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RISK OF INTRODUCTION OF QUARANTINE ORGANISMS: CASE *CLAVIBACTER MICHIGANENSIS* SUBSP. *SEPEDONICUS*

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Abstract

Transmission of plant pathogens and pests across country borders has raised an alert in the EU. Some of them having a severe economic impact, leading for taking precautionary measures such as quarantine of plant materials transported across borders. *Clavibacter michiganensis* subsp. *sepedonicus* (*Cms*) is listed as a quarantine organism in EU. This bacterium causes potato ring rot disease and presents a worldwide threat due to crop losses during vegetation and in storage. Potato tubers from import in Serbia were tested for its presence in accordance with the official EU Council Directive. From the stolon end of 200 tubers (consist one sample), the small core of tissue containing vascular tissue was removed and the heel ends were taken and crashed in sterile phosphate buffer, then centrifuged. Re-suspended pellet was used for immunofluorescence (IF, Loewe Biochemica GmbH), isolation of bacteria and DNA extraction for Polymerase Chain Reaction (PCR) performed with a pathogen-specific primer set PSA-1/PSA-R. As a positive control *Cms* reference strain CFBP 3561 was used. Two samples of ware potato originated from Belarus and Russian Federation in 2019 gave positive results for the presence of *Cms*. Visible internal symptom was observed on several tubers, in form of the vascular ring of tuber when they cut transversely. Bacterial ooze stream when tubers squeezed. The isolation of bacteria was performed from the ooze on Nutrient dextrose agar. Creamy-white, smooth colonies were formed after 3-5 days of incubation at 22 ºC. Restriction fragment length polymorphism (RFLP) of PCR products performed with PSA-1/PSA-R primer pair (502 bp) with enzyme *Bgl*II (fragments 282 and 220 bp in size) confirmed that the isolates belong to *Cms*. Pathogenicity was confirmed on aubergine seedlings, showing typical wilting obtained within 15-20 days after inoculation. Serbia is still free area from *Cms* pathogen due to rejection of contaminated potatoes from import.

Keywords: *health status, plant, disease, bacteria.*

Introduction

Plant quarantine represents a system of regulations established to protect agriculture production from losses caused by harmful organisms. It controls the introduction of harmful insects, mites, nematodes, gastropods, bacteria, phytoplasmas, fungi, viruses and virus-like organisms, and plants into free areas and as such is the best and most effective preventive measure for spreading of quarantine organisms. In the European Union (EU), phytosanitary measures are specified by EC Council Directive 2000/29/EC (EC, 2000), and 300 pests that are marked as quarantine are subjected to quarantine requirements (Schrader and Unger, 2003). There are two lists of quarantine pests recommended by EPPO (European and Mediterranean Plant Protection Organization), A1 list that includes pests absent from the EU, and A2 list with pests that are not widespread in the EU. According to EU standards, Serbian Ministry for Agriculture, Forestry and Water Management through the Plant Protection Directorate proposes Regulations for each harmful organism.

Clavibacter michiganensis subsp. *sepedonicus* Davis. *et al.* (*Cms*) causal agent of potato ring rot is listed in the European Plant Health Directive and may be very destructive to the potato crop. Since it is highly contagious and persistent in plant and soil debris, this bacterium is submitted to strict regulation to avoid its dissemination by plant transport. *Cms* represents a worldwide threat to potato growing and its industry due to crop losses. Losses are direct, during growth and in storage, and indirect, reflected through rejection of infected seed lots and the cost for the control measures, and by loss of export markets or difficulties in opening new markets (Van der Wolf *et al.*, 2005a; Van der Wolf *et al.*, 2005b; Węgierek-Maciejewska *et al.*, 2019; Charkowski *et al.*, 2020). Direct yield losses of over 50% have been estimated from field trials in Norway and the USA (Elphinstone, 2011). In Europe, the economic impact caused by *Cms* is estimated at 15 million Eur per year (Van der Wolf *et al.*, 2005a). Bacterium occurs in cool regions of America, Canada, China, Northern Europe and Russia (Van der Wolf *et al.*, 2005a). According to Charkowski *et al.* (2020) strict regulation in Europe reduced findings in annual surveys, with only occasional findings in some countries (Bulgaria, Czech Republic, Estonia, Finland, Germany, Greece, Hungary, Latvia, Lithuania, Netherlands, Norway, Slovakia, Sweden, Turkey). Isolated former outbreaks have been declared eradicated in Austria, Belgium, Cyprus, Denmark, France, Spain, and the UK (England and Wales) (Charkowski *et al.*, 2020).

