 40 days and examined regularly for symptoms every 8 days. Pure bacterium culture from infected plants was reisolated on nutrient agar (NA) and yeast peptone glucose agar (YPG). The reference strain of Cms, GSPB-Nr. 2823, obtained from the Göttingen Collection of Phytopathogenic Bacteria (GSPB, Göttingen, Germany) was used in the study.

PCR. PCR amplification was performed with Cms 50f and Cms 50r primers [13]. Samples of isolated colonies were boiled for 5 min and centrifuged for 3 min at 12000 g. Ten microlitres of the supernatant was tested in a total volume of 50 µl PCR reaction mix. The PCR reaction mix contained 0.2 µl of Taq polimerase (5 U/µl; Boehringer), 5 µl of 10× PCR buffer (Boehringer), 5 µl dNDP (2 mM of each dATP, dCTP, dGTP, and dTTP), 0,5 µl of each primer (100 nM/ml; Cms 50f: 5'-GAGCGCGATAGAA GAGGAACTC-3'; Cms50r: 5'-TCCTGAGCAACG AACAAGAAAA-3'), 28,8 µl of DEPC water. PCR was run on a Perkin Elmer 9600 thermocycler under the following conditions: 10 cycles of denaturation for 45 s at 94 °C, primer annealing for 60 s at 55 °C, DNR extention 10 s 72 °C followed by 40 cycles of 45 s at 92 °C, 40 s at 55 °C, 15 s at 72 °C and a single final extension 5 min at 72 °C. After the PCR reaction, 15 µl of amplification product was separated on a 1.5% agarose gel stained with 5 µl/100 ml of ethidium bromide and photographed under UV illumination.

RESULTS AND DISCUSSION

Disease distribution and incidence. Since 1998 potato plants and tubers from different parts of the country as well as imported ones have been tested for Cms. In this way more than 1300 samples of potato tubers were tested by IF, of them 350 by ELISA. Samples with symptoms of plant wilting and tuber rotting characteristic to bacterial ring rot were collected from different places of Lithuania. Because of its latent period samples of symptomless tubers were simultaneously involved into the study.

Potato ring rot was recorded in various localities of potato growth. Four localities were revealed in 1998, seven in 1999, and seventeen localities of potato ring rot in total were known in Lithuania by 2001. Seven new localities were found in 2001. The disease was detected in all regions of Lithuania. Bacterial wilt incidence in potato fields reached 5%, while tuber infection in storage places varied in the range of 5–21%.

The pathogen was particularly frequent in the following potato varieties: 'Planta', 'Romano', 'Sante', 'Escort', 'Dietskoselskij', 'Nida', 'Karolin', 'Pemperna', 'Sineglazka', 'Rossella', 'Mirta', 'Helena', 'Bimonda', 'Vindzor', 'Larisa', 'Gloria', 'Aistės', 'Fresco', 'Karatop', 'Ibis', 'Robinta'.

Confirmation of pathogen identity. IF- and ELISA-possitive samples were tested for pathogenicity on eggplants. After inoculation the wilt induced by Cms appeared on leaf edges, on limited areas of leaf and was originated at the leaf-edge. Light-brown colouring covered the damaged parts, which later turned pale and became necrotic. Characteristic symptoms were found on 27 plants. Gram-positive bacteria were isolated from injured tissues on NA and YPG from 16 inoculated eggplants and retested by IF and ELISA.

Twelve potato samples, positive by IF and negative by ELISA, were used in PCR for Cms detection. PCR failure was observed with five samples. In seven cases positive signals were obtained. In the present study PCR amplification based on the primers Cms 50f and Cms50r, allowed a specific and rapid confirmation of Cms from a pure culture of suspicious samples.

Cms has been described as the most damaging bacterium in many parts of the world [19]. Within the EC, Denmark has had problems with the disease for several decades already. In Sweden and Finland the disease was and in some parts still is widespread. The disease was reported in the neighbouring countries Poland and Russia. Unfortunately, there are only few reports on detailed yield losses [5]. Most of the recent papers on crop loss (up to 50%) come from North America [7]. In the EPPO region, the disease appears sporadically and with low levels of infection. One of the reasons for low disease occurrence in this area is the fact that cutting of potato seed and the use of pricker-type planters is not common in Europe. When the tubers are cut, higher levels may also occur (up to 30% of crop loss in France) [10]. The most important means of control are production of disease-free seed following strict certification and testing schemes [16] and sanitation [12].