The main symptoms caused by *Cms* are systemic infection of vascular tissue, interveinal chlorosis, and wilting/epinasty at leaf margins (Sadunishvili *et al.*, 2020; Charkowski *et al.*, 2020). The disease is difficult to detect in the field because the symptoms develop slowly, and latent infections are frequent. On tubers, *Cms* causes well-named ring rot of potato. As the infection usually starts on a tuber via infection in stolon, the infected vascular tissue of the tuber becomes yellowish and cheesy in texture due to bacterial oozing (Bragard *et al.*, 2019). As rot progresses, tuber surface cracks and dark blotches may become visible immediately beneath the periderm (Bragard *et al.*, 2019). The main sources of *Cms* transmission are infected seed potatoes (long distance) or contaminated equipment (Mansfeld-Giese, 1997; Van der Wolf *et al.*, 2005a). Seed damaged by cutting or by equipment can rise up a percentage of the infection to 80% (Van der Wolf *et al.*, 2005a). Bacteria can persist longer than 2 years on surfaces of different materials (iron, wood, rubber, and plastic). Possibility of surviving increases by a low relative humidity of 10%, and a temperature below 10 °C (Van der Wolf *et al.*, 2005a). *Cms* is able to survive in the postharvest debris of crops in the soil, and/or on alternative hosts (weeds) (Franc, 1999; Van der Wolf *et al.*, 2005b).

Management of potato ring rot is relying on the production and distribution of seed potatoes that are free from infection, achieved through strict application of quarantine and seed certification regulations, which involve a zero tolerance for the disease during seed and import inspections (Charkowski *et al.* 2020). In Serbia *Cms* is on List IA part I (Sl.glasnik RS, 2015). Potato tubers from import are being tested for *Cms* presence in accordance with EU Council Directive 93/85/EEC (EU, 1993) and EPPO PM 7/59(1) (OEPP/EPPO, 2006) standards. Quarantine procedure is based on laboratory screening of latent infection of *Cms* in tubers. This work presents cases of potatoes infected with *Cms*, found in the Serbian borders in 2019.

Material and Methods

Extraction of bacteria from plant tissue

Samples consisted of 200 potato tubers were first washed with water to free them from the soil impurities, surface-sterilized with sodium hypochlorite solution, and exposed to visual observations. From the stolon end of tubers, the small core of vascular tissue was removed and the ends were taken and transferred to a disposable maceration bag (Bioreba) where they

were crashed in sufficient volume of 50 mM phosphate buffer (PB). Supernatants were decanted and centrifuged at 7,000 *g* for 15 min, afterwards the pellet was re-suspended in 1 mL of sterile 10 mM PB.

Immunofluorescence (IF)
Undiluted extract of the samples and their 1:10 and 1:100 dilutions in 10 mM PB were subjected to IF assay using the antiserum Loewe Biochemica GmbH. Windows of the immunofluorescence slides were spotted by 20 µL of samples extracts and their dilutions, dried and then fixed. The polyclonal antibody and anti-goat fluorescein isothiocyanate conjugate (FITC) were diluted in PB according to the manufacturer's recommendation. Reference strain *Cms* CFBP 3561 (=PD 406) originated from potato (France) served as a positive control. Samples with the presence of green fluorescing cells with typical *Cms* size and morphology observed under the fluorescence microscope were considered as positive.

Polymerase Chain Reaction (PCR)
Bacterial DNA was extracted directly from potato extracts (Pastrik and Rainey, 1999; Pastrik, 2000). PCR was performed with a pathogen-specific primer set PSA-1 (5'-CTCCTTGTGGGGTGGGAAAA-3′) and PSA-R (5'-TACTGAGATGTTTCACTTCCCC-3′) (Pastrik and Rainey, 1999). PCR thermal conditions were as follows: initial denaturation at 95 °C for 3 min, 10 reaction cycles of 95 °C for 1 min, 64 °C for 1 min, 72 °C for 1 min, then 25 reaction cycles of 95 °C for 30 s, 62 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 5 min. DNA of *Cms* reference strain CFBP 3561 was used as a positive control. The products were visualized on a 1% agarose gel, stained with ethidium bromide. Band presence on the gel was checked under UV light. Amplified PCR products 502 bp in size were considered as positive.

Isolation of bacteria
Tubers from the samples which gave two test positive reactions were cut transversely. Isolation of bacteria was performed directly from the bacterial ooze on Nutrient Dextrose Agar (NDA) and kept at 22 ± 1 °C for 3-5 days. Presumptive colonies that were formed after the incubation period and had the morphology as *Cms* reference strain CFBP 3561 were purified and maintained at - 20 °C in Luria Bertani (LB) broth containing 20% (*v/v*) of sterile glycerol.

Pathogenicity
Pathogenicity of the obtained isolates was tested on young aubergine seedlings, planted in a sterile substrate and grown under the controlled conditions (22-25 °C, natural light and regular watering). Inoculations were performed in the phase of 3rd true leaf stage, appr. 3 weeks after sowing. The bacterial suspension (10^7-10^8 CFU mL^{-1}) was infiltrated in petioles of the 3rd true leaf, with a hypodermic syringe. *Cms* reference strain CFBP 3561 served as a positive control treatment and sterile distilled water as a negative. Inoculated plants were kept in plastic boxes and incubated for two weeks at 22 ± 2 °C and 70-80% humidity. Re-isolation from symptomatic plants was performed on NDA.