It is clear that laboratory testing is an essential feature of any regulatory control, since latent infections must be detected. Thus, the emphasis is being shifted from visual assessments to laboratory testing. Currently, serological techniques such as enzyme-

linked immunosorbent assay and immunofluorescence assays are applied [4, 9]. According to references, the sensitivity of IF is equal to [8], or higher than the sensitivity of ELISA [6]. However, the data are not always reliable due to the cross-reactions with other bacteria [2, 3, 13]. Thus, the use of PCR is promising for the future and opens the way to precise epidemiological studies concerning the development of Cms populations.

The occurrence and distribution of Cms in Lithuania is of great concern for the proliferation of the pathogen. Therefore, national survey and detection programs for the estimation of losses, control and eradication of the pathogen have to be started, and the scientific knowledge of the epidemiology of the pathogen is required.

CONCLUSIONS

1. *Clavibacter michiganensis* subsp. *sepedonicus*, the causal agent of bacterial potato ring rot, was isolated and identified for the first time in Lithuania.

2. The disease incidence reached 5% in the field and 5 to 21% in storage places.

3. National survey and programs for eradication of the pathogen on the basis of scientific knowledge is required, particularly due to the wide distribution of the pathogen in the country.

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References

1. Baranauskaitė L., Vasinauskienė M. Bacterial ring rot of potoes caused by *Clavibacter michiganensis* subsp. *sepedonicus* in Lithuania // Plant Disease. 2001. Vol. 85. No. 10. P. 1119.
2. Calzolari A., Bazzi C., Mazzuchi U. Cross reactions between *Corynebacterium sepedonicum* and *Arthrobacter polychromogenes* in immunofluorescence staining // Potato Research. 1982. Vol. 25. P. 239–246.
3. De Boer S.H. Cross-reaction of *Corynebacterium sepedonicum* antisera with *C. insidiosum, C. michiganense* and unidentificed coryneform bacterium // Phytopathology. 1982. Vol. 72. P. 1474–1478.
4. DeBoer S. H., Wieczorek A., Kummer A. An ELISA test for bacterial ring rot of potato with a new monoclonal antibody // Plant Disease. 1988. Vol. 92. P. 874–878.
5. De Boer S.H. The relationships between bacterial ring rot and North American seed potato export markets // American Potato Journal. 1987. Vol. 64. P. 687–694.
6. De Boer S. H., Stead D. E., Alivizatos A. S., Janse J. O. et al. Evaluation of serological tests for detection of *Clavibacter michiganensis* subsp. *sepedonicus* in composite potato stem and tuber samples // Plant Disease. 1994. Vol. 78. P. 725–729.
7. Easton G. D. The biology and epidemiology of potato ring rot // American Potato Journal. 1979. Vol. 56. P. 459–460.
8. Gudmestad N. C., Baer D., Kurowski C. J. Validating immunoassay test performance in the detection of *Corynebacterium sepedonicum* during the growing season // Phytopathology. 1991. Vol. 81. P. 475–480.
9. Hampton R., Ball E., De Boer S. H. (editors). Serological Methods for Detection and Identification of Viral and Bacterial Plant Pathogens. St. Paul, Minnesota, USA, 1990. 389 p.
10. Lansade M. Recherches sur le flétrissement bactérien de la pomme de terre en France, *Corynebacterium sepedonicum* // Annales de l'Institut National de Réchzrches Agronomiques, Series C (Annales des Epiphyties). 1950. Vol. 1. P. 69–156.
11. Lelliott R. A., Stead D. E. Methods for the diagnosis of bacterial diseases of plants. Oxford, 1987. 216 p.
12. Lynch D. R., Nelson G. A., Kulcsar F. Elimination of bacterial ring rot (*Corynebacterium sepedonicum)* by *in vitro* culture of sprout tissue // Potato Research. 1989. Vol. 32. P. 69–156.
13. Mills D., Russell B. W., Hanus J. W. Specific detection of *Clavibacter michiganensis* subsp. *sepedonicus* by amplification of three unique DNR sequences isolated by substration hybridization // Phytopathology. 1997. Vol. 87. N 8. P. 853–861.
14. Nelson G. A. Long term survival of *Corynebacterium sepedonicum* on contaminated surface and infected potato stems // American Potato Journal. 1980. Vol. 57. P. 595–600.
15. Nelson G. A. *Corynebacterium sepedonicum* in potato: Effect of inoculum concentration on ring rot symptoms and latent infection // Canadian Journal of Plant Pathology. 1982. Vol. 4. P. 129–133.
16. Nelson G. A. Survival of *Corynebacterium sepedonicum* in potato stems and on surfaces held at freezing and above-freezing temperatures. // American Potato Journal. 1984. Vol. 62. P. 23–28.
17. OEPP/EPPO. Quarantine procedures No. 25, *Clavibacter michiganence* subsp. *sepedonicus* – inspection and test methods // Bulletin OEPP/EPPO Bulletin. 1990. Vol. 20. P. 235–254.
18. Pileckis S., Repšienė D., Vengeliauskaitė A. ir kt. Lauko augalų ligos ir kenkėjai. Vilnius, 1994. 456 p.
19. Smith I. M., Charles L. M. F. (editors). Distribution of Maps of Quarantine Pests for Europe Wallingford, U. K., 1998.
20. Vilkaitis V. Augalų apsaugos stoties 1927–1932 m. darbų apyskaita. Kaunas, 1933. P. 45–46.
21. Vinickas Z. Bulvių ligos. Vilnius, 1974. 175 p.

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CLAVIBACTER MICHIGANENSIS SUBSP. *SEPEDONICUS* IŠPLITIMAS LIETUVOJE

S a n t r a u k a

Bulvių žiedinis puvinys, kurį sukelia *Clavibacter michiganensis* subsp. *sepedonicus* (Cms), yra viena pavojingiausių bulvių bakterinių ligų, kelianti nuolatinį pavojų bulvių augintojams. Beveik visos bulves auginančios šalys patiria derliaus nuostolių. Dėl patogeno latentinės fazės liga nepastebimai plinta ir yra sunkiai diagnozuojama.

1998–2001 m. buvo pradėti ir vykdomi Cms bakterijų paplitimo Lietuvoje tyrimai. Ligos židiniai nustatyti visose šalies apskrityse. Pažeidimas sudarė vidutiniškai 5% augalų lauke ir 5–21% bulvių gumbų saugyklose. Bakterijos identifikuotos taikant imunofluorescencinį (IF), imunofermentinį (ELISA) tyrimo metodus, taip pat polimerazės grandininę reakciją (PGR). Bakterijų patogeniškumas patikrintas baklažano lapams.

Raktažodžiai: bulvės, bakterinis žiedinis puvinys, *Clavibacter michiganensis* subsp. *sepedonicus*

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РАСПРОСТРАНЕНИЕ *CLAVIBACTER MICHIGANENSIS* SUBSP. *SEPEDONICUS* В ЛИТВЕ

Р е з ю м е

Кольцевая гниль картофеля, вызываемая *Clavibacter michiganensis* subsp. *sepedonicus* (Cms) бактериями, является широко распространенным заболеванием, имеющим экономическое и международное значение. Почти все страны при возделывании этой культуры неизбежно сталкиваются с потерями урожая.

В 1998–2001 гг. в Литве проводились исследования по распространению возбудителя бактериальной кольцевой гнили картофеля. Установлено, что поражение бактериями в поле составляло в среднем 5%, в хранилищах – 5–21%. Cms бактерии были обнаружены и идентифицированы серологическими методами (IF, ELISA), а также с помощью амплифицирования полимеразной цепной реакции (ПЦР). Патогенность бактерий проверялась на молодых растениях баклажана.

Ключевые слова: картофель, кольцевая гниль картофеля, *Clavibacter michiganensis* subsp. *sepedonicus*