RFLP (Restriction Fragment Length Polymorphism)
For confirmation of isolates and re-isolates identity, RFLP analysis was used with PCR products obtained with PSA-1/PSA-R primer pair and incubation with enzyme *Bgl*II at 37 °C. Obtained amplicons were visualized under UV light on 1.5% agarose gel stained with ethidium bromide.

Results and Discussion

Two samples of ware potato originated from Belarus and Russian Federation in 2019 gave positive results for the presence of ring rot pathogen *Cms* using two diagnostic tests (i) IF test, after showing typical green fluorescing cells (Figure 2), and (ii) PCR with a pathogen-specific primer set PSA-1/PSA-R, after amplifying 502 bp size products. Although the IF test is accessible for detection of *Cms* (Dinesen and De Boer, 1995), non-specific reactions may occur (Van der Wolf *et al.*, 2005a). As a consequence, other tests, such as molecular PCR tests, should be used to verify positive results of IF. Although several PCR assays for *Cms* detection were developed, the most appropriate assays, validated based on specificity and sensitivity, are those given by Pastrik (2000) (Van der Wolf *et al.*, 2005a). According to EPPO distribution maps, *Cms* is present in Russia and Belarus (EPPO, 2020). In Russia, during the period 2015-2018, 39 samples were characterized as *Cms* positive and appeared in five Russian regions (Tver', Irkutsk, Kostroma, Leningrad, and Moscow) (Malko *et al.*, 2019). *Cms* belongs to the list of regulated nonquarantine pathogens in Russia, and after *Phytophthora infestans* it appeared to be most frequent with presence of 29.5% in total surveyed samples (Malko *et al.*, 2019).

A visible internal symptom was observed on a few tubers in two samples, in the form of a vascular ring of tuber when they were cut transversely. Bacterial ooze was expressed after a few seconds, when tuber was squeezed (Figure 1). This is in accordance with the symptom described in the literature (Charkowski *et al.*, 2020; Sadunishvili *et al.*, 2020). From bacterial ooze, white-creamy, smooth colonies were formed 3-5 days after isolation on the NDA (Figure 2). A total of ten representative isolates were subjected (five from each positive sample) to pathogenicity and further identification. In general, isolation of *Cms* from plant tissue is very difficult because it's slowly grown, and it is often overgrown on agar media by saprophytes (De la Cruz *et al.*, 1992; Dinesen and De Boer, 1995). Semi-selective media MTNA (Jansing and Rudolph, 1998) and NCP 88 (de la Cruz *et al.*, 1992) are appeared to be well suited for reliable *Cms* isolation procedure.

Figure 1. Potato ring rot

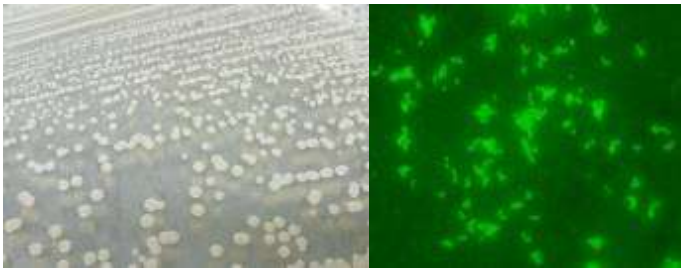

Figure 2. Bacterial colonies of *Cms* on NDA (left) and cells under the fluorescence microscope (right)

Typical wilting was developed on inoculated young aubergine seedlings within 15-20 days for all tested isolates and *Cms* reference strain CFBP 3561. Plants inoculated with sterile distilled water were symptomless. Re-isolation from aubergine plants confirmed the fulfilment of Koch's postulates.

In RFLP analysis, amplifyed products using PSA-1/PSA-R primer pair (502 bp in size) digested with *Bgl*II endonuclease gave the unique, characteristic RFLP pattern (282 and 220 bp in size) for all isolates and reisolates and the *Cms* reference strain CFBP 3561.

Based on the fact that this pathogen occurred in some southern countries, such as Spain, Cyprus, Greece, or neighbouring Bulgaria and Hungary (Charkowski *et al.*, 2020), *Cms* could be able to survive in Serbian environmental conditions. Therefore, strong standards for potato

tuber testing from import and intensive monitoring conducted for potato grown in Serbia is required.

Conclusions

Continuous monitoring of the *Cms* bacterium and rejecting of contaminated potatoes to be imported from the borders in Serbia by the authorities of the Ministry of Agriculture, Forestry and Water Management, gave the results that Serbia is still free area from the ring rot pathogen.

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